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Novel Therapeutic Strategies for Treatment of Castration-Resistant Prostate Cancer

Matthew A. Ingersoll
University of Nebraska Medical Center

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Novel Therapeutic Strategies for Treatment of Castration-Resistant Prostate Cancer

By

Matthew Ingersoll

A DISSERTATION

Presented to the Faculty of the Graduate College in the University of Nebraska

In Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy

Department of Biochemistry and Molecular Biology

Under the Supervision of Professors Ming-Fong Lin and Surinder K. Batra

November 2017

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Parmender P. Mehta, Ph.D.

Yaping Tu, Ph.D.
Prostate cancer (PCa) remains the most commonly diagnosed solid tumor and is the third leading cause of cancer-related death in United States men. While androgen deprivation therapy is the current standard-of-care treatment for metastatic PCa, most patients eventually relapse and develop castration-resistant (CR) tumors, for which there is currently no effective treatment. Therefore, synthesis of novel therapeutic agents and identification of alternative target proteins are necessary to improve treatment. Herein, I investigate the efficacy of novel imidazopyridine and statin derivatives as alternative therapeutic compounds. These molecules not only inhibit androgen receptor signaling, but also block activation of the AKT axis, a mechanism of androgen independence. Furthermore, I investigate the role of p66Shc, a 66 kDa Src and collagen homologue oxidase, in the mechanism of PCa metastatic progression. p66Shc is elevated in clinical PCa as well as multiple PCa cell lines which correspond with advanced CR PCa. Additionally, p66Shc has been demonstrated to promote proliferation in PCa cell lines via generation of reactive oxygen species (ROS). This study is the first to demonstrate p66Shc also regulates PCa cell migration through ROS production and identifies key ROS-sensitive proteins pivotal to its mechanism. Understanding how p66Shc promotes migration may lead to the identification of alternative therapeutic targets for suppression of CR PCa metastatic activity. Overall, this study seeks to support future efforts to generate therapeutic compounds for treatment of metastatic CR PCa.
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<td>antibody</td>
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<tr>
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<td>androgen deprivation therapy</td>
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<td>AI</td>
<td>androgen-independent</td>
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<td>protein kinase B</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
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<td>androgen-sensitive</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
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<td>BAX</td>
<td>bcl-2-like protein 4</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>Bcl-XL</td>
<td>B-cell lymphoma-extra large</td>
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<tr>
<td>CA</td>
<td>constitutively active</td>
</tr>
<tr>
<td>CB</td>
<td>cytochrome B binding</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>cPAcP</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>CR PCa</td>
<td>castration-resistant prostate cancer</td>
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<td>Cys, C</td>
<td>cysteine</td>
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<td>Description</td>
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<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DN</td>
<td>dominant-negative</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>ethylenediamine tetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>Elk-1</td>
<td>ETS domain-containing protein 1</td>
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<td>ErbB-4</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FOXM1</td>
<td>forkhead box protein m1</td>
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<tr>
<td>Gly, G</td>
<td>glycine</td>
</tr>
<tr>
<td>Glu, E</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
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<td>guanosine triphosphate</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<td>HEPES</td>
<td>n-(2-hydroxyethyl)piperazine-n'-(2-ethanesulfonic acid)</td>
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<tr>
<td>HMGCoA</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A</td>
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<td>Hr</td>
<td>hour</td>
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<td>HO-1</td>
<td>heme oxygenase-1</td>
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<tr>
<td>IC\textsubscript{50}</td>
<td>Concentration resulting in 50% inhibition of maximal response</td>
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<td>JNK</td>
<td>c-Jun amino-terminal kinase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<td>LHRH</td>
<td>luteinizing hormone releasing hormone</td>
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<td>Lys, K</td>
<td>Lysine</td>
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<td>Min</td>
<td>minute</td>
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<td>mitogen-activated protein kinase</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>Myc</td>
<td>myelocytomatosis virus oncogene cellular homolog</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
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<tr>
<td>Nrl2</td>
<td>nuclear factor erythroid 2-related factor</td>
</tr>
<tr>
<td>o/n</td>
<td>over night</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
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<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
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<td>PKA/B/C</td>
<td>protein kinase A/B/C</td>
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<td>PCa</td>
<td>prostate cancer</td>
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<td>PSA</td>
<td>prostate-specific antigen</td>
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<tr>
<td>PTB</td>
<td>phosphotyrosine binding</td>
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<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome ten</td>
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<td>PYK2</td>
<td>proline-rich tyrosine kinase 2</td>
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<td>p38</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>p53</td>
<td>tumor-suppressor protein 53</td>
</tr>
<tr>
<td>Raf</td>
<td>rapidly accelerated fibrosarcoma (serine/threonine-specific protein kinases)</td>
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<tr>
<td>Ras</td>
<td>rat sarcoma (small GTP-ase)</td>
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<tr>
<td>RTK</td>
<td>receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>-----------</td>
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<td>serine</td>
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<td>SH2</td>
<td>src homology 2</td>
</tr>
<tr>
<td>Shc</td>
<td>src homology and collagen homologue</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small inhibitory RNA</td>
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<tr>
<td>SR</td>
<td>steroid-reduced</td>
</tr>
<tr>
<td>SOS</td>
<td>son of Sevenless</td>
</tr>
<tr>
<td>Src</td>
<td>steroid receptor coactivator</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SVA</td>
<td>simvastatin hydroxyacid</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>Tyr, Y</td>
<td>tyrosine</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>YAP</td>
<td>yes kinase-associated protein</td>
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Acknowledgements

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Chapter 1

Introduction
1.1 Progression of Prostate Cancer and Castration-Resistance

Prostate cancer (PCa) is the most commonly diagnosed carcinoma and third leading cause of cancer-related death in American men, with 161,360 new cases and 26,730 deaths estimated in 2017 [1,2]. PCa mainly affects older men with the majority of PCa diagnosed in men over the age of 65 and the disease rarely observed in men younger than 40. On average, 1 in every 7 men will be diagnosed with PCa in the course of their lifetime and 1 in every 39 men will die from PCa [2,3]. While the causes of PCa initiation are still being studied, both genetic and environmental factors such as diet, smoking, and exercise, contribute to tumor development [4]. The development of PCa is thought to adhere to the “multi-hit” nature of carcinogenesis in which a number of mutational events eventually lead to tumor initiation and later malignant transformation [3,4]. Typically, PCa is a slow-growing cancer and, as with most solid tumors, localized PCa is generally not lethal and effectively treated by means of surgery or radiation therapy. In some instances, PCa patients may not require treatment if the disease is not expected to affect their normal life span [5,6]. It is not until the tumor becomes metastatic and begins to invade surrounding tissue that it is life threatening. Thus, the ability to distinguish indolent and lethal tumors is the subject of intense focus in the field of PCa research.

The prostate is a glandular organ composed of lumen lined mainly with epithelial cells as well as the occasional neuroendocrine cell, all of which are anchored to a basal membrane [7-8]. Multiple lumen structures are held together by stromal tissue composed of fibroblasts, myoblasts, smooth muscle cells, and neurons in addition to vascular tissues [7]. Importantly, the vast majority of prostate tumors develop from prostate epithelial tissue, meaning they are adenocarcinoma. This distinction is critical because prostate epithelial cells express high levels of androgen receptor (AR) compared to other
prostate tissues, thus tumors of this origin are primarily regulated by androgens in terms of growth and development [9]. Therefore, metastatic PCa is initially treated with androgen deprivation therapy (ADT) in which the body undergoes physical or chemical castration [10]. However, as with the majority of hormone-dependent cancers, many PCa patients relapse into the “hormone-refractory” or “castration-resistant” (CR) form of the disease within a few years (Fig. 1.1) [11]. Despite the tumor’s lack of response to ADT, most CR PCa still maintains some reliance on androgen receptor signaling and androgens for the receptor’s activation [12]. Current therapeutic strategies for CR PCa revolve around AR inhibitors such as enzalutamide, abiraterone acetate, and Casodex. Chemotherapeutic agents like docetaxel, which targets the mitotic spindle complex and inhibits the cell division, are also used to slow the disease’s progression [13-14]. However, the most effective therapies for CR PCa are currently only able to extend patient survival by about 3 months, maintaining a need for more effective treatment options [14]. Thus, because the inhibition of androgen receptor has only marginal effect, new therapeutic targets must be identified. In addition, molecules involved in the process of CR PCa cell proliferation, survival, and migration have the potential to be promising new targets for metastatic PCa. Understanding what regulates PCa cell metastatic progression would allow for the development of new agents to better treat the disease and possibly prevent metastasis from initially occurring. More importantly, knowledge of how the CR phenotype specifically controls growth and migration will aid the development of novel treatment options for patients who have already progressed to end-stage PCa, limiting the disease’s spread to vital organs and further extending survival.
1.2 Key Prostate Cancer Associated Proteins

While many mutations may occur during the development of PCa, deregulation of several protein signaling pathways have been identified as playing a major role in development of castration resistance. The first of these is androgen receptor itself, but also included are proteins directly regulated by androgens, such as p66Shc, and their downstream signaling pathways. Additional proteins regulating cell growth, survival, migration, and overall metastatic progression are also of keen therapeutic interest and include ErbB-2, cPAcP, AKT, and ERK.

1.2A AR and PSA

Human PCa primarily originates from prostate epithelial cells, thus it is characterized as adenocarcinoma and its growth is regulated by androgens [9]. In the male body, the pituitary gland releases luteinizing hormone which stimulates leydig cells in the testes to produce androgens [15]. These are then distributed throughout the body via the bloodstream in the form of testosterone [16]. Upon entering prostate epithelial cells, testosterone is converted to 5α-dihydrotestosterone (DHT) by 5α-reductase in the cytosol [17]. AR, which is normally bound by heatshock proteins (HSP) 27 and 90 in the cytosol, is released upon binding DHT, allowed to form dimers, and enter the nucleus where it acts as a transcription factor for multiple proteins essential to cell growth and survival [18]. In the absence of androgens, prostate epithelial cells undergo apoptosis, thus in the early stages of prostate cancer ADT is very effective at reducing tumor volume (Fig. 1.2) [19-20].

Unfortunately, the majority of PCa patients eventually relapse after initial treatment and becomes hormone-refractory or castration-resistant, meaning the tumor cells are resistant to ADT or no longer require external androgens for growth and development [21]. There are a number of mechanisms through which tumors may obtain
the castration-resistant phenotype. First, while ADT significantly reduces circulating androgen levels, it does not completely abolish androgen production and instead lowers androgen concentrations to about a 10% their original level [22]. In some cases, PCa cells increase AR levels to compensate for lower levels of androgen, sensitizing the cells to even minute concentrations of testosterone [23]. In addition, there are reports that PCa cells can also obtain the complete steroidogenic ability to synthesize their own androgens, bypassing the need of external sources [24]. AR may also become mutated, allowing for receptor promiscuity or constitutive activation, bypassing the need for androgens altogether [25]. Furthermore, AR can be post-translationally modified via phosphorylation by kinases such as PKA, AKT, and MAPK, activating AR and inducing nuclear translocation [26-27]. Finally, activation of key downstream signaling targets of AR, such as ErbB-2, can circumvent the need for AR activation [28].

One of the most pervasive methods for tracking PCa disease progression in patients is monitoring serum levels of prostate-specific antigen (PSA) [29]. PSA is a member of the kallikrein-peptidase family of enzymes whose function is proposed to liquefy semen in the seminal coagulum to allow sperm to swim freely [30-31]. Ordinarily, PSA is regulated by androgen receptor and produced by prostate epithelial cells for secretion into seminal fluid [32]. While PSA is found in high levels in prostate epithelium and seminal fluids, it has relatively very low expression in all other tissues, thus it is a potent biomarker used to measure overall androgen receptor activity in the body [33-36]. After undergoing androgen-ablation therapy, patient serum PSA levels drop dramatically to an undetectable level [35]. Physicians then continue to monitor patient serum PSA levels for an eventual increase, indicating the tumor has relapsed and acquired the castration-resistant phenotype [37].
1.2B p66Shc and Reactive Oxygen Species

Recent studies have shown AR promotes PCa metastatic phenotype in part by increasing levels of cellular reactive oxygen species (ROS) [38]. In part, this is achieved via an AR-mediated increase in levels of p66Shc protein, a 66 kDa proto-oncogene Src and collagen homologue protein, that exhibits oxidase activity and is one of three members of the Shc family, including isoforms p52Shc and p46Shc (Fig. 1.3) [39-40]. All three Shc isoforms possess C-terminal Src homology 2 (SH2), collagen homology 1 (CH1), and phosphotyrosine binding (PTB) domains (Fig. 1.4). While p52Shc and p46Shc are ubiquitously expressed, p66Shc expression is more tightly regulated due to the presence of an alternative promoter which endows p66Shc with additional cytochrome C-binding (CB) and N-terminal collagen homology 2 (CH2) domains and provide it with supplementary functions [41-45]. p66Shc also differs from the other Shc isoforms in that its protein level is up-regulated by androgens that play a critical role in the process of PCa progression [46]. This is achieved via AR-induced stabilization of p66Shc, preventing ubiquitin-mediated degradation and causing an increase in cellular levels [47]. Recently, p66Shc protein is shown to be elevated in clinical PCa tissue samples, higher than in adjacent non-cancerous cells, and its protein level correlates with metastatic potential of PCa cell lines, including the LNCaP cell progressive model [48-49]. Levels of p66Shc have also been found to correlate with acquisition of androgen-independence in a number of PCa cell line models. For example, LNCaP C-33 cells are androgen-sensitive and possess relatively low levels of p66Shc protein [50]. In contrast, as C-33 cells progress to C-81 cells, they become unresponsive to androgen deprivation and exhibit many biochemical properties seen in clinical CR PCa: including functional AR expression, androgen-independent prostate-specific antigen (PSA) secretion, malignancy and proliferation with intracrine growth regulation [48,50-51]. The
LNCaP C-81 cells also possess elevated p66Shc protein level [50]. This data has been further verified using a similar MDA PCa2b cell progressive model in which p66Shc protein level correlates with acquisition of androgen-independence and enhanced metastatic phenotype [46]. Interestingly, AR-null, androgen-independent (AI) PC-3 and DU145 cells possess a much more aggressive metastatic phenotype than LNCaP and MDA PCa2b cells as well as comparatively higher levels of p66Shc [46,50]. This suggests p66Shc is regulated by mechanisms in addition to AR activation.

The p66Shc protein can be regulated at both the transcriptional and post-translational levels. At the transcriptional level, modifications made to the p66Shc promoter, such as DNA methylation or histone deacetylation, result in silencing of p66Shc while demethylation or deacetylase enzymes promote p66Shc transcription [41,52-59]. Moreover, several transcription factors, including Nrf2 and STAT4, are reported to bind to p66Shc-specific promoters and promote transcription [60-61]. Post-translationally, p66Shc contains a number of phosphorylation sites which can modulate its activity or stability. These include sites shared by the other Shc adaptor proteins such as p38 phosphorylation of T386 in the CH1 domain which prevents ubiquitination and S138 phosphorylation by MEK1/PKC in the PTB domain which alters Shc protein activity, preventing it from binding Grb2 [61-64]. p66Shc also possess phosphorylation sites in its N-terminal CH2 domain, which are not shared with p52Shc and p46Shc. These include S54 phosphorylation by p38 preventing ubiquitination and S36 phosphorylation by ERK/JNK that induces cytosol to mitochondria translocation in response to stress stimuli [65-67].

In epithelial cells, p66Shc is localized in both the cytosol and mitochondria [68]. Upon mitochondrial translocation, p66Shc binds and oxidizes cytochrome C, uncoupling the electron transport chain and promoting generation of ROS [44]. ROS are natural by-
products of cellular respiration and contribute to essential signaling pathways; however, when produced in excess, they also readily oxidize proteins, lipids, and nucleic acids, which facilitate cancer development [69-70]. Moreover, ROS are also known to regulate metastatic processes, including proliferation and migration, which are critical to cancer metastasis [71-72].

In addition, p66Shc has been shown to promote phosphorylation and activation of ErbB-2 (HER2) at Y1221/2 and Y1248 by promoting oxidation and subsequent inactivation of cellular prostatic acid phosphatase activity (cPAcP), an authentic protein tyrosine phosphatase and negative regulator of ErbB-2 [75-77]. Downstream targets of ErbB-2, including AKT and ERK, are also shown to be activated by p66Shc [76-77]. These proteins promote cell survival, proliferation, and migration and play a pivotal role in p66Shc's ability to enhance the metastatic potential of CR PCa [75,78-79].

1.2C ErbB-2 and cPAcP

Protein-tyrosine kinases (PTKs) are a large multigene family in humans. These PTKs are involved in key cellular processes regulating differentiation, cellular development, and multicellular communication by transferring a phosphate group from ATP to the hydroxyl group of tyrosine [80]. Moreover, perturbation of PTK signaling results in the development of numerous disease conditions, including cancer [80]. A thorough study of this family of proteins over the past two decades has demonstrated that about ninety genes have been identified in the human genome, along with five pseudogenes [81-82]. Among the 90 identified tyrosine kinases, fifty-eight encode for receptor tyrosine kinases (RTKs) that are further divided into 20 subfamilies. The remaining 32 genes are classified as non-receptor tyrosine kinases and further classified into 10 subfamilies [82].
Results of previous studies suggest that the activation of receptor tyrosine kinase signaling is one of the major mechanisms through which PCa cells promote metastatic phenotype through the activation of subsequent effector pathways such as phosphoinositide 3-kinase (PI3K), protein-kinase B (PKB or AKT) and, mitogen-activated protein kinase (MAPK) and [83]. The human EGF receptor family consists of four distinct RTKs ErbB1-4, each possessing a number of synonymous gene names: EGFR/HER1/ErbB-1, NEU/HER2/ErbB-2, HER3/ ErbB-3 and HER4/ErbB-4. All members of the ErbB family of protein tyrosine kinases possess an extracellular domain, hydrophobic transmembrane section, juxtamembrane segment, an intracellular domain with kinase activity, and a cytoplasmic tail containing potential tyrosine phosphorylation sites [84]. The extracellular portion is further divided into four domains: these include domains I and III which are leucine-rich and responsible for ligand binding, while domains II and IV are cysteine-rich and facilitate dimerization via disulfide bonds. RTKs that exist as monomers are considered to be inactive [85-86]. Following the extracellular domain is a single-pass transmembrane segment consisting of 19-25 hydrophobic residues, a 40-residue juxtamembrane portion which plays a role in dimerization, a protein kinase domain, and a carboxyterminal tail [86]. Interestingly, extracellular domains I and II of ErbB-2 have no ligand-binding function, thus ErbB-2 monomer is potentially inactive [87]. Instead, ErbB-2 relies on heterodimerization with other ErbB family members for ligand-induced activation. Moreover, the protein kinase domain of ErbB-3 in kinase-impaired, thus ErbB-2 and ErbB-3 homodimers are potentially inactive [85,87].

Structurally, ErbB-2 maintains a naturally open conformation in which the dimerization arm of domain II is exposed. This is achieved through the substitution of a glycine residue with proline in domain II as well as a histidine residue with phenylalanine
in domain IV, preventing domain II and IV association in ErbB-2 [88]. While the wild-type ErbB-2 does not normally form homodimers, if the dimerization domain is in the open conformation homodimerization may occur [89]. While other ErbB family members are autoinhibited via a buried dimerization arm, constitutive activation of ErbB-2 is normally prevented by eight disulfide bonds in domain II that are partially disrupted upon heterodimerization with activated ErbB-1/3/4 [90]. Interestingly, it has been shown that in cancer cells overexpressing wild-type ErbB-2, the receptor homodimerizes and self-activates due to the large number of molecules present [91]. However, overexpression of ErbB-2 is rare in PCa. More often, ErbB-2 is reported to be hyper-activated through elevated levels of phosphorylation [50,76].

While ErbB-2 is not directly activated by ligands, indirect activation is achieved via heterodimerization to other ligand-bound ErbB family members. Moreover, ErbB-2 is the preferred heterodimeric partner for other ErbB receptors [92-93]. Several studies suggest that overexpression/mutation/loss of ErbB-2 results in deregulation of dimerization and the downstream signaling of other ErbB family members [94]. In addition, heterodimers containing ErbB-2 induce elevated ligand binding affinity of the partner receptor [95]. The ErbB-2 receptor heterodimerization results in the phosphorylation of specific tyrosine residues in the receptor cytoplasmic tails and subsequent increase in kinase enzymatic activity and phosphorylation of several intracellular targets [96].

Upon heterodimerization, ErbB-2’s cytoplasmic kinase domains are phosphorylated, leading to protein kinase activation. Therefore, the phosphatases that can reverse ErbB-2 phosphorylation are capable of inhibiting its signaling cascade. Cellular prostatic acid phosphatase (cPAcP) is a dual specificity prostate-specific phosphatase that can dephosphorylate tyrosine, serine, and threonine residues with a
preference for p-Tyr [97-99]. In human prostate cells, ErbB-2 has been identified as a target of cPAcP by co-immunoprecipitation experiments that demonstrate the interaction between the two proteins and dephosphorylation of its tyrosyl residues (50,76,99). The loss of cPAcP has also been shown to be an early event in PCa and therefore is likely a prominent cause of ErbB-2 hyper-activation commonly observed in PCa [100]. For example, as androgen-sensitive LNCaP and MDA PCa2b cell lines progress to the androgen-independent castration-resistant phenotype, their levels of ErbB-2 phosphorylation increase while cPAcP levels decrease [75-77,83,97]. Additionally, cPAcP knock-down in androgen-sensitive LNCaP cells results in enhanced ErbB-2 phosphorylation and androgen-independent cell proliferation [76,97]. Similarly, cDNA transfection of cPAcP-deficient androgen-independent LNCaP or cPAcP-null PC-3 cells results in a decrease of ErbB-2 phosphorylation as well as the reacquisition of the androgen-dependent growth phenotype [76]. Importantly, prostate adenocarcinoma archival specimens show decreased cPAcP expression at both an mRNA and protein levels, further supporting a general trend of decreased cPAcP activity and enhanced ErbB-2 activation with PCa progression [76].

1.2 AKT and ERK

Deregulation of protein kinase B (PKB), commonly referred to as “AKT”, is pervasive in PCa progression due to the protein’s regulation of mechanisms critical to metastasis [101]. The AKT signaling pathway promotes cell survival, proliferation, migration, invasion and other biological events associated with carcinogenesis [102-104]. Further, AKT activation via T308 or S473 phosphorylation is primarily controlled by the phosphoinositide 3-kinase (PI3K)/ phosphatase and tensin homolog (PTEN) axis [105-106]. Once activated by RTKs such as ErbB-2, PI3K converts phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) which activates a
number of downstream signaling components, the most notable of which is AKT [107]. Conversely, PTEN catalyzes the opposite reaction, converting PIP3 back into PIP2 and preventing activation of AKT [108]. Indeed, deregulation of this signaling network is very common in PCa with genetic loss and/or mutations in the PI3K/AKT pathway present in up-to 42% of primary prostate tumors and over 90% of metastatic tumors. Loss of tumor-suppressor PTEN function is also considered one of the driving events of PCa development; homozygous and heterozygous PTEN deletions are frequently observed in localized PCa and to an even higher degree in aggressive metastatic tumors [109-111]. Interestingly, cPAcP can also hydrolyze PIP3, and in PTEN-inactive, cPAcP-deficient LNCaP C-81 cells, AKT is hyper-activated by phosphorylation, higher than that in LNCaP C-33 cells that express higher cPAcP. It is thus proposed that cPAcP is a PTEN-functional homologue in prostate epithelia; as such, cPAcP is involved in the early stage of PCa development, while PTEN plays a major role in advanced carcinogenesis [75].

Upon activation, AKT regulates a number of cellular processes critical to PCa progression. For example, AKT promotes cell survival by inactivating pro-apoptotic proteins such as p53, BAX, BAD, YAP, and Caspase-9, while inducing anti-apoptotic proteins such as Bcl-2, BcL-XL, and Survivin [112-114]. AKT also stimulates cell proliferation by enabling the cell to overcome cell cycle arrest at the G1 and G2 phases through activation of Cyclin D1, CDKA, and CDK6 as well as activating other pro-growth signaling molecules such as mTOR [115]. Additionally, AKT promotes angiogenesis through VEGF activation as well as cell migration and invasion though regulation of cadherin proteins, MYC, matrix metalloproteinases, Snail, and FOXM1 [116-119]. AKT also stimulates cholesterol synthesis, which can be used by some advanced prostate tumors for de novo androgen synthesis [120]. Thus, AKT is capable of promoting the
aggressive metastatic phenotype in PCa and has the potential to be a useful therapeutic target.

In PCa, AKT and AR share an overlapping complex signaling network in which each is capable of regulating the other. Through activation of p66Shc, AR promotes an increase in cellular ROS levels that leads to the inactivation of phosphatases such as cPAcP through oxidation of their active domains [77]. By preventing cPAcP inhibition of ErbB-2, AR promotes activation of a number of ErbB-2-regulated pathways. Among these signaling pathways is the activation of the PI3K/AKT pathway [77]. Similarly, AKT can modulate the transcriptional activity of AR through direct phosphorylation. For example, phosphorylation of AR at S210 and S790 by AKT suppresses AR-mediated apoptosis and contributes to PCa survivability [121]. Further, AKT can bind and phosphorylate AR at S213, increasing AR ligand-binding and promoting AR activation and translocation to the nucleus [122]. Moreover, in the event of AR inhibition or androgen-deprivation, the PI3K/AKT pathway is reported to become increasingly activated in PCa cells as a mechanism of androgen-independence, Thus AKT is has the potential to be a useful therapeutic target for CR PCa treatment [122-123].

In addition to AKT, activated ErbB-2 induces the mitogen-activated protein kinase pathway (MAPK), which includes extracellular signal-regulated kinases (ERK) proteins [124,125]. These proteins are frequently reported to be aberrantly activated in PCa and are key regulatory kinases for processes vital to PCa development and progression [50,77,126]. At the cell membrane, activated RTKs initially recruit GRB2 and SOS proteins that facilitate GDP-GTP nucleotide exchange of the RAS protein [127]. RAS then goes on to activate the RAF and then MEK, which finally activates ERK1/2 via T202 and Y204 phosphorylation [127,128]. ERK is a serine-threonine kinase and its isoforms are classified into ERK1 and ERK2 groups according to their coding sequences [129].
Once activated ERK regulates several processes critical to PCa progression such as survival, proliferation, migration via activation of transcription factors such as Elk1 [130-132]. Similar to AKT, ERK and AR signaling pathways undergo cross-talk and feed-back loops [133-134]. Through ErbB-2 regulation, AR is able to promote downstream activation of ERK [133]. In turn, ERK can influence AR through induction of the CREB1 transcription factor that binds AR promoter and enhances its transcription [135]. ERK is also capable of direct phosphorylation of AR and its coregulators, increasing AR activity and promoting its translocation to the nucleus [133,135,136]. Thus, like AKT, ERK is potential therapeutic target for future CR PCa treatment due to its promotion of metastatic phenotype as well as induction of AR signaling.

1.3 Conclusions and Transition

While androgen-sensitive PCa is effectively treated by ADT, therapeutic options for CR PCa remain limited with no adequate treatment available. Moreover, most current treatment strategies for CR PCa continue to focus AR inhibition. Therefore, new therapeutic targets must be identified and the synthesis of novel curative agents is required. Downstream signaling molecules of AR including p66Shc, ErbB-2, AKT, and ERK have the potential to be useful alternative targets for inhibition either alone, or in combination with AR or other proteins. Figure 1.5 summarizes these signaling pathways. Therefore the following studies investigate the effects of novel therapeutic compounds on these proteins’ signaling pathways as well as their ability to promote PCa cell growth and development. We also seek to further elucidate the mechanism through which p66Shc promotes prostate cancer metastatic activity in attempt to identify additional therapeutic targets.
Figure 1.1 Prostate cancer progression over time.

Most prostate cancer arises from prostate epithelial cells and is thus regulated by androgen signaling. Initially, localized prostate cancer is effectively treated by surgery or radiation therapy. However, once the tumor returns or begins to invade surrounding tissues and metastasize, androgen deprivation therapy (ADT) by means of chemical or physical castration is used to treat the patient. At this point the tumor will typically go into remission for a period of 3 to 5 years or longer. However, the majority of patients eventually relapse and develop “hormone-refractory” or “castration-resistant” tumors which are unresponsive to ADT. Typically, while ADT is ineffective, tumors are still regulated by androgen-receptor activation, thus anti-androgen agents such as abiraterone acetate or enzalutamide can be used to treat some patients. Alternatively chemotherapeutic agents such as docetaxel and prescribed. Unfortunately, these methods only extend patient survival by an average of 3 years and median survival rate of castration-resistant prostate cancer patients is 19 months.
Figure 1.2

In the male body, the pituitary gland releases luteinizing hormone which stimulates Leydig cells in the testes to produce androgens. These are then distributed throughout the body via the bloodstream in the form of testosterone. Upon entering prostate epithelial cells, testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase in the cytosol. AR, which is normally bound by heatshock proteins 27 and 90 in the cytosol, is released upon binding DHT, allowed to form dimers, and enter the nucleus where it acts as a transcription factor for multiple proteins essential to cell growth, migration, and survival. In the absence of androgens, prostate epithelial cells undergo apoptosis, thus in the early stages of prostate cancer androgen deprivation therapy ADT is very effective at reducing tumor volume. Androgen deprivation therapy consists of reducing the body’s circulating levels of androgens by about 90% through means of physical or chemical castration.
Figure 1.3 The *Shc1* gene

The *Shc1* gene is localized on chromosome 1 and contains 12,066 base-pairs of DNA, including 13 exons and 13 introns. The *Shc1* locus contains two distinct promoters. The first regulates expression of isoforms *p52Shc* and *p46Shc* that are separated by two different in-frame ATG codons and which splice-out part of the first coding exon contained in *p66Shc*. The second promoter prevents partial loss of the first coding exon and transcribes mRNA encoding the longer *p66Shc* isoform.
Figure 1.4

**Figure 1.4 Molecular domain schematic of p66Shc protein structure.**

All three Shc isoforms (p66, p52, p46) possess C-terminal Src homology 2 (SH2), collagen homology 1 (CH1), and phosphotyrosine binding (PTB) domains. p66Shc has additional cytochrome c-binding (CB) and N-terminal collagen homology 2 (CH2) domains. Rac1 activates p38, which induces phosphorylation of S54 and T386 suppresses ubiquitination and degradation of p66Shc. S36 phosphorylation promotes mitochondrial translocation and pro-apoptotic signaling.
In prostate cancer, AR is activated upon binding DHT and induces stabilization of p66Shc. p66Shc then enters the mitochondria, oxidizing cytochrome C, and inducing generation of ROS which oxidize and inactivate various cytoplasmic phosphatases, including PTEN and cPAcP. cPAcP is then incapable of dephosphorylating ErbB-2, which leads to hyper activation of ErbB-2 and increased activation of ErbB-2 downstream targets including, Rac1, the Ras/Raf/MEK/ERK cascade, and PI3K. Inhibition of PTEN and activation of PI3K lead to increased concentrations of PIP3 and activation of AKT. AKT can directly phosphorylate AR, increasing its activity, as well as induce cholesterol production which some CR PCa cells can use to synthesize androgens de novo, further activating AR. Statins are a class of compounds which inhibit cholesterol synthesis and de novo androgen production. Similarly, ERK can directly phosphorylate AR, increasing its activity, as well as activate transcription factors that increase AR gene transcription. Finally, while Rac1 can be activated by ErbB-2, it can also be directly activated by ROS or indirectly by p66Shc via SOS1. Rac1 can also contribute to ROS generation via binding and activating NOX proteins. Together, these proteins enhance each other’s activation via positive feedback-loops and induce prostate cancer growth, migration, and metastatic progression.

**Figure 1.5 Summary prostate cancer signaling schematic**

In prostate cancer, AR is activated upon binding DHT and induces stabilization of p66Shc. p66Shc then enters the mitochondria, oxidizing cytochrome C, and inducing generation of ROS which oxidize and inactivate various cytoplasmic phosphatases, including PTEN and cPAcP. cPAcP is then incapable of dephosphorylating ErbB-2, which leads to hyper activation of ErbB-2 and increased activation of ErbB-2 downstream targets including, Rac1, the Ras/Raf/MEK/ERK cascade, and PI3K. Inhibition of PTEN and activation of PI3K lead to increased concentrations of PIP3 and activation of AKT. AKT can directly phosphorylate AR, increasing its activity, as well as induce cholesterol production which some CR PCa cells can use to synthesize androgens de novo, further activating AR. Statins are a class of compounds which inhibit cholesterol synthesis and de novo androgen production. Similarly, ERK can directly phosphorylate AR, increasing its activity, as well as activate transcription factors that increase AR gene transcription. Finally, while Rac1 can be activated by ErbB-2, it can also be directly activated by ROS or indirectly by p66Shc via SOS1. Rac1 can also contribute to ROS generation via binding and activating NOX proteins. Together, these proteins enhance each other’s activation via positive feedback-loops and induce prostate cancer growth, migration, and metastatic progression.
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Chapter 2

Materials and Methods
2.1 Materials

RPMI 1640 medium, Keratinocyte SFM medium, DMEM medium, 2',7'-dichlorofluorescin diacetate (DCFDA), gentamicin, anti-p66Shc (#180S0105A) Ab, rhodamine phalloidin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA). FBS and charcoal-treated FBS were obtained from Atlanta Biologicals (Lawrenceville, GA). Molecular biology-grade agarose was procured from Fisher Biotech (Fair Lawn, NJ). Protein molecular weight standard markers, acrylamide, and Bradford protein assay kit were purchased from Bio-Rad (Hercules, CA). Docetaxel was purchased from Aventis Pharmaceutical Products Inc. (Collegeville PA). Anti-AR (#C1411, 1:400), Anti-CDC25B (#D2810, 1:1000), anti-cyclin B1 (#K1907, 1:1000), anti-cyclin D1 (#A2712, 1:1000), anti-BclXL (#F111, 1:1000), anti-Bax (#G241, 1:1000), anti-PCNA (#G261, 1:3000), anti-p53 (#K2607, 1:1000), anti-PSA (#E1812, 1:2000), anti-Survivin (#C271, 1:2000), anti-phospho-ErbB-2 (Y1221/2) (#B2212, 1:1000), anti-ErbB-2 (#E3110, 1:1000), and horseradish peroxidase-conjugated anti-mouse (#C2011, 1:5000), anti-PAcP (#D0209, 1:1000), anti-PYK2 (#F061, 1:1000), anti-Rac1 (#G1905, 1:1000), anti-rabbit (#D2910, 1:5000), anti-goat (#J0608, 1:5000) IgG Abs, and AKT inhibitor (MK2206) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-AKT (Ser473) (#GA160, 1:1000), anti-AKT (#C1411, 1:2000), anti-FOXM1 (#5436S, 1:500), anti-HA-Tag (#C29F4, 1:3000), anti-phospho-mTOR Ser2448 (#5536S, 1:1000), anti-mTOR (#2972S, 1:1000), anti-Caspase 3 (#9665S, 1:1000), anti-PARP (#9532S, 1:1000), anti-phospho-p38 (T180/Y182) (#9211S, 1:1000), and anti-Snail (#3895S, 1:1000), anti-phospho-Stat5 (Y694) (#9351S, 1:4000), and anti-Stat5 (#9363, 1:2000) Abs were from Cell Signaling Technology (Beverly, MA). Anti-HO-1 (#Z04608d, 1:1000) Ab was obtained from ENZO Life Sciences (Farmingdale, NY). Anti-Nrf2 (ab62352, 1:1000) Ab was obtained from Abcam (Cambridge, UK). Anti-GTP-Rac1 (#G052YWF2, 1:1000) Ab
was obtained from New East Biosciences (Malvern, PA). Anti-β-actin (#99H4842, 1:10000), anti-NOX5 (#3116867, 1:1000) Abs, atorvastatin, DHT, propidium iodide, and simvastatin were procured from Sigma (St. Louis, MO). Anti-phospho-PYK2 Y402 (#CDRO0114121, 1:1000) Ab was obtained from R&D Systems (Minneapolis, MN). Anti-Shc (#06-203, 1:5000) Abs was obtained from Upstate Biotech. Inc. (Lake Placid, NY). Anti-β-actin (#99H4842, 1:10000) Ab, FOXM1 inhibitor (FDI-6), Rac1 inhibitor (EHop-016), PYK2 inhibitor (PF-431396), mTOR inhibitor (Rapamycin), N-acetyl-cysteine (NAC), hydrogen peroxide (H₂O₂) was procured from Sigma (St. Louis, MO). DAPI Hard-Mount Medium was obtained from Vector Laboratories (Burlingame, CA). ErbB-2 inhibitor (AG879), PI3K inhibitor (LY-294002), and ERK inhibitor (PD-9805) were obtained from Calbiochem (San Diego, CA).

Imidazopyridine derivatives HIMP (3-phenyl-1-(pyridine-2-yl)imidazo[1,5-a]pyridine), M-Mel (1-(pyridine-2-yl)-3-(m-tolyl)imidazo[1,5-a]pyridine), OMP (1-(pyridine-2-yl)-3-(o-tolyl)imidazo[1,5-a]pyridine), and EtOP (3-(4-ethoxyphenyl)-1-(pyridine-2-yl)imidazo[1,5-a]pyridine) were synthesized and provided by Dr. Xiu Bu as previously described [1,2]. Statin derivative compounds [simvastatin hydroxyacid] (SVA), [8-(3,5-dihydroxy-7-((2-(2-hydroxyethoxy)ethyl)amino)-7-oxoheptyl)-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate] (AM1), and [8-(3,5-dihydroxy-7-((2-(2-hydroxy)ethyl)amino)-7-oxoheptyl)-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate] (AM2) were provided by Dr. Chen’s laboratory, and synthesized based on the structure of simvastatin. For ease of reading, chemical abbreviations are used throughout the text.

**2.2 Cell Culture**

Human prostate carcinoma cell lines LNCaP, MDA-PCa2b, VCaP, PC-3, and DU145 cells were originally purchased from the American Type Culture Collection.
(Rockville, MD, USA). LNCaP, PC-3, and DU145 were routinely maintained in RPMI 1640 medium containing 5% FBS, 2 mM glutamine, and 50 µg/ml gentamicin [3-6]. MDA PCa2b cells were maintained in BRFF-HPC1 medium containing 20% FBS, 2 mM glutamine and 50 µg/ml gentamicin [7-8]. As reported previously, we established LN-AI (C-81) and MDA-AI cells which obtain many biochemical properties of clinical CR PCa including the expression of functional AR as well as PSA secretion and rapid cell proliferation in androgen-depleted conditions [4-6,9]. LNCaP-AI cells also possess the enzymatic capacity to synthesize androgens from cholesterol. Most importantly, both AI cell lines have elevated basal growth rates as well as increased levels of p66Shc protein compared to their respective AS cell lines [3]. VCaP cells were maintained in DMEM medium containing 15% FBS, 2 mM glutamine, 50 µg/ml gentamicin, and 10 µg/ml ciprofloxacin [9,10]. RWPE1 cells were cultured in Keratinocyte-SFM supplemented with bovine pituitary extract (25 µg/ml) and recombinant epidermal growth factor (0.15 ng/ml) along with 50 µg/ml gentamicin. To mimic conditions of clinical ADT, cells were maintained in SR conditions, i.e., phenol red-free RPMI 1640 medium containing 5% charcoal/dextran-treated FBS, 2 mM glutamine, 50 µg/ml gentamicin, and 1 nM DHT.

Imidazopyridine derivatives HIMP, M-Mei, OMP, and EtOP were dissolved in DMSO at 20 mM stock concentrations, stored at -20°C and diluted as needed for experimental conditions in the respective medium. Statin derivatives simvastatin, SVA, AM1, and AM2 were dissolved in DMSO at 20 mM stock concentrations, stored at -20°C, and diluted as needed for experimental conditions in the respective medium.

2.3 Preparation of Cell Lysates and Immunoblot Analysis

All cells were rinsed with ice-cold HEPES-buffered saline, pH 7.0, harvested via scraping, and lysed in ice-cold lysis buffer containing protease and phosphatase inhibitors. Total cellular lysates were prepared as previously described [8,11]. The
protein concentration of the supernatant was determined using a Bio-Rad Bradford protein-assay. For immunoblotting, an aliquot of total cell lysate was electrophoresed on SDS-polyacrylamide gels (7.5%-12%). After being transferred to nitrocellulose membrane, membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 30 minutes at room temperature. Membranes were incubated with the corresponding primary Ab overnight at 4°C. Membranes were then rinsed and incubated with the appropriate secondary Ab for 60 minutes at room temperature. Proteins of interest were detected by an ECL reagent kit and β-actin was used as a loading control. The intensity of the protein bands were analyzed with ImageJ software [8].

2.4 Trypan Blue Exclusion and Membrane Permeability Assays

For cell proliferation experiments under regular conditions, cells were seeded in regular culture medium and allowed to attach for 3 days, then changed to fresh medium containing the respective statin compounds and cultured for an additional 3 days. To determine cell proliferation under SR conditions, all cells were seeded in regular conditions and allowed to attach for 3 days. Cells were then steroid-starved for 48 hours in SR medium, and changed to fresh SR medium containing the noted compound(s) and cultured for an additional 3 days. Control groups received solvent DMSO alone. Cells were harvested via trypsinization and live cell numbers were counted by Trypan Blue dye exclusion assay using a Cellometer Auto T4 Image-based cell counter (Nexcelom, MA, USA). To determine cell membrane integrity, cells were treated as described above and a ratio of blue-stained cells to total counted cells was determined.

2.5 Clonogenic Assay

The clonogenic cell growth assay was conducted as described previously [12-13]. Briefly, LNCaP C-81 cells were plated on the plastic surface of 6-well plates under
regular culture conditions at a density of 2,000 cells per well. Cells were incubated overnight, the unattached cells were removed and remaining cells were fed with fresh regular medium containing 20 µM statin-compounds. Cells were grown for 9 days with a change of fresh medium every 3 days. On the 10th day, the medium was removed and cells were washed with ice-cold HEPES-buffered saline, then attached cells were stained with a 0.2% crystal violet solution containing 50% methanol and counted.

2.6 Soft Agar Assay

The effect of statin agents on anchorage-independent LNCaP C-81 colony growth was assessed by soft agar assay. Briefly, 5 x 10^4 cells were seeded into a 0.25% agarose top layer with a base layer containing 0.3% agarose in 35mm dishes. The day after seeding, cell clusters containing more than one cell were excluded from the study. Cells were then fed with 0.5 mL of fresh regular medium containing the respective statin-agent every 3 days for 6 weeks. The colonies were then stained with a 0.2% crystal violet solution containing 50% methanol and counted.

2.7 Transwell Assay

Cell migration was assessed via Boyden Chamber transwell assay as described previously [14,15]. Cells were plated at a density of 5 x 10^4 cells into the upper chamber of 24-well plate transwell inserts and allowed to migrate for 24 hours. In experiments with small molecule inhibitors, inhibitor compounds were added to the bottom chamber for a final concentration of their IC_{50} in LNCaP cells prior to the addition of cells. After a 24-hour incubation, cells were stained with 0.2% crystal violet solution in 50% methanol and cells remaining in the upper chamber were removed via cotton swab. Cells which had migrated to the lower chamber were counted at 40x magnification under a microscope. For experiments using small molecule inhibitors, to distinguish cell
migration from cell growth the results were normalized to growth inhibition in which 24-hour change in cell migration was divided by 24-hour change in cell growth.

2.8 Cell Adhesion Assay

To determine the effect of imidazopyridine derivatives on PCa cell adhesion to plastic ware surfaces, LNCaP C-81 cells were suspended in 5% FBS 1640 RPMI medium containing 10 μM of respective compounds and incubated for 30 minutes. Cells were then plated in 6-well plates in triplicates at a density of 3x10^3 cells/cm² in respective treatment medium and incubated for an additional hour. Non-attached cells were carefully washed away and the remaining attached cells were stained with a 0.2% crystal violet solution containing 50% methanol. The total number of cells in five fields at 40x magnification per well were counted.

2.9 Flow Cytometry Analysis

2.9.A DCF-DA Assay

Changes in cellular ROS levels induced by statin-compounds in LNCaP C-81 cells were determined via DCF-DA dye analysis [16]. Cells were plated in triplicate at a density of 2 x 10^4 cells per well using 6-well plastic plates and grown under regular conditions for 3 days. Cells were then steroid starved for 48 hours in SR medium before being changed to fresh SR medium containing the noted treatment compound(s) and cultured for an additional 3 days. Control groups received solvent DMSO alone. Cells were harvested and incubated with medium containing 20 μM DCF-DA dye. Determination of cell cycle distribution and DCF-DA fluorescence was carried out using the Becton-Dickinson fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA, USA) at the UNMC Flow Cytometry Core Facility.
2.9.B Propidium Iodide Assay

To determine the compounds’ effects on cell cycle, flow cytometry analysis was conducted as previously described [12]. Briefly, LNCaP C-81 cells were seeded in T25 flasks at a density of 5 x 10^4 cells in regular medium for 3 days, changed to SR medium for 48 hours, and then fed with fresh SR medium containing 20 µM of the specified compound. Cells were harvested after 3 days of treatment, fixed with 70% ethanol, and stained using Telford Reagent at 4°C for 4 hours [17].

2.10 Determination of Cellular Cholesterol

The level of cellular cholesterol was determined using the Abcam cholesterol assay kit [18]. Briefly, LNCaP C-81 cells were plated in 96-well plates at a density of 2 x 10^4 cells per well and allowed to attach overnight. Cells were treated with medium containing 20 µM statin derivatives or solvent alone for 3 days. The medium was then removed and cells were fixed for 10 minutes, followed by 3x wash with cholesterol detection buffer for 5 minutes. In the absence of light, cells were then stained with Filipin III for 1 hour and washed 2x for 5 minutes. Staining was examined via a fluorescence microscope and the relative fluorescence was quantified using Ziess-provided imaging program Zen lite 2012.

2.11 Protein Microarray

Cell lysates were prepared from an equally mixed population of stable p66Shc cDNA-transfected subclones and V1 vector-alone control cells as previously described and sent to Kinexus Protein Profiling Services (Vancouver, BC) where the company performed analysis via the KAM-900P microarray. Results are reported in the format of percent change from control (%CFC) using Z-score transformation.
2.12 Confocal Microscopy and F-actin Staining

Cells were plated on sterile round coverslips at $3 \times 10^4$ cells per coverslip and allowed to attach for 24 hours. Cells were then washed with pre-warmed phosphate-buffered saline and fixed with a 3.7% formaldehyde solution for 10 minutes at room temperature. Cells were then washed, permeabilized with 0.1% Triton X-100 solution for 5 minutes, and again washed before blocking with 1% BSA solution for 30 minutes. At this time, cells were stained with fluorescent phallotoxins, which directly binds to F-actin, in 1% BSA for 20 minutes followed by washing. To stain HA-tagged DN Rac1 transfected cells, cells were then incubated with primary anti-HA-tag Ab in 1% BSA for 1 hour, followed by washing and secondary Ab incubation for 30 minutes and a final wash. Coverslips were then mounted using VectorShield hard-mount medium containing DAPI stain and allowed to set overnight before images were captured via confocal microscopy [14]. The relative area of cell lamellipodia to total cell area was semi-quantified using NIH ImageJ software. Results were repeated in three separate experiments in which 20 cells were quantified (For a total of 60) for each cell-line/treatment [19].

2.13 cDNA and shRNA Transfection

For transient transfection experiments, LNCaP cells were plated at a density of $1 \times 10^4$ cells per cm$^2$ and transfected using Lipofectamine and Plus reagents. Five hours after transfection, the cells were fed with RPMI medium containing 10% FBS for 24 hrs. The cells were then used for transwell assays and whole cell lysates harvested for immunoblot analysis. Stable subclones of LNCaP cells overexpressing p66Shc were established as described previously [3]. DN (T17N) and CA (G12V) Rac1 cDNA were provided by Dr. Yaping Tu's laboratory [19]. For knock-down of p66Shc expression, transient transfection of pSUP-p66 plasmid-based small interfering RNA system targeted against the CH2 domain was used for cDNA transfection as described previously [3].
2.14 Immunohistochemical Staining

The protocol for the usage of human prostate archival specimens was approved by the Institutional Review Board at UNMC. According to tissue availability, we obtained 37 human prostate cancer archival specimens for analyzing p66Shc expression. Of those 37 slides, 33 specimens were identified by H&E staining to contain both benign and malignant tissues on the same slide and were used for the direct comparison of p66Shc protein level in the two tissue types in the same section, as described in our previous study [5]. Immunohistochemical staining was carried out as previously described in Lee et. Al. 2004 [20]. The comparison of expression level of p66Shc protein in paired samples (benign and malignant) on the same tissue section was analyzed using the one population t-test (p<0.05 was considered as significant difference).

2.15 Statistical Analysis

Each set of experiments are conducted in triplicate or duplicate as specified in the figure legend, and experiments are repeated independently at least three times, denoted as n=3x3. All results are presented as mean ± standard error measurement. Correlation coefficient r was calculated using Microsoft Excel. Statistical significance was determined using a paired two-tailed student-t test assuming unequal variance where appropriate unless otherwise stated. p<0.05 was considered statistically significant.
References


Chapter 3

Novel Imidazopyridine Derivatives Possess Anti-tumor Effect on Human Castration-Resistant Prostate Cancer Cells

This chapter is derived from:

3.1 Synopsis

Prostate cancer (PCa) is the third leading cause of cancer-related death afflicting United States males. Most treatments to-date for metastatic PCa includes androgen-deprivation therapy and the second-generation anti-androgens such as abiraterone acetate and enzalutamide. However, a majority of patients eventually develop resistance to these therapies and relapse into the lethal, castration-resistant form of PCa to which no adequate treatment option remains. Hence, there is an immediate need to develop effective therapeutic agents toward this patient population. Imidazopyridines have recently been shown to possess Akt kinase inhibitory activity; thus in this study, we investigated the inhibitory effect of novel imidazopyridine derivatives HIMP, M-Mel, OMP, and EtOP on different human castration-resistant PCa cells. Among these compounds, HIMP and M-Mel were found to possess selective dose- and time-dependent growth inhibition: they reduced castration-resistant PCa cell proliferation and spared benign prostate epithelial cells. Using LNCaP C-81 cells as the model system, these compounds also reduced colony formation as well as cell adhesion and migration, and M-Mel was the most potent in all studies. Further investigation revealed while HIMP primarily inhibits PCa cell growth via suppression of PI3K/Akt signaling pathway, M-Mel can inhibit both PI3K/Akt and androgen receptor pathways and arrest cell growth in the G2 phase. Thus, our results indicate the novel compound M-Mel to be a promising candidate for castration-resistant PCa therapy, and future studies investigating the mechanism of imidazopyridine inhibition may aid to the development of effective anti-PCa agents.
3.2 Background and Rationale

Prostate cancer (PCa) remains the most commonly diagnosed solid tumor and the third leading cause of cancer-related death in United States men, maintaining a need for new effective treatment options [1]. Currently, androgen-deprivation therapy (ADT) is the standard course of treatment for metastatic PCa, however, most PCa patients relapse within 1-3 years and develop CR PCa which is unresponsive to ADT [2,3,4]. In 2004, a combination of docetaxel and prednisone was shown to increase patient median survival by 2-3 months, making it the standard-of-care treatment for CR PCa [5]. Recently, the FDA has approved additional compounds such as novel taxane chemotherapeutic cabazitaxel [6], androgen synthesis inhibitor abiraterone acetate [7], AR signaling inhibitor enzalutamide [8], immunotherapeutic sipuleucel-T [9], and bone micro-environment-targeted radiopharmaceutical alpharadin (Radium-223) for treating CR PCa [10]. However, these treatment options are only able to prolong survival by a few months and the average period of CR PCa patient survival remains less than two years [11]. Despite advancements in post-ADT treatment strategies, CR PCa remains an incurable disease; thus there is a great need for alternative therapeutic options.

While androgen insensitivity can be manifested in multiple ways; one proposed alternative mechanism is the increased activation of Akt signaling under androgen deprived conditions. Akt is known to regulate cell cycle, metabolism, angiogenesis, and cell survival in PCa and its activation may contribute to tumor resistance to ADT and anti-androgens [12,13]. One mechanism through which Akt may contribute to PCa survivability is via modulation of AR signaling. In addition to inducing cell growth, AR also has a role in regulating apoptosis. Upon phosphorylation of AR at Ser-210 and Ser-790 by Akt, AR-mediated apoptosis is suppressed. Through this mechanism, enhanced Akt activity in PCa may contribute to PCa survivability upon ADT [13]. Indeed, genetic
loss and/or mutations in the PI3K/Akt pathway that lead to signal deregulation may present in up-to 42% of primary prostate tumors and over 90% of metastatic tumors, making it a priority next-in-line therapeutic target [14]. Recently, investigations into imidazopyridines, a novel class of compounds containing aromatic aldehydes and a pyridine group, have demonstrated these compounds possess potent Akt kinase inhibitory activity [15-17]. Data shows these compounds have an anti-proliferative effect against CR PCa cells with the ability to simultaneously inhibit AR and PI3K/Akt/mTOR signaling pathways, making them promising therapeutic agents [18].

To investigate imidazopyridines’ efficacy for PCa therapy, the LNCaP progressive cell model, originally characterized in Lin et. al. JBC 1998, was used as the primary cell model in this study. LNCaP C-81 cells are AI, express PSA in the absence of androgens, and gain the ability to synthesize testosterone from cholesterol under SR conditions [19-22]. C-81 cells also possess enhanced proliferation, ability to form colonies, and migratory potential [21,23]. Most importantly, LNCaP C-81 cells retain AR expression and correspond to the expression of AR in the majority of PCa as well as advanced CR PCa [19]. This makes them a superior cell model for therapeutic studies when compared to many other PCa cell lines. Other cell lines selected for this study include MDA PCa2b-AI, PC-3, and RWPE1. Upon passage, MDA PCa2b cells behave similarly to LNCaP cells and shift from AS at low passage to AI at high passage. MDA PCa2b-AI (MDA-AI) cells also retain AR expression and possess enhanced tumorigenicity; this makes MDA-AI and LNCaP C-81 preferable cell models for studying prostate adenocarcinoma. Further, due to the ability of imidazopyridine derivatives to target both Akt and AR pathways, it is prudent to investigate the compounds’ effects on AR-negative PC-3 cells to determine their efficacy in cells which lack classic androgen signaling mechanisms. In addition, PC-3 cell lines are more representative of small-cell
neuroendocrine carcinoma than more clinically predominant adenocarcinoma [24]; therefore this cell line should be used in conjunction with models such as LNCaP and MDA PCa2b cell lines to expand clinical utility. Finally, immortalized benign prostate epithelium RWPE1 cells act as a control to gauge the selectivity of the imidazopyridine derivative compounds (Fig. 3.1). Thus our cell models clearly represent the majority of molecular events observed in clinical implementations of modern PCa therapies.

Our results demonstrate these imidazopyridine derivatives are able to suppress human PCa cell proliferation in a dose- and time-dependent manner. Importantly, compound M-MeI exhibited selective potency against CR PCa cell proliferation in comparison to benign prostate epithelial cells. Furthermore, this compound was also found to inhibit cell migration, adhesion, and in vitro tumorigenicity. Our data is the first to demonstrate the anti-tumor effect of novel imidazopyridine derivatives HIMP, M-MeI, OMP, and EtOP on CR PCa cells and indicates M-MeI to be a promising lead therapeutic agent for future studies.

3.3 Results

3.3.A Dose-Dependent Effect of Imidazopyridine Derivatives on CR PCa cell Proliferation

LNCaP C-81 cells exhibit many biochemical properties as seen in clinical CR PCa, including functional AR expression, AI PSA secretion, and proliferation with intracrine growth regulation [19,21-23] and thus were used as the primary cell model system for testing imidazopyridine compounds. Initially, the dose-dependent effects of HIMP, M-MeI, OMP, and EtOP on LNCaP C-81 cells were tested under regular culture conditions. Cells were treated with 0-10 µM of each compound for 72 hours and cell growth was analyzed via Trypan Blue exclusion assay. Under regular culture conditions, dose-dependent inhibition of cell proliferation was observed for all compounds with
estimated IC\textsubscript{50} values of 6.1 µM (M-Mel), 6.6 µM (EtOP), 7.3 µM (OMP), and 9.3 µM (HIMP) (Fig. 3.2A).

We then examined the effect of the compounds in SR conditions, mimicking ADT conditions. The compounds inhibited cell growth following the dosage response with estimated IC\textsubscript{50} values of 10.2 µM (M-Mel), 10.5 µM (OMP), 11.6 µM (HIMP), and 16.0 µM (EtOP) (Fig. 3.2A). Interestingly, M-Mel had the greatest inhibitory activity under both growth conditions. Though EtOP had comparable inhibition to M-Mel in regular conditions, it had the least effect under SR conditions. HIMP and OMP were also shown to be less effective than M-Mel under both treatment conditions.

3.3.B Selective Anti-Proliferative Effect of Compounds on PCa vs. Immortalized Normal Prostate Epithelial Cells

The suppressive effect of each inhibitor on proliferation was investigated using a panel of cancerous and benign prostate epithelial cell lines. AI PCa cells including AR-positive LNCaP C-81 and MDA PCa2b-AI as well as AR-negative PC-3 cells were chosen as representatives of advanced CR PCa. RWPE1 cells, an immortalized benign prostate epithelial cell line, were used to determine the compounds’ selectivity. After three days of 10 µM treatment under SR conditions, HIMP, M-Mel, and EtOP all displayed selective inhibition of proliferation of cancerous cells with significantly less effect on non-cancerous RWPE1 cells (Fig. 3.2B). Though OMP was effective against C-81 and PC-3 cells, it was comparatively potent against RWPE1 cells. Overall, the results show HIMP and M-Mel were the most selective, inhibiting PCa cell growth significantly more than RWPE1 cells with M-Mel displaying greater inhibition in all cell lines analyzed.
3.3.C Suppression of PCa Tumorigenicity by Imidazopyridine Derivatives

The compounds’ ability to suppress colony formation in LNCaP C-81 cells was initially accessed by in vitro clonogenic assays for anchorage-dependent cell growth. LNCaP C-81 cells were seeded with 20, 200, and 2,000 cells per well in 6-well plates, and then treated with 10µM of each compound. Upon 10-days of treatment, all compounds significantly inhibited clonogenic growth at 2,000 cells per well as shown in Fig. 3.3A for 2,000 cells per well. While M-Mel and EtOP strongly inhibited colony growth, HIMP and OMP were comparatively less potent. Minimal colony formation was observed at densities of 20 and 200 cells per well.

The soft agar colony formation assay was then performed to determine the compounds’ effect on anchorage-independent growth in a 3-dimentional environment. As shown in Fig. 3.3B, cells cultured at a density of 5,000 cells per 35 mm dish for four weeks produced far fewer colonies with smaller colony size when treated with the imidazopyridine derivatives. Compared to control cells treated with solvent alone, M-Mel suppressed colony growth to the greatest extent, reducing the number of colonies by 90 percent with barely visible colony size (Fig. 3.3B). In comparison, EtOP and OMP reduced colony growth with 80 and 70 percent inhibition, respectively, and HIMP had the least effect at about 50 percent inhibition.

To clarify whether these compounds’ effect on colony formation is in part due to the inhibition of cell adhesion, the capacity of HIMP, M-Mel, OMP, and EtOP to influence PCa cell adhesion onto the plastic surface of 6-well plates was then investigated. While these compounds had varying degrees of suppression on the ability of LNCaP C-81 cells to adhere at a density of 50,000 cells per well, a similar inhibitory trend was observed to that of clonogenic and soft agar assays (Fig. 3.3C vs. 3.3A &3.3B). M-Mel had the greatest effect and was able to reduce cell attachment by about 40 percent. While HIMP
and EtOP were also able to significantly inhibit cell adherence, they did so to a lesser extent; OMP was found to have a minimal effect on the C-81 cells’ ability to attach to the plastic surface. Hence, though all compounds belong to the same class of molecules, they influence PCa cell colony formation and adhesion differently.

To investigate the inhibitory ability of these compounds on tumor metastasis, their activity on cell migration was analyzed by transwell migration assay. Interestingly, these compounds were found to have varying degrees of suppression on PCa cell migration. Fig. 3.3D showed that both M-Mel and EtOP were able to significantly reduce LNCaP C-81 cell migration via Boyden Chamber assay over a period of 24 hours by about 30 percent. Comparatively, HIMP and OMP failed to significantly reduce cell migration. Overall, M-Mel was found to exhibit the most potent inhibitory activity on CR PCa cell tumorigenicity.

3.3.D Effect of Imidazopyridine Compounds on Proliferative and Apoptotic Signaling in CR PCa Cells

It is well established that the majority of CR PCa cells express functional AR which is still required for their growth and survival [22,31,32]. To determine how the compounds suppress PCa cell proliferation, we analyzed their effects on proliferative and apoptotic signaling in AR-positive LNCaP C-81 and MDA PCa2b-AI cells under SR conditions. Figs. 3.4A and 3.4B showed that, upon 3-day treatments, 10µM of each compound significantly suppressed LNCaP C-81 and MDA PCa2b-AI cell proliferation.

In LNCaP C-81 cells under SR conditions, though imidazopyridines are known for Akt inhibition, only HIMP and M-Mel inhibited Akt activation as shown by decreased Ser473 phosphorylation (Fig. 3.4C) [15]. Additionally, while all compounds reduced AR protein levels in LNCaP C-81 cells; M-Mel and EtOP were more potent. Importantly, a similar trend was observed in AR-regulated pro-proliferative proteins. M-Mel and EtOP
reduced levels of p66Shc, a 66kDa Src-homologous collagen homologue, cyclin D1, and PCNA, while HIMP and OMP had minimal effects [33-36]. We also analyzed Stat5 phosphorylation at Y694, which aids in the translocation of AR to the nucleus and is a regulator of cyclin D1 synthesis [37,38]. Unexpectedly, M-MeI slightly increased Stat5 activation in C-81 cells while EtOP suppressed activation; HIMP and OMP had no effect. Though all compounds diminished anti-apoptotic Survivin protein, their treatment elevated BclXL, another anti-apoptotic protein [39,40]. The compounds’ effect on p53, a regulator of cell survival and inducer of apoptosis, varied with M-MeI slightly lowering p53 levels, and EtOP slightly increasing them; HIMP and OMP had no significant effect [41].

As shown in Fig. 3.4D, in MDA PCa2b-Al cells under SR conditions, AR inhibition was similar to that of LNCaP C-81 cells: M-MeI and EtOP greatly suppressed AR levels while HIMP and OMP had minimal effects. This also correlated with lower levels of AR-regulated p66Shc, cyclin D1, and PCNA in M-MeI-treated cells, all of which promote cell growth. As seen in C-81 cells, in MDA PCa2b-Al cells HIMP and OMP had no inhibitory effect on Stat5 activation, while EtOP suppressed Y695 phosphorylation. M-MeI, however, strongly reduced Stat5 phosphorylation in MDA PCa2b-Al cells where it had increased it in C-81 cells. Also, while the compounds’ effect on p53 remained similar in MDA-Al cells compared to C-81 cells, their effects on anti-apoptotic Survivin and BclXL proteins were altered. In MDA-Al cells, HIMP, OMP and EtOP increased levels of Survivin while BclXL remained unchanged relative to control cells suggesting these proteins are not essential to growth inhibition. Because Akt phosphorylation at Ser473 in MDA PCa2b-Al cells was undetectable under SR conditions, these cells were instead treated in regular culture medium for three days and all compounds were found to reduce Akt activation (Fig. 3.4E).
In summary, of the four compounds tested, M-Mel was the most potent inhibitor of proliferation in both LNCaP C-81 and MDA PCa2b-AI cell lines under SR conditions. It also consistently reduced AR and AR-regulated proteins as well as Akt Ser473 phosphorylation in both cell lines, suggesting these pathways are involved in imidazopyridine inhibition of CR PCa cell growth.

3.3. Kinetic Effect of HIMP and M-Mel on LNCaP C-81 Cells under SR Conditions

Since HIMP and M-Mel exhibited the most selectively potent activity, these two compounds were investigated further by kinetic analysis in LNCaP C-81 cells under SR conditions for clinical relevance. As shown in Fig. 3.5A, both HIMP and M-Mel began to show significant inhibition of cell proliferation on day three of treatment, and this trend continued through day seven. It should also be noted M-Mel exhibited greater growth suppression than HIMP at every time point analyzed.

Cell lysates were collected from each time point and Western Blot analysis was performed (Fig. 3.5B). Our results showed M-Mel-treated cells had decreased levels of AR and PSA, an androgen-regulated protein, as well as cell cycle proteins cyclin B1 and PCNA as seen at 5-day treatment. Meanwhile, HIMP treatment decreased PSA protein level upon 7-day treatment but not AR protein level. A similar trend was observed in pro-proliferation proteins as HIMP had no effect on cyclin B1 and marginal effect on PCNA. It should be noted, as the level of active unphosphorylated cyclin B1 decreased (lower band), the inactive phosphorylated cyclin B1 protein level increased (upper band) [42]. The data may indicate arrestment of the cell cycle.

We also investigated the effects of HIMP and M-Mel on pro-apoptotic proteins Bax as well as p53 vs. anti-apoptotic Bcl-XL [40]. Treatment with both compounds dramatically increased Bax and p53 protein level by day seven and correlated with the observed decrease in cell proliferation. Interestingly, both compounds initially increased
Bcl-XL levels on days one and three, and had no effect on Bcl-XL as treatment continued (Fig. 3.4B). Thus both compounds seem to induce apoptosis by increasing levels of pro-apoptotic proteins instead of inhibiting anti-apoptotic proteins, while M-Mel also reduces pro-proliferative proteins as well as AR signaling.

To further investigate the kinetic effect of HIMP and M-Mel, cell cycle analysis was performed via flow cytometry. The cell cycle analyses (Fig. 3.5C) revealed that upon 7-day treatment, M-Mel reduced LNCaP cell proliferation by 50% as indicated by the decreased percentage of cell population in S phase. M-Mel also increased the percentage of cells undergoing apoptosis nearly four-fold at day seven relative to control cells. At the same time, HIMP had no significant effect on percentage of cells in S phase and increased the percentage undergoing apoptosis two-fold by day seven. Interestingly, the population of cells treated with M-Mel, but not HIMP, accumulated in G2 phase following the time course of treatment, i.e., only a marginal increase on day 3 and about a 50% increase in day 5 (Fig. 3.6). On day-7, the percentage of cells in G2 phase doubled to about 49% in M-Mel treated cells compared to 24% in control cells (Fig. 3.5C). In parallel, on day 7, the HIMP-treated cells were only slightly increased from about 24% to 27% in G2-phase. These results clearly indicate M-Mel blocks cell cycle at G2 phase.

3.4 Discussion

Given the poor 5-year 29% survival rate of metastatic CR PCa patients, there is a clear need for advancement in treatment alternatives [14]. First line treatment of PCa usually involves ADT by means of surgical castration or chemical castration, such as luteinizing hormone-releasing hormone treatment, coupled with anti-androgens. Treatment with classic anti-androgens such as flutamide or bicalutamide (Casodex), which competitively inhibit androgen binding to AR, can be effective for 1-3 years before
patients subsequently become unresponsive and eventually relapse. Relapse can occur for a number of reasons such as deregulation of AR cofactors, AR overexpression, splicing mutations resulting in constitutively active AR, or mutations allowing AR activation by competitive inhibitors [43]. In the case of the latter, upon discontinuation of flutamide or bicalutamide treatment, patients often experience a period of anti-androgen withdrawal syndrome (AAWS) characterized by a decline in serum PSA levels and tumor regression [43,44]. In some cases, these patients will respond to treatment with alternative anti-androgens, however, patients will again eventually become unresponsive to treatment and develop advanced PCa, commonly referred to as “castration-resistant” (CR) [45]. While more effective anti-androgens such as enzalutamide, which possesses a 5-fold higher binding affinity to AR compared to bicalutamide, are now available, mutations allowing AR activation by enzalutamide have been reported and tumors continue to become CR over time [46]. The therapeutic effect of second generation anti-androgens such as enzalutamide and abiraterone acetate implies most cancer cells, which still express functional AR, require AR signaling to evade traditional regulatory mechanisms to survive androgen deprivation strategies [7,8,47]. Despite the effectiveness of ADT and anti-androgen treatment strategies to delay the progression of PCa, many patients still develop the CR phenotype and thus there is an urgent need for alternative therapeutic targets to AR. Other signaling pathways, such as the PI3K/Akt-mediated cell survival pathway, may act to supplement the lack of AR stimulation under androgen-deprived conditions, bypassing the effect of ADT. Therefore, in this study we investigate novel therapeutic agents which are capable of targeting multiple biochemical pathways critical to CR PCa cell growth and progression.

In this study, four novel imidazopyridine derivatives were investigated to determine their viability as therapeutic agents for CR PCa. LNCaP C-81 cells were
chosen as our primary experimental cell model because they possess many biochemical properties common to CR PCa, including expression of functional AR, AI PSA secretion and proliferation, and expression of enzymes required for the synthesis of androgens from cholesterol [19,21,22,31,47]. We initially showed that all four compounds are effective inhibitors of CR PCa cell growth in the LNCaP C-81 cell line model, though to varying degrees with M-Mel as the most potent under both regular and SR conditions (Fig. 3.2A). Additionally, the effects of the imidazopyridine derivatives on cell proliferation differed between compounds as well as cell lines. Of those compounds investigated, HIMP and M-Mel displayed broad-spectrum growth inhibition of multiple CR PCa cell lines, including both AR-positive and AR-negative cells. Most importantly, these compounds exhibited more potent inhibition of proliferation in PCa cells than benign RWPE1 cells (Fig. 3.2B).

We further investigated the effects of each compound on various biological activities critical to malignant tumor progression. Colony formation, cell adhesion, and migration are vital malignant processes exhibited by many cancer cells. In both anchorage-dependent and anchorage-independent growth assays, M-Mel and EtOP dramatically reduced the number of colonies formed as well as colony size. This may partially be due to M-Mel and EtOP’s ability to inhibit cell adhesion. To investigate the effect of these compounds on cell migration, the transwell migration assay was used to analyze LNCaP C-81 cell motility. Again, M-Mel and EtOP were found to be potent inhibitors of C-81 cell migration while HIMP and OMP had no significant effect. Interestingly, this correlates with M-Mel and EtOP’s ability to reduce AR protein level as shown in Fig. 3.4C where HIMP and OMP fail to influence AR. This suggests AR inhibition may be crucial for the inhibition of these malignant processes. However, while EtOP was an effective inhibitor of PCa tumorigenicity, it was not as effective at growth
suppression as M-Mel. This may be due to EtOP’s failure to prevent Akt activation whereas M-Mel successfully inhibits Akt phosphorylation at S473 (Fig. 3.4C).

The PI3K/Akt signaling pathway is proposed to be pivotal to the growth and survival of CR PCa cells; dysregulation of this pathway is shown to contribute to resistance to treatment [31,48-54]. In clinical studies, for example, treatment with AR inhibitor bicalutamide is shown to progressively increase Akt signaling in patients in correlation with their Gleason grades [51]. In parallel, PCa treated with chemotherapeutic agent docetaxel possess increased Akt activation in patient tumors [52]. Other in vitro studies similarly demonstrated LNCaP cells grown in SR medium for extended periods of time have increased Akt activation which may compensate for a lack of androgen signaling [12]. Indeed, the PI3K/Akt/mTOR pathway is highly activated in CR PCa and emerging studies show inhibitors targeting the PI3K/Akt pathway are rapidly entering into clinical trials [14,53-55]. Therefore, the PI3K/Akt signaling axis is a promising next-in-line therapeutic target and its inhibition in conjunction with ADT and anti-androgens may improve patient survival.

Initially, imidazopyridines have been shown to possess Akt kinase inhibitory activity and are effective suppressors of tumor growth and advancement in a number of carcinomas, including PCa [15,16,18]. Interestingly, while all four derivative compounds inhibited Akt phosphorylation at Ser473 in MDA PCa2b cells; only HIMP and M-Mel inhibited its phosphorylation in LNCaP C-81 cells. It should be noted because Akt signaling in MDA PCa2b-AI cells was too weak to detect under SR conditions, it was observed under regular conditions. It is thus possible the differential inhibition of Akt activation in MDA-AI vs. LNCaP C-81 cells was in part due to the difference between regular and SR growth conditions (Figs. 3.4C-E).
While HIMP is a strong inhibitor of Akt activation in both cells, it fails to consistently down-regulate AR, a long established target of PCa therapy. Supportively, western blot analysis showed HIMP inconsistently affects AR protein levels (Figs. 3.4D and 3.5B), a phenomena which has previously been observed when CR PCa cells are treated with PI3K/Akt inhibitors [12,18]. Furthermore, HIMP had little shift in potency between regular conditions and SR conditions (Fig. 3.2A), suggesting HIMP’s mechanism of inhibition is relatively androgen-independent. In addition, HIMP is a potent inhibitor of Akt phosphorylation at Ser473 and also acts to induce pro-apoptotic p53 and Bax proteins (Figs. 3.4C, 3.4D & 3.5B) [27]. Together, these results suggest HIMP inhibits CR PCa growth by suppressing cell survivability but not androgen signaling.

While both HIMP and M-Mel were found to have promising selective growth suppression, M-Mel was shown to have greater efficacy (Figs. 3.2 and 3.4). M-Mel is a derivative of HIMP, possessing a methyl group on the para position of the benzene ring (Fig. 3.1). This modification apparently allows M-Mel to suppress AR protein level as well as Akt activation; there is also a noticeable change in M-Mel’s IC$_{50}$ value between regular and SR conditions, indicating its mechanism of growth inhibition includes the suppression of AR signaling (Figs. 3.4C-E). Supportively, as shown in Fig. 3.5B, upon M-Mel treatment, there is a consistent, progressive decrease in AR protein. This coincides with a decrease in PSA, a target of AR, as well as p66Shc protein level, a protein regulated by androgens which is involved in cell proliferation and apoptosis (Figs. 3.4C and 3.4D). Downstream pro-proliferative markers, cyclin D$_1$ and PCNA were also down-regulated over-time to a greater extent by M-Mel compared to HIMP. In addition, STAT5 signaling is proposed to be involved in the transition from androgen-sensitive to CR PCa and activated STAT5 can enhance nuclear translocation of AR [40]. Interestingly, as seen in Figs. 3.4C and D, while M-Mel inhibited Stat5 phosphorylation
at Y694 in MDA-AI cells, it increased Y694 phosphorylation in LNCaP cells. This inconsistent effect suggests inhibition of STAT5 activation is not vital to M-Mel's suppression of PCa. Furthermore, M-Mel strongly inhibited the phosphorylation of pro-survival protein Akt (Ser473) (Figs. 3.4C-E) as well as induced pro-apoptotic proteins p53 and Bax (Fig. 3.5B) [27]. Thus, M-Mel's anti-tumorigenic effect on PCa is due to its strong inhibition of both Akt and AR signaling pathways.

Unexpectedly, the kinetic analysis revealed an interesting phenomenon: upon HIMP and M-Mel treatments over time, both compounds, though to a greater extent by M-Mel, decreased the protein level of active unphosphorylated cyclin B₁ (Fig. 3.5B, lower band) and increased the inactive phosphorylated band (upper band) (Fig. 3.5B) [41]. Cyclin B₁, a key regulator of mitosis, is phosphorylated during the G2 phase and becomes unphosphorylated upon the cell reaching M phase [41]. To validate further, we performed cell cycle analysis on HIMP- and M-Mel-treated cells by flow cytometry. Compared to control cells, M-Mel-treated cells had lower percentages of cells in S phase, corresponding with a decrease in cell proliferation, and twice the percentage of cells in the G2 phase. Minimal changes were observed in HIMP-treated cells, although both treatments increased the percentage of cells undergoing apoptosis (Fig. 3.5C). Together, this data indicates these imidazopyridine derivatives function to inhibit the transition of PCa cells between G2 and M phases, though the exact mechanism remains to be elucidated.

In summary, our data shows imidazopyridine derivatives exhibit inhibitory activity of tumorigenicity in CR PCa cells. Each compound displayed differential effects on Akt and AR signaling pathways, and further studies are needed to determine the mechanism by which these molecules suppress the growth of CR PCa. Among the four compounds investigated, M-Mel was found to suppress multiple signaling pathways related to PCa
progression, including classical target AR as well as the Akt survival pathway, making it a promising candidate for future therapeutic studies. Importantly, this compound displays selective growth inhibition, having a significantly greater suppressive effect on PCa compared to benign prostate epithelial cells. Thus, M-Mel can serve as a lead compound for imidazopyridine side-chain modifications, which could yield more potent, selective agents to improve the treatment of CR PCa patients. Future investigation to elucidate M-Mel's specific mechanism of inhibition and in vivo studies will help better determine its potential as a therapeutic agents.
Figure 3.1 Structures of imidazopyridine derivatives. HIMP, 3-phenyl-1-(pyridine-2-yl)imidazo[1,5-a]pyridine; M-Mel, 1-(pyridine-2-yl)-3-(m-tolyl)imidazo[1,5-a]pyridine; OMP, 1-(pyridine-2-yl)-3-(o-tolyl)imidazo[1,5-a]pyridine; EtOP, 3-(4-ethoxyphenyl)-1-(pyridine-2-yl)imidazo[1,5-a]pyridine.
Figure 3.2

A

Cell Growth Ratio to Control

Regular  SR

0  1  5  10 µM

HIMP

B

Cell Growth Ratio to Control

SR

Control LN-C81 MDA-MB PC-3 RWPE1

HIMP

M-Mel

0  1  5  10 µM

OMP

EtOP

0  1  5  10 µM
Figure 3.2 Effects of imidazopyridine derivatives on the proliferation of prostate epithelial cells.

(A) Dosage effect of Imidazopyridine derivatives on LNCaP C-81 cells. Cells were plated in six-well plates at 2 x 10^3 cells/cm^2 in regular medium and grown for 72 hours. One set of cells was then fed with fresh regular medium containing 0, 1, 5, or 10 µM imidazopyridine derivatives with solvent alone for control and grown for an additional 72 hours. Another set of cells was first steroid starved in SR medium for 48 hours then treated with respective compounds in fresh SR media containing 1 nM DHT for 72 hours. All cells were trypsinized and live cell numbers were counted. The experiment was conducted in duplicate wells with 3 sets of independent experiments. The results presented are mean ± SE; n=2x3. *p<0.05 **p<0.005 ***p<0.0005.

(B) Effects of statin-agents on the growth of various PCa cells and immortalized prostate epithelial cells. All cells were plated in six-well plates at the noted density in their respective medium for three days, steroid-starved for two days, then fed with fresh SR medium with 1 nM DHT containing 10 µM imidazopyridine derivatives and grown for three additional days. Cells were trypsinized and live cell number was counted. LNCaP C-81 - 2 x 10^3 cells/cm^2, MDA PCa2b AI - 3 x 10^3 cells/cm^2, PC-3 - 2 x 10^3 cells/cm^2, RWPEI – 7.5 x10^3 cells/cm^2. All experiments were performed in triplicate wells with 3 sets of independent experiments. Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.
Figure 3.3
Figure 3.3 Effects of imidazopyridine derivatives on the tumorigenicity of LNCaP C-81 cells.

(A) Clonogenic assay on plastic wares. LNCaP C-81 cells were plated in six-well plates at densities of 20, 200, and 2,000 cells/well. After 24 hours, attached cells were treated with respective compounds at 10 μM concentrations of imidazopyridine derivatives or solvent alone as control. Cells were fed on days 3, 6, and 9 with fresh culture media containing respective inhibitors. On day 10, cells were stained and the number of colonies counted. The photos of representative colony plates were taken from plates seeded with 2,000 cells/well, and the number of colonies shown was counted also from plates seeded with 2,000 cells/well. Minimal colony formation was observed at densities of 20 and 200 cells/well. Results presented are mean ± SE; n=2x3. ***p<0.0001.

(B) Anchorage-independent soft agar assay. LNCaP C-81 cells were plated at a density of 5 x 10^4 cells/35mm dish in 0.25% soft agar plates. The following day, cells in doublets or greater were marked and excluded from the study. Media were added every three days, and at the end of 5 weeks, colonies formed were stained and counted. Representative images of colonies are shown (above) and the colony number was counted (below). The experiments were performed in duplicate with 3 sets of independent experiments. Results presented are mean ± SE; n=2x3. ***p<0.0001.

(C). Cell adhesion assay on plastic wares. Cells were suspended in treatment media for 30 minutes before being plated in 6-well plates at 3 x10^3 cells/cm² using the same treatment media. Cells were allowed to adhere for one hour, fixed and stained by 0.2% crystal violet solution (50:50, water:MeOH). The total number of cells in five fields at 40x magnification for each well was counted. The experiments were performed in triplicate with 3 sets of independent experiments. Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.01.

(D). Cell migration transwell assay. Cell migration was assessed via Boyden chamber. An aliquot of 5 x 10^4 C-81 cells was seeded in the insert of 24-well plates in media containing 10 μM respective compounds with solvent alone for control in both upper and lower chambers. After 24-hour incubation, the migrated cells were stained and those cells remaining in the upper chamber were removed via cotton swab. Cells which had migrated through to the lower chamber were counted. Representative images are shown at 40x magnification. The experiments were performed in triplicate with 3 sets of independent experiments, and the results presented are mean ± SE; n=3x3, *p<0.05; **p<0.005.
Figure 3.4 Effects of imidazopyridine derivatives on PCa proliferative and apoptotic signaling under SR conditions.

(A) LNCaP C-81 cells were plated in triplicate in T25 flasks at 4 x 10^3 cells/cm² in regular medium, grown for 72 hours then steroid starved for 48 hours. Cells were then treated with 10 µM imidazopyridine derivatives or DMSO as control for an additional 72 hours under SR conditions. Cells were trypsinized and live cell numbers counted via Trypan Blue assay. The experiments were performed in triplicate with 3 sets of independent experiments. *** p<0.0005.

(B) MDA PCa2b-AI cells were grown, treated, and counted under conditions as described above in (A) for LNCaP C-81 cells. Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.005; ***p<0.0005.

(C) LNCaP C-81 total cell lysate proteins were collected from (A) after cell number counting. Those cells were grown in SR conditions and analyzed for phosphorylated Akt and STAT5, as well as total AR, Akt, Shc, p53, cyclin B₁, cyclin D₁, PCNA, Bclₓ, and Survivin protein levels. β-actin protein level was used as a loading control. Similar results were observed in two sets of independent experiments.

(D) MDA PCa2b-AI total cell lysate proteins from (B) after cell number counting. Cells were grown in SR conditions and analyzed for phosphorylated STAT5, as well as total AR, Shc, p53, cyclin B₁, cyclin D₁, PCNA, Bclₓ, and Survivin protein levels. β-actin protein level was used as a loading control. Similar results were observed in three sets of independent experiments.

(E) MDA PCa2b-AI total cell lysate proteins from cells grown in regular conditions were analyzed for phosphorylated Akt and total Akt. β-actin protein level was used as a loading control. Similar results were observed in three sets of independent experiments.
Figure 3.5

A

Cell Growth (Ratio to Control Day 0)

Day 0  Day 1  Day 3  Day 5  Day 7

Control  HIMP  M-Mel

B

HIMP (10µM)  M-Mel (10µM)

AR

PSA

Cyclin B1

PCNA

p53

Bax

Bcl-xl

B-Actin

Day 0  Day 1  Day 3  Day 5  Day 7

kDa

110

34

56

36

53

23

28

42

C

Control  HIMP  M-Mel

Diploid: 100%
Dip G1: 70.93% at 44.84
Dip G2: 29.09% at 84.79
Dip S: 5.18% G2/G1: 1.89
NVC: 5.38
Apoptosis: 0.51%

Diploid: 100%
Dip G1: 67.05% at 44.42
Dip G2: 26.69% at 83.94
Dip S: 5.57% G2/G1: 1.89
NVC: 6.98
Apoptosis: 0.98%

Diploid: 100%
Dip G1: 48.69% at 44.86
Dip G2: 48.82% at 85.23
Dip S: 2.49% G2/G1: 1.90
NVC: 5.29
Apoptosis: 1.95%
Figure 3.5 Kinetic analysis of HIMP and M-Mel’s effects on LNCaP C-81 cells under steroid-deprived conditions.

(A) Cells were plated in six-well plates at 2 x 10^3 cells/cm² in regular medium for three days, then steroid-starved for 48 hours followed by treatment with 10 µM HIMP or M-Mel in SR medium containing 1 nM DHT. Solvent alone was used for controls. On day 0, 1, 3, 5 and 7, one set of cells in duplicates from each group was harvested for live cell counting. Remaining cells were replenished with fresh respective medium. The experiments were performed in duplicates with 3 sets of independent experiments. Results presented are mean ± SE; n=2x3. * p<0.05; ** p<0.005; *** p<0.0005.

(B) Total cell lysate proteins from HIMP- and M-Mel-treated C-81 cells from (A) were collected and analyzed for AR, cPSA, cyclin B₁, PCNA, Bax, p53, and Bcl-X₅ proteins. β-actin protein level was used as a loading control.

(C) Histograms of cell cycle distributions of LNCaP C-81 cells upon 7 days of HIMP and M-Mel treatments. Cells were plated in T25 flasks at 2 x 10^3 cells/cm² in regular medium for three days, then steroid-starved for 48 hours followed by treatment with 10 µM HIMP or M-Mel in SR medium with 1 nM DHT and solvent DMSO alone as control. One set of cells from each group was harvested after 3, 5, and 7 days treatment for flow cytometric analysis. Similar results were obtained from two sets of independent experiments. The data shown were representative results of 7-day treatments.
Figure 3.6 Histograms of cell cycle distributions of LNCaP C-81 cells upon 3 and 5 days of HIMP and M-Mel treatments. Cells were plated in T25 flasks at 2 x 10^3 cells/cm² in regular medium for three days, then steroid-starved for 48 hours followed by treatment with 10 µM HIMP or M-Mel in SR medium with 1 nM DHT and solvent DMSO alone as control. One set of cells from each group was harvested after 3, 5, and 7 days treatment for flow cytometric analysis. Similar results were obtained from two sets of independent experiments. The data shown were representative results of 3- and 5-day treatments.
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Chapter 4

Statin Derivatives as Therapeutic Agents for Castration-Resistant Prostate Cancer

This chapter is derived from:

4.1 Synopsis

Despite recent advances in modern medicine, castration-resistant prostate cancer remains an incurable disease. Subpopulations of prostate cancer cells develop castration-resistance by obtaining the complete steroidogenic ability to synthesize androgens from cholesterol. Statin derivatives, such as simvastatin, inhibit cholesterol biosynthesis and may reduce prostate cancer incidence as well as progression to advanced, metastatic phenotype. In this study, we demonstrate novel simvastatin-related molecules SVA, AM1, and AM2 suppress the tumorigenicity of prostate cancer cell lines including androgen receptor-positive LNCaP C-81 and VCaP as well as androgen receptor-negative PC-3 and DU145. This is achieved through inhibition of cell proliferation, colony formation, and migration as well as induction of S-phase cell-cycle arrest and apoptosis. While the compounds effectively block androgen receptor signaling, their mechanism of inhibition also includes suppression of the AKT pathway, in part, through disruption of the plasma membrane. SVA also possess an added effect on cell growth inhibition when combined with docetaxel. In summary, of the compounds studied, SVA is the most potent inhibitor of prostate cancer cell tumorigenicity, demonstrating its potential as a promising therapeutic agent for castration-resistant prostate cancer.

4.2 Background and Rationale

Prostate cancer (PCa) is the most frequently diagnosed tumor and second leading cause of cancer-related fatality among United States men [1]. The majority of prostate tumors are reliant on androgen signaling for their development and progression, thus ADT is an effective means of treatment and remains the current standard-of-care therapy for metastatic PCa [2,3]. However, most patients ultimately relapse: cancer cells are able to endure the androgen-depleted environment and develop CR tumors for
which the median life-expectancy is less than 19 months. Despite advancements in post-ADT therapy, CR PCa remains an incurable disease maintaining an immediate need for advancement in treatment strategies [4,5].

Though CR tumors are no longer responsive to ADT, the majority continue to rely on AR signaling for growth and progression [3,6-8]. While the mechanism through which tumor cells acquire castration-resistance may vary, one mechanism is the development of intracrine regulation by activating the androgen biosynthesis pathway. [6,7,9,10]. For example, AR-positive LNCaP C-81 cells acquire the complete steroidogenic ability to synthesize androgens from cholesterol and obtain the CR phenotype [11]. This suggests inhibitors of steroid biosynthesis such as statins, which suppress cholesterol production via inhibition of HMG-CoA reductase, can be effective therapeutic agents for this population of tumors [12]. Supportively, ADT combined with simvastatin, a statin compound, reduces tumor growth and delays CR progression in murine models [13]. Moreover, epidemiological studies have reported a significant correlation between statin use and overall reduced risk of PCa diagnosis in addition to decreased development of aggressive, metastatic phenotype. [14]. Meta-analyses of observational studies have found ADT and/or radiotherapy combined with statin treatment resulted in a higher recurrence-free survival rate [15,16]. Together, these findings support the usage of statins to treat CR PCa patients. Importantly, FDA-approved cholesterol lowering drugs, such as simvastatin, possess well tolerated side-effect profiles [17]. This makes statins an ideal treatment option and suggests they can be combined with existing chemotherapeutic agents with minimal additional risk to the patient.

Currently, the mechanism of statin-mediated inhibition of PCa tumorigenicity remains poorly understood; further investigation using clinically-relevant cell line models is required. Moreover, PCa research on statins is frequently conducted using
concentrations well-exceeding what is clinically relevant to achieve significant data, indicating a need for more potent statin derivatives [18]. In this study, we investigate the efficacy of novel statin derivatives SVA, AM1, and AM2 (Fig. 4.1) as therapeutic agents for CR PCa with the goal of producing a more potent compound to effectively treat CR PCa. SVA is a potent metabolite of simvastatin, while AM1 and AM2 are amide modified statin derivatives with lower HMG-CoA reductase inhibition [19]. In addition, we use LNCaP C-81 cells as our primary cell model because they are a useful representative of advanced CR PCa: they express functional AR as well as readily proliferate and secrete PSA under SR conditions which strongly correlates with clinical CR PCa phenotype [20-22]. Furthermore, C-81 cells exhibit intracrine growth regulation in which the cells possess the ability to synthesize androgens from cholesterol [11]. This acquired steroidogenic ability provides a mechanism of escape from ADT and development of castration-resistance. In this study, we demonstrate for the first time that the novel statin compound SVA is a potent inhibitor of CR PCa cells and investigate its mechanism of tumor suppression using clinically relevant cell line models.

4.3 Results

4.3.A Growth Suppressive Effects of Statin Derivatives on PCa Cells

Many reports analyzing the effect of statin agents on CR PCa cells have been carried out using AR-null PCa cells lines such as PC-3 or DU145 [34]. However, most clinical CR PCa retains AR signaling; therefore in this study we used the LNCaP C-81 cell line which possesses active AR and is a more clinically useful model for CR PCa. C-81 cells were initially treated with simvastatin (Fig. 4.2A) or atorvastatin (data not shown) at concentrations from 0-20 µM under regular culture conditions. After 3 days of treatment, cell growth suppression was determined by live-cell counting, and both compounds were found to have similar potency with an IC$_{50}$ of 8.3 µM (Fig. 4.2A).
Western blot analysis showed a similar dosage-response of PCNA protein level, a cell proliferation marker (Fig. 4.2B). Inhibition of cell growth by simvastatin was observed at clinically achievable micromolar concentrations.

In attempt to identify more potent statin derivatives, LNCaP C-81 cells were treated for three days in regular culture medium containing 20 µM of each compound: simvastatin, SVA, AM1, and AM2. Simvastatin and SVA inhibited C-81 cell growth by over 80%, while AM1 and AM2 suppressed growth by 20% and 50%, respectively (Fig. 4.2C). Interestingly, when C-81 cells were treated in SR medium to mimic ADT conditions, the potency of simvastatin decreased with 60% inhibition, while that of SVA, AM1, and AM2 remained unchanged (Fig. 4.2C). Thus, a concentration of 20 µM was found to be suitable for treatment under SR conditions used to conduct all further experiments.

The inhibitors were then tested on a panel of PCa cell lines under SR conditions including: VCaP, PC-3, and DU145 (Fig. 4.2D-F). SVA was the most effective, suppressing androgen-sensitive VCaP cell growth by 80%, followed by simvastatin at 40%, while AM1 and AM2 each had 20% growth inhibition (Fig. 4.2D) [25]. In DU145 and PC-3 cell lines, both of which are androgen-independent and lack AR expression, SVA was the most potent, followed by simvastatin, and AM1 and AM2 exhibited the least inhibitory effect on both cell lines (Fig. 4.2E-F). Then, to determine compound selectivity, the experiment was repeated using the immortalized benign prostate epithelial RWPE-1 cell line. All compounds were found to be less potent against RWPE-1 cells with SVA demonstrating the most selectivity (Fig. 4.2G). Together, the data demonstrates that SVA is the most potent and selective suppressor of both androgen-sensitive and androgen-independent cell growth under SR conditions.
4.3.B Effects of Statin Derivatives on PCa Cell Tumorigenicity

Using the LNCaP C-81 cell line as a model system, we investigated whether statin derivatives can suppress PCa tumorigenicity including cell colony formation and migration. In the clonogenic anchorage-dependent assay, LNCaP C-81 cells were treated with each compound at 20 µM for 10 days. As shown in Figure 4.3A, both simvastatin and SVA were potent inhibitors of colony growth on the plastic-ware surface with over 90% inhibition. Compounds AM1 and AM2 were less potent with about 20% and 55% inhibition, respectively. Colony formation in a 3-dimentional environment was evaluated using the soft agar anchorage-independent assay, in which LNCaP C-81 cells were cultured in an agarose matrix for six weeks. As seen in Figure 4.3B, SVA suppressed colony formation by 90%, while simvastatin reduced colony growth by 60%. AM1 and AM2 each reduced colony growth by 40%. The colony size was also decreased by SVA and simvastatin.

The Boyden Chamber transwell migration assay was used to investigate the effect of these compounds on PCa cell migratory potential. SVA inhibited migration by over 90%, closely followed by simvastatin at 80%. AM1 and AM2 had similar effects with 40% and 45% inhibition, respectively (Fig. 4.3C). Collectively, compound SVA exhibited the most potent inhibitory activity on PCa cell tumorigenicity.

4.3.C Suppression of Cholesterol Synthesis by Statin Derivatives

Clinically, statin derivatives, such as simvastatin, are primarily prescribed to reduce patients’ circulating cholesterol levels. To examine their ability to reduce cholesterol in PCa cells, statin compound-treated LNCaP C-81 cells were stained with Filipin III for cholesterol detection and the fluorescence was semi-quantified via confocal microscopy. As shown in Figure 4.4A, while all compounds significantly reduced cellular cholesterol levels, SVA was the most effective with a 50% reduction of cholesterol level
in treated cells. Unexpectedly, simvastatin and AM1 had a similar 35% reduction in cholesterol level, while AM2 was least effective with only 20% inhibition.

4.3.D Influence of Statin Derivatives on PCa Cell Membrane Integrity

Cholesterol is an integral component of cell membrane stability and fluidity and is highly enriched in lipid rafts which anchor many proteins vital to cell signaling. Therefore, decreased cholesterol levels are expected to destabilize the cell membrane and alter cell signaling. We determined the impact of these compounds on membrane integrity by Trypan Blue dye-exclusion assay. As shown in Figure 4B, SVA induced membrane damage in 30% of treated cells and simvastatin disrupted membranes in 12% of cells, while compounds AM1 and AM2 had no significant impact on membrane permeability. The data together indicates these compounds suppress tumor cells, in part, by reducing cholesterol levels (Fig. 4.4A) and disrupting cell membranes (Fig. 4.4B).

4.3.E SVA Inhibition of Androgen Receptor

Due to the importance of AR function in CR PCa, the effect of SVA on AR was evaluated in LNCaP C-81 cells. Cells were grown in SR medium with or without 10 nM DHT, treated with SVA for 3 days, and whole cell lysates were analyzed via western blot. As shown in Figure 4.4C, under androgen-deprived conditions, SVA functioned as a potent suppressor of AR protein level. However, in the presence of 10 nM DHT, AR was partially protected from SVA blockage. SVA strongly reduced growth-regulator Cyclin B1 protein, independent of the presence of androgens [29]. In parallel, cell growth was greatly suppressed by SVA regardless of the presence of androgens and correlates with Cyclin B1 protein levels (Fig. 4.4D). Thus, SVA effectively inhibits Cyclin B1, a positive cell cycle regulator, and cell proliferation independent of androgen availability.
4.3.F Effect of Statin Derivatives on PCa Cell Cycle

To further investigate the mechanism through which statin derivatives inhibit PCa cell tumorigenicity, we focused our efforts on comparing SVA and AM1 with simvastatin and performed cell cycle analysis on statin-treated LNCaP C-81 cells. Flow cytometry analysis (Fig. 4.4E) revealed the percentage of apoptotic cells rose sharply under SVA and simvastatin treatments to 66% and 27%, respectively, compared to that of only 2% of control and 4% of AM1-treated cells. In addition, SVA and simvastatin treatments greatly increased the percentage of cells in S phase accompanied with a decreased percentage of cells in G2 phase. Thus, SVA and simvastatin induce apoptosis as well as cell cycle arrest in S phase.

4.3.G Effect of Statin Derivatives on Cell Signaling Under SR Conditions

To investigate the mechanism through which the statin derivatives inhibit PCa tumorigenicity under SR conditions, we analyzed their effects on key signaling molecules known to contribute to PCa progression using LNCaP C-81 cells. AR is a major target of anti-PCa agents and remains a vital signaling pathway even in CR PCa cells [27,28]. While all compounds decreased AR protein in C-81 cells, SVA was the most potent followed by simvastatin, and AM1 had only marginal effect (Fig. 4.5A). This correlates with reduced cellular prostate-specific antigen (cPSA) with the exception of AM1 treatment. Interestingly, secreted prostate specific antigen (sPSA) protein level was increased by simvastatin and SVA. In addition, ErbB-2 is a transmembrane tyrosine kinase which regulates AKT and can be activated by androgen signaling in PCa through phosphorylation at Y1221/2 [20-22,30]. Upon treatment with statin derivatives, all agents reduced ErbB-2 Y1221/2 phosphorylation with SVA having the greatest effect. SVA also reduced the total ErbB-2 protein while other compounds had a limited impact (Fig. 4.5A).
Furthermore, while all statin derivatives inhibited AKT phosphorylation at S473, SVA also effectively reduced total AKT protein levels.

Downstream targets of AKT include Survivin and p53, while both AKT and AR can regulate Cyclin B1 [33,35]. AKT upregulates Survivin, an anti-apoptotic protein, and upon treatment, all compounds greatly reduced Survivin protein levels (Fig. 4.5A). Cyclin B1, a cell growth regulator, was strongly inhibited by simvastatin and SVA, but not AM1. Furthermore, p53 is an inducer of apoptosis and is suppressed by AKT; p53 was elevated upon statin compound treatment and its downstream target BAX, another pro-apoptotic protein [36], was elevated only by SVA. Conversely, anti-apoptotic Bcl-xL protein levels were greatly reduced by all statin compounds (Fig. 4.5A). Elevated levels of cleaved PARP and Caspase 3 were also observed in treated cells with SVA having the greatest effect, providing further evidence of induced apoptosis. Together, upon statin compound treatment there is an overall trend of proliferative and anti-apoptotic protein inhibition, while pro-apoptotic proteins were induced.

We investigated whether oxidative stress is involved in statin-induced tumor suppression. Indeed, as shown in Figure 4.5A, NOX5 protein, which functions to generate superoxide at the cell membrane, was greatly increased by SVA treatment. Moreover, while no observable change was found in redox-sensitive NF-κB protein, ROS-response protein Nrf2 and its downstream target HO-1 were highly induced by SVA and to a lesser extent by simvastatin. This may indicate simvastatin and SVA promote oxidative stress resulting in activation of apoptotic pathways.

Statin derivatives also inhibit PCa cell migration (Fig. 4.3C). Since AKT is associated with motility, we analyzed the level of Snail protein, a downstream target of AKT, as well as activation of p38, both of which are associated with cell migration and stress-response [37,38]. Snail was suppressed by both simvastatin and SVA and
correlated with inhibition of AKT activation. Phosphorylation and activation of p38 was inhibited by SVA and AM1, but not by simvastatin (Fig. 4.5A).

To further validate the compounds’ effect on PCa signaling, primary target molecules were also investigated using VCaP cells shown in Figure 4.5B. A similar trend in AR, cPSA, Cyclin B1, Survivin, PARP, Caspase 3, Nrf2, and HO-1 protein levels were observed compared to C-81 treated cells with SVA having the greatest suppressive effect. Interestingly, simvastatin had greater inhibitory effect on total and phosphorylated ErbB-2 than SVA. In addition, while SVA was again the most potent inhibitor of total and phosphorylated AKT, AM1 had a greater inhibitory effect than simvastatin. Overall, a similar trend in inhibition of key functional proteins was observed in both VCaP and LNCaP C-81 cell lines.

4.3.H DCF-DA Dye Analysis of LNCaP C-81 Cells Treated with Statin Derivatives

Upon observing an increase of NOX5 protein level as well as ROS-response proteins Nrf2 and HO-1 in statin-treated LNCaP C-81 cells, we investigated the effect of these compounds on ROS generation. This was semi-quantified by DCF-DA dye and fluorescence was measured via flow cytometry. As shown in Figure 4.5C, both simvastatin and SVA significantly increased cellular levels of ROS, while AM1 had no significant effect. This data demonstrates simvastatin and SVA induce ROS generation, causing oxidative stress in PCa cells under SR conditions which can contribute to growth suppression and apoptosis.

4.3.I LNCaP C-81 Migration in the Presence of Small Molecule Inhibitors

Statin compounds inhibit PCa cell migration (Fig. 4.3C) as well as AKT and p38 pathways (Fig. 4.5A) which are both associated with cell motility. To determine which of these signaling pathways regulate LNCaP C-81 cell migration, transwell assays were
conducted with small molecule inhibitors of p38 (SB202190), AKT (MK-2206), and ErbB-2 (AG879) [39-41]. As shown in Figure 4.5D, inhibition of p38 had no impact on C-81 cell migration; inhibition of ErbB-2 reduced migration by 50%, and inhibition of AKT suppressed migration by over 80%. The results indicate statin compounds inhibit of PCa cell migration primarily through suppression of the ErbB-2/AKT signaling pathway.

4.3. J Growth Suppression via Combined Docetaxel and Statin-Derivative Treatment

Simvastatin has previously been reported to have an added inhibitory effect on PCa cell growth when combined with docetaxel treatment [42]. To determine the interaction between SVA and docetaxel, the compounds were used to treat C-81 cells under SR conditions independently and in combination, and cell growth was determined. Both simvastatin and SVA (5 µM each) were found to possess an added effect on cell growth suppression when combined with docetaxel (1 nM) (Fig. 4.6).

4.4 Discussion

CR PCa remains an incurable disease and an effective therapy is immediately needed. Recent \textit{in vitro} and epidemiological studies revealed that inhibition of cholesterol synthesis by statin derivatives may be an effective treatment strategy for CR PCa [11-16,43]. In the present study utilizing clinically-relevant PCa cell line models, we show for the first time that novel statin derivatives are effective suppressors of CR PCa tumorigenicity through inhibition of both AR and AKT pathways as well as induction of apoptosis. These compounds have the potential to serve as effective therapeutic agents for CR PCa.

In addition to template-compound simvastatin, we investigated the ability of novel statin derivatives SVA, AM1, and AM2 to suppress CR PCa proliferation. We chose
LNCaP C-81 cells as our primary experimental model due to their steroidogenic ability to synthesize androgens from cholesterol in addition to the possession of many biochemical properties common to clinical CR PCa [11,20-22]. While simvastatin is a less potent inhibitor of C-81 cell proliferation under SR conditions compared to regular steroid conditions, the potency of SVA, AM1, and AM2 remains unaltered. This may indicate steroid-deprivation reduces the cells’ ability to activate simvastatin, while SVA, AM1, and AM2 are in active states. Moreover, SVA is the most potent inhibitor of cell growth in all cell lines examined. Notably, the compounds are effective inhibitors of AR-negative DU145 and PC-3 cell growth, which indicates statin derivatives suppress cell proliferation through alternative mechanisms in addition to inhibition of AR signaling. The compounds were also found to have selective inhibition, with all compounds demonstrating reduced potency against benign epithelial RWPE-1 cells as shown in Figure 4.2G. Furthermore, these compounds suppress tumorigenicity including colony formation and migration. Among them, SVA exhibits the most potent suppression of PCa tumor phenotype followed by simvastatin, while AM1 and AM2 had the least effect.

We determined whether these compounds have an impact on AR signaling to test our hypothesis that depriving C-81 cells of cholesterol blocks their ability to synthesize androgen and thus inhibits AR pathways. Statin derivatives were found to reduce AR protein level correlating with a decrease in cellular PSA, despite an increase in secreted PSA. This rise in secreted PSA may be attributed to the loss of membrane stability, allowing PSA protein to leak out of the cell. Moreover, SVA is a potent inhibitor of AR in C-81 cells under SR conditions; however, in the presence of DHT, the impact on AR protein level is reduced (Fig. 4.4C). Unexpectedly, SVA suppression of cell proliferation is only marginally reduced in the presence of androgens, while its impact on AR protein level is greatly diminished (Figs. 4.2C, 4.4D). This data correlates with
observed statin inhibition of AR-null PC-3 and DU145 proliferation (Figs. 4.2E-F) and thus supports the notion that cell growth is suppressed by other mechanisms in addition to AR signaling.

PCa cells possess enriched cholesterol and lipid raft concentrations in comparison to benign cells, and simvastatin has been reported to reduce both lipid raft and cell cholesterol levels in prostate cells [43,44]. The observations in those reports correlate with our data showing a reduction in cellular cholesterol and destabilization of C-81 cell membranes upon statin compound treatment (Fig. 4.4A, 4.4B). Additionally, the compounds’ selective growth inhibition may be attributed to the fact that PCa cells undergo rapid proliferation and more dynamic membrane activity compared to benign cells. Moreover, Adam et al. [45] demonstrated that a cholesterol-sensitive subgroup of AKT, which enhances tumor cell survival and metastatic ability, is enriched in PCa cells and is dependent upon lipid raft availability for activation via phosphorylation. In parallel, a correlation has been reported between decreased AKT activation and prevention of lipid raft formation as a result of simvastatin’s inhibition of cholesterol synthesis [44]. Indeed, our data clearly demonstrates AKT activation is inhibited by statin compounds (Fig. 4.5A, 4.5B) and correlates with loss of membrane integrity (Fig. 4.4B). Collectively, inhibition of AKT signaling is a major mechanism of statin-mediated suppression of PCa cell tumorigenicity.

Inhibition of AKT may be achieved via suppression of ErbB-2, a hyper-phosphorylated and activated transmembrane tyrosine kinase in CR PCa cells which can upregulate AKT by promoting activation via S473 phosphorylation [30, 32]. Emerging studies reveal, in addition to AR, AKT signaling is vital to the progression and development of CR PCa [46,47]. Our data shows statin derivatives inhibit AKT activation as well as reduces total AKT protein levels, correlating with ErbB-2 status and
their effect on tumor phenotype (Fig 4.5A, 4.5B). We propose disruption of the cell membrane can reduce ErbB-2 stability resulting in prevention of AKT activation. Alternatively, we cannot rule-out the possibility that statin-agents directly interact with and inhibit ErbB-2 and/or AKT. These changes in signaling correlate with a large increase of apoptosis and induction of S-phase cell cycle arrest (Fig. 4.4E). Together, the data indicates statin compounds suppress cell survival signaling and induce cell death while SVA has the most potent effect.

Upon observing an elevation of pro-apoptotic proteins and a decrease in survival-associated proteins after statin treatment, we investigated the impact of statin compounds on cellular ROS production and induction of oxidative stress. In Figure 4.5C, a significant increase in cellular ROS is shown in cells treated with simvastatin and SVA. Superoxide generating protein, NOX5, was found to be increased upon treatment with statin derivatives, which may contribute to the elevated ROS level. However further investigation is required to determine the exact mechanism of its involvement [48]. We also observed a rise in ROS-response proteins Nrf2 and its downstream target HO-1 which induces antioxidant mechanisms within the cell (Fig. 4.5A, 4.5B) [49]. While the total protein levels of NFκB, another ROS-sensitive protein, were not altered upon statin-compound treatment, changes in its subcellular localization may occur and requires further study. Collectively, our data shows statin derivatives induce cellular stress in part by increasing ROS which correlates with elevated apoptosis.

In addition to inhibition of proliferation and induction of apoptosis, statin derivatives also reduce cell migratory ability (Fig. 4.3C). Western blot analysis (Fig. 4.5A) revealed the inhibition of a number of motility-related proteins. Moreover, AKT and its downstream target Snail both are strongly inhibited by simvastatin and SVA. While p38 is often associated with cell migration and has decreased activation under SVA and
AM1 treatment (Fig. 4.5A), its inactivation by statins does not correlate with migratory activity (Fig. 4.3C). Furthermore, as shown in Figure 4.5D, p38 inhibition has no impact on C-81 motility. In comparison, ErbB-2 inhibition impedes migration by about 40% and AKT inhibition reduces migration by over 80%. The data together indicates statin derivatives reduce the PCa cell migratory ability and tumorigenicity, in part, through inhibition of the AKT pathway.

In summary, our study uses the clinically relevant LNCaP C-81 cell line model to demonstrate the novel compound SVA is a potent antagonist of CR PCa tumorigenicity and functions to suppress tumor phenotype through concurrent inhibition of AR and AKT pathways. As depicted in Figure 4.7, statin derivatives suppress tumor progression in two ways: first, these compounds inhibit androgen biosynthesis from cholesterol in CR PCa cells which obtain intracrine regulation [11]. In addition, the lack of cholesterol reduces the cell membrane’s integrity which is necessary for ErbB-2 and downstream AKT function. It is also possible the statins physically interact with and suppress AKT. While both AR and AKT signaling contribute to PCa cell tumor phenotype including proliferation and migration, our data indicates inhibition of the AKT pathway is the primary mechanism by which statin agents mediate tumor suppression. Importantly, aberrant AKT signaling is prevalent in a major subpopulation of advanced prostate tumors which endows statin derivatives with broad therapeutic potential. Moreover, statin derivatives are well-tolerated by patients and SVA was found to have an added effect when combined with docetaxel treatment (Fig. 4.6). This implies SVA can be used to supplement docetaxel, which possess more severe side effects, in order to improve patient quality of life. Future studies are required to elucidate the mechanism through which statin derivatives induce oxidative stress and arrest cell cycle in S phase. Further in vivo investigation of SVA will help determine its potential as a therapeutic agent.
Figure 4.1 Structures of statin derivatives. Simvastatin, simvastatin hydroxyacid (SVA), 8-(3,5-dihydroxy-7-((2-(2-hydroxyethoxy)ethyl)amino)-7-oxoheptyl)-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate (AM1), and 8-(3,5-dihydroxy-7-((2-(2-hydroxy)ethyl)amino)-7-oxoheptyl)-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate (AM2).
Figure 4.1

Simvastatin, simvastatin hydroxyacid (SVA), $8\cdot(3,5\cdot$dihydroxy$\cdot7\cdot(2\cdot2\cdothydroxyethoxy)ethyl)amino)\cdot7\cdot$oxoheptyl)$\cdot3,7\cdot$dimethyl$\cdot1,2,3,7,8,8a\cdot$hexahydronaphthalen$\cdot1\cdot$yl 2,2\cdotdimethylbutanoate (AM1), and $8\cdot(3,5\cdot$dihydroxy$\cdot7\cdot(2\cdot2\cdothydroxy)ethyl)amino)\cdot7\cdot$oxoheptyl)$\cdot3,7\cdot$dimethyl$\cdot1,2,3,7,8,8a\cdot$hexahydronaphthalen$\cdot1\cdot$yl 2,2\cdotdimethylbutanoate (AM2).

Figure 4.2
Figure 4.2 Effects of statin derivatives on PCa cell growth.

**(A)** Dosage effect of simvastatin on LNCaP C-81 cells. Cells were plated in six-well plates at 2 x 10³ cells/cm² in regular medium for attachment and grown for 72 hours. Cells were then fed with fresh medium containing 0-20 µM simvastatin with solvent alone for control and grown for an additional 72 hours. Cells were trypsinized and live cell numbers were counted by trypan blue staining. The correlative coefficient of dosage effect was r=0.906; p<0.05. The results presented are mean ± SE; n=2x2. *p<0.05.

**(B)** Cell lysates from (A) were collected for immunoblot analysis of PCNA. β-actin protein level was used as a loading control. Similar results were observed in two sets of independent experiments.

**(C)** Effects of statin derivatives on the growth of LNCaP C-81 cells. Under regular conditions, cells were plated in six-well plates at 3 x 10³ cells/cm² and maintained for 72 hours, then treated with fresh medium containing 20 µM statin-compounds for 72 hours. To mimic steroid-deprived (SR) conditions, cells were plated in six-well plates at 2 x 10³ cells/cm² and grown for 72 hours, steroid-starved for 48 hours, then fed with fresh SR medium with 1 nM DHT containing 20 µM statin-compounds and grown for 72 hours. Cells were trypsinized and live cell number was counted. Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.

Using the same protocol as described in (C), the effect of statin derivatives on PCa cell growth under SR conditions was determined for **(D)** VCaP - 1.5 x 10⁴ cells/cm², **(E)** PC-3 - 2 x 10³ cells/cm², and **(F)** DU145 - 2 x 10³ cells/cm², **(G)** RWPE-1 - 1 x 10⁴ cells/cm². Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.
Figure 4.3

A

B

C

# of Colonies (Ratio to Control)

# of Cells Migrated (Ratio to Control)
Figure 4.3 Effects of statin derivatives on CR PCa tumorigenicity.

(A) Clonogenic assay on plastic wares. LNCaP C-81 cells were plated in six-well plates at 2,000 cells/well. After 24 hours, attached cells were treated with respective compounds at 20 µM or solvent alone as control. Cells were fed on days 3, 6, and 9 with fresh culture media containing respective statin derivatives. On day 10, representative photos of colony plates were taken and the number of colonies counted. Results presented are mean ± SE; n=3x3. **p<0.005 ***p<0.0005.

(B) Anchorage-independent soft agar assay. LNCaP C-81 cells were plated at a density of 5 x 10⁴ cells/35mm dish in 0.25% soft agarose with a base layer of 0.3% agarose. After 24 hours, cells in doublets or greater were marked and excluded from the study. Culture medium containing respective compounds at 20 µM or solvent alone was added every 72 hours, and after 6 weeks, colonies were stained and fixed. Representative images of colonies were taken and the number of colonies counted. Results presented are mean ± SE; n=3x3. **p<0.005 ***p<0.0001.

(C). Cell migration transwell assay. Cell migration was assessed via Boyden chamber assay. 6 x 10⁴ C-81 cells were seeded in the transwell insert of 24-well plates. Medium containing 20 µM of respective compounds or solvent alone for control were placed in the lower chamber. After 24-hour incubation, the migrated cells were stained and those cells remaining in the upper chamber were removed via cotton swab and cells which had migrated through to the lower chamber were counted. Representative images are shown at 40x magnification. Results presented are mean ± SE; n=3x3. **p<0.005 ***p<0.0005.
Figure 4.4

A

Filipin III Staining (Ratio to Control)

B

Trypan Blue (% Stained Cells)

C

D

Cell Growth (Ratio to Control)

E

Control

Simvastatin

SVA

AM1

Diploid: 100%
Dip G1: 81.73% at 44.57
Dip G2: 8.63% at 85.58
Dip S: 9.63% G2/G1: 1.97
%CV: 4.00
Apoptosis: 2.01%

Diploid: 100%
Dip G1: 86.43% at 49.88
Dip G2: 2.12% at 95.78
Dip S: 11.45% G2/G1: 1.92
%CV: 7.15
Apoptosis: 26.52%

Diploid: 100%
Dip G1: 76.62% at 41.86
Dip G2: 0.16% at 84.76
Dip S: 23.22% G2/G1: 1.92
%CV: 8.72
Apoptosis: 66.12%

Diploid: 100%
Dip G1: 86.77% at 46.18
Dip G2: 5.51% at 88.66
Dip S: 7.71% G2/G1: 1.92
%CV: 4.33
Apoptosis: 4.11%
Figure 4.4 Mechanism of statin-derivative suppression of CR PCa tumorigenicity.

(A) Statin derivative’s inhibition of LNCaP C-81 cholesterol synthesis. Cells were plated in 96-well plates at $2 \times 10^3$ cells/cm$^2$ per well in regular medium and allowed to attach over-night then fed with fresh medium containing $20 \mu$M statin derivatives and grown for 72 hours. Cells were stained with Filipin III and examined via fluorescence microscopy. The results presented are mean ± SE; n=5x3. *$p<0.05$ **$p<0.005$ ***$p<0.0005$.

(B) Effects of statin derivatives on cell membrane integrity in LNCaP C-81 cells. Under regular conditions, cells were plated in six-well plates at $3 \times 10^3$ cells/cm$^2$ and maintained for 72 hours, then steroid-starved for 48 hours. After, cells were fed with fresh SR medium with 1 nM DHT containing $20 \mu$M statin derivatives and grown for 72 hours. Cells were trypsinized and stained with Trypan Blue dye and the ratio of blue to total cells counted was recorded. The results presented are mean ± SE; n=3x3. **$p<0.005$ ***$p<0.0005$.

(C) Effect of SVA on AR. LNCaP C-81 cells were treated with $20 \mu$M SVA, 10 nM DHT, or both for 72 hours under SR conditions. Total cell lysate proteins were collected for immunoblot analysis of AR and Cyclin B1 protein levels. β-actin protein level was used as a loading control. Similar results were observed in two sets of independent experiments.

(D) LNCaP C-81 cells were treated with $20 \mu$M SVA, 10 nM DHT, or both for 72 hours under SR conditions. Cells were trypsinized and live cell numbers were counted. The results presented are mean ± SE; n=3x3. ***$p<0.0005$.

(E) Histograms of cell cycle distributions of statin-treated LNCaP C-81 cells. Cells were plated in T25 flasks at $5 \times 10^3$ cells/cm$^2$ in regular medium and maintained for 72 hours, then steroid-starved for 48 hours, followed by treatment with $20 \mu$M of respective compounds in SR medium for 72 hours. Cells were harvested using trypsin, stained with propidium iodide, and cell-cycle was analyzed via flow cytometric analysis. One set of representative data is shown. Similar results were obtained from three sets of independent experiments.
Figure 4.5 Molecular profiling of LNCaP C-81 signaling upon statin-derivative treatment under SR conditions.

(A) Immunoblot analysis of statin-treated LNCaP C-81 cells. Cells were plated in T25 flasks at 5 x 10³ cells/cm² in regular medium, maintained for 72 hours, then steroid starved for 48 hours. Cells were treated with 20 µM of respective compounds and grown for an additional 72 hours under SR conditions. Cells were trypsinized and total cell lysate proteins were collected. Total cell lysates were analyzed for phosphorylated ErbB-2, AKT, and p38 by site-specific phospho-Abs as well as total AR, cPSA, sPSA, ErbB-2, AKT, Cyclin B1, Survivin, BclXL, p53, BAX, PARP, Caspase 3, NOX5, Nrf2, HO-1, NFκB, p38, and Snail protein levels. β-actin protein level was used as a loading control. Similar results were observed in three sets of independent experiments.

(B) Immunoblot analysis of statin-treated VCaP cells. Cells were plated in T75 flasks at 1.3 x 10⁴ cells/cm² in regular medium, maintained for 72 hours, then steroid starved for 48 hours. Cells were treated with 20 µM of respective compounds and grown for an additional 72 hours under SR conditions. Cells were trypsinized and total cell lysate proteins were collected. Total cell lysates were analyzed for phosphorylated ErbB-2 and AKT by site-specific phospho-Abs as well as total AR, cPSA, ErbB-2, AKT, Cyclin B1, Survivin, PARP, Caspase 3, Nrf2 and HO-1 protein levels. β-actin protein level was used as a loading control. Similar results were observed in three sets of independent experiments.

(C) Analysis of ROS with DCF-DA dye in statin-treated LNCaP C-81 cells. Cells were plated in T25 flasks at 1 x 10⁴ cells/cm² and maintained for 72 hours, then steroid-starved for 48 hours, followed by treatment with 20 µM of respective compounds in fresh SR medium for 72 hours. Cells were then incubated with 20 µM DCF-DA for 30 minutes before being harvested and analyzed via flow cytometry. Results presented are mean ± SE; n=3. *p<0.05.

(D) Transwell assay with LNCaP C-81 cells treated with small-molecule inhibitors. Cell migration was assessed via Boyden chamber assay. 6 x 10⁴ C-81 cells were seeded in the transwell insert of 24-well plates. Medium containing small-molecule inhibitors 10 µM SB202190 (p38), 1 µM AG879 (ErbB-2), or 10 µM MK2206 (AKT) was placed in the lower chamber. After 24-hour incubation, the migrated cells were fixed and stained. Cells remaining in the upper chamber were removed via cotton swab and cells which had migrated through to the lower chamber were counted. Results presented are mean ± SE; n=3x3. **p<0.005 ***p<0.0005.
Figure 4.6 Combination treatment of LNCaP C-81 cells with statin-derivatives and docetaxel. Under regular conditions cells were plated in six-well plates at 2 x 10^3 cells/cm^2 and grown for 72 hours, then steroid-starved for 48 hours. Cells were then fed with fresh SR medium with 1 nM DHT containing 5 µM statin derivatives, 1 nM docetaxel, or both and grown for 72 hours. Solvent DMSO alone was used for control. Cells were trypsinized and live cell numbers were counted. The results presented are mean ± SE; n=2x3. **p<0.005 ***p<0.0005.
Figure 4.7

[Diagram showing biological pathways related to tumorigenicity, with labels such as ErbB-2, Statin, Cholesterol, HMG-CoA, HMGCR, p53, Bax, Survivin, Snail, Cyclin B1, and Survival, Migration, Proliferation.]
Figure 4.7 Proposed inhibitory mechanism of statin-derivatives on CR PCa cell tumorigenicity. We propose that statin derivatives suppress CR PCa tumorigenicity through two different mechanisms. (1) First, cholesterol is required for de novo androgen synthesis and subsequent intracrine activation of androgen receptor (AR) in CR PCa cells. Activated AR can induce ErbB-2 phosphorylation and activation as well as cell cycle progression in part via up-regulation of Cyclin B1. (2) In parallel, cholesterol is vital to the cell membrane’s integrity and formation of lipid rafts; their disruption destabilizes the cell membrane as well as ErbB-2 and AKT. Moreover, inhibition of AKT leads to induction of apoptosis through activation of pro-apoptotic p53 and BAX and suppression of pro-survival protein Survivin as well as Cyclin B1. Statin-induced AKT inhibition also leads to suppression of cell migration, in part mediated through Snail. Nevertheless, direct interaction between statin agents and ErbB-2 or AKT is possible. Thus through concurrent inhibition of both AR and AKT signaling pathways in addition to plasma membrane destabilization, statin derivatives induce apoptosis and suppress CR PCa tumorigenicity.
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Chapter 5

p66Shc Regulates Migration of Castration-Resistant Prostate Cancer Cells

This chapter is derived from:

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p66Shc Regulates Migration of Castration-Resistant Prostate Cancer Cells. Submitted
5.1 Synopsis

Metastatic castration-resistant (CR) prostate cancer (PCa) is a lethal disease for which no effective treatment is currently available. p66Shc is an oxidase elevated in patients with PCa and multiple CR PCa cell lines that promotes androgen-independent cell growth through generation of reactive oxygen species (ROS) We hypothesize p66Shc also increases the migratory activity of CR PCa cells through ROS and thus investigate the associated mechanism. Using the transwell assay, our study reveals that the level of p66Shc protein correlates with castration-resistance and cell migratory ability across several PCa cell lines. Furthermore, we show peroxide treatment induces migration of androgen-sensitive cells that express low levels of p66Shc in a dose-dependent manner, while antioxidants inhibit migration. Moreover, stable p66Shc cDNA transfected subclone cells possess increased cell migration which is mitigated upon p66Shc shRNA transfection or expression of oxidase-deficient dominant-negative p66Shc W134F mutant. Protein microarray and immunoblot analyses reveal multiple proteins activated by p66Shc which could play a functional role in cell migration. These include ErbB-2, AKT, mTOR, ERK, FOXM1, PYK2, and Rac1, and their involvement in PCa migration was confirmed using small-molecule inhibitors. The role of Rac1 was further validated using cDNA transfection and, significantly, p66Shc is found to promote lamellipodia formation through Rac1 activation. Together, our results indicate p66Shc not only promotes androgen-independent cell growth in patients with metastatic CR PCa, but we now present evidence that p66Shc increases cell migration through ROS-mediated activation of migration-associated proteins, notably Rac1.

5.2 Background and Rationale

Prostate cancer (PCa) remains the most commonly diagnosed solid tumor and third leading cause of cancer-related death in United States men [1,2]. Localized PCa is
generally not lethal and effectively treated by means of surgery or radiation therapy. It is not until the tumor metastasizes and spreads to vital organs that it becomes life-threatening. While metastatic PCa is initially suppressed by androgen-deprivation therapy (ADT), many PCa patients relapse and develop the lethal castration-resistant (CR) form of the disease for which there is no effective treatment. Thus, new therapeutic targets must be identified. Furthermore, molecules involved in the process of PCa cell migration and proliferation have the potential to be promising biomarkers as well as remedial targets.

p66Shc, a 66 kDa proto-oncogene Src and collagen homologue protein, exhibits oxidase activity and is one of three members of the Shc family of adaptor proteins, including p52Shc and p46Shc [3,4]. p66Shc differs from the other Shc members in numerous ways. For example, p66Shc protein level is regulated through post-translational stabilization via steroids, including androgens, which play a critical role in the process of PCa development [4-6]. While other Shc members are ubiquitously expressed, p66Shc is primarily expressed in epithelial cells and has both cytosolic and mitochondrial localization. Structurally, p66Shc protein has an additional N-terminal CH2 domain which contains serine phosphorylation sites that can regulate p66Shc activity [3,4,7]. For instance, serine 36 phosphorylation by ERK/JNK in response to stress is shown to induce translocation of p66Shc into the mitochondria [8, 9]. In the mitochondrial intermembrane space, p66Shc binds and oxidizes cytochrome C, uncoupling the electron transport chain and inducing production of reactive oxygen species (ROS) [10]. Additionally, p66Shc has been reported to induce Rac1 activation in mouse fibroblasts and breast cancer, though their interaction is unknown in PCa. Rac1 is a key regulator of cell motility and can also increase ROS production via interaction with NOX family of NADPH oxidases [11,12].
ROS molecules are natural by-products of cellular respiration and contribute to essential signaling pathways; local ROS production stimulated by external growth factors and hormones mediates the transduction of signals from the cell membrane to the nucleus through the oxidation and reduction of proteins [13,14]. However, when ROS molecules are produced in excess, they also readily oxidize a number of cellular targets causing DNA, lipid, and protein damage, which facilitate various mutations and cancer development [15]. Furthermore, ROS is known to regulate processes like angiogenesis, cell adhesion, proliferation, and migration, all of which are critical to cancer metastasis [16-19]. Results of several studies have indicated oxidation of protein tyrosine phosphatases mediated by increased cellular levels of ROS can induce cell migration in mouse fibroblasts and more recently in human PCa cells [7,20,21].

Recent studies reveal p66Shc protein levels are increased in prostate, thyroid, ovarian, and colon adenocarcinomas in comparison with corresponding non-cancerous cells [6,22-24]. The level of p66Shc protein is also greater in multiple androgen-independent (AI) PCa cell lines which correspond with advanced metastatic CR PCa. For example, in the LNCaP PCa cell line, androgen-sensitive (AS) LNCaP cells (LNCaP-AS/C-33) possess relatively low levels of p66Shc protein [25,26]. In contrast, as LNCaP cells progress to androgen-independence, meaning they maintain a similar growth rate regardless of the presence of external androgens (LNCaP-AI/C-81), the cells have much higher levels of p66Shc protein on and exhibit many biochemical properties seen in clinical CR PCa [25-28]. These same phenomena are also observed in human MDA-PCa2b PCa cells, which become AI upon passage and possess increased levels of p66Shc; demonstrating the PCa progression in vitro correlates with an increase of p66Shc protein level [25,29-30]. Furthermore, p66Shc induces AI PCa cell growth, at least in part, by increasing cellular levels of ROS [7,25,31]. The current study is the first
to report p66Shc also mediates PCa cell migration, a vital process for tumor metastasis, and we further elucidate its signaling mechanism. Our ultimate goal is to understand the mechanism of PCa progression to metastatic CR PCa in order to identify novel biomarkers and therapeutic targets to aid the development of effective treatment options for this lethal disease.

5.3 Results

5.3.A p66Shc Protein Level Correlates with the CR Phenotype and PCa Cell Migration

We initially analyzed p66Shc protein levels in primary PCa archival specimens to determine its association with the disease. Immunohistochemical staining was performed using 33 specimens in which each specimen contains both benign and cancerous tissues. As shown in Figure 5.1A, p66Shc protein level was found to be elevated in PCa adenocarcinoma with a score of 3.07 ± 0.41 when compared directly to benign regions with a score of 1.21 ± 0.26. Statistical analysis determined p66Shc protein level was significantly elevated in PCa compared to benign tissues with $p < 0.0005$.

We then examined p66Shc protein level across multiple PCa cell lines via immunoblot analysis as well as their migratory potential using the Boyden Chamber transwell assay. As shown in Figure 5.1B, AS LNCaP cells (LNCaP-AS/C-33) have relatively low levels of p66Shc protein and possess correspondingly low migratory activity when compared to AI PC-3 and DU145 cells exhibiting higher levels of p66Shc as well as migration. The correlation between p66Shc protein and migration was then investigated using two independent PCa progression cell line models including AR-positive LNCaP and MDA PCa2b cell lines. As shown in Figures 5.1C-D, across both progressive PCa cell models, p66Shc protein level and migratory activity are elevated in
AI cells in comparison to respective AS cells. Together, the data supports the notion that p66Shc protein level correlates with the AI/CR phenotype as well as cell migratory activity.

5.3.B p66Shc Promotes PCa Cell Migration

To determine p66Shc’s role in PCa cell migration, the migratory activities of parental LNCaP-AS, vector-alone transfected (V1) cells, and stable p66Shc cDNA-transfected S32 and S36 subclones were investigated. As shown in Figure 5.2A, an increase in p66Shc protein resulted in increased migratory activity. A reversal experiment was then conducted in which stable p66Shc cDNA-transfected subclones were transiently transfected with p66Shc shRNA for analysis. For this set of experiments, to diminish the possible effects of variation between stable subclones, three individual p66Shc-stable subclones (S31, S32, and S36) were combined in equal number prior to shRNA transfection. As shown in Figure 5.2B, while mixed subclones exhibited elevated migratory activity, upon shRNA transfection both p66Shc protein levels and cell migration were decreased in a dose-dependent manner. Moreover, LNCaP-AI (C-81) cells, which possess relatively high levels of p66Shc protein as well as migratory activity (Fig. 5.1C), were transiently transfected with increasing amounts of p66Shc shRNA and then analyzed via western blot and transwell assays. As exhibited in Figure 5.2C, upon shRNA transfection both p66Shc protein levels and cell migratory activity decreased in a dose-dependent manner. The data taken together shows p66Shc protein level is associated with cell migratory activity and thus indicates p66Shc can directly regulate PCa cell migration.

5.3.C p66Shc Promotes PCa Cell Migration via Increasing ROS Production

p66Shc is an authentic oxidase and promotes ROS generation, at least in part, via oxidation of cytochrome C in the mitochondria. Moreover, p66Shc-mediated ROS
production induces PCa cell proliferation [31]. To determine whether p66Shc also promotes cell migration through ROS production, we first investigated the effects of ROS on PCa migration. We examined the effect of ROS on cell migration via transwell assay utilizing LNCaP-AS and MDA-AS cells which possess low migratory potential (Fig. 5.1C & 5.1D). As shown in Figure 5.3A, hydrogen peroxide treatment increased cell migration over 24 hours in a dose-dependent manner with 10µM having the optimal effect on both cell lines. To further investigate the effect of ROS on cell migration, a competitive inhibition transwell experiment conducted on the same cell lines using 10µM hydrogen peroxide, 10mM antioxidant N-acetylcysteine (NAC), or combined treatments. While hydrogen peroxide treatment significantly increased cell migration, Figure 5.3B demonstrate its enhanced effect is mitigated by combination treatment with NAC. NAC alone also reduces the basal migratory activity of both cell lines.

ROS can directly increase cell migration, therefore we determined whether p66Shc promotes cell migration via ROS production. We conducted 24-hour transwell assays using p66Shc stable subclones, i.e., S31, S32 and S36, and the corresponding vector-alone control cells were treated with 10mM NAC (Fig. 5.3C). NAC treatment completely mitigated the elevated migratory activities of the subclones, indicating ROS generation is a key mechanism of p66Shc-induced migration. To further explore p66Shc’s reliance on ROS to promote cell migration, LNCaP-AI cells were transiently transfected with the redox-deficient DN mutant p66Shc W134F cDNA [25,31], and cell migration was then analyzed. This p66Shc W134F mutant has been demonstrated to reduce overall cellular ROS levels in PCa cells [7,31]. As shown in Figure 5.3D, upon transfection, LNCaP-AI cell migration decreased in a dose-dependent manner. The migration assay was again conducted using an equally mixed population of p66Shc cDNA-transfected stable subclones and the corresponding vector-alone control. Figure
5.3E shows that upon transfection with the redox-deficient DN p66Shc W134F cDNA, the elevated migration by WT cDNA in the mixed subclone population was mitigated in a dose-dependent manner. Collectively, the data clearly shows that p66Shc promotes PCa migration via its ROS-production mechanism.

5.3.D Identification of p66Shc Down-Stream Targets and Signaling Profile

p66Shc plays a role in regulating PCa cell migration, thus to elucidate its mechanism of action, we analyzed its downstream signaling. To investigate changes in overall protein phosphorylation signaling initiated by p66Shc expression, we prepared whole cell lysates from an equally mixed population of p66Shc cDNA-transfected subclones (S31, S32, and S36) as well as V1 control cells and analyzed the molecular profile via a Kinex™ Antibody Microarray KAM900-P performed by the company. As shown in Figure 5.4A in the format of a percent change-from-control (%CFC) heat-map, a number of proteins had elevated activation through phosphorylation in the p66Shc cDNA-transfected subclones compared to vector-transfected control cells. Interestingly, the phosphatase PTEN, which plays a critical role in advanced CR PCa progression and is responsible for inactivation of some of these proteins, was found to be down-regulated in the subclones. Of the potential downstream-targets, we chose ErbB-2, AKT, mTOR, ERK, and PYK2 for further validation via western blot due to their association with PCa cell migration [38-43]. We also examined ROS-sensitive FOXM1 and its downstream target CDC25B, as well as Rac1 which are redox-sensitive and associated with migration [41,43-44]. We first performed immunoblot analysis to validate key molecules in V1 and mixed cell lysates used in the array analysis (Figure 5.4B) as well as individual subclone cell lysates (Figure 5.4C) to examine possible individual variation. We also analyzed the level of cellular prostatic acid phosphatase, cPAcP, because it is redox-sensitive [7] and shown to function as a prostate-specific tumor-suppressor-gene in part
by inactivating ErbB-2 [28,32,35]; thus its decreased expression is associated with PCa tumorigenicity and clinical progression. Significantly, as shown in Figure 5.4B-C, cPAcP protein level was down-regulated in p66Shc-subclones. Consequently, protein tyrosine kinase ErbB-2 and its down-stream targets AKT/mTOR, ERK, PYK2, and Rac1 were shown to have increased activity in the subclones compared to V1 cells [29-30,45]. The total protein level of FOXM1, which is regulated by AKT and ERK [46], was also elevated in subclones along with its downstream target CDC25B (Fig. 5.4B-C). Additionally, cell proliferation protein Cyclin B1 was also elevated in the subclones. Thus, the array analysis data is validated by western blotting and together the data clearly reveals migration-associated downstream targets of p66Shc.

5.3.E p66Shc Regulates Migration-Associated Proteins via ROS

To further validate down-stream proteins associated with p66Shc/ROS signaling, LNCaP-AI cells and mixed p66Shc subclone cells were transiently transfected with p66Shc shRNA and whole cell lysates were analyzed by western blot. As shown in Figures 5.5A and 5.5B, knockdown of p66Shc has the reverse, dose-dependent effect on each previously identified signaling target in Figure 5.4. To determine whether p66Shc regulates these proteins through ROS production, LNCaP-AI and mixed p66Shc-subclone cells were transiently transfected with increasing amounts of DN p66Shc redox-deficient mutant W134F cDNA (Myc-Tag) for immunoblot analysis. As shown in Figures 5.5C and 5.5D, inhibition of cPAcP protein and activation of all other proteins were found to be dependent on p66Shc’s ability to generate ROS. The ratio of phosphorylated or GTP-activated protein to respective total protein was quantified using the NIH ImageJ software. Collectively, the data validates p66Shc signaling targets via rescue experiments and demonstrates the signaling mechanism’s reliance on p66Shc-oxidase activity.
5.3.F Determination of Functional Molecules in p66Shc-Regulated PCa Cell Migration

We determined the functional molecules that play a critical role in p66Shc-mediated migration by treating p66Shc mixed subclone cells and V1 control cells with small molecule inhibitors and conducted a transwell migration assay (Fig. 5.6A). Due to the potentially significant impact of small molecule inhibitors on both cell proliferation and migration, the results were normalized to the growth inhibition in which cells’ 24-hour change in migration was divided by 24-hour change in growth. While inhibition of most functional proteins decreased the migration of p66Shc-subclones and V1 control cells to a similar level, inhibition of ERK and Rac1 had significantly greater impact on p66Shc subclone cells compared to V1 cells. In addition, as shown in Figure 5.6B, while no impact on cell migration was observed when cells were treated with FOXM1 inhibitor FDI-6 for 24 hours, pretreatments for 24 and 48 hours, i.e., for a total of 48 and 72 hours respectively, selectively inhibited subclone cell migration compared to V1 control cells (Fig. 5.6B). To determine if effects of FDI-6 on cell migration is entirely due to cell growth effect, a similar experiment was conducted measuring the FDI-6’s effect on cell growth over 24, 48, and 72 hours. The results showed that the inhibitory effect of FDI-6 on migration is greater than cell proliferation, thus showing FOXM1 contributes to PCa cell migration (Fig 5.6B).

We then validated the results via a molecular approach. We focused our efforts on determining the role of Rac1 in the mechanism of migration because its inhibition resulted in the greatest impact on the migratory activity of subclones compared to V1 control cells (Figure 5.6A). V1 control and mixed p66Shc subclones were transiently transfected with either vector-alone or HA-tagged dominant negative (DN) Rac1 T17N cDNA and a 24-hour transwell migration assay was then conducted. As shown in Figure
5.6C, while both transfected V1 and subclone cells had significantly reduced migration, subclone cells’ migration was reduced by 67% compared to only 33% of V1 cells. Conversely, parental LNCaP-AS (C-33) cells were transiently transfected with either vector-alone or HA-tagged constitutively active (CA) Rac1 G12V cDNA followed by a 24-hour transwell migration assay. LNCaP-AS cells transfected with CA Rac1 G12V possessed significantly increased migration compared to the vector alone-transfected cells. Western blot analysis of HA-tag was conducted to ensure cDNA transfection. Taken together, the data clearly shows all identified p66Shc-downstream proteins participate in regulating cell migration signaling. Furthermore, cDNA transfection experiments demonstrate Rac1 activation is critical to the mechanism of p66Shc-mediated migration.

5.3.G p66Shc Promotes Lamellipodia Formation via Rac1 Activation

Rac1 is a well-established regulator of lamellipodia formation, which is essential to cell motility [38,47]. Rac1 activation mediates p66Shc-induced migration, therefore we investigated the effect of p66Shc on lamellipodia formation. Initially, LNCaP-AS and –AI as well as V1 control and mixed p66Shc-cDNA transfected subclone cells were immunocytochemically stained with rhodamine phalloidin to visualize F-actin, which is enriched in the lamellipodia, and cells were observed using confocal microscopy (Fig. 5.7A). The ratio of lamellipodia to total cell area was semi-quantified using NIH ImageJ software. Figure 5.7A shows that in the LNCaP progression model, as cells progressed from AS to AI, there is an observed approximately 50% increase in lamellipodia size which correlates with p66Shc protein level and cell migration (Fig. 5.1C). Additionally, p66Shc cDNA-transfected subclones possess about 50% larger lamellipodia compared to V1 control cells (Fig. 5.7A), which correlates with observed migration (Fig. 5.2B). The data together demonstrates that p66Shc expression promotes lamellipodia formation.
To validate whether p66Shc-induced lamellipodia formation is mediated through Rac1, V1 and p66Shc cDNA-transfected stable subclones were transiently transfected with DN Rac1 T17N cDNA. As shown in Figure 5.7B, cells were then stained with rhodamine phallodin to visualize F-actin and with anti-HA-Tag to identify transfected cells. Upon DN Rac1 T17N cDNA transfection, the lamellipodia area of both V1 and p66Shc-overexpressing subclones was reduced by about 17% and 33%, respectively, thus a greater effect was observed in the p66Shc subclones. The combined data demonstrate p66Shc promotes lamellipodia formation in PCa cells through activation of Rac1, which increases their migratory activity.

5.4 Discussion

PCa is not life-threatening until it has metastasized to vital organs. Moreover, AS PCa is effectively treated by ADT, while CR PCa is a lethal disease with limited therapeutic options. Thus, to identify novel therapeutic targets for the treatment of metastatic CR PCa, we investigate the functional molecules that regulate CR PCa cell migration. The p66Shc protein has been demonstrated to promote AI proliferation of PCa cells through generation of ROS and its protein level correlates with acquisition of the CR phenotype of PCa cell lines [6,7,25,31]. Additionally, it is proposed that ROS can promote motility of PCa cells, suggesting p66Shc has the potential to regulate PCa cell migratory activity during metastasis [16-19]. In this study, p66Shc protein levels were found to be elevated in prostate adenocarcinoma tissue compared to the matched benign tissue (Fig. 5.1A). p66Shc protein level also correlates with androgen-independence and migratory activity in PCa cells, both in AR-null PC-3 and DU145 cell lines as well as both AR-positive progression model cell lines (Fig. 5.1B-D) [48,49]. This data reinforces the role of p66Shc in the development of androgen-independence of PCa cells and suggests it promotes the acquisition of aggressive migratory phenotype.
Further analyses demonstrate that p66Shc can regulate PCa cell migratory ability. While p66Shc cDNA transfection of low migratory-potential LNCaP-AS cells results in significantly enhanced migration, knockdown of p66Shc in high migratory-potential LN-AI cells with p66Shc shRNA significantly decreases cell migration in a dose-dependent manner (Fig. 5.2A-B). Moreover, the elevated migratory activity of p66Shc subclones was reduced in a dose-dependent manner upon p66Shc shRNA transfection (Fig. 5.2C). Together, the data demonstrates p66Shc has a key regulatory role in PCa migration.

We next determined the mechanism through which p66Shc regulates the migratory activity of PCa cells. p66Shc has been demonstrated to promote PCa growth via ROS generation, therefore the effect of ROS on AS PCa cell migration was first investigated [25]. Figure 5.3A-B shows hydrogen peroxide can promote the migratory activity of LNCaP-AS and MDA-AS cell lines in a dose-dependent manner, which is mitigated upon antioxidant NAC treatment. To validate that p66Shc promotes PCa migration through its ability to induce ROS generation, the effect of NAC on the migration of p66Shc subclone cells was investigated. NAC can reduce intracellular ROS levels of p66Shc subclones [31], and upon NAC treatment, increased migratory activity of subclones was fully mitigated (Fig. 5.3C). Further, upon transfection with redox-deficient DN p66Shc W134F cDNA, both LNCaP-AI and p66Shc subclone cell migration is reduced in a dose-dependent manner (Fig. 5.3D-E). Transfection of p66Shc W134F cDNA has previously been shown to reduce intracellular levels of ROS [7,31]. Together the data demonstrates the mechanism of p66Shc-induced PCa migration is reliant on p66Shc's oxidase activity and generation of ROS.

To identify the functional proteins involved in p66Shc-mediated migration, a global protein microarray was used to initially identify potential down-stream targets of
p66hc (Fig. 5.4A). The proteins chosen for further validation by immunoblot analysis are based on the findings of previous studies as well as their known association with cell migration. Briefly, p66Shc has been demonstrated to promote phosphorylation and activation of ErbB-2 at Y1221/2 through oxidation/inactivation of cPAcP, a phospho-protein tyrosine phosphatase [7,28-29,35]. This allows for activation of ErbB-2 downstream targets ERK and AKT/mTOR kinases by phosphorylation [7,25]. Though microarray data revealed both FAK and PYK2 adhesion proteins possessed increased activation in subclones, FAK has been shown to be the dominate adhesion molecule in prostate fibroblasts while PYK2 is the dominate protein in CR PCa adenocarcinoma; thus PYK2 was chosen for further study [30]. Moreover, while not included in the microarray, FOXM1 and its down-stream target CDC25B are up-regulated by ERK and AKT and shown to promote the metastatic phenotype in multiple carcinomas, though little is known of its role in PCa [46]. Additionally, Rac1 is a well-established regulator of cell migration and lamellipodia formation. Rac1 has been shown to be regulated by p66Shc in mouse embryonic fibroblasts as well as esophageal and breast carcinomas, however their interaction has not been studied in PCa [11,50,51]. Importantly, all of these proteins are confirmed to be activated by p66Shc in Figures 5.4B and C. To further validate p66Shc-regulation of each protein of interest, immunoblot analysis was conducted on LNCaP-AI and mixed subclone cells transfected with p66Shc shRNA and p66Shc oxidase-deficient mutant DN W134F cDNA. Figures 5.5A-D show all proteins were confirmed to be down-regulated in a dose dependent manner upon p66Shc knock-down and transfection of DN p66Shc W134F cDNA, with the exception of PAcP, a negative growth regulator [35], which was increased as expected. Thus, this data demonstrates these molecules are activated by p66Shc-mediated ROS generation.
The involvement of each protein in PCa cell migration was then investigated to determine its functional role in p66Shc-induced migration. As shown in Figure 5.6A and B, small molecule inhibitors were initially used to screen for the effects of each protein on the migration of p66Shc subclones and results were normalized to their respective 24-hour growth inhibition. While the FOXM1 inhibitor FDI-6 did not significantly inhibit migration over 24 hours, 48-hour pretreatment (72 hours total treatment) with small-molecule inhibitor FDI-6 significantly reduced the migration, but not growth, of subclones compared to V1 control cells. Moreover, p66Shc subclones are more sensitive to ERK and Rac1 inhibition compared to V1 cells, possibly because the cells are now reliant on these signaling pathways due to increased p66Shc levels. Overall, inhibition of Rac1 has the greatest impact on migration, thus, Rac1 was chosen for further validation through cDNA transfection. Shown in Figure 5.6C, upon transfection of V1 and p66Shc subclones cells with DN Rac1 T17N cDNA, cell migration was inhibited by about 30% and 70%, respectively, demonstrating p66Shc is reliant on Rac1 activation to induce PCa migration. Conversely, upon transfection of parental LNCaP-AS cells with constitutively-active Rac1 G12V cDNA, cell migration was increased by about 45%. Notably, there was no significant difference in migration between control V1 and parental LNCaP-AS cells, demonstrating transfection with vector alone has no phenotypic effect. Together, the data reveals while p66Shc-induced activation of multiple proteins contribute to increased PCa cell migration, Rac1 activation plays a mechanistic role.

It is established that the primary mechanism by which Rac1 promotes cell migration is through facilitating the formation of lamellipodia [11]. Therefore, lamellipodia formation was investigated and observed via confocal microscopy where F-actin, which is enriched in cell lamellipodia, was stained with rhodamine phalloidin. As shown in Figure 5.7A, the relative size of lamellipodia compared to total cell area increased as
LNCaP cells progressed from AS to AI, correlating with observed increase in p66Shc protein level and migration (Fig. 5.1C). Similarly, the size of lamellipodia was increased in p66Shc subclones compared to V1 cells, again correlating with an increase in cell migration (Fig. 5.2B). Furthermore, upon transfection of p66Shc subclones with DN Rac1 T17N cDNA, lamellipodia area was reduced by twice as much as V1 cells (Fig. 5.7B), which closely correlates with observed decrease in migration (Fig. 5.6C).

Collectively, the data demonstrates Rac1 activation is vital to the mechanism of p66Shc-induced lamellipodia formation and migration.

In summary, our results show that p66Shc promotes PCa metastatic progression through ROS-dependent induction of cell migration. As Figure 5.8 summarizes, p66Shc can be translocated to the mitochondria where it oxidizes cytochrome C, decoupling the electron transport chain and generating ROS. The increase in cellular ROS leads to inactivation of cPAcP, preventing de-phosphorylation of ErbB-2 and promoting activation of ErbB-2 downstream migration-associated proteins including: ERK, AKT, mTOR, FOXM1, PYK2, and Rac1. Nevertheless, there are additional mechanisms through which p66Shc may activate Rac1, independent of ErbB-2. Interestingly, p66Shc has been reported to activate Rac1 through cytosolic interaction with Son of Sevenless 1 (SOS1) protein [52]. ROS has also been shown to directly mediate Rac1 GDP-GTP nucleotide exchange and thus is another possible mechanism of p66Shc-mediated Rac1 induction [53]. The exact mechanism of p66Shc-Rac1 interaction regulating in PCa migration requires further investigation. We will also elucidate the role and mechanism of several other proteins in p66Shc-mediated PCa metastasis and validate with clinical relevance.

To conclude, this work highlights p66Shc's potential as a PCa biomarker for identification of aggressive metastatic phenotype as well as tumor progression towards
androgen-independence. Importantly, because p66Shc possesses enzymatic activity, it has the potential to be a potent therapeutic target for suppression of tumor proliferation and migration. Moreover, combined inhibition of p66Shc and its downstream target Rac1 may provide an effective method to combat metastatic CR PCa. Understanding p66Shc's involvement in advanced PCa progression will help determine its potential as a therapeutic target, and elucidating its mechanism of intracellular signaling will enable us to design new treatments for metastatic CR PCa.
Fig. 5.1 p66Shc protein level correlates with the CR phenotype and PCa cell migration. (A) Immunohistochemical staining of malignant and matched benign human prostate tissues with anti-p66Shc Ab, n=33. (B,C,D) Immunoblot staining for Shc and loading control β-actin using androgen-sensitive LNCaP (LN-AS/C-33), PC3, DU145 (B), androgen-sensitive (AS) C-33 and androgen-independent (AI) C-81 LNCaP (C) and AS and AI MDA-PCa2b (D) cell lines. The transwell assay was conducted in which cells were seeded in 24-well plate transwell inserts (5x10^4 cells per well) and allowed to migrate for 24 hours. Migrated cells were fixed and stained before counting. Images at 40x magnification. Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.
Fig. 5.2 p66Shc promotes PCa cell migration. (A) Immunoblot staining for Shc and loading control β-actin using LNCaP-AS C-33 cells transfected with vector-alone (V1) or p66Shc cDNA to generate stable subclones S32 and S36. The transwell assay was conducted in which cells were seeded in 24-well plate transwell inserts (5x10^4 cells per well) and allowed to migrate for 24 hours. Migrated cells were fixed with methanol and stained with crystal violet before counting. Images at 40x magnification. (B) V1 or equally mixed population of stable S31, S32, and S36 subclones transiently transfected with 0-6µg p66Shc shRNA or vector alone. Immunoblot staining and transwell assays were conducted as described in (A). (C) AI LNCaP C-81 cells transiently transfected with 0-6µg p66Shc shRNA or vector alone. Immunoblot staining and transwell assays were conducted as described in (A). Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.
Figure 5.3

A LNCaP-AS (C-33)  MDA-AS

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B

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<th>+NAC 10mM</th>
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<td>1.0 ± 0.3</td>
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C

Con 10mM NAC

D

p66Shc W134F cDNA

LN-AI (C-81) (0µg)  LN-AI (C-81) (1µg)

Correlation: r = -0.894

E

p66Shc W134F cDNA

V1 (0µg)  Subclones (6µg)

Correlation: r = -0.871
Fig. 5.3 p66Shc promotes PCa cell migration via ROS. (A) Transwell assay in which androgen-sensitive (AS) LNCaP C-33 or MDA-PCa2b cells were seeded in 24-well plate transwell inserts (5x10^4 cells per well), treated with 0-20µM hydrogen peroxide in the lower chamber, and allowed to migrate for 24 hours. Migrated cells were fixed and stained before counting. (B) Transwell assay as described in (A) in which AS LNCaP C-33 or MDA-PCa2b cells were treated with 10µM hydrogen peroxide, 10mM NAC, or both. (C) Transwell assay as described in (A) using V1 or stable subclones treated with or without 10mM NAC in the lower chamber of the transwell (D,E) Androgen-independent (AI) LNCaP C-81 (D) or V1 and equally mixed population of stable S31, S32, and S36 subclone cells (E) were transiently transfected with 0-6µg of dominate-negative redox-deficient p66Shc W134F cDNA or vector alone. Transwell assays were conducted as described in (A). Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.
Fig. 5.4 Identification of p66Shc down-stream targets by molecular profile. (A) Total cell lysates of V1 or equally mixed population of stable S31, S32, and S36 subclones analyzed via Kinexus KAM-900P protein microarray. Results presented as percent change from control (%CFC) in which red represents an increase and green a decrease in protein levels. (B,C) Immunoblot analysis of total cell lysates from V1 or equally mixed population of stable S31, S32, and S36 subclones used in the protein microarray (B) or V1 and individual stable S31, S32, and S36 subclones (C). Total and phosphorylated or GTP-activated proteins associated with migration were analyzed. β-actin was used as a loading control. n=3.
Figure 5.5

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**Fig. 5.5 p66Shc regulates migration-associated proteins via ROS.** (A,B)
Immunoblot analysis of androgen-independent (AI) LNCaP C-81 (A) or V1 and equally mixed population of stable p66Shc subclones (B) transiently transfected with 0-6µg p66Shc shRNA or vector alone. Total cell lysates were analyzed for previously identified total and phosphorylated or GTP-activated proteins associated with migration. Ratio of phosphorylated or GTP to total protein was quantified using ImageJ software. β-actin was used as a loading control. (C,D) Immunoblot analysis of AI LNCaP C-81 (C) or V1 and equally mixed population of stable subclones (D) transiently transfected with 0-6µg dominate-negative redox-deficient p66Shc W134F cDNA or vector alone. Immunoblot analysis was carried out as described in (A). n=3.
Figure 5.6

A

B

C

Figure 5.6
**Fig. 5.6 Determination of functional molecules in p66Shc-regulated PCa cell migration.** (A) Transwell assay using V1 or equally mixed population of stable subclones treated with small-molecule inhibitors (at IC50 in LNCaP cells) in the lower chamber of the transwell for functional migration-associated proteins previously identified. Cells were seeded in 24-well plate transwell inserts (5x10^4 cells per well) and allowed to migrate for 24 hrs. Migrated cells were fixed and stained before counting. Cell migration is normalized to small-molecule 24 hour growth inhibition. (B) Transwell assay using V1 or equally mixed population of stable subclones treated with 5µM FOXM1 inhibitor FDI-6 (IC50 in LNCaP cells). Cells were treated with FDI-6 in the lower chamber during the 24 hour transwell or with additional 24 or 48 hour pre-treatment (For a total of 24, 48, and 72 hours of FDI-6 treatment). FDI-6 effect on cell growth of V1 or equally mixed population of stable subclone cells was determined. Cells were seeded at 3x10^3 cells/cm^2 and allowed to attach for three days before treated with 5µM FDI-6 for 24, 48, and 72 hrs. Cells were trypsinized and live cell number was counted. (C) V1 or equally mixed population of stable subclones were transiently transfected with HA-Tagged dominate-negative Rac1 T17N cDNA or vector alone. AS LNCaP C-33 cells were transiently transfected HA-Tagged constitutively-active Rac1 G12V cDNA or vector alone. Transwell assay was conducted as described in (A). Successful transfection was determined via immunoblot of whole cell lysates for HA-Tag. β-actin was used as a loading control. Results presented are mean ± SE; n=3x3. *p<0.05; **p<0.001; ***p<0.0001.
Figure 5.7

A

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![Graph](Graph1)

![Graph](Graph2)

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![Graph](Graph3)

![Graph](Graph4)
**Fig. 5.7 p66Shc promotes lamellipodia formation via Rac1 activation.** (A) Androgen-sensitive (AS) C-33 and androgen-independent (AI) C-81 LNCaP, V1, and equally mixed population p66Shc subclone cells were stained with F-actin binding rhodamine phalloidin (Red) to visualize lamellipodia and DAPI (Blue) to detect nuclei. The ratio of lamellipodia to total cell area of 20 randomly selected cells was quantified. (B) V1 and equally mixed population subclone cells were transfected with HA-Tagged DN Rac1 cDNA and stained with F-actin binding rhodamine phalloidin (Red) to visualize lamellipodia, DAPI (Blue) to visualize nuclei, or anti-HA-tag (Green) to visualize dominant-negative Rac1 cDNA transfected cells. The ratio of lamellipodia to total cell area of 20 randomly selected cells was quantified. Results presented are mean ± SE; n=20x3. *p<0.05; **p<0.001; ***p<0.0001.
Fig. 5.8 Proposed mechanism of p66Shc-regulated PCa cell migration. Upon elevation of protein level, p66Shc translocates to the mitochondria where it binds and oxidizes cytochrome c, decoupling the electron transport chain, and generating ROS. Increased cellular ROS oxidizes cellular prostatic acid phosphatase (cPACP), preventing it from dephosphorylating ErbB-2. Phosphorylated ErbB-2 then activates downstream targets PI3K/AKT/mTOR, ERK, FOXM1, PYK2, and Rac1, all of which contribute to PCa cell migration. p66Shc may also activate Rac1 via SOS1 in the cytoplasm or through ROS-mediated Rac1 GDP-GTP nucleotide exchange.
References

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   10.1002/ijc.11621

   10.1016/j.freeradbiomed.2012.03.024


Chapter 6

Conclusions
6.1 Summary

The overall goal of my work is to advance therapeutic strategies for treatment of CR PCa, a currently incurable, lethal disease. Importantly, due to our laboratory’s access to multiple progressive PCa cell line models, we have a unique opportunity to investigate aspects of the disease in a clinically relevant system. In my first body of work, I investigate a panel of novel imidazopyridine derivatives as potential therapeutic agents for CR PCa and investigate their mechanism of inhibition. This group of compounds was selected for their known ability to suppress AKT activation, a primary downstream target of AR which promotes androgen independence as well as aggressive metastatic phenotype. The template compound HIMP was altered through the addition of various of substituent groups in hopes of synthesizing a compound effective at duel inhibition of AKT and AR pathways. The simultaneous inhibition of both pathways is key due to their ability to cross-activate and compensate for suppression of the other.

Initial screening of the compounds’ ability to suppress LNCaP-AI (C-81) cell growth in regular and steroid-reduced conditions revealed compounds M-MeI and EtOP maintain their inhibitory activity, regardless of the presence of androgens. Further screening of these compounds on a panel of castration-resistant cell lines as well as immortalized benign prostate epithelial cells exposed M-MeI’s selective inhibition of CR PCa. Further tumor phenotypic assays confirmed M-MeI as the comparatively superior tumor suppressor and immunoblot analysis show it is a potent inhibitor of both AR and AKT activation. Additional cell-cycle analysis show M-MeI arrests the cell cycle in G2 phase as well as strongly induces apoptosis. However, the precise mechanism of its inhibitory activity remains unclear. Thus, M-MeI shows promise as future therapeutic agent for
treatment of CR PCa due to its selective growth inhibition and duel suppression of both the AR and AKT pathways.

In my second body of work, I investigate the effects of novel statin derivatives on CR PCa metastatic activity. While the imidazopyridines were studied for their ability to combat AKT-mediated androgen-independence, the use of statin derivatives aims to prevent de novo androgen synthesis by PCa cells, an alternative method of over-coming androgen ablation therapy. By preventing cholesterol synthesis, statins effectively deny PCa cells of precursor compounds necessary to synthesize androgens. One of the major draws of statins as therapeutic agents is that their side-effects on humans are well studied and overall exceptionally mild compared to those of other drugs used to treat CR PCa. In this study, template compound simvastatin is synthesized in its constitutively active form (SVA) and several substituents were again added to the structure in attempt to modulate its activity. Significantly, while simvastatin’s growth suppression of LNCaP-AI (C-81) cells is reduced under steroid-deprived conditions, the activities of the novel statin derivatives remain unaltered. Most importantly, while derivatives AM1 and AM2 demonstrated minimal selective inhibition of PCa cell growth compared to benign epithelial cells, SVA was very selective for PCa growth inhibition. Additional assays for colony formation and cell migration revealed SVA was a more potent suppressor of PCa metastatic phenotype than simvastatin, while AM1 and AM2 were less effective. Further evaluation demonstrated SVA was a more potent suppressor of cholesterol synthesis than simvastatin and can induce cell membrane damage via permeabilization. Interestingly, while the addition of androgens can mitigate SVA inhibition of AR, its inhibition of cell growth remains unchanged, pointing to additional mechanisms of PCa tumor suppression
beyond preventing *de novo* androgen synthesis. I speculate this is due to SVA’s damaging effect on the cell membrane by preventing lipid raft formation, which anchors multiple proteins required for pro-tumorigenic signaling. Further immunoblot analysis shows SVA is a more potent inhibitor of AR, ErbB-2, AKT, and multiple pro-proliferative proteins compared to simvastatin. Perhaps most importantly, SVA is demonstrated to have an added effect when combined with chemotherapeutic agent docetaxel. Docetaxel is sometimes used to treat end-stage CR PCa and possesses notoriously toxic side effects. My hope is that novel compound SVA can be used in conjunction with existing therapeutic agents for CR PCa in effort to maintain anti-tumor effects and reduce patient toxicity. Moreover, like the imidazopyridine derivatives, SVA demonstrates potent duel inhibition of both AR and AKT pathways, making it a promising treatment compound.

Finally, in my third body of work I step away from the investigation of novel therapeutic agents and instead focus on studying p66Shc, a protein previously identified to promote androgen-independent growth through the generation of ROS. Our goal is to identify proteins regulating processes critical to PCa metastasis to act as biomarkers for aggressive tumor phenotype and serve as novel therapeutic targets for future compounds. ROS has been shown to promote migratory activity in other tumor cell lines, thus we theorized p66Shc also promotes PCa migration in mechanism similar to its growth regulation. First, p66Shc protein level was demonstrated to not only correlate with PCa progression to androgen independence, but also the migratory activities of various PCa cell lines. Next, p66Shc cDNA transfection was demonstrated to increase migration of androgen-sensitive LNCaP cells, while shRNA transfection reduced migration of both
androgen-independent LNCaP cells and p66Shc over-expressing subclones. p66Shc-mediated migration was then shown to be reliant on generation of ROS. Treatment of p66Shc subclones with antioxidant NAC or transfection with redox-deficient p66Shc mutant W134F cDNA reduced the cells’ migration and similar results were observed using androgen-independent LNCaP cells (C-81). The mechanism of p66Shc-induced migration was further explored using protein microarray and immunoblot analyses to identify key signaling molecules in p66Shc cDNA transfected LNCaP cells. The results were then verified using immunoblot analysis of LNCaP-AI (C-81) and p66Shc subclones transfected with shRNA in which the opposite signaling patterns were observed. Additional immunoblot analysis of LNCaP-AI (C-81) and p66Shc subclones transfected with redox-deficient p66Shc W134F cDNA demonstrated p66Shc regulation of down-stream signaling targets was reliant on its ability to generate ROS. Downstream p66Shc targets ErbB-2, AKT, mTOR, ERK, Rac1, PYK2, and FOXM1 were then demonstrated to contribute to PCa cell migration with Rac1 having the greatest impact. Rac1’s involvement in migration was further verified via cDNA transfection and demonstrated to mediate p66Shc-induced lamellipodia formation using F-actin staining. Overall, the study demonstrated for the first time that p66Shc plays a critical role in the process of CR PCa migration and identifies numerous proteins involved in the mechanism. My hope is that this work supports future efforts to generate therapeutic compounds for CR PCa patients and that these proteins may serve as biomarkers for early detection of aggressive, metastatic PCa.
6.2 Future Directions

While the results of these studies are promising for future treatment of CR PCa, more work is required to apply them to a clinical setting. In the case of both imidazopyridine and statin derivatives, while they selectively suppress PCa tumorigenicity, the precise mechanism of action remains unclear. Imidazopyridines encompass a large class of compounds that bind and inhibit a wide variety of cellular targets. While M-MeI is a potent inhibitor of AR and AKT, it is uncertain whether it binds these proteins directly or suppresses them through inhibition of up-stream regulators. The reason M-MeI arrests the cell cycle at G2 phase is also obscure and additional analysis is necessary. Moreover, while M-MeI shows selectivity in vitro, in vivo experiments are required to test its toxicity, a major concern for potential clinical use. Furthermore, it remains unclear whether imidazopyridine derivatives would be able to reach the tumor in an in vivo system or be readily absorbed by tumor cells. In addition, the concentration of M-MeI required to observe cytotoxic effects is around 10 µM in steroid-reduced conditions. This concentration is likely on the upper end of clinically achievable levels, thus additional side-chain modification to improve the potency of M-MeI is required for future study. Further side-chain modification may also endow M-MeI with additional tumor-suppressing effects. Future studies should also test combinations of M-MeI with other FDA-approved drugs in attempt to produce additive or synergistic therapeutic effects. Finally, while the imidazopyridine derivatives are effect against CR PCa cells, they may also be useful tools for treating other carcinomas. The AKT axis is commonly deregulated in most cancers, thus it is reasonable to suspect M-MeI may be applicable to a wide range of tumors.
Future work on the statin derivatives is similar to that of the imidazopyridine compounds. First, their mechanism of PCa inhibition requires further clarification. While SVA clearly reduces cellular cholesterol levels, we are uncertain whether it inhibits the cells’ ability to synthesize androgens \textit{de novo}. Furthermore, the results indicate inhibition of androgen synthesis is not the driving mechanism of tumorigenicity suppression; instead it is likely that reduction of cellular cholesterol impedes lipid raft formation, which anchors proteins critical to PCa signaling. Future work on these compounds should investigate the effect of statin compounds on lipid rafts and how formation of these structures regulates AKT and AR signaling. In addition, like the imidazopyridines, it is uncertain what SVA is binding to in the cell. While it almost certainly binds and inhibits HMG-CoA reductase, it may also have a number of off-target effects including direct AKT or AR binding. While the toxicity of the drug is expected to be low, \textit{in vivo} experiments are still required to demonstrate its clinical application. Importantly, SVA has an added effect when combined with docetaxel and may have similar properties when used together with other compounds. Docetaxel is also a commonly used chemotherapeutic agent and because SVA inhibits AKT, a widely deregulated protein in multiple carcinomas, it can likely be used to treat multiple cancers in addition to PCa. Finally, as with M-MeI, SVA can be further modified in order to increase potency and potentially give it additional tumor-suppressing properties. Both M-MeI and SVA show exceptional promise as therapeutic agents for CR PCa due to their duel suppression of AR and AKT as well as selective inhibition. Future investigation of their mechanism of inhibition and improvement on their structure will enhance the clinical application of these compounds.
While the investigation of novel therapeutic compounds is important for the future of PCa research, it is also necessary to continue examining the underlying mechanisms of PCa progression towards castration-resistance and aggressive metastatic phenotype. My third body of work demonstrates elevation of p66Shc protein level as a driving force behind CR PCa migration and identifies a number of key proteins in the mechanism, notably Rac1. While the mechanism in which p66Shc activates ErbB-2 and its downstream signaling cascade clearly revolves around oxidation and inactivation of cPAcP, it is not immediately known how p66Shc interacts with Rac1 in PCa. Rac1 may be activated by ErbB-2, ROS, or SOS1-mediated p66Shc binding. Further investigation of their interaction will be the subject of future studies.

Presently, the next step in this investigation is to carry out *in vivo* experiments to determine whether p66Shc similarly induces migration in a mouse model as well as investigate its potential as a therapeutic target. In addition, there is currently no inhibitor compound available for p66Shc, thus this may also be the subject of future work. Moreover, inhibition of p66Shc may not be appropriate for all stages of PCa due to its promotion of ROS generation. Early stages of PCa typically have lower levels of ROS and may benefit from p66Shc-mediated ROS production, thus p66Shc may be a good target for these cells. However, late-stage PCa may have much higher basal levels of ROS, which induces cellular stress and in some cases apoptosis. Inhibition of p66Shc in these cells may actually aid their survival. Therefore, it is reasonable to assume aggressive metastatic PCa cells, which possess elevated p66Shc levels, must also have elevated anti-oxidant molecules to regulate ROS production and keep it at optimal concentrations. This is the current focus of our laboratory’s work. Targeting antioxidant
molecules in metastatic CR PCa has the potential to be an effective therapeutic strategy by impeding their ability to regulate ROS produced by elevated p66Shc levels. In addition, combined inhibition of p66Shc as well as some of its downstream signaling partners such as Rac1, ErbB-2, ERK, AKT, mTOR, and PYK2 may also be an effective means of treatment. Finally, while p66Shc has been identified as a critical promoter of aggressive metastatic phenotype and acquisition of castration-resistance, the manner through which it is regulated in PCa cells is little understood. Only a small number of transcription factors have been verified to bind p66Shc’s promoter and while AR protects p66Shc from ubiquitination and degradation, the mechanism is unclear. Most importantly, the mechanism governing p66Shc translocation into the mitochondria is currently obscure. While ERK/JNK phosphorylation of p66Shc at S36 has been demonstrated to induce mitochondrial translocation, this process is associated with p66Shc-induced apoptosis and not cell growth. Furthermore, the precise mechanism through which p66Shc moves across mitochondrial membrane is a mystery. Thus, a significant amount of work is still required to fully understand how p66Shc functions in PCa and in what manner its signaling mechanism can be exploited for future therapeutic strategies.