RALANITEN INDUCTION OF EXPRESSION OF METALLOTHIONEIN ISOFORMS IS BY A MECHANISM INDEPENDENT OF ANDROGEN RECEPTOR

by

Simon J. L. Teskey

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

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submitted by Simon J. L. Teskey in partial fulfillment of the requirements for

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in Pathology and Laboratory Medicine

Examining Committee:

Marianne D. Sadar, Pathology and Laboratory Medicine

Supervisor

Dana Devine, Pathology and Laboratory Medicine

Supervisory Committee Member

Sharon Gorski, Medical Genetics (UBC), Molecular Biology and Biochemistry (SFU)

Supervisory Committee Member

William Lockwood, Pathology and Laboratory Medicine

Additional Examiner

Nathan Lack, Department of Urologic Sciences (UBC)

Additional Examiner

Additional Supervisory Committee Members:

Christian Steidl, Pathology and Laboratory Medicine

Supervisory Committee Member

Sharon Gorski, Medical Genetics (UBC), Molecular Biology and Biochemistry (SFU)

Supervisory Committee Member

Abstract

Background: Advanced prostate tumors that develop resistance to androgen deprivation therapy are incurable and uniformly fatal. Nonetheless, the manifestation of this lethal form, known as castration-resistant prostate cancer (CRPC), does not preclude most of these tumors from sustained dependence on the androgen receptor (AR) for growth and survival. To inhibit AR pathway signaling, the strategy of all currently approved drugs ultimately converge upon the C-terminus ligand-binding domain (AR-LBD) to target and disrupt AR activation. Ralaniten is a novel, first-in-class drug which binds the AR within its N-terminal domain (AR-NTD). Due to this unique mechanism of action, we predicted that Ralaniten would induce a distinct global response compared to alternative AR-inhibitors. This study initiates the characterization of Ralaniten specific gene expression profiles and unravels the mechanism of induction of an unexpected group of genes from the metallothionein (MT) gene family.

Methods: In vitro experiments were performed in 4 human prostate cancer cell lines with experimentally useful genomic and phenotypic features. Preliminary gene expression data were generated by microarray. Pathway and statistical analyses revealed candidate genes for subsequent investigation. Transcriptional data were validated by qPCR and at the protein level by western blot. Reporter assays for gene activity were conducted after transient transfection of plasmids. Transient siRNA- mediated knockdown experiments assessed involvement of potentially relevant transcription factors. AR NTD inhibitors included Ralaniten, EPI-7170, SINT-1, and LPY26, whereas AR LBD inhibitors included the antiandrogens bicalutamide and enzalutamide. AR transactivation was mediated using the synthetic androgen R1881.

Results: Microarray analyses revealed the MT family to be the most abundantly induced by Ralaniten in the absence of androgen. Induction was experimentally confirmed to be Ralaniten specific. Knockdown experiments implicate a central role for the transcription factor, MTF-1, in the induction of MT genes by Ralaniten, and have ruled out the requirement for the AR and the redox activated transcription factor, Nrf2, in this mechanism.

Conclusions: Ralaniten induced the expression of MT genes by a mechanism independent of expression of AR and Nrf2. MT induction by Ralaniten is exquisitely dependent on the expression of the transcription factor, MTF-1.

Lay Summary

Proteins can be thought of as the action molecules in a cell. The androgen receptor is a protein that makes most prostate tumors grow. Blocking the activity of the androgen receptor is the goal of many therapies for prostate cancer. This strategy works well for a time, but the cancer returns for most patients in a more aggressive form that is resistant to current treatments that use this approach. For patients at this stage of the disease, there is no cure and, sadly, the average life expectancy is 1-2 years. A promising new class of drugs has been developed in the Sadar Lab that blocks the activity of the androgen receptor in a unique way. The purpose of this study was to explore how prostate cancer cells may respond differently to one of these new drugs in comparison to currently used drugs. With this knowledge, more effective versions of these new drugs can be made and used in combination with current treatments to best help prostate cancer patients.

Preface

All the work presented henceforth was conducted in the Sadar Laboratory at the Michael Smith Genome Sciences Centre, BC Cancer Research Institute, unless explicitly stated otherwise. This thesis is the unpublished and independent work by the author, Simon Teskey, and is prepared under the supervision of Dr. Marianne Sadar, Professor, Department of Pathology and Laboratory Medicine, at the University of British Columbia. Ralaniten and ralaniten analogs (including EPI-7170), and the sintokamides were co-discovered by Dr. Marianne Sadar and Dr. Raymond Andersen.

Dr. Sadar and I developed the overall concept and experimental design of this research project, with gratitude for the discussions and critical review provided by the members of the Sadar lab, and especially the keen insight of Dr. Jonathon Obst. Our lab manager, Nasrin R. Mawji, resuscitated frozen cell lines for culture, oversaw reagent orders and offered technical assistance and troubleshooting advice. Dr. Daniel Caley, a former post-doctoral fellow in our lab, provided guidance and inspiration in the initial stages of the project.

For the microarray study, the preparation of samples was led by Dr. Caley and the analysis of the data was headed by Dr. Obst. The reporter assays comparing inhibitors of the AR-NTD were performed by N.R. Mawji. The qPCR experiments and knock-down studies were assisted by Dr. Obst.

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List of Abbreviations

ADT	androgen deprivation therapy
AF1	activation function 1
AF2	activation function 2
ANOVA	analysis of variance
AR	androgen receptor
FL-AR	androgen receptor (full-length)
AR-V7	androgen receptor variant 7
ARE	androgen response element
BADGE	bisphenol A diglycidic ether
BIC	bicalutamide
CRPC	castration resistant prostate cancer
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
EBRT	external beam radiation therapy
ENZ	enzalutamide
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
HPG	hypothalamic-pituitary-gonadal
IC50	half-maximal inhibitory concentration
IL-6	interleukin-6
LBD	ligand-binding domain
LH	luteinizing hormone
MAB	maximal androgen blockade
MRE	metal response element
MT	metallothionein
MTF-1	metal transcription factor 1
NCCN	National Comprehensive Cancer Network
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRF2	nuclear factor erythroid 2-related factor 2
NTD	N-terminal domain
PSA	prostate-specific antigen
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RAL	ralaniten
RRE	redox (antioxidant) response element
R1881	methyltrienolone
SINT1	sintokamide A
STAT3	signal transducer and activator of transcription 3
TAU1	transcriptional activation unit 1
TAU5	transcriptional activation unit 5
UGT	UDP-glucuronosyl transferase

ACKNOWLEDGEMENTS

It is difficult for me to express the profound gratitude and awe that I feel when reflecting on this scholarly odyssey that has transformed my life. I have been graced with immeasurable kindness and generosity which has nurtured a cherished seed within to grow and blossom in defiance of the challenges encountered along the path. This could never have been achieved without the continuous support and guidance of my supervisor, Dr. Marianne Sadar. I would like to extend my deepest appreciation to her for the gift of this opportunity. Thank you for seeing my potential and taking a chance on an unconventional student. Marianne was an endless source of encouragement, patience, and wisdom throughout the duration of this project. Her belief in my abilities allowed me to overcome my self-doubt. The impact of her mentorship on the development of my skills and confidence as a scientist cannot be overestimated. Marianne is truly a blessing in my life, and she will continue to inspire me as I journey onward.

I am extremely grateful to the members of my Supervisory Committee, Drs. Dana Devine, Sharon Gorski, and Christian Steidl, for their constructive criticism and erudite review of my work. Dana was an unwavering ally whose help was instrumental in navigating the landscape of graduate studies. I'd also like to extend my gratitude to the faculty, staff, and graduate student peers from the Department of Pathology and Laboratory Medicine at UBC who have inspired me, provided friendship and various types of support, and ensured a fertile learning environment. I am thankful for the funding provided by the National Cancer Institute of the National Institutes of Health (2RO1CA105304) which made this work possible. I am also grateful for the scholarships awarded to me by UBC Faculty of Medicine Graduate Awards.

I would like to express my warm appreciation to the members of the Sadar lab, past and present, who have been both colleagues and friends during my time at BC Cancer. They have been generous in sharing their expertise and time, and it has been an honor and a pleasure to learn from them. I am immensely fortunate to have been mentored by such an outstanding group of scientists. Rina Mawji has been incredibly helpful and patient in assisting in my training. Much thanks to Teresa Tam and Drs. Jacky Leung, Amy Tien, Jean Wang, Adriana Banuelos,

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Finally, I wish to extend my deepest gratitude to my wonderful family for their continuous and unconditional support throughout my education. Without them, none of this would have been possible. Laura, Plum, Dude, Kate, Joseph, and Rebecca, each of you continue to help me so much. An extra special thanks to Laura and Jacob, my wife and son, and to the little guy soon to arrive. You mean the world to me. I love you from the bottom of my heart.

This dissertation is dedicated to all who maintain the lighthouses,

to ensure that those lost in darkness can return to shore.

Chapter 1. Introduction

1.1 PROSTATE CANCER

1.1.1 The Prostate

1.1.1.1 Structure

Prostate cancer originates from the prostate gland. A male-specific reproductive organ, the prostate is an acorn-shaped and walnut-sized gland located just anterior to the rectum at the base of the bladder with the urethra running through its center[1] (Figure 1.1). The prostate is exquisitely dependent on androgens, male sex hormones primarily derived from the testis, for the regulation of growth and maintenance of homeostasis. Initial development occurs late in embryogenesis from tissue of endodermal origin and concludes with the adult gland only reaching full maturity during puberty as a result of the androgen imperative[2]. Full-length androgen receptor (FL-AR) is a ligand activated transcription factor, found in both the prostate epithelia and stroma, that mediates the effects of androgens[3]. Androgens induce the prostate to begin the process of budding from the urogenital sinus and influence key morphogenic steps including branching, canalization, and cytodifferentiation[4]. The epithelial compartment of the human prostate has three distinct zones (central, peripheral, transition) surrounded by a fibromuscular stroma[5]. The stromal compartment is abundant with fibroblasts, myofibroblasts, and smooth muscle cells, contains infiltrating lymphocytes and macrophages, and is both innervated and vascular[6]. Surrounded by a basement membrane, the two-layered histological architecture of the prostatic parenchyma is organized into acini and ducts composed of luminal, basal, and neuroendocrine cells[7] (Figure 1.2). The flat, cuboidal basal cells form a layer adhering strongly to the basement membrane. Using immunohistochemistry detection, they may

be marked by cytoplasmic expression of cytokeratins 5 and 14, nuclear expression of p63, and only express low levels of AR. Atop this basal layer sit the pseudostratified columnar secretory cells that line the lumen. Immunohistochemistry distinguishes them by expression of cytokeratins 8 and 18, high levels of AR, and the expression of secretory proteins such as the AR regulated prostate specific antigen (PSA)[8]. The sparse neuroendocrine cells are dispersed throughout the gland and do not express AR. Though rare, these cells can be detected by their unique morphology and positive expression of synaptophysin, chromogranin A, and neuron-specific enolase[9].

Prostate epithelial cells rarely undergo cell division during normal tissue homeostasis. Inflammation due to infection (prostatitis) or tissue injury rapidly alters the growth quiescence of prostate epithelia, leading to its rapid proliferation[10]. The generation and maintenance of the apical prostate epithelium is traced to two cell lineages, basal multipotent stem cells and unipotent luminal progenitors[11,12]. Under the influence of androgenic signaling, these cells differentiate into columnar secretory cells which face the lumen and acquire morphological polarity[13]. Following this terminal differentiation, these cells possess a specialized metabolism and gene expression program to enable the production of prostatic fluid[14]. The growth and survival of this distinct phenotype is predominantly maintained via AR signaling, and activation of the AR pathway is conserved subsequent to oncogenic transformation[15–20]. Approximately 95% of prostatic carcinomas begin in these well-differentiated acinar cells and are referred to as prostate adenocarcinoma[21–23].

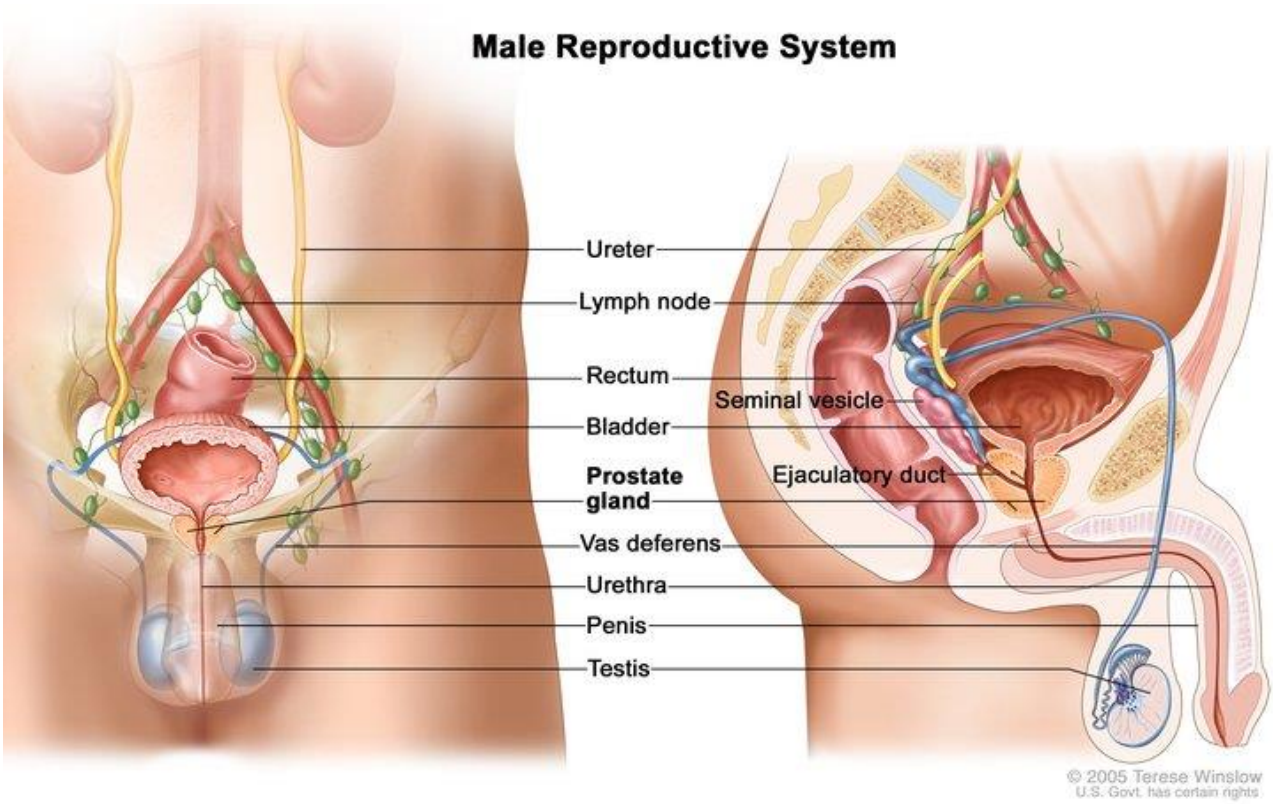
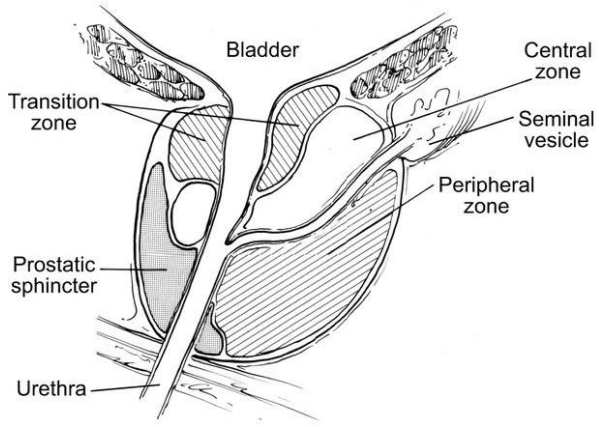


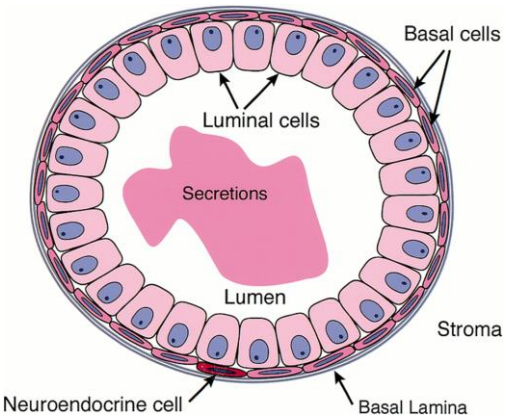
Figure 1.1 Location of the prostate

Anatomy of the male reproductive and urinary systems showing the prostate, testicles, seminal vesicles, bladder, and others. Examples of regional sites within the body associated with tumor dissemination and prostate cancer metastasis include the lymph nodes, pelvic bones, and vertebrae. Reproduced with permission of Terese Winslow. © 2005 Terese Winslow LLC, U.S. Govt. has certain rights.

A. Adult Human Prostate (sagittal section)



B. Normal glandular architecture



C. Prostate Histology

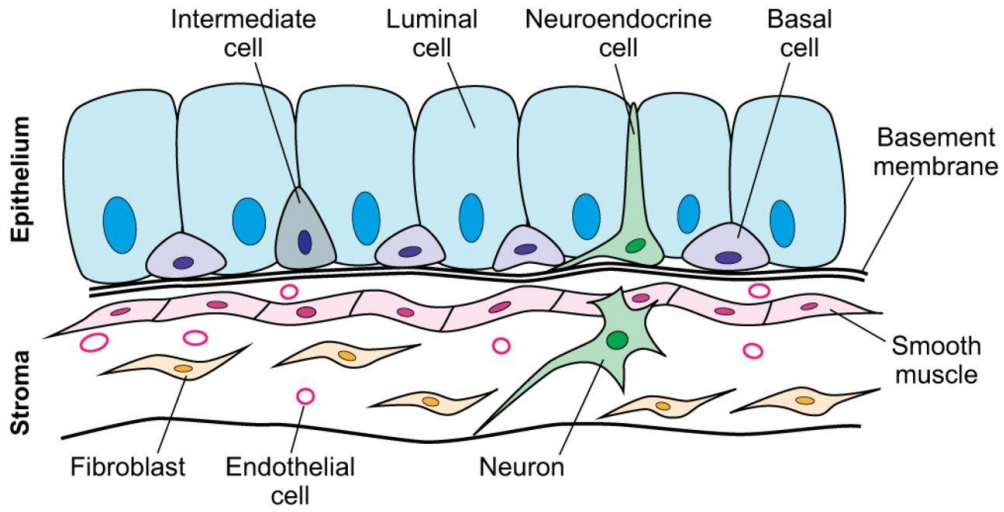


Figure 1.2 Structure of the prostate

(**A**) Anatomical overview the prostate gland with key regions and structures indicated. (**B**) Normal prostate epithelium organizes into discrete, regular shaped, well-formed tubulo-alveolar glands. Malignancy is marked by abnormal, disorganized glandular architecture, luminal cell expansion, and loss of the basal cell layer. (**C**) General histological representation of the adult prostate with examples of cell types. Basal cells, secretory luminal cells, and sparse intermediate and neuroendocrine cell populations comprise the epithelial compartment. Prostate epithelial cells rarely undergo cell division during normal tissue homeostasis. Intermediate cells are in transition and represent basal multipotent stem cells and unipotent luminal progenitors undergoing terminal differentiation into luminal cells. (**A**) and (**C**) are adapted from Figures 1 and 3 from Toivanen and Shen (2017) *Development* 144: 1382-1398. Reproduced with permission of The Company of Biologists via Copyright Clearance Company[24]. (**B**) is reproduced from Abate-Shen and Shen (2000) *Genes and Development* 14: 2410-2434 under license (CC BY-NC-ND 4.0)[25].

1.1.1.2 Functions

As an accessory exocrine gland found solely in males, the role of the prostate is to produce and secrete components of the seminal fluid. Anatomically, the position of the prostatic urethra distal to the bladder facilitates the prevention of retrograde ejaculation. The secretions produced by the prostate maintain liquefaction of the seminal plug and provide nourishment, protection, and lubrication for the sperm. Synthesis of these secretory proteins and nutrients is primarily driven by AR regulation of gene expression and requires the maintenance of a unique metabolic phenotype in the luminal cells[26]. The evidence that functional AR is an absolute requirement for normal prostate development and physiology is unequivocal[27]. Withdrawal of androgen (e.g. castration) results in rapid atrophy and involution of the prostate gland, and in individuals with androgen insensitivity syndrome (AIS) consequent of non-functional AR, the prostate fails to develop altogether[28,29]. Notwithstanding the indispensable role of AR in prostate homeostasis, its dysregulation is a main driver of prostate cancer development and progression, thus establishing AR as a bona fide therapeutic target and the ongoing focus of numerous translationally driven investigational studies[30]. The exceptional sensitivity of the prostate to androgen deprivation is readily apparent and leads to rapid apoptosis of ~90% of luminal cells and a small percentage of basal cells. In the adult prostate, these glands have the capacity to regenerate when androgen is restored. Remarkably, they are able to undergo multiple cycles of regression-regeneration in response to androgen deprivation and androgen-restoration[31]. Application of this principle of androgen sensitivity was first employed therapeutically nearly 80 years ago, demonstrating significant beneficial effects for patients with metastatic prostate cancer[32]. The success of this clinical discovery led the Canadian physician Charles Huggins to be awarded the Nobel Prize in medicine in 1966.

1.1.2 Prostate Cancer

1.1.2.1 Epidemiology

Prostate cancer is the second leading cause of male cancer deaths in the Western world[33]. In recent decades, improved screening correlated with higher incidence rates, though earlier detection has been credited with a reduction in mortality[34]. Roughly 1 in 7 men will be diagnosed with prostate cancer in his lifetime. The current understanding of prostate cancer etiology is multifaceted and complex[35–37]. Both individual biology and lifestyle figure prominently[38]. Perhaps most remarkable, prostate cancer provides possibly the most striking example of age-dependent cancer development among all cancer types[39]. In men under 55 years of age, prostate cancers arise with negligible frequency and most cases (56%) materialize in men over the age of 65. Virtually all cases of prostate cancer (97%) occur in men older than age 50[40,41]. While some factors contributing to the risk of developing prostate cancer are inherent to the individual, others offer motivation for behavioral change[42]. When considering modifiable risk, some of the more salient factors include diet, smoking, physical exercise, and obesity[43–47]. Of the risk factors not amenable to modification, the most well established are advanced age, heredity[48,49], and ethnicity, with higher risk found in men of African descent, moderate risk in Caucasian men, and the lowest risk in Asian men[50,51]. Reinforcing the prominence of dietary influence, Asian men who adopt a typical North American diet see their risk rise to match their Caucasian counterparts[52–56].

1.1.2.2 Diagnosis

A long latency period is characteristic of prostatic neoplasms and many tumors may remain indolent[57,58]. The widespread uptake and utilization of screening practices remains controversial due to concerns surrounding patient overtreatment[59–63]. Indeed, identifying patients that will require treatment remains a major challenge for clinicians. Despite 5-year survival rates of close to 100% following interventions for localized prostate carcinoma, treatment associated morbidities make the decision to pursue active surveillance highly attractive for patients with low risk of progression. Early diagnosis and grading are essential as delay may have lethal consequences[64]. Localized disease is often asymptomatic and possible indicators like changes during urination or sexual activity are near universal in older men and are non-specific for prostate malignancy. On the other hand, more obvious and ominous signs and symptoms, such as bone pain, swelling, or numbness in the lower back, legs or pelvic area, are associated with the incurable, metastatic stage of disease. Thus, most prostate cancers are initially detected during screening with a prostate-specific antigen (PSA) blood test, a digital rectal exam (DRE), or transrectal ultrasound (TRUS)[65].

Patients who screen positive require a biopsy for diagnostic confirmation. Transrectal ultrasonography–guided biopsy has been the standard protocol and, based on criteria including overall prostate volume, involves the removal of between 8 and 20 cores of tissue for evaluation[66]. In recent years, studies have investigated the use of multiparametric magnetic resonance imaging (MRI) in suspected cases of prostate cancer to confirm the necessity of biopsy and report a benefit to including this approach[67,68]. Furthermore, MRI-targeted biopsy with the use of real-time ultrasonographic guidance to select cores specifically from abnormal

areas noted during prior imaging was shown to improve the detection sensitivity of clinically significant lesions and assist discrimination between aggressive and indolent disease compared to standard transrectal ultrasonography–guided biopsy[69,70]. Patient under- or overtreatment remains a challenging clinical issue and, hopefully, a salutary effect will be generated through evolving practical developments like these[71,72].

Biopsies of clinically suspected prostate cancer are sent for histopathologic assessment by a skilled pathologist that may include diagnostic immunohistochemistry[73,74]. The three key criteria which underpin the histologic confirmation of a diagnosis of prostate adenocarcinoma are: hallmark loss of basal cells, atypical nuclei (nuclear enlargement, prominent nucleoli), and abnormal glandular architecture. The characteristic loss of basal cells disturbs glandular structure and likely contributes to a disrupted stromal epithelial relationship. Malignant glands may exhibit nuclear atypia as a manifestation of cellular stress, chromatin remodeling, and altered proliferation[75–77]. Careful assessment of the abnormal growth patterns which predominate within the glandular architecture are fundamental to categorizing tumor histopathology, defined in prostate adenocarcinoma through application of the Gleason pattern scale[78,79]. Scaled from 1 to 5, lower scores are associated with small, well formed, tightly packed glands. As scores increase, epithelial architecture becomes more disorganized with cells spreading out and forming irregular glandular structures. The final Gleason score is calculated by adding the score for the most dominant morphology to the next most common morphological pattern to produce a score from 2 to 10. The Gleason grade provides clinicians with essential prognostic information, along with the radiographically determined clinical TNM stage, to inform treatment planning[80].

1.1.2.3 Treatment

Prostate cancer is a clinically heterogeneous disease and individual patients may face starkly different prognoses. For physicians, minimizing overtreatment of indolent disease and improving outcomes for patients with aggressive disease remains a fundamental tenet of patient care[81]. Risk stratification is an essential tool for guiding decisions regarding the appropriate course of disease management[82]. The assessment of risk is multifaceted and includes consideration of both patient and tumor characteristics[83]. These determinations often combine patient age, number of positive prostate biopsies including the percentage of malignant tissue per core, Gleason score, serum PSA, PSA density, and clinical tumor stage[84]. Based on these factors, the assessments are used to assign patients to one of five categories of risk: very low, low, intermediate, high, and very high. Additional germline genetic testing is recommended for patients with high or very high risk, as well as for patients of any risk category that have either a positive family history of prostate cancer or had biopsy tissue displaying intraductal or cribiform histology. In all but very low risk patients, molecular tumor analysis may also be considered if life expectancy equals or exceed ten years, as biomarker status has proven valuable for assisting treatment selection[85–91]. Patients with intermediate risk or greater are sometimes also referred for further radiologic assessment to enhance screening and characterization of regional or distal metastases. Prostate cancers are notable for prominent osteotropism[92–94]. Spread to the axial skeleton represents the predominant pattern of dissemination with occurrence in over 80% of patients with metastatic disease[95–98]. In addition to bone, metastasis to lymph nodes, liver, and lung are common though less frequent (Figure 1.3). Collectively, these assessments provide a considerable sum of information that is crucial to navigating clinical treatment guidelines for prostate cancer. Integrating modern techniques for disease characterization with more traditional

factors such as life expectancy and risk of death from other causes has improved overall prognostic accuracy.

Clinicians recommend treatment based on both predicted outcome and patient preference to support personalized tailoring of individual patient care[99]. Clinical treatment guidelines for prostate cancer are comprehensive and represent a compendium of evidence-based treatment protocols drawn from a broad therapeutic toolbox. For example, the Clinical Practice Guidelines in Oncology (2020) from the National Comprehensive Cancer Network (NCCN) exhaustively describes the growing armamentarium of approved prostate cancer therapies available to clinicians, defining the specific indications for appropriate patient selection, and meticulously detailing numerous multistep protocols, including contingencies, ranging from active surveillance alone through to multimodality treatment. A thorough review is well beyond the scope of this dissertation. A brief summary of the most current (2020) NCCN Clinical Practice Guidelines for prostate cancer follows below and is the primary source of all information related to treatments throughout this section, with additional citations added where applicable.

The understanding of prostate cancer biology has grown considerably in recent decades yet for individual men, prostate adenocarcinomas trajectories may chart a highly variable course due to a combination of factors such as patient age, clinical tumor stage, and comorbidities at diagnosis, as well as somatic and germline genomic signatures, and the effects associated with choice of treatment[84]. Following diagnosis, a patient-tailored continuum of care is initiated that may last for years, is some cases over a decade, depending on these personal variables. In broad terms, prostate cancer treatments are given with curative intent to patients in earlier stages of the disease and with palliative intent for patients in advanced stages to alleviate symptoms and

potentially prolong survival. Treatments may be given as monotherapies or in combination and include potentially curative local therapies, such as surgery and radiotherapy, which target a specified area and systemic therapies that include hormone therapy, chemotherapy, and immunotherapy[100].

If no metastases are evident at diagnosis, the treatment for men with localized disease is generally selected based on a patient's life expectancy, personal wishes, and initial risk group (very low, low, intermediate favorable, intermediate unfavorable, high, very high, and regional)[101]. Preferring to avoid the side effects of treatment and preserve quality of life, many men assessed as having a lower risk of progression decide to delay treatment when this option is an appropriate alternative[102,103]. Patients that opt for this route are divided into two categories, observation or active surveillance, based on therapeutic goals. Both are closely monitored for symptoms or rising PSA levels that may signify progression, though active surveillance usually includes annual biopsies as well. If evidence of progression is detected, patients on active surveillance initiate potentially curative interventions, while patients on observation convert to palliative ADT, sometimes delaying this for continued monitoring until symptomatic.

For men exhibiting more aggressive disease with higher risk of progression (intermediate unfavorable and above), ADT is given, unless medically contraindicated, in combination with radiotherapy and/or surgery. In patients of lower risk within this higher risk spectrum, radiotherapy is preferred over surgery. Radical prostatectomy is indicated for higher risk patients, entailing complete removal of the prostate, seminal vesicles, some adjacent tissue and often the pelvic lymph nodes as well[104]. In the highest risk patients, this surgery is generally followed by adjuvant radiation therapy, especially when positive surgical margins have been

detected[105]. For patients demonstrating biochemical recurrence (rising PSA) following radical prostatectomy, salvage radiation therapy is indicated[84,106].

Radiation therapy involves two main modalities, external beam radiation therapy (EBRT) and brachytherapy, used individually, concurrently or sequentially. Delivered from sources outside the body, EBRT includes many possible technical approaches and dose fractionation strategies. Standard EBRT, with or without dose escalation, is still commonly used though newer forms of EBRT that employ highly sophisticated technologies continue to be developed and implemented. For instance, intensity modulated radiation therapy (IMRT) involves radiation beams specially shaped to fit the contours of the targeted tumor, thereby enabling greater precision in the delivery of treatment. More of the healthy surrounding tissue is spared from toxicity allowing higher doses of radiation to be used. Stereotactic body radiation therapy (SBRT) utilizes an extremely hypo-fractionated dosing regimen delivered from a combination of angles to precisely sculpt the dose distribution[107]. Employing radioactive sources inside the body, brachytherapy involves either the placement of permanent radioactive seeds which emit energy at a low-dose rate or the temporary insertion of catheters containing radioactive sources which emit energy at a high-dose rate. Brachytherapy is more invasive than any of the ERBT types, in addition to requiring anesthesia. Neoadjuvant ERBT or androgen ablation is a common scenario to make the surgery less invasive and more effective. These primary treatments are often successful, yet 20-30% of patients will have recurrence. Salvage brachytherapy may be recommended for pathologically confirmed local recurrence after previous radiotherapy[108–110].

Despite considerable progress, advanced stage prostate cancer remains lethal and incurable[111]. Patients with evidence of metastasis at diagnosis or those with recurrence after

primary treatment will require systemic therapy[112–116]. A discussion of the chemotherapies, immunotherapies, radiotherapies, and bone-sparing treatments provided with palliative intent is beyond the scope of this thesis[117,118,127–130,119–126]. Most prostate cancers are dependent on an active AR signaling pathway for growth and survival. The first-line treatment for men with metastatic prostate cancer remains ADT to starve malignant cells of natural ligand[131,132]. This is achieved chemically using drugs or surgically via bilateral orchiectomy. In some instances, a strategy termed combined androgen blockade is recommended in which second generation androgen inhibitors (abiraterone, enzalutamide, or apalutamide) are used in conjunction with ADT[133–137]. This profound androgen suppression induces tumor regression and results in significant initial response in most patients[138,139]. Unfortunately, the duration of disease control provided lasts only 18 to 36 months on average[140,141]. Following this, recurrence is inevitable as tumor progress to the state known as castration-resistant prostate cancer (CRPC).

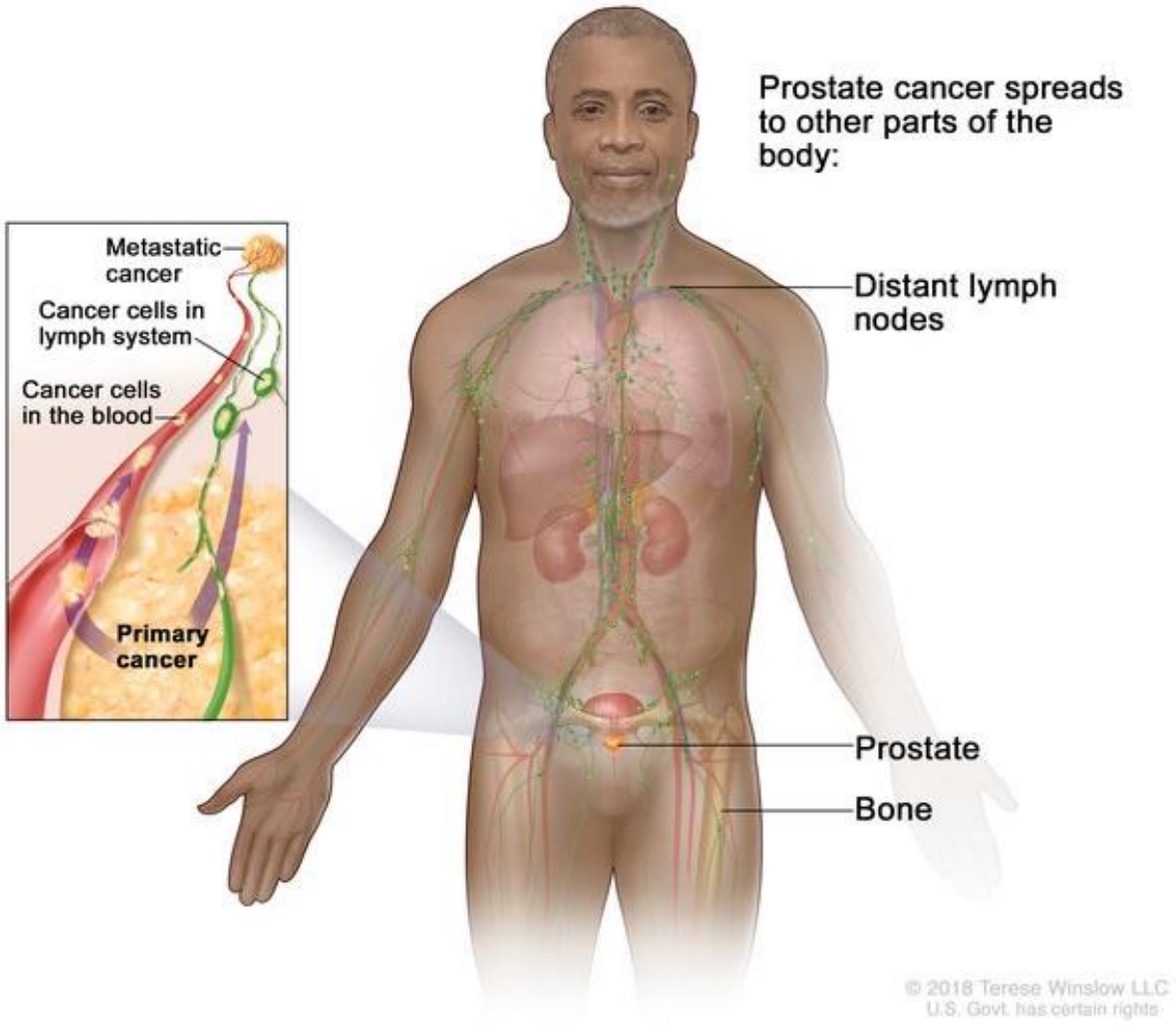


Figure 1.3 Advanced prostate cancer (Stage IV)

- The tumor has grown into tissues beyond the seminal vesicles, with any PSA and any Gleason score, OR
- The tumor has spread to one or more nearby lymph nodes, without metastases, with any PSA and any Gleason score; OR
- The cancer is present in the body beyond the nearby lymph nodes, in tissues like the bone or distant organs, with any PSA and any Gleason score

The inset shows dissemination of cancer cells from the prostate, through the bloodstream and lymphatic system, to form a metastatic lesion elsewhere in the body. Reproduced with permission of Terese Winslow. © 2018 Terese Winslow LLC, U.S. Govt. has certain rights.

1.1.3 Castration-resistant prostate cancer

CRPC emerges, as its name suggests, when tumor progression is evident following chemical or surgical treatment to suppress circulating androgen to castrate levels. This defining feature created confusion in the past based on the mistaken belief that the AR was no longer playing an essential role at this stage. Robust evidence has since overturned this misunderstanding by clearly establishing that CRPC remains reliant on persistent AR signaling[142,143]. Indeed, biochemical recurrence manifests clinically as a rising titer of serum PSA, a hallmark AR-regulated gene, implying AR transcriptional activity continues to be a driver of tumor growth and survival in CRPC patients[144–146]. Treating these patients with second generation androgen inhibitors extends survival, albeit only modestly, thus reinforcing that AR remains active[117,147–154]. Multiple mechanisms of resistance exist which continue to exploit the AR signaling pathway. These include AR overexpression, AR gain-of-function mutations, aberrant expression of co-regulatory factors, and the expression of truncated AR splice variants (AR-Vs) that are constitutively active[16,18,142,155–159] Ongoing research continues to elaborate a more thorough biological understanding of how these mechanisms emerge and function, leading to novel treatments and evidence-based strategies for the sequencing of therapies[20,112,168–171,160–167]. Nonetheless, the salient conclusion evident from these treatment escape mechanisms is that selective pressures continually restore AR pathway signaling, thus confirming the importance of developing new approaches to sufficiently antagonize and sustain blockade of this crucial pathway in the management of CRPC.

1.2 ANDROGEN RECEPTOR

1.2.1 Structure and function

The AR (NR3C4, nuclear receptor subfamily 3, group C, gene 4) is a member of the steroid hormone receptor superfamily[172]. Within this family, phylogenetic studies have shown a relationship among the AR, estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR)[173,174]. These nuclear receptors share a high degree of structural and sequence homology, and are master regulators of distinct, though sometimes overlapping, gene transcription programs[175–177]. Their cognate ligands must cross the plasma membrane to bind these receptors, as they predominantly reside within intracellular compartments, which proceeds readily by passive diffusion due to the lipophilicity of steroid hormones. FL-AR is a ligand-dependent nuclear transcription factor involved in regulating the expression of specific genes. Similar to other nuclear hormone receptors, the structure of AR is comprised of four discrete domains that are functionally distinct: an amino-terminal domain (NTD), a DNA-binding domain (DBD), a carboxy-terminal ligand-binding domain (LBD), and a flexible hinge region (HR) joining the LBD and DBD[178]. The AR-DBD is highly conserved, with roughly 80% sequence homology to the DBD of PR and GR[179]. The AR-LBD shows approximately 50% sequence homology with the LBD of PR and GR[180]. The AR-NTD shares less than 15% sequence homology with the NTD of PR and GR[181].

Mapping studies have revealed the single copy AR gene to reside at the Xq11-12 locus on the long arm of the X-chromosome[182], therefore the AR is a hemizygous gene in human males. Spanning over 90kb of DNA, the AR gene encodes a complementary DNA (cDNA) sequence that contains eight canonical exons interrupted by introns of varying lengths (0.7–2.6

kb). The AR gene (Figure 1.4) expresses a naturally modular 110-kDa protein translated from a 10.6 kb mRNA transcript that results in a polypeptide approximately 919 amino acids long[178]. 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. Exon 1 encodes the full NTD (a.a. 1-558), exons 2 and 3 encode the DBD (a.a. 559-622), and exons 4 through 8 encode the LBD (a.a. 671-919). Encoded within exon 4 is the HR (a.a. 623-670) which shares a nuclear localization sequence (NLS) that overlaps the C-terminal end of the DBD[183,184].

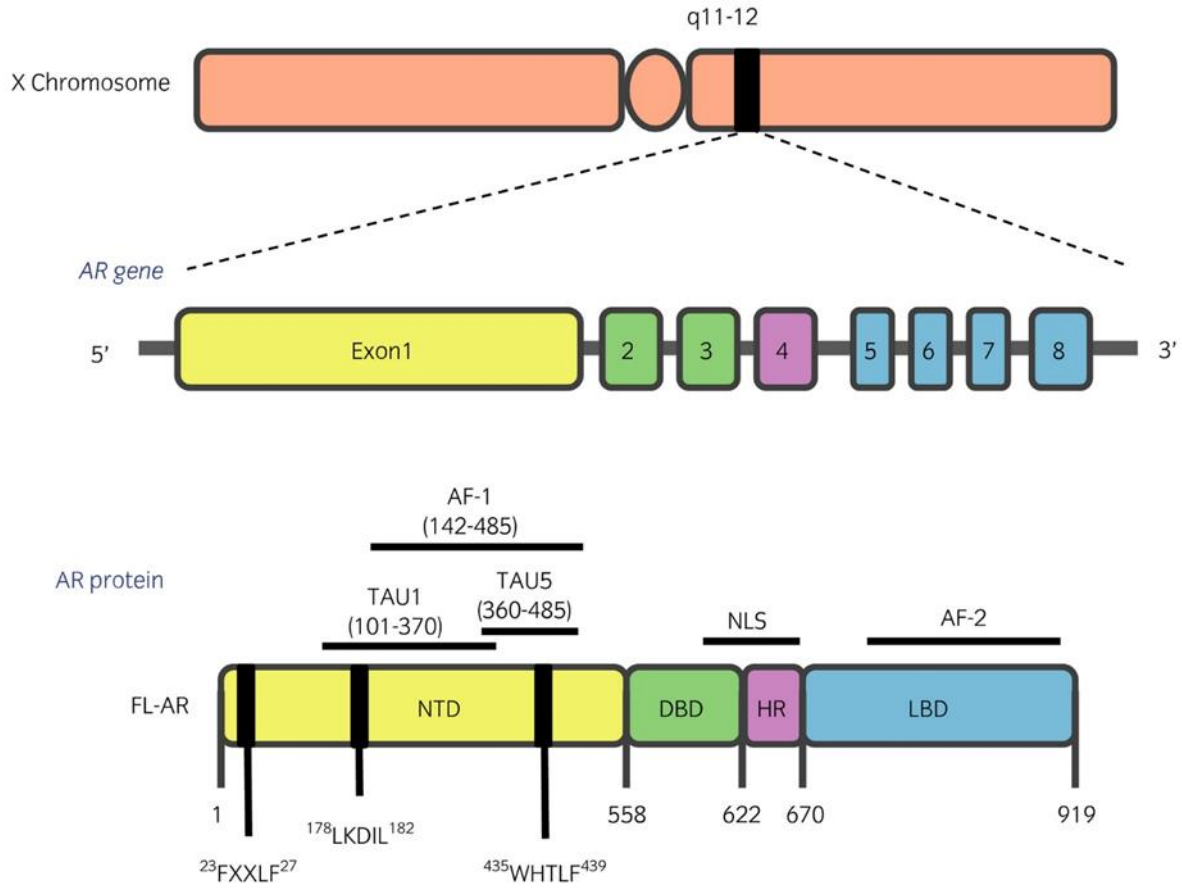


Figure 1.4 AR gene and protein structure

Detailed description follows directly in main text below. Adapted from Figure 2 from Imamura and Sadar (2016). *International Journal of Urology*. 23(8):654-665, under license (CC BY-NC-ND 4.0)[164]

Each structural domain is responsible for different functional aspects of the AR protein[185]. As the indispensable transcriptional engine of the AR, the NTD contains many regulatory regions and post-translational modification sites to augment and direct signaling actions[186]. Activation function 1 (AF-1) includes two overlapping transcription activation units termed TAU-1 (a.a. 100–370) and TAU-5 (a.a. 360–528)[187]. The WHTLF motif (a.a. 433–437) within TAU-5 and the FQNLF motif (a.a. 23–27) are nuclear receptor boxes important for protein-protein interactions[188]. While both TAU-1 and TAU-5 are involved in mediating direct ligand-dependent AR transcriptional activation, the WHTLF motif is essential for constitutive AR activation of truncated AR-Vs that lack the LBD[189]. The AR-DBD contains two α-helical zinc finger structures; one that connects to the hinge region allowing dimerization, stabilization, and DNA recognition, the other interacts with the DNA via hydrogen bonding with nucleotides at the binding site[190]. The AR functions as a homodimer and specifically recognizes and binds genomic regions termed androgen response elements (AREs). Binding of different DNA sequences alters the conformational structure of the DBD in ways characteristic of individual binding locations, thereby assisting in the modulation of receptor activity by presenting surfaces that favor recruitment of different co-regulatory proteins[191]. A canonical nuclear localization signal in the hinge region regulates the nuclear import of the receptor. Unbound to ligand, the AR typically resides in the cytoplasm attached to cytoskeletal elements. Agonist stimulation induces conformational changes in the AR leading to phosphorylation and activation of the receptor, thus exposing the nuclear localization sequence for recognition by importin-α to enable active transport of the AR to the nucleus[192]. The AR-LBD contains the ligand binding pocket and a second transcriptional regulation domain termed activation function 2 (AF-2)[193]. The three-layered architecture of the LBD takes the shape of an "α-helical sandwich" formed from the 11

α-helices and 2 antiparallel β-sheets of its tertiary structure. Agonist binding induces a specific conformational change to the LBD, akin to closing a lid over the binding pocket, which traps and stabilizes the interaction with ligand and exposes the AF2 interface to facilitate regulatory protein-protein interactions. To initiate the N/C interaction that results in AR homodimerization, a deep hydrophobic groove within AF2 preferentially binds the bulky side chains in the 23FQNLF27 core sequence located in the NTD of a second AR molecule[194]. This orientates the dimer in a "head-to-tail" configuration which further stabilizes bound ligand by impeding dissociation, thereby prolonging AR transcriptional activity.

Androgen stimulation of the AR, in cooperation with coregulatory factors, is the classical pathway leading to transcriptional activity of AR target genes. Testosterone and its metabolite 5α-dihydrotestosterone (DHT) are the endogenous ligands of the AR. Under regulation by luteinizing hormone (LH) produced in the anterior pituitary gland, testosterone is synthesized primarily by the Leydig cells in the testes. Hypothalamic control of LH secretion is regulated by gonadotropin-releasing hormone (GnRH)[195]. Pulsatile secretion of endogenous androgens into the general circulation trends closely with the chronobiology of circadian rhythms. The hypothalamic-pituitary-gonadal (HPG) axis accounts for 90-95% of testosterone production, with the remainder synthesized by the adrenal glands[196]. The tissue availability of androgens is influenced by the ratio of free hormone in serum. Virtually all circulating testosterone is protein bound (~98%), mostly to sex-hormone binding globulin (SHBG) or albumin. Free testosterone is a relatively small, highly lipophilic molecule. It readily diffuses into prostate epithelial cells where high expression 5α-reductase enzymes support its metabolism into the more potent DHT[197]. Due to a slower rate of dissociation, DHT binds the AR with a higher affinity resulting in ~5X the potency of testosterone[198].

Following synthesis, the AR forms a complex with numerous heat shock proteins (HSPs) and immunophilins to generate and maintain a receptor with high-ligand-binding affinity. In addition, the interaction with these various chaperone proteins, which include HSP70, HSP27, HSP40, HSP90, and HSP23, greatly helps to prevent AR degradation[199–201]. The AR conformational changes triggered by ligand-binding which expose the NLS and AF-2 also promote dissociation of these chaperones. This allows active AR translocation to the nucleus, homodimerization by N/C interactions, and DNA binding to AREs within the promoter or enhancer regions of AR target genes such as PSA and TMPRSS2. Forming a complex with DNA at the ARE, the AR then directs the organization of the preinitiation transcriptional complex by recruiting members of the basal transcription machinery, including TATA-box-binding protein (TBP), transcription factor IIF (TFIIF), and RNA polymerase II, as well as coregulators such as cAMP-response element-binding protein (CREB)-binding protein (CBP) and p160 proteins to either upregulate (coactivators) or downregulate (corepressors) AR activity[202–204] (Figure 1.5). Chromatin structure largely determines AR access to ARE binding sites and regulation of the AR transcriptional program requires a chromatin landscape favorable to AR access. The histone-modifying enzymes p300 and CRB and the pioneer factors FOXA1, GATA2 and HOXB13 promote open chromatin structure in prostate cancer cells and subsequent AR locoregional binding[205–209]. In summary, AR transcriptional regulation of gene expression is performed through an intricately orchestrated symphony between transcription factor action, chromatin accessibility, DNA sequence, and nucleosome assembly. Modulated by fine tuning through myriad influences, signature patterns in prostate epithelial cells distinguish healthy, transformed and relapsed cells, with cistrome and transcriptome changes tracking alongside progressive stages of the cell cycle during proliferation as well[210].

1.2.2 Target genes

As a master regulator of gene expression, the AR is positioned as an intracellular gatekeeper placed at the threshold of a dramatic cascade of transcriptional events. The signaling pathways under the control of the AR are extensive and diverse, orchestrating a panoply of cellular responses, unique to each cell type, in the broad range of tissues which express AR protein[211]. Using advanced molecular techniques, the annotation of the AR transcriptome has grown beyond 400 genes with functions integrated throughout the complete profile of cytological processes, including cell growth[212] (nutrient uptake, protein synthesis, lipid metabolism)[213–217], proliferation (mitogenic signaling, cell cycle regulation, DNA repair)[218–220], fate (migration, differentiation, senescence)[221], and specialized functions (protein trafficking, secretory vesicle formation, transport of secretory vesicles)[222,223].

The AR transcriptome can be operationally defined as the full spectrum of target genes with expression sensitive to genomic regulation by AR via the presence of one or more AREs within the gene's regulatory regions that recruit AR to modulate transcription. The application of modern experimental techniques such as ChIP-chip, ChIP-Seq, ChIP-exo, high-throughput RNA sequencing and splicing microarrays in the investigation of AR regulated gene expression have highlighted the complexity and versatility of the AR transcriptome[224]. Thousands of AREs have been located within the human genome and these AREs differ between genes in both composition and context to facilitate tissue and cell specific AR function and enable contrasting regulatory functions within a single nucleus[225–227]. The consensus ARE driving most AR target genes is formed by an inverted repeat of two hexameric half-sites with 3 base pairs intervening (5'-AGAACAnnnTGTTCT-3')[172], and this region can also bind DBDs of other class I steroid

receptors (glucocorticoid, progesterone and mineralocorticoid). Genomic data have provided evidence establishing that more AR-specific regulation of certain target genes relies on AR binding to a selective ARE consisting of a half-site of the consensus ARE. It is believed that AR homodimers can bind this shortened element due to the strength of the AR dimer interface and, counterintuitively, less stringent sequence requirements for the 3′ hexamer[228]. AR binds predominantly to AREs found in distant enhancer elements, forms the AR–coactivator complex, and communicates with promoter regions proximal to the transcriptional start site (TSS) of AR-regulated genes through chromosomal looping and RNA pol II tracking[229–231].

When describing AR function within the prostate, an important paradox must be acknowledged which has been described as an "AR malignancy shift [232]." In the differentiated luminal epithelial cells of a healthy prostate, the cell specific role for AR includes maintenance of growth and proliferative quiescence[233] and master regulation of the secretory phenotype. Without the AR acting as a growth suppressor in this context, the physiological levels of circulating testosterone in a non-castrate, adult male would continuously stimulate hyperplastic overgrowth of the gland. During prostatic carcinogenesis, AR signaling converts from a growth suppressor role in normal prostate epithelial cells to acting as an oncogene in prostate cancer cells[234–236], in part due to molecular changes that significantly increase AR protein expression (>5 fold) and alter reciprocal-regulating communication with transcription factors c-MYC and NF-kB[233,237–239]. Of special relevance to AR function after transformation to prostate cancer, it should be noted that the repertoire of genes expressed under the transcriptional control of AR has been demonstrated to vary in cells derived from hormone sensitive cancer as compared to cells derived from CRPC[196,240–243], in part, reflecting the differing characteristic expression signatures of transcriptomes driven by FL-AR and AR-Vs[244–248]. Adding further complexity, the output

from the AR transcriptome is highly dynamic; the transcriptional expression signatures of well established, canonical AR regulated genes do not remain constant across biological states in the prostate, often switching between induction and repression by AR in relation to contexts like embryological vs pubertal development, ligand and nutrient availability, environmental stresses, and oncogenesis vs progression[249–253].

1.2.2.1 Androgen receptor-induced transcriptome

AR activity is indispensable for maintaining prostate function and much of the research to date has focused on investigating genes which are positively regulated by AR. Indeed, studies of androgen-regulated genes report that nearly 50% are involved in the synthesis, folding, modification, and transit of secretory proteins[222,223,254]. The AR is perhaps best known for its role in transcriptional activation of a prototypical AR regulated gene, *KLK3* (PSA), which expresses a secreted protein with protease actions that maintain functional seminal fluid. Other examples of AR genes known for key functions in the prostate include *KLK2, TMPRSS2, PRSS18, SLC2A3,* and *STEAP4*[254]. Interestingly, while the proteins expressed by these genes contribute to healthy prostate function, some may also provide support to pathological processes in prostate cancers. Studies report that through its protease activity, PSA can induce cell migration and epithelial–mesenchymal transition to promote tumor progression[255,256]. *TMPRSS2* encodes a transmembrane serine protease found to be fused to ETS family transcription factors (ex. *ERG* and *ETV1*) in over 50% of patient prostate cancer samples[257–259] and the *TMPRSS2–ETS* fusion is associated with a poor prognosis in localized prostate cancer[260,261]. Many genes induced by AR fluctuate in expression between the pre- and post-neoplastic setting[243]. Examples

illustrating this are genes in the polyamine biosynthesis pathway (*SMS, ODC1, SAT, AMD1, SRM*), ER-stress response pathway (*HRD1, ORP150, PDIR, NDRG1*), and genes associated with glandular development (*TMEPAI, ZBTB10, NKx3.1,* and *ANKH*)[262]. Prostate cancers demonstrate altered lipid metabolism and AR-induced cholesterol/fatty acid biosynthesis pathway genes that are implicated in this process include *AMACR, FASN, FAAH, SREBP2, LDLR, HMGCR, FDFT1, SCAP, MFGE8, APOD, APOL1,* and *PLA2G2A*[213,217]. AR can induce genes that promote AR activation via AR stabilization (*FKBP5*), and coregulator up-regulation (*SRC-2, SRC-3, RNF14, PIAS1, NCOA4*)[263–266]. AR induces genes implicated in numerous pathways and processes that drive prostate cancer progression. AR promotes growth by upregulation of *IGF1, IGFR, VEGFA, FGF8, S100P, c-fos , Drg-1, cav-1, IL6R, RICTOR,* and genes of the *SGK* family, and promotes cell proliferation via the upregulation of genes such as c-*MYC , CDKN1A, CDC2, UBE2C, CDK2,* and *CCND1*[212,267–273]. AR induces DNA damage response genes to assist resilience to prostate cancer therapies and maintains the survival of treatment resistant prostate cells by inducing an array of genes including *PRKCD, RAD54B, XAB1, ERCC8, SEMA3C, PYCR1, GSTT2, CaMKK2,* and *TRPV3*[241,274–278]. Considering the myriad biological processes that AR is involved in, a complete annotation of AR induced genes would be lengthy and beyond the scope of this work. This list is but a partial summary which reviews prominent AR induced genes that have been associated with key processes in prostate cancers.

1.2.2.2 Androgen receptor-repressed transcriptome

An increasing number of studies describe AR functions with respect to silencing transcriptional programs, helping to expand the catalogue of AR-repressed genes[279]. Primarily involving receptor domains other than the LBD, numerous inputs shape the selective regulation of gene expression by AR, though the precise molecular mechanisms that constitute the basis for the transcriptional repression function of AR remain undefined. With the capacity to interact with hundreds of different proteins, AR action can be fine-tuned to align with different contexts. The AR-DNA transcription complex differentially recruits co-activators and co-repressors, though additional factors such as modification by enzymes can be involved in determining if AR target genes are switched on or off[280]. In one study, the authors describe the influence of retinoblastoma protein (Rb) phosphorylation status at the point of recruitment to the AR-DNA transcription complex, especially at regulatory regions of cell-cycle related genes, with hypo-phosphorylated RB implicated in mediating AR target gene repression[281]. In prostate cancer cells, AR downregulates expression of the cell cycle inhibitor gene *CDKN1B*[282] resulting in increased proliferation. The AR repressed transcriptome includes many genes associated with tumor suppressor functions, such as *DEPTOR*[283], *DKK3*[264], and *PDCD4*[284]. For example, the protein encoded by the *DEPTOR* gene binds to mTORC1 and mTORC2 protein kinase complexes to inhibit growth promoting signaling. Epigenetic modifications contribute to shaping the AR transcriptome of prostate cancers as they evolve and progress by altering both chromatin architecture and the DNA of upstream gene regulatory regions. Studies exploring these processes have established the enzyme Lysine-Specific Demethylase 1 (LSD1) as central protein involved in the AR-suppressed gene programs observed in CRPC[285]. Investigations focusing on CRPC reveal important differences in the AR target genes that are repressed by

ligand-bound FL-AR and constitutively active AR-Vs[286], with notable relevance to the emerging, novel inhibitors of the AR-NTD.

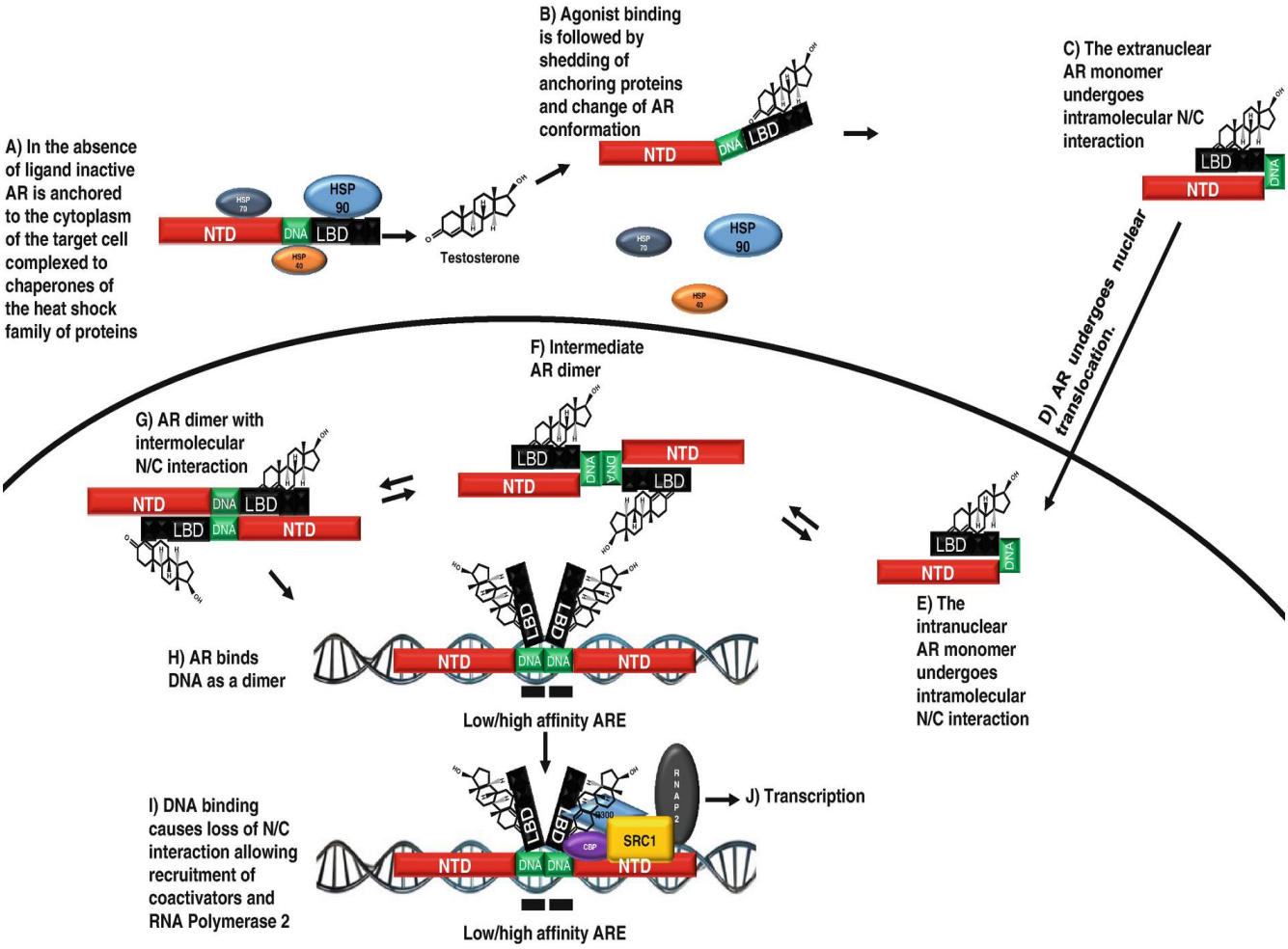


Figure 1.5 Sequence of ligand induced FL-AR transcriptional activation

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1.3 CLINICAL THERAPIES TARGETING THE ANDROGEN RECEPTOR

1.3.1 Androgen-deprivation therapy

Starving the AR of its cognate ligands has been a mainstay of prostate cancer treatment for nearly 80 years. Indeed, androgen deprivation by either pharmacological or surgical means remains the gold standard therapy for prostate cancer patients[288]. As the Leydig cells of the testes are responsible for the synthesis of virtually all circulating androgens, bilateral orchiectomy results in elimination of testicular androgen production followed by serum PSA responses approaching an undetectable threshold (<0.5 ng/mL)[289]. This radical surgery is irreversible and often patients are additionally burdened with significant physical and psychological morbidities[290]. Chemical therapies have demonstrated the capacity to pharmacologically reduce circulating androgens to castrate levels and are a commonly utilized alternative to surgical castration[291]. Strategies that target the HPG axis to achieve gonadal testosterone depletion rely on suppression of the release of LH from the anterior pituitary[292]. Hypothalamic regulation of pituitary LH release is dependent on pulsatile stimulation by GnRH through the hypophysial portal bloodstream. Synthetic analogs of GnRH (LHRH agonists) provide continuous pituitary GnRH receptor stimulation which results in downregulation of these receptors, thus mechanistically exploiting this intrinsic negative feedback loop[293]. Initiating treatment with LHRH agonists causes serum testosterone concentrations to initially rise, potentially stimulating prostate cancer growth and causing pain at metastatic sites, necessitating co-administration of AR antagonists to competitively block the effects of this testosterone flare[294]. Alternatively, LHRH antagonists block the signal necessary to initiate LH release and do not induce a testosterone surge[295]. Whether achieved medically or surgically, profound

androgen suppression is associated with significant morbidities which include metabolic dysregulation leading to cardiovascular disease or diabetes, sexual dysfunction, osteoporosis, muscular atrophy, hot flashes, and mood disorders (depression, anxiety)[296–298]. The considerable clinical benefit gained from reduced tumor burden and prolonged survival outweighs these risks.

ADT is provided with curative intent when given as a component of a comprehensive treatment plan to patients with localized prostate cancers at higher risk of progression, yet between 20-30% of these patients will experience recurrence. When ADT is given to patients with disseminated disease, either those presenting with metastases at initial diagnosis or after recurrence following definitive local therapies, the treatment is provided with palliative intent. Continuous androgen starvation induces adaptive stress responses in prostate cancer cells that insulate against apoptosis and restore mitogenesis[299–302]. Sadly, patient responses at advanced stages of the disease are sustained between one to two years before progression to the stage termed CRPC.

1.3.2 Antiandrogens

CRPC maintains dependence on signaling through the AR pathway despite systemic strategies which profoundly decrease levels of circulating androgens. This implies that androgen-depletion by medical or surgical castration is insufficient to completely inhibit the receptor activity[303]. Resistance to these approaches develop as prostate cancer cells acquire changes that include overexpression of the AR itself and upregulation of enzymes directing androgen biosynthesis, which restore AR activity[158,304–308]. To target these resistance mechanisms, several novel drugs have been developed to either target the AR directly or deplete

extragonadal androgens production via suppression of adrenal and intra-tumoral synthesis pathways[154]. Generally, these agents are added by clinicians to the ADT regimen for CRPC patients in a strategy described as combined or maximal androgen blockade (MAB)[309]. Building on the success of first-generation antiandrogens like bicalutamide and hydroxy-flutamide, newer antiandrogens have emerged including enzalutamide, and more recently, apalutamide and darolutamide[148–151,310–313]. Enzalutamide potently and selectively binds the AR-LBD to competitively displace T and DHT, the natural ligands of AR, and inhibits AR nuclear translocation to disrupt transcriptional activation of AR-regulated genes[314–316]. Abiraterone impairs AR signaling by further deepening the suppression of androgen synthesis. Abiraterone inhibits both the 17α-hydroxylase and 17,20-lyase activities of the CYP17A1 enzyme, theoretically blocking androgen production in all body tissues including tumor[317]. Providing treatment with either class drugs, antiandrogen or androgen synthesis inhibitor, has been clinically demonstrated to offer modest survival benefit for CRPC patients, though utility is limited when these agents are given sequentially to one another[318–320]. Cross-resistance frequently develops between medications targeting a similar therapeutic space and the mechanism of both antiandrogens and androgen synthesis inhibitors ultimately converge on the AR-LBD to exert their effects, thereby compounding the selective pressure for AR aberration following orchiectomy or ADT[321].

1.4 MECHANISMS OF RESISTANCE

1.4.1 Overexpression of the AR gene

Restored AR transcriptional activity drives continued dependence on the AR pathway. The variety of AR aberrations discovered are found almost exclusively in CRPC[322,323], strongly suggesting that these alterations are selected for during therapy. Several mechanisms sustaining AR signaling in CRPC have been described[18,142,158,159,299,324]. AR overexpression is a prominent feature of CPRC cells and a dramatic rise in AR expression may produce hypersensitive responses to residual androgen levels during castrate conditions[305,325]. Increased levels of AR protein may be a consequence of genomic changes, transcriptional upregulation, reduced turnover and increased stability, or any combination of these factors. Profiling of CRPC tumors has revealed recurrent genomic amplifications in both AR gene copy number and upstream AR gene enhancer sequences[326–328]. A recent analysis reported that genomic amplifications of the AR gene and AR distal enhancers led to an increased total number of chromatin interaction modules spanning these regions which contributed to AR overexpression[329]. Further compounding these genomic alterations, conditions that are characteristic of the intracellular environment of CRPC cells such as oxidative stress can induce the activation of transcription factors regulating the AR gene[330,331]. Finally, the signature hyperactivation of inflammation associated transcription factors and cytokine signaling cascades observed in prostate cancer bone metastases and CRPC lesions drives upregulation of AR expression[332].

1.4.2 Gain-of-function mutations

Deprived of androgens and antagonized by small molecules targeting its LBD, the AR may develop gain-of-function mutations which generate a promiscuous receptor to restore AR signaling in prostate tumors[333,334]. These AR mutations are rarely observed in patients with early stage, untreated prostate cancer though their emergence may be hastened consequent to long term ADT and antiandrogens exposure[335,336]. Primary prostate tumors exhibit such profound dependence on the AR signaling pathway that AR blockade may generate sufficient selective pressure to promote the survival of prostate cancer cells that acquire gain-of-function mutations in the AR[337,338]. Indeed, AR mutations are found frequently in tumor samples from patients with CRPC and a variety of mutant variants have been detected and described (i.e. F876L, L701H, W741L/W741C, V715M, and T877A)[143,339,340341]. Predominantly affecting the AR-LBD, these mutations decrease ligand selectivity and render AR responsive to novel stimuli[342–344]. AR transcriptional activity is enabled despite castrate conditions if the mutant AR variant is conferred with the capacity to bind with alternative steroid hormones (glucocorticoid, estrogen, progesterone, adrenal androgens) to permit AR activation[342,343,345,346]. Certain mutations sufficiently alter the LBD to convert non-steroidal antiandrogens from AR antagonists to AR agonists[347–349]. For example, the H875Y, T878A and T878S mutations, all detected in CRPC patients, confer agonist effects on the second generation, non-steroidal antiandrogens enzalutamide and apalutamide[350]. This phenomenon of antagonist-to-agonist switch is believed to be responsible for the benefit observed in some patients that exhibit a withdrawal syndrome after cessation of antiandrogen therapies[351–353]. Individual mutations may engender somewhat selective promiscuity to specific alternate ligands and do not generally confer pan-antagonist resistance. Sensitive assays have been developed to detect tumor DNA that has been shed into

the general circulation by dying prostate cancer cells at tumor margins[350,354–356]. AR gain-of-function mutations detected in plasma DNA may help guide physicians toward rational sequencing of AR pathway inhibitors for CRPC patients.

1.4.3 Aberrant expression of co-regulatory factors

The canonical pathway to trigger AR transcriptional activity at AR target genes via androgen stimulation may be augmented by changes in co-regulatory factor expression and availability[202,204,357]. After progression to CRPC, the expression levels for several AR co-regulators are notably increased in malignant cells, including SRC-1, TIF2, RAC3, p300, CBP, Tip60, MAGE-11, and ARA 70[358–364]. AR coactivator SRC-2 is amplified and the AR corepressors TRAC-1 and SMRT are aberrantly expressed in primary and metastatic disease[357,365]. The altered expression of co-regulatory factors in relapsed prostate cancers is associated with aggressive features and poor outcomes. Providing a mechanism of resistance to ADT and antiandrogens, they enhance AR transactivation of target genes to support AR hypersensitization to depleted androgen availability[366–368]. Demonstrating value as therapeutic targets, investigations are underway to explore methods to antagonize these effects[369–372].

1.4.4 Constitutively active truncated splice variants of AR

Constitutively active, truncated AR variants which lack the AR-LBD are commonly detected in CRPC cell lines and patient tissues[164,373–375]. These are thought to be generated by aberrant regulation of mRNA processing leading to alternative splicing of AR transcripts that

translate into AR-Vs[376–382]. In trying to discern the molecular collaborators involved in driving the production of AR-Vs, studies have implicated epigenetic modifiers, various transcription factors, such as c-MYC, AURKA, and NF-kB, and other proteins (ex. YB-1, Lin28) that play contributing roles[383–392]. As an adaptive mechanism to circumvent androgen ablation, many of these truncated AR isoforms maintain transcriptional function despite loss of the ligand-sensitive AR-LBD to support androgen-independent expression of AR target genes and androgen-independent growth of CRPC cells. A variety of AR-Vs have been detected and characterized[393] and, of these, AR-V7 is shown to be the most commonly expressed[394]. Clinical studies reveal an association between AR-V7 expression in CRPC and resistance to second generation AR inhibitors which have mechanisms targeted to suppression of AR-LBD activation[171,244,324395]. The AR-V regulated transcriptome encompasses a distinct expression signature enriched for the upregulation of cell-cycle genes and the downregulation of tumor suppressor genes[286,396,397]. The emergence of AR-V protein isoforms does not preclude advanced prostate cancers from continued expression of FL-AR[398–400]. Indeed, the activation of AR-FL signaling appears to somewhat suppress the AR-Vs transcriptional signature[401].

Crosstalk between the AR and various cytoplasmic signaling cascades, including those induced by growth factors and cytokines, can enhance transactivation of AR regulated genes through post-translational modification at numerous sites within the AR-NTD[186,402–405]. This mechanism for receptor stimulation is particularly consequential for truncated AR-Vs, as it contributes to the protein stability of these isoforms and ensures robust constitutive transcriptional activity[406–409].

Techniques for the isolation and characterization of anoikis-resistant circulating tumor cells that have migrated and intravasated to the bloodstream are gaining wider clinical utilization for the management of advanced prostate cancer patients. Utilizing AR-V status in prostate cancer cells as a biomarker, these assays are proving helpful in guiding treatment selection and assist prognostication[86,410–416]. Next-generation sequencing and immunohistochemistry analysis of CRPC metastases revealed co-expression of *AR-V3, AR-V7* and *AR-V9,* highlighting the value of AR-NTD inhibitors which suppress AR function by targeting regions common to all AR-Vs[417,418].

1.5 ANTAGONISTS OF THE AR-NTD

Repeated restoration of signaling through the AR pathway by acquisition of mechanisms to subvert AR antagonism reaffirms the importance of AR action in CRPC to motivate discovery of alternative approaches to block AR function. To date, the basis of all major clinical successes toward AR inhibition strategically converge to singularly disrupt ligand-dependent AR activation and the resistance mechanisms briefly summarized above enable malignant prostate cells to circumvent all of them. With every currently approved endocrine treatment inhibiting either ligand production or ligand action, significant therapeutic value clearly exists in the development of drugs that do not mechanistically function through the AR-LBD and which are able to block transactivation of the receptor both in the presence and absence of androgen. The AF-1 subdomain of the AR-NTD is indispensable for both ligand-dependent and ligand-independent transcriptional activity and antagonists directed at this region would be efficacious against the many clinically relevant AR mutants or constitutively active structural variants commonly

detected in CRPC. Targeting the AR AF-1 therapeutically is a challenge, due to its intrinsically disordered nature and lack of enzymatic activity or rigid binding clefts, though substantial progress towards this goal has yielded promising results[419]. Fruitful interrogations of natural compound libraries have confirmed the probative value of high-throughput screening systems when rational drug design is precluded by the absence of a definitive crystal protein structure[420]. Reports describing the discovery and pre-clinical development of the first AR-NTD targeted small molecule inhibitors (EPI-002 and SINT1, Figure 3.2) have provided proof-of-concept that this approach has the potential to overcome the resistance pathways driven by the AR. In recognition of compounds which possess this novel mechanism of action, AR-NTD inhibitors have been assigned a distinct nomenclature to distinguish them from the non-steroidal antiandrogens of the '-lutamide' stem class that inhibit the AR-LBD. The USAN council appointed EPI-002 with the generic name Ralaniten and designated the new stem class '-aniten' for drugs that specifically target and bind the AR-NTD.

1.5.1 Sintokamides

Sintokamides are bioactive chlorinated peptides isolated and purified from the marine sponge *Dysidea sp.* that were identified as potential therapeutic candidates by high-throughput screening of a library of natural marine extracts for inhibitory activity against the AR-NTD[421]. Sintokamide A (SINT1) emerged as a leading compound for further study and characterization[420]. SINT1 demonstrated the ability to block transactivation of the AR NTD in reporter gene-based assays, reduce expression of the AR-regulated gene PSA, and inhibit AR-dependent proliferation of prostate cancer cells in vitro and was shown to impair the growth of

CRPC xenografts in vivo[422]. Mechanistic studies suggest SINT1 antagonizes the transcriptional activities of both FL-AR and AR-Vs and binds to AF-1 in the AR-NTD at a discrete location from ralaniten[423]. The AR directly engages with ~200 distinct proteins and the NTD is rich with sites for post-translational modification and protein- protein interactions[186,202]. Most of these processes enhance the transcriptional function and stability of the AR-NTD[402,424–428]. Different inhibitors may bind unique regions of the AR-NTD, thereby blocking stimulatory pathways associated with the specific binding site of each inhibitor. Thus, the binding location of an individual inhibitor within the NTD can be inferred by mapping the disrupted pathway to its associated site of action. A study by Banuelos et al. (2016) reported that SINT1 has the capacity to block transactivation of AR NTD induced by stimulation of the PKA pathway, but had no effect on IL-6-induced transactivation of AR NTD[422]. Reflecting on the additive effect observed by combination SINT1-ralaniten treatment, the authors suggested that SINT1 and ralaniten bind to different regions of AF-1, noting that previous investigations demonstrated that ralaniten is capable of inhibiting IL-6 induced transactivation[429]. Further pre-clinical development of SINT1 included extensive investigation of the structure-activity relationship, yielding the synthetic analogue LPY26 as a potential drug lead. LPY26 was selected from 29 synthetic analogues/precursors based on the most promising in vitro biological activity[430].

1.5.2 Ralaniten and analogs

Ralaniten is a novel, first-in-class drug which binds the AR-NTD and represents the vanguard in clinical development of the EPI family of compounds (Figure 1.4). Nearly 500 EPI analogs have been methodically evaluated by the Sadar Lab for potential clinical utility,

predominantly for therapeutic application but also as a radiographic tool[431]. The original of these compounds, EPI-067 was isolated from the marine sponge *Geodia lindgreni* and identified by screening a library of natural marine extracts in search of antagonists of the AR-NTD[423]. Closely resembling the chemical structure of bisphenol A diglycidic ether (BADGE), an epoxy resin of industrial provenance, EPI-067 may constitute the biotransformation product of a synthetic contaminant[166]. Of the EPI analogs, the efficacy against AR demonstrated by the racemic mixture referred to as EPI-001 was particularly promising, reinforced by the negligible cross-reactivity toward GR or PR evident in reporter assays. The two chiral carbons contained within the structural scaffold of EPI-001 yield four bioactive stereoisomers; EPI-002 (2R, 20S), EPI-003 (2S, 20R), EPI-004 (2R, 20R), and EPI-005 (2S, 20S). Following a structure-activity analysis to determine the most potent candidate for further development, ralaniten (the EPI-002 isoform) was selected based on a marginally lower IC50 than the three alternative stereoisomers in addition to demonstrating the least toxicity in mouse xenograft studies[165]. In multiple studies, ralaniten inhibited gene expression of the canonical AR-regulated genes *PSA, FKBP5, TMPRSS2*. Reinforcing its status as a bona fide AR-NTD antagonist, ralaniten inhibited expression of signature genes of the AR-V driven transcriptome (*UBE2C, AKT1, and CDC20)* that were unaffected by treatment with bicalutamide and enzalutamide. Pre-clinical in vivo studies revealed that ralaniten significantly reduced the growth of FL-AR driven LNCaP xenografts, as well as AR-V expressing VCaP and LNCaP95 xenografts that model CRPC. Ralaniten was shown to antagonize the transcriptional activities of both FL-AR and AR-Vs by binding to AF-1 in the AR-NTD and blocking the necessary protein-protein interactions required for transactivation[165,166,432–438]. Data from an investigation that utilized nuclear magnetic resonance to analyze the ralaniten-AR interaction confirmed that ralaniten binds AF1 in the AR-

NTD within amino acids 354-448 of TAU-5[439]. In 2015, Ralaniten acetate, an orally active prodrug of ralaniten, entered phase I clinical trials for evaluation in men with end-stage metastatic CRPC (NCT02606123). Inclusion criteria limited patient selection to men who had progressed after enzalutamide or abiraterone and may have had one line of prior chemotherapy. Despite a heavily pretreated patient cohort, ralaniten was well tolerated and responses were evident in patients on higher dosages. Modest declines in serum PSA were observed in some patients and several patients maintained stable disease while continuing ralaniten treatment past one year. Due to poor pharmacokinetics, sustaining plasma concentrations of ralaniten within the target therapeutic window proved challenging and the clinical trial was terminated due to excessive pill burden. Nonetheless, ralaniten served to establish proof-of-concept and validated the feasibility of this novel therapeutic approach. Ongoing research and development of more potent and metabolically stable ralaniten analogs with improved pharmacokinetic profiles continues to yield promising new compounds. The next-generation analog of ralaniten, EPI-7386, has entered Phase I clinical trials in 2020. The unique mechanism provided by small molecule inhibitors of the AR-NTD enriches the clinicians toolbox and offers myriad possibilities for strategic combination with existing treatments. Indeed, preliminary investigations to this effect have reported synergistic results[163,435,437].

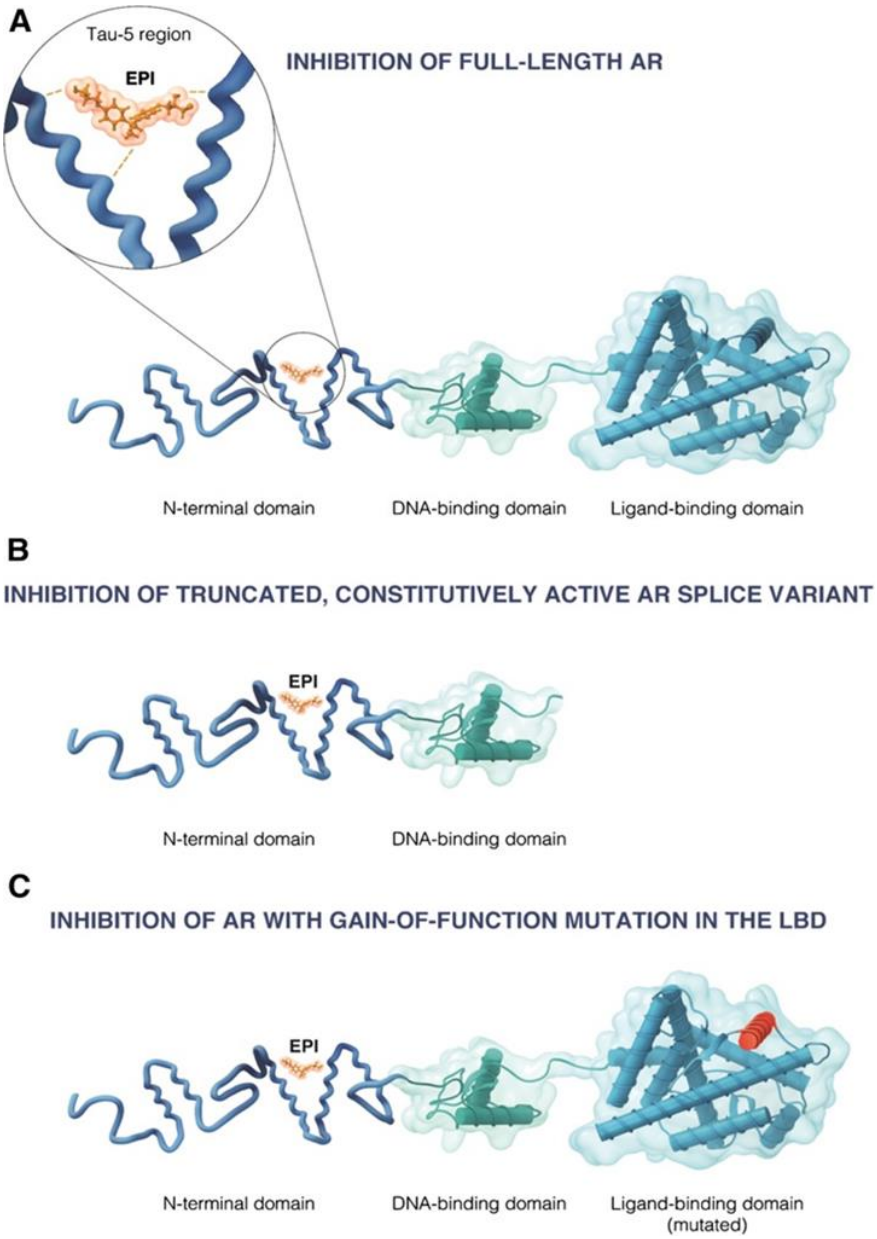


Figure 1.5 Ralaniten and analogs (EPI compounds) have a novel mechanism of action

All currently approved drugs targeting the AR signaling pathway converge in their ultimate site of action at the C-terminus ligand-binding domain (LBD), i.e. enzalutamide. Ralaniten, a novel first-in-class drug, binds the TAU-5 region within activation function-1 of the receptor's N-terminal domain (NTD). This unique mechanism confers ralaniten with the ability to inhibit FL-AR (**A**), as well as the AR isoforms (**B**) and (**C**) that become expressed with resistance to inhibitors of AR LBD (e.g., enzalutamide), such as AR that has acquired gain-of-function mutations and AR with structural alterations that yield constitutively active truncated AR splice variants (AR-Vs). Adapted from Figure 1 from Antonarakis et al. (2016) *The Oncologist* 21: 1427-1435. Reproduced with permission of AlphaMed Press via Copyright Clearance Company[440].

1.6 METALLOTHIONEIN FAMILY

1.6.1 Structure and Function

Metallothioneins (MT) are very small (6-7kDa), highly conserved intracellular proteins that are ubiquitously expressed in living organisms. Responsive to a broad range of inducers, they are non-enzymatic, multifunctional participants in a host of cellular processes and homeostatic control mechanisms[441,442]. DNA mapping studies report the chromosome 16q13 region to encompass the loci of human *MT* genes. These encode polypeptide sequences varying in length from 61-68 amino acids that contain 20-21 cysteines and are devoid of any aromatic or histidine residues[443]. Eleven functional MT proteins have been identified and organized into four isoform groups: MT-1 (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, and MT1X), MT-2 (known as MT2A), MT-3, and MT-4[444]. Present in all tissue types, the MT-1 and MT-2 isoforms are both basally expressed and highly inducible by many factors such as metal ions, glucocorticoids, cytokines, and oxidative stress. MT-3 is predominantly detected within the CNS (neurons, astrocytes in the cortex, hippocampus) where it is a constitutively expressed growth inhibitory factor with limited inducibility[445,446]. Historically considered a tissue specific isoform, MT3 has more recently also been detected in additional tissues including heart and kidney[447], and notably in prostate epithelia after malignant transformation[448,449]. MT-4 shows relatively restricted tissue expression as well. MT-4 is detected in cells of squamous epithelium (mouth, upper gastrointestinal track, skin) and constitutively expressed independently of signal changes[450,451]. MT levels detected in malignant tissues are observed to be highly variable and expression patterns that have emerged following numerous investigation reveal a close

association to tumor type[452]. Based on those findings, MTs have been explored as potential biomarkers for cancer diagnosis and prognosis[453–455].

In the differentiated luminal secretory cells of healthy prostate epithelia, the expression of *MT1* and *MT2* isoforms are required to support both general and specialized functions. Composed of nearly one third cysteines, MTs contain an exceptionally high thiol content that serves to facilitate their main functions, which include the detoxification of heavy metals, buffering oxidative stress, and the storage and inter-protein transfer of zinc[456–461]. These thiol clusters enable individual MT molecules to each complex with up to 12 monovalent or up to 7 divalent heavy metal ions[462] and are the primary repository for labile intracellular zinc. MT bound zinc atoms are easily displaced by other more toxic metals to sequester them and facilitate excretion. Furthermore, the exceptionally low redox potential of the zinc-thiolate bonds contained within MT proteins render them easily disrupted by redox disturbances and oxidative challenges, thereby releasing zinc in the process to restrain inflammation, activate antioxidant programs, and induce transcription of *MT* genes.

Persistent and sustained oxidative stress is a well characterized feature of prostate tumors[463–466]. Acting directly as an antioxidant, sulfhydryl-rich MT may trap electrophiles in a similar manner to reduced glutathione (GSH). MT proteins are 50X more potent than GSH on a molar basis and are exceptional buffers of redox disturbances[467–469]. MTs can be rapidly oxidized by diverse circumstances. These range from interactions with constitutive, mild pro-oxidant factors such as glutathione disulfide (GSSG) or selenium compounds, to more highly reactive metals and alkylating agents, metabolically generated free radicals, and non-radical oxidative stresses. As the stability of the zinc/sulfur network in MT and the relative mobility of

zinc is intrinsically tied to the reducing power of the cell, zinc is released from the thiolate clusters consequent to any of these events.

In the healthy prostate, the differentiated luminal cells express high levels of MT to coordinate the function and storage of accumulated zinc essential to the specialized metabolism and secretory functions of these cells. During oncogenic transformation, malignant prostate cells uniformly lose this distinctive feature of zinc accumulation and, as would be expected, a concomitant loss of *MT* expression is observed, especially in advanced prostate cancers[448,452,470–473]. Exacerbating this, patterns of hypermethylation commonly observed in prostate cancer cells further reduce expression of specific *MT* isoforms[474–476]. MT has been shown to demonstrate tumor suppressive activity in prostate cancers[477]. MT can inhibit the activation of pro-inflammatory cytokines and transcription factors associated with treatment failure and metastasis, such as IL-6, IL-12, TNF-α and NF-kB[478–480]. One meta-analysis found that the loss of the protective effects of MT leads to an escalation of pathogenic processes and carcinogenesis[452].

1.6.2 Transcriptional regulation

The expression of *MT* genes is controlled primarily at the level of transcription. The upstream DNA region on the 5′ end of *MT-1* and *MT-2* genes contains a TATA box core promoter element and numerous cis-acting response elements within the proximal promoter region[450,481–483]. The number of copies and configuration of the promoter response elements vary according to the *MT* isoform to allow sensitive, fine-tuned regulation by trans-acting factors carrying signals from the cellular environment. In general, all *MT* genes contain multiple copies

of a metal-response element (MRE) which binds MTF-1[483,484]; glucocorticoid-response elements (GRE) to which GR binds[481]; and redox (antioxidant)-response elements (RRE) that are binding sites for Nrf1 and Nrf2[485–488] within the regions upstream of the transcriptional start site, and may also include cAMP responsive elements, STAT3 responsive elements, tissue plasminogen activator-responsive elements, and interferon responsive elements[489,490]. Despite the dependency of the prostate on AR function as a master regulator of critical transcriptional programs, the regulation of *MT1* and *MT2* has not been shown to rely on androgen, at least not directly. Investigations exploring a possible role for androgen regulation of *MT3* following the reports that observed an association between prostate cancer and MT3 thus far have failed to conclusively define the relationship due to opposing results[491,492].

1.7 Summary and Research Objectives

1.7.1 Rationale

Prostate cancer is the second leading cause of male-related cancer deaths in the Western world, and with a rapidly aging population, incidence rates are expected to rise significantly. Localized treatments for early stage prostate cancers are often successful, yet between 20 to 40 percent of patients will have recurrence after radical prostatectomy or radiation therapy. Cancer that has disseminated from the primary site requires initiation of systemic therapies to treat the metastatic lesions. Most prostate cancers are dependent on AR for growth and survival, and the AR remains a validated therapeutic target for all stages of disease. Full-length AR (FL-AR) is a ligand activated transcription factor that mediates the effects of androgen and controls a vast transcriptional network. The standard care for advanced prostate cancer is ADT by chemical or

surgical castration. ADT induces disease regression in most patients but is not curative. Unfortunately, clinical responses are temporary, and progression manifests in the lethal form known as CRPC. Additional treatment with chemotherapeutics or second generation nonsteroidal anti-androgens such as enzalutamide may provide a brief window of efficacy before the inevitable emergence of treatment resistance. The discovery and development of additional therapeutic tools to care for these patients represents a major unmet clinical need. All currently approved drugs targeting the AR signaling pathway converge in their ultimate site of action at the C-terminus ligand-binding domain (LBD), i.e. enzalutamide. Ralaniten, a novel first-in-class drug, binds the TAU-5 region within activation function-1 of the receptor's N-terminal domain (NTD). This unique mechanism confers ralaniten with the ability to inhibit FL-AR, as well as the AR isoforms that become expressed with resistance to inhibitors of AR LBD (e.g., enzalutamide), such as AR that has acquired gain-of-function mutations and AR with structural alterations that yield constitutively active truncated AR splice variants (AR-Vs). The remarkable results consistently reported during extensive pre-clinical study of ralaniten merited approval for evaluation in humans. Two analogs of ralaniten have reached first-in-human clinical trials with EPI-7386 currently in phase 1 trials in the USA and Canada for CRPC. Here we investigate the gene expression profile in prostate cancer cells in response to ralaniten to aid in determining potential combination therapies, resistance mechanisms, and off-targets.

1.7.2 Hypothesis and Specific Aims

Due to a unique mechanism of action, we predict that ralaniten will induce a distinct global transcriptional profile compared to non-steroidal antiandrogens. This study initiates

characterization of the ralaniten molecular profile to develop a comprehensive understanding of the cellular responses following treatment with AR-NTD inhibitors, and how this differs from existing AR-LBD inhibitors, in the hope that these insights will be of value for developing the next generation antagonists of the AR-NTD and combination therapies. The aims of this investigation were:

1. Identify genes that are uniquely expressed in response to ralaniten
2. Determine the role of AR in regulating the expression of these unique genes
3. If the mechanism is not dependent on AR, elucidate a possible mechanism

Chapter 2: MATERIALS & METHODS

2.1 Materials

2.1.1 Cell Culture

LNCaP cells were from Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA) and maintained in phenol red-free RPMI 1640 medium supplemented with 5% FBS (VWR, Radnor, PA, USA). LNCaP95 (LN95) cells were provided by Dr. Stephen Plymate (University of Washington, Seattle, Washington) and were maintained in phenol red-free RPMI 1640 medium supplemented with 10% dextran-coated charcoal-stripped FBS. DU145 cells were from Dr. Victor Ling (BC Cancer Research Institute, Vancouver, BC) and maintained in DMEM (Invitrogen, Carlsbad, California) with 10% FBS and supplemented with 2 mM L-glutamine and 1 mM of sodium pyruvate. PC-3 cells were purchased from the American Type Culture Collection (Manassas, Virginia) and maintained in DMEM with 5% FBS and supplemented with 2 mM L-glutamine and 1 mM of sodium pyruvate. All cells used in the experiments were maintained in a humidified incubator at 37°C with 5% CO_2 and passaged in our laboratory not more than 5 to 10 times after resurrection. LNCaP, PC3, and DU145 cells were authenticated by short tandem repeat analysis and tested to ensure that they were mycoplasma free by DDC Medical (Fisher Scientific, Ottawa, Ontario) in September 2013. LNCaP95 cells were not authenticated in our laboratory. Cell lines were routinely tested with the Venor™ GeM Mycoplasma Detection Kit (Sigma-Aldrich) to ensure that they were mycoplasma-free.

LNCaP prostate adenocarcinoma cells were isolated from needle aspiration biopsy derived from a left supraclavicular lymph node metastasis from a 50-year-old Caucasian male in 1977. They

express FL-AR that contains a T877A mutation in its ligand binding domain, rendering it more promiscuous in response to stimulation by steroid hormones other than androgens[493,494].

LNCaP95 cells express functional FL-AR, AR-V7, and potentially other AR splice variants. Growth of the LNCaP95 cell line is androgen-independent and was developed from long-term continuous culture of LNCaP cells in androgen-depleted conditions[495].

PC3 cells were derived from a bone metastatic lesion from a grade IV prostatic adenocarcinoma of a 62-year-old Caucasian male in 1976. These cells are androgen-insensitive and do not express functional AR. PC3 cells have deletion of the PTEN gene. PC3 cells have prostatic neuroendocrine carcinoma features[493,496,497].

DU145 cells are adenocarcinoma cells of prostatic origin from a 69-year-old white male derived from a brain metastasis removed during a parieto-occipital craniotomy [498]. They do not express AR or KLK3 (PSA) and test negative for neuroendocrine markers[493,499].

2.1.2 Compounds and Reagents

Ralaniten was provided by NAEJA (Edmonton, AB, Canada). EPI-7170 and LPY-26 were synthesized by Dr. Raymond Andersen (University of British Columbia). SINT1 is a natural compound extracted and purified by Dr. Raymond Anderson (UBC). Enzalutamide was purchased from OmegaChem (Lévis, QC, Canada). Bicalutamide was a gift from Dr. Marc Zarenda (AstraZeneca, Cambridge, England). The synthetic androgen metribolone (R1881) was purchased from AK Scientific (Mountainview, CA, USA). All other chemicals including

BADGE-H2O2 were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2 Methods

2.2.1 Microarray and analysis

LNCaP cells were seeded in 6-well culture plates ($1.5x10^5$ cells/well) in phenol red-free RPMI 1640 with 5% FBS and incubated for 24 hours to allow attachment. After reaching 60-70% confluence, the cells were serum-starved for 24 hours. Next, cells were treated with enzalutamide, bicalutamide, ralaniten, or DMSO vehicle. Concentrations employed were based upon the IC50s reported for each compound to block AR transcriptional activity Enzalutamide was used at 50X(5 uM) its IC50 (~100 nM), bicalutamide at 50X(10 uM) its IC50 (~200 nM), and the poorly soluble ralaniten compound was limited to 3X(35 uM) its IC50 (~10 uM). After 24 hours treatment, total RNA was extracted, reverse transcribed, and cDNA generated was hybridized to the GeneChip Human Transcriptome Array 2.0 from Affymetrix. Completion of the RT-PCR, cDNA hybridization and chip reading were carried out at CDRD's Target Validation Division at the University of British Columbia (Vancouver, BC, Canada; www.cdrd.ca). The raw signal output was analyzed using GeneSpring software (version 13.1) as recently described by Banuelos et al[500]. Briefly, hierarchical clustering of the data was performed by conducting a 2-way ANOVA with the significance threshold set at 0.05. The Benjamini-Hochberg correction was applied to reduce the false discovery rate (FDR). GSEA version 7.0 software (http://software.broadinstitute.org/gsea/msigdb/index.jsp) was utilized to interrogate differential expression levels between vehicle and drug treatment for each gene, with

analysis based upon the Molecular Signatures Database Set H (Hallmark gene sets, h.all.v7.1.symbols.gmt). Statistical significance was limited to those enrichment gene sets revealed by GSEA as exhibiting a nominal $p < 0.05$ and FDR < 0.05. A pathway analysis was applied to cluster these genes common function or within specific cellular pathways. The inclusion cutoff was set at a minimum of 5 genes per pathway and the determination of significance ($p \leq 0.05$) was calculated based on both the number of genes which appeared in the screen and the magnitude of expression change observed. Next, genes identified by the microarray were ranked by fold change and the top 10 genes were listed individually.

2.2.2 Plasmids and transfections

The MT1G-Luciferase and pGL3-Basic reporter plasmids encoding firefly luciferase were from Dr. Antoine Galmiche (Université de Picardie Jules Verne, Amiens, France) with permission from Dr. Shinichiro Takahashi (Tohoku Medical and Pharmaceutical University, Japan). The pMT1F-Luciferase, pMT2A-Luciferase, and pGL2-Basic reporter plasmids encoding firefly luciferase were from Dr. Carl Séguin (Université Laval, QC, Canada). The MT1G-Luciferase plasmid contains a DNA fragment of the MT1G gene encompassing 5' flanking promoter sequences up to nucleotide –416 upstream of the transcription starting site. The pMT1F-Luciferase plasmid contains a DNA fragment of the MT1F gene encompassing 5' flanking promoter sequences up to nucleotide –1843, whereas the pMT2A-Luciferase plasmid contains MT2A gene 5' flanking promoter sequences up to nucleotide –293. All three *MT*-gene reporter plasmids have been previously described elsewhere[484,501,502]. Pooled siRNA against *AR*, *Nrf2*, *MTF-1*, and non-targeting control were purchased from Dharmacon Research (Lafayette,

CO, USA). Transfections for targeted gene knock-down used Lipofectamine RNAiMAX (Invitrogen) to transfect either 10 nM (AR), 15 nM (Nrf2), or 15 nM (MTF-1) of the siRNA, or matched equimolar concentration of non-targeting control, into cells in Opti-MEM serum free media (Thermo Fisher Scientific). For reporter assays, transfections were performed in serum-free media with Fugene6 or FugeneHD (Promega, Madison, Wisconsin). LNCaP cells seeded in 24-well plates were co-transfected with one of the luciferase reporters (0.25 μg/well) and the corresponding empty vector (0.5 μg/well).

2.2.3 Endogenous gene expression: qRT-PCR assays

For all experiments, after cells were harvested, the extraction of total RNA from the lysate was completed using the RNeasy Micro Kit (Qiagen). These samples were then cleaned from nucleic acid contamination using DNase I Kit, Amplification grade (MilliporeSigma) and reverse transcribed using the High-Capacity RNA-to-cDNA Kit (ThermoFisher Scientific). Diluted cDNA and Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) were combined with gene specific primers. Transcripts were measured by qRT-PCR QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems by Life Technology) and gene expression was normalized to the specified housekeeping genes. The gene specific primer sequences are provided below in Table 2.1.

2.2.4 Western blot analyses

For whole cell analysis, lysates were harvested in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% IGEPAL CA630, 0.1% SDS, 0.5% deoxycholate) containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail TabletsTM (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail TabletsTM (Roche, Laval, Quebec). Using a syringe with a 28-gauge needle, cells were homogenized, and the lysates were then cleared by centrifugation at 15,000 g for 15 minutes.

For separation of cytoplasmic and nuclear fractions, cells were washed in ice cold phosphate-buffered saline (PBS) and resuspended in a hypotonic lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM KCl, 3 mM MgCL2, 0.5% IGEPAL CA-630) with cOmplete EDTA-free protease inhibitor and PhosStop phosphatase inhibitor (Roche). The lysates were incubated for 15 minutes on ice and the nuclei were separated by centrifugation at 500 g for 10 minutes. The pelleted fraction resuspended in RIPA buffer with cOmplete EDTA-free protease inhibitor and PhosStop phosphatase inhibitor and homogenized using a syringe with a 28-gauge needle. Lysates were incubated for 30 minutes at 4°C on a rotating rack and then cleared by centrifugation at 15,000 g for 15 minutes. The concentration of protein was quantified by using a BCA Protein Assay Kit (Thermo Scientific Inc., Rockford, USA). Equal amounts of denatured proteins were separated by 10% SDS-PAGE. Protein was transferred to nitrocellulose (GE Healthcare Life Sciences) or PVDF membrane (Millipore LTD, Cork, IRL) and blocked for 1 hour in 5% weight/volume (w/v) nonfat milk in phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST) prior to incubation with primary antibody. Relevant information regarding specific antibodies are given below in Table 2.2. Antibodies were diluted in PBST

with 5% skim milk and membranes were incubated overnight at 4°C with primary antibody. The following day, membranes were washed three times with PBST, and then incubated with a secondary antibody conjugated to horseradish peroxidase for 1-2 hours at room temperature. After four washes with PBST, chemiluminescence was detected with ECL Prime Reagent (GE Healthcare Life Science, Mississauga, ON). Western blot images were captured using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, California).

2.2.5 Comparison of AR-NTD inhibitors

LNCaP cells were seeded in 24 well culture plates ($3.0x10^4$ cells/well) in phenol red-free RPMI 1640 with 5% FBS and incubated for 24 hours to allow attachment. At 60-70% confluence, the cells were transfected with the reporter plasmid pMT1F-Luciferase, pMT1G-Luciferase, or pMT2-Luciferase. After 16 hours, cells were treated with SINT1 (35 μM), LPY-26 (35 μM), BADGE-2H2O (35 μM), Ralaniten (35 μM) or DMSO vehicle. After 24 hours of treatment, cells were harvested and lysed using passive lysis buffer (Promega, Madison, Wisconsin). Luciferase activity was measured for 10 seconds with the Luciferase Assay System (Promega) and the data were normalized to total protein concentration determined by Bradford assay.

2.2.6 Dose response studies

For the reporter assays, LNCaP cells were seeded in 24 well culture plates ($3.0x10^4$ cells/well) in phenol red-free RPMI 1640 with 5% FBS and incubated for 24 hours. At 60-70%

confluence, the cells were transfected with the reporter plasmid pMT1F-Luciferase, pMT1G-Luciferase, or pMT2-Luciferase. After 16 hours, cells were treated with increasing concentrations of ralaniten or DMSO vehicle. Following 24 hours of treatment, cells were harvested and lysed using passive lysis buffer (Promega, Madison, Wisconsin). Luciferase activity was measured for 10 seconds with the Luciferase Assay System (Promega) and the data were normalized to total protein concentration determined by Bradford assay.

For the qRT-PCR assays, LNCaP cells were seeded in 6 well culture plates ($1.5x10^5$ cells/well) in phenol red-free RPMI 1640 with 5% FBS and incubated for 24 hours to allow attachment. After reaching 60-70% confluence, the cells were serum starved for 24 hours and then treated with increasing concentrations of ralaniten or DMSO vehicle. After 24 hours of treatment, cells were harvested in 1 mL TRIzol reagent (Invitrogen) and mRNA transcripts were isolated and quantified relative to the housekeeping gene SDHA.

2.2.7 Comparison of gene expression responses across cell lines

LNCaP, LNCaP95, PC3, and DU145 cells were seeded separately in 6 well culture plates ($1.5x10^5$ cells/well) each in their respective media specified above including serum and incubated for 24 hours to allow attachment. Media was then removed, switched with serum free media, and cells were serum starved for 24 hours prior to treatment. Cells were treated with enzalutamide, ralaniten, EPI-7170 or DMSO vehicle. For the *MT* isoforms experiment, enzalutamide was used at 5 uM and 20 uM, ralaniten at 5 uM and 35 uM and its more potent analog, EPI-7170 at ~5X (5 uM) its IC50 (~1 uM). For the Nrf2 and Nrf2-regulated genes experiment, cells were treated with enzalutamide (5 μM), ralaniten (35 μM), EPI-7170 (5 μM) or

DMSO vehicle. After 24 hours of treatment, cells were harvested in 1 mL TRIzol reagent (Invitrogen) and mRNA transcripts were isolated and quantified relative to the housekeeping gene SDHA.

2.2.8 Nrf2 nuclear translocation

LNCaP cells were seeded in 10cm culture plates ($1.0x10^6$ cells/plate) in phenol red-free RPMI 1640 with 5% FBS and incubated for 24 hours to allow attachment. After reaching 60-70% confluence, the cells were serum starved for 24 hours. Next, cells were pre-treated for 1 hour with ralaniten (35 uM), enzalutamide (5 uM), bicalutamide (10 uM) or DMSO vehicle before stimulation with 1 nM androgen (R1881) or EtOH control. After 24 hours, cells were harvested, and cytoplasmic and nuclear fractions prepared as described in section 2.2.4. Levels of proteins were determined by immunoblot using antibodies to Nrf2, PSA(*KLK3*), Lamin, and B-actin.

2.2.9 Targeted gene knock down studies

For the qRT-PCR experiments, LNCaP and LNCaP95 cells were seeded in 6 well culture plates ($1.5x10^5$ cells/well) each in their respective media specified above including serum. After incubating for 24 hours to allow attachment, cells were transfected with siRNA or non-targeting control. At 24 hours post transfection, cells were pre-treated with enzalutamide (5 μM), ralaniten (35 μM), EPI-7170 (5 μM) or DMSO vehicle for 16 hours prior to addition of 1 nM R1881 or EtOH vehicle control. After 24 hours of treatment, cells were harvested in 1 mL TRIzol reagent

(Invitrogen) and mRNA transcripts were isolated and quantified relative to the housekeeping gene SDHA.

For the protein expression analysis, LNCaP and LNCaP95 cells were seeded in 10 cm culture plates ($1.0x10^6$ cells/plate) each in their respective media specified above including serum. Following a 24-hour incubation period to allow attachment, cells were transfected with siRNA or non-targeting control. At 24 hours post transfection, cells were pre-treated with enzalutamide (5 μM), ralaniten (35 μM), EPI-7170 (5 μM) or DMSO vehicle for 16 hours prior to addition of 1 nM R1881 or EtOH vehicle control. After 24 hours of treatment, cells were harvested and prepared as described above. Protein expression was determined by immunoblot using antibodies to AR, Nrf2, and B-actin.

2.2.10 Statistical analysis A One- or Two-Way ANOVA statistical test was used to determine significance for all comparisons unless otherwise stated (GraphPad Prism, version 8.0). p-value corrections were applied for all multiple comparisons (Tukey, Sidak or Dunnett as appropriate), and a p-value < 0.05 was considered statistically significant.

Table 2.1 *Gene-specific primer sequences for qRT-PCR experiments.*

Target	Direction	Sequence
AR	FWD	5'-AGGAACTCGATCCTATCATTGC-3'
	REV	5'-CTGCCATCATTTCCGGAA-3'
AR-V7	FWD	5'-CCATCTTGTCGTCTTCGGAAATGTTAT-3'
	REV	5'-TTTGAATGAGGCAAGTCAGCCTTTCT-3'
FKBP5	FWD	5'-CGCAGGATATACGCCAACAT-3'
	REV	5'-GAAGTCTTCTTGCCCATTGC-3'
PSA	FWD	5'-TCATCCTGTCTCGGATTGTG-3'
	REV	5'-ATATCGTAGAGGGGGTGTGG-3'
MT1F	FWD	5'-ACAGAGAGACATGTACAAACCTGG-3'
	REV	5'-GAATGTAGCAAATGGGTCAAGGTG-3'
MT1G	FWD	5'-ATAGAGTGACCCGTAAAATCCAGG-3'
	REV	5'-TAGCAAAGGGGTCAAGATTGTAGC-3'
MT1X	FWD	5'-GTGTTTTCCTCTTGATCGGGAACTC-3'
	REV	5'-TCCATTTCGAGGCAAGGAGAAG-3'
MT2A	FWD	5'-AGATGTAAAGAACGCGACTTCCAC-3'
	REV	5'-AATATAGCAAACGGTCACGGTCAG-3'
MTF1	FWD	5'-CACCCTGTACGTTATCTTCTAGCTC-3'
	REV	5'-CAGTTTCCTTACCACCTCCTAAGTC-3'
SDHA	FWD	5'-CAGCATGTGTTACCAAGCTGT-3'
	REV	5'-CGTGTCGTAGAAATGCCACCT-3'

Table 2.2 *Antibody sources and working concentrations (optimized)*

Target	Species	Supplier	Concentration
AR	Rabbit pAb	Abcam	1:1,000
PSA/KLK3	Rabbit mAb	Cell Signaling	1:1,000
Nrf2	Rabbit pAb	Abcam	1:1,000
Lamin A/C	Mouse mAb	BD Biosciences	1:1,000
β-Actin	Mouse mAb	Sigma-Aldrich	1:5,000
α-mouse	Horse mAb	Cell Signaling	1:10,000
α-rabbit	Goat mAb	Cell Signaling	1:10,000

Chapter 3. RESULTS

3.1 Identification of genes that are uniquely expressed in response to ralaniten

Ralaniten directly binds to AF-1 in the AR-NTD to block essential protein interactions required for transactivation that include the basal transcriptional machinery. Antiandrogens compete with androgen for the C-terminal LBD and interfere with formation of the AF-2 interface for interaction with coactivators to mediate transcriptional activity. One essential interaction blocked by antiandrogens is N/C interaction required for androgen-dependent transactivation. Thus, ralaniten acts via a different mechanism to achieve inhibition of AR signaling compared to antiandrogens. Differences between these two classes of inhibitors on gene expression in the absence of androgen has not previously been reported, thus warranting characterization.

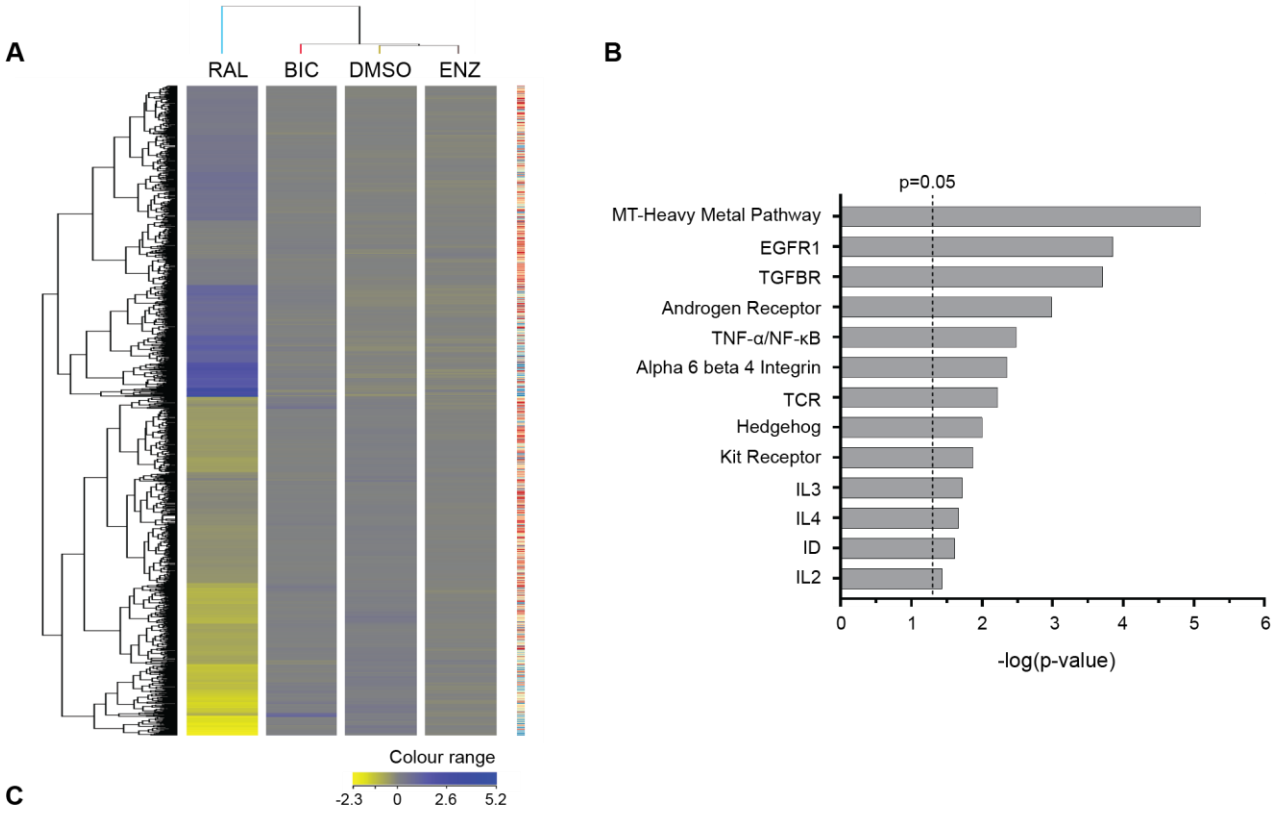
3.1.1 In the absence of androgen, ralaniten has a distinct gene expression signature compared to antiandrogens

To measure differential changes in global gene expression, we employed a whole transcriptome microarray on cDNA isolated from LNCaP cells and compared the effects of ralaniten with the AR-LBD inhibitors, ENZ and BIC, relative to the vehicle control, DMSO (Figure 3.1A). Modeling the typical patient status when treating cases of advanced disease, we focused specifically within the context of therapeutic castration; hence, the experiment was conducted in androgen-free conditions. In addition, this would preclude confounding the data with potential masking effects due to the presence of androgen. Samples were analyzed using

Affymetrix microarray, and hierarchal clustering revealed a subset of genes robustly induced by ralaniten compared to vehicle. Notably, the expression of these genes was not observed to be elevated in samples treated with BIC or ENZ suggesting that this effect was unique to treatment with ralaniten.

Exploring the biological significance of these data further, a pathway analysis determined that these genes clustered within specific cellular pathways or had common functions (Figure 3.1B). Setting an inclusion cut-off at a minimum of 5 genes per pathway, the determination of significance ($p \leq 0.05$) was calculated based on both the number of genes which appeared in the screen and the magnitude of expression change observed. As might be expected of an inhibitor of AR, significant changes were observed in the expression of AR pathway genes. However, the most significantly altered cluster involved genes comprising elements of the MT-Heavy Metal Pathway.

When genes identified by the microarray were ranked by fold-change and listed individually, members of the MT-Heavy Metal pathway comprised a majority within the top 10 candidate genes (Figure 3.1C). The data revealed that for ralaniten within the top 10 most highly induced levels of expression of genes, 6 were isoforms from the *MT1* and *MT2* branches of the *MT* family. All 6 isoforms were upregulated by orders of magnitude in comparison to the treatment conditions with DMSO, BIC, or ENZ. These results revealed that ralaniten uniquely induced expression of *MT1* and *MT2*.



Corrected p-Value	Log_2 FC (DMSO)			Gene ID
	BIC	ENZ	RAL	
0.0028	-0.024	0.413	4.403	**MT1F**
0.0088	-0.085	0.408	4.126	**MT2A**
0.0027	-0.064	0.219	3.660	**MT1G**
0.0130	-0.119	0.282	3.421	**MT1H**
0.0135	0.354	-0.012	3.072	FAM129A
0.0124	-0.059	0.290	3.001	**MT1X**
0.0122	0.260	0.042	2.795	C1orf158
0.0189	0.920	0.119	2.769	SLC7A11
0.0189	-6.84e-4	-0.140	2.649	SLP1
0.0428	0.038	0.232	2.500	**MT1CP**

Figure 3.1 Expression of MT isoforms is positively correlated with ralaniten treatment

(A) Heatmap showing differentially regulated genes in LNCaP cells following treatment with BIC (10 µM), ENZ (5 µM), RAL (35 µM) or DMSO vehicle. Data show two biological replicates per treatment group and are normalized to vehicle. **(B)** Pathway analysis showing genes clustered within specific cellular pathways or with common functions. Inclusion cut-off (≥5 genes/pathway), significance calculated based on both the number of genes which appeared in the screen and the magnitude of expression change observed ($p \leq 0.05$). **(C)** List ranking the top 10 genes which positively correlate with ralaniten treatment. Five genes cluster within the MT family, show significant enrichment, and are specifically associated with ralaniten treatment.

3.2 Induction of MT genes is unique to ralaniten

AR-LBD inhibitors ENZ and BIC have no effect on *MT* gene expression. To investigate whether the induction of expression of *MT1* and *MT2* by ralaniten was unique to ralaniten or a general effect common to AR-NTD inhibitors, we tested SINT-A and LPY-26 which bind to a unique site on the AR-NTD but have no structural similarity to ralaniten, plus BADGE-2H2O, a structural analog of ralaniten with no AR activity (Figures 3.2A and 3.2B). Three reporter gene constructs (pMT1F-luc, pMT2-luc, and pMT1G-luc) were evaluated in transiently transfected LNCaP cells. These constructs contain the promoter regions of these genes with common binding sites for Sp1, NF1, GR, Nrf2, MTF-1, and AP-1 transcription factors and are inducible by metals, hypoxia, oxidative stress, steroid hormones, xenobiotics, and inflammatory cytokine signaling [481,484,490,501–504] (also discussed in Methods section). At equimolar concentrations set to 35 μM for all compounds, only ralaniten induced the activities of these reporters (Figure 3.2C). Neither of the alternate AR-NTD inhibitors nor BADGE-2H2O had any significant activity. Thus, induction of expression of *MT* genes was not a shared effect amongst all AR-NTD inhibitors and instead it was unique to ralaniten. These reporter gene data suggest a mechanism of increased transcription of these genes by ralaniten rather than stabilization of their mRNA.

To validate the induction of expression of *MT* genes shown in the array data, qPCR was performed using cDNA generated by reverse transcription of the total mRNA harvested from LNCaP cells from three independent experiments. Concentrations employed were based upon the IC50s reported for each compound to block AR transcriptional activity. Enzalutamide was used at 50X and 200X its IC50 (~100 nM). Ralaniten concentrations were limited due to poor solubility at 0.5X and 3X its IC50 (~10 μM) and its more potent analog, EPI-7170 at ~5X its

IC50 (~1 μM). ENZ did not induce expression of *MT1* and *MT2* genes at either the high or low concentration (Figure 3.2D). EPI-7170 induced some minimal transcript expression which was not statistically significant. Only ralaniten at 35 μM significantly induced expression of *MT1* and *MT2* genes whereas at a lower concentration of 5 μM had no effect. In addition, primers for the *MT3* isoform were included to determine whether ralaniten induced all or a subset of metallothionein genes. This induction was unique to *MT1* and *MT2* isoforms with ralaniten having no effect on expression of the *MT3* isoform. Overall, these data validate that the gene-specific upregulation of *MT1* and *MT2* families was unique to ralaniten at concentrations required to block AR-transcriptional activity yet did not induce expression of *MT3*.

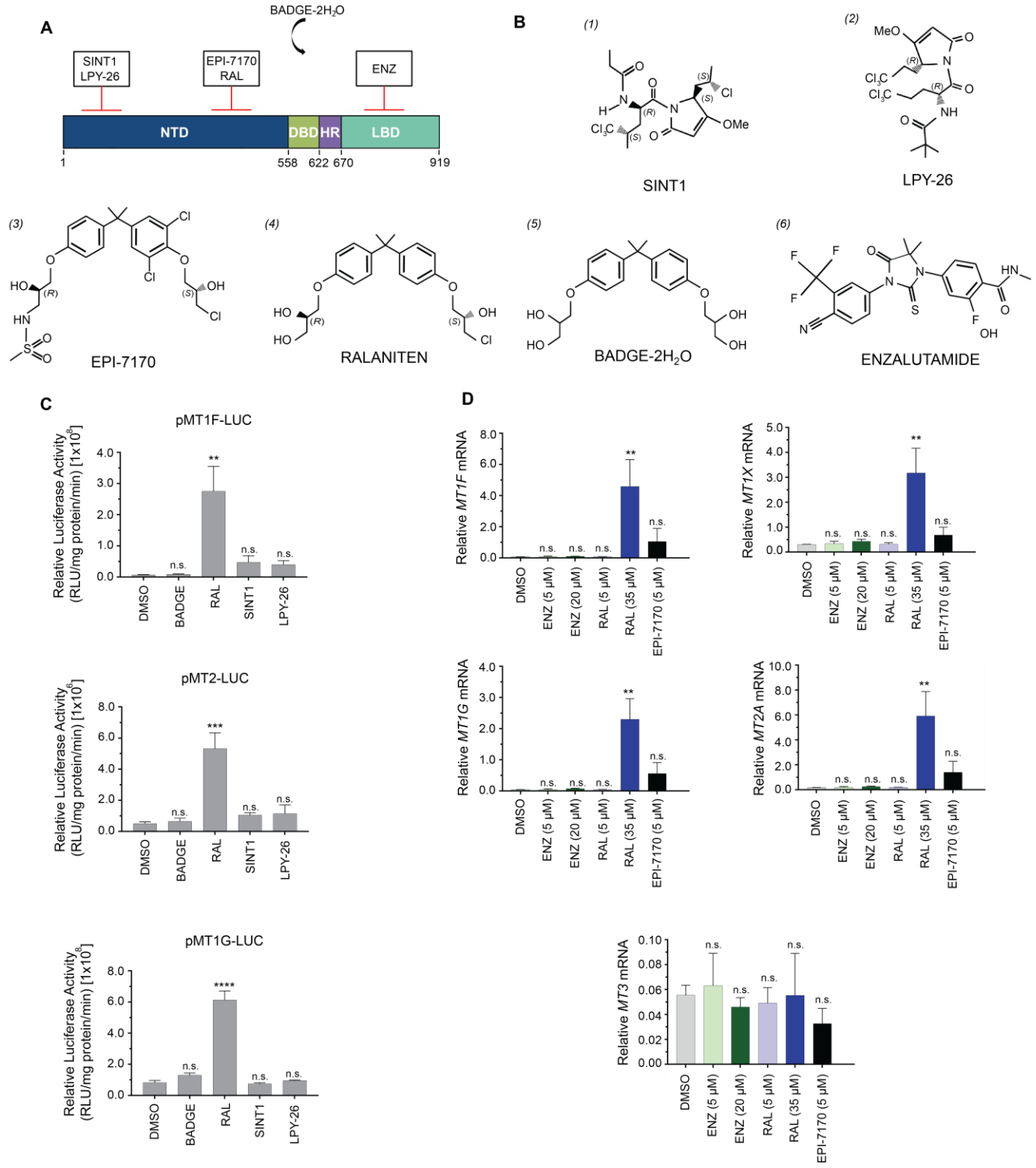


Figure 3.2 Induction of MT gene expression is unique to ralaniten

(**A**) Illustration of the AR region targeted by the various compounds tested. BADGE-2H_2O is inactive and does not inhibit the AR. Antagonists of similar class are grouped together. (**B**) Depiction of the chemical structure for each

compound. (**C**) LNCaP cells transfected with the various MT1/2-luciferase reporters. Cells were subsequently treated with DMSO, BADGE-2H_2O (35 μM), RAL (35 μM), SINT1 (35 μM), or LPY-26 (35 μM) for 24 hrs. Data presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's test applied *post hoc*, *n*=3 independent experiments. (**D**) Transcript levels of *MT1F, MT1G, MT1X, MT2A* and *MT3* normalized to *SDHA* from LNCaP cells treated with ENZ (5 and 20 μM), RAL (5 and 35 μM), EPI-7170 (5 μM) or v/v DMSO. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc, n*=3 independent experiments (*p<0.05; **p<0.01; ***p<0.001; # p<0.0001; n.s., not significant).

3.2.1 Ralaniten induction of MT gene expression is concentration dependent

A concentration dependent relationship between ralaniten and *MT* gene expression was investigated using LNCaP cells transiently transfected with MT-luciferase reporter gene isoform constructs. These experiments showed that increasing concentrations of ralaniten yielded increasing induction of reporter activities (Figure 3.3A). Measurement of endogenous gene expression by qPCR yielded consistent results to the reporter gene constructs. At 25 μM ralaniten, significant induction of *MT1F, MT1G, MT1X*, and *MT2A* were measured and 35 μM further increased these levels (Figure 3.3B). Regarding gene expression, these data support that ralaniten induction of these isoforms trends with elevating concentrations.

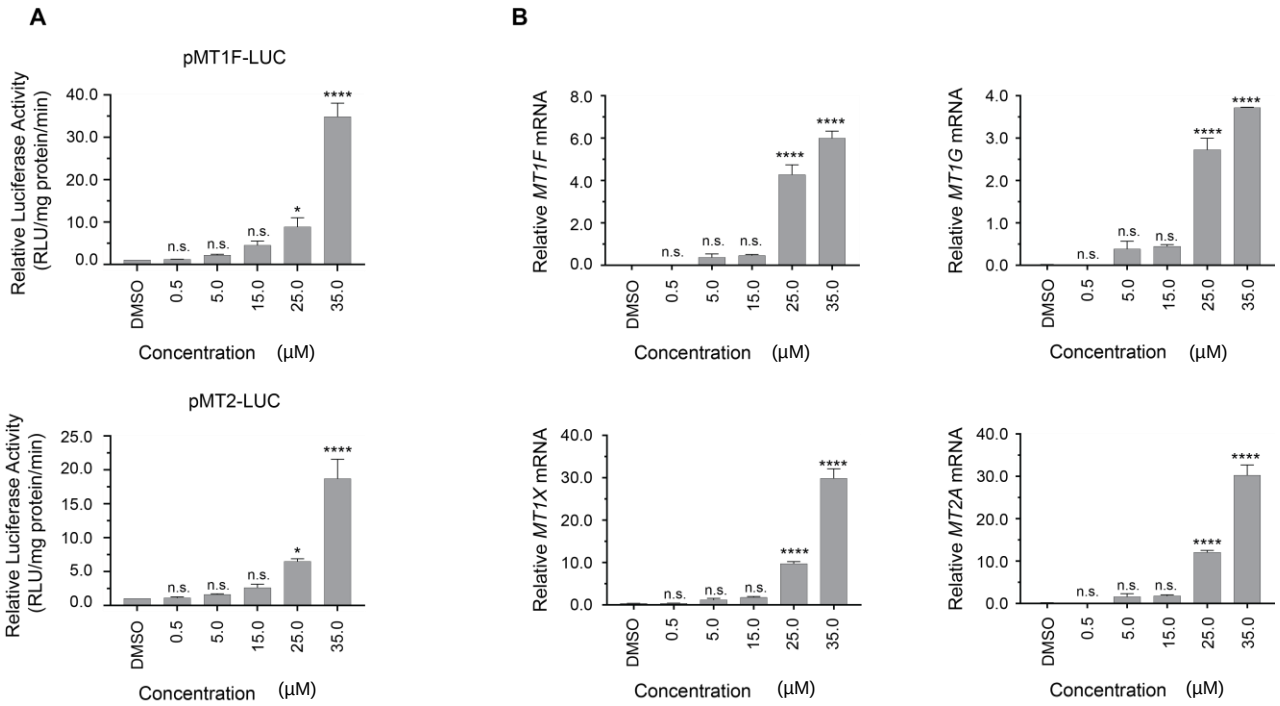


Figure 3.3 Ralaniten dose-response studies

(**A**) LNCaP cells transfected with MT1-luciferase or MT-2-luciferase reporters. Cells were subsequently treated with DMSO or increasing concentrations of EPI-002 (0.5-35 μM) for 24 hours. Data presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's test applied *post hoc*, *n*=3 independent experiments. (**B**) Transcript levels of *MT1F*, *MT1G*, *MT1X*, and *MT2A* normalized to *SDHA* from LNCaP cells treated with DMSO or increasing concentrations of RAL (0.5-35 μM) for 24 hours. Data presented as mean ± SEM and analyzed by two-way ANOVA with Dunnet's test applied *post hoc*, *n*=3 independent experiments.

(*p<0.05; **p<0.01; ***p<0.001; **** p<0.0001; n.s., not significant).

3.3 Induction of MT gene expression by ralaniten in cells that express AR

Despite sharing the same therapeutic target as ralaniten, other inhibitors of the AR did not affect expression of *MT* genes (Figure 3.2). To determine if expression of *AR* impacted ralaniten induction of *MT1* and *MT2* isoforms, a battery of prostate cancer cell lines that differ in expression and dependence upon the AR were examined. In AR-negative DU145 and PC3 prostate cancer cells, ralaniten was generally a poor-inducer of *MT1* and *MT2* gene expression. Statistically significant induction in these cells was only measured for *MT1F* in PC3 cells whereas for AR-positive LNCaP95 cells three of the four isoforms were significantly induced (Figure 3.4). Consistent with what was measured for LNCaP cells (Figure 3.2D), neither enzalutamide nor EPI-7170 had any significant effects (Figure 3.4). These data suggest that the induction of *MT* genes by ralaniten was not unique to LNCaP cells but also included LNCaP95 cells. Both of these cells express functional AR. Poor induction of expression of *MT* genes by ralaniten in AR-negative cells suggested that the mechanism may involve AR or alternatively a sensitivity of the parental LNCaP cells and its subline LNCaP95.

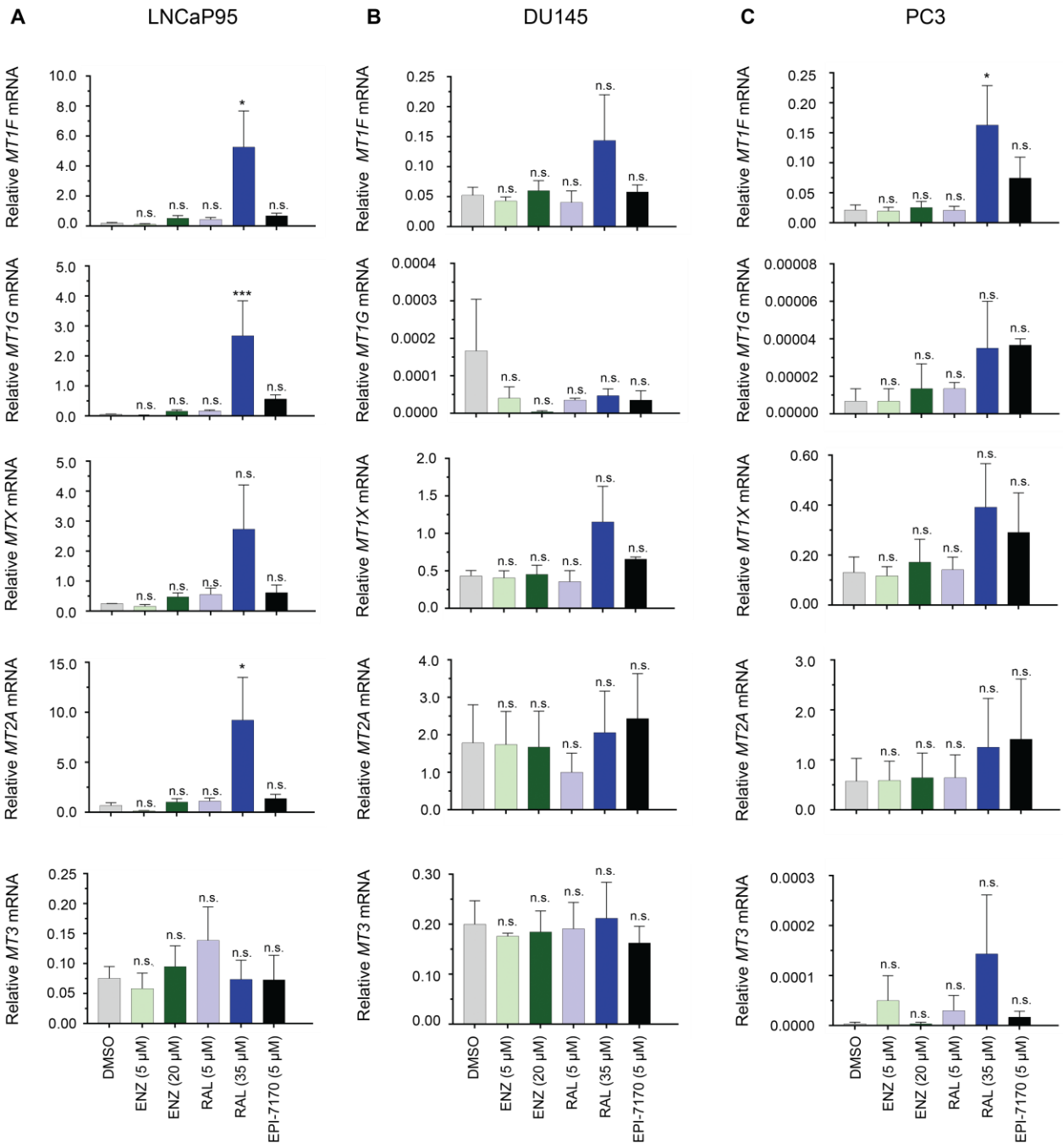


Figure 3.4 MT induction occurs in cell lines with functional, full-length AR

(**A**) Transcript levels of *MT1F, MT1G, MT1X, MT2A* and *MT3* normalized to *SDHA* from LNCaP95, DU145 (**B**), and PC3 (**C**) cells treated with ENZ (5 and 20 µM), RAL (5 and 35 µM), EPI-7170 (5 µM) or v/v DMSO. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc*, *n*=3 independent experiments, (*p<0.05; **p<0.01; ***p<0.001; # p<0.0001; n.s., not significant).

3.3.1 Induction of MT gene expression by ralaniten is by a mechanism independent of AR

To determine whether the AR played a central role in the mechanism of ralaniten induction of MT gene expression, *AR*-targeted knock-down using siRNA was employed in both LNCaP and LNCaP95 cells. ENZ (5 μM) and EPI-7170 (5 μM) were included as controls. Knockdown of AR protein was achieved in LNCaP cells both in the absence and presence of androgen (R1881) and each of the inhibitors (Figure 3.5A). At the transcript level, qPCR experiments confirmed knock down of *AR* mRNA and consistent with previous reports that these cells do not express significant levels of AR-V7 (Figure 3.5B). Knockdown of AR protein levels were sufficient to block androgen-induced levels of two well-characterized AR target genes, *KLK3* (PSA) and *FKBP5*. Knockdown of AR protein had no inhibitory effects on ralaniten-induction of *MT* isoforms (Figure 3.5C). Similarly, in LNCaP95 cells, knockdown of levels of AR and AR-V7 proteins (Figure 3.5D) and mRNAs (Figure 3.5E) were achieved that were sufficient to block androgen-induction of expression *KLK3*/PSA and *FKBP5* genes. Decreasing levels of AR had minimal effect on ralaniten induced expression of *MT* genes (Figure 3.5F). Together these findings suggest that *AR* expression was not required to mediate the upregulation of *MT* genes by ralaniten thereby revealing that this is potentially an off-target effect.

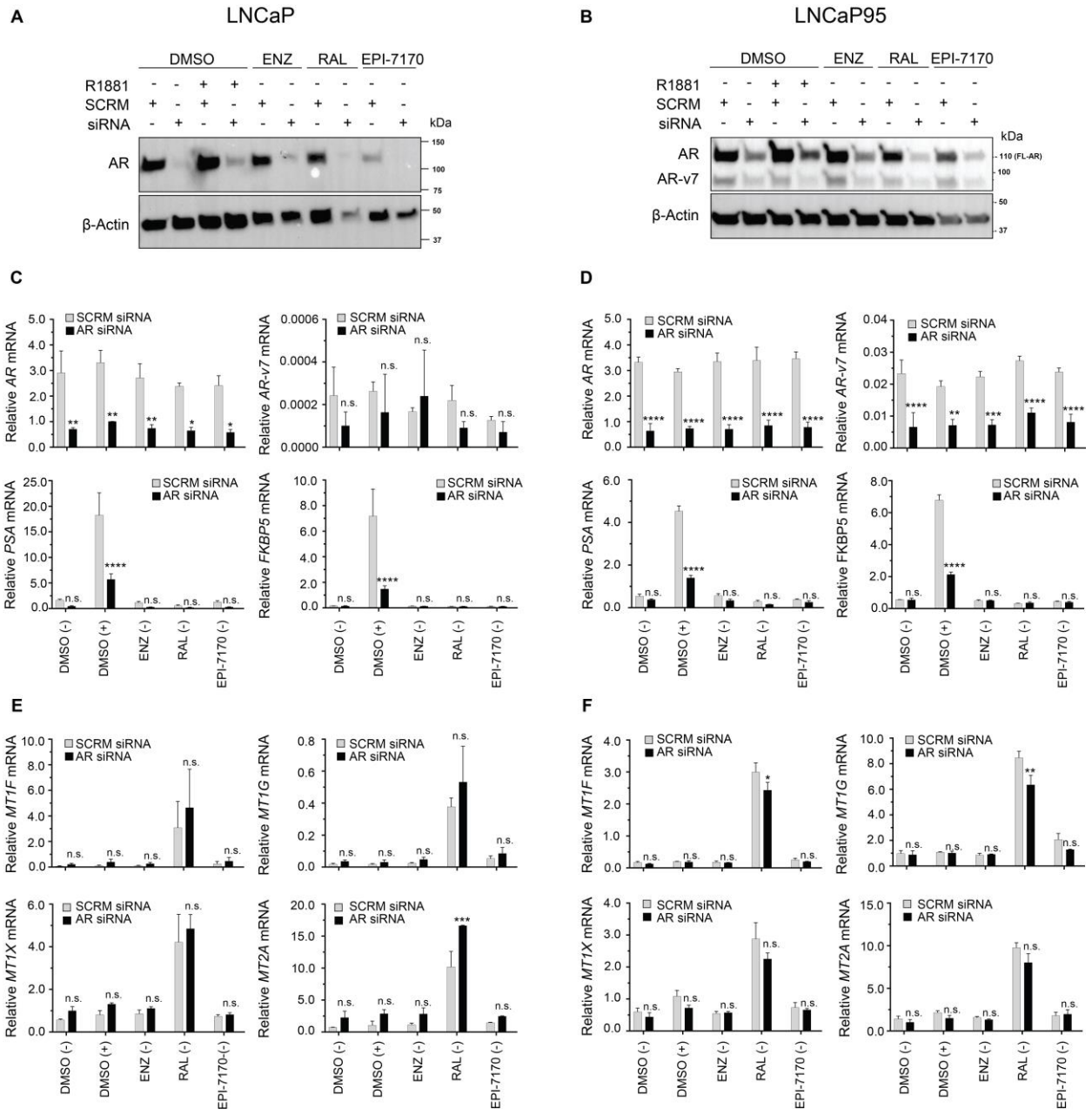


Figure 3.5 MT induction by ralaniten occurs independently of full-length AR

(**A**) Western blots of AR levels in LNCaP and LNCaP95 (**B**) cells transfected with control or AR targeting siRNA (10 nM) for 24h and treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). (**C**) Transcript levels of *AR*, *AR-V7*, *PSA*, and *FKBP5* normalized to *SDHA* from LNCaP and LNCaP95 (**D**) cells treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). Expression of androgen responsive genes are reduced following treatment with siRNA targeting the *AR*. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc*, *n*=3 independent experiments. (**E**) Transcript levels of *MT1F*, *MT1G*, *MT1X*, and *MT2A* normalized to *SDHA* from

LNCaP and LNCaP95 (**F**) cells treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). Expression of MT isoforms are unaffected following treatment with siRNA targeting the *AR*. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc*, *n*=3 independent experiments, (*p<0.05; **p<0.01; ***p<0.001; **** p<0.0001; n.s., not significant).

3.4 Ralaniten impacts the NRF2 pathway

MT genes are transcriptionally regulated by multiple transcription factors including Nrf2. Since each of the MT reporter gene constructs that was induced by ralaniten contain binding sites for Nrf2 (Figures 3.2C and 3.3A), we examined if ralaniten treatment impacted the Nrf2 pathway. Western blot analyses of LNCaP cells showed that ralaniten (35 μM), ENZ (5 μM), and BIC (10 μM) all blocked androgen-induced levels of PSA but only ralaniten increased levels of Nrf2 protein and Nrf2 nuclear accumulation (Figure 3.6A). Levels of *Nrf2* mRNA were not altered by ralaniten or any of the other treatments (Figure 3.6B). Expression of Nrf2-regulated genes were also significantly increased with ralaniten treatment, but not with ENZ. Ralaniten significantly induced the expression of heme oxygenase-1 (*HMOX1*) and *SLC7A11* (Figure 3.6B). Interestingly, EPI-7170 (5 μM) also induced levels of *SLC7A11* transcript in LNCaP cells. To build further insight into the cell line responses observed in Figure 3.4, levels of Nrf2 transcript and expression of some Nrf2 target genes were measured in response to ralaniten, ENZ, and EPI-7170. In response to ralaniten, there were significant increases in levels of *Nrf2* mRNA in both LNCaP95 and PC3 cells, whereas in DU145 cells there was no significant effect (Figure 3.7). Levels of expression of Nrf2 target genes were significantly increased by ralaniten in LNCaP95 cells (*SLC7A11*) and PC3 cells (*HMOX1*, *NQO1*, and *SLC7A11*). Consistent with ralaniten having no effect on levels of *Nrf2* mRNA in DU145 cells, nor were there any significant effects on expression of Nrf2 target genes (Figure 3.7B). In PC3 cells, the results

measured for ralaniten induced expression of *Nrf2* and its target genes (Figure 3.7C) do not correlate with the findings that *MT1G*, *MT1X* and *MT2A* genes are not significantly induced by ralaniten in these cells (Figure 3.4C).

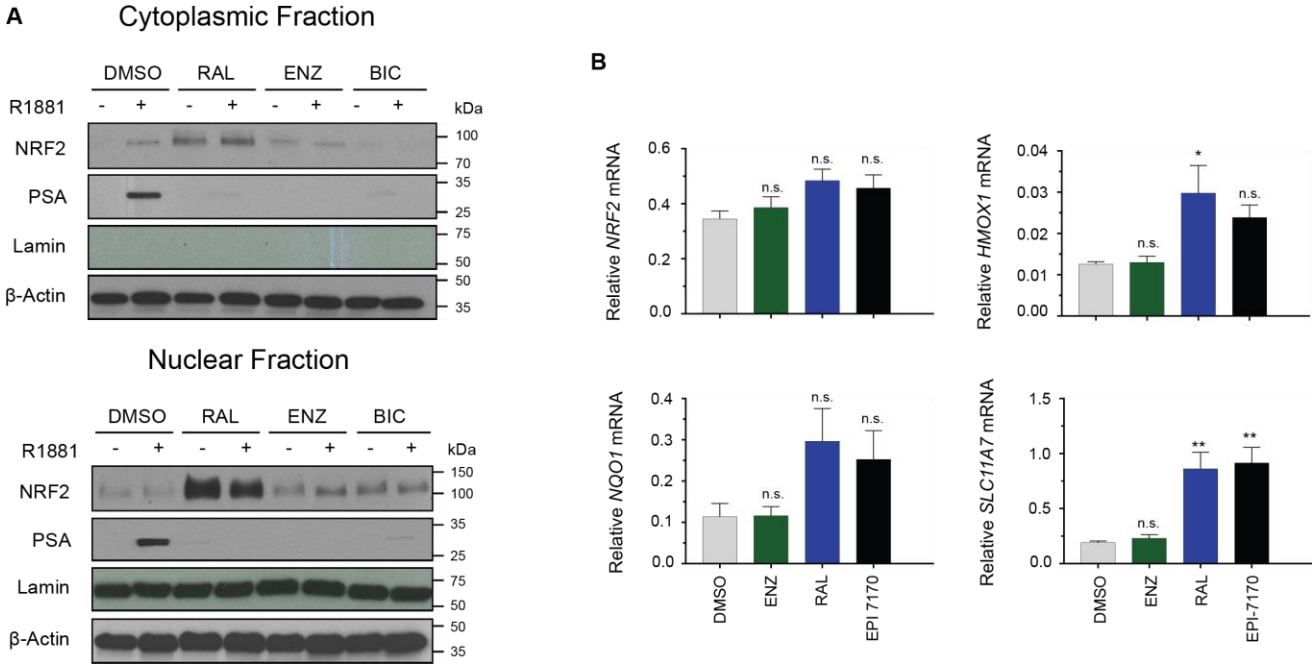


Figure 3.6 Ralaniten impacts the NRF2 pathway

(A) Western blots of NRF2 and PSA levels in LNCaP cells treated with DMSO, RAL (35 μM), ENZ (5 μM), or BIC (10 μM) in the presence or absence of R1881 (1 nM). All inhibitors blocked androgen-induced levels of PSA but only RAL increased NRF2 levels and NRF2 nuclear translocation. **(B)** Transcript levels of *NRF2* and the NRF2-regulated genes *HMOX1*, *NQO1*, and *SLC7A11* normalized to *SDHA* from LNCaP cells treated with ENZ (5 μM), RAL (35 μM), EPI-7170 (5 μM) or v/v DMSO. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied post hoc, n=3 independent experiments, (*p<0.05; **p<0.01; n.s., not significant).

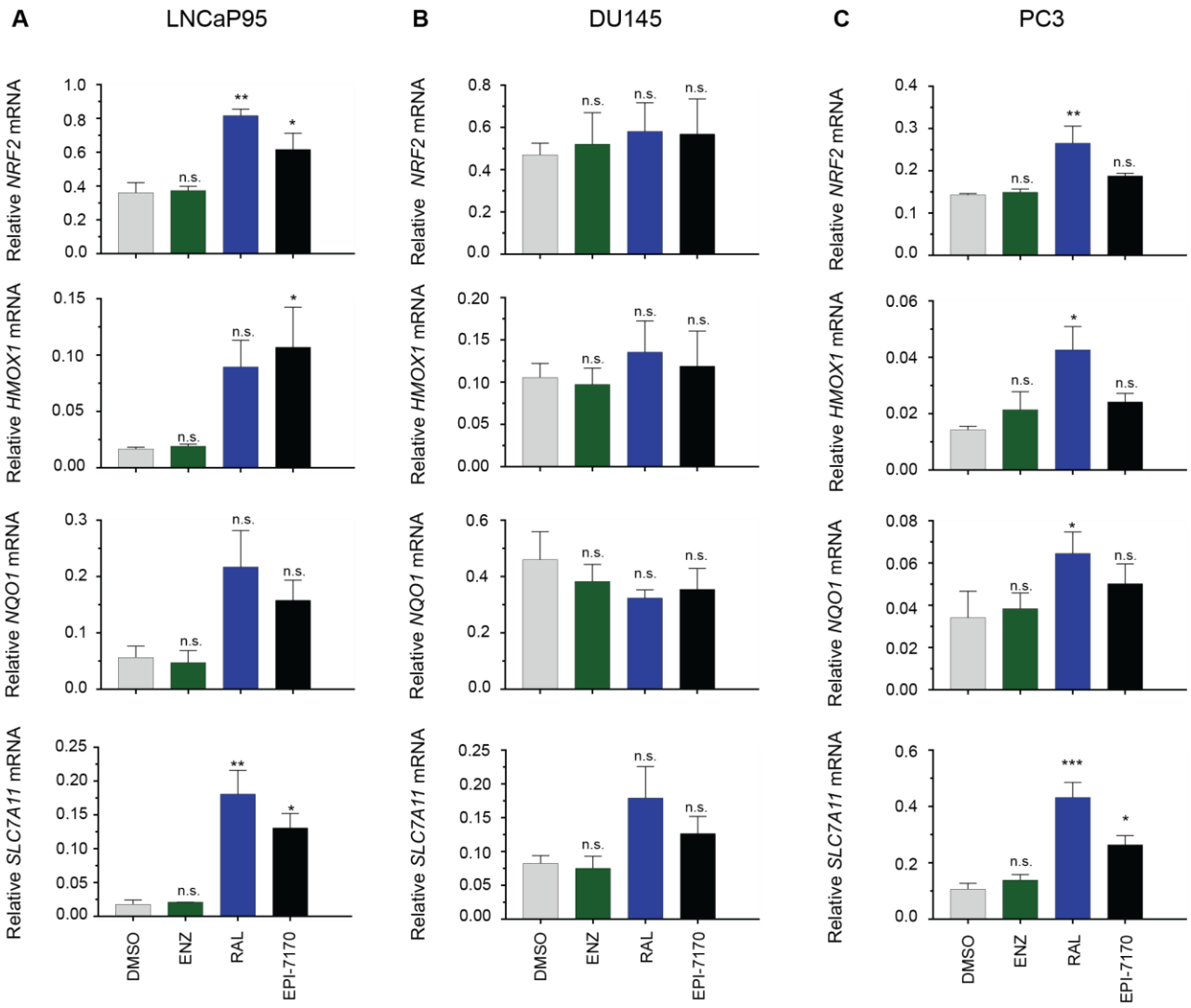


Figure 3.7 NRF2 pathway responses to ralaniten vary in prostate cancer cell lines

(**A**) Transcript levels of *NRF2, HMOX1, NQO1,* and *SLC7A11* normalized to *SDHA* from LNCaP95, DU145 (**B**), and PC3 (**C**) cells treated with ENZ (5 μM), RAL (35 μM), EPI-7170 (5 μM) or v/v DMSO. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc, n*=3 independent experiments, (*p<0.05; **p<0.01; ***p<0.001; n.s., not significant).

3.4.1 Induction of MT gene expression by ralaniten is independent of NRF2

To determine whether Nrf2 played a central role in the mechanism of ralaniten induction of *MT* gene expression, *Nrf2*-targeted knock-down using siRNA was employed in both LNCaP and LNCaP95 cells. Knockdown of Nrf2 protein was achieved in LNCaP cells both in the absence and presence of androgen (R1881) and each of the inhibitors (Figure 3.8A). At the transcript level, qPCR experiments confirmed specific knock down of *Nrf2* mRNA and no effect on levels of *Nrf1* mRNA (Figure 3.8B). Furthermore, knockdown of *Nrf2* levels adequately blocked the expression levels of Nrf-2 target genes, *NQO1* and *SLC11A7*, induced by EPI-7170 and ralaniten. Importantly, knockdown of *Nrf-2* expression had no inhibitory effects on ralaniten-induction of *MT* isoforms (Figure 3.8C). Similarly, in LNCaP95 cells, sufficient knockdown of Nrf-2 protein (Figure 3.8D) and mRNA (Figure 3.8E) levels were achieved to block ralaniten and EPI-7170 induced expression of Nrf-2 target genes (Figure 3.8E). The impact of decreasing levels of Nrf-2 had no inhibitory effect on ralaniten induced expression of *MT* genes (Figure 3.8F). Together these findings suggest that *Nrf-2* expression was not required to mediate the upregulation of *MT* genes by ralaniten.

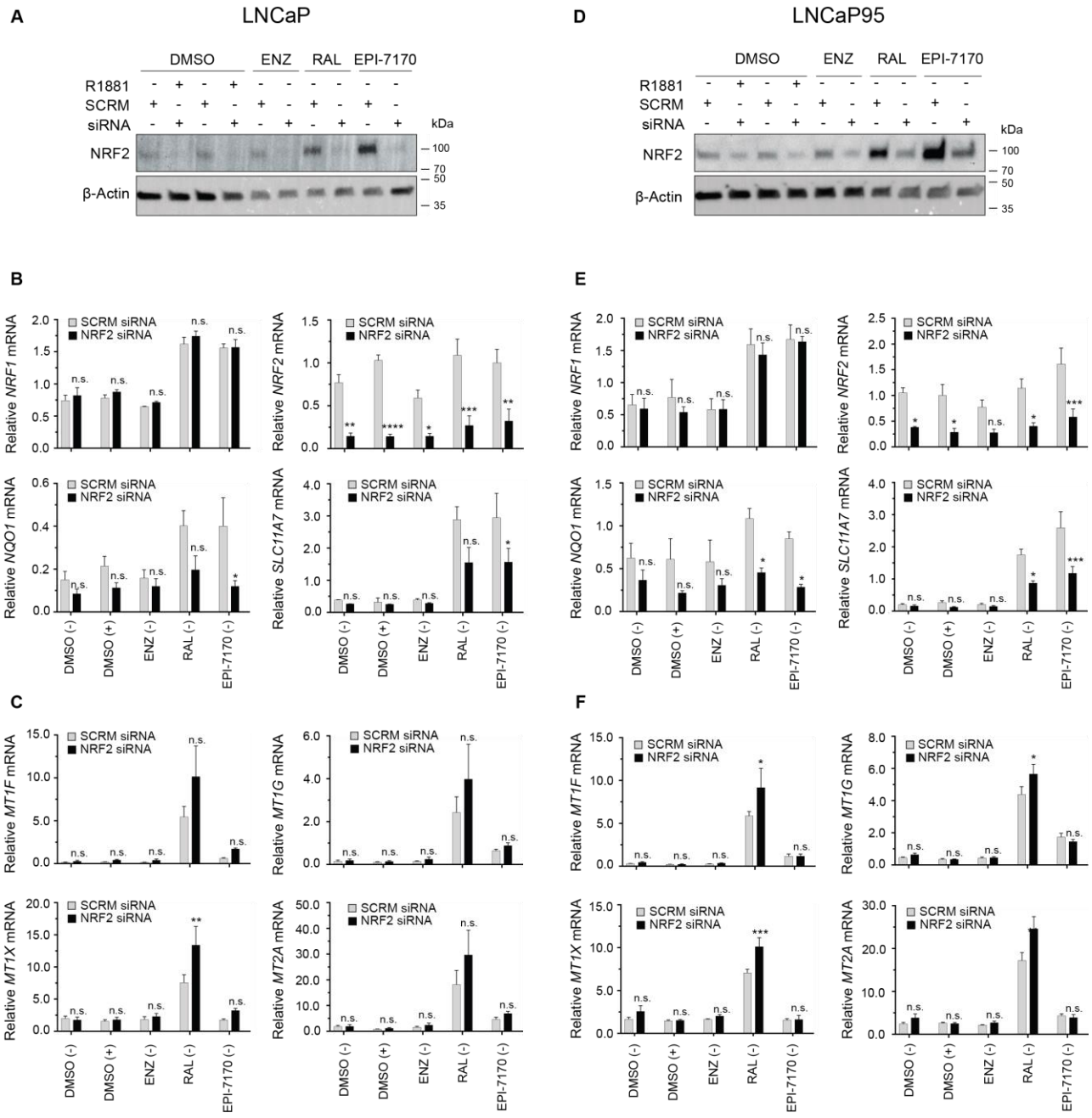


Figure 3.8 MT induction by ralaniten occurs independently of NRF2

(**A**) Western blots of NRF2 levels in LNCaP and LNCaP95 (**D**) cells transfected with control or NRF2 targeting siRNA (15 nM) for 24h and treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). (**B**) Transcript levels of *NRF1, NRF2, NQO1,* and *SLC7A11* normalized to *SDHA* from LNCaP and LNCaP95 (**E**) cells treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). Expression of *NRF1* is unaffected and transcripts of *NRF2* and NRF2-responsive genes are reduced following treatment with siRNA targeting *NRF2*. Data presented as mean ± SEM and analyzed

by two-way ANOVA with Sidak's test applied *post hoc*, *n*=3 independent experiments. (**C**) Transcript levels of *MT1F*, *MT1G*, *MT1X*, and *MT2A* normalized to *SDHA* from LNCaP and LNCaP95 (**F**) cells treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). Expression of MT isoforms are unaffected following treatment with siRNA targeting *NRF2*. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc*, *n*=3 independent experiments,
(*p<0.05; **p<0.01; ***p<0.001; **** p<0.0001; n.s., not significant).

3.5 Induction of MT gene expression by ralaniten is dependent on MTF1

To determine whether the transcription factor MTF1 played a central role in the mechanism of ralaniten induction of *MT* gene expression, *MTF1*-targeted knock-down using siRNA was carried out in LNCaP and LNCaP95 cells. In both the absence and presence of androgen (R1881) and each of the inhibitors, *MTF-1* mRNA levels were adequately suppressed in each cell line following treatment with targeted siRNA (Figure 3.9C). To demonstrate specificity, siRNA treatments targeting either the *AR* or *Nrf-2* demonstrated no effect on *MTF-1* transcripts as expected (Figure 3.9A, B). Strikingly, induction of expression of all *MT* isoforms by ralaniten tested in either cell line was significantly reduced by treatment with siRNA targeting *MTF-1* (Figure 3.9D, E). These results strongly suggest that ralaniten induced *MT* isoforms by a mechanism that was dependent on MTF-1 and independent of AR.

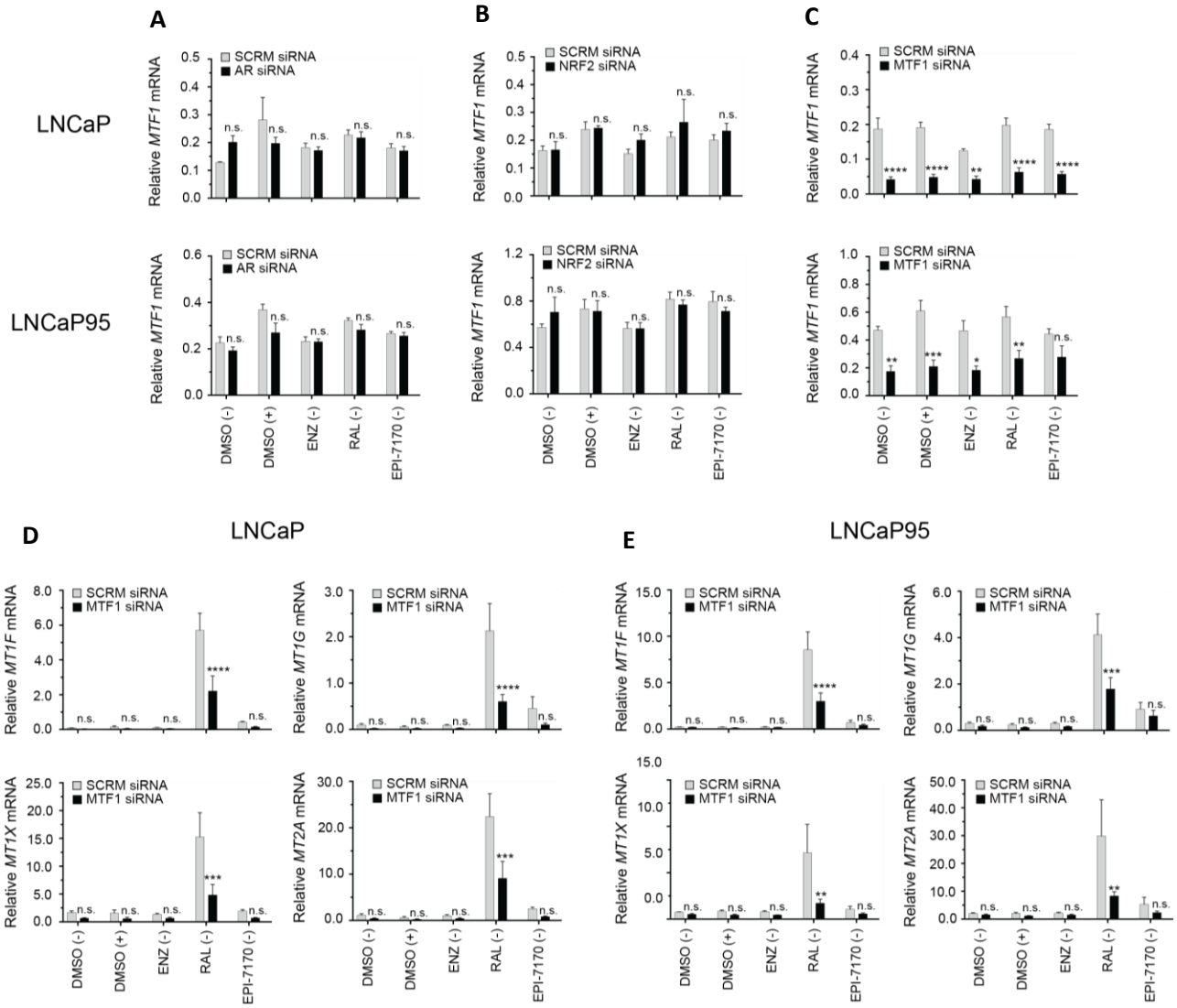


Figure 3.9 MT induction by ralaniten is dependent upon MTF-1

Transcript levels of *MTF-1* normalized to *SDHA* from LNCaP and LNCaP95 cells treated with siRNA (15 nM) targeting the *AR* (**A**), *NRF2* (**B**), or *MTF-1* (**C**) with subsequent treatment of DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). siRNA targeting the *AR* or *NRF2* had no effect upon *MTF-1* expression. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc*, *n*=3 independent experiments. (**D**) Transcript levels of *MT1F*, *MT1G*, *MT1X*, and *MT2A* normalized to *SDHA* from LNCaP and LNCaP95 (**E**) cells treated with DMSO in the presence or absence of R1881 (1 nM), ENZ (5 μM), RAL (35 μM), or EPI-7170 (5 μM). Expression of MT isoforms are significantly reduced following treatment with siRNA targeting *MTF-1*. Data presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's test applied *post hoc, n*=3 independent experiments,
(*p<0.05; **p<0.01; ***p<0.001; **** p<0.0001; n.s., not significant).

4.0 DISCUSSION

4.1 Summary and implications

Most if not all advanced prostate cancer will develop resistance to androgen deprivation therapies with the patient succumbing to metastatic CRPC. For most CRPC patients their disease is still dependent on AR signaling for growth and survival despite castration. The addition of potent second-generation hormonal therapies deepens the suppression of AR activity, yet while initially effective, clinical benefit is of limited duration and resistance soon develops. A myriad of resistance mechanisms exist which contribute to this phenomenon. Salient examples include hyperactivation of AR by mechanisms that include gene amplification or overexpression, the emergence of gain-of-function AR-LBD mutations, and constitutively active AR-Vs which lack the LBD. The AR functions as a master regulator of a vast compendium of genes. To inhibit AR pathway signaling, the strategy of all currently approved drugs ultimately converge upon the AR-LBD to target and disrupt AR activation. Unlike the AR-LBD, the AR-NTD is essential for AR function, and is required to drive the transcriptional activity of FL-AR, AR-Vs, and AR that have acquired LBD gain-of-function mutations.

Ralaniten is a novel, first-in-class drug that suppresses AR activity through binding the AR-NTD. This contrasting approach to inhibition of the molecular target may impact the overall cellular response by interfering with downstream signaling in unique ways. The discrete binding site location of individual inhibitors has the potential to drive drug specific differences in co-activator or co-repressor interactions or may alter post-translational modifications which augment the activity or stability of the AR. Due to its novel mechanism of action, we predicted that ralaniten would induce a distinct global expression profile compared to alternative AR-

inhibitors. Here we initiated the characterization of ralaniten specific gene expression profiles which help to define the molecular changes that occur following ralaniten exposure.

We commenced by looking for a ralaniten-specific gene expression signature. Data generated from the microarray analysis were used to identify differences between AR antagonists. Hierarchal clustering, pathway analysis, and fold-change ranking of upregulated genes from the microarray data distinguished a group of MT isoforms and suggested a potential ralaniten-specific effect when compared to AR-LBD targeting antiandrogens. Utilizing MT-luciferase DNA plasmids that were selected as models based on their promoter sequence concordance with *MT* isoforms drawn from our ranked list, reporter assays demonstrated that induction of *MT* genes was not simply a shared effect among all AR-NTD inhibitors, Furthermore, it could not be explained as a basic feature of molecular structure, as BADGE-H2O2 did not induce expression of the MT reporters. These data strongly suggested that ralaniten induction of *MT* gene expression was unique. In addition, dose-response studies demonstrated a robust concentration dependent relationship between ralaniten and *MT* gene expression. Having deciphered an unexpected group of genes from the *MT* gene family as forming part of a ralaniten gene signature, we set out to begin unravelling the mechanism of induction.

The *MT* promoters that were induced by ralaniten contain binding sites for numerous transcription factors that include: multiple copies of a metal-response element (MRE) which binds MTF-1; glucocorticoid-response elements (GRE) to which GR binds[481]; and redox (antioxidant)-response elements (RRE) that are binding sites for Nrf1 and Nrf2[485,486]. Ralaniten binds the AR-NTD. Thus, investigating AR involvement in *MT* expression by ralaniten was a

natural starting point due to the presence of a GRE within *MT* gene promoters and known promiscuous binding by the AR to some GREs[505–508]. Many steroid receptors show a high degree of homology, particularly in the DNA-binding domain, with phylogenetic studies demonstrating a close relationship between the AR and GR. Indeed, evidence has revealed that the AR and GR share the same chromatin binding sites. Considering that recent studies, notably those focusing specifically on advanced prostate cancer, have demonstrated a GR capacity to regulate genes considered to be AR pathway-specific[509], it is reasonable to surmise that AR too may be capable of affecting GR regulated genes. This provides one possible explanation for *MT* gene expression by ralaniten to occur through an AR-dependent mechanism.

To determine an absolute requirement for any potential AR-dependent mechanism, we contrasted the effect of ralaniten treatment across a panel of prostate cancer cell lines with differing AR expression profiles (Fig. 3.2D, Fig. 3.4). These studies showed that in cells that lacked functional FL-AR, ralaniten was a poor inducer of *MT* genes. These data suggested a possible reliance on AR activity, or potentially a specificity for cells expressing functional FL-AR. To definitively test this hypothesis, knockdown of AR expression demonstrated that ralaniten was capable of increasing MT mRNA even when the AR was significantly decreased (Fig. 3.5E-F). These data implied that ralaniten induced *MT* gene expression by an AR-independent mechanism.

MT genes are inducible by oxidative stress and their promoter regions contain redox (antioxidant)-response elements (RRE) that bind Nrf1 or Nrf2 with comparable affinity to activate transcription[461,485–488,510]. Nrf1 is a constitutively active transcription factor that contributes to *MT* gene regulation through the maintenance of basal *MT* expression levels[511,512].

In contrast, Nrf2 is an inducible transcription factor that readily responds to stimuli upon activation by translocating from cytoplasm to nucleus and inducing target genes to coordinate cellular responses to manifold environmental conditions[513,514]. With tightly regulated basal protein levels and high turnover, Nrf2 is maintained in the cytoplasm in an inactive state bound to its repressor Keap1, an E3 ubiquitin ligase adaptor protein, that continuously targets Nrf2 for proteasomal degradation unless appropriate stimuli activate Nrf2 by causing its release from Keap1[515–517]. Ralaniten treatment produced evidence of Nrf2 pathway responses demonstrated by Nrf2 protein nuclear translocation (Fig. 3.6A) and elevated transcript expression of Nrf2 regulated genes (Fig. 3.6B, Fig. 3.7). Despite this, Nrf2 silencing experiments were ineffective at abrogating *MT* gene induction by ralaniten (Fig. 3.8).

The proximal promoter region of *MT* genes contains multiple MREs that bind MTF-1 to induce gene expression. Gene knock down experiments reduced the transcript levels of all *MT* isoforms tested after MTF-1 silencing and revealed that the mechanism of *MT* gene induction by ralaniten to be MTF-1 dependent. The transcriptional activity of MTF-1 can be induced in response to three main cellular stresses: heavy metal load (Zn^{2+}, Cu^{+}, Cd^{2+}, Pb^{2+}, Ag^{+}, Co^{2+}, Ni^{2+}, Hg^{2+} and Bi^{2+}), hypoxia, and oxidative conditions[461,518–521]. As effects from the presence of any of these metals on MTF-1 transactivation of *MT* genes has been well characterized, it should be noted that ralaniten was cleaned of any heavy metals used in its synthesis. Studies suggest a convergent mechanism mediating oxidative, hypoxic, or metal stress induction of MTF-1 transcriptional activity, with zinc transducing the signal in each case through direct binding to zinc responsive regions within MTF-1[450,456,520–526,457,460,461,467,482,484,518,519]. Considering these findings, additional elaboration is contained in *Future Direction* section.

The potential consequences in prostate cancer of elevated *MT* expression have not been conclusively defined. The clinical significance of *MT* expression is context specific with considerable variation in relevance to treatment response and patient outcome across tumor types[453,454,527]. In some human cancers, MT levels are positively associated with malignancy, tumor grade and progression, and may play a role in radio- and drug resistance[452,528–531]. This relationship appears reversed in other cancer types[532–536] and prostate cancer especially, with the observation that for many *MT* isoforms, expression is significantly downregulated or lost in advanced disease[471,472,475,537]. This trend may represent a survival and growth advantage for prostate malignancies; thus, upregulation of *MT* genes may negate this pro-tumorigenic feature. Interestingly, the specific *MT* isoforms shown to be most responsive to induction by ralaniten treatment may be predicted to offer therapeutic value for prostate cancer patients. *MT1F* has been shown to be oncosuppressive[538] and low expression levels in malignant prostate tumors is associated with perineural invasion and metastasis[472]. *MT1G* is suppressed in CRPC via epigenetic downregulation, loss of heterozygosity, or in response to low zinc levels, portending aggressive disease and a poor prognosis[475]. Expression of the *MT1G* isoform has been associated with reduced proliferation and enhanced apoptosis. Mechanistic studies suggested MT1G indirectly increased TP53 stability and directly enhanced TP53 transactivation of *p21* and *BAX*, leading to cell cycle arrest and apoptosis, respectively[539]. The *MT2A* isoform has been shown to attenuate NF-kB activation[540] with an associated sensitization to clinically relevant chemotherapeutics for CRPC such as docetaxel. Furthermore, high MT2A levels may impair the metabolic signature of malignant prostate cells, noted for their reverse-Warburg phenotype in which the capacity for oxidative phosphorylation and citrate oxidation is restored following m-aconitase disinhibition. MT2A was shown to affect cell respiration by suppressing the

expression of protein subunit II found within Complex IV of the mitochondrial respiratory chain[541]. Downregulation of the *MT1X* isoform is considered a biomarker associated with advanced stage prostate cancer[471]. Ultimately, the upregulation of these *MT* genes may add therapeutic benefit.

Still, whether ralaniten induction of *MT* genes in prostate cancer ultimately results in salutary or deleterious effects on the intended therapeutic goal of AR inhibition remains uncertain. Firstly, do these effects have an impact on ralaniten therapy itself? This study has demonstrated that ralaniten inhibition of AR and ralaniten induction of *MT* genes occur independently. Nonetheless, ralaniten may theoretically bind MT directly, as many cysteines within MT are able to react with relatively weak redox partners[542], possibly via transient interactions. Were this to be occurring, this would provide a resistance mechanism to prostate cancer cells via pharmacokinetic interference since ralaniten would be diverted away from its intended target, the AR-NTD. Consequently, higher dosages of ralaniten would be required to maintain therapeutic levels at the pharmacologically relevant concentration. In previous studies, considerable effort has been expended to define emergent resistance mechanisms to ralaniten after extended treatment[543]. Those studies report that the main resistance mechanisms to ralaniten which develop in prostate cancer cells involve the upregulation of glucoronidating drug metabolism enzymes specific to phase II conjugation, namely members the *UGT* family of genes. Therefore, direct MT binding by ralaniten is not predicted to be a major contributor to the development of ralaniten resistance. However, molecular interactions between ralaniten and MT may occur to some degree.

By inducing *MT* genes, ralaniten may indirectly enhance suppression of AR pathway signaling. Distinct from the direct AR inhibition achieved via binding the AR-NTD, factors driving both the overexpression of AR and ligand-independent induction of AR transcriptional activity may be countered as a result of *MT* expression. Chronic states of oxidative stress and inflammation manifest in most prostate cancers[465,466,544–547], especially at more advanced stages following the development of therapeutic resistance. Indeed, they share highly interconnected biological processes and their joint effects cooperate in maintaining pathological AR signaling[239]. Oxidative stress induces *AR* overexpression by activating numerous transcription factors known to regulate the *AR* gene[332,385,548–553] and inflammation has been associated with the emergence of AR-Vs[383,390,391,554]. Persistent pro-inflammatory signaling, in particular through the IL-6/JAK/STAT pathway[555–558], is frequently observed in prostate cancers[558–561] resulting in increased ligand-independent activation and stabilization of AR through interactions and post-translational modifications of the AR-NTD. MT can counter both inflammation and oxidative stress, thus providing additional clinical utility for AR pathway suppression.

Prostate cancer therapies are frequently combined to produce more complete tumor responses. In certain contexts, MT has been associated with radio- and chemoresistance, and the impact of MT on the efficacy of other hormonal therapies has not been well studied. Concerns regarding the co-application of ralaniten in multimodal approaches to prostate cancer treatment have thus far been shown to be unwarranted. Indeed, exploratory investigations of ralaniten combination with other therapies have produced positive findings, even suggesting potential synergistic effects in some cases[163,435,437,562].

The investigations contained herein establish a foundation for the characterization of ralaniten specific expression profiles. Specifically, they show that:

1) Ralaniten induction of *MT* gene isoforms was distinct from other AR antagonists.

2) Ralaniten induced the expression of *MT* genes by a mechanism independent of expression of AR and Nrf2.

3) *MT* induction by ralaniten was dependent on the expression of the transcription factor, MTF-1.

Chapter 5. CONCLUDING REMARKS

5.1 Study Limitations

This study initiates the characterization of ralaniten-specific expression profiles in prostate cancer cells. While this work expands upon current knowledge to better understand the molecular profile of ralaniten, additional work is necessary to elaborate and ultimately comprehend the implications of the findings. A more comprehensive proteomic analysis, especially protein immune-detection of MTF-1 and MT isoforms, would address some of the current study limitations. Use of a broader panel of cell lines, and perhaps mouse xenografts as well, would enhance the ability to generalize the findings presented herein and predict the potential clinical relevance for patient populations. This issue, in part, stems from a dearth of established and well characterized cell lines which exist for prostate cancer research relative to some other tumor types. This study employed the three most widely utilized human prostate cancer cells (LNCaP, PC3, DU145) and a LNCaP subline (LNCaP95) to explore ralaniten induction of *MT* gene expression in cells with varying AR and AR variant status. Additional insight would be garnered from including other cell lines (e.g. VCaP), and even primary patient samples, though the latter possibility engenders considerable technical and ethical complexities that are somewhat prohibitive for an exploratory investigation such as this one.

5.2 Future Directions

This study is the first to contrast the global transcriptional profile of ralaniten with existing non-steroidal antiandrogens to identify genes that are uniquely expressed in prostate

cancer cells in response to ralaniten. The investigations revealed that ralaniten induction of *MT* gene isoforms was distinct from other AR antagonists, occurred independently of AR, and was dependent on the expression of the transcription factor, MTF-1. While the effect of ralaniten on the transcription of *MT* genes was mediated through MTF-1, it remains uncertain whether this is via direct or indirect interaction. Ralaniten did not induce expression of *MTF-1* mRNA (Fig. 3.9C). The transcriptional activity of MTF-1 is believed to predominantly involve post-translational processes including metal sensing (primarily zinc ions), nuclear translocation, DNA-binding, and transcriptional coactivators interactions. The induction of MTF-1 target genes has been extensively reviewed elsewhere[518]. Findings from numerous studies point to a convergent mechanism mediating oxidative, hypoxic, or metal stress induction of MTF-1 transcriptional activity. The response by MTF-1 to these stresses is most likely indirect, with zinc released from MT transducing the signal in each case through direct binding to zinc responsive regions within MTF-1[450,456,520–526,457,460,461,467,482,484,518,519]. MTF-1 activation is extremely sensitive to shifts in the availability of free intracellular zinc ions to enable finely tuned gene regulatory responses[563].

Concluding that *MT* gene induction by ralaniten is MTF-1 dependent, the critical role played by zinc in the balance between MT and MTF-1[482] raises several pertinent questions. First, do intracellular levels of unbound zinc increase in prostate cancer cells following ralaniten treatment? Employing sensitive free zinc detection assays that utilize fluorescence in intact cells or colorimetric analysis of cell lysates to quantify unbound intracellular zinc ions following ralaniten treatment would contribute helpful data in this regard[564,565]. Second, is zinc required to transduce the signal mediating the indirect interaction between ralaniten and MTF-1? This could be tested in zinc chelation experiments that employ luciferase transcription reporter assays using

the MT-luciferase reporter constructs already described herein and investigating whether chelation of zinc inhibits ralaniten induced transcription of these MTF-1 regulated plasmids[566]. Third, if a requirement for zinc is confirmed, does the expression of *MT* genes following ralaniten treatment reflect an increase in the absolute quantity of intracellular zinc, or merely result from ralaniten induced zinc dissociation from MT causing higher free zinc concentrations to activate MTF-1 transcription? The former scenario would require ralaniten to trigger an influx of zinc, and this seems unlikely as the microarray data did not indicate any elevated expression of genes coding for members of the plasma membrane zinc importer protein (ZIP) family upon ralaniten exposure. Conversely, upon revisiting the analysis of the microarray (data not shown), the results pointed to the opposing trend. The *ZIP10* gene was significantly repressed by ralaniten while the main plasma membrane zinc exporter *ZnT1* was notably upregulated, thereby suggesting overall cellular efflux of zinc. On reflection, this is an intuitive result as both genes are also regulated by MTF-1. Taken together, these findings would be consistent with the latter possible scenario mentioned above. Namely, ralaniten triggers a disruption of the zinc-thiolate clusters in MT leading to zinc release and MTF-1 activation, resulting in *MT* and *ZnT1* gene upregulation and *ZIP10* gene downregulation, as a rapid and specific response to the escalation in unbound intracellular zinc[522,567,568].

If the availability of free zinc to activate MTF-1 was driven by this proposed mechanism, how specifically does ralaniten disrupt the zinc-thiolate bonds in MT proteins to cause zinc release? It is possible ralaniten may interact directly with cysteines of the MT polypeptide via its chlorohydrin moiety. However, in reports from multiple studies, performed in vitro, in vivo, and using patient samples, ralaniten did not appear to be generally reactive or demonstrate alkylation of glutathione[422,431,543,569]. It is important to note here that the chemical structure of the ralaniten

analog EPI-7170 also contains this chlorohydrin group. Though, unlike ralaniten, treatment with EPI-7170 did not affect the expression of *MT* genes. Investigations of structure-function relationships for ralaniten and its analogs revealed that the chlorohydrin group was required for efficacy against the AR[165,166,434,439]. Ralaniten compounds lacking this functional group were unable to inhibit AR activity, nor bind the AF-1 region, in prostate cancer cells. Thus, while the chlorohydrin moiety is essential for ralaniten compounds to interact with AR, it remains questionable whether the induction of *MT* gene expression by ralaniten is reliant on this component of molecular structure. Reflecting on their individual potencies against AR, a comparison of the IC50 values of ralaniten (~10 μM) and EPI-7170 (~1 μM) suggests that structural differences between these analogs may influence the affinity of the chlorohydrin group for AR. Perhaps these intrinsic molecular features are also impacting the affinity of the chlorohydrin group for MT, albeit in an inverse relationship. Alternatively, if the affinity of the chlorohydrin group for MT is the same for ralaniten and EPI-7170, ralaniten induction of *MT* gene expression could simply be a concentration dependant effect. Experiments utilizing biotinylated ralaniten or radio-labeled ralaniten could be undertaken to determine whether a direct MT-ralaniten interaction was occurring.

Redox disturbances to the intracellular milieu may be generated by oxidative stress elsewhere in the cell upon ralaniten treatment, ultimately causing zinc dissociation from MT. Ralaniten metabolism within prostate cells has the potential to generate oxidative stress that indirectly causes the dissociation of zinc from MT. Metabolic processing and detoxification of numerous endo- and xenobiotics, especially drugs and lipophilic molecules, often involves enzymatic pathways localized to the lumen of the endoplasmic reticulum (ER)[570]. To facilitate clearance and prevent interactions that may result in toxicities, these compounds are sequestered

within the ER lumen. As prior studies have defined the prominence of UGT enzymes in ralaniten metabolism[543], it is particularly noteworthy that these proteins are known to reside in the ER anchored to the luminal membrane. Elevated ralaniten concentrations in the ER may disrupt redox homeostasis and alter the overall reducing capacity of the cell, thereby destabilizing the zinc/sulfur network within MT to cause zinc release and activation of MTF-1. In some cases, xenobiotic metabolism creates sufficient ER stress to trigger the unfolded protein response (UPR) through one of three UPR sensors (ATF6, IRE1, PERK) leading to activation of associated pathways[571]. Indeed, evidence from the microarray and gene ranking list clearly suggested that ralaniten induced the PERK pathway. Two non-MT, downstream PERK genes (*SLC7A11, FAM129A*) were found to rank among the top 10 for increased expression following ralaniten treatment (Fig. 3.1C). The *SLC7A11* gene is positively regulated by the transcription factors ATF4 and Nrf2, each mediating a signaling branch of the UPR PERK pathway[572,573]. The *FAM129A* gene is induced by ATF4 downstream of PERK activation[574]. Collectively, these data support the involvement of an indirect mechanism mediating MT zinc release by ralaniten. To confirm if ralaniten induces activation of the ER PERK pathway, phosphorylation levels of PERK could be detected by immunoblot using a phospho-specific PERK antibody, as PERK undergoes trans-autophosphorylation upon ER stress. To determine if the mechanism for ralaniten induction of *MT* gene expression is dependent on the generation of oxidative stress to release zinc from MT and activate MTF-1, experiments could be undertaken in which cells were pre-treated with a redox inhibitor such as N-acetylcysteine before addition of ralaniten.

Overall, the next steps in future studies may continue to build on the foundation provided here. Continued investigation of the distinct cellular responses to ralaniten and its analogs will

assist researchers with discovery and clinicians with the development of strategies and treatment combinations to maximize synergistic effects for the ultimate benefit of prostate cancer patients.

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