Development of novel small molecule inhibitor of androgen receptor to treat castration-resistant prostate cancer

by

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Abstract

Androgen receptor (AR), a transcription factor, is a validated therapeutic target for prostate cancer. All current AR-targeting therapies inhibit the growth of prostate cancer cells by blocking the ligand-binding domain (LBD), where androgen binds to activate the receptor. Unfortunately, these therapies fail to maintain a durable clinical effectiveness, as patients eventually succumb to castration-resistant prostate cancer (CRPC). The clinical onset and progression of most CRPC is accompanied by rising levels of serum prostate specific antigen (PSA), a gene transcriptionally regulated by AR. This indicates aberrant AR transcriptional activity is involved in driving CRPC and conferring therapy-resistance. Therefore, it is imperative to continue the research and development of novel AR inhibitors that can overcome molecular mechanisms underlying aberrant AR transcriptional activity. This dissertation presents three research projects: 1) Discovery of novel AR inhibitors; 2) Evaluation of EPI-002, an AR N-terminal domain (NTD) antagonist; and 3) Generation of a prostate cancer cell line model with resistance to EPI-002.

To discover novel AR inhibitors, candidate compounds identified from high throughput screening were characterized by fluorescent ligand binding assays, AR-driven reporter assays, qPCR gene expression analyses, and proliferation assays. AR NTD inhibitor EPI-002 was evaluated against several mechanisms believed to cause aberrant AR transcriptional activity, including coactivator overexpression, AR gain-of-function mutations, and constitutively active AR splice variants with truncated LBD. To generate a prostate cancer cell line model that developed resistance to EPI-002, LNCaP human prostate cancer cells were cultured under chronic EPI-002 exposure.
First, spongian diterpenoids were discovered as novel antiandrogens that bind to the AR LBD. The diterpenoids blocked androgen-dependent AR transcriptional activity with a structure-activity relationship, reduced androgen-regulated gene expression, and inhibited the proliferation of androgen-sensitive prostate cancer cells. Secondly, EPI-002 was effective against aberrant AR transcriptional activity caused by overexpressed coactivators; AR gain-of-function mutations; and constitutively active AR splice variants such as AR-V7. Importantly, EPI-002 inhibited the growth of CRPC cells driven by AR-V7, whereas antiandrogens had no effect. Finally, a human prostate cancer cell line model resistant to growth inhibition by EPI-002 was generated, allowing future studies to investigate mechanisms of resistance against AR inhibition through the NTD.
Preface

All of the work presented in this dissertation was conducted at the Sadar Laboratory at the Genome Sciences Centre in the BC Cancer Research Centre. This doctoral dissertation describes the independent and original work conducted by me, Yu Chi (Kevin) Yang, and prepared under the supervision of Dr. Marianne Sadar, Professor, Department of Pathology and Laboratory Medicine, at the University of British Columbia. All animal studies and associated methods pertaining to the work presented in this dissertation were approved by the University of British Columbia Animal Care Committee (Certificate # A14-0041). This certificate approves the use of animals for the research projects entitled “Marine natural product drug leads” and “Characterization of EPI-001 for the treatment of prostate cancer”, which include the animal work described in Chapters 2, 3, and 4 of the dissertation.

A version of Chapter 2 has been published [Yang YC, Meimetis LG, Tien AH, Mawji NR, Carr G, Wang J, Andersen RJ, Sadar MD. Spongian diterpenoids inhibit androgen receptor activity. Mol Cancer Ther. 2013 May; 12(5): 621-31]. I was the lead investigator, responsible for the major areas of concept formation, data generation and collection, data analysis, and as well as manuscript composition. Meimetis LG, Carr G, and Andersen RJ provided the compounds for my study. Tien AH completed the IC_{50} determination for LNCaP AR. Mawji NR completed the early compound library screening, resuscitated frozen cell lines for cell culture, managed reagent orders, and provided technical assistance with laboratory equipments. Wang J performed the animal experiments. Sadar MD was the supervisory author on this manuscript and was involved throughout the project in concept formation, data analysis, and manuscript composition.
A version of Chapter 3 has been written as a manuscript. I was the lead investigator, responsible for the major areas of concept formation, data generation and collection, data analysis, and as well as manuscript composition. Banuelos A provided the fold of induction and Western blot analysis of AR with polyglutamine tract. Mawji NR resuscitated frozen cell lines for cell culture, managed reagent orders, and provided technical assistance with laboratory equipments. Wang J completed the animal experiments. Wang J and Kato M performed the immunohistochemistry. Sadar MD was the supervisory author on this manuscript and was involved throughout the project in concept formation, data analysis, and manuscript composition.

Findings in Chapter 4 will be included in a manuscript. I was responsible for the long-term culture and passage of the cell lines. I conducted \textit{in vitro} proliferation assays to confirm the drug resistant phenotype in the cells. I prepared the cells for xenograft inoculation, and assisted with the measurement of tumours/organs/body weights in the animal studies. I analyzed all the data and wrote this chapter. Mawji NR provided early cell line maintenance, resuscitated frozen cell lines for cell culture, managed reagent orders, and provided technical assistance with laboratory equipments. Wang J performed subcutaneous injection of cells as xenografts in mice, castrated the mice, gave treatment, measured tumour volumes and body weights, and harvested the tumours and organs at the end of the study. Tien AH gave treatment to the mice, measured tumour volumes and body weights, and harvested the tumours and organs. Obst J provided the doubling time for the resistant cells. Sadar MD was the research supervisor involved throughout the project in concept formation, data analysis, and also reviewed and edited this chapter.
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CHAPTER 1. INTRODUCTION

1.1 PROSTATE CANCER

1.1.1 Epidemiology and risk factors

Prostate cancer poses a major threat to men’s health worldwide, and accounts for 15% of all male cancers (1). In 2014, there were 256,600 estimated new cases of prostate cancer and approximately 33,480 deaths by prostate cancer, making this disease the second leading cause of cancer-related death for North American men (2, 3). The most important recognized risk factors associated with prostate cancer are age, race, and family history. It is rare for men younger than 40 years old to have prostate cancer, and the incidence rises rapidly with age, with the highest probability of being diagnosed of prostate cancer in men aged 70 years and older (4). The risk of developing and dying from prostate cancer is significantly higher for African-American men than Caucasian men, and African-Americans are more likely to be diagnosed at a younger age and with more aggressive and advanced tumours (5). The reason for the ethnic differences is unclear. Up to 10% of prostate cancer cases are believed to be primarily related to high-risk inherited genetic factors or cancer susceptibility genes, as several studies have suggested that family history is a major risk factor for prostate cancer (6-8). Additional risk factors for prostate cancer may include dietary habits and lifestyle. Despite high incidence, the progression of prostate cancer is relatively slow as compared to other types of cancer such as lung, pancreatic, and colon and rectal cancers. In general, prostate cancer has good survival rates, with a 5-year survival percentage close to 100% for localized disease, which accounts for 81% of cases at the stage of diagnosis (9).
1.1.2 Detection and diagnosis

The mortality rate for prostate cancer has been steadily declining since the early 1990’s, and this positive trend has been attributed to early detection and improved treatments (9). In North America, screening for early detection of prostate cancer commonly involves PSA (prostate specific antigen) testing in conjunction with a DRE (digital rectal examination) (10, 11). PSA is an androgen-regulated serine protease produced by both normal prostate epithelial cells and prostate cancer cells, and it functions to liquefy seminal fluid in order to mobilize spermatozoa flagellum (12, 13). Most healthy men have serum PSA levels under 4 ng/mL, and although PSA level increases with natural aging, a drastic elevation of the PSA level may indicate the presence of prostate cancer (13). During the development of prostate cancer, normal prostate glandular structure is altered because of uncontrolled proliferation of cancerous epithelial cells, which results in a significant loss of basal cell layers, and consequently, a large amount of PSA is released into the blood circulation (13, 14). However, there are controversies surrounding PSA testing, as two large studies showed inconsistent evidence for survival benefit of the screening while revealing the risk of over-diagnosis and over-treatment (15, 16). Nonetheless, PSA testing and DRE are the most commonly used practice for screening and early detection of prostate cancer, and the decision to receive PSA testing and when to get it should be personalized to the patients depending on their risk levels. All men 50 years or older with a life expectancy of at least 10 years are recommended for PSA testing, and men with increased risk for prostate cancer such as those of African descent or with family history of the disease should be offered PSA testing at the age of 40 (10, 11). If prostate cancer is suspected after the initial screening (serum PSA levels > 4 ng/mL and abnormality detected by DRE), a biopsy is done with the aid of transrectal
ultrasound to provide a histological diagnosis regarding the stage and grade of the cancer (10, 11). The Gleason grading system with a scale of 1 to 5 is used for prostate cancer, and 1 indicates low-grade carcinoma while 5 indicates high-grade carcinoma with poorly formed glands (17). A Gleason score is a sum of the grades associated with the most common (primary grade) and the second most common (secondary grade) histological patterns of the specimens (17, 18). Higher scores correspond to more aggressive tumours, which consequently correlate with poorer prognosis (17, 18).

1.1.3 Treatment for localized and advanced prostate cancer

Three forms of therapy are available for localized prostate cancer without metastases to the seminal vesicles, regional lymph nodes, or a distant site: active surveillance (also known as watchful waiting), radiation therapy, and surgery (radical prostatectomy) (18). Active surveillance, which does not require any treatment, is appropriate for patients with a life expectancy of less than 10 years, and also suitable for low-risk patients (PSA level < 10 ng/mL) with low-grade tumours (Gleason score ≤ 6) (18, 19). For high-risk patients with high-grade localized prostate cancer, radiation therapy and radical prostatectomy can effectively eliminate the tumours (18). Figure 1.1 illustrates the typical disease progression and corresponding treatment for prostate cancer.

However, it has been reported that as many as 47% of men developed advanced prostate cancer with distant metastases after receiving radical prostatectomy within an average of 5 years (20). Early pioneering work by Charles Huggins and his colleagues demonstrated the hormonal dependence of advanced prostate cancer, which led to the common practice of androgen deprivation therapy (ADT) to treat the disease (21, 22). ADT
consists of surgical (orchiectomy) or chemical castration that aims to reduce the production of natural androgens such as testosterone (23, 24). Androgens are produced by testes and the adrenal glands, with testes accounting for 90-95% of the production, while adrenal glands contribute 5-10% (23). Androgens are critical for the growth of prostate cancer cells at all stages of the disease, and the pathological and molecular aspects of androgens in prostate cancer will be further discussed in the next sections. Surgical removal of testes is a direct and effective approach to eradicate the primary source of androgens, but it is permanent and some men may find it difficult to psychologically accept the disfiguration. In 1967, the Veterans Administration Cooperative Urologic Research Group indicated that treatment with oral oestrogen diethylstilbestrol (DES) was equally effective as orchiectomy for treating prostate cancer, although DES was associated with significant cardiovascular and thromboembolic adverse effects (25). In 1970s, synthetic peptides were produced to act as agonists of luteinizing hormone-releasing hormone (LHRH or GnRH), which induce the production of testosterone in the testes (26). Chronic administration of LHRH agonists causes downregulation of LHRH receptors, and consequently leads to castrated levels of testosterone, providing an alternative method for castration (27, 28). When compared with DES or orchiectomy, the use of LHRH analogues did not show statistically significant improvement in treating patients with metastatic prostate cancer, but less adverse effects were associated with LHRH analogues (29-31). A number of synthetic LHRH agonists are currently available for clinical treatment of advanced prostate cancer, including goserelin (Zoladex®), leuprolide (Lupron®), and nafarelin (Synarel®). To eliminate “testosterone flare”, which is a major adverse effect of LHRH agonists caused by an initial increase in testosterone production, LHRH antagonists were developed (32). LHRH antagonists directly
bind to the LHRH receptors without initiating a response of testosterone surge, while also resulting in an effective and sustained suppression of testosterone production (33, 34). Currently, degarelix (Firmagon) is the only LHRH antagonist approved to treat advanced prostate cancer in Europe and North America.

Furthermore, maximum androgen blockade (MAB) involving castration (usually either surgical or chemical) and non-steroidal antiandrogens such as bicalutamide has been shown to provide additional clinical benefits including improved survival, when compared to castration alone (35-37). MAB is currently a common practice to treat advanced prostate cancer, meanwhile mitigating testosterone flare caused by LHRH agonists (38, 39). Antiandrogens are compounds that inhibit the androgen receptor (AR), which is an important transcription factor in prostate cancer (40). There will be more discussion about antiandrogens and AR in the later sections and chapters of this dissertation. By lowering serum testosterone level to below 0.5 ng/mL and reducing serum PSA to undetectable level, castration is considered clinically achieved by ADT (41). ADT has numerous adverse effects including vasomotor flushing, loss of libido, impotence, fatigue, gynaecomastia, anaemia, osteoporosis and metabolic complications, but with pharmacologic interventions, many of these adverse effects can be managed (24). According to the most recent National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for prostate cancer, ADT has remained as the primary treatment for high-risk patients with advanced prostate cancer, consistent with literature that has addressed the balance of benefits and risks of ADT (19, 23, 42).
1.1.4 Treatment for castration-resistant prostate cancer

After an initial response to ADT ranging from 12-33 months, most patients would relapse with tumours re-emerging rapidly and PSA levels rising sharply, leading to an aggressive and lethal form of the disease referred to castration-resistant prostate cancer (CRPC) (43-45). The mean survival time for CRPC is approximately 16-18 months (45). In the past 10 years, there have been several major changes in the therapeutic approaches to treat CRPC, due to much improved understanding of the molecular mechanism of the disease. As the only therapy that improved overall survival in CRPC at that time, docetaxel-based chemotherapy was approved by the Food and Drug Administration (FDA) to treat CRPC in 2004, but the prolonged survival only lasted 2-3 months (46, 47). Before 2010, there was no therapy available to treat post-docetaxel CRPC patients (48).

In 2010, cabazitaxel (Jevtana®), a taxane derivative was the first post-docetaxel therapy approved by the FDA to treat metastatic CRPC, as it increased survival for about 2 months (49). Abiraterone (Zytiga®), a small-molecule inhibitor of cytochrome P450 (CYP17) enzyme that plays key roles in adrenal and intratumoral de novo biosynthesis of androgens, was approved in 2011 to treat post-docetaxel metastatic CRPC in combination with prednisone as it increased survival to 3.9 months (50). In 2012, the FDA also approved the use of abiraterone in combination of prednisone as first-line therapy for patients with metastatic CRPC prior to chemotherapy, as studies later provided evidence to support the benefit of such treatment (51). The first immunotherapy in prostate cancer, sipuleucel-T (Provenge®) was approved by FDA in 2010 to treat asymptomatic or minimally symptomatic metastatic CRPC, as it showed a 4.1-month improvement in median survival (52). In 2012, the FDA approved enzalutamide (Xtandi), a second-generation antiandrogen to
treat post-docetaxel metastatic CRPC, based on a trial that demonstrated an improvement in overall survival by 4.8 months (53). A recent trial showed in patients with metastatic prostate cancer prior to chemotherapy, enzalutamide significantly reduced the risk of radiographic progression and death, and postponed the initiation of chemotherapy (54). As a result, in September 2014, the FDA approved the application of enzalutamide as a first-line therapy to treat CRPC patients before chemotherapy. Furthermore, in 2013, radium-223 dichloride (Xofigo®), an alpha emitter, received FDA approval to treat CRPC patients (pre- or post-chemotherapy) with symptomatic bone metastases but no known visceral metastatic disease, as its clinical trial showed a significant improvement in overall survival by almost 3 months (55). The last ten years represent a new era in molecular-targeted drug research and development for CRPC, which had very limited treatment options prior to 2004.

1.1.5 Pathophysiology of prostate cancer

Most prostate cancer is adenocarcinoma, or glandular cancer of the prostate, while the remaining rare types of prostate cancer include neuroendocrine small-cell carcinoma, transitional cell carcinoma, squamous cell carcinoma, and prostatic sarcomas (56-61). The prostate is a male sex gland that produces approximately 30% of all seminal fluid (57). As men age, the prostate gland becomes hyper-proliferative and thereby increasing the risk for prostate cancer (62). Anatomically, the prostate is classified into lobes: anterior, posterior, lateral, and median lobes (63). Pathologically, an adult prostate, which has four distinct glandular regions, is classified into four zones: peripheral, central, transition, and anterior fibro-muscular zones (63). The peripheral and central zones are the two largest components of the prostate gland, with the peripheral zone accounting for up to 70% of the gland, and the
central zone accounting for about 25% (57, 63). It is estimated that around 70-80% of prostate cancer originates from the peripheral zone, which is the sub-capsular portion of the posterior lobe that surrounds the distal urethra (57, 62). The central zone, which surrounds the ejaculatory ducts, accounts for only about 2.5% of prostate cancer, but cancer originated from the central zone tends to be more aggressive and likely to invade the seminal vesicles (57, 62).

The adult prostate is comprised of pseudostratified columnar epithelia containing at least three distinct types of cells: luminal, basal, and neuroendocrine cells (57). Luminal cells are differentiated androgen-dependent and express AR, and these cells constitute the majority of the prostate epithelia and produce prostatic secretory proteins such as PSA (64, 65). Basal cells, found between the luminal cells and the underlying basement membrane, are the second major epithelial cell type in the prostate, and these cells do not produce prostatic secretory proteins and express very low or no AR (65). The neuroendocrine cells are androgen-independent and account for a minor population, and they are dispersed throughout the basal layer (66). Pure small cell carcinoma of neuroendocrine origin is a rare type of prostate cancer, accounting for less than 2% of all prostatic malignancies (58, 67). High-grade prostatic intraepithelial neoplasia (PIN), which involves abnormal and proliferative epithelial cells, is considered to precede the development of prostate adenocarcinoma, but unlike prostate adenocarcinoma, the basal-cell layer remains intact and serum PSA is typically not elevated in PIN (68, 69). For adenocarcinoma of the prostate, luminal cells are generally considered as the cells of origin, because prostate cancer is histologically characterized by an expansion of luminal cells and the destruction of basal cells, but recent studies have suggested that basal epithelial stem cells also play a role in prostate cancer.
initiation (70-73). Nonetheless, most prostate cancer is adenocarcinoma and clinically defined as an androgen-dependent disease, which is why ADT is widely used as the frontline treatment for advanced prostate cancer (23, 38, 56). The other rare types of prostate cancer including small cell (neuroendocrine) carcinoma are only transiently responsive to chemotherapy, because these cells are androgen-independent (58, 74). This dissertation focuses on prostate adenocarcinoma formed by prostate cancer cells that require active androgen receptor for growth and survival.

Androgens such as testosterone mediate their biological signals through AR, which is a transcription factor that regulates the expression of many genes associated with growth and survival (75). In most cases of prostate cancer the tumours express AR and are initially androgen-dependent (44). ADT effectively reduces serum testosterone to a castration-equivalent level to block androgen signaling in the cancer cells, and thereby inhibiting tumour growth (24, 44). However, despite castration-equivalent level of serum testosterone, tumours eventually begin to grow again, usually in correlation with rising PSA level, leading to the onset of the late-stage CRPC, which was formerly termed androgen-independent prostate cancer or hormone-refractory prostate cancer (43).

1.1.6 Molecular biology of castration-resistant prostate cancer

The prostate, which is an androgen-dependent organ, relies on androgen signaling for both physiological and pathological growth and development (75, 76). Androgen mediates its biological actions by binding to its cognate receptor AR, and the androgen bound AR drives the expression of specific sets of genes that are responsible for protein synthesis, secretion, cell-cycle regulation, and other important aspects of cellular biology (76-78). There is
compelling evidence to support a critical role of AR during the progression of prostate cancer. As demonstrated by immunohistochemical analyses and gene expression analyses, AR protein expression is present throughout the progression of prostate cancer from the primary to metastatic CRPC stage, and increased AR mRNA level has been shown to be associated with CRPC progression (79-81). Furthermore, in most cases of CRPC, the clinical onset is accompanied by a rise in serum PSA level following ADT (43). Since the expression of PSA is transcriptionally regulated by AR, it is believed that AR signaling regains functional activity in CRPC despite castrated levels of serum testosterone (43, 82). The notion of restored AR transcriptional activity in CRPC is further supported by recent trials of second-generation hormonal therapies such as abiraterone acetate and enzalutamide, which increase the survival of CRPC patients by targeting androgen-AR signaling (40).

Over the last twenty years, a strong collective research effort has been made to elucidate the molecular mechanisms of reactivated and persistent AR genomic signaling in CRPC. Several mechanisms have been proposed to explain aberrant AR transcriptional activities, as illustrated by Figure 1.2. One is the amplification of the AR gene and increased AR protein expression, which result in hypersensitivity to very low levels of androgens as the case in CRPC (81, 83). In addition, it has been shown that AR gain-of-function mutations can allow activation by nonandrogenic steroidal ligands such as glucocorticoid or even antiandrogens like flutamide, bicalutamide, and enzalutamide (84-88). Overexpression of AR coactivators has also been implicated in aberrant AR activity and prostate cancer progression; for example, steroid receptor coactivator 1 (SRC-1) can enhance androgen-dependent and also facilitate ligand-independent AR transcriptional activities, and the expression of SRC-1 is elevated in advanced prostate cancer (89-95). The AR N-terminal domain (NTD), which
contributes to most if not all of AR transcriptional activity, can be activated in an androgen-independent manner through the stimulation of the cAMP-dependent protein kinase (PKA) pathway, cytokines such as interleukin-6 (IL-6), and by bone-derived factors, indicating a role for alternative AR transactivation in CRPC (96-98). As another explanation for restored AR signaling in CRPC, increased adrenal and intratumoral androgen biosynthesis under castrated conditions has been shown to be capable of maintaining sufficient level of androgen to support AR signaling and tumour growth (99-101). Furthermore, several studies have revealed that constitutively active AR splice variants lacking the C-terminal ligand binding domain (LBD) can confer castration-resistant phenotype in prostate cancer cells and xenograft models (102-105). Importantly, these AR splice variants have emerged as a clinically relevant mechanism underlying aberrant AR activity in CRPC, and the expression levels of the variants are correlated with poor prognosis (106, 107). AR splice variants will be discussed in more details in section 1.2.6.

Furthermore, integrative analyses of the genomic landscape of prostate cancer have shown that besides AR pathway being the most frequently altered pathway, several AR-independent signaling pathways are also implicated in metastatic CRPC (108, 109). There are significant and frequent alterations in the phosphoinositide 3-kinase (PI3K), small GTPase RAS/rapidly accelerated fibrosarcoma (RAF) kianse, and retinoblastoma protein (RB) signaling pathways. For instance, 42% of the metastatic samples (N=37) analyzed were found to harbor a loss of phosphatase and tensin homolog (PTEN), a well known tumour suppressor gene that inhibits the PI3K/AKT signaling pathway (108). Tumour suppressor gene RB1 was also found to be lost in 37% of the metastatic samples, while oncogene KRAS was found to be activated in 32% of the metastases (108). DNA damage and repair pathways
may also be involved in CRPC, as somatic mutations of the tumour suppressor gene TP53 were reported to be the most selectively mutated in metastatic CRPC, while both somatic and germline mutations of BRCA2 were identified (109). Oncogene MYC was revealed to be commonly amplified in prostate cancer, suggesting a role for Myc signaling in the progression of prostate cancer (108, 110). These findings indicate that multiple dysregulated signaling pathways may collectively contribute to the development and progression of CRPC, and therefore precision medicine based on the genetic and molecular alterations of the individual patients will be an effective approach to treat CRPC (109). It is important to note that AR has been reported to have cross-talk activity with multiple signaling pathways, and for example, nongenomic androgen-AR signaling can act as an upstream activator for PI3K and RAS/RAF pathways (111-113). Overall, these studies highlight a therapeutic potential of combination therapy using AR-targeting agents with specific inhibitors of growth signaling pathways.

1.2 ANDROGEN RECEPTOR

1.2.1 Androgen receptor structure and functions

AR is a transcription factor and a member of the nuclear steroid receptor superfamily, also classified as NR3C4 (nuclear receptor subfamily 3, group C, member 4) (114, 115). The nuclear receptor superfamily includes over 100 members, and among this large family of proteins, there are only five known vertebrate steroid receptors: AR, estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and progesterone receptor (PR) (115). The human AR gene is a single-copy X-chromosome gene located at q11-12, and is oriented with the 5’end toward the centromere (116). The gene spans about 90 kilobases
of DNA containing eight exons that encode an mRNA transcript of about 10.6 kb in length (117, 118). The amino acid length of AR can vary because of polymorphic genetic sequences and/or alternative splicing. The most commonly reported full-length AR protein product translated from the 10.6 kb transcript consists of 919 amino acids with a predicted molecular mass of 98.8 kilodaltons (kDa), but when this AR protein is detected using AR-specific antibody on western blots, it appears as a closely spaced doublet at 110 to 112 kDa because of post-translational modifications such as phosphorylation (119, 120). Four distinctive functional domains constitute the full-length AR protein: N-terminal domain (NTD), DNA-binding domain (DBD), hinge region (HR), and a ligand-binding domain (LBD) (121). There is a nuclear localization signal (NLS) sequence overlapping the DBD and HR of the AR (122). Exon 1 encodes the full NTD, exons 2 and 3 encode the DBD, and exons 4-8 encode the LBD (121, 123). Each of the four domains will be separately discussed in sections 1.2.3 to 1.2.5. Figure 1.3 provides a schematic representation of AR gene and protein. The crystal structures of AR DBD and LBD have been solved, but no crystal structures of the full-length AR or the NTD are available. In 2000, the first crystal structure of the AR LBD was solved to show that the AR LBD shares similar 3D structure with other agonist-bound steroid receptors such as PR (124). More details of the structure of AR LBD will be described in a later section. Like other steroid receptors, AR is a soluble protein that functions as a transcription factor, which mediates the biological actions of androgens (76).

The physiological functions of androgen-AR signaling include male embryonic and fetal sexual differentiation and pubertal changes, while in adults, androgen is mainly responsible for maintaining libido, spermatogenesis, muscle mass and strength, bone mineral density, and erythropoisis (123, 125). AR is expressed in cells from various androgen-
dependent tissues such as testis, prostate, and seminal vesicles (79). Expression of AR was also detected in non-genital tissues or cells such as sweat glands and hair follicles of the skin, hepatocytes of the liver, arteries and arterioles, cardiac muscle, acinar cells, myoepithelial cells of the breast, endometrial cells of the uterus, follicular cells of the thyroid gland, adrenocortical cells, and cells in the central nervous system (79, 126). Androgen-AR signaling is responsible for the development of male reproductive system, and the androgen testosterone is a major determinant for sex differentiation during embryology (127). For male sex differentiation, the determination of a testis from the indifferent primodial gonad is the first and critical event in the subsequent development of internal and external male genitalia (128). In mammals, the formation of testis from the primodial gonad is initiated by SRY, a gene that resides on the short arm of the Y-chromosome and encodes for testis-determining factor or sex-determining region Y (SRY) protein (128, 129). The primary role of SRY is to upregulate the gene expression of SOX9, which induces the differentiation of Sertoli cells to drive testis formation (130). The initial formation of testis induces further cellular differentiation that gives rise to steroid-secreting Leydig cells, which are responsible for increasing the level of testosterone to stimulate the differentiation and development of androgen-dependent and AR-expressing tissues such as the vas deferens, epididymis, seminal vesicle, and prostate gland (127). The differentiation and development of male external genitalia is also regulated by androgen in the form of dihydrotestosterone (DHT), the more potent form of testosterone formed after conversion by an enzyme called 5α-reductase (131). Androgens mediate their biological effects in target cells by binding to and activating AR, which is a transcription factor responsible for regulating the expression of genes involved in male reproductive system (76). Using AR knockout (ARKO) mouse models, studies have
revealed the impacts of AR genomic actions on normal male sexual differentiation and development. These impacts include smaller testes located intra-abdominally, appearance of female external genitalia, arrested spermatogenesis, reduced bone volumes, and reduced body weight in ARKO males when compared to normal control males (132, 133).

The importance of androgen-AR signaling for the development and maintenance of the human male reproductive system is evident from studies of hereditary disorders such as androgen insensitivity syndrome (AIS). In individuals with AIS, their AR is partially or completely unable to be activated by androgens possibly caused by loss-of-function AR mutations, or complete or partial AR gene deletion, or abnormal splicing of AR, resulting in a defective AR despite normal serum androgen levels (134). AIS is divided into three classes according to the resulting phenotypes: complete AIS that is indicated by complete phenotypic reversal of male-to-female external genitalia, partial AIS that involves a partially masculinised external genitalia, and mild AIS that has a normal male phenotype but with mild spermatogenic defect and/or reduced secondary terminal hair (135). Defective AR structure and function can also result in spinal and bulbar muscular atrophy (SBMA) or Kennedy’s disease, a severe progressive neurodegenerative disease causing the loss of motor neurons in the muscles of adult men (136). The disease is thought to be caused by an expansion of a trinucleotide (CAG) repeat in the NTD of AR, from a normal average of 21 to over 40 repeats, which leads to aggregation of AR protein in motor neurons and thereby causing neurotoxicity (136, 137). Interestingly, longer and expanded CAG repeats have been shown to be associated with decreased AR transcriptional activity and consequently mild androgen insensitivity, but a study demonstrated that AR loss-of-function was not linked to the clinical symptoms of Kennedy’s disease (138).
1.2.2 Androgen receptor as a transcription factor

Although it is known that androgens can rapidly stimulate kinase-signaling cascades and modulate intracellular calcium levels in a non-genomic manner, the biological effects of androgens are considered to occur predominantly through the genomic effect of AR, which is a transcription factor and master regulator of gene expression (75, 76, 139, 140). This dissertation focuses on the genomic aspect of androgen-AR signaling (Figure 1.4). The androgen-induced AR genomic signaling is responsible for the regulation of many target genes that are involved in important cellular biology such as protein synthesis, metabolism, secretion, cell-cycle regulation, and proliferation (78). Unliganded AR primarily resides in the cytoplasm and is sequestered by association with chaperone molecules such as heat shock proteins (HSPs), which stabilize and maintain the tertiary structure of AR LBD in a particular conformation to allow androgen binding (141, 142). The binding of ligand such as androgen is believed to stabilize AR protein and slow down proteasomal degradation of AR in prostate cancer cells (143, 144). DHT is the most potent natural androgen with greater binding affinity to AR and slower dissociation from AR than testosterone, which is converted into DHT by the enzyme 5α-reductase (145). When androgen binds to the AR LBD, the position of helix 12 in the LBD shifts towards helices 3-5, as a result, serving as a “lid” to stabilize ligand binding and also creating a hydrophobic cleft for binding of the leucine-X-X-leucine-leucine (LXXLL) motif that is present in many nuclear receptor coactivators such as the steroid receptor coactivator (SRC) family (124, 146, 147). However, unlike other nuclear receptors, the hydrophobic cleft in the androgen-bound AR LBD preferentially interacts with the phenylalanine-X-X-leucine-phenylalanine (FXXLF) motif present in the AR NTD and other AR coregulators such as ARA54 and ARA70 (148, 149). The FXXLF motif in the
NTD interacts with the androgen-induced hydrophobic cleft of the LBD to form an intramolecular N-terminal/C-terminal (N/C) interaction (150, 151). The function of intramolecular AR N/C interaction, which occurs in the cytoplasm, is thought to contribute to a delay of androgen dissociation rate, initiation of nuclear localization, and optimization of transcriptional activity (150-152). A tryptophan-X-X-leucine-phenylalanine (WXXLF) motif within the AR NTD can also interact with different regions of the LBD to further stabilize the N/C interaction (151).

The androgen-bound AR translocates into the nucleus by utilizing a nuclear localization signal (NLS) encoded within the DBD and hinge region of AR (122). Androgen binding also causes AR to undergo multiple post-translational modifications including phosphorylation, methylation, acetylation, ubiquitylation, and sumoylation; these modifications are believed to affect AR protein stability, cellular localization, protein-protein interactions, and transcriptional activity (153). Once inside the nucleus, the androgen bound AR undergoes further conformational changes leading to the dimerization of two AR proteins, prior to binding to the DNA of target genes (154, 155). The dimerization of two AR proteins involves intermolecular N/C interaction, which is stabilized by hydrogen bonding between the DBD of the two AR (150, 156). In response to androgen, AR physically binds to specific androgen-responsive elements (AREs), which are DNA sequences usually located within the promoter and/or enhancer regions of AR-regulated genes such as PSA (157). Furthermore, through protein-protein interactions, AR recruits coactivators with chromatin-modifying activities such as steroid receptor coactivator 1 (SRC1) of the p160 family of coactivators and the classical nuclear receptor cofactors CBP/p300 (157-159). An active AR
transcriptional complex is formed when RNA polymerase II joins the AR-coactivator complex, leading to the initiation of transcription (157).

Overall, AR is considered to be an androgen-dependent transcription factor that regulates gene expression (78). However, studies have revealed the presence and significance of an androgen-independent transcriptional program mediated by AR, particularly in the context of CRPC, in which aberrant and persistent AR transcriptional activity continues to drive prostate cancer growth in an androgen-independent manner (160). Possible mechanisms underlying restored and aberrant AR activity in CRPC have been discussed previously in section 1.1.6. Importantly, constitutively active AR splice variants lacking the LBD have been reported to drive a distinct transcriptional program independent of androgen, and the expression of these AR variants have been implicated in the development and progression of CRPC and resistance to current AR-targeting therapies (107, 161, 162). The biology and clinical significance of these AR splice variants will be discussed in section 1.2.6.

### 1.2.3 Amino-terminal domain

Among all the steroid receptors, the amino-terminal domain (NTD) is the least conserved in terms of size and sequence homology, and AR NTD shares less than 15% sequence homology with the closely related GR and PR (115, 163). The NTD of AR, encoded by a single large exon, is the largest domain of AR (164). There are several repeat regions within the AR NTD: polyglutamine between amino acid residues 58 to 89; polyproline between residues 371 to 381; and polyglycine between residues 449 to 472 (118, 119, 164). Because of polymorphic lengths of these repeats, the size and amino acid
sequence numbers of AR and its NTD can vary. The AR with 919 amino acids in total length, 21 polyglutamine repeats, and 24 polyglycine repeats is generally considered to be the wild-type protein (119). In this dissertation, the amino acid sequence numbers of AR are based on the wild-type AR with 919 amino acids, unless noted otherwise. The AR NTD (amino acid 1-558) is considered to be the engine of AR, because the NTD contributes most if not all of the transcriptional activity (122, 165). This is a unique feature of AR, as other steroid receptors predominantly rely on the activation function-2 (AF-2) region located in the LBD for transcriptional activity (123). Within the AR NTD, the AF-1 region (amino acid 142-485) can form protein-protein interactions with AR coactivators, recruit general transcriptional machinery, and retain at least 65% of transcriptional activity of the full-length NTD (166-168). There are two transcription activation units (TAU) in the NTD: TAU1 (amino acid 101-370) and TAU5 (amino acid 360-528) (Figure 1.3), and each TAU has distinct activity depending on the presence of androgen (166). TAU1, with a core sequence of $^{178}$LKDIL$^{182}$, is involved in ligand-dependent AR activation, whereas TAU5 has a core sequence of $^{435}$WHTLF$^{439}$ and is responsible for ligand-independent AR activation (97, 169).

AR NTD also contains a FXXLF motif ($^{23}$FQNLF$^{27}$), which interacts with the androgen bound LBD, forming an intramolecular N/C interaction that is required for androgen-dependent AR transcriptional activity (150, 151).

The AR NTD is intrinsically disordered with a high degree of structural flexibility, because it does not have any stable secondary structure, as opposed to the LBD and DBD which all have a defined structure (123, 170). Due to the lack of stable structure, no crystal structure containing AR NTD has been resolved. Based on the primary amino acid sequence, bioinformatics analysis has predicted that the AR AF-1 region has extensive regions of
naturally disordered structure with four putative helical regions (170, 171). The AF-1 region is believed to have characteristics of collapsed disordered conformation, which allows it to adopt an ordered and globular conformation when bound to specific protein targets or natural osmolytes (171). The propensity of intrinsically disordered AR AF-1 to adopt an ordered structure such as an α-helical conformation in response to specific inducing agents is important for protein-protein interactions between AR NTD and various coactivators, ultimately resulting in the formation of an active AR transcriptional complex (170-174). Overall, AR NTD is essential for transcriptional activity, and its structural flexibility is critical for allosteric regulation of gene expression.

1.2.4 DNA-binding domain and hinge region

The DNA-binding domain (DBD) is the most conserved domain among the steroid receptors, with AR DBD showing about 80% sequence homology to the DBD of GR and PR (164, 175). AR DBD is encoded by exon 2 and 3 (Figure 1.3), and similar to all steroid receptors, consists of two zinc (Zn) finger-like modules that are organized into three α-helices (119). Each Zn-finger contains one zinc ion bound by two cysteine and two histidine residues (176). Within the DBD, the N-terminal Zn finger is involved in direct DNA interaction, whereas the C-terminal Zn finger contains a D box region that is responsible for DNA-dependent AR dimerization (177, 178). Crystal structures have revealed that AR dimerization involves intermolecular N-terminal to C-terminal (N/C) interaction, which is stabilized by van der Waals interactions and hydrogen bonding between the D box regions of the two AR DBD (150, 178). Furthermore, the amino acid residues in the C-terminal Zn finger and a C-terminal extension with 12 amino acids of the AR DBD provide selective
binding to AREs (179). Steroid receptors can bind to DNA elements containing a partial palindrome of the core sequence 5’-TGGTCT-3’ arranged as inverted repeats separated by a three nucleotide spacer (177). AR DBD recognizes specific DNA sequences called androgen response elements (AREs), which are usually found in the promoter or enhancer regions of AR target genes (82, 177, 180). The AREs consist of two hexameric half-sites arranged as a direct repeat separated by a spacer of 3 nucleotide (nnn), with the half-site repeating on the same strand (178). One example of an ARE is the naturally occurring probasin response element 2 (PB-ARE-2) with a sequence of 5’-GGTTCTTGAGTACT-3’ (178, 181). The imperfect direct repeats (5’-GGTTCT-3’ and 5’-AGTACT-3’) offer high affinity for cooperative binding of AR but not the closely related GR, which prefers the inverted repeats (181, 182). Similarly, an ARE sequence 5’-AGAACAGCAAGTGCT-3’ is found in the promoter region of the \( PSA \) gene, which is a well-characterized gene regulated by AR in response to androgen (180).

Encoded by exon 4, the hinge region (HR) acts as a linker between the DBD and LBD (122). The HR contains a nuclear localization signal (NLS) sequence that extends into the C-terminal of the DBD (122). The NLS is a bipartite sequence that can interact with nuclear import factors such as importin-\( \alpha \) in order to facilitate AR nuclear translocation through the nuclear pore complex (183, 184). The interactions of NLS with various import factors are regulated in cells (185). A study has shown that the activity of AR NLS is inhibited by the directly binding of importin 7, which functions as a cytoplasmic retention factor for AR, and androgen binding causes a switch of importin 7 to karyopherin alpha import receptors that mediate nuclear translocation (186).
1.2.5 Ligand-binding domain

The ligand-binding domain (LBD) of steroid receptors exhibits sequence homology ranging from 22% to 55%, and among all the steroid receptors, the human AR LBD shares the most sequence identity with the human PR LBD at 55% (187). Encoded by exons 4-8, the AR LBD is made of 11 α-helices (H) numbered 1 to 12, and 4 short β-strands associated in 2 antiparallel β-sheets (124). The helices are arranged in the typical “helical sandwich” form as in the PR LBD (188). Unlike other steroid receptors, H2 is not present in the AR LBD, but the same numbering was applied to the helices (123). H1 and H3 form one face of the AR LBD, while the second face is made of H6, H7, H10, and H11, and the central layer is composed of H4, H5, the first β-turn, H8 and H9 (123, 124). A hydrophobic ligand-binding pocket (LBP) is formed by H5, the N-terminal region of H3, plus the C-terminal regions of H10 and H11 (124). Upon androgen binding, H12 shifts towards H3-5, and thereby acting as a “lid” for the LBP to stabilize ligand binding (124). This androgen-dependent conformational change also creates a hydrophobic cleft to expose an activation function 2 (AF-2) region, which is involved in protein-protein interactions between AR LBD and nuclear receptor coactivators (124, 146, 147). More importantly, the AR AF-2 prefers to bind with the FXXLF motif located within the NTD over the LXXLL motif found in many coactivators, because the hydrophobic cleft is deep and fits better with the bulky side chain of phenylalanine residues of the FXXLF motif (150, 189). Furthermore, the binding of FXXLF motif can create an intact charge clamp (involving lysine K720 of H3 and glutamate E897 of H12), providing additional stability for the interaction (189). The preferential binding of AR AF-2 with AR NTD is critical for androgen-dependent N/C interaction, which is essential for androgen-induced AR transcriptional activity (150, 152).
The crystal structures of AR LBD complexed with a variety of ligands have been solved, providing a structural basis for agonist-induced AR activation and also antagonist-induced AR inhibition. Natural androgen DHT binds to the LBP created by H3, H5, and H11 of the AR LBD, forming favorable hydrogen bonds with the side chain of several amino acid residues including N705 and R752 (187). Optimal interactions with the LBP determine the affinity and specificity of ligand binding, as demonstrated by the comparison of the structures of DHT bound AR LBD and progesterone bound PR LBD (124, 187). Furthermore, despite having very similar chemical structures, DHT is a more potent androgen in stimulating AR transcriptional activity than testosterone, because when compared with testosterone, DHT binds AR LBD with a 2-fold higher affinity, dissociates from the LBD 3 to 5 times slower, and induces greater AF-2 activity (143, 145, 190). The differences can be attributed to the atomic geometry involved with the hydrogen bonding between R752 of the LBD and ketone group at position C3 of the steroidal compound, as the hydrogen bond formation is more favorable for DHT in terms of atomic angles and distances (191). Binding of agonist such as androgen induces several critical conformational changes of AR, and ultimately results in the formation of an active AR transcriptional complex (150, 157). In contrast, an AR antagonist such as antiandrogen binds the LBD to inhibit AR transcriptional activity by preventing N/C interaction and coactivator recruitment (192). The structural mechanism for the inhibitory effect of antiandrogen has been elucidated, indicating that binding of bicalutamide sterically disrupts the formation of AF-2, which is critical for N/C interaction (193). With important insight gained from crystal structures of AR LBD, structure-based drug design has been a viable approach to discover AR antagonists with the potential to treat prostate cancer or other androgen-dependent diseases (194, 195). The research and development of new
antiandrogens have been active and successful, and section 1.3.2 will describe current antiandrogens in more details.

**1.2.6 Constitutively active androgen receptor splice variants**

Deletion of the LBD can create a constitutively active AR, as shown by early functional mapping experiments (122). The biological relevance of AR with a truncated LBD first came from a Western blot analysis of a relapsed CRPC prostate cancer xenograft model called CWR22, which revealed the endogenous expression of a LBD-truncated AR species with a lower molecular weight of 75-80 kDa compared to the 110 kDa of full-length AR, using an AR NTD specific antibody (196). Later functional characterization demonstrated that the LBD-truncated AR splice variant is a constitutively active receptor, which stimulates the endogenous expression of AR-dependent genes in a ligand-independent manner (104). Additional LBD-truncated AR splice variants were identified from patient specimens, and these AR variants were shown to be constitutively active and confer androgen-independent growth of prostate cancer cells, highlighting the functional and clinical relevance of the AR splice variants (102, 103, 105). The majority of AR variants are encoded by novel cryptic exons originated from intron segments flanking the normal exon regions of the \( AR \) gene, and since the novel exons are present at the mRNA level, it is believed that these naturally occurring AR splice variants are generated by alternative splicing of the AR pre-mRNA (102, 104). However, other studies have indicated that AR splice variants without LBD can also arise from genomic alterations such as the presence of a premature stop codon as a result of somatic nonsense mutations within the \( AR \) gene, or AR intragenic rearrangement or deletion.
of exons that encode the LBD (197-199). The precise mechanism underlying the biosynthesis of AR splice variants remains to be determined.

Currently, a large number of AR splice variants have been identified, but AR-V7 and AR-V567es are considered to be the most clinically relevant, as a study has revealed that they are the most frequently and abundantly expressed AR splice variants found in CRPC bone metastases (106). The relative protein expression level of AR splice variants and full-length AR is heterogeneous in CRPC bone metastases, ranging from 0% to 95% of full-length AR, with a median relative expression of 32% based on a small sample size of \( n=13 \) (106). Compared to hormone-naïve and benign prostate tissues, the mRNA expression levels of AR-V7 and AR-V567es are elevated in CRPC metastatic tissues, and high expression levels of these variants correlate with poorer prognosis and decreased survival (106, 107). Importantly, strong evidence from preclinical and clinical findings has emerged to indicate that these constitutively active AR splice variants are a major mechanism of resistance to androgen deprivation therapy and play a key role in the development of CRPC, as recently reviewed by several groups (200-204).

The most significant functional relevance of constitutively active AR splice variants like AR-V7 and AR-V567es in prostate cancer is their ability to remain transcriptionally active even under androgen-depleted conditions, causing aberrant and persistent AR nuclear signaling commonly observed in CRPC patients with low serum levels of testosterone (102-105). In addition to the canonical androgen-dependent gene expression driven by full-length AR, AR-V7 has been shown to mediate a distinct androgen-independent transcriptional program containing genes involved with cell-cycle regulation, such as \( UBE2C \) and \( CDC20 \) (161). The induction of this expression program and the resulting upregulation of \( UBE2C \)
expression have been implicated in androgen-independent growth of prostate cancer cells, and analysis of clinical samples has revealed that UBE2C protein was indeed overexpressed with a functional role in prostate cancer (160). Besides the differential regulation of a transcriptional program in the absence of androgen, studies have demonstrated that AR-V7 and AR-V567es stimulate the transcriptional activity of full-length AR in an androgen-independent manner, by possibly facilitating AR nuclear translocation (205). Recent clinical findings have provided strong evidence to show that AR-V7 is associated with resistance against the new-generation AR-targeting therapies such as enzalutamide and abiraterone (162). More details regarding the role of AR-V7 in therapy-resistance will be described in section 1.3.3.

1.3 ANDROGEN RECEPTOR AS A THERAPEUTIC TARGET

1.3.1 Androgen receptor as an oncogenic driver of prostate cancer

The normal physiological function of AR as a transcription factor to mediate the biological effects of androgen has been described in early section. The early pioneering work by Huggins first demonstrated the androgen-dependence of prostate cancer, indicating a pathological role of androgen-AR signaling (21). More evidence supporting the critical role of AR during the progression of prostate cancer includes: immunohistochemical analyses that showed AR protein expression is present throughout the progression of prostate cancer from the primary to metastatic CRPC stage, and gene expression analyses that revealed increased AR mRNA level is associated with advanced disease and poor prognosis (79-81). Functional AR nuclear signaling strongly correlates with prostate cancer progression, which provides the
basis of using \textit{PSA}, a well characterized AR-regulated gene as a clinical diagnostic and prognostic biomarker (13, 75).

Furthermore, genomic characterization of prostate cancer at various stages by unbiased next-generation platforms has generated valuable information about the progression of prostate cancer at the genetic and molecular level, which also indicated the profound involvement of AR in the oncogenic transformation of prostate cells. Whole-genome sequencing analyses of early-onset prostate cancer revealed that elevated AR expression and active androgen-AR signaling are associated with the rearrangement of genomic landscape during the development and progression of prostate cancer (206, 207). A recent genomic analyses of tumour biopsies from metastatic CRPC patients showed that the AR pathway is the most frequently altered biological pathway (71\%, or 107/150 patients), with the majority of the alterations being aberrations that directly affect the \textit{AR} gene through amplification and mutation (109). Another evidence of AR as a tumorigenic driver has been shown from the existence of recurring gene fusions involving the AR-regulated \textit{TMPRSS2} to oncogenic transcription factors \textit{ERG} and \textit{ETV1} of the ETS family, highlighting the role of AR as a master regulator of a known oncogenic transcriptional program (208, 209). Analyses of prostate cancer patient tissues revealed that fusion with \textit{TMPRSS2} is the most likely cause for \textit{ERG} and \textit{ETV1} overexpression, providing additional clinical relevance of AR-mediated oncogenic pathways (208).

By itself, AR should be considered as a proto-oncogene with the potential to become an oncogene. The essential characteristic of an oncogene is the ability to transform a normal cell into a cancer cell, under certain circumstances including mutations, genetic or structural alterations, and amplification of the candidate oncogene, which usually encodes a protein that
regulate cell proliferation, apoptosis, or both (210). An AR mutation capable of causing spontaneous cancer initiation and progression has been demonstrated, as transgenic mice expressing the mutant AR E231G rapidly developed prostatic intraepithelial neoplasia, which is the precursor of prostate cancer, and eventually progressed into invasive and metastatic disease (211). The length of polyglutamine tract or CAG repeat sequence within the AR NTD is polymorphic, giving rise to genetically altered AR with a functional impact, as AR with shorter CAG repeats is more transcriptional active (212). Because AR NTD is intrinsically disordered, it is difficult to investigate if variable length of CAG repeats causes structural alterations of the NTD. However, studies have shown that the length CAG repeats affects AR N/C interaction, and since certain structural conformation is required for the N/C interaction, it is likely that variable lengths of CAG repeats result in some exquisite structural alteration of the AR NTD (189, 212, 213). The clinical and pathological relevance of polymorphic AR NTD with variable lengths of CAG repeats has been demonstrated by a population study, which revealed that a shorter CAG repeat sequence was associated with higher risk of total prostate cancer (214). Amplification of AR gene has been well documented in prostate cancer cell lines and patient tissues, though this phenomenon is generally regarded as a treatment-induced mechanism of resistance (81, 83, 215). Finally, in addition to transcriptionally regulating the expression of genes involved in cell-cycle, proliferation, and differentiation as its normal function, AR sustains the growth and survival of prostate cancer cells by controlling central metabolism and biosynthesis (76-78, 216). Taken together, AR has oncogenic potential in prostate cancer, and therefore, targeting the AR is a viable therapeutic approach.
1.3.2 Current androgen receptor targeting therapies

As discussed in the previous sections, AR transcriptional activity is critical for all stages of prostate cancer, including oncogenic development (Section 1.3.1) and recurrent disease progression (Section 1.1.4). Targeting the androgen-AR axis has been a major therapeutic strategy to treat prostate cancer and CRPC, and it will continue to be a key approach (217). All current therapies that target the androgen-AR signaling, such androgen-deprivation therapies (ADT), antiandrogens, and CYP17 inhibitors, essentially act through the LBD of AR (Figure 1.5). ADT consists of surgical (orchiectomy) or chemical castration that aims to reduce the production of natural androgens such as testosterone, and thereby eliminating the ligand-dependent stimulation of AR transcriptional activity (23). ADT is the mainstay of treatment for advanced prostate cancer with distant metastases after receiving radical prostatectomy, but unfortunately ADT is only temporally effective as most patients progress and develop CRPC (24, 45). Possible AR-related mechanisms of resistance overcoming ADT and leading into the onset of CRPC have been described in an earlier section. Antiandrogens are compounds that block AR transcriptional activity by directly interacting with the AR, and all past and current antiandrogens competitively bind to the AR LBD and prevent the binding of androgens (192, 218). Once bound to the LBD, antiandrogens such as bicalutamide sterically disrupts the formation of AR AF-2 region, which consequently inhibits AR N/C interactions and impairs protein-protein interactions with coactivators (192, 193). CYP17 inhibitors such as abiraterone, are designed to deplete androgens by inhibiting the enzymatic activity of CYP17, which plays key roles in adrenal and intratumoral de novo biosynthesis of androgens (219, 220). Because androgen ultimately binds to the LBD to induce AR transcriptional activity, CYP17 inhibitors are considered
indirect AR LBD-targeting therapies, as opposed to antiandrogens that directly bind to the LBD. Nonetheless, second-generation antiandrogen enzalutamide and CYP17 inhibitor abiraterone represent the new-generation AR-targeting therapy approved to treat CRPC, and they have demonstrated significant clinical benefits from their individual trials (50, 53).

Second-generation antiandrogens have been developed with improvements over the first-generation antiandrogens such as flutamide and bicalutamide, which lose their effectiveness to increased expression of AR and gain-of-function AR mutation (87, 215). FIGURE 1.6 shows the chemical structures of antiandrogens and natural androgens. The new antiandrogens such as enzalutamide and ARN-509, have higher binding affinities for AR LBD when compared to bicalutamide, while maintaining receptor specificity for AR (218, 221). Importantly, enzalutamide and ARN-509 are able to maintain full antagonistic activity, in contrast to bicalutamide and flutamide that can stimulate AR transcriptional activity in the absence of androgen (218). In addition, these second-generation antiandrogens are effective against AR gain-of-function mutation W741C, which converts bicalutamide into a pure agonist (218). AR W741C was isolated from a patient with acquired resistance to bicalutamide (87). Abiraterone inhibits CYP17, an enzyme expressed in testicular, adrenal, and prostate cancer tissues (222). The enzymatic activity of CYP17 is responsible for the production of dehydroepiandrosterone (DHEA) and androstenedione, which are the precursors of testosterone (219). Despite a significant decrease of androgens in the body by ADT, active AR transcriptional activity continues to drive tumour growth in CRPC, possibly due to intratumoral androgen biosynthesis, which provides low but sufficient levels of androgens to activate AR (43, 223). By blocking androgen biosynthesis pathway, abiraterone
effectively inhibits androgen-dependent AR transcriptional activity to exert anti-tumour activity in CRPC patients (50, 51).

1.3.3 Mechanisms of resistance to abiraterone and enzalutamide

Unfortunately, most CRPC patients treated with the next-generation AR-targeting therapy abiraterone or enzalutamide will eventually develop resistance and succumb to the disease (224-226). Studies using *in vitro* and *in vivo* models indicated that resistance to abiraterone is associated with an upregulation of CYP17 expression, as the cancer cells attempt to increase androgen biosynthesis (227). This observation is supported by analyses of tumour biopsies from CRPC patients after CYP17 inhibitor therapy, which showed markedly elevated intratumoral expression of CYP17 (227). By using human CRPC xenograft models, one group revealed that the molecular adaptations to counter CYP17 inhibition by abiraterone may also involve increased expression levels of full-length AR and AR splice variants with truncated LBD (227). Possible mechanisms of resistance to enzalutamide have been investigated, and one of which involves a missense mutation from phenylalanine to leucine at position 876 (F876) in the ligand binding pocket of the AR LBD (228). The AR F876L mutation was first identified from a reporter-based mutagenesis screen, and preclinical studies showed that AR F876L converts enzalutamide into an AR agonist to rescue the growth inhibition by enzalutamide (88, 228). The clinical relevance of the AR F876L mutation is demonstrated by the detection of the mutant AR in plasma DNA from progressive CRPC patients failing second-generation antiandrogen therapy (229). Induction of glucocorticoid receptor (GR) expression has been proposed to confer resistance enzalutamide, as GR replaces AR to drive a similar but distinguishable transcription program
Preclinical studies showed that GR inhibition may have therapeutic benefit to treat enzalutamide-resistant CRPC (231). However, since GR overexpression was only observed in samples from a small subset of patients who responded poorly to enzalutamide, the clinical significance of GR-mediated resistance to enzalutamide remains to be validated (230, 232).

Clinical studies on the efficacy of abiraterone on CRPC patients progressed after enzalutamide treatment reported modest responses and brief duration of effect (224, 225). The clinical activity of enzalutamide on CRPC patients progressed after abiraterone treatment was shown to be limited as well (226, 233, 234). These observations suggest cross-resistance between abiraterone and enzalutamide, which may involve common mechanisms of resistance. Constitutively active AR splice variants have been implicated in resistance to abiraterone and enzalutamide. In a recent study, AR-V7 mRNA levels in circulating tumours cells from men with metastatic CRPC who received either abiraterone or enzalutamide were quantitatively analyzed (162). The study found positive AR-V7 expression in 12 of 31 enzalutamide-treated patients (39%) and 6 of 31 abiraterone-treated patients (19%) (162). Further analyses on each treatment group showed the presence of AR-V7 is associated with therapeutic resistance, as the AR-V7-positive patients had lower PSA response rates, shorter PSA progression-free survival, shorter radiographic progression-free survival, and shorter overall survival when compared to the AR-V7-negative patients (162). From bone marrow biopsies of enzalutamide-treated patients with bone metastatic CRPC, another study also reported that the presence of AR-V7 is associated with primary resistance to enzalutamide (235). Consistent preclinical findings also support the ability of AR splice variants to confer resistance to enzalutamide and abiraterone, as a group showed that AR-V7 transcriptional activity is unaffected by enzalutamide and drives enzalutamide-resistant growth in CRPC.
cells (236). In addition, another group demonstrated that abiraterone induces the expression of AR splice variants including AR-V7 in human CRPC xenografts, suggesting the production of AR splice variants is a natural adaptive response by CRPC cells in order to resist AR-targeting therapies (237). Taken together, these findings indicate that constitutively active AR splice variants may be a common mechanism of resistance to enzalutamide and abiraterone.

1.4 INHIBITORS OF THE AMINO-TERMINAL DOMAIN OF ANDROGEN RECEPTOR

1.4.1 Discovery of EPI-001 and identification of EPI-002 as the lead compound

EPI-001 is a small-molecule compound and an analogue of a compound isolated from the marine sponge *Geodia lindgreni* (238). EPI-001 is the hydrolyzed metabolite of bisphenol A diglycidic ether (BADGE) (238). The presence of BADGE-related compounds in the marine sponge is presumably due to bio-accumulation of industrial materials. The discovery of EPI-001 is significant because it was the first small-molecule reported to inhibit AR NTD, as supported by the findings that EPI-001 does not compete with androgen for binding to the AR LBD, but rather it interacts with the AF-1 region within the NTD (238). EPI-001 inhibits both androgen-dependent and androgen-independent AR transcriptional activities, and demonstrates receptor specificity as it has no effect on the activities of related steroid receptors GR and PR (238). EPI-001 inhibits androgen-induced growth of human prostate cancer cells *in vitro* and *in vivo* (238). Importantly, in castrated mice bearing LNCaP xenografts to model CRPC growth, EPI-001 causes significant tumour regression without producing toxicity to the hosts, highlighting the potential for EPI-001 to treat CRPC (238).
Pharmacokinetic studies demonstrated an ideal profile of EPI-001 by oral dosing, which has 86% bioavailability, approximately 3.3 hours of half-life, and a slow clearance rate of 1.75 l/h/kg (239). As required by FDA, EPI-001, which is a mixture of 4 stereoisomers with 2 chiral carbons at the 2 and 20 positions, needs to have each stereoisomer evaluated for efficacy because biological activity among stereoisomers may vary (239). The stereoisomer EPI-002 (2R and 20S configuration) showed a potent inhibition of AR transcriptional activity in vitro and caused regression of LNCaP CRPC tumours in vivo without reducing the body weight of mice (239). Therefore, EPI-002 is chosen to be the lead compound for further studies. Figure 1.7 shows the chemical structures of the EPI compounds.

1.4.2 Mechanism of action for EPI compounds

EPI-001 including EPI-002, or collectively referred to as EPI compounds, inhibit AR transcriptional activity by a unique mechanism of action (Figure 1.8), as opposed to all current AR-targeting therapies, which act through the LBD (Figure 1.5). Antiandrogens inhibit AR transcriptional activity by competitively binding to the LBD, and thereby preventing androgen binding to activate the receptor. In contrast, EPI compounds block AR activity by binding to the NTD with specificity, as steady-state fluorescence emission spectra revealed that EPI-001 alters the folding of AR AF-1 but not GR AF-1 (239). In vitro ligand competition assay showed that EPI compounds do not bind to the AR LBD, whereas antiandrogen expectedly binds to and competes with androgen binding (238, 239). Using Click-chemistry in living cells to compare active EPI analogs containing a chlorohydrin group and inactive EPI analogs lacking the chlorohydrin, two crucial pieces of information regarding how EPI compounds bind to AR NTD were obtained: active EPI compounds
covalently bind to the AR NTD in an irreversible manner, and the chlorohydrin group is required for the covalent binding (239). Findings from additional biochemical experiments suggested a possible binding mechanism for EPI compound, which initially involves a fast reversible binding to a basic site within the AF-1 region of AR NTD, followed by a slow rate-limiting epoxide formation, and finally the epoxide reacts irreversibly with a nucleophilic site within the AF-1 to form a covalent bond (239). Although the exact binding site of active EPI compounds in the AR AF-1 has yet to be determined, the above results have provided strong evidence to show that EPI compounds covalently bind to AR NTD.

Once bound to AR NTD, EPI compounds disrupt androgen-induced AR N/C interaction, similar to the effect of antiandrogen bicalutamide (238). Protein-protein interactions between AR and coactivator CBP can be induced by androgen or IL-6 in ligand-dependent or ligand-independent mechanism, respectively, but regardless of the presence of androgen or IL-6, EPI compounds effectively inhibit the physical interaction between AR and CBP in prostate cancer cells (238). Using in vitro recombinant protein binding assays, EPI compounds decrease the interactions between endogenous AR NTD and CBP as expected, and in addition, the interaction between AR NTD and the large subunit of general transcription factor TFIIF (RAP74) is also reduced by EPI compounds (238). Chromatin immunoprecipitation (ChIP) assay showed that EPI compounds block AR from binding to AREs on target genes such as PSA and TMPRSS2 (238). As a result, EPI compounds inhibit androgen-dependent AR transcriptional activity, leading to a significant decrease of expression of genes regulated by androgen-AR signaling in prostate cancer cells and tumour xenografts in mice (238, 239). Besides inhibiting androgen-dependent AR signaling, EPI compounds block androgen-independent AR transcriptional activity stimulated by alternative
protein kinase pathway or cytokine IL-6 (238). Furthermore, in vivo studies demonstrated that EPI compounds effectively inhibit the growth of androgen-dependent and castration-resistant prostate cancer.

1.4.3 Other inhibitors of androgen receptor amino-terminal domain

AR NTD contains the most if not all of AR transcriptional activity, highlighting the therapeutic potential of targeting the NTD to treat prostate cancer (121, 122). Furthermore, inactive AR NTD decoy molecules lacking the DBD and LBD can effectively block AR activity, and reduce both androgen-dependent and androgen-independent prostate cancer growth in vivo, validating the NTD as a therapeutic target (240). However, because the AR NTD is an intrinsically disordered domain in a pre-molten globular structure, drug discovery to this domain is very challenging (121, 241). In addition to the EPI compounds described in the previous section, only a few inhibitors of AR NTD have been reported. Sintokamid A, a chlorinated peptide isolated from marine sponge Dysidea sp., inhibits androgen-independent transcriptional activity of AR NTD in prostate cancer cells (242). The mechanism of action for Sintokamid A remains to be determined, and further studies are needed to validate its specificity and efficacy in vivo. Niphatenone B, a glycerol ether initially isolated from the marine sponge Niphates digitalis, covalently binds to the AF-1 regions of the AR NTD, but lack specificity as it also binds to the AF-1 of GR (243, 244). Functionally, Niphatenone B acts as an antagonist of AR NTD by inhibiting AR N/C interactions and blocking both androgen-dependent and androgen-independent AR transcriptional activities, and as a result, Niphatenone B attenuates the proliferation of AR-expressing prostate cancer cells (243, 244). Despite the ability of Niphatenone B to inhibit constitutively active AR splice variant AR-
V567es, further chemical studies indicated that the structural scaffold of Niphatenone compounds is not ideal for further drug development (243). As of today, EPI-002 remains as the most promising small-molecule inhibitor of AR NTD with therapeutic potential to treat prostate cancer.

1.4.4 Inhibitors of androgen receptor splice variants

One clear advantage of AR NTD inhibitors over antiandrogens or CYP17 inhibitors is the ability to inhibit constitutively active AR splice variants with truncated LBD, which are emerging as a clinically significant mechanism of resistance to current AR-targeting therapies (162). Indeed, studies demonstrated that AR NTD inhibitors EPI-002 and Niphatenone B can effectively inhibit constitutively active AR splice variant AR-V567es, in addition to inhibiting the full-length AR (239, 243). Chapter 3 of this dissertation will present more results showing EPI-002 also inhibits the androgen-independent and enzalutamide-resistant transcriptional activity of AR-V7, and reduces the growth of an antiandrogen-resistant CRPC model that is driven by AR-V7.

A few other compounds have been reported to inhibit AR splice variants, despite the lack of evidence for them to specifically target AR NTD. A group reported that niclosamide, a drug used to treat tapeworms, inhibits AR-V7 and reduces prostate cancer growth in vitro and in vivo (245). The mechanism for niclosamide to inhibit AR-V7 transcriptional activity is by decreasing AR-V7 protein expression via a proteasome-dependent protein degradation pathway (245). Similar to niclosamide, another group identified ASC-J9 as an AR degradation enhancer that can reduce the protein expression of full-length AR and AR splice variants, and thereby inhibiting the transcriptional activity of both AR species (246). By
promoting AR degradation, ASC-J9 can also effectively reduce the aggregation of AR with expanded polyglutamine tract, providing a viable therapeutic strategy to treat spinal and bulbar muscular atrophy or Kennedy disease (247). Currently, niclosamide and ASC-J9 are still undergoing preclinical evaluation as potential therapeutic agents for prostate cancer, and further characterization is needed to determine if these compounds directly target AR NTD. In addition, small-molecule inhibitors of AR DBD have been recently identified, and preclinical studies have demonstrated the ability of these inhibitors to block the transcriptional activity of AR splice variants (248, 249). Targeting AR DBD could be another viable approach to inhibit the AR splice variants, but since AR DBD is the most conserved domain among the steroid receptors, the specificity and off-target activity of AR DBD inhibitors need to be carefully investigated. Overall, as constitutively active AR splice variants with truncated LBD have been shown to play significant roles in the progression of CRPC and resistance to current AR-targeting therapies, there is an urgent and unmet demand for inhibitors of AR splice variants.

1.5 EXPERIMENTAL MODELS FOR PROSTATE CANCER

1.5.1 Cell lines

Cell culture is extremely valuable to drug research and development, providing a biologically relevant platform for essential preclinical studies of therapeutic compounds. For prostate cancer, many cell lines have been established from clinical samples or clonal derivatives of previously established lines, and different cell lines are models of different clinical phenotypes of prostate cancer. This section will describe several commonly used
prostate cancer cell lines, including all cell lines used in experiments pertaining to this
dissertation.

LNCaP cells, probably the most commonly used cell line in prostate cancer, were
originally isolated from lymph node metastases of a 50-year-old Caucasian man with prostate
cancer (250). LNCaP cells express endogenous and functional AR, and the growth of these
cells is androgen-sensitive (251, 252). The AR in LNCaP cells contains a gain-of-function
T877A mutation, resulting in a promiscuous AR that responds to other steroids to promote
growth and survival (85, 253). Some strains of LNCaP cells can proliferate without
androgens, and a few sublines have been derived from long-term androgen deprivation,
including LNCaP-AI, LNCaP-abl, and LNCaP95 cell lines. Details about LNCaP-AI and
LNCaP-abl cells can be found in a review by Sobel and Sadar (254). Of particular interest,
LNCaP95 cells endogenously express both the full-length AR and AR splice variants
including AR-V7, and studies revealed that these AR species regulate distinct transcriptional
programs (161). LNCaP95 cells represent a clinically relevant model to study AR-V7 and
evaluate AR inhibitors. VCaP is another cell line that expresses endogenous full-length AR
and AR splice variants. VCaP cells were derived from xenograft of a vertebral metastatic
lesion of a patient with hormone-refractory prostate cancer (255). VCaP cells predominantly
express full-length wild-type AR, and when compared to LNCaP cells, VCaP cells have a 20-
fold amplification of the full-length AR gene based on genome copy number analysis of AR
coding exons (199, 256). VCaP cells have been used to examine the ability of antiandrogens
to maintain effective inhibition of overexpressed AR (218). Similar to LNCaP95 cells, the
protein expression level of AR splice variants is lower than that of the full-length AR in
VCaP cells, but upon suppression of full-length AR, the expression of AR splice variants
increases (161). Furthermore, 22Rv1 is a cell line derived from a relapsed tumour of the CWR22 xenograft, which was originally isolated from human primary prostatic carcinomas (257, 258). Despite the expression of functional full-length AR and detectable levels of PSA, the growth of 22Rv1 cells is weakly stimulated by androgen, possibly because of the constitutively active AR splice variants that are also expressed in 22Rv1 cells (104, 258).

LNCaP, LNCaP95, and VCaP cells were used in experiments pertaining to this dissertation, and the findings and discussion are presented in later chapters.

There are also AR-negative prostate cancer cell lines. PC-3 and DU-145 cells do not express functional AR, and therefore their growth is androgen-independent and androgen-insensitive (254). PC-3 cells were isolated from a lumbar vertebral metastasis of a 62-year-old Caucasian man (259). DU-145 cells, the first prostate cancer cell line to be established in tissue culture, were derived from a brain metastasis of a 69-year-old Caucasian man with prostate cancer and lymphocytic leukemia (260). In the research and development of AR-targeting therapeutic agents, these AR-negative cells are commonly used as negative controls to show specificity for androgen-AR axis, as PC-3 cells was used in experiments for this dissertation.

1.5.2 In vivo models

Preclinical studies using appropriate in vivo models are crucial for validating and evaluating the efficacy of therapeutic compounds. Several commonly used prostate cancer in vivo models were established from xenografts of cell lines, such as LNCaP, VCaP, and PC-3 cells. As an in vivo model, LNCaP xenografts in mice demonstrate accurate correlation between tumour volume and serum PSA level (261). LNCaP xenografts in mice are a good
model for the clinical progression from androgen-sensitive to castration-resistance, as reflected by the initial decrease in PSA (both serum and tumour) levels resulted from castration followed by a progressive increase to reach the pre-castration levels within 4 weeks after castration (262). These features make LNCaP xenograft a valuable in vivo model to test AR inhibitors including enzalutamide and EPI compounds (218, 238, 239). VCaP xenografts can also grow in castrated mice, and studies showed that castration induces the expression of constitutively active AR splice variants in VCaP tumours (263). The ability of EPI-002 to inhibit the growth of CRPC that expresses amplified full-length AR and functional AR splice variants has been demonstrated by using VCaP in vivo model (239).

Because PC-3 cells do not express functional AR and their growth is androgen-independent, they are used to validate the in vivo specificity of AR inhibitors, which should not affect the growth of PC-3 xenografts (238).

In additional to xenografts of prostate cancer cell lines, xenografts of primary human tumours have been used as in vivo models to study the molecular biology underlying prostate cancer progression. Two xenografts of human tissues are described here, because they have been used to investigate the relationship between constitutively active AR splice variants and CRPC. LuCaP 86.2 xenograft was derived from a prostate cancer bladder metastasis, and contains both full-length AR and AR-V567es mRNA, with AR-V567es being the predominant AR species expressed (105, 107). In agreement with the notion that constitutively active AR splice variants can drive castration-resistant growth, studies demonstrated that LuCaP 86.2 xenografts do not respond to castration and behave as an in vivo model for CRPC (105). On the other hand, LuCaP 136 xenograft, derived from ascites cells, expresses a mix of full-length AR and AR-V567es with the full-length AR being the
predominant AR species (105). Consistent with its AR expression profile, studies showed LuCaP 136 xenografts display a modest response to castration, which only inhibits the tumour growth dependent on full-length AR (105).

1.5.3 Drug-resistant models

Experimental drug-resistant models are important to study the molecular mechanisms underlying clinical therapy resistance. First-generation antiandrogen bicalutamide was once the most prevalent antiandrogen used in the clinic to treat prostate cancer. Several bicalutamide-resistant prostate cancer cell lines have been established. LNCaP-cxD was the first bicalutamide-resistant prostate cancer cell line, and it was generated from the parental LNCaP cells cultured in the presence of bicalutamide with androgen-depleted medium (264). Within only 6-13 weeks of \textit{in vitro} exposure to bicalutamide, parental LNCaP cells started to grow in the presence of bicalutamide (264). Studies showed bicalutamide acts as an agonist to promote proliferation in LNCaP-cxD cells, as supported by consistent \textit{in vitro} and \textit{in vivo} data (264). The molecular mechanism underlying bicalutamide resistance in LNCaP-cxD cells was determined to be an AR gain-of-function mutation involving codon 741 in the LBD (264). The mutation W741C or W741L allows AR to be activated by bicalutamide, and thereby conferring resistance (264). LNCaP-Bic, another bicalutamide-resistant cell line, was generated after prolonged treatment with bicalutamide under low level of androgen for 3 months (265). In addition to bicalutamide-resistance, studies revealed the growth of LNCaP-Bic cells is androgen-independent (265). Because LNCaP-Bic cells do not overexpress AR and their AR does not show an enhanced transcriptional activity when compared to the AR in parental LNCaP cells, the mechanism of bicalutamide resistance in the LNCaP-Bic cells was
thought be independent of androgen-AR signaling pathway (265). A different group also established a bicalutamide-resistant LNCaP-BC2 cell line from long-term bicalutamide treatment (266). But unlike the androgen-independent LNCaP-Bic cells, studies demonstrated that LNCaP-BC2 cells are sensitive to androgen for growth, and overcome bicalutamide inhibition via an AR-dependent mechanism involving AR overexpression and androgen hypersensitivity (266). The AR from LNCaP-Bic and LNCaP-BC2 cells was each sequenced and compared to AR from parental LNCaP, and no difference was found, indicating mutation was not involved (265, 266).

Enzalutamide is a second-generation antiandrogen used to treat CRPC. Compared to the first-generation, the second-generation antiandrogens are developed with key improvements, including the abilities to overcome AR overexpression and AR LBD mutations that confer resistance to bicalutamide such as the AR W741C or W741L (218). Despite an initial effectiveness, most patients who responded to enzalutamide have eventually developed resistance and progressive disease (224, 225). To investigate the potential mechanism of resistance, Korpal and colleagues developed a LNCaP prostate cancer cell model with spontaneous resistance to enzalutamide (88). From AR expression analysis and global gene expression analysis of AR pathway activity, it was concluded that the mechanism of enzalutamide resistance in these cells neither involves AR overexpression nor significant change of AR-signaling pathway (88). By whole-transcriptome sequencing, a novel recurrent AR mutation F876L was identified from the enzalutamide-resistant cells, and further studies confirmed that the AR F876L is a gain-of-function mutation, which converts enzalutamide into an agonist and thereby promotes enzalutamide-resistant growth in prostate cancer cells (88). Another group led by Joseph also identified the AR F876L mutation by
developing and characterizing LNCaP cells resistant to the second-generation antiandrogens enzalutamide and ARN-509 (229). Importantly, this group provided clinical evidence for AR F876L, as the mutant was detected in plasma DNA from ARN-509 treated patients with progressive CRPC (229).

A few chemotherapy-resistant prostate cancer cell lines were generated to investigate the molecular mechanisms of resistance against docetaxel, which was the only therapy that improved CRPC survival before 2010 (46, 47). Docetaxel-resistant prostate cancer cell lines DU-145 R and 22Rv1 R were established over a period of 6 months by gradually increased concentrations of docetaxel (267). Studies revealed that the classical drug pump P-glycoprotein is overexpressed in the resistant DU-145 R and 22Rv1 R cells when compared with their respective age-matched parental cells, and the resistance to docetaxel-induced apoptosis in these two cell lines can be reversed by using a P-glycoprotein inhibitor (267). Interestingly, P-glycoprotein is neither expressed in the age-matched parental PC-3 cells nor the docetaxel-resistant PC-3 cells, and P-glycoprotein inhibitor has no effect on docetaxel-induced apoptosis in the parental and resistant PC-3 cells (267). Further studies elucidated that the mechanism underlying docetaxel resistance in PC-3 cells involves transcription factor NF-κB, as treatment of docetaxel significantly increases NF-κB transcriptional activity in the PC-3 resistant line when compared to the parental line, and the resistance can be effectively reversed by using an inhibitor of NF-κB (267).
1.6 RESEARCH SUMMARY

1.6.1 Overview of dissertation

Prostate cancer is a major health issue for men worldwide. AR is a transcription factor that regulates gene expression, and many AR-target genes are involved in cellular activities such as differentiation, protein synthesis, and proliferation (76). AR is also a validated therapeutic target in prostate cancer, because AR signaling is a major driver of tumour growth and survival (217). Even in CRPC where serum testosterone remains at castrated level, AR transcriptional activity is aberrantly restored to play a key role in the development and progression of CRPC, as supported by clinical evidence that targeting the AR leads to improved survival of CRPC patients (48). All current AR-targeting therapies for CRPC act through the LBD of AR, either by directly binding to the LBD to compete against androgen binding, or by inhibiting the biosynthesis of androgens. In contrast, EPI-002 inhibits the AF-1 region of AR NTD, which contains the majority if not all of AR transcriptional activity (166, 239). It has been previously shown that EPI-002 covalently and specifically binds to AR NTD, and inhibits androgen-independent AR transcriptional activity mediated by the NTD in additional to blocking androgen-dependent AR transcriptional activity (238, 239).

In the remainder of this dissertation, Chapter 2 describes the discovery and characterization of novel AR inhibitors called spongian diterpenoids, which act as antiandrogens to inhibit AR transcriptional activity by competitively binding to AR LBD. However, because constitutively active AR splice variants lacking the LBD are emerging as a major mechanism underlying resistance to all current AR-target therapies including antiandrogens, inhibitors of AR NTD have the advantage of inhibiting both full-length AR and the AR splice variants. Therefore, in Chapter 3, the focus shifts to the preclinical
evaluation of AR NTD inhibitor EPI-002. Results are presented and discussed to show that EPI-002 has the potential to be effective against all known molecular alterations believed to cause aberrant AR transcriptional activity that is pathologically associated with CRPC.

Chapter 4 reports the generation of a human prostate cancer cell line model resistant to EPI-002 after chronic exposure to the drug. This drug-resistant model is a valuable tool to investigate possible mechanisms of resistance to AR NTD inhibitors like EPI-002. Finally, Chapter 5 provides a conclusion of all research findings presented in this dissertation, and highlights the significance of this dissertation in terms of how it may contribute to the future of drug research and development for prostate cancer.

1.6.2 Hypotheses and objectives

AR-targeting agents such as antiandrogens have therapeutic benefit to treat most CRPC, but all current AR-targeting therapies act through AR LBD and have limited clinical activity. In contrast, I hypothesize that EPI, an antagonist of AR NTD will be capable of overcoming AR-related molecular mechanisms presently proposed to drive aberrant AR transcriptional activity. Furthermore, I hypothesize that prostate cancer cells will develop resistance against this AR NTD inhibitor after prolonged exposure.

In addition to testing the hypotheses, there are three research objectives: 1. to characterize novel AR inhibitor spongian diterpenoids and elucidate the mechanism of action for these compounds; 2. to evaluate AR NTD inhibitor EPI-002 against several AR-related molecular alterations proposed to affect AR transcriptional activity including mechanisms underlying aberrant AR transcriptional activity; and 3. to generate a human prostate cancer
cell line model that has developed resistance to EPI-002 after long-term culture in the presence of EPI-002.

Objective 1 is addressed by the following specific aims: 1) to determine if the spongian diterpenoids target the LBD or NTD of AR; 2) to test if the diterpenoids block AR transcriptional activity and inhibit gene expression regulated by AR; 3) to examine if the diterpenoids have target specificity for AR; and 4) to demonstrate if the diterpenoids reduce the in vitro proliferation of prostate cancer cells and have in vivo effect on androgen-dependent tissue. The materials and methods and results pertaining to Objective 1 are presented and discussed in Chapter 2 of this dissertation.

Objective 2 is addressed by the following specific aims: 1) to assess if EPI-002 maintains effective inhibition of AR transcriptional activity when several known AR coactivators are individually overexpressed in prostate cancer cells; 2) to examine if EPI-002 inhibits the transcriptional activity of several clinically relevant AR gain-of-function mutations; 3) to investigate if EPI-002 blocks the transcriptional activity of constitutively active AR splice variant AR-V7; 4) to determine if EPI-002 inhibits the in vitro cell growth and in vivo tumour growth of a CRPC model that is driven by AR-V7; 5) to evaluate EPI-002 against polymorphic AR NTD with variable lengths of polyglutamine tract; and 6) to test if EPI-002 blocks increased AR transcriptional activity caused by cytoplasmic AR variant AR23. All the materials and methods and results pertaining to Objective 2 are presented and discussed in Chapter 3.

Objective 3 is addressed by the following specific aims: 1) to establish a LNCaP cell line with chronic exposure to EPI-002 by long-term culture in the presence of EPI-002; 2) to determine if resistance has developed in these cells by performing in vitro proliferation
assay; and 3) if resistant *in vitro* proliferation to EPI-002 is observed, to validate the resistance by using *in vivo* xenografts of these cells in an animal study. The materials and methods and results pertaining to Objective 3 are described and discussed in Chapter 4.
Most prostate cancer is androgen-dependent. Serum PSA level is a commonly used biomarker to monitor the progression of prostate cancer, because it correlates with tumour growth. Localized prostate cancer can be effectively treated by active surveillance, radiation therapy, or radical prostatectomy. However, many men will develop advanced prostate cancer with distant metastases after receiving the primary treatment for localized disease. Androgen deprivation therapy (ADT) is the mainstay of treatment for advanced disease. ADT consists of orchiectomy or LHRH analogs (surgical or chemical castration, respectively), which aims to reduce the production of testosterone. Antiandrogens including flutamide and bicalutamide are also used in combination with orchiectomy or LHRH analogs to achieve maximum androgen blockade. Unfortunately ADT is only temporarily effective, as most patients will progress into castration-resistant prostate cancer (CRPC) within 12-33 months. CRPC is lethal with a mean survival time of approximately 16-18 months. Currently there are several therapeutic agents available for CRPC, including the next-generation hormonal therapy abiraterone and enzalutamide, which target the androgen-AR signaling. However, prostate cancer cells have inevitably developed resistance to these therapeutic agents, and thereby limiting their clinical activity.
Figure 1.2. Mechanisms for aberrant AR transcriptional activity.

Compelling evidence supports the notion that aberrant AR transcriptional activity is a major driver of most CRPC. There are several molecular mechanisms proposed to explain the aberrant AR activity despite castrated testosterone level: 1. Amplification of the AR gene and overexpression of AR protein level, providing hypersensitivity to low levels of androgen; 2. AR gain-of-function mutations that allow the AR to be activated by non-androgenic steroidal ligands such as glucocorticoid and convert antiandrogens into agonists; 3. Overexpression of AR coactivators that can enhance androgen-dependent and also promote ligand-independent AR transcriptional activities; 4. Androgen-independent AR transactivation through its N-terminal domain (NTD), such as cytokine IL-6 that can stimulate AR transcriptional activity in the absence of androgen; 5. Increased adrenal and/or intratumoral androgen biosynthesis, generating a low but sufficient level of androgen to support AR transcriptional activity; and 6. AR splice variants with truncated ligand-binding domain (LBD), which has the potential to be constitutively active regardless of the presence of androgen.
Figure 1.3. Schematic representation of AR gene and protein.

The human AR gene is located at q11-12 on the X-chromosome. The gene spans about 90 kilobase of DNA containing eight exons that encode an mRNA transcript of about 10.6 kilobase in length. The most commonly published full-length AR protein product contains 919 amino acids with four distinctive functional domains: N-terminal domain (NTD), DNA-binding domain (DBD), hinge region (HR), and ligand-binding domain (LBD). Exon 1 encodes the full NTD, exons 2 and 3 encode the DBD, and exons 4-8 encode the HR and LBD. Two motifs involved in protein-protein interactions and AR N-terminal/C-terminal (NC) interactions are located in the NTD: FXXLF and WXXLF motifs. The NTD also contains an activation function-1 (AF-1) region, and two transcription activation units (TAU1 and TAU5). A nuclear localization signal (NLS) sequence is found in the HR, with part of it extending into the DBD. The LBD contains an AF-2 region, which androgen binds to induce interaction between AF-2 with the FXXLF motif of the NTD, resulting in intramolecular AR N/C interaction.
Figure 1.4. Androgen-induced AR transcriptional activity.

Testosterone (T) is converted to dihydrotestosterone (DHT) by 5α-reductase. DHT is the most potent natural androgen. Once bound by DHT, AR dissociates from chaperone molecules such as heat shock proteins (HSP) and undergoes conformational changes including intramolecular AR N/C interaction. DHT-bound AR also undergoes post-translational modifications, such as phosphorylation, methylation, and acetylation. DHT binding causes AR to translocate into the nucleus. Two DHT-bound AR molecules form a dimer by intermolecular AR N/C interaction, and bind to specific DNA sequences called androgen-response elements (ARE), which are generally located in the promoter and/or enhancer regions of AR-regulated genes. Through protein-protein interactions, AR recruits coactivators such as CBP/p300 and the p160 family of coactivators, which have chromatin-modifying activities to facilitate gene transcription. Finally, an active AR transcriptional complex is formed when RNA polymerase II (Pol II) is recruited, leading to the initiation of transcription.
Androgen deprivation therapy (ADT) consists of surgical or chemical (LHRH agonist or antagonist) castration, which is aimed to eliminate the production of androgen (A) such as testosterone, and thereby blocking androgen-dependent activation of AR through the ligand-binding domain (LBD) where androgen binds to. Similarly, CYP17 inhibitors such as abiraterone are designed to deplete androgens by inhibiting androgen biosynthesis. Therefore, ADT and abiraterone are considered to indirectly target the AR LBD. Antiandrogens like enzalutamide directly bind to the LBD with high affinity and inhibit AR transcriptional activity by competing against androgens from binding.
Figure 1.6. Chemical structures of antiandrogens and androgens.

Flutamide and bicalutamide are the first-generation antiandrogen, whereas enzalutamide and ARN-509 represent the second-generation. Enzalutamide has obtained FDA approval to treat CRPC before or after chemotherapy, and ARN-509 is an investigational drug currently undergoing a Phase III clinical trial to treat non-metastatic CRPC. Note that these antiandrogens are non-steroidal in structure, and bicalutamide is structurally related to flutamide while enzalutamide is structurally related to ARN-509. Testosterone is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT is the most potent natural androgen to stimulate AR transcriptional activity. R1881 is a potent synthetic non-aromatizable androgen commonly used in experiments. Androgens are steroidal compounds.
Figure 1.7. Chemical structures of EPI compounds.

EPI-001 is a small molecule compound with a molecular weight (MW) of 395 daltons. EPI-001 contains 2 chiral carbons at the 2 and 20 positions, giving rise to 4 stereoisomers with different R and S configurations. EPI-002 (2R, 20S) has a potent \textit{in vitro} inhibition of AR transcriptional activity, and causes \textit{in vivo} regression of CRPC xenografts without significantly affecting the body weight of mice. Therefore, EPI-002 is selected to be the lead compound to undergo further studies as a potential therapeutc agent for prostate cancer.
In contrast to all current AR-targeting therapies, which act through the ligand-binding domain (LBD), EPI-002 binds to the intrinsically disordered N-terminal domain (NTD) of AR. EPI-002 binds to the AF-1 region within the NTD, potentially disrupting a conformation of AF-1 that is required for critical protein-protein interactions. As a result, EPI-002 inhibits AR N/C interaction and prevents the interaction between AR and coactivators such as CBP/p300. Although EPI-002 does not affect AR nuclear translocation, it blocks AR from binding to the androgen-response elements (ARE) of target genes, and thereby inhibits AR transcriptional activity.

Figure 1.8. Mechanism of action for EPI-002 to inhibit AR.
CHAPTER 2. Discovery and characterization of novel antiandrogens

2.1 INTRODUCTION

Localized prostate cancer can be effectively managed by active surveillance, surgery, or radiation therapy (18). Unfortunately as many as 47% of patients with localized disease will progress into advanced prostate cancer with metastasis and require systemic therapy (20). Prostate cancer is primarily an androgen-dependent disease, as demonstrated by the pioneer studies of Dr. Huggins, who was awarded the 1966 Nobel Prize for Physiology and Medicine for the discovery of hormonal treatment for certain types of cancers, including prostate cancer (21). Therefore, androgen deprivation therapy (ADT) by either chemical or surgical castration is the primary treatment for advanced prostate cancer (38, 44). ADT is designed to reduce levels of testicular androgen, and thereby suppressing the activity of androgen receptor (AR) that drives the growth and survival of prostate cancer (40, 75). After an initial and effective response to castration, resistance will inevitably occur with the development of lethal metastatic castration-resistant prostate cancer (mCRPC). In spite of reduced levels of testicular androgen, there is compelling evidence to indicate that AR plays a key role in CRPC (43, 140). The clinical onset of most CRPC patients is accompanied by a rise in serum prostate-specific antigen (PSA) level, and because PSA is an AR-regulated gene, AR activity is believed to be functionally restored in CRPC (43, 82).

AR functions as a ligand-activated transcription factor to regulate gene expression in response to androgen (76). Binding of androgen to the ligand-binding domain (LBD) of AR initiates a series of conformational changes that promote the N-terminal/C-terminal domain.
interaction (N/C interaction), leading to the dissociation of AR from chaperone molecules such as the heat shock proteins and resulting in post-translational modifications (150, 151, 153). The androgen-bound AR translocates into the nucleus, where it dimerizes with another AR before binding to the DNA of target genes (154, 155). AR directly binds to specific DNA sequences called androgen-response elements (ARE) that are present in the regulatory regions of its target genes (82, 178). The DNA-bound AR recruits coactivators with chromatin-modifying activities, and when RNA polymerase II joins the AR-coactivator complex, an active AR transcriptional complex is formed to initiate transcription (157-159). Many AR-regulated genes are involved in important cellular biology such as protein synthesis, metabolism, secretion, cell-cycle regulation, and proliferation (76, 78). In prostate cancer, the expression and function of AR are essential for the growth and survival of the cancer cells (75, 268). Importantly, AR transcriptional activity is aberrantly restored despite low levels or the absence of androgen, as seen in most cases of CRPC (43, 269). Molecular mechanisms suspected to be involved in the aberrant and persistent AR activity in CRPC include: 1) AR gene amplification and/or increased expression of AR (83, 215, 270); 2) gain-of-function mutations in AR that result in activation by non-androgenic steroidal ligands and antiandrogens (264, 271); 3) increased expression of AR coactivators (90, 92, 272, 273); 4) ligand-independent activation of AR by alternative pathways such as the cAMP-dependent protein kinase, interleukin-6, and other factors (96-98, 274, 275); 5) adrenal or intratumoral de novo synthesis of androgens (101); and 6) expression of constitutively active AR splice variants with truncated LBD (102, 276). Therapies that can effectively inhibit AR transcriptional activity will have significant clinical benefits in the treatment of prostate cancer.
Currently, AR is therapeutically targeted by ADT, CYP17 inhibitors, and antiandrogens. ADT involves orchiectomy (surgical castration) or LHRH analogues (chemical castration), and aims to decrease the level of testicular androgen, which binds to the AR LBD and activates the receptor (38, 44). CYP17 is an enzyme essential for androgen biosynthesis, and CYP17 inhibitors such as abiraterone effectively reduces the adrenal or intratumoral level of androgen, and thereby preventing androgen-induced AR transcriptional activity in CRPC (50, 101, 277). ADT and CYP17 inhibitors are considered to be indirect AR-targeting therapies. Antiandrogens such as bicalutamide and enzalutamide competitively bind to AR LBD to antagonize the action of androgens, and thereby directly blocking AR transcriptional activity (192, 193, 218). Unfortunately, prostate cancer cells have acquired resistance against first-generation antiandrogens including bicalutamide, and therefore, second-generation antiandrogens such as enzalutamide have been developed and approved to treat metastatic CRPC (54, 218, 278). Despite significant survival improvement by enzalutamide, no antiandrogen can stop CRPC progression (224, 225). However, once an antiandrogen fails, changing to an alternative second-line antiandrogen can be clinically effective with improved survival (279, 280), thereby supporting the quest to discover additional antiandrogens to treat prostate cancer.

Here, the research objective is to characterize novel AR inhibitor spongian diterpenoids and elucidate the mechanism of action for these compounds, involving four specific aims: 1) to determine if the spongian diterpenoids target the LBD or NTD of AR; 2) to test if the diterpenoids block AR transcriptional activity and inhibit gene expression regulated by AR; 3) to examine if the diterpenoids have target specificity for AR; and 4) to demonstrate if the
diterpenoids reduce the *in vitro* proliferation of prostate cancer cells and have *in vivo* effect on androgen-dependent tissue.

**2.2 MATERIALS AND METHODS**

**2.2.1 Spongian diterpenoids**

T1 (spongia-13(16),14-dien-19-oic acid) was isolated from a crude extract of a marine sponge based upon its activity in a cell-based high-throughput screening assay for compounds that inhibit AR transcriptional activity. T1 belongs to a family of compounds called spongian diterpenoids. T2 and T3 are semisynthetic compounds produced by reducing either the furan or 17β carboxylic acid groups in T1, respectively. The diterpenoid compounds used in experiments pertaining to this dissertation were provided by Dr. Andersen (Department of Chemistry, Department of Earth Ocean & Atmospheric Sciences, University of British Columbia).

**2.2.2 Cell lines**

LNCaP human prostate cancer cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen™ by Life Technologies, Carlsbad, CA). PC-3 cells were maintained in DMEM with 5% (v/v) FBS. CV-1 monkey kidney cells were maintained in MEM medium with 10% (v/v) FBS and 1% (v/v) L-glutamine. VCaP cells were maintained in DMEM containing 10% (v/v) FBS. These cell lines were obtained from the American Type Culture Collection (ATCC). LNCaP95, an androgen-independent cell line derived from LNCaP cells exposed to chronic androgen depletion, were maintained in RPMI 1640 containing 10% (v/v) dextran-coated charcoal-stripped serum (CSS). The
LNCaP95 cells were kindly provided by Dr. Plymate (University of Washington, Seattle, WA), who has previously published studies using these cells (161). All cells were maintained in culture for no more than 10 to 15 passages and regularly tested to ensure they were mycoplasma-free.

2.2.3 Chemical reagents

Metribolone, or R1881, is a synthetic androgen purchased from Perkin-Elmer (Wellesley, MA). Antiandrogen bicalutamide was from AstraZeneca (London, UK). 4-pregnene-3,20-dione was from Steraloids Inc (Newport, RI). All other chemicals were obtained from Sigma-Aldrich (St. Louis MO), unless stated otherwise.

2.2.4 Proliferation assays

Two methods were used to measure cellular proliferation: bromodeoxyuridine (BrdU) incorporation and AlamarBlue® reduction. The BrdU incorporation method detects proliferating cells that are undergoing S phase, in which Brdu is incorporated into newly synthesized DNA. The AlamarBlue® reduction method relies on the living cell’s natural ability to reduce resazurin and convert it into resorufin, which generates bright red fluorescence. For BrdU incorporation, LNCaP cells (5,000 cells/well) were plated in RPMI 1640 containing 0.5% FBS, and PC-3 cells (2,000 cells/well) were plated in DMEM with 5% FBS and then changed to serum-free media the next day. 96-well Falcon Primaria tissue culture plates were used for plating. Both LNCaP and PC-3 cells were pre-treated with DMSO vehicle, bicalutamide, T1, T2, or T3 for 1 hour, and then treated with 0.1 nM R1881. LNCaP and PC-3 cells were labeled with BrdU at the indicated times after treatment. All
media were removed and the plates were dried in a Hybaid oven at 60°C for 1 hour. BrdU ELISA kit (Roche Applied Science, Mannheim, Germany) was employed for BrdU labelling and ELISA assay, following the manufacturer’s protocol.

For AlamarBlue® (Invitrogen™) reduction, 96-well Falcon Primaria tissue culture plates were used to plate LNCaP cells (5,000 cells/well) in RPMI 1640 containing 0.5% FBS, PC-3 cells (2,000 cells/well) in DMEM containing 5% FBS, LNCaP95 cells (7,500 cells/well) in RPMI containing 5% dextran-coated CSS, and VCaP cells (8,000 cells/well) in DMEM containing 5% dextran-coated CSS. One day after plating, LNCaP and PC-3 cells were treated, and the media in PC-3 cells was changed to serum-free DMEM. LNCaP95 and VCaP cells were treated two days after plating. For treatment, all cells were pre-treated with vehicle control, bicalutamide, or each diterpenoid for 1 hour before addition of 0.1 nM R1881. At indicated time points, AlamarBlue® was added to the cells for 2 hours at 37°C, and then the reduction of AlamarBlue® in living cells was quantified by measuring fluorescence intensity at 570nm excitation and 585nm emission wavelengths using Infinite® M1000 (TECAN). The fluorescence intensity measured is proportional to the number of living cells.

2.2.5 Plasmids and transfection for luciferase assays

The expression plasmid containing human full-length wild-type androgen receptor (AR), pSV-AR0 was from Dr. Brinkmann (Erasmus University, Rotterdam, The Netherlands) (281). The AR-driven reporter PSA (6.1kb)-luciferase has been previously described (97, 282). Another AR-driven reporter ARR3-tk-luciferase reporter has also been used for published studies (283). Human progesterone receptor (PR) expression plasmid
pCR3.1-hPR and PR-driven reporter PRE-E1b-Luc were provided by Drs. Smith and Weigel (Baylor College of Medicine, Houston, TX). Human glucocorticoid receptor (GR) expression plasmid was from GeneCopoeia (Germantown, MD), and GR-driven reporter pGRE-Luc construct was from Panomics (Fremont, CA).

All transfections for luciferase assays were performed in phenol red-free media. LNCaP cells (1 X 10^5 cells/well) were plated at 60-70% confluence in 12-well plates using RPMI 1640 with 10% FBS. 24 hours later, Lipofectin (Invitrogen™) was used to transfect the cells with ARR3-tk-luciferase reporter (for AR transcriptional activity), or PRE-E1b-Luc and PR expression plasmid (for PR transcriptional activity), or pGRE-Luc and GR expression plasmid (for GR transcriptional activity) under serum-free conditions by gently removing the media containing FBS. 24 hours after transfection, the cells were pre-treated with compounds under testing for 1 hour prior to addition of 1 nM R1881, 10 nM Preg (4-pregnene-3,20-dione), or 10 nM DEX (dexamethasone). The treated cells were incubated for 48 hours with ligands before harvesting with Passive Lysis Buffer (Promega, Madison, WI). Luciferase activities were measured by using the Luciferase Assay System (Promega) with the aid of a multiplate luminometer (EG&G Berthold, Wildbad, Germany). The relative luminescent unit per second (RLU/s) was normalized to the amount of protein in each sample measured by Bradford protocol. For wild-type AR transcriptional activity, CV-1 cells (3.0 X 10^4 cells per well) were plated in 24-well plates. Fugene6 (Roche) was used for the co-transfection of 0.25 µg of ARR3-tk-luciferase reporter plasmid and 0.125 µg of pSV-AR0 per well. 24 hours later, cells were pre-treated with the compounds for 1 hour prior to addition of 1nM R1881. Cells were harvested 24 hours after treatment for luciferase assay as described above.
For mammalian two-hybrid N/C interaction assays, CV-1 cells (3.0 X 10^4 cells per well) plated in 24-well plate were co-transfected using Fugene6 with 0.25 µg/well 5XGAL4-Luc reporter vector, 0.25 µg/well VP16-AR-NTD that encodes the VP16 transactivation domain fused to amino acid residues 1-565 of AR N-terminal domain (NTD), and 0.25 µg/well GAL4DBD-AR-LBD that contains the Gal4 DNA-binding domain (DBD) fused to amino acid residues 628-919 of the wild-type AR ligand-binding domain (LBD). Transfected cells were incubated for 24 hours and then pre-treated with the compounds for 1 hour prior to the addition of 1 nm R1881 or ethanol control. Cells were harvested 24 hours later for luciferase assay as described above.

2.2.6 Endogenous expression of androgen-regulated genes

LNCaP cells (180,000 cells/well) were plated in 6-well plates in RPMI 1640 with 10% FBS. After 1 day or the cells attached, the media was gently replaced with serum-free RPMI media, and the cells were incubated for 48 hours prior to pre-treatment for 1 hour with DMSO vehicle, bicalutamide, or each of the diterpenoids before addition of 1 nM R1881. VCaP cells (300,000 cells/well) were plated in 6-well plates in DMEM with 5% dextran-coated CSS. Two days after plating, VCaP cells were treated in the same manner as LNCaP. Total RNA was isolated after 48 hours of treatment (for LNCaP) and 16 hours of treatment (for VCaP) by using RNeasy® Micro Kit (QIAGEN, Valencia, CA), and subsequently reverse transcribed to cDNA by SuperScript® III First-Strand Synthesis System for RT-PCR (InvitrogenTM). Diluted cDNA and gene-specific primers were combined with Platinum® SYBR® Green qPCRSuperMix-UDG with ROX (Invitrogen™), and the transcripts were measured by quantitative real-time (qRT)-PCR (ABI PRISM®, Applied Biosystems by Life
Technologies, Carlsbad, CA). qRT-PCR (QPCR) was performed in triplicates for each biological sample using microAmp® optical 384-well reaction plate (Life Technologies). Expression levels were normalized to housekeeping gene GAPDH. Primers used were previously described (238, 284, 285).

2.2.7 In vitro ligand binding assay

Androgen Receptor, Progesterone Receptor, and Estrogen Receptor alpha PolarScreen Competitor Assay kits (Invitrogen™) were employed following the manufacturer’s protocol. Serial dilution was done for each small molecule, and solvent was compensated to ensure equal volume percentage of DMSO and ethanol in each sample. Fluorescence polarization at excitation wavelength 470 nm and emission at 535 nm was measured in Greiner 384 black clear bottom plates by Infinite ® M1000 (TECAN, Grödig, Austria).

2.2.8 Calculations for IC_{50} and EC_{50}

Transcriptional activities of wild-type AR transfected into CV-1 cells and LNCaP endogenous AR with a T877A mutation were measured after treatment of varying concentrations of the diterpenoids in cell-based luciferase assays by transient transfection as described above. Nonlinear regression fit for Log[inhibitor] vs. response curves were generated using Prism (GraphPad Software, La Jolla, CA) to calculate the values for half-maximal inhibitory concentration (IC_{50}) for each diterpenoid’s inhibition of AR transcriptional activity. Data from the in vitro ligand binding assays were plotted as Nonlinear Regression Fit for One-site Competition curves by Prism (GraphPad Software), and the half-maximal effective concentration (EC_{50}) values were calculated from the binding
curves. The modeling equation used for both the IC$_{50}$ and EC$_{50}$ curves was $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{\text{X} - \text{LogIC}_{50}})$.

### 2.2.9 Western blot analysis

Whole-cell lysates were collected by solubilizing cells in RIPA buffer containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablet™ (Roche). The protein concentration of samples was quantified using BCA Protein Assay Kit (Thermo Scientific Inc., Rockford, IL). Western blot analysis of protein lysates used anti-androgen receptor antibodies PG-21 (MILLIPORE, Temecula, CA), or N-20 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were re-probed with monoclonal mouse anti-β-actin antibody (ab8226 from Abcam, Cambridge, MA).

### 2.2.10 Animal studies

12-week-old male nonobese diabetic-severe combined immunodeficiency (NOD-SCID) intact mice were bred and maintained in the Animal Care Facility at the British Columbia Cancer Agency Research Center. All animal experiments were approved by the University of British Columbia Animal Care Committee, and the ethical regulatory standards were strictly followed. The mice were divided into three groups: DMSO control ($n = 10$), bicalutamide (BIC, $n = 8$), and T3 ($n = 8$). BIC and T3 were administered by oral gavage at 10 mg/kg daily for a total of 13 doses. Initial and final body weights were recorded for each mouse. Mice were sacrificed 1 day after the final treatment, and tissues were collected and weighed. Prism (GraphPad Software) was used to generate Whisker plots for the weight of seminal vesicles.
and testes for each treatment group. Change in the body weight was calculated from the
difference between initial and final weight as a percentage of initial weight.

2.2.11 Statistics

Statistical analysis was performed using GraphPad Prism (version 6.01; GraphPad
Software). Comparisons between groups were done with unpaired 2-tailed Student’s t test,
unless otherwise noted. The differences were considered statistically significant with a P
value less than 0.05.

2.3 RESULTS

2.3.1 Diterpenoids bind to AR LBD and disrupt AR N/C interaction

Diterpenoids T1, T2, and T3 share an unsubstituted tricyclic perhydrophenanthrene ABC
ring system fused to a 5-membered D ring with the physiologic AR ligand
dihydrotestosterone (DHT) as well as the synthetic androgen R1881(Figure 2.1). The
chemical structures of diterpenoids (Figure 2.1) resemble steroidal androgens, and thereby
suggesting that they may physically interact with AR LBD. To determine if these
diterpenoids bind to AR LBD and if so, to assess their binding affinities, we performed in
vitro ligand competitor binding assays using recombinant wild-type AR LBD. R1881 had
strong affinity for AR LBD with a half-maximal effective concentration (EC50) calculated at
5.3 nM (Figure 2.2A). Compared to R1881, T1 showed weaker binding with an EC50 of 27.1
µM, whereas T2 was much weaker at 398.4 µM. However, T3 which has the carboxylic acid
functionality present in T1 reduced to a primary alcohol displayed an improved binding
affinity with an EC50 of 1.6 µM, which was comparable with that achieved by bicalutamide
at 1.1 µM (Figure 2.2A). Bicalutamide is a nonsteroidal antiandrogen used clinically to block AR activity and inhibit androgen-dependent proliferation of prostate cancer cells (286).

Furthermore, AR N-terminal/C-terminal interaction (N/C interaction) is required for androgen-dependent AR transcriptional activity, and the mechanism for antiandrogen such as bicalutamide to inhibit AR transcriptional activity involves blocking androgen-induced AR N/C interaction (150, 192, 193). To investigate if diterpenoids behave like antiandrogen and inhibit androgen-induced AR N/C interaction, the mammalian two-hybrid system was employed using wild-type AR LBD. Indeed, T1 and T3 significantly inhibited androgen-induced N/C interaction (Figure 2.2B). Inhibition of N/C interaction by T3 was comparable to that achieved with bicalutamide, consistent with their similar binding affinities for AR LBD. T2 had no effect on AR N/C interaction, which can be explained by its poor binding affinity to the LBD. Together, these data reveal that a structure-activity relationship clearly exists among the diterpenoids, and T1 and T3 inhibit AR N/C interaction by directly binding to AR LBD.

2.3.2 Diterpenoids inhibit AR transcriptional activity

To confirm that the diterpenoids inhibit AR transcriptional activity, human prostate cancer LNCaP cells were used to conduct luciferase reporter assays using an AR-driven reporter ARR3-tk-luciferase (ARR3-LUC). Androgen-induced ARR3-LUC reporter activity was potently blocked to approximately baseline levels by T1 and T3 at 10 µM concentration, and this inhibition was comparable to that achieved with an equal concentration of bicalutamide (Figure 2.3A). Interestingly, 10 µM T2 also significantly inhibited AR transcriptional activity with about 50% inhibition, although T2 was less potent than
bicalutamide, T1 or T3. Dose-response curves (Figure 2.3B) estimated the half-maximal inhibitory concentration (IC$_{50}$) for inhibition of endogenous AR transcriptional activity in LNCaP cells to be 0.21 µM for bicalutamide, 4.2 µM for T1, 0.41 µM for T3, and greater than or equal to 10 µM for T2. Together these data indicate that these diterpenoids were effective in blocking transcriptional activity of endogenous AR in LNCaP cells with T2 being less potent than T1 and T3.

The endogenous AR in LNCaP cells has a T877A mutation in the LBD (AR-T877A) that alters both ligand specificity and affinity (253). Therefore, to investigate the effect of diterpenoids on wild-type AR transcriptional activity, AR-negative CV-1 cells were transiently co-transfected with ARR3-LUC and an optimized amount of an expression vector for wild-type human AR that resulted in a physiologically relevant expression level of AR protein (Figure 2.8D). Surprisingly, at 10 µM, T1 and T2 had no significant effect on the transcriptional activity of wild-type AR activated by androgen, while T3 caused potent inhibition that was comparable to bicalutamide (Figure 2.3C). The IC$_{50}$ for wild-type AR in CV-1 cells was 0.39 µM for bicalutamide and 0.96 µM for T3, whereas T1 had an IC$_{50}$ greater than 30 µM and the IC$_{50}$ for T2 could not be calculated due to no inhibition in this concentration range (Figure 2.3D). These IC$_{50}$ values are consistent with the trends observed for the potency of compounds on wild-type AR activity at the single concentration of 10 µM (Figure 2.3C). The binding affinities of the diterpenoids for wild-type AR LBD (Figure 2.2A) also agree with the inhibition of the diterpenoids on the transcriptional activity of wild-type AR, as well as the IC$_{50}$ values for wild-type AR. Taken together with the results from section 2.3.1, these studies provide important characterization of the diterpenoids, demonstrating that
T1 and T3 are antiandrogens, which inhibit AR transcriptional activity by directly binding to AR LBD and blocking AR N/C interaction.

### 2.3.3 Diterpenoids reduce endogenous expression of AR-regulated genes

AR regulates the transcription of hundreds of genes in prostate cells with several well-characterized genes, such as *PSA, KLK2, FKBP5*, and *TMPRSS2*, which have been shown to have functional AREs (287-290). To test the effects of diterpenoids on endogenous expression of androgen-regulated genes, qRT-PCR was used to measure the levels of these transcripts in cells exposed to 10 µM of each diterpenoid. First, LNCaP cells with mutated AR T877A were tested. Among the 3 compounds, T1 was consistently the most effective inhibitor of androgen-induced gene expression with a potency comparable with or better than bicalutamide (Figure 2.4A). T3 also inhibited androgen-induced gene expression, whereas T2 showed no significant effects on this set of genes. Importantly, in the absence of androgen, T1 and T2 decreased basal levels of transcripts, whereas T3 significantly increased basal expression, suggesting that T3 may be a partial agonist for mutated AR-T877A similar to bicalutamide (85). Levels of transcripts of androgen-regulated genes are sensitive to changes in the levels of AR. Therefore, the levels of AR were measured in LNCaP cells treated with the diterpenoids. In the absence of androgen, levels of AR mRNA were significantly decreased by both T1 and T3 (Figure 2.4B). The decrease in AR mRNA level by T1 was consistent with the observed decrease in AR protein level by this compound in the absence of androgen by a currently unknown mechanism (Figure 2.4C). However, in the presence of androgen, the diterpenoids had no significant effect on AR mRNA and protein levels, thereby
suggesting that the inhibition of androgen-induced transcription of AR–regulated genes is not caused by decreased levels of AR.

Analysis of AR–regulated gene expression was next examined in VCaP cells that endogenously express wild-type AR. As expected from previous studies that revealed VCaP cells express lower mRNA level of PSA as compared to LNCaP cells (256), levels of PSA transcript in VCaP cells were weakly increased in response to androgen, and neither bicalutamide nor any of the diterpenoids significantly reduced PSA mRNA levels (Figure 2.4D). However, FKBP5 and TMPRSS2 transcript levels were robustly increased in response to androgen and both were significantly blocked by bicalutamide and T3, whereas T1 and T2 had no significant effects. These data are consistent with the trends observed from wild-type AR activity using a reporter gene assay in CV-1 cells (Figure 2.3C). Overall, results from the AR-driven reporter assays and endogenous expression of AR-regulated genes demonstrate that the diterpenoids inhibit androgen-dependent AR transcriptional activity.

2.3.4 Diterpenoids demonstrate target specificity for AR

AR is a member of the steroid receptor superfamily, and its LBD shares substantial sequence homology with the LBDs of progesterone receptor (PR) and glucocorticoid receptor (GR) at 55% and 51%, respectively (123, 291). Some antiandrogens such as bicalutamide also inhibit PR transcriptional activity (291). During the preclinical characterization and evaluation of novel inhibitors, it is important to examine the specificity for the target. To test receptor specificity of diterpenoids for AR, the transcriptional activities of PR and GR were examined using their respective reporter gene constructs transiently transfected into LNCaP cells, which do not endogenously express PR and GR. Induction of
PR transcriptional activity by its ligand 4-pregnene-3,20 dione (Preg) was inhibited by bicalutamide and RU486, a potent PR inhibitor, as well as by T1 and T3, whereas T2 showed no significant effect (Figure 2.5A). The 3 diterpenoids and bicalutamide had no effect on the transcriptional activity of GR induced by dexamethasone (Figure 2.5B). These results suggest that diterpenoids have some specificity for AR and the closely related PR, but they do not affect GR transcriptional activity. Consistent with T1 and T3 inhibiting PR transcriptional activity, these compounds competed for the PR LBD as shown using the *in vitro* ligand competitor assay with recombinant human PR LBD (Figure 2.5C). RU486 had strong binding affinity to PR LBD with an EC$_{50}$ of 45 nM, comparable to Preg at 35 nM, whereas bicalutamide and T1 had similar EC$_{50}$ values at 5,100 and 5,600 nM, respectively. T3 had a binding affinity for PR LBD at about 500 nM, which agreed with its potent inhibition on PR transcriptional activity (Figure 2.5A). The EC$_{50}$ for T2 could not be assessed due to low activity and poor solubility at high concentrations. Consistent with estrogen receptor α (ERα) having little sequence homology to AR, none of the AR ligands (R1881, bicalutamide, and the diterpenoids) had any binding affinity for ERα within the concentration range tested (Figure 2.5D).

2.3.5 Diterpenoids inhibit androgen-dependent proliferation of prostate cancer cells

To examine if the diterpenoids have antiproliferative effect, LNCaP human prostate cancer cells that express functional full-length AR were treated with vehicle, or 10 μM of bicalutamide (positive control), or 10 μM of each diterpenoid. As expected, 0.1 nM of R1881 led to increased proliferation in LNCaP cells, as measured by BrdU incorporation (Figure 2.6A). Both T1 and T3 inhibited androgen-dependent proliferation with T1 being
significantly more effective than T3 and comparable with bicalutamide, whereas T2 had no effect (Figure 2.6A). To determine the specificity of T1 and T3 for attenuating AR–dependent proliferation, PC-3 human prostate cancer cells were used because these cells do not express functional AR. Consistent with PC-3 cells not being dependent on AR activity for growth, 0.1 nM R1881 did not alter their proliferation. Importantly, none of the diterpenoids inhibited proliferation of these cells (Figure 2.6B), suggesting that these diterpenoids selectively inhibit cell proliferation driven by androgen-AR signaling. Viability assays using AlamarBlue® mirrored the proliferation data shown above, and thus supporting and confirming the proliferation data (Figures 2.6C and 2.6D). A time course study with PC-3 cells treated with each diterpenoid in the presence or absence of R1881 demonstrated androgen-independent growth that was not inhibited by the diterpenoids, further supporting the diterpenoids do not cause general cytotoxicity (Figure 2.7).

Furthermore, effect of diterpenoids on prostate cancer cell growth was investigated using two other cell lines with clinically relevant characteristics. LNCaP95 cells express endogenous AR splice variants with truncated LBD in addition to full-length AR, and both AR species are functional and drive distinct transcriptional programs (161). Viability assay showed the androgen-independent growth of LNCaP95 cells, and as a result, neither bicalutamide nor diterpenoids inhibited their growth (Figure 2.8A). Consistent with previous studies, AR-driven reporter assay in LNCaP95 cells indicated the presence of a functional full-length AR, which was induced by R1881 and inhibited by bicalutamide, T1 and T3 (Figure 2.8B) (161). T2 had no significant effect on androgen-induced ARR3-LUC activity in this cell line. VCaP cells have overexpressed full-length wild-type AR and also express endogenous AR splice variants (161, 199). While the growth of VCaP cells is androgen-
inducible, bicalutamide and diterpenoids did not inhibit VCaP cell growth (Figure 2.8C), consistent with previous studies that showed VCaP growth is poorly inhibited by antiandrogens (218). Elevated expression of full-length AR in VCaP cells and the presence of AR splice variants in VCaP and LNCaP95 cells were confirmed by Western blot (Figure 2.8D). Together these data suggest that T1 and T3 specifically inhibit the growth of cells that are dependent on full-length AR and that these diterpenoids are not generally cytotoxic.

2.3.6 Diterpenoid T3 demonstrates in vivo on-target activity

Blocking the androgen axis in vivo results in atrophy of androgen-dependent tissues such as the seminal vesicles, and thereby serving as an indication of on-target activity for inhibitors of androgen-AR axis (292, 293). Of the 3 diterpenoids tested, T3 was consistently the most potent inhibitor of wild-type AR and was therefore chosen for in vivo evaluation for effects on benign tissue that harbors wild-type AR. Mature male mice treated with 13 daily oral doses of T3 had a significant decrease in seminal vesicle weight (Figure 2.9A), which was consistent with the properties of an antiandrogen. No changes in the testes weight or body weight were observed (Figures 2.9B and 2.9C), and thereby indicating a relatively specific effect of T3 on androgen-dependent tissue as opposed to it merely being toxic, consistent with the results obtained from the growth assays in the previous section.

2.4 DISCUSSION AND CONCLUSION

AR is a ligand-activated transcription factor that plays an important role in prostate cancer. Developing therapeutic agents to directly block AR transcriptional activity for the treatment of prostate cancer has yielded FDA approved drugs including nonsteroidal
antiandrogens such as bicalutamide and enzalutamide. Here, furanoditerpenoid spongia-13(16),-14-dien-19-oic acid, or T1 was originally isolated from a crude extract of a marine sponge based upon its activity in our screen for inhibitors of AR transcriptional activity. T1 belongs to a family of steroidal compounds called spongian diterpenoids, which are commonly found in marine sponges and shell-less mollusks that feed on the sponges (294). T2 and T3 are semisynthetic compounds produced by reducing either the furan or 17β carboxylic acid functionalities in T1, respectively. Spongian diterpenoids have been previously investigated for in vitro antiviral and antitumour activities (295). This is the first report that diterpenoids have antiandrogen activity as shown by (i) binding to AR LBD and blocking AR N/C interaction, (ii) blocking AR transcriptional activity and inhibiting the expression of AR-regulated genes, (iii) having receptor specificity for AR with no effect on GR transcriptional activity and no effect on the growth of cells that do not expression functional AR, and (iv) inhibiting androgen-dependent in vitro growth of prostate cancer cells and reducing the weight of androgen-dependent tissue in vivo while having no effect on the body weight of mice. Our studies revealed that the mechanism of action of diterpenoids to inhibit AR transcriptional activity involves direct binding to the AR LBD as shown by in vitro ligand competition binding assays. Consistent with other antiandrogens that block AR N/C interaction induced by androgen (296), the diterpenoids T1 and T3 also inhibited this critical interaction required for androgen-dependent AR transcriptional activity.

AR mediates the effects of androgen, which is the major mitogen for prostate cancer, thereby providing the rationale for targeting AR for the treatment of prostate cancer. Here, furanoditerpenoids T1 and T3 inhibited androgen-dependent proliferation of LNCaP human prostate cancer cells that are androgen-sensitive and express functional AR. The
antiproliferative effect of T1 and T3 was not observed in PC-3 cells that do not have a functional AR, thereby indicating potential specificity of these diterpenoids for cells that are dependent on AR for growth and survival. In agreement with this interpretation, administration of T3 to mature male mice reduced the weight of androgen-dependent seminal vesicles while having no effect on body weight. T3 had no effect on the weight of testis, similar to bicalutamide and consistent with other inhibitors of the androgen axis (297). Other data supporting specificity of the diterpenoids for blocking AR transcriptional activity include that they did not broadly inhibit transcription and translation or affect all steroid hormone receptors as indicated by a lack of effect on the transcriptional activity of the structurally related GR. However, similar to bicalutamide, T1 and T3 inhibited PR transcriptional activity whereas T2 did not. PR is not known to be associated with any essential biologic function in mature men and bicalutamide, a PR inhibitor, has been approved to treat prostate cancer for many years with an acceptable safety profile (286). Consistent with its relatively modest biologic activity, T2 showed very weak binding affinity to AR and PR. However, both T1 and T3 were shown to bind to AR and PR, while T3 clearly showed stronger binding affinity, which was comparable to bicalutamide for AR. Together these data suggest these spongian diterpenoids are novel antiandrogens with T3 having the best potency of the 3 compounds. Further structure–activity relationship studies of the diterpenoids are worth pursuing with the intention of developing more potent derivatives of T3, which could be evaluated and optimized for the potential clinical application to treat prostate cancer and/or other diseases involving the androgen-AR axis.

LNCaP cells have a mutated AR LBD (T877A), which reduces ligand specificity as well as alters ligand affinity and dissociation rates (253, 298). Thus, inhibitory properties of
compounds that bind to AR LBD can be altered by mutations within this domain. When comparing the properties of the 3 diterpenoids on wild-type AR versus mutated AR-T877A, T1 activity was the most affected by this mutation. T1 had no effect on wild-type AR transcriptional activity with accompanying poor inhibition of N/C interaction and higher \( IC_{50} \) and \( EC_{50} \) values. However, with the mutated AR-T877A in LNCaP cells, T1 became a potent inhibitor of both androgen-dependent proliferation and AR transcriptional activity with an \( IC_{50} \) of 4.2 \( \mu \)M. Generally, T2 had poor activity, whereas T3 had good activity regardless of whether the AR had the T877A mutation. T3 was synthesized with the intention of maintaining the furan ring in T1 but modifying the 17\( \beta \) carboxylic acid group to determine whether a compound could be generated to provide better binding affinity to AR LBD and consequently become a more potent inhibitor. From \textit{in vitro} ligand competition binding assays, the \( EC_{50} \) for T3 is 1.6 \( \mu \)M, which indeed showed approximately 16-fold higher affinity for AR LBD than T1 (27.1 \( \mu \)M), making T3 comparable to bicalutamide (1.1 \( \mu \)M). Improved affinity of T3 compared with T1 probably involves specific interactions with a set of well-conserved amino acid residues in the LBD of AR. Hydrophobic interactions between the perhydrophenanthrene skeleton of the ligand and the amino acid residues within the ligand-binding pocket are critical for binding as well as hydrogen-bonding which would impact binding affinity. Comparing with other nonsteroidal small-molecule inhibitors of the AR LBD such as bicalutamide and enzalutamide, the diterpenoids represent a novel class of chemical compounds with antiandrogen activity.

The molecular target for antiandrogens is the LBD of full-length AR, and thus it is important to note that antiandrogens cannot inhibit the growth of prostate cancer cells driven by constitutively active AR splice variants with truncated LBD, as bicalutamide and
diterpenoids did not inhibit the growth of androgen-independent LNCaP95 cells that express the AR splice variants. Since more evidence has emerged to suggest that these AR splice variants are associated with the progression of CRPC and resistance to current therapies targeting the androgen-AR axis, AR inhibitors capable of blocking both the full-length AR and AR splice variants with truncated LBD will have a therapeutic advantage over antiandrogens (102, 162, 276, 299). Nonetheless, the discovery of novel antiandrogens like the diterpenoids and future research and development of antiandrogens still have clinical significance, as it has been demonstrated that once an antiandrogen fails, changing to an alternative second-line antiandrogen can provide additional survival improvement for some prostate cancer patients (279, 280). Importantly, the new-generation antiandrogen enzalutamide provides significant survival improvement in metastatic CRPC patients, and thus it has been approved by FDA to treat metastatic CRPC either as the first-line therapy prior to chemotherapy or second-line therapy after chemotherapy (54, 278). Furthermore, to compare antiandrogens head-to-head, a randomized phase II clinical trial has been conducted to evaluate the efficacy of enzalutamide versus bicalutamide in metastatic CRPC patients without previous chemotherapy (300). The trial achieved its primary endpoint by demonstrating that enzalutamide significantly increased progression-free survival by nearly 10 months when compared to bicalutamide in these patients, according to recent news releases by Medivation, Inc. and Astellas Pharma Inc., the companies co-developing enzalutamide. These latest clinical findings support the future research and development of novel antiandrogens with the goal to improve potency and efficacy. Overall, there is a significant niche for antiandrogens within the treatment landscape of CRPC.
Figure 2.1. Chemical structures of diterpenoids, androgens, and bicalutamide.

Chemical structures of spongian diterpenoids (T1, T2, and T3), dihydrotestosterone (DHT), synthetic androgen R1881, and antiandrogen bicalutamide. Steroidal compounds have 4 rings that are labeled A, B, C, and D, as illustrated on T1 in red.

Acknowledgement: Mawji NR completed the early compound library screening that led to the identification of T1.
Figure 2.2 Diterpenoids bind to AR LBD and disrupt AR N/C interaction.

(A) Recombinant AR LBD was tested for the binding affinity of the diterpenoids by measuring fluorescence polarization (mP) with an excitation wavelength of 470 nm and emission wavelength of 535 nm, as described in Materials and Methods. Serial dilution was performed for synthetic androgen R1881, antiandrogen bicalutamide (BIC), and each diterpenoid. A representative plot from at least 3 independent assays is shown, and error bars represent the mean ± SEM. (B) CV-1 cells cotransfected with 5XGAL4-Luc reporter vector, VP16-AR-NTD, and GAL4DBD-AR-LBD (wild-type) were pre-treated for 1 hour with 10 µM BIC, T1, T2, T3, or vehicle (VEH), prior to the addition of 1 nM R1881 for 24 hours. Luciferase assay was performed. Error bars represent the mean ± SEM, n=5 independent experiments. Statistical analyses were done to compare drug-treated groups to VEH-treated in the presence of R1881, unless otherwise noted. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 2.3. Diterpenoids inhibit AR transcriptional activity.

(A) LNCaP cells were transfected with ARR3-LUC and treated with 10 µM of bicalutamide (BIC), T1, T2, T3, or vehicle (VEH) in the absence or presence of 1 nM R1881 (− or +) for 48 hours. (B) LNCaP cells transfected with ARR3-LUC were treated with diterpenoids at 10 µM, 1 µM, 0.1 µM, and 0 µM in the presence of 1 nM R1881 for 48 hours. (C) CV-1 cells were transiently cotransfected with pSV-AR0, the expression vector for wild-type AR, and ARR3-LUC, prior to treatment with 10 µM of BIC, diterpenoids, or VEH in the absence or presence of 1 nM R1881 (− or +) for 24 hours. (D) CV-1 cells transiently cotransfected with pSV-AR0 and ARR3-LUC were treated with diterpenoids at 10 µM, 5 µM, 2.5 µM, 1 µM, and 0 µM in the presence of 1 nM R1881 for 24 hours. Luciferase assay was performed. Error bars represent the mean ± SEM, n = 3 independent experiments. Statistical analyses were done to compare drug-treated groups to VEH-treated in the presence of R1881, unless otherwise noted. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001.

Acknowledgement: Tien AH completed the IC50 determination for LNCaP AR and generated Figures 2.3 (A) and (B).
Figure 2.4. Diterpenoids reduce endogenous expression of androgen-regulated genes.

(A) mRNA levels of four androgen-regulated genes (PSA, KLK2, FKBP5, and TMPRSS2) in LNCaP cells were measured by qRT-PCR using gene-specific primers, as described in Materials and Methods. LNCaP cells were pre-treated for 1 hour with 10 µM of bicalutamide (BIC), T1, T2, T3, or vehicle (VEH), prior to the addition of 1 nM R1881 (black bars and +) or ethanol control (white bars and -) for 48 hr. Endogenous expression of AR in LNCaP cells under the same treatment conditions as mentioned above were detected by (B) qRT-PCR for AR mRNA levels and (C) Western blot analysis for AR protein levels. (D) Levels of PSA, FKBP5, and TMPRSS2 mRNAs were also measured in VCaP cells treated the same as LNCaP cells. Levels of expression of each gene were normalized to the mRNA levels of housekeeping gene GAPDH. Error bars represent the mean ± SEM, n = 3 independent experiments. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001.
(A) LNCaP cells were transiently cotransfected with an expression plasmid for full-length human progesterone receptor-beta (PRβ) and a PR-driven (PRE) luciferase reporter. Cells were pre-treated for 1 hour with 10 µM of bicalutamide (BIC), RU486, each diterpenoid, or vehicle (VEH), prior to the addition of 10 nM 4-pregnene-3,20 dione (Preg) (black bars and +), or ethanol control (white bars and -). (B) LNCaP cells were transiently cotransfected with an expression plasmid for full-length human glucocorticoid receptor (GR) and GR-driven (GRE) reporter, and then pre-treated for 1 hour with 10 µM of BIC, each diterpenoid, or VEH, prior to the addition of 10 nM of dexamethasone (Dex). 48 hours after treatment, luciferase assay was performed as described in Materials and Methods. Error bars represent the mean ± SEM, n ≥ 3 independent experiments. Statistical analyses were done to compare drug-treated groups to VEH-treated in the presence of ligand, unless otherwise noted. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001. (C) Recombinant human PR LBD and (D) recombinant human full-length estrogen receptor-α (ERα) were tested for the binding affinity of the diterpenoids by measuring fluorescence polarization (mP). A representative plot from at least 3 independent assays is shown for each receptor.
Figure 2.6. Diterpenoids inhibit androgen-dependent proliferation of prostate cancer cells.

(A) Proliferation of LNCaP cells after 4 days of treatment, and (B) PC-3 cells after 3 days of treatment. Proliferation assays employed BrdU incorporation and ELISA as described in Materials and Methods. Both cell lines were pre-treated for 1 hour with 10 µM of bicalutamide (BIC), each diterpenoid, or vehicle (VEH), prior to the addition of 0.1 nM R1881 (black bars and +) or ethanol control (white bars). Using AlamarBlue® assays (as described in Materials and Methods), growth of (C) LNCaP cells after 4 days, and (D) PC-3 cells after 3 days was quantified. These cells were treated the same as the proliferation assays. Error bars represent the mean ± SEM, n = 4 independent experiments. Statistical analyses were done to compare drug-treated groups to VEH-treated in the presence of R1881, unless otherwise noted. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 2.7. Diterpenoids do not affect androgen-independent cell growth.

PC-3 cells were pre-treated for 1 hour with 10 μM of bicalutamide (BIC), each of the diterpenoids, or vehicle (VEH), prior to the addition of 0.1 nM R1881 or ethanol control. PC-3 cells were assessed for BrdU incorporation at day 1, 2, and 3 post-treatment. Proliferation in the presence of (A) ethanol and (B) R1881 were plotted. One representative experiment is shown here.
Figure 2.8. Effect of diterpenoids on LNCaP95 and VCaP cells.

(A) Growth of LNCaP95 cells after 3 days of treatment of 10 µM bicalutamide (BIC), each diterpenoid, or vehicle (VEH) in the presence of 0.1 nM R1881 (black bars and +) or ethanol (white bars and --) was quantified by AlamarBlue® assay. (B) LNCaP95 cells transfected with ARR3-LUC were pre-treated for 1 hour with 10 µM of BIC, each diterpenoid, or VEH, prior to the addition 1 nM R1881 (black bars and +) or ethanol control (white bars and --) for 24 hours. AR transcriptional activity was measured by luciferase assay. (C) Growth of VCaP cells after 5 days of the same treatment as LNCaP95 cells was quantified by AlamarBlue® assays. Error bars represent the mean ± SEM, n = 3 independent experiments. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001. (D) Endogenous AR protein expression levels from VCaP, LNCaP, and LNCaP95 cells in the presence of 1 nM R1881 were examined, and as well as ectopic expression level of wild-type AR protein in transiently transfected CV-1 cells treated with 1 nM R1881 and used to test AR transcriptional activity. The AR N-20 antibody was used in the Western blot to detect both the full-length AR and AR variant, and β-actin was used as a loading control. A representative blot is shown here.
Figure 2.9. Diterpenoid T3 shows *in vivo* on-target activity.

Male NOD-SCID intact mice were treated with DMSO, or bicalutamide (BIC), or T3 at 10 mg/kg body weight for two weeks. Tissues were collected, and their weights were measured. The weight of seminal vesicle (A) and testes (B) were plotted as Whisker plots. Body weight (C) was plotted as the percentage change between initial and final weights. Error bars represent the mean ± SEM, n = 8 to 10 as indicated. Statistical analyses were done to compare drug-treated groups with DMSO control group, unless otherwise noted. Student’s t test: *p < 0.05; **p < 0.01; ***p < 0.001.

Acknowledgement: Wang J conducted the animal study.
CHAPTER 3. Preclinical evaluation of EPI-002, an androgen receptor N-terminal domain inhibitor for the treatment of castration-resistant prostate cancer

3.1 INTRODUCTION

Most prostate cancer cells require functional androgen receptor (AR) transcriptional activity for survival and growth, and therefore, targeting AR signaling has become a major therapeutic approach to treat the disease (75, 140). Androgen deprivation therapy (ADT), designed to block the androgen-AR axis by reducing the production of natural androgen testosterone via castration, is the standard treatment for advanced prostate (44, 301). ADT is initially effective in inhibiting AR transcriptional activity and managing tumour burden, but within 12-33 months most patients treated with ADT relapse with a more aggressive and lethal form of the disease referred to as metastatic castration-resistant prostate cancer (CRPC) (44, 45).

Patients with metastatic CRPC have poor prognosis with a mean survival time of only 16-18 months (45, 302). Despite having castrated levels of serum testosterone, persistent or aberrantly restored AR transcriptional activity is believed to play a key role in the progression of CRPC, as the clinical onset of CRPC is defined by a rapid rise in the level of prostate-specific antigen (PSA), which is a gene regulated by AR (43, 48, 301). There are several AR-related molecular alterations that have been proposed as mechanisms underlying aberrant AR transcriptional activity. One is the amplification or overexpression of AR that provides hypersensitivity to low levels of androgens (270, 303). Gain-of-function mutations

within the ligand binding domain (LBD) of AR have been shown to promote activation by nonandrogenic steroids and convert antiandrogens into agonists (86-88). It has been revealed that ligand-independent activation of the AR N-terminus domain (NTD) can maintain functional AR transcriptional activity in the absence of androgen (96-98). Studies have demonstrated that overexpression of AR coactivators can enhance androgen-dependent and also promote ligand-independent AR transcriptional activities (90, 92, 95, 273, 304). Increased adrenal and intratumoral de novo synthesis of androgens, which provide a low but a sufficient level of androgens to sustain AR transcriptional activity under castrated conditions, have been implicated with CRPC progression (99-101). Finally, constitutively active AR splice variants with truncated LBD have been shown to drive a distinct transcriptional program from the full-length AR, and these AR splice variants confer castration-resistant phenotype in prostate cancer cells and xenograft models (102, 103, 105, 161).

In recent years, strong effort has been made to develop new-generation inhibitors of the androgen-AR axis, adopting improvements to overcome some of the aforementioned mechanisms underlying aberrant AR transcriptional activity. Abiraterone and enzalutamide represent the new-generation therapeutic agents that block androgen-AR signaling, and both have been approved by FDA to treat metastatic CRPC. Abiraterone is a CYP17 inhibitor that decreases adrenal and/or intratumoral androgen biosynthesis that are believed to stimulate AR transcriptional activity under castrated conditions, thereby improving the survival of metastatic CRPC (51, 305). Enzalutamide is a second-generation antiandrogen that inhibits AR transcriptional activity by directly and competitively binding to AR LBD, providing survival benefits to patients with metastatic CRPC (53, 54, 218). Despite their different
mechanisms of action, both abiraterone and enzalutamide essentially target the LBD of AR. Unfortunately, CRPC tumours will eventually become resistant to these drugs. Studies have revealed possible molecular mechanisms conferring resistance to abiraterone and enzalutamide. Elevated levels of intratumoral androgen biosynthesis and overexpression of enzymes involved in steroidogenesis have been reported as possible molecular adaptations to counter CYP17 inhibitors such as abiraterone (227). As for resistance to enzalutamide, several mechanisms have been proposed, including the selection of AR gain-of-function point mutation that converts enzalutamide from an antagonist into agonist, and substitution of glucocorticoid receptor (GR) to drive the growth of enzalutamide-resistant prostate cancer cells (88, 230). Importantly, constitutively active AR splice variants with truncated LBD have been reported as a common mechanism of resistance to both abiraterone and enzalutamide, as supported by preclinical and clinical evidence (162, 227, 236). Since aberrant AR transcriptional activity remains as a key driver of CRPC and current AR-targeting agents are limited by drug-resistance, it is imperative to continue the search for new AR inhibitors.

The discovery and development of new antiandrogens certainly has potential clinical benefits, as exemplified by the successful trial of the second-generation antiandrogen enzalutamide to treat metastatic CRPC. Furthermore, although ADT and antiandrogens cannot prevent the progression CRPC, once an antiandrogen fails, changing to an alternative second-line antiandrogen can effectively improve survival for some patients (306-308). While inhibitors targeting the AR LBD such as antiandrogens will continue to have a key role to treat prostate cancer, AR inhibitors that target the NTD may also have a significant
and unique clinical niche within the treatment landscape of prostate cancer. In this chapter, we shift the focus onto the evaluation of an AR inhibitor that targets the NTD.

The AR NTD is an intrinsically disordered domain and contains most if not all of AR transcriptional activity, and early work has validated the NTD as a viable therapeutic target for prostate cancer (122, 165, 309, 310). We have previously discovered a novel small-molecule compound EPI, which effectively inhibits the growth of CRPC xenografts in mice (238). EPI binds to the AR NTD and thereby disrupts critical protein-protein interactions between AR and coactivators, consequently inhibiting both androgen-dependent and androgen-independent AR transcriptional activities (238, 239). Importantly, EPI specifically targets AR without causing general toxicity in vivo, and possesses ideal pharmacokinetic features with a bioavailability of 86% and a slow clearance rate of 1.75 l/h/kg (238, 239). EPI-001 is a mixture of four stereoisomers, and the single stereoisomer EPI-002 (2R, 20S) had a potent in vitro and in vivo activity without causing significant body weight loss in mice (239). Therefore, EPI-002 was selected as the lead compound to move forward into clinical development.

Here, we aim to test the hypothesis that the AR NTD inhibitor EPI-002 can effectively inhibit aberrant AR transcriptional activity caused by known and proposed AR-related molecular mechanisms as described above. It has been previously shown that EPI-002 is effective in blocking androgen-independent AR transcriptional activity induced by protein kinase A pathway or cytokine IL-6, reducing the growth of VCaP xenografts that have overexpressed full-length AR, and inhibiting the transcriptional activity of constitutively active AR splice variant AR-V567es (238, 239). Therefore, Chapter 3 of this dissertation will describe the investigation to evaluate the effect of EPI-002 against aberrant AR
transcriptional activity in four specific aims: 1) to determine if EPI-002 maintains effective inhibition of AR transcriptional activity when several AR coactivators are individually overexpressed in prostate cancer cells; 2) to examine if EPI-002 inhibits the transcriptional activity of several clinically relevant AR gain-of-function mutations; 3) to investigate if EPI-002 blocks the transcriptional activity of constitutively active AR splice variant AR-V7 that has a truncated LBD; 4) to determine if EPI-002 inhibits the \textit{in vitro} cell growth and \textit{in vivo} tumour growth of a CRPC model that is driven by AR-V7. In addition, EPI-002 will be evaluated against two other AR-related molecular alterations that have been shown to increase AR transcriptional activity: polymorphic AR with variable polyglutamine tract (CAG repeats) in the NTD, and the exclusively cytoplasmic AR variant AR23 that contains an insertion of 23 amino acids between the two zinc fingers in the DBD (311, 312).

3.2 MATERIALS AND METHODS

3.2.1 Plasmids and reagents

AR-driven luciferase reporter genes PSA (6.1kb)-LUC and Probasin-LUC have been described previously (96, 313). The expression plasmid for full-length wild-type human AR pSV-AR0 was a gift from Dr. Brinkmann (Erasmus University, Rotterdam, The Netherlands) (122). Plasmids containing polymorphic human ARs with variable lengths of polyglutamine tract (CAG-repeats) were from Dr. Mark Trifiro (Lady Davis Institute for Medical Research, Quebec) (314). The expression plasmids containing AR mutants derived from patients treated with antiandrogens were provided by Dr. Robins (University of Michigan Medical School, Ann Arbor, MI) (315). For coactivator expression plasmids, wild-type p300 was provided by Dr. Tindall (Mayo Clinic, Rochester, MN), whereas SRC-1, SRC-2, and SRC-3 were
provided by Dr. Weigel (Baylor College of Medicine, Houston, TX) (90, 91, 316, 317). Metribolone, or R1881, synthetic androgen was purchased from Perkin-Elmer (Wellesley, MA). Bicalutamide was from AstraZeneca (London, UK). All other chemicals were purchased from Sigma, unless stated otherwise.

3.2.2 Cell lines

LNCaP human prostate cancer cells were maintained in RPMI-1640 supplemented with 10% (v/v) FBS (Invitrogen™ by Life Technologies, Carlsbad, CA). LNCaP95 cells were provided by Dr. Plymate (University of Washington, Seattle, WA). LNCaP95 cells are androgen-independent and were derived from parental LNCaP cells under prolonged androgen depletion. LNCaP95 cells were maintained in RPMI-1640 containing 10% (v/v) dextran-coated charcoal-stripped serum (CSS). COS-1 cells were maintained in RPMI-1640 with 10% (v/v) FBS. Cell lines were obtained from American Type Culture Collection unless otherwise noted. Cells were resuscitated immediately before experiments. All cells were maintained in culture no more than 10 to 15 passages and regularly tested to ensure they were mycoplasma-free.

3.2.3 Transfection and reporter assays

Transient transfection using AR-driven luciferase reporters was performed to determine the transcriptional activities of endogenous AR in LNCaP cells and ectopically expressed AR in COS-1 cells. LNCaP cells (5 x 10^4 cells/well) or COS-1 cells (2.5 x 10^4 cells/well) were plated in 24-well plates and incubated for one day, and on the second day, transfection was done in serum-free and phenol red-free media. LNCaP cells were transfected with constant
amount of AR-driven reporter PSA (6.1kb)-LUC and various amounts of coactivators using Lipofectin (Invitrogen™). COS-1 cells were transfected with constant amounts of AR-driven reporter PB-LUC and expression vectors for AR mutants (100ng/plate) or polymorphic AR with various CAG repeats (100ng/plate) using Fugene6 (Promega, Madison, WI). On day 3, cells were treated with inhibitors in the presence or absence of synthetic androgen R1881. After incubation at 37°C and 5% CO₂ for 48 hours for LNCaP cells or 24 hours for COS-1 cells, the cells were collected with 1X Passive Lysis Buffer (Promega). Luciferase activities were measured by using the Luciferase Assay System (Promega) with the aid of GlowMax® 96 microplate luminometer (Promega).

3.2.4 Cell proliferation assay

LNCaP95 cells (7,500 cells/well) were plated in 96-well Falcon Primaria tissue culture plates in RPMI supplemented with 10% charcoal-stripped FBS (CSS) and then changed to serum-free media 2 days later for treatment. Cells were pre-treated with 10 µM antiandrogens (bicalutamide or enzalutamide), or 25 µM EPI-002 for 1 hour, then treated with 0.1 nM synthetic androgen R1881. Cellular proliferation was assessed 2 days after treatment by incubating with 100 µM of BrdU labeling agent for 2 hours at 37°C. All media was removed and the plates were dried in a Hybaid oven at 60°C for 1 hour. Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Applied Science, Mannheim, Germany) was applied following the manufacturer’s protocol, and ELISA was performed 30 minutes after substrate addition at two wavelengths (370 nm and 492 nm).
3.2.5 Western blot analysis

Whole-cell lysates were prepared by lysing cells in RIPA buffer containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablet™ (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets™ (Roche). The protein concentration of each sample was quantified using BCA Protein Assay Kit (Thermo Scientific Inc., Rockford, IL). High molecular weight proteins such as p300 and SRC family co-activators were run on NOVEX NuPAGE 3-8% Tris-Acetate Gel (Invitroge™), and AR was separated on 4-20% Mini-PROTEAN® TGXTM Gel (Bio-Rad, Hercules, CA). SRC-1, SRC-2 (TIF2), and SRC-3 (AIB-1) were probed with purified mouse anti-SRC-1, anti-TIF2, and anti-AIB-1, respectively (BD Biosciences, Franklin Lakes, NJ). p300 was probed using p300 antibody (C-20) (Santa Cruz Biotechnology, Dallas, TX). AR was probed by anti-AR antibody N-20 (Santa Cruz Biotechnology) or PG-21 (MILLIPORE, Temecula, CA). Membranes were also probed for loading control β-actin using monoclonal mouse anti-β-actin antibody (ab8226 from Abcam, Cambridge, MA).

3.2.6 Endogenous expression of genes regulated by androgen receptor

LNCaP95 cells (150,000 cells/well) were plated with 10% CSS RPMI in 6-well plates. Two days later, the media was changed to serum-free media. After 24 hours of serum starvation, cells were treated with 10 µM enzalutamide or 25 µM EPI-002 for 1 hour, then treated with 0.1 nM R1881. 45 hours after treatment, total RNA was extracted by using RNeasy® Micro Kit (QIAGEN, Valencia, CA), and subsequently reverse transcribed to cDNA by SuperScript®III First-Strand Synthesis System for RT-PCR (Invitrogen™). Diluted cDNA and gene-specific primers were combined with Platinum ® SYBR® Green qPCR SuperMix-
UDG with ROX (Invitrogen™), and the transcripts were measured by quantitative real-time (qRT)-PCR ABI PRISM 7900 Sequence Detection System (ABI PRISM®, Applied Biosystems by Life Technologies). qRT-PCR was performed separately in triplicates for each biological sample. Gene expression levels were normalized to housekeeping gene RPL13A. Primers have been described (107, 238).

3.2.7 Xenografts and animal study

Male NOD-SCID mice at 6-8 weeks old were subcutaneously injected with LNCaP95 cells (10 million cells per site) using Matrigel (Becton Dickinson, New Jersey) in the flanks. Mice were castrated when tumour volumes reached approximately 100 mm³. Treatment started one week after castration. Mice bearing LNCaP95 xenografts were randomized into three groups and administered with 100 mg/kg body weight of EPI-002 twice daily, 50 mg/kg body weight of enzalutamide once daily, or vehicle by oral gavage. Tumours were excised 2 days after the last treatment. All animal studies conformed to the relevant regulatory and ethical standards. All experiments involving animals were approved by the University of British Columbia Animal Care Committee.

3.2.8 Immunohistochemistry

For immunohistochemical staining, sections 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 min, followed by washing in PBST three times. Sections were then incubated with super blocking buffer for 30 min to prevent the non-specific bindings of antibodies and then with anti-AR
PG-21 (1:200; MILLIPORE) and anti-Ki-67 (1:50; Dako) at 4°C overnight. This was followed by incubation with biotinylated secondary antibodies for 30 min and avidin–biotin peroxidase complex for 30 min at room temperature. Antigen was detected with 3,3-diaminobenzidine and counterstaining with hematoxylin. For TUNEL staining, The ApopTag® Fluorescein In Situ Apoptosis Detection Kit (MILLIPORE) was used. Cells that were positive for Ki67 or TUNEL staining were counted from different xenografts for each treatment.

3.2.9 Statistics

Statistical analysis was performed using GraphPad Prism (version 6.05; GraphPad Software). Comparisons between two groups were performed with unpaired 2-tailed Student’s t test, and comparisons between three or more groups were performed using One-way or two-way ANOVA with indicated multiple comparisons test. A p value less than 0.05 was considered statistically significant for differences between groups.

3.3 RESULTS

3.3.1 EPI-002 maintains effective inhibition of AR transcriptional activity despite elevated levels of coactivators

AR coactivators interact with AR to enhance androgen-dependent and androgen-independent AR transactivation, and overexpressed coactivators cause aberrant AR transcriptional activity (318-320). Elevated levels of AR coactivators such as the p160 steroid receptor coactivator (SRC) family proteins (SRC-1, SRC-2, and SRC-3) and CBP/p300 are associated with prostate cancer progression and poor prognosis (90, 92, 95,
To test if EPI-002 could still effectively inhibit AR transcriptional activity when several well-known AR coactivators were overexpressed, luciferase reporter-assays were performed in LNCaP cells transiently co-transfected with increasing amounts of AR coactivators and AR-driven reporter PSA (6.1kb)-LUC. We observed that the overexpression of each of the SRC p160 family proteins at varying amounts caused dose-dependent increase of androgen-induced AR transcriptional activity, resulting in a maximum approximately two-fold increase when compared to controls with endogenous expression levels of the coactivators (Figure 3.1A, B, and C). These observations are consistent with previously published data showing overexpressed SRC-1 led to dose-dependent increase of androgen-dependent AR transcriptional activity measured by luciferase assay using the same AR-driven reporter gene (320). Despite the increased AR transcriptional activity caused by overexpressed coactivators of the SRC proteins, enzalutamide and EPI-002 maintained effective and consistent inhibition of AR activity, as indicated by the unchanged percentage of inhibition. Similarly, overexpressed p300 led to a dose-dependent enhancement of androgen-induced AR transcriptional activity in LNCaP cells with a three-fold maximum increase (Figure 3.1D). Both enzalutamide and EPI-002 effectively inhibited the increased AR transcriptional activity by p300 overexpression. EPI-002 kept a constant inhibition on the activation of PSA6.1-LUC reporter gene in the presence of elevated levels of p300, resulting in a significantly increased percentage of inhibition (Figure 3.1D). Western blot analysis confirmed that each coactivator was overexpressed in LNCaP cells as compared to the endogenous expression levels (Figure 3.1).
3.3.2 EPI-002 inhibits transcriptional activities of AR with clinically relevant mutations

According to the latest updates from the AR mutation database, most of the 159 treatment-related AR mutations found in prostate cancer tissues are single-base substitutions due to somatic mutations, with the majority (~45%) occurring within the LBD, whereas about 30% have been found in the NTD (321). To assess the effectiveness of EPI-002 against transcriptional activity of several clinically relevant AR gain-of-function mutations including mutations found in both the NTD and LBD, we performed AR-driven reporter assays using AR-negative COS-1 cells transiently transfected with each of the AR mutants and treated with different inhibitors (Figure 3.2). First, we examined two AR NTD gain-of-function mutations derived from patients treated with antiandrogens: E255K and W435L, which have been shown to increase AR stability and enhance N-C interaction, respectively (315). We showed that EPI-002 significantly inhibited the androgen-dependent transcriptional activity of these two AR NTD mutations, and the level of inhibition achieved was similar to that of the wild-type AR (Figure 3.2A and B), indicating that these NTD mutations did not impair the effectiveness of EPI-002. Next, we tested several AR LBD gain-of-function mutations including V715M, R761G, H874Y, and T877A, and we found that EPI-002 also significantly blocked the androgen-induced transcriptional activity of these mutants. Furthermore, we showed that first-generation antiandrogen flutamide failed to significantly block androgen-dependent transcriptional activity in H874Y and T877A, consistent with previous studies that reported these two mutants conferred resistance to flutamide (86, 322). We also observed significant partial agonist property of bicalutamide and particularly flutamide, as these first-generation antiandrogens stimulated substantial AR transcriptional activity in the absence of androgen for wild-type AR and several mutants (Figure 3.2A). Importantly, the second-
generation antiandrogen enzalutamide and EPI-002 did not stimulate AR activity in the absence of androgen, both behaving as a pure AR antagonist. Enzalutamide showed a moderately better inhibition on androgen-dependent AR transcriptional activity than EPI-002, achieving statistical significance in the wild-type AR and AR T877A (Figure 3.2B). The expression levels of each AR mutant transiently transfected in COS-1 cells were examined using Western blot, and the protein expression levels were comparable with the endogenous expression if LNCaP cells, thus indicating physiologically relevant levels of AR (Figure 3.2C).

3.3.3 EPI-002 blocks gene expression differentially regulated by full-length AR and constitutively active AR splice variant V7

Constitutively active AR splice variants with truncated LBD such as AR-V7 have emerged as a clinically relevant mechanism of resistance underlying CRPC, and the expression of AR-V7 is associated with a poor prognosis and resistance to current AR-targeting therapies (106, 107, 162). It has been shown that AR-V7 drives a distinct transcriptional program including cell-cycle related genes, as opposed to the canonical program regulated by full-length AR (161). Because AR-V7 lacks the LBD, any LBD-targeting therapies including antiandrogens would not be able to inhibit its transcriptional activity. To validate the therapeutic benefit of AR NTD inhibitor EPI-002, we tested the effect of EPI-002 on the transcriptional activity of AR-V7 in LNCaP95 cells, which express both functional full-length AR and AR-V7 (161). We confirmed the expression of full-length AR and AR-V7 in LNCaP95 cells by QPCR using gene-specific primers, and found that AR-V7 was the predominant variant (Figure 3.3A). Luciferase reporter assay in LNCaP95 cells
showed that antiandrogens and EPI-002 effectively inhibited androgen-dependent activation of AR-driven reporter PSA6.1-LUC (Figure 3.3B). Consistent with results from the reporter assay, the mRNA expression levels of canonical androgen-dependent and AR-regulated genes such as *PSA* and *FKBP5* were strongly inducible by R1881, and their expression levels were significantly inhibited by both enzalutamide and EPI-002 (Figure 3.4A). However, the mRNA expression levels of *UBE2C*, *CDC20*, and *AKT1*, which are genes differentially driven by constitutively active AR splice variants including AR-V7, were androgen-independent and enzalutamide-insensitive (Figure 3.4B). Importantly, EPI-002 significantly reduced the expression levels of genes regulated by AR-V7 regardless of the presence of androgen (Figure 3.4B). Furthermore, the mRNA levels of full-length AR and AR-V7 were not significantly affected by enzalutamide or EPI-002 (Figure 3.4C). Western blot analysis showed that protein levels of full-length AR and AR-V7 were not decreased by enzalutamide or EPI-002 (Figure 3.4D). Our results demonstrated the ability of EPI-002 to inhibit the transcriptional activities and gene expression programs regulated by both the full-length AR and AR-V7.

### 3.3.4 EPI-002 attenuates the growth of LNCaP95 cells that are androgen-independent and enzalutamide-resistant

To examine the effect of EPI-002 on CRPC that express constitutively active AR splice variants conferring growth advantage and therapy resistance, we used LNCaP95 cells as a xenograft model. LNCaP95 cells express endogenous full-length AR and AR variants with AR-V7 being the predominant species, as shown in Figure 3.3A. Synthetic androgen R1881 did not induce the growth of LNCaP95 cells, confirming the proliferation of these cells is
androgen-independent (Figure 3.5A). Regardless of the presence of androgen, antiandrogens such as bicalutamide and enzalutamide did not have any effect on the proliferation of LNCaP95 cells, and thereby supporting that the proliferation of LNCaP95 cells is resistant to antiandrogens. Importantly, treatment of EPI-002 significantly reduced the proliferation of LNCaP95 cells when compared with vehicle controls (Figure 3.5A). In castrated male SCID mice bearing LNCaP95 xenografts, treatment of control, or enzalutamide at 50mg/kg body weight, or EPI-002 at 100mg/kg body weight (bid dosing) was daily delivered into the animal by oral gavage. EPI-002 significantly attenuated the growth of LNCaP95 xenografts, whereas enzalutamide did not have any significant effect on the growth (Figure 3.5B). By the end of the 28-day study, tumours treated with EPI-002 were significantly smaller than those treated with enzalutamide, and in fact EPI-002 reduced the tumour size by more than 50% compared to the control (Figure 3.5C). These animal studies demonstrated the efficacy of EPI-002 on the growth of castration-resistant and enzalutamide-resistant LNCaP95 tumours, and are consistent with the suggestion that these tumours were driven by constitutively active AR splice variants with truncated LBD. Immunohistochemistry analysis of these same harvested xenografts (Figure 3.6A) revealed that EPI-002 significantly decreased proliferation as indicated by the lowered number of Ki67 positive cells (Figure 3.6B), while significantly induced apoptosis as indicated by the increased number of TUNEL positive cells (Figure 3.6C). In contrast, enzalutamide had no effect on the proliferation and apoptosis in these xenografts. Together these data support the efficacy of EPI-002 on the growth of castration-resistant and enzalutamide-resistant tumours by a mechanism that is consistent with blocking the transcriptional activities of both full-length AR and constitutively active AR splice variants.
3.3.5 EPI-002 inhibits transcriptional activities of AR influenced by other AR-related molecular alterations

The human AR gene contains a polymorphic CAG-repeat sequence within its exon 1, resulting in a variable length of polyglutamine tract in the NTD with a median of 23 repeats (323). Because EPI-002 directly binds to the AR NTD, it is important to examine if variable lengths of the NTD would affect the inhibition of EPI-002 on AR transactivation activity. We tested EPI-002 on AR with 0, 12, 20, 40, or 49 glutamine repeats. Similar to the effect observed with wild-type AR (21 glutamine repeats), EPI-002 significantly inhibited androgen-induced AR transcriptional activity regardless of the length of polyglutamine tract, and as expected, enzalutamide also showed strong inhibition of AR (Figure 3.7A). Importantly, the effectiveness of EPI-002 to inhibit AR transcriptional activity remained unchanged against different ARs with variable lengths of NTD, as indicated by the consistent percentage of inhibition that was unaffected by the length of polyglutamine tract (Figure 3.7B). Furthermore, in agreement with previously published studies, our data confirmed the inverse correlation between the length of polyglutamine tract and AR transcriptional activity (Figure 3.7C) (311, 323, 324). To ensure the levels of AR transfected into COS-1 cells were within a physiological range, Western blot analysis (Figure 3.7D) revealed that the AR expression levels were comparable with the endogenous AR level in LNCaP cells that have 26 polyglutamine repeats (325). The AR with the longest polyglutamine tract (AR CAG49) had the lowest protein expression levels, which could explain its decreased androgen-dependent transcriptional activity. Treatment of EPI-002 reduced the expression levels of AR transfected in COS-1 cells, which could also account for the decreased AR transcriptional activity (Figure 3.7D). However, our earlier studies have demonstrated that reducing AR
protein level is not a mechanism by which EPI compounds inhibit AR transcriptional activity in LNCaP cells (238). Therefore, it might be a cell-specific effect of EPI-002 that decreased the protein levels of transiently expressed AR in COS-1 cells. Together, these results provided an important evaluation of EPI-002 that binds the AR NTD, indicating the inhibition of AR activity by EPI-002 was not affected by the polymorphic variable lengths of polyglutamine tracts.

In addition, we also examined the effect of EPI-002 on aberrant AR transcriptional activity in the presence of AR23, which is an AR variant detected from a patient with metastatic CRPC (312). AR23 contains an insertion of 23 amino acids between the two zinc fingers in the DNA-binding domain as a result of abnormal splicing, and has been reported to be exclusively cytoplasmic and insensitive to androgen on its own (312). However, when AR23 is coexpressed with full-length AR in prostate cancer cells, a significantly elevated androgen-dependent AR transcriptional activity was observed (312, 315). Here, we found that AR23 caused a nearly 2-fold increase of androgen-induced AR transcriptional activity when transfected into LNCaP cells and expressed with an approximate ratio of 2:1 with the endogenous AR expression level as shown in Figures 3.8A and 3.8C. The ability of AR23 to further enhance endogenous AR transcriptional activity in LNCaP cells has been previously reported (312). Importantly, we demonstrated that both enzalutamide and EPI-002 significantly blocked the increased AR transcriptional activity caused by the expression of AR23 (Figure 3.8A). However, the presence of AR23 slightly mitigated the inhibition by EPI-002 (Figure 3.8B). Taken together, these results highlight the ability of both enzalutamide and EPI-002 to maintain effective inhibition of androgen-dependent AR transcriptional activity despite the presence of AR23 that can enhance AR activity.
3.4 DISCUSSION AND CONCLUSION

Aberrant AR transcriptional activity is believed to be a major driver of the growth and survival of most CRPC tumours (40, 43, 140). Therefore, it is crucial for all current and prospective AR-targeting therapies to have the ability to overcome mechanisms underlying such AR transcriptional activity, in order to deliver effective and durable responses in treating CRPC (40, 217, 326). In contrast to all current AR-targeting therapies that act through the LBD of AR, our focus here was to develop and evaluate inhibitors of the AR NTD. The AR NTD is an intrinsically disordered domain possessing most if not all of AR’s transactivation capacity (166). Previously, we identified the small-molecule EPI-002, which inhibits AR by binding to the NTD (238, 239). To further evaluate the therapeutic potential of EPI-002 to treat CRPC, we investigated the effectiveness of EPI-002 against several molecular alterations proposed as mechanisms underlying aberrant AR transcriptional activity. Here, the specific aims outlined in Section 3.1 are fulfilled, and our preclinical studies demonstrate that 1) EPI-002 maintains an effective inhibition of aberrantly increased AR transcriptional activity caused by elevated expression levels of coactivators; 2) EPI-002 is effective against several clinically relevant gain-of-function AR mutations; 3) EPI-002 significantly blocks the transcriptional activity of constitutively active AR-V7; and 4) EPI-002 inhibits the growth of an antiandrogen-resistant CRPC model that is driven by AR-V7. Additionally, we showed that EPI-002 significantly blocks AR transcriptional activities influenced by two other AR-related molecular alterations: polymorphic AR NTD with variable lengths of polyglutamine tract, and the expression of cytoplasmic AR variant AR23.

The p160 SRC family of coactivators directly interact with AR and other coregulators during the assembly of AR transcriptional complex, and their functions include chromatin
modifications and recruitment of other components of the general transcriptional machinery (319, 327). Studies have demonstrated that overexpression of each of the SRC coactivators increased AR transcriptional activity in prostate cancer cells (304, 319, 320, 328). The expression levels of the SRC coactivators have been shown to positively correlate with prostate cancer progression and poor prognosis (90, 94, 304). Consistent with previous studies, we showed that overexpression of SRC-1 in LNCaP cells increased androgen-dependent AR transcriptional activity in a dose-dependent manner to a maximum of approximately two-fold (320). Similarly, overexpressed SRC-2 and SRC-3 also led to enhanced androgen-dependent AR transcriptional activity. Despite the increased AR transcriptional activity caused by overexpressed SRC coactivators, both enzalutamide and EPI-002 maintained effective inhibition of AR, as indicated by unaltered percentage of inhibition of AR-driven reporter activity under different amounts of the coactivator transfected. Furthermore, CBP and p300 are related coactivators sharing many similarities, and both interact with transcription factors including AR, to facilitate transcription via histone acetylation (329-331). We chose p300 to evaluate the effect of EPI-002 because a study indicated that p300 is dominant over CBP for regulating androgen-induced gene expression in prostate cancer cells (332). The expression level of p300 has been reported to correlate with prostate cancer progression (92). We showed that overexpressed p300 resulted in an increase of AR transcriptional activity in LNCaP cells using PSA (6.1kb)-LUC reporter gene, which is consistent with a previous study (333). Importantly, both enzalutamide and EPI-002 effectively inhibited the increased AR transcriptional activity by p300 overexpression. Interestingly, EPI-002 was more effective against increased AR activity by p300 overexpression than that by the overexpression of SRC family of coactivators. Further
studies are needed to explain this finding. Overall, these results (Section 3.3.1) suggest that both enzalutamide and EPI-002 can effectively inhibit AR transcriptional activity in the presence of overexpressed coactivators.

AR point-mutations have been identified in prostate cancer patients treated with antiandrogens, and many of these mutations confer gain-of-function and resistance (315). Here, we tested EPI-002 on several clinically relevant AR gain-of-function mutations derived from patients treated with antiandrogens. EPI-002 effectively inhibited all of these AR mutations, including two NTD mutations E255K and W435L. Importantly, EPI-002 acted as a pure AR antagonist, unlike the first-generation antiandrogens bicalutamide and flutamide, which stimulated AR transcriptional activity in the absence of androgen. The second-generation antiandrogen enzalutamide potently inhibited all AR mutants tested here, and also behaved as a pure AR antagonist. The mechanism of action for antiandrogens like bicalutamide and enzalutamide involves competing against androgens to bind to the LBD and sterically disrupting the formation of activation function-2 (AF-2), which is essential for androgen-dependent AR transcriptional activity (150, 193). Therefore, even the new and improved antiandrogen enzalutamide is vulnerable towards any structure-altering mutations that affect ligand-specificity and cause conformational changes to alleviate steric hindrance (228). In fact, recent studies have reported that an AR F876L point-mutation converts enzalutamide into an AR agonist to promote growth and survival, and this mutation is clinically relevant as it was detected in patients treated with second-generation antiandrogen (228, 229, 334). Based on the notion that EPI-002 binds to the NTD and supported by our results (Section 3.3.2), we hypothesize that AR NTD inhibitor such as EPI-002 would
overcome the enzalutamide-resistant AR F876L mutation and all other mutations of the AR LBD conferring resistance to antiandrogens.

Constitutively active AR splice variants with truncated LBD such as AR-V567es and AR-V7 have been shown to confer resistance to current AR-targeting therapies, which all target the LBD of AR (227, 236, 335). Importantly, clinical studies have revealed that the expression of AR-V7 in circulating tumour cells is associated with resistance to abiraterone and enzalutamide in metastatic CRPC patients (162). Here, we demonstrated the superiority of the AR NTD-inhibitor EPI-002 over antiandrogen enzalutamide in blocking the transcriptional activities of both full-length AR and AR-V7. In LNCaP95 cells that endogenously express both functional full-length AR and AR-V7, EPI-002 significantly reduced the mRNA expression levels of androgen-independent genes previously shown to be distinctively regulated by AR-V7, such as UBE2C, CDC20, and AKT1, while enzalutamide did not have any effect. As for the canonical androgen-regulated genes such as PSA and FKBP5, both enzalutamide and EPI-002 effectively inhibited their expression levels. These findings (Section 3.3.3) are consistent with microarray data obtained by specific knockdown of full-length AR and AR-V7, which showed that AR-V7 drives a distinctive androgen-independent transcription program than the full-length AR (161). Many of the genes differentially regulated by AR-V7 are involved in cell-cycle regulation and proliferation, and some of these genes such as UBE2C are associated with CRPC progression (160, 161). UBE2C plays a critical role in the inactivation of M-phase checkpoint, and its expression levels have been shown to correlate with CRPC progression (160, 336).

Consistent with the EPI-002 reducing the expression levels of UBE2C in LNCaP95 cells, EPI-002 also significantly inhibited the androgen-independent proliferation of these
cells, while antiandrogens bicalutamide and enzalutamide had no effect. We used LNCaP95 xenografts in castrated male mice to recapitulate CRPC tumour growth driven by constitutively active AR splice variants that confer resistance to enzalutamide, and we showed that EPI-002 significantly inhibited the growth of LNCaP95 tumours while induced apoptosis (Section 3.3.4). This clearly demonstrates the advantage of an AR NTD inhibitor such as EPI-002 over LBD-targeting agents like enzalutamide. Our results from the LNCaP95 CRPC model are supported by previous studies using VCaP xenografts that also express constitutively active AR splice variants, as EPI-002 was more effective in attenuating the castration-resistant growth of VCaP tumours than bicalutamide (239). Furthermore, induction of GR expression has been reported to confer resistance to enzalutamide, as GR substitutes AR to drive a similar but distinguishable transcription program to sustain the growth and survival of CRPC cells (230). We have previously demonstrated that EPI-002 neither bind to GR nor alter GR transcriptional activity (238, 239). Thus, the fact that the growth of LNCaP95 cells was effectively inhibited by EPI-002 suggests that these cells do not heavily rely on GR for enzalutamide-resistance, but rather may rely on an AR-dependent mechanism of resistance involving constitutively active AR splice variants with truncated LBD. Also, the clinical significance of GR-mediated resistance to enzalutamide remains to be determined, as GR overexpression was only observed in samples from a small subset of patients who had initial poor responses to enzalutamide (230, 232). In contrast, a clinical study of 31 enzalutamide-treated CRPC patients has reported that a substantial number of these patients (39%; 12 out of 31) had detectable AR-V7 mRNA in their circulating tumour cells, and the presence of AR-V7 was significantly associated with enzalutamide resistance (162). Overall, constitutively active AR splice variants with truncated LBD have emerged as
a clinically significant mechanism underlying CRPC progression and resistance to current AR-targeting therapies, and thereby highlighting the need for AR NTD inhibitors such as EPI-002.

Molecular alterations within the AR LBD such as point mutations and truncation are unlikely to reduce the effectiveness of the AR NTD inhibitor EPI-002, as supported by our results discussed above. However, it is critical to evaluate the effectiveness of EPI-002 against molecular alterations within AR NTD, because these alterations may affect the binding of EPI-002 to the NTD. Here, we demonstrated that EPI-002 maintained significant and consistent levels of inhibition on AR transcriptional activity regardless of the length of polyglutamine tract, which is located within AR NTD (119, 176). The length of polyglutamine tract has a functional impact on AR transactivation, as AR with a shorter tract is more transcriptionally active in response to androgen than those with longer tracts (311, 323, 324). The inverse relationship between the length of polyglutamine tract and AR activity was also observed in our study. Clinically, AR protein with shorter polyglutamine tracts have been shown to correlate with increased risk of developing prostate cancer and having an aggressive disease (337). Results from our preclinical study suggest that EPI-002 would be effective in patients with polymorphic AR NTD containing variable lengths of polyglutamine tract.

AR23, detected from a metastatic CRPC patient, is produced by aberrant splicing that results in an insertion of 69 nucleotides encoding 23 amino acids between the two zinc fingers of the DBD (312). AR23 has been shown to be exclusively cytoplasmic and insensitive to androgen on its own, but when coexpressed with full-length AR in prostate cancer cells, it significantly increased AR transcriptional activity in response to androgen.
Here, our results demonstrated that EPI-002 significantly inhibited the elevated androgen-dependent AR transcriptional activity caused by the expression of AR23. We noticed that the effectiveness of EPI-002 was slightly impaired by the expression of AR23, whereas enzalutamide was not affected. The reduced inhibition of EPI-002 may be caused by the increased amount of drug target in the cells, as the exogenously expressed AR23 (with a ratio of 2:1 to the endogenous AR) also has the NTD where EPI-002 binds to. Alternatively, overexpressed AR23 may reduce the accessibility to the binding site for EPI-002. The inhibition by enzalutamide was not affected by overexpressed AR23, possibly due to the binding site for enzalutamide, which is located in the LBD and remains relatively more accessible. Taken together, our results (Section 3.3.5) indicate that EPI-002 maintained effective inhibition of AR transcriptional activity in spite of variable lengths of polyglutamine tract and aberrantly spliced AR23, which represent molecular alterations within the NTD and DBD of AR, respectively.

In this chapter, our results support the hypothesis that an AR NTD inhibitor such as EPI-002 can effectively inhibit aberrant AR transcriptional activity caused by known and proposed molecular mechanisms. Although EPI-002 significantly blocked androgen-dependent transcriptional activity of full-length AR, the in vitro inhibition by EPI-002 was generally less potent than that by enzalutamide. This difference could be attributed to the different binding mechanisms between enzalutamide and EPI-002, as well as the structural difference between their drug targets. Enzalutamide is an antiandrogen that reversibly binds to AR LBD with high affinity, and thus its inhibitory effect on AR may be rapidly delivered. In contrast, a proposed mechanism for EPI to bind AR NTD involves the formation of an irreversible covalent bond, which is dependent on a slow rate-determining intermediate step.
Furthermore, since AR LBD is a structurally ordered domain with an open conformation for ligand binding, the binding pocket for antiandrogen is readily accessible (187, 193). On the other hand, EPI-002 is an inhibitor of the intrinsically disordered AR NTD, thereby possibly requiring more time and additional biochemical reactions to bind (239). However, the difference in effectiveness between antiandrogen and EPI-002 seems to be limited to in vitro conditions, as EPI-002 was previously demonstrated to be as effective as antiandrogen in inhibiting the in vivo growth of CRPC LNCaP tumours (239).

Importantly, EPI-002 shows the unique ability to inhibit both the full-length AR and constitutively active AR splice variants lacking the LBD, whereas all current AR-targeting therapies only inhibit the full-length receptor. Our preclinical evaluation of EPI-002 demonstrates the potential clinical advantage of targeting the AR NTD to treat CPRC as opposed to targeting the LBD, highlighted by EPI-002 effectively inhibiting castration-resistant and antiandrogen-resistant tumours driven by constitutively active AR splice variants. As the AR splice variants are emerging as a clinically significant mechanism underlying CRPC progression and resistance to all current AR-targeting therapies, AR NTD inhibitors like EPI-002, should have an important clinical niche in the current and future therapeutic landscape of CRPC.
Figure 3.1
Figure 3.1. EPI-002 maintains effective inhibition of increased AR transcriptional activity caused by overexpressed coactivators.

LNCaP cells were transfected with AR-driven luciferase reporter PSA6.1-LUC and various amounts of coactivators (ng/well in 6-well plates). Cells were then pretreated with vehicle (VEH), 10µM enzalutamide (ENZ), or 25µM EPI-002 (EPI) for 1 hr before the treatment of 1nM R1881 for 48 hr. Luciferase activities were measured, and values were plotted after normalized to vehicle-ethanol treatment for (A) SRC1, (B) SRC2, (C) SRC3, and (D) p300. For each coactivator, percentage of inhibition by the treatments was plotted using vehicle-R1881 treatment for normalization. Overexpression of each coactivator in LNCaP cells was confirmed by Western blot analysis using specific antibodies, and β-actin was used as a loading control. Bar graphs are mean ± SEM with n = at least 3 independent experiments. One-way ANOVA Dunnett’s multiple comparisons test was used to compare the percentage of inhibition by each treatment at the indicated amount of coactivator transfected to the respective control without coactivator overexpression; significance was observed only in p300 overexpression; *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3.2

A

B

C

COS-1 transfection

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Figure 3.2. EPI-002 blocks transcriptional activity of several clinically relevant AR mutants.

COS-1 cells were transfected with PB-luciferase reporter and expression vector containing wild-type (WT) AR or mutant AR. Cells were pretreated with vehicle (VEH), 10µM enzalutamide (ENZ), 10µM bicalutamide (BIC), or 10µM flutamide (FLU), or 25µM EPI-002 (EPI) for 1 hr prior to treatment of 1 nM R1881 or ethanol for 24 hr. (A) Luciferase activities were measured, and values were plotted after normalized to vehicle-ethanol treatment for WT AR and each mutant. Bar graphs are mean ± SEM with n = at least 3 independent experiments. One-way ANOVA Dunnett’s multiple comparisons test was used for statistical analyses comparing each treatment group to the respective vehicle control in the absence or presence of R1881; *p < 0.05; **p < 0.01; ***p < 0.001. (B) Percentage of inhibition by the treatment of ENZ or EPI was calculated based on the vehicle-R1881 treatment and plotted. Bar graphs are mean ± SEM with n = at least 3 independent experiments, and Student’s t test was used for statistical analyses comparing ENZ and EPI for each AR; *p < 0.05; #p < 0.001. (C) The protein expression levels of WT AR and each AR mutant transiently transfected in COS-1 cells and endogenous AR expression in LNCaP cells were shown in a representative western blot using AR-N20 antibody, and β-actin was used as a loading control.
Figure 3.3. LNCaP95 cells express both full-length AR and AR-V7, and contain a functional androgen-AR signaling.

(A) LNCaP95 cells were pretreated with vehicle (VEH), 10µM enzalutamide (ENZ), or 25µM EPI-002 (EPI) for 1 hr prior to treatment of 1 nM R1881 or ethanol for 48 hr. Levels of mRNA were measured and quantified for full-length (FL) AR, AR-V567es, and AR-V7. A representative plot is shown. (B) LNCaP95 cells transfected with AR-driven luciferase reporter PSA6.1-LUC were pretreated with VEH, 10µM ENZ, or 25µM EPI for 1 hr prior to treatment of 1 nM R1881 or ethanol for 48 hr. Luciferase activities were measured, and values were plotted after normalized to vehicle-ethanol treatment. Bar graphs are mean ± SEM with n = 3 independent experiments. One-way ANOVA Dunnett’s multiple comparisons test was used for statistical analyses comparing each treatment group to the respective vehicle control in the absence or presence of R1881; *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3.4. EPI-002 blocks gene expression differentially regulated by full-length AR and AR splice variant V7.

LNCaP95 cells were pretreated with vehicle (VEH), 10µM enzalutamide (ENZ), or 25µM EPI-002 (EPI) for 1 hr prior to treatment of 1 nM R1881 or ethanol for 48 hr. Levels of mRNA were measured and quantified for (A) canonical androgen and AR-regulated genes PSA and FKBP5, (B) AR variant-regulated genes UBE2C, CDC20, and AKT1 (C) full-length (FL) AR and AR-V7. Levels of expression for each gene were relative to the mRNA levels of housekeeping gene RPL13A, and normalized to the vehicle control without R1881. Bar graphs are mean ± SEM with n = at least 3 independent experiments. One-way ANOVA Tukey’s multiple comparisons test was used for statistical analyses comparing the treatment groups with each other in the absence or presence or R1881; *p < 0.05; **p < 0.01; ***p < 0.001. (D) AR protein expression levels were examined by western blot using AR-N20 antibody, and β-actin was used as a loading control.
Figure 3.5. EPI-002 inhibits the *in vitro* proliferation and *in vivo* tumour growth of LNCaP95 cells.

(A) LNCaP95 cells were pretreated with vehicle (VEH), 10µM bicalutamide (BIC), 10µM enzalutamide (ENZ), or 25µM EPI-002 (EPI) for 1 hr prior to treatment of 0.1 nM R1881 for 48 hr (2 days). Proliferation of LNCaP95 cells 2 days after treatment was measured by bromodeoxyuridine (BrdU) incorporation. Bar graphs are mean ± SEM with n = at least 3 independent experiments. Two-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses comparing each treatment group to every other group, and the relevant comparisons with statistical significance were indicated; ****p < 0.0001. (B) Tumour growth of LNCaP95 xenografts was calculated as volume percentage normalized to day 0 when treatment began. Mice bearing LNCaP95 xenografts were divided into 3 groups and treated daily with control (n=3), ENZ (n=3), or EPI (n=4) by oral gavage for 26 doses. (C) Tumour volumes analyzed at day 28 when harvested. Photographs of representative tumours are shown. Bar graphs are mean ± SEM with n = the number of tumours in each group. One-way ANOVA Tukey’s multiple comparisons test was used for statistical analyses comparing each treatment group with every other group; *p < 0.05; **p < 0.01; ***p < 0.001; N.S. = no statistical significance.

Acknowledgement: Wang J conducted the animal study.
Figure 3.6

A

HE

AR (PG-21)

Ki67

TUNEL

B

C

% of Ki67 positive cells

% of TUNEL positive cells
Figure 3.6. EPI-002 reduces proliferation and induces apoptosis in LNCaP95 xenografts.

(A) Immunohistochemistry of representative xenograft tumours stained for hematoxylin and eosin (HE), AR, Ki67 and TUNEL. Scale bars (red) indicate 20 µm. (B) % of Ki67 and (C) % of TUNEL positive cells were counted in sections from xenografts for each treatment. At least 1000 cells per xenograft were counted. Total number of cells counted: 2361 (Control, Ki67), 2658 (EPI-002, Ki67), 2409 (ENZA, Ki67), 1209 (Control, TUNEL), 1210 (EPI-002, TUNEL) and 1137 (ENZA, TUNEL). Bar graphs are mean ± SEM with n = 3 different xenograft sections. One-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses comparing the drug treatment groups to the control; *p < 0.05; **p < 0.01.

Acknowledgement: Wang J and Kato M completed the immunohistochemistry and generated the images in Figure 3.6 (A).
Figure 3.7

A

B

C

D
Figure 3.7. EPI-002 inhibits AR transcriptional activity regardless of variable CAG repeats (polyglutamine tract) within the AR NTD.

COS-1 cells were transfected with PB-luciferase reporter and expression vector containing polymorphic AR with variable CAG repeats. Cells were pretreated with vehicle (VEH), 10µM enzalutamide (ENZ), or 25µM EPI-002 (EPI) for 1 hr prior to treatment of 1 nM R1881 for 24 hr. (A) Luciferase activities for AR with polymorphic CAG repeats were plotted in percentage of PB-LUC activation using values normalized to the vehicle-R1881 treatment. One-way ANOVA Dunnett’s multiple comparisons test was used for statistical analyses comparing each treatment group to the vehicle-R1881 control; *p < 0.05; **p < 0.01; ***p < 0.001. (B) Inhibition by ENZ or EPI was normalized to vehicle-R1881 treatment and plotted as normalized percentage of inhibition. (C) R1881-induced PB-LUC activity under vehicle treatment was plotted for each AR with variable length of polyglutamine tract. One-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses comparing each AR with every other AR. The only comparison with statistical significance is indicated; *p < 0.05; **p < 0.01; ***p < 0.001. Bar graphs are mean ± SEM with n = at least 3 independent experiments. (D) The expression level of each polymorphic AR in COS-1 cells treated with DMSO vehicle (D) or EPI-002 (E) in the presence of R1881 were examined and compared to LNCaP endogenous levels by western blot using AR-N20. A representative blot is shown. β-actin was used as a loading control. Acknowledgement: Banuelos A provided the fold of induction of each AR in Figure 3.7 (C), and generated the western blot in Figure 3.7 (D).
Figure 3.8. EPI-002 blocks AR transcriptional activity in the presence of AR23.

LNCaP cells were transfected with AR-driven luciferase reporter PSA6.1-LUC and expression vector containing AR23. Cells were pretreated with vehicle (VEH), 10µM enzalutamide (ENZ), or 25µM EPI-002 (EPI) for 1 hr prior to treatment of 1 nM R1881 or ethanol for 48 hr. (A) Luciferase activities were measured, and values were plotted after normalized to vehicle-ethanol treatment as fold of induction. Two-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses comparing each treatment group to every other group, and the statistical significance is indicated for drug-treated groups to their controls, unless otherwise noted; *p < 0.05; **p < 0.01; ***p < 0.001. (B) Inhibition by ENZ or EPI was calculated by normalizing to the vehicle-R1881 control and plotted as normalized percentage of inhibition. Two-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses comparing each treatment group to every other group, and the relevant statistical significance is indicated; *p < 0.05; **p < 0.01; ***p < 0.001. (C) The expression level of AR23 transfected into LNCaP cells in this study was examined and compared to LNCaP endogenous levels by western blot using AR-N20, and β-actin was used as a loading control. A representative western blot is shown. Bar graphs are mean ± SEM with n = 4 independent experiments.
CHAPTER 4. Generation of a human prostate cancer cell line model that is resistant to AR NTD inhibitor EPI-002

4.1 INTRODUCTION

Currently, targeted therapy and chemotherapy are two major modalities of treatment for cancer. However, drug resistance is a significant cause of treatment failure, thereby limiting the clinical effectiveness of these therapies. Despite different mechanisms of action between targeted therapy and chemotherapy, the mechanisms of resistance to these two therapies largely overlap (338). The cancer cells can be intrinsically resistant to certain therapies because resistance-conferring factors are originally present in these cells (339). Alternatively, cancer cells can acquire drug resistance during the course of treatment, in which the cells that are initially sensitive to therapy gradually become resistant (340, 341). Since tumours can contain a high degree of cellular and molecular heterogeneity, resistance can also arise from therapy-induced selection and enrichment of a small subpopulation of cancer cells that are intrinsically resistant to certain therapies (342). Due to the complexity of cancer biology, drug resistance remains as a major challenge to current cancer research. However, various molecular mechanisms underlying resistance to cancer drugs have been elucidated. These include: reduction of the intracellular levels of active drug compounds by promoting drug efflux and affecting drug metabolism (343-346); alterations related to drug targets such as gain-of-function mutation and amplification (215, 229, 347); activation of alternative pro-survival signaling pathway as adaptive responses to therapies (348, 349); and inactivation of apoptosis to evade drug-induced cell death (350, 351).

In prostate cancer, both targeted therapy and chemotherapy are standard of care treatments (19). Docetaxel, a taxane-based drug, is a chemotherapeutic agent commonly used
to treat metastatic CRPC (19, 46, 47). However, docetaxel can only prolong CRPC survival by 2-3 months, as the cancer cells eventually develop resistance (46, 47, 352). Studies using docetaxel-resistant prostate cancer cells revealed that overexpression of the classical drug pump P-glycoprotein was involved with resistance against docetaxel (267). P-glycoprotein, also known as multidrug resistance protein 1, is an ATP-dependent efflux pump believed to play a key role in conferring resistance against chemotherapeutic agents in many types of cancer (338, 343). In addition, transcription factor NF-κB was shown to be associated with docetaxel resistance prostate cancer cells without P-glycoprotein expression (267). These studies were conducted using androgen-independent prostate cancer cells that do not express functional androgen receptor (AR). Androgen is the primary mitogen for prostate cancer cells, and mediates its biological signal through AR, which is a transcription factor that regulates the expression of many genes associated with the growth and survival (40, 75). Clinically, only a minority of prostate cancer is driven by androgen-independent cells such as small cell (neuroendocrine) carcinoma, which is transiently responsive to chemotherapy (58, 74). In most cases of prostate cancer, the cancer cells express AR and rely on AR transcriptional activity for growth and survival, highlighting AR as a primary therapeutic target for prostate cancer (44, 353). In fact, AR-targeting therapies have formed the mainstay of treatment for the disease. All current AR-targeting therapies, which include androgen-deprivation therapy (ADT), antiandrogen, and CYP17 inhibitor, are designed to prevent androgen from binding to AR ligand-binding domain (LBD), thereby blocking AR transcriptional activity (217).

Despite providing significant clinical benefits, the current AR-targeting therapies will fail because prostate cancer cells will inevitably develop resistance. ADT has been the
standard first-line treatment for advanced prostate cancer for decades, and it works by reducing the levels of androgen (19, 44). But within 12-33 months most patients will develop resistance to ADT, and consequently progress into castration-resistant prostate cancer (CRPC), which is lethal and incurable (43). The clinical onset of most CRPC is accompanied by a rise in serum prostate-specific antigen (PSA) level (43). Since PSA is a gene transcriptionally regulated by AR, this indicates that AR transcriptional activity is aberrantly restored in CRPC, despite reduced levels of androgens by ADT (40, 43, 82). The notion that AR remains functional in CRPC has led to the development and approval of the new-generation AR-targeting therapies abiraterone and enzalutamide, and each of them has been demonstrated to significantly improve the survival of metastatic CRPC (50, 51, 54, 278). Unfortunately, the effectiveness of abiraterone and enzalutamide is limited by resistance yet again, as patients will develop progressive disease after showing transient responses (224-226, 354). Preclinical and clinical studies have provided consistent findings to suggest that the resistance against ADT, abiraterone, and enzalutamide commonly involves AR-related molecular alterations, which are capable of driving aberrant AR transcriptional activity. These molecular alterations include: amplification of the AR gene and increased AR protein expression, which result in hypersensitivity to very low levels of androgens as the case in CRPC (81, 83); AR gain-of-function mutations can allow activation by nonandrogenic steroidal ligands such as glucocorticoid or even antiandrogens like flutamide, bicalutamide, and enzalutamide (84-88); overexpression of AR coactivators that can enhance androgen-dependent and ligand-independent AR transcriptional activities (89-95); androgen-independent transactivation of the AR N-terminal domain (NTD), which contains the most if not all of AR transcriptional activity, through protein kinase (PKA) pathway, cytokines such
as interleukin-6 (IL-6), and by bone-derived factors (96-98, 166); increased adrenal and intratumoral androgen biosynthesis under castrated conditions to maintain a sufficient level of androgen for AR signaling (99-101); and expression of constitutively active AR splice variants with truncated LBD that are capable of driving a distinct transcriptional program than the full-length AR (102-105, 161).

In contrast to all current AR-targeting therapies which act through the LBD, EPI-002 inhibits AR by binding to the NTD (238, 239). As presented and discussed in Chapter 3 of this dissertation, EPI-002 has the ability to overcome all AR-related mechanisms that have been shown to confer resistance to current AR-targeting therapies, including mutations of the AR LBD and constitutively active AR splice variants lacking the LBD. Here, we aim to develop a prostate cancer cell line model resistant to EPI-002. Despite the effective inhibition of AR transcriptional activity by EPI-002, we hypothesize that prostate cancer cells will eventually develop resistance to EPI-002. To test this hypothesis, three specific aims are outlined: 1. to establish a LNCaP cell line with chronic exposure to EPI-002 by continuously culturing in the presence of EPI-002; 2. to determine if resistance has developed in these cells by performing in vitro proliferation assay; and 3. if resistant in vitro proliferation to EPI-002 is observed, to validate the resistance by using in vivo xenografts of these cells in an animal study. Whether or not prostate cancer cells develop resistance to EPI-002, the findings will provide important insight into the long-term effects of AR suppression by an inhibitor of the NTD.
4.2 MATERIALS AND METHODS

4.2.1 Cell culture

Parental LNCaP human prostate cancer cells were maintained in RPMI 1640 phenol red-free media supplemented with 5% (v/v) fetal bovine serum (FBS) (Invitrogen™ by Life Technologies, Carlsbad, CA). To generate a LNCaP cell subline with long-term EPI-002 treatment (LNCaP-EPI), the parental LNCaP cells (started at passage #39) were cultured in RPMI 1640 phenol red-free media with 5% FBS in the presence of 25 µM EPI-002. Once every 3 days, EPI-002 was replenished in these long-term treatment cells. The cells were passaged with a dilution ratio of 1/10 (3ml cells into 27ml media containing 25 µM EPI-002) using T175 Corning™ cell culture flask. Between passage #116 to #118, the LNCaP-EPI cells were used for in vitro proliferation assays and in vivo xenografts.

4.2.2 Proliferation assay

Bromodeoxyuridine (BrdU) incorporation was applied to measure cellular proliferation. Parental LNCaP or LNCaP-EPI cells were plated at 5,000 cells/well in phenol red-free RPMI 1640 containing 5% FBS using 96-well Falcon Primaria tissue culture plates. The plated cells were allowed one day to attach to the wells. The cells were then treated with DMSO vehicle, or increasing concentrations of EPI-002 for three days before BrdU labeling. After two hour incubation at 37°C for BrdU incorporation, media were removed and the plates were dried in a Hybaid oven at 60°C for 1 hour. Proliferation was quantitatively measured using BrdU ELISA kit (Roche Applied Science, Mannheim, Germany) for BrdU labelling and the subsequent ELISA assay following the manufacturer’s protocol.
4.2.3 Xenografts and animal studies

Male NOD-SCID mice, 6-8 weeks old, were subcutaneously inoculated with parental LNCaP or LNCaP-EPI cells (10 million cells per site) using Matrigel (Becton Dickinson, New Jersey) in the flanks. After implantation, xenografts were allowed to grow until reaching approximately 100 mm$^3$ in size, and then the mice were surgically castrated. Treatment of drugs started seven days after castration, and mice were randomized into three groups. Mice were administered daily with vehicle control, enzalutamide at 10mg/kg body weight, or EPI-002 at 200mg/kg body weight by oral gavage. Over the duration of the experiment, tumour volumes were regularly measured. Because LNCaP xenograft tumours usually take on a spheroid geometry, we measured the tumour volume based on the formula: $V = \text{length} \times \text{width} \times \text{height} \times 0.5236$. All animal studies conformed to the relevant regulatory and ethical standards. All experiments involving animals were approved by the University of British Columbia Animal Care Committee.

4.2.4 Statistics

Statistical analysis was performed using GraphPad Prism (version 6.05; GraphPad Software). Comparisons between three or more groups were performed using either One-way or Two-way ANOVA as indicated. A p value less than 0.05 was considered statistically significant for differences between groups compared.
4.3 RESULTS

4.3.1 LNCaP cells with chronic exposure to EPI-002 developed resistance to growth inhibition by EPI-002

Long-term culturing of parental LNCaP cells in the presence of 25 µM EPI-002 generated the LNCaP-EPI subline. To determine if the LNCaP-EPI cells developed resistance to EPI-002 after chronic exposure, we used BrdU to label the cells that were actively replicating their DNA during S-phase, and then measured cell proliferation. EPI-002 caused a dose-dependent inhibition of proliferation of parental LNCaP cells, with a half-maximal inhibitory concentration (IC$_{50}$) of approximately 26 µM (Figure 4.1A). EPI-002 also resulted in a dose-dependent inhibition of proliferation of LNCaP-EPI cells, but with an IC$_{50}$ of about 43 µM (Figure 4.1A). Moreover, when compared to parental LNCaP cells, LNCaP-EPI cells had significantly more proliferating cells in the presence of increasing concentrations of EPI-002, which was consistent with microscopic images showing cells treated with 25 µM or 50 µM of EPI-002 (Figure 4.1B). Next, we wanted to examine if LNCaP-EPI could maintain resistance to EPI-002 when exposure to EPI-002 was discontinued for an extended period of time. We split a flask of LNCaP-EPI cells into two. The cells in one flask were cultured in normal media without EPI-002 for three consecutive passages (21 days, approximately 15 doubling times), and we referred these cells as LNCaP-EPI-STOPPED$_{P3}$. In parallel, the cells in the other flask were cultured and passaged with the presence of EPI-002 according to the same procedures for the generation of the LNCaP-EPI subline. We measured the proliferation of these cells and compared it with the proliferation of parental LNCaP cells. The LNCaP-EPI-STOPPED$_{P3}$ cells demonstrated significant resistance to growth inhibition by EPI-002 at increasing concentrations of EPI-002, with an IC$_{50}$ value comparable to that of
the LNCaP-EPI cells with continuous exposure to EPI-002 (Figure 4.2). Together, these results indicate that during the course of chronic exposure to EPI-002, the LNCaP-EPI cells developed significant resistance to growth inhibition by EPI-002, and the resistance was maintained for at least three passages (21 days, approximately 15 doubling times) in the absence of EPI-002.

4.3.2 Tumour incidence and growth were increased in intact mice bearing LNCaP-EPI xenografts.

LNCaP-EPI cells were inoculated subcutaneously into the flanks of NOD-SCID male intact mice, and a separate group of mice was inoculated with parental LNCaP cells in the same manner. For both xenograft models, the same number of cells (10 x 10⁶) was implanted per site. To investigate the growth characteristics of the LNCaP-EPI xenograft model in the presence of androgen, we examined tumour incidence in intact mice using the criteria of a tumour being approximately 80 mm³ in volume. The tumour incidence was much higher in the LNCaP-EPI xenograft model when compared with the parental LNCaP model. At 37 days after implantation, tumour incidence was 100% (25 out of 25) in mice bearing LNCaP-EPI xenografts, whereas tumour incidence remained at zero (0 out of 12) in mice bearing parental LNCaP xenografts (Figure 4.3A). Mice bearing parental LNCaP xenografts eventually had 92% (11 out of 12) tumour incidence 63 days after implantation. The elevated tumour incidence in intact mice bearing the LNCaP-EPI model was consistent with increased tumour growth rate (Figure 4.3B). The LNCaP-EPI tumours increased in mean volume from 76.6 ± 2.6 to 103.5 ± 5.6 mm³ in 3 days. In contrast, the parental LNCaP tumours increased from 78.2 ± 4.3 mm³ to 100.6 ± 4.1 mm³ in 8 days. These results indicated that LNCaP-EPI
tumours had a faster growth rate than parental LNCaP tumours in intact mice. Furthermore, castration did not cause the tumours from either xenograft model to regress (Figure 4.3B), which is consistent with the castration-resistant phenotype of LNCaP xenografts as previously reported (262).

4.3.3 LNCaP-EPI tumours were resistant to EPI-002 but sensitive to enzalutamide

Castrated mice bearing LNCaP-EPI xenografts were randomized into three treatment groups for daily oral dosing of vehicle control, enzalutamide, or EPI-002. The growth of LNCaP-EPI tumours in mice treated with EPI-002 at 200 mg/kg body weight was resistant to EPI-002 throughout the study duration, with a final tumour volume significantly larger than that of the vehicle treated group (466.5 ± 59.8 mm³ vs. 352.2 ± 49.5 mm³; p<0.01) (Figure 4.4A). At a daily dosing of 10 mg/kg body weight, enzalutamide has been previously shown by others to effectively inhibit the growth of parental LNCaP tumours in castrated mice (355). Here, enzalutamide at the same dosing was also effective against the growth of LNCaP-EPI tumours, resulting in a final tumour volume of 105.6 ± 14.6 mm³ (Figure 4.4A). To serve as a control for the effectiveness of EPI-002, castrated mice bearing parental LNCaP xenografts were randomized into two groups to receive daily oral dosing of vehicle or EPI-002 at 200 mg/kg body weight, the same dosing that failed to inhibit the growth of LNCaP-EPI-002 tumours. As expected, the growth of parental LNCaP tumours was potently inhibited by EPI-002, as the final tumour volume for EPI-002 treated group was significantly smaller than that of the vehicle treated group (155.7 ± 20.3 mm³ vs. 367.8 ± 56.9 mm³; p<0.01) (Figure 4.4B). At the end of the study, LNCaP-EPI tumours were extracted and weighed. Consistent with the final tumour volumes shown in Figure 4.4A, EPI-002 did not
reduce the weight of LNCaP-EPI tumours, whereas enzalutamide significantly decreased the weight of these tumours (Figure 4.4C). We found that regardless of the treatments, there were no differences between the initial and final body weights of all mice used in the study (Figure 4.4D). In addition, we did not observe any behavioral changes related with the treatments, and the treatments did not significantly alter the weights of liver, spleen, and kidneys, indicating the absence of toxicity in the animals used for this study.

4.4 DISCUSSION AND CONCLUSION

The human prostate cancer cell line LNCaP is one of the most commonly used cell lines in prostate cancer research. LNCaP cells express endogenous and functional AR, and their growth is androgen-sensitive (251, 252). Derived from parental LNCaP cells chronically maintained under androgen-depleted conditions intended to simulate the clinical AR suppression by ADT, LNCaP-abl and LNCaP95 were used to investigate the resistance against ADT and the consequential development of CRPC. Results from these two preclinical models suggest that aberrant AR transcriptional regulation and AR splice variants are involved in the resistance to ADT and development of CRPC (160, 161). Clinical studies have revealed that aberrant AR transcriptional activity and AR splice variants are indeed associated with therapy resistance and disease progression (51, 54, 105, 107). In addition, LNCaP cells were used to study the mechanisms of drug resistance against antiandrogens. Bicalutamide is a first-generation antiandrogen, and several bicalutamide-resistant prostate cancer cell lines have been reported, including LNCaP-cxD and LNCaP-BC2. The mechanism underlying bicalutamide resistance in LNCaP-cxD cells was determined to be an AR gain-of-function mutation, W741C or W741L, which allows AR to be activated by
bicalutamide (264). The AR W741C mutation was also found in a prostate cancer xenograft in vivo model derived from a bicalutamide-treated patient, highlighting its clinical relevance (87). Furthermore, the AR from LNCaP-BC2 cells did not have any treatment-induced mutation from prolonged exposure to bicalutamide. The AR in these cells was instead overexpressed and hypersensitive to low levels of androgens, consistent with a previous report that AR overexpression can be the sole mechanism underlying antiandrogen resistance in prostate cancer xenograft models (215, 266). Amplification of the AR gene and increased AR protein expression have been shown in clinical samples from therapy-resistant prostate cancer (81, 83). Importantly, studies on therapy-resistant prostate cancer models derived from LNCaP cells have elucidated clinically relevant and significant mechanisms of resistance to AR-targeting therapies, thus providing the rationale of using the LNCaP model to investigate potential resistance mechanisms to novel AR inhibitors.

Unlike all current AR-targeting therapies that act through the AR LBD, EPI-002 is an antagonist of AR NTD, which is an intrinsically disordered domain that contains the majority if not all of AR transcriptional activity (166, 238, 239). Here, we derived a LNCaP-EPI subline from parental LNCaP cells exposed to long-term and continuous treatment of EPI-002 in the presence of 5% FBS. We maintained these cells in the presence of serum with androgens, because we wanted to examine the resistance mechanisms developed against AR inhibition through the NTD. A culture condition that contains EPI-002 in serum-free media would select mechanisms that confer resistance to AR suppression as a result of targeting both NTD and LBD. In the present study, we used BrdU incorporation to measure the effect of EPI-002 on proliferation. We determined that the IC₅₀ value for EPI-002 in parental LNCaP cells was approximately 26 µM, comparable with the previously reported data.
showing a reduction of cells undergoing S-phase in parental LNCaP cells treated by 25 µM EPI-002 (239). In contrast, the IC₅₀ for EPI-002 in the LNCaP-EPI cells was 43 µM, substantially higher than that of the parental LNCaP cells, indicating that the LNCaP-EPI cells became more tolerant to the antiproliferative effect of EPI-002. Furthermore, we found that the resistance to EPI-002 in LNCaP-EPI cells is relatively stable, as suggested by the data showing that upon re-exposure to EPI-002 after three passages (21 days, approximately 15 doubling times) without EPI-002, the LNCaP-EPI-STOPPEDP3 cells still displayed significant resistance to EPI-002 and had an IC₅₀ value comparable to that of the LNCaP-EPI cells continuously cultured in the presence of EPI-002. For future experiments to determine how long the resistance could be sustained or if the resistance is permanent, LNCaP-EPI cells cultured without EPI-002 for longer than three passages are required for this future experiment.

The *in vitro* resistance to EPI-002 in the LNCaP-EPI cells was confirmed by *in vivo* studies using these cells as xenografts in mice. It is important to note that the same number of cells (10 x 10⁶) per site was inoculated into the mice for both parental LNCaP and LNCaP-EPI xenograft models, and therefore, any differences in growth observed between the two models should not be caused by different starting numbers of cells. Drug treatment in this study did not cause apparent toxicity to the mice, as indicated by no changes in the body and organ weights of the mice. In our study, both the parental and LNCaP-EPI tumours treated with vehicle continued to grow after the mice were castrated, in agreement with the previously demonstrated castration-resistant phenotype of LNCaP xenografts (262). The growth of LNCaP-EPI tumours displayed resistance to EPI-002 at a daily oral dosing of 200 mg/kg body weight, whereas the same dosing of EPI-002 significantly inhibited the growth
of parental LNCaP tumours. Since the final volume of LNCaP-EPI tumours in mice treated with EPI-002 was significantly larger than that of the vehicle-treated group, future studies will be conducted to determine if the LNCaP-EPI cells have evolved to become capable of utilizing EPI-002 for growth advantage. Interestingly, enzalutamide completely inhibited the growth of LNCaP-EPI tumours in castrated mice. Enzalutamide is a second-generation antiandrogen that blocks AR transcriptional activity by competing against androgens from binding to the LBD (218). The fact that the LNCaP-EPI tumours were sensitive to antiandrogen such as enzalutamide suggests that AR transcriptional activity was a key driver of the growth and survival of LNCaP-EPI tumours in castrated mice. Therefore, an AR-related mechanism may be involved that confers resistance to EPI-002 in the LNCaP-EPI cells. Several mechanisms of resistance to enzalutamide in CRPC have been elucidated. They include constitutively active AR splice variants with truncated LBD, AR gain-of-function mutation F876L within the LBD, and induction of glucocorticoid receptor (GR) to maintain growth and survival (88, 230, 236). The potent effectiveness of enzalutamide against the LNCaP-EPI tumours indicates that the potential AR-related mechanism of resistance to EPI-002 in the LNCaP-EPI cells does not involve the aforementioned mechanisms underlying enzalutamide resistance. Long-term passage of LNCaP cells might have produced molecular alterations that caused increased tumour incidence and tumour growth in intact mice regardless of prolonged EPI-002 treatment, and further studies are needed to investigate this.

Overall, we have obtained consistent results from in vitro proliferation and in vivo tumour growth to show that LNCaP-EPI is a cell line model resistant to EPI-002, an AR inhibitor that binds to the NTD. To our knowledge, this is the first report of a prostate cancer model that developed resistance to chronic AR suppression through the NTD. Resistance
mechanisms underlying long-term AR inhibition through the LBD have been elucidated by using relevant LNCaP models, and the findings have made significant impact on the development of new-generation AR-targeting therapies. The molecular mechanisms underlying resistance to EPI-002 in the LNCaP-EPI cells are currently being investigated by our group. Studies based on the LNCaP-EPI model have the potential to make important contributions to the development of the next-generation AR-targeting therapies.
Figure 4.1

A

Proliferation after 72hr treatment

Normalized % BrdU Incorporation

EPI-002 [μM]

B

Parental LNCaP
72hr EPI-002 treatment

LNCaP-EPI
72hr EPI-002 treatment

0 μM

25 μM

50 μM
Figure 4.1. LNCaP-EPI cells demonstrate significant resistance to growth inhibition by EPI-002.

(A) Parental LNCaP and LNCaP-EPI cells were plated in 96-well plates and treated with increasing concentrations of EPI-002 for 3 days in normal culture media containing 5% FBS. Proliferation assays using BrdU incorporation and ELISA were performed to measure cell proliferation. Proliferation measured in vehicle-treated cells was used for normalization, and normalized percentage of BrdU incorporation was plotted for each cell line. Data points are connected to show inhibition curves. Dotted lines illustrate the IC$_{50}$ for each cell line. Each data point is the mean ± SEM with n = at least 3 independent experiments. Two-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses to compare each data point with every other point, and relevant comparisons are indicated by dotted brackets; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. (B) Representative pictures of cells treated with various concentrations of EPI-002 for 3 days are shown.
Parental LNCaP cells, LNCaP-EPI cells continuously cultured in the presence of EPI-002, and LNCaP-EPI STOPPED_{P3} cells cultured in normal media without EPI-002 for 3 passages were plated in 96-well plates and treated with increasing concentrations of EPI-002 for 3 days in normal culture media containing 5% FBS. Proliferation assays using BrdU incorporation and ELISA were performed to measure cell proliferation. Proliferation in vehicle-treated cells was used for normalization, and normalized percentage of BrdU incorporation was plotted. Data points are connected to show inhibition curve. Each data point is the mean ± SEM with n = at least 3 independent experiments. Two-way ANOVA Bonferroni’s multiple comparisons test was used for statistical analyses to compare each data point with every other point, and relevant comparisons are indicated by dotted brackets. In each bracket, statistical significance is noted (in the same order) for parental LNCaP vs. LNCaP-EPI, and parental LNCaP vs. LNCaP-EPI STOPPED_{P3}; *p<0.05; **p < 0.01; ***p < 0.001.
Figure 4.3. Increased tumour incidence and tumour growth in intact mice bearing LNCaP-EPI xenografts.

(A) Parental LNCaP or LNCaP-EPI cells were subcutaneously grafted into intact mice at 10 million cells per xenograft. Tumour incidence was recorded as the percentage of tumours with a volume approximately 80 mm$^3$ out of $n = 12$ for parental LNCaP tumours, and $n = 25$ for LNCaP-EPI tumours. (B) Tumour volume was measured for both the parental LNCaP ($n=8$) and LNCaP-EPI ($n=25$) tumours in intact mice until 7 days after castration. Arrows indicate the time of castration. Each data point is the mean ± SEM of the tumours. Acknowledgement: Wang J conducted the animal study.
Figure 4.4

A. LNCaP-EPI tumour growth

B. Parental LNCaP tumour growth

C. LNCaP-EPI tumour weight

D. Body weight

E. Liver, Spleen, Kidneys
Figure 4.4. LNCaP-EPI tumours are resistant to EPI-002 but sensitive to enzalutamide in castrated mice.

(A) Growth of LNCaP-EPI tumours in castrated mice administered with vehicle (VEH), enzalutamide (ENZA) at 10 mg/kg body weight, or EPI-002 (EPI) at 200 mg/kg body weight by oral gavage. The number of tumours in each treatment group is indicated by n. (B) Growth of parental LNCaP tumours in castrated mice administered with vehicle (VEH), or EPI-002 (EPI) at 200 mg/kg body weight by oral gavage. The number of tumours in each treatment group is indicated by n. (C) Weight of LNCaP-EPI tumours harvested at the end of study was measured and compared between the treatment groups. Each dot represents an individual tumour. (D) Body weight and (E) organ weight of mice administered with each treatment by oral gavage. Each dot represents an individual mouse. Mice were randomized into 3 treatment groups. VEH: n = 8; ENZA: n = 8; EPI: n = 8. T0: body weight before treatment started; Tf: body weight 1 day after the last treatment. One day after the last treatment, organs were harvested and weighed. Two-way ANOVA Bonferroni’s multiple comparison test was used for statistical analyses for the tumour volumes, whereas One-way ANOVA Bonferroni’s multiple comparison test was used for statistical analyses for tumour weights, as well as the body and organ weights. Bars represent the mean ± SEM; **p < 0.01; ***p < 0.001; ****p<0.0001. No significance (ns) for the body weights between T0 and Tf in each treatment group and organ weights between treatment groups. Acknowledgement: Wang J and Tien AH conducted the animal study.
CHAPTER 5. Conclusions

5.1 RESEARCH SUMMARY

Prostate cancer remains as a major health risk for men around the world. AR transcriptional activity is required for the growth and survival of most prostate cancer cells, and thus AR-targeting therapies have become a standard of care treatment for prostate cancer (76, 217). CRPC is the late-stage and an aggressive form of the disease, and currently CRPC is lethal and incurable. Compelling evidence from basic and clinical research indicates that the development and progression of CRPC is still dependent on AR transcriptional activity, highlighting a significant role for AR-targeting therapies in the treatment landscape of CRPC (40, 48, 217). Abiraterone and enzalutamide represent the new-generation AR-targeting drugs, and each has been approved to treat CRPC as the first-line therapy prior to chemotherapy, or second-line therapy for CRPC patients progressed after chemotherapy (50, 51, 53, 54). Despite providing an initial clinical effectiveness, enzalutamide and abiraterone will fail to stop the progression of CRPC, and eventually patients will succumb to the disease. Therefore, it is imperative to continue to search for new drugs to target the AR.

In Chapter 2, we identified furanoditerpenoid spongia-13(16),-14-dien-19-oic acid, or T1 from a crude extract of a marine sponge based upon its activity in our screening assays for compounds that block AR transcriptional activity. T1 belongs to a family of steroidal compounds called spongian diterpenoids, which are commonly found in marine sponges and shell-less mollusks that feed on the sponges (294). T2 and T3 are semisynthetic compounds produced by reducing either the furan or 17β carboxylic acid functionalities in T1, respectively. Although some other spongian diterpenoids were previously evaluated for in
v/ vitro antiviral and antitumour activities, there were no studies that investigated the therapeutic potential of spongian diterpenoids in prostate cancer (295). Here, for the first time we demonstrated that the diterpenoids are novel antiandrogens with a structure-activity relationship. The diterpenoids inhibited androgen-dependent AR transcriptional activity by binding to AR LBD and blocking androgen-induced AR N/C interaction. Consistent with the inhibition of AR-driven luciferase reporter activity, the diterpenoids reduced the expression of several AR-regulated genes including PSA, and FKBP5. We demonstrated that the diterpenoids specifically inhibited AR without affecting the transcriptional activity of related receptor GR. Similar to bicalutamide, which is an approved antiandrogen used to treat advanced prostate cancer, diterpenoids showed binding affinity for PR LBD and thereby inhibited PR transcriptional activity. The diterpenoids attenuated androgen-dependent in vitro proliferation of prostate cancer cells without decreasing the proliferation of androgen-independent cells, indicating these compounds did not cause general cytotoxicity. Furthermore, T3, the most potent diterpenoid compound, showed on-target in vivo activity by significantly reducing the weight of androgen-dependent tissue such as the seminal vesicles with no toxicity in mice. However, as expected for antiandrogens, the diterpenoids did not inhibit the proliferation of prostate cancer cells that is driven by constitutively active AR splice variants with truncated LBD.

In Chapter 3, the focus shifts to a small molecule inhibitor of AR NTD called EPI-002. Our previous work has demonstrated that EPI-002 specifically binds to the AF-1 region of the AR NTD (238, 239). Here, we investigated the effectiveness of EPI-002 against several molecular alterations proposed as mechanisms underlying aberrant AR transcriptional activity, including increased expression of AR coactivators, AR gain-of-function mutations,
and constitutively active AR splice variants with truncated LBD (89, 161, 315). EPI-002 maintained an effective and consistent inhibition on aberrant AR transcriptional activity caused by overexpression of coactivators such as the p160 SRC family of coactivators and p300. EPI-002 overcame several clinically relevant AR mutations that confer gain-of-functions including enhanced AR N/C interaction, increased AR protein stability, and resistance to the first-generation antiandrogens such as flutamide and bicalutamide. EPI-002 blocked the transcriptional activity of AR splice variants with truncated LBD, and consequently reduced gene expression regulated by the AR variants. EPI-002 inhibited the antiandrogen-resistant proliferation of LNCaP95 cells, as these cells are believed to rely on AR splice variants for growth. Using the LNCaP95 cells as a xenograft model in mice, we recapitulated the in vivo growth of CRPC that is driven by AR splice variants and resistant to current AR-targeting therapies. Consistent with the results from the LNCaP95 cell proliferation study, EPI-002 significantly attenuated the castration-resistant growth of LNCaP95 tumours in castrated mice, whereas enzalutamide had no effect.

Furthermore, we evaluated EPI-002 against two other AR-related molecular alterations that have been shown to affect AR transcriptional activity: polymorphic AR NTD with variable lengths of polyglutamine tract and cytoplasmic AR variant AR23. The length of polyglutamine tract has a functional impact on AR transactivation, as AR with a shorter glutamine tract is more transcriptionally active in response to androgen than those with longer tracts (311, 323, 324). We demonstrated that EPI-002 maintained significant and consistent inhibition on AR transcriptional activity regardless of the length of the polyglutamine tract. AR23 is another AR variant detected from a metastatic CRPC patient, and is produced by aberrant splicing that results in an insertion of 69 nucleotides encoding 23
amino acids between the two zinc fingers of the DBD (312). AR23 has been shown to be exclusively cytoplasmic and insensitive to androgen on its own, but when it is coexpressed with full-length AR in prostate cancer cells, it causes a substantially elevated androgen-dependent AR transcriptional activity (312, 315). Here, our results showed that EPI-002 significantly inhibited the elevated androgen-dependent AR transcriptional activity caused by the expression of AR23.

In Chapter 4, we generated the LNCaP-EPI subline from parental LNCaP cells cultured under prolonged exposure to EPI-002. Using BrdU incorporation to measure proliferation, we showed that the IC$_{50}$ for EPI-002 in LNCaP-EPI was 43 µM, whereas the IC$_{50}$ for EPI-002 in parental LNCaP cells was 26 µM. The substantially increased IC$_{50}$ in LNCaP-EPI cells indicates that these cells have developed resistance against growth inhibition by EPI-002. In addition, we showed that the resistance to EPI-002 in the LNCaP-EPI cells was relatively stable, as discontinuation of EPI-002 exposure for three passages did not affect the resistance of these cells to EPI-002 upon re-exposure. Next, we conducted in vivo studies to confirm the resistance to EPI-002 in the LNCaP-EPI cells. First, we found that treatment-naïve intact mice bearing LNCaP-EPI xenografts had increased tumour incidence and tumour growth, as compared to the mice bearing parental LNCaP xenografts. In castrated mice bearing LNCaP-EPI xenografts, the growth of LNCaP-EPI tumours in mice treated by EPI-002 at 200 mg/kg body weight displayed a complete resistance to EPI-002 throughout the study duration, with a final tumour volume significantly larger than that of the vehicle treated group (466.5 $\pm$ 59.8 mm$^3$ vs. 352.2 $\pm$ 49.5 mm$^3$, n = 8 for each; p<0.01). As a control, the growth of parental LNCaP tumours in castrated mice was potently inhibited by EPI-002 at the same dosing as compared to the vehicle treated group (155.7 $\pm$ 20.3 mm$^3$ vs. 367.8 $\pm$
Interestingly, antiandrogen enzalutamide was effective against the castration-resistant growth of LNCaP-EPI tumours, suggesting that the growth and survival of the LNCaP-EPI cells were dependent on the transcriptional activity of full-length AR. Overall, with consistent results from *in vitro* proliferation and *in vivo* tumour growth, we demonstrated that the LNCaP-EPI is a cell line model that developed resistance to EPI-002, an inhibitor of AR NTD.

**5.2 FUTURE STUDIES**

For the diterpenoids, potential future studies should focus on further investigation regarding their structure-activity relationship to see if a more potent antiandrogen can be generated from the diterpenoids. In addition, since a close structural homology exists between AR and PR, the diterpenoids also inhibited PR transcriptional activity. Therefore, the development of novel PR inhibitors derived from the diterpenoids could be worthy of consideration, as PR antagonists have a role in medical termination of pregnancy.

There are additional future studies that could be conducted to provide a more comprehensive preclinical evaluation of the AR NTD inhibitor EPI-002. One experiment is to test the effect of EPI-002 against the clinically relevant AR mutant F876L that has been shown to convert enzalutamide into an agonist (88, 229). Although we have presented consistent results that demonstrated the effectiveness of EPI-002 against other clinically relevant AR gain-of-function mutations within the LBD and as well as the NTD, it would provide a direct evidence of EPI-002 overcoming an enzalutamide-resistant AR mutant if we had included the F876L mutant in our studies. Site-directed mutagenesis will be applied to generate this mutant. Another study is to investigate the effect of EPI-002 against elevated
intratumoral androgen biosynthesis that has been implicated with resistance to abiraterone, which is an inhibitor of CYP17 (227). We have previously shown that EPI-002 was effective against elevated androgen levels in in vitro conditions (239). To compare EPI-002 and abiraterone head-to-head and demonstrate the therapeutic advantage of EPI-002 over abiraterone in vivo, we would need to evaluate the effectiveness of EPI-002 in the relapsed and castration-resistant VCaP xenograft model described to possess resistance against CYP17 inhibition (227).

To elucidate the molecular mechanisms underlying resistance to EPI-002, future studies will include the analyses of the LNCaP-EPI tumours extracted from the in vivo studies that showed the LNCaP-EPI xenograft model was resistant to EPI-002 but sensitive to enzalutamide. AR from these resistant tumours will be sequenced and compared to the AR from parental LNCaP tumours, as this will reveal if any AR mutation occurred from chronic exposure to EPI-002. If an AR mutation is detected, functional assays such as AR-driven reporter assay will be employed to determine if the mutation confers resistance to EPI-002. From the tumour samples (LNCaP-EPI and parental LNCaP), the protein and mRNA expression levels of AR and several relevant AR coactivators will be examined by Western blots and QPCR, respectively. If a significant change of expression level is revealed for AR or coactivators, IHC will be performed to verify the change. The mRNA expression levels of AR-V7 and AR-V567es will be measured, even though our in vivo studies indicated that that the growth of these tumours were not dependent on these constitutively active AR splice variants with truncated LBD. To determine if EPI-002 was still inhibiting AR transcriptional activity in the LNCaP-EPI model, the mRNA expression levels of several AR-regulated genes will be measured in the cells and tumour samples, including PSA, FKBP5, and
Furthermore, Affymetrix™ microarray analysis will be performed with an unbiased and focused approach to investigate other possible molecular alterations associated with chronic EPI-002 exposure, including AR-independent changes in gene expression and signaling pathways that may be related to EPI-002 resistance in the LNCaP-EPI cells. For the microarray analysis, LNCaP-EPI, parental LNCaP, and age-matched LNCaP cells with long-term passage in the presence of vehicle control will be included in the study. If significant changes are observed, QPCR would be employed to validate the results.

5.3 RESEARCH SIGNIFICANCE

This dissertation describes studies that provided important insights into the research and development of prostate cancer drugs. The basis of all current AR-targeting therapies is hormonal therapy, which suppresses AR transcriptional activity through the LBD where androgen hormone binds to activate the receptor. Despite providing significant clinical benefits, all current AR-targeting therapies will eventually fail, thereby highlighting the importance of developing novel and more effective inhibitors of AR.

First we identified novel antiandrogens from spongian diterpenoids. T3, the most potent compound out of the diterpenoids we studied, demonstrated significant in vitro and in vivo inhibition of AR transcriptional activity, comparable with bicalutamide. Bicalutamide is a first-generation antiandrogen that once was the most prevalent AR inhibitor approved to treat advanced prostate cancer, and currently, bicalutamide is still commonly used to treat the disease. It is impressive that we showed T3 was as effective as bicalutamide, considering the fact that T3 did not undergo extensive lead compound optimization. With further preclinical development, it is possible to derive novel and potent antiandrogens from the diterpenoids.
Although the clinical activity of antiandrogens is temporary, the discovery of novel antiandrogens such as the diterpenoids is still valuable and has therapeutic potential, because it has been demonstrated that once an antiandrogen fails, switching to an alternative second-line antiandrogen can provide additional survival improvement for prostate cancer patients (279).

In our evaluation of AR NTD inhibitor EPI-002, EPI-002 was effective against various clinically relevant molecular mechanisms that have been shown to alter AR transcriptional activity. Some of these mechanisms cause aberrant AR transcriptional activity, which is a major driver of CRPC. Constitutively active AR splice variants with truncated LBD have emerged as a clinically significant mechanism underlying the development and progression of CRPC (102, 103, 105). Importantly, these AR splice variants are associated with therapeutic resistance to abiraterone and enzalutamide, which are the only AR-targeting drugs currently available to treat CRPC (162). Therefore, the ability for a future AR-targeting therapeutic agent to inhibit the AR splice variants is of particular importance in the treatment of CRPC. Our studies demonstrate the advantage of AR NTD inhibitors such as EPI-002 over antiandrogen that targets the AR LBD, as EPI-002 significantly blocked the transcriptional activities of both full-length AR and AR splice variants, whereas enzalutamide only worked against full-length AR. Furthermore, EPI-002 reduced the in vitro proliferation and in vivo tumour growth of a CRPC model that is driven by AR splice variants and resistant to enzalutamide. These findings indicate a unique and significant therapeutic niche for AR NTD inhibitors such as EPI-002 to treat CRPC patients whose tumours rely on AR splice variants for survival and growth. Taken together, our results
provide compelling evidence to support the further research and development of inhibitors of AR NTD to treat CRPC.

Finally, we generated the first human prostate cancer model that developed resistance to chronic AR suppression through the NTD. Studies of the resistance mechanisms underlying long-term AR inhibition through the LBD have made significant impact on the development of new-generation AR-targeting therapies. The second-generation antiandrogen enzalutamide was developed with the improvements to overcome AR overexpression and AR gain-of-function mutations that conferred resistance against the older antiandrogens such as bicalutamide and flutamide. However, the effectiveness of enzalutamide is limited by AR-related mechanisms of resistance including AR mutation and AR splice variants, both of which have been reported from basic research as well as clinical studies (88, 162, 229, 236). We have highlighted the ability of AR NTD inhibitors such as EPI-002 to effectively overcome these two mechanisms underlying resistance to enzalutamide. Here, the LNCaP-EPI cells that showed both in vitro and in vivo resistance to EPI-002 can be used as a valuable preclinical model to study the molecular resistance mechanisms related to the inhibition of AR NTD by EPI-002. Studies using the LNCaP-EPI model have the potential to make important contributions to the development of the next-generation AR-targeting therapies.


