A MODEL OF ACQUIRED RESISTANCE TO THE NOVEL AR-NTD INHIBITOR RALANITEN REVEALS RESISTANCE OCCURS VIA A SELECTIVE METABOLISM PATHWAY

by

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A MODEL OF ACQUIRED RESISTANCE TO THE NOVEL AR-NTD INHIBITOR RALANITEN REVEALS RESISTANCE OCCURS VIA A SELECTIVE METABOLISM PATHWAY

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ABSTRACT

Background: Inhibition of the androgen receptor (AR) is the mainstay treatment for advanced prostate cancer. While initially effective, the disease ultimately progresses to metastatic castration-resistant prostate cancer, which is lethal. Ralaniten specifically targets the AR N-terminal domain (NTD). This allows for sustained inhibition in the context of constitutively active AR-splice variants and mutations which often drive resistance to current therapies. Ralaniten acetate is the pro-drug of ralaniten, and the first AR-NTD inhibitor to enter clinical trials (NCT02606123, ESSA Pharma, Inc.). Here we generated and characterized a model of acquired ralaniten resistance to aid in the development of next generation AR-NTD inhibitors.

Methods: A resistant cell line (LNCaP-RAL\(^R\)) was created by serially passaging parental LNCaP cells in the presence of ralaniten. Resistant cells were challenged with ralaniten and existing AR antagonists to assess growth and sensitivity of the resistant line in vitro and in vivo. LNCaP-RAL\(^R\) cells were subject to global microarray profiling, and findings were validated using qRT-PCR, western blot and functional assays.

Results: Ralaniten displayed antitumour activity in LNCaP but not LNCaP-RAL\(^R\) xenografts due to its reduced ability to block AR mediated gene transcription. Interestingly, LNCaP-RAL\(^R\) cells retained sensitivity to antiandrogens and AR knockdown by targeted siRNA, implying growth remains driven by AR signalling. Interrogation of microarray data revealed candidate genes (UGT2B family) associated with ralaniten resistance. These genes are involved in drug metabolism (glucuronidation), and knockdown of UGT2B isoforms was sufficient to restore
sensitivity to ralaniten in resistant cells. LC/MS and ion spectra data from serum samples collected from patients, revealed that ralaniten is glucuronidated in humans. EPI-045 (which is resistant to glucuronidation) significantly inhibited AR mediated transcription and proliferation in LNCaP-RAL\textsuperscript{R} cells - both \textit{in vitro} and \textit{in vivo}.

**Conclusions:** We have generated a model of acquired ralaniten resistance, and demonstrated that selective modification of ralaniten can reduce its glucuronidation. LNCaP-RAL\textsuperscript{R} cells remain dependent upon AR signalling, and are sensitive to both EPI-045 and antiandrogens used clinically. This work highlights the potential for combination or sequential therapy following ralaniten resistance, and provides a model with which to test next generation AR-NTD inhibitors.
LAY SUMMARY

The androgen receptor is the main driver of advanced prostate cancer; and many therapies currently available attempt to prevent it from becoming activated. Unfortunately resistance to these drugs will inevitably occur, and there is no cure for late stage prostate cancer. Our lab has developed a brand new class of drugs which should be effective in the context of resistance to existing therapies. Our lead compound ralaniten, was recently accepted into clinical trials due its success in the laboratory. Despite the potential that ralaniten holds for the treatment of advanced prostate cancer, it is possible that resistance will develop against this drug as well. Therefore this work focused on pre-emptively predicting how resistance might arise, and as a result we are actively developing backup compounds. Here we present a potential resistance mechanism to ralaniten, and identify a new drug which may be used to combat it.
PREFACE

All of the work presented in this dissertation was performed at the Sadar Laboratory at the Genome Sciences Centre in the BC Cancer Research Centre unless explicitly stated otherwise. This doctoral dissertation is the independent and original work by me, Jonathon Obst, and is prepared under the supervision of Dr. Marianne Sadar, Professor, Department of Pathology and Laboratory Medicine, at the University of British Columbia. Blood plasma specimens used in this work were deidentified samples obtained from patients enrolled in Phase I clinical trial (NCT02606123), which received Research Ethics Board approval at all participating sites. All patients provided written informed consent prior to inclusion in the study. All experiments involving animals were conducted in compliance with, and the approval of, the Animal Care Committee of the University of British Columbia (A18-0077).

A version of Chapters 2-4 have been written as a manuscript and has been submitted to the Journal of Clinical Investigation. [Obst JK, Wang J, Jian K, Williams D, Tien AH, Mawji NR, Tam T, Yang YC, Andersen RJ, Chi KN, Montgomery B, Sadar MD. Modeling acquired ralaniten resistance to promote drug discovery and development in prostate cancer. J Clin Invest. 2018. Submitted]. I was responsible for the major areas of concept formation, data generation and collection, data analysis and manuscript composition. Sadar MD was the supervisory author on this manuscript and was involved throughout the project in concept formation, data analysis and manuscript composition.
Chapter 1. Figures 1.1, 1.2, 1.5 and 1.9 are used with permission from applicable sources.

Chapter 2. Wang J and Tien A performed the animal experiments. Tam T generated samples for AR sequencing (sequencing performed at the NAPS core unit at the University of British Columbia). Mawji NR and Yang YC were responsible for early maintenance of the resistant cell line, and Mawji NR resuscitated frozen cell lines for culture, managed reagent orders and provided technical assistance as well.

Chapter 3. Figures 3.1 and 3.2 A are used with permission from applicable sources. Mawji NR performed the lentiviral transduction of LNCaP cells, resuscitated frozen cell lines for culture, managed reagent orders and provided technical assistance.

Chapter 4. Andersen RJ, Williams D and Jian K provided experimental compounds including glucuronidated standards, and performed the HPLC and $^1$H NMR experiments at the Andersen Laboratory, Departments of Earth, Ocean & Atmospheric Sciences at the University of British Columbia. Chi KN and Montgomery B oversaw the clinical trial and provided samples for metabolic analysis (performed at RMI Laboratories, North Wales, Pennsylvania, USA). Wang J performed the animal experiments. Mawji NR resuscitated frozen cell lines for culture, managed reagent orders and provided technical assistance.
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<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AUA</td>
<td>American Urology Association</td>
</tr>
<tr>
<td>ADT</td>
<td>androgen deprivation therapy</td>
</tr>
<tr>
<td>AIS</td>
<td>androgen insensitivity syndrome</td>
</tr>
<tr>
<td>AF1/2</td>
<td>activation function 1/2</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AR(^{FL})</td>
<td>androgen receptor (full-length)</td>
</tr>
<tr>
<td>AR(^{-V7})</td>
<td>androgen receptor variant 7</td>
</tr>
<tr>
<td>AR(^{v567es})</td>
<td>androgen receptor variant exons 5,6,7 skipped</td>
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<td>ARE</td>
<td>androgen response element</td>
</tr>
<tr>
<td>AS</td>
<td>androsterone</td>
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<tr>
<td>BADGE</td>
<td>bisphenol A diglycidic ether</td>
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<tr>
<td>BCR</td>
<td>biochemical recurrence</td>
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<tr>
<td>BIC</td>
<td>bicalutamide</td>
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<td>BPH</td>
<td>benign prostatic hyperplasia</td>
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<td>BPA</td>
<td>bisphenol-A</td>
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<tr>
<td>CAD</td>
<td>continuous androgen deprivation</td>
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<tr>
<td>CRPC</td>
<td>castration resistant prostate cancer</td>
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<tr>
<td>CTCs</td>
<td>circulating tumour cells</td>
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<tr>
<td>CTE</td>
<td>C-terminal extension</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<td>DDI</td>
<td>drug-drug interactions</td>
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<td>DHEA</td>
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<td>DHEA-S</td>
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<td>5α-dihydrotestosterone</td>
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<td>digital rectal examination</td>
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<tr>
<td>EBRT</td>
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<td>ENZ</td>
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<tr>
<td>EUA</td>
<td>European Urology Association</td>
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<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
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<td>glucocorticoid receptor</td>
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<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
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<td>HR</td>
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<td>hinge region</td>
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<td>intermittent androgen deprivation</td>
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<td>IR3</td>
<td>three-nucleotide spaced inverted repeat</td>
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<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
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<td>lutenizing hormone</td>
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<td>maximal androgen blockade</td>
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<td>mineralocorticoid receptor</td>
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<td>NES</td>
<td>nuclear export signal</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>NTD</td>
<td>N-terminal domain</td>
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<tr>
<td>PFS</td>
<td>progression free survival</td>
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<tr>
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<td>PR</td>
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<tr>
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<tr>
<td>rPFS</td>
<td>radiological progression free survival</td>
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<tr>
<td>TAU1/5</td>
<td>transcriptional activation unit 1/5</td>
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<td>TRUS</td>
<td>transrectal ultrasound</td>
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<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
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<tr>
<td>UDPGA</td>
<td>Uridine diphosphate glucuronic acid</td>
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<tr>
<td>UGT</td>
<td>UDP-glucuronosyl transferase</td>
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I am forever grateful for the tremendous support I have received throughout this journey. I am truly blessed to have so many kind people in my life who have made my PhD experience a positive and exciting one.

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This dissertation is dedicated to

~ those who seek to know, for the sake of knowing ~
Chapter 1. Introduction

1.1 PROSTATE CANCER EPIDEMIOLOGY

1.1.1 Disease risk factors and etiology

Prostate cancer is most often diagnosed at an early stage when the disease is localized and remains organ confined. At this stage, the prognosis is very favorable with 5-year survival rates approaching 100% [1–3]. Due in part to advancements in screening practices and early detection of the disease, the 5-year survival rate across all combined stages of prostate cancer has increased from 68% to ~99% over the past 25 years [1–3]. Nonetheless the overall incidence of prostate cancer has also concurrently risen, largely owing to both increased screening practices and an ageing population. Indeed, prostate cancer represents the second most common cancer in men worldwide, with an estimated global incidence of 1.1 million diagnoses (15% of cancer cases in men) in 2012 [4]. The vast majority of cases occur in developed countries [2–6] with 180,890 new diagnoses and 26,120 deaths predicted annually in the United States alone [1].

Prostate cancer is typically slow growing and primarily affects the elderly. As such the disease is very rarely observed in men less than 40 years of age; although the risk of developing prostate cancer increases dramatically for men aged 70 and older [3–5,7]. While age remains the strongest risk factor, others have been identified including race and family history indicating a genetic component with respect to disease etiology. This is evidenced by the fact that significant differences in risk of acquiring prostate cancer exist between different ethnic groups. African Americans represent the most at-risk group, with a 50-60 fold higher incidence rate than Chinese men (who embody the lowest risk group) [5,7,8]. Similarly, the impact of family history on disease risk has been described. A retrospective study comparing cancer incidence rates in
mono- and dizygotic twins revealed that ~42% of the risk of developing prostate cancer may be attributable to genomic factors [9]. While these data clearly point to an inheritable factor(s) playing an important role prostate cancer, other putative non-genomic risk factors have also been identified. Studies have shown that migrants who emigrate from ancestral countries associated with lower incidence rates to Western states are at a higher risk of developing prostate cancer more in line with men from Western societies [5]. Additionally, traditionally low-risk countries are seeing increases in prostate cancer incidence independent of increased screening practices, and likely reflect lifestyle changes associated with Westernization [8]. This suggests environmental factor(s) such as diet/exercise, occupation, obesity and alcohol/tobacco consumption may also play important roles in prostate cancer etiology [5,7].

1.1.2 Prostate anatomy and physiology

The prostate is a small secretory organ made up of exocrine glandular epithelial cells as well as fibromuscular stroma tissue [10–13]. The prostate is an encapsulated organ surrounded by collagen, elastin and smooth muscle. It functions to assist in the maturation of semen, specifically with respect to preventing coagulation and maintaining alkalinity of the ejaculate [11,14]. In man, the prostate undergoes most of its development during puberty and is considered mature by 20 years of age [13]. While prostate weight remains relatively stable for a time after reaching maturity, growth slowly resumes once the individual reaches the fourth or fifth decade of life [11,13,15].

The prostate is organized into discrete zones [12,16,17] with the peripheral and central zones of the glandular compartment making up the majority (70% and 25% respectively) of the total prostate [16]. The remainder of the glandular component is composed of the transitional
zone (surrounding the prostatic urethra) and peri-urethral glands [12,16,17]. Most prostate cancers (~60-70%) originate in the peripheral zone which lies to the posterior of the gland adjacent to the rectal wall [10,12]. Approximately 10-20% develop in the transitional zone, and tumours rarely (~5%) arise in the central zone [12,16].

There are three distinct types of cells which form the prostate epithelium. Luminal cells are secretory epithelial cells and line the secretory ducts of the prostate. These are separated from the surrounding stroma and basement membrane by a layer of basal cells [10,16,18,19]. Neuroendocrine cells are the third cell type residing in prostate epithelium, and though rare, are found in all zones of the prostate interspersed among basal cells. Each of these cell types can be histologically distinguished based upon cellular morphology and/or expression of cellular specific biomarkers which aid in the classification of prostatic carcinoma subtypes [6,18,19].

Most human prostate cancers involve the secretory luminal cells and retain at least some glandular structure [10,12,18,19]. These are classified as adenocarcinomas and are androgen responsive expressing functional androgen receptor (AR) and secrete prostate-specific antigen or PSA [10,12,19]. Conversely, basal cells are thought to only express low levels of AR, do not secrete PSA or other prostatic secretory molecules, and have been proposed to contain some stem-like properties [10,12,18–21]. Neuroendocrine cells express neurological markers such as neuron-specific enolase and chromagranin A, and often have dendritic processes [16]. They also lack AR expression and do not respond to androgens [7,16,18,22]. Tumours originating from neuroendocrine cells are rare, however patients diagnosed with neuroendocrine prostatic carcinoma tend to have aggressive disease and poor responses to current therapies [18,22].
1.1.3 Detection and diagnosis of prostate cancer

In Western countries the lifetime risk of developing prostate cancer is ~14.3% [2–4,7,23–25], therefore several methods have been developed to aid in early detection of the disease. The most common include the digital-rectal examination (DRE), the PSA blood test, and transrectal ultrasound (TRUS). Any indication of disease usually necessitates a needle biopsy guided using TRUS to histologically confirm the presence of cancerous cells [23–26].

A DRE is a standard screening procedure to investigate prostatic abnormalities, specifically prostate enlargement and detection of growths. The majority (~70%) of prostatic carcinomas originate in the peripheral zone of the prostate [5,7] and may be detected by a DRE, provided the tumour is ~0.2 cm$^3$ in volume or greater [27]. However, the test is not perfect and it has been shown that tumours up to 0.5 cm$^3$ may be missed and in some cases, smaller tumours may actually represent clinically relevant disease [28].

Alternatively, PSA (also known as kallikrein-3/KLK3) is a glycoprotein enzyme secreted into the ejaculate by epithelial cells of the prostate [29]. PSA expression is transcriptionally regulated by the AR (which is a known driver of most prostate cancers), and detection of PSA is therefore used as a surrogate for AR activity. PSA levels are often elevated in the serum of prostate cancer patients as the tumour breaches organ confines and secretes PSA into the blood [7,14,24,27,30,31]. A threshold of 4.0 ng PSA/mL of blood is typically used to stratify patients for additional testing and/or treatment. The PSA test was originally approved by the FDA in 1986 as a means to observe cancer progression in men who had been previously diagnosed with the disease [14,32]. In 1994, the PSA test was approved to be used in conjunction with the DRE to screen asymptomatic men for prostate cancer. As a result, PSA screening has been directly
implicated in the dramatic increase in prostate cancer diagnoses seen since 1986 [7,14,25,27,30–32].

For men who have a suspicious DRE and/or a PSA level $\geq 10.0$ ng/mL, a TRUS guided needle biopsy is generally prescribed to confirm the presence of prostate cancer (a PSA rise of 0.75 ng/mL/year can also be used when PSA level is between 4.1 and 10 ng/mL) [24,25,27]. When deciding whether to perform a biopsy, the patients family history, age and race are also considered. Typically 10-12 cores of prostate tissue are removed for histological analysis to determine Gleason score and relative differentiation. These metrics are used alongside TNM staging to diagnose and classify the disease, guiding therapeutic decisions [6,24–28].

Often, prostate cancer is preceded by a condition termed prostatic intraepithelial neoplasia or PIN [5–7,10,12,18,33–35]. PIN is undetectable by DRE, nor is it associated with elevated PSA levels, and therefore must be diagnosed by biopsy and histological examination [33]. PIN is stratified into low- or high-grade designations. Low-grade PIN is evidenced by the expansion of epithelial cells lining existing ducts, however is histologically similar to normal prostate tissue [36]. Conversely, high-grade PIN is closely associated with prostate cancer, and has a predictive value of nearly 25% for contracting prostatic adenocarcinoma [12,33]. Indications of high-grade PIN include enlargement of the nucleus (with or without distinct nucleoli), as well as expansion of the luminal epithelial cells, emergence of disorganized glands (in terms of secretory luminal and basal cell separation), and increased microvessel density [6,12,33–36]. Patients who have high-grade PIN are closely monitored with repeat biopsies, however prophylactic treatment is not appropriate, and interventions are only administered upon disease progression [33-35].
1.1.4 Treatment of localized prostate cancer

Patients with localized disease who opt to receive medical intervention typically undergo radiation therapy or surgery to remove the prostate. Two types of radiation therapy are generally used: brachytherapy and external beam radiation therapy (EBRT) with or without dose escalation [24,26]. Brachytherapy is a form of radiotherapy wherein small encapsulated radioisotopes are implanted directly into the prostate near the tumour. This allows for the targeted release of short-range radiation to specifically kill cancerous and surrounding tissue, but impedes its release into general circulation. Alternatively, EBRT refers to high energy x-rays which are targeted at the tumour from a source outside of the body. Adverse reactions to radiation therapy can be fairly common and include bowel irritability (diarrhea and/or painful bowel movements, bowel urgency, and hemorrhoids), as well as decreases in urinary and sexual functions (frequent urinary urges, incontinence and erectile dysfunction) [37–39].

Radical prostatectomy (RP) involves surgically removing the prostate as well as some surrounding tissue, namely the seminal vesicles and part of the urethra within the prostate. Some significant side effects are commonly associated with this procedure, specifically erectile dysfunction, infertility and urinary incontinence [40–43] which may have a detrimental effect upon quality of life. Very rarely other serious side effects can occur including injury to surrounding organs (rectum) or death as a result of surgery [41,42]. Both radiotherapy and RP are considered curative interventions for localized disease, however there is as yet insufficient clinical evidence to support prescribing one treatment over the other [37,44,45]. While radiation therapy has less association with adverse urological effects than RP, it is not without side effects of its own and the patient's personal choices must be considered when prescribing treatment [37–39,41,43,46].
Due to both the low lifetime risk of death from prostate cancer (~3%) compared to the risk of contracting the disease (~14%), and the associated negative effects from the treatment itself, some men may opt for observation (either active surveillance or watchful waiting) rather than definitive treatment [2,3,7,10–13]. The key difference between these two methods of observation, is that patients undergoing active surveillance receive periodic PSA/DRE examinations and additional biopsies as required. Directed treatment (typically radical prostatectomy or radiation therapy) though delayed, is still considered curative and is provided upon disease progression, or the threat of progression [24–27,47–50]. Watchful waiting on the other hand, is instead reserved for patients with low risk prostate cancer, but also a low life expectancy. It rather focuses on treating symptoms related to the disease, and therefore additional screening is deferred until symptoms arise, triggering biopsies and/or palliative treatment [24,50].

Based upon two large-scale randomized clinical trials (PIVOT, ref 42; and SPCG-4, refs 40,47) current guidelines recommend active surveillance or watchful waiting only for very low-/low-risk disease (PSA <10 ng/mL, Gleason score ≤6, organ confined disease) provided patients have a life expectancy of ≤5 years (watchful waiting) or ≤10 years (active surveillance) [24,26]. For patients with low- or intermediate-grade disease and >10 year life expectancy, either RP or radiation therapy is recommended, especially if the disease is expected to progress [24,26,50]. Patients with high-risk disease (extracapsular disease with or without local lymph node infiltration and/or distant metastases) will require much more aggressive therapies discussed in detail later in this dissertation.
1.1.5 Pathology and prognosis for advanced prostate cancer

Despite the fact that prostate cancer tends to be slow growing and indolent, and may not always warrant medical intervention, an estimated 4% of men will present with metastatic disease at diagnosis [51,52] and approximately 20-30% will experience recurrence following primary treatment [52–57]. Recurrence is often first evidenced by rising serum PSA levels which generally precede clinical symptoms of metastatic disease (Figure 1.1), a phenomenon termed biochemical recurrence (BCR). While progression to aggressive metastatic disease is precluded by BCR, the existence of BCR itself may simply indicate local failure. In this case, some patients may undergo salvage radiation therapy following RP with curative intent [52,54–58]. Unfortunately, salvage therapy is not always curative and inevitably the majority of patients progress despite subsequent treatment [59].

Patients who have distant metastases or are at risk of clinical progression will require much more aggressive and systemic therapy, therefore identifying high risk patients and differentiating between them is critical at this stage [59]. A wide range of metrics are used to guide patient stratification, and PSA kinetics, Gleason score and time from initial treatment to BCR have all been positively associated with clinical progression [54–56,58,59]. Current imaging technology (namely CT and bone scans) are also used to compliment PSA testing and confirm distant disease, but have a relatively high threshold for detection and may not detect disease in men with PSA levels <10 ng/mL [59]. MRI and PET scans have better sensitivity, however also have a higher cost, and still micro metastasis may exist and yet remain below the limit of detection [52].

While the 5-, 10- and 15-year survival rates for patients with localized or regional prostate cancer are very favorable (99%, 98% and 96% respectively), once the cancer has
disseminated, survival rates are much more dismal (5-year survival rate = 29%, ref. 60). Prostate cancer often metastasizes to the bone causing osteoblastic bone lesions [61,62] and may lead to pathologic fractures. These understandably induce considerable acute and chronic pain, and many of these metastatic fractures may never heal [62]. Spine metastases have been reported following autopsy in up to 87% patients with advanced disease [61], and those affecting the vertebrae can lead to spinal cord compression and paraplegia if surgical intervention is not administered quickly [62]. Patients with metastatic prostate cancer will ultimately succumb to their disease and although palliative treatment is available, many of the symptoms of advanced prostate cancer have a severe impact on the patient's quality of life [62]. Most prostate cancers are driven by AR signalling and antagonizing this pathway is the primary focus of current therapies. Androgen deprivation therapy (ADT) remains the gold standard of care for patients with metastatic disease, and will be one of the major focuses presented later in this dissertation (section 1.3.2).

1.2 THE ANDROGEN RECEPTOR: STRUCTURE AND FUNCTION

1.2.1 The androgen receptor is a nuclear steroid receptor

The vast majority of prostate cancer is adenocarcinoma, and is histologically defined as the destruction and infiltration of the basal layer by expanding luminal cells [10,12,18,19]. As mentioned previously, these cells are androgen responsive and rely upon functional AR signalling. The AR is a member of related nuclear steroid hormone family of receptors which also include the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR) and the oestrogen receptors α/β (ER-α/ER-β) [63–65]. These nuclear steroid receptors are soluble, ligand-activated transcription factors which function primarily as master
regulators for a large number of genes. They are mainly activated by steroid hormone ligands which diffuse across the cell membrane. Ligand binding induces conformational changes in the AR leading to phosphorylation and activation of the receptor, exposing a nuclear localization sequence and allows its active transport to the nucleus. The AR functions as a dimer and recognizes specific genomic regions termed androgen response elements (AREs). DNA binding mediates recruitment of coactivators and transcriptional machinery to induce the transcription of target genes [63–65].

The single copy AR gene is roughly 90 kb containing 8 exons, and is located at q11-12 on the X-chromosome [65]. The full-length AR (ARFL) is generally reported as being composed of 919 amino acids (a.a) forming three distinct structural domains and a hinge region. Exon 1 encodes the entire N-terminal domain (NTD, a.a 1-558), exons 2 and 3 encode the DNA-binding domain (DBD, a.a 559-622) and exons 4-8 encode the ligand-binding domain (LBD, a.a 671-919). The short hinge region (HR, a.a 623-670) is encoded within exon 4 and shares a nuclear localization sequence with the C-terminal end of the DBD [65,66]. Some discrepancy exists with respect to reported size and amino acid numbering, due to the existence of two polymorphic regions within the NTD (discussed in more detail in section 1.2.4). Wild-type AR is defined herein as having 21 polyglutamine repeats, 24 polyglycine repeats and a total length of 919 amino acids as described by Lubahn et al. [67] and is shown in Figure 1.2.

1.2.2 The androgen receptor ligand-binding domain

The AR-LBD (a.a 671-919) displays variable sequence homology (~22-54%) between the other human nuclear receptors. Despite this, the 3D structures of the steroid receptor LBDs are strikingly similar implying a common mechanism of action [68,69]. The AR-LBD contains
11 \(\alpha\)-helices (numbered H1-H12 excluding H2) and 2 antiparallel \(\beta\)-sheets arranged into a prototypical "helical sandwich". Agonist binding induces a conformational change where H12 is folded over top of the pocket, trapping and stabilizing the ligand [68–70]. This change in structure exposes the hydrophobic activation function 2 (AF2) subdomain which, for most nuclear receptors, serves as a docking site for coactivators expressing LxxLL motifs such as SRC1, TIF2 and AIB1 [71–75].

The AR however, is unique in that the vast majority of transcriptional activity is mediated through protein-protein interactions which occur in an alternative region in the N-terminal domain [73,74,76–78]. While the AR-LBD indeed contains an AF2 region which is exposed following ligand binding and activation of the receptor, it is much more important in mediating homodimerization between two AR molecules. Coactivators containing LxxLL motifs have been shown to only weakly bind AR AF2 [73,74,78], and selectively mutating these LxxLL motifs had little effect on their ability to increase AR transactivation [73,79]. Instead, the AR AF2 preferentially binds a \(^{23}\)FQNLF\(^{27}\) core sequence found in a second AR molecule (located in the NTD), which orientates the dimer in a "head-to-tail" manner (Figure 1.3, refs. 75–78,80). This N/C interaction stabilizes ligand-binding and slows the rate of dissociation, allowing for sustained AR transcriptional activity [75–78,81].

The natural ligands of the AR are the male sex hormone testosterone and its metabolite 5\(\alpha\)-dihydrotestosterone (DHT). 90-95\% of testosterone is secreted by the Leydig cells of the testes in a pulsatile fashion, with the remainder synthesized by the adrenal glands [82]. In the general circulation, testosterone is generally sequestered by sex-hormone binding globulin (\(~40\%)\) or albumin (\(~60\%)\) with \(~2\%) representing free unbound hormone [64,65]. Prostate epithelial cells readily take up free testosterone where it is metabolized by 5\(\alpha\)-reductase into its
most potent form, DHT [64,65,83,84]. DHT binds the AR with a higher affinity than testosterone (and has a slower dissociation rate), and overall is ~2-10 fold more potent than testosterone [65]. Ligand binding to the AR induces conformational changes and phosphorylation of the receptor [85–87] mediating its dimerization and activation.

1.2.3 The androgen receptor hinge region and DNA-binding domain

The hinge region (a.a 623-670) is a short amino acid sequence linking the LBD with the DBD. Site directed mutagenesis studies have shown that residues spanning 628-657 are essential for hormone mediated nuclear translocation of the AR [88–91]. The nuclear localization signal (NLS) has been further mapped to residues 617-633 and spans the C-terminal end of the DBD and N-terminal end of the hinge region [89,90]. The proposed mechanism of nuclear import/export of the AR is as follows: In the absence of agonist, the AR is localized in the cytoplasm bound to a complex of chaperone proteins (specifically HSP70, HSP90, p23, and immunophilins) which prime the AR for ligand binding [64,92]. Hormone binding to the LBD induces a conformational change exposing both the AF2 region and the NLS within the hinge region. This allows for release from chaperone molecules, as well as recognition of the NLS by importin-a, facilitating the active transport of the AR into the nucleus [64,89,93]. Once inside the nucleus two AR molecules homodimerize and form a complex with DNA, by recognizing specific consensus sequences termed androgen response elements (AREs) within the promoter or enhancer regions of target genes. Coactivators and transcriptional machinery are recruited to mediate the transcription of target genes. Finally, a nuclear export signal (NES) has also been described which is located in the LBD in close proximity to the ligand-binding pocket [94]. This
suggests that ligand binding sequesters this functional domain and upon ligand disassociation, the NES is exposed providing a mechanism of terminating AR signalling [93,94].

The DBD (a.a 559-622) shows the most conservation across species (and also with other human steroid receptors) with 100% sequence homology seen between rat and human AR-DBD, and 56%-79% homology between human nuclear steroid receptors [67,95]. Class I nuclear steroid receptors (AR, GR, MR and PR) all recognize specific inverted repeats organized as two palindromic, hexameric half-sites which are separated by a short three nucleotide spacer (5'-AGAACAnnnTGTTCT-3') [95–102]. This DNA sequence is also referred to as a three-nucleotide spaced inverted repeat (IR3) and is recognized by all Class I steroid receptors [96,97,100,103]. These hormone response elements are generally located within promoter or enhancer regions of target genes.

Like the other Class I nuclear receptors, the AR-DBD contains three α-helices which form a hydrophobic core [103]. Additionally, the AR-DBD can be thought of as being made up of two subdomains; each containing a single highly conserved zinc finger which facilitate AR binding in the major groove of DNA [65,95,98]. These are created by two sets of four cysteine residues which each coordinate a single Zn$^{2+}$ ion. The amino terminal zinc finger is presumed to assist in sequence recognition and guides the receptor to consensus response elements. Recognition of the IR3 response elements is mediated by a 5 amino acid sequence (GSCKV) termed the "P-box" contained within this first zinc finger [96,97,100,103]. The function of the second zinc finger is to stabilize the AR/DNA complex by forming intramolecular hydrophobic interactions with the first zinc finger, as well as intermolecular interactions between the AR/AR homodimer. This DNA dependent homodimerization of two AR molecules occurs through
cooperative binding, utilizing a region termed the "D-box" located in the second zinc finger of the DBD [96,98,100,101,103].

With such a high degree of homology between the DBDs of related steroid receptors - both in terms of sequence conservation and DNA consensus site recognition - a major focus of interest is how cells containing multiple species of nuclear receptor could regulate target genes in a species-specific manner. Localized or tissue specific expression of steroid receptors, steroid metabolism, and chromatin structure can only partially account for differential gene regulation by individual nuclear receptors. Several groups have therefore explored nuclear receptor DNA sequence specificity in more depth. The existence of AR specific response elements (androgen response elements/AREs) have been discovered and are generally not recognized by other steroid receptors [95,97,98,103]. A third functional region in the DBD called the C-terminal extension (CTE) or T-box, allows for recognition of AREs as compared to general IR3 binding sites, and was first described in the AR-specific rat probasin promoter [98,102]. Interestingly, swapping specific regions of the DBD between the AR and GR revealed that the CTE/T-box lies within the second zinc finger and part of the hinge region, and is independent of the DNA recognition function of the N-terminal zinc finger [96,98]. Finally, it is highly likely that protein-protein interactions between the AR-NTD and coregulatory molecules also aid in AR specific gene regulation; both in stabilizing the AR/DNA complex, but also indirectly by inhibiting other nuclear receptors from competing for IR3 binding sites.

1.2.4 The androgen receptor N-terminal domain

The NTD encompasses the majority of the AR protein (a.a 1-558), and it is this region which is mainly responsible for interacting with coregulatory proteins and transcriptional
machinery - allowing the AR to function as a master regulator of gene transcription [79,91,104,105]. Unlike the other AR functional domains, the NTD is largely disordered and shares little sequence homology with the other nuclear steroid receptors [65,91,95,104,106,107]. Due to the lack of inherent organization in the NTD, the 3D crystal structure has yet to be solved.

There exist two separate polymorphic regions within the NTD comprised of polyglutamine (CAG) and polyglycine (GGC) repeats. The polyglutamine (poly-Q) tract begins at codon 58, and extends for an average of 21 amino acids ± 2 repeats [95]. An increase in the number of repeats has been shown to be associated with greater NTD helical structure and a concurrent decrease in AR transactivation activity [64]. Kennedy's disease occurs in patients with 40-62 poly-Q repeats in this region and is characterized by spinal and bulbar muscular atrophy following neuronal cell apoptosis due to insufficient AR signalling [95,97,108]. Conversely, a decrease in the poly-Q tract is associated with increased AR signalling potential and may increase the risk for developing prostate cancer [64,95,97,109].

All nuclear steroid receptors contain an activation function 1 (AF1) domain within the NTD [106]. However as described earlier, the AR appears to be unique in that it relies upon this subdomain to a much greater extent than the other related steroid receptors which primarily utilize the AF2 [65,73,110]. This was shown by the fact that an AR mutant lacking the LBD retained transcriptional activity which approached that of wild-type AR activated with ligand [91]. Additionally, gene reporter assays have shown that the AR AF1 fragment (a.a 142-485) fused to a heterologous DNA binding domain retains ~65% of AR-NTD transcriptional activity [79]. Deletion mapping of the NTD has not only confirmed the importance of AF1, but lead to the discovery of two overlapping transcriptional activation units (TAU-1 and TAU-5) which lie within this subdomain. These are responsible for ligand-dependent, and ligand-independent
activation of the AR respectively, and both TAU-1 (a.a 101-370) and TAU-5 (a.a. 360-485) seem to have a specific core sequence which is integral to their function [104]. For TAU-1 this sequence is \(^{178}\text{LKDIL}^{182}\), whereas for TAU-5 it is \(^{435}\text{WHTLF}^{439}\) [64,66,75–77,95]. The \(^{435}\text{WHTLF}^{439}\) sequence also appears to mediate N/C homodimerization although in a region outside of the AF2 subdomain in the LBD [75].

The NTD binds a surprising number of structurally diverse proteins, and ~200 putative binding partners have been described [111]. These include coactivators (CBP/p300, SRC1, TIF2, AIB1, MAGE-11), corepressors (SMRT and SMAD3), transcriptional machinery (TFIIF, TFIIH and P-TEFb complexes) and many others [70,73,76,77,79,97,105,107,111–113]. It is thought that the ability of the NTD to interact with so many binding partners stems from the large regions of intrinsic disorder found within the NTD. The AF-1 region has been experimentally shown to adopt localized regions of ordered helical structure in response to protein-protein interactions [64,97,107,112,113]. It is therefore proposed that this domain exists with limited secondary and little to no tertiary structure (collapsed disorder), but is rather primed for forming complexes with numerous coregulatory proteins [64,107,113]. The intrinsic plasticity of the NTD is what allows the AR to adopt distinct conformations and binding patterns based upon specific cellular and environmental contexts, endowing the AR with exquisite control over target gene regulation [64,112–114].

1.2.5 Function of the androgen receptor

The function of the AR on a cellular level is to mediate strict control over the expression of genes (Figure 1.4). Approximately 400 genes have been suggested to be influenced either positively or negatively by the AR [114–117], and microarray data generated from this study
supports this observation. Unsurprisingly, the function of downstream AR targets varies significantly and their expression is determined by a host of different factors not least of which include, stage of development, cellular type and context (such as coactivator expression, cell cycle, chromatin structure, etc.). Accordingly, the AR regulates a myriad of cellular responses ranging from mitogenic and proliferative signals, lipid metabolism, cellular migration and induction of cellular senescence and differentiation [115–118]. The vast number of diverse signalling pathways under the control of the AR is reflected in the wide range of tissues which express AR protein, and underscores its importance as a master regulator of gene expression. Varying levels of AR protein has been found by immunohistochemistry in cardiac valves, brain (specifically the hypothalamus), skeletal muscle, spinal cord, skin, thymus and other organs [119–124].

However, the role of the androgen receptor on a physiological level is best characterized in its ability to initiate the male phenotype during embryonic development, and promote secondary sexual characteristics at puberty [11,13,110,116,119-121,124]. Unsurprisingly, the tissues which are most sensitive to androgens and have the highest expression of the AR tend to be male genital tissue (specifically the prostate, seminal vesicles and epididymus) [119,121]. Nowhere is the profound role that the AR plays in male sexual development more obvious, than in patients with androgen insensitivity syndrome (AIS). AIS is characterized by the inability of the AR to become activated in the presence of androgen due to silencing mutations in key regions of the receptor, AR gene deletion, or defects in AR splicing. These patients are genotypically male (46, XY), with testes (usually undescended) and express high levels of circulating testosterone. Despite this, patients are also phenotypically female with external female genitalia and body type, although internal organization of female reproductive tissue is
generally lacking [116,125–127]. The role that the AR plays in AIS was inferred as early as 1950 when it was discovered that despite the addition of exogenous testosterone, patients with AIS failed to develop male characteristics [125,127]. Indeed AIS is highly heterogeneous, and the severity of the disorder is now understood to be directly correlated with the relative ability of the AR to partially respond to androgen stimulation [68,80,125,126].

It has long been recognized that the prostate gland relies heavily upon the androgen stimulation, and as such is a good model for studying AR signalling pathways. Numerous studies have shown that AR mediated transcription is required for the normal development and maturation of the gland [11,13,15,128]. For example, castration of sexually mature animals causes a significant increase in cells undergoing apoptosis in the prostate, and leads to the involution and atrophy of the gland. These changes are reversible, as adding exogenous testosterone is sufficient to induce proliferation and growth of the prostate in dogs which have been castrated [15]. Additionally, female mouse embryos exposed to exogenous androgens develop prostates [11,128].

Since discovering the importance that the AR plays in promoting the growth and development of the normal prostate, intense study has also sought to describe its role in driving proliferation in prostate cancer. The remainder of this dissertation will focus on aberrant AR signalling and its effect upon prostate cancer initiation and progression, as well as the targeted treatment modalities which aim to antagonize the AR signalling axis.

1.2.6 The role of the androgen receptor in prostate cancer

The AR was first shown to be directly implicated in maintaining prostate cancer in 1941 when Charles Huggins and Clarence Hodges demonstrated that either castration or
administration of estrogen were effective in treating metastatic disease in patients, while exogenous testosterone injection increased tumour burden [129]. To this day, chemical or surgical castration as a means to starve the AR of agonist remains the gold-standard as a frontline therapy; both for patients who present with metastatic disease, (or in some cases intermediate to high-risk localized disease), and those who fail primary therapy [24,26,27,50]. This systemic treatment regimen is termed androgen deprivation therapy (ADT) and though considered palliative, provides immediate benefit for the vast majority of patients.

More recent reports have since confirmed the role of the AR in driving prostate cancer growth, and the molecular mechanisms which mediate the enhanced signalling potential of the AR are well documented and described. AR gene amplification, gain-of-function mutations, constitutively active AR splice variants, increased coactivator expression and decreased length of NTD CAG repeats have all been shown to be associated with an increased relative risk of developing prostate cancer, disease progression, increased tumour burden, and/or resistance to AR-targeted therapy in the clinical setting [109,130–136]. Nearly all patients receiving ADT display a rapid - albeit temporary - reduction in tumour burden [129,131,137]. However, xenograft studies have shown sustained androgen depletion promotes AR overexpression and promiscuity, sensitizing tumours to castrate levels of agonist [138]. Furthermore, although patients eventually relapse following ADT, their tumours often continue to express elevated levels of AR [139], and many patients do receive additional benefit from sequential AR-targeted therapies [140,141]. These studies confirm that the AR has oncogenic potential and imply that treatment failure is often due to insufficient silencing of AR signalling, rather than the tumour bypassing this pathway altogether. Thus the AR has been definitively established as a bona fide drug target - both in the context of early, and in late stage prostate cancer.
1.3 CASTRATION-RESISTANT PROSTATE CANCER

1.3.1 The hypothalamic-pituitary-gonadal signalling axis

In the male, the prostate is a downstream target of a complex hormonal signalling pathway, which functions to minutely regulate a host reproductive and sexual functions. Termed the hypothalamic-pituitary-gonadal (HPG) axis, this pathway is directly responsible for the initiation of puberty, spermatogenesis, steroidogenesis, development and maturation of male sexual organs and many other important physiological functions [122]. Briefly, and with respect to its relationship with the prostate, the signalling pathway is described below, and illustrated in Figure 1.5.

The hypothalamus initiates the signalling cascade by releasing gonadotropin-releasing hormone (GnRH) in a pulsatile fashion, stimulating specialized receptors upon the anterior pituitary gland. The pituitary gland secretes luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the general circulation which target Leydig or Sertoli cells respectively, both found in the testis. LH mediates the production and secretion of testosterone while FSH stimulates spermatogenesis and also initiates silencing of the signalling pathway by inducing the production of inhibin, and sex hormone-binding globulin protein. Both testosterone and inhibin participate in a negative feedback loop, primarily by targeting the hypothalamic neurons (preventing GnRH secretion), and the pituitary (blocking FSH/LH secretion) respectively [122]. As described in section 1.2.2, free testicular testosterone is taken up by prostate epithelial cells and metabolized to DHT; stimulating growth of the prostate via direct action upon the AR [64,65,83,84].
1.3.2 Androgen deprivation therapy

Bilateral orchiectomy or surgical castration, is an effective means of eliminating testicular androgens and reducing serum PSA to undetectable levels (<0.5 ng/mL). However this procedure is permanent and the physical and psychological effects are often detrimental to the patient. An alternative practice, termed chemical or medical castration, uses LHRH agonists (GnRH analogs) which exploit the inherent negative feedback loop of the HPG axis. In this case, chronic exposure to exogenous GnRH analogs reduces GnRH receptors on the pituitary. This eventually decreases LH (and downstream testosterone) secretion by desensitizing the signalling axis to endogenous stimuli [124,142,143]. A side effect of this method of ADT is that the signalling axis is initially stimulated by the treatment, and can result in as much as a 10-fold increase in circulating LH and a subsequent surge in testosterone secretion [142,143]. Known as a "testosterone flare" this effect may aggravate symptoms and cause considerable pain, especially if the disease has disseminated to the bone. To mitigate the adverse side effects of GnRH analogs, drugs which specifically target the AR-LBD (antiandrogens) are administered in an effort to competitively prevent androgen binding [24,124,142–144]. While most patients do eventually reach castrate levels of testosterone (≤50 ng/dL), it has been reported that as many as 12% fail to meet this threshold [124,144].

GnRH agonists currently represent the standard for chemical castration and are preferred over orchiectomy, however GnRH antagonists are also being investigated as possible alternatives [144]. These drugs act by preventing GnRH binding to receptors on the pituitary and therefore have an immediate effect on reducing LH and testosterone levels (<72 hours vs. 7-21 days). They do not induce the flare response seen with GnRH agonists, and therefore do not require concurrent treatment with antiandrogens [142–144]. However, no long-term survival benefit
associated with GnRH antagonists versus agonists has yet been reported [144]. Additionally serious allergic reactions can result from repeated intramuscular injections [142].

It has been known for quite some time that while the testes produce the vast majority of testosterone (~95%), the adrenal glands synthesize androgens as well. These are namely the testosterone precursor molecules androsterone (AS), dihydroepiandrosterone (DHEA) and dihydroepiandrosterone sulphate (DHEA-S) which are converted to testosterone and DHT in peripheral tissues and prostate epithelial cells [145–148]. To prevent adrenal androgens from being able to activate the AR, antiandrogens or the CYP450 inhibitor ketoconazole can be coadministered in combination with ADT (either surgical or chemical) to completely prevent AR signalling. Termed maximal androgen blockade (MAB), it is associated with a modest decrease (8%) in the risk of mortality compared to castration alone [146].

For patients with locally advanced disease (extracapsular or invasion to local lymph nodes) survival benefits (both at 5- and 10-yrs) were seen in several phase III randomized trials comparing ADT plus radiation therapy compared to radiation therapy alone [143,149]. Additionally a role for adjuvant ADT following radical prostatectomy has been clearly defined especially in the setting of local lymph node infiltration [149]. However, significant adverse effects are associated with ADT including hot flashes, loss of libido/erectile dysfunction, bone and muscular degeneration, cardiovascular disease and increased risk of diabetes [124,143,149,150]. Therefore the use of intermittent androgen deprivation (IAD) therapy has been examined as a way to mitigate adverse side effects while maintaining anti-tumour efficacy. Several studies comparing IAD with continuous androgen deprivation (CAD) have indicated a similar efficacy between them with greater tolerability associated with IAD, although this
appears to be restricted to patients with a lower disease burden and who do not have metastases [50,124,151].

Nonetheless, CAD remains primarily prescribed as a first line therapy for patients with symptomatic metastatic lesions [143,148,149,152]. Both surgical and chemical ADT provide rapid improvements in quality of life and a decrease in tumour burden for the vast majority (~90%) of patients bearing metastases [149]. Therefore ADT remains the gold standard for treating patients with advanced disease [148,149]. Regardless of the method of ADT (surgical or chemical), this treatment is considered palliative in the metastatic setting, as the cancer will inevitably progress within ~20 months despite castrate levels of androgens [124,149,153]. At this stage the disease is termed castration-resistant prostate cancer (CRPC) and is uniformly lethal with an average life expectancy of only 18 months once diagnosed [149,154].

1.3.3 Molecular mechanisms leading to castration-resistant prostate cancer

Most CRPC is defined by the ability of the tumour to continue to rely upon AR transcriptional activity despite castrate levels of androgens. This is often first evidenced by a concurrent rise in serum PSA levels at the onset of new or worsening symptoms following ADT [155–157]. The implication of the AR in driving progression to a castration-resistant phenotype, is also demonstrated by the fact that AR gain-of-function mutations, AR expression levels, and intracellular androgens are highly enriched in CRPC metastases [134–137,158]. From these discoveries, it is now widely understood that most CRPC is dependent upon AR signalling, and recurrence following ADT is due to insufficient antagonization of the pathway. The molecular evolution of CRPC depends upon subtle alterations of the AR signalling pathway which allow it
to maintain signalling potential in the absence of agonist, and these will be expanded upon briefly (Figure 1.6).

Increased AR protein expression has been observed in xenograft models, and has been shown to sensitize tumours to minute levels of circulating androgens [138]. Amplifications of the AR gene occur in as many as 30% of metastases in CRPC patients following ADT [134,136,159]. Notably this genomic alteration was absent in matched untreated primary tumours within the same patients, strongly indicating that this is a mechanism of acquired resistance to ADT rather than an early event leading to disease initiation [136,159]. Amplification of the AR allows cells to be hypersensitive to low levels of androgen, driving disease progression and likely promotes metastasis as well [148].

Intratumoral synthesis of androgens from adrenal precursors also provides a means for sustained AR transcriptional activity. Several reports have indicated that intratumoral androgen levels (specifically testosterone and DHT) are elevated in CRPC and are well within the threshold to sufficiently activate the AR. In the case of testosterone, intratumoral concentrations were comparable to those found in untreated tumours [158,160]. Accordingly microarray data on metastatic tumour tissue isolated from bone biopsies revealed that gene transcripts involved in the synthesis of testosterone and DHT from adrenal androgen precursors were upregulated compared to primary tumours (namely ARK1C3, SRD1A5, and HSD3B2) [145]. This work has been confirmed by others analyzing mRNA transcripts in postmortem samples comparing ADT treated metastases and untreated primary tumours [158,161]. Furthermore enzymes which metabolize and deactivate DHT have also been shown to be downregulated in CRPC [161]. Finally Locke et al. observed de novo synthesis of DHT from cholesterol occurring entirely within the tumour itself in mouse xenograft experiments [162].
As mentioned previously, the AR is able to directly interact with ~200 different proteins. The relative expression levels of some AR coactivators and corepressors have been shown to be altered in response to ADT and correlate with advanced disease and prognosis [163–165]. It has been suggested that overexpression of coactivators such as TIF2, SRC1, AIB1 and CBP may sensitize the AR to low (castrate) levels of androgens. This is supported by the fact that siRNA knockdown of coactivators decreased androgen dependent cellular proliferation of cancerous, but not untransformed cells [164]. Additionally while TIF2 and SRC1 levels were shown to fall following castration in a xenograft model, expression eventually began to increase closely paralleling tumour recurrence [163]. Similarly, two of the best characterized AR corepressors (NCoR1 and SMRT) have been shown to compete with the p160 coactivator family for binding sites on the AR [165]. Loss of corepressor expression represents another mechanism that prostate cancer cells may exploit to resist androgen deprivation and antagonization by antiandrogens.

Molecules such as interleukins (IL-6), peptide growth factors (EGF, IGF-1 and KGF), and the diterpine forskolin (FSK, which is a direct adenylyl cyclase agonist) may induce ligand-independent activation of the AR through downstream phosphorylation of S650 of the AR and/or activation of its coactivators [87,166–168]. This has been shown by the ability of these molecules to induce transcription of reporter constructs using AR specific promoters, and also to promote proliferation of prostate cancer cells in the absence of androgens [87,166–169]. Phosphorylation of the AR-NTD at Y267 and Y534 by Src kinase in response to growth factor stimulation [170], IL-6 mediated activation of SRC-1 cofactor [166] or STAT3 [167] has also been demonstrated. Additionally, STAT3 has been shown to physically associate with AR-NTD and may act as an AR transcriptional coactivator in the absence of androgens [167]. Finally,
both androgen and EGF stimulation of prostate cancer cells has been demonstrated to induce the expression of IL-6 and STAT3, suggesting an autocrine or paracrine positive feedback loop may be utilized by prostate cancers in an androgen depleted environment [171].

Gain-of-function mutations creating a promiscuous receptor represent another means of escape by the tumour from castrate levels androgens. While the frequency of AR mutations is quite low in primary tumours, clinical samples isolated from patients following ADT have revealed a relatively high proportion of mutated AR [138,167–170]. Unsurprisingly the majority of mutations associated with prostate cancer are localized to the AR-LBD (i.e., L701H, V715M, W741L/W741C, F876L and T877A) and decrease the specificity for ligand [176]. These mutants are often able to bind other steroid hormones (estrogen, progesterone and glucocorticoids) and can mediate transcriptional activation in the absence of native ligand [172,174,175,177]. Long term treatment with antiandrogens (for example when administering MAB) can also act as a selective pressure for the generation of AR mutants able to use these non-steroidal drugs themselves as agonists [137,174,175,177–179]. This is best evidenced by the existence of the "antiandrogen withdrawal syndrome." The effect is seen in a subset of patients whereby tumours paradoxically regress following the temporary removal of antiandrogens from their treatment regimen; highlighting the reliance of the tumour upon AR gain-of-function mutations [137,147,150,172,178,179].

Lastly, structural alteration of the AR embodies yet another possible mechanism of resistance to ADT therapy. Approximately 25 unique AR species have been found in human tissue and cell lines, and are thought to be derived from posttranscriptional splicing of cryptic exons within the AR pre-mRNA, or genomic alterations resulting in premature stop codons
and/or alternative reading frames [180–183]. While some of these are non-functional, others are translated and have transcriptional activity [180,184–187].

Most notable are the species in which the LBD is lost leaving only the DBD and NTD, and of these, AR variants AR-V7 and ARv567es remain the most clinically relevant [180,182,184–186,188–190]. These represent constitutively active receptors which are predominantly localized in the nucleus irrespective of androgen stimulation, are not responsive to antagonizing agents, and can promote androgen independent growth [180,184–186,190]. Whole genome microarray data has revealed these variants were capable of initiating gene transcription of a subset of classic full-length AR (ARFL) targets in the absence of androgen. Furthermore, both variants were able to regulate an additional cohort of genes which were not associated with androgen induced AR signalling, and are related to cell cycle, steroid synthesis, sterol metabolism, proliferative and anti-apoptotic functions [184–186]. Interestingly it appears that these variants are also able to physically bind ARFL and promote its nuclear translocation and transactivation in the absence of androgens [186,191]. It has also been shown that androgen activated ARFL negatively regulates the expression of AR-V7 in variant expressing cell lines. Conversely, an increase in variant expression is seen under androgen depleted conditions, or when the ARFL LBD is antagonized by antiandrogen treatment [182,184]. These results strongly suggest AR variants play a protective role in maintaining prostate cancer, and an inherent mechanism of regulation exists to promote their expression when prostate cells are exposed to a low androgen environment.

Indeed ectopic AR-V7 or ARv567es expression allows androgen dependent prostate cancer cell lines to grow under androgen depleted conditions, and this has been confirmed in vivo using castrate hosts [186,190]. Targeted shRNA with specificity against AR-V7 abrogated castration-
resistant growth both *in vitro* and *in vivo* without affecting androgen dependent proliferation [190]. It has also been widely reported that both AR-V7 and AR\(^{V567E}\) are often enriched in metastases taken from patients who have relapsed following ADT compared to hormone therapy-naive tumours [180,182,186,189]. Accordingly the expression of AR-variants are correlated with poor prognosis, decreased survival [189] and, as will be discussed in the following section, resistance to current AR-targeted therapies.

The progression of most advanced prostate cancer to CRPC continues to be reliant upon functional AR signalling despite castrate levels of androgens. Importantly, while the AR-LBD may be mutated or even lost altogether as a means to adapt to low levels of androgens, structural alteration of the AR-NTD remains largely unchanged. Furthermore current targeted therapies for CRPC all directly or indirectly aim to prevent activation of the AR by inhibiting androgen binding. Therefore many of the mechanisms described above which promote CRPC development, are also relevant mechanisms of resistance to these drugs as well. As a result, treatment with AR targeted therapies often act as a selective pressure to promote further alterations allowing ligand-independent activation of the AR. Sadly there is no cure for CRPC, and all current therapies will inevitably fail due development of primary or acquired resistance. Despite this significant progress has been made, and the last eight years alone have seen marked improvements in treatment options available for patients with advanced prostate cancer. The following section will address the current therapeutic landscape as well as the mechanisms of resistance which plague these treatments.
1.4 CURRENT THERAPIES FOR THE TREATMENT OF CASTRATION-RESISTANT PROSTATE CANCER

1.4.1 Taxane, chemo-, immuno- and radiotherapies

Once patients have progressed on ADT and develop metastatic CRPC the prognosis is poor with an average life expectancy of 18 months following diagnosis [149,154]. The last decade has ushered in several novel therapeutics which add to the arsenal of treatment options for patients. Due to their success in clinical trials, some of these (namely docetaxel and abiraterone) have also been investigated in the context of combination therapies in conjunction with ADT as a means to delay CRPC onset. However the following sections will primarily focus on treatment options for CRPC patients who have already progressed on ADT. The clinical trials which demonstrated their utility are briefly described below, and are summarized in Table 1.1.

Taxane-based chemotherapies are the only class of chemotherapeutic to demonstrate a survival advantage in the context of CRPC [192–195], and are now often used as a first line treatment following a CRPC diagnosis [196]. Taxanes work by stabilizing microtubules, inhibiting their disassembly. This prevents chromosomal separation, inducing cell cycle arrest and initiation of apoptosis. The anti-apoptotic protein BCL-2 is inactivated by phosphorylation, while p53 is activated following taxane treatment, which sensitize cells to apoptotic signals [194]. Additionally, AR translocation to the nucleus is thought to be mediated by microtubules. Microtubule stabilization following taxane therapy may sequester the AR to the cytoplasm and provide an additive effect to ADT [195]. Currently, docetaxel is the standard first line chemotherapeutic and has been shown to give both prolonged overall survival (OS), and positive PSA response as compared to mitoxantrone in two large scale randomized clinical trials. SWOG 99-16 found a 1.9 month survival benefit (OS, 17.5 months vs. 15.6 months; hazard ratio for
death [HR] = 0.80; 95% CI 0.67-0.97; p=0.02) while TAX 327 reported an increase of 2.4 months (OS 18.9 months vs. 16.5 months; HR = 0.76; 95% CI 0.62-0.94; p=0.009) [195,196]. Based on these trials, 75 mg/m² docetaxel every 3 weeks plus prednisone at 5 mg/day was approved for use after progression on ADT in 2004.

Resistance to docetaxel is thought to occur via mutations in microtubule binding sites or upregulation of β-tubulin isoforms which restrict docetaxel binding [197], deregulation of anti-apoptotic proteins [198], and cellular efflux of the drug by the overexpression of P-glycoprotein drug transporter proteins [199]. Following progression on docetaxel, cabazitaxel (a second line taxane chemotherapy) may be given as of 2010. The results of a randomized phase III trial (TROPIC) demonstrated an overall survival benefit of 2.4 months (OS 15.1 months vs. 12.7 months; HR = 0.70, 95% CI 0.59-0.83; p<0.0001) and favorable progression free survival (PFS) when administered at a dose of 25 mg/m² every 3 weeks compared to a docetaxel regime [192,196].

For patients who have metastatic CRPC but are asymptomatic or only have very mild symptoms, Sipuleucel-T immunotherapy may be administered instead of docetaxel [196]. This form of therapy is an autologous cancer immunotherapy whereby the patients dendritic cells are extracted and primed with prostatic acid phosphatase (a prostate specific antigen) and expanded. The activated immune cells are then reinfused in the patient. Based upon the results of the IMPACT clinical trial which showed a median survival benefit of 4.1 months compared to placebo (OS 25.8 months vs. 21.7 months; HR = 0.78; 95% CI 0.61-0.98; p=0.03; ref. 202), Sipuleucel-T therapy was approved by the FDA for use in minimally, or asymptomatic CRPC patients in 2010.
Radium-223 represents a targeted therapy against bone metastases which are a common malignancy in CRPC. It is estimated that 90% of men with CRPC will eventually develop bone metastases which are a major detriment to the patients quality of life and are a significant cause of death [201]. Radium-223 is a calcium mimetic which causes it to be selectively taken up by bone tissue which is being rapidly turned over in metastases. It emits high energy $\alpha$-particle radiation causing DNA-damage induced apoptosis of nearby cells [201]. The ALSYMPCA clinical trial investigating radium-223 has shown positive effects in terms of decreased bone pain, PSA response and a 3.6 month survival benefit (OS 14.9 months vs. 11.3 months, HR = 0.70 95% CI 0.58-0.83; p<0.001) compared to standard of care in patients with symptomatic CRPC [201]. Based upon these favorable data radium-223 was approved for use in patients with symptomatic and metastatic disease to the bone before or after docetaxel in 2013 [196].

1.4.2 Steroidogenesis inhibitors

Intratumoral synthesis of androgens from adrenal androgen precursors as a mechanism of resistance to ADT has been previously described. In 2011 the first inhibitor of steroidogenesis (abiraterone acetate) was approved by the FDA for use in patients with CRPC following chemotherapy, and expanded in 2012 for use as a first line therapy [196]. Abiraterone acetate is a selective and non-reversible CYP17A1 inhibitor. CYP17A1 is an enzyme belonging to the cytochrome P450 superfamily, and has both 17α-hydroxylase and C17,20-lyase functionality. It is expressed in steroidogenic tissues and mediates synthesis of 17-hydroxypregnenolone from pregnenolone, and 17-hydroxyprogesterone from progesterone utilizing 17α-hydroxylase function. CYP17A1 further modifies these substrates into testosterone precursors DHEA and androstenedione respectively. Androstenedione is directly converted to testosterone by
HSD17B3 in peripheral tissue including prostate epithelial cells [173]. CYP17A1 and downstream steroidogenic enzymes have been shown to be overexpressed in CRPC with increased expression in metastases compared to primary tumours [173]. A recent study has also shown that a potent metabolite of abiraterone (Δ^4-abiraterone/D4A) may inhibit steroidogenesis enzymes (3βHSD and SRD5A) in addition to CYP17A1[202]. 3βHSD and SRD5A are both necessary for the backdoor conversion of DHEA into DHT. Therefore abiraterone acetate is predicted to potently prevent adrenal and tumoural synthesis of androgens, and acts as an indirect inhibitor of the AR-LBD. As CYP17A1 is also involved in cortisol biosynthesis, coadministration of prednisone is required to offset the adverse effects caused by compensatory mineralocorticoid accumulation [203].

In 2011 data from a large scale randomized clinical trial (COU-AA-301) was released demonstrating treatment was associated with a significant increase in overall survival of 3.9 months (OS 14.8 months vs. 10.9 months; HR = 0.65; 95% CI 0.54-0.77; p<0.001) in patients who had received previous docetaxel [204]. Additional benefits associated with treatment (1000 mg/day abiraterone acetate plus 5 mg prednisone twice a day) versus prednisone alone were also seen with respect to time to PSA progression, PFS and PSA response rate [204]. This finding was significant as this study clearly demonstrated that even in the context of previous chemotherapy, hormonal therapies could still be effective - further implicating the AR as a relevant target in CRPC. Abiraterone acetate plus prednisone was approved in the post chemotherapy setting by the FDA that same year.

A follow up study (COU-AA-302) investigated the efficacy of abiraterone acetate plus prednisone in chemotherapy naive patients. When the final data was released, a significant improvement in both primary endpoints (OS and radiological-PFS/rPFS) was seen (OS, 34.7
months vs. 30.3 months; HR = 0.81; 95% CI 0.70-0.93; p=0.0033). An advantage was also observed with respect to delayed onset of symptoms and opiate use [140], and the use of abiraterone was expanded to include its use as a first line therapy in 2012. The role for abiraterone acetate is now clearly defined for patients who have metastatic CRPC both with and without prior chemotherapy [140,196,205].

1.4.3 Antiandrogens

Antiandrogens are compounds which competitively inhibit androgen ligand activation of the AR by targeting the AR-LBD. Non-steroidal antiandrogens were first described in the late 1960s and early 1970s as alternatives to steroidal antiandrogens which had detrimental side effects (namely loss of libido and impotence) due to their ability to agonize other related hormone receptors [206]. While not used clinically until the 1980's, nonsteroidal antiandrogens quickly gained favor due to their specificity with respect to AR inhibition without activating or antagonizing the other receptors, and are considered pure antiandrogens. Flutamide was the first pure antiandrogen discovered, and its derivatives nilalutamide and bicalutamide also represent first generation non-steroidal antiandrogens [206]. The first generation antiandrogens are generally used in combination with ADT to achieve maximal androgen blockade, or as a monotherapy instead of ADT to preserve sexual function in non-metastatic patients [27,196].

Enzalutamide was one of the first second generation antiandrogens to be developed, and the first to be approved for clinical use. Preclinical evidence demonstrated enzalutamide had a 5-8 fold greater affinity for the AR-LBD than bicalutamide, and while bicalutamide induced AR nuclear translocation, enzalutamide was able to sequester the AR to the cytoplasm [207]. Furthermore, enzalutamide was shown to be effective using in vitro and in vivo models of CRPC.
displaying AR overexpression and AR gain-of-function mutant W741C, both of which turn bicalutamide into an AR agonist [207]. Based on these results the phase III randomized trial AFFIRM was conducted in 2012 to examine whether enzalutamide monotherapy could be utilized in the post chemotherapy setting in CRPC patients.

At a dose of 160 mg/day, enzalutamide demonstrated a 4.8 month overall survival benefit (OS 18.4 months vs. 13.6 months; HR = 0.63; 95% CI 0.53-0.75; p<0.001) and significant responses were seen for all secondary end points (time to PSA progression and PSA response, rPFS, quality of life, and time to first skeletal event; ref 141). This led to the approval of enzalutamide as a second line therapeutic following docetaxel chemotherapy in CRPC patients in 2012. Two years later, following the success of the PREVAIL clinical trial, enzalutamide was approved by the FDA for indication as a first line therapy for men with CRPC as well [196]. This trial demonstrated statistically and clinically significant benefits for both primary endpoints (OS and rPFS) as well as all secondary endpoints. Both AFFIRM and PREVAIL were terminated early to allow all patients the opportunity to receive enzalutamide treatment due to highly encouraging results at interim analysis [141,208].

Apalutamide (previously ARN-509) represents the most recent addition of second generation antiandrogens to receive FDA approval for the treatment of CRPC. Preclinical studies were highly encouraging, with apalutamide displaying a greater efficacy profile than either enzalutamide or bicalutamide in a CRPC murine xenograft model overexpressing the AR [209]. Apalutamide prevented agonist induced AR N/C interaction, AR nuclear translocation, and DNA binding. Potent antiandrogenic effects of apalutamide were also observed in xenografts in intact male mice, as well as in androgen dependent reproductive organs (epididymus and prostate) in intact dogs. This finding indicates that apalutamide would be
effective in the context of castration-sensitive disease and represents an alternative to first
generation antiandrogens used in combination with ADT. In following, the phase III clinical
trial (SPARTAN) investigated the effect of apalutamide versus placebo treatment in combination
with continuous ADT, on metastasis-free survival or death in patients with high risk CRPC but
without metastases [210]. Treatment with apalutamide was associated with a significant
decrease (~70%) in the risk of death or metastases and the median metastases-free survival was 2
years longer than those on placebo (40.5 months vs. 16.2 months; HR = 0.28, 95% CI 0.23-0.35;
p<0.001). Apalutamide was approved for indication in patients with high risk CRPC (but no
evidence of metastases) in early 2018.

1.4.4 Resistance to AR-targeted therapy

The importance of continued AR signalling in driving and maintaining CRPC has long
been recognized and is best evidenced by the survival benefits seen in CRPC patients treated
with hormonal therapies - namely abiraterone acetate, enzalutamide and apalutamide. However
while disease progression and death are delayed with these treatments, they are not curative and
de novo or acquired resistance is inevitable [140,141,204,208]. Rising serum PSA levels often
correspond with disease progression, alluding to restored AR transcriptional activity despite
targeted treatment. Because most CRPC remains dependent upon AR signalling, and both
hormonal therapies and ADT directly or indirectly target the AR-LBD, many of the mechanisms
which lead to failure of ADT also mediate resistance to abiraterone and enzalutamide. As in the
progression from castration-sensitive to CRPC disease, treatment failure stems from incomplete
AR blockade and restoration of AR transcriptional signalling.
Intratumoral synthesis of androgens has been recognized as a potential mechanism exploited by tumours to maintain intracellular DHT levels following inhibition by CYP17A1 inhibitors such as abiraterone or ketoconazole [66,211–213]. Mice bearing xenografts which had developed acquired resistance to abiraterone demonstrated a compensatory increase in CYP17A1 expression restoring AR transcriptional activity [212,213]. This finding was further validated in clinical samples showing that tumour biopsies isolated from patients following treatment with a CYP17A1 inhibitor, demonstrated increased expression levels of CYP17A1 relative to treatment naive tumours [212]. These studies indicate that the tumour itself is capable of supplying itself with sufficient levels of androgens utilizing precursor molecules, and that it is not wholly reliant upon adrenal production of DHEA following surgical or chemical castration. Furthermore, treatment with CYP17A1 inhibitors may provide selective pressure for the increased expression of genes involved in steroidogenesis such as CYP17A1, CYP11A1 and AKR1C3 [212].

Modification of the AR in terms of gain-of-function mutations, constitutively active splice variants or AR amplification represent additional common resistance mechanisms to hormonal therapy. AR-LBD mutations have been reported to occur with a frequency approaching 10-20% in patient samples following treatment with antiandrogens [137,214,215], and increases to ~60% of patients if amplification of the AR is included [216,217]. This often confers an antagonist-agonist switch, or allows the AR to maintain signalling potential by overcoming competition for androgen binding respectively. The former is most clearly seen by the existence of a withdrawal effect whereby the cancer regresses following discontinuation of the antiandrogen in a subset of patients. Strikingly, this effect occurs more frequently in patients who are treated with antiandrogens for a prolonged period of time [179], and a withdrawal effect has been observed with respect to all antiandrogens used in the clinic - with AR-LBD mutations
specific to the antiandrogen used [137,174,178,179,218–220]. While sequential use of alternative antiandrogens is generally efficacious in the short term, modification of the ligand-binding pocket by the cancer is detrimental to all classes of antiandrogens. Collectively these observations strongly indicate that specific alteration of the AR-LBD may result from the selective pressure by antiandrogen treatment itself.

AR splice variants are becoming increasingly recognized as a clinically relevant means of overcoming inhibition by both antiandrogens and steroidogenesis inhibitors. As described previously, both AR-V7 and AR\(^{v567es}\) represent the two most clinically relevant variants, both of which lack the LBD, are ligand independent and constitutively active [180,184–186,190]. Therefore they allow the continued expression of an AR transcriptional program and drugs which bind the LBD (such as enzalutamide) or synthesis of androgen (abiraterone) would be ineffective in the context of tumours expressing these variants. Indeed numerous reports have demonstrated that splice variant expression plays a causative role in resistance to both antiandrogens and abiraterone using both \textit{in vitro} and xenograft models of CRPC [185,213,221,222]. AR-V7 specifically has been further clinically characterized in an effort to support its use as a biomarker to predict patient response to AR targeted agents, and therefore guide treatment decisions [130]. This prospective study captured circulating tumour cells (CTCs) in patients treated with either abiraterone or enzalutamide and determined AR-V7 status by qRT-PCR. The authors found that prior targeted therapy (i.e., enzalutamide in the abiraterone cohort and vice versa) was associated with positive AR-V7 status in CTCs. Importantly, no patients who were positive for AR-V7 had a PSA response (defined as \(\geq50\%\) decline from baseline, maintained for 4 weeks) following treatment with either abiraterone (0 of 6 men) or enzalutamide (0 of 12 men). Conversely, the majority of patients who were AR-V7 negative and
treated with either agent demonstrated favorable PSA responses (ENZ: 10 of 19 men, 53%; p=0.004; ABI: 17 of 31 men, 55%; p=0.004) [130]. Furthermore statistically significant negative associations between AR-V7 positivity and all secondary endpoints (PSA progression-free survival, clinical or radiographic progression-free survival and overall survival) were observed even after multivariable models were applied to correct for prior drug use and AR\textsuperscript{FL} mRNA levels [130]. Though only a small number of patients were followed, this study provides strong evidence for AR-V7 to be used as a biomarker due to its association with resistance to both abiraterone and enzalutamide.

Prostate cancer is by nature a heterogeneous disease and it is highly likely that multiple mechanisms of resistance may exist simultaneously within the same patient, and even within the same tumour. Nonetheless it is telling that numerous alterations found in prostate cancer converge upon the AR signalling axis. Furthermore, many of these are specifically associated with the LBD in terms of gain-of-function mutations as well as major structural alterations. Ligand independent activation of the AR has also been shown to occur via NTD phosphorylation mediated by alternative signalling pathways and/or aberrant expression and activity of coactivators [223]. An alternative strategy therefore involves targeting the AR-NTD using small molecule inhibitors. These drugs would be likely be effective in the context of the resistance mechanisms outlined above, which are all common to therapies which directly or indirectly target the LBD. As such, AR-NTD inhibitors may prove a powerful tool for the treatment of patients with CRPC. Our lab has identified the first AR-NTD antagonists which specifically bind the TAU-5 region within the AF-1, a microdomain essential for mediating AR interaction with coactivators and transcriptional activity (as described in section 1.2.4).
1.5 NEW HORIZONS FOR AR-TARGETED THERAPY: N-TERMINAL DOMAIN INHIBITORS

1.5.1 Targeting the Achilles' heel of the androgen receptor: Proof-of-principal

While an inhibitor of the NTD has obvious advantages over traditional antiandrogens in terms of overcoming the mechanisms of resistance which have been described, others exist as well. The NTD has the least sequence homology (<15%) of any structural domain between the human steroid receptors; therefore any inhibitor specifically targeting this region is not predicted to show cross reactivity between the other related nuclear receptors, especially GR and PR \[65,106,107\]. Within the NTD lies the AF-1 subdomain, a region containing TAU-1 and TAU-5 which are necessary for ligand-dependent and ligand-independent transcriptional activity respectfully. A small molecule inhibitor of the NTD would inhibit transactivation of the receptor both in the presence and absence of androgen, as well as in the context of many clinically relevant AR mutants or structural variants. Targeting a distinct region of the AR also opens the possibility of combining antiandrogen treatment with an AR-NTD antagonist, and may give a synergistic or additive response in patients with CRPC. It is now widely accepted that the vast majority of CRPC remains critically dependent upon AR transcriptional activity for mitogenic and survival signals. Deletion experiments have confirmed that the AR-NTD harbors nearly all transcriptional activity while the LBD is disposable. Therefore targeting the AR-NTD represents a novel strategy with significant potential for extending the therapeutic window for patients with CRPC (Figure 1.7).

To prove that targeting the NTD could prove effective, our lab previously demonstrated that a construct encoding the AR-NTD could be used as a decoy molecule to inhibit endogenous AR\textsuperscript{FL} signalling by competing for coregulatory proteins required for activation and
transcriptional activity. AR1-558 encodes the entire NTD but lacks the DBD and LBD and has no putative nuclear localization sequence [224]. Both stable and transient expression of AR1-558 was sufficient to prevent both androgen-independent transactivation by FSK or IL-6, and androgen activation in LNCaP cells which is an AR dependent human prostate cancer model [224]. Importantly, lentiviral delivery of AR1-558 to established LNCaP xenografts resulted in tumours which were 55% smaller than xenografts treated with empty vector, and significant differences in tumour volume were seen in as little as 5 days following initial treatment [224]. This study was the first to definitively show that a decoy molecule corresponding to the AR-NTD could block prostate cancer growth both in intact and castrate xenograft models. This was significant as it suggested that a role for AR-NTD inhibition exists in both early and advanced prostate cancer. Through this work the way was paved for development of the first small molecule inhibitors of the AR-NTD.

1.5.2 Challenges in targeting a disordered domain

As described previously, the AR-NTD exists in a collapsed disordered or molten-globular conformation with limited secondary structure, and lacking a discrete tertiary configuration. It is only following interaction with binding partners that isolated regions of the NTD adopt a relatively stable structure and assume a more helical conformation [106,113]. As the AR directly interacts with ~200 unique proteins [111] it is not surprising that unlike the rest of the AR, the crystallized structure of the NTD has not yet been solved. This represents a major hurdle in drug development as the strategy of rational drug design is often guided using 3D models of drug-target interactions, which in this case are unavailable. Therefore a high-throughput screening system is required to identify potential candidates, and each must be individually tested
empirically which is labor intensive. In addition, while many of the coactivators and transcriptional machinery which bind the AR have been mapped to the AF1 region in the AR-NTD, finding single compound(s) which can inhibit the majority of possible interactions will inevitably prove challenging. This is why until recently efforts to antagonize the AR were exclusively geared toward preventing androgen activation through the LBD; an interaction which has long been understood and structurally well defined. In 2010 as a result of a large scale drug screening program, the first AR-NTD antagonist EPI-001 was published [169].

1.5.3 EPI-001 has a novel mechanism of action

EPI-001 was first identified by screening a library of marine sponge extracts for their ability to inhibit both ligand-dependent and independent AR-NTD transactivation. EPI-001 is a derivative of bisphenol A diglycidic ether (BADGE) isolated from the marine sponge Geodia lindgreni and has a unique chemical structure compared to antiandrogens and androgens (Figure 1.8). EPI-001 demonstrated potent activity against a construct containing the Gal4DBD fused to the AR-NTD (AR1-558) stimulated with both FSK and IL-6. This reporter assay confirmed that EPI-001 specifically targeted the AR-NTD, as the antiandrogen bicalutamide had no effect [169]. Further evidence for an EPI-001/NTD interaction comes from analyzing steady-state fluorescence emission spectra when EPI-001 was coincubated with the AR-AF1 NTD fragment [169]. Increasing concentrations of synthetic androgen R1881 did not influence its ability to inhibit endogenous AR FL in reporter assays [225], nor could high concentrations of EPI-001 competitively inhibit binding of a fluoromone ligand as shown by a fluorescence polarization assay [169]. These results are in direct contradiction from what is seen with antiandrogens, and further support EPI-001 as having a novel mechanism of action [225].
The binding site for EPI-001 has been mapped via NMR using a fragment of the AF-1 region (AR\textsubscript{142-448}) which contains the majority of TAU-1 and TAU-5. Interestingly, it was shown that EPI-001 specifically targets TAU-5 in a highly selective and reproducible way, and no chemical shift in TAU-1 was seen even at a concentration of 350 µM EPI-001 [226]. It was also demonstrated that the entirety of residues 354 to 448 of the AF-1 are required for EPI-001 binding; when peptides corresponding to the regions 341-371, 391-414, and 426-446 were used independently, EPI-001 failed to bind [226,227]. As large chemical shifts were found in all three of these regions, this finding implies that the specificity of EPI-001 for TAU-5 is related to the structural conformation which TAU-5 adopts [226,227].

Biotinylated probes of EPI analogs have been used to independently confirm binding of EPI compounds to the AF-1 region in living cells, and that some secondary structure of the AF-1 is required for EPI-001 to bind. Furthermore it was demonstrated that the chlorohydrin group was necessary as probes lacking this functional group were unable to inhibit AR activity, nor bind the AF-1 region [169,225,226].

The AR-NTD interacts with a host of coregulators and transcriptional machinery which allows it to function as a master regulator of gene expression. As discussed previously, the core TAU-5 sequence \textsuperscript{435}WHTLF\textsuperscript{439} is also thought to be important in supporting AR homodimerization by interacting with the LBD in a second AR molecule [75]. This N/C interaction is thought to stabilize androgen binding and slow its dissociation rate [75–78,81]. Previously well described protein-protein interactions between the AR-NTD and coregulators (CBP) and transcriptional machinery (RAP74) could be experimentally inhibited by EPI-001 pre-treatment. Additionally, androgen induced AR N/C interactions were also significantly
reduced [169]. These experiments confirm that EPI-001 binds the NTD and is sufficient to impair biologically relevant protein-protein interactions with the AR.

Early experiments examining the mechanism of EPI-001 binding to TAU-5 suggested that this occurred in a two-step reaction, requiring the secondary hydroxyl group contained on C20 [169,225,226]. These in vitro studies propose a binding mechanism composed of a rapid reversible interaction followed by slow covalent binding step (Figure 1.9, ref 225). However, a subsequent report published by our group found that this is likely not the case in vivo. This study examined the possibility of using a radio-labelled EPI analog to specifically identify the presence of AR splice-variants in mice bearing xenografts. Biodistribution data showed that the accumulation of radio-labeled EPI found in AR-V7 positive xenografts decreased over time, and by extension a lack of covalent binding to the AR-NTD [228]. It was postulated that due to the relatively fast turnover seen in vivo ($t_{1/2}$ ≈ 3 hrs), the slow covalent binding step was not permitted.

As class I nuclear receptors, the PR and GR display high sequence homology with the AR, specifically with respect to the DBD and to a lesser extent, the LBD as well [68,69,95]. However, despite that less than 15% sequence homology exists between the NTD of the AR and other steroid receptors, there are protein interactions with coactivators and basal transcriptional machinery are common to all three [110,111]. The specificity of EPI-001 for the AR-NTD was demonstrated using reporter assays under the control of ER, PR or GR response elements, as EPI-001 did not inhibit the transcriptional activity of these steroid receptors [169,228].
1.5.4 Functional characterization of EPI-001 and EPI-002

Previous reports have shown that EPI-001 inhibits the formation of a DNA/AR complex with the promoter and enhancer regions of androgen induced genes PSA and TMPRSS2 [169]. EPI-001 was also able to significantly inhibit AR transcriptional activity and androgen dependent proliferation in vitro and in vivo [169,225].

EPI-001 has two chiral carbons (C2 and C20) and therefore exists as a racemic mixture of four stereoisomers termed EPI-002 through EPI-005 (Figure 1.10). Each stereoisomer was individually examined to determine if differences between bioactivity exist between them. All of the four stereoisomers are able to interact with the AF-1 region to a similar extent as the racemate [226], and were able to significantly inhibit both androgen dependent and independent AR transcriptional activity in vitro [225]. However EPI-002 (2R,20S) and EPI-005 (2S,20S) consistently had the greatest potency which was confirmed using a LNCaP CRPC xenograft model. Due to the more favorable toxicity profile, EPI-002 was pursued as a lead compound [225].

As described in previous sections, constitutively active AR splice variants and gain-of-function mutations represent clinically important mechanisms of resistance to hormonal therapy. As EPI-002 selectively binds the AR-NTD, it is predicted to retain efficacy in both of these contexts. Accordingly an AR-driven reporter assay using cells transfected with various AR mutations demonstrated that unlike flutamide, or bicalutamide, EPI-002 did not have agonist activity in the absence of androgen and was able to inhibit agonist stimulated AR transcriptional activity [221]. Additionally cells expressing either ectopic AR\textsuperscript{v567es}, or endogenous AR-V7 were resistant to antiandrogens bicalutamide and enzalutamide, both in terms of variant transcriptional activity and cellular proliferation - regardless if androgen was present or not. Conversely,
sensitivity to EPI-002 was unaffected by the presence of either AR splice variant [169,221,228]. Similarly, mice bearing xenografts expressing endogenous AR-V7 treated orally with EPI-002 had tumours which were significantly smaller than mice treated with bicalutamide [225] or enzalutamide [221].

1.6 RESEARCH SUMMARY

1.6.1 Dissertation theme and outline

Prostate cancer is a significant health issue affecting a ~1.1 million men annually worldwide [4]. While significant progress has been made for the majority of patients with low grade disease, for men with CRPC the outlook remains bleak. The AR has long been recognized as a primary driver of CRPC in nearly all cases despite castrate levels of androgens, thereby validating it as a bona-fide drug target. Indeed significant - albeit temporary - reductions in tumour burden are seen in CRPC patients treated with hormonal AR-targeted therapies. These will all ultimately fail as they universally directly or indirectly target the AR-LBD and numerous escape mechanisms involving LBD alterations may be exploited by tumour cells, rendering this treatment strategy ineffective (Figure 1.11).

Because of this, much interest has arisen in developing small molecule inhibitors to the NTD, although progress has been slow due to the disordered nature of the domain, and lack of solved crystal structure. Despite this challenge, our lab has developed and characterized the first AR-NTD inhibitors which have demonstrated excellent potency in the context of antiandrogen resistant models. Based upon the aforementioned and other favorable preclinical data, an orally active prodrug of EPI-002 (ralaniten-acetate) was the first AR-NTD inhibitor to be accepted into Phase I clinical trials in November 2015 (ClinicalTrials.gov identifier: NCT02606123, ESSA
Pharma, Inc.). Ralaniten (previously EPI-002) represents a major step forward in the field of prostate cancer as it is a first-in-class small molecule inhibitor of the AR-NTD and will hopefully provide the framework for the development of additional next-generation inhibitors.

This dissertation aims to address the possibility that resistance to such a molecule may arise with chronic exposure. By pre-emptively modeling resistance mechanisms in the lab, we will be better equipped to combat them in the clinic. Chapter 2 details the generation of a cell line with acquired resistance to ralaniten from parental LNCaP cells, and the associated experiments which confirmed biological resistance had occurred. The selection of clones for additional characterization from the heterogeneous line is described. A highly resistant clone (D7) was chosen for use in all future experiments and was termed LNCaP-RAL\textsuperscript{R}. The initial characterization of this line in terms of AR transcriptional activity and AR structural modification are also outlined. Finally, Chapter 2 concludes with the discovery of a resistant signature following analysis of a whole transcriptome microarray, and briefly introduces the UGT2B genes which came out of that analysis.

The UGT2B family is the focus of Chapter 3. These genes are associated with drug metabolism, and ralaniten is predicted to be a substrate of UGT2B mediated glucuronidation. UGT2B enzymatic expression and activity in each cell line are highlighted as are experiments demonstrating that selective knockdown of these genes can restore sensitivity to ralaniten in the resistant LNCaP-RAL\textsuperscript{R} line. Chapter 3 closes with a final experiment showing that ectopic overexpression of UGT2B15 is sufficient to reduce the efficacy of ralaniten in a previously sensitive line. Chapter 4 opens with experiments to identify the functional group(s) on ralaniten which are targets for glucuronidation. Data showing that metabolism of ralaniten by glucuronidation occurs in humans given ralaniten orally is also presented. The primary focus
however, is on an analog of ralaniten (EPI-045) which is resistant to this metabolic pathway and provides evidence suggesting that it is an effective AR-NTD inhibitor in the resistant line. Chapter 4 concludes with experiments using EPI-045 in a mouse xenograft model and suggests that developing analogs of ralaniten may optimize clinical responses to AR-NTD inhibitors. Finally, Chapter 5 concludes the dissertation by discussing all the results presented herein in the context of drug resistance in advanced prostate cancer. It addresses the significance and drawbacks of this work and identifies how it may contribute to the advancement of future drug development and discovery.

1.6.2 Hypothesis and specific aims

CRPC by and large remains dependent upon AR transcriptional activity despite castrate levels of androgens, and therapies targeting the AR do display efficacy even if it is temporary. Unfortunately, both intrinsic and acquired resistance to targeted hormonal therapies remain a major challenge in combating CRPC. The discovery of small molecule inhibitors targeting the AR-NTD represent a significant achievement and may one day be used to treat advanced CRPC which is resistant to current therapies. Despite the potential ralaniten holds, there exists the possibility that resistance will develop in response to chronic treatment as well. Therefore, I hypothesize that prostate cancer cells will develop acquired resistance to ralaniten treatment following prolonged exposure. Additionally, I hypothesize that the AR will remain functional and continue to drive the proliferation of cancer cells which have become resistant to ralaniten, and that alternative AR-targeted strategies will remain effective in this context.

The hypotheses will be tested by focusing on the following research objectives: 1). to create a cell line modeling acquired resistance to ralaniten, and confirm that resistance is
recapitulated in a mouse xenograft model; 2). to thoroughly characterize the resistant line and elucidate a molecular mechanism(s) of ralaniten resistance; 3). to validate any resistance mechanisms through functional studies; and 4). to identify and test additional compounds or strategies which may circumvent ralaniten resistance. Objective 1 is addressed by the following specific aims: 1) to establish a cell line resistant to ralaniten derived from long term culture of parental LNCaP cells with ralaniten; 2) confirm resistance has developed using proliferation experiments in vitro; and 3) validate findings by demonstrating biological resistance is retained in a xenograft study. The relevant materials, methods and results are presented in Chapter 2. Objective 2 is addressed by the following specific aims: 1) to confirm the role that the AR plays in driving proliferation of the resistant line; 2) to determine if alternative AR-targeted therapeutics (antiandrogens) remain effective in the context of ralaniten resistance 3) to assess if AR modification (i.e. splice variants, gain-of-function mutations, over expression) are associated with ralaniten resistance; 4) to examine global gene expression to find a resistant signature and identify potential pathways for further characterization; and 5) to validate microarray findings through mRNA and protein expression, as well as functional characterization experiments. The relevant materials, methods and results are presented in Chapters 2 and 3. Objective 3 is addressed by the following specific aims: 1) to restore sensitivity to ralaniten through antagonizing pathways associated with ralaniten resistance; and 2) to reduce sensitivity to ralaniten through ectopic expression of genes associated with ralaniten resistance. The relevant materials, methods and results are presented in Chapter 3. Finally, objective 4 is addressed by the following specific aims: 1) to identify alternative compound(s) which are predicted to retain efficacy in the context of resistant mechanisms; 2) to assess the ability of backup compound(s) to effectively antagonize the AR signalling pathway in vitro; and 3) to validate the efficacy of
backup compounds using a mouse xenograft model. The relevant materials, methods and results are presented in Chapter 4.
### Table 1.1  Practice-changing trials of treatments for metastatic prostate cancer that improve survival

<table>
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<tr>
<th>Trial and Registration No.</th>
<th>Treatment</th>
<th>Study Treatment</th>
<th>Control</th>
<th>Median Overall Survival (months)</th>
<th>Hazard Ratio for Death (95% CI)</th>
<th>Year of Initial Report</th>
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<td>ADT</td>
<td>n.r.</td>
<td>0.61 (0.49-0.75)</td>
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<td>0.70 (0.47-1.04)</td>
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<td>TAX 327 d</td>
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<td>MIT and PRED</td>
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<th>Hazard Ratio for Death (95% CI)</th>
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<td>0.65 (0.58-0.83)</td>
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a Adapted from Table 1 with permission from Sartor and de Bono (2018). *NEJM.* 378(7):645-657. Copyright Massachusetts Medical Society [233].

b Abbreviations: ABI, abiraterone; ADT, androgen-deprivation therapy; APA, apalutamide; CAB, cabazitaxel; DOC, docetaxel; EST, estramustine; ENZ, enzalutamide; MIT, mitoxantrone; PRED, prednisone; CI, confidence interval; n.r., not reached.

c Median overall survival was a secondary endpoint; the primary endpoint was metastasis-free survival.

d There is no trial registration number for TAX 327.
Figure 1.1. PSA progression predicts treatment failure

Rising levels of serum PSA often preclude initial diagnosis of prostate cancer. When the disease is localized, surgery or radiation therapy are often curative and are associated with a dramatic decrease in PSA and tumour burden. A subset of patients will relapse indicated by a subsequent rise in PSA levels. Though no longer organ confined, at this stage the disease remains reliant upon AR signalling. Therefore androgen deprivation therapy is prescribed to delay cancer progression. Rising PSA following surgical or chemical castration is a hallmark of CRPC, and all treatments given at this stage are palliative. Adapted from Figure 1 from Imamura and Sadar (2016). International Journal of Urology. 23(8):654-665. Reproduced with permission of Wiley via Copyright Clearance Center [66].
The human AR spans ~90 kDa and is located at q11-12 on the X-chromosome. The AR gene is composed of 8 exons which make up four distinct functional domains. The N-terminal domain (NTD) (a.a. 1-558) contains the activation function 1 (AF1) domain and two transcriptional activation units TAU-1 and TAU-5 responsible for ligand-dependent and -independent transcriptional activity respectively. Each has a core sequence integral for its function (LKDIL\textsuperscript{27} and WHTLF\textsuperscript{439}). The NTD also contains two short motifs (FxxLF and WxxLF) which are involved in forming protein-protein interactions with the ligand-binding domain (LBD). A nuclear localization signal (NLS) is exposed following agonist binding and exists within the hinge region (HR) and the C-terminal region of the DNA-binding domain (DBD). The LBD recognizes androgen ligand, and ligand binding induces a conformational change to expose the AF2. However unlike other steroid receptors in which the AF2 serves as a docking site for coactivators, in the case of the AR, the AF2 is primarily involved in mediating intra- and intermolecular N/C interactions with FQNL\textsuperscript{27}. Adapted from Figure 2 from Imamura and Sadar (2016). International Journal of Urology. 23(8):654-665. Reproduced with permission of Wiley via Copyright Clearance Center [66].
Homodimerization of two AR monomers is mediated through androgen binding to the LBD, and dimerization servers to stabilize ligand by reducing the disassociation rate of DHT. The AF2 region within the LBD is formed following androgen binding from α-helices 3,4 and 12 and exposes a hydrophobic cleft which for most steroid receptors, recognize coactivator proteins bearing LxxLL consensus sequences. The AR AF2 however, is unique in that it preferentially binds the $^{23}$FQNLF$^{27}$ sequence found in the intrinsically disordered NTD. Mutational studies have revealed that a second sequence ($^{433}$WHTLF$^{437}$) also contributes to the N/C interaction, although this occurs in a region outside of the AF2. **DBD**, DNA-binding domain; **LBD**, ligand-binding domain; **NTD**, N-terminal domain.
Testosterone enters prostate cells by passive diffusion across the plasma membrane and is irreversibly converted into the more potent metabolite DHT by 5α-reductase. The AR exists in the cytoplasm bound to heatshock and immunophilin chaperone proteins. DHT binding to the LBD induces phosphorylation of the AR as well as conformational changes releasing the AR from chaperone proteins and exposing a nuclear localization sequence. This allows the AR to be shuttled into the nucleus where it forms a homodimer with a second DHT-bound AR molecule. The dimer recognizes specific DNA sequences located within the enhancer or promoter regions of target genes termed androgen response elements (ARE). The AR dimer also mediates recruitment of coregulators and transcriptional machinery to facilitate the transcription of target genes.
Synthesis and secretion of testosterone is controlled by a reciprocal feedback system beginning with the release of GnRH from the hypothalamus. Specialized receptors on the anterior pituitary gland respond to GnRH stimulation by initializing the synthesis and secretion of LH and FSH into the circulation. Both of these hormones act upon cells within the testes and induce testosterone production and spermatogenesis respectively. Testosterone and inhibin B both act upon the pituitary gland to downregulate the expression of receptors which respond to GnRH stimulation. Chemical castration using GnRH agonists exploit this negative regulation by disrupting the pulsatile nature of GnRH secretion; however these drugs are also associated with a testosterone surge which occurs due to initial stimulation of the pathway. Adapted from Figure 2 from Drudge-Coates (2009). *International Journal of Urological Nursing*. 3(3):85-92. Reproduced with permission of Wiley via Copyright Clearance Center [234].
CRPC remains driven by AR and is evidenced by the fact that it is precluded by rising PSA following surgical or chemical castration, and also that additional AR targeted therapies remain transiently effective following ADT. The molecular mechanisms shown above have all been identified as a means of prostate cancer cells to restore AR transcriptional activity in the absence of testicular androgens. **CoA/R**, coactivators, corepressors; **DBD**, DNA-binding domain; **LBD**, ligand-binding domain; **NTD** N-terminal domain.
AR-NTD inhibitors (black diamond) do not prevent ligand binding as they target a region distinct from the LBD. Instead, transcriptional activity is prevented by an inhibition of critical protein-protein interactions between the AR and coactivators, bridging factors, and transcriptional machinery. Inhibitors of the AR-NTD are also capable of blocking ligand-independent trans-activation through alternative signalling pathways (IE. cytokines or growth factors) unlike molecules which target the LBD. AR-NTD inhibition has been shown to prevent AR N/C interaction, homodimerization and formation of AR-DNA complex. DBD, DNA-binding domain DHT, dihydrotestosterone; LBD, ligand-binding domain.
Figure 1.8. Chemical structures of AR agonists and antagonists used in the clinic

Testosterone and DHT are the natural steroidal ligands of the AR, while R1881 is a synthetic androgen often used in the laboratory due to its stability. Antiandrogens all competitively inhibit AR transcriptional activity by binding the AR-LBD. Abiraterone and ketoconazole indirectly target the AR by inhibiting the CYP17 mediated generation of testosterone precursor molecules by the adrenal glands or in some cases, by the tumour itself. In this way the amount of androgen available is reduced, preventing AR signalling. Ralaniten (previously EPI-002) represents the first small molecule inhibitor of the AR-NTD to advance into clinical trials. These types of molecules block essential protein-protein interactions from occurring between the AR and coregulatory proteins, but do not prevent ligand binding to the AR-LBD.
Figure 1.9 EPI-001 binds the AR TAU5 domain

A model illustrating the proposed mechanism of EPI-001 binding to the TAU5 region of the AR-NTD. While covalent binding of EPI-001 has been observed using *in vitro* experiments over a long period of time, *in vivo* data suggests that binding is temporary and reversible. Adapted from Figure 7 from Myung *et al.* (2013). *Journal of Clinical Investigation.* 123(7):2948-2960. Reproduced with permission of American Society of Clinical Investigation via Copyright Clearance Center [225].
Figure 1.10. Chemical structures of the stereoisomers of EPI-001

EPI-001 is a racemic mixture made up of 4 unique stereoisomers due to two chiral carbon atoms (C2 and C20). EPI-002 (ralaniten) was chosen as a lead compound for further testing and characterization due to its potency and more favorable toxicity profile compared to the other stereoisomers.
Figure 1.11. Ralaniten is predicted to retain efficacy against mechanisms of resistance to current AR targeted therapies

Several resistance mechanisms are common to therapies targeting the AR-LBD (ADT, antiandrogens and CYP17A1 inhibitors) and mediate the restoration AR transcriptional activity. These include AR gene amplifications, sensitizing the AR to reduced levels of androgens; intratumoral androgen synthesis; gain-of-function mutations (specifically in the LBD), which generate a promiscuous receptor; and constitutively active AR splice variants which lack a LBD. Ralaniten has a unique mechanism of action and has been shown to retain efficacy in the context of these resistance mechanisms. Therefore a clinical role for small molecule inhibitors of the AR-NTD is clearly defined in the post abiraterone or enzalutamide setting. AA, antiandrogen; RAL, ralaniten.
Chapter 2. Generating and Characterizing a Model of Acquired Ralaniten Resistance

2.1 INTRODUCTION

While the number of drugs which have become available for the treatment of CRPC has increased in recent years, primary or acquired resistance to these compounds is inevitable. Cellular models have been utilized in the laboratory to great effect in recapitulating resistance strategies exploited by tumours, and have directly accelerated the development of additional therapies used clinically [192,207,235]. For example, in vitro modeling of acquired docetaxel resistance revealed that overexpression of MDR-1 (encoding the drug efflux pump, P-glycoprotein) was a common mechanism [235,236]. This led to the development and eventual FDA approval of cabazitaxel which is a poor P-glycoprotein substrate [192,237]. Additionally, the second generation antiandrogens enzalutamide and apalutamide were first identified as lead compounds following the demonstration that they had increased potency compared to bicalutamide in a prostate cancer model which overexpressed the AR [207]. Both of these compounds have since received FDA approval and are now used in the CRPC setting.

Due to the fact that prostate cancer is by nature a highly heterogeneous disease, clinical responses to these therapies often vary between patients. Therefore modeling mechanisms of resistance can also identify biomarkers (such as AR-V7, AR\textsuperscript{W741C}, AR\textsuperscript{F876L}) which can be used to predict responses to a given therapy [185,218,220,238,239]. These laboratory studies can

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provide invaluable information and pave the way for more individualized care based upon the patient's unique tumour biology [130].

Small molecules targeting the AR-NTD is a widely recognized strategy which if successful could have broad applicability for extending treatment options to patients who have progressed on existing AR targeted therapies. Our lab has previously demonstrated that ralaniten is capable of inhibiting androgen-dependent transcriptional activity as well as growth of prostate cancer cell lines which are resistant to enzalutamide and express AR-variant [221]. In following, ralaniten entered into a Phase I/II clinical trial in November, 2015 (NCT02606123, ESSA Pharma, Inc.) specifically in men with mCRPC in the post abiraterone and/or enzalutamide setting.

While a niche for AR-NTD inhibitors is clearly defined, albeit underexploited, there are as yet no models of resistance to such a therapy. Therefore it is impossible to predict what the therapeutic landscape might look like once resistance arises to these drugs. Addressing this is of the utmost importance in an effort to develop more potent and effective second generation compounds. While this is the primary goal and focus of this thesis, another intriguing question is whether resistance to ralaniten occurs independently of classical resistance mechanisms associated with standard of care hormonal therapies (i.e. enzalutamide and abiraterone). Based upon differences between the mechanisms of action for an AR-NTD inhibitor and those targeting the LBD, we would not expect this to occur (illustrated in Figure 1.11). However this may indicate that existing therapies could have reciprocal activity following treatment with an AR-NTD inhibitor. Additionally, our lab is also actively pursuing studies focusing on the efficacy of using combination therapies utilizing both classic antiandrogens and ralaniten. Should these drugs become routinely used, the optimal sequencing and timing of administration within the
context of AR targeted therapy will have to be deduced. Extensive work and research will need to be completed to adequately answer this question, and is outside the scope of this dissertation. However this thesis does begin to lay the groundwork for future studies by asking if existing antiandrogens retain efficacy in the context of ralaniten resistance.

Choosing an appropriate cell line to model acquired resistance is imperative to gaining data which will be both meaningful and useful. Prostate cancer is a heterogeneous disease and numerous clinical phenotypes have been modeled using cell lines established from clinical samples or from clonal derivatives of existing cell lines. Historically, the most widely used cell lines have been DU-145, PC-3, LNCaP and their derivatives [240–242]. The DU-145 cell line was first established from a brain metastases excised from a 69 year-old patient with advanced prostate cancer in 1975. DU-145 cells are epithelial prostate cells described as a moderately differentiated adenocarcinoma [243]. While DU-145 cells are able to form tumours in nude or SCID mice and readily metastasize, they do not express the AR nor the classical AR regulated gene PSA at either the mRNA or protein level, and their growth is not dependent upon androgen stimulation [240,243]. The PC-3 cell line was derived from a vertebral metastasis in a 62 year-old man in 1979 [244]. Like DU-145 these cells readily form tumours in mice, however they also lack AR expression and are unresponsive to androgen stimulation [240]. While both of these cell lines are useful for modeling late stage mCRPC, the fact that they do not express functional AR makes them less desirable (except as negative controls) when looking at the efficacy of AR-NTD inhibitors.

LNCaP cells are by far the most widely used cell line in prostate cancer research [242], and were first isolated from a lymph node metastasis from a 50 year-old patient in 1977 [245]. As a result, this cell line is extremely well characterized and has been used to generate a host of
While substantially slower growing than either DU-145 and PC-3 cells, the LNCaP cell line expresses AR at both the mRNA and protein level, as well as classic AR regulated genes including PSA [246], allowing transcriptional activity of the AR to be easily measured. Interestingly, LNCaP cells harbour a mutation within a key region of the AR-LBD (T877A) which generates a promiscuous receptor able to bind, and be stimulated by, a variety of steroids as well as the antiandrogen flutamide [247]. As such, these cells exhibit AR-dependent growth and are capable of forming tumours in mice, however to a lesser degree than PC-3 or DU-145 and require coinjection with Matrigel for effective tumour take [240].

While used much less frequently than the three aforementioned cell lines, there are a number of cell lines and sublines which are nonetheless important in research. While many others exist two important ones include LNCaP95 and VCaP due to their expression of AR-variant. Therefore these represent clinically relevant models in which to study the effects of compounds against a background of constitutively active AR-splice variants and AR amplification respectively [184,248]. While these cell lines were not used outside of positive controls for variant expression in this study, others in our lab have focused on these lines to demonstrate the superiority of ralaniten compared to existing antiandrogens [221,225].

In this work, LNCaP cells were used as a parental line to generate a model of acquired ralaniten resistance. LNCaP cells retain a functional AR transcriptional program and express classic AR-regulated genes allowing for the quick and robust measurement of the effect of AR-NTD inhibition in living cells. Much of the initial characterization of ralaniten was completed in this cell line, and our lab has worked extensively with LNCaP cells throughout its development [169,221,225,228]. This provided a clear and well defined baseline with which to compare any
future characterization which would be carried out in the resistant line, both at the transcriptional level as well as cellular proliferation in vitro and in vivo.

Here, the primary research objective was to generate a model of acquired resistance to ralaniten, and to begin the initial characterization in order to identify possible mechanisms of escape. We hypothesized that despite the sensitivity of LNCaP cells to ralaniten, acquired resistance would occur with chronic treatment. Furthermore, due to the importance of the AR in driving the growth of these cells, we further hypothesized that resistant cells would retain a functional AR transcriptional program (i.e. that an AR bypass mechanism would not be identified). To test these hypotheses the following specific aims were established: 1) to generate a cell line modeling acquired ralaniten resistance derived from long term culture of parental LNCaP cells with ralaniten; 2) to confirm resistance has developed using proliferation experiments in vitro; 3) to validate findings by demonstrating biological resistance is retained in a xenograft study; 4) to confirm the role that the AR plays in driving proliferation of the resistant line; 5) to determine if alternative AR-targeted therapeutics (antiandrogens) remain effective in the context of ralaniten resistance; and 6) to assess if AR modification (i.e. splice variants, gain-of-function mutations, over expression) are associated with ralaniten resistance.

Small molecule inhibitors targeting the AR-NTD have tremendous promise by potentially embodying a new and powerful tool in the fight against prostate cancer. By modeling how cancers might evolve in response, we can begin to pre-emptively identify and address potential shortcomings in an effort to optimize clinical responses.
2.2 MATERIALS AND METHODS

2.2.1 Cell culture

LNCaP cells were maintained in phenol red-free RPMI 1640 (Invitrogen), supplemented with 5% heat-inactivated fetal bovine serum (FBS; VWR). A cell line resistant to ralaniten was established by exposing parental LNCaP cells with increasing concentrations of ralaniten beginning in September, 2012 (media supplemented with ralaniten was replenished every 3-4 days). The resulting line displayed stable growth at 25 µM ralaniten, however it was initially a heterogeneous culture. Therefore, highly resistant clones were selected by serial dilution and challenged with increasing concentrations of ralaniten up to 50 µM (September, 2014). Of the six clones which were isolated and expanded, clone D7 had the fastest doubling time in the presence of 50 µM ralaniten and was chosen for further characterization (hereafter referred to as LNCaP-RAL\textsuperscript{R}). LNCaP-RAL\textsuperscript{R} cells were maintained in RPMI 1640 supplemented with 5% FBS and 50 µM ralaniten. Cellular doubling time was estimated by the following equation:

\[ D_T = \frac{T^* \ln (2)}{\ln \left( \frac{C_f}{C_i} \right)} \]  \hspace{1cm} (2.1)

- \( D_T \) is the doubling time (in hours)
- \( T^* \) is incubation time (in hours)
- \( C_f \) is cell number at end of incubation time
- \( C_i \) is cell number at beginning of incubation time

Media was replaced once weekly with fresh compound. The remaining clones were cryopreserved in liquid nitrogen. All cells were regularly tested to ensure that they were
mycoplasma-free (VenorGem Mycoplasma Detection Kit, Sigma-Aldrich) and were authenticated by short tandem repeat analysis in June, 2017. Cells were resuscitated immediately prior to experimentation, and maintained for no more than 10 to 15 passages.

2.2.2 Chemicals and compounds

Metribolone (R1881) was purchased from AK Scientific (Mountainview, CA, USA), antiandrogens bicalutamide was a kind gift from Dr. Mark Zarenda (AstraZeneca) and enzalutamide was purchased from Omega Chem (Lévis, QC, Canada). Ralaniten was provided by Naeja-RGM (Edmonton, AB, Canada), and EPI-045 and EPI-002053 were synthesized by Dr. Raymond Andersen at UBC (Vancouver, BC, Canada). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2.3 Proliferation and dose response assays

Crystal violet staining was utilized to measure cellular proliferation. LNCaP or LNCaP-RAL^c^ cells were seeded onto 96 well tissue culture plates in RPMI 1640 supplemented with 5% charcoal-stripped FBS at a density of 5,000 cells/well. Cells were given 24 hours to attach to the plate prior to treatment. Cells were pre-treated with DMSO vehicle or ralaniten (25 µM) for 1 hour before stimulation with synthetic androgen R1881 (0.1 nM). Plates were harvested every 24 hours and cell number was estimated against a standard curve (0-30,000 cells) which were seeded on plates 24 hours prior to harvesting.

To harvest plates, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 minutes at room temperature. Wells were washed 3x with dH$_2$O and stained with 0.1% crystal violet for 10 minutes at room temperature, followed by three more wash steps with dH$_2$O. Dye was solubilised by addition of 1% SDS and incubating plates on an orbital
shaker for 30 minutes at room temperature. Absorbance was measured using a VersaMax plate reader (Molecular Devices) at 595 nm.

For the dose response assays, 5,000 cells/well were seeded onto 96-well plates as described above. After given 24 hours to attach, cells were pre-treated with DMSO vehicle, ralaniten (0.1, 0.5, 1, 5, 10, 25, 35 µM), bicalutamide (0.05, 0.1, 0.5, 1, 5, 10, 25 µM) or enzalutamide (0.01, 0.05, 0.1, 0.5, 1, 5, 10 µM) for one hour. Cells were then stimulated with R1881 (0.1 nM) or EtOH vehicle. Plates were harvested 96 hours following treatment as described above. Data was background subtracted and normalized to DMSO/R1881 treatment to calculate androgen dependent growth.

2.2.4 Gene and protein expression assays

For western blot experiments, 1x10⁶ cells were seeded onto 10 cm plates in media contain 5% FBS for 24 hours to allow cells to attach. Media was then removed and replaced with RPMI 1640 lacking serum to starve cells of androgens for 24 hours. Cells were pre-treated with ralaniten (35µM for 16 hours), enzalutamide (5 µM for 1 hour), or DMSO vehicle (16 hours) before stimulated with 1 nM R1881 or EtOH vehicle. Lysates were harvested 24 hours after R1881 stimulation in RIPA buffer containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablets™ (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets™ (Roche). The concentration of protein was quantified by using a BCA Protein Assay Kit (Thermo Scientific Inc., Rockford, USA) and samples were separated by 10% SDS-PAGE. Protein was transferred to PVDF membrane (Millipore LTD, Cork, IRL) and blocked for 1 hour in 5% skim milk in TBST prior to incubation with primary antibody. Relevant information regarding specific antibodies are given in Table 2.1.
For the qRT-PCR and microarray experiments, 1.5x10^5 cells/well were seeded onto 6-well plates and treated identically as for the protein expression assay. 24 hours following R1881 stimulation, cells were harvested in 1 mL/well TRIzol reagent (Invitrogen) and total RNA was extracted using the RNeasy Micro Kit (Qiagen). RNA was cleaned using DNase I kit, Amplification Grade (Millipore, Sigma) and reverse transcribed using the High-Capacity RNA-to-cDNA Kit (ThermoFisher Scientific). Diluted cDNA and Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) were combined with gene specific primers (sequences shown in Table 2.2). Transcripts were measured by qRT-PCR QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems by Life Technology) and gene expression was normalized to the house keeping gene SDHA.

2.2.5 AR cloning and sequencing

Total RNA (1 µg) from LNCaP-RALR cells was treated with DNase I followed by reverse transcription using Superscript III First Strand Synthesis Kit (Invitrogen) with 2.5 µM oligo(dT)20. PCR amplifications were performed with Platinum DNA Taq Polymerase High Fidelity (Invitrogen). PCR amplifications were completed in 20 µL reactions with 1U Taq DNA High Fidelity Polymerase, 2 mM MgSO₄, 0.2 mM deoxynucleotide mix, and 2% DMSO with primers covering either the entirety of the AR (AR̂FL) or the LBD (AR̂LBD) with the following sequences:

AR̂FL (FWD) 5'-AGGGGAGGCGGGGTAAGGGAAGTA-3'
(REV) 5'-CATGAGCTGGGGTGGGGAAATAGG-3'

AR̂LBD (FWD) 5'GCGAAATGGGCCCTGGATGGAT-3'
(REV) 5'-CATGAGCTGGGGTGGGGAAATAGG-3'
The PCR cycle conditions consisted of 94°C for 2 minutes, followed by 30 cycles of a denaturing step (94°C, 15 sec), an annealing step (60°C, 30 sec) and an elongation step (68°C, 1.5 minutes). The reaction was terminated with a final extension step at 68°C for 5 minutes. PCR products were electrophoresed in a 0.8% agarose gel. Corresponding DNA fragments were isolated and cloned into PCR 2.1 TOPO cloning vector using the TOPO TA cloning kit (Invitrogen) and transformed into chemically competent TOPO10 cells according to manufacturer's protocol. Transformant plasmids containing DNA inserts were sequenced at the NAPS core unit at the University of British Columbia (Vancouver, BC, Canada; https://naps.msl.ubc.ca).

2.2.6 siRNA transfection and proliferation assays

For experiments examining protein or mRNA expression, cells were plated on 10 cm (1x10^6 cells/dish) or 6-well (1.5x10^5 cells/well) plates respectively in complete media for 24 hours prior to transfection. Pooled siRNA against AR (L-003400-00-0005) or non-targeting control (D-001810-10-05) were purchased from Dharmacon Research (Layfayette, USA). After 24 hours, media was removed and replaced with Opti-MEM (Gibco) containing 10 nM siRNA/transfection reagent complexes (Lipofectamine RNAiMAX Transfection Reagent, Invitrogen). 24-96 hours post transfection, protein or mRNA was harvested and analyzed as previously described.

For the proliferation assays, cells were plated on 24-well plates (3.0x10^4 cells/well) and transfected as described above. After 24 hours, cells were treated with 0.1 nM R1881 or EtOH. After 72 hours post treatment, cells were fixed and stained with 0.1% crystal violet as described
previously. Absorbance was read using a VersaMax Microplate Reader (Molecular Devices) at 595 nm.

### 2.2.7 Xenografts and ethics approval

Male NOD/SCID mice at 6 to 8 weeks of age were subcutaneously injected with LNCaP or LNCaP-RAL\textsuperscript{R} cells (1x10\textsuperscript{7} cells/site) using Matrigel (Becton Dickinson). Once tumours reached \(~100\text{ mm}^3\), mice were castrated and treatment began one week later. Mice were randomized into treatment groups receiving ralaniten (200 mg/kg), enzalutamide (10 mg/kg) or vehicle control (5% DMSO/1% CMC/0.1% Tween-80) once daily by oral gavage. Mouse body weight and tumour volume (defined as volume = length x width x height x 0.5236) were regularly recorded and tumours were excised 2 days after the last treatment. The University of British Columbia Animal Care Committee approved all experiments involving animals and these studies conformed to relevant regulatory and ethical standards.

### 2.2.8 Gene and protein expression in xenografts

To analyze tumour gene expression, tumours were flash frozen and \(~100\text{ mg}\) were pulverized using a motor and pestle under liquid nitrogen. The samples were added to 1 mL TRIzol reagent (Invitrogen) and homogenized using a pre-chilled dounce tissue grinder. RNA was extracted and reverse transcribed as detailed previously. For immunohistochemistry (IHC) experiments, 5\(\mu\text{m}\) thick sections of tumour tissue were cut from formalin fixed paraffin-embedded tissues and deparaffinized in xylene and rehydrated in alcohols and distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 minutes, followed by washing in PBS three times. Incubation with the following primary antibodies: anti-
AR N-20 (1:300; Santa Cruz) and anti-PSA C19 (1:500; Santa Cruz) were incubated at 4°C overnight. Antigen was detected with 3,3-diaminobenzidine and counterstained with hematoxylin.

### 2.2.9 Global gene expression analysis

To characterize the gene expression profile of the resistant line, total RNA was extracted from both LNCaP and LNCaP-RALR cells treated with ralaniten (35 µM), enzalutamide (5 µM) or DMSO vehicle and stimulated with either 1 nM R1881 or EtOH vehicle. RNA was reverse transcribed, and the resulting cDNA was hybridized to the GeneChip Human Transcriptome Array 2.0 from Affymetrix. RT-PCR, cDNA hybridization and chip reading was carried out at CDRD's Target Validation Division at the University of British Columbia (Vancouver, BC, Canada; www.crd.ca). Analysis of raw signal output was done using GeneSpring software (version 13.1). Clustered data was generated by conducting a 2-Way ANOVA on the data with a significance threshold set at 0.05. The Benjamini-Hochberg correction was applied to reduce the false discovery rate.

### 2.2.10 Statistical analysis

A One- or Two-Way ANOVA statistical test was used to determine significance for all comparisons unless otherwise stated (Graphpad Prism, version 7.0). p-value corrections were applied for all multiple comparisons (Tukey, Sidak or Dunnett as appropriate), and a p-value < 0.05 was considered statistically significant.
2.3 RESULTS

2.3.1 Generating a model of acquired ralaniten resistance

To understand how mechanisms of acquired ralaniten resistance might arise following chronic exposure, we created a cell line which could achieve stable growth in the presence of inhibitory concentrations of ralaniten. LNCaP cells were chosen as a parental line, as these cells are well characterized, express functional AR and classic AR regulated genes, and readily form tumours in mice [240,249]. Long-term culture of parental LNCaP cells in media supplemented with increasing concentrations of ralaniten led to the generation of a ralaniten resistant subline which showed stable growth at 25 µM ralaniten (Figure 2.1 A). To confirm that resistance was stable, both cell lines were challenged with 25 µM ralaniten and cellular proliferation was measured over the course of 96 hours. Ralaniten caused a significant reduction in cell number in parental LNCaP cells, but not in the resistant line (Figure 2.1 B).

The resulting resistant line represented a heterogeneous population of cells, in which multiple unique resistance mechanisms might be represented, and some cells are likely to be more resistant than others. Therefore we isolated highly resistant clones for further characterization. Cells from the resistant line were seeded onto 96-well plates and serially diluted until wells contained ~750 cells in media containing 25 µM ralaniten (wells containing fewer cells did not display sufficient growth for further expansion). Cells were plated an additional two times (at 750 cells/well) once reaching confluence to have a better chance of isolating homogenous colonies. Colonies which grew the fastest were chosen for expansion and were subjected to increasing concentrations of ralaniten (35 µM - 50 µM) and six clones were isolated which demonstrated stable growth at 50 µM (Figure 2.1 C) and expanded. The doubling times for each clone were deduced using equation 2.1, and of these, clone D7 grew the fastest in
the presence of 50 µM ralaniten (Figure 2.1 D). We chose this clone for further characterization and as such, it will be referred to as LNCaP-RAL\textsuperscript{R} for the remainder of this dissertation.

2.3.2 LNCaP-RAL\textsuperscript{R} cells retain sensitivity to alternative AR antagonists and require functional AR\textsuperscript{FL} for proliferation

To definitively demonstrate that the LNCaP-RAL\textsuperscript{R} cell line was indeed resistant to ralaniten, dose escalation experiments were performed to calculate the IC\textsubscript{50} of ralaniten in each cell line. There was a highly significant difference in androgen dependent growth between LNCaP-RAL\textsuperscript{R} and LNCaP cells with each having an IC\textsubscript{50} of 37.77 µM and 16.91 µM respectively (Figure 2.2 A). Interestingly, when dose escalation experiments were repeated for antiandrogens bicalutamide and enzalutamide (which antagonize the AR by competitively binding the LBD), no significant difference was observed at any concentration of drug tested (Figure 2.2 B). This implies that cross-resistance between different compounds targeting the AR did not occur, and that functional AR\textsuperscript{FL} is still driving the growth of LNCaP-RAL\textsuperscript{R} cells.

We next explored drug activity in the context of inhibiting AR regulated gene transcription. Cells were pretreated with vehicle, ralaniten or enzalutamide and stimulated with synthetic androgen R1881 or EtOH vehicle. As expected, neither ralaniten nor enzalutamide had any effect upon AR mRNA levels in either cell line. In LNCaP cells, both ralaniten and enzalutamide were highly effective in reducing R1881 stimulated levels of AR regulated genes (KLK2, PSA, NKX3.1, FKBP5 and RHOU) to a similar degree as non-stimulated samples (Figure 2.3 A). In contrast, ralaniten was less effective in its ability to inhibit AR mediated gene transcription in LNCaP-RAL\textsuperscript{R} cells. While KLK2 and NKX3.1 did show statistically significant reduction in gene expression with ralaniten pre-treatment, this was to a lesser extent than seen in LNCaP cells. Enzalutamide was highly effective in both cell lines, agreeing well with previous
dose response data. These trends were also observed at the protein level (i.e. stable AR levels across treatments/cell lines and LNCaP-RAL\textsuperscript{R} cells demonstrating sensitivity to enzalutamide but not ralaniten; Figure 2.3 B).

Collectively, these data strongly indicate that LNCaP-RAL\textsuperscript{R} cells remain dependent upon AR\textsuperscript{FL}. To test this we transiently knocked down AR expression using targeted siRNA and measured the proliferative ability of each cell line. AR protein levels were undetectable by 24 hours and remained so up to 96 hours in cells treated with siRNA (Figure 2.4 A). Cells which were treated with siRNA targeting the AR and stimulated with R1881 displayed significantly blunted levels of proliferation which closely matched cells treated in the absence of androgen stimulation (Figure 2.4 B). While not surprising, this experiment confirmed the continued reliance of LNCaP-RAL\textsuperscript{R} cells upon AR\textsuperscript{FL} to drive cellular proliferation, allowing us to eliminate AR bypass as a mechanism of resistance.

Interestingly, although detectable, expression of PSA both at the protein and mRNA level was dramatically reduced in LNCaP-RAL\textsuperscript{R} cells across treatments (Figure 2.3 A-B). As yet a rationale of how, or by what mechanism this occurs, has not been deduced. However, it may involve epigenetic silencing of the PSA gene as the AR is clearly driving the growth of these cells, and is functioning similarly to the parental line with respect to other AR regulated genes. As LNCaP-RAL\textsuperscript{R} cells were generally used within a window of ~ 7 passages before resurrecting new stocks, this expression profile (i.e. similar levels of AR expression compared to LNCaP cells, no detectable AR-variant, FKBP5 expression levels that are sensitive to enzalutamide but not ralaniten, and very low PSA expression) was used as a way to quickly validate the resistant line before continuing with additional experiments.
2.3.3 Biological resistance to ralaniten is retained in LNCaP-RAL\textsuperscript{R} xenografts

We next replicated our findings in a mouse xenograft model. To this end, castrated mice bearing LNCaP-RAL\textsuperscript{R} xenografts were randomized into three treatment groups receiving compounds once daily by oral gavage: ralaniten (200 mg/kg), enzalutamide (10 mg/kg) or vehicle (5% DMSO/1% CMC/0.1% Tween-80). Mice bearing LNCaP xenografts were also employed as a control for ralaniten sensitivity, and have been previously shown to be highly sensitive to enzalutamide given at 10 mg/kg [209].

LNCaP-RAL\textsuperscript{R} xenografts displayed enhanced growth rates and tumorigenicity compared to parental LNCaP xenografts, evidenced by a reduced time to tumour incidence despite identical numbers of cells injected at each tumour site (LNCaP-RAL\textsuperscript{R}: 37 days/100% incidence vs. LNCaP: 63 days/92% incidence). Additionally, the in vivo study agreed well with previous observations in that LNCaP xenografts were highly sensitive to ralaniten while LNCaP-RAL\textsuperscript{R} xenografts were unresponsive. Importantly, tumour growth was dramatically reduced in mice bearing LNCaP-RAL\textsuperscript{R} xenografts treated with enzalutamide (Figure 2.5 A-C). The change in body weight of mice from the beginning of the experiment until its completion was monitored carefully to ensure drug related toxicity was minimized. No treatments were associated with a significantly altered weight of the liver, spleen nor kidneys indicating off target effects were minimal (Figure 2.5 D-E).

qRT-PCR and IHC experiments conducted on LNCaP-RAL\textsuperscript{R} tumours harvested at the end of the experiment confirmed that AR\textsuperscript{FL} expression was maintained both at the protein (Figure 2.6 A) and mRNA (Figure 2.6 B) levels. In agreement with earlier experiments, classical AR\textsuperscript{FL} regulated genes were reduced by enzalutamide but not ralaniten, however PSA expression was restored (Figure 2.6 A-B), indicating that expression may be context dependent.
Collectively these data confirm that LNCaP-RAL\textsuperscript{R} xenografts retain biological resistance to ralaniten and imply that the antitumour effects of enzalutamide are directly related to its ability to effectively antagonize the AR.

2.3.4 Acquired ralaniten resistance occurs independently of AR structural alterations

Common resistance mechanisms to current therapies often converge upon the AR signalling pathway. These include AR structural alterations which generate constitutively active splice variants [130], gain-of-function mutations [137,214,215], and AR gene amplification and protein overexpression [216,217]. Therefore we first looked at these potential resistance mechanisms in an attempt to at least rule these out and focus our efforts on other potential mechanisms if need be. First, AR and AR-V7 mRNA and protein expression levels were quantified using qRT-PCR and western blot (using an antibody which specifically recognizes the AR-NTD) respectively. LNCaP 95 and VCaP cells were used as positive controls for AR-variant and AR amplification respectively [184,248]. AR levels remained stable between cell lines both at the mRNA and protein level as seen in previous experiments. Additionally, no evidence for the existence of AR splice variants could be discovered (Figure 2.7 A-B). This was expected as AR overexpression [209] and expression of AR splice variants [130] are validated resistance mechanisms against enzalutamide, and LNCaP-RAL\textsuperscript{R} cells retain sensitivity to antiandrogens.

Gain-of-function mutations in the AR have also been clinically shown to contribute to resistance to CYP171A inhibitor abiraterone as well as antiandrogens [137,154,218,250]. We next cloned the AR isolated from LNCaP-RAL\textsuperscript{R} cells to be sequenced and compared to parental LNCaP cells. As ralaniten specifically binds the TAU-5 domain within the AF-1 of the AR-
NTD [169,225–227], we expected that should a mutation be identified, it would reside in or near this region. No mutation within the AR-NTD was seen, however the well characterized T877A lesion was detected in all clones sequenced. This mutation is endogenous to LNCaP cells [212,221,251,252] which are sensitive to ralaniten, therefore this mutation has little bearing on ralaniten resistance (Figure 2.7 C). A second mutation within the C-terminal LBD (Q783L) was also identified in 2 of 26 clones which were sequenced. Due to the low mutational frequency, we believe that this was more likely an error introduced during the PCR amplification and cloning rather than a bona-fide resistance mechanism.

2.3.5 Identifying a resistant signature using a whole transcriptome microarray

To better guide our efforts in pursuing a resistance mechanism, we employed a whole transcriptome microarray (containing 67,110 probe sets) using cDNA samples isolated from LNCaP and LNCaP-RALR cells treated as in the qRT-PCR experiments. Initially, the subset of protein coding genes which were induced by androgen stimulation were identified and the ability of enzalutamide or ralaniten to inhibit their expression was assessed. In this way, the ability of each AR inhibitor to prevent AR-mediated gene transcription was quantified on a global level. For this analysis, protein coding genes which displayed a ≥ 2.55 fold increase in expression in the presence of DMSO/R1881 vs. DMSO/EtOH were defined as androgen stimulated. Using this method, 113 and 138 genes were identified for LNCaP and LNCaP-RALR cells respectively, with 74 genes common to both cell lines. Within these subsets, we next identified genes which were inhibited ≥ 2 fold by either ralaniten or enzalutamide in the presence of R1881. This analysis confirmed that on global scale, the ability of ralaniten to effectively inhibit AR mediated transcription was largely blunted in LNCaP-RALR cells compared to LNCaP cells (LNCaP-
RAL\textsuperscript{R}: 21.8\% of genes inhibited; vs. LNCaP: 68.2\% of genes inhibited). Conversely, enzalutamide was highly effective in both lines, inhibiting the majority of AR regulated genes (LNCaP-RAL\textsuperscript{R}: 84.1\% of genes inhibited; vs. LNCaP: 94.7\% of genes inhibited; Figure 2.8 A). As implied with the xenograft study, this experiment indicates that resistance to ralaniten is directly related with its inability to adequately inhibit AR transcriptional activity, and specifically the regulation of genes involved with growth and survival.

Hierarchical clustering identified a cohort of genes which were specifically upregulated across treatments in LNCaP-RAL\textsuperscript{R} compared to LNCaP cells (Figure 2.8 B). These genes are part of a subfamily (UGT2B) belonging to the \textit{UDP-glucuronosyltransferase} superfamily of enzymes with Phase II metabolic functionality. While the UGT2B pathway will be the primary focus of Chapter 3, briefly these enzymes function to mediate the conjugation of glucuronic acid to suitable substrates. This renders the target physiologically non-functional and highly hydrophilic allowing its excretion via the biliary or renal pathways [253,254]. UGT2B enzymes specifically target nucleophilic hydroxyl moieties of a wide variety of lipophilic substrates including drugs, environmental pollutants, steroid hormones and others [253–258]. While mainly expressed in the liver and gastrointestinal tract, UGT2B15, UGT2B17 and UGT2B28 are also found in the prostate and have been shown to act upon steroid hormones to maintain homeostasis [253,254,256–260].

We first validated the microarray data which showed levels of UGT2B genes were specifically elevated in LNCaP-RAL\textsuperscript{R} cells compared to parental LNCaP cells by using qRT-PCR and western blot assays in additional samples (Figure 2.8 C-D). UGT2B isoforms were highly enriched in the resistant line compared to parental LNCaP cells at both the mRNA and protein level. \textit{UGT2B11} and \textit{UGT2B28} were strongly induced by androgen in LNCaP-RAL\textsuperscript{R}
cells but poorly expressed in parental cells regardless of androgen. Consistent with previous reports that \textit{UGT2B15} and \textit{UGT2B17} are androgen-repressed genes, we too observed that expression of these genes were inhibited by synthetic androgen R1881, and that enzalutamide partially restored levels of expression (Figure 2.8 C; ref 258-260).

\subsection*{2.4 DISCUSSION AND CONCLUSIONS}

Here we have successfully created a model of acquired ralaniten resistance in an effort to pre-emptively begin development of additional inhibitors to the AR-NTD. The resulting resistant cell line was created following long-term culture of parental LNCaP cells in media containing increasing concentrations of ralaniten until stable growth in the presence of inhibitory concentrations of ralaniten was observed. Highly resistant clones were selected and expanded, with clone D7/LNCaP-RAL\textsuperscript{R} chosen for further characterization. Importantly, in creating the resistant line, LNCaP-RAL\textsuperscript{R} cells were maintained in the presence of serum containing androgens. This ensured that resistance mechanisms which evolved over long-term culture were specifically against chronic AR-NTD inhibition only, preventing alternative mechanisms arising through combined suppression of the AR-NTD and AR-LBD. Accordingly, the initial characterization of the cell line using both in vitro and in vivo experiments revealed that cross-resistance between ralaniten and antiandrogens (which act through competitive inhibition of the AR-LBD) did not occur. This phenomenon may not have happened had LNCaP-RAL\textsuperscript{R} cells been generated in an androgen-free environment. The implication of this is that should small molecule inhibitors targeting the AR-NTD make their way into clinical applications (especially in the pre-ADT setting), that existing compounds targeting the AR-LBD may prove effective if or when acquired resistance to ralaniten develops.
Our model indicates that the extent that ralaniten is able to block AR transcriptional activity was shown to be closely related to its antiproliferative and antitumour effects. Most notable was the microarray analysis which demonstrated that the majority of AR regulated genes which were inhibited by ralaniten in the parental LNCaP line were no longer in the resistant line. Conversely, enzalutamide was highly effective in preventing AR mediated gene expression and again retained antiproliferative effects in both cell lines. This is not wholly surprising given that the majority of prostate cancers continue to rely upon the AR transcriptional program even at the castration-resistant stage [134–137,158]. In conjunction with the AR knockdown experiments, these data clearly show the continued reliance of LNCaP-RAL\textsuperscript{R} cells upon maintaining a functional AR transcriptional program, allowing us to rule out AR bypass as a mechanism of resistance in this model. This is further evidence that alternative strategies targeting the AR could prove efficacious in the context of acquired ralaniten resistance.

In attempting to identify the primary method of resistance leading to acquired ralaniten resistance, we endeavored to eliminate (or quickly identify) some of the most common potential mechanisms which are associated with current AR-targeted therapies. These often converge upon the AR itself and include AR gene amplification and overexpression [216,217], gain-of-function mutations [137,218,261], and expression of constitutively active AR splice variants [130,184,185,213]. We did not expect any of these mechanisms to arise, as each has been shown to be associated with resistance to antagonists of the AR-LBD, and we observed sensitivity to enzalutamide in the resistant line.

Secondly, ralaniten specifically binds the TAU-5 region within the AF-1 in the AR-NTD [225–227], a region which contains roughly 65% of the transcriptional activity of the AR [79,104]. Due to the fact that the AR-NTD is integral to the function of the AR, we also did not
expect to find structural variations or mutations in this region as this could very well lead to a transcriptionally dead receptor. Additionally, ralaniten has been previously shown to inhibit AR-variant driven transcriptional activity and proliferation [169,221,225]. Furthermore we have also previously demonstrated the ability of ralaniten to block the transcriptional activity of AR gain-of-function mutants in the LBD using luciferase reporter constructs [221]. In following, no significant differences could be discovered when comparing the LNCaP-RAL R line to parental LNCaP cells within the context of any of these AR modifications.

Instead we discovered that genes associated with drug metabolism and excretion were preferentially expressed in the resistant line using a whole transcriptome microarray. These genes were UGT2B isoforms which function to catalyze the conjugation of glucuronic acid onto lipophilic substrates including xenobiotics and drugs [253–258]. Additional samples validated the microarray findings and confirmed that LNCaP-RAL R cells had increased expression of these genes both at the mRNA and protein level. A mechanism of resistance involving the selective metabolism of ralaniten agrees well with the data presented thus far, in that in this way any modification to the AR transcriptional program is unnecessary as ralaniten is simply deactivated and excreted from the cells. As will be discussed in much greater detail in Chapter 4, ralaniten is indeed a substrate for UGT2B mediated glucuronidation, containing three hydroxyl groups which could be potentially modified.

Bicalutamide exists as a racemate of two stereoisomers; with nearly all antiandrogenic activity residing in the (R)-enantiomer and little to no activity in the (S)-enantiomer [262,263]. While (S)-bicalutamide is rapidly and directly glucuronidated, (R)-bicalutamide must first be hydroxylated by CYP450. While glucuronidation does occur, it happens much more slowly and to a far lesser extent [262–264]. Furthermore, glucuronidation of bicalutamide has been
experimentally shown to occur predominantly through the activity of UGT2B7, UGT1A8 and UGT1A9, with UGT1A9 having dominance relative to the others [263]. In our work, we identified UGT2B11, UGT2B15, UGT2B17 and UGT2B28 as the major isoforms expressed in the resistant line. Thus the fact that we observed sensitivity to bicalutamide in the resistant line is not contradicted by previous studies. Similarly, elimination of enzalutamide occurs primarily through hepatic mediated phase I metabolism by CYP2C8 and CYP3A4 [265]. This also agrees with our observations that LNCaP-RAL\textsuperscript{R} cells retain sensitivity to this second generation antiandrogen as well.

The data presented herein point to a mechanism of acquired resistance to ralaniten involving its preferential metabolism and clearance via glucuronidation. The UGT2B pathway will be the primary focus of Chapter 3. There data will be discussed which support that silencing UGT2B isoforms may be sufficient to restore sensitivity to ralaniten in the resistant line, and ectopic expression may lead to the development of resistance in cells previously sensitive to ralaniten.
Table 2.1  *Antibody sources and working concentrations.*

<table>
<thead>
<tr>
<th>Target</th>
<th>Species</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
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<td>RB</td>
<td>Santa Cruz</td>
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</tr>
<tr>
<td>FKBP5</td>
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<td>Santa Cruz</td>
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</tr>
<tr>
<td>PSA (C19)</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>HA-Tag</td>
<td>RB</td>
<td>Cell Signalling</td>
<td>1:1,000</td>
</tr>
<tr>
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<td>MS</td>
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<td>α-mouse</td>
<td>HS</td>
<td>Cell Signalling</td>
<td>1:10,000</td>
</tr>
<tr>
<td>α-rabbit</td>
<td>GT</td>
<td>Cell Signalling</td>
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</tr>
<tr>
<td>α-goat</td>
<td>DK</td>
<td>Santa Cruz</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

*a* Abbreviations: **RB**, rabbit; **GT**, goat; **MS** mouse; **HS**, horse; **DK**, donkey.
<table>
<thead>
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<th>Target</th>
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Figure 2.1. Generation of a model of acquired resistance to ralaniten

(A) Representative photographs at 20X magnification of the parental LNCaP and the initial resistant cell line once stable growth in the presence of 25 µM ralaniten was demonstrated. The resistant line was generated following continuous passage with increasing concentrations of ralaniten up to 25 µM. Cells were split once weekly, and received fresh media containing ralaniten twice weekly. (B) Growth curves of parental LNCaP and Resistant cell lines demonstrate acquired resistance had developed. Data are presented as mean ± SEM and significance was determined using a two-way ANOVA with Sidak's test to correct for multiple comparisons. n=3 independent experiments, #p<0.0001, n.s. not significant. (C) As the resistant line was heterogeneous, highly resistant clones were isolated and challenged with increasing concentrations of ralaniten (50 µM). Representative photographs of individual clones treated with 50 µM ralaniten are shown at 20X magnification. (D) Doubling times of each of the clones were measured once stable growth was seen in presence of 50 µM ralaniten. Clone D7 was chosen further characterization (hereafter referred to as LNCaP-RALR) as it had the fastest doubling time. Data are presented as mean ± SD, n=3 independent experiments.
Figure 2.2. LNCaP-RAL<sup>R</sup> cells retain sensitivity to antiandrogens in vitro

(A) Dose response curves of LNCaP and LNCaP-RAL<sup>R</sup> cells treated with ralaniten, bicalutamide or enzalutamide and stimulated with 0.1 nM R1881. The IC<sub>50</sub> for each compound was calculated for both cell lines. **RAL:** LNCaP = 16.91 µM, LNCaP-RAL<sup>R</sup> = 37.77 µM, p=0.0046. (B) LNCaP-RAL<sup>R</sup> cells had similar sensitivity to antiandrogens as parental LNCaP cells indicating cross-resistance did not occur. **BIC:** LNCaP = 8.01 µM, LNCaP-RAL<sup>R</sup> = 7.14 µM; p=0.539; **ENZ:** LNCaP = 0.92 µM, LNCaP-RAL<sup>R</sup> = 1.01 µM; p=0.660. *n=4* independent experiments. IC<sub>50</sub> values were calculated using a linear (RAL and BIC) or non-linear regression and interpolating where the line crossed 50% growth. Significance was determined between the two cell lines for individual concentrations of each compound by two-way ANOVA followed by Sidak's test to correct for multiple comparisons. *p<0.05; **p<0.01; *p<0.0001; n.s., not significant.
Figure 2.3. Ralaniten no longer inhibits AR transcriptional activity in LNCaP-RAL<sup>R</sup> cells

(A) Transcript levels of AR, KLK2, PSA, NKX3.1, FKBP5 and RHOU normalized to levels of SDHA transcript from LNCaP and LNCaP-RAL<sup>R</sup> cells treated with ralaniten (35 µM), enzalutamide (5 µM) or DMSO control and stimulated with R1881 (1 nM). LNCaP-RAL<sup>R</sup> cells show reduced sensitivity to ralaniten compared to LNCaP cells, yet AR transcriptional activity is impaired following treatment with an antiandrogen. n=3 independent experiments, data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Dunnett's test to correct for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; # p<0.0001; n.s., not significant. (B) Protein levels of AR and AR regulated genes from whole cell lysates treated as in A.
Figure 2.4  **LNCaP-RAL** \(^R\) cells require AR for proliferation

(A) Western blots of AR levels in LNCaP and LNCaP-RAL\(^R\) cells transfected with control or AR siRNA (10 nM) for 24-96 hours. (B) Crystal violet assay measuring proliferation of cells 96 hours post transfection with either scrambled or AR siRNA (10 nM) and stimulated with 0.1 nM R1881. \(n=3\) independent experiments, data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Dunnett's test to correct for multiple comparisons. **p<0.01; ***p<0.001; #p<0.0001; n.s. not significant.
Figure 2.5. LNCaP-RAL\textsuperscript{R} cells retain biological resistance to ralaniten and sensitivity to enzalutamide in vivo

(A) LNCaP and LNCaP-RAL\textsuperscript{R} tumour growth in castrated mice treated daily with ralaniten (200 mg/kg), enzalutamide (10 mg/kg) or vehicle by oral gavage. Tumours were harvested 2 days after last treatment. Data presented as mean ± SEM and significance was determined by two-way ANOVA followed by Sidak's test (LNCaP) or Tukey's test (LNCaP-RAL\textsuperscript{R}) to correct for multiple comparisons. (B) Representative photographs of LNCaP-RAL\textsuperscript{R} tumours. Scale bars: 10 mm. (C) Weight of LNCaP-RAL\textsuperscript{R} tumours harvested from mice at the termination of the experiment. (D) Body weight change over the course of the experiment. (E) Weight of organs harvested at completion of experiment. Data presented as mean ± SEM and significance was determined by two-way ANOVA followed by Tukey's test to correct for multiple comparisons for C-E. p<0.05; **p<0.01; ***p<0.001; # p<0.0001; n.s., not significant.
Figure 2.6. AR transcriptional activity in LNCaP-RAL\textsuperscript{R} xenografts is not blocked by ralaniten

(A) Real-time PCR of AR, FKB5, RHOU, and PSA transcript normalized to levels of SDHA transcript harvested from LNCaP-RAL\textsuperscript{R} tumours (\(n=8\) tumours/sample for all genes except FKB5, \(n=6\) for RAL). Data are presented as mean ± SEM and significance was determined by one-way ANOVA followed by Dunnett’s test to correct for multiple comparisons. *\(p<0.05\); **\(p<0.01\); ***\(p<0.001\); n.s., not significant. (B) Representative examples of IHC from LNCaP-RAL\textsuperscript{R} tumours showing AR and PSA protein expression levels.
Figure 2.7. LNCaP-RAL\textsuperscript{R} cells do not express AR-variants or harbour novel mutations

(A) Transcript levels of AR or AR-V7 normalized to levels of \textit{SDHA} transcript from LNCaP and LNCaP-RAL\textsuperscript{R} cells treated with ralaniten (35 µM), enzalutamide (5 µM) or DMSO and stimulated with 1 nM R1881. Untreated LNCaP95 and VCaP cells were used as positive controls for variant expression. \textit{n}=3 independent experiments, data are presented as mean ± SEM. (B) Protein levels of AR and AR-variant were detected using an antibody recognizing the AR-NTD. Whole cell lysates were harvested from cells treated as in A. (C) Cartoon of the AR gene and protein showing functional domains. Sequencing the entire AR did not reveal any mutations in the NTD, although two were picked up in the LBD (N692S and H776R) in addition to the T877A mutation native to LNCaP cells. (D) When 26 additional bacterial clones were sequenced to confirm the authenticity of these mutations, only a new Q783L mutation was detected in more than one clone (2 of 26). The T877A mutation was seen in all 26, indicating that the others were likely the result of erroneous mistakes during the PCR process.
Figure 2.8. Global gene expression analysis reveals that UGT2B expression is associated with acquired ralaniten resistance

(A) Analysis of global gene expression identified genes which were specifically induced by R1881 (LNCaP=113; LNCaP-RAL<sup>R</sup>=138). The Venn diagrams show the percentage of androgen induced genes in this subset whose expression decreased by ≥ 2 fold in the presence of enzalutamide or ralaniten. (B) Heatmap representing differentially regulated genes between LNCaP-RAL<sup>R</sup> (group 1/blue) and LNCaP (group 2/black) cells treated with ralaniten (35 µM), enzalutamide (5 µM) or DMSO vehicle and stimulated with 1 nM R1881 or EtOH vehicle. Four UGT2B isoforms (UGT2B11, UGT2B15, UGT2B17, and UGT2B28 were identified which were highly enriched in the resistant line (between 10- and 18-fold increase) compared to matched LNCaP samples (n=2 per treatment group). (C) Transcript levels of UGT2B isoforms normalized to levels of SDHA transcript in additional samples validated microarray data. n=3 independent experiments, and data are presented as mean ± SEM. (D) Microsomes harvested from untreated parental LNCaP and LNCaP-RAL<sup>R</sup> cells revealed increased basal expression of UGT2B isoforms in the resistant line.
Chapter 3. Resistance to Ralaniten is Mediated via Metabolism by UGT2B Enzymes

3.1 INTRODUCTION

UDP-glycosyltransferases (UGT) comprise a superfamily of enzymes which function primarily to detoxify lipophilic substrates by phase II metabolism [254,266–268]. These genes are found in all kingdoms of life; from fungi and bacteria to plants and vertebrates, highlighting their evolutionary importance in clearing xeno- and endobiotics [267–269]. Thousands of structurally diverse substrates from a variety of sources are recognized by UGT enzymes, including drugs, environmental pollutants and carcinogens, as well as steroids, bile acids, vitamins, hormones and dietary constituents [254]. All UGT genes function by catalyzing the conjugation of sugars to lipophilic substrates using uridine diphospho- (UDP) based sugar cofactors as illustrated in Figure 3.1. Indeed UGT genes share a similar protein structure and all have a common 44 amino acid sequence which is thought to aid in recognition of UDP [268]. With some rare exceptions, conjugation results in the substrate containing significantly less biological activity than the parental compound. Furthermore, the conjugate has increased polarity and molecular weight, facilitating its excretion via the biliary or renal pathways [254,266–268,270].

Across mammals, 117 members have been identified, of which 22 protein coding genes are expressed in humans [268–270]. These are divided into four main families termed UGT1,

UGT2, UGT3 and UGT8. In humans, enzymes belonging to the UGT1 and UGT2 families typically utilize uridine diphospho-glucuronic acid (UDPGA) as a cofactor [267,268,270], however UGT3 and UGT8 have evolved to use alternative sugars such as UDP-glucose, UDP-galactose and UDP-xylose [267,270]. The UGT1 and UGT2 families contain nearly all of individual isoforms expressed in humans (19 of 22 members), and can be further divided into subfamilies UGT1A, UGT2A and UGT2B. These are the most widely studied due to both their pervasiveness as well as their preferential functionality in recognizing and detoxifying drugs as compared to UGT3 and UGT8 [267]. For the remainder of this dissertation, UGT will be used to specifically refer to members of the human UGT1 and UGT2 families.

UGTs are transmembrane glycoproteins which are generally localized to the endoplasmic reticulum (ER) with ~95% of the protein contained within the lumen [271–274], although some isoforms have been identified which exist within the nuclear membrane as well [275]. Several UGT proteins have been shown to form both homo- and heteroligomers with other isoforms, and it has been proposed that this may aid in expanding the number of substrates which could be recognized [266,272]. Despite the fact that discrete substrate preferences exist between UGT isoforms, a considerable amount of overlap has also been demonstrated. This observation supports the hypothesis that oligomerization between separate isoforms may influence substrate selectivity [266,267,272,276]. Additionally, oligomerization may also assist with the formation of channels, mediating the active transport of UDPGA cofactor into the ER lumen. While this has yet to be experimentally confirmed, this would theoretically improve the efficiency of glucuronidation by ensuring substrate and UDPGA cofactor were in close proximity [266].

As stated previously, a major focus when characterizing the function of UGT enzymes in the literature revolves around their ability to mediate the detoxification and excretion of
therapeutic drugs [253,267,276–282]. In fact it has been estimated that 1/10 of the top 200 drugs prescribed are cleared primarily by direct glucuronidation [281]. This presents a major challenge when developing novel compounds, specifically in ensuring sufficient bioavailability is maintained within a therapeutic range. However, additional consideration is also required for drugs cleared by glucuronidation with respect to drug-drug interactions (DDI). As significant overlap in substrate specificity exists between UGT isoforms, coadministration of compounds which also undergo UGT mediated clearance can lead to adverse effects and drug associated toxicity [267,281,283]. This is especially important considering that some UGT enzymes also are specifically involved in detoxification of endogenous substances, such as bilirubin by UGT1A1 [253,267,276,281].

Due to their specialization in detoxifying lipophilic compounds, nearly all UGT isoforms may be found to some extent in hepatic tissues, as the liver is the primary clearance organ [253,267,276,279]. However on the whole, UGT genes show a wide range of tissue distribution and individual isoforms can be concurrently expressed in extraheptaic tissues including the gastrointestinal tract, lungs, brain, kidneys, skin and others [253,272,276,279]. The differential expression of specific isoforms is thought to be regulated by epigenetic mechanisms such as promoter hypermethylation [267]. Additionally, the relative amount of TA repeats contained within promoter regions [253] and tissue-specific transcription factors [267], have also been demonstrated to regulate relative UGT expression. This reflects the diversity that exists within UGT isoforms with respect to functions which are independent of detoxification. For example, enzymes part of the UGT2A subfamily are often found in the olfactory tissues and are specialized to conjugate a wide variety of odorants, terminating the olfactory signal [256,271,284]. Additionally, several UGT2B genes have also been shown to regulate
homeostasis of steroidal hormones and may also be found in hormone sensitive tissues including the prostate [257–260,285]. While the expression of UGT2B enzymes within the prostate is traditionally thought to primarily play a role in regulating the levels of androgens [256,260,285], this pathway could theoretically be exploited by tumours to also aid in the elimination of therapeutic drugs. We discovered that LNCaP-RAL\textsuperscript{R} cells had elevated UGT2B expression compared to parental LNCaP cells. As ralaniten is a small lipophilic molecule it could potentially be a suitable substrate for UGT2B enzymes. We therefore postulated that increased clearance of ralaniten mediated by UGT2B activity might be a mechanism of resistance to ralaniten.

The overall objective was to confirm whether enhanced glucuronidation was causatively associated with acquired ralaniten resistance, and if preventing this from occurring could restore sensitivity to ralaniten. To answer these questions, we attempted to: 1) validate that enzymatic activity in the LNCaP-RAL\textsuperscript{R} line correlated with increased transcript and protein expression as compared to parental LNCaP cells; 2) to restore sensitivity to ralaniten through selective knockdown of UGT2B genes; and 3) to induce resistance to ralaniten through ectopic expression of these genes in the ralaniten-sensitive parental LNCaP cell line.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Cell culture

LNCaP cells were maintained in phenol-red free RPMI 1640 (Invitrogen) supplemented with 5\% heat-inactivated FBS (VWR). LNCaP-RAL\textsuperscript{R} and other clones derived from the initial resistant line were maintained in phenol red-free RPMI 1640 supplemented with 5\% heat-inactivated FBS and 50 \(\mu\)M ralaniten. LNCaP-GFP, LNCaP-2B15 and LNCaP-2B17 clones
were maintained in phenol red-free RPMI 1640 supplemented with 5% heat-inactivated FBS and 1.5 μg/mL puromycin (Sigma). Cells were resuscitated immediately prior to experimentation, and maintained for no more than 10 to 15 passages. Cells were periodically tested to ensure they were mycoplasma-free.

### 3.2.2 Lentiviral transduction of LNCaP-GFP, -2B15 and -2B17 cells

Stable expression of UGT2B15-HA, UGT2B17-HA, and GFP-HA was achieved by lentiviral infection. Recombinant lentiviral particles were produced by the cotransfection of 293T cells with 10 μg of expression vector UGT2B15-HA (LV352002), UGT2B17-HA (LV352008) or GFP-HA (LV032) in combination with 2nd Generation packaging mix (LV003) and transfected using LentiFectin™ transfection reagent (G074) according to the manufacturer’s instructions. All components were purchased from Applied Biological Materials (ABM, Richmond, Canada), and the vector system was pLenti-GIII-CMV-C-term-HA. 48 hours post transfection, media was collected and concentrated by centrifugation at 25,000 rpm for 90 minutes at 4°C.

LNCaP cells were transduced by plating 3x10⁵ cells/well in 6-well plates and infecting with respective lentiviral particles (MOI: ~10⁵-10⁶; at a ratio of 1:1). After 24 hours of incubation at 37°C at 5% CO₂, media was removed and replaced with complete media. Following a further 24 hour incubation, media was changed once more to that containing 1.5 μg/mL puromycin to begin selection of transduced cells. After 7 days of selection, cells from 10 unique colonies for each expression vector were chosen for further expansion and aliquots were removed for initial confirmation of protein and mRNA expression. Clones were stored in liquid nitrogen.
3.2.3 Gene and protein expression assays

To examine endogenous levels of UGT2B mRNA in parental and resistant clones, LNCaP, LNCaP-2B15, LNCaP-2B17, LNCaP-GFP, B7, D7, G7, E9, F9 and G9 cells (1.5 x 10^5 cells/well) were plated on 6-well plates in respective growth media. After 24 hours, cells were harvested in 1 mL/well TRIzol reagent (Invitrogen) and total RNA was extracted, cleaned, reverse transcribed and measured using qRT-PCR QuantStudio 6 Flex Real-Time PCR System as described in section 2.2.4. Gene expression was normalized to the house keeping gene SDHA, and sequences for gene-specific primers are shown in Table 2.2.

In the experiment examining sensitivity to DHT in LNCaP and LNCaP-RAL^R cells, (1.5x10^5 cells/well) were seeded onto 6-well plates and serum starved for 24 hours prior to pre-treatment with DMSO vehicle or ralaniten (35 µM) for 16 hours before stimulation with synthetic androgen R1881 (1 nM), DHT (1 nM) or EtOH vehicle. Cells were harvested in 1 mL/well TRIzol reagent (Invitrogen), reverse transcribed, and measured using qRT-PCR QuantStudio 6 Flex Real-Time PCR System as described in section 2.2.4. Gene expression was normalized to the house keeping gene SDHA, and sequences for gene-specific primers are shown in Table 2.2.

3.2.4 Proliferation and dose response assays

For the dose response assay using resistant clones, 5,000 cells/well were seeded onto 96-well plates as described previously. After given 24 hours to attach, cells were pre-treated with DMSO vehicle, or ralaniten (0.1, 0.5, 1, 5, 10, 25, 50 µM) for one hour. For experiments involving lentiviral transduced cells, 0.1, 1, 10, 25, 35 µM ralaniten was used. Cells were then stimulated with R1881 (0.1 nM) or EtOH vehicle. Cells were stained with 0.1% crystal violet 96
hours following treatment as described in section 2.2.3. Absorbance was read using a VersaMax Microplate Reader (Molecular Devices) at 595 nm. Data was background subtracted and normalized to DMSO/R1881 treatment to calculate androgen dependent growth.

Crystal violet staining was utilized to measure cellular proliferation for the DHT sensitivity experiment. LNCaP or LNCaP-RAL<sup>R</sup> cells were seeded onto 96-well tissue culture plates in RPMI 1640 supplemented with 5% charcoal-stripped FBS at a density of 5,000 cells/well. Cells were given 24 hours to attach to the plate prior to treatment. Cells were pre-treated with DMSO vehicle or ralaniten (25 µM) for 1 hour before stimulation with synthetic androgen R1881 (0.1 nM), DHT (0.1 nM) or EtOH vehicle. Plates were harvested every 24 hours and cell number was estimated against a standard curve (0-30,000 cells) which were seeded on plates 24 hours prior to harvesting. Cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma) 96 hours following treatment as described in section 2.2.3. Absorbance was read using a VersaMax Microplate Reader (Molecular Devices) at 595 nm.

3.2.5 Harvesting microsomes and quantifying UGT2B enzymatic activity

Livers were removed from euthanized mice and immediately processed. All steps were carried out on ice. Approximately 3.0x10<sup>8</sup> cultured cells (LNCaP, LNCaP-RAL<sup>R</sup>, LNCaP-2B, and LNCaP-GFP) were washed with PBS, pelleted and homogenized using a 28.5 gauge needle. Microsomes were isolated using sequential, high speed centrifugation. Buffers and a protease cocktail were supplied with the Microsome Isolation Kit (Biovision, Milpitas, USA) and the procedure was carried out according to manufacturer's instruction.

A bicinchoninic acid assay was used to determine protein concentration of microsomes prior to beginning further experiments. UGT2B activity was quantified by measuring the
amount of proluciferin substrate consumed by glucuronidation using the UGTglo Kit (Promega, Madison, USA) according to manufacturer's instruction. The reaction conditions consisted of 50 µg of microsomes isolated from each cell line, 100 µM substrate, 5 mg/mL alamathecin, 50 mM TES (pH 7.5), 8 mM MgCl₂ and 4 mM UDPGA to a total volume of 40 µL and incubated at 37°C for 2 hours. 1X luciferin detection reagent was added and luminescence was measured using a GloMax® 96 microplate luminometer (Promega, Madison, USA). For experiments measuring enzymatic activity of lentiviral transduced cells, the fluorometric UGT Activity Assay kit (Biovision, Milpitas, USA) was used according to manufacturer's instruction. Reaction conditions consisted of 35 µg of microsomes isolated from each cell line, 40 µM UGT substrate, 1X reaction buffer and 1X UDPGA to a total volume of 100 µL. Fluorescence was measured using a Safire² Multi-Mode Plate Reader (Tecan, Männendorf, Switzerland). Measurements were taken every 2 minutes for 60 cycles with Ex/Em=415/502 nm and the bandwidth was set at 20 nm.

### 3.2.6 siRNA transfection and proliferation assays

For experiments examining protein or mRNA expression, cells were plated on 10 cm (1x10⁶ cells/dish) or 6-well (1.5x10⁵ cells/well) plates respectively in normal culture media for 24 hours prior to transfection. Pooled siRNA against UGT2B15 (L-020194-02-0005), UGT2B17 (L-020195-00-0005) and non-targeting control (D-001810-10-05) were purchased from Dharmaco Research (Layfayette, USA). After 24 hours, media was removed and replaced with Opti-MEM (Gibco) containing 15 nM siRNA/transfection reagent complexes (Lipofectamine RNAiMAX Transfection Reagent, Invitrogen). 24-96 hours post transfection, protein or mRNA was harvested and analyzed as described in section 2.2.4 Gene expression was normalized to the
house keeping gene SDHA, and sequences for gene specific primers are shown in Table 2.2. Antibodies used for detection of proteins and their concentrations are given in Table 2.1.

For the proliferation assays, cells were plated on 24-well plates (3x10^4 cells/well) and transfected as described above. After 24 hours, cells were treated with 25 µM ralaniten and 0.1 nM R1881 or equal volume EtOH vehicle. After 72 hours post treatment, cells were fixed and stained with 0.1% crystal violet as described previously. Absorbance was read using a VersaMax Microplate Reader (Molecular Devices) at 595 nm.

3.2.7 Statistical analysis

A One- or Two-Way ANOVA statistical test was used to determine significance for all comparisons unless otherwise stated (Graphpad Prism, version 7.0). p-value corrections were applied for all multiple comparisons (Tukey, Sidak or Dunnett as appropriate), and a p-value < 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 LNCaP-RAL^R cells have increased UGT2B activity

We confirmed that increased levels of UGT2B expression observed in the resistant LNCaP-RAL^R cell line was correlated with enzymatic activity. UGT enzymes are primarily localized in the ER, therefore the microsomal fraction was isolated from both parental LNCaP and LNCaP-RAL^R cells which were grown in their normal culture media. UGT enzymatic activity was quantified by using a luciferase based assay system (UGT-Glo™, Promega) illustrated in Figure 3.2 A. Briefly, the assay exploits the fact that proluiciferin is a substrate for UGT2B mediated glucuronidation. In the absence of UDPGA cofactor (or UGT enzyme), the
substrate remains unconjugated, and subsequent incubation with luciferase produces light. Conversely, glucuronidation of proluciferin prevents its activation by luciferase. The difference in luminescence in samples incubated in the presence and absence of UDPGA can be calculated, and used to quantify the percentage of substrate consumed. Additionally, the antibiotic alamathecin was added to the reaction mixture. Alamathecin is a pore forming peptide and has been shown to increase UGT2B mediated conjugation by 2-3 fold due to the increased exposure of the enzymatic active site (which is located within the lumen of the ER) to both substrate and UDPGA cofactor [286].

We found that microsomes harvested from LNCaP-RAL R cells had nearly a 6-fold increase in enzymatic activity compared to parental LNCaP cells (40.31% substrate consumed vs. 6.48% respectively, p=0.0003; Figure 3.2 B). This experiment clearly indicates that the UGT2B isoforms found to be overexpressed in the LNCaP-RAL R cell line are functional, and lends support to our hypothesis that increased UGT2B activity is causatively associated with acquired ralaniten resistance in this cell line. However, this assay is a cell-free based experiment, and the methodology necessitates that cells be killed in order to extract the microsomes. We wanted to at least indirectly demonstrate that increased UGT2B enzymatic activity was increased in living LNCaP-RAL R cells.

To this end we took advantage of the fact that UGT2B15 and UGT2B17 (which were the isoforms which were most highly expressed in LNCaP-RAL R cells) both function to maintain androgen homeostasis by modulating DHT concentration through glucuronidation [256,258,260,287,288]. Conversely, the synthetic androgen R1881 is much more stable and is resistant to glucuronidation [289]. This being the case, we hypothesized that LNCaP-RAL R cells would have less sensitivity to DHT than parental LNCaP cells. To test this, the ability of both
DHT and R1881 to induce transcription and cellular proliferation were examined. No significant differences could be determined between either DHT and R1881 within LNCaP cells, with respect to either transcription or proliferation. Conversely, LNCaP-RAL$^R$ cells were not sensitive to DHT while responding strongly to R1881 stimulation (Figure 3.3 A-B). Collectively these data demonstrate that LNCaP-RAL$^R$ cells have increased UGT2B activity compared to LNCaP cells.

3.3.2 UGT2B expression correlates with ralaniten resistance in highly resistant clones

Having identified that UGT2B levels and enzymatic activity were increased in LNCaP-RAL$^R$ cells, we next set out to look at UGT2B expression levels in the other highly resistant clones isolated as described in sections 2.2.1 and 2.3.1. Furthermore, we were interested to determine if UGT2B expression was related to the degree of ralaniten resistance (as determined by respective IC$_{50}$ in each clone).

For the gene expression experiment, clones were cultured in their native media (RPMI 1640 supplemented with 5% FBS and 50µM ralaniten) and harvested once cells reached ~80% confluency. While AR levels were stable across both clones and parental LNCaP cells, expression of UGT2B isoforms varied, although were still much higher than parental LNCaP cells (Figure 3.4 A). This suggests that cellular changes inducing increased UGT2B expression occurred prior to the isolation of individual clones from the resistant line. Therefore enhanced UGT2B expression in response to chronic ralaniten exposure might be a relatively early event. In a parallel experiment, clones were challenged with increasing concentrations of ralaniten in order to compare the relative sensitivities of each clone. No significant differences in ralaniten IC$_{50}$ were observed. However there was a trend in which clones (namely E9 and F9 which had
the lowest UGT2B expression) levels were also the most sensitive to ralaniten (Figure 3.4 A-C). Collectively these results imply that acquired ralaniten resistance is associated with elevated UGT2B expression.

3.3.3 Knockdown of UGT2B genes is sufficient to restore sensitivity to ralaniten in LNCaP-RAL R cells

We next attempted to discern whether or not overexpression of UGT2B genes was causatively associated with acquired resistance to ralaniten. To adequately address this question we endeavoured to restore sensitivity to ralaniten by targeted siRNA knock down of UGT2B15 or UGT2B17, as these isoforms showed the greatest expression in LNCaP-RAL R cells (Figure 2.8 C-D). The specificity of each siRNA pool was tested against the expression of the four main UGT2B isoforms (UGT2B11, UGT2B15, UGT2B17 and UGT2B28). While expression of UGT2B11 and UGT2B28 were unaffected by treatment with either siRNA, some cross-reactivity was initially observed between each siRNA with respect to UGT2B15 and UGT2B17 (Figure 3.5). This was not altogether surprising as UGT2B15 and UGT2B17 have ~95% sequence homology [256,287], although this prevented our ability to distinguish the specific contributions of each gene.

We confirmed that protein levels of UGT2B15 and UGT2B17 could be effectively silenced for the entire duration prior to beginning the proliferation experiment (Figure 3.6A). When additional samples were treated with ralaniten, we found that attenuation of expression of either UGT2B15 or UGT2B17 was sufficient to restore sensitivity. Importantly, the concentration of ralaniten used (25 µM) was significantly less than the concentration that LNCaP-RAL R cells are routinely exposed to in culture conditions (50 µM). Conversely, cells which were treated with a non-targeting scrambled control siRNA remained resistant to ralaniten.
(Figure 3.6 B). No additive or synergistic effect was measured when both siRNAs were used concurrently, likely due to the cross-reactivity between the two siRNA pools. This experiment reconfirmed the continued reliance of LNCaP-RAL\(^R\) cells upon AR\(^FL\) to drive cellular proliferation, and demonstrated that expression of \(UGT2B15\) or \(UGT2B17\) is causatively associated with resistance.

### 3.3.4 Ectopic expression of UGT2B15 is sufficient to reduce sensitivity to ralaniten

Finally we determined whether aberrant UGT2B15 and/or UGT2B17 expression was sufficient to induce resistance to ralaniten, or at least reduce sensitivity to it. To this end, parental LNCaP cells were transduced with a recombinant lentivirus containing an expression vector coding for HA-tagged human UGT2B15, UGT2B17 or GFP under the control of the constitutively active CMV promoter. Following puromycin selection, 10 colonies were picked from cells transduced with UGT2B15 or UGT2B17, and 2 from GFP for initial characterization and expansion. \(UGT2B15\) and \(UGT2B17\) mRNA expression in each clone was used to quickly identify a subset of potential clones for further analysis (Figure 3.7 A). From this experiment, clone C (LNCaP-GFP); clones B, D, F and H (LNCaP-2B15); and clones D, E and G (LNCaP-2B17) were chosen. Interestingly, while \(UGT2B15\) was expressed at levels equal to, or exceeding those found in LNCaP-RAL\(^R\) cells, \(UGT2B17\) expression was substantially lower across clones transduced with either construct (Figure 3.7A). Next we looked at protein levels in the eight clones compared to LNCaP-RAL\(^R\) cells (Figure 3.7 B) in an effort to further reduce the total number of clones to a manageable number. We used UGT2B mRNA and protein levels in conjunction with how easily each clone was able to be cultured in the presence of puromycin to decide on which clones to continue to maintain. In the end, LNCaP-GFP_C, LNCaP-2B15_F,
LNCaP-2B15_H, LNCaP-2B17_D and LNCaP-2B17_E were selected and used in future experiments. Aliquots from all 22 clones initially isolated were expanded and stored in liquid nitrogen.

We further characterized the clones in terms of UGT2B expression and activity. As was seen in our initial screening, LNCaP-2B15_H displayed the most robust expression of UGT2B mRNA and protein levels of all the clones. While LNCaP-2B17_E appeared to have greater mRNA expression than LNCaP-2B17_D, the latter had higher protein levels (Figure 3.8 A-B). Therefore LNCaP-2B15_H and LNCaP-2B17_D were expanded in preparation for extraction of microsomes to assay for enzymatic activity. Both LNCaP-2B15_H and LNCaP-2B17_D demonstrated enhanced ability to glucuronidate the fluorescent substrate compared to LNCaP-GFP_C. However there was also a significant difference in activity compared to LNCaP-RAL\(^R\) cells (2B15_H, \(p=0.0220\); 2B17_D, \(p=0.0005\)) possibly due to the influence of additional UGT2B isoforms which are expressed in the LNCaP-RAL\(^R\) cell line (Figure 3.8 C).

Having determined that the ectopic UGT2B enzymes that were expressed in the transduced clones were capable of glucuronidating substrate, tested if this would have any effect upon the relative sensitivity of each clone to ralaniten. To this end we challenged clones with escalating doses of ralaniten and compared each IC\(_{50}\) to that of control LNCaP-GFP_C cells. LNCaP-GFP_C cells displayed an IC\(_{50}\) of 18.15 \(\mu\)M, comparable to past experiments using parental LNCaP cells (16.91 \(\mu\)M; Figure 2.2 A). While all transduced clones had reduced sensitivity to ralaniten, significant differences were only observed for LNCaP-2B15_H and LNCaP-2B17_D (Figure 3.9 A-B).

The LNCaP-RAL\(^R\) cell was generated by serial passaging of LNCaP cells in escalating concentrations of ralaniten until cells ultimately demonstrated stable growth at a concentration of
50 µM ralaniten. Lastly, we looked at the ability of transduced cells to tolerate equivalent levels of ralaniten. 1.5x10⁵ cells were plated in native media (RPMI 1640 plus 5% FBS and 1.5 µg/mL puromycin; transduced clones or RPMI 1640 plus 5% FBS; LNCaP-RAL<sup>R</sup>) supplemented with 50 µM ralaniten. Images were captured 24 and 96 hours after plating. Consistent with previous observations, both LNCaP-2B15_H and LNCaP-2B17_D were less sensitive to ralaniten than LNCaP-GFP_C; with LNCaP-2B15_H demonstrating the greatest tolerance to ralaniten. Nonetheless, both clones were still more sensitive to ralaniten than LNCaP-RAL<sup>R</sup> cells (Figure 3.9 C).

3.4 DISCUSSION AND CONCLUSIONS

Whole transcriptome analysis revealed that UGT2B genes were preferentially upregulated in LNCaP-RAL<sup>R</sup> cells. Similarly, we observed that enzymatic activity in the resistant line was also increased. Microsomes harvested from LNCaP-RAL<sup>R</sup> cells had nearly a 6-fold increase in enzymatic activity as compared to those isolated from the parental line. Three of the isoforms which were identified (UGT2B15, UGT2B17 and UGT2B28) have been previously shown to normally be expressed in the prostate where they function to maintain hormone homeostasis [253,254,256–260]. In following, we found that the resistant line had reduced sensitivity to DHT than the parental line, supporting that UGT2B enzymatic activity was increased in living cells.

Due to the involvement of UGT2B enzymes in mediating the detoxification and elimination of xenobiotics, we postulated that their increased expression in the resistant line could be causatively related to acquired ralaniten resistance. To test this we employed targeted siRNA against the two isoforms which were most highly expressed in the resistant line
(UGT2B15 and UGT2B17) and challenged the resistant line with 25 µM ralaniten. LNCaP-RAL\textsuperscript{R} cells are routinely cultured in the presence of 50 µM ralaniten and demonstrate stable growth in this context. We found that knockdown of UGT2B15 and/or UGT2B17 was sufficient to re-establish sensitivity to ralaniten and its ability to inhibit androgen dependent growth of the resistant line. Although some ambiguity exists between the exact contribution of each isoform as significant cross-reactivity was found between the two siRNA pools.

We also attempted the reverse experiment, to induce resistance in a previously sensitive line by forcing the ectopic expression of UGT2B genes we hypothesized were responsible for driving resistance to ralaniten. While we were successful in our efforts with respect to UGT2B15 expression, unfortunately no clones expressed UGT2B17 to comparable levels seen in LNCaP-RAL\textsuperscript{R} cells. Despite this, we were still able to detect significant differences in ralaniten sensitivity between clones expressing UGT2B15 and the control LNCaP-GFP clone. Collectively these results further implicate at least UGT2B15 in mediating acquired resistance to ralaniten.

The ability of tumour cells to specifically exploit the glucuronidation pathway as a means of attaining drug resistance has been previously established across a range of cancer types. For example, overexpression of UGT isoforms has been shown to occur as a mechanism of acquired resistance in following sustained exposure to ribavirin and cytarabine in AML [290], adriamycin and epirubicin in melanoma [291], and SN-38 [280] in lung cancer cell lines. Additionally, \textit{de novo} resistance to NU/ICRF 505 and SN-38 was observed in the HT29 colon cancer cell line (which has elevated glucuronidation activity) compared to control HCT116 cells [277,278]. In these studies siRNA knock down [291], or co-treatment with an isoform specific substrate to competitively inhibit glucuronidation [278], was sufficient to restore sensitivity to these drugs.
Strikingly, a recent report by Zahreddine et al. has shown enhanced glucuronidation as a mechanism of acquired resistance to ribavirin may also occur in patients with AML as well [290]. Elevated UGT1A levels were observed following ribavirin treatment and were closely associated with treatment failure. This group was able to demonstrate that GLI1 stabilized UGT1A protein levels, and attenuation of GLI1 both reduced UGT1A expression and restored sensitivity to ribavirin in their resistant models [290]. From the results of this study, an ongoing Phase II clinical trial (NCT02073838) is investigating the use of combining the GLI1 inhibitor vismodegib with ribavirin in patients with AML [292]. These studies clearly illustrate the potential impact of UGT enzymes on drug clearance, lending support to the validity of our work.

As mentioned previously the prostate expresses several UGT2B isoforms, and these are also found in LNCaP cells as well [255,257,285,286]. Therefore a mechanism of simply increasing the expression of these genes in response to ralaniten treatment could theoretically be achieved more easily compared to other resistance strategies such as AR mutation (especially within the NTD), or becoming reliant on an alternative signalling pathway. Resistance involving the preferential metabolism of ralaniten agrees well with our previous observations that LNCaP-RALR cells continue to rely heavily upon the AR transcriptional pathway.

We have successfully generated a model of acquired ralaniten resistance, illustrating the potential of prostate cancer cells to adapt to chronic AR-NTD inhibition. Nonetheless, this strategy has significant therapeutic potential especially for CRPC patients who have relapsed on traditional AR-targeted therapies. Ralaniten represents a first-in-class targeted therapy, and is the sole selective AR-NTD inhibitor to advance to clinical trials; thus it is a powerful tool for studying advanced prostate cancer. Therefore we are aggressively pursuing alternative small molecule inhibitors in an effort to fully realize the potential of these new compounds.
Figure 3.1 Mechanism of glucuronidation by UDP-glucuronosyltransferase (UGT) enzymes

The human UGT1 and UGT2 family of enzymes catalyze the Phase II metabolism reaction termed glucuronidation. Using uridine diphosphoglucuronic acid (UDPGA) as a cofactor, glucuronic acid is conjugated to a suitable nucleophilic functional group (i.e. aliphatic alcohols, phenols, carboxylic acids, aromatic and aliphatic amines, thiols and acidic carbon atoms) contained upon lipophilic substrates. The resulting glucuronide conjugate is highly polar and generally physiologically non-functional. Thus glucuronidation is primarily a detoxifying mechanism and promotes the excretion of lipophilic compounds via the biliary or renal pathways. Adapted from Figure 1 from Rowland et al. (2013). International Journal of Biochemistry and Cell Biology. 45(6):1121-1132. Reproduced with permission of Elsevier via Copyright Clearance Center [267].
Figure 3.2. UGT2B activity is increased in LNCaP-RAL\textsuperscript{R} cells

(A) Cartoon illustrating rationale behind enzymatic activity assay. The proluciferin substrate contains a hydroxyl group and is a target for glucuronidation by UGT2B enzymes. \textit{d}-cysteine converts the proluciferin substrate into luciferin which produces light when incubated with luciferase. Glucuronidation of the proluciferin substrate prevents light from being produced and therefore the amount of substrate consumed can be quantified. Reproduced with permission from Promega Corporation. © 2018. All Rights Reserved. (B) UGT2B enzymatic activity quantified using microsomes harvested from LNCaP and LNCaP-RAL\textsuperscript{R} cells. \textit{n}=4 independent experiments, and data are presented as mean ± SEM. Significance was determined by unpaired Student’s T-test. ***\textit{p}<0.001.
Figure 3.3. LNCaP-RAL\textsuperscript{R} cells have reduced sensitivity to DHT

(A) Transcript levels of \textit{FKBP5}, \textit{KLK2}, \textit{PSA} and \textit{RHOU} normalized to levels of \textit{SDHA} transcript from LNCaP and LNCaP-RAL\textsuperscript{R} cells treated with 1 nM R1881, DHT or v/v EtOH. DHT is a substrate for UGT2B15 and UGT2B17 and LNCaP-RAL\textsuperscript{R} cells are much less sensitive to it than synthetic R1881. \textit{n}=3 independent experiments, and data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Dunnett’s test to correct for multiple comparisons.

(B) Growth curves for both cell lines show the ability of R1881 or DHT to stimulate androgen dependent proliferation. \textit{n}=3 independent experiments, and data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Tukey’s test to correct for multiple comparisons. *\textit{p}<0.05; **\textit{p}<0.01; ***\textit{p}<0.001; # \textit{p}<0.0001; n.s., not significant.
**Figure 3.4. Enhanced UGT2B expression occurs in clones resistant to ralaniten**

(A) Real-time PCR of AR, *UGT2B7, UGT2B11, UGT2B15, UGT2B17*, and *UGT2B28* transcript normalized to levels of *SDHA* transcript harvested from untreated LNCaP and individual clones isolated from the resistant cell line. While some variability is seen with regards to UGT2B expression between the clones, all have higher expression than LNCaP cells. Conversely, AR levels continue to remain stable across clones. *n*=3 independent experiments, and data are presented as mean ± SD. (B) Dose response curves of LNCaP and resistant clones treated with ralaniten, and stimulated with 0.1 nM R1881. The IC$_{50}$ values were calculated using a linear regression and interpolating where the line crossed 50% growth. (C) Graph of ralaniten IC$_{50}$ values for each resistant clone compared to LNCaP. Clone D7 is LNCaP-RAL$^R$. 
Cells treated with 15 nM siRNA targeting *UGT2B15* and *UGT2B17* efficiently silenced mRNA expression. Significant cross-reactivity was observed with respect to *UGT2B15* and *UGT2B17*, however no other isoforms were silenced by siRNA treatment. *n*=4 independent experiments, and data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Dunnett’s test to correct for multiple comparisons. *p*<0.05; **p*<0.01; ***p*<0.001; #p*<0.0001; n.s., not significant.
Figure 3.6. Biological resistance to ralaniten is dependent upon expression of UGT2B15 or UGT2B17

(A) Western blots of UGT2B15/17 protein levels in LNCaP-RAL cells transfected with 15 nM control, UGT2B15 (top), or UGT2B17 (bottom) siRNA for 24-96 hours. (B) Crystal violet assay measuring proliferation of cells 96 hours post transfection with either scrambled, UGT2B15 and/or UGT2B17 siRNA (15 nM). 24 hours after transfection, cells were treated with ralaniten (25 µM) or DMSO vehicle and stimulated with R1881 (0.1 nM). Silencing of either UGT2B15 or UGT2B17 was sufficient to restore sensitivity to ralaniten. n=3 independent experiments, and data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Dunnett's test to correct for multiple comparisons. *p<0.05; **p<0.01; n.s., not significant.
Figure 3.7. Initial characterization of lentiviral transduced clones

(A) Real-time PCR of UGT2B15 and UGT2B17 mRNA transcript of normalized to levels of SDHA transcript harvested from untreated clones isolated following lentiviral transduction and LNCaP-RAL$^R$ cells. $n=1$ biological sample run in triplicate, data is represented as mean ± SEM. Based upon the initial screen, eight clones with the highest UGT2B expression were chosen for characterization of protein expression levels and to further isolate potential candidates. (B) Western blots of ectopic and endogenous UGT2B15/17 in LNCaP-RAL$^R$ and lentiviral transduced clones. GFP_C; 2B15_F; 2B15_H; 2B17_D; and 2B17_E were chosen for further analysis.
Figure 3.8. Ectopic UGT2B expression has enzymatic activity in lentiviral transduced clones

(A) Real-time PCR of UGT2B11, UGT2B15, UGT2B17, and UGT2B28 mRNA transcript of normalized to levels of SDHA transcript harvested from untreated clones isolated following lentiviral transduction and LNCaP-RALR cells. n=3 independent experiments and data are presented as mean ± SEM. Significance was determined by one-way ANOVA followed by Dunnett's test to correct for multiple comparisons. *p<0.05; **p<0.01; #p<0.0001; n.s., not significant. (B) Western blots of ectopic and endogenous UGT2B15/17 in LNCaP-RALR and lentiviral transduced clones. (C) UGT2B enzymatic activity quantified using microsomes harvested from LNCaP-RALR, 2B15_H, 2B17_D and GFP_C cells. n=3 independent experiments, and data are presented as mean ± SEM. Significance was determined by two-way ANOVA followed by Tukey's test to correct for multiple comparisons. ***p<0.001; #p<0.0001.
Figure 3.9. Ectopic expression of UGT2B15 reduces sensitivity to ralaniten

(A) Dose response curves of showing androgen dependent growth of LNCaP-RAL\textsuperscript{R} and lentiviral transduced clones treated with ralaniten and stimulated with 0.1 nM R1881. IC\textsubscript{50} values were calculated using a linear regression and interpolating where the line crossed 50% growth. (B) IC\textsubscript{50} values plotted for each cell line. \textbf{L-GFP_C}: 18.15 µM, \textbf{LNCaP-RAL\textsuperscript{R}}: 39.68 µM, p=0.020; \textbf{L-2B15_F}: 25.94 µM, p=0.088; \textbf{L-2B15_H}: 32.48 µM, p=0.049; \textbf{L-2B17_D}: 28.14 µM, p=0.047; \textbf{L-2B17_E}: 25.71 µM, p=0.091. \textit{n}=5 independent experiments, and data are presented as mean ± SEM. (C) Representative photographs of cells exposed to 50 µM ralaniten supplemented in native media at 24 and 96 hrs post treatment. Magnification is 10X.
Chapter 4. Bypassing Glucuronidation to Overcome Acquired Ralani
ten Resistance³

4.1 INTRODUCTION

UGT2B15 and UGT2B17 were the UGT isoforms which had the greatest expression in LNCaP-RAL\(^R\) cells, and we have demonstrated that their overexpression was causatively associated with ralaniten resistance. UGT2B15 and UGT2B17 are well characterized enzymes and have been shown to specialize in catalyzing O-glucuronidation, specifically targeting hydroxyl functional groups on target substrates [256,287,288]. Ralaniten contains three hydroxyl groups which are potential targets for UGT2B mediated glucuronidation (Figure 4.1 A-B). Furthermore, the clearance of bisphenol-A (BPA) has been shown to be primarily cleared by glucuronidation via UGT2B15 activity [255]. Though lacking any androgenic or estrogenic activity, ralaniten is itself a metabolite of BPA and shares some structural resemblance [169]. Taken together with the data generated thus far, there is a strong indication that ralaniten is preferentially metabolized by one or more UGT2B isoforms, allowing resistant cells to maintain AR transcriptional activity.

There are two main strategies which could be employed to restore sensitivity to ralaniten in the resistant cell line. The first involves suppressing UGT2B activity to allow enhanced exposure of cells to unconjugated compound. We have already demonstrated that this is effective in principal using targeted knockdown of \textit{UGT2B15} and \textit{UGT2B17}. However using

this strategy in a clinical context is problematic due to both the widespread tissue distribution of UGT2B genes, and the fact that wide substrate specificity exists with regard to both endogenous and exogenous substances - including carcinogens [253,276,293,294]. While some drugs which inhibit specific UGT isoforms do exist, they are typically competitive inhibitors being substrates themselves. Therefore these are mostly used as probes to identify specific isoforms involved in laboratory drug-drug interaction studies, rather than being utilized to specifically inhibit UGT activity in the clinical setting [253,279,283,293–295].

Alternatively, modifying the original drug scaffold could be a strategy to reduce levels of metabolism by glucuronidation. Altering existing functional groups has been shown to optimize drug stability by preventing UGT mediated elimination [296,297]. The molecular changes do not necessarily have to be significant as even stereochemistry has been shown to have a profound effect upon the ability of UGT enzymes to metabolize substrates [262–264,298–300].

Ralaniten was first identified as the lead clinical candidate following a large scale screening program of potential AR-NTD inhibitors [169]. As we had the benefit of having previously developed a library of compounds (some of which had undergone initial characterization), we decided to see if one of these might show resistance to UGT2B mediated glucuronidation. In this way we could avoid potential problems associated with nonspecific inhibition of UGT2B enzymes. Furthermore, while also attempting to overcome acquired ralaniten resistance, we aimed to accelerate the development of back-up compounds which show increased stability compared to ralaniten.
4.2 MATERIALS AND METHODS

4.2.1 Cell culture

LNCaP cells were maintained in phenol-red free RPMI 1640 (Invitrogen) supplemented with 5% heat-inactivated FBS (VWR). LNCaP-RALR cells were maintained in phenol red-free RPMI 1640 supplemented with 5% heat-inactivated FBS and 50 µM ralaniten. PC3 cells were maintained in DMEM supplemented with 5% heat-inactivated FBS. LNCaP-GFP, LNCaP-2B15 and LNCaP-2B17 clones were maintained in phenol red-free RPMI 1640 supplemented with 5% heat-inactivated FBS and 1.5 µg/mL puromycin (Sigma). Cells were resuscitated immediately prior to experimentation, and maintained for no more than 10 to 15 passages. Cells were periodically tested to ensure they were mycoplasma-free.

4.2.2 Detection and isolation of glucuronidated EPI compounds

The ability of ralaniten or its analog EPI-045, to competitively inhibit the glucuronidation of luciferin substrate was determined by repeating the UGTglo assay in the presence of each compound. Reactions consisted of 5 µg microsomes (isolated from mouse livers), 50 µM substrate, 50 mM TES (pH 7.5), 8 mM MgCl₂, 4 mM UDPGA and 2mM ralaniten, EPI-045 or equal volume DMSO vehicle to a total volume of 40 µL. The reaction mixture was incubated at 37°C and plates were read at 15 minutes, 30 minutes, 60 minutes, 90 minutes and 120 minutes. 1X luciferin detection reagent was added and luminescence was measured using a GloMax® 96 microplate luminometer (Promega, Madison, USA).

Detection and isolation of glucuronidated compounds was carried out using HPLC. Microsomes isolated from mouse liver were incubated with ralaniten or EPI-045 in reaction buffer for 16 hours at 37°C. The reaction mixture contained 50 µg mouse liver, 15.8 µg of
respective compounds, 4 mM UDPGA, 50 mM TES (pH 7.5), 8 mM MgCl₂, 25 µg/mL alamathecin and 30U β-glucuronidase or v/v phosphate buffer (3.24 mM, pH 6.8) to a total volume of 200 µL. Following incubation, the reaction mixture was centrifuged for 15 minutes at 12,000 relative centrifugal force (rcf) and the supernatant was stored on ice until the samples were analyzed via C₁₈ reversed-phased HPLC using an InertSustain 5 µM column (25 X 0.4 cm). An isocratic gradient system of 67:33 was employed containing (0.05% TFA/H₂O)/MeCN for 28 minutes followed by a linear gradient to 11:9 (0.05% TFA/H₂O)/MeCN over an additional 20 minutes (flow rate 1 mL/min), with UV detection at 201 nM. Synthetic ralaniten G-1, G-2, and G-3 and EPI-045 G-1 and G-2 glucuronides were used as standards for retention time comparison and coinjection (Figure 4.1 A-D).

Pure samples of the mono-glucuronides of ralaniten following incubation (G-1, G-2, G-3) were obtained by HPLC separation on a CSC-Insertsil 150A/ODS 2, 25 x 0.94 cm column using isocratic elution at 2 mL/min with either MeCN: H₂O (0.05% TFA) = 35%: 65% (ralaniten G-1 and ralaniten G-2) or MeCN: H₂O (0.05% TFA) = 32.5%: 67.5% (ralaniten G-3) monitored at 230 nM. EPI-045 consisted of a mixture of four stereoisomers. Consequently, the mono-glucuronides EPI-045 G-1 and EPI-045 G-2 were obtained as mixtures of diastereomers after C₁₈ reversed-phase HPLC using a InertSustain 5 µm, 25 x 1.0 cm column with 13:7 (0.05%TFA/H₂O)/MeCN as eluent and UV detection at 201 nm. The diastereomeric mixtures of EPI-045-G-1i and EPI-045-G-1ii, and EPI-045-G-2i and EPI-045-G-2ii were isolated. The constitutions of EPI-045-G-1i, EPI-045-G-1ii, EPI-045-G-2i and EPI-045-G-2ii have been unambiguously assigned, but their absolute configurations have not been assigned. The constitutions of each of the pure mono-glucuronides was confirmed by detailed analysis of their 1D and 2D nuclear magnetic resonance (NMR) and high resolution mass spectra (HRMS). The
proton NMR spectrum of synthetic ralaniten glucuronides was compared with the ralaniten metabolite isolated following biological incubation with mouse liver microsomes.

4.2.3 Detection of ralaniten metabolites in human hepatocytes and clinical samples

Sample preparation and analysis of ralaniten metabolites following incubation with human hepatocytes and those isolated from patient serum, were performed by RMI Laboratories. For the human hepatocyte experiment, 10 µM of ralaniten was incubated with hepatocytes in incubation media (inVitroGRO KHB buffer, IVT) for 4 hours at 37°C. The sample was quenched and centrifuged at 13,000 rpm and the supernatant was analyzed by UPLC-HRMS using a C18 reverse-phased Phenomenex Kinetex BP 1.7 µM column (2.1 x 50 mm) and run on a Waters ACUITY I-Class system. An isocratic gradient system of 90:10 for 0.5 minutes; 58:42 for 3 minutes; 45:55 for 13 minutes; 25:75 for 1.5 minutes; 5:95 for 1 minute; and 90:10 for 1 minute was employed containing (0.1% formic acid/H2O)/(0.1% formic acid/MeCN) at a flow rate of 0.5 mL/min. Product ion spectra were acquired by a separate UPLC-HRMS/MS run to assign structures of metabolites by MS/MS conducted on a Waters Xevo G2 XS system.

For identification of ralaniten metabolites in patients, plasma samples were pooled using the Hamilton pooling method. 50 µL were quenched with two volumes of ACN and centrifuged for 5 min at 13,000 rpm and the supernatant from each sample was analyzed by LC/MS using the same conditions as outlined above. Product ion spectra were acquired by a separate LC-MS/MS run to assign structures of metabolites by MS/MS conducted on a Waters Xevo G2 XS system.
4.2.4 Dose response assays

5,000 cells/well were seeded onto 96-well plates as described previously. After given 24 hours to attach, cells were pre-treated with DMSO vehicle, ralaniten, EPI-002053 (synthetic RAL-G-3/M15) or EPI-045 (0.1, 0.5, 1, 5, 10, 25, 35 µM) for one hour. For experiments involving lentiviral transduced cells, 0.1, 1, 10, 25, 35 µM EPI-045 was used. Cells were then stimulated with R1881 (0.1 nM) or EtOH vehicle. Cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet 96 hours following treatment as described in section 2.2.3. Absorbance was read using a VersaMax microplate reader (Molecular Devices) at 595 nm. Data was background subtracted and normalized to DMSO/R1881 treatment to calculate androgen dependent growth.

For the initial characterization of EPI-045, 2,000 cells/well were seeded onto 96-well plates. Following a 24 hour incubation cells were pre-treated with DMSO vehicle, ralaniten or EPI-045 (0.1, 0.5, 5, 15, 35 µM) for one hour. Cells were stimulated with R1881 (0.1 nM) or EtOH vehicle. 72 hours post treatment, cell viability was measured using the Alamar Blue assay. 10 µL Alamar blue was added directly to wells and cells were incubated at 37°C for 2 hours. Plates were read using an Infinite M1000 microplate reader (TECAN) with Ex/Em=570/585 nm and bandwidth set at 5 nm. Data was background subtracted and normalized to DMSO/R1881 treatment to calculate androgen dependent growth.

4.2.5 Protein and gene expression assays

The ability of EPI-002053 and EPI-045 to inhibit AR transcriptional activity was assessed by measuring expression of classical AR regulated genes at the protein and mRNA level following treatment with EPI-002053, EPI-045 or ralaniten in the presence of R1881. For
experiments involving EPI-002053, only LNCaP cells were used. For gene expression, LNCaP and LNCaP-RAL$^R$ cells (1.5x10$^5$ cells/well) were seeded onto 6-well plates. After incubation for 24 hours at 37°C to allow cells to attach, media was removed and cells were serum starved for 24 hours prior to pre-treatment with DMSO vehicle, ralaniten (35 µM), EPI-002053 (35 µM) or EPI-045 (35 µM) for 16 hours before stimulation with synthetic androgen R1881 (1 nM) or EtOH vehicle. Cells were harvested in 1 mL/well TRIzol reagent (Invitrogen), reverse transcribed, and measured using qRT-PCR QuantStudio 6 Flex Real-Time PCR System as described in section 2.2.4. Gene expression was normalized to the expression of housekeeping gene SDHA, and sequences for gene-specific primers are shown in Table 2.2.

For protein expression, 1x10$^6$ cells/well were seeded onto 10 cm plates, serum-starved and treated as above. 24 hours after R1881/EtOH treatment, cells were harvested in RIPA buffer and separated by SDS-PAGE as outlined in section 2.2.4. Antibodies and concentrations used are shown in Table 2.1.

4.2.6 Xenografts and ethics approval

Male NOD/SCID mice at 6 to 8 weeks of age were subcutaneously injected with LNCaP or LNCaP-RAL$^R$ cells (1x10$^7$ cells/site) using Matrigel (Becton Dickinson). Once tumours reached ~100 mm$^3$, mice were castrated and treatment began one week later. Mice were randomized into treatment groups receiving ralaniten (50 mg/kg), EPI-045 (50 mg/kg) or vehicle control (15% DMSO/30% PEG-400) every other day tail vein injection. Mouse body weight and tumour volume (defined as volume = length x width x height x 0.5236) were regularly recorded and tumours were excised 2 days after the last treatment. The University of British Columbia
Animal Care Committee approved all experiments involving animals and these studies conformed to relevant regulatory and ethical standards.

4.2.7 Ki67 and TUNEL staining in LNCaP-RAL\textsuperscript{R} xenografts

Sections (5 µm thick) were cut from formalin fixed paraffin-embedded tissues and deparaffinized in xylene and rehydrated in alcohols and distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 minutes, followed by washing in PBS three times. Sections were incubated with anti-Ki67 (Dako envision system HRP/DAB kit) at 4°C overnight. Antigen was detected with 3,3-diaminobenzidine and counterstained with haematoxylin. The ApopTag® Fluorescein In Situ Apoptosis Detection Kit (MILLIPORE) was used for the TUNEL assay. Cells positive for Ki67 or TUNEL staining were counted from three different sections of three different xenografts for each treatment. Ki67: 2732 (vehicle); 2514 (ralaniten) and 2619 (EPI\textsuperscript{-045}) cells in total were counted. For TUNEL staining, 3123 (vehicle); 3538 (ralaniten) and 3400 (EPI\textsuperscript{-045}) cells were counted.

4.2.8 Gene and protein expression in LNCaP-RAL\textsuperscript{R} xenografts

To analyze tumour gene expression, tumours were flash frozen and ~100 mg were pulverized using a motor and pestle under liquid nitrogen. The samples were added to 1 mL TRIzol reagent (Invitrogen) and homogenized using a pre-chilled dounce tissue grinder. RNA was extracted and reverse transcribed as detailed previously. For IHC, 5µM thick sections of tumour tissue were cut from formalin fixed paraffin-embedded tissues and deparaffinized in xylene and rehydrated in alcohols and distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 minutes, followed by washing in PBS three times.
Incubation with the following primary antibodies: anti-AR N-20 (1:300; Santa Cruz) and anti-UGT2B15 (1:100; Abcam) were incubated at 4°C overnight. Antigen was detected with 3,3-diaminobenzidine and counterstained with haematoxylin.

4.2.9 Statistical analysis

A One- or Two-Way ANOVA statistical test was used to determine significance for all comparisons unless otherwise stated (Graphpad Prism, version 7.0). p-value corrections were applied for all multiple comparisons (Tukey, Sidak or Dunnett as appropriate), and a p-value < 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 EPI-045 is resistant to UGT2B mediated O-glucuronidation

We identified and confirmed that LNCaP-RALR cells had exploited the glucuronidation pathway as a means to deactivate and excrete ralaniten. Specifically, this was mediated through the overexpression of UGT2B15 and/or UGT2B17 which preferentially recognize hydroxyl functional groups on target substrates and catalyze O-linked glucuronidation [256,287,288]. We anticipated that the primary hydroxyl group on C1 would be the principal target for glucuronidation due to its presumed ease of access by UGT2B enzymes. Therefore we pursued EPI-045, a drug candidate previously identified in a large scale screen for AR-NTD inhibitors. EPI-045 is structurally very similar to ralaniten, yet predicted to show resistance to UGT2B mediated glucuronidation due to the conversion of the primary alcohol on C1 to a methoxy functional group (Figure 4.1 C). The preliminary characterization of EPI-045 was done by others in our lab prior to the development of the LNCaP-RALR cell line. Like ralaniten, EPI-045
specifically targets the AR and does not inhibit the growth of the AR-negative PC3 cell line (Figure 4.2 A). The IC\textsubscript{50} of EPI-045 and ralaniten in LNCaP cells were very similar confirming that modification of the primary hydroxyl moiety does not abrogate its ability to block AR mediated proliferation.

To test the hypothesis that EPI-045 was resistant to glucuronidation, we first repeated the UGTglo assay. For this preliminary experiment we attempted to determine whether mouse liver microsomes coincubated with ralaniten, EPI-045, or DMSO vehicle could competitively inhibit luciferin substrate consumption. Repeated measurements were taken to observe the rate of consumption over time. As expected, ralaniten reduced the amount of substrate consumed as it is also presumed to be a substrate for UGT2B mediated glucuronidation. Conversely EPI-045 had no effect implying that it is not metabolized by these enzymes (Figure 4.2 B).

Next, we attempted to validate our findings by using HPLC to identify individual glucuronide conjugates formed following incubations of ralaniten or EPI-045 with mouse liver microsomes in the presence and absence of uridine 5'-diphosphoglucuronic acid (UDPGA) cofactor. β-glucuronidase is an enzyme which catalyzes the hydrolysis of glucuronic acid from glucuronidated substrates, and was used to confirm that peaks identified using HPLC chromatograms were in fact glucuronides. A single peak corresponding to ralaniten-glucuronide was detected when microsomes were incubated with UDPGA, and this peak disappeared following co-treatment with β-glucuronidase (Figure 4.3, top row). The small peaks detected from EPI-045 treated microsomes that were incubated with UDPGA also disappeared with β-glucuronidase treatment. These data suggest that EPI-045 may be a substrate for UGT2B mediated glucuronidation but to lesser degree than ralaniten (Figure 4.3, bottom row).
4.3.2 Ralaniten is glucuronidated at the secondary alcohol on C20

To further understand how ralaniten was being metabolised, we next attempted to delineate which hydroxyl specie(s) contained on ralaniten was the primary target for glucuronidation. The HPLC experiment was repeated so that the glucuronide fraction could be isolated and analyzed by $^1$H NMR and compared to individual synthetic monoglucuronides of ralaniten (Figure 4.4). We expected that the primary alcohol on C1 would be the main target moiety, given that we had previously shown that EPI-045 did not inhibit glucuronidation of luciferin substrate. Surprisingly we instead found that the glucuronide product corresponded to RAL-G-3, a metabolite formed following conjugation on the secondary alcohol at C20 (Figure 3.9). This finding helps explain why EPI-045 may be partially glucuronidated, however considering that EPI-045 also contains this functional group, this does raise the question as to why it is glucuronidated to a lesser extent than ralaniten. A possible explanation is that altering the primary alcohol restricts recognition or binding of EPI-045 by UGT2B enzymes due to changes in the hydrophobicity of the compound compared to ralaniten. Further work will be required to definitively understand this mechanism.

Additional characterization of ralaniten metabolites was carried out independently of our work by the Contract Research Organization RMI laboratories. Consistent with the data presented here, profiling of ralaniten metabolites using UPLC-HRMS/MS following incubation with human hepatocytes revealed that one of the major pathways of ralaniten clearance in humans was by direct glucuronidation. Strikingly, the most abundant metabolite (M15) was equivalent to RAL-G-3, as the glucuronide was also formed from the secondary alcohol on C20 (Figure 4.5).
A Phase I clinical trial investigating ralaniten acetate (EPI-506, the pro-drug of ralaniten) in the post-enzalutamide or post-abiraterone setting was completed in November 2017. Ralaniten acetate was given orally at escalating doses to determine the safety profile, maximum tolerated dose, pharmacokinetics and preliminary indications of anti-tumour activity (ClinicalTrials.gov identifier: NCT02606123, ESSA Pharma, Inc.). The maximum dose tested of 3600 mg/day was well tolerated and imparted PSA responses in some patients, however it was unfortunately rapidly metabolized leading to poor pharmacokinetic properties. As a result, the trial ultimately was terminated prior to beginning Phase II due to excessive pill burden. Plasma samples collected from patients treated at 3600 mg/day were analyzed by LC/MS and were used to identify the major metabolites of ralaniten. Ion spectra of the metabolite products was obtained using UPLC-HRMS/MS to profile individual metabolites (also carried out by RMI laboratories). Importantly the glucuronide conjugate M15, was again identified and represented the second most abundant metabolite (Figure 4.6). This implies that hepatic mediated glucuronidation of ralaniten may be a significant mechanism for its clearance. Collectively these data clearly demonstrate that ralaniten is a good substrate for glucuronidation, that this seems to preferentially involve the alcohol group on C20, and occurs readily in humans.

4.3.3 Glucuronidated ralaniten is incapable of inhibiting AR transcriptional activity

Glucuronidation is typically thought of as a detoxifying pathway, and the biological activity of the substrate is reduced due to both the steric hindrance of the glucuronide adduct, as well as its increased rate of excretion from the cell. An important exception is the opioid morphine. Formed by UGT2B7 metabolism, morphine-6β-glucuronide has an approximately a 100-fold increase in pharmacological activity than the parent compound [301]. Therefore we
wanted to confirm that glucuronidation of ralaniten did in fact eliminate its ability to function as an AR-NTD inhibitor.

EPI-002053 was synthesized by our colleagues at the University of British Columbia, and is structurally identical to both RAL-G-3 (isolated via HPLC following incubation with liver microsomes) and M15 (the ralaniten glucuronide identified in human plasma samples). LNCaP cells were used for these experiments, as they have been repeatedly shown to be sensitive to ralaniten. While ralaniten treatment was sufficient to reduce androgen stimulated gene transcription at both the mRNA and protein level, equivalent treatment with EPI-002053 had no effect (Figure 4.7 A-B). Importantly neither was EPI-002053 able to inhibit the androgen-dependent growth of LNCaP at any concentration tested. Conversely, ralaniten was associated with a dose-dependent decrease in cellular proliferation (Figure 4.7 C). Unsurprisingly, these data confirm that glucuronidation of ralaniten abrogates its activity, and supports our hypothesis that acquired resistance to ralaniten is mediated through metabolism by UGT2B enzymes.

4.3.4 EPI-045 is effective in inhibiting growth of cells resistant to ralaniten

Due to the fact that EPI-045 was glucuronidated to a lesser extent than ralaniten, we aimed to further characterize this AR-NTD inhibitor in its ability to inhibit AR transcriptional activity in both LNCaP and LNCaP-RAL\textsuperscript{R} cells. EPI-045 was able to significantly inhibit expression of AR regulated genes both at the mRNA and protein level (Figure 4.8 A-B). This agrees well with the earlier data which indicate that modification of the primary hydroxyl moiety does not reduce its ability to inhibit the AR. Importantly, LNCaP-RAL\textsuperscript{R} cells also displayed sensitivity to EPI-045 supporting its ability to evade glucuronidation. The ability of EPI-045 to inhibit androgen dependent growth was reconfirmed and no significant difference in sensitivities
were found with respect to the IC$_{50}$ in either cell line (LNCaP: 17.79 µM vs. LNCaP-RAL$^R$: 14.70 µM; p=0.96, Figure. 4.8 C). These results show that the sensitivity to EPI-045 by LNCaP-RAL$^R$ cells is not due to increased potency compared to ralaniten, but rather from its ability to avoid glucuronidation.

We next tested the efficacy of EPI-045 in its ability to inhibit AR transcriptional activity, and androgen-dependent growth in the lentiviral transduced clones which we had previously generated and characterized. With respect to inhibiting AR transcriptional activity, ralaniten was less effective than was EPI-045 at preventing the expression of AR regulated genes for both 2B15_H and 2B17_D cells. Comparatively, the control GFP_C clone remained sensitive to both ralaniten and EPI-045 (Figure 4.9 A). In following with previous observations, some sensitivity to ralaniten was seen when compared to LNCaP-RAL$^R$ cells. Therefore while expression of ectopic UGT2B15 achieved here is able to reduce the efficacy of ralaniten, it is not sufficient to mediate complete resistance. This implies that expression of additional UGT2B isoforms are also required as seen in the LNCaP-RAL$^R$ line. Finally we also assessed the ability of EPI-045 to inhibit androgen-dependent growth of the transduced clones. As expected, all demonstrated approximately equivalent sensitivity to EPI-045, and no significant differences were seen between clones or LNCaP-RAL$^R$ cells (Figure 4.9 B-C).

Following the success of EPI-045 to inhibit androgen-dependent growth and AR transcriptional activity in LNCaP-RAL$^R$ and lentiviral transduced cells, we next attempted to test the potency of EPI-045 in vivo. Castrated mice bearing LNCaP-RAL$^R$ xenografts were randomized to receive ralaniten (50 mg/kg), EPI-045 (50 mg/kg), or vehicle every other day by tail vein injection. Drugs were administered in this way to limit the amount of first-pass metabolism, and also to avoid differences in oral bioavailability between the two compounds.
EPI-045 had significant anti-tumour activity compared to both vehicle (p<0.0001) and ralaniten (p=0.0016, Figure 4.10 A-B), and both ralaniten and EPI-045 were well tolerated at 50 mg/kg (Figure 4.10 C). The superiority of EPI-045 over ralaniten was further demonstrated as markers associated with proliferation (Ki67) and apoptosis (TUNEL) were decreased and increased respectively, compared to levels in tumours harvested from mice treated with either vehicle or ralaniten (Figure 4.10 D-E). Conversely, LNCaP xenografts responded equally well to either treatment. Interestingly ralaniten was also associated with a small yet significant reduction in tumour volume in mice bearing LNCaP-RALR xenografts compared to vehicle treatment (p=0.0263). This was in contrast to what we observed when ralaniten was dosed orally (Figure 2.5 A), and may be partially due to hepatic clearance of ralaniten when given orally versus intravenously. Despite this, the data presented here indicate that EPI-045 has increased potency versus ralaniten in LNCaP-RALR xenografts which is due to its ability to resist glucuronidation

Neither ralaniten nor EPI-045 had any significant effect on the protein levels of AR or UGT2B15 as measured by IHC in harvested tumours (Figure 4.11 A). Conversely, EPI-045 significantly reduced levels of KLK2, FKBP5 and RHOU mRNAs in harvested tumours whereas ralaniten had no effect (Figure 4.11 B). Collectively, these results support previous data which showed inhibiting AR transcriptional activity was sufficient to block proliferation in LNCaP-RALR cells. Furthermore, these experiments reveal that EPI-045 is able to avoid glucuronidation by UGT2B enzymes and yet remains an AR antagonist despite minor modifications of its scaffold. Thus using rational drug design, we were able to overcome acquired ralaniten resistance in the LNCaP-RALR cell line. With this work, we have established a proof-of-principal which may be used to guide the development of second generation AR-NTD inhibitors which will ideally demonstrate enhanced stability and efficacy compared to ralaniten.
Furthermore, the LNCaP-RAL\textsuperscript{R} cell line is an established model which can be utilized to challenge these inhibitors against a background of enhanced glucuronidation even as they are being developed.

4.4 DISCUSSION AND CONCLUSIONS

LNCaP-RAL\textsuperscript{R} cells remain dependent upon AR transcriptional activity to mediate mitogenic and proliferative signalling. Acquired resistance to ralaniten occurs due to the enhanced expression of UGT2B enzymes which function to eliminate substrates by glucuronidation. As hydroxyl groups represent the target moiety on potential substrates for both UGT2B15 and UGT2B17, we used an analog of ralaniten (EPI-045) which had the primary hydroxyl group modified to restrict UGT2B mediated glucuronidation. While we expected that glucuronidation would occur on the primary alcohol, the data presented thus far strongly indicate that this is not the case. Instead, we found that ralaniten is preferentially glucuronidated on the secondary alcohol on C20, and also occurs in patients who were given ralaniten orally.

Members of the CYP450 family have long been recognized of critical importance with respect to drug metabolism. Indeed the first crystal structure of a member of the CYP450 was published in 1985 [302], and as of 2016 a further 22 human isoforms had been solved [303]. These studies have proven invaluable in generating models to predict substrate and inhibitor selectivities of preclinical compounds. Conversely while investigations into the characterization of UGT enzymes are becoming more widespread, they have yielded fewer insights into substrate preference by comparison. Currently, the only human UGT member for which a crystal structure exists remains the C-terminal portion of UGT2B7 which was published in 2007 [272]. This is exacerbated due to the broad and overlapping substrate specificities which is often observed
across UGT members [304]. Furthermore, the C-terminal domain contains significant homology across members of the UGT family likely due to its involvement in recognizing UDPGA. However it is the highly variable N-terminal domain which has been suggested to mediate substrate specificity [272,305].

Despite these challenges, several *in silico* models for individual UGT1A and UGT2B isoforms have been developed based upon quantitative structural-activity relationships (QSAR), and has been aided by the crystallization of several soluble plant and bacterial UGT homologs [304–308]. This ligand based approach has led to the creation of databases of known and suspected substrates based upon ligand pharmacophore properties. Interestingly it has been reported (at least with UGT1A6) that substrate binding to the active site may not always necessitate its glucuronidation [306]. This implies that subtle changes in ligand chemistry may have a profound effect upon its ability to be eliminated by a particular UGT isoform. In following several other studies investigating pharmacophore modeling of potential ligands found that molecular geometry and 3D spatial orientation, stereochemistry, hydrophobicity and electrostatic interactions can significantly affect levels of glucuronidation [304–308]. Importantly, even relatively minor differences in regions other than the sites of glucuronidation may be sufficient to positively or negatively influence the ability of UGT isoforms to metabolize a given substrate [305].

Therefore, while EPI-045 is highly similar to ralaniten (and retains the preferred hydroxyl target), conversion of the primary hydroxyl group to a methoxy moiety does profoundly influence its hydrophobicity (Ralaniten: CLogP = 2.7958, EPI-045: CLogP = 4.7136). We have shown that EPI-045 is glucuronidated to a lesser extent than ralaniten and propose that this is due
to reduced recognition and/or binding of EPI-045 to UGT2B enzymes. The experiments required to definitively confirm this however, are outside the scope of this dissertation.

The mechanism of action of ralaniten involves binding to the TAU-5 region within the AF-1 of the AR-NTD [225,227]. This is a two-step chemical reaction in which the chlorohydrin group is critically important (see 1.5.3 and Figure 1.9; refs. 221,223). As ralaniten is preferentially glucuronidated on C20, it is highly unlikely that this metabolite retains any activity. However it was necessary to confirm that this is the case as isolated substrates (namely morphine) show potentiated activity following glucuronidation [301]. We tested this using a synthetic glucuronide conjugate (EPI-002053) which is chemically equivalent to the glucuronides which were isolated experimentally (RAL-G-3) or identified in human plasma (M15). Unsurprisingly we found that glucuronidation of ralaniten is sufficient to completely abrogate its ability to antagonize the AR.

As this work was being done, a Phase I/II clinical trial investigating ralaniten acetate (the orally active pro-drug of ralaniten) in the post abiraterone or enzalutamide setting was undertaken independently of our study. While demonstrating a favorable safety profile, the trial was ultimately terminated at the conclusion of the Phase I portion in November, 2017. This was due to the excessive pill burden which was required as a result of the rapid metabolism of ralaniten. As is shown in Figure 4.6, the major routes of clearance appears to be oxidation of the primary alcohol, as well as direct glucuronidation of the secondary alcohol on C20. This confirms that ralaniten is a substrate for glucuronidation in humans and is susceptible to first pass metabolism.

In this case, glucuronidation most likely took place in the livers or GI tracts of patients rather than intratumorally as a resistance mechanism to ralaniten. However this also underscores
another important facet to the work presented thus far. In attempting to overcome acquired resistance to ralaniten, we began to further characterize an alternative AR-NTD inhibitor which could be resistant to glucuronidation. In doing so, we have begun to lay the groundwork in developing second generation molecules which have greater stability and potentially greater potency than ralaniten. This has implications for not only overcoming acquired ralaniten resistance, but also for guiding future drug design with respect to reducing hepatic mediated metabolism. Furthermore, we now have a well defined cell line which overexpresses these metabolic enzymes. LNCaP-RAL$^R$ cells can be used as a model to test additional backup compounds against a background of enhanced glucuronidation. In this way we can quickly screen potential compounds and prioritize the development of potential clinical candidates which demonstrate an ability to inhibit AR transcriptional activity in this context.

We have shown that EPI-045 is able to inhibit the growth of LNCaP-RAL$^R$ cells both \textit{in vitro} and \textit{in vivo}. Its efficacy is derived from its ability to maintain sustained AR antagonism as it shows greater resistance to glucuronidation than ralaniten. Despite its enhanced potency in LNCaP-RAL$^R$ cells, EPI-045 was not chosen for further development due in part to its reduced solubility in water and by extension, lower oral bioavailability compared to ralaniten. Therefore this was primarily a proof-of-principal study showing that specific modification of the scaffold of ralaniten can restrict its metabolism by UGT2B enzymes without significantly affecting its function as an AR-NTD inhibitor.

All current AR-targeted therapies available for men with mCRPC will eventually fail, and the disease remains incurable. Small molecule inhibitors targeting the AR-NTD have tremendous potential to expand the therapeutic landscape for many of these patients. The results from the work presented here, as well as those from the clinical trial have provided important
lessons which can be used to optimize the development of backup compounds. As such we are actively investigating alternative AR-NTD inhibitors, in an effort to efficiently identify new clinical candidates which show greater potency and stability than ralaniten. It is our hope that targeted AR-NTD inhibition will soon become an important addition to the treatment options available to men with mCRPC.
Figure 4.1 Structures of ralaniten, EPI-045 and their potential glucuronide metabolites

(A) Ralaniten contains three hydroxyl functional groups and so could form three unique glucuronide metabolites.  (B) Each potential ralaniten monoglucuronide was synthesized and used as internal controls for HPLC experiments so as to assign the structure of the metabolite isolated following incubation with liver microsomes.  (C) Chemical structures of EPI-045, and (D) its potential glucuronide metabolites used for HPLC experiments.
Figure 4.2. EPI-045 antagonizes the AR and does not compete with luciferin substrate for UGT2B activity

(A) Dose response curves showing androgen stimulated growth of LNCaP and PC3 cells treated with ralaniten or EPI-045 and stimulated with 0.1 nM R1881. IC₅₀ values were calculated using a linear regression and interpolating/extrapolating where the line crossed 50% growth. **LNCaP; RAL: 9.15 µM, EPI-045: 6.65 µM, p=0.194. PC3; RAL: 145.3 µM, EPI-045: 119.9 µM, p=0.862. n=3 independent experiments for each cell line, and data are presented as mean ± SEM. (B) Ralaniten, but not EPI-045 competes with luciferin substrate for consumption by UGT2B enzymes in mouse liver microsomes. n=3 independent experiments, and significance was determined by two-way ANOVA followed by Dunnett's test to correct for multiple comparisons. *p<0.05, ** p<0.01.
Figure 4.3. HPLC chromatograms of ralaniten or its analog EPI-045 and their glucuronides

HPLC chromatograms show glucuronidated products following incubation in the presence of UDPGA cofactor. While some minor peaks are observed in samples incubated with EPI-045, the intensity is substantially lower than that seen in those incubated with ralaniten. Cotreatment with β-glucuronidase is sufficient to remove glucuronides from substrate, and thus only peaks indicating unbound drugs are seen.
Figure 4.4. Glucuronidation of ralaniten occurs primarily on the secondary alcohol bound to C20

$^1$H NMR spectra of synthetic ralaniten G-3 (100 µg/600µL) (blue) overlaid with that of the glucuronide isolated following biological incubation of 250 µg ralaniten/UDPGA with mouse liver microsomes (red) at 600 MHz in DMSO-d$_6$. RAL-G-3 represents the metabolite formed following glucuronidation of ralaniten on the secondary alcohol on C20.
Glucuronidation is a major mechanism of ralaniten clearance in humans

Detection of ralaniten metabolites using LC/MS following incubation with human hepatocytes. Product ion spectra was acquired by a separate UPLC-HRMS run to assign structures of metabolites by MS/MS. M15 represents a glucuronide metabolite equivalent to RAL-G-3, with the hydroxyl group on C20 as the target moiety for glucuronidation.
Figure 4.6. A ralaniten-glucuronide metabolite is detectable in the serum of patients treated with ralaniten

LC/MS chromatographs showing ralaniten metabolites isolated from pooled human plasma samples. Patients were treated with 3600 mg ralaniten acetate daily. M15 represents a glucuronide metabolite equivalent to RAL-G-3, as determined by subsequent LC-MS/MS.
Glucuronidated ralaniten has no activity in LNCaP cells

Figure 4.7. Glucuronidated ralaniten has no activity in LNCaP cells

(A) Transcript levels of AR, KLK2, PSA, FKBP5, RHOU, and NKX3.1 normalized to levels of SDHA transcript harvested from LNCaP cells treated with RAL (35 µM), EPI-002053 (35 µM) or v/v DMSO vehicle and stimulated with 1 nM R1881. EPI-002053 is a synthetic glucuronide conjugate of ralaniten and lacks any activity against AR transcriptional activity. *p<0.05; **p<0.01; ***p<0.001; #p<0.0001; n.s., not significant. (B) Protein levels of AR and AR regulated genes from whole cell lysates (LNCaP) treated as in A. (C) Dose response curves measuring androgen-dependent growth of LNCaP cells treated with escalating concentrations of ralaniten or EPI-002053 and stimulated with 0.1 nM R1881. IC\textsubscript{50} values were calculated using a linear regression and interpolating/extrapolating where the line crossed 50% growth. RAL: 14.78 µM, EPI-002053: 691.4 µM, p=0.007. n=4 independent experiments, and data are presented as mean ± SEM.
Figure 4.8. EPI-045 effectively inhibits AR signalling in LNCaP-RAL<sup>R</sup> cells <i>in vitro</i>

(A) Transcript levels of AR, NKX3.1, FKBP5 and RHOU normalized to levels of SDHA transcript from LNCaP and LNCaP-RAL<sup>R</sup> cells treated with EPI-045 (35 µM), RAL (35 µM) or v/v DMSO and stimulated with 1 nM R1881. AR transcriptional activity is inhibited by EPI-045 in both cell lines. <i>n</i>=3 independent experiments, and significance was determined between the two cell lines for individual concentrations of each compound by two-way ANOVA followed by Sidak's test to correct for multiple comparisons. ***p<0.001; # p<0.0001; n.s., not significant. (B) Protein levels of AR and AR regulated genes from whole cell lysates treated as in A. (C) Dose response curves of the growth of LNCaP and LNCaP-RAL<sup>R</sup> cells treated with EPI-045 and stimulated with 0.1 nM R1881. IC<sub>50</sub> values were calculated using a linear regression and interpolating where the line crossed 50% growth. **LNCaP**: 17.79 µM, **LNCaP-RAL<sup>R</sup>**: 14.70 µM, p=0.966. <i>n</i>=4 independent experiments, and data are presented as mean ± SEM.
Figure 4.9 EPI-045 effectively inhibits AR transcriptional activity in LNCaP-GFP, -2B15 and -2B17 cells in vitro

(A) Transcript levels of AR, PSA, FKBP5, and RHOU normalized to levels of SDHA transcript from lentiviral transduced and LNCaP-RALR cells treated with EPI-045 (35 µM), RAL (35 µM) or v/v DMSO and stimulated with 1 nM R1881. Ectopic expression of UGT2B15 reduces sensitivity of AR regulated genes to ralaniten treatment while EPI-045 retains potency. n=3 independent experiments, and significance was determined by two-way ANOVA followed by Dunnett's test to correct for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; #p<0.0001; n.s., not significant. (B) Dose response curves of showing androgen dependent growth of LNCaP-RALR and lentiviral transduced clones treated with EPI-045 and stimulated with 0.1 nM R1881. IC50 values were calculated using a linear regression and interpolating where the line crossed 50% growth. (C) IC50 values plotted for each cell line. L-GFP_C: 18.35 µM, LNCaP-RALR: 20.53 µM, p=0.533; L-2B15_F: 20.78 µM, p=0.602; L-2B15_H: 18.58 µM, p=0.912; L-2B17_D: 19.66 µM, p=0.912; L-2B17_E: 20.04 µM, p=0.967. n=5 independent experiments, and data are presented as mean ± SEM.
Figure 4.10. EPI-045 inhibits growth of LNCaP-RAL\textsuperscript{R} xenografts

LNCaP and LNCaP-RAL\textsuperscript{R} tumour growth in castrated mice treated every other day with ralaniten (50 mg/kg), EPI-045 (50 mg/kg) or vehicle. To reduce first pass metabolism and hepatic mediated glucuronidation, drugs were administered by tail vein injection. Tumours were harvested 2 days after last treatment. (B) Representative photographs of LNCaP-RAL\textsuperscript{R} tumours. Scale bars: 10 mm. (C) Body weight change over the course of the experiment. (D) IHC from LNCaP-RAL\textsuperscript{R} tumours showing Ki67 and TUNEL/DAPI staining. (E) Quantification of proliferative and apoptotic indices from LNCaP-RAL\textsuperscript{R} tumours. Data are presented as mean ± SEM and significance was determined by two-way ANOVA followed by Tukey's test (A,C) or a one-way ANOVA followed by Dunnnett's test (E) to correct for multiple comparisons. *p<0.05; **p<0.01; # p<0.0001; n.s., not significant.
Figure 4.11. EPI-045 inhibits AR transcriptional activity in LNCaP-RAL\textsuperscript{R} xenografts

(A) IHC from LNCaP-RAL\textsuperscript{R} tumors showing AR and UGT2B15 staining. (B) Real-time PCR of AR, PSA, FKBP5 and RHOU transcript normalized to levels of SDHA transcript harvested from LNCaP-RAL\textsuperscript{R} tumors (n=8 tumors/sample except RAL, n=7). Data are presented as mean ± SEM. *p<0.05; **p<0.01; # p<0.0001; n.s., not significant.


Chapter 5. Conclusions

5.1 RESEARCH SUMMARY

Prostate cancer afflicts more than a million men worldwide annually [4] and continues to represent a major challenge for both caregivers and patients. Due to increased screening practices, the disease is most often diagnosed at an early stage and can often be effectively managed. In many cases treatment is not required as most men will die with the disease rather than from it [7,309]. Unfortunately approximately 4% of patients present with metastatic disease, and a further 20-30% will require hormonal therapy following progression after receiving first-line treatment [7,52,211].

The vast majority of prostate cancer is classified as adenocarcinoma and is driven by aberrant AR signalling [18,129]. The AR is a modular, ligand activated transcription factor which is responsible for the exquisite regulation of hundreds genes; many involved with proliferation, survival and differentiation [114,117]. Therefore surgical or chemical castration (ADT) is initiated as a first line therapy for patients with metastatic disease in an effort to starve the AR of androgen [129]. While ADT is associated with an immediate benefit for the vast majority of patients, this treatment is considered palliative as the cancer inevitably adapts to castrate levels of androgens and transitions to CRPC within 2-3 years. CRPC is uniformly lethal and once diagnosed, patients have a life expectancy of only 18 months [149,154]. As such, prostate cancer remains the second leading cause of cancer related mortality in North American men [1,60].

Despite this, CRPC has been clearly demonstrated to continue to rely upon AR transcriptional activity, and the number of hormonal therapies available for patients in this
setting has increased in the past 5 years [233]. Tragically none are curative, and survival benefits typically do not extend past several months due to the emergence of de novo or acquired resistance [233]. Importantly, resistance mechanisms typically converge upon the AR signalling axis. Specifically these include enhanced expression of steroidogenesis genes such as CYP17A1 allowing the tumour to synthesize androgens [162,212,213]; AR gain-of-function mutations generating a promiscuous receptor able to used antiandrogens as ligands [137,218,220]; AR amplification/overexpression which permit the activation of the AR despite low levels of ligand [216,217]; and AR splice variants lacking the LBD which are constitutively active [130,213]. Because all hormone based therapies currently available are exclusively targeted against the AR-LBD, the aforementioned resistance strategies employed by the tumour are not necessarily exclusive or specific for a given treatment. This is problematic as cross-resistance can occur limiting the efficacy of second-line targeted therapies [310–313].

An alternative strategy involves targeting the AR-NTD. This region contains most if not all transcriptional activity, and unlike the LBD its presence is vital for a functional receptor [91,104]. Small molecule inhibitors targeting the NTD have been demonstrated to be highly effective in blocking AR transcriptional activity and growth of prostate cancer xenograft models [169,221,224,225]. This strategy also has the added benefit of retaining efficacy in the context of the resistance mechanisms identified above [221]. Therefore a large clinical niche exists for small molecule inhibitors of the AR-NTD as they could potentially be used prior to, following or in conjunction with, current hormonal therapies. Our lab has developed the first such agent, a compound termed ralaniten. Due to the success of ralaniten in preclinical laboratory studies, an orally active prodrug (ralaniten-acetate) was accepted into a Phase I/II clinical trial in November 2015.
This dissertation aimed to accelerate the development of additional AR-NTD inhibitors by creating and characterizing a model of acquired resistance to ralaniten. In Chapter 2, we provided evidence that chronic treatment with ralaniten lead to the generation of a resistant subline which could demonstrate stable growth in the presence of inhibitory concentrations of ralaniten. This line was termed LNCaP-RAL\(^R\) and formed the foundation of this thesis. Interestingly, we found that acquired ralaniten resistance occurred independently of common resistance mechanisms, and that LNCaP-RAL\(^R\) cells retained sensitivity to alternative compounds targeting the AR-LBD. This has exciting implications for the clinical prospects of AR-NTD inhibitors, as these data support that cross-resistance between AR-NTD and AR-LBD antagonists did not occur in our model. Furthermore, this also confirms the continued reliance of these cells upon the AR signalling pathway which is a well studied and clinically validated drug target. Molecular characterization of this cell line revealed that LNCaP-RAL\(^R\) cells had substantially increased expression of several UGT2B isoforms. These genes are a part of a large superfamily of enzymes which function to mediate the conjugation of glucuronic acid to substrates. This Phase II metabolic pathway is primarily an irreversible detoxification and clearance mechanism, and we postulated that LNCaP-RAL\(^R\) cells were exploiting these genes in order to neutralize and eliminate ralaniten.

In Chapter 3, we validated that the UGT2B genes which we previously identified were causatively associated with ralaniten resistance. Elevated UGT2B expression was found in all clones isolated from the original heterogeneous resistant line, and there was a trend between relative UGT2B mRNA levels and IC\(_{50}\) of ralaniten. This implies that ralaniten resistance is associated with UGT2B expression and that increasing UGT2B expression levels may be a relatively early event following chronic ralaniten exposure. Targeted knockdown of \textit{UGT2B15}
or UGT2B17 was sufficient to restore sensitivity to ralaniten supporting their role in mediating resistance to ralaniten.

UGT2B15 and UGT2B17 are preferentially involved in O-glucuronidation recognizing hydroxyl groups on suitable substrates [256,287,288]. Ralaniten has three hydroxyl groups which are potential targets for conjugation of glucuronic acid, and agrees well with our model of acquired resistance. We therefore attempted to further demonstrate that these genes were causatively associated with resistance by forcing the ralaniten sensitive LNCaP cell line to express ectopic UGT2B15 or UGT2B17 and testing whether these cells would lose sensitivity to ralaniten. While we were able to successfully induce expression of ectopic UGT2B15, UGT2B17 was not expressed in any transduced clone to the comparable levels seen in LNCaP-RALR cells. Nonetheless, we were able to show that sensitivity to ralaniten could be reduced compared to control cells. Collectively these data clearly implicate UGT2B15 and, to a lesser extent UGT2B17 as being causatively associated with acquired resistance to ralaniten.

Finally in Chapter 4 we proposed that resistance could be overcome by using an analog of ralaniten which is resistant to UGT2B mediated O-glucuronidation. We initially hypothesized that this would occur on the primary hydroxyl group as this is presumably the most accessible moiety. Therefore we pursued EPI-045 which has the primary hydroxyl converted to a methoxy functional group. Despite being glucuronidated to far lesser extent than ralaniten, we were surprised to find that ralaniten was instead glucuronidated on the secondary hydroxyl group on C20, as this is also contained on EPI-045. Our findings were confirmed by RMI laboratories, a contract research organization. An identical glucuronide metabolite of ralaniten was identified following a study investigating metabolite characterization - both following incubation with human hepatocytes as well as in the plasma of patients treated with ralaniten acetate.
Importantly this shows that ralaniten is a substrate for glucuronidation and occurs in humans. We also provided evidence that glucuronidated ralaniten possess little to no activity with respect to antagonizing the AR or restricting androgen induced growth. This supports our hypothesis that LNCaP-RAL\textsuperscript{R} cells have selectively upregulated UGT2B genes for the purpose of ralaniten detoxification and clearance.

Lastly we demonstrated that the potency of EPI-045 was nearly identical to ralaniten in parental LNCaP cells indicating that the change in scaffold had minimal effect upon its ability to antagonize the AR-NTD. In following EPI-045 was able to significantly inhibit the AR transcriptional activity and androgen dependent growth of LNCaP cells \textit{in vitro} and \textit{in vivo}. These data provide a proof-of-principal suggesting that rational drug design can be utilized to specifically avoid UGT2B mediated clearance.

\section*{5.2 STUDY LIMITATIONS AND FUTURE DIRECTIONS}

This study is the first to detail the creation and characterization of a model of acquired resistance to any AR-NTD inhibitor. While this work provides invaluable insight into the evolution of prostate cancer, a drawback is the fact that only a single cell line (LNCaP) was used to create the original resistant line from which LNCaP-RAL\textsuperscript{R} was derived. This unfortunately limits our ability to generalize the findings presented herein and predict whether or not increased UGT2B expression by the tumour itself would represent a clinically relevant resistance mechanism. Part of the problem stems from the relatively few established and well characterized cell lines which exist for prostate cancer research. Two of the three most widely used (PC3 and DU145) lack AR expression and would not be appropriate for our studies. Others while commonly used, can be slow growing increasing the difficulty in establishing resistant clones.
through selection with anti-mitotic agents. Even with LNCaP cells, a widely used and highly characterized cell line, it took >1.5 years to generate the heterogeneous resistant line from which LNCaP-RAL\textsuperscript{R} and the other clones were derived.

The LNCaP cell line provided a robust model for measuring the ability of ralaniten to effectively inhibit AR transcriptional activity, as this represents an AR-dependent cell line. While generating resistance in the setting of androgen is perhaps not the most relevant in terms of clinical applicability (any AR-NTD inhibitor used clinically would initially be used in the CRPC setting), this approach made it much easier to characterize and compare the resistant line to its parental line; especially during the early stages of this study. For future studies involving resistance to alternative AR-NTD inhibitors, LNCaP-95, VCaP, C4-2B or CWR22RV1 which more closely recapitulate the CRPC setting \cite{314} could be used in tandem.

We attempted to address this issue by generating stable clones engineered to overexpress UGT2B15 or UGT2B17. In this way we wished expand the impact of our model to test whether these genes were sufficient to influence ralaniten sensitivity in cells other than LNCaP-RAL\textsuperscript{R}. While we were able to generate clones which had UGT2B15 expression levels at or exceeding those found in LNCaP-RAL\textsuperscript{R} cells, unfortunately UGT2B17 expression was far lower. Despite this, we were able to show that sensitivity to ralaniten was reduced in clones which had elevated levels of UGT2B15. In the future, repeating the lentiviral transfection to isolate clones with higher UGT2B17 protein levels could further expand the scope of this work.

We have 5 additional clones which were isolated from the original heterogeneous cell line. Overexpression of UGT2B isoforms was identified in all clones to some extent, indicating that this mechanism arose in the resistant line prior to the isolation of individual clones. However it is possible that alternative changes have occurred between clones which also
influence the relative sensitivity to ralaniten. While each clone was initially characterized with respect to mRNA expression of several UGT2B isoforms and relative ralaniten sensitivity, much more could be done to tease out any other differences between them. It may prove useful to sequence the AR of these clones to identify the presence of any AR mutations which may have arisen independently of increased UGT2B expression. Looking at global gene expression between the clones would allow us to identify subtle differences and could possibly help identify additional alterations which are associated with ralaniten resistance. Finally over the course of generating the resistant line, we stored consecutive generations of cells in liquid nitrogen as a way to preserve our progress. As we now have identified a resistant signature in LNCaP-RAL\textsuperscript{R} cells, these stored cells now represent a resource we could exploit to identify when overexpression of the various UGT2B isoforms first arose, and how long it took following first exposure to ralaniten.

Lastly, our findings were independently corroborated by the fact that glucuronide metabolites of ralaniten were identified in human plasma samples taken from patients who participated in the clinical trial. Ultimately ralaniten acetate did not proceed to Phase II due to excessive metabolism and our group is now involved in developing an alternative lead candidate. Another important consideration is that some UGT2B isoforms (namely UGT2B15 and UGT2B17) are negatively regulated by the AR \cite{258-260}. Indeed increased protein expression of both UGT2B15 and UGT2B17 within the prostate were found to be associated with ADT in prostate cancer specimens compared to untreated patients, and may be associated with biochemical recurrence \cite{260,315}. This regulatory mechanism means that treatments targeting the AR signalling axis may inadvertently also increase UGT2B protein expression in tumour
tissue. Therefore developing novel AR-NTD antagonists which are resistant to this mechanism of clearance is of great importance in order to maximize anti-tumour efficacy.

The LNCaP-RAL® cell line represents an opportunity to challenge these new potential compounds in the context of enhanced glucuronidation. Second generation AR-NTD inhibitors with greater potency and stability can be prioritized and screened against this validated and characterized cellular model. This application represents the most useful aspect of our work, and will hopefully serve to accelerate the development of additional AR-NTD inhibitors.

5.3 RESEARCH SIGNIFICANCE

Current targeted therapies for the treatment of CRPC all centre on suppressing AR transcriptional activity through directly or indirectly antagonizing the AR-LBD. While initially effective, de novo and acquired resistance is common, and all hormonal treatments will ultimately fail. Thus there is an urgent and unmet need to develop alternative therapies which have efficacy in the context of clinically relevant resistance mechanisms. Of particular concern are the emergence of constitutively active AR splice variants which lack the LBD. Associated with the development and progression of CRPC, AR splice variants are increasingly implicated with resistance to hormonal therapy [130,316]. The ability of ralaniten to remain effective in the context of major mechanisms of resistance (namely AR splice variants and LBD gain-of-function mutations) has definitively demonstrated that a therapeutic niche exists for small molecules targeting the AR-NTD [221]. While unfortunately the reality is that resistance to these new therapies will likely inevitably arise, we have proactively begun to lay the groundwork by modeling acquired resistance to ralaniten in an effort to promote development and discovery of second generation AR-NTD inhibitors. As such, this study represents the first to characterize
and describe a mechanism of acquired resistance to the only AR-NTD inhibitor to be tested in clinical trials.

In this work, we identified that resistance to ralaniten occurred through a pathway which was independent of modification to the AR-LBD, while remaining dependent upon functional AR transcriptional activity. The implication of this was that cross-resistance to existing AR-targeted therapies did not occur. We have also identified a subset of genes (UGT2B) whose expression was causatively associated with ralaniten resistance. Furthermore, we have provided evidence that resistance could be overcome by altering the chemistry of ralaniten such that it reduces Phase II metabolism by UGT2B enzymes. Glucuronidation occurs extensively in the liver and gastrointestinal tract, and first pass metabolism of ralaniten was a major factor leading to its inability to generate sustained clinical responses. Small molecules targeting the AR-NTD nonetheless have significant therapeutic potential, and we are actively pursuing additional lead compounds. This study has provided a model with which to challenge second generation AR-NTD inhibitors against a background of excessive glucuronidation. Therefore our work will hopefully speed the development of additional small molecules inhibitors of the AR-NTD which may one day become instrumental in the treatment of mCRPC.
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