CHOLESTEROL METABOLISM AS A TARGET IN CASTRATION-RESISTANT PROSTATE CANCER

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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ABSTRACT

Despite clinical benefits of existing prostate cancer treatments, patients continue to develop therapeutic resistance. Persistence of androgen receptor pathway activity is attributed to several mechanisms associated with resistance, including intratumoral androgen receptor agonist synthesis from the precursor cholesterol. Cholesterol has been correlated to poor outcomes in patients and clinically the use of cholesterol synthesis inhibitors, statins, improves prostate cancer survival. The expression of the high-density lipoprotein-cholesterol receptor, scavenger receptor B1 (SR-B1), is elevated in castration-resistant prostate cancer models and has been linked to poor survival of patients. The overarching hypothesis of this thesis is that cholesterol modulation, through either synthesis or uptake inhibition, will impact essential signaling processes impeding the proliferation of prostate cancer. Clinically statin use was found to improve the overall survival of metastatic castration-resistant prostate cancer patients receiving the androgen synthesis inhibitor abiraterone. In vivo experiments demonstrated the ability of statins to impede post-castration biochemical recurrence and reduce tumor growth and androgen receptor agonist synthesis in LNCaP-derived xenograft tumors. SR-B1 was found to be overexpressed in clinical samples from both local and metastatic prostate cancer. Antagonism of SR-B1 in steroid responsive C4-2 cells decreased cholesterol uptake and growth and induced cell cycle arrest. Initially attributed to an observed decrease in de novo steroid synthesis and androgen receptor activity, the inability of exogenous steroid to restore cellular proliferation or androgen receptor activity indicated steroid independent cellular arrest. As such, cellular stress and nutrient deprivation responses were assessed and SR-B1 antagonism was found to induce both autophagy and endoplasmic reticulum stress markers. Given the steroid-independent manner of SR-B1 antagonism mediated cellular arrest, the effects of SR-B1 antagonism on androgen independent PC-3 cells was assessed and found to result in robust cellular death in vitro and decreased growth of xenograft tumors. These findings demonstrate that the reduction of cellular cholesterol availability can impede prostate cancer proliferation through both decreased steroid-synthesis and steroid-independent mechanisms providing a potential therapeutic target for the treatment of prostate cancer.
LAY SUMMARY

Prostate cancer is managed by therapies that block the production of androgen sex hormones, or inhibit activation of the androgen receptor. Resistance to these therapies is inevitable due to several adaptations, including the tumor acquiring the ability to produce its own androgens. In addition to the production of androgens, cholesterol is essential for numerous cellular functions needed to maintain metabolism for rapid tumor growth. The research described in this thesis demonstrates that antagonizing the cancers ability to obtain cholesterol impedes tumor growth and the production of essential androgens, while clinically cholesterol lowering drugs improve outcomes of patients with advanced stage prostate cancer. Lastly a key protein for the import of cholesterol into the cell, scavenger receptor B1 is identified as a novel therapeutic target in advanced prostate cancer. Highly expressed in prostate cancer, the inhibition of scavenger receptor B1 reduces cancer growth through the reduction of androgens and other mechanisms. In summary, the findings of this thesis highlight the potential of targeting cholesterol metabolism as an approach to prostate cancer treatment.
PREFACE

This thesis was conducted at the University of British Columbia (UBC) and Vancouver Prostate Centre (VPC) with support from clinicians and researchers at the British Columbia Cancer Agency (BCCA). Contributions of collaborators are acknowledged below.

Chapter 2: The work assessing clinical outcomes of prostate cancer patients at the British Columbia Cancer Agency has become a component of a multi-institutional international study recently accepted for publication in the peer-reviewed journal *Oncotarget*. The British Columbia portion of the study was carried out under the supervision of Dr. Bernhard J. Eigl (BCCA). Database construction was performed by Dr. Jenn Locke (VPC) and myself. Univariate statistical analysis was performed by me and multivariate statistical modelling was performed by Dr. Gregory Pond (McMaster University). Data interpretation described herein is my own while interpretations found in the multi-institutional study were collaborative among listed authors. All clinical work was carried out in accordance with human ethics protocol: H13-01851 CRPC Retrospective Hormonal Agents Database.

The *in vivo* experiments described in the latter half of the chapter have been published and peer-reviewed in the journal *Prostate Cancer and Prostatic Diseases* in the year 2016. All experiments were designed and conducted by myself under the supervision of Dr. Kishor M. Wasan and Dr. Michael E. Cox. General assistance was provided by Mr. Ankur Midha and Dr. Yubin Guo, while LC-MS assistance was provided by Mr. Andras Szeitz and Mr. Hans Adomat under the supervision of Dr. Tara Klassen and Dr. Emma S. Guns respectively. All animal experiments in this chapter were carried out in accordance with UBC animal ethics protocol: UBC ACC A12-0211.

Publications:


Chapter 3: The experiments assessing the therapeutic potential of targeting SR-B1 are currently undergoing peer-review for publication. All experiments described in this chapter were designed and conducted by myself under the supervision of Dr. Kishor M. Wasan and Dr. Michael E. Cox unless otherwise stated. Immunohistochemical staining was conducted courtesy of Dr. Colm Morrissey and scored by Dr. Gang Wang and Dr. Fatemah Derakshan. Access to the Shanghai Cohort mRNA expression database was provided courtesy of Dr. Collin Collins and access to the Fred Hutchinson Rapid Autopsy mRNA expression database courtesy of Dr. Colm Morrissey. Fluorescent imaging was conducted with the assistance of Mr. Jonathan Frew.

Publications:


Chapter 4: The work assessing BLT-1 use in vivo will in part be published alongside findings from Chapter 3 currently undergoing peer-review for publication. All experiments were designed and conducted by myself with assistance from Dr. Emma S. Guns under the supervision of Dr. Kishor M. Wasan and Dr. Michael E. Cox. General animal support was provided by Ms. Mei Chin and LC-MS support and expertise provided by Mr. Hans Adomat. All animal experiments in this chapter were carried out in accordance with UBC animal ethics protocol: UBC ACC A16-0072.

Publications:

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<tbody>
<tr>
<td>4E-BP1/2</td>
<td>eIF-4E Binding Proteins</td>
</tr>
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<td>ABCA1</td>
<td>ATP-Binding Cassette Transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-Binding Cassette Transporter G1</td>
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<tr>
<td>ACAT</td>
<td>Acyl-CoA Cholesterol Acyltransferase</td>
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<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
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<td>ADT</td>
<td>Androgen Deprivation Therapy</td>
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<td>Albumin</td>
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<td>Alanine Transaminase</td>
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<td>AMP-Activated Protein Kinase</td>
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<td>AMY</td>
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</tr>
<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen Response Element</td>
</tr>
<tr>
<td>Atg12</td>
<td>Autophagy-related Protein 12</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve</td>
</tr>
<tr>
<td>BCR</td>
<td>Biochemical Recurrence</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding Immunoglobulin Protein</td>
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<tr>
<td>BLT-1</td>
<td>Block Lipid Transport-1</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>BUN</td>
<td>Blood Urea Nitrogen</td>
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<tr>
<td>C/EBP</td>
<td>Ccaat-Enhancer-Binding Proteins</td>
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<td>CA</td>
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<td>CaMKK-β</td>
<td>Calmodulin-Dependent Protein Kinase Kinase 2</td>
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<td>CL_{int-app}</td>
<td>Apparent Intrinsic Clearance</td>
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<td>Clu</td>
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<td>Cmax</td>
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<td>Creatinine</td>
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<td>CRPC</td>
<td>Castration-Resistant Prostate Cancer</td>
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<td>CTLA4</td>
<td>Cytotoxic T Lymphocyte-associated Antigen 4</td>
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<td>DBD</td>
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<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
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<td>DHEAS</td>
<td>Dehydroepiandrosterone Sulfate</td>
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<td>DiI</td>
<td>1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate</td>
</tr>
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<td>Dimethyl Sulfoxide</td>
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<td>Digital Rectal Exam</td>
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<td>EBRT</td>
<td>External Beam Radiation Therapy</td>
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<td>Abbreviation</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>eIF2α</td>
<td>Eukaryotic Initiation Factor 2</td>
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<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
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<tr>
<td>ETS</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FPKM</td>
<td>Fragments per Kilobase of Transcript per Million Mapped Reads</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<td>Mammalian Target of Rapamycin</td>
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<td>NAFLD</td>
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<td>NEPC</td>
<td>Neuroendocrine Prostate Cancer</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells</td>
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<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>RFU</td>
<td>Corrected Florescence</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
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<td>Site 1 Protease</td>
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<tr>
<td>S2P</td>
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</tr>
<tr>
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<td>S6 Kinase 1</td>
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<tr>
<td>SC</td>
<td>Shanghai Cohort</td>
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<td>SCAP</td>
<td>SREBP Cleavage-activating Protein</td>
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<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
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<tr>
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<td>Steroidogenic Factor-1</td>
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<tr>
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<td>Sonic Hedgehog</td>
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<tr>
<td>SpiP</td>
<td>Sphingosine-1-Phosphate</td>
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<td>Squalene Monooxygenase</td>
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<tr>
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<td>Scavenger Receptor B1 Knock Down</td>
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<td>Steroidogenic Acute Regulatory Protein</td>
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<tr>
<td>TBIL</td>
<td>Total Bilirubin</td>
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<tr>
<td>TBS-T</td>
<td>Tris-buffered Saline, 0.1% Tween 20</td>
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<td>TCGA</td>
<td>The Cancer Genome Atlas-PRAD</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
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<td>Tumor-Node-Metastasis</td>
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<td>TNF Receptor-Associated Factor 2</td>
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<tr>
<td>TRAMP</td>
<td>Transgenic Adenocarcinoma Mouse Prostate</td>
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<td>Urogenital Sinus Epithelium</td>
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<td>Urogenital Sinus Mesenchyme</td>
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<tr>
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<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<td>-------------------------------------------------</td>
</tr>
<tr>
<td>ULDL</td>
<td>Ultra Low Density Lipoprotein</td>
</tr>
<tr>
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<td>Unc-51 Like Autophagy Activating Kinase</td>
</tr>
<tr>
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<td>Very Low Density Lipoprotein</td>
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<td>Wheat Germ Agglutinin</td>
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<td>X-box Binding Protein 1</td>
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DEDICATION

This work is dedicated to my loving parents John and Darlene, brothers Sam and Jonah, partner Irene, grandparents Roy and Doreen, the farm girls Aunt Eileen and Aunt Sharon, the Brodeur-Eyers and Afonsos and lastly my dearly departed grandparents Donald and Barbara. Although she will never see the culmination of my years of graduate studies Grammy G’s loving impact will forever be with me.
CHAPTER 1: INTRODUCTION

1.1 Prostate cancer epidemiology

An estimated 206,200 Canadians will have been diagnosed with new cases of cancer in 2017\(^1\). Of these new cases 21,300 will be cancers of the prostate accounting for 20.7% of the new cancers in men and making prostate cancer (PCa) the 4\(^{th}\) most diagnosed form of cancer following cancers of the lung, colorectal region and breast. A vast majority of men diagnosed with the disease are over the age of 50 years (>99.9%) and approximately 85% of patients are diagnosed after the age of 65 years, while a majority of men aged above 85 years show histological evidence of PCa\(^2\). The relative five and ten year survival rates for prostate cancer are 99% and 96% respectively largely due to the near 100% survival rates of those diagnosed with local disease\(^3\). However, those with distant disease have a relative 5 year survival rate of only 29%. In total this translates to an expected 4,000 deaths due to prostate cancer in 2017 in Canada.

Both environmental and genetic factors have been deemed to play contributory roles in the risk of developing PCa. The presence of PCa within the immediate family has been shown to increase the risk of developing PCa, such that having one first-degree relative with the disease doubles PCa risk and two first-degree relatives increasing risk by up to 11-fold\(^2,4\). Racial and ethnic background further appears to have association with PCa risk with African-Americans having amongst the highest rates and in general the lowest rates being found among Asian populations. Although contributory effects of culture and environment cannot be fully eliminated several potential genetic links have been identified\(^5-7\). Environmental factors can include diet, tobacco use and occupational exposure to chemicals such as pesticides\(^8,9\). Dietary studies have found a correlation between the consumption of red-meat as well as high-fat diets to be positively associated with the development of PCa\(^10-12\). While in contrast the consumption of tomato and soy based products has been correlated to a reduction in the risk of developing PCa\(^2,13\).
1.2 Structure and development of the prostate

The prostate is an exocrine gland in the male reproductive system that functions to secrete alkaline fluids contributing to the motility, and liquefaction of seminal fluids promoting successful fertilization\textsuperscript{14,15}. The adult prostate consists of three regions, the central zone, the transition zone and the peripheral zone each of different embryologic origin and structurally contains epithelial ductal regions supported by smooth muscle stroma. The prostate gland originates from the urogenital sinus (UGS), in contrast to the majority of male accessory sex gland that develop from the Wolffian ducts\textsuperscript{16}, and occurs during the second and third trimester of pregnancy involving cross signaling between two components of the UGS, the UGS mesenchyme (UGM) and the UGS epithelium (UGE)\textsuperscript{16,17}. Essential to the differentiation and development of the prostate is the expression of the androgen receptor (AR), a nuclear hormone receptor activated by natural ligands testosterone, and the more potent testosterone derivative dihydrotestosterone (DHT). Produced predominantly in the testes of developed men, and to a smaller extent by the adrenals and ovaries of women, androgen activation of the AR induces the expression of a large number of genes\textsuperscript{18,19}.

Androgens initially act on the UGM, where AR activation initiates paracrine signaling that drives the outgrowth of UGE derived epithelial buds into the surrounding UGM\textsuperscript{20}. The precise pattern of this branching leads to the development of the mature glandular secretory epithelium\textsuperscript{21}. The developing UGE will in turn promote UGM differentiation into the fibroblast and smooth muscle tissue observed in the developed prostate. Several paracrine signaling pathways have been identified as playing important roles in the development of the prostate including sonic hedgehog (SHH), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ) and insulin-like growth factor (IGF)\textsuperscript{16}.

1.3 Androgen receptor

Although not the primary focus of this thesis the AR is considered to be the central driver of PCa and the focus of therapeutic approaches to disease management for patients with disseminated disease. In brief, the AR belongs to the steroid hormone group of nuclear receptors which also includes the estrogen
receptor, and the glucocorticoid receptor among others. The 110 kDa protein, similar to other nuclear receptor family members contains three major functional domains, the N-terminal domain (NTD), the DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD). The NTD contains the activation function -1 regions essential for AR transactivation, while the highly conserved DBD interacts with the DNA promoter and the LBD provides a binding pocket for androgens. Once bound by DHT, or other weaker activating androgens, displacing heat-shock proteins and binding importin-α the AR translocates into the nucleus. Within the nucleus AR dimers bind to the androgen response element (ARE) promoting the transcription of growth and survival genes.

1.4 Androgens and steroid hormones

Beyond the prostate, androgens and similar steroid hormones are an important part of the endocrine system responsible for proper sexual development and function, metabolic homeostasis and also help to regulate inflammation and immunity. Androgens are 19 carbon, four carbon ring structures that are synthesized from the precursor cholesterol (Figure 1.1). Testosterone, containing a ketone and hydroxyl functional group on opposing ends, is the primary circulating androgen in males with significant levels of DHT and dehydroepiandrosterone sulfate (DHEAS) also being consistently detectable. Controlled by the hypothalamic-pituitary-adrenal-gonadotropic axis, androgen production takes place primarily in the Leydig cells of the testes with the adrenal cortex also producing DHEA. Initially, the hypothalamus releases luteinizing hormone-releasing hormone (LHRH) which stimulates the pituitary to in turn release luteinizing hormone (LH) and adrenocorticotropin hormone (ACTH). LH then stimulates testosterone production in the testes while ACTH stimulates the adrenals to produce DHEA. Testosterone is synthesized by the initial uptake of cholesterol into the mitochondria of steroidogenic cells by steroidalogenic acute regulatory protein (StAR) followed by side-chain cleavage of cholesterol into pregnenolone by CYP11A1 (P450scc). This is followed by hydroxylase and lyase steps by CYP17A1 and reduction steps by 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD resulting in end product testosterone.
Figure 1.1: Pathway of testosterone and DHT synthesis
The synthesis of AR-activating androgens testosterone and DHT from precursor cholesterol through the standard (blue) and back-door pathways (red) displaying steroid structures and primary enzymes.

Circulating testosterone is mostly bound to serum sex hormone-binding globulin and albumin\textsuperscript{37,38}, with debate surrounding the relative importance of free diffusion and protein bound endocytosed cellular testosterone uptake\textsuperscript{39,40}. Once testosterone enters the prostate cell it is converted to the more potent AR activating androgen DHT by 5α-reductase functioning to remove the Δ4,5 carbon double bond\textsuperscript{41}. Other
substrates for 5α-reductase include progesterone, androstenedione and aldosterone. 5α-reductase inhibitors finasteride and dutasteride have been used to treat benign prostatic hyperplasia and hair loss in men.\(^{42,43}\)

DHT is generally not highly excreted from tissue and is metabolized in the peripheral tissue in which it is generated.\(^{44,45}\) Phase I metabolism of DHT by various isoforms of 3α/β-HSD and 17β-HSD enzymes generates several constituents including androstenedione and androsterone.\(^{46-48}\) However, potentially all of these tissues also possess the required HSD isoforms to convert the metabolized constituents back into DHT and expression of phase I enzymes vary significantly between prostatic cell types.\(^{48-51}\) It is believed that these constituents as well as DHT are capable of undergoing phase II metabolism elimination. The UDP-glucuronosyltransferase (UGT) family of glucuronosyltransferases catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to various targeting molecules, including androgens, generally resulting in a more polar and water soluble molecule for easy excretion.

The expression and role of individual UGTs in prostate cancer is still a debated topic with implicated isoforms including UGT2B15 and UGT2B17.\(^{52,53}\)

### 1.5 Prostate cancer development

Tumorigenesis and malignant cancer development has long been understood to be a genetic disease in which genetic mutations drive pre-malignant cells to overcome an inherent system of checks and balances eventually leading to unabridged growth.\(^{54}\) Famously summarized by Hanahan and Weinberg, these genetic mutations can be stratified into specific categories critical for malignant transformation.\(^{55,56}\) Termed “Hallmarks of Cancer” these phenomena include sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis.

Although PCa is generally considered a cancer-type with low mutational burden and substantial heterogeneity, several oncogenic mutations are consistently observed.\(^{54}\) In fact, recent sequencing efforts have indicated that up 74% of primary prostate cancers can be stratified into one of seven subtypes.
characterized by either gene fusions or mutations. Gene fusion events focus primarily on the E26 transcription specific (ETS) family of transcription factors (ERG, ETV1, ETV4, and FLI1) largely with androgen regulated genes including TMPRSS2, SLC45A3, and NDRG1. While common mutations observed are SPOP, FOXA1, and IDH1. Other common, but not of the defined subtypes, drivers include loss of functional phosphatase and tensin homolog (PTEN), p53 and retinoblastoma protein (pRB) tumor suppressors. A vast majority of PCas including those with AR driven mutations and those with non-AR related mutations express significant levels of the AR and validated AR target genes. This understanding combined with the success of therapeutics that interrupt AR signaling and the mechanisms by which the cancers evade these AR targeted therapies, discussed below, highlight the understanding that a majority of PCas require sustained AR signaling to maintain proliferative signaling.

1.6 Diagnosis, Gleason grading and staging of prostate cancer

Initial PCa diagnosis generally consists of two important tests, the digital rectal exam (DRE) and serum prostate specific antigen (PSA) measurement. A DRE allows for the detection of an enlarged prostate and presence of abnormal nodules. The subjective nature of the test can lead to large amounts of inter-examiner variability and is further limited due to it primarily addressing the dorsal prostate. Serum PSA although used to aid in the diagnosis of PCa is primarily used to monitor changes in cancer growth and progression as a correlate for monitoring tumor burden. PSA itself is an AR regulated serine protease part of the kallikrein protease family, produced by prostatic epithelial tissue and secreted into the lumen where it functions to cleave semenogelin I/II aiding in seminal fluid liquefaction. Normally serum levels of PSA are low but can be elevated in response to several factors including PCa, benign prostatic hyperplasia, prostatitis, urinary tract infection or trauma. Abnormal findings with these tests are normally a precursor to a prostate biopsy for histopathological confirmation of cancer.

Originally developed in 1966 by Donald Gleason the Gleason grading system has since been used to evaluate the prognosis of men diagnosed with PCa. Grading is based on the histological examination of hematoxylin and eosin stained prostate biopsy specimens. The specimens are graded from 1,
characterized by a well-contained nodular lesion with well-differentiated glands, to grade 5 characterized by poorly differentiated, stromal invasive cells often lacking glandular formation. As the obtained specimens often harbor multiple Gleason patterns the Gleason scoring system was developed in which the most common, primary, grade is combined with the second most common, secondary, grade yielding a sum score with a higher number indicating a poorer prognosis. However, importantly the presence of high-grade tertiary pattern is scored as the second component even if it is the third most present pattern.

Updates to the traditional Gleason grading system have been made, most notably in two distinct forms. In 2005 the modified Gleason system was introduced by the International Society of Urologic pathology. In summary the modified system essentially removed both 1 and 2 Gleason scores, citing low reproducibility and inability to properly diagnose by needle biopsy and shifted certain growths including cribriform growths from Gleason grade 3 to 4. These changes led to an increase in the diagnosis of high Gleason grade cancers and is generally considered to correlate better to clinical stage and patient outcome. In 2016, the second major update to the Gleason grading system was proposed in which patients are stratified into “Gleason grade groups” based on the correlation of specific Gleason scores to progression free survival rates. The update included five groups of increasing odds of recurrence following primary therapy. Grade group one includes all Gleason scores less than or equal to 6, grade group two includes Gleason scores of $3 + 4 = 7$, grade group three includes Gleason scores of $4 + 3 = 7$, Gleason group four includes Gleason scores equal to 8 and grade group five includes Gleason scores of 9 or higher. The five year biochemical recurrence free percentages following radical prostatectomy are 96% for grade group one, 88% for grade group two, 63% for grade group three, 48% for grade group four and 26% for grade group five.

Beyond Gleason scoring the tumor-node-metastasis (TNM) staging system for the classification of malignant tumors is a widely used standardized system for the classification of cancer. The system describes the primary tumor (T) on a scale of 1 to 4 depending on the extent of external tissue invasion, spread to the lymph node (N) generally ranked from 1 to 3 based on regional or distant lymph node involvement and metastasis (M) present (1) or absent (0). The findings of this clinical staging process
combined with the Gleason scoring of the biopsy results provide critical information in guiding treatment decisions and prognosis.

1.7 Local therapies for prostate cancer

Often times patients with low risk localized disease will defer active treatment opting instead for an active surveillance approach involving increased frequency of PSA and DRE testing and prostate biopsy\textsuperscript{67}. The increasing popularity of this approach has helped decrease instances of over-treatment and the associated adverse effects. For local cancers considered too high risk for a surveillance approach, based on factors described above, the potentially curative approaches of radical prostatectomy or radiation therapy are generally considered. Radical prostatectomy involves the complete removal of the prostate and surrounding tissues with the objective of removing all cancers tissue. This approach is generally considered for lower risk patients with disease that remains encapsulated to the prostate due to concerns over the ability to clear all cancerous tissue surgically in more disseminated disease\textsuperscript{68}. The procedure is associated with a 10 year progression free survival rate of 88\% in patients with low to intermediate risk disease\textsuperscript{69}. Radiation therapy involves the use of ionizing radiation to kill malignant cells and is generally considered for patients with extracapsular disease. External beam radiation therapy (EBRT), in which ionizing radiation is externally applied to cancer, is associated with a 10 year progression free survival rate of 90\% and is the most common approach for intermediate to high risk PCa patients\textsuperscript{70}. Brachytherapy, the insertion of sealed source radiation beads into or adjacent to the cancerous prostatic tissue is also a treatment option for generally low-risk PCa patients.

For men with no evidence of metastatic disease these local therapies successfully remove or kill a vast majority of the tumor leading to the observed high progression free survival rates. These numbers are further bolstered by the heterogeneous nature of primary PCa tumors in that only an extremely limited number of PCa cells possess the required mutations to escape the primary site and proliferate as a distant metastasis\textsuperscript{71}. However, the highly unstable nature of these rapidly dividing cells drives genomic evolution of populations of cells that not only display metastatic potential but also allow for the development of
therapeutic resistance\textsuperscript{72}. If these primary therapies fail to remove all cells with metastatic potential or if metastatic populations already exist, whether dormant or of undetectable size, the likelihood of recurrence is increased. For the approximately 20\%-30\% of surgery or radiation therapy patients that do eventually recur following local treatment or those that present with high-risk localized or metastatic disease on diagnosis systemic approaches are required\textsuperscript{73,74}.

\textbf{1.8 Androgen deprivation therapy}

Of those patients requiring systemic approaches, whether with confirmed metastatic disease or progressed local disease, androgen deprivation therapy (ADT) has long been the mainstay approach, functioning to reduce AR-mediated cell signaling through the reduction of circulating gonadal testosterone\textsuperscript{57,75,76}. Used as a catch-all term for the reduction of circulating testosterone different approaches have been used since the advent of the therapy. These approaches include surgically through bilateral orchiectomy, or with small molecule LHRH agonist and antagonists\textsuperscript{75,76}. LHRH acting agents, such as goserelin or leuprolide, decrease signaling through the hypothalamic-pituitary-adrenal-gonadotropin axis leading to a near total reduction in testosterone production and similar or slightly improved survival rates to orchiectomy but may result in increased adverse events\textsuperscript{77,78}. With the non-invasive and reversible nature of small molecule approaches compared to surgery, LHRH agents are the most common and preferred method of ADT.

ADT is believed to induce a therapeutic response in approximately 90\% of patients and provide varying progression-free and overall survival benefits depending on previous treatment and its use as a primary, adjuvant or neoadjuvant therapy\textsuperscript{79}. In men with advanced disease ADT has approximate progression free and overall survival times of 16 months and 36 months respectively, however beneficial effects may be limited to quality of life improvements as opposed to true survival benefits\textsuperscript{75,80,81}. However, when given as an adjuvant to EBRT, ADT has been shown to improve outcomes in a number of studies\textsuperscript{68,75,82}, with increases in five year overall survival by up to 26\% compared to solo radiation therapy and increases in five year disease free rate of up to 37\%. As such the National Comprehensive
Cancer Network (NCCN) recommends ADT plus EBRT for patients with high-risk disease. Despite these described benefits ADT, when given alone or with EBRT, the approach is not considered curative as a majority of patients will eventually suffer disease recurrence into what is termed castration-resistant prostate cancer (CRPC). Traditional findings place the median overall survival rate between 12 months and 18 months for patients with CRPC, however recent investigations of the modern CRPC landscape indicate median overall survival times could be up to 40 months or higher. This large improvement in median overall survival is likely a result of the growing number of therapies available to CRPC patients and the improved implementation of said therapies described below.

1.9 CRPC and mechanisms of progression

As described above PCas that fail primary ADT are termed CRPC. The multiple mechanisms by which the cancer overcomes ADT are still being elucidated and remain an intense area of research. The observed increase in PSA, an AR regulated protein, and the observed successes of abiraterone and enzalutamide in castrate-resistant progression reflects the continued activation of the AR despite castrate-level serum testosterone and as such a majority of CRPC mechanisms revolve around the AR axis.

Increased expression of the AR has been correlated to CRPC via gene amplification and may be present in up to 30% of CRPCs. Further, increased activation and expression of the AR through the loss of pRB has been demonstrated as a potential driver of castrate-resistant progression. pRB is a critical negative regulator of tumor development through the suppression of cell cycle progression via complex mechanisms including the suppression of activator E2Fs. The loss of pRB expression has been demonstrated in up to 73% of CRPCs and is thought to both lead to increased AR-target gene occupancy and AR mRNA dysregulation leading to increased AR expression. Efforts of impeding AR activity directly have been made through the use of current generation AR antagonists, enzalutamide, and traditional agents, such as flutamide and bicalutamide.

Several alterations directly to the AR have been proposed as drivers for castrate-resistant progression. Thought to increase in frequency through the selective pressure of anti-androgens AR
mutations may be present in up to 50% of CRPCs. These mutations include alterations in the NTD altering cofactor binding, and mutations in the LBD conferring a loss of ligand specificity. The first described mutation to drive a loss in specificity was a threonine to alanine (T877A) substitution in LNCaP based models which has since been confirmed in up to 30% of bone metastases. This mutation allows for AR activation in response to estrogens, progestins and the activated form of flutamide. Further mutations have since been documented allowing activation by alternative steroid molecules, estrogens, corticosteroids and androgen precursor progesterone.

The identification of splice variants provided a further AR based explanation for the development of CRPC. Several of these variants result in the deletion of the exons coding the LBD leading to a constitutively active AR lacking the LBD. These splice variants are thought to be upregulated in the presence of anti-androgens such as enzalutamide conferring resistance to multiple levels of androgen targeted therapies. Further, AR based mechanisms include alterations in post-translational modification and changes in co-regulator function. An in depth discussion of these mechanisms is described by Karantanos et al. Efforts to overcome these splice variants include the development of DBD antagonists such as EPI-506 currently undergoing early clinical trials.

Several mechanisms of castration-resistant progression independent of AR pathway activity have been proposed. C-myc expression is known to play a critical role in prostate cancer development, has been shown to be amplified in up to 72% of CRPCs and prostate cancer cell growth under ADT conditions has been correlated to c-myc expression. Alterations in the phosphoinositide 3-kinase (PI3K) signaling pathway, notably PTEN loss, are also thought to play a role with very high incidence of alterations in metastatic cancers and lead to alterations in protein kinase B (AKT) and mammalian target of rapamycin (mTOR) signaling. Several compounds targeting these pathways have progressed to early clinical trials, including PX-866 and GSK2636771 as PI3K inhibitors and GSK2141795 and AZD5363 as AKT inhibitors, but to date none have achieved clinical success.

Despite the number of proposed mechanisms of castration-resistance, the concept of de novo steroidogenesis has proven to be perhaps the most clinically validated and actionable. Despite continued
castrate serum levels of testosterone it has been found that intratumoral levels of both testosterone and
DHT remain similar to levels found in hormone-naïve cancers\textsuperscript{121}. Further, evidence suggests that essential
enzymes in the androgen synthesis pathway are stimulated within the tumor microenvironment and that
PCa bone metastases are capable of converting adrenal derived androgens to testosterone and DHT\textsuperscript{122-125}. Mechanisms by which the tumor can synthesize DHT bypassing testosterone have also been identified
often referred to as the “backdoor pathway” (Figure 1.1)\textsuperscript{126,127}. From which precursor the late stage
androgens in CRPC are derived is currently a debated topic with likely multiple contributing pathways.
Testicular androgens have been demonstrated to potentially only account for as little as 60\% of the total
androgen content of the prostate with the remainder likely a result of the conversion of adrenal derived
DHEA to testosterone and DHT (Figure 1.2)\textsuperscript{128-130}. This understanding has led to the belief that adrenal
derived DHEA is the predominant source of testosterone precursor following ADT\textsuperscript{131-133}. However, a
significant portion of the research describing the phenomenon of \textit{de novo} steroidogenesis has been
performed in mouse based xenograft models\textsuperscript{123,126,134}. Mice are reported to not secrete DHEA or other
androgens from their adrenals which highlights the ability of testosterone production, at least in
experimental models, to be derived from precursor cholesterol within the tumor\textsuperscript{123,135-138}. While not fully
elucidated it appears likely that tumor testosterone production occurs from both cholesterol and adrenal
derived DHEA. The importance of these mechanisms is emphasized by the success of abiraterone
clinically, further described below\textsuperscript{139-141}.

1.10 Other androgen receptor pathway targeted treatments

In most cases advanced PCa is treated by an LHRH agent in concert with the use of an AR
inhibitor to reduce testosterone related side effects and further aid in the shutdown of AR
signaling\textsuperscript{57,75,76,142}. Common traditional AR inhibitors include flutamide and bicalutamide which
competitively bind to the LBD of the AR preventing activation and nuclear translocation. In theory the
addition of AR inhibitors to ADT prevent AR activation by remaining gonadal derived androgens, non-
gonadal derived androgens and mute any effects of the testosterone flair associated with LHRH agents.
However, the extent to which this combinatorial approach correspond to an improvement in overall survival is minimal at best\textsuperscript{80,143-145}. The inability of these agents to improve the survival of men undergoing ADT has been attributed primarily to both a lack of potency and concerns of partial agonism of mutated AR\textsuperscript{146,147}. Despite uncertainty surrounding survival benefits AR inhibitors are believed to aid in pain relief and other side effects associated with testosterone flare\textsuperscript{148}. As such traditional AR antagonists are still commonly prescribed, however with the recent successes of current generation AR antagonist enzalutamide and CYP17A1 inhibitor abiraterone the treatment landscape is rapidly changing.

\textbf{Figure 1.2: Mechanisms of cellular cholesterol and androgen metabolism in CRPC}

Cholesterol is taken up into the cell through membrane transporters LDLr and SR-B1 and synthesized via the mevalonate pathway and HMGCR. Cellular cholesterol can be converted for storage as cholesteryl ester by ACAT, metabolized from cholesteryl ester to cholesterol by HSL and excreted from the cell by ABCA1. Prior to conversion to DHT and AR-activation testosterone can be obtained by the cell through uptake from circulation or synthesis from circulation derived DHEA, or cellular cholesterol.
Enzalutamide, first approved for clinical use in 2012, has come to represent the first of potentially several current generation AR antagonists. Driving candidate selection for a novel AR antagonist was the attempt to account for the shortcomings of traditional AR antagonists such that pre-clinically enzalutamide (IC_{50}: 36 nM) demonstrates at least four-fold higher binding affinity for the AR compared to bicalutamide (IC_{50}: 159 nM) and further impairs nuclear translocation and coactivator recruitment of the AR appeasing concerns over agonist activity in mutated AR\textsuperscript{147}. Although improving on the potency of traditional antagonists’ enzalutamide still pales in comparison to the potency of the primary AR activating agonist DHT (IC\textsubscript{50}: 5.1 nM)\textsuperscript{147}. Clinically enzalutamide has been shown to significantly increase both the progression free and overall survival of men with metastatic CRPC in both pre and post chemotherapy settings\textsuperscript{149,150}. The AFFIRM trial showed an improved overall survival from 13.6 months to 18.4 months with enzalutamide in metastatic CRPC patients that had previously received docetaxel chemotherapy while the PREVAIL trial demonstrated a 51\% increase in 12 month progression free survival rate in chemotherapy naïve metastatic CRPC patients receiving enzalutamide\textsuperscript{150,151}. As such enzalutamide is seeing use in both pre-chemotherapy and post-chemotherapy patients. Clinical trials are ongoing testing the effectiveness of enzalutamide for first line use alongside androgen deprivation therapy (ENZAMET, NCT02446405).

Abiraterone is a current generation inhibitor of CYP17A1, thereby inhibiting the synthesis of AR activating androgens. A pregnenolone analogue originally developed by the Institute of Cancer Research in London following the limited success of the anti-fungal ketocanazole in the 1990s which demonstrated limited inhibition of CYP17A1 abiraterone was abandoned due to the widespread belief that post-ADT recurrent PCa, then referred to as hormone-refractory disease, proliferated independently of the need for androgens and further concerns of adrenal insufficiency with the inhibition of CYP17A1. However, as the community’s understanding of the continued importance of AR-mediated signaling in post-ADT recurrent PCa evolved, interest returned and following changes in licensing and successful clinical trials, abiraterone was approved for clinical use in 2011. Initially shown to improve the survival of men with metastatic CRPC following chemotherapy, similarly to enzalutamide, it has since been demonstrated to be
efficacious in a pre-chemotherapy context\textsuperscript{75,141,151,152}. The COU-AA-301 trial demonstrated an improvement in median survival of 15.8 months versus 11.2 months with the use of abiraterone in post-chemotherapy metastatic PCa while the COU-AA-302 trial showed improved median progression free survival of 16.5 months versus 8.3 months in chemotherapy naïve metastatic CRPC patients\textsuperscript{153-155}. As such both abiraterone and enzalutamide are currently in use as late-stage agents for men suffering from metastatic CRPC in both a pre- and post-chemotherapy setting. However, recent reports from the STAMPEDE and LATITUDE trials have demonstrated the potential of abiraterone as an adjuvant agent co-administered with LHRH agents. The STAMPEDE trial reported a 13.9 month improvement in mean failure free survival time with abiraterone and ADT compared to ADT alone. While the LATITUDE trial found an improvement of 18.2 months in median progression free survival time with the addition of abiraterone to ADT in patients with advanced PCa\textsuperscript{139,140}. These promising results indicate that abiraterone use at the time of ADT will likely soon see widespread implementation.

Despite the benefits observed with both enzalutamide and abiraterone several mechanisms of resistance have been proposed for the eventual failure observed in several patients. These mechanisms include the upregulation of CYP17A1 and the AR, as well as the proliferation of populations possessing altered AR function including mutations allowing for activation by pre-CYP17A1 steroid precursors or constitutively active AR splice variants\textsuperscript{156,157}. As with standard ADT, The potential role of constitutively active AR splice variants, through the loss of the LBD, has been considered as a mechanism of resistance\textsuperscript{158}. Interestingly recent evidence has demonstrated that patients possessing the constitutively active AR splice variant AR-V7 can still show therapeutic response to both enzalutamide and abiraterone, perhaps highlighting the heterogeneous nature of the disease\textsuperscript{159}. Although the improvements in survival and quality of life seen with the current generation of AR pathway targeted therapeutics should not be discounted, the continued inability to fully interrupt AR signaling despite the multiple approaches trialed drives a need for further therapies.
1.11 Chemotherapeutic approaches to CRPC treatment

For patients that fail or are ineligible for AR targeted therapies the main approaches are anti-neoplastic chemotherapeutic agents. Chemotherapeutic agents consist of a large family of anti-cancer drugs used for a large range of cancer types and situations. Despite generally being considered poor responders to chemotherapeutics, PCa patients do see limited benefit to specific agents making them common clinical agents. Mitoxantrone, a topoisomerase inhibitor, was the first approved chemotherapeutic for use in PCa in 1996. Although known to have little effect on overall survival mitoxantrone was shown to provide palliative benefits and improved PSA response rates\textsuperscript{160,161}. In 2004 mitoxantrone was replaced after the taxane based docetaxel demonstrated improved overall survival in patients with CRPC. In phase III trials the compound was shown to improve survival of patients by approximately 3 months compared to mitoxantrone\textsuperscript{162-165}. Docetaxel, and other taxanes, function by binding and stabilizing microtubule filaments which in turn physically prevent cellular division leading to a forced mitotic arrest and cell death\textsuperscript{166}. The use of docetaxel with first-line ADT for highly advanced PCa was demonstrated to improve median overall survival by more than 12 months in the CHAARTED and STAMPEDE trials\textsuperscript{167,168}. As such, the NCCN now includes within its guidelines the use of docetaxel with EBRT and ADT for fit men with high risk disease. Although docetaxel remains the most commonly used chemotherapeutic for CRPC the second generation taxane cabazitaxel, designed for docetaxel-resistant cancers, was approved in 2010. Unlike other taxanes cabazitaxel is not a substrate of p-glycoprotein (MDR1) a known small molecule efflux protein driver of therapeutic resistance and has demonstrated a survival benefit of approximately 2.4 months over mitoxantrone in CRPC patients previously treated with docetaxel\textsuperscript{169-171}. Further chemotherapeutics such as platinum and anthracycline-based compounds have shown little or no survival benefit in PCa and as such don’t see regular use in the clinic\textsuperscript{172,173}. 
1.12 Immunotherapies and other systemic treatments for CRPC

Beyond AR axis targeted therapies and chemotherapeutics a handful of other therapeutics have been approved and seen sporadic use in the clinical treatment of CRPC. With bone being the most common site for the development of metastatic lesions in PCa several bone targeted therapies have been developed\(174\). These therapies are largely used in palliative approaches as bone metastasis often result in severe pain alongside nerve compression and myelosuppression which can have significant impacts on patient quality of life. These therapies include the RANKL targeted human monoclonal antibody, denosumab, for bone loss and various radiopharmaceuticals. These radiopharmaceuticals include \(^{89}\)Sr and \(^{153}\)Sm which emit \(\beta\)-particles and \(^{223}\)Ra which emits \(\alpha\)-particles. Demonstrated to improve survival by approximately three months and have comparably minimal side effects, \(^{223}\)Ra is the most commonly used bone targeted agent for patients suffering from severe pain due to bone metastasis\(^{162,175,176}\).

The recent advent of clinically approved immunotherapies has highlighted the ability to activate the immune system to recognize cancer cell antigens and induce anti-tumor responses\(^{177,178}\). Despite the successes of immunotherapy treatment for other cancers to date only one immunotherapy has been approved for the treatment and demonstrated improved survival of metastatic CRPC patients. The reason for the relatively poor success of immunotherapies in PCa is not fully understood and likely varies between the range of differing immunotherapy approaches but has been attributed to a highly immunosuppressive tumor microenvironment and low mutational burden\(^{179}\). Approved for minimally asymptomatic metastatic patients by the FDA in 2010 and demonstrating an approximate four months improvement on median survival, sipuleucel-T remains the only approved immunotherapy in PCa\(^{180}\).

Using an autologous approach, blood mononuclear cells are transfected with recombinant human prostatic acid phosphatase (PAP), an enzyme expressed in approximately 95% of prostate cancers, and granulocyte-macrophage colony stimulating factor, a known immune cell activator\(^{181-184}\). This process results in active autologous antigen presenting cells and also contains T cells, B cells and natural killer cells which are infused into the patient leading to an anti-PAP immune response\(^{181,185}\).
Other immunotherapeutic approaches are being investigated for use in PCa. Cytotoxic T lymphocyte-associated antigen 4 (CTLA4), an important negative regulator of regulatory T cell responses which has demonstrated efficacy in melanoma, was trialed for use in PCa but did not show improvements in overall survival despite initially promising effects on PSA declines and antitumor activity\textsuperscript{186-189}. Programmed cell death protein 1 (PD-1), another immune checkpoint inhibitor, is currently being investigated with tempered expectations following the failure of CTLA4 for use in PCa with similarly mixed initial results\textsuperscript{190,191}. PROSTVAC is viral-based immunovaccine currently in phase III trials for use in metastatic CRPC. The regimen includes the initial delivery of vaccinia vectors containing PSA and immune stimulatory molecule transgenes followed by successive boost shots in a fowlpox vector\textsuperscript{192,193}. This approach leads to a strong immune response to the viral proteins, including PSA, resulting in the creation of anti-PSA cytotoxic T cells capable of attacking tumor cells. Phase II trials of PROSTVAC demonstrated an 8.5 month improvement in median overall survival of patients with metastatic CRPC compared to those receiving control empty vectors\textsuperscript{194}.

1.13 Cholesterol structure and distribution

As androgen axis targeting remains central to the management of CRPC the essential steroid precursor, cholesterol, must be considered as part of a comprehensive understanding of the disease. Cholesterol is a multifunctional lipid that plays numerous essential cellular functions including in the context of CRPC serving as a precursor molecule for the synthesis of AR driving androgens. Consisting of 4 hydrocarbon rings and a hydroxyl group and carbon chain on opposite ends, cholesterol is an important component of the cellular membrane helping to regulate membrane fluidity and participates in several membrane trafficking and signaling processes\textsuperscript{195}. Further, cholesterol functions as a precursor to steroids, bile acids and vitamin D\textsuperscript{195}. Our understanding of cellular cholesterol trafficking by in large comes from studies involving fibroblasts and only more recently have intercellular differences come to light\textsuperscript{195}. The study of cholesterol transport in vertebrates has been, for the most part, conducted in mice or
rats, providing valuable insight but coming with significant caution due to the differing lipid profile between mice, rats and humans\(^{196}\).

Within the mammalian cell cholesterol is stored in the form of hydrophobic cholesteryl esters in lipid droplets and non-ester cholesterol distributed heterogeneously between cellular membranes, accounting for 20-25\% of the membranes lipid content within the plasma membrane and similarly high concentrations in the endocytic recycling compartment, trans-Golgi compartment and to a lesser extent the endoplasmic reticulum (ER)\(^ {197-199}\). The rigid four ring sterol backbone of cholesterol results in its positioning adjacently to saturated lipid chains in internal and external membranes, increasing lateral lipid ordering resulting in a less fluidic section of the membrane\(^ {200}\). The clustering of these lipids and cholesterol in small area make up important cell signaling interfaces known as lipid rafts\(^ {195}\). Lipid rafts host numerous different proteins and cell signaling families including glycosylphosphatidylinositol-anchored proteins, cholesterol-linked and palmitoylated proteins, such as hedgehog, and doubly acylated proteins including Src-family kinases or the $\alpha$-subunits of heterotrimeric G proteins\(^ {201-203}\). The presence of the sterol sensing domain on several critical membrane embedded and anchored cholesterol regulatory proteins including HMG-CoA reductase (HMGCR), sterol regulatory element-binding protein cleavage-activating protein (SCAP) and Niemann-Pick C1 (NPC1) provides a binding site for cholesterol\(^ {204}\). However, the promiscuous nature of the domain means that cholesterol may not be a common ligand and only SCAP has been shown to specifically bind cholesterol\(^ {205}\).

### 1.14 Cellular cholesterol metabolism

Cells can obtain new cholesterol through two mechanisms, cholesterol synthesis through the mevalonate pathway and the uptake of circulating cholesterol (Figure 1.2). The mevalonate pathway functions to produce cholesterol, isoprenoids and other sterols from acetyl-CoA which itself is a result of glucose, glutamine or acetate consumption\(^ {206}\). The rate limiting enzyme in the mevalonate pathway is the integral ER membrane protein HMGCR, notably the target of the statin class of drug molecules\(^ {207,208}\). HMGCR and other enzymes in the mevalonate pathway including HMG-CoA synthase and squalene
synthase are regulated at the transcriptional level by sterol regulatory element binding protein (SREBP). SREBP is itself a membrane bound protein that resides in the ER and is transported to the Golgi in sterol-deplete conditions bound to COPII vesicles. This translocation of SREBP is prevented by sterol induced conformational changes in the SCAP and insulin induced gene 1 (INSIG) chaperones, in which INSIG functions as an ER anchor while SCAP functions as an escort for SREBP to the Golgi. Once within the Golgi SREBP is sequentially cleaved by the site 1 (S1P) and site 2 (S2P) proteases which in turn release the NTD of SREBP allowing for its entrance into the nucleus and to function as a transcription factor. Conversely the transcription factor liver x receptor (LXR) functions to remove cholesterol from the cell increasing biliary sterol secretion. Active in the presence of oxysterols LXR target genes include several important cholesterol metabolism proteins including ATP-binding cassette transporter-A1 (ABCA1) and -G1 (ABCG1) which efflux cholesterol from the cell as well as CYP7A1 the first and rate limiting enzyme in conversion of cholesterol into bile acids. Once cholesterol is fully synthesized it is rapidly removed from the ER largely by non-vesicular mechanisms that bypass the Golgi. The cholesterol can be targeted towards the plasma membrane, into the mitochondria by the StAR protein for steroid synthesis, or esterified by acyl-CoA cholesterol acyltransferase (ACAT) for storage in lipid droplets.

The inhibition of HMGCR, and thus cholesterol synthesis, by statins leads to increased tissue uptake of circulating LDL and thus decreased circulating cholesterol. Initially isolated from the mold *Penicillium citrinum* in the 1970s, the statin class of therapeutics now includes seven clinically used agents that have become the most commonly used agents for the treatment of hypercholesterolemia. As such these compounds have an established role in primary and secondary cardiovascular prevention being shown to improve all-cause mortality and decrease the incidence of major coronary events. Beyond the reduction of cholesterol production, the inhibition of HMGCR is known to result in a number of unintended effects largely through the reduction of HMG-CoA-cholesterol intermediates. The inhibition of HMGCR by statins results in reduced synthesis of mevalonate, the precursor for isoprenoid generation including farnesyl pyrophosphate and geranyl pyrophosphate. These isoprenoids notably aid in the...
anchoring of Ras and Rho proteins to the plasma membrane and help facilitate prostate cancer proliferation\(^ {225,226}\).

The second mechanism by which cells obtain cholesterol is by uptake from circulating lipoproteins largely through the low density lipoprotein receptor (LDLr) and scavenger receptor B1 (SR-B1). The LDLr is a type I transmembrane protein which resides on the plasma membrane with an external facing N-terminal ligand binding domain and a cytosol facing C-terminal domain that regulates endocytosis and intracellular transport\(^ {227}\). Circulating LDL particles bind to the LDLr after which the particle is endocytosed by clathrin coated vesicles and transported to acidic endocytic compartments where acid lipase hydrolyses cholesteryl esters into free cholesterol which are then moved out of the endosome by NPC1 and Niemann-Pick C2 (NPC2) proteins\(^ {228,229}\). The LDLr is regulated similarly to HMGCR through SREBP and LXR mediated ubiquitination and lysosomal degradation and is ubiquitously expressed with high expression in the liver and steroidogenic tissues\(^ {230,231}\).

1.15 Scavenger Receptor B1

Scavenger receptor class B type 1 (SR-B1), encoded by the \(SCARB1\) gene, is a highly glycosylated cell surface receptor that contains two transmembrane domains located near to the cytosolic N- and C-terminal domains\(^ {232-234}\). Functioning to selectively uptake cholesterol from circulating high density lipoprotein (HDL) SR-B1 is ubiquitously expressed, similarly to LDLr, but shows particularly high expression in the liver as well as steroidogenic tissues including the adrenal glands, ovaries and testes\(^ {235,236}\). Importantly with regard to the work described in this thesis, SR-B1 appears to be differentially regulated between the liver and steroidogenic tissues. In steroidogenic cells SR-B1 appears to be primarily regulated by trophic hormones (LH, follicle stimulating hormone [FSH], ACTH)\(^ {232,233,236-238}\). While the expression of SR-B1 in liver cells can be effected by dietary fats and pharmacologic agents notably fibrates, on top of hormonal control\(^ {236,239}\). Prolonged adrenal stimulation by ACTH has been shown to induce SR-B1 expression in adrenocortical cells and reduce hepatic expression in mice and rats likely indicating a mechanism for preferential channeling of cholesterol to the adrenal tissue\(^ {233,240,241}\). On a
cellular level, SR-B1 has been shown to be regulated in rather a complex manner including genetic transcription factor binding sites for SREBP, steroidogenic factor-1 (SF-1), LXR, liver receptor homolog 1 and others. Evidence demonstrates that SR-B1 regulation by trophic hormones may be dependent on cAMP/protein kinase A signaling stimulating transcriptional function of SF-1 and Ccaat-enhancer-binding proteins (C/EBP), while cholesterol regulation of SR-B1 has also been demonstrated through SREBP and LXR functions implicating multiple signaling pathways participation in SR-B1 expression.

Beyond cholesterol uptake SR-B1 and HDL have been implicated in several cellular signaling processes. SR-B1 has been shown as a critical receptor in the ability of HDL to induce activation of endothelial nitric oxide synthase (eNOS) leading to, at least in part, the athero-protective effects associated with HDL. The activation of eNOS in turn leads to increased concentrations of nitric oxide (NO) known to have a number of effects leading to improved cardiovascular health including vasodilation, endothelial regeneration, and inhibition of leukocyte chemotaxis. Further, the presence of HDL and eNOS activation are thought to prevent SR-B1 mediated induction of apoptosis in endothelial cells. Several pathways have been identified as potential mechanisms for this SR-B1 dependent signaling. HDL is known to carry several lipids beyond cholesterol including lipid soluble vitamins and sphingolipids. Although insufficient on its own, sphingosine-1-phosphate (Sp1P) when associated with HDL has been shown to be involved in the activation of eNOS. The mechanism by which Sp1P or other SR-B1 inducers drive eNOS activation is believed to be through the PI3K signaling pathway. HDL binding to SR-B1 has been shown to lead to the activation of Src which in turn activates both the PI3K/AKT and RAS/ERK1/2 pathways resulting in eNOS activation through serine phosphorylation. Although the relationship between SR-B1 and these signaling pathways has largely only been established in endothelial cells, aberrations in the PI3K pathway and the RAS pathway, have been identified and implicated in advanced PCa and highlight the importance of their consideration when studying SR-B1 in the context of PCa.
1.16 Lipoproteins and cholesterol transport

Cholesterol is transported throughout the blood stream complexed within lipoproteins, both on the surface as native cholesterol and within the core of these single phospholipid membrane structures, as cholesteryl-ester, that function to transport hydrophobic constituents. Present on the membrane of lipoproteins, apolipoproteins provide functional identity determining their roles within the human body and although most lipoproteins carry multiple apolipoproteins only the primary functional constituents will be discussed here. Lipoproteins are classified by the inverse relationship of size/density and are generally classified as HDL, LDL, very low density lipoprotein (VLDL) and ultra low density lipoprotein (ULDL), also known as chylomicrons. Cholesterol is taken up from the gut into enterocytes where it is packaged into chylomicrons with other lipids before the chylomicrons enter the lacteals and the bloodstream through the thoracic duct\textsuperscript{257}. These chylomicrons have the ApoB48 lipoprotein; a truncated form of the apolipoprotein B (ApoB) and primarily consist of triglycerides. The triglyceride content of these chylomicrons is metabolized by lipoprotein lipase resulting in circulating high cholesterol chylomicron remnants which are then endocytosed by the liver\textsuperscript{258}. VLDL is secreted from the liver containing similarly high levels of triglyceride and the LDLr recognized full form ApoB100\textsuperscript{257}. The triglycerides within these particles are similarly metabolized by lipoprotein lipase resulting in smaller particles sometimes referred to intermediate density lipoprotein (IDL) and eventually primarily cholesterol containing LDL\textsuperscript{257,258}. These now smaller LDL particles, often oxidized, are considered important factors in the development of atherosclerosis in which the particles are retained in the arterial wall eliciting an immune response and leading to lesion development\textsuperscript{259}. The oxidation of either the lipid or apolipoprotein constituents of LDL can lead to conformational changes preventing uptake via LDLr and instead the uptake by scavenger receptors\textsuperscript{260,261}. This shift in lipoprotein-receptor interaction manifests itself in the early stages of atherosclerosis as newly differentiated macrophages uptake large quantities of oxidized-LDL derived cholesterol through highly expressed scavenger receptors, leading to the creation of foam cells a fundamental part of atherosclerotic plaque formation\textsuperscript{260}. 
The Apo-AI containing HDL plays a vital role in reverse cholesterol transport\textsuperscript{257,262}. The process where there is net cholesterol movement from peripheral tissues to the liver. Produced by the liver HDL, in general, receives cholesterol through Apo-AI interaction with ABCA1 of peripheral tissues and releases cholesterol to tissues expressing SR-B1, however SR-B1 has been demonstrated to facilitate bidirectional flux of cholesterol under varying conditions\textsuperscript{262,263}. The mechanism of HDL-SR-B1 mediated cholesterol uptake is not fully understood, but it was initially believed that the SR-B1, unlike LDL and the LDLr, does not degrade HDL but does mediate selective cholesteryl ester uptake from HDL and in some cases LDL in the absence of holo-HDL uptake\textsuperscript{264-266}. However, further study of the HDL-SR-B1 interaction has demonstrated that the HDL particle may be internalized in a non-clathrin-coated vesicle manner in which cholesterol-deplete HDL is secreted after uptake\textsuperscript{267,268}.

1.17 Cholesterol and prostate cancer

Cholesterol has long been observed and studied in the field of cancer with first acknowledgments of significant cholesterol accumulation taking place in the early 20\textsuperscript{th} century and speculation of a role in PCa malignancy beginning in 1940s by G. Swyer and colleagues\textsuperscript{269,270}. Further insights have since come from a wealth of epidemiological evidence. Early studies produced confounding results with several studies finding that cholesterol lowering in fact increased cancer incidence\textsuperscript{271,272} while others found no association\textsuperscript{273,274}. K. Solomon and M. Freeman more recently reviewed fifty two population studies that reported cholesterol and total cancer incidence or mortality\textsuperscript{275}. Thirty two of these studies found an inverse association between cancer risk and cholesterol level while sixteen showed no association. Interestingly the authors note that there did not appear to be an absolute level of cholesterol associated with cancer but rather that the low cholesterol cohort, relative to cohort average, in any population has a greater prevalence of cancer. Recent evidence has pointed towards a positive association between cholesterol and PCa. Men with high blood-cholesterol levels have more recently been found to have an increased risk of PCa\textsuperscript{276,277} and prostate cancer mortality\textsuperscript{278}. Further, cholesterol levels are elevated in post-ADT patient serum\textsuperscript{279,280}. At issue is a distinction of cause and effect from these epidemiologic
observations and has led to the hypothesis that the presence of cancer reduces circulating cholesterol, as opposed to the implication that low cholesterol somehow drives the development of cancer.

Investigations into understanding the specific alterations to cholesterol metabolism within the tumor setting have generated evidence demonstrating that hypercholesterolemia is associated with increased tumor growth, AKT activation and tumoral androgens in xenograft models, while hypercholesterolemia was further found to increase tumor volume, progression, and incidence in the TRansgenic Adenocarcinoma Mouse Prostate (TRAMP) model of spontaneous PCa. SR-B1 expression, for which elevated expression has previously been associated with aggressive characteristics and poor prognosis of breast cancer, and clear cell renal carcinoma, may be a key alteration in the development of PCa. Specifically SR-B1 expression has been found to be associated with high Gleason grade primary cancers and that high expression was correlated with decreased disease-specific survival in patients. Further, SR-B1 expression was correlated with androgen synthesis enzymes 3β-HSD and 17β-HSD and mTOR complex 1 target ribosomal protein S6. Pre-clinically elevated SR-B1 expression is observed in CRPC derivatives of LNCaP, and with western diet induced tumor development in the TRAMP model. The siRNA silencing of SR-B1 has further been shown to decrease PSA secretion and cell viability in C4-2 cells. These findings underlie the apparent potential in targeting cholesterol metabolism as a mechanism for impeding the proliferation of PCa.

The popularity of the statin class of therapeutics used in a preventive fashion for cardiovascular disease has allowed for fundamental clinical insight into their effect on PCa. The findings of several studies have led to the belief that statins may also play a role as anti-cancer agents in PCa via both cholesterol and pleiotropic non-cholesterol mediated effects. From an epidemiological perspective several attempts have been made to understand the effect of statins on both the occurrence of PCa and the clinical outcomes of those diagnosed with PCa. There is a vast body of literature with regards to total PCa incidence producing conflicting results. Several studies have found an inverse association between statin use and PCa risk, but despite these findings a plurality of studies have found no association and further three studies found a positive association. A recent meta-analysis of observational
studies found a 7% reduction in the risk of total PCa with statin use\textsuperscript{314}, while meta-analyses that included randomized controlled trials of cardio-preventive statin use found no association between statin use and overall PCa incidence\textsuperscript{315-317}. Concerns about diet interventions, statin choice and short follow-up time have been raised as potential explanations for the difference between observational and randomized trials\textsuperscript{293}.

Despite the uncertainty as to whether statins impact overall PCa incidence there is growing evidence that statin use may reduce the incidence of advanced PCa. As with total incidence, there is confounding evidence with regards to statins effect on advanced PCa. Studies finding a lack of association\textsuperscript{308,309,318-321} and those finding an inverse association between advanced PCa and statin use have been reported in numerous populations\textsuperscript{295-298,300,304,305,310,322,323}. A 2012 meta-analysis of observational studies found that statin use was associated with a 20% reduction in risk of advanced PCa\textsuperscript{314}. Beyond the potential impact of statins on PCa incidence and confounding results with regard to statins effect on time to progression\textsuperscript{324-326} several studies have found that statins have a beneficial impact on the survival of patients with PCa\textsuperscript{324,327-331}. One such study further noted that patients using statins prior to diagnosis displayed a larger decrease in PCa mortality compared to those who initiated statin treatment post-diagnosis (HR, 0.55; 95% CI, 0.41 to 0.74 vs HR, 0.82; 95% CI, 0.71 to 0.96)\textsuperscript{329}. In a preclinical setting, simvastatin and lovastatin have been found to reduce cell viability through an induction of apoptosis and G\textsubscript{1} phase cell cycle arrest and induce AR-positive LNCaP and AR-negative PC-3 and DU145 cells. While atorvastatin, simvastatin and rosvuvasatin have all been shown to reduce the metastatic potential of PC-3 cells\textsuperscript{332,333}. Further, lovastatin reduced cellular adhesion through down-regulated Rho-signaling in colon carcinoma and cerivastatin reduced tumor vascularization in a murine lung cancer model\textsuperscript{334,335}. Although not without contradiction, the wealth of evidence including consistent preclinical findings appears to demonstrate the possibility of targeting cholesterol metabolism, in this case through statins but also potentially through other cholesterol metabolism proteins, as therapeutic target in PCa.
1.18 Research rationale

Despite successes of recent therapies, PCa remains a lethal disease. The understanding of the role of *de novo* steroidogenesis, whether adrenally or tumor derived, from precursor cholesterol as a major mechanism of resistance to ADT drove the creation novel AR-targeted therapies including abiraterone. These findings combined with growing evidence of the importance of cholesterol in PCa development and progression highlights a potential avenue for novel therapies in PCa. Statins have been shown to very slightly reduce circulating testosterone\textsuperscript{336,337}, however, this decrease is thought to be negligible in the context of non-inhibited gonadal production and other studies have demonstrated no effect\textsuperscript{338,339}. What is not understood is whether statins directly affect CRPC steroidogenesis in the context of castrate levels of circulating testosterone, particularly given that a high-fat, high cholesterol diet has been found to increase intratumoral levels of androgens in LNCaP xenograft\textsuperscript{283}, or whether they potentiate the effect of other steroidogenesis inhibitors. Given the clinically accessible and historically successful nature of statins, the majority of research revolving around cholesterol-based therapeutics for PCa has focused on cholesterol synthesis targeting. Alternatively, the role of SR-B1 in providing cholesterol for steroid synthesis and its correlation to increased disease-specific PCa mortality provides an intriguing basis for its development as a therapeutic target. As such developing a more in-depth understanding of the clinical expression of SR-B1 across the disease state and determining the effects of inhibition pre-clinically are essential initial steps in determining whether SR-B1 is a viable target for future PCa treatments.

1.19 Hypothesis

Cholesterol modulation, through either synthesis or uptake inhibition, will impact essential signaling processes impeding the growth of CRPC.

Specific Aims:
(1) Determine whether statins act synergistically with abiraterone in a clinical setting and whether these compounds have the ability to impact *de novo* steroidogenesis.
(2) Assess the expression of SR-B1 across the PCa disease state and determine whether it is a viable cholesterol metabolism target for PCa therapy through reduced *de novo* steroidogenesis or other mechanisms.

(3) Assess the potential of the small molecule SR-B1 inhibitor BLT-1 as a potential basis for therapeutic development.
CHAPTER 2: THE IMPACT OF STATIN USE ON DE NOVO STEROIDOGENESIS AND CLINICAL OUTCOMES OF CRPC PATIENTS RECEIVING ABIRATERONE

2.1 Specific aim and rationale

As described above, there has been a vast amount of research on the effect of statins on PCa. Clinically, statins are believed to improve PCa prognosis improving incidence of aggressive disease and decreased mortality. Pre-clinically statins have been demonstrated to impede PCa progression, including reduced PSA secretion, proliferation, adhesion and invasion. Now seeing widespread clinical use, abiraterone is a known inhibitor of de novo steroidogenesis but recent evidence into whether the use of concomitant statins improves outcomes for these patients is inconclusive. Further, if statins do improve CRPC patient outcomes it was unknown whether this effect would be solely due to established consequences of limiting cholesterol such as impacted membrane synthesis and signaling or whether statins possessed the ability to directly impact the synthesis of AR-driving steroids.

The studies described in this chapter aimed to elucidate the effect of statin use on the survival and progression of metastatic CRPC patients receiving abiraterone therapy. To perform this research, a database generated from the province-wide British Columbia Cancer Agency was analyzed for survival and progression data. Further, the effect of statins on de novo steroidogenesis was assessed pre-clinically using an LNCaP based xenograft mouse model. The progression and growth of castrate LNCaP tumors were assessed alongside measurements for androgen production.

2.2 Methods

2.2.1 Analysis of abiraterone patients at the British Columbia Cancer Agency

Patient characteristics and response information was obtained from the British Columbia Cancer Agency and used to generate a dataset of men being treated with abiraterone for locally advanced or metastatic CRPC. Laboratory reports, British Columbia Cancer Agency pharmacy records and consultation reports were used to generate the dataset. Information on statin use was obtained by patient
claims and admission and consultation reports. Summary statistics were used to describe patient characteristics and responses and the Kaplan-Meier method was used to estimate overall survival times. Cox proportional hazards regression was used to investigate prognostic factors of overall survival and logistic regression was used to investigate prognostic factors of PSA response\textsuperscript{344}. A multivariable model was constructed including factors which had nearly complete data and biological rationale for inclusion and was used to assess the impact of statins. PSA response was assessed as both a decline of $\geq 30\%$ and $\geq 50\%$ relative to baseline. Charlson index comorbidities were weighted according to Table 2.1\textsuperscript{345}.

**Table 2.1: Comorbidity score weighting of Charlson Index.**

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<td></td>
<td>Congestive heart failure</td>
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</tr>
<tr>
<td>2</td>
<td>Hemiplegia</td>
</tr>
<tr>
<td></td>
<td>Renal disease</td>
</tr>
<tr>
<td></td>
<td>Diabetes - end organ damage</td>
</tr>
<tr>
<td></td>
<td>Tumor without metastasis</td>
</tr>
<tr>
<td></td>
<td>Leukemia</td>
</tr>
<tr>
<td></td>
<td>Lymphoma</td>
</tr>
<tr>
<td>3</td>
<td>Severe liver disease</td>
</tr>
<tr>
<td>6</td>
<td>Metastatic solid tumour</td>
</tr>
<tr>
<td></td>
<td>AIDS</td>
</tr>
</tbody>
</table>

2.2.2 LNCaP xenografts

All animal experiments described in this chapter were carried out in accordance with the University of British Columbia’s Committee on Animal Care and approved protocol A12-0211 held by Dr. Kishor Wasan at the Faculty of Pharmaceutical Sciences, UBC. Athymic nude mice (Crl:NU-Foxn1\textsuperscm; Harlan, Indianapolis, IN) aged 6 to 8 weeks-old were inoculated on two sites of the hind flank with 2 x
10^6 LNCaP cells (LNCaP cells cultured in RPMI + 10% FBS, 1% penicillin-streptomycin) in 100 µL matrigel (BD Biosciences, Franklin Lakes, NJ).

Once weekly, serum PSA levels were measured using the ELISA-based Cobas e 411 analyzer (Roche Diagnostics, Indianapolis, IN). Serum was obtained by performing tail vein bleeds with samples being collected in hematocrit tubes and spun in a hematocrit centrifuge at 13,000 RPM for 4 min. 15 µL of serum was diluted with 135 µL of phosphate-buffered saline (PBS) prior to performing the assay.

Once serum PSA exceeded 50 ng/mL, mice were castrated by an experienced animal technician and randomized into control “standard” diet (Open Standard Diet containing 40 mg/kg cholesterol, Research Diets Inc., New Brunswick, NJ), or a simvastatin diet (Open Standard Diet containing 0.1% [w/w] simvastatin [Sanis, Dieppe, New Brunswick, Canada]). At 8 weeks post-castration, anesthetized animals were exsanguinated by cardiac puncture to collect blood, from which serum was prepared, and tumors were in part harvested, flash frozen with liquid nitrogen and in part fixed in 10% buffered formalin solution over night before being transferred to 70% ethanol for long term storage. Throughout the course of the experiment weekly measurements of body weight, tumor volume and PSA were performed. PSA measurements were performed as described above and tumor volume was measured using calipers and the equation: Tumor Volume = length x width x height x 0.5326^{346}.

2.2.3 Simvastatin and simvastatin hydroxy acid LC-MS

50 µL of serum obtained via cardiac puncture was added to 200 µL of water with 50 µL of 10 ng/mL internal standard (lovastatin and lovastatin hydroxy acid, IS). This was extracted using 2 mL of methyl tert-butyl ether (MTBE) and vortexed thoroughly. The samples were then placed in the -80 °C freezer for 10 min to freeze the aqueous phase. The solvent phase was then decanted into separate tubes and dried down using a nitrogen blow-down evaporator. The dried down sample was reconstituted in 30/70 water/methanol plus 5 mM ammonium acetate, for analysis of 20 µL injectable aliquot.

Simvastatin and simvastatin hydroxy acid were both quantitated using liquid chromatography-mass spectrometry (LC-MS). This system consisted of an Agilent 1290 Infinity Binary Pump, a 1290
Infinity Sampler, a 1290 Infinity Thermostat and a 1290 Infinity Thermostatted Column Compartment (Agilent, Mississauga, Ontario, Canada) connected to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer equipped with a Turbo Spray source (AB Sciex, Concord, Ontario, Canada). Data was acquired using the Analyst 1.5.2 software. Separations were carried out using a Waters Acquity UPLC C18 1.7 µm 2.1 x 100 mm column (Waters Corp., Milford, MA, USA) maintained at 30 °C and the autosampler tray temperature was maintained at 10 °C. The isocratic mobile phase consisted of water (10%; Solvent A) and methanol (90%; Solvent B) with both solvents containing 5 mM ammonium acetate. The flow rate was 0.2 mL/min and the run time 4.0 min. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using polarity switching. Simvastatin and lovastatin were monitored as their ammonium adducts in positive ionization (ES+) mode. The retention times for simvastatin and lovastatin were 2.75 min and 2.53 min, respectively, and for simvastatin hydroxy acid and lovastatin hydroxy acid 2.37 min and 2.25 min, respectively.

2.2.4 Creatine kinase assay

The creatine kinase fluorometric assay (Cat. #: 700630, Cayman Chemical, Ann Arbor, MI) was performed according to manufacturer's instructions. For each sample, standard, and control 10 µL of serum obtained via cardiac puncture and 50 µL of diluted buffer was added to a well of the 96 well plate. 10 µL of ADP followed by 10 µL of “Enzyme Mixture” were then added to the well before initiating the reaction with 20 µL of creatine phosphate. The plate was incubated for 15 min at 37°C, and followed by the addition of 80 µL of ammonium acetate and 20 µL of formaldehyde detector. The plate was then incubated for 15 min at room temperature and read on a plate reader (excitation wavelength: 365, emission wavelength 465) to determine the average fluorescence of each standard, control and sample.

The fluorescent value for the 0 uM standard solution was subtracted from each of the standards, controls and samples to produce the corrected fluorescence (RFU). The corrected fluorescence values for each of the standards was plotted to create a standard curve. The creatine kinase activity of each sample was then calculated using the following equation:
Creatine Kinase (U/L) = \left( \frac{RFU}{\text{Slope of standard curve} \left( \frac{RGU}{\mu M} \right)} \right) \times \text{sample dilution}

2.2.5 Alanine transaminase assay

The alanine transaminase colorimetric assay (Cat. #: 700260, Cayman Chemical) was performed according to manufacturer’s instructions. In each well of a 96 well plate 150 µL of “substrate”, 20 µL of “cofactor” and 20 µL of sample or control were added. The plate was then incubated for 15 min at 37°C. The reaction was then initiated by adding 20 µL of “ALT initiator” to each of the wells. The plate was then immediately placed in a plate reader and each well measured at 340 nm every min for 5 min.

To determine the change in absorbance (\(\Delta A_{340}\)) per min the absorbance values were plotted as a function of time and the slope of the linear portion of the curve was used. The following equation was then used to calculate ALT activity:

\[
ALT \text{ activity (U/mL)} = \left( \frac{\Delta A_{340}/\text{min} \times 0.21 \text{ mL}}{4.11 \text{ mM}^{-1} \times 0.02 \text{ mL}} \right) \times \text{sample dilution}
\]

2.2.6 Cholesterol LC-MS

Cholesterol levels were assessed and quantified by LC-MS as previously described\(^{347}\). Tumors (50–150 mg) were homogenized (Precellys Tissue Homogenizer, Rockville, MD) for 2 x 20 sec at 6000 rpm in nine volumes of water. IS (deuterated cholesterol) was added and homogenates were extracted twice with 90/10 MTBE/methanol. Extracts were dried and pooled under vacuum and reconstituted in 400 µL of 1/5 acetyl Cl/CHCl\(_3\). 50 µL serum samples were similarly processed without the initial homogenization. These samples were then vortexed and dried down using a CentriVap (Labconco, Kansas City, MO) before being dissolved in 70/30 methanol/CHCl\(_3\). All samples were analyzed using a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer (Waters, Milford, MA). Separations were carried out on a Waters 2.1x100mm BEH 1.7 µM C18 column using an acetonitrile/0.1M ammonium acetate 9/1 isopropanol mobile phase gradient. Data was collected in ES+ multiple reaction monitoring (MRM) mode and processed with Quanlynx (Waters) prior to
exporting to Excel (Microsoft, Redmond, WA) for additional normalization to weights and volumes as required. Quantification was by area under curve (AUC) ratio of standard to IS.

2.2.7 Steroid LC-MS

Intratumoural and serum androgen levels were assessed and quantified by LC-MS as previously described. Two volumes of water were added to tumours (50-150 mg) and homogenized (Precellys Tissue Homogenizer) for 2 x 20 sec at 6000 rpm. IS (20 pg/40 pg deuterated testosterone/DHT; C/D/N isotopes) was added and homogenates were twice extracted by 30 min vortexing with 2 mL of 60/40 hexane/ethyl acetate. Extracts were pooled, dried using a CentriVap, reconstituted in 50 µL of 50 mM hydroxylamine and incubated 1 hour at 65˚C. 50 µL serum samples were similarly processed without the initial homogenization. Resulting steroid-oxime derivatives were analyzed using a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer (Waters). Separations were carried out with a 2.1 x 100 mm BEH 1.7 μM C18 column, mobile phase water (A) and acetonitrile (B) (gradient: 0.2 min, 25% B; 8 min, 70% B; 9-12 min, 98% B; 12.2 min, 25% B; 14 min run length). Data was collected in ES+ by multiple reaction monitoring with instrument parameters optimized for the mass-to-charge ratios and corresponding fragments of the oxime-steroids for all keto-steroids in the androgen synthesis pathway. Data processing was done with Quanlynx (Waters) and exported to Excel (Microsoft) for additional normalization to weights and volumes as required. Quantification was by AUC ratio of standard to IS. Deuterated testosterone was used as the IS with all analytes other than DHT for which deuterated DHT was used.

2.2.8 Quantitative PCR

RNA was isolated from homogenized (Precellys Tissue Homogenizer; 2 x 20sec, 6000 rpm) tumors using a TRIzol (Life Technologies, Burlington, Ontario, Canada) chloroform extraction according to manufacturer instructions. TRIzol (1 mL) was added to 50-100 mg of homogenized tissue and incubated for 5 min after which 0.2 mL of chloroform was added and incubated for a further 3 min prior to centrifugation for 15 min at 12,000 g, 4 ºC. The aqueous phase was separated and incubated with 0.5
35 mL of isopropanol for 10 min prior to centrifugation for 10 min at 12,000 g, 4 °C. The pelleted RNA was then washed with 1 mL 75% ethanol pelleted and suspended in 50 µL RNase-free water. cDNA was synthesized from the RNA using a SuperScript® II Reverse Transcriptase kit (Life Technologies) according to manufacturer instructions. First strand synthesis was carried out with 50 – 250 ng random primers, 0.5 mM dNTP mix and 1 ng - 5 µg total RNA incubated at 65 °C for 5 min. Following this, 1x First-strand buffer, 10 mM DTT and 40 U RNaseOUT were added to the mixture and incubated for 2 min at 42 °C. 200 units of SuperScript® II Reverse Transcriptase was then added to the solution prior a 5 min incubation at 42 °C followed by heat inactivation at 70 °C for 15 min. 2 U of RNase H was then added and incubated at 37 °C for 20 min. Using this cDNA qPCR was performed on an ABI 7900HT (Life Technologies) using FastStart Universal SyberGreen Master mix (Roche) and a Qiagen (Toronto, Ontario, Canada) SYBR Green probe targeted to the gene of interest. The following thermocycler conditions were used for qPCR: 50 °C for 2 min, 95 °C for 10 min, 40x: 95 °C for 15 sec, 60 °C for 1 min, 95 °C for 15 sec, 60 °C for 15 sec, 95 °C for 15 sec.

2.3 Results

2.3.1 Statin use improves the overall survival and PSA declines of men receiving abiraterone for locally advanced or metastatic CRPC

There is limited and conflicting evidence as to the effect of statin use on the outcomes of patients receiving abiraterone for CRPC. Analysis of existing clinical data retrospectively can provide valuable insight into whether prospective studies are feasible. The British Columbia Cancer Agency is a comprehensive cancer institute that provides cancer care through the operation of six regional centers to the population of British Columbia. Data used to calculate patient survival, PCa progression and general health was obtained from the British Columbia Cancer Agency and compared by statin status. In total 301 patients receiving abiraterone were included in the study of which 84 patients were statin users. The specific statins used by these patients included 5 different types: atorvastatin (n = 45), lovastatin (n = 2), pravastatin (n = 2), rosuvastatin (n = 24), simvastatin (n = 11). The patient median age was 74 and 68.6%
of which had a Gleason score of ≥ 8. Patients were maintained on abiraterone for a median time of 5.6 months and had a median overall survival time of 12.1 months. Further, information on the characteristics of these patients can be found in the summary statistics (Table 2.2).

Table 2.2: Baseline characteristics and outcomes of British Columbia abiraterone patients

<table>
<thead>
<tr>
<th>Statistic</th>
<th>N (%)</th>
<th>N</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concomitant Statins</td>
<td>N (%) Yes</td>
<td>301</td>
<td>84 (27.9)</td>
</tr>
<tr>
<td></td>
<td>Atorovastin</td>
<td>45</td>
<td>(53.6)</td>
</tr>
<tr>
<td></td>
<td>Lovastatin</td>
<td>2</td>
<td>(2.4)</td>
</tr>
<tr>
<td></td>
<td>Pravastatin</td>
<td>2</td>
<td>(2.4)</td>
</tr>
<tr>
<td></td>
<td>Rosuvastatin</td>
<td>24</td>
<td>(28.6)</td>
</tr>
<tr>
<td></td>
<td>Simvastatin</td>
<td>11</td>
<td>(13.1)</td>
</tr>
<tr>
<td>Age at path. diagnosis</td>
<td>Mean (std dev)</td>
<td>300</td>
<td>66.1 (8.9)</td>
</tr>
<tr>
<td></td>
<td>Med. (range)</td>
<td></td>
<td>65 (45, 95)</td>
</tr>
<tr>
<td>Age at Abi start date</td>
<td>Mean (std dev)</td>
<td>301</td>
<td>73.8 (9.8)</td>
</tr>
<tr>
<td></td>
<td>Med. (range)</td>
<td></td>
<td>74 (45, 96)</td>
</tr>
<tr>
<td>Gleason Score</td>
<td>N (%) ≥8</td>
<td>258</td>
<td>177 (68.6)</td>
</tr>
<tr>
<td>Charlson Score</td>
<td>N (%) ≥7</td>
<td>298</td>
<td>156 (52.4)</td>
</tr>
<tr>
<td>PSA at Diagnosis</td>
<td>Med. (range)</td>
<td>278</td>
<td>27 (0.2, 9832)</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>Med. (range)</td>
<td>228</td>
<td>122 (8.9, 2189)</td>
</tr>
<tr>
<td>LDH</td>
<td>Med. (range)</td>
<td>80</td>
<td>241 (108, 2598)</td>
</tr>
<tr>
<td>Albumin</td>
<td>Med. (range)</td>
<td>102</td>
<td>3.7 (2.4, 5)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Med. (range)</td>
<td>291</td>
<td>4900 (100, 81500)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Med. (range)</td>
<td>290</td>
<td>1135 (200, 30640)</td>
</tr>
<tr>
<td>Neutrophils/Lymphocyte Ratio</td>
<td>Med. (range)</td>
<td>290</td>
<td>4.1 (0.19, 34.5)</td>
</tr>
<tr>
<td>Change in PSA, Diagnosis to Abi</td>
<td>Med. (range)</td>
<td>276</td>
<td>48.3 (-6798, 7926)</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi</td>
<td>Med. (range)</td>
<td>300</td>
<td>71.2 (4.2, 324.5)</td>
</tr>
<tr>
<td>Months, Diagnosis to Metastases</td>
<td>Med. (range)</td>
<td>236</td>
<td>43.5 (-6.3, 306.8)</td>
</tr>
<tr>
<td>Months, Diagnosis to CRPC</td>
<td>Med. (range)</td>
<td>193</td>
<td>44.7 (2.5, 203.3)</td>
</tr>
<tr>
<td>Months, ADT to Abi</td>
<td>Med. (range)</td>
<td>291</td>
<td>49.0 (1.1, 298.8)</td>
</tr>
<tr>
<td>Months, Metastases to Abi</td>
<td>Med. (range)</td>
<td>237</td>
<td>21.2 (0.2, 164.8)</td>
</tr>
<tr>
<td>Months, CRPC to Abi</td>
<td>Med. (range)</td>
<td>193</td>
<td>23.1 (0.3, 183.5)</td>
</tr>
<tr>
<td>Months, Docetaxel to Abi</td>
<td>Med. (range)</td>
<td>172</td>
<td>7.7 (-42.8, 86.5)</td>
</tr>
<tr>
<td>Months of ADT (1st line)</td>
<td>Med. (range)</td>
<td>131</td>
<td>9.5 (0, 169.9)</td>
</tr>
<tr>
<td>Months of ADT (1st to last ever)</td>
<td>Med. (range)</td>
<td>279</td>
<td>38.1 (0, 294.4)</td>
</tr>
<tr>
<td>Months, Docetaxel Duration</td>
<td>Med. (range)</td>
<td>169</td>
<td>3.8 (0, 83.8)</td>
</tr>
<tr>
<td>Prior Prostatectomy</td>
<td>N (%) Yes</td>
<td>290</td>
<td>53 (18.3)</td>
</tr>
<tr>
<td>Prior Radiation</td>
<td>N (%) Yes</td>
<td>292</td>
<td>91 (31.2)</td>
</tr>
<tr>
<td>Opiates</td>
<td>N (%) Yes</td>
<td>301</td>
<td>60 (19.9)</td>
</tr>
<tr>
<td>Metastases</td>
<td>N (%) Any</td>
<td>301</td>
<td>237 (78.7)</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>193</td>
<td>(64.1)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>7</td>
<td>(2.3)</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>5</td>
<td>(1.7)</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>0</td>
<td>(0)</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>92</td>
<td>(30.6)</td>
</tr>
</tbody>
</table>
A univariate analysis of statin vs non-statin use was performed to determine effects on overall survival and PSA declines of patients taking abiraterone. Of the 217 non-statin users 5 patients were still alive at the time of data collection while 7 of the statin patients remained alive. Statin users were found to have improved overall survival having a median survival of 16.2 months compared to 11.3 months for non-statin users (Hazard Ratio: 0.79, 95%CI = 0.62 – 1.02, log-rank test: p = 0.078; Gehan-Breslow-Wilson Test: p = 0.022 Table 2.3, Figure 2.1). Although failing to reach statistical significance the percentage of patients who saw a PSA response to abiraterone was increased in statin users (47.6%) compared to non-statin users (37.6%, p = 0.10, Table 2.3). Time on abiraterone (Statin: 7.2 months; Non-statin: 5.3 months; p = 0.078) and change in PSA at nadir (Statin: -41.0%; Non-statin: -30.1%; p = 0.086) were also increased in statin users, but as with PSA response did not reach statistical significance (Table 2.3). Despite the difference in the number of PSA responders and the relative change in the response that was observed with statin users, no difference was observed in the number of patients who saw borderline PSA responses of >30% drop or robust PSA responses >50% drop in PSA at specific 4, 8 and 12 week time points (Table 2.4).
Figure 2.1: Statin use prolongs survival of CRPC patients on abiraterone
A univariate retrospective analysis was performed on a cohort of CRPC patients treated with abiraterone comparing overall survival of statin (blue line) and non-statin (black line) users. Statin users had a median overall survival time of 16.2 months compared to non-statin users at 11.3 months (HR: 0.79; log rank test: p = 0.078; Gehan-Brislow-Wilcoxon test: p = 0.022).

Table 2.3: Outcomes by Statin Status of abiraterone patients

<table>
<thead>
<tr>
<th>Statistic</th>
<th>No Statins</th>
<th>Statins</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>217</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>PSA Response N (%)</td>
<td>81 (37.3)</td>
<td>40 (47.6)</td>
<td>0.10</td>
</tr>
<tr>
<td>PSA at Baseline Median (range)</td>
<td>120.0 (0.5, 7149)</td>
<td>90.6 (0.2, 7938)</td>
<td>0.23</td>
</tr>
<tr>
<td>Change in PSA at Nadir Median (range)</td>
<td>-30.1 (-99.9, 1000)</td>
<td>-41.0 (-99.6, 174.5)</td>
<td>0.086</td>
</tr>
<tr>
<td>Months to Nadir Median (range)</td>
<td>1.5 (0, 17.4)</td>
<td>2.3 (0, 13.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>Months of Abi Median (range)</td>
<td>5.3 (0.3, 49.7)</td>
<td>7.2 (0.4, 47.5)</td>
<td>0.069</td>
</tr>
<tr>
<td>Overall Survival N (% Deaths)</td>
<td>210 (96.8)</td>
<td>79 (94.0)</td>
<td></td>
</tr>
<tr>
<td>Median (95% CI)</td>
<td>11.3 (9.3, 12.7)</td>
<td>16.2 (13.2, 18.8)</td>
<td></td>
</tr>
<tr>
<td>6-mo OS (95% CI)</td>
<td>74.7 (68.3, 80.0)</td>
<td>82.1 (72.1, 88.8)</td>
<td></td>
</tr>
<tr>
<td>1-year OS (95% CI)</td>
<td>45.8 (39.1, 52.3)</td>
<td>63.1 (51.8, 72.4)</td>
<td></td>
</tr>
</tbody>
</table>

LR: 0.078, GBW: 0.022

Table 2.4: PSA Declines of >30% and >50% by statin status of abiraterone patients

<table>
<thead>
<tr>
<th>Statistic</th>
<th>No Statins</th>
<th>Statins</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA &gt;30%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>73 (39.3%)</td>
<td>30 (46.8%)</td>
<td>0.31</td>
</tr>
<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>70 (43.7%)</td>
<td>35 (51.5%)</td>
<td>0.31</td>
</tr>
<tr>
<td>12 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>74 (49.3%)</td>
<td>30 (48.4%)</td>
<td>1.00</td>
</tr>
<tr>
<td>PSA &gt;50%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>43 (23.2%)</td>
<td>22 (34.4%)</td>
<td>0.098</td>
</tr>
<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>54 (33.7%)</td>
<td>27 (39.7%)</td>
<td>0.45</td>
</tr>
<tr>
<td>12 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>59 (39.3%)</td>
<td>23 (37.1%)</td>
<td>0.87</td>
</tr>
</tbody>
</table>
Multivariate analyses were performed to adjust for age, prior prostatectomy, radiation, time from diagnosis, neutrophils-lymphocyte ratio and presence of metastases as prognostic factors for overall survival and PSA response. The effect of statins on overall survival remained borderline by Cox regression analysis (n = 279, hazard ratio = 0.78, 95% CI = 0.59-1.04, p = 0.087; Table 2.5). Interestingly when Gleason score was included in the multivariate analysis, despite a smaller sample size, significant improvement in overall survival for statin users was observed (n = 241, hazard ratio = 0.71, 95% CI = 0.52 – 0.96, p = 0.024). Similar to overall survival, the percentage of patients who saw PSA responses to abiraterone with statin use trended towards improvement but remained borderline with regards to statistical significance when assessed by a logistic regression analysis (multivariable odds ratio = 1.59, 95% CI = 0.91 - 2.78, p = 0.11, Table 2.6).

The Charlson Index was used to assess patient comorbidities and overall health. The total patient dataset had 156 (52.4%) patients with a Charlson score of ≥7 (Table 2.2) with the most common comorbidity, excluding the presence of metastasis, being hypertension effecting 140 (46%) patients (Table 2.7). Statin users had a median Charlson score of 7, which was increased, compared to non-statin users who had a median score of 6 (p < 0.001). Further, 87 (40.1%) non-statin users compared to 68 (80.9%) statin users had Charlson scores of seven or greater (p < 0.001) (Table 2.8). Specifically, statin users had significantly increased rates of hypertension (statin: 61 [72.6%]; no statin: 79 [36.4%], p<0.001), myocardial infarction (statin: 9 [10.7%]; no statin: 4 [1.8%], p = 0.002), peripheral disease (statin: 12 [14.2%]; no statin: 4 [1.8%], p < 0.001), cerebrovascular disease (statin: 5 [6.0%]; no statin: 2 [0.9%], p=0.02), dementia (statin: 4 [4.76%]; no statin 1 [0.5%], p=0.02) and diabetes (statin: 25 [29.8%]; no statin: 16 [7.4%], p < 0.001, Table 2.7). The use of statins concomitantly with abiraterone indicated a trend to improved prognosis, but failed to reach statistical significance in a number of categories. What appears to be clear is that those patients taking statins are in considerably poorer health as measured by the Charlson index, potentially acting as a confounding factor when attempting to determine effects on overall survival.
Table 2.5: Prognostic factors for overall survival determined by multivariate Cox regression analyses for British Columbia abiraterone patients

<table>
<thead>
<tr>
<th>Type</th>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age / decade</td>
<td>1.06 (0.94, 1.20)</td>
<td>0.32</td>
</tr>
<tr>
<td>Gleason Score ≥8 vs &lt;8</td>
<td>1.20 (0.92, 1.58)</td>
<td>0.18</td>
</tr>
<tr>
<td>Prior Prostatectomy Y vs N</td>
<td>0.75 (0.55, 1.04)</td>
<td>0.081</td>
</tr>
<tr>
<td>Prior Radiation Y vs N</td>
<td>1.12 (0.87, 1.45)</td>
<td>0.36</td>
</tr>
<tr>
<td>Bone Metastases Y vs N</td>
<td>0.98 (0.77, 1.25)</td>
<td>0.88</td>
</tr>
<tr>
<td>Liver Metastases Y vs N</td>
<td>3.19 (1.49, 6.83)</td>
<td>0.003</td>
</tr>
<tr>
<td>Lung Metastases Y vs N</td>
<td>1.55 (0.64, 3.77)</td>
<td>0.33</td>
</tr>
<tr>
<td>Brain Metastases Y vs N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LN Metastases Y vs N</td>
<td>1.20 (0.93, 1.54)</td>
<td>0.16</td>
</tr>
<tr>
<td>Any Metastases Y vs N</td>
<td>1.01 (0.76, 1.33)</td>
<td>0.97</td>
</tr>
<tr>
<td>Opiates Y vs N</td>
<td>0.81 (0.61, 1.09)</td>
<td>0.16</td>
</tr>
<tr>
<td>Baseline PSA Log-transform</td>
<td>1.18 (1.10, 1.26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Months of ADT Log-transform</td>
<td>0.73 (0.65, 0.83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi Log-transform</td>
<td>0.81 (0.71, 0.92)</td>
<td>0.002</td>
</tr>
<tr>
<td>Alkaline Phosphatase Log-transform</td>
<td>1.55 (1.32, 1.82)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDH Log-transform</td>
<td>3.13 (1.95, 5.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin Log-transform</td>
<td>0.38 (0.25, 0.59)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils Log-transform</td>
<td>1.75 (1.34, 2.28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lymphocytes Log-transform</td>
<td>0.66 (0.53, 0.82)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NL Ratio Log-transform</td>
<td>1.62 (1.36, 1.92)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Concomitant Statins Y vs N</td>
<td>0.79 (0.61, 1.03)</td>
<td>0.079</td>
</tr>
</tbody>
</table>

Multivariable – N=241

<table>
<thead>
<tr>
<th>Age / decade</th>
<th>1.23 (1.05, 1.45)</th>
<th>0.013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleason Score ≥8 vs &lt;8</td>
<td>0.89 (0.64, 1.23)</td>
<td>0.47</td>
</tr>
<tr>
<td>Prior Prostatectomy Y vs N</td>
<td>1.23 (0.80, 1.88)</td>
<td>0.36</td>
</tr>
<tr>
<td>Prior Radiation Y vs N</td>
<td>1.54 (1.09, 2.16)</td>
<td>0.015</td>
</tr>
<tr>
<td>Confirmed Metastases Y vs N</td>
<td>1.11 (0.79, 1.57)</td>
<td>0.54</td>
</tr>
<tr>
<td>Baseline PSA Log-transform</td>
<td>1.18 (1.09, 1.28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi Log-transform</td>
<td>0.61 (0.49, 0.75)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NL Ratio Log-transform</td>
<td>1.63 (1.35, 1.97)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Concomitant Statins Y vs N</td>
<td>0.71 (0.52, 0.96)</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Multivariable – N=279

<table>
<thead>
<tr>
<th>Age / decade</th>
<th>1.29 (1.10, 1.50)</th>
<th>0.002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior Prostatectomy Y vs N</td>
<td>1.29 (0.88, 1.89)</td>
<td>0.20</td>
</tr>
<tr>
<td>Prior Radiation Y vs N</td>
<td>1.60 (1.17, 2.20)</td>
<td>0.004</td>
</tr>
<tr>
<td>Confirmed Metastases Y vs N</td>
<td>1.08 (0.80, 1.46)</td>
<td>0.60</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi Log-transform</td>
<td>0.64 (0.53, 0.77)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NL Ratio Log-transform</td>
<td>1.60 (1.34, 1.91)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Concomitant Statins Y vs N</td>
<td>0.78 (0.59, 1.04)</td>
<td>0.087</td>
</tr>
</tbody>
</table>
Table 2.6: Prognostic factors for PSA response (50% decline from baseline) determined by multivariate logistic regression analyses for British Columbia abiraterone patients

<table>
<thead>
<tr>
<th>Type</th>
<th>Odds Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age / decade</td>
<td>1.15 (0.90, 1.45)</td>
<td>0.26</td>
</tr>
<tr>
<td>Gleason Score ≥8 vs &lt;8</td>
<td>0.80 (0.47, 1.37)</td>
<td>0.41</td>
</tr>
<tr>
<td>Prior Prostatectomy Y vs N</td>
<td>1.49 (0.82, 2.71)</td>
<td>0.20</td>
</tr>
<tr>
<td>Prior Radiation Y vs N</td>
<td>1.26 (0.76, 2.09)</td>
<td>0.37</td>
</tr>
<tr>
<td>Bone Metastases Y vs N</td>
<td>1.03 (0.63, 1.66)</td>
<td>0.92</td>
</tr>
<tr>
<td>Liver Metastases Y vs N</td>
<td>0.24 (0.03, 2.03)</td>
<td>0.19</td>
</tr>
<tr>
<td>Lung Metastases Y vs N</td>
<td>2.26 (0.37, 13.75)</td>
<td>0.38</td>
</tr>
<tr>
<td>Brain Metastases Y vs N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LN Metastases Y vs N</td>
<td>0.55 (0.32, 0.92)</td>
<td>0.023</td>
</tr>
<tr>
<td>Confirmed Metastases Y vs N</td>
<td>0.90 (0.51, 1.58)</td>
<td>0.71</td>
</tr>
<tr>
<td>Opiates Y vs N</td>
<td>1.28 (0.72, 2.26)</td>
<td>0.40</td>
</tr>
<tr>
<td>Baseline PSA Log-transform</td>
<td>0.96 (0.83, 1.10)</td>
<td>0.53</td>
</tr>
<tr>
<td>Months of ADT Log-transform</td>
<td>1.59 (1.23, 2.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi Log-transform</td>
<td>1.30 (1.01, 1.67)</td>
<td>0.046</td>
</tr>
<tr>
<td>Alkaline Phosphatase Log-transform</td>
<td>0.91 (0.67, 1.24)</td>
<td>0.55</td>
</tr>
<tr>
<td>LDH Log-transform</td>
<td>0.89 (0.40, 2.00)</td>
<td>0.78</td>
</tr>
<tr>
<td>Albumin Log-transform</td>
<td>1.53 (0.70, 3.36)</td>
<td>0.29</td>
</tr>
<tr>
<td>Neutrophils Log-transform</td>
<td>0.62 (0.39, 0.99)</td>
<td>0.047</td>
</tr>
<tr>
<td>Lymphocytes Log-transform</td>
<td>1.05 (0.70, 1.56)</td>
<td>0.82</td>
</tr>
<tr>
<td>NL Ratio Log-transform</td>
<td>0.78 (0.57, 1.06)</td>
<td>0.11</td>
</tr>
<tr>
<td>Concomitant Statins Y vs N</td>
<td>1.53 (0.92, 2.54)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Multivariable – N=241

<table>
<thead>
<tr>
<th>Type</th>
<th>Odds Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age / decade</td>
<td>1.05 (0.74, 1.49)</td>
<td>0.77</td>
</tr>
<tr>
<td>Gleason Score ≥8 vs &lt;8</td>
<td>1.40 (0.72, 2.74)</td>
<td>0.33</td>
</tr>
<tr>
<td>Prior Prostatectomy Y vs N</td>
<td>1.67 (0.72, 3.87)</td>
<td>0.23</td>
</tr>
<tr>
<td>Prior Radiation Y vs N</td>
<td>1.29 (0.65, 2.57)</td>
<td>0.47</td>
</tr>
<tr>
<td>Confirmed Metastases Y vs N</td>
<td>1.19 (0.59, 2.43)</td>
<td>0.63</td>
</tr>
<tr>
<td>Baseline PSA Log-transform</td>
<td>0.99 (0.83, 1.16)</td>
<td>0.86</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi Log-transform</td>
<td>1.19 (0.76, 1.85)</td>
<td>0.46</td>
</tr>
<tr>
<td>NL Ratio Log-transform</td>
<td>0.80 (0.57, 1.13)</td>
<td>0.21</td>
</tr>
<tr>
<td>Concomitant Statins Y vs N</td>
<td>1.69 (0.93, 3.06)</td>
<td>0.087</td>
</tr>
</tbody>
</table>

Multivariable – N=279

<table>
<thead>
<tr>
<th>Type</th>
<th>Odds Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age / decade</td>
<td>1.09 (0.79, 1.51)</td>
<td>0.59</td>
</tr>
<tr>
<td>Prior Prostatectomy Y vs N</td>
<td>1.50 (0.72, 3.10)</td>
<td>0.28</td>
</tr>
<tr>
<td>Prior Radiation Y vs N</td>
<td>1.06 (0.57, 1.98)</td>
<td>0.86</td>
</tr>
<tr>
<td>Confirmed Metastases Y vs N</td>
<td>0.99 (0.53, 1.83)</td>
<td>0.96</td>
</tr>
<tr>
<td>Months, Diagnosis to Abi Log-transform</td>
<td>1.16 (0.79, 1.70)</td>
<td>0.45</td>
</tr>
<tr>
<td>NL Ratio Log-transform</td>
<td>0.79 (0.57, 1.09)</td>
<td>0.15</td>
</tr>
<tr>
<td>Concomitant Statins Y vs N</td>
<td>1.59 (0.91, 2.78)</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Table 2.7: Breakdown of Charlson Index scoring by statin status

<table>
<thead>
<tr>
<th>Charlson Score:</th>
<th>Statistic</th>
<th>No Statins</th>
<th>Statins</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>N (%)</td>
<td>79 (36.4)</td>
<td>61 (72.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>N (%)</td>
<td>4 (1.8)</td>
<td>9 (10.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>Congestive Heart Failure</td>
<td>N (%)</td>
<td>3 (1.4)</td>
<td>3 (3.6)</td>
<td>0.35</td>
</tr>
<tr>
<td>Peripheral Disease</td>
<td>N (%)</td>
<td>4 (1.8)</td>
<td>12 (14.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cerebrovascular Disease</td>
<td>N (%)</td>
<td>2 (0.9)</td>
<td>5 (6.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>Dementia</td>
<td>N (%)</td>
<td>1 (0.5)</td>
<td>4 (4.76)</td>
<td>0.02</td>
</tr>
<tr>
<td>Chronic Pulmonary Disease</td>
<td>N (%)</td>
<td>7 (3.2)</td>
<td>0 (0.0)</td>
<td>0.20</td>
</tr>
<tr>
<td>Connective Tissue Disease</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Ulcer Disease</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Mild Liver Disease</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>1 (1.2)</td>
<td>0.28</td>
</tr>
<tr>
<td>Diabetes</td>
<td>N (%)</td>
<td>16 (7.4)</td>
<td>25 (29.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemiplegia</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Renal Disease</td>
<td>N (%)</td>
<td>4 (1.8)</td>
<td>2 (2.4)</td>
<td>0.67</td>
</tr>
<tr>
<td>Diabetes - organ damage</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Tumor without metastasis</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Leukemia</td>
<td>N (%)</td>
<td>1 (0.5)</td>
<td>1 (1.2)</td>
<td>0.48</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Severe Liver Disease</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Metastatic Solid Tumor</td>
<td>N (%)</td>
<td>207 (95.4)</td>
<td>78 (92.8)</td>
<td>0.40</td>
</tr>
<tr>
<td>AIDS</td>
<td>N (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.8: Baseline characteristic comparison of abiraterone patients by statin status

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Statistic</th>
<th>No Statins</th>
<th>Statins</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at path. diagnosis</td>
<td>Mean (std dev)</td>
<td>65.3 (9.3)</td>
<td>68.1 (7.7)</td>
<td>0.018</td>
</tr>
<tr>
<td>Age at Abi start date</td>
<td>Median (range)</td>
<td>65 (45, 95)</td>
<td>63 (53, 90)</td>
<td>0.001</td>
</tr>
<tr>
<td>Gleason Score</td>
<td>Mean (std dev)</td>
<td>73 (10.2)</td>
<td>77 (7.9)</td>
<td>0.90</td>
</tr>
<tr>
<td>Charlson Score</td>
<td>Median (range)</td>
<td>72 (45, 96)</td>
<td>77 (59, 94)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PSA at Diagnosis</td>
<td>Median (range)</td>
<td>29 (0.2, 9832)</td>
<td>23 (0.65, 2000)</td>
<td>0.04</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>Median (range)</td>
<td>121 (8.9, 2189)</td>
<td>122 (25, 1707)</td>
<td>0.53</td>
</tr>
<tr>
<td>LDH</td>
<td>Median (range)</td>
<td>239 (108, 2598)</td>
<td>254 (142, 289)</td>
<td>0.54</td>
</tr>
<tr>
<td>Albumin</td>
<td>Median (range)</td>
<td>4 (2.4)</td>
<td>4 (3.5)</td>
<td>0.54</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Median (range)</td>
<td>4800 (400, 81500)</td>
<td>4900 (100, 10700)</td>
<td>0.62</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Median (range)</td>
<td>1130 (200, 30640)</td>
<td>1140 (200, 17400)</td>
<td>0.91</td>
</tr>
<tr>
<td>Neutrophils/Lymphocyte Ratio</td>
<td>Median (range)</td>
<td>4.0 (0.2, 34.5)</td>
<td>4.4 (0.2, 33.5)</td>
<td>0.76</td>
</tr>
</tbody>
</table>
Abiraterone is an established inhibitor of androgen synthesis by CYP17A1 blockade, however, the mechanism by which statins may potentiate the effect of abiraterone as described above or other PCa treatments is yet to be fully understood. The ability of statins to reduce the availability of pre-cursor cholesterol used for androgen synthesis would seem a natural mechanism for impeding castration-resistance and potentiation of the effects of abiraterone but had yet to be empirically established. As such the following research describes in vivo based experiments analyzing the effect of simvastatin on de novo steroidogenesis and castration-resistant progression of an LNCaP-based xenograft model.

2.3.2 Oral administration of simvastatin resulted in clinically relevant serum concentrations and reduced serum cholesterol

Crucial to the ability to make inferences from xenograft based mouse models on potential therapeutics is the use of clinically relevant concentrations. In order to determine whether the levels of simvastatin and its active metabolite simvastatin hydroxy acid were physiologically relevant to those seen in humans receiving simvastatin the concentrations of both were measured via LC-MS from castrated mice with established LNCaP xenograft tumors treated with or without simvastatin. Serum assessed from the simvastatin diet cohort at 8 weeks post castration had an average simvastatin concentration of 3.29 ± 5.03 ng/mL and simvastatin hydroxy acid of 19.36 ± 11.34 ng/mL (Figure 2.2A). These levels were comparable to clinical levels of simvastatin in humans taking 40 mg P.O., which ranged from 1.0–10.0 ng/mL.

As simvastatin is an established inhibitor of cholesterol synthesis to ensure efficacious concentrations were achieved both circulating and tumor cholesterol was assessed. Circulating cholesterol levels were decreased in the simvastatin group (3.50 ± 0.47 µg/ml) compared to the control diet group (4.20 ± 0.62 µg/ml, p = 0.032, Figure 2.2B) while no change in tumor cholesterol was observed (simvastatin: 1.98 ± 0.177 mg/g, Standard: 1.90 ± 0.176 mg/g, p > 0.05, Figure 2.2C). These findings suggest that simvastatin reached clinically relevant and efficacious concentrations.
2.3.3 Simvastatin administration does not result in overt toxicity

Although robust toxicity would be unlikely at the described concentrations of simvastatin, to ensure the absence of potentially confounding alterations in mouse dietary habits and statin induced toxicity, body weights were monitored once weekly (Figure 2.2D) and common toxicity markers were assessed at end point (Figure 2.2E,F). The average body weight of the simvastatin treated mice was ~7% less than that of the control diet over the treatment period (1 to 8 weeks post-castration) but neither group displayed progressive weight loss. The statin group had a linear regression slope of $-0.177 \pm 0.0847$ g per week and the control group of $0.037 \pm 0.0648$ g per week over the treatment period. These body weights were within the range of historically measured weights of castrated tumor bearing nude mice$^{351}$. The two most common toxicities associated with simvastatin are myopathy and liver dysfunction$^{352}$. As such two common serum markers for these conditions were selected, creatine kinase (CK) for myopathy and alanine transaminase (ALT) for liver dysfunction. For both CK (Statin: $289.51 \pm 79.41$ U/L vs. Control: $282.32 \pm 36.79$ U/L, Figure 2.2E) and ALT (Statin: $0.044 \pm 0.027$ U/mL vs. $0.053 \pm 0.026$ U/mL, Figure 2.2F) levels were indistinguishable between mice receiving the statin diet and those receiving the control diet. These combined results indicate that the mice were not suffering any overt toxicity from the simvastatin treatment.

2.3.4 Simvastatin administration reduces tumor volume

To assess the in vivo efficacy of simvastatin administration on castration-resistant progression and tumor growth LNCaP xenografts were established on two sites subcutaneously in nude mice. Weekly tumor volume and PSA measurements were taken and mice were castrated once PSA exceeded 50 ng/ml. Following castration mice were randomized into standard control or simvastatin diet cohorts and maintained for an additional 8 weeks. Tumor volume measurements were normalized to body weight to account for the ~7% difference in average body weight between the two groups (Figure 2.3A). The tumor volume of the control diet group had increased by more than 2-fold at 6 weeks post-castration and 4-fold at 8 weeks post castration compared to the tumor volume at the time of castration with an apparent
Figure 2.2: Oral simvastatin administration results in efficacious and clinically relevant serum concentrations in the absence of toxicity

The impact of dietary simvastatin on serum statin and cholesterol levels, muscle and liver toxicity and body weight was assessed. (A) Circulating simvastatin (SV) and the active metabolite simvastatin hydroxy acid (SV-HA) levels were measured at experimental endpoint by LC-MS (mean ± SEM, n=9). (B) Total serum cholesterol was measured at experimental endpoint by LC-MS. The simvastatin (statin) diet group had significantly reduced serum cholesterol compared to the standard diet group (Standard, simvastatin: n = 6, standard: n = 10, mean ± SEM, p = 0.032, T-Test). (C) Tumor cholesterol was measured from flash frozen tumors obtained at experimental end point. Tumor cholesterol levels were unchanged between the simvastatin diet group and the standard group (simvastatin: n = 10, standard n = 9, mean ± SEM, t-test p > 0.05, T-Test). (D) Weekly body weigh measurements were made during the course of the experiment. The simvastatin diet group (open circles, n = 10) was ~7% lighter than the standard diet group (closed circles; n = 11) however neither group displayed progressive weight loss over the course of the experiment. (E) Serum creatine kinase activity was assessed by enzymatic assay as a measure of muscle toxicity in terminal serum samples. No difference was observed between the standard diet group (n = 11) and the simvastatin diet group (n = 9, mean ± SEM, p > 0.05, T-Test). (F) Alanine transaminase (ALT) was assessed by enzymatic assay as a measure of liver toxicity. The simvastatin diet group (n = 9) had slightly elevated levels of ALT but no significant difference was observed when compared to the standard diet group (n = 11, mean ± SEM, p > 0.05, T-Test).
exponential increase observed starting at 5 weeks post-castration. In contrast, the statin treated group did not reach a 2-fold increase of castration tumor volume until 8 weeks post-castration. Tumor volume growth rates were assessed by log-transforming tumor volume measurements and fitted versus time by linear regression (Figure 2.3B). The growth rate of LNCaP xenograft tumors in castrated mice were significantly reduced in the simvastatin treated group (0.0648 log mm$^3$/g per week ± 0.0061) compared to the control diet group (0.0921 log mm$^3$/g per week ± 0.0055, Figure 2.3C).

Figure 2.3: Simvastatin suppresses post-castration LNCaP xenograft growth
Weekly tumor volume measurements were conducted using calipers for 8 weeks after castration. Mice were castrated (week 0) when serum PSA values exceeded 50 ng/mL. (A) Weekly body weight-normalized tumor volume measurements. Following castration mice were randomized to either simvastatin diet (Statin, open circles, n = 10) or control diet (Standard, closed circles, n =11) for 8 weeks (mean ± SEM). (B) Weekly tumor volume measurements (weeks 1 to 8) were log transformed and assessed by linear regression to determine slope and extrapolate growth rate. Solid lines represent the best linear fit of mean log-transformed tumor volume (control diet, $y = 0.0921x + 0.9729$, $r^2 = 0.978$, slope = 0.0921 log mm$^3$/week ± 0.0055; Simvastatin diet, $y = 0.0648x + 0.9061$, $r^2 = 0.941$, slope = 0.0648 log mm$^3$/week ± 0.0066). (C) The log$_{10}$ body weight-normalized tumor volume (TV/BW (mm$^3$/g)) growth per week (mean ± SEM) was calculated from linear regression of tumors from the simvastatin (Statin) diet or control diet (Std) groups. The tumor volume growth rate was significantly reduced in the simvastatin treated group compared to the statin group ($p = 0.008$, T-Test).
2.3.5 Simvastatin treatment suppresses intratumoral androgen accumulation and alters androgen receptor activity

As the expression of PSA is regulated through AR activity and the androgen response element (ARE), PSA is regularly used as marker for AR activity pre-clinically. Clinically serum PSA is an accepted measure of tumor burden and disease progression in men being managed for prostate cancer. Generally two successive PSA increases is deemed PSA progression clinically however a strict definition has yet to be determined\(^{353}\). Due to the high variability and rapid tumor growth and progression seen in the LNCaP xenograft model this definition is not ideal. Therefore, here we define PSA progression as the time to two doublings (400%) of nadir serum PSA for each respective mouse. By three weeks post castration it was observed that 5 of 11 standard diet mice had reached PSA progression and 10 out of 11 mice reached PSA progression by 6 weeks post castration (Figure 2.4). Conversely, within the simvastatin treated group only 1 of 10 mice had reached PSA progression at three weeks post castration, 6 out of 10 mice by 6 weeks post castration and 3 of 10 mice never reached PSA progression over the 8 week study period (Figure 2.4). Performing a Kaplan-Meier analysis to statistically assess time to PSA progression the standard diet group progressed significantly faster than the simvastatin diet group (hazard ratio = 0.45, Figure 2.4).

Figure 2.4: Simvastatin suppresses PSA progression in castrated LNCaP xenograft mice
Time to PSA progression was defined as two PSA doublings (400%) of the nadir post-castration and serum PSA levels were measured once weekly over the course of the study. PSA nadir was defined as the lowest weekly serum PSA level following castration, which occurred at either one or two weeks post castration. The fraction of the group yet to undergo PSA progression (percent below 400% nadir PSA) over the 8-week post-castration time course are presented (statin = simvastatin diet group; standard =
control diet group). The simvastatin diet group had significantly prolonged time to recurrence compared with the control diet group (Log-rank, p = 0.049, hazard ratio = 0.45).

In order to assess the effect of simvastatin on post castration de novo steroidogenesis the levels of testosterone, DHT and cholesterol-androgen intermediates was assessed by LC-MS in serum and xenograft tissue collected 8 weeks post castration (Figure 2.5 & Figure 2.6). The tumoral levels of steroidal intermediates were indistinguishable between the standard and simvastatin diet groups except for pregnenolone which was modestly increased (Figure 2.5). Despite this, intratumoral testosterone (Standard: 0.28 ± 0.08 ng/mL, Simvastatin: 0.11 ± 0.03 ng/mL) and DHT (Standard: 0.55 ± 0.16 ng/mL, Simvastatin: 0.21 ± 0.06 ng/mL) levels were significantly reduced in simvastatin treated mice compared to the standard diet cohort (Figure 2.6A). Waterfall plot analysis of the testosterone and DHT results highlight that the highest steroid levels observed in the simvastatin diet group were approximately proportional to the median level in the standard diet group (Figure 2.6C,D). Paralleling the results of the intratumoral steroid analysis the observed testosterone concentration in serum was half in the simvastatin diet group compared to the standard diet group but did not reach statistical significance (Standard: 0.06 ± 0.02 ng/mL, Simvastatin: 0.03 ± 0.01 ng/mL, Figure 2.6B,C). Serum DHT levels were below the lower limit of quantification.

The expression of AR mRNA was assessed through SYBR green qPCR at end point. The simvastatin diet group appeared to trend toward reduced expression of AR, however, expression levels were statistically indistinguishable from the standard diet group (Figure 2.7A). Taken in total, the reduced intratumoral tumor androgen accumulation, and PSA secretion indicate that simvastatin may reduce CRPC tumor growth through inhibitory effects on de novo steroidogenesis.

2.3.6 Simvastatin treatment alters cholesterol metabolism

Further to the assessment of circulating and tumor based cholesterol the expression of key cholesterol metabolism proteins SR-B1 and HMGCR were assessed. The mRNA expression of HMGCR and SR-B1 was assessed by SYBR green qPCR. The simvastatin diet and standard diet groups were
indistinguishable for HMGCR expression (Figure 2.7C). While SR-B1 expression was increased seven fold in the simvastatin treated group compared to the standard diet group (Figure 2.7B).

Figure 2.5: Simvastatin does not alter androgen precursor accumulation in castrated LNCaP xenograft bearing mice
Androgen levels were assessed from tumor homogenates via LC-MS in the simvastatin diet group (Statin, n = 10) and the control diet (Standard, n = 10) group (Mean ± SEM). Concentration of pregnenolone was modestly increased in the simvastatin diet group (p = 0.01, ANOVA with Sidak’s Test).
Figure 2.6: Simvastatin suppresses intratumoral and serum steroid accumulation in castrated LNCaP xenograft bearing mice

Androgen levels were assessed by LC-MS from both tumor homogenates and terminal cardiac puncture serum. (A) Tumor testosterone ($p = 0.031$, T-Test) and DHT ($p = 0.030$, T-Test) levels were significantly reduced in the simvastatin diet group (Statin, $n = 10$) compared to the standard diet group (Standard, $n = 10$, mean ± SEM, T-Test). (B) Serum testosterone was slightly reduced in the simvastatin diet group (Statin, $n = 10$) compared to the standard diet group (Standard, $n = 10$) but did not reach statistical significance ($t$-test, $p > 0.05$, mean ± SEM). Waterfall plots were generated for tumor testosterone (C), tumor DHT (D) and serum testosterone (E) with each column representing an individual castrated LNCaP xenograft mouse.
Figure 2.7: Simvastatin administration alters cholesterol metabolism in the absence of changes to AR expression

qPCR analysis was performed on tumors obtained at study end to assess AR, SR-B1 and HMGCR mRNA expression. (A) AR expression showed a trend towards decreased expression in the simvastatin (statin) group compared to the standard diet (standard) group but did not reach statistical significance (p > 0.05, T-Test; mean ± SEM). (B) SR-B1 expression was significantly increased in the simvastatin diet group compared to the standard diet group (p = 0.013, T-Test; mean ± SEM). (C) HMGCR expression was highly variable but did not display any significant difference between the simvastatin and standard diet groups (p > 0.05, T-Test; mean ± SEM).

2.4 Discussion

The use of the lipid-lowering class of therapeutic agents known as statins has been shown to reduce risk of and mortality from cardiovascular disease. With this understanding statins continue to be prescribed to millions of at risk North American men every year. With the significant demographic overlap between those suffering from prostate cancer and those receiving statins, middle aged to senior aged men, gaining an epidemiological perspective on the effect of these statins at first glance appears straight forward. However, with the rapidly changing landscape of prostate cancer treatment, including the advent of PSA screening, novel therapeutics and our further understanding of treatment altering disease heterogeneity, the picture rapidly becomes obscured. This natural disease heterogeneity and treatment induced heterogeneity alongside the risks and comorbidities that drive the prescription of statins to patients are, perhaps, the reasons that more broad studies have failed to find any association between statin use and risk of cancer development. Despite this, several studies have consistently found that statin use correlates to significantly improved outcomes after the diagnosis of

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prostate cancer\textsuperscript{293}. A meta-analysis of several of these studies concluded that statin use correlated to a 22\% reduction in the risk of metastases (HR: 0.78), a 24\% decrease in the risk of both all-cause and prostate cancer specific mortality (HR: 0.76)\textsuperscript{357}.

The success of both abiraterone and enzalutamide to improve the survival of metastatic CRPC patients brought the role of tumor based \textit{de novo} steroidogenesis, whether from adrenally derived DHEA or tumor cholesterol, as a mechanism of ADT resistance into the spotlight\textsuperscript{123,124,141,150,325}. This sentiment is furthered by the recent findings of the LATITUDE and STAMPEDE clinical trials studying the use of abiraterone at the beginning of ADT. In the LATITUDE clinical trial the median length of progression free survival was 33.0 months in the abiraterone group and 14.8 months in the placebo group (HR: 0.47)\textsuperscript{140}. The STAMPEDE trial reported that the three year failure free survival rate was 75\% in the abiraterone group and 45\% in the ADT-only group (HR: 0.29)\textsuperscript{139}. These findings emphasize the realization that the deactivation of gonadal androgen synthesis through LHRH targeting therapeutics is largely insufficient to fully inhibit the tumoral AR signaling axis and that the addition of further steroidogenic inhibiting therapeutics is essential. Despite these benefits, resistance to abiraterone and other androgen synthesis inhibitors is common place and thought to be largely due to amplification of enzymes, namely CYP17A1, that result in intratumoral accumulation of higher order steroids\textsuperscript{156,358}. Further, abiraterone-resistant CRPCs can gain CYP17A1\textsuperscript{-}independence by becoming responsive to steroid precursors such as pregnenolone and progesterone indicating that despite initial successes in halting AR signaling the combination of ADT and abiraterone is again insufficient to continually inhibit AR activation\textsuperscript{359,360}.

What is yet to be fully understood is whether statins, either by reducing the availability of the primary steroid precursor cholesterol, or by other mechanisms, will potentiate the beneficial effects of abiraterone through reduced steroidogenesis and AR activity or via other nutrient deprivation mechanisms. A recent retrospective report of 187 patients in European Urology Focus found improved overall survival times (HR: 0.51) for patients taking statins and abiraterone as well as increased PSA declines compared to patients only taking abiraterone\textsuperscript{342}. The publication reported an increased percentage
of abiraterone patients saw PSA responses when using statins at 8 weeks, 12 weeks and 16 weeks following the start of treatment. Further, a second retrospective study was published in mid-2017 in two patient cohorts from the Dana-Farber Cancer Institute, 224 patients, and John’s Hopkins University, 270 patients\(^3\). The Dana-Farber Cancer Institute cohort displayed a trend toward improved survival (HR: 0.79) but failed to reach statistical significance and the John’s Hopkins University showed no difference with statin use (HR: 0.89) as described by the authors. Here we report a validation study on a cohort of patients from the British Columbia Cancer Agency receiving abiraterone for metastatic or locally advanced CRPC. A clear, however muted in comparison to the findings of Di Lorenzo et al. (2017), improvement in survival was observed in the British Columbia cohort (HR: 0.79) reaching statistical significance using the Gehan-Breslow-Wilcoxon test (p = 0.022) but not the log-rank test (p = 0.078). This differences likely being a result of the Gehan-Breslow-Wilcoxon test being better powered to detect early differences in survival distributions compared to the log-rank test\(^3\). Complete records on 279 patients allowed for multivariable modeling adjusting for age, prostatectomy, radiation, time from diagnosis, neutrophils-lymphocyte ratio and presence of metastases. The effect of statin use on overall survival remained borderline when using this model (p = 0.087) and did achieve statistical significance when assessing the 241 patients that had available Gleason grade information allowing for its inclusion in the multivariate model (p = 0.024). Unlike the European Urology Focus report only a very minor increase in the percentage of patients who saw PSA responses at 8 and 12 weeks were observed with statin use. However, both the number of patients who saw PSA response at any time and relative extent of PSA decline to nadir were increased but failed to reach statistical significance (p = 0.10 and p = 0.086).

When assessed in the multivariable model similar borderline effects were observed for the ability of concomitant statin use to predict a PSA response in abiraterone patients. Taken as a whole these findings appear to indicate a trend of improved outcomes for men taking statins concomitantly with abiraterone although to a lesser extent than the patients observed by Di Lorenzo et al (2017)\(^3\). The reason for the observed differences between the two datasets is likely a result of several compounding factors. Both are limited to a relatively small sample size and come from different population centers,
British Columbia versus Italy. Contributing factors could include differing standards of care, ethnic backgrounds, economic status, and dietary habits (western diet vs Mediterranean diet). Further, there are specific factors of note in the British Columbia dataset that could have confounded the potential benefits of statins. The average age of statin users was 4 years older than that of non-statin users at the time of the patient starting abiraterone. The statin treated group, perhaps expectedly due to their use of statins, had a significantly higher comorbidity burden and were in general in poorer health as indicated by the Charlson index scoring. Although, the individual aspects of the index are likely underpowered to make definitive conclusions there is a clear trend towards poorer cardiovascular health through the increases in hypertension, myocardial infarction, peripheral disease and diabetes in the statin treated population. These factors although not directly related to prostate cancer specific mortality do play a role in patient overall survival and should not be ignored when analyzing retrospective studies of this nature.

These confounding factors combined with the heterogeneity of the disease state likely act to mute differences between statin and non-statin users likely requiring a high number of patients to establish statistical significance. As such, the results described within this chapter were combined into a larger multi-institutional study that has been published in the peer-reviewed journal *Oncotarget*. This study assessed 598 patients across eight Centers receiving abiraterone or enzalutamide. The results of the combined study found a statistically significant increase in median overall survival of 7.9 months. Further, multivariate analysis found both a statistical significant decrease of 53% in the risk of death and statistically significant increase of 63% in potential for a > 30% PSA decline within the first 12 months of abiraterone treatment. These findings help to further validate the trends observed within the British Columbia Cancer Agency cohort indicating the beneficial role of statins in patients undergoing abiraterone treatment for metastatic CRPC. With the mounting evidence for the beneficial role for statins in both prostate cancer and abiraterone treated prostate cancer a prospective clinical trial seems warranted. This would importantly allow for the correction of the potential confounding variable discussed above such as patient general health and treatment history and help garner a true understanding of the potential role of statins in the prostate cancer therapeutic landscape.
As the effectiveness of statins on improving prostate cancer outcomes clinically is becoming clearer the necessity to understand the mechanism by which statins impact prostate cancer becomes evident. Several separate mechanisms have been proposed including the inhibition of SLCO2B1\textsuperscript{325}, geranylgeranyl pyrophosphate and farnesyl pyrophosphate synthesis inhibition\textsuperscript{363} and a novel mechanism described here in which reduced synthesis of precursor cholesterol impedes androgen synthesis and AR activation in CRPC\textsuperscript{364}. In order to evaluate this mechanism of beneficial statin use in prostate cancer we performed an LNCaP xenograft study assessing the effect of simvastatin administration on castration-resistant progression. The validity of oral administration through diet incorporation of simvastatin was assessed by measuring circulating concentrations of simvastatin and simvastatin hydroxy acid. Further, the common simvastatin toxicity markers CK and ALT were assessed to measure liver and muscle toxicities. The observed serum levels of simvastatin were proportional to those seen in humans taking a relatively high but clinically acceptable dose of 40 mg daily simvastatin\textsuperscript{350}. The ability to obtain this dose in the absence of significant changes in toxicity markers indicates that any changes in cancer progression of the LNCaP xenografts occurred at clinically achievable doses.

The determination of the best animal model for studies of cholesterol and lipid metabolism has been a debated topic with rabbits, rats and mouse models all having significantly differing lipid profiles to that of humans\textsuperscript{196}. Mice specifically are known to have lipoprotein profiles primarily composed of circulating HDL while the human lipoprotein profile is LDL dominant\textsuperscript{196}. Statin treatment in these mice does not consistently alter circulating cholesterol levels but has been reported to lower cholesterol production in peripheral tissues (reviewed by Solomon and Freeman\textsuperscript{365}). Undoubtedly these differing lipid profiles handicap the ability of one to make clinically based inferences, however, despite these differences a decrease in serum cholesterol was measured in the simvastatin diet group indicating that efficacious levels of the drug were reached. The reduction of serum cholesterol did not translate to a reduction of total tumor cellular cholesterol levels. This relatively unsurprising finding is likely due to the large discrepancy in cellular free cholesterol and cholesterol otherwise sequestered in the plasma membrane. It has been reported that up to 80% of cellular cholesterol resides in the plasma membrane and the majority
of the remainder being distributed among internal membranes leaving only a limited pool of free cholesterol to be used as a precursor for other purposes including de novo steroidogenesis\textsuperscript{366}. Earlier work studying the effect of simvastatin on PCa cells \textit{in vitro} also did not report measurable changes in cellular cholesterol\textsuperscript{340}. 

Previously, Zheng \textit{et al.} reported the ability of atorvastatin to decrease post-castration LNCaP xenograft growth concluding that the decreased tumor growth was a result of increased apoptosis\textsuperscript{367}. Here we report a reduction in the AR ligands testosterone and DHT and observe that the time to PSA progression following castration was significantly increased in the simvastatin treated group. These findings are indicative of repressed AR reactivation and signaling correlating to delayed castration-resistant progression of the xenografts corroborating previously reported findings that simvastatin treatment decreases PSA production in C4-2 cells \textit{in vitro}\textsuperscript{340}. Conversely it has been shown that a hypercholesterolemia diet in LNCaP xenograft mice promoted elevated circulating cholesterol levels that was in turn correlated to increased \textit{de novo} steroidogenesis through elevated intratumoral testosterone and expression of CYP17A1\textsuperscript{283}. These findings correlate to clinical findings that suggest the capacity of tumors to acquire \textit{de novo} steroidogenic potential is increased in bone metastases by the increased expression of the factors responsible for the uptake of cholesterol from high-density lipoprotein (SR-B1) and low-density lipoprotein (LDLr) and steroid synthesis\textsuperscript{368}. Taken as a whole these studies indicate the importance of cholesterol availability in promoting CRPC progression through intratumoral steroidogenesis.

Despite these findings, we cannot rule out the potential anti-cancer activities of prolonged reduction of cholesterol availability and simvastatin administration that may have impacted non-AR based mechanisms including non-cholesterol mediated pleiotropic effects\textsuperscript{365}. The ability of tumors to synthesize testosterone directly from an available cholesterol pool and our ability to modify that mechanism through reduction of said cholesterol pool does not preclude the ability of these tumors to uptake testosterone synthesis intermediates to drive AR reactivation and castration-resistant progression. A recent report concluded that the beneficial effects seen clinically with statin use may be due to the ability of some statin
class molecules to inhibit the uptake of DHEAS through SLCO2B1\textsuperscript{325}. Although potentially clinically important, adult mice do not produce adrenal DHEAS\textsuperscript{369} making it likely irrelevant in mouse based xenograft studies and making the finding of reduced androgen accumulation in simvastatin treated mice more consistent with a cholesterol depletion based phenomenon. Lastly, the nude mice used in these studies generally have circulating serum testosterone levels of 0.1 – 0.4 ng/mL prior to castration and undetectable levels post-castration\textsuperscript{347}. The ability of castration to deplete serum testosterone was confirmed by the low levels observed in the castrated mice. The presence of still detectable concentrations of serum testosterone in the castrated hosts was likely the result of leakage from the highly vascularized and rapidly growing LNCaP xenografts present at the time of euthanasia.

The beneficial effect of statins in treating prostate cancer clinically would likely be due to a combination of several mechanisms including both AR-dependent and independent mechanisms of cellular homeostasis. These mechanisms are of relevance to the current landscape of prostate cancer treatment with the potential of statins to interfere with various mechanisms of resistance to current therapeutics including the shuttling of androgens through the “back-door” pathway for testosterone synthesis and mutant AR activation by pregnenolone to overcome abiraterone treatment\textsuperscript{370,371}. The findings described in this chapter demonstrate the potential of statins in treating CRPC alongside currently in use second-line AR axis inhibitors. Statins are the most commonly used therapeutic method of lowering cholesterol in the clinic. However, the upregulation of SR-B1 in response to statin administration described here, as well as it’s upregulation in models of CRPC\textsuperscript{289,290}, highlight the multifaceted approach of the cancer cell to obtain cholesterol and implicate SR-B1 as a major mechanism of cholesterol regulation in PCa. As such, an alternative approach to targeting cholesterol through SR-B1 antagonism will be described in the following chapter.
CHAPTER 3: SR-B1 A NOVEL TARGET FOR PROSTATE CANCER TREATMENT THROUGH ANDROGEN RECEPTOR PATHWAY-DEPENDENT AND –INDEPENDENT MECHANISMS

3.1 Specific aim and rationale

In comparison to the body of research regarding statin use in PCa, our understanding of the potential of SR-B1 targeting as a therapeutic approach to PCa treatment remains limited. Initial evidence demonstrated the upregulation of SR-B1 with castrate-resistant progression of pre-clinical LNCaP xenografts. Clinically SR-B1 has recently been associated with high Gleason grade primary cancers and correlated with decreased disease-specific survival. Further, SR-B1 expression was correlated with androgen synthesis enzymes 3β-HSD and 17βHSD, and the mTOR target, ribosomal protein S6, indicating potential links to de novo steroidogenesis and critical metabolism regulatory pathways. Despite, encouraging initial clinical evidence, further research was needed to confirm and expand our understanding of the expression of SR-B1 across the PCa landscape. As such, clinical tumor samples were assessed for major cholesterol metabolism proteins by immunohistochemical (IHC) and mRNA expression analysis. Preliminary work by the Wasan & Cox research groups targeting SR-B1 pre-clinically indicated the ability to suppress PSA secretion and reduce viability of C4-2 CRPC cells. Remaining unresolved was the impact of SR-B1 antagonism on critical proliferative cellular functions such as androgen synthesis and nutrient homeostasis, or in which disease context would targeting SR-B1 be most effective. As such, the studies described in this chapter aimed to elucidate the effects of SR-B1 inhibition by both interfering RNA and small molecule techniques in both the steroidogenic and steroid-independent models of PCa.

3.2 Methods

3.2.1 Immunohistochemical and mRNA expression analysis of clinical samples

IHC staining of the Prostate Cancer Donor Rapid Autopsy Program at the University of Washington (UWRA, Seattle, WA) samples was performed courtesy of the Dr. Colm Morrissey group in the Department of Urology at University of Washington, using a 1:500 dilution of SR-B1 primary
antibody (AB52629, Abcam, Cambridge, United Kingdom) and a pH 9.0 antigen retrieval process. mRNA expression data for SCARB1 (SR-B1), LDLr and HMGCR was obtained from several databases for assessment of general expression, and the comparison of expression between cancerous prostatic tissue and normal prostatic tissue as well as CRPC and neuroendocrine prostate cancer (NEPC). Expression data from the Shanghai cohort (SC) was obtained courtesy of Dr. Colin Collins (Vancouver Prostate Centre) for 28 patients with paired normal prostatic tissue and localized cancerous samples. Expression data from the The Cancer Genome Atlas-PRAD (TCGA) dataset was acquired through the National Cancer Institute GDC Data Portal for 375 localized cancer patients and 48 samples of normal prostatic tissue. Expression data from the UWRA was obtained courtesy of Dr. Colm Morrissey for 83 rapid autopsy patients. Expression data from the Weill Cornell (WC) dataset was acquired courtesy of Dr. Himisha Beltran (Weill Cornell) for 78 advanced stage prostate cancer patients.

3.2.2 Prostate cancer cell lines

The human CRPC cell lines C4-2 and PC-3 were maintained in phenol red-free Roswell Park Memorial Institute (RPMI) 1640 media (Life Technologies) supplemented with 5% fetal bovine serum (FBS, Life Technologies) and Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) with 10% FBS (Life Technologies) respectively in a 5% CO2/37 °C incubator.

3.2.3 Antibodies

Information on sources, species of origin, and dilutions primary antibodies used can be found in Table 3.1.

**Table 3.1: Antibody specifications for primary antibodies used in western blotting**

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3.2.4 RNA-interference transfection protocol

Cells were seeded into 6-welled plates unless stated otherwise and transfected two days post seeding. The transfection media consisted of RPMI or DMEM +1%FBS. Lipofectamine RNAiMAX transfection reagent and either Stealth RNAi duplexes targeting SR-B1 (SRB1-KD, Oligo ID HSS101571: AUAAUCCGAAUCUGUCUUGAAGGG, Cat. No. 1299001) or Lo GC Negative Control duplexes (NC, Cat. No. 12935-110) were combined in Opti-MEM I reduced serum media for 20 min prior to addition to transfection media to a final concentration of 10 nM RNAi and 0.25% Lipofectamine (All purchased from Life Technologies). Cells were incubated with transfection media for 5 h following which cells were allowed to recover overnight in Opti-MEM I. The following day the media was changed to either RPMI-1640 with 5% charcoal-dextran stripped FBS (CSS, Life Technologies) to replicate androgen deprived conditions for C4-2 cells, or DMEM with 10% FBS for PC-3 cells. For experiments containing DHEA, DHEA was added to the culture medium of cells transfected with either the negative control or the SR-B1 targeted siRNA as described above 1 day post-transfection. Unless otherwise stated all assays were conducted at 4 days post-transfection as a previously established time point for nadir of SR-B1 expression in C4-2 cells by the group.

3.2.5 BLT-1 treatment protocol

Cells were seeded into 6-welled plates unless stated otherwise. Three days post seeding the media was transferred to either RPMI-1640 with 5% CSS for C4-2 cells or DMEM with 10% FBS for PC-3 cells and either dimethyl sulfoxide (DMSO, vehicle) or the specified concentration of BLT-1 was added. Unless otherwise specified all assays were conducted at 3 days post treatment initiation to mirror the siRNA treatment method for time spent in CSS supplemented media and replicate as close as possible the time of SR-B1 antagonism between the two methods.

3.2.6 HDL-cholesterol uptake assay

Studies performed to approximate the cellular uptake of HDL-derived cholesterol were conducted using the fluorescent lipid 1,1’-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI,
excitation max: 549 nm, emission max: 565 nm) from labelled HDL particles (DiI-HDL) similar to previously published methods\textsuperscript{378-381}. For siRNA treated cells the assay was performed 4 days post-transfection while BLT-1 treated cells were treated overnight prior to assay start. Cells were incubated in standard media containing 0.5% bovine serum albumin (BSA) and DiI-HDL (10 μg HDL protein per mL, Alfa Aesar, Haverhill, MA) for 2 h in a tissue culture incubator (37 °C/5% CO\textsubscript{2}). Following incubation cells were washed with PBS and harvested by trypsinization. Cells were then twice washed via centrifugation (1,000 rpm, 5 min) and suspended in PBS prior to being subjected to fluorescence activated cell sorting (FACS) analysis on a BD FACSCANTO II flow cytometer (Beckman, Dickinson & Company, Mississauga, Canada). Excitation was performed using a 488 nm laser line and fluorescence was detected using the PE channel (585/42 bandpass). Mean fluorescent intensity data were analyzed using BD FACSDiva software (Beckman, Dickinson & Company). Gating was performed based on forward (FSC) and side (SSC) scatter characteristics according to previously published methods\textsuperscript{382}. Mean fluorescent intensity was normalized to the mean fluorescent intensity of non-treated cells incubated with DiI-HDL.

3.2.7 Quantitative PCR

RNA was isolated from cells using a TRIzol (Life Technologies) chloroform extraction according to manufacturer instructions. 1 mL of TRIzol was used to suspend cells and incubated for 5 min. 0.2 mL of chloroform was then added and incubated for 3 min prior to centrifugation (15 min, 12,000 g, 4 °C). The aqueous phase was separated and incubated with 0.5 mL of isopropanol for 10 min prior to centrifugation (10 min, 12,000 g, 4 °C). The RNA precipitate was pelleted then washed with 1 mL 75% ethanol, pelleted and suspended in 25 μL RNase-free water. cDNA was synthesized from the RNA using a SuperScript\textsuperscript{®} II Reverse Transcriptase kit (Life Technologies) according to manufacturer instructions. First strand synthesis was carried out with 50 – 250 ng random primers, 0.5 mM dNTP mix and 1 ng - 5 μg total RNA, incubated at 65 °C for 5 min. 1x First-strand buffer, 10 mM DTT and 40 U RNaseOUT were then added to the mixture and incubated for 2 min at 42 °C. 200 U of SuperScript\textsuperscript{®} II Reverse
Transcriptase were added prior to 42 °C incubation for 5 min and inactivation by heating to 70 °C for 15 min. 2 U of RNase H was then added to each tube and incubated at 37 °C for 20 min. qPCR was performed on an ABI 7900HT (Life Technologies) using FastStart Universal SyberGreen Master mix (Roche) and commercially available Qiagen SYBR Green probes targeted to SR-BI (QT00033488), HMGCR (QT00004081), PSA (QT00027713) and NKX3.1 (QT00202650). The following conditions were used: 50 °C for 2 min, 95 °C for 10 min, 40x: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 °C for 15 sec, 95 °C for 15 sec.

3.2.8 IncuCyte Zoom cellular growth assay

The Incucyte Zoom live-cell analysis system (Essen Bioscience, Ann Arbor, MI) consists of a tissue culture incubator with built-in imaging equipment capable of imaging tissue culture plates undisturbed and tracking cellular growth within individual plate wells. Cells were either siRNA transfected two days post seeding or treated with BLT-1 three days post seeding. Plates were then placed into the Incucyte Zoom incubator (Essen Bioscience) and phase contrast imaging with internal software processing was used to generate confluency readings taken every 6 h. Confluency readings were then used to generate cell growth curves over time.

3.2.9 Live/Dead cytotoxicity assay

The Live/Dead Cytotoxicity assay (Invitrogen, Carlsbad, CA) was carried out according to manufacturer instructions. Following BLT-1 treatment or siRNA transfection cells were harvested with trypsin, pelleted (1,000 rpm, 5 min) and suspended in PBS containing 100 nM calcein AM and 8 µM ethidium homodimer-1. Cells were then incubated at room temperature, away from light for 20 min prior to FACS analysis on a BD FACSCANTO II flow cytometer. Excitation was performed using a 488 nm laser line, green fluorescence was detected using the FITC channel (530/30 bandpass) and red fluorescence was detected using the 7-AAD channel (670 longpass). Data were obtained and analyzed using BD FACSDiva software. Gating was performed based on FSC and SSC characteristics. Cells were then stratified by quadrant gating into two categories: those who displayed high green fluorescence...
indicating intracellular esterase activity (living cells), and those who displayed high red fluorescence indicating damaged or permeable membranes (dead or dying cells).

3.2.10 Cell cycle analysis

Cell cycle analysis was performed using the propidium iodide (PI) based method as previously described\textsuperscript{384-386}. Samples previously fixed with ethanol and washed in PBS were suspended in DNA staining buffer (50 μg/mL PI, 0.1 mg/mL RNAse A, 0.05% Triton X-100, PBS) for one hour in the dark at room temperature. Immediately after staining, samples were subjected to FACS analysis on a BD FACSCANTO II flow cytometer (Beckman, Dickinson & Company). A 488 nm laser line was used for excitation and fluorescence was detected using the PE channel (585/42 bandpass). Data were obtained and analyzed using BD FACSDiva software (Beckman, Dickinson & Company). Gating was performed based on FSC and SSC characteristics and interval gates were used to separate cell cycle phase fractions by DNA content according to established methods\textsuperscript{386}.

3.2.11 Western blotting

Whole cell lysates were prepared by incubating cells with ice cold modified radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Roche) for 5 min prior to collection using cell scraper. Lysed cells in RIPA buffer were centrifuged (8,000 g, 10 min) and the supernatant recovered. Protein concentrations of cell lysates were measured using the Pierce BCA Protein Assay (Fisher, Hampton, NH) or Bradford Protein Assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA) using a semi-dry transfer apparatus (Trans-Blot Turbo, Bio-Rad) in the presence of Towbin transfer buffer (20% methanol supplemented running buffer). Following protein transfer membrane blocking with 5% BSA in tris-buffered saline, 0.1% Tween 20 (TBS-T) for 1 hour at room temperature was performed. Primary antibody incubation was carried out overnight at 4 °C and followed by secondary antibody incubation (Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling
[7074]; IRDye® 800CW Goat anti-Mouse IgG, Li-Cor [925-32210]) for 1 hour at room temperature. In between each step post-transfer the membrane was washed 3 times for 5 min in TBS-T. Membranes imaging was performed either by enhanced chemiluminescence detection (Supersignal West Pico, Thermo Fisher, Waltham, MA) or on an Odyssey CLx infrared imaging system using Odyssey software version 3.0 (LI-COR, Lincoln, NE).

3.2.12 Fluorescent microscopy

C4-2 cell morphology following siRNA transfection was examined by fluorescent microscopy. Cells were cultured on coverslips (Fisher) in 6-well plates and transfected as described previously. Cells were then washed with PBS and fixed with 10% buffered formalin (Fisher) for 15 min at 37 °C. Cells were then washed again and blocked in 10% goat serum, TBS supplemented with background sniper blocking reagent (Biocare Medical, Concord, CA) for 1 hour at room temperature. Cells were then stained with wheat germ agglutinin (WGA) labeling to highlight membranous cellular organelles using WGA from *Triticum vulgaris* conjugated to Alexa Fluor 647 (WGA-647, Life Technologies) at a final concentration of 10 μg/mL. Cells were incubated in WGA-647 diluted in TBS-T for one hour at room temperature, protected from light. Cells were then washed in TBS and coverslips were mounted and counterstained with Vectashield antifade mounting medium with DAPI (Vector Laboratories, Burlington, ON). Imaging was performed on the Zeiss 780 confocal microscope (Toronto, Canada).

3.2.13 Steroid analysis by LC-MS

Cellular androgen levels were assessed and quantified from ~100 mg cell pellets following either transfection or BLT-1 treatment by LC-MS similar to previously described. To obtain sufficient material for analysis cells were seeded into five T75 cell cultures flasks (~4.2 x 10⁹ cells) and treatment groups merged prior to LC-MS analysis. Internal standard (20 pg/40 pg deuterated testosterone/DHT; C/D/N isotopes, IS) was added and pellets were twice extracted by 30 min vortexing with 60/40 hexane/ethyl acetate. Extracts were pooled, dried using a CentriVap (Labconco), reconstituted in 50 μL of 50 mM hydroxylamine and incubated 1 hour at 65 °C. Resulting steroid-oxime derivatives were analyzed
using a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer (Waters). Separations were carried out with a 2.1x100 mm BEH 1.7 µM C18 column, mobile phase water (A) and acetonitrile (B) (gradient: 0.2 min, 25% B; 8 min, 70% B; 9-12 min, 98% B; 12.2 min, 25% B; 14 min run length). Data was collected in ES+ mode by multiple reaction monitoring with instrument parameters optimized for the mass to charge ratios and corresponding fragments of the oxime-steroids for all keto-steroids in the androgen synthesis pathway. Data processing was performed with Quanlynx (Waters) and exported to Excel (Microsoft, Redmond, WA) for additional normalization to pellet weights as required. Quantification was by AUC ratio of standard to IS. Deuterated T was used as IS with all analytes other than DHT for which deuterated DHT was used and a curve of 6 calibration standards (0.01-10 ng/mL) was generated (R²> 0.98) for determination of steroids concentrations in the test samples.

3.2.14 PSA secretion quantification

PSA secreted into media was quantified for transfected or BLT-1 treated cells using an electrochemiluminescent immunoassay on a Cobas e 411 analyzer (Roche Diagnostics) and analyzed according to manufacturer’s instructions. 150 µL of media was analyzed using the fully automated procedure. The Cobas e 411 analyzer is validated for clinical use and determining PSA concentrations ranging from 0 – 100 ng/mL.\(^{387}\)

3.2.15 Statistical analyses

Statistical analyses on all data were performed using GraphPad Prism software version 6 (GraphPad, La Jolla, CA). Two-sided Student’s t-tests and ANOVA with Tukey’s multiple comparisons test were used to determine differences between treatment groups. Means of the data sets were considered to be statistically significantly different if p < 0.05.

3.3 Results

3.3.1 SR-B1 is highly expressed in primary, metastatic and neuroendocrine prostate cancer
In order to assess the expression of SR-B1 across the PCa disease state IHC staining of rapid autopsy specimens from the UWRA was performed and confirmed via analysis of multiple clinical mRNA expression datasets. This included samples of cancerous prostate and patient matched adjacent normal prostatic tissue from 127 patients as well as terminal rapid autopsy specimens of varying metastatic sites including bone, lymph node, liver and lung from 71 patients. The tissue specimens containing stromal and epithelial cells displayed a range of SR-B1 staining, with high expression samples staining predominantly along the plasma membrane of epithelial cells with stromal adjacent epithelial cells appearing to show the highest expression (Figure 3.1). Each sample was scored by an experienced independent pathologist with a score between 0 and 3 (0 = no staining, 1 = low staining, 2 = moderate staining, 3 = high staining). For normal prostatic tissue only 24% of samples were scored as moderate to high staining for SR-B1 while 71% of local prostate cancer samples were scored as moderate to high staining. Similarly, to local PCa, metastatic samples showed high levels of SR-B1 staining. Moderate to high staining of SR-B1 was scored in 57% of bone, 77% of liver, 84% of lymph node and 84% of lung samples.

Clinical mRNA expression data for SCARB1 (SR-B1), LDLr and HMGCR was collected from multiple datasets to analyze the expression level of important cholesterol metabolism proteins across the disease state. In total four different datasets were analyzed, two allowed for the comparison of expression levels in normal prostate to cancerous prostate (SC and TCGA datasets, Figure 3.2), one reporting expression information from metastatic tumors (UWRA Dataset, Figure 3.2) and two allowing for the comparison of CRPC to NEPC (UWRA and WC Datasets, Figure 3.3). Both databases assessed for the comparison of normal prostatic tissue to cancerous prostatic tissue showed increased expression of SR-B1, decreased expression of LDLr and a minor, but not significant, decrease in HMGCR in the cancerous tissue (Figure 3.2A,B: SC: SCARB1: +18%; LDLr: -47%; HMGCR: -21%, n = 27; TCGA: SCARB1: +68%; LDLr: -23%; HMGCR: -10%, normal n = 48, cancer n = 375). In these primary cancers SR-B1 expression was nearly double that of LDLr and four times HMGCR expression for the SC database (Mean Counts: SCARB1: 2341, LDLr: 1267, HMGCR: 586, n = 27, Figure 3.2A) contrasting with the TCGA
database for which SR-B1 was the lowest expressed, although only slightly, of the three proteins (Mean fragments per kilobase of transcript per million mapped reads [FPKM]: SCARB1: 9.47, LDLr: 11.48, HMGCR: 11.31, n = 375, Figure 3.2B). The analysis of metastatic prostate cancer tissue, through the UWRA database showed a high expression of SR-B1 compared to the other proteins (Mean FPKM: SCARB1: 28.50, LDLr: 9.03 HMGCR: 11.50, n = 83). SR-B1 expression was highest in liver metastasis, while bone metastasis had the lowest expression. Due to the differing protocols and units used in these mRNA expression databases comparisons were not made between databases. Further, differing probe-mRNA affinities for each target examined could impact the differences observed between SCARB1, LDLr and HMGCR expression and as such should be considered when making conclusions.

Figure 3.1: SR-B1 expression is increased in local and metastatic prostate cancer
(A) Representative IHC staining for SR-B1 of University of Washington rapid autopsy samples across the prostate cancer landscape and (B) pathological scoring by an independent pathologist. (Normal/NP = Normal prostatic tissues, Cancer/PCa = Localized prostate cancer, Liver Met. = Liver metastasis, LN Met. = Lymph node metastasis, Rib Met. = Rib bone metastasis, Adrenal Met. = Adrenal metastasis (p < 0.001, Chi-square).
Figure 3.2 SR-B1 expression is increased in prostate cancer

The mRNA expression levels of important cholesterol metabolism proteins were assessed from available expression datasets. (A) normalized mRNA sequencing counts were analyzed from the Shanghai Cohort. SR-B1 (SCARB1) was more highly expressed than both LDLr and HMGCR (Left, n = 27, p < 0.001, ANOVA with Tukey’s Test) and cancerous prostatic tissue (PCa) had increased expression of SR-B1 (p < 0.001) and decreased expression of LDLr (p = 0.002) when compared to matched normal prostatic tissue (Normal)(Right, n = 28, ANOVA with Sidak’s Test). (B) Analysis of the mRNA expression reads (FPKM) from the TCGA database found that SR-B1 (SCARB1) expression was lower than that of LDLr and HMGCR (Left, n = 375, p < 0.001, ANOVA with Tukey’s Test). The expression of SR-B1 (p < 0.001) was increased in cancerous prostatic tissue (PCa) and LDLr (p < 0.001) expression decreased when compared to normal prostatic tissue (Normal)(Right, Cancer: n = 375, Normal: n = 48, ANOVA with Sidak’s Test). (C) mRNA expression (FPKM) analysis of metastatic tumors from the University of Washington rapid autopsy dataset SR-B1 (SCARB1) was more highly expressed than LDLr and HMGCR (Left, n = 83, p < 0.001, ANOVA with Tukey’s Test). (Right) The expression of SR-B1 (SCARB1), LDLr and HMGCR by site of metastasis. The expression of SR-B1 was higher in liver (p = 0.008) than...
bone and lymph node (LN, p < 0.001) tissue (n = 83, ANOVA with Sidak’s Test). For box and whisker plots: middle line = median, box = 25th to 75th percentile, bars = min. to max.

To assess SR-B1 expression in NEPC two databases were assessed, UWRA and WC datasets which sequenced and stratified metastatic prostate cancer into adeno-CRPC and neuroendocrine-CRPC. Both of the databases showed increased expression of SR-B1 in the absence of changes to LDLr and HMGCR expression (UWRA: SCARB1: +49%; LDLr: -8%, HMGCR: -9%; CRPC n = 65, NEPC n = 18, Figure 3.3A; WC: SCARB1: +66%; LDLr: -10%; HMGCR: -17%, CRPC n = 34, NEPC n = 15, Figure 3.3B). These results taken as whole suggest an increased expression of SR-B1 in primary, metastatic and neuroendocrine prostate cancers highlighting its potential as a therapeutic target in CRPC.

Figure 3.3 SR-B1 expression is increased in NEPC
The mRNA expression levels of important cholesterol metabolism proteins in CRPC and NEPC were assessed from available expression datasets. (A) Using the University of Washington rapid autopsy database SR-B1 expression was found to be increased in NEPC (CRPC: n = 65, NEPC: n = 18, p < 0.001, ANOVA with Sidak’s Test). (B) Using the Weill Cornell database SR-B1 expression was found to be increased in NEPC (CRPC: n = 34, NEPC: n = 15, p = 0.036, ANOVA with Sidak’s Test). For box and whisker plots: middle line = median, box = 25th to 75th percentile, bars = min. to max.

3.3.2 SR-B1 antagonism alters cholesterol metabolism of castration-resistant C4-2 cells
As a preliminary assessment into the viability of targeting SR-B1 in PCa initial experiments were performed to validate potential approaches to SR-B1 antagonism. Both siRNA knockdown and small molecule inhibition with block lipid transport-1 (BLT-1) were assessed for their ability to reduce HDL-derived cholesterol uptake by the uptake of fluorescent lipid-DiI with flow cytometry. In order to interpret this effect each sample was normalized to the fluorescent uptake of non-treated (NT) cells and presented as a percent change in uptake for treated cells. The SR-B1 targeted siRNA reduced fluorescent uptake by
39% (NC: 89.9 %MFI/NT, 55.0 %MFI/NT*100, n = 3, Figure 3.4A) and BLT-1 by 62% in C4-2 cells (Veh: 90.07 %MFI/NT*100, BLT-1: 34.3 %MFI/NT*100, n =3, Figure 3.4A). To confirm the ability of SR-B1 targeted siRNA to reduce expression SR-B1 mRNA expression was measured by qPCR in C4-2 cells. The targeted siRNA reduced mRNA expression by 65% compared to scramble control (n = 3, Figure 3.4B). Further confirmation of the effect of the siRNA treatment on SR-B1 expression can be found in the western blots seen in Figure 3.9B. Due to the ability of simvastatin to induce SR-B1 expression as described Chapter 2 (Figure 2.7B) the effect of SR-B1 knockdown on HMGCR expression was assessed by qPCR. C4-2 SR-B1 knockdown cells had an increase of HMGCR mRNA of 217% (n = 3, Figure 3.4C). The induction of SR-B1 expression with HMGCR inhibition as described in Chapter 2 and the increase in HMGCR expression described here may indicate a compensatory mechanism by which the cell shifts the pathway through which it obtains cholesterol.

Figure 3.4 SR-B1 antagonism inhibits cholesterol uptake and induces HMGCR expression (A) SR-B1 siRNA silencing or BLT-1 treatment C4-2 cells followed by DiI-HDL incubation and mean fluorescence measured using flow cytometry. siRNA knockdown (SRB1-KD) and BLT-1 treatment in C4-2 cells (n = 3, siRNA: p = 0.019, BLT-1: p = 0.0013, ANOVA with Sidak’s Test, mean ± SEM) reduced DiI-HDL uptake compared to negative siRNA control (NC) and DMSO vehicle (Veh.) respectively. Expression of SR-B1 mRNA (B) (n = 3, p < 0.001, T-Test, mean ± SEM) assessed by qPCR, was reduced and expression of HMGCR (C) (n = 3, p = 0.012, T-Test, mean ± SEM) increased following anti-SR-B1 siRNA (SRB1-KD) treatment compared to negative control (NC) in C4-2 cells.

3.3.3 SR-B1 antagonism inhibits cellular proliferation of C4-2 cells

To determine if the effects on cholesterol metabolism described above affected the proliferation of the prostate cancer cells an Incucyte Zoom based cellular growth assay was performed. The confluency of plated cells treated with siRNA transfection or BLT-1 was measured every 6 h. C4-2 cells grew to
almost 30% confluency when treated with the scramble control (NC) and despite seeing modest growth initially cells treated with the targeted siRNA rapidly arrested (Figure 3.5A). The respective growth rates were 0.192 ± 0.012 %/hour and 0.030 ± 0.005 %/hour for the scramble and targeted siRNA respectively (n = 3, Figure 3.5C). BLT-1 treated C4-2 cells displayed progressive growth arrest with increasing concentrations of BLT-1. A treatment of 1 µM BLT-1 resulted in a slight decrease in cell growth (0.365 ± 0.007 %/hour), 10 µM resulted in only very modest growth (0.087 ± 0.004 %/hour) and 20 µM treatment caused near total growth arrest (0.019 ± 0.005 %/hour) when compared to the vehicle (0.440 ± 0.015 %/hour, n = 3, Figure 3.5B,D).

**Figure 3.5 SR-B1 antagonism inhibits C4-2 cell growth**

C4-2 cells were (A) transfected with SR-B1 silencing siRNA (SRBI-KD) or negative control (NC) or treated with (B) BLT-1 or vehicle (right) prior to incubation in the IncuCyte Zoom system. Confluency measurements were taken every 6 hours (n = 3, mean ± SEM). Linear regression was used to calculate the slope of each treatment in order to determine growth rate (C)(D). siRNA silencing (p = 0.0002, T-Test) and BLT-1 (p = 0.0053, ANOVA with Tukey’s Test) at concentrations greater than 10 µM significantly reduced cell growth.

To determine the phenotypic cause of the observed arrested growth, a Live/Dead cytotoxicity assay was performed. The assay combined two fluorescent dyes, calcein AM and ethidium homodimer-1. Calcein AM, a membrane penetrable dye, is rapidly converted into a fluorescent metabolite by cytosolic
esterases of living cells while ethidium homodimer-1, a membrane non-penetrable dye, becomes fluorescent when binding cellular DNA of porous membraned dead cells. C4-2 cells did not exhibit a significant decrease in calcein AM or increase in ethidium homodimer-1 staining indicating no induction of cell death with SR-B1 silencing (NC: 17.6 ± 1.43%, KD: 24.3 ± 6.53%, n = 3, Figure 3.6A) but a significant induction was observed with BLT-1 treatment at 10 µM (45 ± 3.54%) and 20 µM (79.2 ± 0.13%) compared to the vehicle control (27.5 ± 5.77%, n = 3, Figure 3.6A). The apparent lack of cell death in response to SR-B1 silencing or low concentrations BLT-1 combined with significant decreases in cell growth indicates the likely induction of cell cycle arrest while the observed cell death at higher concentrations of BLT-1 appears to indicate either on-target or off-target toxicity.

**Figure 3.6 SR-B1 antagonism induces cell cycle arrest and death**

(A) C4-2 cells were transfected with SR-B1 silencing siRNA (KD) or negative control (NC) or treated with BLT-1 (BLT) or vehicle (Veh.). Following treatment, incubation with the Live/Dead cytotoxicity kit reagents was performed and measured using flow cytometry. The number of dead cells was significantly increased at BLT-1 concentrations of 10 µM and 20 µM (n = 3, p < 0.001, ANOVA with Tukey’s Test, mean ± SEM) but not with siRNA SR-B1 knockdown. The proportion of cells in different cell cycle phases was determined by PI staining and flow cytometry. In C4-2 cells the (B) siRNA knockdown induced an accumulation of cells in the G0-G1 phase (n = 3, p < 0.001, ANOVA with Sidak’s Test, mean ± SEM), while (C) BLT-1 treatment induced accumulation of cells in the sub G0 phase and a corresponding decrease of cells in the G2-M phase (n = 3, p < 0.001, ANOVA with Sidak’s Test, mean ± SEM).
3.3.4 SR-B1 antagonism can induce sub $G_0$ and $G_0–G_1$ cell cycle accumulation

To gain an understanding of the mitotically inactive but apparently intact and functioning cells as described above cell cycle analysis was performed using a PI based cell cycle assay. In C4-2 cells, the silencing of SR-B1 expression induced a modest but significant $G_0–G_1$ phase accumulation (NC: 47.1 ± 1.48%, SRB1-KD: 58.1 ± 1.23%, n=3, Figure 3.6B) and an approximate doubling in sub $G_0$ phase accumulation but failed to reach statistical significance (NC: 2.33 ± 0.67%, SRB1-KD: 5.53 ± 1.36%, n=3, Figure 3.6B). Treatment with BLT-1 induced an increasing sub $G_0$ phase accumulation for cells treated with 5 µM and 10 µM BLT-1 (Veh: 2.1 ± 0.39%, 5 µM BLT-1: 13.5 ± 5.83%, 10 µM BLT-1: 18.4 ± 5.73%, n = 4, Figure 3.6C). These results appear to confirm cytotoxicity as evidenced through sub $G_0$ accumulation with high concentration of BLT-1 while further suggesting a $G_0–G_1$ cell cycle arrest with siRNA silencing to explain the absence of mitotic activity observed in the growth assay.

3.3.5 SR-B1 antagonism reduces cellular androgen accumulation and AR activity in steroidogenic C4-2 cells

Given that ligand driven AR-activation is considered the primary driver of PCa proliferation and that cholesterol serves as the primary upstream precursor for the synthesis of AR activating androgens and *de novo* steroidogenesis, the effects of impaired cholesterol procurement on androgen accumulation were assessed as a potential cause of the observed cell cycle arrest$^{388,389}$. The methods by which the cells can obtain the cholesterol necessary for androgen synthesis include cellular cholesterol synthesis or uptake from extracellular sources through LDLr and SR-B1. SR-B1 specifically is known to provide HDL-cholesterol to steroidogenic tissues and previous work from our group has demonstrated a reduction in PSA secretion following SR-B1 knockdown$^{235,290}$. In Chapter 2, the decreased accumulation of testosterone and DHT in castrated LNCaP tumors with the inhibition of cholesterol synthesis by statins was described (Figure 2.6)$^{364}$. As such, cellular androgen levels and markers of AR activation were examined in C4-2 cells cultured in charcoal stripped media as a steroidogenic model$^{390}$. Testosterone and DHT levels were measured by LC-MS. Testosterone levels were decreased by nearly 60% in SR-B1 knockdown cells compared to the scramble control (NC: 0.062 ± 0.0129 ng/mL/mg, SRB1-KD: 0.025 ±
0.0079 ng/mL/mg, n = 3, Figure 3.7A) while BLT-1 treatments of 5 µM and 10 µM reduced testosterone by approximately 50% and 65% respectively but failed to reach statistical significance (Veh: 0.189 ± 0.0693 ng/mL/mg, 5 µM BLT-1: 0.091 ± 0.0287 ng/mL/mg, 10 µM BLT-1: 0.063 ± 0.0134, n =3, Figure 3.7A). DHT levels were significantly decreased with SR-B1 knockdown and BLT-1 treatment. The knockdown resulted in a decreased concentration of DHT by more than 80% (NC: 0.087 ± 0.0278 ng/mL/mg, SRB1-KD: 0.015 ± 0.0076, n = 3, Figure 3.7B) and decreases of 70% and 80% for 5 µM and 10 µM BLT-1 treatment respectively (Veh: 0.251 ± 0.0507 ng/mL/mg, 5 µM BLT-1: 0.076 ± 0.0149 ng/mL/mg, 10 µM BLT-1: 0.048 ± 0.0096 ng/mL/mg, n = 3, Figure 3.7B). These findings indicate, similarly to cholesterol synthesis inhibition, that SR-B1 antagonism whether by siRNA or BLT-1 treatment can reduce the accumulation of the androgenic AR ligands testosterone and DHT. However, whether the reduced cellular concentration of these androgens actually translated to reduced AR activity needed to be confirmed.

The mRNA expression of PSA and NKX3.1, AR regulated genes, was assessed to determine whether the decrease in cellular androgen levels translated to decreased activation of the AR. PSA mRNA expression was reduced by 86% (n = 3) and NKX3.1 by 43% (n = 3) indicating decreased AR activation likely due to the reduction in AR ligands (Figure 3.7C). This finding was further confirmed by a significant decrease in PSA secretion for both the SR-B1 knockdown and dose dependent decrease with BLT-1 reaching significance with 5 µM and 10 µM BLT-1 (NC: 0.0055 ± 0.00038 ng/mL/µg, SRB1-KD: 0.0011 ± 0.00013 ng/mL/µg, n = 3 BLT-1 IC50: 1.07 µM, n = 3 Figure 3.7D). The decreased expression of AR regulated genes as well as decreased PSA secretion suggest that the decreased accumulation of AR-activating androgens is in fact translating to decreased activation of the AR for both siRNA silencing and BLT-1 treatment. Taken in total these findings indicate that SR-B1 antagonism negatively impacts the ability of C4-2 cells to undergo de novo steroidogenesis and drive AR activation in the absence of extracellular androgens.
Alterations in androgen accumulation and AR activation were assessed in C4-2 cells. Testosterone (A) and DHT (B) levels following siRNA transfection (left) or BLT-1 (right) treatment were measured by LC-MS from ~100 mg cell pellets normalized to protein content. Testosterone accumulation was significantly reduced with siRNA knockdown and trended towards decrease with BLT-1 treatment (siRNA: n = 3, p = 0.0384, T-Test; BLT-1: n = 3, p = 0.1844, ANOVA with Tukey’s Test, mean ± SEM). DHT accumulation was significantly reduced with both siRNA knockdown and BLT-1 treatment (siRNA: n = 3, p = 0.0336, T-Test; BLT-1: n = 3, p = 0.0070, ANOVA with Tukey’s Test, mean ± SEM).

Expression of AR regulated PSA and NKX3.1 mRNA was assessed by qPCR. Both PSA (n = 3, p < 0.001, T-Test, mean ± SEM) and NKX3.1 (n = 3, p < 0.001, T-Test, mean ± SEM) mRNA was significantly reduced with SR-B1 knockdown. (D) PSA secretion into media was assessed using the Cobas e 411 analyzer. PSA secretion was significantly reduced with siRNA knockdown (n = 3, p < 0.001, t-test, mean ± SEM) and with 5 µM and 10 µM BLT-1 treatment (n = 3, p < 0.001, ANOVA with Tukey’s Test, mean ± SEM).

3.3.6 SR-B1 knockdown phenotype is not rescued by exogenous steroid

In order to determine whether the anti-proliferative effects of SR-B1 antagonism in C4-2 cells were primarily a result of decreased presence of cellular AR activating androgens experiments were performed in the presence of the exogenous steroid DHEA. DHEA is an androgen intermediate in the testosterone synthesis pathway primarily produced by the adrenal glands. Although a weaker activator of
the AR than T or DHT, DHEA serves primarily as endogenous precursor to more potent AR activating androgens, in theory replacing the need for pre-cursor cholesterol for androgen synthesis\textsuperscript{391}. The knockdown of SR-B1 in the absence of DHEA resulted in a decrease of 80.1\% in PSA secretion compared to control (NC: 0.0055 ± 0.00038 ng/mL/µg, SRB1-KD: 0.0011 ± 0.00013 ng/mL/µg, n = 3 Figure 3.8A). A concentration of 2.5 \textmu M DHEA was capable of inducing robust androgen receptor activation in negative control treated cells as measured by PSA secretion (NC: 0.0055 ± 0.00038 ng/mL/µg, NC + DHEA: 0.0465 ± 0.00617 ng/mL/µg, n = 3, Figure 3.8A). The silencing of SR-B1 expression in the presence of DHEA resulted in a 74\% decrease in PSA secretion compared to the negative control treated cells in the presence of DHEA (SRB1-KD + DHEA: 0.0121 ± 0.00105 ng/mL/µg, Figure 3.8A). Although the addition of DHEA resulted higher absolute PSA secretion, as would be expected from steroidogenic cells growing in otherwise androgen-replete medium, the relative decrease in PSA secretion observed with SR-B1 silencing remained nearly unchanged (80\% vs 74\% decrease) in the presence or absence of external steroid.

If AR activation is sufficient to drive C4-2 proliferation then the addition of exogenous steroid with SR-B1 silencing should avert the induction of the G\textsubscript{0} – G\textsubscript{1} cell cycle arrest observed with SR-B1 silencing alone. SR-B1 cells in the absence of DHEA had an increase of 16\% in the G\textsubscript{0} – G\textsubscript{1} phase fraction, assessed by PI staining, compared to negative control whereas SR-B1 knockdown cells in the presence of DHEA had an increase of 20\% in the same fraction compared to negative control in the presence of DHEA (G\textsubscript{0} – G\textsubscript{1} phase fraction: NC: 70.0 ± 0.96\%, SRB1-KD: 86.4 ± 0.63\%, NC + DHEA: 64.5 ± 1.25\%, SRB1-KD + DHEA: 84.4 ± 1.05\%, n = 3, Figure 3.8B). Lastly, clusterin (Clu), used as a marker of autophagy described below, was assessed by immunoblotting and it was found that SRB1-KD cells in the presence of DHEA still displayed a robust induction of Clu expression compared to the negative control (Figure 3.8C). The induction of G\textsubscript{0} – G\textsubscript{1} cell cycle arrest in the presence and absence of exogenous steroid indicates that AR activity alone is insufficient to drive C4-2 proliferation. The inability of exogenous steroid to fully restore AR activation or prevent cell cycle arrest with SR-B1 silencing suggests that the arrested phenotype is not solely driven by alterations to the AR axis.
Figure 3.8 Arrested SRBI knockdown phenotype is not rescued by exogenous steroid

siRNA-transfected C4-2 cells were cultured in the presence or absence of DHEA (2.5 μM). (A) PSA secretion into media was assessed using the Cobas e 411 analyzer. PSA secretion was significantly reduced with siRNA knockdown both in the presence and absence of DHEA (n = 3, p < 0.001, ANOVA with Tukey’s Test, mean ± SEM). (B) The proportion of cells in different cell cycle phases was determined by PI staining and flow cytometry. Similar levels of sub G₀ and G₀ – G₁ accumulation were observed in the presence and absence of DHEA (n = 3, p < 0.001, ANOVA with Tukey’s Test, mean ± SEM). (C) Representative western blot analysis of autophagy pathway marker Clu. Clu expression was increased in both the presence and absence of DHEA (n = 3).

3.3.7 SR-B1 antagonism induces cell stress and autophagy pathways

While consistently suppressing growth and androgen accumulation the addition of exogenous steroid failed to reverse the SR-B1 antagonized phenotype, therefore other cell stress features that might account for the growth suppression observed by both SR-B1 antagonizing methods were considered. As such, induction of autophagy was considered as a potential mechanism for the observed growth arrest. Cancer cells undergoing nutritional or other external stresses have been demonstrated to employ several mechanisms to evolve and adapt\(^{388,392}\). Autophagy, one such survival mechanism, is a process in which the cell recycles and degrades unnecessary cellular constituents. Initial evidence of autophagy was discovered serendipitously during the visualization of SR-B1 knockout cells by fluorescent microscopy.
using WGA as a membrane stain. The silencing of SR-B1 expression appeared to induce the accumulation of perinuclear vacuoles easily visible in Figure 3.9A. The presence of membranous vacuoles has been demonstrated to be a hallmark of autophagy which functions as the center of lysosomal degradation of cellular components. To further evaluate whether cells were undergoing autophagy the induction of Clu expression was assessed as a validated molecular marker. Clu is a molecular chaperone known to be upregulated during cellular stress that functions to prevent protein aggregation and assist in LC3-I/II lipidation thereby promoting survival and autophagic flux. Mature Clu levels were robustly induced by both the siRNA knockdown and the BLT-1 treatment indicating a strong induction of autophagy (Figure 3.9B). Induction of an autophagic phenotype is further supported by work from Ankur Midha who demonstrated increased LC3-I/II lipidation in response to SR-B1 knockdown.

**Figure 3.9 SR-B1 antagonism induces cell stress and autophagy pathways**

(A) C4-2 cells were transfected with SR-B1 siRNA (KD) or negative control (NC) and stained with wheat germ agglutinin to image intracellular membrane structures by confocal microscopy and compare levels of ER/Golgi blebbing and vacuole formation. (B) Representative western blot analysis of autophagy and ER stress pathway markers following transfection as above, or BLT-1 (BLT) or DMSO vehicle (Veh) treatment in C4-2 cells (n = 3).

Although independent mechanisms of autophagy regulation have been found mTOR is generally considered to be the primary regulator. Within nutrient starved conditions the dephosphorylation and therefore inactivation of mTOR leads to the downstream activation of autophagy pathways. As such,
the levels of mTOR phosphorylation were assessed through western blotting in C4-2 cells and were noticeably decreased when cells were treated with SR-B1 targeted siRNA or treated with the BLT-1 compound (Figure 3.9B). There are several different types of cellular stress that can lead to mTOR regulated autophagy including low glucose and ATP through AMPK. Altered cholesterol metabolism is known to be involved in ER stress mechanisms and disrupted lipid equilibriums have been shown to induce the unfolded protein response (UPR). The UPR, generally in response to an accumulation of misfolded proteins, activates adaptive pathways that attenuate general protein translation, increase molecular chaperone expression and induce cell cycle arrest in an attempt to alleviate the ER stress.

This process has further been demonstrated to induce autophagic activity through both mTOR mediated and mTOR independent pathways. As such, binding immunoglobulin protein (BiP) an essential chaperone and regulator of ER stress was assessed alongside inositol-requiring enzyme 1 alpha (IRE1α) a known inducer of ER stress chaperones through the unconventional splicing x-box binding protein 1 (XBP1). It was found that both siRNA knockdown and high concentrations of BLT-1 (10 µM and 20 µM) can induce BiP expression, while comparatively modest inductions of IREα were also observed (Figure 3.9B). Taken as a whole these results appear to indicate that SR-B1 antagonism induces a strong autophagic phenotype that is at least in part through the activation of ER stress pathways and the inhibition of mTOR activity.

Due to the decreasing detection of the internal structural protein actin, used here as a loading control, detected in Figure 3.9A, methods of protein quantification were compared to insure that misleading results were not being obtained. The two compared methods were the Pierce BCA Protein Assay Kit and the Bradford Protein Assay, both performed according to manufacturer instructions (Figure 3.10). The BCA protein assay indicated lower concentrations of protein than the Bradford assay at higher concentrations of BLT-1 (5 µM, 10 µM and 20 µM) however neither assay showed a large progressive decrease of protein concentration with increasing concentration of BLT-1 that could account for the large decrease in actin expression. Further, 10 µM BLT-1 treatment leads to a large increase in detectable levels of Clu despite the decrease in actin detection (Figure 3.9B). This indicates that the decreasing
intensity of the actin band was not likely due to decreased protein loading but perhaps due to the cellular death processes that were indicated in Figure 3.6.

![Comparison of protein quantification assays](image)

**Figure 3.10 Comparison of protein quantification assays**
The concentrations of whole cell lysates prepared for western blots were assessed and compared using both the BCA and Bradford based protein quantification assays.

3.3.8 SR-B1 antagonism reduces cholesterol uptake and induces robust cell death in androgen independent PC-3 cells

As the reduction of androgen accumulation appeared to play a limited role on the observed antiproliferative effects of SR-B1 antagonism, PC-3 cells, generally considered to be AR negative, were chosen as a non-steroidogenic model to assess the impact of SR-B1 antagonism on cellular proliferation by non-AR dependent pathways. As with C4-2 cells, the ability of SR-B1 antagonism to reduce HDL-derived cholesterol uptake was assessed in PC-3 cells. The SR-B1 targeted siRNA reduced fluorescent uptake by 81% in PC-3 cells (NC: 142.5 %MFI/NT, KD: 26.6 %MFI/NT, n = 3, Figure 3.11) and 10 µM BLT-1 treatment reduced uptake by 38% in PC-3 cells (Veh: 103.2 %MFI/NT*100, BLT-1: 64.9 %MFI/NT*100, n = 3, Figure 3.11)
Figure 3.11 SR-B1 antagonism inhibits cholesterol in PC-3 cells
SR-B1 siRNA silencing or BLT-1 treatment in PC-3 cells followed by DiI-HDL incubation and mean fluorescence measured using flow cytometry. siRNA knockdown (SRB1-KD) and BLT-1 treatment in PC-3 (n = 3, siRNA: p < 0.001, BLT-1: p = 0.021, ANOVA with Sidak’s Test, mean ± SEM) reduced DiI-HDL uptake compared to negative siRNA control (NC) and DMSO vehicle (Veh.) respectively.

To again assess the effects of SR-B1 antagonism on cell growth an Incucyte Zoom based cellular growth assay was performed. PC-3 cells transfected with the scramble control grew to nearly 80% confluency over the time course of the experiment while cells treated with SR-B1 targeted siRNA showed no growth over the same time course, never surpassing 20% confluency (Figure 3.12A). This translated to a growth rate of 0.560 ± 0.096 %/hour for the scramble treated cells and -0.002 ± 0.009 %/hour for the SR-B1 knockdown cells (n = 3, Figure 3.12C). When treated with BLT-1 PC-3 cells show a significant and near complete growth arrest for each dose of BLT-1 tested including from the lowest dose tested at 1 µM BLT-1 (0.075 ± 0.002 %/hour) in comparison to the vehicle only treated cells which grew to approximately 50% confluency by the end of the experiment (0.407 ± 0.018 %/hour, Figure 3.12B,D).

The silencing of SR-B1 expression in PC-3 cells resulted in the death of 78.0 ± 3.81% of cells compared to only 11.1 ± 3.85% of scramble transfected cells (n = 3, Figure 3.13A) as assessed by Live/Dead cytotoxicity assay. The BLT-1 treated cells did not display a significant induction of cell death except for at 20 µM (29.0 ± 7.00%) when compared to the vehicle treated cells (3.6 ± 0.51%, n = 4, Figure 3.13A). These findings translated to a large accumulation of cells in the sub-G0 phase (NC: 7.1 ± 0.56%, SRB1-KD: 66.5 ± 4.87%, n = 5, Figure 3.13B) consistent with the robust cell death observed in Figure 3.13A as measured by cell cycle analysis. The BLT-1 treated cells displayed a significant
accumulation of cells in the G₀ – G₁ phase when treated with 5 µM and 10 µM BLT-1 concentrations, although modest compared to the differences seen with the siRNA transfection (Veh: 67.8 ± 2.4%, 5 µM BLT-1: 78.7 ± 2.81%, 10 µM BLT-1: 80.5 ± 1.76%, n = 5, Figure 3.13C). Given the inability of exogenous steroid to revert the SR-B1 antagonized phenotype, alongside the observation of a robust response of PC-3 cells, the importance of non-AR mediated effects to SR-B1 antagonism indicates further the likely impact of nutrient starvation and induction of cellular stresses.

**Figure 3.12 SR-B1 antagonism inhibits cell growth in PC-3 cells**
PC-3 cells were (A) transfected with SR-B1 silencing siRNA (SRBI-KD) or negative control (NC) or (B) treated with BLT-1 or vehicle prior to incubation in the IncuCyte Zoom system. Confluency measurements were taken every 6 hours (n = 3, mean ± SEM). Linear regression was used to calculate the slope of each treatment in order to determine growth rate (C)(D). siRNA silencing (p = 0.0043, T-Test) and BLT-1 (p = 0.0028, ANOVA with Tukey’s Test) at concentrations 1 µM or higher significantly reduced cell growth.
Figure 3.13 SR-B1 antagonism induces robust cell cycle arrest and death in PC-3 cells

PC-3 cells were transfected with SR-B1 silencing siRNA (KD) or negative control (NC) or treated with BLT-1 (BLT) or vehicle (Veh.). (A) Following treatment, incubation with the Live/Dead cytotoxicity kit reagents was performed and measured using flow cytometry. The number of dead cells was significantly increased in the siRNA knockdown cells and at a BLT-1 concentration of 20 µM (n = 3, p < 0.001, ANOVA with Tukey’s Test, mean ± SEM). The proportion of cells in different cell cycle phases was determined by PI staining and flow cytometry. In PC-3 cells the (B) siRNA knockdown induced accumulation of cells in the sub G₀ phase and corresponding decrease of cells in the G₀-G₁ and G₂-M phases (n = 5, p < 0.001, ANOVA with Sidak’s Test, mean ± SEM), (C) while BLT-1 treatment induced accumulation of cells in the G₀-G₁ phase and a corresponding decrease in the G₂-M phase (n = 5, p < 0.001, ANOVA with Sidak’s Test, mean ± SEM).

3.3.9 SR-B1 co-targeting can amplify the effects of singular agents

As SR-B1 knockdown was demonstrated to induce expression of HMGCR (Figure 3.4C) indicating a potential mechanism of compensation for the lost HDL-derived cholesterol the effect of co-targeting SR-B1 and HMGCR was assessed. Simvastatin and BLT-1 were used in combination and the effects on PSA secretion in C4-2 cells were assessed. Simvastatin concentrations of 1 µM and 10 µM did not, by themselves, lead to a significant decrease in PSA secretion, although 10 µM simvastatin did
display a modest decrease (n = 3, Figure 3.14A). When combined with BLT-1, however, a near 50% decrease in PSA secretion was observed with 0.1 µM BLT-1 in the presence of 1 µM simvastatin as compared to the 1 µM BLT-1 required to reach a comparable decrease as a singular agent (n = 3, Figure 3.14A). These results demonstrate the essential role of these mechanisms to provide cholesterol as a metabolite and indicate that targeting multiple mechanisms by which cells obtain cholesterol may potentiate the effects of solo targeting.

Figure 3.14 SR-B1 co-targeting can amplify the effects of singular agents
Potential strategies for co-targeting SR-B1 were assessed. (A) PSA secretion with SR-B1 inhibition by BLT-1 and HMGCR inhibition with simvastatin was measured by the Cobas e 411 analyzer. The addition of simvastatin sensitized cells to BLT-1 treatment (right, n = 3, p < 0.001, Extra sum of squares F-test, mean ± SEM) at concentrations of simvastatin that themselves did not decrease PSA (left, n = 3, p = 0.369, ANOVA, mean ± SEM). (B) The percentage of cells in the sub G₀ phase following transfection and treatment with autophagy inhibitor chloroquine (CQ) was assessed by propidium iodide staining and flow cytometry. Cells transfected with SR-B1 knockdown showed significantly increased accumulation in the sub G₀ phase compared to the negative control at 80 µM and 100 µM CQ (n = 2, p < 0.001, ANOVA with Sidak’s Test, mean ± SEM).

With the induction of autophagy and other cell stress mechanisms becoming a mainstay of therapeutic resistance in oncology, methods of inhibition for these mechanisms are becoming more prominent. Chloroquine, a small molecule lysosomal alkalizer known to inhibit the binding of the lysosome and autophagosome, has been a staple in preclinical investigation of autophagy inhibition and serves as an established agent for a proof-of-principle assessment in SR-B1 antagonized cells. To determine whether the inhibition of autophagy would result in a more robust cellular death in SR-B1 knockdown C4-2 cells sub-G₀ phase accumulation was measured with increasing concentrations of chloroquine. The percentage of SR-B1 knockdown cells in the sub-G₀ drastically increased with
increasing chloroquine concentration reaching 36% with 100 µM chloroquine (Figure 3.14B). Although, modest increases in sub-G₀ fraction were observed with negative control transfected cells the SR-B1 knockdown cells had a significantly higher percentage of cells in the sub-G₀ phase at 80 µM and 100 µM chloroquine (n =2). Although limited by replicate number these preliminary results indicate the ability to induce a robust cell death response by inhibiting the autophagy response of SR-B1 antagonized cells.

3.4 Discussion

Selective cholesterol ester uptake through SR-B1 is a critical way cells acquire precursor cholesterol for steroid hormone biosynthesis in steroidogenic tissues. The receptor further signals the growth and survival of both non-steroidogenic endothelial cells and breast cancer cells. Research into the role of SR-B1 in cancer has related its expression to aggressive characteristics in multiple cancer types including breast cancer and clear cell renal carcinoma. In prostate cancer, clinically elevated SR-B1 expression is correlated with high risk PCa and decreased disease-specific survival. Further, elevated SR-B1 expression is observed in CRPC derivatives of LNCaP, and with increased tumor growth in the TRAMP model. These results implicate SR-B1 as an actionable target for managing CRPC.

In this thesis the increased expression of SR-B1 in the progression from normal prostatic tissue to cancerous localized disease through both IHC analyses of radical prostatectomy specimens and available expression databases is described. Specimens derived from radical prostatectomy allowed for matched comparisons of cancerous tissue and adjacent-normal prostatic tissue. The SC dataset was generated from the sequencing of paired samples of normal prostatic and cancerous tissue obtained from the same patient similarly allowing for a direct comparison of expression between the tissues. Although conflicting on which cholesterol metabolism protein was most highly expressed in localized disease both the SC and TCGA datasets showed increased expression of SR-B1 in cancerous tissue, and perhaps just as importantly, a stark decrease in LDLr expression indicating a potential shift in the mechanism by which the cancerous prostatic tissue acquires cholesterol compared to non-cancerous prostatic tissue.
These findings, alongside the evidence of high expression of SR-B1 in metastatic tissues, correlate with the findings by Schorghofer et al. who reported the high expression of SR-B1 in high Gleason grade primary tumors from US Biomax (Rockville, MD) tissue samples. Together with the understanding that cholesterol accumulation underlies prostate cancer aggressiveness, these findings strongly implicate SR-B1 as a major factor in the progression of the disease. Notably however, a recent 2017 publication in Carcinogenesis correlated squalene monooxygenase (SQLE) with lethal prostate cancers in the Health Professional Follow-up Study, the Physicians’ Health Study and the Swedish Watchful Waiting Study. Although generally not considered to serve the same regulatory function as SR-B1, LDLr or HMGCR, SQLE catalyzes an essential step, downstream of HMGCR, in cholesterol synthesis and has been a target for the development of hypercholesterolemia therapeutics. Recently, SQLE has been identified as a potential oncogenic driver of breast cancer. The growing number of cholesterol metabolism proteins implicated in the proliferation of various cancers indicates that systems or pathway approaches to analysis, such as gene set enrichment analysis, would provide valuable understanding of global cholesterol metabolism alterations.

NEPC comprises a small, but highly aggressive and lethal, subset of PCa patients. Although a strict definition is not agreed upon, NEPC is generally considered to take on small cell carcinoma like features, may or may not express classical IHC markers (chromogranin, synaptophysin, etc.) and is often AR negative. In rare cases de novo NEPC has been identified but in the context of modern PCa management, a vast majority of cases arise as variant of recurrent CRPC including in response to current generation androgen receptor pathway inhibitors enzalutamide and abiraterone. The mechanism by which adenocarcinoma becomes NEPC remains a complex and unanswered question but several factors and pathways have been implicated including MYCN, cAMP, BRN2 or loss of p53, pRB or REST. Here we report the increased expression of SR-B1 in post-ADT NEPC compared to standard CRPC as assessed in both the UWRA and WC datasets. NE differentiated cells have been shown to have accumulation of free cholesterol as well as close relationship between NE differentiation and cholesterol rich lipid membranes. Whether the increased expression of SR-B1 is necessary for providing
cholesterol to facilitate essential NE differential signaling pathways, or a larger pool of available cholesterol is required for the rapid cell division associated with aggressive prostate cancers such as NEPC, or other undetermined reasons is unknown, but may provide an intriguing avenue for further research.\textsuperscript{419}

The finding that SR-B1 antagonism leads to differential responses between PCa models belies the heterogeneous nature of the disease. The cell lines used within represent, as far as a single immortalized cell line can, two very different types of recurrent metastatic prostate cancer. The C4-2 cell line is a derivative line of the well-known LNCaP cell line, itself taken from a biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with metastatic CRPC. C4-2 cells were generated through the inoculation and growth of LNCaP cells in a castrated athymic mouse resulting in a line capable of androgen independent growth.\textsuperscript{420} These cells are p53 positive and maintain AR activation and signaling even in serum depleted conditions through \textit{de novo} steroidogenesis.\textsuperscript{358} PC-3 cells were obtained from a bone metastasis of a 62 year old Caucasian male.\textsuperscript{421} Unlike C4-2 and LNCaP cells, PC-3 cells do not express the AR making them fully androgen-independent and further do not express functional p53.\textsuperscript{422,423} Interestingly SR-B1 knockdown in PC-3 cells resulted in robust cellular death in contrast to C4-2 cells which entered an autophagic phenotype. This finding would emphasize an extra-steroidal mechanism to the effects seen.

Autophagy is a highly conserved mechanism by which cells regulate several homeostatic processes including the removal of protein aggregates and intracellular toxins as well regulating cytoplasmic biomass.\textsuperscript{424} Autophagic responses to cancer treatments are a common survival mechanism employed by cancer. This includes responses to ADT and androgen pathway inhibitors, taxanes and kinase inhibitors.\textsuperscript{425-428} PC-3 cells are capable of entering into an autophagic state following treatment with direct autophagy inducing agents including the proteasome inhibitor MG 132 and mTOR inhibitor rapamycin.\textsuperscript{392} However, the strong induction of cell death as opposed to autophagy in PC-3 cells is likely a result of a distinct regulatory environment compared to C4-2 cells.\textsuperscript{429} One major distinction between the two cell lines is the absence of p53 in PC-3 cells, a transcription factor which is known to positively and
negatively regulate autophagy through, at this point, poorly understood mechanisms\textsuperscript{430}. Notably p53 activation inhibits mTOR, a master regulator of autophagy known to inhibit autophagy in its activated state\textsuperscript{394,431,432}. Further, the effect of ER stress on p53 activation has been enigmatic with evidence demonstrating both p53 activation and deactivation within ER stressed environments\textsuperscript{433,434}. Although mechanistic understanding of the role of p53 in autophagy is poorly understood, the absence of p53 in PC-3 may have impeded the cells ability to enter an autophagic state in a similar manner to C4-2 cells. The lack of p53 or other regulatory alterations may be providing an opportunity to maintain a proliferative state under impaired nutrient conditions due to SR-B1 loss. This would result in cells replicating and dividing with insufficient nutrients to maintain viability leading to the robust cellular death response observed. In contrast if C4-2 cells maintain a stronger nutrient regulatory environment, highlighted by active p53, allowing them to enter into an autophagic state through functional stress response mechanisms avoiding large amounts of cellular death. More general potential reasons for the differing responses to SR-B1 antagonism may include the differing environment, treatment stage or progression status from which the studied cell lines were obtained. In particular bone metastases, the origin site of PC-3, have been noted for high levels of cholesterol and cholesterol metabolism proteins potentially indicating an increased dependence on cholesterol\textsuperscript{368}.

BLT-1 is an established small molecule inhibitor of SR-B1. The molecule was first identified by high-throughput screening of inhibition of SR-B1 selective lipid uptake using DiI-HDL\textsuperscript{435}. Interestingly the compound was found not to inhibit, rather enhance, the binding of HDL to SR-B1 and in turn prevent the transfer of cholesterol or cholesteryl ester across the membrane\textsuperscript{435}. The treatment of PC-3 and C4-2 cells with BLT-1 was able to successfully replicate the growth arrest observed with the siRNA knockdown. However, different responses were observed with cell cycle and Live/Dead analysis both within SR-B1 targeting methods of a single cell line and the cellular response to BLT-1 between C4-2 and PC-3 cells. Interestingly the treatment of PC-3 cells with BLT-1 did not result in a similar robust cell death as seen with SR-B1 knockdown. This is potentially explained by the inability of BLT-1 to fully shut down the transfer of cholesterol by the SR-B1 receptor due to issues relating to affinity binding. Despite
this, BLT-1 does appear to induce autophagy in a similar manner to the siRNA knockdown in C4-2 cells, as measured through decreased mTOR activation and increased Clu expression while also demonstrating similar markers of ER stress. Further, BLT-1 had similar effects on cellular androgen accumulation confirming the importance of SR-B1 derived cholesterol in androgen synthesis.

What cannot be fully accounted for when using a small molecule inhibitor such as BLT-1 is potential off-target binding and effects. A notable potential off-target is CD36 also a member of the Scavenger Receptor B family which has several natural ligands including oxidized phospholipids, oxidized low-density lipoproteins and long chain fatty acids. The inhibition of CD36 could prevent the accumulation of nutrients essential for proliferation, notably fatty acids, potentially resulting in a similar nutrient deprived phenotype. CD36 and SR-B1 maintain a high level of structural homology including similarities in the ligand binding area of the protein. The homology of SR-B1 and CD36 is such that HDL can readily bind to CD36 receptors but lacks the structure to facilitate transport of HDL-derived cholesterol. The cysteine 384 residue located in the tunnel domain of SR-B1 has been shown to be essential for cholesterol uptake inhibition by BLT-1, a residue that is absent from CD36. Despite the lack of cysteine 384 negating the ability of BLT-1 to inhibit cholesterol transfer, what is not known is the ability of BLT-1 to bind CD36 or whether it impedes other CD36 functions such as long chain fatty acid uptake. The potential off target effects described here or other unknown effects may be the reason for the difference in response to BLT-1 as compared to the siRNA knockdown.

As described in Chapter 2 the success of both abiraterone and enzalutamide in managing metastatic CRPC patients brought the role of de novo steroidogenesis in ADT resistance into the spotlight. The initial success of these therapies, despite eventual resistance, displays the continued potential of novel AR pathway therapies. Here we describe how SR-B1 inhibition reduces androgen accumulation and AR activation in steroidogenic C4-2 cells. If the inhibition of HDL-derived cholesterol uptake through SR-B1 was in fact impacting androgen accumulation by impeding de novo steroidogenesis there would be high potential to overcome several proposed mechanisms of resistance to other androgen pathway inhibitors. This would include amplification of enzymes, such as CYP17A1, that result in
intratumoral accumulation of higher order steroids and CYP17A1-independence by becoming responsive to steroid precursors\textsuperscript{156,358-360}. However, the inability of DHEA to reverse the effects of SR-B1 knockdown indicates that the observed effects are due in part to factors other than solely \textit{de novo} steroidogenesis antagonism. Specifically, the inability to return PSA secretion to levels seen in non-SR-B1 antagonized cells may indicate that the reduction of androgens was not through reduction of precursor or that the extra-steroidal effects of SR-B1 antagonism impede the ability of the cell to convert DHEA to more potent androgenic AR ligands. The ability of androgens, through AR activation, to negatively regulate autophagic activity under what would normally be considered sub-optimal environmental conditions, such as culturing in charcoal stripped serum, was initially considered\textsuperscript{441,442}. The reduction of androgen accumulation through \textit{de novo} steroidogenesis antagonism could potentially have lead the induction of autophagy. However, the inability of external steroid to revert said autophagy phenotype makes this unlikely, and further provides evidence that the observed autophagy and anti-proliferative effects of SR-B1 antagonism were largely external to the effects observed within the AR pathway.

The ability of prostate cancer cells obstructed from obtaining cholesterol to compensate via other mechanistic sources provides an intriguing basis for combination therapy. As described in Chapter 2, castrated mice bearing LNCaP xenografts being treated with simvastatin to inhibit HMGCR upregulate SR-B1 and SR-B1 antagonized C4-2 cells similarly upregulate HMGCR expression. These changes in expression, as well as the entrance of the cell into a nutrient deprived autophagic state, indicate that, at least, the C4-2 cancer cell is able to detect cholesterol depleted conditions and attempt to induce compensatory changes. Theoretically, therefore impeding the cells ability to use such compensatory mechanisms by co-targeting both SR-B1 and HMGCR or other cholesterol metabolism proteins may amplify the anti-proliferative effects seen with individual approaches. Here we describe early preliminary data co-targeting SR-B1 and HMGCR to potentiate the anti-PSA effects seen with the individual inhibitors. However, further studies are required to determine the effect of co-targeting on cellular proliferation, cell death and autophagic induction.
The targeting of autophagy alongside autophagy-inducing primary treatment is a commonly employed technique including, to date, unsuccessful attempts at the development of clinical stage autophagy inhibitors for PCa treatment\textsuperscript{443}. Preclinical research has commonly used CQ as an autophagy inhibitor, including co-targeting to induce more robust cell death effects in PCa\textsuperscript{444,445}. Here we show that the inhibition of autophagy with cholesterol in SR-B1 antagonized cells can result in a robust cellular death as measured through an accumulation of the sub G\textsubscript{0} cell phase fraction. This result highlights autophagy as a potential co-target with SR-B1 to significantly impair prostate cancer growth by reducing the cells ability to overcome stress results in a more robust cellular death.

Although the two above described potential co-targets for SR-B1 have been rationally identified and provide promising future avenues of investigation, the advent of modern high throughput techniques allows for genomic screening for synergistically lethal targeting. With the advent of CRISPR/Cas9 silencing and the development of stable Cas9 expressing cell lines the potential of screening such libraries for lethal co-targets of BLT-1 treated or SR-B1 knockdown cells is promising\textsuperscript{446,447}. In addition to providing potential co-targets, these screening techniques would likely provide valuable insight into which cell signaling pathways are perturbed through SR-B1 antagonism.

The findings presented in this chapter have identified the high likelihood of SR-B1 being an important protein for the progression and survival of prostate cancer, the inhibition of which leads to distinct anti-proliferative effects likely through both steroidal and non-steroidal mechanisms. Further examination of the mechanisms altered by SR-B1 loss and the investigation of its potential as a therapeutic target is warranted.
CHAPTER 4: EVALUATION OF BLT-1 AS AN IN VIVO INHIBITOR OF SR-B1

4.1 Specific aim and rationale

Central to the process of target evaluation and drug development is establishing the “drugability” of the target in an in vivo setting. Such investigations generally involve research into the pharmacokinetic and toxicity profiles of candidate compounds. As both interfering RNA and small molecule approaches to targeting SR-B1 displayed high efficacy in vitro (Chapter 3), this chapter assessed the potential of the small molecule inhibitor BLT-1 as candidate for both further research in vivo and as a proof of principal for the development of SR-B1 targeted therapeutics. Specifically, the pharmacokinetic and toxicity profiles of BLT-1 were assessed to determine whether potentially efficacious circulating concentrations could be achieved in the absence of significant toxicity. These concentrations were then tested in xenograft based efficacy studies to confirm the ability of BLT-1 to impede tumor growth in vivo.

4.2 Methods

4.2.1 Microsomal assay

Working stocks of BLT-1, VPC-13574 and enzalutamide were prepared in DMSO to 1 mM. VPC-13574 is a compound originally designed as an inhibitor of the Binding Function 3 (BF3) domain of the AR\textsuperscript{48,49}. For the purposes of this chapter it was used as an in house metabolically labile positive control. Compounds were added to 100 mM potassium phosphate buffer (pH 7.4) to a final concentration of 1 µM followed by the addition of mouse liver microsomes (Xenotech, Kansas City, KS) to a final concentration of 0.15 mg/mL. “NADPH regenerating solution” (BD Biosciences, Franklin Lakes, NJ) was then added and the incubation started in a 37 °C water bath. Aliquots were taken and combined with stopping solution (Acetonitrile+0.05% formic acid) for analysis by LC-MS at specified time points.

4.2.2 Pharmacokinetic and toxicity analysis

All animal experiments in Chapter 4 were conducted in accordance with the University of British Columbia’s Committee on Animal Care and approved protocol A16-0072 held by Dr. Emma Guns at the Vancouver Prostate Centre.
Athymic nude mice (Crl:NU-Foxn1\textsuperscript{nu}; Harlan) were dosed by oral gavage with either 25 mg/kg or 50 mg/kg BLT-1. Due to the highly hydrophobic nature of BLT-1 propylene glycol was used, as a previously established vehicle for hydrophobic compounds, to allow for complete dissolution of the compound\textsuperscript{450}. Blood samples were obtained (approximately 50 µL per sample) via capillary collection of tail vein bleeds at the following time points: 0, 0.5, 1, 1.5, 2, 4, 8 and 24 h post dosing. Capillaries were spun by centrifuge at 3000 g for 10 min to isolate serum for analysis. Following the final tail vein bleed mice were exsanguinated by cardiac puncture to collect blood from which serum was prepared, and organs were in part flash frozen and in part fixed in 10% buffered formalin solution over night before being transferred to 70% ethanol for long term storage.

Toxicity analysis was performed using collected terminal serum and the VetScan VS2 (Abaxis, Union City, CA) and VetScan Comprehensive Diagnostic Profile (Abaxis) according to manufacturer instructions\textsuperscript{451}. The Comprehensive Diagnostic Profile includes quantification of serum albumin (ALB), alkaline phosphatase (ALP), alanine transaminase (ALT), amylase (AMY), total bilirubin (TBIL), blood urea nitrogen (BUN), calcium (CA), phosphorus (PHOS), creatinine (CRE), glucose (GLU), sodium (NA+), potassium (K+), total protein (TP), globulin (GLOB).

4.2.3 BLT-1 analysis by LC-MS

Serum samples for each of the time points collected were subjected to LC-MS analysis. Sample extraction was performed in which 2 µL of 1 µM VPC-13226 IS was combined with 8 µL of serum and 22 µL of acetonitrile (ACN) prior to vortexing for 10 sec. Precipitate was pelleted by centrifugation at 20,000 g for 5 min and supernatant transferred to LC vials for analysis. Standards were prepared in a similar fashion with standard/IS spiked mouse serum (2 µL standard/2µl IS/20µl ACN) while parallel standards made up in 67% ACN were used to characterize matrix effects. Samples and standards were analyzed using a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer (Waters). Separations were carried out with a 2.1x100 mm BEH 1.7 µM C18 column (Waters) and a 50-98% acetonitrile (ACN) gradient from 0.2-3 min (0.3 mL/min) used for separation
(with 1 min 98% ACN flush and a 2 min re-equilibration; 6 min run length; 0.1% formic acid throughout). Data was collected in ES+ mode and compounds were detected by multiple reaction monitoring with mass to charge (m/z) transitions of 242.1>166 and 242.1>95.9 for BLT1 and m/z 256.1>110.8 for IS. Retention times for BLT-1 and IS were 3.16 min and 2.83 min respectively. Data analysis was performed with Quanlynx (Waters) with AUC of BLT-1 normalized to IS AUC. Comparisons with acetonitrile based and serum based standard extracts indicated high extraction efficiency (>90%) but substantial ion suppression with BLT-1 (~50%), therefore serum-based standards were used for the generation of the standard curve with 7 calibration standards (4 nM to 4 µM, R²>0.99) for the determination of BLT-1 concentration in test samples.

4.2.4 Xenograft experiments

For C4-2 xenografts, athymic nude mice (Crl:NU-Foxn1null; Age: 12 weeks; Harlan) were surgically castrated and allowed to recover and for circulating testosterone to nadir for 2 weeks prior to inoculation on two sites of the hind flank with 2 x 10⁶ C4-2 cells (cultured in RPMI + 5% FBS, 1% penicillin/streptomycin) in 100 µL matrigel (BD Biosciences). Tumor volume was calculated by caliper measurements and the equation: Tumor Volume = length x width x height x 0.5326. Once tumor volume exceeded 100 mm³ mice were given 50 mg/kg BLT-1 (vehicle: propylene glycol) by oral gavage daily. Treatment was continued for 6 weeks or until tumor burden exceeded 10% of body weight or weight loss exceeding 20%. At the time of euthanasia, mice were exsanguinated by cardiac puncture following cervical dislocation under CO₂ to collect blood from which serum was prepared. Tumors were in part flash frozen and in part fixed in 10% buffered formalin solution over night before being transferred to 70% ethanol for long term storage. Serum was isolated from whole blood as described above.

For PC-3 xenografts, athymic nude mice (Crl:NU-Foxn1null; Age: 16 weeks; Harlan) were inoculated on one site of the hind flank with 2 x 10⁶ PC-3 cells (cultured in DMEM + 10% FBS, 1% penicillin/streptomycin). Tumor volume measurements were taken twice weekly as described above and once tumor volume exceeded 100 mm³ mice were treated with 25 mg/kg BLT-1(vehicle: propylene
glycol) by oral gavage daily. Treatment was continued for four weeks or until tumor burden exceeded 10% of body weight or weight loss exceeding 20%. Post-euthanasia treatment of animals was the same as with C4-2 xenografts.

### 4.3 Results

#### 4.3.1 BLT-1 is rapidly metabolized by mouse liver microsomes

Prior knowledge regarding the effects of BLT-1 use *in vivo* was limited to the reported ability of 1 mg/kg oral BLT-1 to impede chylomicron formation in Syrian golden hamsters which detailed no pharmacokinetic or robust toxicity analysis. Given this paucity of available information initial experiments were performed to gain an understanding of the stability of BLT-1 *in vivo* and further how the compound would fair in use for xenograft experiments. A microsomal stability assay in which the compound is incubated in the presence of mouse liver microsomes was performed (Figure 4.1). Enzalutamide was used as a known metabolically stable control compound and was by in large not metabolized by the microsomes as there was still 97.7% fraction remaining after 60 min incubation with liver microsomes. VPC-13574 was rapidly metabolized with only 14.7% fraction remaining after 60 min incubation, translating to a half-life of 22 min. In comparison BLT-1 was metabolized even more rapidly than the VPC-13574 positive control with only 8.14% fraction remaining at 45 min incubation and having a half-life of 8 min when incubated with the mouse liver microsomes. The apparent intrinsic clearance ($CL_{int-app}$) was estimated for each compound using the equation:

$$CL_{int-app} = \left( \frac{0.693}{t_{1/2}} \right) \left( \frac{Vol. \ of \ incubation}{mg \ microsomal \ protein} \right) \left( \frac{mg \ of \ microsome}{g \ of \ liver} \right) \left( \frac{liver \ weight \ (g)}{body \ weight \ (kg)} \right)$$

Literature values of 45 mg/g for (mg of microsome / g of liver) and 87 g/kg of (liver weight / body weight) were used, while the reaction volume was 0.5 mL with 0.075 mg microsomal protein. The $CL_{int-app}$ of VPC-13574 was 822.12 mL/min/kg and 2260.92 mL/min/kg for BLT-1. Such a high $CL_{int-app}$ would not be considered ideal for the development of the compound as a lead candidate, and indicate that BLT-1 is likely not an ideal candidate for use in *in vivo* experiments. These results further indicate BLT-1
would require relatively high dosing to achieve the efficacious levels observed in the \textit{in vitro} experiments, approximately \(~5\ \mu\text{M} \) for C4-2 cells and \(~1\ \mu\text{M} \) for PC-3 cells as described in Chapter 3. However, the microsomal stability assay works under the assumption that the compound is completely protein unbound in serum and that the liver would have ample access to free compound, which is unlikely given the highly hydrophobic nature of BLT-1\textsuperscript{454}. Given the limited alternative options and considerations to the limitations of the microsomal study, mouse based pharmacokinetic studies were performed to determine if sufficient circulating concentrations could be obtained for the purposes of pre-clinical study of SR-B1 inhibition.

![Figure 4.1 BLT-1 is rapidly metabolized by liver microsomes](image)

**Figure 4.1 BLT-1 is rapidly metabolized by liver microsomes**

BLT-1 was incubated in the presence of mouse liver microsomes and concentration was measured over time. Enzalutamide (Enza) and VPC-13574 were used as non-labile and labile controls, respectively (\(n = 1\)).

4.3.2 Oral administration of BLT-1 results in relevant serum concentrations

To determine if actionable levels of circulating BLT-1 could be achieved, despite the rapid metabolism by mouse microsomes, mice were dosed with either 25 mg/kg or 50 mg/kg by oral gavage and serum samples obtained to develop a pharmacokinetic profile. Mice dosed with 25 mg/kg BLT-1 had a \(C_{\text{max}}\) of 552.5 ng/mL (2.28 \(\mu\text{M}\)) at 0.5 hours post-dose the first measured dose (Figure 4.3A). The elimination half-life was 10.4 hours with the final measured concentration at 24 hours post dose being
relatively low at 0.783 ng/mL (0.189 µM) resulting in an AUC\textsubscript{0-\infty} of 3971.2 ng*hr/mL. Mice dosed with 50 mg/kg had an only slightly higher Cmax of 640.1 ng/mL (2.65 µM) and a much longer absorption phase reaching Cmax at 2 hours post-dose (Figure 4.2A). With 50 mg/kg oral dosing an elimination half-life of 7.2 hours and an AUC\textsubscript{0-\infty} of 3913.5 ng*hr/mL was observed.

**Figure 4.2 BLT-1 reduces survival of C4-2 xenografts**

(A) Serum samples from mice dosed with 50 mg/kg BLT-1 were assessed for BLT-1 concentration over time and used to calculate basic pharmacokinetic parameters (n = 3). C4-2 xenografts were established on the hind flank of athymic nude mice. Mice were dosed with either 50 mg/kg BLT-1 (n = 9) or vehicle (n = 7) and body weight (B) and tumor measurements were taken weekly (C). (D) Survival of mice being treated with vehicle and BLT-1. BLT-1 treated mice had significantly decreased survival time compared to vehicle treated mice (Hazard Ratio = 0.16, 95%CI = 0.04 – 0.63, p = 0.009, Log-Rank).

4.3.3 50 mg/kg BLT-1 induces overt toxicity in C4-2 tumor bearing mice

The use of C4-2 xenograft mice would have provided an ideal model for the testing of BLT-1 \textit{in vivo}. Notably a majority of the SR-B1 targeted work has been within the C4-2 model resulting a larger understanding of the cellular reaction to SR-B1 antagonism. Further, although the reduction of androgen
accumulation in C4-2 was found not to be a primary driver of the SR-B1 antagonized phenotype, an understanding of the ability of BLT-1 to reduce tumoral androgen accumulation would still provide valuable insight into placing SR-B1 among other successful AR-axis targeted therapeutics. However, the minimum efficacious concentration of BLT-1 in C4-2 appears to be ~5 µM as described in Chapter 3. As such the highest tested dose of 50 mg/kg (Cmax: 2.65 µM) was selected in order to achieve as close to efficacious circulating concentrations as possible.

To best model the castration-resistant environment mice were castrated and allowed to recover for two weeks in order to allow sufficient time for the metabolism of remaining serum testosterone prior to tumor inoculation. Once tumor volume reached 100 mm$^3$ treatments began for a planned six weeks. Of the nine mice that started BLT-1 treatment, none survived past four weeks of treatment with only four of the mice surviving to the four-week time point. One mouse was euthanized due to tumor volume size approaching an inhumane limit while the eight remaining were euthanized due to body weight loss at differing points over the four weeks. Of the seven mice treated with the vehicle four survived past the four week time point with three having to be euthanized due to body weight loss. The effect of this body weight loss on the overall survival of the two treatment groups is exemplified in Figure 4.2D. Both groups showed a decrease of approximately 2 g mean body weight over the treatment period (Figure 4.2B). However, the number of mice being euthanized due to body weight loss and removed from the pool over the course of the experiment means that the mean body weight does not adequately represent the severe body weight loss being observed. Terminal serum samples were used to complete a comprehensive toxicity profile of 14 markers. The BLT-1 treated mice had significantly elevated levels of ALB, ALP, ALT, TBIL and TP alongside significant decrease in GLU levels (Table 4.1). Altered serum concentrations of these markers could indicate dysfunction among several organs and processes in the body including kidney disease for ALB and TP, and diabetic and nutritional disorders with GLU and TP. However, the group observed as a whole appears to indicate that mice treated with BLT-1 were experiencing significant liver toxicity. Each of the significantly altered markers is known to be effected in
response to liver damage. In particular ALB, ALP and ALT are generally considered strong indicators of liver dysfunction.451,455

Table 4.1 Assessment of common serum toxicity markers following repeated BLT-1 dosing in mice

*CRE levels were only detectable at >18.0 mmol/L, as such 18 mmol/L was used to represent all values less than or equal to 18 mmol/L. **K+ levels were only quantifiable at < 8.5 mmol/L, as such 8.5 mmol/L was used to represent all values greater than or equal to 8.5 mmol/L.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Vehicle (SD)</th>
<th>25 mg/kg BLT-1 (SD)</th>
<th>50 mg/kg BLT-1 (SD)</th>
<th>P - Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB (g/L)</td>
<td>45.2 (3.69)</td>
<td>42.3 (2.06)</td>
<td>50.3 (5.35)</td>
<td>0.006</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>42.7 (12.10)</td>
<td>45.2 (15.04)</td>
<td>86.1 (42.91)</td>
<td>0.004</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>47.8 (15.41)</td>
<td>37.2 (12.12)</td>
<td>131.6 (141.46)</td>
<td>0.047</td>
</tr>
<tr>
<td>AMY (U/L)</td>
<td>1161.9 (489.96)</td>
<td>952.2 (180.25)</td>
<td>1243.0 (168.39)</td>
<td>0.387</td>
</tr>
<tr>
<td>TBIL (mmol/L)</td>
<td>4.8 (0.72)</td>
<td>4.6 (0.52)</td>
<td>6.3 (1.03)</td>
<td>0.001</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>6.8 (0.93)</td>
<td>6.8 (0.64)</td>
<td>6.1 (1.26)</td>
<td>0.325</td>
</tr>
<tr>
<td>CA (mmol/L)</td>
<td>2.9 (0.23)</td>
<td>2.8 (0.13)</td>
<td>2.9 (0.18)</td>
<td>0.911</td>
</tr>
<tr>
<td>PHOS (mmol/L)</td>
<td>2.8 (0.66)</td>
<td>3.2 (0.24)</td>
<td>2.5 (0.77)</td>
<td>0.144</td>
</tr>
<tr>
<td>CRE (mmol/L)</td>
<td>18.7 (2.60)*</td>
<td>19.5 (3.67)*</td>
<td>18.0 (0.00)*</td>
<td>0.614*</td>
</tr>
<tr>
<td>GLU (mmol/L)</td>
<td>13.4 (2.44)</td>
<td>13.7 (2.21)</td>
<td>8.4 (2.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NA+ (mmol/L)</td>
<td>167.4 (5.95)</td>
<td>169.1 (6.18)</td>
<td>169.3 (1.03)</td>
<td>0.700</td>
</tr>
<tr>
<td>K+ (mmol/L)</td>
<td>8.5 (0.00)**</td>
<td>8.5 (0.00)**</td>
<td>8.5 (0.00)**</td>
<td>----**</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>58.8 (4.80)</td>
<td>57.6 (5.16)</td>
<td>67.6 (5.28)</td>
<td>0.003</td>
</tr>
<tr>
<td>GLOB (g/L)</td>
<td>14.0 (3.22)</td>
<td>15.5 (3.51)</td>
<td>17.3 (4.03)</td>
<td>0.183</td>
</tr>
</tbody>
</table>

Despite the obvious toxicity associated with 50 mg/kg dosing of BLT-1 tumor volumes were assessed over the course of the treatment. Mice were randomized into treatment groups as tumors exceeded 100 mm³ with both treatment groups having a median inoculation to treatment time of 5 weeks. Retrospective comparison of initial and final tumor volume showed that mice undergoing vehicle treatment had a mean initial tumor volume of 161.1 mm³ and grew to a mean of 1305.0 mm³ by week four of the treatment period. The BLT-1 treated mice had an initial mean tumor volume of 160.1 mm³ and a mean tumor volume of 1925.8 mm³ four weeks into the treatment period (Figure 4.2C). The growth of the tumors for both groups appeared to closely mirror each other for a majority of the experiment. To confirm, linear regression analysis was performed on the two treatment groups to determine whether growth rate was significantly impacted by BLT-1 administration. The observed growth rate of the BLT-1 treated group was slightly higher than the vehicle treated group however the two were statistically
indistinguishable (Vehicle: 310.0 ± 27.69 mm³/week; BLT-1: 364.7 ± 64.38 mm³/week, p = 0.415).

Although the robust toxicity observed with 50 mg/kg oral administration of BLT-1 significantly hampers the ability to reach sound conclusions on the effects of BLT-1 on tumor volume it appears unlikely that 50 mg/kg BLT-1 is sufficient to impede C4-2 tumor growth.

4.3.4 BLT-1 administration reduces PC-3 tumor growth

As 1 µM BLT-1 was sufficient to arrest *in vitro* PC-3 growth (Figure 3.5) and concerns of toxicity at high concentrations of BLT-1, as observed with the 50 mg/kg dose in C4-2 tumor bearing mice (Table 4.2), the lower dose of 25 mg/kg, which resulted in a potentially efficacious Cmax of 2.28 uM, was selected for the PC-3 based xenograft experiments. Mice inoculated with PC-3 tumors began daily oral dosing of 25 mg/kg BLT-1 when tumor volume reached 100 mm³. A majority of both BLT-1 (7 of 13 mice) and vehicle treated (7 of 12 mice) reached dosing tumor volume by one week post inoculation with all mice starting treatment by 1.5 weeks post inoculation. Of the mice receiving vehicle treatment 10 of 12 successfully completed all four weeks on treatment. One mouse was euthanized after 2.5 weeks due to body weight loss and another died of undetermined causes overnight 1.5 weeks into treatment. Of mice receiving BLT-1 treatment 8 of 13 mice successfully completed all four weeks on treatment. One mouse was euthanized after 3.5 weeks due to body weight loss, another at 2 weeks due to sickly appearance and behaviour and one found dead of unknown causes after 2 weeks treatment. The remaining two mice were sacrificed due to tumor ulceration and total tumor volume at 3 weeks and 2.5 weeks treatment respectively. Mice treated with BLT-1 displayed an average body weight of ~2 g less than that of the vehicle treated group but neither group displayed treatment wide progressive weight loss (Figure 4.3B).

As with the C4-2 xenografts, terminal serum samples were used to complete a comprehensive toxicity profile of 14 common markers. Mice treated with 25 mg/kg BLT-1 did not display significantly increased or decreased expression of any of the assessed markers (Table 4.1). These findings indicate that likely only 3 of the BLT-1 treated mice, the two euthanized for body weight and sickly appearance and the one mouse found dead, experienced major toxicities. However, in a similar manner two mice
receiving vehicle treatment, one euthanized due to body weight loss and one found dead, were also likely experiencing toxicity. The similarity in toxicity rates indicate that BLT-1 was not inducing toxicity at 25 mg/kg and that the observed toxicities were probably due to vehicle based toxicity or issues related to the presence of rapidly growing xenografts.

**Figure 4.3 BLT-1 reduces PC-3 xenograft tumor growth**

(A) Serum samples from mice dosed with 25 mg/kg BLT-1 were assessed for BLT-1 concentration over time and used to calculate basic pharmacokinetic parameters (n = 3). PC-3 xenografts were established on the hind flank of athymic nude mice. Mice were dosed with either 25 mg/kg BLT-1 (n = 13) or vehicle (n = 12) and body weight (B) and tumor measurements were taken twice weekly (C). (D) Time to tumor volume doubling in vehicle and BLT-1 treated tumors. BLT-1 tumors had significantly increased time to tumor volume doubling (Hazard Ratio = 0.19, 95%CI = 0.06 – 0.60, p = 0.004, Log-Rank).

Mice receiving both vehicle and BLT-1 treatment displayed progressive tumor growth over the 4 week experiment (Figure 4.3C). Vehicle treated mice had a mean tumor volume of 106.8 mm³ at the start of treatment and a four week tumor volume of 799.3 mm³ while BLT-1 treated mice had an initial mean
tumor volume of 103.25 mm$^3$ and a four week tumor volume of 554.8 mm$^3$. While initial tumor volumes were indistinguishable ($p = 0.669$), linear regression was performed to assess the growth rates of each treatment group during treatment as previously published by our group and described in Chapter 2$^{364}$. Mice receiving BLT-1 treatment had significantly reduced tumor growth rate compared to the vehicle treatment group, growing approximately 30% slower (Vehicle: $156.6 \pm 10.74$ mm$^3$/week; BLT-1: $110.3 \pm 9.28$ mm$^3$/week, $p = 0.005$). Tumor volume doubling times were assessed between the two groups. BLT-1 treated mice had significantly longer tumor doubling times than vehicle treated mice (Hazard Ratio = 0.19, 95%CI = 0.06 – 0.60, Figure 4.3D). By one week of treatment 50% of mice treated with vehicle had doubled in tumor volume with all mice in the group reaching double the initial tumor volume at 2 weeks of treatment. In comparison, it took two weeks for 50% of mice treated with BLT-1 to reach double tumor volume and not all mice reached this threshold until 4 weeks of treatment. These results indicate that BLT-1 dosed at 25 mg/kg orally is capable of reducing PC-3 tumor volume growth in the absence of overt toxicity.

4.4 Discussion

Despite a substantial number of publications on BLT-1 use in vitro, in vivo based publications remain scarce$^{452,456-458}$. The apparently singular publication (Lino et. al, 2015) features the oral dosing of BLT-1, likely as mixture rather than solution due to use of PBS as a vehicle given our anecdotal experience finding an inability to solubilize BLT-1 in PBS, focused on the effects of BLT-1 administration on chylomicron formation from the gut and lacked any systemic analysis of BLT-1$^{452}$. Here we report for the first time a drug metabolism – pharmacokinetic assessment of the compound as well as studies into the efficacy of the compound in limiting xenograft tumor growth. BLT-1 is composed of three structural features; the cyclopentyl, linear alkyl and thiosemicarbazone moieties$^{454}$. Both the thiosemicarbazone and linear alkyl appear to be critical to the potency of the molecule while the cyclopentyl moiety has little apparent impact on activity. The predictably high hydrophobicity (cLogP = 4.41) also appears to be essential for inhibitory activity$^{454}$. Although this high cLogP does not surpass the
outlined boundary of cLogP = 5 set out by Lipinski’s “rule of five”, the most general of guidelines for determining drug like qualities of a molecule, it’s hydrophobicity does present potential issues with regard to suitable vehicles and methods of delivery, absorption and free drug concentration\textsuperscript{459,460}. This understanding highlighted the reasoning for using propylene glycol as a safe, miscible vehicle, as opposed to the published PBS vehicle, in an effort to provide consistent dosing and ideal conditions for absorption\textsuperscript{450,452}.

Microsomal stability assays are a commonly used component of the absorption, distribution, metabolism and excretion (ADME) studies performed to assess a compounds potential in pre-clinical drug discovery and development. The microsomal fraction of livers contains major drug metabolism proteins including CYP and UGT enzymes and is commonly used to predict the intrinsic clearance of the compound in humans\textsuperscript{461}. The extremely rapid metabolism and high intrinsic clearance of BLT-1 observed following incubation in the presence of mouse liver microsomes indicates that the compound would likely be similarly rapidly metabolized within the serum. Rapid hepatic metabolism is a common occurrence of highly hydrophobic compounds a phenomenon observed here\textsuperscript{462}. Despite the rapid metabolism observed within the microsomal study with 25 mg/kg and 50 mg/kg oral administration of BLT-1 sufficient peak serum concentrations (>1 µM) were obtained in pharmacokinetic studies. Although at Cmax the required concentration to inhibit PC-3 growth was observed the BLT-1 was fairly quickly removed from circulation meaning that at once daily dosing the amount of time BLT-1 levels remained in the therapeutic window were likely minimal. Serum concentrations never reached the required \textit{in vitro} concentration to fully arrest growth of C4-2 cells. Although potentially efficacious levels were reached for PC-3 cells, even if only briefly, the protein unbound fraction is unknown.

Lipophilic compounds, like BLT-1, commonly display high amounts of protein binding\textsuperscript{462}. A high level of protein binding could highlight several important implications from the observed data. If BLT-1 is highly protein bound the fraction available for the liver to rapidly metabolize would be minimal compared to the microsomal assay, an assay based on the assumption that compound is completely unbound. The inability of the liver to access unbound BLT-1 would in turn translate to slower metabolism...
and increased half-life. Concurrent with reduced access of BLT-1 to metabolizing enzymes, access of BLT-1 to tumor-based or other SR-B1 would be reduced. If this was the case the lack of available unbound drug to act on and inhibit SR-B1 would have significant implications with regards to the traditional understanding of the “free-drug hypothesis” which states only free unbound drug at the site of action can act on target. However, the importance of low protein binding in candidate drugs is increasingly being called into question. This would be further complicated by evidence that BLT-1 may act in a largely irreversible manner on SR-B1.

The rapid metabolism observed with the microsomal assay and high hydrophobicity alongside the ability to only briefly reach the potential therapeutic window in pharmacokinetic studies indicate that BLT-1 is not an ideal candidate beyond in vitro studies for SR-B1 inhibition. However, the lack of well classified options means that at the time it remained the best option for assessing in vivo efficacy by xenograft experiments. Here we report for the first time the ability of SR-B1 antagonism through BLT-1 administration to reduce CRPC tumor growth as demonstrated in the PC-3 xenografts. The significant reduction in tumor volume growth and increase in tumor volume doubling time was successfully achieved but likely muted by the inability to achieve efficacious serum concentrations for extended period of time with once daily dosing. Notably, however, the doubling of the dose to 50 mg/kg appeared to have a muted effect on AUC and Cmax but a significant induction of toxicity meaning that increasing a single daily dose is likely impossible. Unknown is what level of toxicity would be observed in mice dosed twice daily at 25 mg/kg or lower concentrations likely providing increased time in the potential therapeutic window. What appears clear is that maximum tolerated dose of BLT-1 lies somewhere between 25 mg/kg and 50 mg/kg via oral gavage and that the apparent therapeutic window varies from narrow in the case of PC-3 xenografts to non-existent in the case of C4-2 xenografts.

The toxicity observed when mice were dosed with 50 mg/kg manifested both in significant weight loss and alterations in circulating concentrations of several serum toxicity markers. The significant increase in particularly ALT, ALP and TBIL indicate that liver toxicity is the primarily observed form of toxicity and leading to significant weight loss in the mice. What is unknown is whether the toxicity is
related to on-target effects of SR-B1 inhibition or unrelated off-target effects. With the importance of the liver for both drug metabolism and cholesterol metabolism determining the mechanism by which hepatotoxicity occurred is likely an arduous task. The liver is considered to be one of the highest expressers of SR-B1 where the membrane protein plays a critical role in reverse cholesterol transport functioning to uptake cholesterol from circulating HDL for bile acid synthesis and redistribution\textsuperscript{236,464}. The idea that the liver may therefore be suffering from a lack of cholesterol seems unlikely due to the liver’s multiple mechanisms of obtaining cholesterol including direct blood flow from newly obtained digestive cholesterol as well as cholesterol synthesis\textsuperscript{464}. What is perhaps more likely is an increase to toxic levels of liver based cholesterol. Although generally observed for its ability to provide cholesterol to the cell SR-B1 maintains bidirectional flux allowing for the uptake of cholesterol to HDL from cells with high levels of cholesterol\textsuperscript{465,466}. Non-alcoholic fatty acid liver disease (NAFLD) is a common liver disorder present in obese patients. The disease is characterized by the accumulation of fat in the liver and is associated with hepatitis, fibrosis and cirrhosis, conditions which in turn often lead to the differential expression of the toxicity markers observed with BLT-1 treatment\textsuperscript{467}. Although only corollary and potentially only a side-effect of greater dyslipidemic processes, low-HDL has been associated with NAFLD potentially indicating a role in HDL for helping to remove accumulating cholesterol in the liver\textsuperscript{468}. It is possible that the inhibition of cholesterol flux to HDL with BLT-1 had similar effects to low circulating HDL resulting in cholesterol accumulation and NAFLD-like toxicity in the liver.

BLT-1 itself appears to be a poor candidate for further work in animals or humans due to stability and toxicity issues. However, the ability of the compound to impede PC-3 tumor growth in spite of the issues highlights the potential and serves as a proof of principle for the targeting of SR-B1 clinically to impede CRPC growth. As alluded to previously, despite BLT-1 being the most robustly studied inhibitor of SR-B1 other compounds have recently been shown to inhibit HDL-derived cholesterol uptake through SR-B1\textsuperscript{469,470}. Notably, ML278 was a recent result of a high-throughput screen and the development of a structure activity relationship for compounds targeted to SR-B1\textsuperscript{469,470}. ML278 displays a ten-fold increased potency for the inhibition of SR-B1 compared to BLT-1 and displays no significant toxicity in
Chinese hamster ovary (CHO) cells. Further, the compound appears to bind in reversible manner and displays high selectivity to SR-B1 but does still does experience a high level of protein binding due to its hydrophobicity. These improvements over the current most common inhibitor BLT-1 indicate that ML278 may be an ideal option for further investigations into targeting SR-B1 in both \textit{in vitro} and \textit{in vivo} settings.
CHAPTER 5: DISCUSSION AND CONCLUSIONS

Our understanding of cholesterol metabolism on both cellular and systemic levels remains a continually evolving and complex facet of human biology\textsuperscript{257}. This fact combined with the now well-developed appreciation for the heterogeneous nature of PCa and cancer in general drive the necessity for cautious conclusions to the described findings. The overarching hypothesis of this thesis was that cholesterol modulation, through either synthesis or uptake inhibition, will impact essential signaling processes impeding the proliferation of CRPC.

To assess the effects of cholesterol synthesis inhibition on CRPC Chapter 2 focused the effects of statin use with the current generation CRPC therapeutic abiraterone, alongside pre-clinical work assessing the ability of statins to impede the accumulation of AR-activating androgens. In the BCCA patient cohort receiving abiraterone, statin use was associated with a trend towards improved outcomes, however only reaching statistical significance when merged into a larger multi-institutional study. Pre-clinically the ability of statins to reduce the accumulation of AR-activating androgens in a castrated LNCaP xenograft model was demonstrated. The central understanding derived from the chapter is that statins are capable of impacting the ability of CRPC to drive AR activation and as such may provide a mechanism by which statin use potentiates the beneficial effects of abiraterone or other AR axis targeted therapies.

The second portion of the thesis focused on the assessment of the expression cholesterol uptake protein SR-B1 in PCa and the preclinical effects of SR-B1 antagonism in Chapter 3, while Chapter 4 contained an assessment of the \textit{in vivo} potential of the small molecule SR-B1 inhibitor BLT-1 in CRPC. It was found that SR-B1 was highly expressed in clinical prostate cancer and that pre-clinically inhibition leads to distinct anti-proliferative effects likely occurring through both steroidal and non-steroidal mechanisms and while BLT-1 itself may not serve as an ideal candidate for SR-B1 targeting due to inherent dose limiting toxicity, it has the capacity to decrease \textit{in vivo} tumor growth. Although the results of both cholesterol synthesis and uptake inhibition are promising several limitations must be taken into account when forming conclusions.
5.1 Limitations and alternatives

5.1.1 Pleiotropic effects of statin therapy

The most pressing limitations include the multiple effects of HMGCR inhibition through statin use and the ability of statins to inhibit the DHEA transporter SLCO2B1. As described in Chapter 2, the inhibition of HMGCR through statins reduces not only the ability of the cell to synthesize cholesterol but also HMG-CoA-cholesterol intermediates. These include non-steroid isoprenoid intermediates such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate\(^{363}\). Integral molecules for the post-translational modifications of several proteins including GTP-bound proteins Ras and Ras-like proteins these isoprenoids aid in sub-cellular localization and intracellular trafficking\(^{471}\). These alterations in isoprenoid synthesis are believed to be responsible for a number of the non-cholesterol mediated effects of statins. This includes the induction of eNOS expression, activation of the PI3K/Akt pathway and modulation of immune responses\(^{471-475}\). These effects, if present, could result in unaccounted for pleiotropic effects when using statins as a mechanism of cholesterol inhibition.

However, perhaps the most relevant pleiotropic effect of statins is their ability to reduce DHEA uptake through the inhibition SLCO2B1, presenting an intriguing potential method by which statins may impede steroidogenesis and AR activation by mechanisms superfluous to cholesterol synthesis. As discussed in Chapter 2, it appears unlikely that this mechanism played a significant role in the xenograft experiments as adult male mice are known to not produce DHEA\(^{369}\). Yet, this mechanism must be considered when attempting to dissect mechanisms for the observed benefits of statin use in PCa patients. Pre-clinically alternative approaches to targeting cholesterol, such as SR-B1 antagonism, and non-HMGCR cholesterol synthesis inhibition exist. The targeting of downstream enzymes in the mevalonate pathway would in theory avoid interruptions in geranylgeranyl pyrophosphate and farnesyl pyrophosphate production and potentially prevent confounding inhibition of SLCO2B1. The inhibitors of squalene synthase, SQLE and oxidosqualene cyclase all present potential options for reducing cellular cholesterol synthesis\(^{476}\). SQLE, as described in Chapter 3, has been correlated to poor outcomes in breast and
Further, the inhibition of SQLE with terbinafine has been shown to decrease cell viability in a number of breast cancer cell lines. These compounds would almost certainly present with their own set of confounding factors but would provide valuable comparators to the effects seen with statins.

### 5.1.2 Alternative mechanisms of cholesterol targeting

Beyond targeting cholesterol synthesis, alternative mechanisms of cholesterol reduction have been explored in this thesis through antagonism of SR-B1 as discussed in Chapter 3. However, further mechanisms of cholesterol mediation are worthy of consideration. Clinically available cholesterol modulators include fibrates, niacin and cholestyramine. Fibrates are known to activate peroxisome proliferator-activated receptors leading to expression of lipid metabolism genes and have been shown to have a neutral effect on cancer outcomes. Niacin functions through the G protein coupled receptors niacin receptor 1/2 inhibiting cAMP production and leading to a reduction in liver derived lipoprotein production and cholestyramine is a bile acid sequestering agent preventing gut reabsorption and leading to increased bile acid synthesis from plasma cholesterol. Notably, each of these approaches is less direct than either synthesis or uptake inhibition for reducing cholesterol and as such would likely come with significant confounding variables.

### 5.1.3 Pleiotropic effects of SR-B1 antagonism

Although the effects of the inhibition of SR-B1 are less well understood than that of HMGCR inhibition, there are still several potential limitations. The primary function of SR-B1 is the uptake of extracellular cholesterol, but the protein further plays a functional role in cell signaling pathways such as eNOS-mediated signaling which is in turn known to interact with established oncogenic pathways as described in Chapter 1. Particularly the role of Src- and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-mediated signaling in cancer proliferation is well established and as such, perturbations to these pathways via SR-B1 antagonism should be considered as mechanisms for the anti-proliferative effects and warrant investigation with future work.
As detailed in Chapter 4, the use of BLT-1 comes with significant concerns for toxicity. Although the on target effect of BLT-1 was confirmed through the inhibition of HDL-mediated cholesterol uptake in Chapter 3 what is not known are sites of off-target binding and to what extent they influence the observed results. Further, it is unknown if the observed toxicity is a result of on-target toxicity, likely due to the high expression of SR-B1 in liver tissue, or off-targeted effects. In order to help understand the contribution of on/off-target effects on both toxicity and efficacy the use of alternative small molecule inhibitors, such as ML278 described in detail in Chapter 4, which share little structural similarity to BLT-1 could be used in a similar fashion.

**5.2 Future directions**

The results described in this thesis provide a fundamental backbone for further investigation into the potential of cholesterol based therapeutic targets in PCa and CRPC progression. This section will outline potential future experiments which may provide answers to remaining questions and further build on the knowledge developed within the literature and here.

**5.2.1 Statins and PCa**

As our understanding of the role of statins in a clinical setting continues to expand and evidence continues to grow and dictate under which circumstances a clinical trial may prove successful the continually changing landscape of PCa provides further avenues of investigation. With the success of abiraterone administration alongside ADT in the STAMPEDE and LATITUDE trials the approach has been approved for widespread use within the clinic. In Chapter 2, a trend towards improved outcomes was observed with statin use alongside abiraterone in the post-ADT setting. Once combined into a larger multi-institutional study both abiraterone and enzalutamide patients were found to have improved outcomes with statin use. Investigation into the effects of statin use in the STAMPEDE and LATITUDE trials may elucidate further benefits of statin use. Further, the strict inclusion and follow up criteria and highly detailed medical record keeping associated with such clinical trials would provide an ideal dataset for analysis.
The growing body of epidemiological research finding improved outcomes for patients receiving statins across multiple stages of PCa emphasizes its potential clinical use. However, by nature epidemiological studies are limited by the risk of systemic error, bias and inability to fully account for confounding variables. This has led to calls for prospectively designed trials as an essential step prior to the clinical introduction of statin use for PCa\textsuperscript{293,481}. Determining which disease state such a trial should focus on remains an unanswered question. Recently, L. Mucci and P. Kantoff proposed initial investigations in high-risk non-metastatic patients undergoing ADT, using primary endpoint as metastases free survival as surrogate marker for overall survival\textsuperscript{481}. Although there is limited evidence demonstrating prolonged time to progression with statin use at the time of ADT, the effect of statins on metastases free survival is unknown and its rationale relies on its surrogacy for overall survival\textsuperscript{325,482}. This approach has the advantage of having an anchored and rationalized start point for statin use while using metastases free survival as opposed to overall survival expedites the evaluation of statins as an adjuvant therapy. With increasing evidence, including the findings described here, that statin use may improve outcomes of patients receiving abiraterone and enzalutamide an adjuvant post-ADT approach may provide an alternative trial strategy. Due to the relatively short survival time of men on post-ADT abiraterone or enzalutamide this approach would have a manageable time length while having overall survival as primary endpoints, consistent with where statins appear to show the most dependable benefits. However, the length of time required for a patient to see positive effects with statin use is unknown. It is possible that prolonged statin use is required for beneficial effects which may be potentially unobtainable at such late stage CRPC. Despite potential pitfalls of these approaches their undertaking would provide crucial information to guide the use of statins as therapeutic agents in PCa.

5.2.2 SR-B1 and PCa

Despite the promising results obtained from quantifying SR-B1 expression clinically in Chapter 3 further analysis could be performed to validate and expand on the findings described here and the earlier report that SR-B1 expression is elevated in high Gleason grade PCa and linked to decreased disease-free
survival\textsuperscript{288}. Analysis of the correlation of SR-B1 expression with patient outcomes could be performed using one of the available predictive TMAs. These arrays include a Vancouver Prostate Centre in-house designed TMA and the Canary Prostate Cancer Tissue Microarray designed to identify biomarkers for recurrence-free survival\textsuperscript{483}. The arrays consist of radical prostatectomy derived specimens and corresponding patient follow up allowing for direct comparison of protein expression to clinical outcome. Specifically this would allow for the analysis of the role of cholesterol metabolism proteins in biochemical recurrence as well as assessment of expression across the disease state.

In this thesis the results of targeting SR-B1 in two established PCa cell lines are described, however, determining the expression of SR-B1 and the effects of targeting in further cell lines may provide valuable insight into the role of SR-B1 in PCa. Models could include the AR driven, but androgen-independent cell lines LN-AI, LN95 and 22Rv1. The androgen independence of these lines is attributed to increased LBD-deficient AR splice variant, AR-V7, expression and should therefore not be dependent on cholesterol for \textit{de novo} steroidogenesis but remain AR driven\textsuperscript{484}. The apparent increased expression of SR-B1 in NEPC mRNA datasets and sensitivity of PC-3 to antagonism described in Chapter 3 provides an intriguing basis for further investigation for a role of SR-B1 in neuroendocrine differentiation or proliferation. NEPC models: H660, and enzalutamide-resistant LNCaP-derived 42D and 42F cells\textsuperscript{415,485} can be used as both a direct test of the transcript predictions of increased SR-B1 expression in NEPC and a model for analyzing SR-B1 antagonism in NEPC. Further, PCa cells that maintain steroidogenic potential and AR expression such as VCaP would provide further context to effects on steroidogenic potential\textsuperscript{358}. While the DU145 cell line is AR-negative, and despite expressing some NE markers is considered a non-NE model which provides a comparative model to PC-3 cells\textsuperscript{386}.

Although the use of immortalized PCa cell lines provide valuable tools for evaluating potential therapeutic targets these models are limited by selective pressures including adaptions to continued proliferation in a non-tumor environment leading to poor correlation to clinical outcome\textsuperscript{487,488}. The advent of patient derived xenografts maintained in murine hosts provides a powerful new approach to evaluating therapeutic potential across the heterogeneity of the disease. These xenograft tumors, directly implanted
from patient to mouse, are thought to better replicate clinical PCa avoiding the genetic drift observed within immortalized cell lines\(^{488}\). Several research institutions have developed patient derived xenograft programs, including the Vancouver Prostate Centre\(^{489}\) and the University of Washington\(^{490}\), either of which would be ideal candidates for experiments investigating SR-B1 antagonism. Examining the effects of SR-B1 antagonism in either the cell lines described above or patient described derived xenografts would provide insight into the role of SR-B1 in PCa and which PCa phenotype is most impacted by its antagonism.

Beyond an understanding of where SR-B1 antagonism would be most significant further investigation into the mechanism of the observed anti-proliferative effects is warranted for both cholesterol synthesis and uptake inhibition. The findings in this thesis describe the ability to impede PCa growth and progression through the limiting of cholesterol availability and propose a novel potential contributory mechanism being reduced precursor availability for \textit{de novo} steroidogenesis. However, the inability of DHEA administration to revert the effects of SR-B1 antagonism indicates that although reducing steroid availability, at least by SR-B1 antagonism, is not primarily responsible for the arrested phenotype. This finding emphasized the potential impact of more general nutrient-deprived stress mechanisms driven as a result of impacted cholesterol accumulation. In order to adapt to cellular stresses such as nutrient deprivation both cancerous and non-cancerous cells employ several mechanisms of adaptation allowing for continued survival in harsh tumor microenvironments. Specifically intracellular cholesterol is generally regulated through SREBP which under cholesterol-deplete conditions induces the expression of proteins responsible for cholesterol uptake and synthesis\(^{491}\). Activity of SREBP pathway should therefore be examined as the mechanism by which SR-B1-antagonized cells upregulate HMGCR expression and vice versa with SR-B1 overexpression following statin inhibition of HMGCR. For cells unable to replenish cholesterol cellular dysfunction could impact function through several processes including impaired membrane synthesis and lipid-raft mediated signaling leading to the manifestation of cell stress responses\(^{492-494}\).
Autophagy functions as a robust mechanism by which PCa copes with several cellular stresses including ADT, chemotherapy and nutrient deprivation which would otherwise be lethal. This thesis describes what appears to be stark induction of autophagy in SR-B1 antagonized cells. Although initial results indicate the role of reduced mTOR signaling as a driver of autophagic phenotype further characterization of the mechanism of the observed stress should be investigated. These include downstream mTOR signaling pathways including S6 Kinase 1 (S6K1), the eIF-4E binding proteins (4E-BP1/2) or Unc-51 like autophagy activating kinase (ULK1/2) through which mTOR mediated signaling drives its regulatory effects. External to mTOR–regulated autophagy AMP-activated protein kinase (AMPK) is a critical energy/nutrient regulatory protein, which functions as a sensor to depleted ATP conditions within the cell promoting catabolic processes to generate more ATP and has been connected to metabolic processes including autophagy. Capable of activating autophagy through both ULK1 phosphorylation and mTOR inhibition AMPK activity has been shown to be reduced by androgen-driven reduced expression of liver kinase B1 (LKB1) and could play a role in SR-B1 driven autophagy.

Autophagy can manifest in response to several disruptions to cellular signaling processes. This thesis describes the apparent induction of ER-stress and the UPR in response to SR-B1 antagonism as measured through increased expression of BiP and IRE1α. ER-stress is generally thought to be result off a disruption of protein homeostasis but has also been demonstrated in response to disrupted lipid homeostasis. Further, ER stress is known to induce autophagy which could mechanistically explain the SR-B1 antagonized phenotype. There are several proposed mechanisms of ER stress mediated autophagy that could be investigated to assess the connection including eukaryotic initiation factor 2 (eIF2α)-autophagy-related protein 12 (Atg12) expression, IRE1α-TNF receptor-associated factor 2 (TRAF2)- c-Jun N-terminal kinase (JNK) expression and measuring cellular Ca²⁺ and calmodulin-dependent protein kinase kinase 2 (CaMKK-β).

Although generally considered to be cholesterol-poor, increased tumoral cholesterol has been demonstrated in mitochondria and may indicate a role in sustained oncogenic signaling. It has been postulated that this increased cholesterol plays a role in facilitating hypoxia-inducible factor 1-alpha
(HIF1α) activity and mitochondrial function in the hypoxic environment generally observed in cancer\cite{498}. The loss of this cholesterol could result in decreased HIF1α signaling and increased hypoxic stress leading to the observed induction of autophagy with SR-B1 antagonism\cite{498,499}. To assess this, general measurements of mitochondrial function could be taken including ATP production and reactive oxygen species concentration or more specific assessment of HIF1α signaling.

Cholesterol constitutes a critical component of lipid rafts, membrane domains essential for several proliferative signaling pathways\cite{500}. These include receptor tyrosine kinases insulin-like growth factor 1 receptor (IGFR) and epidermal growth factor receptor (EGFR) lipid raft signaling and the small GTPase Ras family with established roles in prostate cancer proliferation\cite{500,503}. Both IGFR and EGFR are known to induce cancer proliferation through PI3K/AKT and Ras/MAPK signaling pathways the disruption of which can lead to the induction of autophagy\cite{504,505}. PI3K functions to phosphorylate phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2) to PI[3,4,5]P3 leading to a conformational change inducing AKT binding and activation leading to a proliferative phenotype through many different branches including increased growth through mTOR activation, increased nutrient metabolism through the inhibition of glycogen synthase kinase 3 (GSK3), and decreased apoptotic signaling through NF-KB activation\cite{506}. Ras is a small GTPase which in its active state recruits and activates a kinase signaling cascade through RAF and MAPK kinases leading to the phosphorylation of numerous targets including pro-proliferation transcription factors Fos, Jun and Myc\cite{507,508}. These pathways are considered some of the most critical oncogenic pathways the disruption of which through impaired lipid raft formation could lead to reduced proliferative signaling and the induction of autophagy. However, as described in Chapter 1, SR-B1 is capable of inducing PI3K/AKT and Ras/MAPK signaling via Src independent of its effects on cellular cholesterol\cite{255,256}. The examination of the activity of these pathways would provide insight into their role in the induction of autophagy in SR-B1 antagonized cells however determining whether the altered activity of these pathways is a result of cholesterol mediated or independent effects would be necessary. Both fluorescent probe and detergent resistance assessment could be used to directly assess membrane formation in SR-B1 antagonized cells\cite{509,510}. In general, understanding alterations in cellular
signaling processes that drive the observed autophagic phenotype in response to SR-B1 antagonism
provide not only valuable mechanistic insight into the role of SR-B1 in PCa but may also guide the design
of novel small molecule SR-B1 inhibitors and provide rational candidates for synthetic lethal co-targeting.

As described in Chapter 4, one of the major potential issues for further development of BLT-1 is
the significant toxicity associated with use in vivo. The identification of novel small-molecule inhibitors
of BLT-1 would therefore be of high priority. The recently established SR-B1 inhibitor ML278 presents
an intriguing option for this approach. Notably the likely reduction in toxicity and increase in potency
could significantly improve the therapeutic window over that observed with BLT-1 making ML278 more
suitable for use in vivo. Prior to use in vivo, however, ML278 or any other potential inhibitor would need
to be studied in vitro in a similar manner to the experiments described in Chapter 3.

Synthetic lethal co-targeting can be approached by either high-throughput identification or
rational design as a method to increase the cytotoxicity of individual cholesterol metabolism targeted
agents.\textsuperscript{446,447} In theory high-throughput approaches would employ genome-wide knockout libraries in
concert with SR-B1 antagonism to identify candidates resulting in robust cytotoxicity. While examples of
the potential of rationale co-targeting of SR-B1 with either HMGCR or autophagy inhibitors were
demonstrated in Figure 3.12. Chloroquine and hydroxychloroquine, clinically used prophylactically
against malaria, inhibit the lysosomal degradation of cellular components of autophagic vacuoles and are
commonly employed as an in vitro autophagy inhibitor\textsuperscript{511}. Clinically, a number of trials are currently
underway combining chloroquine or hydroxychloroquine with standard therapy in an effort to prevent
therapy induced autophagic resistance in several cancer types. Of those trials that have reported, largely in
limited numbers of glioblastoma multiforme patients, minimal to modest improvements in outcomes have
been reported\textsuperscript{512-519}. Although the clinical potential of chloroquine or hydroxychloroquine appears limited,
there use for pre-clinical proof of principal experiments would still be valuable. Further, more potent
autophagy inhibitors have been developed which may demonstrate superior co-targeting than traditional
agents\textsuperscript{520}. Notably, SR-B1 antagonized cells displayed significant upregulation of Clu (Figure 3.8). As
described previously, Clu is a stress-activated chaperone that regulates protein homeostasis by preventing
protein aggregation, and enhancing autophagosome biogenesis, inhibiting apoptosis and promoting survival of PCa\textsuperscript{392,521}. In order to determine if Clu expression affects SR-B1-antagonism-induced cell stress SR-B1 antagonized cells could be targeted with either Clu-targeted siRNA or OGX-011 antisense oligonucleotide. Evaluating these co-targeting approaches or those identified through high-throughput approaches in a similar manner to the experiments described in this thesis would likely provide therapeutic options for inducing a more robust cytotoxic response in SR-B1 antagonized cells. The research proposed here would further the understanding of the role and potential of targeting cholesterol metabolism in PCa, through expanding both the knowledge of clinical expression and impact and effects of targeting cholesterol metabolism pre-clinically.

**5.3 Conclusions**

For patients diagnosed with advanced local or metastatic CRPC the mainstay treatment remains ADT generally through the use of LHRH targeting agents in combination with an AR inhibitor such as bicalutamide. For those patients that recur following ADT treatment options remain limited. Currently only the anti-androgens abiraterone and enzalutamide and chemotherapeutic docetaxel are in regular use and providing significant although limited improvements in patient survival. With taxane-based chemotherapies remaining the most commonly used non-AR axis based agent in PCa treatment the discovery of the role of androgens and their use as a therapeutic target remains the central thesis in the treatment of PCa. The success and failure of AR axis targeting agents in PCa has driven investigation into the understanding of multiple complex mechanisms of therapeutic resistance, in turn leading to modifications of approach in how novel AR axis therapeutics are developed. The continual success and eventual failure of AR axis targeted therapeutics belays both the critical importance and the robust adaptive potential of the pathway. The continued resistance to these therapies due to AR centered mechanisms would in theory direct focus to targeting other PCa signaling pathways. However, the paucity of non-AR axis targeted therapies highlights the difficulty of such approaches.
The multifunctional sterol, cholesterol, underlies several essential cellular processes and its role in human disease is continually developing across multiple ailments. The role of cholesterol in cancer and more specifically PCa is intently being investigated from multiple angles and appears to have an important role in the aggressiveness and proliferation of the disease. Here a therapeutic approach where in the multifaceted role of cholesterol in steroid driven and steroid independent cellular proliferation is leveraged as a mechanism for halting CRPC growth. The ability of multiple approaches to cholesterol targeting to impede steroid accumulation integrates the approach with other clinically successful AR axis targeted therapies. This approach of limiting pre-cursor cholesterol for androgen synthesis would likely in turn obstruct several mechanisms of resistance to existing AR-axis targeted therapies, including CYP17A1 over expression and AR hypersensitivity and promiscuity. However, precursor reduction, as with all clinically approved AR-axis therapies, would be limited by resistance mechanisms that preclude steroid accumulation for AR activation or growth such as constitutively active AR splice variants and neuroendocrine differentiation for which steroid reduction would be insufficient to impact CRPC growth. This understanding emphasizes the importance of the findings described within this thesis that although cholesterol targeting does reduce androgen accumulation, external mechanisms, likely revolving around nutritional stress, significantly contribute to the arrest of CRPC growth. This is exemplified by both the inability of exogenous steroid to reverse the cholesterol antagonized phenotype and the significant impact of that antagonism in AR negative models of CRPC. This multifaceted approach to targeting CRPC provides a unique opportunity to tackle a large subset of the heterogeneous recurrent CRPCs.

Beyond establishing the potential window in which cholesterol targeting may prove to be a viable therapeutic target, by examining the outcomes of patients receiving abiraterone while on statins, this thesis further attempts to confirm the “drugability” of a novel therapeutic target in SR-B1. The circulating concentrations of cholesterol, regularly at superfluous levels, observed in western populations alongside its role in the development of atherosclerosis have driven the development of several cholesterol targeted therapeutics, namely statins. Although often overstated, side effects of statin use do exist but in general statins are considered to be safe for use and demonstrate the feasibility of targeting
cholesterol and more specifically HMGCR in a clinical setting\textsuperscript{224}. This clinical use of statins allows for the relatively simple investigation of its effects on PCa outcomes as demonstrated here and provides the ability to develop rationale and design for prospective clinical trials in a relatively straightforward manner. If a positive role for statin use in PCa treatment was confirmed it would provide a low toxicity, low cost method for improving the survival of PCa patients. However, statins do not necessarily provide the best method of cholesterol antagonism for the treatment of PCa but rather the most convenient. The apparent overexpression of SR-B1 in PCa, its correlation to survival and the comparative specificity of expression versus HMGCR may indicate a more favorable target for cholesterol modulation in PCa treatment. Here not only is the ability to impede PCa growth through SR-B1 antagonism demonstrated but further the viability of SR-B1 targeting \textit{in vivo} was assessed. Although issues of hydrophobicity and toxicity at high concentrations were observed with the specific small molecule, BLT-1, the ability to reach efficacious concentrations was demonstrated and highlights the potential for other SR-B1 targeted small molecules.

The findings described here contribute to existing literature on the role of cholesterol metabolism in PCa and can function as a base for further investigation into the topic. The targeting of cholesterol metabolism for the treatment of PCa has the potential to provide a multifaceted approach to dealing with the highly heterogeneous nature of the recurrent disease state.
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