Androgen Receptor Modulation by non-Androgenic Factors and the Basal Transcription Factor TAF1

by

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Abstract

There is considerable evidence to suggest that progression of prostate cancers to castration-resistant is due to inappropriate activation of the androgen receptor (AR) and hence, the AR is a good target for the treatment of this disease. In this study we used two approaches to investigate AR activation and inhibition.

First, we developed high-throughput, non-invasive, cell-based screening assays to rapidly and biologically assess factors that modulate prostate cancer growth and affect AR activity. Using these assays, we found that differentiated osteoblast-like condition media enhanced prostate cancer cell growth, but not AR activity. In addition, we applied this system to screen compounds, selected through *in silico* approaches against a crucial pocket on the AR protein. The application of our *in silico* tools and our cell based screening assay resulted in identification of 17 compounds out of 4 millions that can inhibit AR activity. Importantly, some of these compounds are more potent than bicalutamide, which is one of the most potent antiandrogen drugs currently used to treat patients with metastatic prostate cancer.

In the context of AR transcription target genes, the presence of AR coactivators is essential for AR activity. Using the repressed transactivator yeast two-hybrid system, we found that TATA binding protein-associated factor 1 (TAF1) interacted with the AR. In tissue microarrays, TAF1 was shown to steadily increase with duration of neoadjuvant androgen withdrawal and with progression to castration resistance. GST pull-down assays established TAF1/AR interaction and co-immunoprecipitation and ChIP assays revealed colocalization of TAF1 and AR on the prostate specific antigen (PSA) promoter in prostate cancer cells. With respect to modulation of AR activity, while full-length TAF1 showed enhancement of both AR and some generic gene transcriptional activity, selective AR coactivator activity was demonstrated in transactivation

experiments using cloned TAF1 N-terminal kinase and ubiquitin-activating/conjugating (E1/E2) domains. In keeping with AR coactivation by the E1/E2 domain, TAF1 was found to greatly increase the cellular amount of poly-ubiquitinated AR. In conclusion, our results indicate that TAF1 is a coactivator of AR and its overexpression could be part of a compensatory mechanism adapted by cancer cells to overcome reduced levels of circulating androgens.

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List of Abbreviations

ADMET	drug absorption, distribution, metabolism, excretion and toxicity
AF 1	activation function 1
AF 2	activation function 2
AJCC	American joint committee on cancer
AIB1	amplified in breast cancer 1
AR	androgen receptor
ARR_2PB	androgen responsive probasin-derived promoter
ARA70	androgen receptor activator 70
ARE	androgen response elements
Bag-1L	Bag-1 isoform
BF-3	binding Function-3
BPH	benign prostatic hyperplasia
BRE^u	upstream TFIIB-recognition element
BRE^d	downstream TFIIB-recognition element
CAF	CBP associated factor
CBP	CREB binding protein
Cdc37	cell division cycle 37 homolog
ChIP	chromatin immunoprecipitation
CM	conditioned media
CSS	charcoal stripped serum
CTK	C-terminal kinase
DBD	DNA-binding domain
DCE	downstream core element
DDC	L-dopa decarboxylase
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOX	doxycycline
DPE	downstream promoter element
DRE	digital rectal exam
EGFP	Enhanced green fluorescent protein
eHITS	Electronic high throughput screening
ERK-2	Extracellular signal-regulated kinase 2
E1/E2	ubiquitin activating/conjugating
EV	empty vector
FACS	Fluorescence activated cell sorting
FBS	fetal bovine serum
FLF	flufenamic acid
FSH	follicle stimulating hormone
FSK	forskolin
FXXLF	F, phenylalanine; X, any amino acid; L, leucine
GAK	Cyclin G-associated kinase

GST	glutathione S-transferase
GTF	general transcription factor
GnRH	gonadotropin-releasing hormone
HAT	histone acetyl transferase
H & E	haematoxylin and eosin
HG-PIN	high grade prostatic intraepithelial neoplasia
HSP	heat shock proteins
IL-4	interleukin-4
IL-6	interleukin-6
Inr	initiator
IP	immunoprecipitation
K	Lysine
LBD	ligand-binding domain
LH	luteinizing hormone
LH-RH	leuteinizing hormone-releasing hormone
LigX	ligand explorer
LXXLL L,	leucine; X, any amino acid
MMFF94x	forcefield Merck molecular force field
MOE	molecular operating environment
MTE	motif ten element
MDM2	murine double minute 2
MS	main screen
NEDD4	neuronal precursor cell-expressed developmentally downregulated 4
NF1	negative cofactor 1
NF2	negative cofactor 2
NHT	neoadjuvant hormone therapy
NLS	nuclear localization sequence
NTD	NH2-terminal domain
NTK	N-terminal kinase
OPLS	optimized potentials for liquid simulations
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDB	protein data bank
PEST	P, proline; E, glutamic acid; S, serine; T, threonine
PIC	preinitiation complex
PIN	prostatic intraepithelial neoplasia
PRK-1	protein kinase C related kinase
PSA	prostate specific antigen
PTEN	phosphatase and tensin homologue
PTM	post-translational modification
Poly-G	poly-glycine
Poly-P	poly- proline
Poly-Q	polyglutamine
P-S	penicillin-streptomycin
PVDF	polyvinylidene difluoride membrane
P450scc	P450 side-chain-cleavage
P450c17	17α-hydroxylase

RFU	relative fluorescence unit
RLU	relative luciferase unit
RMSD	root mean standard deviation
RPMI	Roswell Park Memorial Institute medium
RTA	reverse yeast two-hybrid system
R1881	Metribolone or methyltrienolone
SHBG	sex hormone-binding globulin
Smad	signalling mothers against decapentaplegic
SP	standard precision
SRC	steroid receptor coactivator
StAR	steroidogenic acute regulatory protein
STAT3	signal transducer and activator of transcription 3
SD	structural data
SDS	sodium dodecyl sulfate
TAF	TATA binding protein associated factors
TAF1	TATA binding protein Associated Factor 1
TATA	TATA box
Tau	transcriptional activation units
TBP	TATA binding protein
TF II	Transcription factors II
TIF2	transcriptional intermediary factor 2
TGF	transforming growth factor
Tip 60	Tat-interactive protein 60 kDa
TRAP220	thyroid hormone receptor associated protein mediator complex
TRIAC	3,3",5-triiodothyroacetic acid
TSGP	tumour susceptibility gene product
T3	triiodothyronine
UBCH7	human ubiquitin-conjugating enzyme 7
UICC	International union against cancer
USA	upstream stimulatory activity
VSV-G	vesicular stomatitis virus envelope glycoprotein G
WXXLF	W, tryptophan; F, phenylalanine; X, any amino acid; L, leucine
3β-HSD-II	3β-hydroxysteroid dehydrogenase
17β-HSD-III	17β-hydroxysteroid dehydrogenase

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I would like to dedicate this thesis to my beautiful wife,

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And my lovely sons,

Ryan Parsa

and

Aiden Aria Tavassoli

I thank you from the bottom of my heart for the sacrifices and the support that lead to finishing this chapter of our lives. I am very much looking forward to starting our new adventure.

Co-authorship statement

The contents of this dissertation are derived from my research study that I have accomplished to graduate from the PhD program at UBC. This is to confirm that Peyman Tavassoli is the first author of all publications included in this thesis and that he has the prime role in designing the experiments, performing the research, data analysis and preparation of the manuscripts of these publications.

Dr. Paul Rennie was the investigator of the research projects, described herein. He closely supervised all experimental designs and approaches and critically reviewed all manuscripts included in this dissertation. The main idea of the role of bone-related factors in modulating androgen receptor activity and in progression of prostate cancer was initially introduced by Dr. Mira Ray (former PhD student with Dr. Rennie) and then modified by Peyman Tavassoli. Dr Art Cherkasov at the department of Urologic Sciences (UBC) founded the *in silico* drug discovery platform at the Prostate Centre at Vancouver General Hospital. With his collaboration, *in silico* drug discovery has been used to screen a virtual chemical library for compounds that bind to the BF-3 pocket on the androgen receptor. Peter Axerio-Cilies (a PhD student in Dr. Cherkasov's lab) performed *in silico* screening using the combined power of modern computers and wrote the corresponded material and results of chapter 3.

Dr. Latif Wafa (former PhD student in our lab) and Helen Cheng (research assistant in our lab) isolated the TAF1 protein using the repressed transactivator yeast two-hybrid system. Dr. Wafa also played a senior role in the experimental design and data interpretation of the chapter 4. Dr. Martin Gleave at the department of Urologic Sciences (UBC) founded the human prostate cancer tissue microarray and collaborated with our lab. Dr. Ladan Fazli (research pathologist in the Vancouver Prostate Centre) performed tissue microarray staining and visually scored the array. Robert Bell (biostatistician in the Vancouver Prostate Centre) performed statistical analysis of the NHT-tissue microarray data. Dr. Amina Zoubeidi **(**a postdoc in Dr. Gleaves's lab) and Dr. Rob Snoek (former research associate in our lab) provided critical comments.

Chapter 1-Introduction, hypothesis and specific aims

1.1 The prostate gland

1.1.1 Anatomy and physiology

The human prostate gland is one of the male sex accessory tissues, which include the prostate, seminal vesicles, and bulbourethral glands (1). The normal human adult male gland is a horse chestnut-shaped structure, surrounds the urethra immediately below the base of the bladder and is located posterior to the inferior of symphysis of pubis, superior to the urogenital diaphragm, and anterior to the rectum (1) (Fig. 1.1). The prostate measures approximately 5cm x 4cm x 3cm and weights 20g between ages 20 and 50 years (1,2).

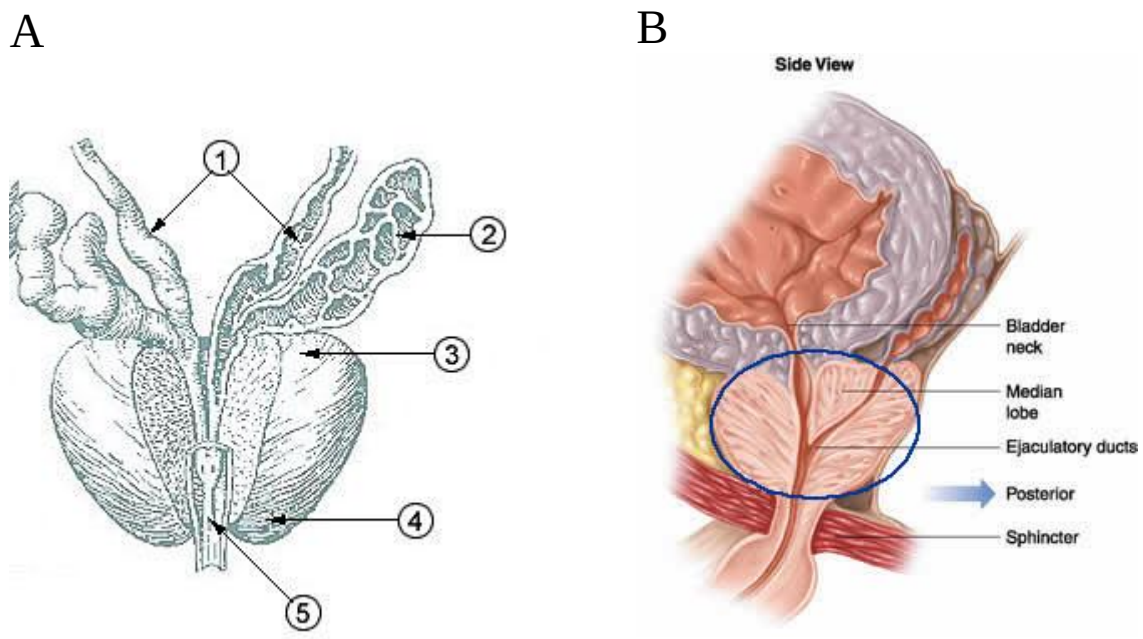

Figure 1.1 – Anatomical relationship of prostate in frontal (A) and sagittal views (B). Labels: Vas deferan (1); Seminal vesicle (2); Base of prostate (3); Apex of prostate (4); Prostatic urethra (5). From http://www.malecare.com/prostate-cancer_42.htm.

The main function of the prostate is the production and secretion of factors that comprises of one half or two thirds of the 3 mL volume of the ejaculate (1). The prostatic fluid itself is composed of potassium, zinc citric acid, spermine, amino acids, prostaglandins, and enzymes such as acid phosphatase, lactic dehydrogenase, prostate-specific antigen (PSA), and β-glucuronidase (1). These secretory products enhance fertility by promoting sperm viability and motility. Physically, through mass effect, musculature and location, the prostate may contribute to control the urine outflow from the bladder. However, the prostate gland is not a vital organ as the entire gland can be removed without critical consequences (3).

1.1.2 Development

The prostate is derived from the urogenital sinus and is first recognizable at 9 to 10 weeks of embryonic stage (2). At this time, testosterone from the fetal testis stimulates the growth of endodermal buds into the urogenital sinus mesenchyme. Under the influence of androgens, the urogenital sinus mesenchyme induces the urogenital sinus epithelium to undergo prostatic ductal morphogenesis and differentiation (4). Prostatic development is a complex androgen-dependent process involving a coordinated set of events occurring in both epithelial and stromal compartments of the gland (5). Whereas epithelial differentiation is dependent on the urogenital sinus mesenchyme in the presence of androgen, stromal differentiation is in turn dependent on the presence and differentiation state of the developing prostatic epithelium (6). This development enters a resting period until puberty. In this period the size of gland remains stable at about 1g (3). The pubertal period is marked by substantial androgen-driven increase in gland size that reaches 20g around age 20. The size of prostate gland remains constant for about 30 years and then increases from age 50 due to hyperplasia, inflammation and atrophy in its transitional zone (see section 1.1.3 below) (3).

1.1.3 Histology

The prostate is composed of multiple secretory acini and ducts that drain into prostatic urethra. Each glandular acinus is composed of the epithelial cells and fibromuscular stroma compartments. Based on different microscopic structures within the gland, the prostate is divided into three zones: the peripheral zone, the transition zone and the central zone (Fig 1.2). The peripheral zone makes up about 70% of the entire prostate gland and consists of evenly distributed ducts and acini but irregular in size and shape. The stroma in this zone is loosely woven and fibromascular. The transitional zone accounts for 5% volume of the prostate and is similar to the peripheral zone, but the stroma is more compact with interlacing smooth muscle bundles. The central zone, which makes up about 25% of the total gland is more densely arranged than the peripheral and transitional zones. The epithelial-stromal ratio in the central zone is 2:1, compared with 1:1 for the other two zones (2). Interestingly, diseases differentially affect these zones. For instance, prostate cancer tends to occur in the peripheral zone, whereas, benign hyperplasia uniformly originates from the transitional zone.

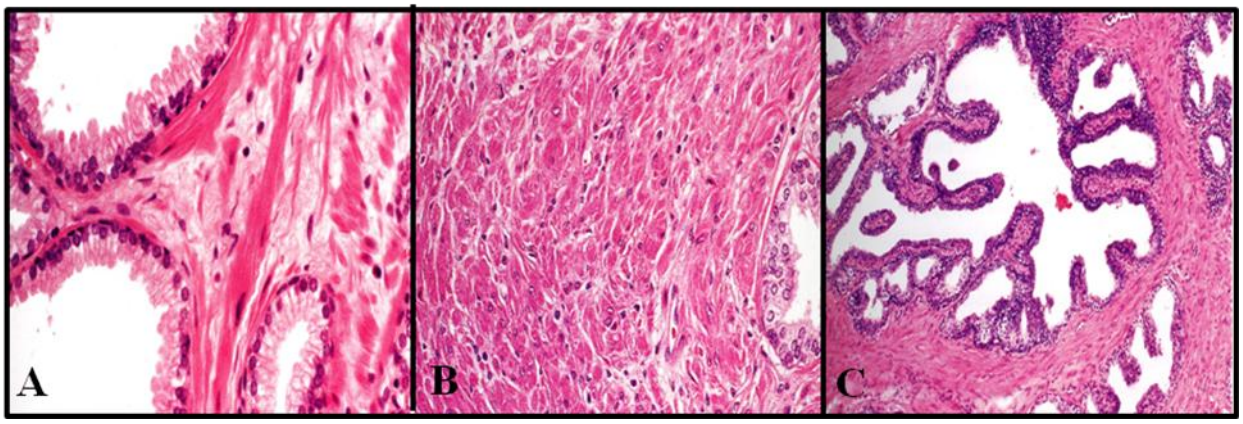

Figure 1.2 – Histology features of three zones in the human prostate. (A) Normal adult peripheral zone: The stroma contains smooth muscles and fibroblasts; **(B)** Normal adult transitional zone: The stroma is comprised of densely concentrated smooth muscle bundles; **(C)** Normal adult central zone: Glands with prominent ridges with stroma cores. From Prostate Pathology, © 2003 American Society for Clinical Pathology and © 2003 ASCP Press (3).

The normal epithelium of the prostate consists of two cell layers: a luminal or secretory cell layer and a basal cell layer (Fig. 1.3). A third cell type in the normal prostate epithelium is the neuroendocrine cells (7). The basal cell layer is virtually a continuous layer that separates the luminal cells from the basement membrane and its absence is an important indicator in invasive prostate cancer (3). The immunophenotype of basal cells is characteristic and different from luminal cell layer (Table 1.1). Currently, the most important diagnostic marker of basal cells is the monoclonal antibody 34βE12 that binds to keratin 1, 5, 10, 14 (8,9). The basal cells are the dominant proliferative cell type in the normal prostate and it is proposed that the basal cell population harbors stem cells (10,11).

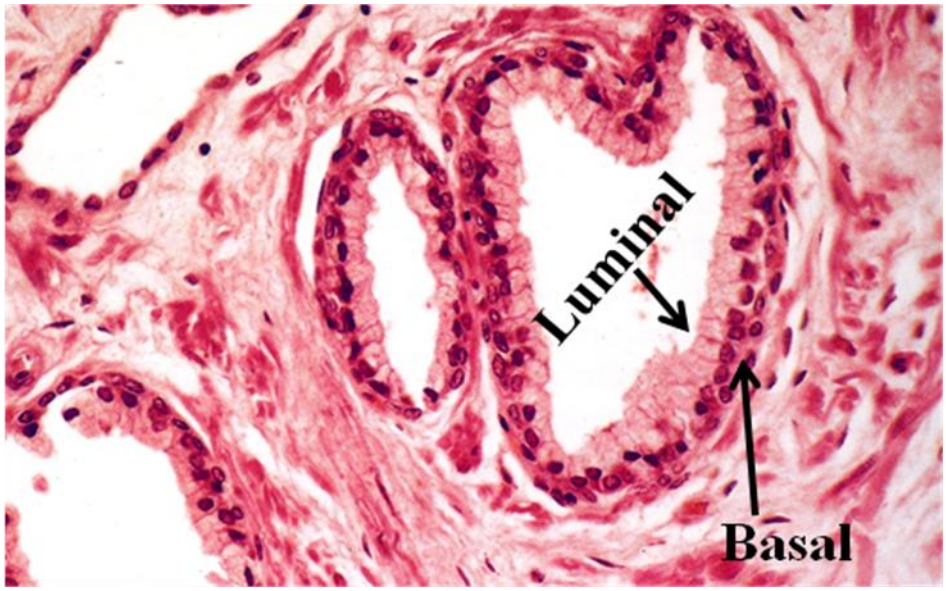

Figure 1.3 - Normal prostatic glands with two cell layers – an inner luminal cell layer and outer basal cell layer. Modified from Prostate Pathology © 2003 American Society for Clinical Pathology and © 2003 ASCP Press (3).

Even though luminal cells outnumber basal cells 3 to 1 in the normal prostate (2,12), proliferative basal cells constitute 70% to 80% of the proliferating benign epithelium (2,12). Overall, the proliferative index of normal prostatic epithelium is extremely low with 0.2% of

cells proliferating per day and that is relatively balanced by a 0.2% cell death rate (13). This translates into continuous replacement or turnover every 500 days (13).

The secretory or luminal cells of the normal prostate glandular epithelium account about 70% of the epithelial volume. The immunophenotype of the luminal cells demonstrates the epithelial, secretory, androgen-responsive and weakly proliferative nature of this cell type (Table 1.1). Among the secretory products of these cells, the most important diagnostic marker is PSA. The luminal cells in the prostatic epithelium show diffuse and strong immunohistochemical staining for PSA. Antibody reactions against the proliferation markers such as Ki-67 antigen reveal that only 20% to 30% of proliferating cells in the normal prostatic epithelium are luminal cells with a proliferation index of 0.12% (10,11).

	Cell Type		
Marker	**Basal Cell**	**Luminal cell**	**Neuroendocrine Cell**
Pan-cytokeratin	+	+	+
CK 5,6,10,13,14	+		±
CK 8,18	– to ±	+	+
PSA	–	+	+ or –
PAP	–	+	+
AR	–	+	+ or –
GST-pi	+	–	NA
bcl-2	+	–	–
Chromogranin A	–	–	+
MSA, SMA	–	–	–
S-100	–	–	–
Vimentin	–	+	NA
P63	+	–	NA
CD40, CD44H	+	–	NA
CD10, CD57, CDw123	–	+	NA

Table 1.1- Normal prostatic epithelial cells immunophenotype.
Cytokeratin (CK); PSA = Prostate Specific Antigen; PAP = Prostate Acid Phosphatase; GST-pi = Glutathione-S-transferase-pi; MSA = Muscle-specific actin; SMA = Smooth-muscle actin; NA = Not available. Modified from (3).

Neuroendocrine cells are terminally differentiated cells that constitute about 0.4% of the total adult prostatic epithelial cell population. These cells are recognizable by neuroendocrine markers such as chromogranin A, enolase, serotonin, calcitonin, bombesin and L-dopa decarboxylase (DDC) (14). By immunohistochemical detection, neuroendocrine cells are found at the highest concentration in the periurethral regions and prostatic ducts (7). Most of neuroendocrine cells are more basally situated without luminal extensions (closed type), whereas a few cells extend to the luminal surface (so called open type). The functional significance of neuroendocrine cells in normal prostate is not clear, but it seems likely that the neroendocrine products of these cells influence neighboring cells through the elaborate dendritic process via a paracrine effect (15). Autocrine and endocrine influences may also be in operation (16).

The stroma in the prostate consists chiefly of smooth muscle cells and fibroblasts with branching of blood and lymph vessels as well as nerve bundles (2,17). In addition to structural support, the normal function of smooth muscle is to contract upon parasympathetic neural stimulation to promote secretion (3). Furthermore, stromal cells modulate normal prostatic epithelium development, growth, and differentiation via dynamic and reciprocal stromal-epithelial interactions (3).

1.2 Androgens and the prostate

Androgens, which are essential for development of male sexual organs and secondary sex characteristic, regulate growth, differentiation, and maintenance of prostatic tissues (18). The two most important androgens in this respect are testosterone and 5α-dihydrotestosterone (DHT). While acting through the androgen receptor (AR), each androgen has its own specific role during male sexual differentiation. Testosterone is directly involved in the development and

differentiation of Wolffian duct derived structures (epididymides, vasa deferentia, seminal vesicles and ejaculatory ducts), whereas DHT, a metabolite of testosterone, is the active ligand in a number of other androgen target tissues, like the urogenital sinus and tubercle and their derived structures (prostate gland, scrotum, urethra, penis) (19,20). The interaction of both androgens with the androgen receptor is different. Testosterone has a two-fold lower affinity than DHT for the androgen receptor and the dissociation rate of testosterone from the receptor is five-fold faster than of DHT (21). However, testosterone can compensate for this "weaker" androgenic potency during sexual differentiation and development of the Wolffian duct structures via high local concentrations due to diffusion from the nearby positioned testis. In the more distally located structures, like the urogenital sinus and urogenital tubercle the testosterone signal is amplified via conversion to DHT (21).

1.2.1 Endocrinology of androgens

Androgen is the generic term for any natural or synthetic compound that promotes the development of male secondary sexual characteristics. They belong to the group of steroid hormones and derived from cholesterol. The major circulating androgen is testosterone, which is secreted from the leydig cells within the testes. The leydig cell differentiation and the initial early testosterone biosynthesis in the foetal testes are independent of luteinizing hormone (LH) (22-24). During testis development the production of testosterone comes under the control of LH which is produced by the pituitary gland. The synthesis and release of LH is under control of the hypothalamus through gonadotropin-releasing hormone (GnRH) and inhibited by testosterone via a negative feedback mechanism (25) (Fig 1.4).

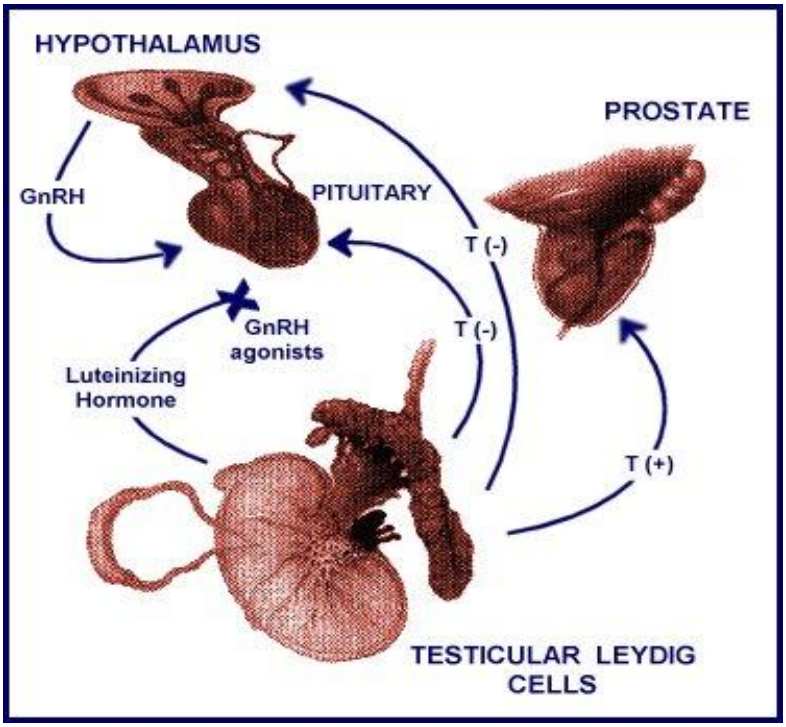

Figure 1.4 - The hypothalamus pituitary gonadal axis. Activation of androgen signalling begins in the hypothalamus where gonadotropin-releasing hormone (GnRH) is produced. GnRH acts on the anterior pituitary to stimulate the release of LH, FSH. They are subsequently released into circulation, eventually inducing the testes to produce testosterone (http://www.prostaphil.com/trg-3cop.html).

The biosynthetic conversion of cholesterol to testosterone involves several discrete steps, of which the first one includes the transfer of cholesterol from the outer to the inner mitochondral membrane by the steroidogenic acute regulatory protein (StAR) and the subsequent side chain cleavage of cholesterol by the enzyme P450 side-chain-cleavage (P450scc) (26). This conversion, resulting in the synthesis of pregnenolone, is a rate-limiting step in testosterone biosynthesis. Subsequent steps require several enzymes including, 3β-hydroxysteroid dehydrogenase (3β-HSD-II), 17α-hydroxylase (P450c17) and 17β-hydroxysteroid dehydrogenase (17β-HSD-III) (Fig 1.5). It has to be noted that LH enhances the transcription of genes that encode a range of enzymes in the steroidogenic pathway and that continued LH stimulation results in Leydig cell hyperplasia and hypertrophy (27,28). In the normal male, the episodic nature of LH stimulation is likely to avoid prolonged periods of Leydig cell refractoriness to LH stimulation (29). Approximately 95% of circulating testosterone in males is

produced by Leydig cells. However, the adrenal gland can also serve as an alternative source of androgen production in both sexes. The adrenal glands synthesize and secrete the weak androgens such as dehydroepiandrosterone (DHEA) that could be converted into testosterone in peripheral tissues (30).

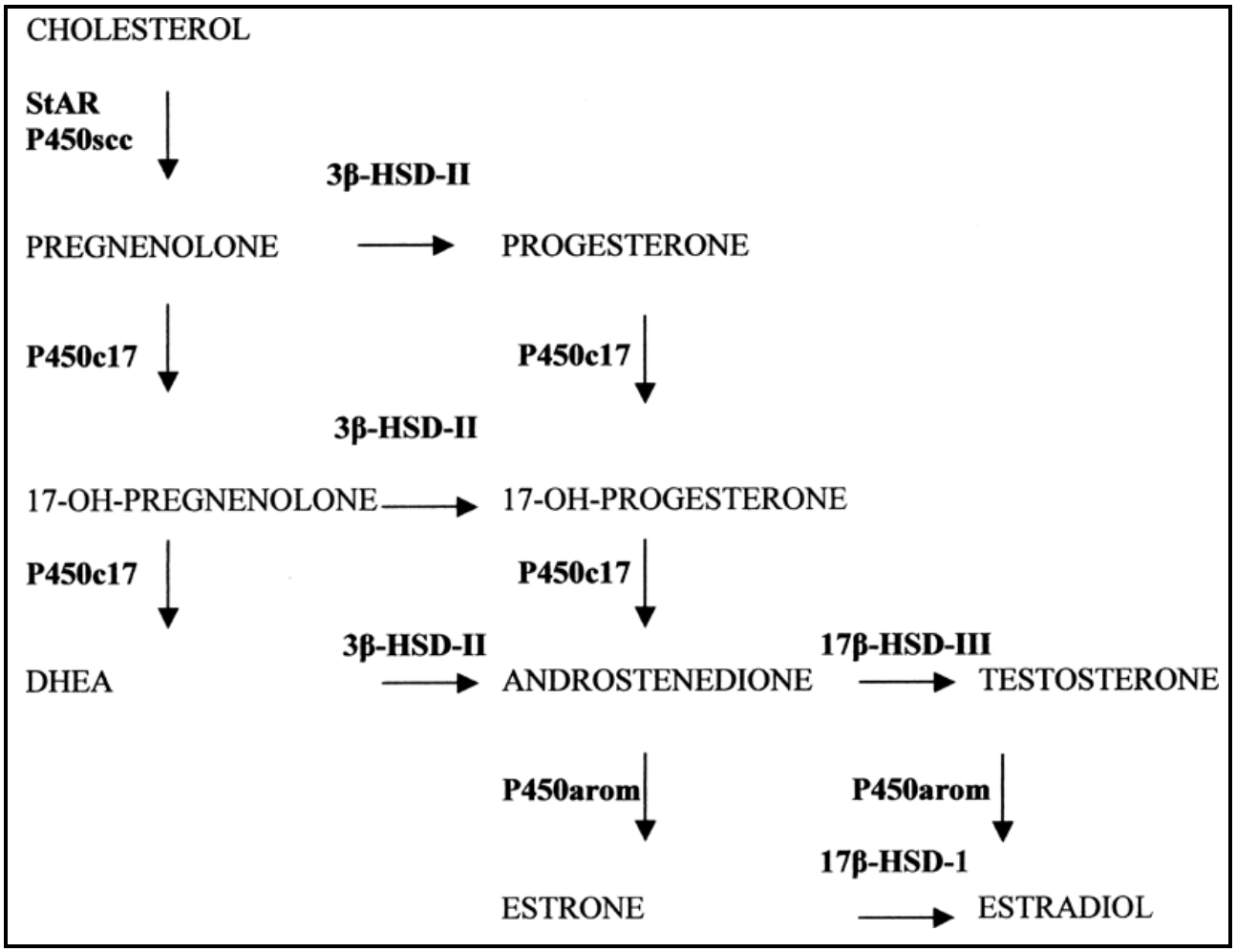

Figure 1.5- Steroid hormone biosynthesis. Testosterone is produced from a cholesterol precursor in Leydig cells. One of the key rate limiting reactions in the production of testosterone is the conversion of cholesterol to pregnenolone by the P450 side-chain-cleavage enzyme ($P450_{SCC}$). This reaction occurs in the mitochondria. Pregnenolone is eventually converted to DHEA (dehydroepiandrosterone) in the endoplasmic reticulum. From Androgen Physiology (31).

1.2.2 Androgen metabolism

Once testosterone is synthesized, it diffuses out of the Leydig cell and into the bloodstream with smaller amounts secreted into lymphatics and tubule fluid. Circulating testosterone at concentrations higher than its aqueous solubility binds avidly to sex hormone-binding globulin (SHBG) (60- 70%) or to albumin with a lower affinity (20-30%). Only about 1% of total testosterone remains free and unbound to carrier proteins, which equates to a serum concentration of 1 nM (1).

Testosterone undergoes metabolism to bioactive metabolites and to inactivated oxidized and conjugated metabolites for urinary and/or biliary excretion. A small proportion of circulating testosterone is metabolized to biologically active metabolites in specific target tissues to modulate biological effects. This process includes both an activation pathway converting testosterone to DHT (androgen amplification) as well as a diversification pathway whereby the enzyme aromatase produces estradiol from testosterone, which is capable of activating estrogen receptors. In the prostate, free testosterone that is not bound to SHBG or albumin, but diffuses through the plasma membrane of target cells where it is converted irreversibly to DHT by the 5α-reductase enzyme. There are two distinct 5α-reductase genes in humans, each encoding a biochemically distinct isozyme (32). The type 1 isozyme is present at low levels in the prostate and is not involved in androgen dependent male sexual differentiation (33), whereas the type 2 isozyme is the predominant 5α-reductase in androgen target tissues, including the prostate (34). An important issue is whether eliminating intra-prostatic androgen amplification, by inhibition of 5α-reductase, can prevent prostate disease. A major 10 year chemoprevention study randomizing nearly 19,000 men over age 55 years without known prostate disease to daily treatment with an oral 5α-reductase inhibitor, finasteride, or placebo observed a cumulative 25% reduction at 7

years of treatment in early stage, organ-confined low grade prostate cancer. While not designed to determine survival benefit, there was an apparent “stage shift” towards higher grade, but still organ-confined, cancers possibly a medication effect on tumour histology or an observation bias because the smaller prostate the higher chance to detect tumour (35). These findings highlight the importance of androgen amplification within the prostate in the origin of cancer during the long latent pre-malignant phase. However, routine chemopreventative use of prostatic 5α-reductase inhibition has not applied yet and probably needs more clinical studies (36).

The biological action of androgen is initiated by the binding of testosterone or DHT to its cognate receptor, the AR, causing its activation (Fig. 1.6). AR itself, when it is not bound to its ligand, is localized in the cytoplasm as part of an inactive complex that includes heat shock proteins 70, 90 and 40. These proteins have an important role to prevent premature AR degradation and to maintain an AR conformation that is accessible to the ligand (37-39). Upon binding to its specific ligand, the AR undergoes a conformational change that results in a more compact and stable form of the receptor. Activated AR dissociates from heat shock proteins and translocates to the nucleus, where it interacts with DNA androgen response elements as a homodimer to influence transcription of downstream genes (40). The active DNA-bound AR can recruit coregulator proteins and basal transcriptional machinery to regulate gene-specific expression that leads to changes in the growth and survival of target cells (41,42).

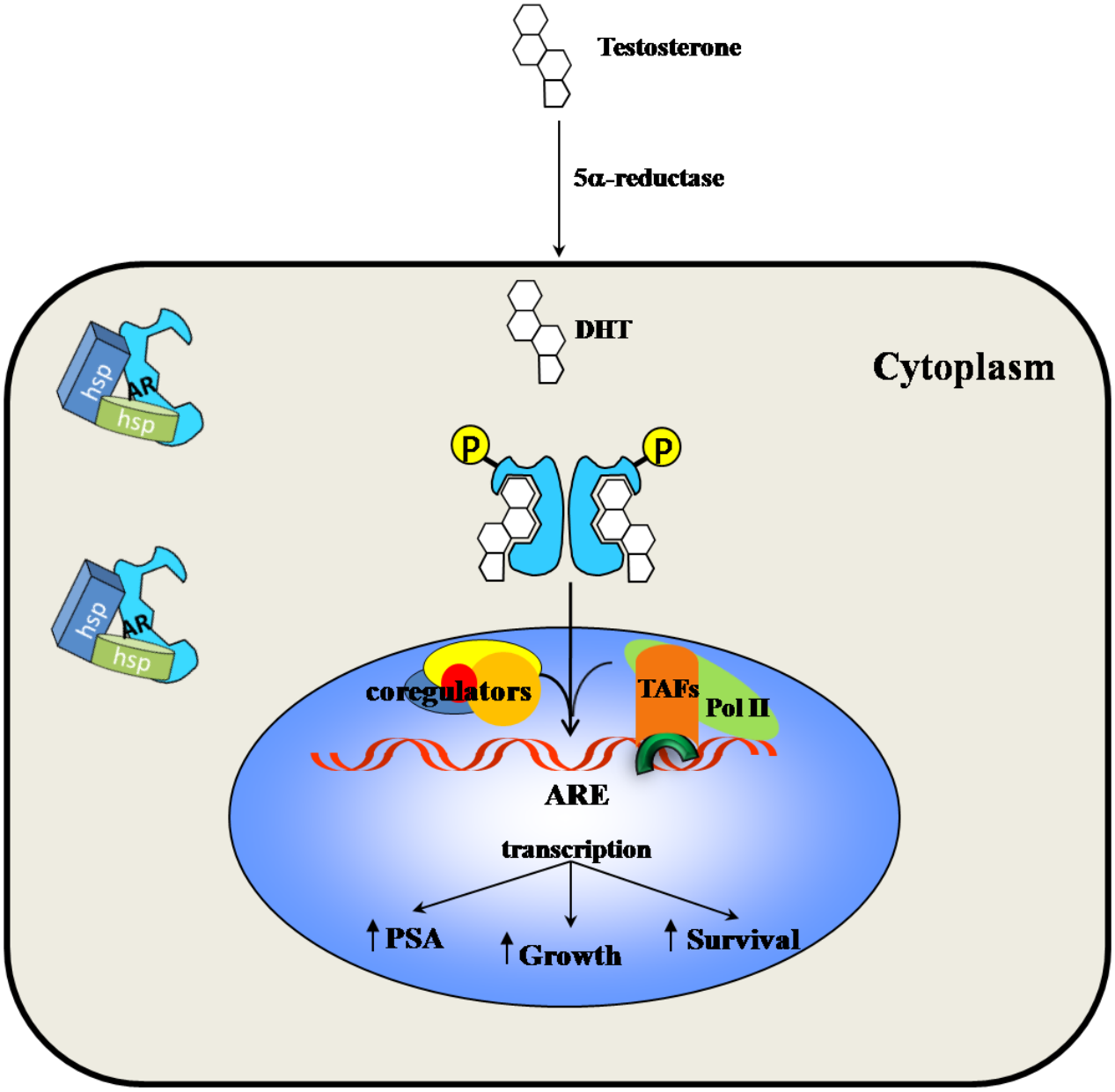

Figure 1.6 – Androgen receptor signalling. Testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase inside the cell. Activation of AR is achieved through binding of cognate DHT ligand to the ligand binding domain of AR, which induces a cascade of events that include conformational changes and phosphorylation (P) of the receptor, as well as dissociation from heat shock proteins (hsp), receptor dimerization, nuclear translocation and binding to DNA androgen response elements (ARE) within the promoters/enhancers of target genes, such as PSA. At all stages of AR action, the receptor can interact with coregulator (coactivators and corepressors) proteins that modulate its transcriptional activity. TAFs = TATA binding associated factors; PolII = RNA polymerase II.

1.3 Prostate cancer

1.3.1 Epidemiology

Prostate cancer is the most commonly diagnosed non-skin cancer among men in Western countries and one of the leading causes of cancer-related death. In Canadian men in 2008, prostate cancer will continue as the leading type of cancer, with an estimated 24,700 newly diagnosed cases, resulting in approximately 4,300 deaths (43). The incidence of prostate cancer is increasing around the world, in part because of the aging of the population, but the age-adjusted incidence is also increasing (44,45). The age-adjusted incidence rates are highest in North America, Australia, and New Zealand, Europe, and the Caribbean, where the Jamaican men have the highest incidence in the world (46). In contrast, rates are far lower in Asia, and the difference between North America and China is greater than 80-fold. Both genetic (non-modifiable) and environmental (modifiable) factors have been implicated in this dissimilarity. Established non-modifiable risk factors for prostate cancer include older age, a family history of prostate or breast cancer and race (47). Risk is the greatest among African American men, followed by white individuals and Asians. However, environmental components seem to be a major factor because the movement of low-risk Asian men to high-risk geographic areas (North America) results in a marked increase in prostate cancer (48).

Many environmental risk factors, including androgens, diet, physical activity, sexual factors, inflammation, and obesity, have been implicated, but their roles in prostate cancer aetiology remain unclear. The evidence that very low level of androgens prevent prostate cancer is substantial. For example, the incidence of prostate cancer is low among men with cirrhosis, which is associated with testicular atrophy (49) and long-term diabetic patients who may have a

low level of testosterone (50). Also, androgen inhibition or ablation causes the regression of prostate cancer early on (51). In experimental models, human prostate tumours implanted into castrated mice do not grow (52) and prostate tumours develop in rats who received testosterone subcutaneously (53,54).

The concept that variation in androgenicity influences prostate cancer risk extends to studies of polymorphisms in androgen-related genes. The most consistent results have been shown for the AR (55). The first exon of AR, encoding the N-terminal domain responsible for transactivational control (56), contains a polymorphic region of CAG repeats encoding polyglutamine (poly-Q) (see section 1.4.4 for more detail). The mean repeat number of CAGs is about 22 in whites and 20 in African-American, and the normal range is from 6 to 39 over several racial or ethnic groups (57,58). Shorter length of the CAG repeats correlate with increased AR transcriptional activity *in vitro* (59) and longer CAG repeats are associated with androgen insensitivity syndrome (60). Epidemiological studies of prostate cancer and AR gene CAG repeat length showed a moderately increased risk of prostate cancer in men with shorter CAG repeats (61).

1.3.2 Clinical presentation and diagnosis

Prostate cancer usually does not cause symptoms until late in the course of disease. Local symptoms such as an increase in frequency and difficult urination are due to the pressure effect of the enlarged prostate onto the bladder neck or urethra. Acute urinary retention and hematuria are uncommon and also non-specific in prostate cancer. Rectal invasion, renal failure secondary to ureteral obstruction, and impotence (due to extraprostatic extension into the adjacent neurovascular bundles) are rare and late manifestations of highly advanced local disease (62).

The first symptoms of prostate cancer can be caused by metastasis. Regional pelvic lymph nodes and bones are the most common anatomic metastatic sites. In fact, over 84% of patients with advanced disease show skeletal metastases (63). Bone involvement in these patients consistently produces significant symptoms such as pain or pathologic fracture at metastatic sites (64). Currently, most of the time, prostate cancer is clinically detected through abnormal digital rectal exam (DRE) and serum PSA. A DRE could miss a substantial proportion of prostate cancers, is poorly reproducible and subjective, with high inter-examiner variability (65). Nevertheless, it is a clinically important part of physical examination that is useful in prostate cancer detection and staging (62).

PSA expression is not, as the name PSA indicates, specific for the prostate gland. It has also been detected in male and female normal periurethral glands (66), normal endometrium (67) pancreas, salivary glands (68) and breast tissue (69). PSA is normally secreted by the prostatic luminal cells into seminal fluid, where concentrations, at around 1 to 5 mg/mL, are about 1 million times greater than normal serum level, which is usually less than 4 ng/mL. In addition to normal tissue, many non-prostatic tumours appear to express detectable PSA including breast and ovarian cancers (69,70), renal cell, colon and liver carcinomas (71). This lack of specificity does not decrease the value of PSA in clinical urology and diagnostic pathology. Serum PSA is clearly the best predictor of a histologic diagnostic adenocarcinoma in needle biopsy (72). The positive predictive value [1] of a PSA level > 4 ng/mL for a histologic diagnosis of carcinoma in needle biopsy is 31% to 51% (72,73). This value is increased to 42% to 72% when the DRE is suspicious for prostate cancer (72,73).

[1-] Positive Predictive Value = the probability that disease is present with an abnormal test

1.3.3 Staging and grading

Once prostate cancer is diagnosed, tumours have to be staged and graded. Prostate cancer stage is defined as anatomic extension of cancer within and out of the gland. The goals in staging of prostate cancer are: i) to rationally select treatment approach and ii) to predict prognosis. The typical system to stage prostate cancer is the TNM classification. TNM is based on the size of a primary tumour (T), regional lymph node involvement (N), and distant metastasis (M). The latest TNM classification for prostate cancer that is adopted by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) in 2002 (74) is the most widely used in clinical practice and the medical literature (Table 1.2). Clinical staging initiates after a histologic diagnosis of prostate cancer is established. DRE, radiologic imaging, serum PSA and sometimes prostatic acid phosphatase, histologic grading and surgical lymphadenectomy are most commonly used to determine prostate cancer extension (62). However, pathologic T staging (see Table 1.2) usually requires histologic examination of radical prostatectomy tissues, including the prostate gland and seminal vesicles (74). Pathologic stage strongly influences therapeutic outcome and decision making for men with localized prostate cancer (62).

The histologic grade of a neoplasm is often dictated by the degree of differentiation of the neoplastic cells. Currently, the most widely used grading system worldwide is the Gleason system (75). This grading system is based entirely on the histologic pattern of arrangement of carcinoma cells in haematoxylin and eosin (H&E) stained prostatic tissue sections (76). Gleason grade determines the extent of gland differentiation and stromal invasion using a scoring system of 1 to 5, which is appointed for the most predominant staining pattern (primary pattern)

Primary tumour, Clinical (T)				
TX	Primary tumour cannot be assessed			
T0	No evidence of primary tumour			
T1	Clinically inapparent tumour not palpable nor visible by imaging			
T1a	Tumour incidental histologic finding in 5% or less of tissue resected			
T1b	Tumour incidental histologic finding in more than 5% of tissue resected			
T1c	Tumour identified by needle biopsy (eg, because of elevated PSA)			
T2	Tumour confined within prostate [1]			
T2a	Tumour involves one half of one lobes or less			
T2b	Tumour involves more than one half of one lobe but not both lobes			
T2c	Tumour involves both lobes			
T3	Tumour extends through the prostate capsule			
T3a	Extracapsular extension (unilateral or bilateral)			
T3b	Tumour invades seminal vesicle(s)			
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, and/or pelvic wall			
Primary tumour, Pathologic (pT)				
pT2 [1]	Organ confined			
pT2a	Unilateral, involving one half of the lobe or less			
pT2b	Unilateral, involving more than one half of one lobe but not both lobes			
pT2c	Bilateral			
pT3	Extraprostatic extension			
pT3a	Extraprostatic extension			
pT3b	Seminal vesicle invasion			
pT4	Invasion of bladder, rectum			
[1] There is no pathologic T1 classification				
Regional Lymph Nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph nodes metastasis			
N1	Metastasis in regional lymph nodes or nodes			
Distant Metastasis (M)				
MX	Distant metastasis cannot be assessed			
M0	No distant metastasis			
M1	Distant metastasis			
M1a	Non-regional lymph node(s)			
M1b	Bone(s)			
M1c	Other site(s)			
Stage Grouping				
Stage I	T1a	N0	M0	G1(Gleason score 2-4)
Stage II	T1a	N0	M0	G2, 3-4 (Gleason score 5-10)
	T1b	N0	M0	Any G
	T1c	N0	M0	Any G
	T1	N0	M0	Any G
	T2	N0	M0	Any G
Stage III	T3	N0	M0	Any G
Stage IV	T4	N0	M0	Any G
	Any T	N1	M0	Any G
	Any T	Any T	M1	Any G

Table 1.2 – TNM staging scheme.

and the second most common pattern. The sum of these two grades is referred to as the Gleason score (or Gleason sum) and can range from 2 to 10. Generally, a well-differentiated tumour is assigned a low score of 2-4, a moderately differentiated tumour is designated as score 5-7 and a score of 8-10 denotes a poorly differentiated tumour. Patients with higher Gleason scores have a poorer clinical outcome, with a greater risk of progression of cancer to metastatic disease.

1.3.4 Treatment

A primary factor in choosing an effective treatment approach for prostate cancer is the stage of disease. Surgical treatment, radiation therapy, cryosurgery, or active surveillance all are available options for patients with localized prostate cancer (e.g., stages I and II). However, for advanced prostate cancer in which the disease is not confined to prostate, hormone therapy is the cornerstone of treatment. This therapy is known by many other names, including androgen ablation, combined hormone blockade, or androgen deprivation. Hormone therapy is used in five situations – i) to prolong survival, ii) to slow down the spread of cancer cells that have escaped (secondary or adjuvant therapy), iii) to ease pain caused by the spread of the cancer (palliative therapy), iv) to enhance the effect of radiotherapy v) to shrink the prostate and the tumour before a procedure, in order to reduce the likelihood of escape (neoadjuvant hormone therapy (NHT)).

Huggins and Hodges pioneered the use of androgen ablation in prostate cancer about seventy years ago and demonstrated that prostate tumour growth was dependent on androgens (77). Bilateral orchiectomy (surgical castration) led to relief of prostate cancer symptoms in about 80% of patients and was therefore adopted as the “gold standard” of hormone therapy. The rationale behind the use of orchiectomy is reducing circulating testosterone levels by 95% at

once. However, due to the psychosocial side effects (physical loss, impotence, decreased libido) of this procedure it is not a routine treatment approach for patients with advanced prostate cancer. As an alternative approach and by exploiting the leuteinizing hormone secretion pathway (section 1.2.1), Leuteinizing Hormone-Releasing Hormone (LH-RH) agonists, such as Leuprolide, reduce circulating testosterone to levels that no longer support growth and maintenance of prostate cells, resulting in medical castration. The only serious side effect with this method is the surge in the serum testosterone level at the time of first administration, which may result in the exacerbation of clinical symptoms. To avoid this problem, antiandrogen therapy should be initiated in combination of LH-RH agonists for the first three weeks to suppress androgen flare up.

Antiandrogen agents are another class of hormone therapy that compete with DHT for AR. Most patients with advanced prostate cancer will receive an antiandrogen at some point during treatment. There are two types of androgen antagonists: steroidal and non-steroidal. Steroid-based antagonists, such as cyproterone acetate, compete with androgens for binding to the ligand binding pocket within the AR. 3 mg/day of cyproterone acetate can prevent flares caused by the first injection of LH-RH agonists (78). However in contrast to non-steroidal antagonists, these steroidal compounds have significant progestational activity. Non-steroidal compounds, such as flutamide, nilutamide, and bicalutamide (Casodex) also prevent androgen flares. They facilitate the assembly of inactive AR protein complexes to DNA at the proximal promoter of PSA without involving the promoter enhancer region (79) (see section 1.5.2). Among the non-steroidal compounds, only the pharmacological profile of bicalutamide allows use as monotherapy (150 mg/ day) or combination therapy (50 mg/ day) (80). Flutamide and nilutamide can result in elevation of plasma testosterone level. This elevation overrides the interaction

between antiandrogen and AR, making treatment difficult when they are used as monotherapy (81). Despite the availability and use of a combination of treatments to block androgen, progression of advanced disease to hormone-refractory prostate cancer (also known as the castration- resistant state) is inevitable.

1.3.5 Progression of prostate cancer to castration-resistant state

As discussed above, the growth and maintenance of the prostate gland is dependent on the presence of testosterone and DHT. Initially, prostate cancer is also dependent on androgens for growth, and in its early stages the disease is treatable by radical prostatectomy or by radiation ablation of the prostate gland. However, once cancer cells escape the prostate capsule and disseminate to distant sites, the disease is much more difficult to treat. Unfortunately, approximately a third of prostate cancer patients present with advanced disease (82). Advanced prostate cancer is treated by androgen withdrawal, when radical prostatectomy alone is no longer an adequate option. Removal of hormone by androgen ablation therapy prevents the growth promoting effects of androgens, leads to apoptosis of cancer cells and ultimately results in tumour regression. The decrease in tumour burden following androgen ablation occurs during the androgen-dependent stage of prostate cancer when the tumour still requires androgens for survival and growth. Unfortunately, this form of therapy offers limited aid, and the average range of overall survival is less than 2-3 years (83). For reasons that are not fully understood, prostate cancer cells switch from an androgen-dependent to castration-resistant state, in which cells are able to bypass requirement for the androgenic growth signal, and grow in an uncontrolled fashion. As a result, tumour burden and prognostic disease markers such as PSA increase dramatically (Figure 1.7).

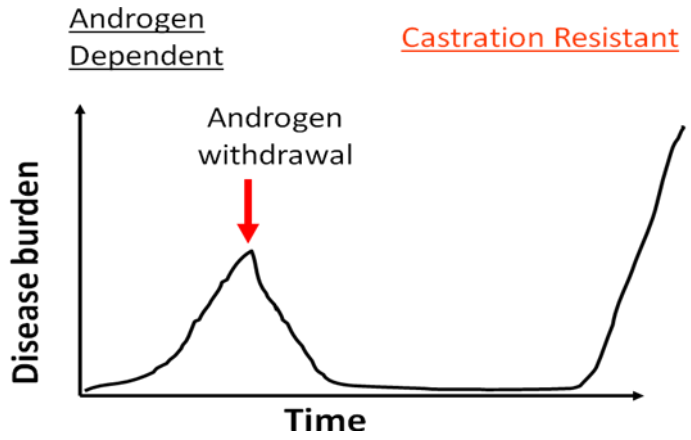

Figure 1.7 - Progression to castration resistance. Upon androgen ablation treatment of prostate cancer, an initial recovery of the disease occurs; however, progression towards castration resistance is inevitable, and ultimately fatal in most cases.

Since androgens are essential to the survival of prostate cells, a major question is how a prostate cell survives after androgen-ablation therapy. Recent findings from a number of investigators suggest that the AR plays a critical role in the development of androgen-refractory prostate cancer (84-87). In over 80 % of locally advanced castration-resistant prostate tumours, high levels of nuclear AR have been observed (88); and in bone metastases, the amount of AR present is often higher than in primary tumours (89). There is evidence that in most cases some form of inappropriate activation of AR is linked to recurrent growth of prostate cancers (87,90). Indeed, a study by Chen et al showed that the AR gene is the only gene that is consistently up-regulated during tumour progression in different experimental models of androgen refractory prostate cancer (91).

Generally, the molecular mechanisms by which progression transitions from androgen dependence to castration-resistant state can be divided into two pathways – those bypassing the AR and those functioning through the receptor. These are not mutually exclusive and frequently coexist in castration-resistant prostate cancer (92) (Figure 1.8).

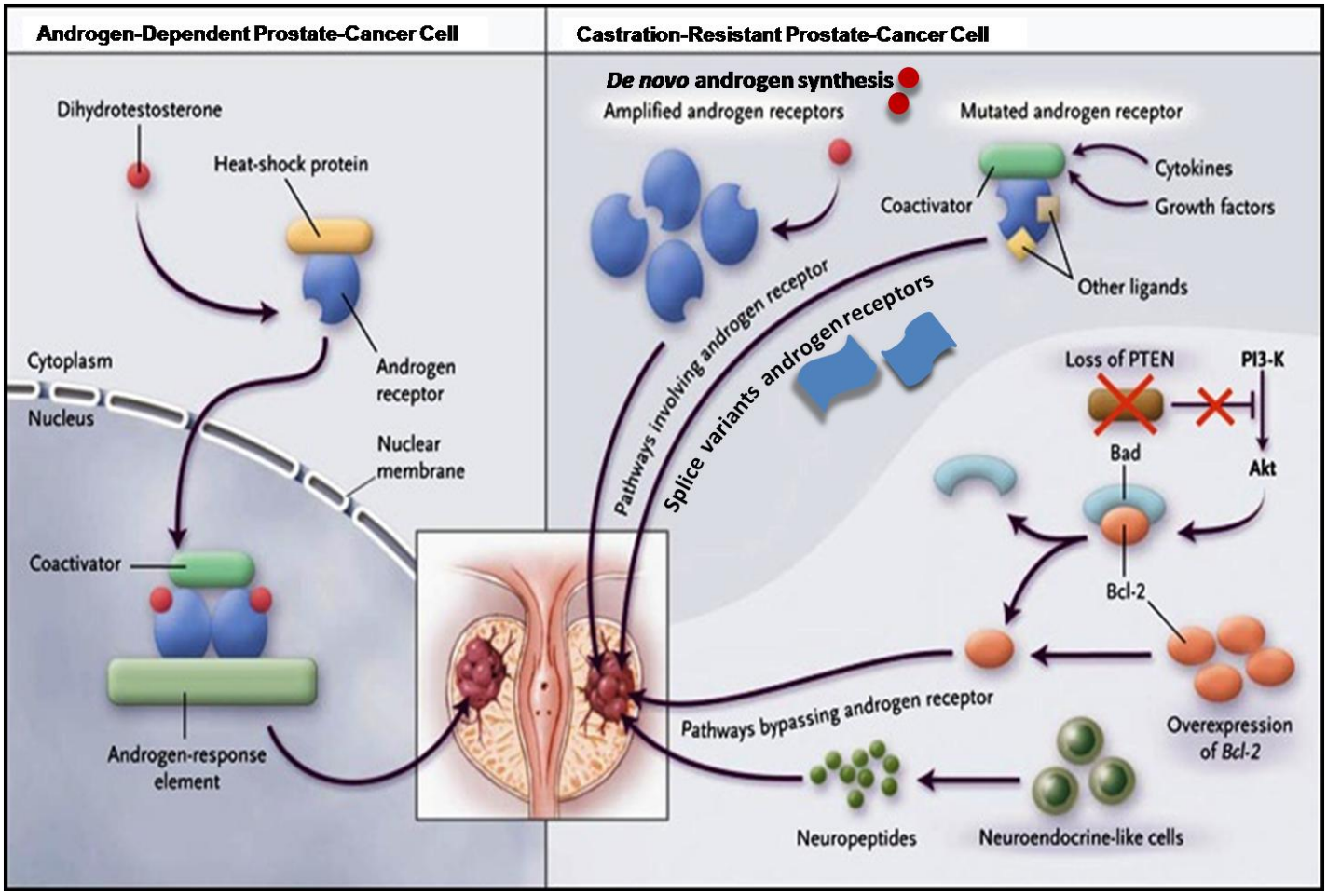

Figure 1.8 – Androgen dependent and castration-resistant progression of prostate cancer. During androgen-dependent progression, prostate cancer cells depend on androgen/AR axis for growth and survival (left panel). During castration-resistant progression, two main pathways support growth and survival of cancer cells (right panel) –one involves AR and the other one bypasses it. Modified from (92) Copyright © *[2004] Massachusetts Medical Society. All rights reserved*.

One important pathway, which completely bypasses AR, is related to the deregulation of apoptotic genes, such as the tumour suppressor gene *PTEN* [1] and the antiapoptotic gene *Bcl-2.* PTEN inhibits the phosphatidylinositol 3-kinase pathway. Activation of this pathway stimulates the *Akt* protein (protein kinase B), which inactivates several proapoptotic proteins, thus enhancing cell survival. The inactivation of PTEN is considerably more likely to occur in

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[1-] Phosphatase and Tensin homologue

castration-resistant prostate cancer, where the loss of PTEN increases Akt activity and blocks apoptosis. One of the main targets of Akt is Bcl-2, which is released from its bound protein (called Bad) upon Akt activation, increasing cell survival. Overexpression of Bcl-2 has been implicated in the progression to hormone-refractory prostate cancer.

Neuroendocrine differentiation of prostate cancer cells is another pathway that circumvents AR toward transition to castration-resistant state. Although neuroendocrine cells are seen in normal prostate and androgen-dependent disease, they are more common in hormone-refractory prostate cancer. The low rate of proliferation of neuroendocrine cells allows them to survive treatment with most chemotherapeutic agents, as well as endocrine and radiation treatments. In addition, neuroendocrine cells secrete neuropeptides such as serotonin and bombesin, which can increase the proliferation of neighbouring cancer cells, thereby allowing progression of castration-resistant prostate cancer (93).

The pathways involving AR-mediated survival of prostate cancer cells include *i)* amplification or mutations of the receptor (94,95), *ii)* deregulation of growth factors or cytokines (96-100), *iii)* autocrine production of active androgens in the cancer cells (101,102), *iv)* alteration of coactivator expression (103-105), and *v)* expression of alternatively spliced variants of the AR that lack the ligand binding domain and are constitutively active (106,107). These pathways cause activation of the AR in a ligand reduced or independent manner. In order to understand the molecular processes that take place during disease progression through AR, first, the structure and function of the receptor will be discussed and the relevant AR-mediated survival pathway will be explained in the corresponding sections.

1.4 Androgen receptor structure and function

As mentioned earlier, androgens are steroid hormones that carry out their function through the AR. The human AR gene is located in the q11-12 region of the X chromosome, spans 90 kb and is comprised of 8 exons (108). The AR coding region of the mRNA product is 2.7 kb in length, with 5' and 3' un-translated regions of 1.1 and 6.9 kb respectively (109,110). The AR cDNA codes for a 919 amino acid protein with an apparent molecular weight of 110 kDa. The protein is a ligand-dependent transcription factor that is capable of regulating expression of genes involved with growth and differentiation of the prostate gland. The AR belongs to a family of steroid receptors along with the glucocorticoid, progesterone, and mineralocorticoid receptors. They share similar domain structures and mechanism of action. Steroid receptors, including the AR, have three functional domains: an NH_2-terminal domain (NTD) that contains the transcriptional activation function 1 (AF-1), a central DNA-binding domain (DBD), and a COOH-terminal ligand-binding domain (LBD), which is linked to the DBD by a hinge region and contains the transcriptional activation function 2 (AF-2) (Figure 1.9) (111). The characteristic of each domain of AR will be discussed below.

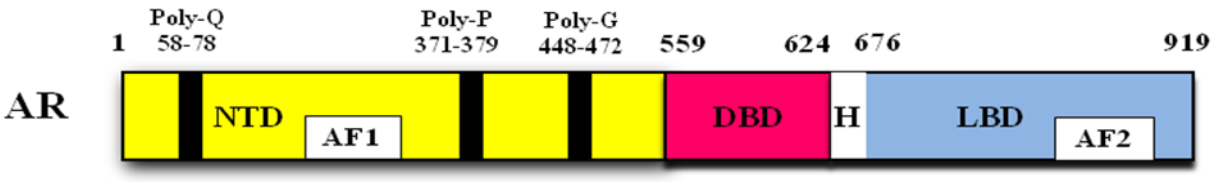


Figure 1.9 - Functional domain structure of androgen receptor. Androgen receptor contains functional domains that include an N-terminal domain (NTD), a DNA binding domain (DBD), a hinge region (H) and a ligand binding domain (LBD). Transcriptional activation functions 1 and 2 (AF1 and AF2) are located in the NTD and LBD, respectively.

1.4.1 The Androgen receptor ligand-binding domain

The COOH-terminal LBD of steroid receptors operate similarly by binding their cognate ligand within a central LBD pocket. The LBD of AR spans amino acids 616-919. Within the LBD, there is a hydrophobic pocket that accepts the androgen ligands, such as testosterone and DHT. The AR-LBD is highly conserved between different species (human, rat, and mouse) and ranges in its degree of homology (20-55%) with LBDs of other steroid receptor family members (112,113). The role of AR-LBD is of particular importance for prostate cancer, because it is the primary target of current androgen ablation therapies. Although there are potent androgen antagonists available in clinic, mutations in the AR-LBD allow for promiscuity of ligand binding, which can result in inappropriate activation of AR by non-androgenic compounds (114). Over 30% of prostate tumours possess AR mutations and several different AR variants have been identified which exhibit loss of receptor specificity in the absence of conventional ligands (115). The majority of these mutations affect the ligand binding pocket and are clustered in three main areas of the LBD, which includes amino acids 670-678, 710-730, and 874-910 (87). Three of the most commonly identified variants in tumours are H874Y, T877A, T877S. The AR T877A mutation has been in well known, because it is found in a human prostate cancer cell line (LNCaP), as well as cases of advanced prostate cancer. Overall, compared to wild type AR, the above mutations sensitize the receptor to adrenal androgens or other steroid hormomes. This is most likely due to the recruitment of different coactivators, which permit the AR to bind other steroid ligands and allows antagonists to act as agonists for activation of AR in an androgen-depleted environment (116,117).

The three-dimensional structure of AR-LBD bound to synthetic androgen R1881 has been determined (118). It contains a series of α-helices similar to other steroid receptors, from helix 1-

12, but lacks helix 2 found in other family members. These helices are arranged in an anti-parallel three-layer sandwich motif to create the hydrophobic ligand-binding pocket. The strategy of targeting this pocket with pharmaceutical drugs has limitations since the ligand binding pocket is small and restricted and it is not remodelled upon ligand binding (119). A total of 18 amino acid residues, situated within each of 11 AR-LBD helices, are responsible for direct interactions with the ligand, with helix 12 performing as a flexible lid to stabilize the ligand within the LBD pocket. Precise positioning of helix 12 is essential for activation of AR by its ligand (120), and may also be important for AR inactivation by antagonists (121). Upon hormone binding, the LBD changes conformation to trap the ligand and form the AF-2 surface that interacts with *LXXLL*[1] motif of classical coactivators (122,123). AF-2 also interacts with *FXXLF*[2] and *WXXLF*[3] motifs within the N-terminus of AR. This intermolecular interaction is unique among steroid receptor family members and crucial for AR transactivation probably due to decreasing AR /ligand dissociation (124). The FXXLF motif of AR-NTD mediates interaction with AF-2, while the WXXLF motif in the AR-NTD interacts with a region of LBD that is outside of the AF-2 domain. These two sites allow for dimerization of activated AR molecules in a head-to-tail configuration and stabilize ligand binding in the hydrophobic pocket, which keeps AR in an active conformation. In addition to its role in stabilizing active AR, the NH_2/COOH interaction also regulates LXXLL mediated coactivator interactions with AR, such as the steroid receptor coactivator (SRC) family of coactivators. The relatively conserved central region of SRC family contains three LXXLL motifs that are responsible for interaction of ligand-bound AR. The FXXLF and WXXLF sequences in AR-NTD compete with LXXLL coactivator motifs and govern coactivator interactions with AR-LBD (124,125).

Since AF-2 is crucial in AR transcriptional activity, it is an attractive target site for the development of small molecules to suppress receptor activity (126-128). Recently, the Binding Function-3 (BF-3) pocket adjacent to the AF-2 domain has been identified as a novel site that allosterically influences coregulator association with AF-2 (119). Blocking this pocket by small molecules has been shown to inhibit AR activity (119). Targeting the BF-3 pocket is more favourable than ligand binding pocket since it is an outsized pocket that is modified upon binding of coactivators or small molecules (119). In addition, it is a site of mutation in prostate cancer patients and androgen insensitivity syndrome. Mutations at Gln-670, Ile-672, Leu-830 enhance AR transcriptional activity in prostate cancer (129-131), while mutation at Arg-840, Asn-727 reduce SRC-2 binding to AR and suppress its activity (132). In this thesis, several compounds that potentially fit into the BF-3 pocket and inhibit androgen receptor activity have been identified (discussed in Chapter 3).

1.4.2 The androgen receptor DNA-binding domain and hinge region

The DBD and hinge region of AR span amino acids 560-623 and 624-676 respectively (111). These regions are responsible for multiple functions that include: i) binding to DNA at consensus sequences in the promoter/enhancer region of AR-regulated genes (133), ii) dimerization of active AR molecules (134), and iii) nuclear localization of activated receptors (135). In addition, the DBD of AR interacts with proteins that make up the basal transcriptional apparatus as well as putative transcriptional coregulators (122). The DBD of AR and other steroid receptors are composed of two conserved zinc finger motifs that interact with DNA regulatory sequences. For AR, these DNA sequences are known as androgen response elements (ARE), in the promoters of androgen-regulated genes (Figure 1.10) (136,137). The ARE is made up of inverted palindromic sequences containing two half-sites, separated by a 3 nucleotide spacer (5'-

GGA/TACAnnnTGTTCT-3') (126). The first NH_2-terminal zinc finger of the DBD is responsible for recognizing ARE sequences and for binding specifically to AREs in the major groove of DNA, while the second zinc finger stabilizes receptor-DNA interactions (138,139). This second zinc finger of AR-DBD could influence receptor binding to AR-specific ARE (140).

The hinge region of AR contains a nuclear localization sequence (NLS) (amino acids 613-633) that targets the activated receptor to the nucleus (141). The bipartite NLS consists of two clusters of basic amino acids separated by ten amino acids (135). The lysine residues within the hinge region (K630, 632 and 633) that are acetylated upon receptor activation are believed to be important in nuclear translocation since Lys-to-Ala mutations of these residues disrupt nuclear trafficking of activated AR (142).

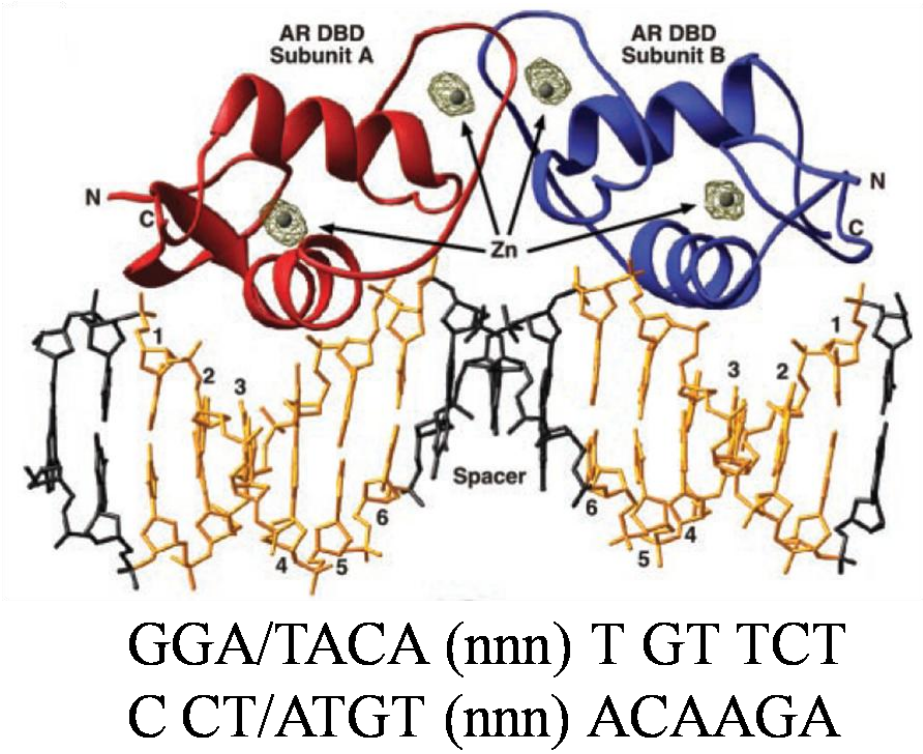


Figure 1.10 - Schematic of an AR dimer bound to an ARE (From (143) © 2004, National Academy of Sciences, USA).

1.4.3 The androgen receptor NH_2-terminal domain

The NTD of AR spans amino acids 1-559 and occupies over half of the receptor's primary sequence of 919 amino acids (144). The AR-NTD has unique features among steroid receptor family members and has the highest degree of amino acids variability with less than 15% amino acid sequence homology with the other steroid receptor-NTDs. It encodes the AF-1, which contains two transcriptional activation units (Tau) referred to as Tau1 and Tau5. The Tau1 region (amino acids 141-338) is essential for ligand-dependent transactivation of AR, while the Tau5 region (amino acids 360-528) irrespective of the presence of ligands shows constitutive transcription of AR-NTD once the AR-LBD is deleted (145). Unlike Tau-1, the Tau-5 is a signal-dependent transactivation site that requires signalling events from the protein kinase C related kinase (PRK-1) for activation (146).

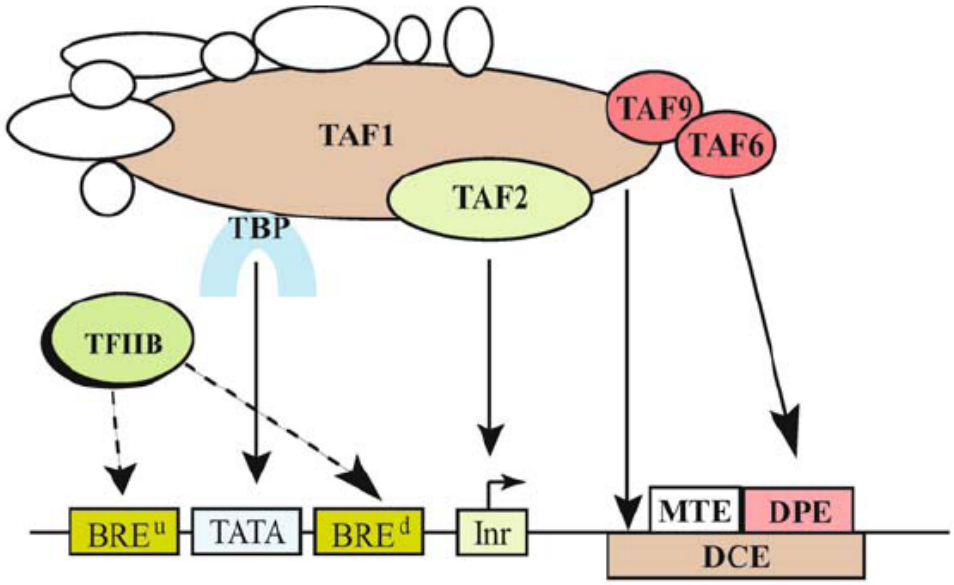
The AR-NTD contains three different homo-polymeric amino acid repeats that are not present in other steroid receptor-NTDs. These are poly-glutamine (poly-Q), poly- proline (poly-P) and poly-glycine (poly-G) repeats (Fig. 1.9). The poly-Q tract is found at amino acid 59, and has a normal range of 17-29 residues, the poly-P tract is 9 residues long starting at amino acid 327, and the poly-glycine tract is 24 residues in length starting at amino acid 449 (109). The precise function of these three repeats is not known; however, extensive research has been performed on the poly-Q tract to explain its role in AR action. It has been shown that transcriptional activity of AR has an inverse relationship with length of the poly-Q tract (147). The length of AR-poly-Q tract could also influence the interaction of AR with its coregulatory proteins that interact directly with AR to regulate AR-mediated transcription (148). As mentioned earlier (section 1.3.1), shortening of poly-Q tract of AR to 17 amino acids or less has been associated with an increased risk of prostate cancer (61).

The unique sequences and features of the AR-NTD make this region attractive for AR-specific protein interactions, which in turn may be important for directing AR-specific responses. Therefore, identifying novel protein partners that interact with the AR-NTD may elucidate the mechanism by which cells are able to achieve AR-specific responses to androgenic ligands. Our laboratory has identified several novel AR-NTD interacting proteins by using the N-terminus of AR as bait in the reverse yeast two-hybrid system (RTA) (14,149). In Chapter 4, one of these proteins, referred as TATA binding protein Associated Factor 1 (TAF1) will be introduced and its effect on AR be discussed.

1.5 Androgen receptor transcriptional activity

1.5.1 Mechanism of general transcription

The initiation of RNA polymerase II-directed transcription is a multistep process requiring the coordinated interactions of many proteins. Basal factors assembled proximal to the transcription start site, activators bound to more distal enhancer sequences, and coactivators that function to bridge these two groups all make important contributions to transcriptional regulation. The transcription process per se can be divided in the following steps: preinitiation complex (PIC) assembly on the core promoter, transcription initiation, promoter clearance, elongation, and termination (150,151). In order to initiate transcription, the core promoter serves as a platform for the assembly of PIC and is composed of seven characteristic DNA sequences located at around – 30 bp in the promoter (Fig. 1.11). PIC consists of RNA polymerase II (pol II) and Transcription Factors IIs (TFII), including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and



Core Promoter Element	Position	Consensus Sequence (5' to 3')	Bound Protein
BRE^u	-38 to -32	(G/C)(G/C)(G/A)CGCC	TFIIB
TATA	-31 to -24	TATA(A/T)A(A/T)(A/G)	TBP
BRE^d	-23 to -17	(G/A)T(T/G/A)(T/G)(G/T)(T/G)(T/G)	TFIIB
Inr	-2 to +5	PyPyAN(T/A)PyPy	TAF1/TAF2
MTE	+18 to +29	C(G/C)A(A/G)C(G/C)(G/C)AACG(G/C)	n.a.
DPE	+28 to +34	(A/G)G(A/T)CGTG	TAF6/TAF9
DCE	3 subelements +6 to +11 +16 to +21 +30 to +34	core sequence: S_I CTTC S_{II} CTGT S_{III} AGC	TAF1

Figure 1.11 - Recognition of core promoter elements by TFIID and TFIIB. The upper figure depicts the interactions between TFIID and TFIIB with the seven core promoter elements. The table in the lower panel lists the consensus sequence and positions for each of these core promoter elements. n.a., not available. Built from (152). BRE^u = upstream TFIIB-recognition element; TATA = TATA box; BRE^d = downstream TFIIB-recognition element; Inr = initiator; MTE = motif ten element; DPE = downstream promoter; DCE = downstream core element
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are collectively defined as general transcription factors (GTFs). PIC formation usually starts with TFIID binding to the TATA box, initiator, and/or downstream promoter element (DPE) found in most core promoters, followed by the entry of other TFIIs and pol II through either a stepwise assembly or a preassembled holoenzyme pathway (150,151). Formation of this promoter-bound complex is sufficient for a basal level of transcription. However, for activator-dependent transcription, such as AR, general cofactors are often required to transmit regulatory signals between gene-specific activators and the general transcription machinery.

Three classes of general cofactors, including TATA binding protein (TBP) associated factors (TAFs), Mediator, and upstream stimulatory activity (USA)-derived positive cofactors (PC1/PARP-1, PC2, PC3/DNA topoisomerase I, and PC4) and negative cofactor 1 (NC1/HMGB1), normally function independently or in combination to fine-tune the promoter activity in a gene-specific or cell-type-specific manner. In addition, other cofactors, such as TAF1, BTAF1, and negative cofactor 2 (NC2), can also modulate TBP or TFIID binding to the core promoter (152). In general, these cofactors are capable of repressing basal transcription when activators are absent and stimulating transcription in the presence of activators (152). It has to be noted that the assembly of coactivator or general transcription complexes is itself a dynamic and cell-specific process, with signal transduction pathways regulating the composition of specific complex components. Apparently, cell type specific components of coregulator complexes and general transcriptional apparatus have been evolved as a mechanism to accommodate more complex programs of tissue-specific and gene-selective transcription (153,154). For example, the p160 family of coactivator proteins nucleates the assembly of multiple, distinct complexes containing diverse enzymatic activities and functions to coactivate several classes of signal-dependent transcription factors (155). In the case of the SRC-3 within

the p160 family, six phosphorylation sites have been shown to be required for coactivation of AR, but not all of these sites are required for coactivation of NF-κB (156). Furthermore, different combinations of site-specific phosphorylations of SRC-3 are necessary for regulation of endogenous genes involved in inflammation or transformation (156). Biochemical studies support the concept that modulation of SRC-3 phosphorylation alters its interactions with potential activator/coactivator partners, allowing it to function as a regulatory integrator for diverse signalling pathways. For instance, phosphorylation of several residues of SRC-3 is required for its effective interaction with CBP (CREB binding protein) (156-158). In addition, many proteins in the general transcription apparatus are functionally specialized core promoter factors such as the subunits of TFIID complex. It has been shown that the subunits of TFIID are specialized for core promoter recognition, catalysis of protein modification, and targeting to specifically acetylated nucleosomes (159).

1.5.2 Androgen receptor transcriptional activity

AR-mediated transactivation also follows the general transcription mechanism but with some unique features. AR transcriptional activity is a multi-step process that includes ligand binding, nuclear translocation and AR/DNA interactions. Every step in this process is regulated and coordinated to ensure success and specificity of the AR response. Several alterations of these steps have been documented that explain changes in AR activity, which may be important for the progression of prostate cancer (160). To initiate AR transcription, chromatin structure around the promoter region of AR target genes needs to be recognized. Acetylation at the lysine residues in the NH_2-terminal domain of the core histones destabilizes the DNA-histone contacts in the nucleosomes and allows chromatin to be accessible to AR (161). Coregulator proteins, such as SRC-1, 2 and 3, CBP, p300, P/CAF (CBP associated factor) possess histone acetyl transferase

(HAT) activity (162-169) (see Table 1.3). In addition to HAT, the SWI/SNF complexes disrupt the DNA-histone interaction within the nucleosomes in an ATP-dependent manner. Upon DNA binding, AR actively recruits coactivator complexes and components of basal transcriptional machinery to influence the expression of its target genes (Fig. 1.12).

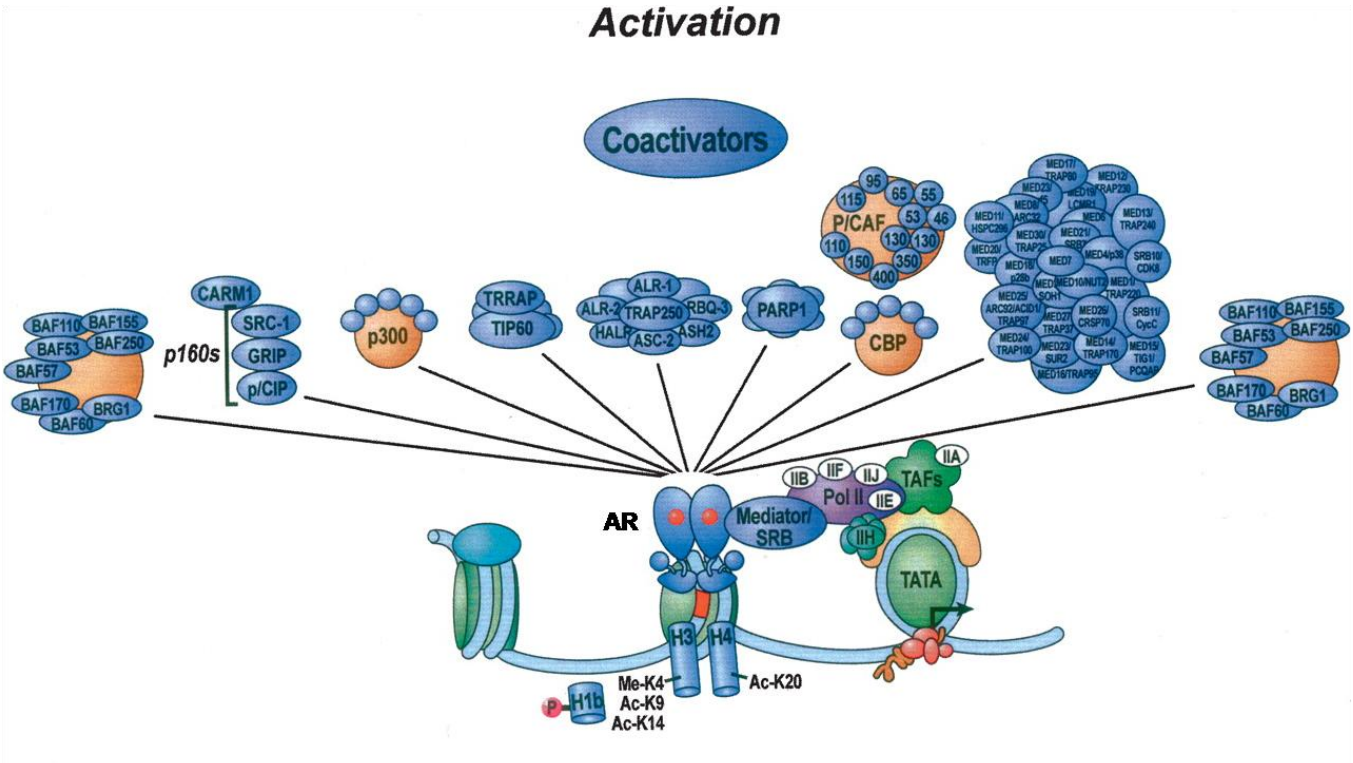

Figure 1.12 - The coactivator matrix. Androgen receptor (AR) binds to *cis*-active elements in promoters and enhancers of target genes and activates transcription in a ligand-dependent manner. Transcriptional activation requires the actions of many, multisubunit coactivator complexes that are recruited in a parallel and/or sequential manner. Enzymatic activities associated with specific components of coactivator complexes result in nucleosome remodeling and covalent modifications of histone tails, such as histones H3K4 methylation, H3K9 and H3K9 acetylation, H4K20 acetylation, and phosphorylation of the linker histone H1b. Me = Methylation; Ac = Acetylation; (Modified from (170) with permission from Cold Spring Harbor Laboratory Press, Copyright 2006).

To gain a better understanding of AR transactivation, the transcriptional mechanism of AR on the PSA promoter has been the subject of detailed research. The PSA promoter contains three AREs; two AREs at the proximal and the third ARE at the enhancer promoter. The AREI and

AREII within the proximal promoter of the PSA are at position -170 and - 400 (171), whereas the AREIII is identified within a potent core enhancer element located 4.2 kb upstream of transcription start site (172) (Fig. 1.13). In response to DHT, AR is recruited to the AREs in both the enhancer and the promoter. Reporter gene assays demonstrate that mutations in the ARE-I and the ARE-II result in 80 and 50% reduction, respectively, while mutations in the ARE-III result in 99% reduction of the PSA promoter activity (172). This indicates that the ARE-III in the enhancer region is absolutely required for optimal PSA gene expression.

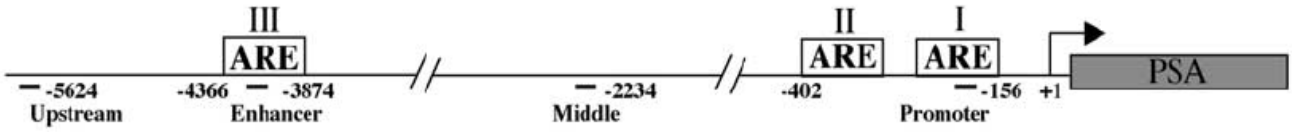

Figure 1.13 - Schematic diagram of the PSA gene regulatory region. Open boxes depict putative androgen response elements (ARE).

It has been well documented that in addition to the AR protein, the HAT, Mediator, and pol II protein complex are also recruited to the PSA enhancer and/or proximal promoter in response to short term (0.5 to 4 h) androgen stimulation (173-175). The proteins that directly bind nuclear receptors to enhance (*coactivators*) or inhibit (*corepressors*) receptor-mediated transcription are called *coregulators*. They differ from the general and specific transcription factors in that they do not affect the basal rate of transcription and typically do not bind to DNA (105). Recently, it has been shown that the occupancy of TBP and AR coactivator complexes on the PSA regulatory regions increase gradually upon androgen stimulation and peaks at 16 h, and then slowly

declines following longer stimulation (176). Coactivators, such as CBP and SRC-2, are also recruited to all three AREs upon androgen induction (174). However, corepressors, such as NCoR and SMRT, are recruited to the ARE-1 and ARE-II, but not to the ARE-III, and only in the presence of the antiandrogen bicalutamide, indicating that antiandrogen bound AR is actively engaged in gene repression by recruiting histone deacetylase activity (174). Neither corepressors nor coactivators were recruited to the AREs in the absence of ligands (174).

As mentioned in section (1.3.5), amplification of AR is one of the mechanisms, which leads progression of prostate cancer to the castration-resistant state. This has been shown by *in situ* hybridization and KI67 labelling in one third of prostate cancer patients who had undergone androgen ablation therapy (177). AR amplification enhances AR at the mRNA and protein levels and therefore could augment its transcriptional activity (178). This is probably an adaptive response to castration levels of androgens in the circulation.

1.5.3 Post-translational modifications of the androgen receptor

Post-translational modifications (PTMs), as a result of intra-cellular signalling events, also influence AR activity. They can alter AR stability, subcellular localization, protein-protein interactions and transcriptional activity (179). The three known modifications for AR include phosphorylation, acetylation and ubiquitination (180-183). AR is phosphorylated both in its active and inactive state. However, activation by its cognate ligand results in a general increase in AR phosphorylation, which suggests activation is accompanied by phosphorylation on novel sites, including six serine residues of AR (Ser16, 81, 256, 308, 424, and 650) (184). Phosphorylation of AR by protein kinases, such as Akt and MAPK is also involved in enhancing AR activity (185-188). Akt, a Ser/Thr kinase, phosphorylates AR on Ser213 and Ser791, and

enhances AR transactivation in high passage LNCaP cells, following stimulation with insulin growth factor 1 (IGF-1) (186). MAPK (Erk-2 [1]) phosphorylates AR at Ser514 and enhances the interactions of AR coactivators and therefore increases AR transcriptional activity (185,188). Phosphorylation of AR with expanded polyglutamine tracts also regulates receptor viability and activity. Phosphorylation at Ser514 only occurs when the poly-Q tract far exceeds normal length (more than 112Q), which may target the receptor for caspase-3 cleavage and degradation by the ubiquitin-proteosome pathway (189). TAF1, a novel AR coactivator, possesses a bipartite serine kinase activity that could potentially phosphorylate AR.

Acetylation of lysine residues in the hinge region of AR (630, 632 and 633) is another PTM that has a major impact on AR stability and activity. These lysine residues are located adjacent to the bipartite nuclear localizing signal (NLS) and are acetylated in a ligand-dependent manner by coregulator proteins, such as p/CAF, p300 and TAT-interactive protein (Tip60) (180,190,191). Mutational analysis has shown that impaired acetylation at these sites inhibits nuclear translocation of AR in response to ligand and reduces the AR transactivation response (142). Acetylation of AR at the same lysine residues also enhances transcriptional coactivator protein interactions, which ultimately results in enhanced transactivation of AR-regulated genes (192). We also showed that the HAT domain of TAF1 binds to all three domains of AR but most strongly to the N-terminus. The detail of this interaction is discussed in Chapter 4.

The third PTM that is important for regulation of AR activity and relevant to this study is ubiquitination of AR. Ubiquitination is a reversible PTM that mediates the covalent conjugation of ubiquitin to protein substrates. Ubiquitin is a highly conserved protein of seventy-six amino acids that is expressed in all eukaryotic cells. It contains a di-glycine motif at the C-terminal end,

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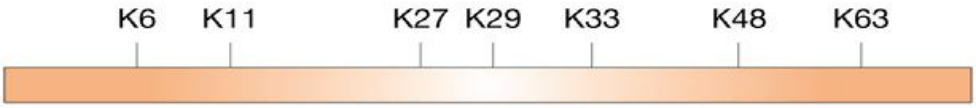
[1]- Extracellular signal-regulated kinase 2

A



B

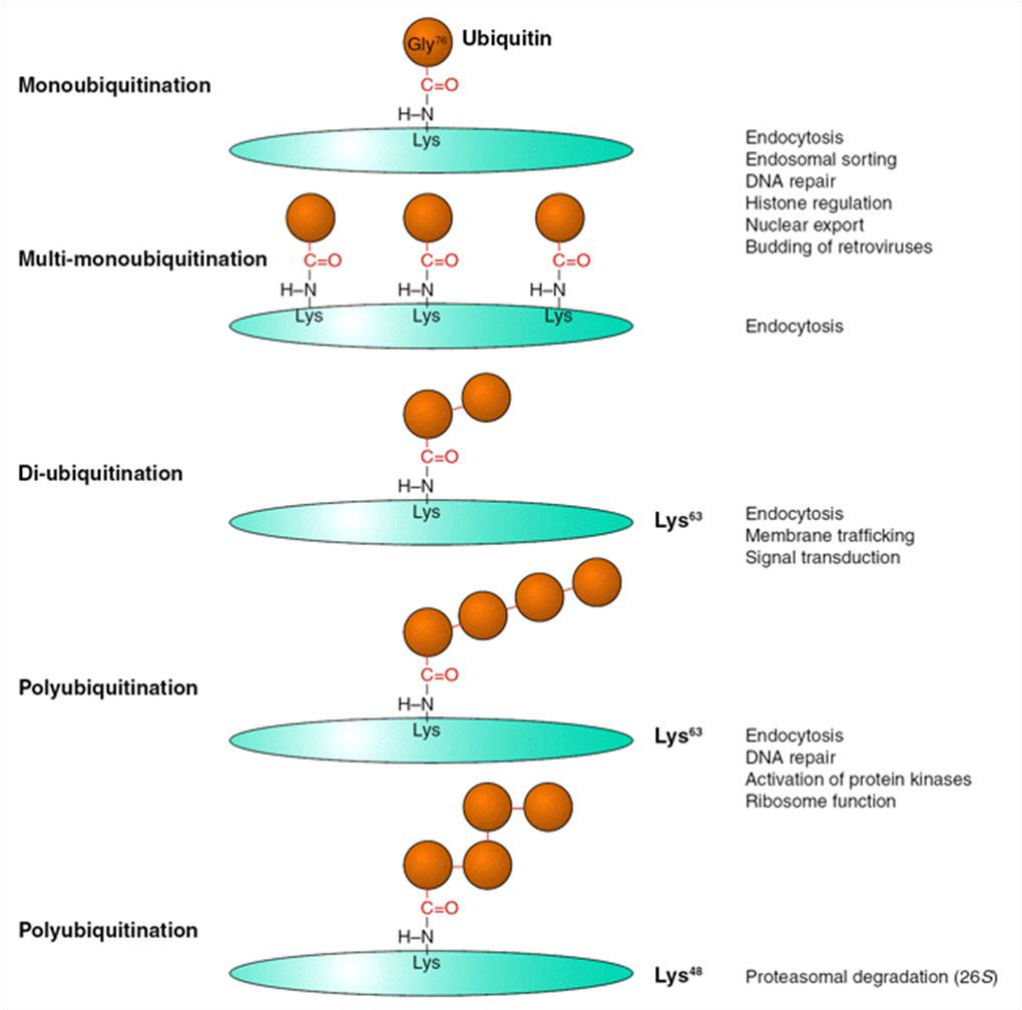


Figure 1.14 – Type of ubiquitin conjugation. (A) Schematic diagram of ubiquitin protein and its seven Lysines (K), which can be involved in chain ubiquitination. **(B)** Schematic representation of the different ubiquitin modifications with their functional roles. (From (193) © 2007, with permission from American Society for Pharmacology and Experimental Therapeutics)

in which the last residue, Gly76, can be conjugated to the ε-amino group of a lysine residue in a target protein (Fig. 1.14). The mechanism of ubiquitination involves the sequential action of several enzymes. In the initial step, the E1 ubiquitin-activating enzyme forms a thioester bond between its catalytic cysteine and the carboxyl group of Gly76 of ubiquitin in an ATP-dependent manner. The ubiquitin molecule is then transferred to an E2 ubiquitin-conjugating enzyme, which also forms a thioester bond between its cysteine and ubiquitin. Finally, ubiquitin is transferred to a lysine residue of the substrate with the help of an E3 ubiquitin ligase. There are only a small number of E1 enzymes, fewer than sixty E2 enzymes and more than 400 E3 ligases in the human genome (194). Attachment of a single ubiquitin moiety to a single lysine on a substrate results in mono-ubiquitination. Mono-ubiquitination of several lysine residues of a protein results in multi-mono-ubiquitination (Figure 1.14B). Ubiquitin itself contains seven lysine residues, all capable of conjugating ubiquitin (Figure 1.14A). Additional ubiquitin molecules can be attached to the lysine residues in ubiquitin itself, resulting in the formation of di-ubiquitin and poly-ubiquitin chains on a single lysine of the substrate. Ubiquitination can affect localization, activity, structure, interaction partners of proteins, or signalling for proteasome degradation (195,196). For instance, poly-ubiquitination at Lys48 serves as the recognition signal by the proteasome and targets proteins for the proteasomal degradation (196). In contrast, Lys63-linked ubiquitin chains do not target proteins to proteasome, but play important roles in interactions with protein machineries involved in endocytic trafficking, inflammatory response, protein translation, and DNA repair (196).

AR is also a substrate for mono- and poly-ubiquitination. Poly-ubiquitination of AR represses, while AR mono-ubiquitination enhances its transcriptional activity. The putative *PEST* [1] sequence located in the hinge region of AR is probably involved in ubiquitination-related AR

[1-] P, proline; E, glutamic acid; S, serine; T, threonine

degradation (197). The E3 ligases, *Mdm2*[1] and *NEDD4*[2] promote AR polyubiquitination (198,199), while heat shock proteins 90 and 27 protect AR from degradation (200-202). *TSG101*[3] also regulates ubiquitination of AR by inducing AR monoubiquitination, resulting in enhanced AR transactivation (203). In addition, the *UBCH7*[4], which is an E2 conjugase, coactivates AR in a ligand-dependent manner (204). Hereby, we have shown that TAF1 can enhance AR transcriptional activity through its ubiquitin activating/conjugating domain (E1/E2) and is able to poly-ubiquitinate AR (discussed in Chapter 4).

1.5.4 Coregulators of androgen receptor in the prostate

As discussed earlier, the transcriptional activity of AR is modulated by coregulators that have a significant influence on a number of functional properties of AR, including the ligand specificity as well as the DNA binding capacity. When coactivator levels increase, AR function most likely will change as a consequence. For example, an environment enriched for coactivators could make AR more sensitive or responsive to low levels of agonist (205), or allow promiscuous activation of AR by abundant yet low-affinity androgenic ligands, such as the adrenal androgens androstenedione and DHEA (206). At least 169 AR-coregulators have been identified (105), most of them were isolated through yeast-two-hybrid screens with discrete AR receptor domains as bait. AR-coactivators are broadly divided into two main categories – classical or type I and non-classical or type II coactivators. The classical coactivators can be subclassified based on their functional characteristics: (i) histone modifiers (e.g., CBP/p300, CARM1/PRMT5), (ii) coordinators of transcription (e.g., TRAP/DRIP), (iii) DNA structural

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[1]-Murine double minute 2
[2]-Neuronal precursor cell-expressed developmentally downregulated 4
[3]-Tumour susceptibility gene product 101
[4]-Human ubiquitin-conjugating enzyme 7

modifiers (e.g., SWI/SNF/BRG1). Type II coactivators are chaperone proteins that can coordinate AR stabilization or nuclear translocation mainly through protein-protein interaction or PTM. Table 1.3 shows a summarized list of AR-coactivators, their interaction domains and known function.

Accumulating data indicates that androgens control over approximately 30% of coregulator genes in prostate cancer cells (104,207-209). Hence, an aberrant regulation or expression of AR coregulators may contribute to androgen and AR-related diseases including prostate cancer (103,210,211). A precise understanding of the roles and mechanisms of individual coregulators may provide valuable information in designing rational therapy against prostate cancer. In this thesis, the up-dated information regarding AR coregulators and their potential roles in prostate cancer progression is discussed.

1.5.4.1 Type I AR-coactivators and their role in prostate cancer

The first identified and most extensively understood of AR coregulators are the p160 coactivators. This small family consists of three 160-kDa proteins, namely steroid receptor coactivator 1 (SRC-1), transcriptional intermediary factor 2 (TIF2) and glucocorticoid receptor interacting protein 1 (SRC-2), and amplified in breast cancer 1 (AIB1/SRC3) (165,168,212,213). The p160 coactivators have been shown to interact with TAU-5 of the AR NTD, an interaction that is influenced by the TAU-1 and AF-2 surface (214,215).Their recruitment directly increases AR transactivation through intrinsic histone acetyltransferase activity (169), and indirectly by acting as platforms for the recruitment of secondary coactivators possessing chromatin remodelling and protein acetyltransferase capabilities, such as CBP/p300 (216,217). CBP/p300 has been shown to contribute to transcriptional activation by remodelling chromatin and

Coactivator	AR-interacting Domain(s)	Function	References
Type I/Classical Coactivators			
i. Histone Modifying Proteins			
SRC-1/NCoA-1	All domains	Unlike other nuclear receptors which interact with SRC-1 through their LBD, AR interacts through its NH2-terminal and DBDs. Enhances AR NH2/COOH-terminus interactions. Interacts with CBP/p300. General nuclear receptor coactivator. Possesses weak acetyltransferase activity.	(122,211,218)
SRC-2/GRIP-1/TIF-2	NTD, DBD	General nuclear receptor coactivator. Mutations of AR that interrupt NH2/COOH domain interactions also disrupt AR interactions with TIF2.	(105,125,219,220)
SRC-3/RAC-3		Also enhances transcription by TR, PR, and RAR. Interacts with CBP/p300. Possesses acetyltransferase activity.	(125,176,221)
p300/CBP	NTD, DBD	Facilitates AR NH2/COOH-terminus interaction. Possesses acetyltransferase activity. Interacts with members of the SRC family. Coactivates multiple transcription factors.	(155,176,222)
p/CAF	NTD, DBD	p/CAF is part of a large multiprotein complex, and the HAT activity of the p/CAF complex is significantly higher toward nucleosomal histones than p/CAF alone. p/CAF interacts with SRC-1, SRC-3, and CBP proteins.	(155,180,192,222)
Tip60	Hinge-LBD	Member of the MYST/SAS family of histone acetyltransferases. Acetylates AR; also coactivates PR and ER.	(190,223,224)
CARM1/PRMT5	Not defined	Catalyzes methylation of histone H3 at Arg-17; directs methylation of histone H4 at Arg-3.	(225-228)
NSD1/ARA267	NTD, LBD	histone methyltransferase activity with a specificity for H3-K36 and H4-K20.	
LSD1	All domains	All three assemble on chromatin, resulting in demethylation of mono-, di-, and trimethyl H3K9 and stimulation of AR-dependent transcription.	(229,230)
JHDM2A	All domains		(231)
JMJD2C	Not defined		(230)
ii. Coordinators of Transcription			
MEDI/TRAP220	LBD	Links AR to the core promoter and the general transcriptional machinery.	(215,232,233)
TFIIF	NTD	Associates with RNA pol II and prevents RNA poly II from nonspecific interaction to DNA; Also regulates transcriptional elongation.	(234)
TFIIH	NTD	Unwinds the DNA template around the transcription initiation site and regulates the early elongation/promoter-clearance steps.	(235)
RNA Pol II subunit	LBD		(236)
iii. DNA Structural Modifiers			
BAF57	Full-length AR	An accessory component of SWI/SNF chromatin remodeling complex	(237)
ARIP4	AR zinc-finger		(238,239)
SRCAP	Not identified	functions as a coactivator for CREB-mediated transcription	(240)

Coactivator (continued)	AR-interacting Domain(s)	Function	References
Type II/Non-Classical Coactivators			
i) Molecular Chaperones and Cochaperones			
Hsp90	LBD	Translocates the AR to the nucleus	(241,242)
Hsp70	LBD	Maintains AR in a high-affinity ligand-binding state; Recruited with the Bag-1L and AR to promoter regions of AR target genes	(241-243)
Hsp40	LBD	Necessary for hormone binding to the AR; Mutations in Hsp40 result in a reduction of AR-Hsp70 complex formation and defects in AR folding	(242,244)
Cdc37	LBD	A molecular chaperone associated with the folding of protein kinases; An Hsp90-associated protein involved in AR trafficking.	(105,245)
ii) AR Stabilization			
Bag-1L	NTD, LBD	Functions as both an E3 ubiquitin ligase and a cochaperone;	(153,243, 246)
ARA70	LBD	Stabilize the ligand-bound receptor; facilitates nuclear translocation.	(247,248)
DDC	All domains	An enzyme in dopamine and serotonin production pathway; Binds to AR-LBD and enhances affinity and capacity of AR for androgen; Activates AR through its enzymatic activity	(249)
Nuclear Translocation			
Caveolin-1	NTD, LBD	Component of caveolae membranes; involved in many signal transduction pathways; increases AR transactivation and nuclear localization of phosphorylated AR	(250,251)
Filamin	Hinge domain	Dynamic actors in the cytoskeleton remodelling	(252,253)
Gelsolin	DBD, LBD		(253,254)
Ran/ARA24	NTD	Responsible for the nuclear export of importin proteins; enhancing the AR N-C interaction in the nucleus	(148,255, 256)
Supervillin	NTD, DBD	Slows cell spreading (migration) by facilitating myosin II activation at the cell periphery.	(241,257, 258)
iii) Unknown mechanism			
ARA55	LBD	Associated with focal adhesion complexes; functions as an AR coactivator in stromal cells and is necessary for effective androgen induction of the stromal paracrine factor.	(259-261)
ARA160	NTD	Binds to AR-NTD and can enhance AR transactivation cooperatively with ARA70 coactivator in prostate cancer cells.	
ART-27	NTD	Binds to the NTD of AR and enhances transcription in prostate cancer cells	(262,263)
Cdc25	Full-length AR	Is a cdk-activating phosphatase that activates cyclin/cdk complexes by removing inhibitory Thr-14 and Tyr-15 phosphates on cdk which, in turn, mediate the cell cycle progression.	(264)
GAK	All domains	A member of actin regulating kinase family; Involves in clathrin coated vesicle endocytosis; Promotes tumour intake and progression	(265-267)

Table 1.3 – Type I and II coactivators of AR. An updated list of AR coactivators, known to be involved in prostate cancer progression. Herein, they are categorized based on their function.

recruiting the basal transcription factors, TFIIB and TBP (165,268). Nucleosome remodelling complexes such as SWI/SNF are subsequently recruited followed by a multisubunit Mediator complex that serves to bridge DNA-bound transcription factors with the general transcriptional machinery, particularly RNA polymerase II (216,232). In addition, the AR may directly interact with members of the transcription machinery, including TFIIF and TFIIH, which are involved in promoter clearance and transcription elongation (234,235).

Immunohistochemical evaluation of the three p160 coactivators in human prostate cancer samples demonstrated overexpression of SRC-1 in approximately half of non-treated prostate cancers, and high levels of both SRC-1 and SRC-2 in the majority of castration-resistant tumours (206). Additionally, a correlation between increased levels of SRC-3 and prostate tumour grade and stage has been identified in prostate cancer patients (269,270). In tissue prostatectomy samples, p300 levels correlated with *in vivo* proliferation, extraprostatic extension, and seminal vesicle involvement at prostatectomy, as well as prostate cancer progression after surgery (271). Levels of CBP were highly expressed in advanced prostate cancer and, specifically, in tissues from patients who were resistant to NHT (272). These two observations are of particular interest given a report that p300 and CBP can acetylate AR as discussed in section 1.5.3.

The Tat-interactive protein 60 kDa (Tip60) is another type I coactivator that has also been implicated in prostate cancer progression and the development of a castration-resistant state following long-term NHT. Originally identified as a coactivator for the human immunodeficiency virus type I-encoded TAT protein, this ligand-dependent coactivator acts on the androgen, progesterone and estrogen receptors (190). Tip60 has been found concentrated in the nucleus in biopsies of hormone-refractory disease and increased levels and nuclear

accumulation of Tip60 have been observed following androgen withdrawal in CWR22 xenografts[1] and LNCaP cells. While levels of Tip60 decrease with androgen treatment and localize to the cytoplasm (224), a recent independent study indicates that Tip60 is downregulated in metastatic cancer cells at both RNA and protein levels (273), which may represent a random tumour-specific change, or indicate different roles of this coregulator in AR transcriptional activity at different stages of prostate cancer progression.

Thyroid hormone receptor associated protein Mediator complex (MEDI/TRAP220) is a unique AR-coactivator since its presence is indispensable in AR-mediated transcription of the PSA gene (176). It has been shown that the knockdown of this Mediator subunit in prostate cancer cells markedly inhibits transcription from androgen-responsive AR target genes, decreases androgen-dependent and -independent cellular proliferation, and increases apoptosis. Importantly, MED1/TRAP220 is overexpressed in both AR-positive and -negative prostate cancer cells lines, as well as in clinically localized prostate cancers, thus suggesting that MED1/TRAP220 hyperactivity might promote prostate oncogenesis (233).

1.5.4.2 Type II AR-coactivators and their role in prostate cancer

1.5.4.2.1 AR-chaperone complexes

Although chaperone proteins were initially identified by their accumulation in response to cellular stress, they are able to recognize and bind to hydrophobic regions on unfolded or partially folded proteins preventing their irreversible aggregation and promoting cycles of chaperone-mediated folding instead (274). In the case of the AR, chaperones configure the ligand-binding domain into a relatively stable, partially unfolded, inactive intermediate with a

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[1]- An *Ex vivo* human tumour model for prostate cancer gene therapy

high-affinity for DHT (275,276). Hormone binding to the complex permits the folding of the AR into an active conformation. Binding of ligand, followed by the dissociation of the receptor-chaperone complex and activation of the receptor is viewed as the general regulatory mechanism of AR signalling (276). However, molecular chaperones remain important players in the events downstream of receptor activation (242).

An increase in specific AR-chaperone proteins has been documented for prostate cancer. For instance, heat shock protein (Hsp) 27 is highly expressed in hormone-refractory tumours and its expression is correlated with Gleason score (277). In addition, the expression level of Hsp27 in diagnostic biopsy samples is directly correlated with preoperative serum PSA levels. Accordingly, the biochemical recurrence and recurrence-free survival in patients with strong HSP27 staining is shorter than in those with weak expression (277,278).

The cell division cycle 37 homolog (Cdc37) is another chaperone protein that has shown increased expression in carcinomas. It functions down-stream of hormone-binding as an Hsp90-associated protein involved in AR trafficking. Mutant forms of Cdc37 induce defects in AR transactivation while leaving AR protein levels unaltered (245). Overexpression of Cdc37 in animal models has been associated with abnormalities such as prostatic epithelial cell hyperplasia and dysplasia (279). In addition, targeting Cdc37 inhibits multiple signalling pathways and induces growth arrest in prostate cancer cells (280).

1.5.4.2.2 Coactivators that stabilize AR

The Bag-1 isoform (Bag-1L) is an AR-cochaperone that binds directly to the TAU 5 domain in the AR. Its function as a coactivator relies on its association with Hsp70 to interact with the AR, and loss of this interaction domain markedly suppresses its ability to stimulate AR-mediated

transactivation. Moreover, Bag-1L as well as Hsp70 are recruited with the AR to promoter regions of AR target genes (243). Importantly, Bag-1L harbors a ubiquitin-like domain that facilitates association of Bag-1L with the proteasome, enabling Bag-1L to function as a coupling factor between the chaperone and proteolytic complex (153). The basal cells of a benign prostate normally express Bag-1L, but the epithelial cells do not. However, in prostate cancer, the opposite is observed; where Bag-1L is expressed in malignant epithelial cells and is no longer expressed in the basal cells. The distribution of Bag-1L is similar to Hsp70, which may act synergistically to increase AR transcriptional activity (243).

The androgen receptor activator 70 (ARA70/ELE1) interacts with the AR-LBD via an FXXLF motif and with the N-terminal AF-1 region of AR in an FXXLF-independent manner (281,282). ARA70 can enhance AR ligand-dependent transcriptional activation, possibly by interacting with TFIIB (283). This protein exists as two isoforms that are generated by alternative splicing: full-length ARA70α (70 kDa) and an internally spliced variant ARA70β (35 kDa). Although either isoform enhances transcription activation function of the AR, stimulatory effects on proliferation and invasion were seen only in cells in which the beta form was overexpressed (284). Accordingly, in contrast of ARA70α, the expression of ARA70β is increased in human prostate cancer samples, suggesting a clinically relevant role for the latter protein.

DDC, a neuroendocrine marker in some human cancers (285-287), is an AR-coactivator protein and its role in prostate cancer progression has been recently discovered by our laboratory (14). DDC directly interacts with all domains of AR, but shows affinity for the AR-LBD and has been shown to enhance receptor transcriptional activity *in vitro* and *in vivo* (149). It facilitates

AR ligand binding and requires its enzymatic activity for coactivation function (249). DDC has been found to be co-expressed with AR in neuroendocrine-phenotype prostate cancer and its expression is increased in castration-resistant tumours and with hormone therapy (14).

Gelsolin is an actin-binding protein that also binds the AR-LBD and DBD during nuclear translocation and enhances AR transactivation in a ligand-dependent manner (254). Immunohistochemical analysis of tissues representing prostate adenocarcinoma, prostatic intraepithelial neoplasia (PIN), and benign prostatic hyperplasia (BPH) has demonstrated decreased expression of Gelsolin when compared with levels in non-proliferative tissues (288). However, it has been found by others that its expression level was increased in LNCaP cells, in LNCaP xenografts, and in human prostate tumours following androgen depletion (254).

ARA24/Ran interacts with the TAU-1 region of the AR-NTD and such binding decreases with increasing poly-Q length (148). It has been shown that ARA24 enhances AR transactivation by enhancing the AR N-C interaction in the nucleus (256). The involvement of ARA24 in prostate cancer progression is uncertain. The ARA24 RNA expression has been found elevated in the early stages of primary prostate cancer in one study (289) but in another study no significant differences have been reported between the BPH and different stages of cancers (290).

1.5.4.2.3 AR-Coactivators with unknown mechanisms of action

ARA55/Hic-5 is a member of the group 3 subfamily of LIM domain proteins[1], and is preferentially expressed in prostate stromal cells (289). ARA55 binds to the AR in a ligand-dependent manner through its C-terminal LIM domains and results in increased AR activity

[1]-LIM domain proteins are protein structural domain, consisting of two zinc finger binding domains. They are named after the initial discovery in the proteins LinII, Isl-I, and Mec-3.

and altered specificity of receptor binding to alternate ligands (259,260). Expression of ARA55 mRNA is found to be lower in non-malignant prostate tissue compared with adjacent malignant tissue (291) and lower in castration-resistant tumours compared with untreated tumours or BPH. Increased expression of ARA55 has been associated with both shorter recurrence-free survival and overall survival in castration-resistant prostate cancer patients (261,292).

AR trapped clone-27 (ART-27) is another AR coactivator that binds to NTD of AR. While ART-27 was shown to enhance androgen-mediated transcription of PSA, enforced expression in LNCaP cells inhibited proliferation in response to androgen (263). Immunohistochemical studies indicate that ART-27 protein is expressed in normal differentiated prostate epithelial cells and benign and premalignant (HG-PIN) epithelium. Levels of ART-27 drastically decreased in prostate cancer specimens compared with non-malignant tissue, possibly reflecting the state of glandular differentiation (263). In fact, the functions of ART-27 are consistent with tumour suppressor/pro-differentiation genes and may explain why the expression of ART-27 decreases dramatically during prostate cancer progression (103).

The tyrosine phosphatase proteins, Cdc25A and Cdc25B, are also found to be AR-coregulators that interact with the AR and modulates AR-dependent transcription (264,293). They primarily function as key regulators in cell cycle progression and DNA damage response in eukaryotes. Cdc25A suppresses while Cdc25B enhances AR transcriptional activity that is independent of its cell cycle function (264). Overexpression of Cdc25A has been observed in prostate cancer cell lines and human prostate cancer with expression correlated with both the Gleason score and metastatic potential of prostate cancer (293). Cdc25B has also been shown to

be overexpressed in cancer cell lines and its expression level correlated with histological prostate tumour grade and frequently with more poorly differentiated tumours (264,294,295).

Cyclin G-associated kinase (GAK) is another protein identified by our laboratory that directly interacts with the AR, most strongly in the presence of hormone, and its expression level can increase with prolonged NHT and in LNCaP xenografts following castration (266,296). However, the outcome of GAK/AR interaction and its effect on AR transcriptional activity is still uncertain (265,266,296).

1.5.5 Ligand reduced or independent activation of AR

AR is among the transcription factors whose activity is influenced by signal transduction cascades and disruption of the normal interaction between signal transduction and AR transactivation may contribute to the progression of prostate cancer. Here, we focus on growth factors or cytokines that influence prostate cancer through modulation of AR activity.

IGF-1 and epidermal growth factors activate AR independent of androgens. An increase in the proportion of Her2[1]-positive prostate tumours in patients receiving combined androgen ablation therapy before prostatectomy compared with patients treated by prostatectomy alone has been observed (297). A further increase in Her2-positive cases was seen in patients with metastatic, hormone refractory prostate cancer (297). Apparently, Her2 enhances AR activity through the PI3/Akt signal transduction and at least partly through the MAPK pathway (188,298). In addition to Her2, IGFs have been implicated in the pathogenesis, cell proliferation, and cell survival in many neoplasms including prostate cancer (299-301). IGFs exert their activity predominantly via the type I IGF receptor (IGF-IR), which is present on prostate cancer cells. Among IGFs, IGF-1

[1]-Human Epidermal growth factor Receptor 2

has been shown to activate AR via MAPK and/or PI3/Akt pathways. Studies have suggested that an elevation of serum IGF-I is associated with a 2- to 4-fold increase in prostate cancer risk. This epidemiologic data further suggests the role of IGF-1 in prostate cancer development potentially through AR modification.

TGF-β is another factor that has a role in prostate cancer progression and is abundantly stored in bone extracellular matrix. The clinical observation that an elevation of serum TGF-β is associated with elevated serum PSA (302,303) suggests that interaction between the TGF-β pathway and AR transcriptional activity may occur. TGF-β family members regulate proliferation, growth arrest, differentiation, and apoptosis of prostatic stromal and epithelial cells, as well as the formation of osteoblastic metastases. Cancer cells become refractory to the growth inhibitory activity of TGF-β due to the loss or mutation of transmembrane receptors or intracellular TGF-β signalling effectors during tumour initiation (304). During prostate tumour progression to metastatic disease, TGF-β ligand overexpression results in pro-oncogenic rather than growth suppressive effect. The mediators of TGF-β signalling are the Smad proteins[1] that function as phosphorylation-regulated transcription factors. It has been shown that the transfection of Smad3 enhances AR transactivation in PC3 and LNCaP human prostate cancer cell lines (305).

The cytokine interleukin-6 (IL-6) has also been shown to increase AR activity in the absence of androgens and this occurs through the MAPK and STAT3[2] signalling pathways. Elevated serum levels of IL-6 have been found in patients with metastatic prostate cancer, particularly those with hormone refractory disease (306-308), suggesting that IL-6 may play a role in the progression of prostate cancer.

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[1]-Signalling Mothers Against Decapentaplegic; set of evolutionary conserved proteins initially discovered in drosophila

[2]-Signal transducer and activator of transcription 3

Interleukin-4 (IL-4) also enhances AR activation in the absence of androgen, probably through the Akt pathway, and stimulates growth of androgen-sensitive prostate cancer cells (309). Recently it has been found that IL-4 activates AR through enhanced expression of CBP/p300 and its histone acetyltransferase activity (310). The elevated serum levels of IL-4 in patients with castration-resistant prostate cancer further suggest that this cytokine may also play an important role in disease progression. Taken together, the activation of AR by growth factor or cytokine signalling in a ligand-independent or ligand-reduced manner highlights the role of these factors in progression of prostate cancer to castration-resistant state.

Another important pathway that is crucial in development and progression of prostate cancer and functions through AR is Wnt signalling. Both human cancers and mouse models have confirmed that mutations or altered expression of components of this pathway are associated with prostate tumours (311). Furthermore, it has been shown that overexpression of AR can activate the Wnt-beta catenin pathway (99). Upon Wnt signalling, AR and beta catenin (a coactivator of AR) are recruited to the promoter and enhancer regions of the PSA gene. Interestingly, physiological levels of androgens inhibit these effects. In this way, AR overexpression has been shown to promote prostate cell proliferation in a ligand-independent manner (99).

1.6 Scope of dissertation

1.6.1 Hypothesis

An accumulating body of evidence suggests that AR is the key player in the development and progression of prostate cancer to castration-resistant disease. Inappropriate activation of AR in

advanced disease is likely to give cancer cells a growth advantage in androgen-depleted conditions which may, in part, be due to changes in accessory proteins or factors that regulate AR transactivation. For this reason, the main hypothesis of this thesis is as follows: **Inappropriate activation of the AR with its coactivators may associate with prostate cancer progression.**

1.6.2 Specific experimental goals

We have employed two approaches to test this hypothesis:

1. Investigation of factors that can alter AR activity
2. Examination of aberrant expression of AR-coregulators that can enhance AR activity

1. Investigation of factors that can alter AR activity - As discussed in section 1.3, the bone is the most common site of metastasis and AR expression level has often increased in bone metastatic samples of prostate cancer patients (63,89). Bone tissue continually undergoes a process of remodelling and in the bone microenvironment, prostate carcinomas are exposed to a novel array of factors derived from bone marrow, osteoblasts, or from bone matrix products. The bone extracellular matrix is rich in a variety of growth factors, including morphogenetic proteins, heparin-binding fibroblast growth factor, platelet-derived growth factor, insulin-like growth factors, TGF-β, and various cytokines (312). Because several of these factors have been shown to have an impact on either chemotaxis or growth of prostate cancer cells, we were interested in determining their effect on AR activity. Hence, we hypothesized that inappropriate AR-responses to these non-androgenic factors in the bone contribute to the progression of prostate cancer. Accordingly, to test this hypothesis, the following specific aims were planned: i) To

create an *in vitro* bone model by which AR transcriptional activity can be assessed (Chapter 2). This was established by using an osteoblast-like cell line referred as SaOS-2 cells, which is derived from human osteosarcoma. These cells are capable of inducing bone formation and as such are a model for osteoblast behaviour. ii) To generate a stably infected prostate cancer cell line and appropriate control that express fluorescence (Enhanced Green Fluorescent Protein (EGFP)) upon androgen stimulation (Chapter 2). Using these cell lines, AR activity and growth of prostate cancer cells can be monitored for different treatment conditions in a high throughput and non-invasive manner. Although we have shown that the conditioned media of terminally differentiated SaOS-2 cells is able to promote the growth of prostate cancer cells, we were unable to detect any influence on AR activity upon conditioned media treatment. Nevertheless, the establishment of this novel bioassay led us to screen hundreds of compounds that are selected via *in silico* drug discovery for anti-AR activity (Chapter 3).

2. **Examination of aberrant expression of AR-coregulators that can enhance AR activity –** As described in section 1.4, the AR has the most variable N-terminus domain among the steroid receptor family. However, this region has not been routinely used for identifying AR interacting proteins. This is because the AR-NTD has an intrinsic transactivation activity that makes it unsuitable for conventional yeast two hybrid assays. To circumvent this problem, our laboratory has used a reverse yeast-two hybrid system (repressed transactivator; RTA) with AR-NTD as bait to identify novel AR-interacting proteins. One of the proteins that has been isolated through this system is TATA binding protein Associated Factor 1 (TAF1) (Chapter 4). The research described in this part of the thesis has three specific experimental goals: i) to determine the relevance of TAF1 expression level in prostate cancer progression; ii) to confirm TAF1/AR

interaction through other protein interaction assays; iii) to assess the role of TAF1 in AR-mediated transactivation and the molecular mechanism by which TAF1 modify AR activity.

The first aim of this project was completed by assessing the expression profile of TAF1 during disease progression in patients who had undergone varying lengths of NHT prior to radical prostatectomy or autopsy using our *in house* NHT tissue microarrays. The results suggested that TAF1 expression increases with prolonged hormone treatment and with progression to castration-resistant prostate cancer. To achieve the second objective of this study, *in vitro* and *in vivo* protein interaction experiments were performed. The results confirm that TAF1 directly interacts with N-terminus of AR (by GST pull-down assay), reveal that TAF1 and AR interact in the nucleus of prostate cancer cells (by co-immunoprecipitation), and suggest that TAF1 is associated with AR at the proximal promoter of the PSA upon treatment with hormone (by ChIP assay). To complete the third goal, the effects of overexpression and knock-down of TAF1 on AR transactivation were investigated. This revealed that in the presence of hormone, the expression level of TAF1 correlates with AR activity. The next set of experiments focused on post translational modification of AR by TAF1. In regard to three known enzymatic functions of TAF1, it was determined that TAF1 is able to unbiquitinate AR both *in vivo* and *in vitro*.

In summary, we developed non-invasive, cell-based screening assays to rapidly and biologically assess factors, drugs, or complex mixtures that modulate prostate cancer growth and affect AR activity. Using this system, we found a dozen novel compounds that inhibit AR activity probably via binding to a pocket of AR that is different from the pocket that conventional anti-androgen drugs bind. In addition, we found that TAF1 is a specific coactivator of AR that binds and differentially enhances AR transcriptional activity most likely through

ubiquitination of AR. Accordingly, an increase in TAF1 expression during NHT therapy could be a compensatory mechanism adapted by cancer cells to overcome lack of circulating androgens. The latter study provides a clearer understanding of AR-mediated transcription, as well as a better perception of the contribution of coactivators in progression of prostate cancer to castration-resistant disease.

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Chapter 2

Rapid, non-destructive, cell-based screening assays for agents that modulate growth, death, and androgen receptor activation in prostate cancer cells [1]

2.1 Introduction

The development and proliferation of prostate cancer in its early stages depends on the presence of androgens and androgen receptor (AR) (1). There is considerable evidence that even with androgen ablation, AR still plays an important role in progression of castration-resistant prostate cancer (1-4). The activation of AR at this stage may be due to the compensatory mechanisms of the multiplicity of endogenous factors such as co-regulators, cytokines or growth factors (5-11), or due to the effect of exogenous factors. For example, it has been reported that many chemicals present in the environment can mimic, antagonize or interfere with physiological actions of endogenous hormones such as androgens (12). These exogenous substances have been called endocrine disruptors and include environmental contaminants (13-16), designer androgens (17,18), and natural products (19,20). There is evidence that some of these endocrine disruptors can cause cancers in humans, including prostate cancer (21,22). With a continually growing list of endogenous and exogenous substances that can alter AR

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Dr. Rob Snoek (former research associate with Dr. Rennie) provided critical comments. Dr. Mira Ray (former PhD student with Dr. Rennie) came up with an innovative idea and wrote a grant proposal for this research. Dr. Leticia Gomez Rao from the department of Medicine at the University of Toronto collaborated with our lab to establish the *in vitro* bone model system.

transcriptional activity or prostate cancer cell growth, there is a need for a high throughput method to assess these activities.

Typically, AR transcription assays involve transient transfection of cell lines with one or more plasmids expressing AR and a reporter gene, followed by treatment with a variety of agents and subsequent measurement of reporter gene expression. However, transient transfection assays may not accurately reflect evenly distributed, steady state levels of receptor, as transfection efficiencies can vary greatly between cells and between replicate assays. Also, these assays usually require a large number of cells, costly transfection materials and several experimental manipulations. In addition, transient transfections have time-limited responsiveness since the transgene is usually lost within 72 hr. For cell growth assays, procedures for determining cell number at given time points usually are lethal to the cells and hence, to obtain growth curves, one needs to perform several parallel yet independent experiments.

To circumvent these problems, we have created a novel approach to screen for agents that modulate prostate cancer cell growth or AR activity using stably transformed prostate cancer cells expressing an Enhanced Green Fluorescence Protein (EGFP) reporter under a constitutive or AR-regulated promoter. These assays are relatively high throughput, versatile, and far less variable than transient transfection techniques. While others have also developed *in vitro* bioassays using stable cell lines to quantify AR activity (21,23,24), the advantage of our system is that one can monitor and screen multifactorial components such as conditioned media, growth factors, and potential therapeutic treatments over a time course in a rapid and non-destructive manner and thereby find the optimal treatment conditions with respect to impact on prostate cell growth and AR activation.

2.2 Material and methods

2.2.1 Cell lines, media and additives

LNCaP human prostate adenocarcinoma cells and 293T human embryo kidney cells were obtained from American Type culture collection (ATCC, Manasa, VA). PC3 human prostate cancer cells that stably express androgen receptor ($PC3(AR)_2$) were a kind gift from Dr. T.J. Brown, (University of Toronto, Ontario, Canada). LNCaP cells were grown in RPMI 1640 medium supplemented by 5% heat inactivated fetal bovine serum (FBS), and Penicillin-Streptomycin (P-S) 1% (Life Technologies, Inc., Gaithersburg, MD). $PC3(AR)_2$ and 293T cells were maintained in DMEM medium with 10% FBS and 1% P-S. Osteoblast-like SaOS-2 cells (ATCC) were cultured and induced to differentiate as previously described (25). Briefly, cells were initially cultured in Ham's F-12 medium (Life Technologies) containing 28 mM HEPES pH 7.4, 1.4 mM $CaCl_2$, 2 mM glutamine, 10% FBS, 1% P-S. This is further supplemented with 10 nM dexamethasone and 50 μg/ml ascorbic acid. At day 8, 10 nM β-glycerophosphate was added and this addition was repeated at every medium change until mineralization, at approximately day 15. Subsequently, the media was switched to 5% charcoal stripped serum (CSS) supplemented Ham's F-12 media as above except without ascorbic acid and dexamethasone, for another 24 hr. Conditioned media (CM) from this source was stored at -80°C prior to experimentation. SaOS-2 differentiation was validated by alkaline phosphatase and Von Kossa staining, as before (26).

2.2.2 Generation of stable cell lines expressing EGFP

A lentiviral approach was used to generate either stable, constitutive EGFP-expressing LNCaP cells (LN-CMV-EGFP cells) or $PC3(AR)_2$ cells (PC3-CMV-EGFP) as described

previously (27). Briefly, 1.5 x 10^6 293T cells were seeded onto 10 cm plates and co-transfected the following day by calcium phosphate precipitation method (Profection Mammalian Transfection Systems, Promega, Madison, WI) with 10 μg of lentiviral vector, Lv-CMV-EGFP; 10 μg of pCMVDR8.2, containing viral assembly sequences; and 5 μg of pMD.G, carrying the vesicular stomatitis virus envelope glycoprotein G (VSV-G). After 16 hr incubation, the medium was changed to 10%FBS-DMEM. The virus containing medium was collected after 48 hr, passed through a 0.45 mm filter to remove debris and then stored at -80˚C. For lentiviral infection, LNCaP or PC3$(AR)_2$ cells were seeded onto 10 cm plates. When cell confluence was ~ 60%, 5 ml of virus containing medium was added. After 16 hr, the media was switched to 5% FBS RPMI and, two days after infection, cells expressing the EGFP transgene were checked under a fluorescent microscope.

Similar procedures were used for generating stable, AR-regulated EGFP-expressing LNCaP cells (LN-ARR_2PB-EGFP) using an androgen responsive probasin-derived promoter (ARR_2PB) instead of a CMV promoter for the transfer plasmid (Lv-ARR_2PB-EGFP). The ARR_2PB promoter, a gift from Dr. R. Matusik (28), contains two copies of probasin androgen response region (PB ARR; -244 to -96) upstream of the minimal promoter and is the most potent form of PB promoter that has been shown to retain prostate-specific and hormone-regulated EGFP expression in LNCaP cells (27).

2.2.3 Selecting for homogeneous EGFP expressing cells

To obtain populations of cells that show relatively homogeneous fluorescence, LN-CMV-EGFP, PC3-CMV-EGFP and AR-activated LN-ARR_2PB-EGFP cells were sorted for high fluorescence levels using Fluorescence Activated Cell Sorting (FACS) at the Biomedical

Research Centre of the University of British Columbia (Vancouver, BC, Canada). Briefly, about 2 X 10^7 trypsinized cells were re-suspended in cold PBS (Phosphate Buffered Saline) supplemented with 0.02 mM EDTA and ranked based according to their EGFP expression. The highest fluorescence expressing cells (the top 50%) were collected and cultured for subsequent assay development.

2.2.4 Cell proliferation assays

LN-CMV-EGFP cells were seeded into 96-well black plates (Nalge Nunc) using 5% FBS, phenol red-free RPMI medium (Gibco) at different cell densities (5000-20,000 cells per well) and assessed non-destructively over time by measuring fluorescence (EGFP) using a Fluoroskan Ascent FL (Thermo-Labsystem, Helsinki, Finland) at excitation and emission of 485 and 518 nm, respectively every 24 hr and verified by fluorescent microscopy. Mean relative fluorescence unit (RFU) values were obtained from 8 replicates of each assay condition. For treatment with CM from SaOS-2 cells, LN-CMV-EGFP cells were re-suspended with 50 or 100% of CM or control (5% CSS SaOS-2 media) and seeded at 20,000 cells/well. Fluorescent cells were assessed over time using the Fluoroskan. Cell numbers were determined at the termination of each experiment (day 4) using the MTS cell proliferation assay (CellTiter 96® AQueous One Solution Reagent, Promega). In brief, 20 µl of the reagent was added to attached cells in each well of the 96-well plate containing 100 µl media and incubated for 2 hr at 37°C in 5% CO_2. Light absorbance of formazan was measured at 490 nm on a 96-well plate reader equipped with KC4 software (Biotek Instruments, INC., Winooski, VT).

2.2.5 Measurement of apoptotic cells

Mitoxantrone was purchased from Sigma Chemicals (St. Louis, MO). Stock solutions of mitoxantrone (1 mg/m1) were prepared with PBS and diluted to the required concentrations prior to each experiment. 100 μl of 5% FBS phenol red-free media RPMI containing 20,000 LN-CMV-EGFP cells were seeded into each well of a 96-well black plate. 24 hr later, cells were treated with different mitoxantrone concentrations ranging from 0 to 10 μM. Fluorescence was measured at time 0 and the cells were also monitored visually under a fluorescent microscope. After 48 hr, the media were gently transferred into a new multi well plate and 100 μl of fresh media was added to the original plate, followed by fluorescence measurements of both plates. At the termination of each experiment, a MTS cell number assay was performed.

2.2.6 Androgen receptor transactivation assays

LN-ARR$_2$PB-EGFP cells were grown in 5% CSS RPMI for 5 days to deplete cells of bio-available androgen and to reduce their EGFP expression to background levels. This was confirmed by fluorescent microscopy. Cells were then seeded at a density of 20,000 cells/well in 96-well black plates in 5% CSS phenol red-free RPMI, grown overnight, subsequently treated with different concentrations of the potent synthetic androgen, R1881 (methyltrienolone, Dupont, Boston, MA) and followed by fluorescent measurements every 24 hr. At day 3, experiments were terminated and MTS assays were performed. Using analogous protocols, 20 ng/ml interleukin-6 (IL-6) (Calbiochem-Novabiochem Corp., San Diego, CA), 25 μM forskolin (FSK) (Sigma), and 0.1-1 μM dichlorvos (AccuStandard, New Haven, CT), with or without R1881, were also tested in this system. For experiments with bicalutamide (Casodex, a gift from AstraZeneca), LN-ARR$_2$PB-EGFP cells were grown for 5 days in CSS, seeded at 20,000

cells/well and incubated with bicalutamide (0-50 μM) overnight. Following this, the cells were treated with 1 nM R1881 and then after 72 hr assessed for fluorescence and cell number as described above.

2.2.7 Statistical analysis

Statistical analyses were performed using Student's *t* tests with JMP IN software (version 4.0.2.-Academic) and values were given as the mean (± SD) of 3 independent experiments.

2.3 Results

2.3.1 EGFP expression directly correlates with the number of cells

LNCaP cells were infected by lentiviral vector Lv-CMV-EGFP to create the LN-CMV-EGFP cell line. In these cells, the constitutively active CMV promoter drives EGFP expression, which is visible under fluorescent microscopy and quantifiable by Fluoroskan fluorometry. Using this cell line, we sought to establish and to validate a fluorescence assay to estimate changes in prostate cancer cell numbers in response to various growth promoting or inhibiting agents. Due to differences in the relative multiplicity of infection of individual cells, variable expression of EGFP from low to high intensities was observed. To achieve a homogeneous population of LN-CMV-EGFP cells and therefore a robust growth assay system, these cells were sorted for high fluorescence expression by FACS. The sorted cells had a comparatively homogeneous pattern of EGFP expression as determined by a fluorescent microscope and remained stable expressers of EGFP even after more than 12 passages. Since the CMV promoter is constitutively active, higher EGFP expression levels is presumably due to a higher number of cells rather than any change in fluorescent intensity per cell. To test for a direct correlation between fluorescence and cell

number, fluorescent LN-CMV-EGFP cells and wild-type LNCaP cells were seeded at different densities in 96 well plates and the amount of fluorescence measured every 24 hr. Fig. 2.1A shows that there was an increase in relative fluorescence units (RFUs) as the seeding density of the LN-CMV-EGFP cells was increased. After an initial delay of 24 hr, the RFU values in each well containing LN-CMV-EGFP cells increased in a time-dependent manner, such that there was a linear increase ($R^2 > 0.90$) between 24-96 hr. To verify that the RFU increases indeed reflected increased cell numbers, MTS assays were performed at 96 hr. As expected, the number of both EGFP and non-EGFP expressing cells increased by day 4 in proportion to the number of cells initially seeded (Fig. 2.1B). To determine if the amount of EGFP fluorescence directly correlated with the number of cells, the corresponding cell numbers and RFU at day 1 and 4 were plotted. As shown in Fig.2.1C there is a linear correlation between cell number and RFU up to 30,000 cells/well ($R^2 = 0.99$).

PC3 human prostate cancer cells, which were infected by Lv-CMV-EGFP were also tested in a similar fashion. These cells were seeded at densities of 2000 to 8000 cells/well and fluorescence was measured every 24 hr as described above (Fig. 2.1D). Again, after an initial 24 hr delay, there was an apparent linear relationship between RFU levels and incubation time ($R^2 > 0.98$). After four days, cell numbers were determined by MTS assays and compared to RFU values, with a linear correlation between RFU and cell number estimates seen over the range of 2000 to 17,000 cells/well ($R^2 = 0.99$) (data not shown). Overall, the linear correlation between EGFP expression and cell numbers in both LNCaP and PC3 EGFP-expressing cells indicated that these cells could provide the basis for a direct assay to non-destructively measure changes in cell number over time in living prostate cancer cells in response to various growth modulators or inhibitors.

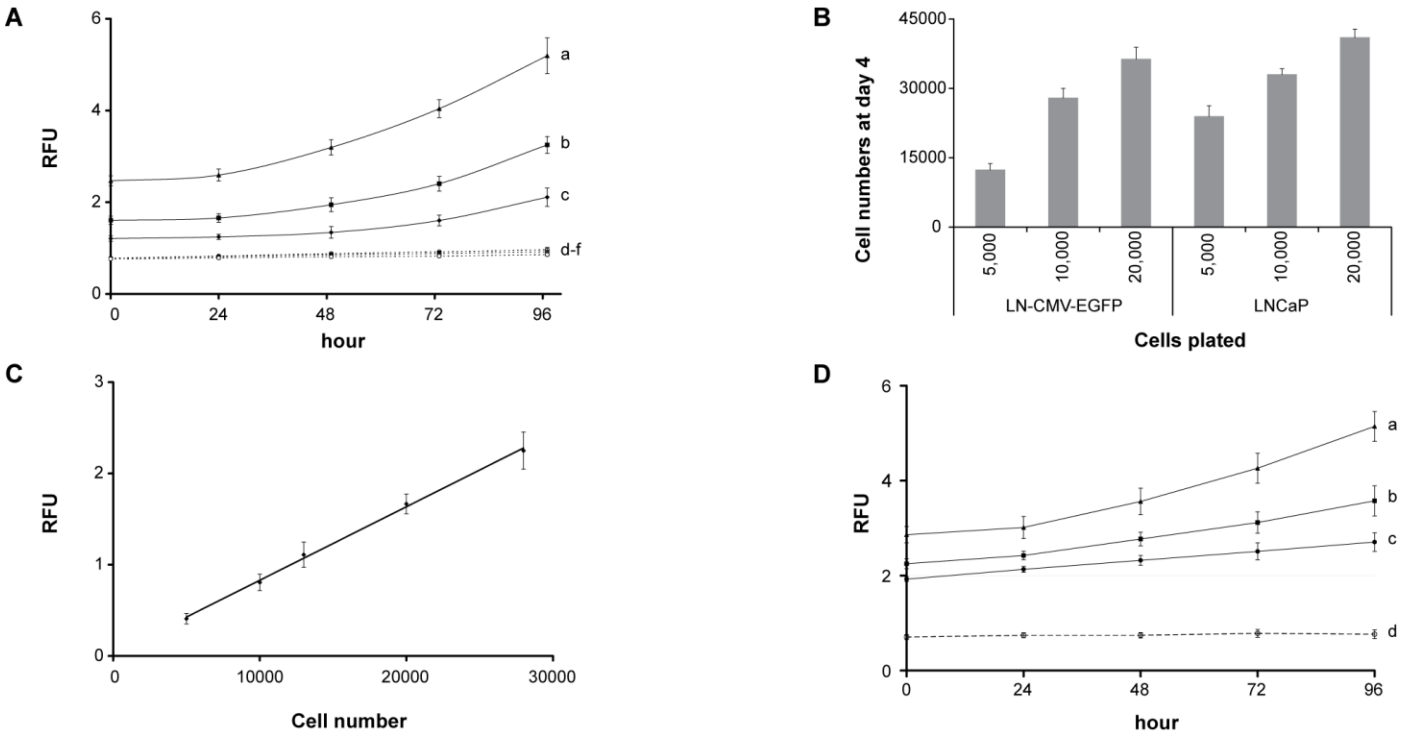

Figure 2.1: Correlation of EGFP fluorescence with cell number. A: LN-CMV-EGFP cells were seeded in 96 black well plates at different densities (**a**) 20,000, (**b**) 10,000, (**c**) 5,000 cells/well versus LNCaP cells at the corresponding numbers (**d-f**) using 5% FBS phenol red free RPMI. The EGFP was measured by fluorometry every 24 hr. B: Cell number assay using a MTS method at day 4 of both LN-CMV-EGFP and LNCaP cells based on seeded cell numbers. C: Plot of fluorescence (RFU) vs. LNCaP-CMV-EGFP cell numbers D: PC3-CMV-EGFP cells were seeded at different densities (**a**) 8,000, (**b**) 4,000, (**c**) 2,000 cells/well versus (**d**) media into a 96-well plate and the fluorescence was measured every 24 hr. The RFU values are given as the mean (± SD) of 3 independent experiments.

2.3.2 Simultaneously screening for cell death and cell viability

Having demonstrated that this fluorescence assay could be used for quantitation of prostate cancer cells under growth promoting circumstances, we next tested whether it could accurately estimate decreasing cell numbers in response to a cytotoxic agent. Mitoxantrone is an anthraquinone antineoplastic agent that has been extensively used to treat castrate-resistant

prostate cancer (29) and has been shown to induce apoptosis in LNCaP cells (30). To validate whether our cell-based assays could determine a dose response to mitoxantrone, LN-CMV-EGFP cells were plated at 20,000 cells/well and then treated with mitoxantrone concentrations ranging from 0 to 10,000 nM. After 48 hr, light microscopy revealed that wells with >1000 nM mitoxantrone contained a mixture of alive and dead cells with some cells having apoptotic features such as cell shrinkage and nuclear fragmentation (data not shown). Under the fluorescent microscope, it was observed that viable, adherent LN-CMV-EGFP cells had EGFP fluorescence that was concentrated inside the cells, resulting in bright fluorescence wherever there was a live cell. By comparison, dead cells had lysed and their EGFP proteins had diffused into media. This diffused EGFP resulted in an elevated fluorescence background that could be easily seen with the fluorescent microscope. Since only upon cell death did EGFP appear in the media, the amount of fluorescence in the media should be an index of cell death. Accordingly, after 48 hr treatment with mitoxantrone, the media from each well was transferred to a new plate and RFUs were measured. As is evident in Fig. 2.2A, only background fluorescence was detected in the media after treatment with 0 to 100 nM mitoxantrone. However, significant media RFU was measured at >1000 nM suggesting extensive cell death ($p < 0.05$). At 10,000 nM of mitoxantrone, all the cells were dead as determined by fluorescent microscopy and further validated by MTS assays.

To measure the proportion of living cells in the same samples, fresh media was added and RFUs measured in adherent cells. To confirm that the RFU values obtained were correlated with the number of surviving cells, MTS assays were performed and compared to the corresponding RFUs (Fig. 2.2B). At 0 nM mitoxantrone, the RFU levels of live cells had increased 30% in comparison to the average RFUs measured at the time of seeding (2.26 ± 0.14). Between 10-100

nM of mitoxantrone treatment, cells appeared to be in a quiescent state, since their mean RFU values were not significantly changed in comparison to that at seeding time and the MTS assay performed on these cells showed no significant difference in cell numbers between seeding time and after 48 hr treatment. However, at 1000 nM mitoxantrone, there was 30% less RFU than at seeding time, consistent with a 30% decrease in cell numbers determined by the MTS assay. As the concentration of the drug increased, the RFU levels decreased in the same proportion as the cell number, such that at 10,000 nM mitoxantrone no live cells were detectable. It should be noted that there is an apparent reciprocal relationship between the RFU values measured in the surviving cells and that in the media, where the fluorescent contribution is from dead cells and that this assay enables one to estimate the relative number of both living and dead cells in the same sample.

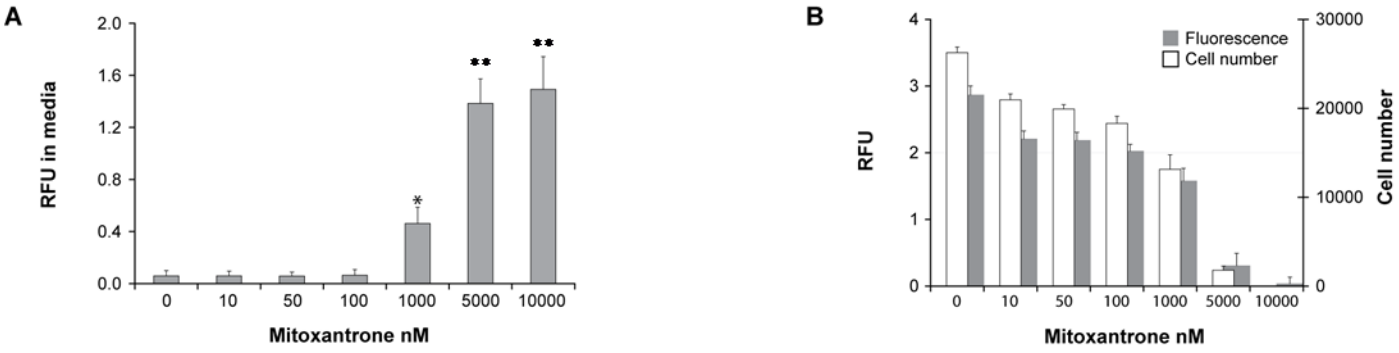

Figure 2.2: Screening for cell viability and cell death using LN-CMV-EGFP cells. A: 20,000 LN-CMV-GFP cells were seeded in each well of a 96 black well plate overnight. Cells were then treated with mitoxantrone ranging from 10 nM to 10,000 nM for 48 hr. The media was then transferred to a new 96-well plate. The diffused EGFP (RFUs) in the media from lysed cells was measured. * indicates a significant difference ($p < 0.05$) at a 1000 nM in comparison to lower levels. ** indicates a $p < 0.05$ compared to *. B: 100 µl of fresh media was added to each well and the EGFP from the attached viable cells measured. After the fluorescence was measured, a MTS assay was performed to determine the cell numbers/well. Both fluorescence and cell numbers at various mitoxantrone levels were plotted. The RFU values are given as the mean (± SD) of 3 independent experiments.

2.3.3 Osteosarcoma (SaOS-2) conditioned media increases LN-CMV-EGFP cell proliferation

Having developed an assay that quantifies both cell proliferation and cell death, we next evaluated a biological sample with an unknown potential for growth modulation. *In vitro* and *in vivo* studies have shown that bone factors can accelerate human prostate cancer growth (6,31-36). SaOS-2, a human osteoblast-like osteosarcoma cell line is capable of producing a variety of bone factors (osteoprotegrin, osteonectin, osteocalcin, bone sialoprotein, TGF-β, IGF-1, IL-6, IL-1) and mineralization in the presence of β-glycerophosphate (6,37-39). Accordingly, we tested conditioned media (CM) from differentiated SaOS-2 cells in our assay system. Fig. 2.3A shows the RFUs measured over time for cells treated with different concentrations of steroid-depleted (charcoal-stripped) CM. While following the first 24 hr there were no significant differences among treatment conditions, after 48 hr, differences in fluorescence were detectable and became significant with 100% CM compared to non-CM at day 3 ($p< 0.05$). After 4 days, a significant RFU difference over control ($p< 0.05$) was also seen in experiments with 50% CM. The increased RFUs with 50% and 100% CM at day 4 were about 1.3- and 1.4-fold, respectively. This was confirmed by a MTS assay, which showed a similar fold increase in cell number (Fig. 2.3B). Taken together, these results indicate that, in a single time-course experiment, this assay can be used to non-destructively monitor the presence of secreted mitogens in complex mixtures (ie CM) and evaluate their relative potency in stimulating prostate cancer cell growth.

2.3.4 A cell-based assay to measure changes in AR activity

Since the AR plays a key role in prostate cancer growth and survival, we sought to establish a cell-based assay for evaluation of agents capable of modulating endogenous AR activation in

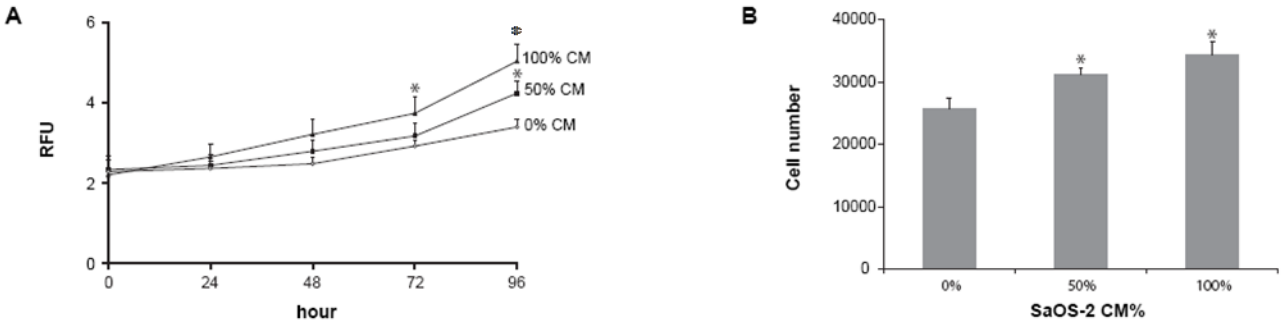

Figure 2.3: Effects of osteoblastic conditioned media (CM) on EGFP expression in LN-CMV-EGFP cells. A: 20,000 LN-CMV-EGFP cells were seeded in a 96-well plate either suspended in 50% or 100% differentiated SaOS-2 CM media, or media alone (0% CM). The amount of fluorescence was measured every 24 hr. B: Cell number assay (MTS) was performed at day 4. Asterisks show significant differences in cell numbers of treatment conditions over media alone ($p < 0.05$). The RFU values are given as the mean (± SD) of 3 independent experiments.

living prostate cancer cells. A lentivirus having an androgen responsive probasin-derived promoter upstream of an EGFP reporter gene (ARR_2PB-EGFP) was used to make the LNCaP cell line LN-ARR_2PB-EGFP. EGFP expression in this system is regulated by AR and thus, increased fluorescence should indicate increased AR transcriptional activity. To test this, LN-ARR_2PB-EGFP cells were first grown in charcoal stripped media to deplete cells of bio-available androgen. As before (27), EGFP fluorescence declined over time and by day 5, no fluorescent cells were observed (data not shown). This lag period is likely due to the time it takes for i) remaining androgens to be metabolized, ii) the AR to become transcriptionally inactive after translocation into cytoplasm, and iii) all endogenous EGFP protein to be degraded. When EGFP fluorescence was undetectable, LN-ARR_2PB-EGFP cells were incubated with various concentrations of the potent synthetic androgen R1881 at concentrations ranging from 0.01-10 nM (Fig. 2.4A). After 24 hr of hormone treatment, cells exposed to 1 nM or higher concentrations of R1881 showed an increased amount of EGFP expression, which was visually

confirmed by fluorescent microscopy. The EGFP expression levels increased over time in a dose dependent manner and showed significant differences between 0.1 and 1 nM hormone at 48 hr ($p< 0.001$) and with 0.1 and 0.05 nM at 72 hr ($p< 0.001$). On a per cell basis, the mean RFU value at day 3 indicated that there was a higher fluorescence as the R1881 concentration increased (Fig. 2.4B). This indicates that the observed increase in EGFP expression was due to enhanced AR activation and not due to cell growth.

We next tested the effect of bicalutamide, an anti-androgen and antagonist of AR (40), to determine if this assay could also measure inhibition of androgen induced EGFP expression. Fig. 4C shows changes over time in RFU values in LN-ARR$_2$PB-EGFP cells exposed to increasing concentrations of bicalutamide in the presence of R1881. With 10 μM bicalutamide there is a slight suppression of RFU levels after 3 days, whereas a significant decrease ($p<0.05$) is seen at this time point when the drug concentration is raised to 25 μM. By comparison, a significant inhibition of AR activity is observed after only 2 days of treatment with 50 μM bicalutamide with a >90% decrease in androgen-induced RFU seen at 3 days. Thus, this assay can readily measure dose-dependent effects of agents that are able to decrease AR activity.

2.3.5 Assessment of non-steroidal activation of AR

Having established an assay that is able to monitor endogenous AR activity in living cells, we next tested whether the effects of non-steroidal modulators of AR could also be assessed. Since we had found that CM from differentiated SaOS-2 cells could induce growth of LNCaP cells in a steroid depleted environment (Fig. 2.3A), we sought to determine whether this growth response was mediated via AR transactivation. Similar to that observed with the LN-CMV-EGFP cells, LN-ARR$_2$PB-EGFP cells exhibited significant enhancement of growth at day 3 when treated

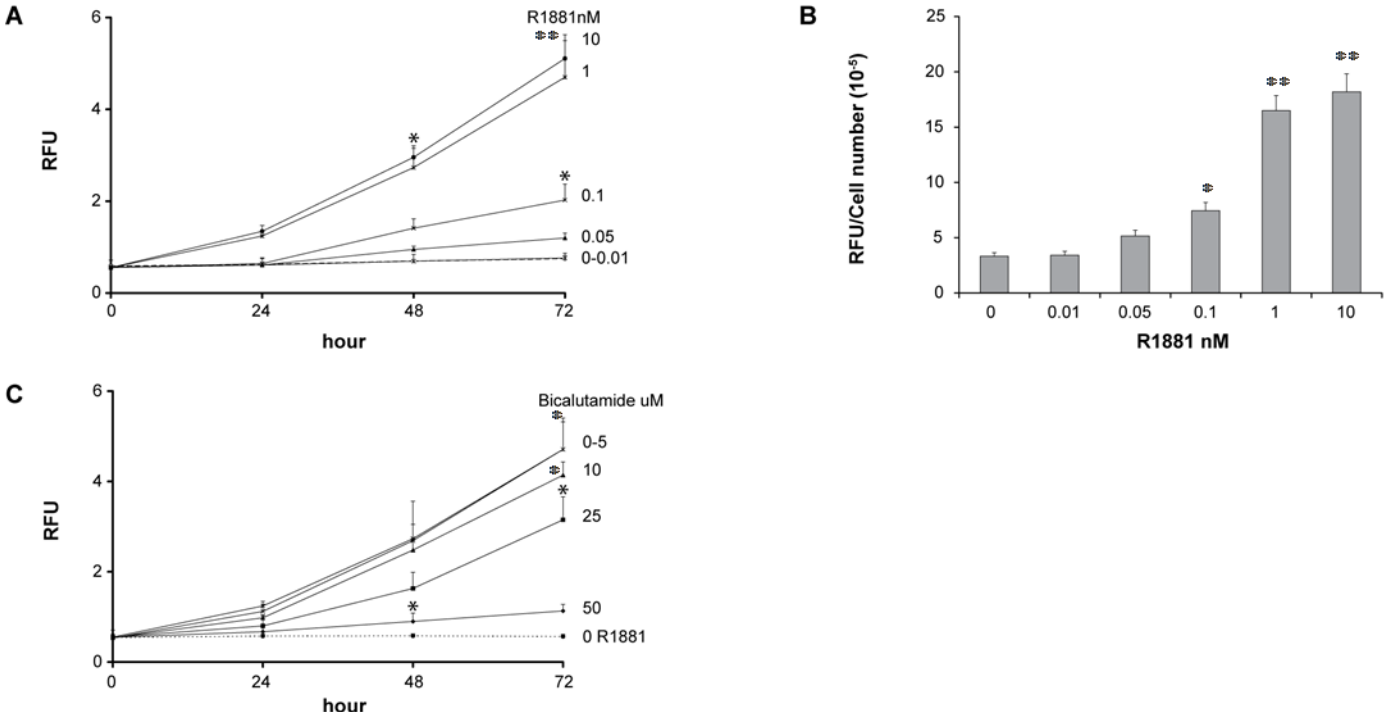

Figure 2.4: Measurement of AR activity with LN-ARR2PB-EGFP cells. A: After 5 days growing in charcoal stripped serum (CSS) RPMI, 20,000 LN-ARR2PB-EGFP cells/well were seeded in a 96 black well plate and incubated with R1881 ranging from 0.05 nM to 10 nM of R1881. Fluorescence was measured every 24 hr. * shows the significant difference between 0.1 and 1 nM R1881 at 48 hr ($p < 0.05$) and 0.1 and 0.05 at 72 hr ($p < 0.05$). ** indicates a $p<0.05$ compared to 0.1 nM of R1881. B: After 72 hr cell numbers were determined using a MTS assay. The mean fluorescence/treatment at 72 hr was normalized to mean cell number/treatment and plotted against R1881 concentration. * indicates a $p<0.05$ compared to 0.05 and ** indicates a $p<0.05$ compared to 0.1 nM R1881. C: After 5 days growing in CSS, LN-ARR2PB-EGFP cells were seeded at 20,000 cells/well and incubated overnight with bicalutamide from 0-50 µM before treatment with 1 nM R1881. Asteristiks show significant difference with bicalutamide treatment at 48 hr and 72 hr, respectively compared to 0 nM R1881. The RFU values are given as the mean (± SD) of 3 independent experiments.

with 100% CM in the absence of hormone (as detected by a MTS assay, $p < 0.05$). However, no significant changes of AR-induced fluorescent activity were seen ($p > 0.05$), even using CM concentrated to 400% by centrifugal filter devices (500MW cut-off). When combined with R1881, the amount of fluorescence seen could be attributed entirely to the presence of androgen (data not shown). Thus osteoblast CM induced growth of LNCaP cells through an AR-independent mechanism.

It has been reported that both IL-6 and FSK can modulate the AR transcriptional activity (5,7,8,11,41-44). Both were added to LN-ARR$_2$PB-EGFP cells to determine if they could change the endogenous AR activity in this system. Neither reagent modulated AR activity in the absence of R1881, nor at R1881 concentrations lower than 0.1 nM (data not shown). However, as shown in Fig. 2.5A, in the presence of $\geq$ 0.1 nM R1881, a 72 hr exposure to 25 μM FSK increased RFUs 1.5-fold relative to R1881 alone ($p < 0.01$), consistent with reported observations of this androgenic priming requirement (41,43,44). Conversely, IL-6 (20 ng/ml) in the presence of 0.1 nM R1881 resulted in a 40% decrease in fluorescence ($p < 0.01$) (Fig. 2.5A). This is in agreement with Jia et al (42), who also observed an inhibition of AR transactivation with IL-6. Cell numbers at day 3 were determined by MTS assays and showed no significant differences among the above treatment conditions (data not shown), indicating fluorescence measurements reflected AR activity and not cell growth. Thus, FSK enhanced and IL-6 inhibited endogenous AR transcriptional activation in the presence of 0.1 nM or 1 nM R1881.

We next tested the utility of this assay for ascertaining the effects of an environmental chemical contaminant on our androgen regulated EGFP cells. Dichlorvos is a potential carcinogenic pesticide (45) and also a possible AR modulator through yet undetermined mechanism(s) (46). This pesticide was used to treat LN-ARR$_2$PB-EGFP cells in the presence or absence of androgen. In the absence of R1881, different concentrations of dichlorvos did not affect AR activity (data not shown). After 48 hr in the presence of 0.1 nM R1881, 1 μM dichlorvos significantly enhanced 0.1nM R1881 induction of EGFP ($p < 0.05$), whereas fluorescence enhancement by 0.1 μM of dichlorvos became significant by day 3 ($p < 0.01$) (Fig. 2.5B). Thus, dichlorvos is ineffective alone, but is able to enhance AR activity in the presence of

low androgen levels. A future application of this system could be to screen various chemical compounds or natural products for modulation of AR activity.

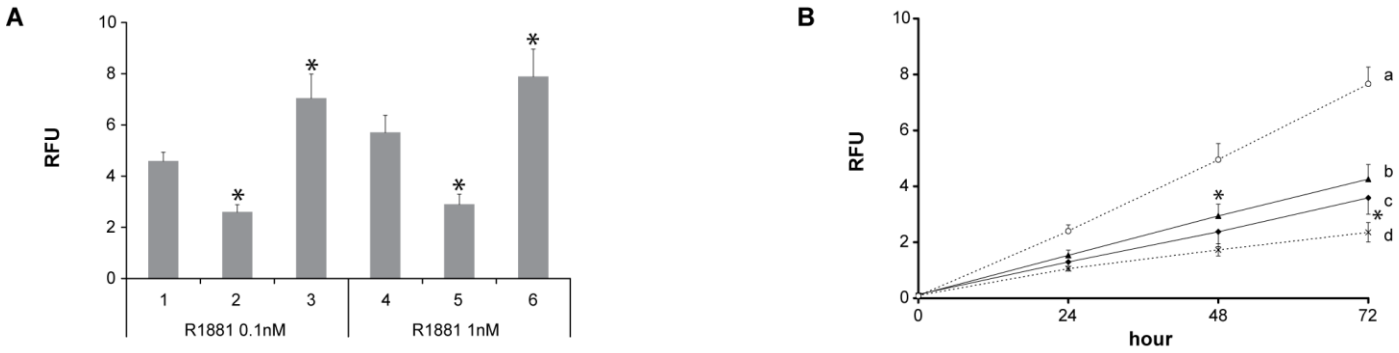

Figure 2.5: Effects of FSK, the pesticide dichlorvos, and IL-6 on fluorescence levels in ARR2PB-EGFP cells. A: After 5 days growing in CSS, LN-ARR2PB-EGFP cells were seeded at 20,000 cells/well and treated with 0.1 or 1 nM R1881 alone (1 & 4), or in addition with 20 ng/ml IL-6 (2 & 5), or 25 µM forskolin (3 & 6). The fluorescence, representing AR activity was measured every 24 hr. At t = 72 hr, in the presence of 0.1 or 1 nM R1881, fluorescence expressions of IL-6 and forskolin are significantly different over control ($p < 0.05$). B: LN-ARR2PB-EGFP cells were treated with (**a**) 1 nM R1881, (**b** & **c**) 1 and 0.1 µM dichlorvos in the presence of 0.1 nM R1881 respectively, or (**d**) 0.1 nM R1881 alone. The RFU values are given as the mean (± SD) of 3 independent experiments. * indicates a $p<0.05$ compared to 0.1 nM R1881 at 48 and 72 hr respectively.

2.4 Discussion

Since there is an expanding number of chemicals, biological substances and cellular extracts that can alter prostate cancer cell growth and AR transcriptional activity, a high throughput, non-destructive method to identify these agents and to asses their activities in viable cells was developed using LNCaP and PC3 cell lines that had been lentiviral-treated to stably express fluorescent EGFP reporters. To monitor cell growth or AR enhancement, EGFP was either driven by a constitutive CMV promoter (LN-CMV-EGFP or PC3-CMV-EGFP cells) or by an ARR_2PB androgen responsive promoter (LN-ARR_2PB-EGFP cells) respectively.

After lentiviral infection of LNCaP or PC3 human prostate cancer cells with Lv-CMV-EGFP and subsequent enrichment of high fluorescence expression levels by FACS sorting, we found that, after 24-96 hr culturing in multi-well plates, there was a direct linear relationship (R^2 = 0.99) between levels of fluorescence and cell number (Fig. 2.1). To evaluate the accuracy and practical utility of this high throughput assay for assessment of agents that kill or inhibit the growth of prostate cancer cells, we tested the effects of a known cytotoxic drug, mitoxantrone (30,47), and found that there was direct concordance between the patterns and quantitation of cell death and apoptosis measured by both our non-destructive assay and MTS (non-viable) estimates (Fig. 2.2). Similarly, our fluorescent assay enabled accurate and reproducible measurement of prostate cell proliferating effects of complex mixtures such as osteoblast CM from differentiated SaOS-2 cells which induced a small (30-40%), yet statistically significant ($p<0.05$), increase in cell proliferation (Fig. 2.3), in agreement with reports that the bone environment is supportive both in survival and metastatic growth of prostate cancer cells (6,31-36). In contrast to its growth promoting effects, CM did not alter endogenous AR activity, implying that the cell proliferation induced by osteoblast CM was through AR-independent pathways. Overall, our cell-based screening method can robustly measure factors that even subtly influence prostate cancer cell proliferation or cell death and therefore should be particularly useful for large scale, simultaneous assessment of multifactor treatments at different concentrations and time points, saving both time and costs. Furthermore, the non-destructive nature of this 96-well assay would enable multiple dynamic measurements of cellular and molecular responses to the treatments, replacing several individual experiments.

The practical utility of applying a viable, cell-based assay to measure chemical and environmental agents that can modulate AR activity was tested by using lentiviral vectors

containing a probasin-derived AR-responsive promoter to create the LNCaP cell line, LN-ARR$_2$PB-EGFP. Using these cells, we found that R1881, a potent synthetic androgen and AR agonist (48), could reproducibly induce EGFP expression in a concentration- and time-dependent manner (Fig. 2.4) over a large number of passages. As anticipated, bicalutamide, an effective anti-androgen known to block AR activation (40), was readily evaluable in this system (Fig. 2.4). Because these cells are both stable AR-inducible expressers and enriched by FACS selection, responses are far less variable than estimates using transient transfection systems.

While screening for established androgenic and anti-androgenic compounds validated this AR-bioassay, LN-ARR$_2$PB-EGFP cells also provided an opportunity to assess the effects of non-steroidal drugs or compounds that may modulate the AR-dependent gene transcription. There are conflicting reports as to whether the FSK can directly activate the AR in a ligand-independent fashion or whether it requires some form of androgen priming to see this agonisitic effect (43). Similarly, it remains unresolved whether the cytokine IL-6 is an androgen-independent agonist (5,8,11) or an antagonist (42) of AR. Since our AR functional screening assay is stable, reproducible, relies on a chromosomally integrated AR reporter system and is not confounded by transient transfection manipulations, we felt that it could provide a more physiological judgment of these questions. While neither chemical was observed to modulate AR activity in the absence of androgen in charcoal-stripped serum, in the presence of 0.1 nM or higher concentrations of R1881, IL-6 significantly inhibited AR activity whereas FSK stimulated AR transcriptional activity of EGFP (Fig. 2.5).

To broaden the screening application of our cell-based AR functional assay, we tested whether the pesticide dichlorvos could modulate AR activity in a prostate cancer cell. As with

IL-6, there is some controversy as to whether this environmental contaminant is an AR agonist as demonstrated in transient transfections using LNCaP prostate cancer cells (49) or antagonist as shown in similar experiments with Chinese hamster ovary cells (46). Our results clearly support an agonist role for dichlorvos, at least in the presence of priming amounts of androgen and in the context of human prostate cancer cells. In general, these experiments support the efficacy of using a stable, reproducible and chromatin-integrated AR-inducible reporter system for assessing the impact of drugs and chemicals on endogenous AR activity in prostate cancer cells. Furthermore, to assist in uncovering the mechanism of action of AR-interfering compounds, one can use this fluorescent assay to monitor AR activity over time in multiple sets of viable prostate cancer cells to determine the optimal time points and conditions for subsequent analyses such as gene expression microarrays or signal transduction assays.

2.5 Conclusions

The lentiviral-created, cell-based fluorescent assay systems developed in this study enable rapid, reproducible, time-dependent, and high-throughput screening and monitoring of chemicals or complex mixtures for their capacity to influence prostate cancer cell growth/death or AR-mediated signaling, under a variety of treatment conditions in a single experiment. After validation, we used these assays to illustrate the cytotoxicity of mitoxantrone, the AR-independent growth-promoting activity of CM from differentiated osteoblasts (SaOS-2 cells), the androgen priming requirement for FSK activation and IL-6 inhibition of AR activity, and finally, that the pesticide dichlorvos enhances AR transcription activity in a androgen-dependent manner.

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Chapter 3

Identification and development of a new class of drugs to treat advanced prostate cancer [1]

3.1 Introduction

Androgen receptor (AR) plays a key role in development of prostate cancer and progression to castration-resistant disease. Hence, AR knock down has been proposed as an additional therapy after failure of androgen ablation (1). Several groups including our laboratory have used RNA approaches to knock down the AR as a method to inhibit the growth of hormone-refractory prostate cancer (2-5). However, to deliver RNAi into the human cancer cells remains yet to be resolved. Another approach to downregulate or eliminate AR signalling pathway would be using antiandrogens (6-12). Although the currently marketed antiandrogens, such as bicalutamide and flutamide, prolong survival in some cases, they are generally ineffective in treatment of patients with advanced prostate cancer (13). In addition, all of the available antiandrogens in clinics have been observed to convert to agonists mainly through mutation in the AR-ligand binding pocket (14,15). For these reasons, additional therapies or targets are critically needed in order to decrease the current mortality rate from prostate cancer. In searching for new targets on the AR molecule, the Fletterick laboratory has targeted the AF-2 of AR (16). AF-2 is formed in response

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[1] A version of this chapter will be submitted for publication. Peyman Tavassoli, Peter Axerio-Cilies, Art Cherkasov, Paul S. Rennie, Identification and development of new class of drugs to treat advanced prostate cancer.

Dr Art Cherkasov at the department of Urologic Sciences (UBC) founded the *in silico* drug discovery platform at the Prostate Centre at Vancouver General Hospital. Peter Axerio-Cilies (a PhD student with Dr. Cherkasov) performed *in silico* screening and wrote the corresponded material and results, described herein.

to ligand binding, which facilitates binding of coregulators, such as the SRC family to the AR. Only 6 amino acids (V284, K288, I302, K306, L454, and E457) in the AF-2 are crucial for its function (17), and these form a hydrophobic cleft that binds short α-helical peptides in coactivators (16). Compounds that bind AF-2 should disrupt AR/coactivator interactions (18). Moreover, they could inhibit intermolecular interaction between the AR-LBD and NTD required for optimal AR transcriptional activity (18). Using structural x-ray screens for AR-interacting compounds, Fletterick's laboratory found a small molecule binding site on AF-2 referred as BF-3 (binding function-3) (Fig. 3.1). They suggested that compounds, such as flufenamic acid (FLF), 3,3",5-triiodothyroacetic acid (TRIAC), and triiodothyronine (T3), that bind to the BF-3 will modulate coregulator recruitment and suppress AR activity (16). Upon binding with 3,3",5-triiodothyroacetic acid (TRIAC), BF-3 goes inward and changes the secondary structure of AR (AF-2), therefore, distort coactivator binding site, such as ARA 70.

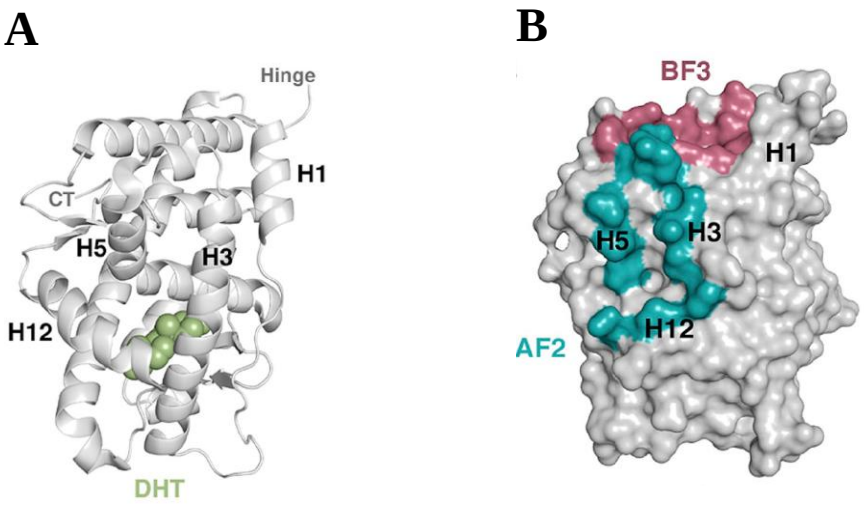


Figure 3.1 – **The AF-2 and BF-3**. BF-3, next to AF-2 site (LXXLF motif for coactivators) is an outward pocket. **A)** Schematic of AR LBD showing location of DHT, key AF-2 helices 3, 5, and 12, and H1. **B)** Space-filling model showing residues in AF-2 (cyan) and BF-3 (red) (From (16) © 2007, National Academy of Sciences, USA).

Following this discovery, we scanned over 50 million available compounds for potential interactions with the BF-3 cavity on the AR using *in silico* drug discovery tools. *In silico* or structure-based computational methods are valid tools for analyzing the properties of chemical compounds for such properties as toxicity, biological activity, or interactions with macromolecules, such as the AR. Structure-based computational methods mainly can be used for drug design or screening of large chemical libraries targeted towards a biological receptor with known three dimensional structure (19,20). The *in silico* screen is a repetitive selection process from large chemical libraries targeted towards a biological receptor and often proceeds through multiple cycles before optimized leads are identified. This task is facilitated significantly by the advent of high performance computing environments, data management software and internet to offer the advantage of delivering new drug candidates more quickly and at lower costs in comparison to traditional drug discovery methods (21-23). Generally, *in silico* drug discovery process can be divided into three stages (Fig. 3.2). Stage I includes identification of a potential therapeutic drug target and building a heterogeneous small molecule library to be tested against it. This is followed by the development of a virtual screening protocol initialized by docking of the small molecules from the library. The next step is binding affinity prediction/scoring and optimization of the set of molecules until the desired binding affinity is achieved. Following this, molecular simulations can be performed in a select few cases to obtain a more realistic appreciation of binding affinity and its dependence on solvent, salt and dynamics. This way, a set of molecules with desired affinity are selected as better binders to the target and termed as 'hits'.

In Stage II, these selected hits are checked for specificity by docking at binding sites of other known drug targets. The hits that score best for only the target of interest and poorly for all other targets are selected as specific binding molecules. In Stage III, these selected hits are subjected to

detailed *in silico* profiling studies of drug absorption, distribution, metabolism, excretion and toxicity (ADMET) and those molecules that pass these studies are termed as 'leads' (24-26). For instance, Captopril (anti hypertensive), Dorzolamide (treatment for glaucoma), Saquinavir, zanamivir (anti flu), LY517717 (anti cloting factor) and TMI-005 (anti rheumatoid arthritis) are medications that have been discovered through *in silico* application and currently are used clinically or under evaluation in phase II clinical studies (27,28).

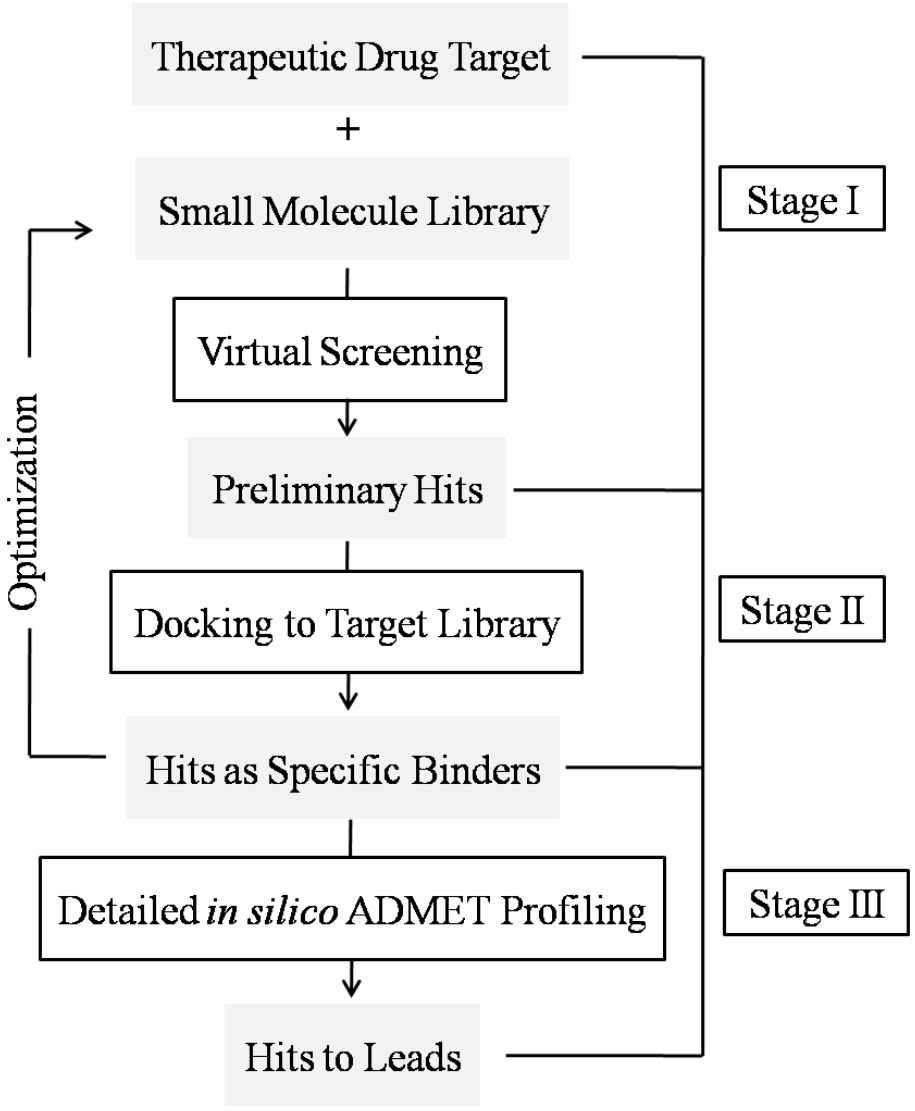


Figure 3.2 – A flowchart outlining a plausible generalized structure-based *in silico* drug discovery strategy. Modified from (25)

Using *in silico* drug discovery tools, out of 4 million purchasable chemicals, 220 compounds were scored the best for binding to the BF-3 pocket. To determine how these compounds are biologically active in comparison to bicalutamide, the LN-ARR2PB-EGFP cell line (29) was then used to screen them for AR transcriptional activity and cell toxicity. We found 17 compounds suppressed AR activity, some of them even more efficiently than bicalutamide.

3.2 Materials and methods

3.2.1 ZINC chemical database preparation

Approximately ~4 million structures in structural data format (SD format) from the ZINC-5.0 compound library (30) were imported into a molecular database using Molecular Operating Environment (MOE) (31). These structures were adjusted i.e. all inorganic components were removed, and all ionizable groups were coordinated with pH= 7.0 conditions. Next, this database was energy-minimized using the '*MMFF94x forcefield'* (Merck Molecular Force Field) (32) and exported in SD format for use by the '*Glide'* (an approach for rapid, accurate docking and scoring) (33,34) and '*eHITS'* (Electronic High Throughput Screening) (35,36) docking programs.

3.2.2 Molecular docking

The Maestro suite (37), a visual media software was used to prepare the human AR structure obtained from the Protein Data Bank (PDB) (38) for molecular docking. The BF-3 binding site was defined by the binding mode of TRIAC which was crystallographically resolved with the LBD of the human AR crystal structure (PDB code: 2PIT). Hydrogen atoms were added and adjusted where necessary to the corresponding AR crystal structure model. Protein and ligand charges have been assigned by the OPLS (optimized potentials for liquid simulations) molecular mechanics force field method (39). The Glide-score and eHITS-score algorithms were

implemented to establish the predicted binding poses and relative energies of the molecules with Glide 1.64.2.5 standard precision (SP) docking for the main screen and eHITS-2008 docking for the second screen, using default settings for both. The ligands were docked to the AR model into the BF-3 binding pocket which was defined as 10 Å radius surrounding the TRIAC molecule, (x= 16.967922 y=18.261421 z=28.947824). Approximately 4 million compounds obtained from the ZINC-5.0 compound library were docked into the BF-3 site using SP-Glide. Glidescore cutoffs of -6.0 were implemented and these molecular structures were then subsequently re-docked into the BF-3 site using the eHITS docking program. All calculations were done by a Linux cluster of 5 Intel Xeon dual processors at 3.2 GHz with 2 Gb of RAM. Graphic manipulations and analysis of the docking experiments were performed by the Maestro Graphical User Interface, version 4.1.012, for Linux operating systems.

3.2.3 Scoring functions

A. Binding affinity (pKi[1])

The Molecular Operating Environment (MOE) estimated pKi was calculated for each ligand using the scoring.svl script available through the SVL exchange service (40). For this, hydrogen atoms were added to the AR model and the partial charges were calculated with the OPLS force field (39). The Gasteiger partial charges (partial charge property of each atom) were calculated for the structures that passed the docking cutoff. The estimated pKi for these structures were calculated by choosing the dock_pKi descriptor with default settings for the molecular database.

[1-] The affinity is usually quantified as the equilibrium dissociation constant, Ki. The Ki is the concentration of the competing ligand that will bind to half the binding sites at equilibrium, in the absence of ligand or other competitors. The subscript "i" is used to indicate that the competitor inhibited ligand binding. Ki is usually shown as the log(Ki) value (pKi). If the pKi is high, therefore, the affinity of the receptor for the inhibitor is high.

B. Ligand explorer (LigX)

LigX module was used to account for the receptor/ligand flexibility where the receptor atoms near the ligand produced by Glide/eHITS docking programs and the ligand itself are succumb to the MMFF94x forcefield energy minimization as implemented in MOE. LigX calculations were applied to all the structures that passed the docking cutoffs. Tether restraints were set to 10 for the receptor to discourage gross movement and all other parameters were set to default. Subsequently, pKi binding affinity score was generated after energy minimization to predict the ligands that showed the best binding characteristics defined mainly by the energy of hydrogen bonds and hydrophobic interactions.

C. Root mean standard deviation (RMSD)

The root mean standard deviation (RMSD) was calculated between the Glide poses and the eHITS poses to evaluate the docking consistency and reliability and thus establish the most probable binding pose for a given ligand. The MOE RMSD values were calculated for each ligand using the rmsd_mol.svl script available through the SVL exchange service. The RMSD was added to the voting "consensus" system, where ligands were assigned a 1 vote for a RMSD value of < 3.0 Å and a 0 vote for > 3.0 Å.

3.2.4 Similarity searching (SS)

The 2D similarity was done for the compounds that were proven previously to bind to the BF-3 site which were FLF, TRIAC, and T3 (16). The similarity searching was applied to the ZINC-5.0 compound library which is a ~4 million compound database (30). The *MACCS fingerprint* was applied as a tool that represents certain structural features of a molecule using a list of binary values (0 or 1). This fingerprint contains 166 bits indicating the presence of specified structural fragments in the molecular graph representation. The Tanimoto coefficient was then used to

select similar molecules as implemented in MOE (31,41). The Tanimoto coefficient is a statistic used for comparing the similarity between two vectors of *n* dimensions by finding the angle between them. A T_c similarity measure was calculated for each ligand based on formula 1. T_c cutoffs were implemented based on a > = 75% similarity or a T_c value of 0.75 between the target and database molecule.

3.2.5 Screening for androgen receptor activity and cell toxicity

LN-ARR$_2$PB-EGFP cells were used to screen 220 compounds against AR transcriptional activity (29). Briefly, cells were grown in 5% charcoal stripped serum (CSS) RPMI for ~ 5 days to deplete cells from bio-available hormone and hence reduce their basal EGFP fluorescence. After visual verification with a fluorescent microscope, cells were treated with 50 µM of selected chemicals (dissolved in DMSO), or bicalutamide (positive control) in the presence of 0.1 nM R1881 and seeded at 20,000 cells/well in 96-well black plates (Nalge Nunc) using 5% CSS, phenol red-free RPMI medium (Gibco). For a quality control of this system, vehicle, 0.1 nM, and 1 nM R1881 treatment were also set up within each plate, respectively. The fluorescence was measured every 24 hr for 3 days using a *fluorometer (*Fluoroskan Ascent FL). At the end of each experiment, cell viability was measured by MTS assays as described before (29).

3.2.6 Transient transfection assays

HeLa cells that constitutively express AR (HeLa-AR) (42) were seeded in 24-well plates and at 90% confluency transfected with 125 ng/well of pARR3-tk-Luc, as a reporter using lipofectamine 2000 reagent (Invitrogen). Cells were incubated with the transfection mix for 16 h at 37°C and subsequently replenished with 5% CSS RPMI containing 1 nM R1881, or 1 nM R1881 and 50 µM of each compound or 50 µM bicalutamide for 24 h before harvesting for

luciferase assay and Western blot analysis. Luciferase activity was normalized to protein concentration as quantified by BCA protein assay. Each assay was done in triplicate, and experiments were repeated three times.

3.3 Results

3.3.1 BF-3 docking

For the main screen we considered ~4 million commercially available compounds from the ZINC-5.0 compound library (30). These compounds were optimized and docked into the AR-BF-3 pocket using the Glide 1.64.2.5 docking module (33,34); cut-off filters were implemented based on Glide scores of -6.0, which corresponded to 20% of the top "hits" from the main screen (MS). This resulted in a dataset of ~500,000 molecules which were then re-docked into the same BF-3 binding cavity using the eHiTS docking software program (33-36). Other scoring functions or predictors were calculated for this dataset to filter out compounds that exhibited poor binding characteristics towards the BF-3 binding site on AR. The first predictor calculated for each ligand was the dock_pKi parameter computable by the MOE scoring svl.script. The dock_pKi is an estimated pKi value defined mainly by the energy of hydrogen bonds and hydrophobic interactions. The second predictor used was the binding affinity score generated by the ligand explorer (LigX) module in MOE, where the receptor atoms near the ligand and the ligand itself are energy minimized and ultimately scored based on the dock_pKi parameter. Based on the four scores, we implemented a voting scheme where, for each score, a value of 1 was assigned to the ligands in the top 10% while all other ligands were given a value of 0. Then the four binary votes from Glide score, eHITS score, and pKi (31), and ligand explorer (ligX) (31), were added together for each ligand and, in turn, the ligands were ranked by their voting "consensus" score, where a score of four was the maximum optimal value.

At the next stage, the 500,000 compound dataset was complied and analyzed in order to assess the consistency and reliability of the docking poses between the Glide and eHITS docking programs. For this, the root mean standard deviation (RMSD) was calculated to establish the most probable binding pose for a given ligand with respect to the BF-3 binding cavity. The fifth predictor RMSD (between Glide pose and eHITS pose) was added to the voting system, where ligands were assigned a 1 vote for a RMSD value of < 3.0 Å and a 0 vote for > 3.0 Å. In addition, a 2D similarity search was done on FLF, TRIAC and T3 that were known previously to bind to the BF-3 site of AR. The similarity searches on FLF, TRIAC, and T3 were computed against the ZINC-5 compound library, which contained ~4 million compounds. A T_c similarity score was generated by means of the Tanimoto coefficient by using the MACCS fingerprints as implemented in MOE. Cut-off values of > 0.75 (75%) were used and this generated a dataset of ~2000 analogous compounds. Amongst ~2000 ligands in the dataset, 500 with the highest vote totals (compounds voted 4 or 5 times with the criteria mentioned above) were visually inspected and disqualified according to whether Glide and eHITS predicted a dissimilar pose; whether lipophilic moieties were exposed into the solvent phase and based on their overall binding characteristics. This method refined and reduced the number of candidates to 220 compounds from the total number of molecules screened and these were recognized as "virtual leads" which were then selected for experimental testing.

3.3.2 Assessment of selected chemicals on androgen receptor transcription activity

Having selected 220 compounds that potentially bind to the BF-3 pocket on the AR, we next tested whether these chemicals could influence AR activity and cell viability using LN-ARR_2PB-EGFP cells. After growing in hormone depleted condition, cells were treated with either 50 µM of a compound or bicalutamide in the presence of 0.1 nM R1881. Cells were then visually

monitored and the fluorescence, as representative of AR activity, was measured every 24 hr. At day 3, MTS assays were performed to measure relative cell viability in each treatment conditions over controls. The criteria to select a compound were as follows: i) a decrease in AR activity > 50%; ii) not overt cell toxicity or at least having the same effect on cell viability as bicalutamide. If AR activity suppression was associated with decreased cell viability, different concentrations of those particular compounds were used to find the lowest concentrations at which at least > 50% of AR activity was suppressed and the effect on cell viability was comparable to bicalutamide. Table 3.1 shows the list of 17 compounds categorized based on their effect on AR activity – more than 85% and between 50-85% AR inhibition. Interestingly, TRIAC (#128) that was originally reported by the Fletterick lab is among the selected compounds. The effect of each compound on cell morphology after 3 days treatment and concentration at which this phenomenon was observed has been also shown in table 3.1.

3.3.3 Effects of chemicals on androgen receptor levels and activity

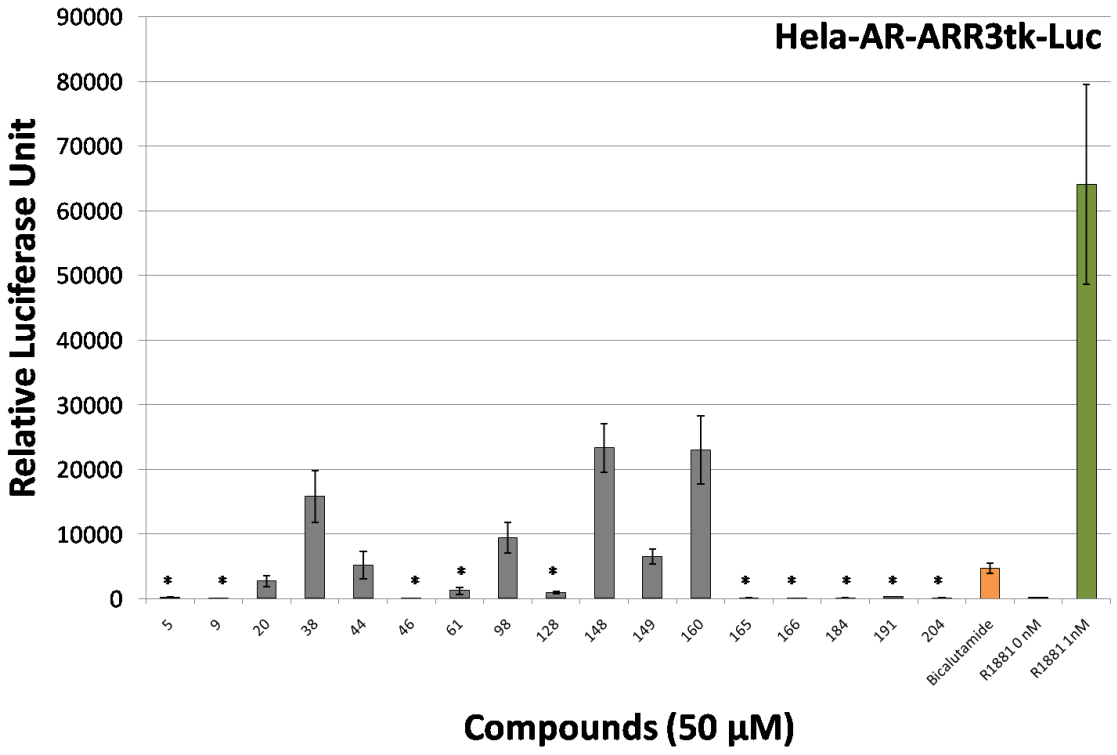
Transient transfection assays are the conventional method to quantify the AR activity levels. Hence, we next wanted to validate the results of our screening and find out the relative potency of each compound relative to bicalutamide. HeLa-AR cells, which constitutively express the wild type AR, as opposed to the mutated AR found in LNCaP cells were used for this purpose. After transfection with an androgen-responsive luciferase reporter as readout, cells were treated with 1 nM of R1881 and 50 µM of each compound or 50 µM bicalutamide for 24 hr before harvesting for luciferase assay and Western blot analysis. Fig. 3.3A showed that all 17 compounds inhibited AR activity by > 50%, and seven of these chemicals demonstrated strong anti-AR activity that considerably exceeded the activity of bicalutamide (Fig. 3.3B). Fig. 3.3C showed that the expression level of the AR protein was not changed with different treatment conditions except in

at least one case (shown in duplicate as #'s 9 and 46), the AR protein is actually knocked out by the test chemical. Together, these results confirmed our previous observation described above and highlighted the potency of each compound in inhibition of the wild type AR activity.

Label	Concentration µM	Effect on cell morphology
Suppress AR transactivation more than 85%		
148	50	No effect
61	40	"
149	50	"
128 (TRIAC)	50	"
98	50	Cell aggregation
184	50	"
Suppress AR transactivation between 50-85%		
5	20	No effect
9	20	Rounded cells
46	20	"
160	50	Forming colony
165	50	"
166	50	"
38	50	Forming crystal b/w cells
20	50	No effect
44	50	"
191	50	Not effect on cell morphology but slightly lower cell number
204	50	Slightly clumping of the cells

Table 3.1 – Screening of 220 compounds for AR activity using LNCaP-ARR2PB-EGFP cells. LN-ARR$_2$PB-EGFP cells were grown in 5% CSS RPMI for ~ 5 days to deplete cells from bio-available hormone and hence losing their EGFP fluorescence. After visual verification with fluorescent microscope, cells were treated with a particular compound plus 0.1 nM synthetic androgen R1881 and then seeded at a density of 20,000 cells/well in a 96-black well plate followed by fluorescent measurement every 24 hr. At day 3 the experiment was terminated and cell viability assay performed to assess any potential toxicity.

A


Hela-AR-ARR3tk-Luc
Relative Luciferase Unit
90000
80000
70000
60000
50000
40000
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20000
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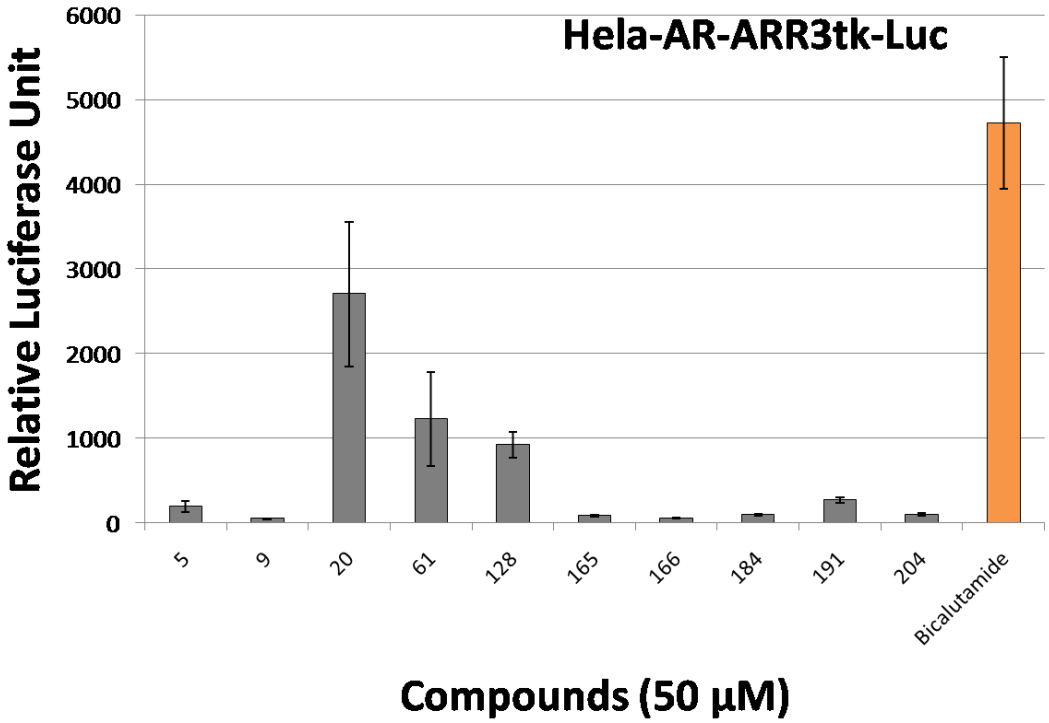
5
9
20
38
44
46
61
98
128
148
149
160
165
166
184
191
204
Bicalutamide
R18810 nM
R18811nM
Compounds (50 µM)

B


Hela-AR-ARR3tk-Luc
Relative Luciferase Unit
6000
5000
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3000
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128
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191
204
Bicalutamide
Compounds (50 µM)

C

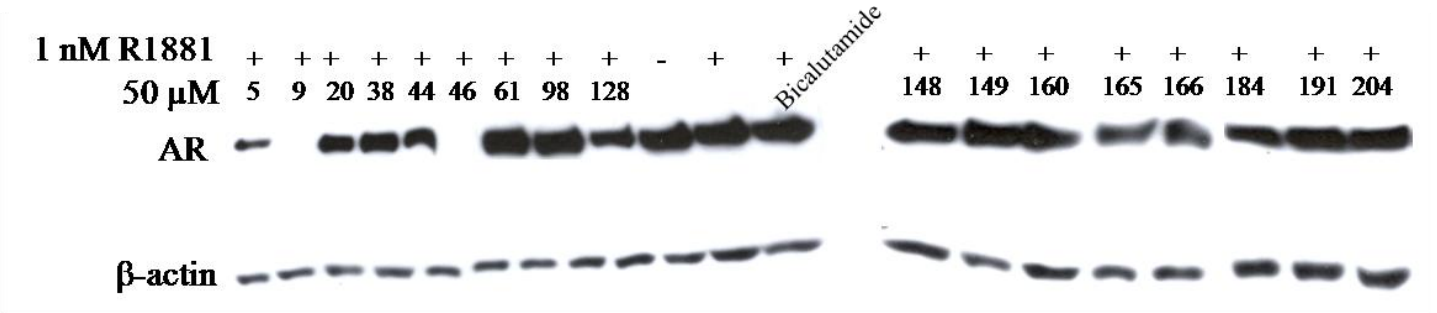

Figure 3.3- Effects of selected compounds on AR activity. A) HeLa cells that constitutively express AR (HeLa-AR) were transfected with the reporter, pARR3-tk-Luc, and then treated with 1 nM of R1881 and 50 µM of each compound or 50 µM bicalutamide for 24 h before harvesting for luciferase assay. Luciferase units (RLU) values are given as the mean (± SD) of 3 independent experiments. * indicates a $p<0.05$ compared to bicalutamide. **B)** Compounds that are more active than bicalutamide were shown at different scale. **C)** Western blot showing AR and β-actin protein level of Hela-AR cells after 24 hr treatment with 50 µM selected compounds or bicalutamide.

3.4 Discussion

Progression to castration resistance is the lethal end stage of prostate cancer, for which there is currently no cure. The understanding of the molecular mechanisms responsible for the prostate cancer transition to castration-resistant state remains a challenge, both therapeutically and experimentally. However, a large amount of evidence indicates that even at the hormone-refractory stage the functional AR signalling is essential for the growth of tumour cells and that more potent antiandrogens or AR antagonists could be an appropriate therapeutic approach for patients who failed androgen ablation therapies (43). Several antiandrogen drugs are currently in clinical use. However, they are limited by their relatively lack of potency and efficacy. Hence, efforts for the development of secondary therapies targeting multiple mechanisms of retained AR signalling have been pursued (44-49). In addition, other approaches, including anti-AR antibody,

hammerhead ribozymes, RNAi and chemicals such as LAQ824 and 3,3'-diindolylmethane have been proposed and tested for their abilities to reduce the level of AR, resulting in growth inhibition and in some cases apoptosis in castration-resistance prostate cancer cell lines (3,43,50-55). In studying the three-dimensional structure of the AR, a novel pocket referred to as the BF-3 was discovered (16). The BF-3 is a known target for mutations in prostate cancer (56) and located near, but distinct from, the ligand binding site that is normally targeted by conventional antiandrogen drugs such as bicalutamide. Compounds that interact with the BF-3 exert indirect effects on AF-2 to inhibit coregulator binding, which makes the BF-3 an attractive pharmaceutical target for blocking AR activity.

Most new drugs are traditionally identified and developed by the pharmaceutical industry. Typically, new drug candidates are selected using automated systems to empirically screen thousands, or even millions, of compounds against the target (usually a protein) of interest in an effort to identify a single potential lead compound. This approach is very costly, as only one or two of the multitude of chemicals screened will become lead compounds, and most of those fail subsequent testing. Thus, large scale industrial high-throughput screening routinely used by pharmaceutical companies typically yields less than a 0.1% success rate (57,58). In contrast, the use of a rationalized, computer-aided approach enables us to achieve a success rate as high as 30% (59).

Using *in silico* methods, we selected the top 220 compounds out of ~4 million commercially available chemicals that bind to the BF-3 pocket on AR. They were initially tested for ability to suppress AR function using our established cell-based screening assays (29). We found that 17 out of 220 compounds did exhibit some inhibiting effect on AR. Based on the MTS assays and cell morphologies, these 17 compounds were also found to be not toxic at the concentrations

tested (Table 3.1). This result has been validated with transfection assays (Fig. 3.3) with seven of these chemicals showed strong anti-AR activity that drastically exceeded the activity of bicalutamide, one of the most potent antiandrogen drugs currently used to treat men with metastatic prostate cancer. While these compounds have biological activity in both cytotoxicity and AR activity assays their binding to the BF-3 site of the AR is postulated based upon the results of our computational docking model and requires validation biophysically. This remains to be further investigated using a bioacore system in conjunction with NMR based x-ray crystallography to ensure definitive binding site validation. It has to be noted that all of the *in vitro* activity based assays have been carried out using DMSO as a vehicle. In order to assess potency and efficacy of theses compounds in animal model, alternative solvents need to be explored for lead compounds. Once suitable vehicles are determined for dosing, both oral and intravenous routes of administration will be evaluated for pharmacokinetic and pharmacodynamic studies before testing in human xenograft models.

Although more research is required to certify the potency and efficacy of these selected compounds, the results showed here indicate that virtual screening can be performed rapidly and at low cost, and researchers can now screen almost 4 million commercially available ZINC-5.0 compound library for binding to the BF-3 site on AR using a rational, computer-aided approach, followed by rapid cell-based screening assays to identify the most potent AR inhibitors. Collectively, this method is indeed a very promising beginning with a provisional patent applied for and with the expectation that a new class of anti-AR drugs will emerge from this work for the treatment of castration-resistant metastatic prostate cancer.

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Chapter 4

TATA binding protein-associated factor 1 (TAF1) binds and enhances androgen receptor transcriptional activity inducing receptor ubiquitination and is overexpressed with hormone ablation therapy [1]

4.1 Introduction

The androgen receptor (AR) plays a crucial role in the growth and development of prostate cancer (1,2). In the absence of androgens, the AR exists in the cytoplasm as a transcriptionally inactive multiplex of chaperones (3). Upon androgen binding, it undergoes a conformational change, forms homodimers and is translocated into the nucleus where it binds cognate response elements upstream of target genes (4,5). The AR recruits coregulator proteins, chromatin-remodeling complexes (6-8) and also components of the general transcription machinery including TFIIF (9) and TFIIH (10), that ultimately results in initiation and elongation of transcription.

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[1] A version of this chapter has been submitted and provisionally accepted in the Molecular Endocrinology journal for publication. Peyman Tavassoli**,** Latif A Wafa, Helen Cheng, Amina Zoubeidi, Ladan Fazli, Martin Gleave, Robert Snoek, Paul S. Rennie (2009) TATA binding protein-associated factor 1 (TAF1) binds and enhances androgen receptor transcriptional activity inducing receptor ubiquitination and is overexpressed with hormone ablation therapy, Molecular Endocrinology.

Dr. Latif Wafa (former PhD student with Dr. Rennie) and Helen Cheng (research assistant with Dr. Rennie) isolated the TAF1 protein using the repressed transactivator yeast two-hybrid system. Dr. Wafa also played a senior role in the experimental design and data interpretation of this manuscript. Dr. Amina Zoubeidi **(**a postdoc with Dr. Gleave) and Dr. Rob Snoek (former research associate with Dr. Rennie) provided critical comments. Dr. Snoek also purified FLAG-AR from HeLa-AR cells. Dr. Ladan Fazli (a research pathologist at the Vancouver Prostate Centre) performed tissue microarray staining and visually scored the array. Dr. Martin Gleave at the department of Urologic Sciences (UBC) founded the human prostate cancer tissue microarray and collaborated with our lab. Robert Bell (biostatistician at the Vancouver Prostate Centre) performed statistical analysis of the NHT-tissue microarray data.

The AR is a member of the steroid receptor family that shares common functional domains and structures (11,12). This family of receptors has (i) a ligand-binding domain (LBD) located in the C-terminal region (ii) a hinge region (iii) a centrally located DNA binding domain (DBD), and (iv) an N-terminal domain (NTD). Between members of this family, the NTD domain has the highest degree of amino acid sequence variability, suggesting that this region has a major role in AR-specific transcription regulation (13-16). To identify novel NTD-interacting proteins, we employed the Tup1 repressed transactivator (RTA) yeast two-hybrid system (17), which has previously been used by our laboratory to identify L-dopa decarboxylase and cyclin G-associated kinase as AR-interacting proteins (18,19). Using this system, TATA binding protein-Associated Factor 1 (TAF1) was identified as a previously unreported AR-interacting protein.

TAF1 (formerly referred to as TAF II250 or CCG1) is part of the TFIID complex (Transcription Factor IID), which consists of TATA binding protein (TBP) and approximately 15 TBP associated factors (TAFs). TAFs, including TAF1, mediate activator-dependent transcription in a promoter and tissue specific manner (20-22). The TAF1 gene contains 38 exons which span 98 kb of genomic DNA on chromosome X and encode approximately 6 kb mRNA. The TAF1 protein possesses intrinsic protein kinase activity (23), histone acetyltransferase activity (HAT; (24)), and ubiquitin-activating/conjugating activity (E1/E2; (25)). The TAF1 kinase is bipartite, consisting of N- and C-terminal kinase domains (NTK and CTK, respectively). TAF1 is capable of autophosphorylation as well as specific phosphorylation of TFIIF (23), p53 (26), and the MDM2 proto-oncogene (27). TAF1 also binds and modulates transcriptional activity of proteins such as c-Jun (28), MDM2 (29), and cyclin D1 (30) which are known to influence AR activity and hence prostate cancer progression (31-33).

In this study, the expression of TAF1 was assessed in prostate cancer tissue at different stages of androgen withdrawal by neo-adjuvant hormone therapy (NHT) using tissue microarray analysis. The result showed that increased TAF1 expression was associated with duration of NHT, suggesting a role for TAF1 in castration-resistant prostate cancer. GST pull-down assays were performed and confirmed that TAF1 binds to the NTD of AR through the E1/E2 and HAT domains. In addition, we demonstrated by co-immunoprecipitation and ChIP assays that TAF1 and AR bind in the nucleus and associate with the androgen response element (ARE) in the proximal promoter of the prostate specific antigen (PSA) gene in the presence of androgen. We also found that TAF1 acts as a coactivator and enhances AR transcriptional activity through its NTK and E1/E2 domains without influencing the general transcriptional activity of a non-androgen responsive promoter. By using in-cell and *in vitro* ubiquitination assays, our findings also suggest that TAF1 can ubiquitinate AR.

4.2 Material and methods

4.2.1 NHT tissue microarrays and immunohistochemistry

Archival formalin-fixed, paraffin-embedded human prostate tumour specimens were used to construct a human prostate cancer tissue microarray of hormone naive, NHT-treated and castration-resistant samples as described previously (18,34). Briefly, a total of 112 samples were obtained from radical prostatectomy, transurethral resections or warm autopsy. Specimens were chosen so as to represent various treatment durations of androgen withdrawal therapy ranging from no treatment (n=21), < 3 months (n=21), 3-6 months (n=28), > 6 months (n=28), and castration-resistant tumours (n=14). Cancer sites in donor paraffin blocks were identified by a pathologist (L. F.) using matching H&E reference slides, and the tissue microarray was constructed in triplicate cores, each 0.6 mm in diameter. For staining, tissue sections mounted on

the slides were de-paraffinized by xylene, rehydrated through ethanol washes, and then permeabilized in a solution of 0.02% triton-X 100. Slides were then steamed in citrate buffer (pH =6), cooled for 30 min, washed in PBS, and incubated in 3% hydrogen peroxide for 10 min to promote antigen retrieval. After blocking in 3% BSA for 30 min, slides were then incubated overnight with anti-TAF1 (abcam, ab17360) or IgG as a negative control at a working dilution of 1/2000 in 1% BSA. The following day, the slides were washed extensively with PBS, and developed using the LSAB+ kit detection system (Dako Corporation, Mississauga, Canada). Nova Red chromogen was applied, and hematoxylin counterstaining was performed (Vector Laboratories Inc, Burlingame, CA).

4.2.2 Plasmid construction

pCS2+HA-hTAF1, kindly given by Dr. R. Tjian (University of California) and Dr. Wong (University of Washington), was used as a template to sub-clone four TAF1 fragments (NTK_{52}-1441, $HAT_{1442-3102}$, $E1/E2_{22215-3777}$, $CTK_{4252-5730}$) into pcDNA3.1-V5/His expression vectors (TOPO1-TA Expression Kit, invitrogen). The following primers were used to PCR amplify each domain of TAF1: the NTK domain: Forward: CACCATGGGACCCGGCTGCGATTTG; Reverse: AGTCATGCTAGAAGGAAGCCAGCC; the HAT domain: Forward: CACCATGAATGCGAT GGCTTACAATGTT; Reverse: ACGAAGGTCTGCATCTGTTCCTGT; the E1/E2 domain: Forward: CACCATGGTAAAGAACTATTATAAACGG; Reverse: TCGCATCTCTTCCCGATGTTGTTC; the CTK domain: Forward: CACCATGGACCCTATGGTGACGCTGTCG; Reverse: TTCATCAGAGTC CAAGTCACTGTC. The full-length human AR (pcDNA3.1-hAR) and GST-fusion constructs of AR were generated as described before (18).

4.2.3 GST pull-down assay

GST fusion proteins of various AR domains (NTD_{1-559}, $DBD_{541-665}$, $LBD_{649-919}$) were expressed in the BL21 cells *Escherichia coli* strain, purified and immobilized onto glutathione-agarose beads (Sigma) (18,19). Purified GST-AR domains were analyzed by SDS-PAGE followed by Coomassie Blue staining to ensure equimolar amounts of each fusion protein were used, as described before (19). Using the Quick Coupled T7 TNT *in vitro* transcription/translation kit (Promega Corporation), [^{35}S]-Methionine-labeled human full length TAF1 and the four truncated TAF1 peptides were generated and incubated with equimolar amounts of GST-AR fusion proteins that were pre-coupled to glutathione-agarose beads. GST alone was used as a negative control to assess nonspecific interactions of radiolabeled proteins. Binding reactions were carried out for 2 hr at 4°C in GST-binding buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 5 mM MgCl2, 1 mM EDTA and 0.05% NP-40). GST-beads were then washed 4 times with ice-cold binding buffer, re-suspended in protein sample buffer (400 mM Tris Cl, pH, 8.8, 2% SDS and 5% β-mercaptoethanol), and then resolved by SDS-PAGE electrophoresis and analyzed by autoradiography. Dried gels were also analyzed using Phosphorimaging screen. Quantity One software was used to obtain [CNT∗mm2] data (counts/mm2) for radiolabelled protein bands. All pull-downs were done in triplicate and the average [CNT∗mm2] was normalized as a percentage input bound.

4.2.4 Cell culture

LNCaP human prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manasa, VA) and grown in RPMI 1640 media supplemented with 5% fetal bovine serum (FBS) (GibcoBRL). PC3 human prostate cancer cells (ATCC) and HeLa cervical cancer

cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. All cells were maintained at 37°C in 5% CO2.

4.2.5 Co-immunoprecipitation and Western blot

LNCaP cells were plated on 10-cm dishes and grown to 70% confluency. Cells were then transfected with pCS2+HA-TAF1 vector (6 μg/dish) using Lipofectin Reagent (Invitrogen) according to the manufacturer's instruction. After 16 hr incubation with transfection mix at 37°C, medium was changed to 5% charcoal stripped serum (CSS) RPMI and incubated for a further 24 hr to deplete cells of bio-available hormone. Cells were then treated with or without 1 nM R1881 for another 4 h before harvest. Using an Active Motif Co-IP kit (Carlsbad CA), nuclear proteins were extracted and then incubated with DNase according to manufacturer's protocol. 0.1 mg of nuclear extracts (10%) as quantified by BCA assay (PIERCE Biotechnology) was saved as inputs. 1 mg of nuclear proteins was then incubated with a mouse monoclonal anti-TAF1 antibody (Santa Cruz, 63B) or with an equivalent amount of normal mouse IgG (negative control) overnight. Recombinant Protein A/G–agarose (Santa Cruz) beads were used to immunoprecipitate antibody–protein complexes. Beads were washed four times with Active Motif washing buffer (150 mM NaCl, 1% detergent) and then re-suspended in 2×SDS sample buffer. Associated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, U.S.A.) as described before (19). Membranes were blocked in 5% skim milk in Tris-buffered saline (20 mM Tris/HCl, pH 7.6/140 mM NaCl) prior to incubation with the appropriately diluted primary antibody. AR and TAF1 were detected using mouse monoclonal antibodies (Santa Cruz, AR-441, TAF1-63B, respectively). Blots were developed using horseradish peroxidase-conjugated secondary antibody and the ECL chemiluminescence kit (Amersham Biosciences).

4.2.6 Chromatin immunoprecipitation assay

LNCaP cells were grown for three days in 5% CSS RPMI and then untreated or treated with 1 nM of R1881 for 4 hr. Cells were subsequently cross-linked with paraformaldehyde and sonicated to achieve a DNA smear between 200-1000 bp. Chromatin immunoprecipitation (ChIP) assay was performed using EZ ChIP kit according to the manufacturer's protocol (Upstate) on the β-actin and PSA genes, as previously described (35). For PCR, 2 µl out of 50 µl DNA extraction were used in 25 cycles of amplification. The primer sequences were as follows: the proximal promoter of the PSA: forward: TCTGCCTTTGTCCCCTAGAT, reverse: AACCTTCATTCCCCAGGACT; the non-promoter region of PSA: forward: CTGTGCTTGGAGTTTACCT GA, reverse: GCAGAGGTTGCAGTGAGCC; and β-actin primers: forward: TCCTCCTCTTCCTCAATCTCG, reverse: AAGGCAACTTTCGGAACGG. AR-C-19 and TAF1-63B antibodies (Santa Cruz) were used for chromatin immunoprecipitation.

4.2.7 Transcriptional assays

Cells were seeded in six-well plates and, for PC3 cells, grown to 90% confluency. Using Lipofectin Reagent (Invitrogen), cells were co-transfected with increasing amounts of pHA-hTAF1, 1.5 µg/well full length AR (pAR6), 0.2 µg/well pARR3-tk-Luc and 0.1 µg/well pRLtk-Renilla. For LNCaP cells, cells were co-transfected at 70% confluency with 1 µg/well pPSA-Luc (-6,100/+12; gift from Dr. J. Trapman, Erasmus University, Rotterdam, the Netherlands), and 1 µg/well pHA-TAF1 in addition to pRLtk-Renilla as mentioned above. The total amount of DNA was adjusted to 3 µg/well using vector (pCS2+). Cells were incubated with the transfection mix for 16 hr at 37°C and subsequently replenished with 5% CSS RPMI containing 1 nM R1881 or vehicle alone. Transfected cells were cultured for an additional 24 hr prior to harvesting for luciferase and Western blot analysis. For siRNA-mediated TAF1 reduction, two 21 nucleotide

siRNA duplexes with 3´dTdT overhangs corresponding to TAF1 mRNA (5´-AAGACCCAAACAACCCCGCAT-3´and 5´-AACTACGACTACGCTCCACCA-3´) (26) were synthesized and purchased from Applied Biosystem (Ambion) together with control siRNA. At 40% confluency PC3 or LNCaP cells were co-transfected with 5 and/or 10 nM of siRNA duplexes, pRLtk-Renilla and pARR3tk-Luc or pPSA-Luc as described above, except cells were allowed to grow for 48 hr in 5% CSS media ± 1 nM R1881. Luciferase activity was normalized to protein concentration as quantified by BCA protein assay. Each assay was done in triplicate, and experiments were repeated at least three times.

4.2.8 Purification of His-tagged ubiquitin conjugates

LNCaP cells, grown in 5% CSS RMPI for 24 hr, were co-transfected with 2 µg of plasmid expressing His-tagged Ubiquitin (pHis6-ubiquitin, ATCC) and 6 µg of pHA-TAF1, pV5-E1/E2, or empty vector using Lipofectin Reagent. After 16 h, the medium was changed to 5% CSS RPMI ± 1 nM R1881 for 24 hr followed by 6 h treatment with 10 µM MG132 (Calbiochem) or vehicle. Cells were then scraped and lysed in 1 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris/HCl pH 8). 5% of the cell lysate was used as input and the remainder was mixed with 50 µl Ni2+-NTA-agarose beads (Qiagen) as described before (36). The mixture was allowed to rotate at 4ºC for 3 hr and a magnet holder was used to pull-down His6-ubiquitinated conjugates. Beads were then washed four times with RIPA buffer and re-suspended in 2×SDS sample buffer. Associated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane and blotted for antibodies against AR (Santa Cruz, AR-441), MDM2 (Santa Cruz), HA (Covance), V5 (Invitrogen), and β-actin (Sigma).

4.2.9 Ubiquitination assay

LNCaP cells were cultured, harvested and then lysed in RIPA lysis buffer. Nuclear proteins of HeLa cells were extracted as described elsewhere (37). Briefly, HeLa cells were harvested into cold phosphate buffered saline (PBS) and ~10^7 cells were pelleted and resuspended in 500 µl cold hyotonic buffer (10 mM HEPES-KOH pH 7.9 at 4ºC, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF). The cells were incubated on ice for 10 min, and then vortexed for 10 seconds. The sample was centifuged, the supernatant discarded, and the pellet was resuspended in 100 µl of hypertonic buffer (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min for high salt extraction. The supernatant fraction, which contains nuclear proteins, was subjected to IP using antibody against TAF1 or IgG and a modified in-solution ubiquitination assay was performed (38). Briefly, 1 mg protein of the whole cell lysate from LNCaP cells was incubated in HEMG buffer (final concentration: 25 mM Hepes–KOH, pH 8.0, 12.5 mM MgCl2, 0.1 mM EDTA, 400 mM KCl, 10% glycerol) containing 1 mM ATP and 5 µl ^{35}S-ubiquitin in the presence of immunoprecipitated TAF1 or control mouse IgG. After rotating for 1 hr at room temperature, AR was immunoprecipitated and subjected to autoradiography or Western blot analysis with antibodies against AR (Santa Cruz, N-20) and ubiquitin (Santa Cruz, Ubi-1).

***In vitro* kinase assay**

AR was purified from HeLa cells that stably express FLAG-tagged AR (FLAG-AR) using an anti-FLAG affinity column (Sigma-Aldrich). Kinase assays were carried out as described previously (39). In brief, HeLa cells were transfected with expression vectors for HA-TAF1 and grown in complete media (DMEM + 5% FCS) for 48 hr prior to harvesting for whole cell protein

extracts. Cell lysis was carried out in RIPA buffer, 1X protease inhibitor cocktail (Complete™, Roche Applied Science) and 2 μM microcystin (Biomol Research Laboratories)) for 30 minutes on ice, followed by centrifugation at 13,000 rpm to remove cellular debris (20 minutes, 4ºC). Pre-clearing of protein lysates was accomplished with normal mouse IgG (Santa Cruz Biotechnology, Inc.) bound to Protein A/G beads (Santa Cruz Biotechnology, Inc.). TAF1 was then immunoprecipitated with 4 μg of anti TAF1 antibody (Santa Cruz) over night at 4ºC; normal mouse IgG (4μg) was used as negative control. Immunocomplexes were pulled-down with Protein A/G beads and unbound protein was washed away with lysis buffer. Protein complexes were washed an additional 2 times with Kinase Wash Buffer (50 mM Hepes pH 7.0, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM Na_3VO_4, 2 μM microcystin). A typical 25 μL kinase reaction included: Protein A/G-bound TAF1 (or the IgG negative control), 50 mM Hepes pH 7.0, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM Na_3VO_4, 2 mM NaF, 2 μM microcystin, 200 mM [γ^{32}P]-ATP and/or FLAG-AR. Kinase reactions occurred for 20 minutes at 30ºC and were terminated by addition of 5X SDS sample buffer followed by boiling at 95ºC for 5 minutes. Phosphorylated protein were resolved by SDS-PAGE and analyzed by phosphorimagery (Typhoon™, Amersham Biosciences). ERK enzyme (New England Biolab) was used as a positive control.

4.2.10 Statistical analysis

Student's t-test (two sided) was used for statistical analysis of transactivation assays and GST pull-down assays. Wilcoxon rank test was used for tissue microarray analysis. $p < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 TAF1 expression increases with prolonged hormone treatment and with progression to castration-resistant prostate cancer

Recent evidence suggests that AR-specific gene regulation may occur as a consequence of interactions with unique coregulatory proteins (40). Since the NTD of AR is the least conserved nuclear receptor domain, protein interactions in this region may dictate receptor-specific coregulation capacity. To identify novel AR-interacting proteins, the NTD of AR was used as bait in the RTA yeast two-hybrid system (17) to screen a LNCaP prostate carcinoma cell line cDNA library. The RTA system allows for screening with a bait protein that has intrinsic transactivation function, such as the NTD of AR (19). We have identified several clones that code for AR-interacting proteins, one of which was the COOH-terminus of TAF1 [(a.a. 1118-1893) (bp: 3406- 6138), GenBankTM accession number NM 004606].

We next wanted to identify whether TAF1 has a role in advanced prostate cancer. The TAF1 expression level during disease progression was assessed in patients who had undergone varying lengths of neo-adjuvant hormone therapy (NHT) prior to radical prostatectomy or autopsy using NHT tissue microarrays (18,41). Each NHT array is comprised of 336 tumour biopsies, which were obtained from triplicate cores of 112 tumours. A control tissue microarray slide was incubated with normal IgG, as described in Material and Methods. Staining intensity was scored visually by a pathologist on a scale from 0 to 3, ranging from no staining (score 0) to very intense staining (score 3) (18,34). Fig. 4.1A shows representative histology pictures of four test groups (<3 months NHT, 3-6 months NHT, >6 months NHT, and castration-resistant state) and Fig. 1B shows visual scoring analysis of the whole NHT array. Interestingly, we found the longer the NHT treatment, the higher the level of TAF1 protein. TAF1 expression of individual cores,

was compared between the different treatment groups and its level was found to be significantly higher in the 3-6 months NHT over the untreated group (Fig. 4.1B). Furthermore, there was an additional increase in TAF1 expression with longer NHT and with castration-resistant progression. Thus, increased levels of TAF1 expression are associated with progression to castration-resistant, and may have potential clinical value as a biomarker or a therapeutic target for advanced prostate cancer.

4.3.2 TAF1 interacts with the N-terminus of AR mainly through its HAT and E1/E2 domains

To confirm the AR/TAF1 interaction and to determine the domains involved, GST pull-down assays were performed using GST-fusion protein with AR/NTD$_{1\text{-}559}$, DBD$_{541\text{-}665}$, or LBD$_{649\text{-}919}$ (Fig. 4.2). Purified GST-AR domains were verified for purification and concentration by SDS-PAGE followed by Coomassie Blue staining (Fig. 4.2A). Equimolar amounts of non-degraded protein were determined for each of the GST protein products (* in Fig. 4.2A) and used in pull-down assays to assess relative TAF1 binding to AR domains, as described before (19). Radiolabeled TAF1 and TAF1 truncated domains were generated *in vitro* and analyzed by SDS-PAGE followed by autoradiography (Fig. 4.2B). The presence of multiple bands for radiolabeled proteins is due to the presence of several ATG sites within hTAF1 cDNA. Radiolabeled TAF1 (Fig. 4.2B, lane 1) was allowed to interact with GST protein or GST-AR fragments bound to agarose beads. As shown in Fig. 4.2C (top row), TAF1 did not interact with GST alone (lane 2), whereas it did bind to all domains of AR (lanes 3-5), with the strongest interaction being with the NTD of AR (lane 3).

GST pull-down experiments were repeated with radiolabeled TAF1 domains (NTK, HAT, E1/E2, and CTK proteins; Fig. 4.2B) to identify domains essential for interaction between TAF1

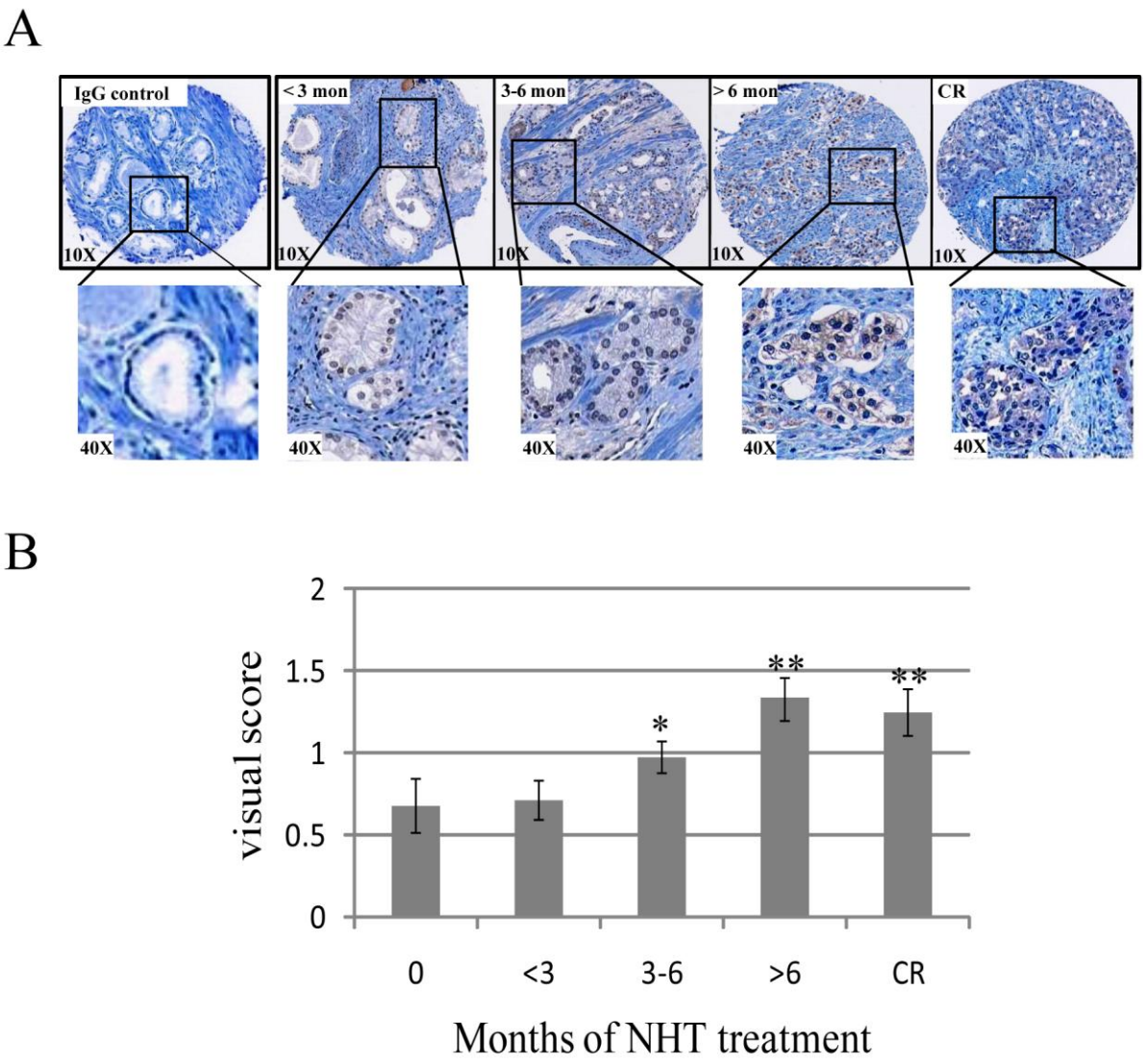

Figure 4.1. Tissue microarray analysis TAF1 expression in prostate cancer. *(A)* A NHT tissue microarray was stained with an antibody that recognizes TAF1 (abcam). Staining intensity was scored from 0 to 3 by a pathologist. Slides were visualized under 10X magnification and further magnification (40X) of delineated areas is shown. *(B)* Visual score of samples with TAF1 staining intensity of 0–3 is given for each treatment group. * indicates that there is significant difference over 0 months. ** indicates that there is significant difference over 3-6 months.

and AR (Fig. 4.2C, rows 2-5). All domains except the NTK of TAF1 bind to GST fusion AR proteins with different affinities. We performed the above pull-down assay repeatedly with two more independent experiments and calculated an average percentage of total input bound to the GST–AR domains ([CNT*mm2] bound/[CNT*mm2] input), as described in materials and

methods. A summary of the quantified GST pull-down data (percentage of the input bound) for these TAF1 truncations and the full-length protein is shown in Fig. 4.2D. Similar to the full-length protein, HAT and E1/E2 domains interact most strongly with the NTD of AR, but the HAT domain binds this region with 1.5-times higher affinity. Although the CTK domain was originally identified as binding to the N-terminus of AR by the RTA assays, this domain interacts more strongly to the DBD and LBD domains of AR. Taken together, these data demonstrate that TAF1 interacts directly with AR, confirming our RTA screening result. In addition, mapping the interaction domains of TAF1 and AR suggests that the HAT, E1/E2 and CTK domains of TAF1 are all involved in binding to AR. The pattern of binding of HAT and E1/E2 domains is similar to that seen with full length TAF1, suggesting TAF1/AR interaction through these domains.

4.3.3 TAF1 interacts with AR within a prostate cancer cell line

Human TAF1 protein has a molecular weight of 250,000 and is ubiquitously expressed in normal tissues, with highest expression levels in the spleen and testes (42). TAF1 is expressed in nuclei of all prostate cancer cell lines including LNCaP human prostate cancer cells (data not shown). Since there are strong interactions between TAF1 and AR *in vitro* and both are expressed in LNCaP cells, these cells were used to confirm TAF1/AR interaction by co-immunoprecipitation assays. LNCaP cells were first transfected with HA-TAF1 and treated with or without 1 nM of the synthetic androgen R1881. Using the Active Motif Co-immunoprecipitation (IP) kit, nuclear extracts of cells were subjected to IP with an anti-TAF1 antibody and analyzed by Western blot for AR and TAF1 (Fig. 4.3A). The levels of TAF1 expression are the same in the presence or absence of hormone (lanes 1 & 5, upper panel) or after pull-down (lanes 4 & 8, upper panel). However, the total amount of nuclear AR is increased upon hormone treatment (lane 5 relative to lane 1, lower panel) and there is more AR co-

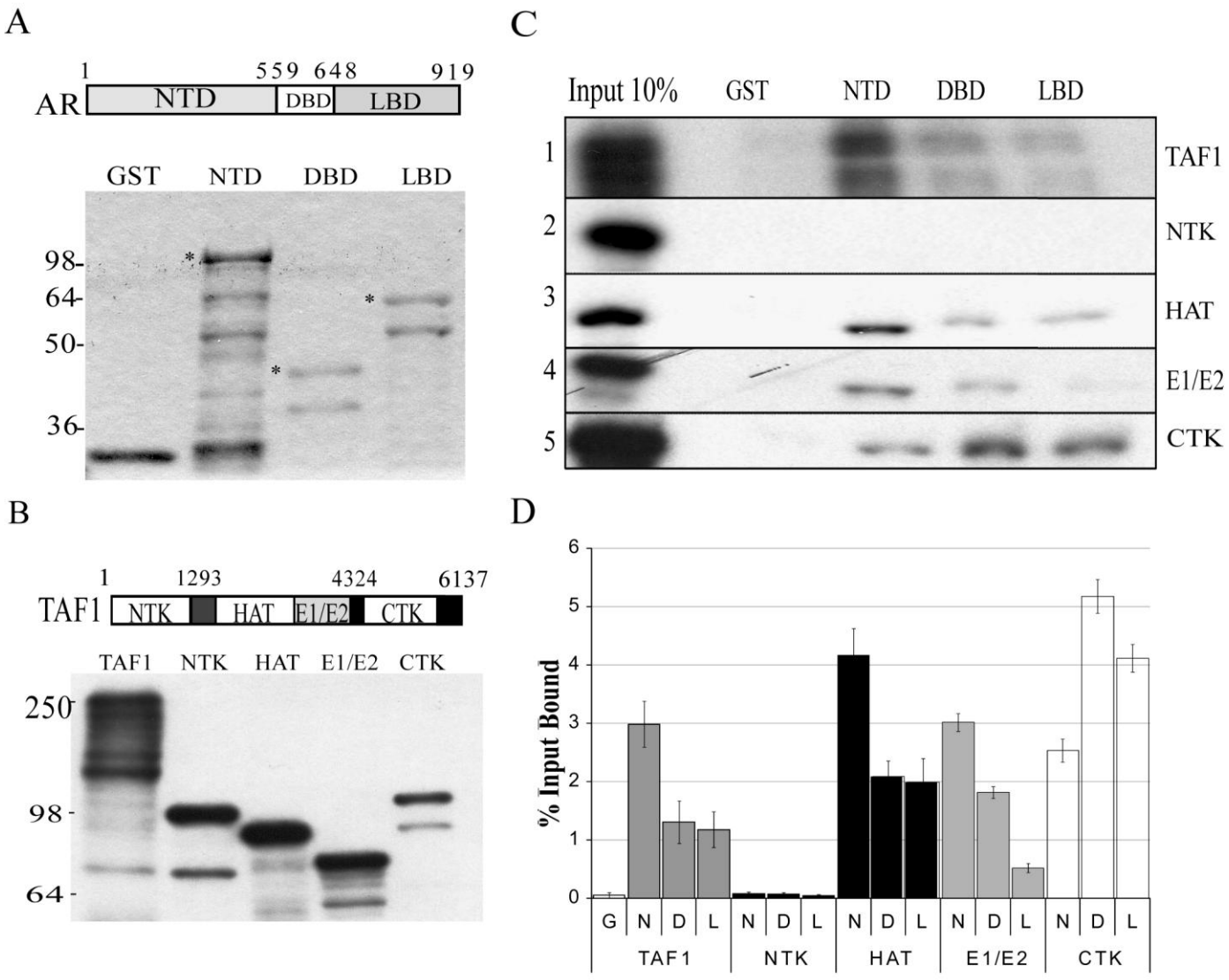

Figure 4.2. TAF1 binds AR through HAT and E1/E2 domains *in vitro*. *(A)* GST-fused AR domains (N-terminus =NTD, DNA binding domain =DBD, ligand binding domain =LBD) were expressed in E. Coli BL21 and purified using glutathione beads. Fusion protein-bound beads were eluted with sample buffer and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. The eluent in each case was run alongside known amounts of BSA (ranging from 250 to 1000 ng) to generate a standard curve for protein concentrations. Equimolar amounts of non-degraded proteins (*) were used in GST-pull down assays. *(B)* [^{35}S] Radiolabeled TAF1 and its domains (N-terminal kinase (NTK), Histone acetylation (HAT), Ubiquitin activating conjugating (E1/E2), and C-terminal kinase (CTK) were generated using *in vitro* Transcription/Translation kit and autoradiographed. *(C)* GST pull-down assay. Equivolume of [^{35}S] labeled TAF1, [^{35}S] labeled NTK, [^{35}S] labeled HAT[^{35}S] labeled E1/E2, [^{35}S] labeled CTK were incubated with GST-AR fragments bound to agarose beads. GST alone coupled to agarose beads was used as negative control. *(D)* Dried gels were also analyzed using a Phosphorimaging screen. Quantity One software was used to obtain data (counts/mm2) for radiolabelled protein bands. All pull-downs were done in triplicate, normalized as a function of the percentage input bound, and averaged. G =GST; N = NTD; D = DBD; L =LBD.

immunoprecipitated with TAF1 in the presence of hormone (lane 8 compared to lane 4, lower panel).

Since TAF1 and AR interact in the nucleus and TAF1 is an essential player in the transcriptional activity of a number of genes (43), we next investigated whether TAF1 can associate with AR on the promoter of PSA using ChIP assays (Fig. 4.3B). DNA and proteins of untreated LNCaP cells or from those treated with 1 nM R1881 were cross linked, lysed, sonicated, and then immunoprecipitated with anti-TAF1 antibody (row 2), AR (row 3), or an equivalent amount of normal rabbit/mouse IgG as negative controls (rows 4 & 5 respectively). The DNA was purified and used as a template for PCR of the proximal PSA promoter (left panel). The result indicated that TAF1, like AR, was indeed associated with the PSA promoter and that this association was found to occur only with androgen treatment. To determine whether the binding of TAF1 to the PSA promoter is specific, the purified DNA obtained after TAF1 IP was subjected to PCR for a non-promoter region of the PSA promoter (middle panel), as well as for β-actin promoter (right panel). The absence of significant PCR products in these regions indicate that TAF1 binding to the PSA promoter was promoter- and sequence-specific.

4.3.4 TAF1 enhances transcriptional activity of the AR

To determine the impact of AR-TAF1 interaction on AR transcriptional activity within cells, transient transfection assays were carried out in PC3 cells, which were co-transfected with increasing amounts of full-length TAF1 expression vector (pHA-TAF1), a constant amount of full-length AR expression vector, along with the pARR3tk-Luc reporter plasmid. Transfected cells were stimulated with 1 nM R1881or vehicle for 24 hr and analyzed for luciferase activity (Fig. 4.4A). In the absence of exogenous TAF1, AR activity in PC3 cells was increased by 50-fold (± 7) with the addition of androgen (1 nM R1881). Increasing amounts of TAF1 enhanced

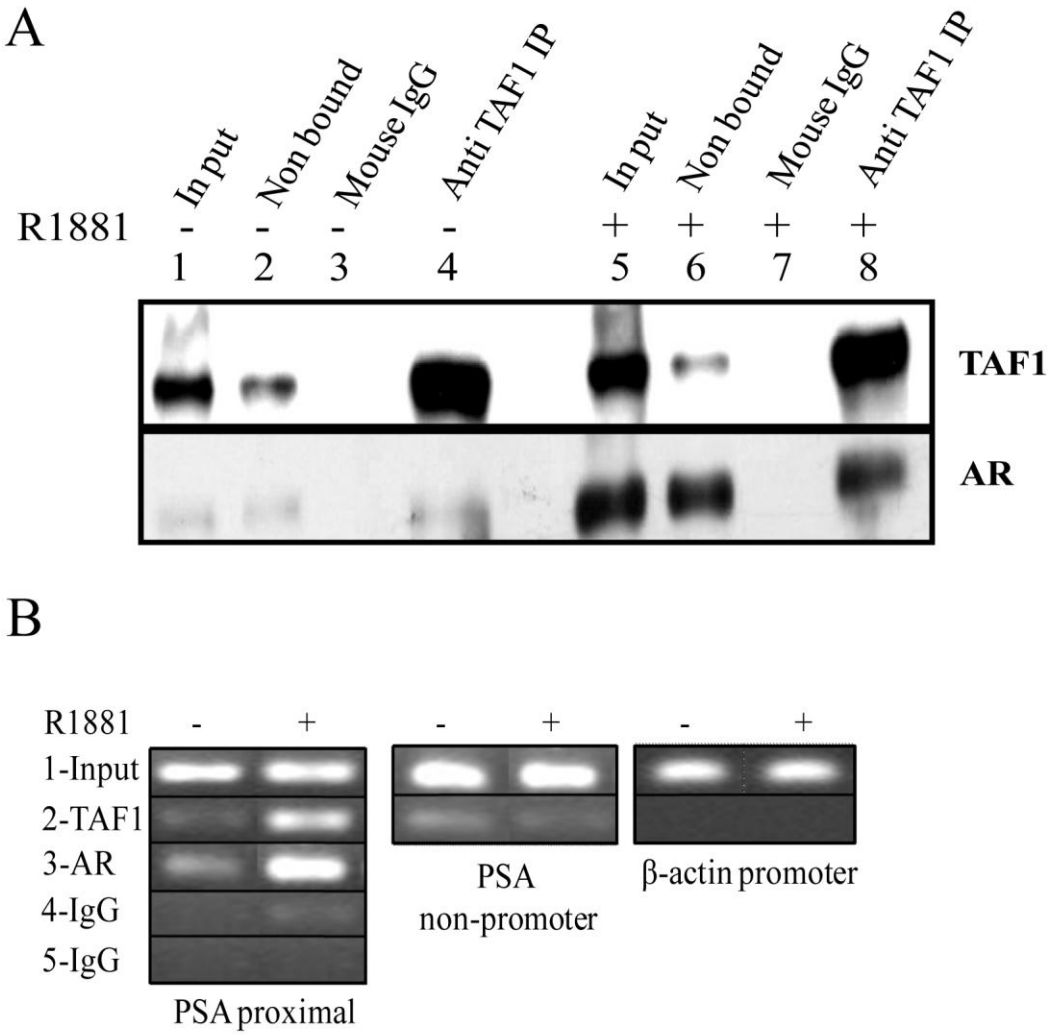

Figure 4.3. TAF1 interacts with AR within a prostate cancer cell line. *(A)* LNCaP cells were transiently transfected with HA-TAF1, grown in 5% charcoal stripped serum media (CSS) for 24 hr and then treated ± 1 nM R1881 (synthetic androgen) for 4 hr. Nuclear proteins were extracted and TAF1 was immunoprecipitated (IP) followed by Western blot analysis, probing with anti AR or TAF1 antibodies. Lanes 1 & 5 = Input (10%); lanes 2 & 6 = non-precipitated; lanes 3 & 7 = mock IgG IP; lanes 4 & 8 = anti TAF1 IP. *(B)* LNCaP cells ± 1 nM R1881 were cross linked, DNA sheared and then the protein/DNA complexes were immunoprecipitated with anti TAF1 or anti AR and the PSA proximal promoter PCR amplified. The non-promoter region of PSA and β-actin primers were used as negative controls for TAF1 enriched protein/DNA complexes.

AR transactivation by up to an additional 2.6-fold (± 0.5) ($p < 0.005$) in a dose-dependent manner when overexpressed in the presence of hormone. To confirm the results, LNCaP cells, which contain AR, were also transfected with pHA-TAF1 and the PSA-Luc reporter. Again, TAF1

could enhance AR transcriptional activity up to 2.3-fold (± 0.7) in the presence of hormone (Fig. 4.4B). To further explore the role of TAF1 in AR-mediated transcription, these transactivation assays were repeated as described above except siRNA duplexes were added to knock down endogenous TAF1 protein (Fig. 4.4C & D). Decreasing the amount of endogenous TAF1 suppressed AR transactivation in the presence of hormone in a dose-dependent manner by 3-fold (± 0.6) ($p < 0.005$) in AR transfected PC3 cells using a pARR3tk-Luc reporter, and by 2.1-fold (± 0.3) in LNCaP cells using the PSA-Luc reporter. Western blot analysis confirmed that TAF1 protein levels were modulated according to increasing levels of its expression construct or siRNA duplexes (Fig. 4.4). In addition, TAF1 protein levels are directly related to the endogenous PSA expression in LNCaP cells (Fig 4B & D). Together, these results suggest that the expression level of TAF1 correlates with AR activity.

4.3.5 AR transcriptional activity is significantly enhanced by N-terminal kinase or ubiquitin activating/conjugating domains

Since TAF1 is a member of the general transcription machinery complex, it is not surprising that it exerts its influence on promoters of different genes. In our transactivation assays, we found that increasing or decreasing TAF1 levels also resulted in increased or decreased thymidine kinase-renilla activity (pRLtk-Renilla). To differentiate the effect of TAF1 on AR from TAF1's general effects on transcription and to determine which TAF1 domains are specifically involved in AR activation, we created expression vectors of the TAF1 domains and repeated the transfection experiments in LNCaP cells using the PSA-Luc reporter. In contrast to full-length TAF1, none of the individual TAF1 domains had any effect on the generic renilla construct with its pRLtk promoter, implying that general transcription is not affected (Fig. 4.5A). By comparison, while HAT and CTK domains had no significant effect on AR activity, NTK

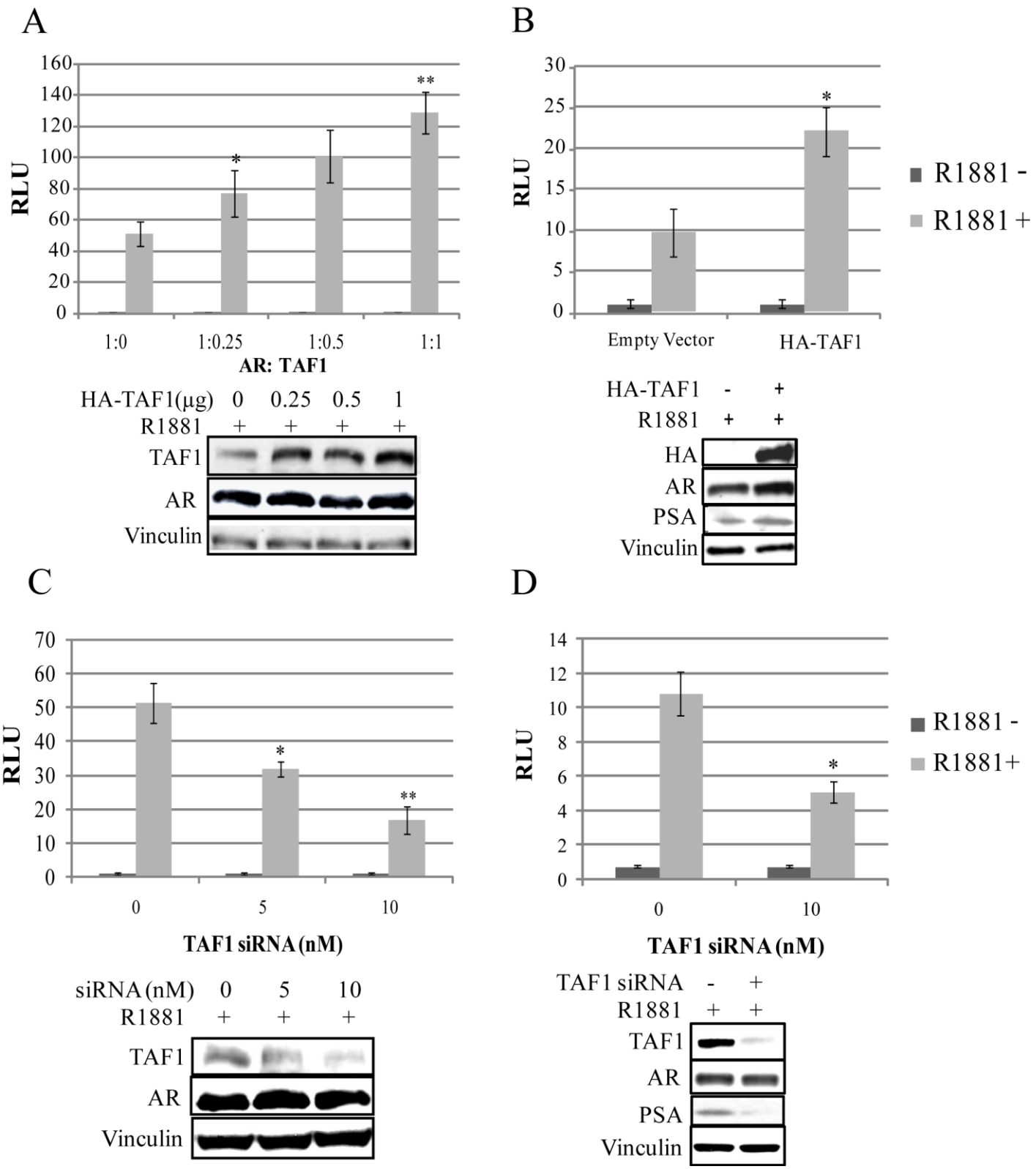

Figure 4.4. TAF1 modulates AR transactivation. PC3 cells were co-transfected with 1.5 μg/well full length AR (pAR6) and 0.2 μg/well pARR3-tk-Luc reporter, 0.1 μg/well pRLtk-Renilla, and increasing amounts of pHA-TAF1 *(A)* or increasing amounts of TAF1 siRNA duplexes *(C)*. LNCaP cells were co-transfected with 1 μg/well pPSA-Luc, 0.1 μg/well pRLtk-Renilla and 1 μg/well pCS2+HA-hTAF1 *(B)* or 10 nM TAF1 siRNA duplexes *(D)*. Transfected cells were grown in the presence or absence of 1 nM R1881 for 24 hr *(A & B)* or 48 h *(C & D)* before harvesting for luciferase assay and Western blot analysis. Luciferase units (RLU) are expressed relative to protein values for each sample. All luciferase values are given as the mean (± SEM) of triplicate readings. Graphs are representative of the 3 independent experiments. * indicates a $p<0.05$ compared to empty vector control. ** indicates a $p<0.05$ compared to *.

and the E1/E2 domains of TAF1 did enhance AR activity in an androgen dependent manner (Fig. 4.5B). NTK significantly enhanced AR transactivation by 2.4-fold (± 0.5), which is almost as much as the full-length TAF1. However, E1/E2 domain had an even greater effect, enhancing AR activity over 3.4-fold (± 0.6). Fig. 4.5C shows the relative expression levels of full-length TAF1 or its individual domains to AR and β-actin proteins.

Since NTK enhances AR transactivation, we tested the effect of apigenin, a TAF1 protein kinase inhibitor (26,27), on AR transcriptional activity. Using the MTS assay, we found that apigenin at 5 μM does not affect LNCaP cell viability (data not shown). This concentration was then used to treat LNCaP cells transfected with the PSA-Luc reporter. As shown in Fig. 4.6A, AR activity is suppressed up to 4.3-fold (± 0.6) in the presence of apigenin. We next tested whether TAF1 could phosphorylate AR, using *in vitro* kinase assays. TAF1 pulled down from HeLa cells with antibody was incubated with affinity purified FLAG-AR protein from HeLa-AR cells (44) in the presence of [γ32P]-ATP. Although we were able to show TAF1 autophosphorylates consistent with reports by others (23), we could not detect AR phosphorylation by TAF1 (Fig. 4.6B).

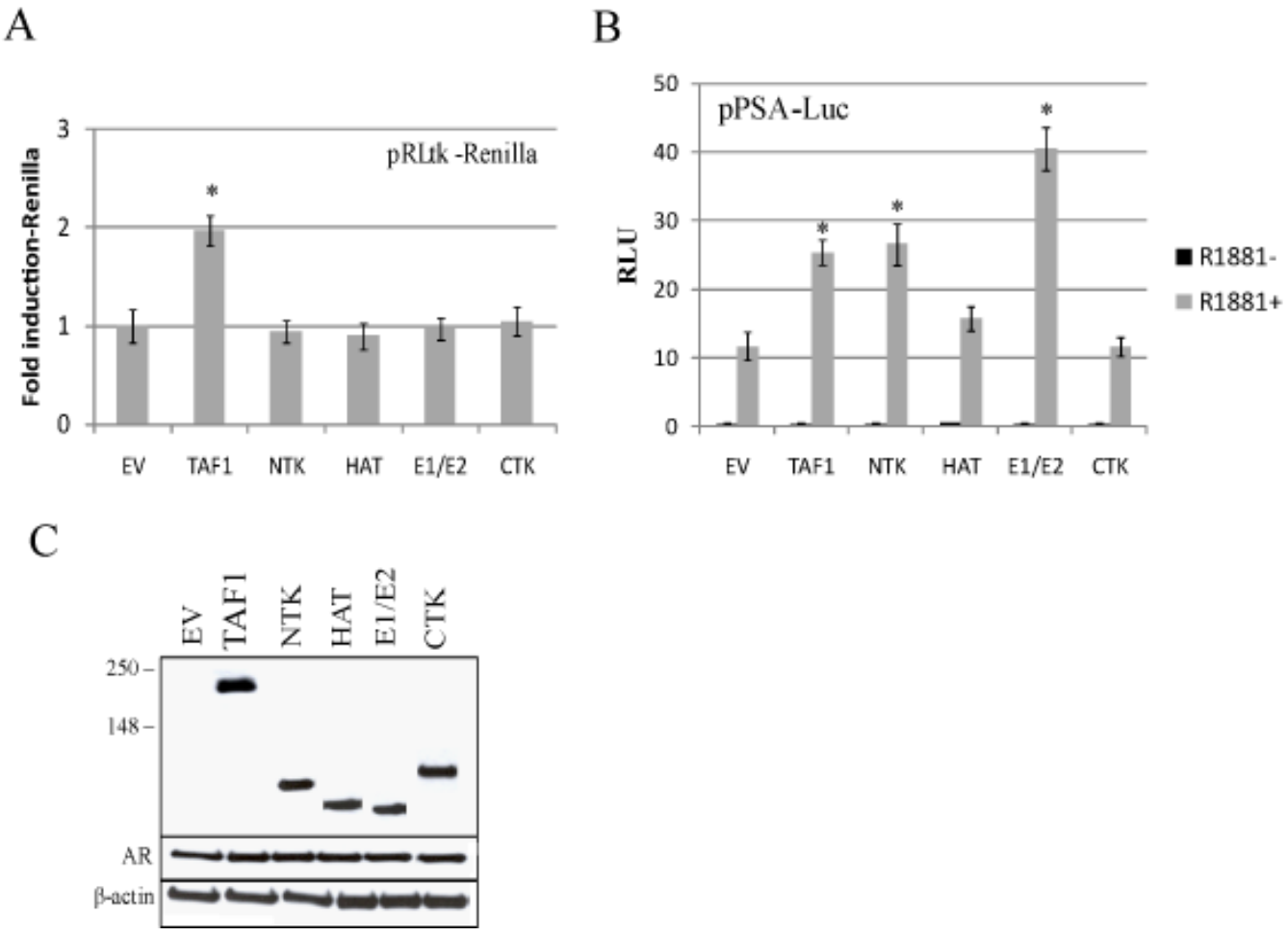

Figure 4.5. N-terminal kinase and ubiquitin activating/conjugating domains of TAF specifically enhance AR transactivation. LNCaP cells were co-transfected with either pHA-TAF1 or one of its four domains (pV5-NTK, pV5-HAT, V5-E1/E2, or pV5-CTK) (1 ug/well) and the pPSA-Luc and pRLtk-Renilla. 1 ug/well empty vector (EV) was used as a control. Transfected cells were grown in the presence or absence of 1 nM R1881 for 24 hr before harvesting for luciferase assay. *(A)* Fold-induction of renilla units in the presence of R1881 versus the empty vector plotted for TAF1 or its individual domains. *(B)* Luciferase units were normalized to protein plotted for PSA-Luciferase. * indicates a $p<0.05$ compared to empty vector control. *(C)* Western blot analysis for AR, β-actin, empty vector (EV), TAF1, NTK, HAT, E1/E2 and CTK.

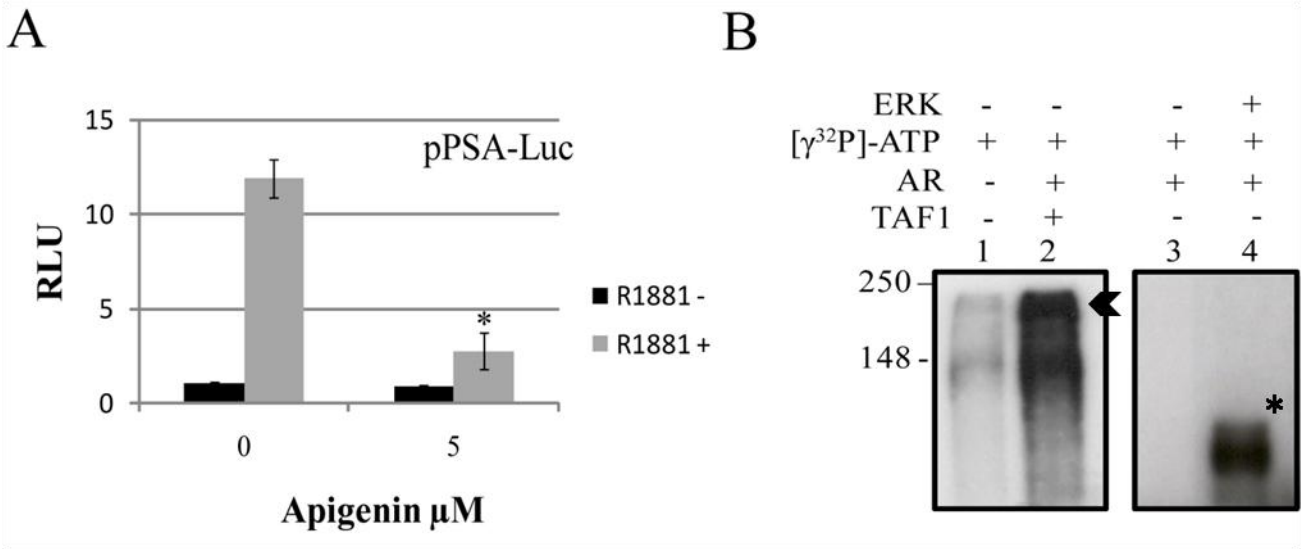

Figure 4.6. Kinase activity of TAF1. *(A)* LNCaP cells were co-transfected with the pPSA-Luc and pRLtk-Renilla. Transfected cells were treated ± 1 nM R1881 and 5 µM apigenin or vehicle for 24 hr before harvesting for luciferase assay. Luciferase units were normalized to protein and fold-induction of luciferase activity was plotted against vehicle treatment. *(B)* Transfected HeLa cells with HA-TAF1were subjected to pull-down with anti-TAF1 antibodies or IgG followed by immunoprecipitation. The protein complexes were then incubated with [γ^{32}P]-ATP alone (lane 1) and FLAG-AR (lane 2) at 30ºC for 20 min. Mock enzyme (lane 3) or ERK (lane 4) were incubated in kinase buffer with the presence of [γ^{32}P]-ATP and FLAG-AR as controls. The arrow head and asterisk show phosphorylated TAF1 and AR respectively.

4.3.6 AR is ubiquitinated by TAF1

Since NTK does not bind to AR and the CTK and HAT domains do not enhance AR transcriptional activity, we focused on the E1/E2 domain, which binds to AR and has the most profound effect on its transactivation. Ubiquitination is a post-translational modification that mediates the covalent conjugation of ubiquitin to protein substrates. The functional role of ubiquitination was originally considered to be the targeting of proteins to the proteasome for degradation. However, it is now known that ubiquitination regulates many other processes in the cell, including membrane trafficking, DNA repair, and transcription depending on which lysine is ubiquitin-conjugated (45)**.** AR is also a direct target for mono- and poly-ubiquitination (32,46).

To address whether TAF1 can ubiquitinate AR, LNCaP cells were cultured in 5% CSS media for 24 hr and then co-transfected with pHis6-Ubiquitin and either pHA-TAF1 or empty vector. Cells were then treated with 5% CSS media with or without 1 nM R1881 followed by 6 hr treatment with vehicle or MG132, a proteasome inhibitor. To show the His-ubiquitin conjugated status of AR in the presence and absence of MG132, after saving 5% input (Fig. 4.7A, lower panels), His-conjugated proteins were purified followed by a Western blot with antibody against AR. Fig. 4.7A (lanes 1 & 2) shows that in the absence of MG132 and hormone, there is no His-conjugated AR. However, very faint bands appear in the presence of hormone and are strongest with overexpression of TAF1 (lanes 4 versus 3). Lanes 5-8 show the same order of experiments in the presence of proteasome inhibitor. As expected, there are no ubiquitination of AR in the absence of hormone while the total amount of polyubiquitinated AR is increased with MG132 and R1881 treatment when TAF1 is overexpressed (lanes 8 versus 7). Since MDM2 (an E3 ligase) is involved in the poly-ubiquitination of AR (32), we wanted to see if the MDM2 protein could also be detected in this set of experiments. Hence, the same membrane was blotted with antibody against MDM2. As shown in Fig. 4.7A (middle panels), the more ubiquitinated AR, the more MDM2 within the protein complex, indicating that TAF1 induces ubiquitination of AR through MDM2.

Given that the E1/E2 domain of TAF1 enhances AR activity, we wanted to know if this domain could also ubiquitinate AR in LNCaP cells in the same condition as mentioned above, except the truncated E1/E2 of TAF1 was transfected instead of the full-length protein. Fig. 4.7B shows a drastic increase in the level of ubiquitinated AR once the E1/E2 is expressed in the presence of MG132 and R1881 (lane 4 versus 3). Together, these experiments indicate that TAF1 can ubiquitinate AR and its E1/E2 domain is sufficient to ubiquinate AR.

To assess whether TAF1 is able to directly ubiquitinate AR, an *in vitro* ubiquitination assay was performed (38). As an abundant source of endogenous TAF1, HeLa cells were subjected to pull-down with anti-TAF1 antibodies and immobilized on agarose beads. As a source for AR and E3 ligases, proteins were extracted from LNCaP cells and incubated with radiolabeled ubiquitin, 10 nM ATP and TAF1 or mock IP (negative control) at room temperature. After 60 min, AR was immunoprecipitated and subjected to autoradiography (Fig. 4.7C; left panel) and Western blot analysis for AR and ubiquitin (Fig. 4.7C; right panel). Lanes 1 and 2 are 5% inputs after incubation with ATP and ubiquitin in the absence or presence of TAF1, respectively. They show a smear of ^{35}S-ubiquitinated proteins, indicating *in vitro* ubiquitination has occurred. In the absence of TAF1, after AR antibody pull-down, two separate faint bands can be seen (lane 3) implying ^{35}S-ubiquitinated AR. However, in the presence of TAF1, there is a profound increase in the amount of radiolabeled AR (lane 4, arrow heads), indicating that TAF1 plays a direct role in the extent of ubiquitination of AR. Lanes 5-10 show a Western blot of the same experiment. Lanes 5 and 6 are 5% of inputs before IP of AR (open arrow shows non-ubiquitinated AR). Lanes 7 & 8 are immunoprecipitations of AR following incubation with ATP and radiolabeled ubiquitin. The presence of multiple bands with higher molecular weight than AR protein suggests ubiquitination of AR that is enhanced in the presence of TAF1 (lane 8 versus 7, arrows). To confirm these bands were ubiquitin-conjugated AR, the same membrane was blotted with ubiquitin antibody showing the same corresponding high molecular weight bands (lanes 9 & 10). Accordingly, the presence of TAF1 increases the amount of ubiquitinated AR (lanes 8 versus 7 & lanes 10 versus 9), confirming involvement of TAF1 in AR ubiquitination. Overall, these results validate our *in vivo* data and highlight the direct role of TAF1 in the ubiquitination of AR.

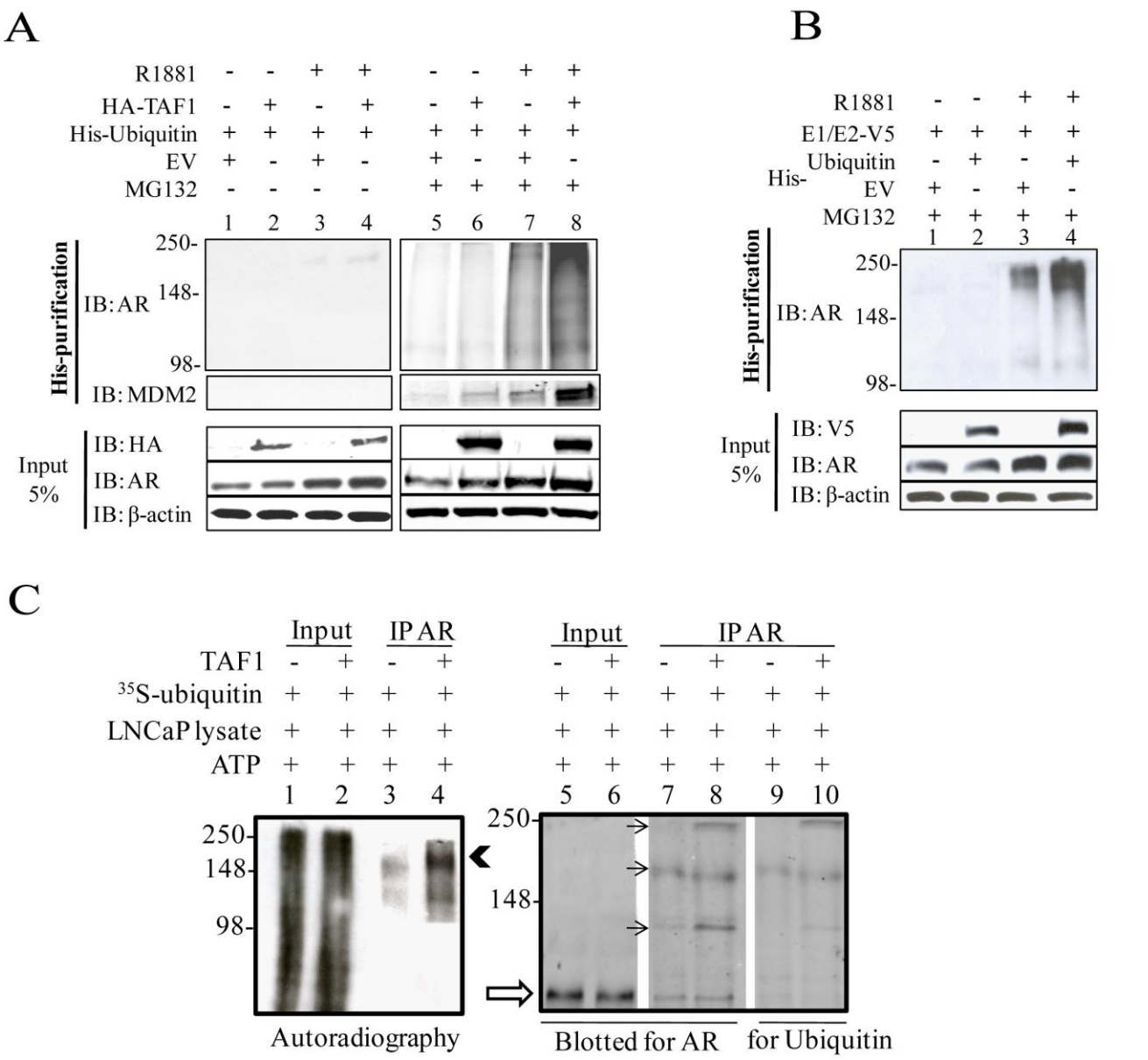

Figure 4.7. TAF1 ubiquitinates AR. *(A)* LNCaP cells were transfected with 2 µg pHis$_6$-ubiquitin and either 6 µg of pHA-TAF1 or empty vector. Cells were then treated with 5% CSS RPMI ± 1 nM R1881 followed by 10 µM MG132 or vehicle for 6 hr. After harvesting and lysing the cells in RIPA buffer, 5% of cell lysate was used as an input (lower panels) and the remainder was mixed with 50 µl Ni^{2+}-NTA-agarose beads. The mixture was rotated at 4ºC for 3 hr and then affinity pulled down followed by Western blot analysis for AR and MDM2 *(upper and middle panels)*. The input was blotted for HA, AR and β-actin. *(B)* Experiments were designed as above, except cells were transfected with the pE1/E2 instead of the full length protein. *(C)* Nuclear extracts of HeLa cells were subjected to IP using antibody against TAF1 or IgG. LNCaP cell lysate was incubated with 1 mM ATP, ^{35}S-ubiquitin in the absence (lanes 1, 3, 5, 7 and 9) or presence of TAF1 IP (lanes 2, 4, 6, 8 and 10) in HEMG buffer. After 1 hr incubation at room temperature, AR was immunoprecipitated followed by autoradiography (left panel) and Western blot for AR and ubiquitin (right panel). Lanes 1, 2, 5 and 6 = Input (5%) from LNCaP lysate before IP of AR. Arrow heads and arrows show ubiquitin-conjugated AR. Open arrow shows non-ubiquitinated AR.

4.4 Discussion

Many possible molecular mechanisms responsible for the development of castration-resistant prostate cancer have been proposed. Most involve retention of AR expression (47). The up-regulation of AR-target genes and overexpression of AR at the protein and mRNA levels support the notion that AR activity is altered in castration-resistant states (48-51). There are a variety of molecular alterations that could lead to continued or amplified AR signaling following surgical or medical castration. However, the most commonly occurring mechanisms for progression to castration-resistant are likely to be epigenetic, involving ligand-independent (or ligand-reduced) activation of AR through autocrine production of active androgens in the cancer cells, convergence of cell signaling pathways and/or altered activity and expression of AR coregulators (49,52-56). With respect to signaling pathways, several growth factors and cytokines including IGF-1, EGF, KGF, and IL-6 have been implicated, with some evidence that their activity is mediated directly or indirectly through participation of kinases such as MAPK, PKA and PKC (57-60). Using a short-hairpin RNAi to knock down AR, we reported that about 75% of ligand-independent AR-transcriptional activity ascribed to these factors required the direct participation of AR (61). In addition to phosphorylation, changes in other post-translational modifications of AR such as acetylation (62) and sumoylation (63) may also play a major role in ligand-independent activation of AR and development of castration-resistant disease. Furthermore, the levels of androgens that persist in the prostate after castration (64) may be sufficient to activate AR under conditions of aberrant expression of specific coactivator proteins (65). Hence, an understanding of coregulator protein functions is required for a full comprehension of AR transactivation in both normal and neoplastic prostate cells.

Using the RTA yeast two-hybrid system TAF1 was identified as a novel AR-NTD-interacting protein and this direct interaction was confirmed with full-length TAF1, using GST pull-down assays (Fig. 4.2C). We also found that TAF1 expression levels were increased in prostate cancer patients who underwent NHT treatment for more than 3 months (Fig. 4.1). Although some non-specific staining was observed within the cytoplasm of prostate cancer cells, stonger nuclear staining for TAF1 and the lack of cytoplasmic staining in our control slides suggest the TAF1 expression pattern in this array is valid. Mapping of the TAF1 and AR interacting domains showed that the HAT and E1/E2 domains bind strongly to AR-NTD, mimicking the full length TAF1. The CTK domain that was originally isolated by the RTA system interacts with all AR domains, but most strongly with the AR-DBD. In contrast, NTK does not have affinity for any AR domains, further indicating the specificity of these interactions (Fig. 4.2C & D). It has been reported by others that the N-terminus of TAF1 binds to the concave surface of TBP and consequently inhibits TBP/ TATA box contact, hence repressing transcription (28,66). However, binding of activators, such as c-Jun with the N-terminus of TAF1 releases this inhibition, resulting in transcription initiation (67). Accordingly, the ability of TAF1 to interact with AR through multiple domains other than NTK suggests that TAF1 may play a role in modulating AR folding and one can speculate that, upon interaction with AR, the NTK release from the concave surface of TBP will initiate transcription. This hypothesis is supported by the fact that upon overexpression of TAF1 in both PC3 and LNCaP cells in the presence of nuclear AR (hormone induced activation), AR activity is increased (Fig. 4.4A&B), whereas siRNA knock down of TAF1 suppresses AR activity (Fig. 4.4C&D).

Co-immunoprecipitation assays of the nuclear extracts of LNCaP cells demonstrate that the interaction between AR and TAF1 occurs in the presence of hormone (Fig. 4.3A). Since TAF1 is

a component of the general transcription machinery within the TFIID complex and directly associates with AR to modulate AR activity, we explored whether TAF1 co-localizes with AR at the PSA promoter. Using ChIP assays with LNCaP cells, we found that TAF1 is associated with an ARE in the proximal promoter of the PSA gene (Fig. 4.3B).

The essential nature of TAF1 can be attributed to its broad requirement during RNA-pol-II-dependent transcription (68). Indeed, about 18% of hamster cell line genes are TAF1-dependent (43,69). In our studies, we also found that TAF1 can modulate the transcription of non-androgen responsive reporters. To show the specificity of TAF1 on AR, various domains of TAF1 were tested for their inability to modulate non-androgen responsive reporters in transient transfection assays (Fig. 4.5A). Although TAF1 is a component of the transcriptional machinery and is able to modulate pRLtk-Renilla, none of its individual domains influenced this non-androgen responsive promoter. However, TAF1 through its NTK and E1/E2 domains appears to be a coactivator of AR, enhancing AR transcription (Fig. 4.5B).

The AR is a substrate for phosphorylation, acetylation and ubiquitination (6,32,46,70,71) and this makes AR a potential substrate for TAF1, which possesses all of the above enzymatic activities. We did not determine whether TAF1 can acetylate AR because no significant changes on AR activity were seen in transfection assays when the HAT domain was overexpressed (Fig. 4.5B). The NTK appears to be involved, since apigenin, a TAF1 kinase inhibitor (26,27) can suppress AR transcriptional activity by 4.3-fold (Fig. 4.6A), but we were unable to observe direct AR phosphorylation by TAF1, which suggests that TAF1 induces phosphorylation of intermediate proteins to enhance AR activity.

It has been reported that TAF1 can mono-ubiquitinate histone 1(H1) (38) and is involved in poly-ubiquitination of p53 (26,27). Since E1/E2 has the most profound effect on AR activity, we

also sought to determine whether ubiquitination of AR can be increased as a consequence of TAF1 overexpression. Interestingly, in the presence of proteasome inhibitor and expression of His-ubiquitin, both TAF1 and its E1/E2 domain enhance the total amount of ubiquitinated AR in within a prostate cancer cell line (Fig. 4.7A, lane 8 versus 7).

In addition, the E1/E2 domain alone is able to increase the total amount of ubiquitinated AR (Fig. 4.7B). This supports our transactivation data (Fig. 4.4B), in which the E1/E2 domain enhances AR activity more than 3-fold. Interestingly, TAF1 can ubiquitinate AR even in the absence of proteasome inhibitor within the cells (Fig. 4.7A, lane 4) and *in vitro* (Fig. 4.7C). Since the majority of TAF1-induced poly-ubiquitinated AR is accumulated after the proteasome inhibition and that poly-ubiquitinated AR is not functional (32,72), there are at least two possible mechanisms that could explain how TAF1 enhance AR transcriptional activity. First, TAF1 can poly-ubiquitinate AR through lysine 48, causing proteasome degradation of AR mainly through MDM2 (Fig. 6A & B). This would induce AR turnover and consequently enhance AR transcriptional activity. Second, TAF1 may induce AR poly-ubiquitianation on other lysine sites, such as K6 or K27, as recently reported with the RNF6 protein and AR (73) . This type of AR-poly-ubiquitination does not lead to AR degradation, as MDM2 does. In contrast, it can enhance AR activity through modulation of AR binding proteins/chromatin, as it was shown with p53- and Met4-poly-ubiquitination (74,75). These alternative mechanisms will be explored further in future studies.

In conclusion, our results suggest that TAF1 is a coactivator of AR that binds and enhances AR transcriptional activity most likely through ubiquitination of AR. Accordingly, an increase in TAF1 expression during NHT therapy for advanced prostate cancer, especially with treatment

extended over 6 months, could be a compensatory mechanism adapted by cancer cells to overcome lack of circulating androgens.

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Chapter 5

General discussion, conclusions and future directions

Several clinical observations followed by molecular pathology evidence from human or animal model prostate cancer samples indicate that AR signalling is active and required for castration-resistant prostate cancer (Sections 1.4 & 1.5). PSA is an exclusively AR-dependent gene and is commonly used as a biomarker for disease activity. PSA declines upon androgen ablation therapy and its subsequent rise mainly indicates the relapse of prostate cancer. A significant body of evidence indicates that tumour cells at this stage are still dependent on AR signalling, even in the most advanced hormone-refractory prostate cancer (1-5). Several mechanisms used by tumour cells to keep the AR signalling active despite androgen ablation therapy and/or in the presence of AR antagonist. These mechanisms include *i)* point mutation in AR ligand binding pocket that can result in activation by other steroid hormones or nonandrogenic factors (4) (Section 1.4.1); *ii)* amplification of the AR gene and AR protein overexpression (6) (Section 1.5.2); *iii)* expression of alternatively spliced variants of the AR that are constitutively active (7,8); *iv)* aberrant expression of AR-coregulators (9-11) (Section 1.5.4); *v)* changes in the expression of enzymes involved in steroidogenesis and de novo steroid synthesis (12,13); *vi)* "cross-talk" with other signalling pathways that promote post-translational modification of AR (14-18) (Section 1.5.3). Therefore, targeting AR transactivation is critical to achieve chemotherapeutic response in castration-resistant prostate cancer. Accordingly, recent data have shown that many patients will respond to a second round of hormone therapy, suggesting that targeting AR with novel agents could suppress or delay tumour growth (19,20)

and potentially increase survival (21). In the current research project, the following two approaches that modulate AR activity have been explored (Chapters 3 and 4).

1. Establishment of screening assays for agents that modulate growth, death, and AR activation in prostate cancer cells and its application for selecting AR antagonist compounds
2. Investigation of TAF1 as a novel AR-coactivator that is overexpressed in advanced prostate cancer

5.1 Establishment of screening assays for agents that modulate growth, death, and AR activation in prostate cancer cells and its application for selecting AR antagonist compounds

Genetically modified LNCaP cells that stably express EGFP either constitutively or upon AR activation (Section 2.2.2) were treated with a variety of agents, and then monitored by fluorescence and MTS assays for dose-dependent changes in cell number and AR activity (22). The assays were validated for rapid, fluorescence-based, quantitative measurement for the presence of growth and AR modulators (Section 2.3). Using these assays, we found that osteoblast conditioned media enhanced prostate cancer cell growth, but not AR activity (Section 2.3.4). After priming with androgen (<1 nM R1881), forskolin or the pesticide dichlorvos, AR activity was enhanced, whereas it was inhibited by interleukin 6.

These non-destructive, cell-based assays enabled rapid systematic monitoring of the effects of drugs or complex mixtures on prostate cancer cell growth and /or AR activity in real-time. It has to be emphasized that the AR-reporter in this system is chromosomally integrated, which makes it a more appropriate model than transient transfection assays. However, there are some technical

aspects that limit its widespread application. First, the expression of EGFP as a read-out decreases after the 10^{th} passage or repeated freezing and thawing. To circumvent this problem, cells have to be sorted for high fluorescence levels by FACS after several passages. In addition, this system would be more robust if a low passage LNCaP cell line is used for viral infection. The second limitation is the lack of sensitivity. Even though the above screening assay is reproducible and represents a more physiological condition (Section 2.4), the relative fluorescent unit expression is far less than relative luciferase unit as a reporter assay for AR activity. Hence, it is unable to differentiate subtle changes in AR activity and cannot be replaced by conventional transient transfection assays.

This system has been used to screen potential AR antagonist compounds, which were selected through computer-aided drug design (Chapter 3). Due to limitations in targeting the ligand binding pocket on AR (Section 1.4.1), Fletterick et al conducted functional and x-ray screens to identify compounds that bind the AF-2 on the AR surface (23). They discovered the BF-3 pocket as a target for inhibiting AR activity (Section 3.1). Using *in house* developed components of an *in silico* drug discovery platform, 220 top-scored compounds were selected (Section 3.4.1). These chemicals were screened for AR inhibition and cell toxicity using LN-ARR_2PB-EGFP, described above. We found that 17 out of the 220 compounds did exhibit some inhibiting effect on the AR. Based on MTS assays and cell morphology, these 17 compounds were also found to be non-toxic at the concentrations tested (Table 3.1). This result has been validated with transfection assays (Fig. 3.1 A), and seven of these chemicals showed strong anti-androgen receptor activity that significantly exceeded the activity of bicalutamide, one of the most potent antiandrogen drugs currently used to treat men with metastatic prostate cancer (Fig. 3.1 B).

The next step in this study will be to prove that these chemicals bind directly to the BF-3 site and to determine their orientation and structural interactions. With the collaboration with Dr. Fletterick, the initial binding assessment will be done primarily using a Biacore system (www.biacore.com) and the specific orientation of the most active chemicals will be determined using high throughput x-ray screening (23), where the compounds are soaked with AR ligand binding domain crystals (24). The primary objective will be to determine exactly how the currently most active chemicals fit into the BF-3 pocket and to use this information for future design of even more potent derivative drugs. Based on the results, further refinement of the computational approaches will be performed in a way that will allow ranking of the 17 compounds at the top of the list. In addition, possible structural changes in the BF-3 pocket upon ligand binding (molecular dynamic) will be explored to improve predictions of induced fits. In simple terms, the molecular dynamic approach allows recreation of 'cartoon-like' movements of a compound in a protein cavity during their mutual molecular recognition and binding. Such presentations allow isolation of various 'snapshots' of a target protein's structure corresponding to its state before, during and after ligand binding. These 'snapshots' of the BF-3 binding site will be used to identify configurations of the target that have a higher affinity toward the 17 chemicals possessing the inhibiting effect on the AR. This will improve the accuracy of the process when the ~50 million chemicals are re-run through the refined *in silico* pipeline. It is expected that the second run will yield more active leads against the AR and will bring more insight into the mechanisms of BF-3 binding and AR inhibition by the developed drug leads.

The next phase of this research will then be to determine the LC50 (50% lethal concentration) of our 17 lead candidate anti-androgen compounds. MTS assays will be performed after treatment of both androgen-dependent (LNCaP & LAPC-4) and androgen-independent (C4-2,

PC-3 & DU145) prostate cancer cell lines with increasing concentrations of test compounds. These experiments should reveal the relative sensitivity to these compounds of prostate cancer cells with functional ARs and determine whether these compounds mediate cell killing through the AR. Once *in vitro* toxicology and efficacy studies are verified, the effects of non-toxic candidate drugs (and potential derivatives) on inhibiting AR activity and growth of prostate cancer tumours grown *in vivo* in immunocompromised mice could be performed (19,20,25). It is expected that the most effective chemical for inhibiting androgen receptor activity will also be the most potent in reducing serum PSA levels, inhibiting tumour growth, and delaying or preventing progression to the lethal castration-resistant state.

5.2 Investigation of TAF1 as a novel AR-coactivator that is overexpressed in advanced prostate cancer

In the second approach, we aimed to find the proteins that bind to the AR, modulate AR transcriptional activity, and are involved in prostate cancer progression. In Chapter 4, the finding that TAF1 is a novel AR-coactivator has been introduced. Using the repressed transactivator yeast two-hybrid system, we found that TAF1 interacted with the AR (26). In tissue microarrays, TAF1 was shown to steadily increase with duration of neoadjuvant androgen withdrawal and with progression to castration resistance (Section 4.3.1). GST pull-down assays established that TAF1 bound through its acetylation and E1/E2 directly to the AR N-terminus (Section 4.3.2). Co-immunoprecipitation and ChIP assays revealed colocalization of TAF1 and AR on the prostate specific antigen promoter in LNCaP prostate cancer cells (Section 4.3.3). With respect to modulation of AR activity, overexpression of TAF1 enhanced AR activity several-fold, whereas siRNA knockdown of TAF1 significantly decreased AR transactivation (Section 4.3.4).

While full-length TAF1 showed enhancement of both AR and some generic gene transcriptional activity, selective AR coactivator activity by TAF1 was demonstrated in transactivation experiments using cloned N-terminal kinase and E1/E2 functional domains (Section 4.3.5). In keeping with specific AR coactivation by the ubiquitin activating and conjugating domains, TAF1 was found to greatly increase the cellular amount of poly-ubiquitinated AR (Section 4.3.6). In conclusion, our results indicate that increased TAF1 expression is associated with progression of human prostate cancers to the lethal castration-resistant state. Since TAF1 is a coactivator of AR that binds and differentially enhances AR transcriptional activity, its overexpression could be part of a compensatory mechanism adapted by cancer cells to overcome reduced levels of circulating androgens.

With the discovery of increased TAF1 expression levels in advanced prostate cancer and its influence on AR activity, a next step in assessing the potential role of TAF1 in prostate tumour growth and progression would be to test the effects of its inducible overexpression or knockdown in our LNCaP tumour model. The LNCaP xenograft model generated in athymic nude mice provides a reproducible *in vivo* experimental system for monitoring molecular and genetic events associated with growth, regression and progression to castration resistance (27,28). We will create LNCaP cell lines that have DOX-inducible overexpression of TAF1 as well as inducible shRNA knockdown of endogenous TAF1 plus appropriate empty vector or non-specific controls, as we have reported in detail elsewhere (19,29). After *in vitro* testing of the effects on cell growth and PSA expression in the absence and presence of DOX, these lines, together with stable, non-specific or empty vector controls, will be inoculated and grown in groups of 6 nude mice in the LNCaP tumour model, exactly as described before (19,27,28). At various time points before and after castration, we will test the effects of TAF1 expression level (by treating ± DOX

in the drinking water) on the rate of growth, regression, and PSA-progression to castration resistance. As before, statistical analyses will be performed after the first set of experiments and animal numbers will be altered accordingly in consultation with our biostatistician (R. Bell). We will monitor the status and cellular localization of AR under these treatment conditions and also perform immunohistochemistry and Western blot analyses to ensure that TAF1 expression is modulated as expected. These experiments should uncover any role of TAF1 in progression to castration-resistant prostate cancer.

One of the biggest issues when working on TAF1 is to differentiate between the role of TAF1 in the general transcriptional machinery from its influence on the AR. This is even more of a concern when TAF1 is considered as a therapeutic target. To avoid this problem, one could identify the sequences on the AR where TAF1 binds. Using *in silico* drug design discovery, we could screen our virtual compound library for small molecules that bind to this particular site on the AR molecule. We would next screen the top-selected compounds for AR inhibition followed by validating the physical binding using X-ray crystallography as described above. The compounds that are selected by this approach would interrupt the TAF1/AR interaction and the coactivator function of TAF1 would therrby be disrupted without a major influence on cell viability.

With respect to cell growth, since TAF1 has a role in cell cycle progression and repression of apoptosis (30,31), the overexpression of TAF1 might facilitate cell proliferation through mechanisms other than AR. To address this concern, we will generate another inducible prostate cancer cell line that does not have an AR, such as PC3 cells (PC3-TAF1). We will then compare the growth of PC3-TAF1 to PC3-DEST as a negative control *in vitro* and then in the athymic

nude mice. The result of the latter experiment should answer whether TAF1 can influence tumour progression in an AR independent manner. Another issue is that complete TAF1 knockdown may be lethal, although we rarely see >80% knockdown using this system (19). Recently, we have been able to titrate the expression level of shRNAs by varying the level of DOX administered (data not shown), and if necessary, we will use this strategy to modulate the cellular levels of TAF1.

Although we were unable to show AR phosphorylation by TAF1 through *in vitro* kinase assays, that could be due to inadequacy of cell-free assays. As an alternative, an *in vivo* phosphorylation assay could be performed (32). The DOX-inducible LNCaP-TAF1 cells (as described above) will be treated with combinations of ± R1881 and ± DOX in the presence of ^{32}P orthophosphate before cell lysis and AR pulldown. Phosphorylation of AR will then be analyzed by SDS-PAGE, followed by autoradiography. A stronger ^{32}P band compared to controls would suggest that increased TAF1 is involved in increased AR phosphorylation.

5.3 Significance

The limiting factor in the survival of a patient with prostate cancer is the rate of progression to castration resistance (33,34). In order to have any impact on mortality rates from this disease, we need to understand how cancers will progress to a hormone-refractory state and to develop ways to prevent, delay, or treat the hormone-refractory phenotype. Since the AR plays a key role in progression of prostate cancer to advanced stages, the overall goal of this research was to determine new targets through which the AR activity could be inhibited and to develop better treatments for castration-resistant prostate cancer.

The data presented in this thesis shows our two different approaches for inhibiting AR function. First, by co-application of our cell-based screening assays for AR activity and our relatively unique *in silico* drug discovery program, we identified several compounds that inhibit AR activity even more efficient than the current conventional drugs. Second, we found that TAF1 is a novel AR-coactivator that directly binds to and coactivates the AR, and that this AR coactivation activity is distinct from TAF1's generic transcriptional enhancement of gene activity. Furthermore, we show that TAF1 is overexpressed in human prostate cancers during progression to castration resistance, whose overexpression could be part of a compensatory mechanism adapted by castration-resistant prostate cancer cells to overcome reduced levels of circulating androgens. These research projects will be the subject of future studies, which will provide possible novel drugs for treating advanced prostate cancer. We hope by using the small molecules that we are currently developing for the BF-3 pocket, and in future for the sites on which TAF1 binds to the AR, a new class prostate cancer drugs will be developed to create a more powerful total androgen blockade than is currently possible. This will extend the remission period of hormone sensitive or castration-resistant prostate cancer.

5.4 References

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