

**EPI-002 and Enzalutamide Combination Therapy as a Potential Therapeutic  
Benefit for Castration-Resistant Prostate Cancer Patients**

by

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## **Abstract**

Development of castration-resistant prostate cancer (CRPC) is thought to be dependent on androgen receptor (AR) transcriptional activity. Resistance to current therapies is linked to constitutively active AR splice variants that lack the ligand-binding domain (LBD). Metastatic tumours heterogeneously express varying levels of AR splice variants to full-length AR (fl-AR). AR splice variant V7 is frequently expressed and correlated with poor prognosis in patients. Since current therapies such as enzalutamide target the fl-AR LBD, which is absent in AR variants, an amino-terminal domain (NTD) inhibitor that blocks transcriptional activities of all AR species may significantly improve survival of CRPC patients. EPI-002 binds to the AR NTD and inhibits transcriptional activity of AR. Hypotheses tested in this thesis include: (1) EPI-002 inhibits transcriptional activity of endogenous and exogenous V7 in human prostate cancer cell lines, and (2) combination therapy of EPI-002 and antiandrogen enzalutamide demonstrates greater inhibition of transcriptional activities of mixed AR populations than each treatment alone.

V7 transactivation was measured with EPI-002 treatment in AR-negative PC3 cells. Combined transcriptional activities with probasin-, PSA- and ARR3-luciferase AR-driven reporters were measured in androgen-sensitive LNCaP cells ectopically expressing V7, and androgen-independent LNCaP95 cells endogenously expressing V7. EPI-002 and enzalutamide monotherapies and in combination were evaluated with mixed AR populations in LNCaP and LNCaP95. Cell growth of LNCaP95 was measured with combination treatment.

EPI-002 inhibited constitutive transcriptional activity of V7. EPI-002 monotherapy and in combination with enzalutamide demonstrated greater inhibition of transcriptional activities of V7 expressed approximately 1:1 with fl-AR in LNCaP than enzalutamide monotherapy. With low

V7 levels compared to fl-AR, enzalutamide monotherapy and in combination with EPI-002 generally showed greater inhibition of AR transcriptional activities in LNCaP95 than EPI-002 monotherapy. It was observed that V7 activity demonstrated differential gene regulation of AR-driven reporters employed. Proliferation of LNCaP95 cells was inhibited by EPI-002 monotherapy and in combination with enzalutamide, with enzalutamide monotherapy having no effect.

Data shown here begins to reveal: 1) proof-of-principle that AR NTD inhibitor EPI-002 blocks all AR species in a heterogeneous tumour population; and 2) a potential therapeutic benefit of CRPC patients treated with EPI-002 in combination with enzalutamide.

## **Preface**

This thesis is the unpublished and independent work by the author, Erica Osbourne, and is prepared under the supervision of Dr. Marianne Sadar at the BC Cancer Agency Genome Sciences Centre. This original thesis is part of an investigation into the clinical efficacy of small molecule drug EPI-002 on the human androgen receptor and its alternatively spliced variants. EPI-002 was co-discovered by Dr. Marianne Sadar and Dr. Raymond Andersen. EPI-002 was prepared by members of Dr. Raymond Andersen's laboratory in the departments of Chemistry and Earth Ocean & Atmospheric Sciences at the University of British Columbia.

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## **Chapter 1: Introduction**

### ***1.1 Prostate cancer***

#### **1.1.1 Incidence & diagnosis of prostate cancer**

Prostate cancer is the most common cancer among Canadian men. With 23,600 men diagnosed in 2014, it is estimated that one in eight males will develop prostate cancer in his lifetime [1]. Risk factors for developing prostate cancer include: age, family history, genetics, race, infection or inflammation, diet, and lifestyle [2]. The incidence of prostate cancer also increases with aging [3]. It is most frequently diagnosed in Canadian males between 60-69 years of age, representing 40% of newly diagnosed cases of prostate cancer each year [1]. Though incidence is high among the population, progression of prostate cancer is relatively slow. Fewer men will die from prostate cancer than are diagnosed with the disease, at one in 28 Canadian men, and most are aged 80 years and older [1]. Fortunately, since the 1990's the mortality rate has been declining each year due to improved treatments for advanced disease and early detection [4].

According to Canadian guidelines, initial screening for prostate cancer is conducted by both a physical DRE (digital rectal exam) and PSA (prostate-specific antigen) blood test [5]. Each of these tests have different sensitivities and specificities, thus they are used in conjunction. DRE examines for asymmetry of the prostate and the presence of hard nodules that can indicate prostate cancer growth; however DRE alone can be unreliable and show findings consistent with benign prostatic hyperplasia (BPH) [5]. Positive prediction from DRE increases as blood PSA levels increase. This is why PSA blood tests are taken multiple times over a period of time and monitored for a significant increase [5, 6]. PSA is produced primarily by epithelial cells that line

the prostate ducts in the gland and is secreted in seminal fluid [7, 8]. Immunological studies have detected PSA in prostatic tissues (normal, BPH, cancerous), seminal fluid, and blood serum in humans [8]. It is a serine protease that cleaves gel proteins to liquefy seminal fluid, which results in the mobilization of spermatozoa flagellum [7, 9]. Normal levels of PSA in blood circulation have an upper limit of 4 ng/mL, though this upper limit for normal levels increases with age [6, 10]. When prostate cancer develops, elevated levels of PSA are released into the blood due to disruption of normal gland architecture, such as loss of basal cell layer and disordered polarity of proliferating epithelial cells [11, 12]. Thus, diagnostic PSA tests are used to detect the risk of prostate cancer development and also to monitor for response to treatment or for tumour progression. PSA is currently the most reliable serum marker for prostate cancer, though it has poor specificity [5]. Since PSA levels can fluctuate from other causes not cancer-related such as exercise, sex, pressure from DRE, medication or prostatitis, PSA blood tests are not currently recommended in Canada as a population-based screening test [6]. It is also not offered as a standardized test to men because of the risk of over-diagnosis of clinically indolent tumours, over-treatment of the disease, and morbidity from treatment [6]. Prostate cancer screening has many risks and benefits, thus the decision to take blood PSA tests for early detection should be individualized [5]. Initial screening should be offered to all men 50 years of age with at least a 10 year life expectancy, and men at a higher risk with family history or African descent should be offered screening at 40 years of age [5].

Once DRE and PSA testing detects that a man is at increased risk for having prostate cancer, a transrectal ultrasound-guided biopsy is conducted to obtain prostate samples for pathological assessment [5, 10]. The biological significance of a patient's prostate cancer is predicted based on tumour volume, PSA density, clinical stage, and grade (Gleason score) [5].

### **1.1.2 Development and treatment of castration-resistant prostate cancer**

Cancer is characterized by sustained proliferation, resistance of normal cell death, evasion of growth suppressors, replicative immortality, angiogenesis, and activation of metastasis all within a favourable tumour microenvironment [13]. Almost all cases of prostate cancer are adenocarcinomas. They are histologically characterized by uncontrolled proliferation of malignant secretory luminal epithelial cells with a lack of basal cells [14]. Rare cases of prostate cancer are of small cell neuroendocrine carcinomas and constitute less than 1% of all prostate cancer [14]. Adenocarcinoma cells express PSA and their proliferation is indirectly measured by an increase in PSA serum levels, whereas neuroendocrine carcinoma have negligible levels of PSA [6]. With early screening, adenocarcinoma of the prostate is primarily detected at the clinically localized stage of the disease and confined within the prostate capsule [15].

Treatment options for primary localized prostate cancer are watchful waiting (active surveillance), radical prostatectomy (surgical removal of the prostate), and radiation therapy (Figure 1.1). Men that have an early-stage, low risk cancer are advised to the watchful waiting strategy to reduce over-treatment of the disease. With this strategy patients receive close follow-up to monitor for clinical progression on a DRE and a rapid PSA doubling time [5]. Though watchful waiting has shown high 10 year overall survival, radical prostatectomy reduces the risk of metastases and prostate cancer-specific mortality [16]. Most men that receive radical prostatectomy are considered cured of prostate cancer. Unfortunately, 20-40% of these men will progress to a biochemical recurrent stage, which is indicated by a secondary rise of PSA and relapsed tumour burden approximately 2-10 years after primary therapy [17].

Recurrent tumour burden after localized therapy is androgen-dependent. This discovery was made by Canadian Charles Huggins in 1941 by treating patients with metastatic prostate cancer with surgical (orchiectomy) or chemical androgen deprivation therapy (ADT) [18]. Androgen is a hormone produced mainly in the testes that plays a critical role in the maintenance of prostate growth, which will be discussed further in the next section [19]. Chemical ADTs reduce serum levels of androgen and block androgen synthesis. Current Food and Drug Administration (FDA) approved ADTs that target the hypothalamic-pituitary-testicular androgen axis include: 1) gonadotropin-releasing hormone (GnRH) agonists and antagonists such as leuprolide and degarelix, respectively, which cause a negative feedback on GnRH and inhibit release of luteinizing hormone; 2) cytochrome P450 (CYP)17A1 enzyme inhibitors that block conversion of cholesterol precursor to androgen, such as ketoconazole and abiraterone acetate; and 3) 5 $\alpha$ -reductase inhibitors that block conversion of testosterone (T) into the more potent metabolite dihydrotestosterone (DHT), such as finasteride and dutasteride [20, 21]. Another class of therapies for prostate cancer are synthetic antiandrogens that do not decrease serum androgen levels. These compounds bind to the androgen receptor (AR) and competitively inhibit interactions with androgen [21]. Antiandrogens include steroidal compounds such as cyproterone acetate, and non-steroidal compounds such as flutamide, bicalutamide and enzalutamide [20, 21]. Androgen ablation plus antiandrogens provide effective maximal AR blockade to patients and PSA levels decline to castrate levels (<0.5 ng/mL). However, responses are transient lasting approximately 1 to 3 years and eventually all patients will progress to castration-resistance prostate cancer (CRPC) [11]. CRPC presents as a recurrent rise in PSA levels and progression of tumour growth despite low levels of androgen in serum.

CRPC is the lethal form of prostate cancer and most patients succumb to the disease within approximately 2 years [20]. CRPC manifests from mechanisms of resistance to ADT either by reactivation of the androgen signaling axis through its effector protein the AR, which will be discussed in detail later, or upregulation of other cellular pathways controlling growth [20]. The standard first-line therapy approved in 2004 to show prolonged survival in symptomatic patients is docetaxel chemotherapy combined with corticosteroids [20]. Symptomatic progression is indicative of prostate cancer metastases to distant parts of the body such as lymph nodes and bones. Since 2010 many drugs have been FDA approved after demonstrating increased survival post-docetaxel. Cabazitaxel, a taxane derivative microtubule inhibitor, was the first post-docetaxel therapy to be approved in 2010 with approximately 2 months increased survival [22]. Since then Sipuleucel-T immunotherapy vaccine was approved in 2010 for asymptomatic patients and in 2013 the first radiopharmaceutical radium-223 was approved for symptomatic patients with bone metastases [22]. Though there have been many advances in novel therapeutics for chemotherapy-refractory CRPC patients, abiraterone acetate combined with prednisone or enzalutamide have demonstrated to extend survival the most by approximately 5 months [23, 24]. Abiraterone acetate in chemotherapy-naïve patients has shown increased progression free survival in the COU-AA-302 study; however it did not reach overall survival statistical significance [25]. Nonetheless, it has been FDA approved in the pre-docetaxel setting and the PREVAIL trial for enzalutamide use in chemotherapy-naïve patients is ongoing.

### **1.1.3 Pivotal role of the androgen receptor in castration-resistant prostate cancer**

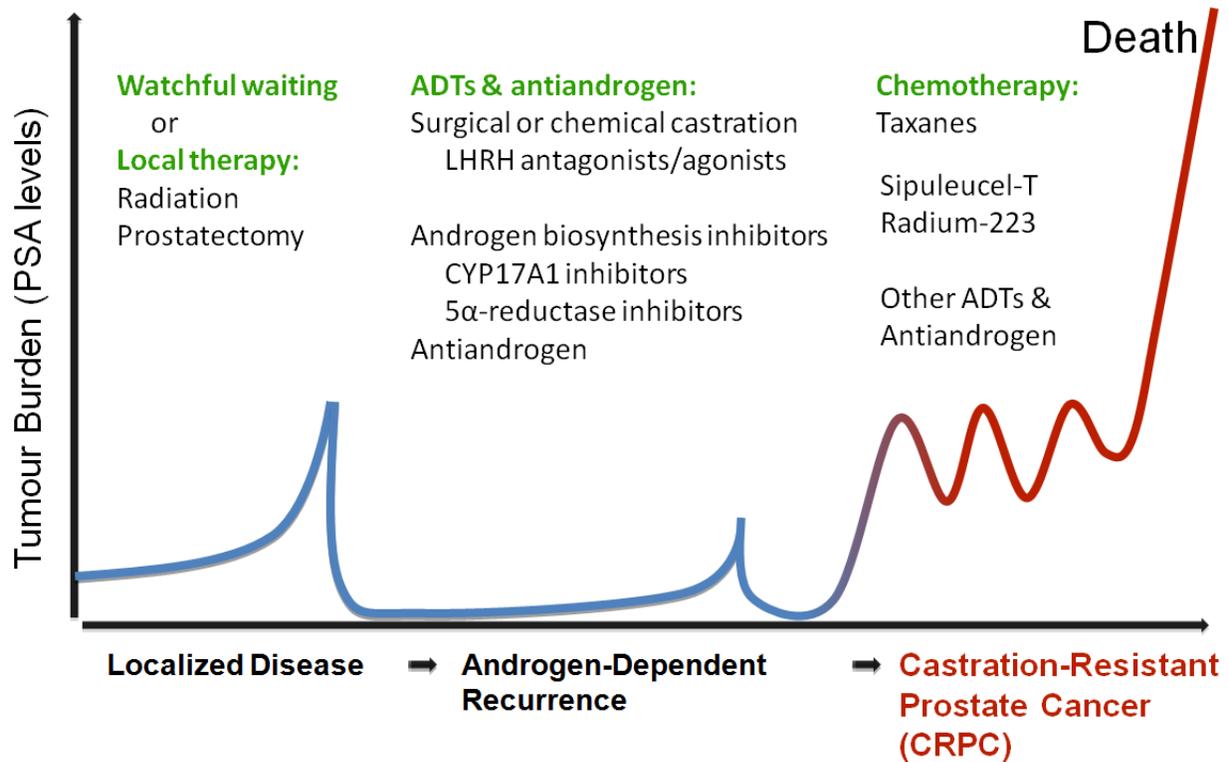
The normal development, growth and maintenance of the prostate is dependent on the activity of the AR [26]. The AR is a ligand-dependent transcription factor that is part of the nuclear steroid hormone receptor family [27]. Transcriptional activity of the AR drives growth and progression of prostate cancer by upregulating AR target genes for cellular proliferation and survival (discussed later in detail). Androgens, T or the more potent DHT, mediate AR signaling and cause a cellular response [28]. Synthesis and secretion of T is regulated by the hypothalamic-pituitary-testicular androgen axis [29]. This axis involves a negative-feedback loop to regulate levels of circulating T in the serum [29]. T and DHT are made from a cholesterol precursor that play a critical role in the development of the prostate gland, male phenotype embryogenesis, and maintenance of male physical characteristics [26, 30]. T is predominantly produced by Leydig cells in the testes and binds to plasma proteins in the blood for transport throughout the body [31]. Approximately 5% of circulating T in the blood is produced from the adrenal glands and can contribute to AR activation [32]. Free steroid reaches the AR by passively diffusing across the plasma cellular membrane, where it is converted from T to DHT by  $5\alpha$ -reductase in the cytoplasm [33]. The  $5\alpha$ -reductase enzyme is differentially expressed in various organs of the body with high expression in testes, prostate, seminal vesicles and bone marrow [34]. The interaction of T or DHT with AR stimulates its activity and initiates downstream cellular effects (discussed further later). Under normal conditions AR activity is controlled and maintains growth and differentiation of the prostate; however in prostate cancer AR is hyperstimulated and signaling becomes aberrant [35].

Clinical monitoring of serum PSA levels has demonstrated that the androgen-AR signaling axis plays an important role in prostate cancer. Transcription to direct synthesis of PSA is positively regulated by ligand-activated AR bound to androgen-response elements (ARE) located in the promoter and enhancer regions of the *PSA/KLK3* gene [36, 37]. Co-operative binding of multiple ARs to ARE regions of the *PSA/KLK3* gene accounts for its strong androgen-dependent activity [38]. Rising PSA levels are concomitant with increased tumour burden at initial diagnosis and also with recurrent metastatic disease. Blockade of AR activity with ADT in patients results in a decrease of serum PSA levels and improved symptoms. This indicates AR as a key player in the progression of prostate cancer. Consequently, resistance to ADT results in a recurrent rise in PSA levels indicative of AR reactivation and restored transcription of the *PSA/KLK3* gene. Furthermore, adenocarcinoma luminal cells express AR and their growth is inhibited by ADT, whereas ADT is ineffective at blocking growth of neuroendocrine cells that do not express AR. This provides proof of principle that AR drives prostate cancer progression.

Until recently, CRPC was regarded as an androgen-independent disease [39]. Patients responding to ADT treatment post-castration exemplify that the AR remains active and androgen-sensitive in the progression from hormone-dependent to castration-resistant disease. Abiraterone acetate and enzalutamide effectively decrease recurrent PSA levels in most patients with CRPC. This evidence suggests that further blockade of the AR and its signaling pathway remains to be a relevant therapeutic target to treat CRPC.

#### 1.1.4 Androgen receptor mechanisms of resistance to androgen deprivation therapies

There are several proposed mechanisms of resistance the AR adapts in order to sustain transcriptional activity and develop into castrate-resistant tumours [35, 40]. Deregulation of the AR is the most common mechanism of adaptation found in patients, either through overexpression of the AR protein or amplification of the *AR* gene, which makes AR hypersensitive to androgen levels [41, 42]. Gain-of-function somatic mutations in the *AR* gene to produce a promiscuous receptor that binds to other steroids (e.g. progesterone) can reactivate AR [43]. Also, increased expression of (CYP)17A1 in CRPC tumours can use cholesterol precursors for *de novo* intratumoural steroid synthesis to reactivate AR [44]. These mechanisms mentioned so far target ligand-dependent activation of AR, but alterations in cofactors can increase transcription of AR whether enhancing ligand-dependent activity or inducing ligand-independent activity of AR [35]. An increase in co-activator and a decrease in co-repressor proteins have shown to both mediate AR reactivation [45-47]. Furthermore, aberrant post-translational modifications of the AR including phosphorylation, acetylation, and SUMOylation can induce transactivation of AR target genes in the absence of ligand [48]. Lastly, alternatively spliced AR variants have been identified that are truncated and lack a binding pocket for androgen and antiandrogens [49, 50]. These AR splice variants are constitutively active and currently no therapies in the clinic can inhibit their activity [51]. The AR splice variants present a significant clinical challenge and will be discussed in detail in Section 1.4.



**Figure 1.1. Growth and progression of prostate cancer to CRPC.**

Adapted from Figure 1 from © Heidenreich, A et al. (2013). Rise and fall of serum prostate-specific antigen (PSA) is concomitant with tumour burden in men. PSA rises at the onset of prostate cancer at time of initial diagnosis. First-line radiation or surgical localized therapy cures most men; however recurrent increase in PSA indicates regrowth of cancer. At the androgen-dependent disease state, androgen deprivation therapy (ADT) reduces androgen produced by the testes and adrenals. Eventually the cancer will progress to a castration-resistant phenotype, resulting in biochemical failure. At this stage treatments are given to the patients to reduce pain and increase survival, as PSA continues to rise prior to an eventual death. *European Urology*. 64(1): Page 260-265. By permission from Elsevier Ltd.

## ***1.2 Androgen receptor***

### **1.2.1 Structure & function of the androgen receptor**

AR regulates transcription and belongs to the steroid receptor family [27]. This class of receptors includes glucocorticoid (GR), progesterone (PR), and mineralocorticoid receptors (MR), which all bear structural homology to AR [27]. The human *AR* gene is a single-copy X chromosome gene located at q11-12 and spans more than 90 kilobases (kb) of DNA [52]. The complementary DNA (cDNA) sequence encodes a protein approximately 919 amino acids (aa) long [53]. Its predicted molecular mass is 98,845 Daltons (Da); however when the AR protein is run on an acrylamide gel it can be detected as a doublet at 110- to 112-kDa due to phosphorylation [52, 54]. The AR protein-coding region comprises 8 exons, which encode four main functional domains of the AR (Figure 1.2) [51, 55]. The AR domains include: a variable amino(NH<sub>2</sub>)-terminal domain (NTD) that modulates transcriptional activity, a highly conserved DNA-binding domain (DBD) that binds to AREs, a flexible hinge region (HR), and a carboxyl(COOH)-terminal ligand binding domain (LBD) that contains a ligand-binding pocket [51, 55, 56]. A nuclear localization signal (NLS) overlaps the DBD and HR of the AR [56]. The variable NTD accounts for the discrepancy between nucleotide and amino acid numeration of AR in the literature. Amino acid sequence numbers vary due to different numbers of glycine (G) and glutamine (Q) repeats in the poly-G and poly-Q sequences in the NTD. Thus, reported aa length of AR protein can range, with 910 and 919 aa lengths being the most commonly published [51, 55]. The 919 aa AR is composed of 24(G) and 21(Q) repeats [57]. The AR protein is modular with exon 1 encoding the full NTD, exons 2 & 3 encoding the DBD, and exons 4-8 encoding the LBD [19]. The AR DBD is approximately 70 aa long and is highly conserved, with

83% and 80% sequence homology to PR and GR, respectively [58]. The second most conserved region of the AR is the LBD. This domain showed 53% to 50% sequence homology with the LBD of PR and GR, respectively [58]. Lastly, the NTD and HR are highly variable in size and aa composition compared to related receptors. The NTD shares less than 15% sequence homology with PR and GR [27]. Each AR domain structure and function will be discussed in detail in subsequent sections.

Immunohistochemical localization and expression of AR was conducted with various human tissue samples [59]. High expression was confirmed in genital tissues including the testes, prostate, seminal vesicles, and bone marrow, which are important organs for male sexual differentiation [59]. AR expression was also present in non-genital tissues such as the skin, salivary glands, sweat glands, hair follicles, cardiac muscle, vascular and gastrointestinal smooth muscle, thyroid follicular cells, adrenal cortical cells, and regions in the brain [59]. Hereditary disorders such as androgen insensitivity syndrome (AIS) have demonstrated the importance of AR function for development and maintenance of the male reproductive system [30]. Individuals with AIS have normal serum androgen levels but the AR is mildly to fully defective in mediating the hormone signal [19]. AIS can be partial or complete and range from mild virilization defects to complete male-to-female phenotypic sex reversal [30].

Loss in AR signaling can also result in Kennedy's disease. It is a severe neurodegenerative disease resulting in the loss of motor neurons in the muscles of male adults [19]. The exact cause of this disease is not fully understood; however the expression of transcriptionally weak AR may contribute [30]. The disease is characterized by the formation of AR protein aggregates in the muscle cells of the spine and bulbar [30]. Decreased AR

transcriptional activity is due an extended poly-Q tract in the NTD [30]. Normal healthy individuals have a repeat of 9-36(Q), with an average of 21, whereas men with an expansion over 40(Q) repeats develop neurotoxicity [30].

### 1.2.2 Transcriptional target genes

After translation of the AR protein, it quickly becomes phosphorylated in the cytoplasm and associates with heat shock proteins (Hsps) into a heteromeric complex with Hsp90, Hsp70 and Hsp56 [60]. Hsps regulate not only protein folding and stability of AR but also bind and cover the NLS to prevent protein import into the nucleus, in the absence of androgen [60]. The canonical pathway of AR activity, also called genomic activation, begins with high affinity binding of the LBD with DHT (Figure 1.3) [58, 61]. This induces a conformational change in AR structure to dissociate from the Hsps, expose the NLS, and stimulate cytoplasmic shuttling to the nuclear membrane [60]. NTD to LBD interactions within one AR molecule have shown to inhibit transcriptional activity; however NTD to LBD interactions between two AR molecules increases transactivation of AR target genes [62]. Thus, the AR forms a homodimer via antiparallel amino- to carboxy- (N/C) interaction [62]. The AR homodimer enters the nucleus where it binds to AREs in the promoter and enhancer regions of target genes [63]. AREs contain a core recognition sequence of 5'-TGTTCT-3' and AR binds to direct repeats of this sequence with a three nucleotide spacer [63]. AREs are recognized exclusively by AR, but hormone response elements (HREs) are also present that contain indirect repeats of the core recognition sequence that are inclusive to AR, GR, and PR [63]. While the DNA sequences play an important role to where AR binding occurs in the genome, transcriptional activity of the AR NTD is also dependent on the recruitment of gene-specific co-activators or co-repressors [63]. The expression of these co-regulatory proteins is cell- and tissue-specific.

The first androgen-regulated gene to be characterized and constructed into an AR-driven reporter plasmid was the probasin *PB* gene from the rat prostate [64]. Probasin is a ligand-carrier

protein expressed in the epithelial cells of the prostate [64]. Some examples of human androgen-regulated genes in the prostate are: *PSA/KLK3* which encodes for kallikrein-3 or commonly known as PSA, *TMPRSS2* a transmembrane serine protease, *FKBP5* an immunophilin protein that binds with AR-Hsp complexes, and *CREB3L4* for CREB (cAMP responsive binding protein) [65, 66]. Canonical genes regulated by androgen-liganded AR are mostly related to prostate differentiation, biosynthesis, metabolism and secretion, which are all essential for maintenance and growth of the prostate [66].

AR-specific genes can also be regulated via ligand-independent pathways, in the absence of androgen [67]. AR can be activated by: 1) cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8); 2) signaling with intracellular kinases such as mitogen-activated protein kinase (MAPK), cAMP-dependent protein kinase A (PKA), and protein kinase B also known as Akt; 3) growth factors such as insulin-like growth factor (IGF-1) and epidermal growth factor (EGF); and 4) alternatively spliced AR variants [67].

### 1.2.3 Ligand-binding domain

Segment deletions of the AR have shown that the full LBD is essential for binding to DHT [51]. Subcellular localization of the AR is also dependent on the presence of the carboxy-terminal end of the LBD, which interacts with Hsps to cover the NLS [51, 60]. Deletion of the full LBD forms a constitutively active AR mutant (1-653 aa) that is predominantly nuclear [51]. The crystal structures of AR LBD and DBD have been resolved and show that 12  $\alpha$ -helices and a small  $\beta$ -sheet comprise the LBD [61]. The  $\alpha$ -helices are arranged into three layers forming a sandwich-like structure with a ligand-binding pocket in the interior [61]. Upon androgens interacting with the hydrophobic pocket of the LBD,  $\alpha$ -helix 12 structurally forms a lid over the pocket to stabilize the ligand and also provides a platform along with  $\alpha$ -helices 3, 4, and 5 for protein-protein interactions with co-regulatory proteins [68]. This platform exposed has an activation function region (AF2) containing an LxxLL aa motif [68]. AF2 recruits co-activators with FxxLF aa motifs to interact with the LxxLL aa motif [68]. The AF2 is critical for protein-protein interactions with the ligand-binding, amino-to-carboxy (N/C) interaction, and stability of the AR homodimer [69]. Functional analysis studies have demonstrated the AF2 shows minor transcriptional activity in the absence of the AR NTD [69]. Thus, AR transcriptional activation does not require AF2.

#### 1.2.4 DNA-binding domain and hinge region

The DBD is composed of two zinc (Zn) finger-like modules each encoded by exon 2 and 3, organized into three  $\alpha$ -helices [58, 63]. Each Zn-finger contains one zinc ion liganded by two cysteine and two histidine residues [27]. The first  $\alpha$ -helix contains the P-box, responsible for recognition of HRE half-site sequence specificity, and is inserted into the major groove of DNA [63]. The other two  $\alpha$ -helices are for DNA specificity of the steroid receptor binding [63]. The AR, GR and PR have the same P-box aa motif, which indicates the P-box locates the HREs in the genome [63]. The second Zn-finger mediates protein-protein interactions with the second AR molecule for homodimerization [63]. Once the first AR molecule has bound to the half-site HRE sequence 5'-TGTTCT-3' with high affinity, this enables co-operative binding of the second AR dimer to either the inverted (less AR specificity; GR recognizable) or direct (high AR specificity; no GR binding) repeat [63]. Two AR proteins bind per one ARE sequence, and AREs can be found upstream or downstream from the transcription start site of a gene [70].

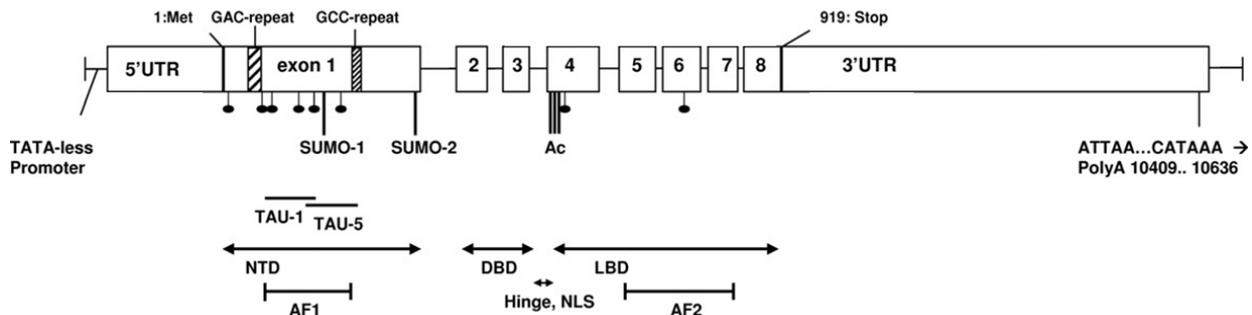
The HR is a flexible linker between the DBD and LBD that is encoded by exon 4 [52, 71]. The HR contains the NLS that also spans into the carboxy-terminal end of the DBD [52]. The bipartite NLS interacts with  $\alpha$ - and  $\beta$ -importin proteins to translocate through the nuclear core complex into the nucleus [71].

### 1.2.5 Amino-terminal domain

The AR NTD (1-558 aa) is encoded by one, large exon and is the transcriptional engine of AR [51, 72]. Almost the entire NTD is necessary for full transcriptional activity when compared to ligand-bound wild-type AR [55, 73]. Deletion of large parts of this domain completely inactivates AR activity [51]. An activation function region (AF1) located in the NTD harbours AR transcriptional activity [51, 55, 73]. Two transcription activation units (TAUs) exist within the AF1 (142 to 485 aa) that are crucial for AR-dependent gene regulation, TAU1 (101 to 370 aa) and its overlapping region TAU5 (360 to 528 aa) [73]. Interestingly, each TAU transcriptional activity depends on whether an agonist occupies the LBD or not. With the LBD present and agonist bound, the activity of the full-length AR is mediated 50% by TAU1 [73]. However, when the LBD is truncated or in the absence of agonist binding, the constitutive transcriptional activity is mediated by TAU5 and shifts away from TAU1 [73]. Core sequence motifs <sup>178</sup>LKDIL<sup>182</sup> in TAU1 and <sup>435</sup>WHTLF<sup>439</sup> in TAU5 are thought to be responsible for ligand-dependent and ligand-independent activation of the AR, respectively [69, 74]. Transcriptional activity of the AR can also be affected by N/C interaction with the FxxLF motif in the NTD, which interacts with the LxxLL motif in the AR AF2 [75].

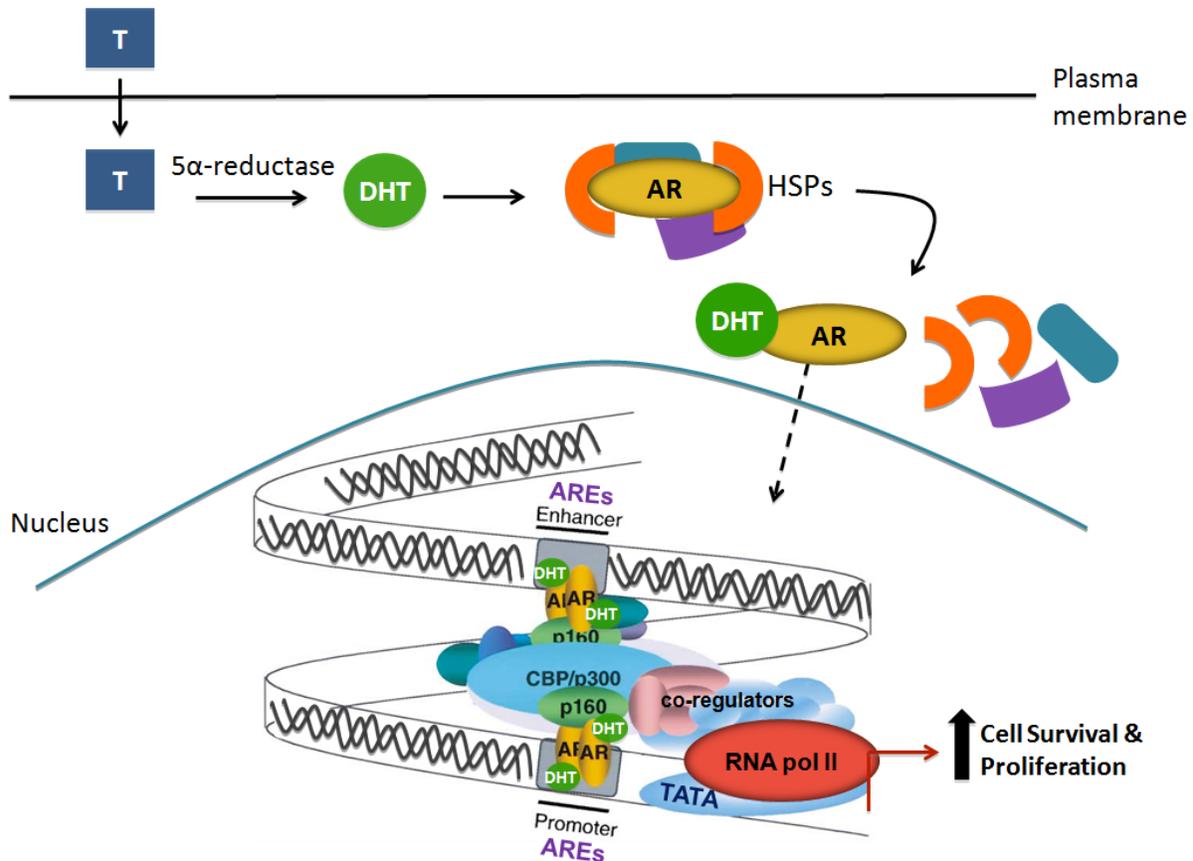
The AF1 is important for forming essential protein-protein interactions with the p160 family steroid receptor co-activators 1-3 (SRC1-3), AR trapped clone 27 (ART27), CBP/p300, and the large subunit RAP74 of the transcription factor IIF subunit 1 (TFIIF) to initiate transactivation of target genes [46, 76-78]. These co-ordinated and ordered interactions facilitate recruitment of RNA polymerase II holoenzyme to form the AR transcription complex at the promoter [70]. Binding of co-regulatory proteins to the AR AF1 induces structure within the

NTD that is favourable for interaction with RNA polymerase II and production of messengerRNA (mRNA). This intrinsically disordered structure of the AR NTD will be described in Section 1.5. Lastly, the conformation of AR NTD appears to allosterically affect DNA-binding affinity of the DBD for AREs in target genes, indicating inter-domain communication within the AR [79].



**Figure 1.2. Schematic representation of AR gene and protein.**

Adapted from Figure 1 from © Waltering, KK et al. (2012). Structure of AR gene comprises 8 exons which encode 4 main functional domains. UTR = untranslated region. AR protein contains an amino-terminal domain (NTD) that has an activation function 1 (AF1) region, a DNA-binding domain (DBD), a flexible hinge region, and a carboxy-terminal ligand-binding domain (LBD) that has an activation function 2 (AF2) region. The AF1 has two transactivation units (TAU1&5) with important amino acid motifs for activity. Poly-glutamine (CAG) tract and poly-glycine (GCC) tract are localized in or near the AF1 region. The nuclear localization signal (NLS) spans the hinge and part of DBD. Locations of post-translational modifications of the synthesized AR protein are indicated: phosphorylation sites marked with pinheads, SUMOylation sites as SUMO-1 & 2 and acetylation sites marked Ac. *Molecular and Cellular Endocrinology*. 360(1): Page 38-43. By permission from Elsevier Ireland Ltd.



**Figure 1.3. Canonical pathway of AR signal transduction.**

Adapted from Figure 7 from © Shang, Y et al. (2002). Testosterone (T) enters the plasma membrane and is converted into dihydrotestosterone (DHT) by 5α-reductase. Binding of DHT to AR induces a conformational change and dissociates from heat shock proteins (Hsps). AR is then able to translocate into the nucleus, homodimerize with another AR species, and bind to target genes via AREs in enhancer and promoter regions. AR regulates gene expression by coordinated and ordered recruiting of co-regulatory proteins (CBP/p300, SRC1-3, and others) to the AR NTD, and general transcriptional machinery to the TATA box and transcription start site. *Molecular Cell*. 9(3): Page 601-610. By permission from Elsevier Ltd.

## ***1.3 Antiandrogens in castration-resistant prostate cancer***

### **1.3.1 Enzalutamide mechanism of action**

Enzalutamide, previously known as MDV3100, is a second-generation antiandrogen that was developed to target CRPC associated with AR overexpression (Figure 1.4) [41, 42, 80, 81]. AR gene amplification or increased protein expression of AR were sufficient for AR resistance mechanisms against ADT in mouse xenografts [82, 83]. First-generation antiandrogen bicalutamide demonstrated agonism of AR, therefore novel antiandrogens were developed to target AR overexpression [82, 83]. Competition binding curves with radiolabeled  $16\beta[^{18}\text{F}]$ fluoro- $5\alpha$ -DHT (18-FDHT) in LNCaP cells overexpressing wild-type AR showed enzalutamide to have a half maximal inhibitory concentration ( $\text{IC}_{50}$ ) of  $21.4\pm 4.4$  nM compared to 18-FDHT natural ligand at  $11.5\pm 2.0$  nM and bicalutamide at  $160\pm 29$  nM [81], which is the concentration needed to effectively reduce AR biological activity by one half.

Enzalutamide is a potent inhibitor of *PSA/KLK3* gene expression and AR-driven reporter gene activity in AR overexpressing LNCaP cells [81]. Furthermore, enzalutamide inhibited *in vitro* growth of VCaP prostate cancer cells that have endogenous AR gene amplification [81]. This growth suppression was reversed by co-treatment with R1881, a synthetic androgen, demonstrating that enzalutamide is a reversible, competitive inhibitor of the AR LBD [81]. Unlike previous antiandrogens, enzalutamide blocks AR activity at multiple steps in the signaling pathway. Its mechanism of action is to: 1) inhibit androgen from binding to AR LBD with higher affinity than bicalutamide; 2) prevent binding of AR complex to DNA; 3) impede recruitment of co-activators to the LxxLL motif in the AR AF2 region; and 4) reduce the

efficiency of AR complex nuclear translocation [81]. However, in the absence of androgen enzalutamide induces nuclear translocation of AR [84, 85]. Enzalutamide most importantly induced regression of AR overexpressing LNCaP xenograft tumours in castrated male mice, whereas bicalutamide only retarded growth [81]. Overall data supports that enzalutamide is a potent reversible inhibitor to the AR LBD, does not show agonistic activity, and is substantially superior to its predecessor first-generation antiandrogens.

### **1.3.2 Enzalutamide treatment in castration-resistant prostate cancer patients**

In the phase 1-2 study that assessed safety and efficacy of enzalutamide 140 patients with CRPC were given oral doses from 30 mg to 600 mg daily [86]. The majority of men had metastatic disease (78%) and at least two previous lines of hormonal therapy (75%), and around half had previously received chemotherapy [86]. The terminal half-life in patients, which is the time taken for the drug to reach half concentration in the blood plasma after equilibrium, was an average of 1 week [86]. Antitumour activity was observed at all tested doses, and time to PSA progression was greater in the chemotherapy-naïve compared to chemotherapy-pretreated group (41 weeks and 20 weeks, respectively) [86]. However, the maximum tolerated dose was 240 mg daily with main side effects of headache, hot flashes and fatigue [86]. Three patients developed seizures at higher doses, which was concerning so the dosage was reduced to 160 mg daily for phase 3 trials [86].

The phase 3 AFFIRM international, double-blind, placebo-controlled trial commenced in September 2009 [24]. A total of 1,200 men with metastatic CRPC post-chemotherapy received either 160 mg once daily of enzalutamide or placebo [24]. Overall survival was significantly improved by approximately 5 months and all secondary endpoints (time to PSA progression, quality of life, pain palliation, time to first skeletal-related event) were improved with enzalutamide compared to placebo [24]. Unfortunately, even with reduced dosage of enzalutamide compared to phase 2 trials five patients experienced seizures (0.9% of patients) [24]. It was postulated that risk factors, such as pre-existing brain metastases, co-treatment with lidocaine and brain atrophy due to alcoholism, reduced the threshold of seizures in these patients [24]. Even so, animal studies suggest that enzalutamide may have off-target effects and inhibit  $\gamma$ -

aminobutyric acid (GABA)-gated chloride ion channels, therefore patients with increased risk of seizures cannot take enzalutamide [87-89]. Enzalutamide was the first therapy to demonstrate improved overall survival and had a favourable risk-benefit profile. Thus, August of 2012 the FDA approved its use in the post-chemotherapy setting of metastatic CRPC [90].

A number of clinical trials are currently recruiting and ongoing to evaluate enzalutamide in a range of patient populations and in the pre-chemotherapy setting [87]. The most anticipated results are from the PREVAIL phase 3 trial comparing enzalutamide to placebo in chemotherapy-naïve metastatic CRPC patients, which has been completed though findings unpublished as of yet [87]. Retrospective studies that evaluate optimal sequential treatment of abiraterone acetate and enzalutamide in metastatic CRPC patients have been investigated. In two studies that treated patients with abiraterone acetate post-docetaxel and post-enzalutamide failure, time to progression (serum PSA incline or symptomatic) was minimally improved at 2.7 months with modest antitumour activity for a small subset of patients [91, 92]. Thus, enzalutamide monotherapy provided better progression-free survival than subsequent abiraterone acetate treatment [91, 92]. It is postulated that this cross-resistance could be due to promiscuous AR activity by an enzalutamide-activated AR mutant [93]. Similar to the abiraterone studies, preliminary data suggested that enzalutamide provided limited benefit for the post-docetaxel and post-abiraterone acetate patient population [94-96]. The European Association of Urology was the first to publish its study with thirty-five men treated with subsequent enzalutamide after failure of docetaxel and abiraterone [94]. Of the 16 men that were initially abiraterone-sensitive, seven patients had a subsequent  $\geq 50\%$  decline in PSA with enzalutamide [94]. A median of 4.9 months increased survival in a small subset of patients suggests that sequential therapy of enzalutamide provides modest clinical improvement [94]. Cross-resistance of promiscuous

progesterone-responsive AR mutants could potentially contribute to minimal activity [44]. Combination therapy of enzalutamide with abiraterone acetate is currently being assessed in a phase 2 trial in bone metastatic CRPC patients. However, there are concerns of men developing recurrent disease faster than receiving either monotherapies since both of these therapies target the AR LBD [87]. Enzalutamide in combination or sequential treatment with novel drugs targeted towards other steps in the AR signaling pathway, such as the AR NTD, might provide antitumour benefit for CRPC patients. Though as of yet this is unknown.

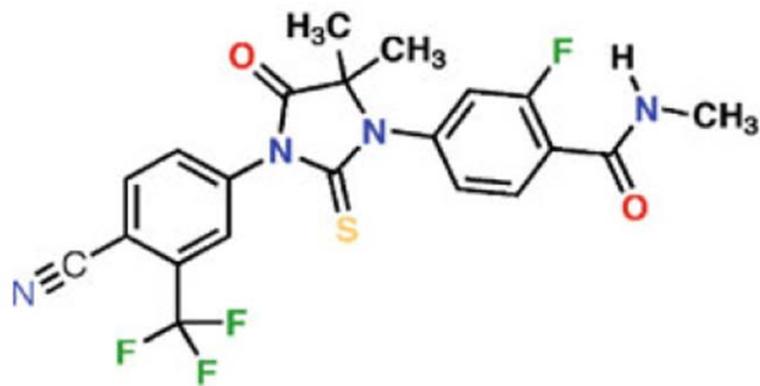
### 1.3.3 Mechanisms of resistance against enzalutamide

Enzalutamide was designed to target AR overexpression and to have higher affinity to the AR LBD than bicalutamide [81]. Despite the successful response and PSA decline in patients, CRPC tumour cells continued to grow and the AR regained its ability to signal and promote cancer cell survival in low androgen environments. One AR mechanism of resistance against enzalutamide treatment is an adaptive shift towards alternative splicing of AR pre-mRNA to form truncated variants that lack a LBD [97]. In one study, growth and transcriptional activity of an AR reporter gene was measured in the *22Rv1* androgen-independent prostate cancer cell line with treatment of enzalutamide or bicalutamide [97]. *22Rv1* endogenously expresses the full-length AR and alternatively spliced truncated AR variants that lack the full LBD [97]. Antiandrogen treatment inhibited androgen-induced AR transcriptional activity, which indicates on-target inhibition of full-length AR, though antiandrogen treatment did not inhibit growth of these cells [97]. Upon selective knockdown of the AR variants via transient transfections of short interfering RNA strands (siRNA), antiandrogen treatment completely inhibited constitutive AR reporter activity and reduced androgen-independent growth. On the basis of these findings, knockdown of truncated AR variants restores efficacy of antiandrogen drug therapy and presence of AR variants lacking the LBD are sufficient to reactivate the AR signaling pathway [97].

Another mechanism of resistance to enzalutamide is the emergence of a missense mutation from phenylalanine to leucine at position 876 (F876L) in the ligand binding pocket of the AR [98, 99]. One group developed LNCaP cell lines that were long term cultured for 90 days in 1  $\mu$ M enzalutamide and growth-resistant clones were selected and expanded for further analysis [99]. Ectopic expression of the discovered F876L AR mutant into androgen-sensitive

LNCaP cells displayed agonistic activity with treatment of enzalutamide [93]. The F876L AR mutant has been found in patients and *in vivo* xenografts post-treatment with enzalutamide, thus it is suggested that this missense mutation in the AR LBD is sufficient to confer resistance to enzalutamide and drive CRPC growth [93, 98].

Lastly, upregulation of the glucocorticoid receptor (GR) has been proposed to be a mechanism of resistance to enzalutamide [100]. Charles Sawyer's group showed that in a small subset of poor responding patients to enzalutamide AR target genes were reactivated by GR overexpression [100]. However, GR activity on AR target genes has been established for years [101]. AR DNA-binding domain is 77% identical to that of the GR and most AREs bind with high affinity to both GR and AR [63, 101]. Furthermore, patients regularly take glucocorticoids (such as prednisone) to slow disease progression, improve pain control, and offset side effects of chemotherapy [102]. This has shown to improve symptoms, not cause dramatic decrease in functional status [102]. Thus, further studies need to be conducted to support GR activity as an AR resistance mechanism. As of yet treatment with corticosteroids and GR overexpression is not sufficient to drive cancer and mediate progression of tumours post-enzalutamide therapy [102]. Overall these mechanisms of resistance to enzalutamide should be taken into consideration upon selecting drugs for combination therapy. Ideally combination therapies with enzalutamide should strategically target multiple steps of the AR signaling pathway and anticipate production of AR splice variants and mutations in the AR LBD as resistance to ADT.



Enzalutamide

**Figure 1.4. Structure of enzalutamide.**

Adapted from Figure 1B from © Sadar, MD (2012). Chemical structures of AR LBD-targeted antiandrogen for treating CPRC. Enzalutamide (MDV3100) thiohydantoin derivative reversibly binds to AR LBD and competitively inhibits androgen from binding to ligand-binding pocket. *The World Journal of Urology*. 30(3): Page 311-318. By permission from Springer Basel AG.

## ***1.4 Alternatively spliced androgen receptor variants***

### **1.4.1 Clinical relevance of alternatively spliced androgen receptor variants**

Alternatively spliced AR variants were first discovered in the androgen-independent cell line, 22Rv1, derived from a CWR22 prostate cancer xenograft relapsed after androgen ablation [103]. Two different AR isoforms with truncated LBD were found to be constitutively active, promote expression of AR-dependent target genes, and mediate proliferation of cells in androgen deprived conditions [103]. Soon thereafter other groups identified multiple functional AR isoforms in hormone-refractory prostate cancer patient specimens that retained the AR NTD and DBD but lacked the LBD [49, 50]. These truncated AR isoforms were expressed at higher levels in CRPC metastatic tissue, compared to hormone-naïve and benign prostate tissues, indicating that their upregulation promoted progression of CRPC [49, 50, 104].

AR variants are encoded by novel cryptic exons derived from intron segments flanking the canonical exon regions of the *AR* gene [49, 50, 103]. Since the novel exons are present at the mRNA level, it is believed AR variants are produced by alternative splicing of the AR pre-mRNA (Figure 1.5) [103]. Other groups have found that LBD-truncated AR variants can also originate from alterations at the genomic level, by either somatic nonsense mutations within the *AR* gene to produce a stop codon or through gene rearrangements to delete exons in the coding region that encode LBD [105, 106]. Translation of alternatively spliced mRNA yields AR variant protein products that via Western blot analysis migrate at a lower molecular weight than the 110 kDa full-length AR (fl-AR) protein [49, 50, 107]. LBD-truncated AR variants are predominately nuclear and their transcriptional activity is not regulated by androgens or antiandrogens [105].

Since the year 2008 a large number of AR splice variants have been identified in CRPC patient tumour samples, though AR V7 (also called AR3) and AR V567es are the most clinically relevant [49, 107, 108]. Hörnberg et al. examined bone metastases that were obtained from 40 patients that were hormone-naïve or castration-resistant. In the CRPC bone metastases, V7 and V567es were the most frequently and abundantly expressed AR variants. V7 and V567es mRNA transcripts were detected in 100% and 23%, respectively, of the 30 CRPC bone metastases samples [108]. In 10 hormone-naïve bone metastases samples V7 and V567es mRNA levels were detected at 80% and 0%, respectively. Intriguingly, there is a discrepancy between the abundance of variant protein compared to its mRNA output [108]. Despite relatively low levels of AR variant mRNA (0.4-1.0% of fl-AR mRNA), AR variant protein expression constituted a range from 0% to a maximum of 95% of fl-AR protein expression [108]. Bone metastases with high V7 mRNA were found to express AR variants at approximately 1:1 with fl-AR on an immunoblot. Patients with high mRNA expression of AR V7 had decreased survival and poorer prognosis than other CRPC patients with low AR variant mRNA levels [108]. Similarly, patients with detectable mRNA levels of AR V567es or detection of both V7 and V567es transcripts (AR-V high) had decreased survival than CRPC patients with low AR variant levels. Remarkably, 33% of the 30 castration-resistant patients expressed AR variant protein levels comparable to fl-AR (i.e. 1:1 levels in the AR-V high bone samples) [108]. A separate study measured AR variant and fl-AR transcripts in prostate, lymph node, lung, liver and bone specimens for frequency of expression [107]. Over 50% of the tissues expressed one or more AR splice variants in the presence of fl-AR [107]. This indicates AR variants are frequently expressed concomitantly with fl-AR, and AR variant levels relative to fl-AR are expressed as a mixed population in metastases. Overall, many studies demonstrated expression of AR variants

with fl-AR in tumour samples and xenografts is heterogeneous [66, 104, 107, 108]. However, one xenograft called LuCaP 86.2 derived from a human primary tumour predominantly expresses AR splice variants with little fl-AR protein [107]. AR V7 and V567es transcripts have also been identified in androgen-independent cell lines including 22Rv1, VCaP, LNCaP95 and even at low levels in androgen-sensitive cell lines such as LNCaP [50, 103, 104, 107]. Recently, AR splice variants transcripts have been detected in breast cancer cell lines and normal human tissue samples [109].

AR splice variants are an important mechanism of resistance to study because they are upregulated in xenografts and cell lines after abiraterone acetate and enzalutamide treatment [66, 97, 110]. *In vitro* studies showed that LNCaP95 cells treated with enzalutamide or grown in androgen depleted conditions increased the expression of endogenous AR splice variants [66]. Conversely, in the presence of synthetic androgens expression of constitutively active AR variants decreased [66]. This supports that an important mechanism of drug resistance to ADT is an adaptive shift toward AR variant-mediated signaling when the fl-AR LBD is inactive. Furthermore, V567es and V7 AR splice variants display differential sensitivity to taxane treatment (i.e. microtubule inhibitor) in CRPC [111]. Since V567es contains a HR domain that interacts with microtubules for nuclear translocation, taxane treatment was able to impede V567es from the nucleus and inhibit transcriptional activity [111]. However, V7 was not sensitive to taxane treatment because it lacks the HR domain and canonical NLS. As a result, V7 accumulated in the nucleus and remained transcriptionally active post-taxane treatment *in vitro* and *in vivo* [111]. It is imperative to elucidate the role of AR splice variants in CRPC because their expression alone drives tumour growth, they are upregulated with treatment of

antiandrogens and ADTs, and their transcriptional activity is independent of androgen [50, 97, 110, 112].

### 1.4.2 AR V567es

AR V567es transcript was first identified and characterized in the LuCaP 86.2 and 136 xenografts obtained from men with CRPC metastases after prolonged exposure to ADT [107]. The AR V567es mRNA is alternatively spliced to skip exons 5, 6, and 7, which encode the LBD, with a frameshift mutation in exon 8 to produce a stop codon [107]. Consequently, AR V567es protein has the full NTD, DBD, hinge region, and a unique carboxy-terminus 10 aa sequence [107]. There currently is no antibody specific to the AR V567es protein. V567es protein is detected at a molecular weight of ~80 kDa using an antibody specific to the AR NTD [107]. V567es demonstrates constitutive transactivation, increased sensitivity of the fl-AR in the presence of androgens, and enhanced tumour growth in the absence of androgens [107].

Co-immunoprecipitation assay results have shown that AR V567es potentially binds and functionally interacts with fl-AR as a heterodimer [107]. This protein interaction was validated further with findings that V567es increases the stability of fl-AR in cell lines [107]. Gene expression analysis of V567es activity confirmed upregulation of AR-specific genes, such as *PSA/KLK3*, *TMPRSS2*, and *FKBP5*, in the absence of androgens [107]. Though V567es is one of the most frequently detected AR splice variants in CRPC patients, V7 has an increased expression at the mRNA level, constitutes up to 95% of the fl-AR protein level, and is the only AR splice variant with a developed antibody to its unique C-terminus [108]. For these reasons the prostate cancer field tends to focus on characterizing AR V7 activity in CRPC experimental models. All other AR splice variants are detected at the mRNA level using specific primers and can be identified as truncated AR protein products on an immunoblot with a broad binding NTD-directed antibody.

### 1.4.3 AR V7

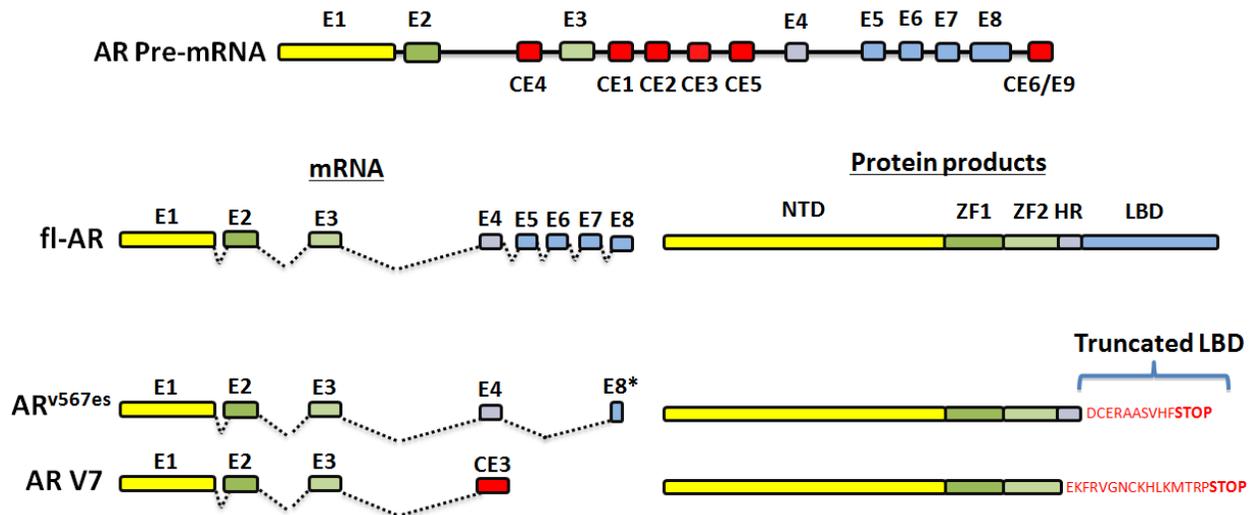
AR V7 is the most characterized out of the 25 AR splice variants identified to date in human tissues and prostate cell lines [113]. AR V7 mRNA is generated by contiguous joining of exon 1, 2, 3, and an alternatively spliced cryptic exon 3 (CE3) from an intronic region of AR [50]. AR V7 translated protein product contains the NTD and DBD, but lacks the HR and LBD [50]. The premature termination codon downstream of the DBD produces a unique 16 aa long sequence that is used to develop an antibody specific to the AR V7 protein [50]. Its predicted protein length is approximately 628 aa long and can be detected on an immunoblot at a molecular weight of ~75 kDa [50]. AR V7 has constitutive transcriptional activity, activates AR-regulated genes such as *PSA/KLK3*, *TMPRSS2*, and *FKBP5*, and promotes prostate cancer tumour growth under androgen-depleted conditions [49, 50]. In LNCaP xenograft tumours ectopic expression of AR V7 confers castrate-resistant growth [49]. Conversely, knockdown of AR V7 in 22Rv1 xenografts attenuates their growth [49]. Many studies have shown AR V7 to localize primarily to the nucleus in the presence and absence of androgens [49, 50, 112, 114]. However AR V7 mRNA transcript lacks the exon 4-encoded HR that spans part of the NLS. Site directed mutagenesis experiments demonstrated that NLS activity is reconstituted in AR V7 and independent from canonical NLS activity [115]. Residues in the unique 16 aa sequence restore the bipartite NLS and are sufficient for strong, basal nuclear localization [115].

AR V7 splice variant may physically interact and form heterodimers with the fl-AR protein. Watson et al. have postulated the AR V7 requires fl-AR to confer anchorage-independent growth *in vitro*, which was blocked by enzalutamide [116]. However, direct physical interaction with fl-AR and AR V7 proteins has yet to be confirmed using co-

immunoprecipitation. Studies have shown that expression of AR V7 protein is upregulated with the treatment of enzalutamide and that AR V7 induces a differential gene set with selective knockdown of fl-AR in 22Rv1 cells [49, 66, 97]. Therefore, not only can AR V7 activate androgen-regulated fl-AR canonical genes, but also its own transcriptional program is enriched for cell-cycle genes [66]. AR variant-regulated genes include *AKT1*, *UBE2C*, *CDC20*, *CDK1*, *CyclinA2*, and *UGT2B17*, which mostly encode cell-cycle proteins tightly regulated in the M phase [49, 66]. Recently, Cao et al. detected complexes of AR V7 and fl-AR on AREs of androgen-regulated genes in a chromatin-immunoprecipitation assay, with an AR V7-specific antibody and a fl-AR-specific (C-terminal) antibody [117]. AR V7 and fl-AR co-occupied the AR-regulated *PSA/KLK3* gene promoter, whereas AR V7 alone occupied the promoter of *UBE2C* in 22Rv1 cells [117]. Upon knockdown of fl-AR, V7 binding to the *PSA/KLK3* promoter was diminished suggesting that V7-mediated induction of AR-driven genes depended on a mutual ARE-binding of the two AR species [117].

While V7 drives CRPC progression, its transcript level does not predict recurrence in patients with indeterminate risk [118]. Men with Gleason grade 3 or grade 4/5 prostate cancer with high levels of AR V7 did not recur more often or with a shortened time to biochemical recurrence compared to men with low V7 transcript levels [118]. Thus, the functional role of AR V7 is not fully understood in CRPC disease. AR V7 transcript is also expressed in normal human tissues with highest levels in the spleen, placenta, lung, and brain [109]. As of yet the functional role of AR V7 expression in normal tissue is unknown and further studies should be conducted to elucidate AR pre-mRNA transcriptional splicing events in healthy males [109].

The molecular mechanisms by which AR V7 is generated remains unclear. One study showed that RNA splicing of AR V7 was upregulated by enhanced transcription at the *AR* gene locus and was closely associated with increased transcription initiation and elongation rates [119]. Another recent study showed that generation of V7 transcript was enhanced relative to full-length AR mRNA transcript from the *AR* gene promoter and was rapidly transcribed immediately after androgen deprivation [120]. This induction of V7 mRNA might be temporary and levels are potentially stabilized after a shift in AR variant-mediated signaling [120]. Moreover, AR pre-mRNA was associated with increased recruitment of RNA splicing factors, such as U2AF65 and ASF/SF2 to the 3' splice site, under androgen-deprived conditions [119]. Potentially both of these mechanisms could contribute to increased AR V7 production in CRPC tumours.



**Figure 1.5. Alternatively spliced mRNA transcripts of full-length AR, AR V567es and AR V7.**

Adapted from Figure 2 from © Haile, S & Sadar, MD (2011). Top: AR pre-mRNA transcribed from AR gene locus. Bottom left: Putative exon coding regions (E) and cryptic exons derived from intronic regions (CE) of the AR pre-mRNA alternatively spliced and joined to form the innate full-length AR transcript and V567es and V7 mRNAs. Full-length AR contains exons 1-8. V567es is alternatively spliced to skip exons 5-7 with a missense stop codon in exon 8 (\*). V7 contains exons 1-3 and a novel cryptic exon 3. Bottom right: Translated protein products of full-length and variant AR mRNA transcripts. V567es contains HR, whereas V7 lacks exon 4 protein coding region. Unique carboxy-terminus amino acid sequence in red shown for both truncated variants V7 and V567es. Cellular and Molecular Life Sciences. 68(24): Page 3971-3981. By permission from Springer Basel AG.

## ***1.5 EPI compounds in castration-resistant prostate cancer***

### **1.5.1 Intrinsically disordered AR NTD**

With co-crystal structure analysis both the isolated AR LBD and DBD demonstrate compact globular  $\alpha$ -helical structure. In contrast, the NTD lacks stable secondary structure and conforms as an intrinsically disordered protein (IDP) domain [61, 121, 122]. IDPs, as a whole unit or minimal regions of a protein, have biological function yet lack stable secondary or tertiary protein structure in physiological *in vitro* conditions [123]. IDPs are important for cell cycle regulation and signal transduction, and it is thought that the disordered regions enable high protein turnover for tight control of cell processes [123, 124]. Due to the highly dynamic nature of IDPs in solution and their structural plasticity, it is difficult to determine the exact structure of the AR AF1 transactivation domain. Using methods such as circular dichroism, Fourier Transformed Infrared spectroscopy and secondary structure prediction analysis the AR AF1 region in the NTD has approximately 13% helical structure, with four predicted regions of  $\alpha$ -helices (Figure 1.6) [122, 125]. Though this number is difficult to quantify since the AR AF1 region can fold into a more stable conformation upon interactions with co-regulatory proteins, general transcription machinery or osmolytes [122, 125]. The AF1 fragment forms helical structures in the presence of a natural osmolyte, trimethylamine N-oxide (TMAO) or with binding partner RAP74 [122, 125]. The addition of TMAO or RAP74 co-regulatory protein induced a 3-fold increase in  $\alpha$ -helical secondary structure of the AF1 fragment [125]. Experiments conducted with the AF1 fragment in the presence of RAP74 demonstrated that this region has a molten-globule like conformation referred to as 'collapsed disorder' [78]. This

suggests that the AR NTD most likely exists in a conformation favourably primed for binding partners, then induces folding to stabilize its structure upon interaction with other proteins [78].

Studies have shown the AR AF1 region adopts helical conformations on its own. However, mutant fragments generated of the full AR NTD demonstrated that the AF1 secondary structure can be modulated by other factors [79, 126, 127]. Binding of the AR DBD to AREs resulted in allosteric changes in the intrinsic fluorescence emission spectrum for four tryptophan residues within the AR NTD/AF1 region [79]. This intercommunication between the AR DBD and AR NTD modulated the structure of the NTD into an induced protease-resistant conformation [79]. Furthermore, deletions of the large poly-Q tract in the AR NTD results in a polypeptide with less  $\alpha$ -helical structure [126]. Consequently, expanding the poly-Q tract (Q repeats greater than 40), as occurs in Kennedy's disease where the AR becomes aggregated in the cell, resulted in an increase in  $\alpha$ -helical structure [126]. Lastly, it is suggested that post-translational modifications of the AR AF1 region, such as phosphorylation, render the AR into a configuration more susceptible to proteolytic degradation upon loss of structure [127].

Though the intrinsic disorder of the AR NTD/AF1 region is important for its function, its structural plasticity in solution makes structure-based drug design difficult. As mentioned previously, the AR NTD mediates transcriptional activity with sequences crucial for androgen-dependent gene regulation, collectively called the AF1 region [55, 73]. Deletion of the NTD resulted in a transcriptional weak or silent receptor protein, which makes it a very attractive drug target for abolishing AR activity in CRPC [55, 73]. A study conducted with AR NTD decoy molecules (1-558 aa) demonstrated the importance of blocking transcriptional activity of the NTD that drives prostate cancer growth [128]. The overexpressed intact NTD was able to

competitively bind co-regulatory interacting proteins required for activation of the fl-AR and decrease transcriptional activity of an AR-responsive reporter [128]. Thus, targeting the AR NTD transcriptional engine with an inhibitor could greatly decrease prostate cancer proliferation and effectively reduce tumour growth in CRPC patients.

### 1.5.2 EPI-001/EPI-002 mechanism of action

Small molecule EPI-001 is a first-in-class inhibitor of the intrinsically disordered AR NTD [129]. EPI-001 is a structural derivative of BADGE (*Bisphenol A Diglycidic Ether*) and is a modified compound from extracts originally isolated from the marine sponge *Geodia lindgreni* [129]. Presumably the BADGE-related compounds were bioaccumulated in the marine sponge due to contaminated seawater from industrial waste [129]. A library of BADGE analogues were screened in cell-based assays for inhibition of AR NTD transcriptional activity in LNCaP cells [129]. EPI-001 (BADGE.HCl.H<sub>2</sub>O) had the most potent, specific activity with desirable drug-like qualities. EPI-001 is a mixture of four stereoisomers with 2 chiral carbons at the 2 and 20 positions (Figure 1.7) [84]. Due to FDA requirements, each stereoisomer needs to be evaluated for efficacy since biological activity among stereoisomers can differ [84]. Using AR-driven reporter assays IC<sub>50</sub> values were calculated for each stereoisomer. EPI-001 mixture was effective at inhibiting NTD-directed transcriptional activity with an IC<sub>50</sub> of 12.63±4.33 μM, with the stereoisomer EPI-002 having the most potent activity with 7.40± 1.46 μM [84]. EPI-002 has a configuration of 2R, 20S and effectively inhibited AR transcriptional activity with 3 different AR-driven reporters [84].

EPI-001/EPI-002 has a unique mechanism of action compared to antiandrogens used in the clinic. *In vitro* studies showed that EPI compounds: 1) inhibit NTD transcriptional activity; 2) covalently bind to the AR AF1 region; 3) block androgen-stimulated N/C interaction 4) block AR DNA-binding to AREs on target genes; 5) reduce protein-protein interactions of co-regulators and transcriptional machinery with AR NTD; and 6) do not prevent nuclear translocation of AR [84, 129]. AR inhibition with EPI compounds was unaffected in LNCaP

cells treated with increasing concentrations of R1881 [84]. This demonstrates that EPI compounds do not target the AR LBD. In contrast, elevated levels of R1881 reversed enzalutamide inhibitory effects [81]. Fluorescent polarization assay also showed that EPI-001 does not compete with R1881 fluoromone for AR LBD fragment, whereas bicalutamide antiandrogen inhibited R1881 binding [129]. EPI-001 binds covalently to the AR AF1 region and has shown to cause a shift in steady-state fluorescence spectra of local tryptophan and tyrosine amino acid residues [129].

In order to bind covalently to the intrinsically disordered AR NTD region, EPI-001 requires some secondary structure of the AF1 [84]. The amount of EPI covalently bound to AF1 is time-, dose-, and temperature-dependent [84]. This covalent binding is specific to the AR AF1 region and EPI-001 does not bind to the GR AF1 [129]. Since the AR NTD region shares less than 15% homology with GR and PR, EPI-001 did not inhibit the transcriptional activities of these receptors [129].

EPI-001/EPI-002 bound to AR AF1 effectively inhibits both ligand-dependent (androgen bound LBD) and ligand-independent (absence of androgen) transactivation. In the absence of androgen the AR NTD can be activated by alternative pathways including cAMP-dependent protein kinase A (PKA) activity, or interleukin-6 (IL-6) or epidermal growth factors [128, 130, 131]. EPI-001/EPI-002 inhibited transactivation of AR NTD (1-558 aa) induced by forskolin, which induces PKA activity, or by IL-6 [129]. Other protein-protein interactions that drive transcriptional activity of the AR NTD in the presence or absence of R1881 are CBP (CREB-binding protein), which acts as a scaffold for transcriptional machinery and stabilizes binding to AREs, and RAP74, the subunit of TFIIF that associates with RNA polymerase II and the TATA

box [76, 77]. EPI compounds inhibit interaction with CBP and RAP74 with the AR AF1 region [129]. Lastly, EPI-001 blocked gene expression of androgen-regulated genes such as *PSA/KLK3* and *TMPRSS2* in response to R1881 in LNCaP cells [129]. Thus, *in vitro* studies have demonstrated that EPI compounds bind and target the AR NTD and inhibit transcriptional activity of the fl-AR just as effectively as its LBD-targeted competitor enzalutamide. However, these compounds interact with the AR in very different ways including site of binding (AR NTD AF1 region vs. LBD pocket), binding mechanism (irreversible vs. reversible), time to bind to AR (moderately slow vs. moderately fast), potency (IC<sub>50</sub> values), disruption of protein-protein interactions (NTD-associated transcriptional machinery vs. FxxLF co-regulators), and mechanism of action (nuclear translocation). The differential chemistry and effect on prostate cell biology of both EPI-002 and enzalutamide compounds should be considered upon testing models of various CRPC patient populations or as a combination therapy.

### 1.5.3 EPI-001/EPI-002 treatment in castration-resistant prostate cancer models

In order to reduce tumour burden of CRPC patients it is essential to decrease proliferation and induce apoptosis of tumour cells. EPI-001/EPI-002 inhibited *in vitro* proliferation of androgen-dependent LNCaP prostate cancer cells and androgen-independent 22Rv1 prostate cancer cells in the presence and absence of androgen [129]. Cell cycle analysis showed that EPI-002 inhibited LNCaP cells actively proliferating in S-phase (mitosis and DNA synthesis) [84]. This data supports that EPI compounds target AR-dependent proliferation of prostate cancer cell lines that mimic CRPC progression.

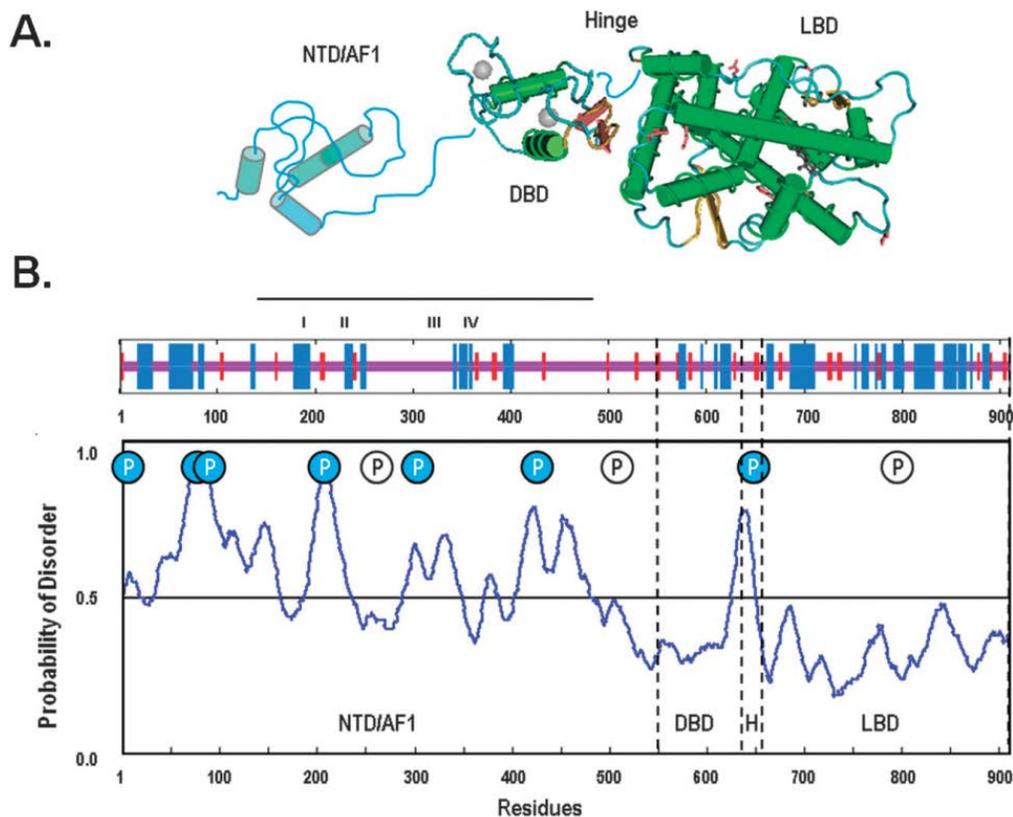
Most importantly, EPI-001 blocked *in vivo* tumour growth in the presence of androgen demonstrating that EPI targets the androgen-axis [129]. Intact mice were implanted with subcutaneous LNCaP xenografts and intravenously injected with EPI-001. EPI-001 significantly reduced tumour growth and increased apoptosis. Furthermore, EPI-001 reduced tumour burden in a CRPC mouse model with no apparent toxicity [129]. Mice with subcutaneous LNCaP xenografts were castrated then treated with EPI-001 either intravenously (50 mg/kg body weight) or intratumourally (20 mg/kg body weight). Tumours appeared less bloody with significantly reduced volume with both forms of injection [129]. Body weight and behaviour of the mice did not change with response to EPI-001 injections compared to vehicle treatments indicating no toxicity [129]. Pathological analysis of other harvested organs such as the heart, liver and spleen confirmed no signs of toxicity [129]. Notably, castrated mice orthotopically bearing LNCaP xenografts showed decreased serum PSA levels upon treatment of EPI-001 with concomitant decrease in tumour volumes [129]. Pharmacokinetic studies have shown that EPI-001 has a half-life of 3.3 hours [129]. Overall, EPI-001/EPI-002 is effective at reducing tumour burden *in vivo*

under castrate and non-castrate conditions, indicating that EPI compounds block the AR NTD transcriptional activity driving progression of CRPC. EPI compounds may benefit CRPC patients that are failing hormonal therapies targeting the AR LBD.

One patient population that would benefit from EPI compounds is that which has heterogeneous tumours with mixed populations of fl-AR and AR splice variants. *In vitro* studies with AR V567es in COS1 monkey kidney cells that do not express AR, demonstrated that EPI-002 reduced constitutive transcriptional activity in the presence and absence of R1881 [84]. Transcriptional activities of V567es co-expressed with endogenous fl-AR in LNCaP cells was inhibited by EPI-002 treatment both in the presence and absence of R1881 [84]. Not surprisingly, antiandrogens had no effect on transcriptional activity of AR V567es since it lacks an AR LBD [84]. In a CRPC *in vivo* model, oral dosing of EPI-002 (200 mg/kg body weight) inhibited growth of VCaP xenografts in castrated mice, which express AR splice variants [84]. This is an advantage of a novel AR NTD inhibitor since EPI compounds are able to block the activities of alternatively spliced variants of the AR, which is a mechanism adapted by the AR to confer resistance against current hormonal therapies used in the clinic. Based on novel data presented thus far, EPI-002 treatment may benefit patients at many stages of prostate cancer. At non-castrated intact conditions EPI-002 can reduce tumour burden with its direct interference with AR signaling, but also at the CRPC level when tumour populations are androgen-independent and can express heterogeneous populations of full-length and AR splice variants. As of yet EPI-002 has not undergone clinical studies, however phase I trials for safety and efficacy are anticipated early next year (2015).

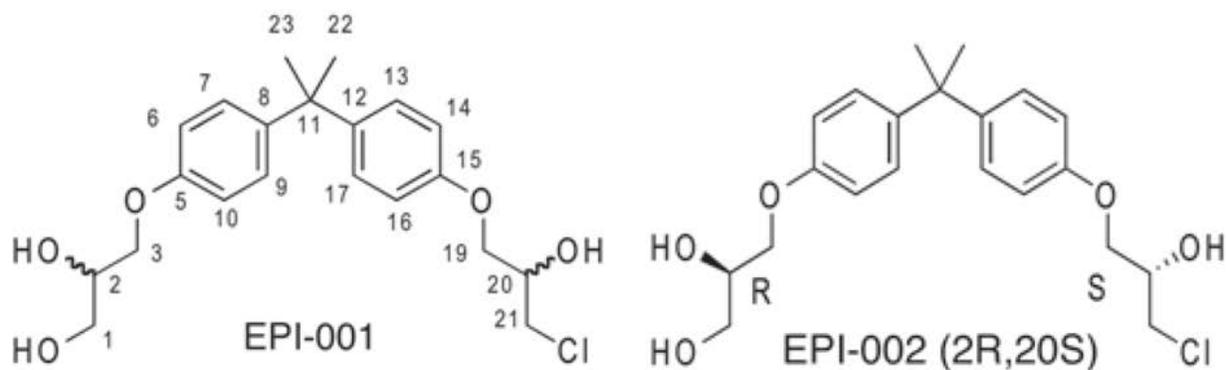
#### **1.5.4 Mechanisms of resistance against EPI compounds**

The exact residue(s) in the AF1 (142-485 aa) of AR NTD that EPI-002 covalently binds to is currently under investigation. AF1-EPI-002 binding studies will help predict potential AR mechanisms of resistance against EPI-002 monotherapy in patients. Since AR transcriptional activity is dependent on the TAU regions in the AF1, any base pair mutations would abolish all AR transcription and render it a silent receptor protein. Furthermore, the flexibility of the intrinsically disordered AR NTD requires that other co-regulators co-operate and bind together to induce the "collapsed disorder" transcriptionally primed structure [78]. Thus, gain-of-function mutations to a flexible backbone to overcome transcriptional blockade would be unlikely. Overall, the propensity of AR NTD to develop gain-of-function mutations against irreversibly bound EPI-002 is theoretically low, though possible mechanisms of resistance should be considered.



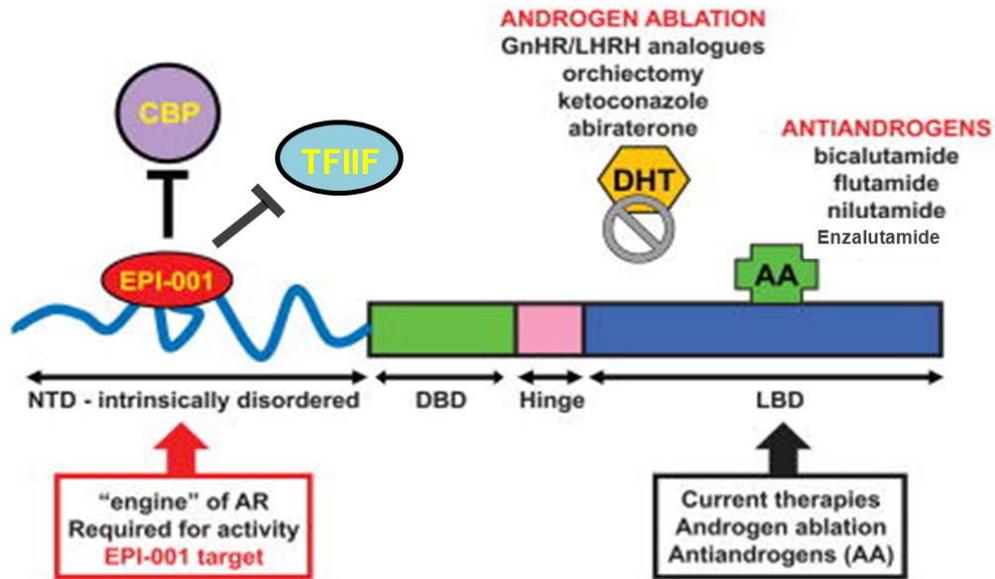
**Figure 1.6. Intrinsically disordered AR AF1 region.**

Figure 2A&B from © McEwan, IJ (2012). A) Modular domain organization and structure of AR protein. Representation of 3D  $\alpha$ -helical structure of LBD and DBD with the intrinsically disordered NTD/AF1 region. B) Upper panel: Prediction of secondary structure content in human AR with blue and red bars representing  $\alpha$ -helices and  $\beta$ -strands, respectively. Lines I-IV indicate locations of 4 putative helices in the AF1. Lower panel: Plotted peaks represent high probability ( $>0.5$ ) of intrinsic disorder within the AR protein. Known phosphorylated serines are indicated with 'P' and ones present in intrinsic disordered regions are denoted by blue filled circles. Post-translational modifications may mediate interactions with binding partners to form the 'collapsed disorder' conformation of AF1. *Molecular Biosystems*. 8(1): Page 82-90. By permission from The Royal Society of Chemistry.



**Figure 1.7. Structure of EPI-001 mixture and stereoisomer EPI-002.**

Adapted from Figure 1A from © Myung, JK et al. (2013). Chemical structures of AR NTD small molecule inhibitors for treating CPRC. EPI-001 compound (BADGE.H<sub>2</sub>O.HCl) mixture of four stereoisomers and the most potent stereoisomer EPI-002 (2R, 20S configuration) irreversibly bind to AR AF1 region. *The Journal of Clinical Investigation*. 123(7): Page 2948-2960. By permission from American Society for Clinical Investigation.



**Figure 1.8. Therapeutic approach for absolute AR blockade.**

Adapted from Figure 1A from © Sadar, MD (2011). EPI compounds bind to AR NTD to block protein-protein interactions and inhibit transcriptional activity. Enzalutamide and other AR LBD inhibitors block DHT binding to ligand-binding pocket of full-length AR. *Cancer Research*. 71(4): Page 1208-1213. By permission from American Association for Cancer Research.

## *1.6 Hypotheses and specific aims*

CRPC is the regrowth of malignant cells accompanied in the majority of cases by a persistent rise in PSA that results from resistance to current therapies. Growth and progression of CRPC are thought to be dependent on the transcriptional activity of the AR [51, 55]. The AR requires the NTD for transactivation of androgen-regulated genes [73]. Current antiandrogens, such as enzalutamide, target the AR LBD but only provide a temporary reduction in tumour burden before AR signaling is restored [23, 24, 49, 107]. Resistance to current AR LBD-directed therapies is developed through adaptation events such as alternative splicing of AR pre-mRNA to form truncated AR variants that lack the full LBD [35, 49, 50, 107]. At this time no therapies exist to block AR splice variant transcriptional activity. Hence, small molecule inhibitors of the AR NTD are the only compounds that could directly target truncated AR splice variants and improve survival of CRPC patients.

AR splice variants are upregulated in CRPC patient metastases and promote androgen ablation-independent growth [49, 104, 108]. V7 and V567es are the most prominent and clinically relevant AR splice variants since they are correlated with poorer prognosis and decreased survival [49, 104, 108]. V7 and V567es are primarily nuclear in the cell, constitutively active, and do not vary in transcriptional activity upon stimulation of androgen nor antiandrogen treatment [49, 107, 114]. Furthermore, AR splice variants activate a distinct transcriptional program enriched for cell-cycle genes, which may contribute to resistance to hormonal therapies and disease progression [66, 107]. V7 expression is frequently concomitant with fl-AR in CRPC patients and is expressed as a heterogeneous AR population between and within metastatic tumours [104]. V7 activity may be regulated by its interaction with fl-AR, and recent data has

shown that V7 occupancy on androgen-regulated AREs is mutually-dependent on fl-AR binding [116, 117]. Interestingly, V7 independently occupies promoters of AR variant-driven cell-cycle genes and does not require fl-AR for activity [50, 66, 117]. Thus, the interaction and transcriptional activity of V7 in the presence of androgen-inducible fl-AR has yet to be fully characterized in CRPC models. Elucidation of V7 transcriptional activity with fl-AR in a heterogeneous population could help assess potential therapeutic agents, alone or in combination, for CRPC patients. Taken these findings and questions together, two hypotheses were tested in this thesis:

**Hypotheses:**

**A.** NTD antagonist EPI-002 inhibits transcriptional activity of endogenous and exogenous AR V7 in human prostate cancer cell lines.

**B.** Combination therapy of EPI-002 and antiandrogen enzalutamide demonstrates greater inhibition of transcriptional activity of mixed AR populations than each treatment alone.

The following specific aims were addressed.

**Aims:**

**1.** To characterize the transcriptional activity of endogenous and exogenous AR V7 with fl-AR using various prostate cancer cell lines and evaluate the effect of NTD antagonist EPI-002.

**2.** To evaluate the effect of combination therapy on the transcriptional activity of fl-AR and AR V7 with enzalutamide and EPI-002, further supported with the evaluation of cell growth of cell lines expressing mixed populations of AR and truncated splice variants.

## Chapter 2: Materials & Methods

### 2.1 Cell culture

Human prostate cancer PC3 cells are androgen-insensitive and do not express functional AR [132]. PC3 cells were obtained from American Type Culture Collection (ATCC; Bethesda, MD, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin (FBS and antibiotics from Gibco, Life Technologies). PC3 cells have prostatic neuroendocrine carcinoma features and were derived from a bone metastatic lesion from a grade IV prostatic adenocarcinoma of a 62-year-old Caucasian male in 1976 [132, 133]. LNCaP cells are androgen-sensitive and express endogenous fl-AR [134]. Human prostate cancer LNCaP cells were provided by Dr. L.W.K. Chung (Cedar-Sinai Medical Center, CA, USA) and maintained in phenol red-free Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco, Life Technologies) supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. LNCaP adenocarcinoma cells were derived from the left supraclavicular lymph node metastasis from a 50-year-old Caucasian male in 1977 [135]. LNCaP95 cells express functional fl-AR, AR V7, and AR splice variants [50]. Growth of the LNCaP95 cell line is androgen-independent and was developed from long-term continuous culture of LNCaP cells in androgen-depleted conditions since the year 1995 [50]. Human prostate cancer LNCaP95 cells were provided by Dr. Stephen Plymate (University of Washington School of Medicine, WA, USA) and maintained in phenol red-free RPMI medium 1640 with 10% charcoal stripped serum (CSS), 100 units/mL penicillin, and 100 µg/mL

streptomycin. CSS is incubated with charcoal to remove the majority of hormones from the serum. All cells were grown at 37°C with 5% CO<sub>2</sub>.

## ***2.2 Expression plasmids and transfection reagents***

pcDNA3.1 empty vector plasmid and pAR V7 expression plasmid containing AR V7 cDNA, AR gene exons 1-3 and 3b cryptic exon, were gifts from Dr. Stephen Plymate [50]. PSA(6.1kb)-luciferase reporter plasmid encodes nucleotides -6000/+12 relative to transcription start site of the human *PSA/KLK3* gene and includes the PSA promoter, with AREII (-395 to -376) and AREI (-170 to -156), and enhancer regions with AREIII (-4148 to -4134) [131, 136]. Probasin-luciferase reporter plasmid encodes -286/+28 of the rat probasin (*PB*) gene and includes AREI (-236 to -223) and AREII (-140 to -117) in the promoter [137]. ARR3-tk-luciferase reporter plasmid consists of three repeats of rat probasin AREs (-244/-96) ligated in tandem into the *HindIII* site of the pT81 luciferase vector [138, 139]. The multiple probasin AREs of the ARR3-luciferase reporter are linked upstream to a thymidine kinase (tk) minimal promoter. PC3 cells were transiently transfected using FuGENE6 (Promega, Madison, WI, USA) transfection reagent according to manufacturer's protocol. LNCaP and LNCaP95 cells were transiently transfected using Lipofectin (Invitrogen, Burlington, ON, Canada) according to manufacturer's protocol. The total amount of plasmid DNA transfected into cells was predetermined and optimized to 18 µg/plate.

### ***2.3 Luciferase reporter assay***

PC3 cells with passage numbers from 3 to 18 were seeded in BD Falcon 6-well culture plates ( $2.5 \times 10^5$  cells/well; Becton Dickinson Biosciences, Mississauga, ON, Canada) and left to adhere for 24 hours in 5% FBS DMEM. Cells reached ~60-70% confluence before their media was replaced with serum-free DMEM followed by transient co-transfection of 6  $\mu$ g of probasin-luciferase reporter gene construct and 50-400 ng/well of AR V7 expression vector or pcDNA3 empty vector in serum-free conditions. PC3 cells were treated 5 hours post-transfection with EPI-002 (25  $\mu$ M), provided by Dr. Raymond Andersen (Earth, Ocean & Atmospheric Sciences, University of British Columbia, Canada), or dimethyl sulfoxide (DMSO; Sigma Aldrich Canada Co., Oakville, ON, Canada) carrier alone and left to incubate for an additional 24 hours prior to harvesting. LNCaP cells with passage numbers from 39 to 45 were seeded in 12-well culture plates ( $8.0 \times 10^4$  cells/well; Greiner Bio One, Monroe, NC, USA) and left to adhere for 24 hours in 10% FBS RPMI medium 1640. Cells reached ~60-80% confluence and were transiently co-transfected with 6  $\mu$ g of AR-driven luciferase reporter plasmids and 450 ng/plate of AR V7 expression vector or pcDNA3 empty vector in serum-free and phenol red-free RPMI medium 1640. LNCaP cells were pre-treated 5 hours post-transfection with EPI-002 (25  $\mu$ M), enzalutamide (5  $\mu$ M; OmegaChem, Saint-Romuald, QC, Canada), EPI-002 (25  $\mu$ M) and enzalutamide (5  $\mu$ M) combination therapy or DMSO alone. Cells incubated for 16 hours prior to addition of 1 nM synthetic androgen methyltrienolone (R1881; AK Scientific, Union City, CA, USA) or ethanol vehicle alone. Cells were incubated for an additional 48 hours before harvesting. LNCaP95 cells with passage numbers from 3 to 11 were seeded in 12-well culture plates ( $1.2 \times 10^5$  cells/well; Greiner Bio One) and left to adhere for 48 hours in 10% CSS phenol

red-free RPMI medium 1640. At ~60-80% cell confluency LNCaP95 cells were transiently transfected with 6 µg of AR-driven luciferase reporter plasmids to measure endogenous AR V7 activity, or co-transfected with AR-driven reporters and either 1.2 µg/plate AR V7 plasmid DNA, 0.03 µg/plate of AR V7 plasmid DNA or 1.2 µg/plate pcDNA3 plasmid DNA to measure ectopic AR V7 activity. All transient transfections were conducted in serum-free, phenol red-free RPMI medium 1640. Cells were pre-treated 5 hours post-transfection with EPI-002 (25 µM), enzalutamide (5 µM), EPI-002 (25 µM) and enzalutamide (5 µM) combination therapy or DMSO (ATCC). Sixteen hours later cells were treated in the presence or absence of 1 nM R1881 and left to incubate for an additional 48 hours prior to harvesting.

PC3 cells were washed with cold phosphate-buffered saline (PBS) prior to cell lysis. PC3, LNCaP and LNCaP95 cells were harvested in Passive Lysis Buffer (Promega), then Firefly Luciferase Assay System was employed (Promega) according to manufacturer's protocol. Relative Luminescence Units (RLU) in cell lysates were detected for 10 seconds using Promega GloMax-Multi Detection Luminometer (Promega). Protein concentrations of the cell lysates were determined by the Bradford method using Bio-Rad Protein Assay kit (Mississauga, ON, Canada) and the VersaMax Tunable Microplate Reader (Molecular Devices). Luciferase activities were normalized to protein levels and expressed as RLU/mg of protein/min. Cells were visualized using Zeiss Axioplan 2 Microscope (Carl Zeiss, Toronto, ON, Canada). At least 3 independent experiments were performed with each containing triplicate technical replicates.

## ***2.4 Western blot analyses for protein expression***

Cell lysates conducted in parallel to luciferase reporter assays were collected in RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 20 mM sodium fluoride, 20 nM sodium molybdate, cOmplete mini EDTA free (Roche) and phosSTOP (Roche) protease inhibitors. Protein was quantified using Pierce BCA Protein Assay kit (Thermo Scientific, Ottawa, ON, Canada), according to manufacturer's protocol. Protein lysates (5 or 10 µg of total protein/lane) were separated by 8% SDS-polyacrylamide gel electrophoresis (Bio-Rad). For visualization using the rabbit raised polyclonal primary antibody anti-AR N20 (Santa Cruz Biotechnologies, Dallas, TX, USA), proteins were transferred to Hybond C extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) and blocked with 5% milk in PBS-T (phosphate-buffered saline and 0.05% Tween-20) for 1 hour. Incubation with anti-AR N20 antibody was carried out overnight at 4°C (1:1000 diluted in 1% milk in PBS-T). For visualization using the mouse raised monoclonal primary antibody anti-AR V7 (A&G Pharmaceuticals, Columbia, MD, USA), proteins were transferred to Immobilon-PVDF membrane (Millipore, Billerica, MA, USA) and blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 hour. Incubation with anti-AR V7 antibody was carried out overnight at 4°C (1:400 dilution in 2.5% milk in PBS-T). Equal loading of protein for all blots was determined by probing with mouse anti-β-actin (1:7000 dilution; Abcam, Toronto, ON, Canada). AR protein and β-actin levels were detected using Electro-chemi luminescence (ECL) kit (Amersham). Densitometric analyses of AR V7 protein bands relative to fl-AR were quantified using ImageJ software. Western blot analyses were performed for at least 3 independent experiments.

## ***2.5 Cell proliferation BrdU immunoassay***

LNCaP95 cells were seeded in BD Falcon 96-well plates ( $7.8 \times 10^3$  cells/well) and left to adhere for 48 hours in 10% CSS phenol red-free RPMI medium 1640. Cells were then pre-treated with EPI-002 (25  $\mu$ M), enzalutamide (5  $\mu$ M), EPI-002 (25  $\mu$ M) and enzalutamide (5  $\mu$ M) combination therapy or DMSO in serum-free conditions. Five hours later cells were treated with 0.1 nM R1881 or vehicle control. BrdU (5-bromo-2'-deoxyuridine) labeling reagent (Roche Diagnostics, Laval, QC, Canada) was added to the cells at 48 hours, 72 hours and 96 hours post-R1881 treatment and incubated for 2 hours at 37°C. Cells were then dried in Hybaid oven (Thermo Scientific) for 30 minutes and stored at 4°C until ELISA (enzyme-linked immunosorbent assay). ELISA was conducted according to manufacturer's protocol (Roche), and absorbance of the samples was measured at 370 nm with reference wavelength at 492 nm using the VersaMax Microplate Reader. LNCaP95 cells were visualized using Zeiss Axioplan 2 Microscope. Cell proliferation was measured in 3 separate experiments with six technical replicates each treatment.

## ***2.6 Statistical analyses***

Student's *t*-test, one-way ANOVA, two-way ANOVA, and post-hoc multiple comparisons tests were performed by GraphPad Prism (Version 6.01 for Windows; La Jolla, CA, USA). Differences were considered statistically significant at *P* values less than 0.05.

## Chapter 3: Results

### *3.1 EPI-002 inhibited constitutive AR V7 transcriptional activity.*

EPI-001, the mixture of four stereoisomers, inhibits the AR NTD-directed transcriptional activity [84, 129]. To evaluate the efficacy of EPI-002, the most potent stereoisomer of EPI-001, on inhibition of AR V7 transcriptional activity an AR-negative cell line must be used. The choice of cell line is very important since V7 is cytosolic in AR-negative DU-145 cells and AR-positive VCaP cells when treated with synthetic androgen, R1881 [50, 114]. PC3 cells were used to evaluate EPI-002 inhibition on V7 activity since this cell line does not express any functional AR, V7 is nuclear localized, and it is of prostate cancer origin [50, 133]. PC3 cells were transiently transfected with a range of V7 expression plasmid (50 ng to 400ng/well in a 6 well plate) then treated 5 hours later with EPI-002 (25  $\mu$ M) or DMSO control. Cell lysates were collected to determine protein levels of ectopic V7 by immunoblot analysis. Transfected levels of V7 were compared to levels of endogenous fl-AR in whole cell lysates from LNCaP cells to achieve approximately 1:1 expression. Western blot analysis confirmed 100 ng/well of V7 plasmid DNA in a 6 well plate ectopically expressed in PC3 cells was comparable to physiological levels of fl-AR expressed in LNCaP cells (Figure 3.1A). Visualization with an antibody specific to the first 20 amino acids of the AR NTD (AR N20) detected AR V7 protein migration at approximately 75-80 kDa molecular weight and fl-AR at approximately 110 kDa [50, 140]. Equal loading was confirmed by probing to  $\beta$ -actin housekeeping protein.

Transcriptional activity was measured in PC3 cells co-transfected with a constant concentration of V7 expression plasmid (100 ng/well in a 6 well plate) and AR-driven reporter

probasin-luciferase. Average fold induction of constitutive V7 activity [ $5.66 \pm 1.62$  standard deviation (SD)] was within reported range of 4-8 folds (Representative Figure 3.1B) [50, 112]. Constitutive activity was set to 100% and percent inhibition was calculated. EPI-002 inhibited V7 transcriptional activity approximately 35% in four independent experiments ( $P \leq 0.0001$ ; Student's *t*-test) (Figure 3.1C). Empty vector control plasmid, pcDNA3, produced high background luciferase activity in PC3 cells. To reduce background activity dual-luciferase assay system was employed and other cell lines were tested (CV-1 parental and SV40 transformed COS1 monkey kidney cells). Despite attempts at optimization, non-specific luciferase activity remained high with empty vector, although this effect was not seen in transfected LNCaP or LNCaP95 cells.

### ***3.2 EPI-002 inhibited ectopically expressed AR V7 transcriptional activity in the presence of fl-AR.***

The Sadar group previously showed that EPI-002 blocks AR transcriptional activity in LNCaP cells [84]. LNCaP cells are androgen-sensitive and express endogenous fl-AR [134]. First, levels of ectopic V7 and fl-AR protein in LNCaP cells were measured by Western blot analyses. This was to ensure the levels of transfected V7 were expressed at physiological levels and examine possible effects on AR protein levels from treatments. LNCaP cells were transfected with a constant concentration of V7 expression plasmid (450 ng/plate in a 12 well plate) or empty vector pcDNA3. Cells were pre-treated 5 hours post-transfection with either EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Sixteen hours later cells were then treated with 1 nM R1881 to activate the fl-AR LBD, or ethanol vehicle. Immunoblots were probed with antibody specific to the AR NTD and equal loading was determined with probing for  $\beta$ -actin housekeeping protein. Levels of AR V7 ectopically expressed in LNCaP cells were comparable to maximal physiological levels of AR splice variants expressed in CRPC tumours, at a ratio of 1:1 (Representative Figure 3.2A). Densitometric quantification of AR levels showed V7 was expressed 104% of fl-AR in the absence of R1881 and 82% of fl-AR in the presence of R1881. The presence of androgen increased fl-AR levels, as expected [54]. Furthermore, treatment with inhibitors did not affect AR levels compared to DMSO control.

Transcriptional activity of ectopic V7 at approximately 1:1 levels of fl-AR was characterized. LNCaP cells were co-transfected with a constant concentration of V7 expression

plasmid (450 ng/plate in a 12 well plate) or empty vector pcDNA3 with three AR-driven reporters: probasin-luciferase, PSA-luciferase or ARR3-luciferase. First, percent of V7 transcriptional activity in the absence of androgen was calculated for each reporter. Constitutive DMSO treated V7 activities were set to 100%. Statistical analyses were performed by GraphPad prism to determine if either monotherapies or combination therapy decreased V7 activity compared to DMSO control. With probasin-luciferase EPI-002 monotherapy and in combination with enzalutamide decreased V7 activity, with enzalutamide monotherapy having no effect (Figure 3.2B) ( $P < 0.0001$ ; Two-way ANOVA post-hoc Dunnett's test). Similarly, EPI-002 monotherapy and in combination with enzalutamide blocked V7 activity with PSA-luciferase, with enzalutamide monotherapy having no effect (Figure 3.2C) ( $P < 0.05$ ; Two-way ANOVA post-hoc Dunnett's test). Both monotherapies and combination therapy did not have an effect on V7 activity with ARR3-luciferase (Figure 3.2D) (*ns*; Two-way ANOVA post-hoc Dunnett's test).

Upon addition of R1881 to transfected LNCaP cells, the induction of AR-driven reporters was measured with endogenous fl-AR only and with AR V7 in the presence of androgen-activated fl-AR. Average fold induction of AR species with each reporter were calculated from three independent experiments, though one representative experiment is shown. Probasin-luciferase is a rat AR-driven gene reporter and average fold induction by R1881-stimulated fl-AR was  $922 \pm 709$  SD (Representative Figure 3.3A). In the absence of androgen V7 constitutively activated probasin-luciferase ( $1399 \pm 1116$  SD average fold induction), and in the presence of androgen enhanced the transcriptional activities of R1881-treated fl-AR with V7 ( $2155 \pm 1588$  SD average fold induction). With PSA-luciferase, a canonical AR human gene reporter, endogenous fl-AR induced reporter activity by an average fold induction of  $191 \pm 117$  SD in the presence of androgen (Representative Figure 3.3B). Interestingly, V7 showed less constitutive transactivation

with PSA-luciferase compared to R1881-induced fl-AR only activity ( $57 \pm 12$  SD average fold induction), but also suppressed transcriptional activities of R1881-treated fl-AR with V7 ( $89 \pm 2$  SD average fold induction). ARR3-luciferase is a synthetic reporter with 3 repeats of the probasin rat promoter, and R1881-stimulated fl-AR in LNCaP induced activity with an average fold induction of  $9818 \pm 15301$  SD, which is within reported range of approximately 10 times probasin reporter activity [137] (Representative Figure 3.3C). In contrast to PSA-luciferase, V7 activity in the absence of R1881 greatly amplified the ARR3 reporter compared to R1881 stimulated fl-AR only activity ( $44375 \pm 66698$  SD average fold induction), and enhanced the transcriptional activities of R1881-treated fl-AR with V7 ( $105224 \pm 166271$  SD average fold induction). This demonstrates differential activation of AR-driven reporters with AR V7 in the presence of fl-AR. For all 3 AR-driven reporters, EPI-002 monotherapy and EPI-002 in combination with enzalutamide effectively inhibited transcriptional activity of fl-AR only, and the combined transcriptional activities of fl-AR and ectopic AR V7 in the presence and absence of R1881 (Figures 3.3A-C). Most importantly, combined V7 and R1881-stimulated fl-AR activities treated with enzalutamide monotherapy were inhibited to levels equivalent to V7 in the absence of R1881. This demonstrates that for all 3 AR-driven reporters LBD-targeted enzalutamide inhibited solely fl-AR and the remaining activity probably represents V7 constitutive activity.

Next, percent inhibition of R1881-treated fl-AR activity only and combined transcriptional activities of AR V7 in the presence of R1881-treated fl-AR were calculated for at least three independent experiments for each AR-driven reporter. R1881-treated fl-AR and R1881-treated fl-AR with V7 activities were set to 100% to calculate percent inhibition. Statistical analyses were performed by GraphPad Prism to determine if combination therapy is

better than either monotherapies on V7 ectopically expressed LNCaP cells (Figure 3.3D-F). EPI-002 in combination with enzalutamide was found to inhibit endogenous fl-AR only activities of probasin-luciferase and PSA-luciferase as effectively as either monotherapies (*ns* =not statistically significant ( $P>0.05$ ); Two-way ANOVA post-hoc Tukey's test) (Figure 3.3D & E). However, enzalutamide monotherapy and combination therapy demonstrated significantly greater percent inhibition of fl-AR only activity of ARR3-luciferase reporter than EPI-002 monotherapy ( $P<0.05$ ; Two-way ANOVA post-hoc Tukey's test) (Figure 3.3F). Upon ectopically expressing V7 in the presence of fl-AR, EPI-002 in combination with enzalutamide demonstrated significantly greater inhibition of combined V7 and fl-AR probasin-luciferase and PSA-luciferase reporter activities than enzalutamide monotherapy ( $P<0.01$  and  $P<0.0001$ , respectively; Two-way ANOVA post-hoc Tukey's test) (Figure 3.3D & E). EPI-002 with enzalutamide treatment did not show better efficacy than EPI-002 or enzalutamide monotherapies on combined AR species activity with ARR3-luciferase reporter (*ns*; Two-way ANOVA post-hoc Tukey's test) (Figure 3.3F). All 3 AR-driven reporters demonstrated similar percent inhibition trends with fl-AR only activity and combined V7 and fl-AR activities. Overall these findings suggest that EPI-002 contributes most of the inhibition of AR species activity when used in combination with enzalutamide when V7 levels to fl-AR levels are approximately 1:1.

Lastly, microscopy pictures of LNCaP cells treated with EPI-002 and enzalutamide combination therapy were taken prior to cell harvest to evaluate morphology and possible toxicity (Figure 3.4). Treatment with synthetic androgen R1881 caused cells to appear enlarged and rounded, as opposed to the elongated and asteroid shaped vehicle-treated cells, as expected

[141]. Cells treated with combination therapy did not display signs of toxicity. However, notably less cells were present than either monotherapies or DMSO control.

### ***3.3 EPI-002 inhibited endogenously expressed V7 transcriptional activity in the presence of fl-AR.***

After investigating the effect of EPI-002 when ectopic V7 levels to fl-AR are approximately 1:1, EPI-002 was evaluated in human prostate LNCaP95 cells that express endogenous fl-AR, AR V7, and AR splice variants. LNCaP95 cells express a functional fl-AR protein yet their growth is androgen-independent. This cell line was derived from the parental LNCaP and continuously cultured in hormone-depleted CSS media since the year 1995 [50]. Previous immunoblot analyses have shown that LNCaP95 cells express AR variants at levels 1/10<sup>th</sup> the amount of fl-AR in androgen-deprived conditions, and upon R1881 addition levels of V7 and AR variants decrease relative to fl-AR [66]. LNCaP95 cells were pre-treated with EPI-002 (25 µM), enzalutamide (5 µM), EPI-002 and enzalutamide (25 µM and 5 µM, respectively) combination therapy or DMSO as control then treated with 1 nM R1881 or vehicle 16 hours later. After 48 hours in serum-free conditions cells were harvested for protein expression and luciferase activity. Levels of AR splice variants and fl-AR in treated LNCaP95 cells were measured by immunoblot analysis to examine effects from treatments (Representative Figure 3.5). Immunoblots were probed with AR-NTD directed antibody and equal loading was determined with probing for β-actin protein. Endogenous AR splice variant and V7 protein levels were expressed at approximately 1/10th the amount of fl-AR, similar to previous studies [66, 119]. Furthermore, AR variant and V7 protein expression increased with suppression of fl-AR signaling upon vehicle treatment and following enzalutamide treatment, which coincides with findings of other investigators [66].

To characterize transcriptional activities of endogenous V7 and AR variants in the presence of fl-AR LNCaP95 cells were transfected with three AR-driven reporters: probasin-luciferase, PSA-luciferase or ARR3-luciferase. EPI-002 monotherapy effectively inhibited all AR activity and in combination with enzalutamide in the presence and absence of R1881 (Representative Figures 3.6A-C). Contrary to expected, AR variant and V7 transcriptional activities were almost negligible in the absence of R1881 compared to combined LBD-stimulated fl-AR and V7 activity, which has also been seen in published findings [66]. Since LNCaP95 cell growth is androgen-independent and assumed to be driven by constitutively active AR splice variant signaling, endogenous V7 basal luciferase levels in the absence of R1881 were expected to be higher than observed in the reporter assays.

To determine if combination therapy is better than either monotherapy on endogenously expressed mixed population of V7 and fl-AR, percent inhibition of R1881-treated transcriptional activities was calculated for at least three independent experiments for each AR-driven reporter. Statistical analyses were performed by GraphPad Prism. EPI-002 in combination with enzalutamide demonstrated greater percent inhibition of combined fl-AR and endogenous AR variant activities of probasin-luciferase, PSA-luciferase, and ARR3-luciferase reporters than EPI-002 monotherapy ( $P < 0.0001$  for all 3 reporters; One-way ANOVA post-hoc Sidak's test) (Figure 3.6D-F). In the presence of R1881, these results suggest that enzalutamide contributes most of the inhibition of AR activity when V7 levels are much lower than fl-AR. Combination of EPI-002 with enzalutamide was no better than enzalutamide monotherapy when endogenous V7 levels are much lower than fl-AR levels with androgen responsive gene reporters.

### ***3.4 EPI-002 and enzalutamide combination therapy inhibits transcriptional activities of V7 at all varied levels relative to fl-AR.***

Thus far it has been demonstrated that EPI-002 inhibits V7 transcriptional activity when levels are 1:1 relative to fl-AR and when V7 is expressed much lower than fl-AR. To evaluate the efficacy of combination therapy at varied levels of V7 relative to fl-AR LNCaP95 cells were transiently transfected with V7 expression vector at "low ectopic V7 levels" and "high ectopic V7 levels". Desired levels of low ectopic V7 expression were visibly little to no different than empty vector transfected levels of variant AR on an immunoblot, yet reporter assays showed increased transcriptional activity. Moreover, desired levels of high ectopic V7 expression were visible at approximately 1:1 levels with fl-AR on an immunoblot. To achieve varied levels of V7 relative to fl-AR, LNCaP95 cells were transfected with constant concentrations of low V7 plasmid DNA (0.03 µg/plate in a 12 well plate) or high V7 plasmid DNA (1.2 µg/plate). Cells were pre-treated with EPI-002 (25 µM), enzalutamide (5 µM), EPI-002 and enzalutamide (25 µM and 5 µM, respectively) combination therapy or DMSO as control and treated with 1 nM R1881 or vehicle 16 hours later. After 48 hours in serum-free conditions cells were harvested for protein expression and luciferase activity. Levels of V7 relative to fl-AR in LNCaP95 cell lysates were analyzed by immunoblot. Western blots were probed with an antibody directed to the AR NTD and with an AR V7 antibody (Representative Figure 3.7A&B). Anti-AR V7 antibody detects an epitope specific to the unique 16 amino acids long cryptic exon 3b. Equal loading was determined by normalization to β-actin housekeeping protein for both AR NTD and AR V7 antibodies. Densitometric quantification of AR levels confirmed that low ectopic V7 levels in the absence of R1881 were approximately 21% of fl-AR expression, which were close to endogenous V7 and AR variant expression levels at approximately 9% (Figure 3.7A). High

ectopic AR V7 levels constituted approximately 88% of fl-AR levels and were visibly approximately 1:1 on an immunoblot. With the treatment of R1881, AR variant levels decreased relative to fl-AR as expected (endogenous V7 at 4%, low ectopic V7 at 6%, and high ectopic V7 at 74% to fl-AR levels) (Figure 3.7B). Both high and low ectopic V7 levels were within physiological range. The anti-AR V7 antibody validated that V7 protein in LNCaP95 contains the alternatively spliced cryptic exon 3b of the AR gene and that it was not a degradation product of fl-AR protein.

Transcriptional activity of varied levels of V7 relative to fl-AR in LNCaP95 cells were measured using three AR-driven reporters: probasin-luciferase, PSA-luciferase or ARR3-luciferase (Representative Figures 3.8A-C). EPI-002 monotherapy and in combination with enzalutamide inhibited all AR transcriptional activity at varied levels of V7 expression (Figures 3.8A-C). At low levels of ectopic V7, basal V7 transcriptional activity with all 3 AR reporters increased in the absence of R1881. Though upon addition of R1881, transcriptional activity was predominantly fl-AR which was expected since fl-AR protein levels are much higher than V7 levels. With low ectopic V7 levels in the presence of R1881, probasin reporter activity was higher than empty vector control (Figure 3.8A), whereas PSA and ARR3 reporter activities did not change relative to empty vector control (Figure 3.8B & C). At high levels of ectopic V7, basal V7 transcriptional activity further increases in the absence of R1881 compared to low ectopic V7 levels. As expected, with levels of V7 approximately 1:1 with fl-AR the combined transcriptional activity was predominantly V7 driven. Similar to results in LNCaP cells, higher levels of V7 showed suppressed PSA reporter activity and enhanced ARR3 reporter activity (Figure 3.8B & C).

To determine if EPI-002 in combination with enzalutamide inhibits mixed populations of fl-AR and V7 activities, percent inhibition of R1881-treated transcriptional activities was calculated for at least three independent experiments for each AR-driven reporter. Statistical analyses were performed by GraphPad Prism. Results showed that the level of V7 relative to fl-AR had an impact on the efficacy of both monotherapies in LNCaP95 cells (Figures 3.9A-C). In the presence of androgen at low ectopic V7 levels, EPI-002 in combination with enzalutamide demonstrated greater percent inhibition than EPI-002 monotherapy for probasin-luciferase and ARR3-luciferase ( $P < 0.001$  for both; Two-way ANOVA post-hoc Tukey's test) (Figure 3.9A & C). However, at high ectopic V7 levels EPI-002 in combination with enzalutamide showed greater percent inhibition than enzalutamide monotherapy for probasin-luciferase, PSA-luciferase and ARR3-luciferase ( $P < 0.0001$ ,  $P < 0.05$ , and  $P < 0.001$ , respectively; Two-way ANOVA post-hoc Tukey's test). Similar to results in LNCaP cells, percent inhibition with combination therapy was no greater than EPI-002 monotherapy when V7 is expressed 1:1 with fl-AR (*ns* for all AR-driven reporters; Two-way ANOVA post-hoc Tukey's test). Most importantly these results show that EPI-002 and enzalutamide combination therapy inhibits mixed AR species at varied levels of V7 relative to fl-AR in the presence of androgen.

Since the effect of both monotherapies and combination therapy on R1881-treated mixed AR population transcriptional activities were determined, it was of interest to investigate the effect of inhibitors on constitutive AR variant activity in the absence of androgen. The same three independent experiments used for percent inhibition were analyzed for percent AR variant activity only in the absence of R1881 for each AR-driven reporter (Figure 3.9D-F). Constitutive DMSO treated AR variant activities were set to 100% for each level of V7. Thus, upon reference of the figures as V7 expression increases from empty vector to high ectopic V7 so does the fold

induction. Statistical analyses were performed by GraphPad Prism to determine effect of both monotherapies and combination therapy compared to DMSO control. Probasin reporter was induced moderately by increasing levels of V7 (fold induction: empty vector at  $1\pm 0$ , low ectopic V7 at  $26.48\pm 9.76$ , high ectopic V7 at  $215.63\pm 66.14$ ), whereas ARR3 reporter was enhanced by increased V7 levels (fold induction: empty vector at  $1\pm 0$ , low ectopic V7 at  $390.24\pm 225.50$ , high ectopic V7 at  $5170.02\pm 508.54$ ) and PSA barely induced, for one experiment was suppressed, PSA reporter with increasing levels of V7 (fold induction: empty vector at  $1\pm 0$ , low ectopic V7 at  $4.92\pm 5.25$ , high ectopic V7 at  $7.63\pm 6.18$ ). At endogenous AR variant levels neither monotherapies or combination therapy significantly inhibited AR constitutive activity compared to DMSO control for probasin-luciferase and PSA-luciferase (Figure 3.9D&E). Constitutive endogenous AR activity with ARR3-luciferase was decreased by enzalutamide monotherapy and combination therapy ( $P<0.05$  and  $P<0.001$ , respectively; Two-way ANOVA post-hoc Dunnett's test) (Figure 3.9F). At low ectopic V7 levels neither monotherapies or combination therapy significantly decreased AR constitutive activity with probasin-luciferase and ARR3-luciferase compared to DMSO control activity (Figure 3.9D & F). With the PSA reporter EPI-002 monotherapy was the only inhibitor to significantly decrease low V7 activity in the absence of androgen ( $P<0.05$ ; Two-way ANOVA post-hoc Dunnett's test) (Figure 3.9E). With high ectopic V7 levels in the absence of R1881 EPI-002 monotherapy decreased AR variant percent activity with PSA-luciferase and ARR3-luciferase reporters ( $P<0.05$  for both; Two-way ANOVA post-hoc Dunnett's test) (Figure 3.9E & F). Whereas, high ectopic V7 levels treated with combination therapy in the absence of androgen decreased both probasin-luciferase and PSA-luciferase activities ( $P<0.05$  and  $P<0.01$ ; Two-way ANOVA post-hoc Dunnett's test) (Figure 3.9D & E). Overall the results suggest the effect of monotherapies and combination therapy on constitutive

AR activities depends on AR-driven reporter gene, the level of V7 expressed, and androgen status.

**3.5 EPI-002 alone and in combination with enzalutamide inhibits androgen-independent growth of cells expressing endogenous V7, whereas enzalutamide has no effect.**

Results shown here have demonstrated that combination therapy inhibits fl-AR and AR variant transcriptional activities. Next the effect of combination therapy was evaluated on cell proliferation using a castrate-resistant cell line. The Sadar group previously showed that EPI-001 blocks fl-AR driven androgen-sensitive proliferation of LNCaP cells in the presence of R1881 [129]. EPI-001 also blocked cell proliferation of androgen-independent human prostate 22Rv1 cells, which express fl-AR and many AR splice variants, whereas antiandrogen bicalutamide had no effect [49, 129]. To evaluate whether combination therapy with EPI-002 and enzalutamide would be greater than either monotherapies cell growth was measured using LNCaP95 cells. LNCaP95 growth is mediated by AR splice variant transcriptional activity. LNCaP95 cells were pre-treated with EPI-002 (25  $\mu$ M), enzalutamide (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO control and treated 5 hours later with 0.1 nM R1881 or vehicle control. Cells were kept in serum-free conditions and BrdU (5-bromo-2'-deoxyuridine) labeling reagent was added to the cells for 2 hours at 48 hours post-R1881 addition. BrdU is a thymidine analogue that is incorporated into newly synthesized DNA upon replication of proliferating cells. Cells were dried and immunohistochemistry was performed for three independent experiments using enzyme-linked immunosorbent assay (ELISA). Statistical analyses were performed by GraphPad Prism to compare the effect of inhibitors to each other and to DMSO control. As expected EPI-002 monotherapy blocked cell proliferation of LNCaP95 cells in the presence and absence of R1881, with enzalutamide monotherapy having no effect ( $P < 0.001$  and *ns*, respectively; Two-way ANOVA post-hoc Sidak's test) (Figure 3.10A). Combination therapy effectively inhibited cell proliferation in the presence and absence of

R1881, but was not statistically greater than EPI-002 monotherapy ( $P < 0.0001$  and *ns*, respectively; Two-way ANOVA post-hoc Sidak's test). Based on doubling time and limitations of confluency at 72 hours, 48 hours was selected for measuring proliferation (Figure 3.10B).

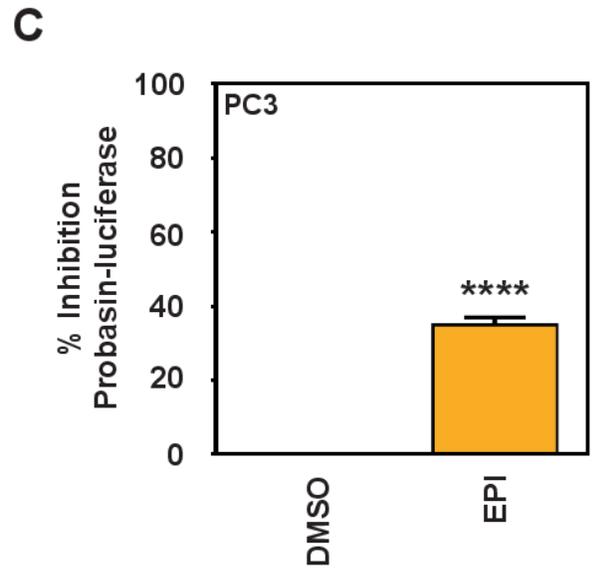
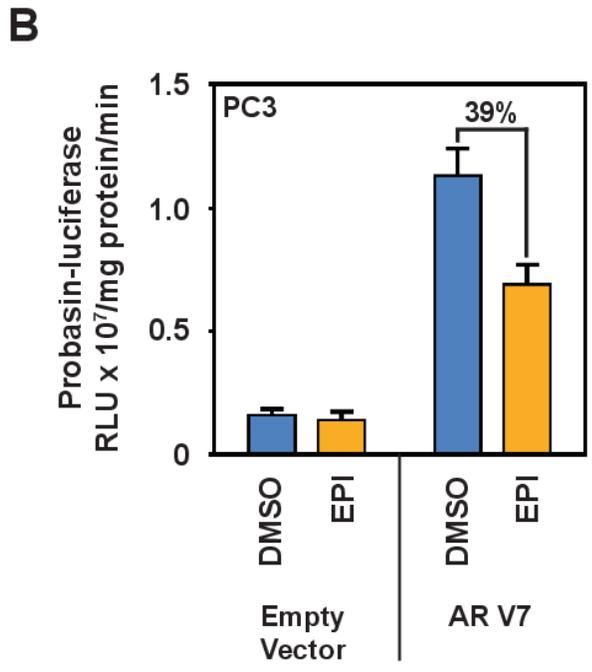
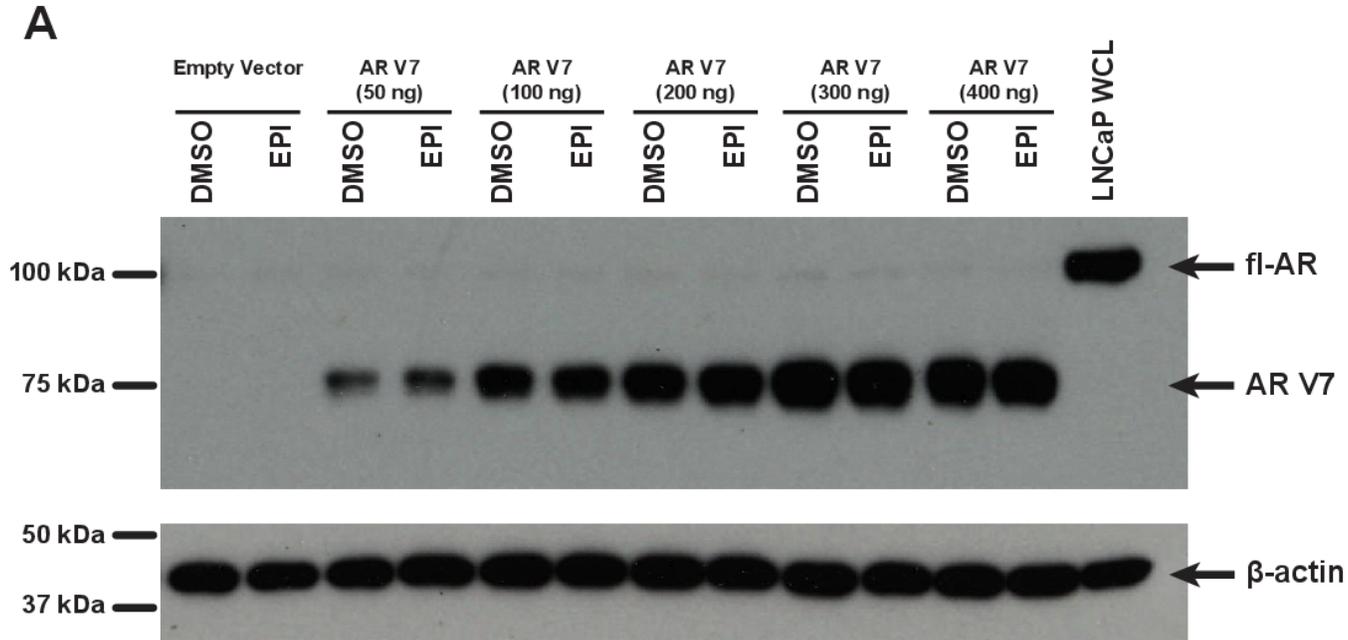
**Figure 3.1. EPI-002 inhibited constitutive transcriptional activity of AR V7 ectopically expressed in PC3 cells.**

A) Levels of AR V7 (100 ng) ectopically expressed in PC3 cells were comparable to level of endogenous fl-AR in whole cell lysate (WCL) from LNCaP cells. Cells were treated with EPI-002 (25  $\mu$ M) or DMSO as control. A representative Western Blot with 10  $\mu$ g of protein loaded to each lane. Western Blot analyses was conducted for at least 3 independent experiments. AR proteins were detected using AR N20 antibody. Equal loading was determined by normalization to  $\beta$ -actin housekeeping protein. Empty vector pcDNA3 as control.

B) Transcriptional activity of AR V7 in PC3 cells using probasin-luciferase reporter. Cells treated with EPI-002 (25  $\mu$ M) or DMSO as control. Four independent experiments conducted. Error bars represent  $\pm$  standard deviation (SD) with 3 technical replicates from one representative. Relative luminescence units (RLU) and empty vector pcDNA3 as control.

C) The average percent (%) inhibition of AR V7 transcriptional activity with probasin-luciferase reporter. DMSO treated constitutive AR V7 activity was set to 100% to calculate % inhibition. Four independent experiments conducted. Error bars represent  $\pm$  standard error of mean (SEM). Unpaired Student's *t*-test. \*\*\*\*  $P \leq 0.0001$

Figure 3.1



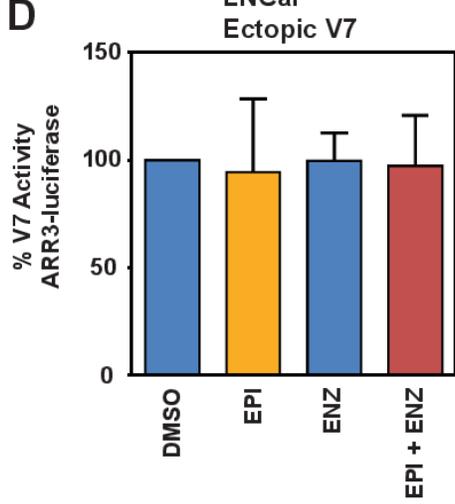
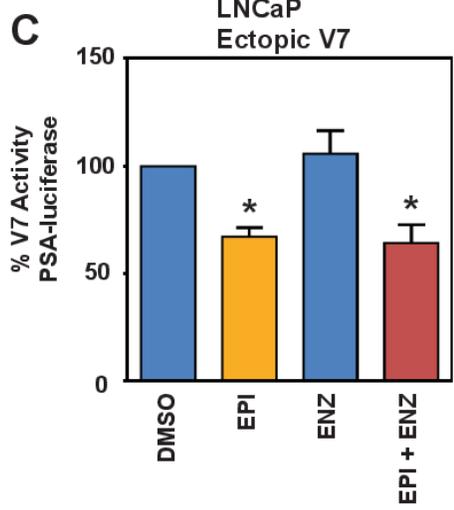
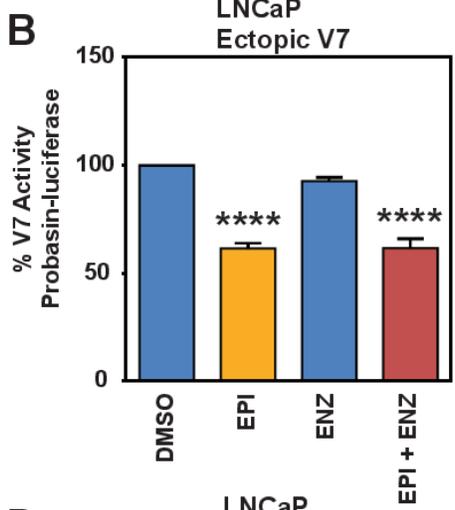
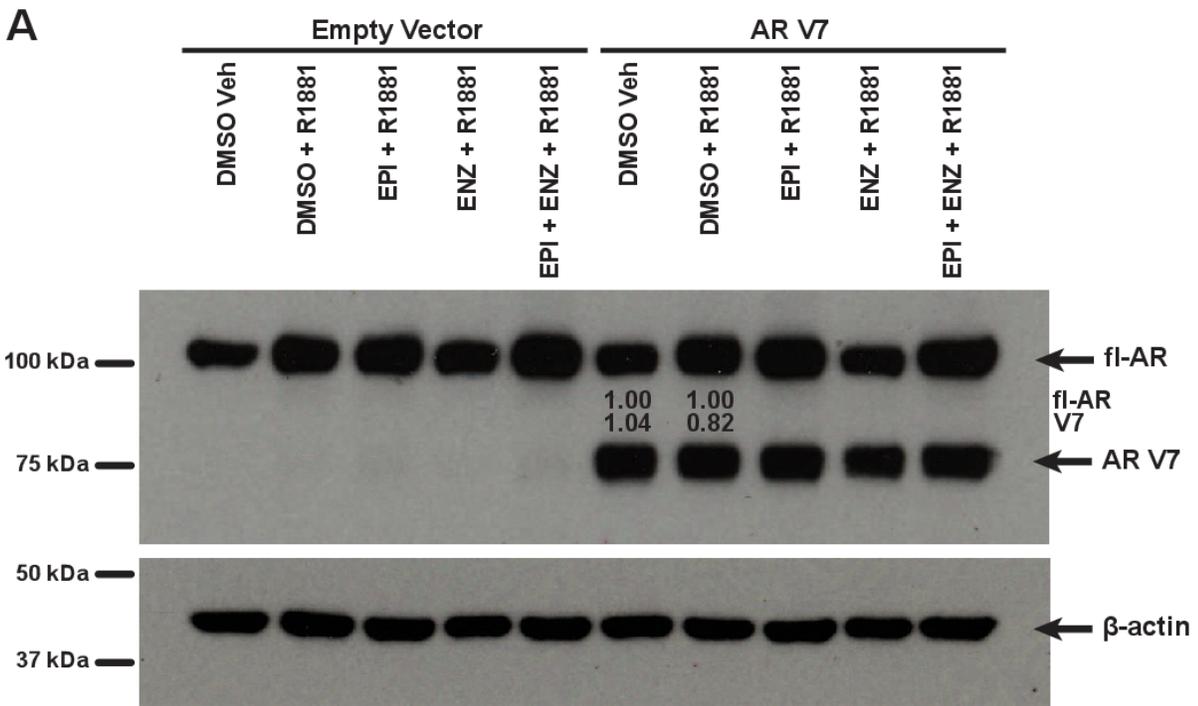
**Figure 3.2. Levels of AR V7 in LNCaP cells were comparable to physiological levels.**

A) Levels of AR V7 ectopically expressed in LNCaP cells were comparable to endogenous fl-AR levels. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Proteins were analyzed in the absence (Veh) and presence of R1881 (1 nM).

Densitometric quantification of relative AR levels was determined by ImageJ. A representative Western Blot with 5  $\mu$ g of protein loaded to each lane. Western Blot analyses was performed for at least 3 independent experiments. AR proteins were detected using AR N20 antibody. Equal loading was determined by normalization to  $\beta$ -actin housekeeping protein. Empty vector pcDNA3 as control.

B) Probasin-luciferase, C) PSA-luciferase, & D) ARR3-luciferase. The average percent (%) transcriptional activity of AR V7 in the absence of R1881 ectopically expressed in LNCaP cells. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Constitutive AR variant DMSO treated activities were set to 100%. Error bars for all graphs represent  $\pm$  SEM from three independent experiments. Two-way ANOVA, post-hoc Dunnett's multiple comparisons test. *ns* = not statistically significant; \* $P$ <0.05; \*\*\*\* $P$ ≤0.0001

Figure 3.2

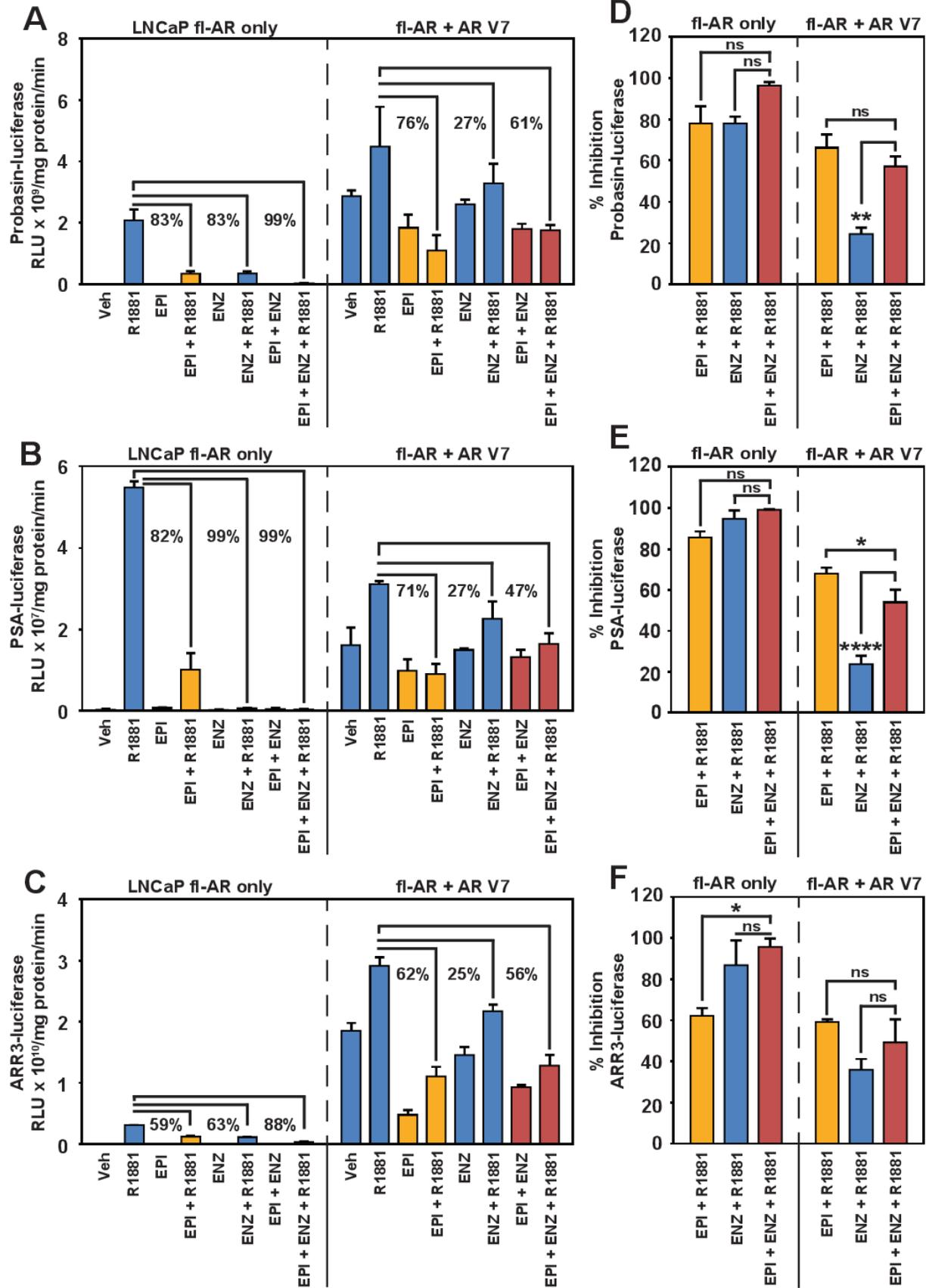


**Figure 3.3. EPI-002 alone and in combination with enzalutamide inhibited transcriptional activities of fl-AR only, and fl-AR with ectopic AR V7 (1:1) in LNCaP cells.**

A) Probasin-luciferase, B) PSA-luciferase, & C) ARR3-luciferase. Transcriptional activity of ectopic AR V7 in LNCaP cells. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Relative luminescence units (RLU) measured in the absence (Veh) and presence of R1881 (1 nM). Minimum of 3 independent experiments conducted for all 3 AR-driven luciferase reporters. Error bars represent  $\pm$  SD with 3 technical replicates from one representative experiment. LNCaP fl-AR only samples transfected with empty vector pcDNA3 as control.

D) Probasin-luciferase, E) PSA-luciferase, & F) ARR3-luciferase. The average percent (%) inhibition of R1881-treated fl-AR activity only and R1881-treated combined transcriptional activities of fl-AR and AR V7 in LNCaP cells. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. R1881 at 1 nM. R1881-treated fl-AR and R1881-treated fl-AR with V7 DMSO treated activities were set to 100% (not plotted) to calculate % inhibition of drugs. Error bars represent  $\pm$  SEM for three independent experiments. LNCaP fl-AR only samples transfected with empty vector pcDNA3 as control. Two-way ANOVA, post-hoc Tukey's multiple comparisons test. *ns* = not statistically significant; \* $P$ <0.05; \*\* $P$ <0.01; \*\*\*\* $P$ <0.0001

Figure 3.3



**Figure 3.4. LNCaP cells displayed no obvious signs of toxicity with EPI-002 and enzalutamide combination treatment.**

LNCaP cells treated with EPI-002 (25  $\mu$ M), enzalutamide (5  $\mu$ M), EPI-002 and enzalutamide in combination (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO control. Cells visualized in the absence (Veh) or presence of R1881 (1 nM). Microscopy pictures taken at 400X magnification just prior to cell harvest of luciferase activities (48 hours post-treatment). R1881-treated cells appeared enlarged and rounded (\*), as opposed to the elongated and asteroid shaped (arrow) vehicle-treated control cells. Empty vector pcDNA3 as control.

Figure 3.4

LNCaP Morphology

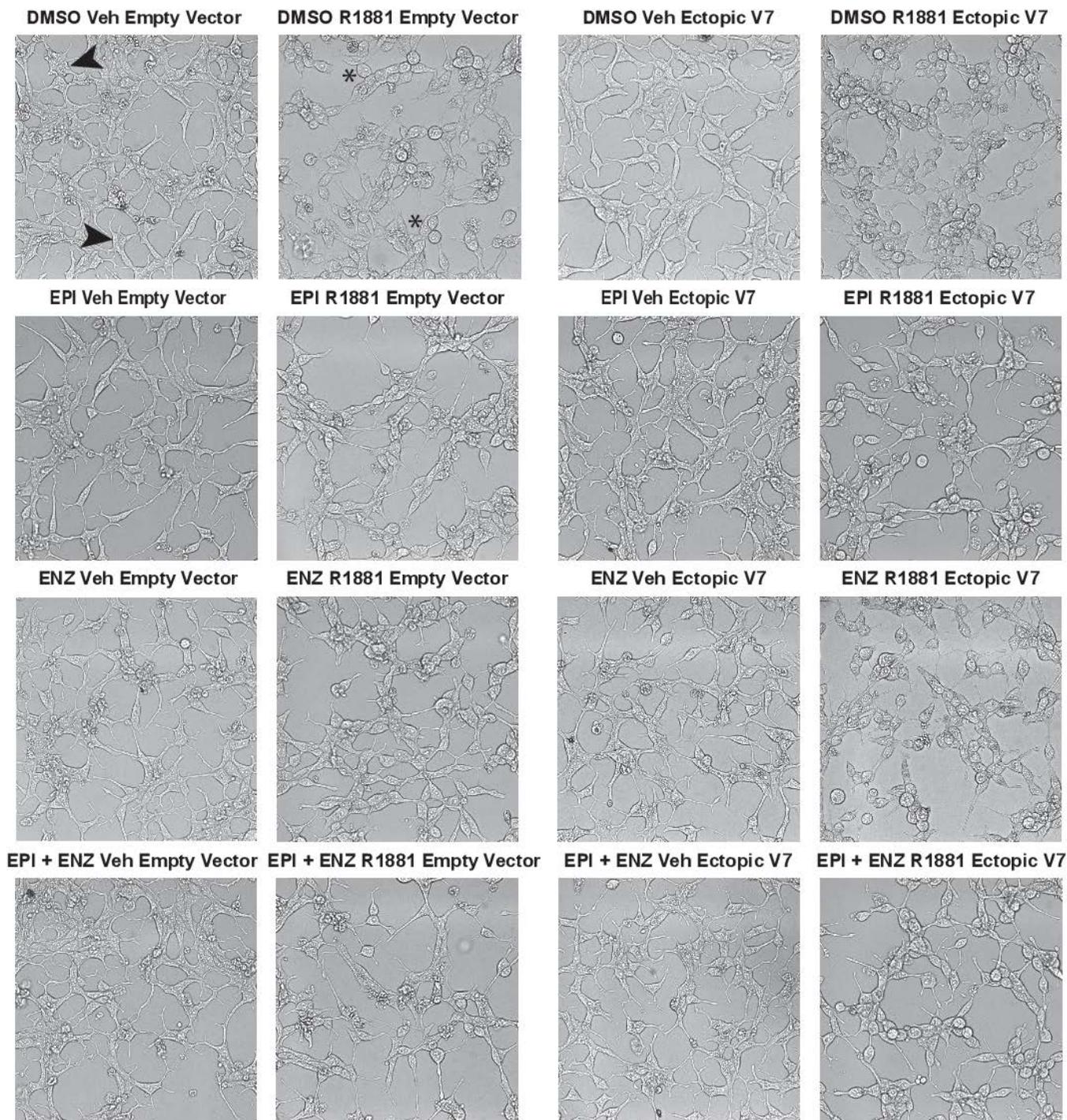
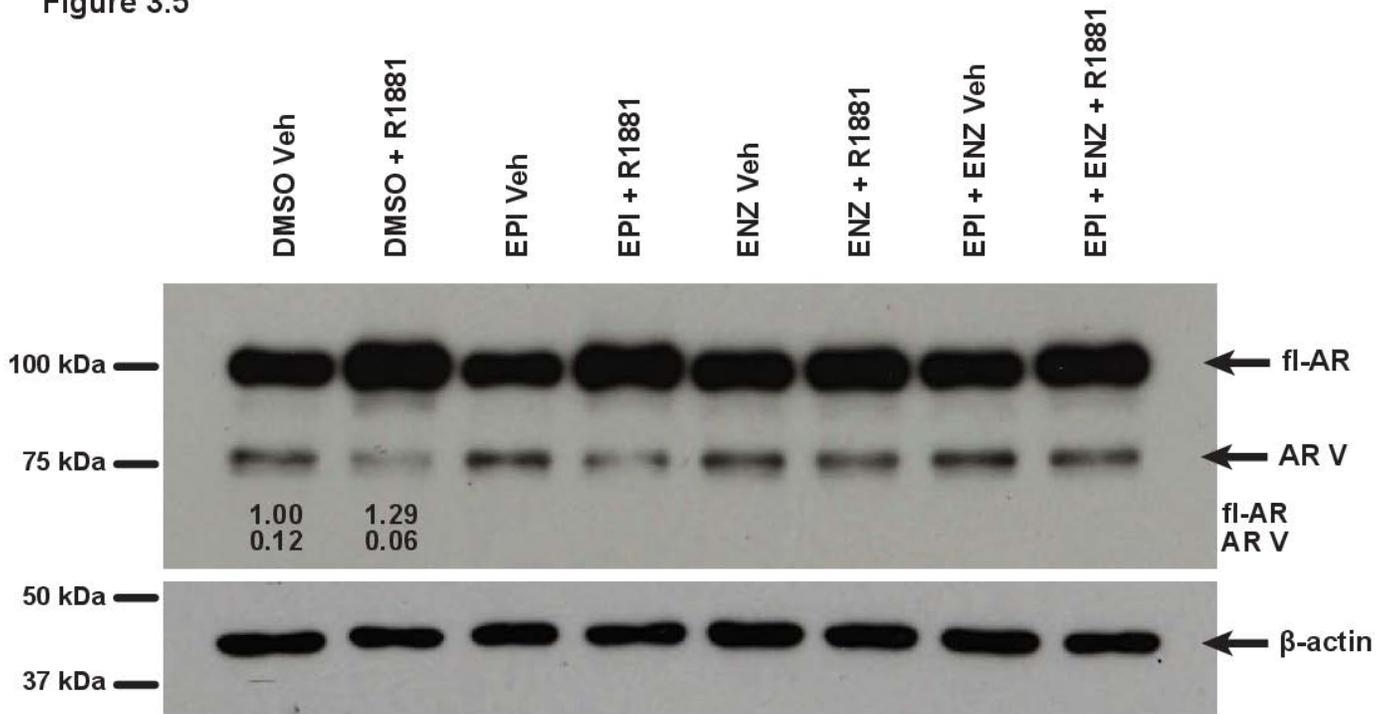


Figure 3.5



**Figure 3.5. Levels of AR variants in androgen-independent LNCaP95 cells.**

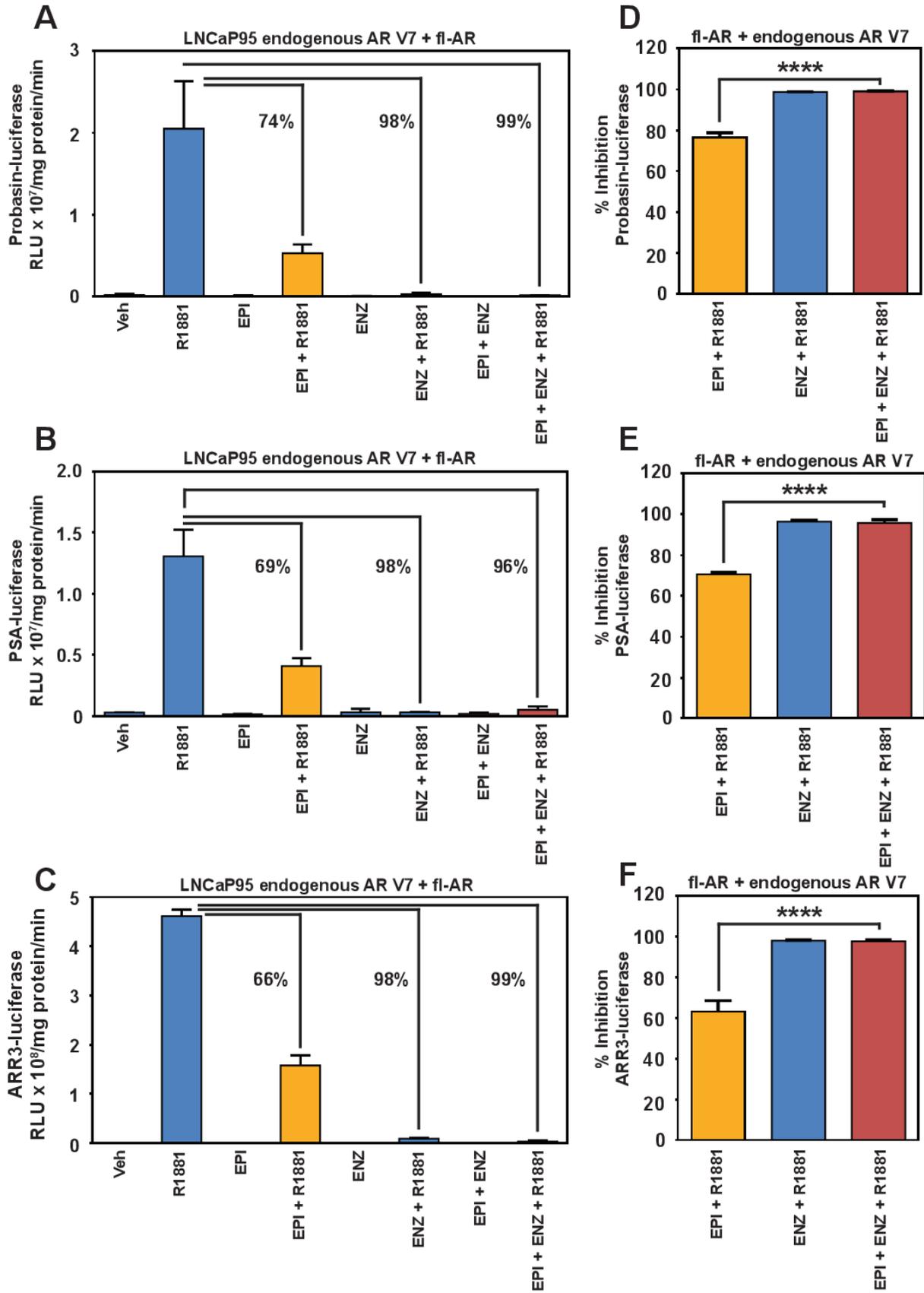
Levels of AR splice variants (AR V) endogenously expressed in LNCaP95 cells were approximately 1/10th of fl-AR protein levels. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Proteins were analyzed in the absence (Veh) and presence of R1881 (1 nM). Western Blots were loaded with 5  $\mu$ g of protein to each lane. Western Blot analyses was performed for at least 3 independent experiments. AR proteins were detected using AR N20 antibody. Densitometric quantification of relative AR levels was determined by ImageJ. Equal loading was determined by normalization to  $\beta$ -actin housekeeping protein.

**Figure 3.6. EPI-002 inhibited endogenous AR V7 and fl-AR combined transcriptional activities alone and in combination with enzalutamide in LNCaP95 cells.**

A) Probasin-luciferase, B) PSA-luciferase, & C) ARR3-luciferase. Transcriptional activity of endogenous AR V7 in LNCaP95 cells. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Relative luminescence units (RLU) measured in the absence (Veh) and presence of R1881 (1 nM). Minimum of 3 independent experiments conducted for all 3 AR-driven luciferase reporters. Error bars represent  $\pm$  SD with 3 technical replicates from one representative experiment.

D) Probasin-luciferase, E) PSA-luciferase, & F) ARR3-luciferase. The average percent (%) inhibition of R1881-treated combined transcriptional activities of fl-AR and endogenous AR V7 in LNCaP95 cells. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. R1881 at 1 nM. R1881-treated fl-AR with AR variant DMSO treated activities were set to 100% (not plotted) to calculate % inhibition of drugs. Error bars represent  $\pm$  SEM from three independent experiments. Two-way ANOVA, post-hoc Tukey's multiple comparisons test. *ns* = not statistically significant; \*\*\*\**P*<0.0001

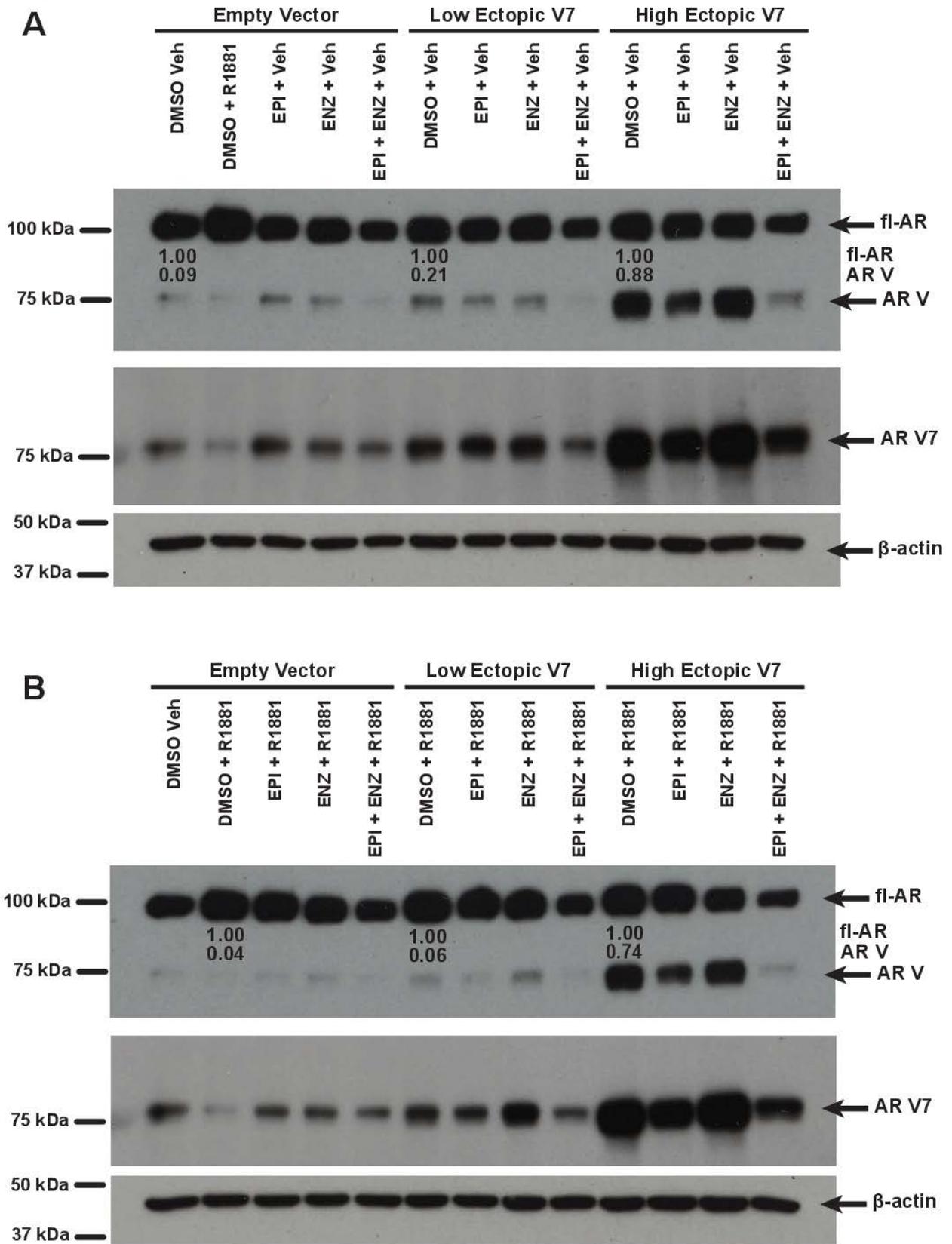
Figure 3.6



**Figure 3.7. V7 protein endogenously and exogenously expressed in LNCaP95 cells is alternatively spliced AR variant and not a degradation product of fl-AR.**

A) Ethanol (Veh) treated samples. B) R1881 (1 nM) treated samples. Levels of varied AR V7 ectopically expressed in LNCaP95 cells (from left to right): endogenous V7 levels with empty vector (pcDNA3), low ectopic V7 levels (0.03 µg/plate of AR V7 plasmid), and high ectopic V7 levels (1.2 µg/plate of AR V7 plasmid). Cells were treated with EPI-002 monotherapy (25 µM), enzalutamide monotherapy (5 µM), EPI-002 and enzalutamide combination therapy (25 µM and 5 µM, respectively) or DMSO as control. Densitometric quantification of relative AR levels was determined by ImageJ. A representative Western Blot with 5 µg of protein loaded to each lane. Western Blot analyses was performed for at least 3 independent experiments. AR variants (AR V) and fl-AR proteins were detected using AR N20 antibody (top blots of A and B). AR V7 only was detected using anti-AR V7 antibody (middle blots of A and B). Equal loading was determined by normalization to β-actin housekeeping protein (bottom blots of A and B).

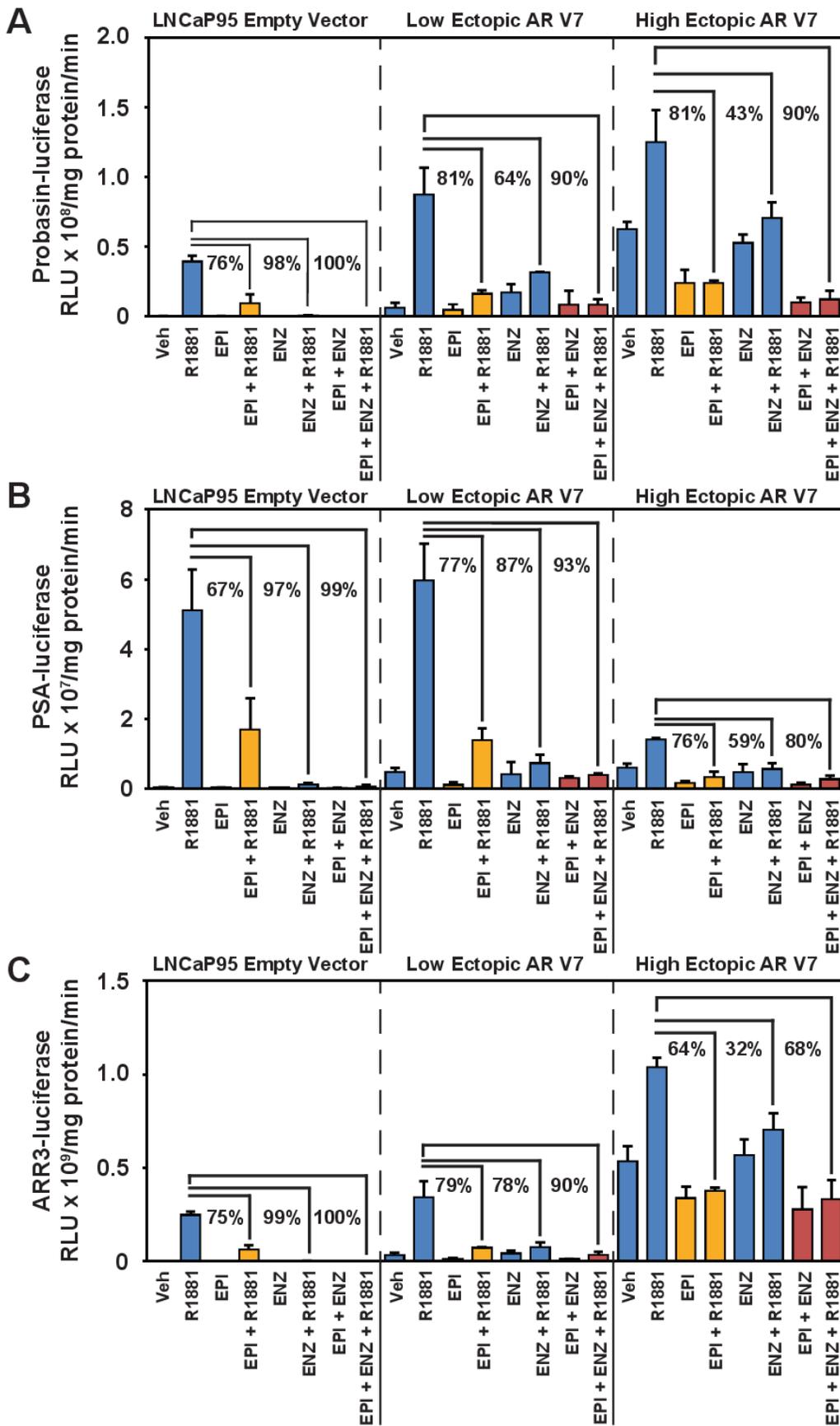
Figure 3.7



**Figure 3.8. Levels of V7 relative to fl-AR have an impact on efficacy of either monotherapies in LNCaP95 cells.**

A) Probasin-luciferase, B) PSA-luciferase, & C) ARR3-luciferase. Transcriptional activity of varied levels of AR V7 ectopically expressed in LNCaP95 cells (from left to right): endogenous V7 levels with empty vector (pcDNA3), low ectopic V7 levels, and high ectopic V7 levels. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Relative luminescence units (RLU) measured in the absence (Veh) and presence of R1881 (1 nM). Minimum of 3 independent replicates conducted for all 3 AR-driven luciferase reporters. Error bars represent  $\pm$  SD with 3 technical replicates from one representative experiment.

Figure 3.8

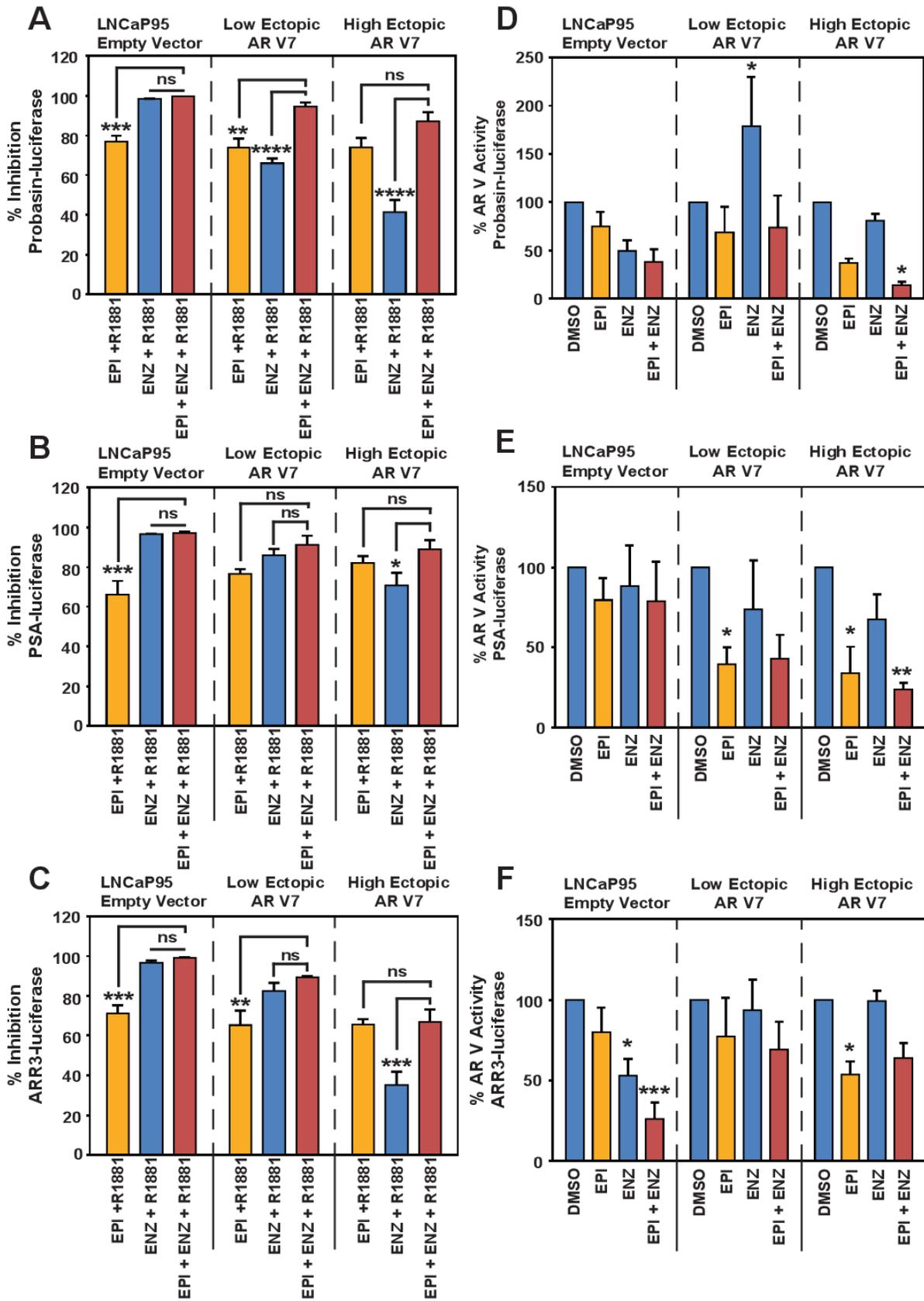


**Figure 3.9. EPI-002 and enzalutamide combination therapy inhibits all AR species at varied levels of V7 relative to fl-AR in LNCaP95 cells.**

A) Probasin-luciferase, B) PSA-luciferase, & C) ARR3-luciferase. The average percent (%) inhibition of R1881-treated combined transcriptional activities of fl-AR and varied levels of AR V7 ectopically expressed in LNCaP95 cells. From left to right: endogenous V7 levels with empty vector (pcDNA3), low ectopic V7 levels, and high ectopic V7 levels. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. R1881 at 1 nM. R1881-treated fl-AR with constitutive AR variant DMSO treated activities were set to 100% (not plotted) to calculate % inhibition of drugs. Error bars represent  $\pm$  SEM for three independent experiments. Two-way ANOVA, post-hoc Tukey's multiple comparisons test. *ns* = not statistically significant; \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001; \*\*\*\* $P$ <0.0001

D) Probasin-luciferase, E) PSA-luciferase, & F) ARR3-luciferase. The average percent (%) transcriptional activity of varied AR variant (AR V) levels in the absence of R1881 ectopically expressed in LNCaP95 cells. From left to right: endogenous V7 levels with empty vector (pcDNA3), low ectopic V7 levels, and high ectopic V7 levels. Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Constitutive AR variant DMSO treated activities were set to 100% for each expression level of V7. Fold induction of DMSO comparator increases with the level of V7 (i.e. DMSO control for empty vector < low ectopic V7 < high ectopic V7). Error bars represent  $\pm$  SEM from three independent experiments. Two-way ANOVA, post-hoc Dunnett's multiple comparisons test. *ns* = not statistically significant; \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001

Figure 3.9

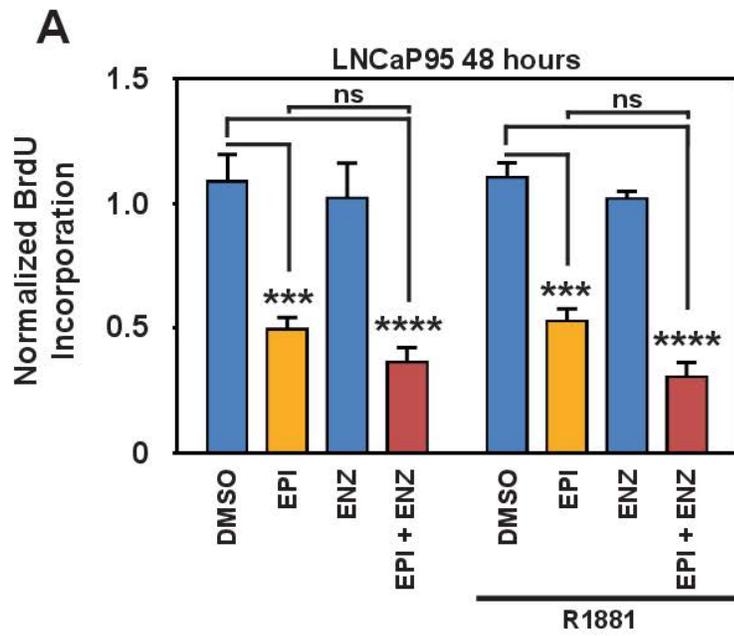


**Figure 3.10. LNCaP95 cell proliferation was inhibited by EPI-002 alone and in combination with enzalutamide, with enzalutamide monotherapy having no effect.**

A) LNCaP95 cell proliferation in the presence and absence of R1881 (0.1 nM). Cells were treated with EPI-002 monotherapy (25  $\mu$ M), enzalutamide monotherapy (5  $\mu$ M), EPI-002 and enzalutamide combination therapy (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO as control. Error bars represent  $\pm$  SEM from three independent experiments. Two-way ANOVA, post-hoc Sidak's multiple comparisons test. *ns* = not statistically significant; \*\*\* $P$ <0.001; \*\*\*\* $P$ <0.0001

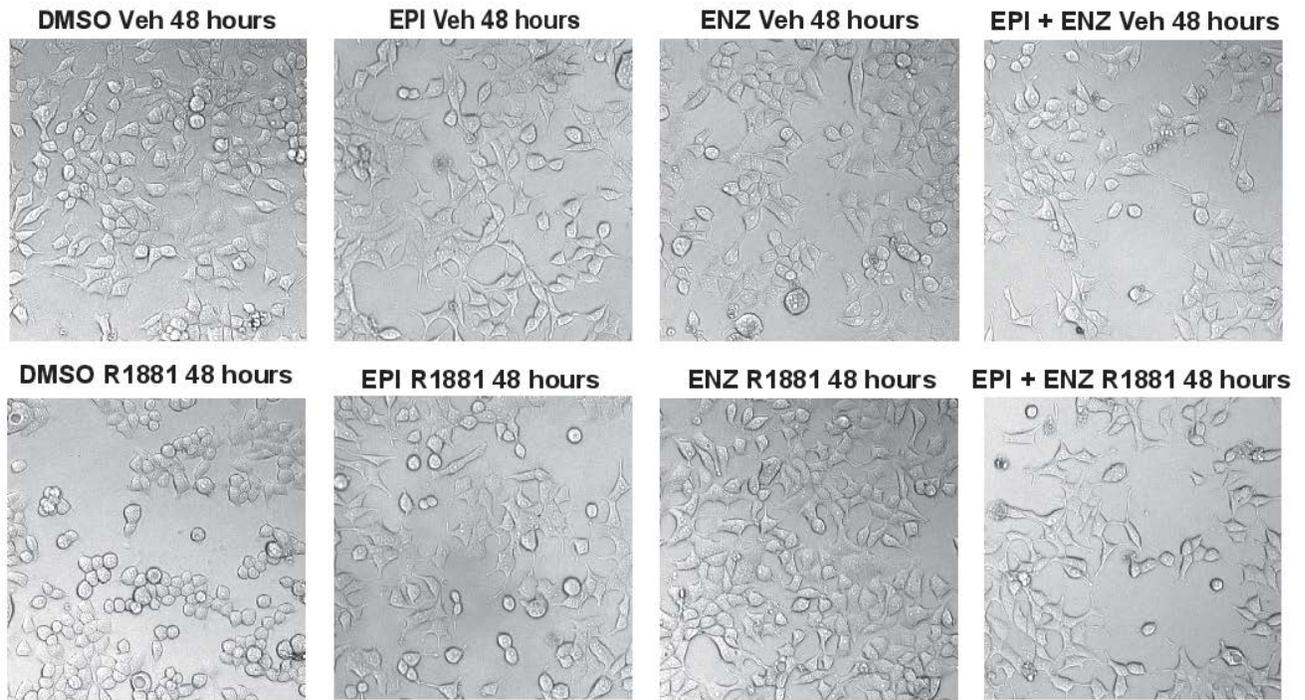
B) LNCaP95 cells treated with EPI-002 (25  $\mu$ M), enzalutamide (5  $\mu$ M), EPI-002 and enzalutamide in combination (25  $\mu$ M and 5  $\mu$ M, respectively) or DMSO control. Cells visualized in the absence (Veh) and presence of R1881 (0.1 nM) at 48 hours post-treatment. Microscopy pictures taken at 100X magnification prior to BrdU addition. Cells treated with combination therapy did not display apparent signs of toxicity.

Figure 3.10



**B**

LNCaP95 Cell Proliferation



## Chapter 4: Discussion

### *4.1 EPI-002 and V7 transcriptional activity*

Currently there is no cure for CRPC. Unfortunately, most men who are diagnosed with metastatic CRPC succumb within approximately 2 years. The AR adapts many mechanisms of resistance to evade blockade from hormonal therapies and continue its transactivation of survival genes [35, 40]. The pitfall of clinically available hormonal therapies is that they target the AR LBD and not the NTD [81, 142]. Enzalutamide and abiraterone both prevent, directly or indirectly, DHT interaction with LBD to inhibit fl-AR transcriptional activity. One resistance mechanism that circumvents this inhibition of drugs targeting the AR LBD is by alternatively splicing the AR pre-mRNA to exclude exons encoding the LBD [49, 50, 104, 107]. As a result of this, translated protein products are truncated and constitutively active [112]. All functional AR species require the NTD [51, 55]. The NTD harbours the transcriptional activity of the AR and drives growth and progression of prostate cancer tumours. The AR remains a central drug target for CRPC since it is expressed and functional in more than 99% of prostate cancers [14]. Thus, it is evident to block the engine of AR, the NTDs of full-length and its splice variants, in order to inhibit growth and induce apoptosis of cancer cells. This would reduce tumour burden and potentially extend survival of men with CRPC. EPI-001 is a first-in-class inhibitor of the AR NTD [129]. EPI compounds bind specifically and covalently to the AF1 region of the AR NTD and block transcriptional activity [84, 129]. Covalent binding of EPI-001 requires some structure of the intrinsically disordered AF1 region [84]. The most potent stereoisomer of EPI-001 mixture is EPI-002 [84].

The first goal of my work was to characterize V7 transcriptional activity in the presence of fl-AR with canonical AR-driven reporters. Prior to assessing mixed populations of V7 and fl-AR, it was first established that EPI-002 blocked AR V7 transactivation alone. To our knowledge, data shown here demonstrated for the first time that EPI-002 can inhibit AR splice variant V7 constitutive transcriptional activity. Fold induction of V7 activity in an AR-negative cell line was comparable to reported values [50, 112]. The focus of this experiment was to show EPI-002 inhibition of V7 at a physiologically and clinically relevant level of expression. In previously published data from Sadar and colleagues, EPI compounds blocked AR V567es and artificially made AR 1-653 aa deletion mutant transcriptional activities with AR-driven reporters [84, 129]. This further supports specificity of EPI-002 to the AF1 region of V7 and could possibly extend to other truncated AR variants yet to be analyzed.

Transcriptional activities of V7 in the presence of fl-AR were then evaluated. The LNCaP cell line was ideal for characterizing exogenous V7 transcriptional activity because V7 protein could be maintained at expression levels equivalent to endogenous fl-AR levels. V7 expression at a ratio of approximately 1:1 with fl-AR is the highest level of V7 detected in CRPC bone metastases [108]. Furthermore, V7 transcriptional activity could be measured in isolation of fl-AR by depriving cells of androgen and theoretically localizing fl-AR to the cytosol. Conversely, supplementing LNCaP cells ectopically expressing V7 with synthetic androgen should localize all AR species to the nucleus and their combined transcriptional activities could be measured. As expected, V7 constitutively activated all three AR-driven reporter genes in the absence of androgen. This was consistent with previously published findings [50, 112, 115]. Interestingly, V7 transcriptional activity in the presence of androgen-stimulated fl-AR demonstrated promoter-dependency. V7 enhanced transactivation of probasin and ARR3 reporter genes greater than fl-

AR induction alone, yet suppressed full androgen induced activation of the PSA reporter gene construct. Similar results were shown by Cao et al. with ectopic V7 attenuation of PSA gene expression in the presence of androgen-induced fl-AR in LNCaP cells [117]. Regardless of V7 gene-dependent activity, EPI-002 effectively inhibited combined transcriptional activities of fl-AR and truncated V7 at approximately 1:1 expression levels with all AR-driven genes tested.

Endogenous V7 transcriptional activity was then assessed in the presence of fl-AR. AR variant protein levels are  $1/10^{\text{th}}$  fl-AR levels in LNCaP95 cells [66]. LNCaP95 was used for characterizing endogenous V7 activity because it has a similar genetic profile to its parental LNCaP cell line, which allows comparison of V7 gene reporter activities more relevant than in 22Rv1 or VCaP cell lines. Though LNCaP95 growth is dependent on constitutive AR splice variant signaling, the fl-AR retains sensitivity to androgen. This enabled characterization of transcriptional activity of low V7 levels in the absence of androgen, and also combined activity in the presence of androgen-stimulated fl-AR. Surprisingly, endogenous V7 basal transcriptional activity was low and barely detectable with AR-driven reporters. V7 transactivation in the absence of androgen was expected to be higher than observed. It was demonstrated that when V7 levels are low compared to fl-AR, differential gene induction of AR-driven genes is not observed. Upon stimulation of androgen, functional fl-AR provided most of the induction of AR-driven reporters. Similar findings were also obtained when Hu et al. employed a PSA gene reporter assay in LNCaP95. Their work revealed weak induction of endogenous V7 in the absence of androgen [66]. Importantly, EPI-002 inhibited combined transcriptional activities of V7 expressed at much lower levels than fl-AR with all canonical AR-driven genes.

To complete characterization of V7 activity, varied levels of ectopic V7 were expressed relative to fl-AR. With increasing concentrations of exogenous V7 in LNCaP95 cells, V7 demonstrated differential gene activation with canonical AR-driven genes similar to results in LNCaP. V7 suppressed transactivation of PSA and enhanced activities of probasin and ARR3 reporters. From this we can propose that canonical AR-gene activation by V7 may be dependent on the amount of protein expressed in the cell relative to fl-AR and also by AREs present in the target gene. In addition, EPI-002 inhibited transcriptional activities of all AR V7 levels relative to fl-AR ectopically expressed in LNCaP95.

To our knowledge, this is the first study to look at transcriptional activation of varied levels of AR V7 relative to fl-AR using multiple AR-driven reporters. Overall, results shown here support the first hypothesis tested that EPI-002 inhibits endogenous and exogenous AR V7 in the presence of fl-AR in various human prostate cancer cell lines. Discovery of an AR NTD transcriptional inhibitor of both the fl-AR and truncated V7 is of importance since currently all clinical therapies target the AR LBD. Blockade of both AR species by EPI-002 in a heterogeneous population could significantly improve survival of CRPC patients, since V7 expression is frequently concomitant with fl-AR in patient metastatic tumours [104, 107].

## ***4.2 Combination therapy and mixed AR populations***

The second goal of my work was to determine if combination therapy of EPI-002 and enzalutamide would demonstrate greater inhibition of transcriptional activity of mixed AR populations than each treatment alone. The efficacy of combination therapy on fl-AR activity was first assessed. LNCaP cells treated with combination therapy generally demonstrated no greater inhibition of fl-AR activity than either monotherapy for all AR-driven reporters. This was not surprising since previously published data demonstrated both drugs strongly inhibit fl-AR transcriptional activity in androgen-sensitive cell lines [81, 129]. Most importantly, cells did not display apparent signs of toxicity indicating specific target binding of drugs to AR. With V7 levels expressed approximately 1:1 with fl-AR, it was demonstrated that combination therapy exhibited greater inhibition of all AR transcriptional activities than enzalutamide monotherapy. However, combination therapy did not demonstrate greater inhibition than EPI-002 monotherapy. Overall, when levels of V7 are expressed at a ratio of 1:1 with fl-AR the NTD covalent-binding EPI-002 compound provided most of the AR inhibition when in combination with enzalutamide.

Combination therapy in LNCaP95 was then evaluated with AR variants and V7 endogenously expressed 1/10<sup>th</sup> the levels of fl-AR. With much lower levels of AR V7 relative to fl-AR, induction of AR-driven reporters was almost all mediated by fl-AR activity since V7 induction was negligible. Combination therapy demonstrated greater inhibition of all AR transcriptional activities than EPI-002 monotherapy. Combination therapy was not greater than enzalutamide monotherapy when V7 levels were much lower than fl-AR. As shown here with

androgen-driven reporters, LBD-targeting enzalutamide provided most of the AR inhibition when in combination with EPI-002 when the mixed AR population is predominantly fl-AR.

Combination therapy was then evaluated with varied levels of V7 ectopically expressed in LNCaP95 relative to fl-AR. At levels of V7 slightly higher than endogenous levels combination therapy generally demonstrated greater inhibition of combined transcriptional activities than EPI-002 monotherapy, though, combination was no greater than enzalutamide monotherapy for all three AR-driven reporters at this expression level of V7. Of significant interest was the shift in efficacy of both monotherapies upon increasing concentrations of V7. At high ectopic levels of V7 in LNCaP95, with V7 expressed at approximately 1:1 with fl-AR, combination therapy effect was significantly greater than enzalutamide monotherapy. High ectopic V7 in LNCaP95 treated with combination did not show greater inhibition of transcriptional activities than EPI-002 monotherapy. The drug efficacy profile shown here was comparable to data obtained in LNCaP cells with high V7.

Lastly, combination therapy was assessed on cell growth of androgen-independent LNCaP95. This was important to confirm downstream inhibition of AR transactivation of survival genes that drive prostate cancer cell growth. Sadar and colleagues previously reported EPI-001 inhibited androgen-independent growth of 22Rv1 cells while antiandrogens were ineffective [129]. Growth of 22Rv1 and LNCaP95 cell lines progress in the presence of antiandrogens because AR splice variants induce cell-cycle genes [49, 66]. In both the presence and absence of androgens, EPI-002 monotherapy and combination therapy inhibited LNCaP95 cell growth compared to control. Inhibition with EPI-002 and enzalutamide in combination was

not statistically different than EPI-002 monotherapy. As expected, enzalutamide monotherapy was ineffective.

Taken as a whole, results shown here broadly do not support the second hypothesis tested that combination therapy inhibits mixed AR transcriptional activities better than either treatment alone. Using AR-driven reporters, the effectiveness of enzalutamide on a mixed AR population appeared to be dependent on the level of V7 expressed. Enzalutamide displayed greater inhibition when AR populations expressed low V7 and transcriptional activity was predominately contributed by fl-AR. However, EPI-002 effectively inhibited all AR species at all levels of V7 tested relative to fl-AR. The ideal CRPC therapy needs to inhibit all NTD-driven AR transcriptional activity, induce apoptosis and reduce tumour growth. Work presented in this thesis demonstrates that combination therapy generally achieved maximum inhibition of AR transcriptional activity and cell growth.

### ***4.3 AR V7 transcriptional landscape in castration-resistant prostate cancer***

Potential reasons why V7 differentially regulates canonical AR-driven reporter genes, such as probasin and PSA, might be due to a spectrum of the following: 1) variation of ARE sequences in the promoter and enhancer regions, 2) possible heterodimer interactions with other steroid nuclear receptors, 3) site-directed recruitment of co-regulators to TAU1 or TAU5 depending on AR species/presence of LBD, and 4) gene-dependent and cell-dependent recruitment of co-regulators to AF1 regions of V7.

The probasin reporter construct encodes -286/+28 bps from the probasin (*PB*) gene and includes AREI (-236 to -223) and AREII (-140 to -117), that function co-operatively in the promoter region [64, 137]. PB-AREII is a direct repeat of the 5'-TGTTCT-3' HRE consensus sequence and has a high affinity and selectivity for AR DBD binding [63, 101]. PB-AREI contains the HRE consensus sequence, but has a low affinity for AR and non-specificity for other hormone receptors [63]. Matusik and colleagues tested -244/+28 probasin gene promoter sequence and artificially-made adjacent duplicates of this sequence and found that both reporters (i.e. ARR<sub>2</sub>PB) were induced by AR and GR activation [143]. In comparison, the PSA reporter construct has -6000/+12 of the *PSA/KLK3* gene with an enhancer (AREIII) that synergistically co-operates with two AREs in the promoter region and is essential for full activation of *PSA/KLK3* promoter activity [131, 136]. PSA-AREI (-170 to -156) and AREIII (-4148 to -4134) have high affinity and selectivity for AR DBD binding, whereas AREII (-395 to -376) has a low affinity binding of AR [136]. Further analysis of the AREIII enhancer region mapped five additional low affinity ARE binding sites, between -4366 to -3870, that work co-operatively as an enhancer to androgen-regulate *PSA/KLK3* gene transcription [144]. Both PR and GR cannot

activate the *PSA/KLK3* enhancer [144]. Overall, the PSA reporter gene requires more AR species bound to its high affinity, AR-specific AREs to co-operatively activate luciferase activity, whereas the probasin reporter gene can be transcriptionally activated by fewer AR species bound to its AREs or co-operatively by other nuclear receptors with conserved DBDs, such as GR or PR. Since V7 retains the full intact DBD as its fl-AR protein, theoretically V7 species would bind to AREs of the PSA reporter gene more readily than probasin. Despite harbouring fewer ARE sequences with high affinity to AR binding, probasin and ARR3 reporter genes are enhanced by V7 whereas PSA reporter gene is suppressed. To note, the rat *PB* and human *PSA/KLK3* genes *in vivo* may have other AREs outside of the reporter constructs used in this study. Thus, V7 differential regulation of canonical AR-driven reporter genes must be due to other factors as well.

AR V7 may form heterodimer interactions with fl-AR protein [49, 116]. Recently in a study by Cao et al. fl-AR was found to localize to the nucleus in the absence of androgen along with AR splice variants, V7 and V567es [117]. This suggests that V7 facilitates nuclear localization of fl-AR and that there is a possible physical interaction. Further studies with chromatin binding on AREs showed that V7 and fl-AR co-occupy the *PSA/KLK3* promoter and co-occupancy was unaffected by androgen or enzalutamide treatment. Knockdown of fl-AR or V7 reduced binding of the other AR species, thus co-occupancy on AR-driven reporters is most likely mutually-dependent. This may help explain why V7 induction of an AR-driven reporter in AR-negative PC3 cells is quite low (~6-fold) compared to reporter induction of V7 in LNCaP cells endogenously expressing fl-AR in the absence of androgen. Cao et al. also found that enzalutamide reduced nuclear localization of fl-AR by approximately 50% in the presence of androgen, but had no effect on variant or fl-AR localization in the absence of androgen [117].

The inability of enzalutamide to reduce fl-AR nuclear translocation in androgen-deprived conditions further supports the speculation that there is some heterodimer interaction between fl-AR and V7 as a survival mechanism in the absence of androgen. Furthermore, V7 heterodimer interactions could be also possible with other steroid nuclear receptors to induce differential transcription of AR-driven genes. Other nuclear steroid receptors, such as GR and PR, share conserved sequence homology of their DBD with the AR. Published reports show that GR and PR can also activate AR-driven genes [63, 101, 143, 145, 146]. Therefore, it is possible that V7 can mediate activation of AR-driven reporter genes through co-occupying AREs with other steroid receptors, like GR and PR, in the absence of nuclear localized AR due to enzalutamide inhibition. To date, no studies have been published investigating V7 heterodimer interaction with other steroid nuclear receptors.

V7 transcriptional activity of AR-driven reporter genes can be regulated by recruitment of co-regulators to its NTD/AF1 region. Co-regulators can have site-specific interactions within the AF1 region and influence transcriptional activity at target gene promoters. TAU1&5 are present in the AF1, and upon deletion of the LBD the AR transcriptional function is shifted mostly to the TAU5 site [73]. Implications of this finding are that TAU1 is required for full-agonist-stimulated fl-AR activity, and TAU5 confers constitutive AR V7 activity in the absence of a LBD [73]. This shift to a specific-localized sequence of the AF1 region may be due to co-regulators that converge and have protein-DNA interactions with the TAU5 sequence, which in turn might cause a distinct set of other specialized protein-protein interactions with the general transcriptional machinery. To date, few groups have investigated co-regulators specific for AR splice variant activity [147, 148]. One group demonstrated that a co-activator, Vav3, co-immunoprecipitated with AR V7 protein and enhanced nuclear translocation of AR splice

variants [147]. However, whether Vav3 binds and interacts directly with TAU5 of AR V7 or indirectly via protein-protein interactions is unknown. Another group looked at the p160 co-activator SRC-1 that is known to localize to the AR NTD, and found that disrupting protein-protein interactions of SRC-1 decreased both AR V7 and fl-AR transcriptional activity of AR-driven PSA reporter [148]. SRC-1 binds to TAU5 and the AF2 surface in the AR LBD [149, 150]. SRC-1, along with other p160 co-activators, are histone acetyltransferases and their interaction with AR acts as a platform for recruitment of secondary co-activators that possess chromatin remodeling (ex. CBP/p300), protein acetyltransferase (ex. pCAF), and protein methyltransferase activity (ex. CARM1 and PRMT1) [46, 149]. Other co-activators that interact and localize to the AR TAU5 sequence include: p160 family members SRC-3 [45, 150, 151] and SRC-2 also called TIF2/GRIP1 [46, 150], the large subunit RAP74 of the TFIIF [76], CBP/p300 [77], and STAT3 [130, 149]. Claessens and colleagues demonstrated that the integrity of TAU5 in the AR NTD is a prerequisite for p160 recruitment [152]. Deletion of TAU5 in the full AR or the isolated AR NTD prevented co-activation by SRC-1 [152]. This necessary TAU5 sequence for co-regulator recruitment in truncated AR species, such as AR splice variants V7 and V567es, could extend to other co-activators that have been previously shown to localize to the 360 to 528 aa AR region. Furthermore, the same group showed that the TAU1 core attenuated recruitment of p160 co-activators binding to TAU5 in the full-length AR [152]. This supports the proposal that a shift to TAU5 mediated signaling in AR and an overexpression of TAU5-region binding co-activators could potentially influence and preferentially mediate AR V7 transcriptional activity of AR-driven promoters over TAU1-driven fl-AR.

Lastly, co-regulators recruited to the V7 AF1 region can be differentially regulated depending on the target gene and cell type. Co-activators that are necessary to initiate

transcriptional activity of AR-driven genes may be different than those recruited to cell-cycle genes. Since AR-driven genes may potentially be co-occupied and mutually-dependent on both V7 and fl-AR in the absence of androgen, co-regulators recruited to the TAU1 of the AF1 region may play an important role in differential regulation of canonical AR-driven reporter genes [117]. Co-regulator recruitment to TAU1 sequence requires the presence of TAU5 for proper DNA-protein conformational binding [152]. The full AF1, both TAU1 and TAU5 regions, are necessary for full activation of AR [73, 152]. Thus, it is plausible that overexpression of co-activators or co-repressors known to bind to this TAU1 region can influence AR gene regulation. Co-activators that interact and localize to the AR TAU1 sequence include: ARA24/RAN, STUB1/CHIP, ART27, Zimp10, and BRCA1&2 [149]. Generally, overexpression of the aforementioned co-activators has shown to enhance transactivation of androgen-bound AR [149]. Furthermore, two well-characterized co-repressors called nuclear receptor co-repressor 2 (NCoR1) and the silencing mediator of retinoid and thyroid hormone receptor SMRT/NCoR2, are recruited and localized to the 142-370 aa region of TAU1 [149]. Agonist- and antagonist-bound AR interaction with NCoR proteins suppresses activation of AR-driven genes, such as *PSA/KLK3* [149, 153]. Overall, not only is there differential regulation in which co-activators or co-repressors are recruited to the TAU1 region, but also complexity in which AR species these co-regulators are localized to. Both unliganded fl-AR and V7 potentially bind to AREs together in canonical AR-driven genes [117]. Thus, recruitment of co-regulators could interact with either the strong, active TAU1 region of the unliganded fl-AR or to its mutually binding partner AR V7 with a weak TAU1 region, though still active. It is important to note that co-regulators recruited to the AF2 sequence in the fl-AR LBD would likely not play a role in reporter gene regulation of AR V7. This is because androgen-binding of fl-AR is required for a conformational change to

expose the LxxLL aa motif of AF2 for critical protein-protein interactions [68]. Also, AR V7 independently induces a distinct set of genes associated with cell-cycle in androgen deprived conditions [66]. Cell-cycle genes, such as *CDC20*, *UBE2C*, *CDK1*, and *AKT1*, are upregulated in androgen-independent prostate cancer growth and not in androgen-sensitive LNCaP cells in the presence of DHT [66, 154]. Wang et al. investigated ubiquitin-conjugating enzyme E2C (*UBE2C*) gene and found that FoxA1, GATA2, Oct1 and MED1 (TRAP220) co-regulators are all recruited specifically to the *UBE2C* enhancer regions in androgen-independent cell lines. Significantly less FoxA1, GATA2, and MED1 co-regulators and RNA polymerase II were localized to the *UBE2C* enhancer and promoter regions, respectively, in LNCaP androgen-sensitive cell lines compared to androgen-independent cell lines [154]. Lastly, the activities of human (*PSA/KLK3*) and murine (*PB* and *ARR3*) AR-driven reporter genes were compared, thus species-dependent expression of co-regulators could play a role. Human and rat prostate cells are not identical, therefore differential gene induction with PSA human-based reporters and probasin rat-based reporters may be due to different expression profiles of co-regulators recruited to these reporters in human prostate cancer cells, as opposed to rat prostate cells. In conclusion, differential recruitment of co-regulators to various target genes may play a role in V7 gene-dependent transcriptional activity.

Here we postulate how AR splice variant V7 might be differentially regulating AR-driven reporters when it is expressed 1:1 with fl-AR. However, endogenous V7 in LNCaP95 displayed negligible levels of transcriptional activity in the absence of androgen. This low induction may be due to the use of a canonical fl-AR reporter gene, as opposed to a cell-cycle V7-driven reporter gene. The employment of AR variant-driven reporters in LNCaP95 would help uncover the discrepancy between androgen-independent V7-driven cell proliferation and lack of V7

induction in reporter assays. This is potentially ambiguous in interpreting drug efficacies since the effect of EPI-002 and enzalutamide monotherapies or in combination with AR variant-driven reporters was not measured in this study. Furthermore, this would provide further evidence that the effectiveness of enzalutamide monotherapy on mixed AR populations is dependent on levels of AR V7 present. The V7-upregulated *UBE2C* or *CDC20* promoters are both potential gene reporters to confirm constitutive basal activity in LNCaP95. Moreover, EPI-002 significantly decreased transcript levels of V7-driven genes, whereas antiandrogen bicalutamide had no effect [84]. Gene expression analysis from Myung et al., the differential gene set enrichment of V7 from Hu et al., and the work discussed in this thesis all together demonstrate that the major limitation in characterizing V7 transcriptional activity with fl-AR is the lack of a V7 specific reporter gene [66, 84].

#### ***4.4 Clinical significance***

Transcriptional activity of fl-AR throughout the progression of prostate cancer is complex and involves many factors at various levels of regulation to converge and co-operate. Upon AR binding to AREs on target genes, protein-protein interactions are initiated, which induce remodeling of chromatin, recruitment of basal transcriptional machinery, and initiation of RNA polymerase activity [70, 149]. With the increased production of truncated AR V7 in a mixed population with fl-AR, it is crucial to understand how these AR species are transcriptionally activating target genes, evading current therapies in the clinic, and reducing survival of CRPC patients. It is essential to target V7 and all constitutively active AR splice variants because they promote CRPC tumour growth independently of fl-AR by driving transcriptional activity of AR-driven genes in the absence of androgen [49, 50]. V7 is one of the most important AR splice variants to block activity since it is abundantly expressed in CRPC patient metastases, and is correlated with poor prognosis in patients [50, 108]. Furthermore, expression of V7 is frequently concomitant with fl-AR indicating patients have metastatic tumours with heterogeneous populations of these AR species [104, 107, 108]. It is important to study V7 activity at varying levels of expression relative to fl-AR because V7 expression is upregulated with treatment of ADTs and can potentially be rapidly generated from the *AR* gene shortly after androgen deprivation [66, 97, 110]. With work presented in this thesis, three major findings are contributed and impact treatment of CRPC patients. These include: 1) motive to investigate novel prostate cancer biomarkers to detect V7-expressing tumour progression; 2) proof-of-principle that a small molecule inhibitor to the AR AF1/NTD inhibits all AR species in

a heterogeneous tumour population; and 3) a potential therapeutic benefit for CRPC patients with treatment of EPI-002 in combination with enzalutamide antiandrogen.

With characterizing V7 transcriptional activity, it was discovered that V7 suppresses activation of PSA at 1:1 levels relative to fl-AR in both LNCaP and LNCaP95 cell lines. This is of clinical significance since PSA gene reporter is supposedly the most reliable serum biomarker used for diagnostic tests for disease progression and recurrence in patients [5, 11]. Upon application of findings in this thesis to clinical situations androgen-stimulated fl-AR alone would show an increase on a serum PSA test; however V7 expressed at high levels in tumours would show less of a response on a PSA test and potentially give a false-negative result. Results shown here justify the need and urgency to discover novel biomarkers of prostate cancer disease progression and AR signaling reactivation for all AR species.

Next, the employment of a small molecule inhibitor to the AR AF1 region would theoretically inhibit all AR species present in a heterogeneous population. Results shown here demonstrate that EPI-002 effectively blocked transcriptional activity of V7 which is present at the highest level in CRPC patient tumours [108]. This shows that EPI-002 monotherapy effectively inhibits all AR transcriptional activity irrespective of V7 level expressed relative to fl-AR. EPI-002 as a monotherapy in patients could target all mixed AR populations within a heterogeneous tumour and between separate metastases within the same patient. To our knowledge, this thesis demonstrated the first AR AF1 inhibitor to target and block transcriptional activity of mixed populations of constitutive active truncated AR V7 and full-length AR species.

The most important and clinically relevant finding of this thesis is that combination of enzalutamide and EPI-002 did not generally show greater inhibition nor less inhibition than

either monotherapies. These results alongside EPI-002 inhibition of fl-AR activity with elevated V7, shows the EPI-002 compound is providing most of the inhibitory activity. Verification using a V7-driven cell-cycle gene reporter would conclusively provide more evidence to support that EPI-002 is effective at inhibiting V7, while enzalutamide is not. If cells were treated with EPI-002 at an increased concentration all AR transcriptional activity would show better inhibition than combination therapy of EPI-002 with enzalutamide at 25  $\mu$ M and 5  $\mu$ M, respectively. Though *in vitro* studies have shown that EPI-002 at high concentrations is not feasible due to insolubility in aqueous solution. Safe drug doses of EPI-002 used in animals are 50 mg/kg body weight every two days intravenously or 200 mg/kg body weight daily via oral gavage, which both achieve approximately equivalent blood concentrations as *in vitro* studies (25  $\mu$ M). At these doses, EPI-002 reduced tumour burden of mice with subcutaneous VCaP, which express V7 at high levels [84]. This has been shown with on target activity provided by decreased V7 transcription in harvested xenografts. Higher doses of EPI-002 monotherapy or EPI-002 in combination with an antiandrogen have not yet been tested *in vivo*. However, a combination of EPI-002 dosed with enzalutamide could theoretically provide effective maximal AR blockade and regress tumours, with the advantage of targeting the AR at different steps in the signaling pathway (NTD and LBD). However, results presented here did not show better inhibition with combination therapy, it was demonstrated that EPI-002 in combination with an antiandrogen would still provide a therapeutic benefit for CRPC patients and may extend survival and decrease tumour burden. This thesis revealed that EPI-002 in combination with enzalutamide: 1) blocked V7 and fl-AR combined transcriptional activity; 2) demonstrated specificity for AR; and 3) inhibited proliferation of androgen-independent LNCaP95 cell lines. *In vivo* studies with animals would allow evaluation of apoptosis and tumour reduction, which ultimately provides evidence

of benefiting patients and determines advancement of combination therapy to clinical studies in humans.

## Chapter 5: Future Directions

EPI compounds supersede antiandrogens in their ability to inhibit activity of truncated LBD AR splice variants, gain-of-function mutations in AR LBD, and ligand-independent activation of AR NTD via cytokine and PKA activation. Antiandrogens fail to inhibit androgen-independent cell lines due to increased activation of cell-cycle genes via upregulation of AR splice variants. The most potent stereoisomer EPI-002 effectively inhibits cell growth of V7 expressing cell lines and gene expression of cell-cycle genes, including *UBE2C* [84].

Of interest for future directions, is first to determine the exact residues and location where EPI compounds are binding and how many molecules may be covalently binding to the AF1 region. EPI-002 covalently bound to AF1 peptides are currently being investigated using mass spectrometry.

Since this thesis demonstrated preliminary data that supports a potential therapeutic benefit for combination therapy, the next step would be conducting animal studies with treatment of EPI-002 with enzalutamide. This is to confirm that doses of both compounds would complement each other and provide maximal AR blockade with reducing side effects in animals, such as seizures, and possibly minimize resistance mechanisms. Future studies could also investigate EPI-002 with other compounds in combination that might provide better inhibition than EPI-002 monotherapy.

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