THE EFFECT OF *SALSOLA TUBERCULATIFORMIS* BOSTCHANTZEV ON THE ANDROGEN AND GLUCOCORTICOID RECEPTORS IN PROSTATE CANCER CELL LINES.

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at the University of Stellenbosch.

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Date: December 2017

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SUMMARY:

This study describes:

1.	The preparation of a biologically active methanol extract from *S.tuberculatiformis.*

2.	The demonstration of biological activity for both the crude S.tuberculatiformis extract and Compound A.

3.	The effects of the crude S.tuberculatiformis extract and Compound A on nuclear translocation of the androgen and estrogens receptors in three different prostate cancer cell-lines.

4.	Cell viability assays demonstrating the potency (IC_{50}) for Compound A and the crude *S.*tuberculatiformis extract between the three prostate cancer cell-lines.

5.	Caspase assays to demonstrate apoptosis for the crude *S.*tuberculatiformis extract and Compound A in prostate cancer cell-lines.

OPSOMMING:

Hierdie studie beskryf:

1. Die bereiding van 'n biologies-aktiewe kru metanolekstrak uit *S.tuberculatiformis.*

2. Die bepaling van die biologiese aktiwiteit vir beide die kru metanolekstrak uit *S.tuberculatiformis* en verbinding A.

3. Die effek van die kru S.tuberculatiformis ekstrak en verbindingA op die translokasie van die glukokortikoïed- en androgeenreseptore in drie verskillende prostaatkanker sel-lyne.

4. Sellewensvatbaarheidstoetse (IC50) met drie prostaatkankersellyne om die toksisiteit van die kru metanolekstrak uit *S.tuberculatiformis* en verbinding A te bepaal.

5. Kaspasetoetse in prostaatkanker sel-lyne om die apoptotiese effek van die kru metanolekstrak uit *S.tuberculatiformis* en verbinding A te demonstreer

Dedicated to the ones I love…

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Contents

LIST OF FIGURES:

LIST OF TABLES:

ABBREVIATIONS:

AAM	African-American men
AAT	Androgen ablation therapy
AF-1	Activation function 1
AF-2	Activation function 2
AP-1	Activator protein 1
AR	Androgen receptor
ARE	Androgen-response elements
BCA	Bicinchoninic acid assay
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CAM	Caucasian-American men
CANSA	Cancer Association of South Africa
CO	Carbon monoxide
CPD	p160, p300/CREB binding protein
CpdA	Compound A
CREB	CRE-binding protein
CRPC	Castration resistant prostate cancer
CYP11B1	Cytochrome P450-dependent 11β-hydroxylase
CYP11B12	Aldosterone synthase
DBD	DNA-binding domain
DD	Death domain
Dex	Dexamethasone
DHT	5α-dihydrotestosterone
DISC	Death inducing signalling complex

DOC	Deoxycorticosterone
E	Estrogens
ECACC	European Collection of Cell Cultures
EI	
ER	Estrogens receptor
ERKs	Extracellular signal-regulated kinases
ERS	Endoplasmic reticulum stress
ESMS	Electrospray mass spectrometry
FA	Fluocinolone acetonide
FABMS	Fast atom bombardment mass spectrometry
FADD	Fas-Associated protein with Death Domain
FCS	Foetal calf serum
FLIP	FLICE inhibitory protein
GC	Gel chromatography
GR	Glucocorticoid receptor
H	Hinge region
HAT	Histone acetyltransferase
HPLC	High performance liquid chromatography
HRE	Hormone-response elements
HS	High spin state
HSPs	Heat shock proteins
IAP	Inhibitors of apoptosis proteins
IBs	NFκB inhibitors
IGF-1	Insulin-like growth factor-1
IKK	IB kinase
JNKs	c-Jun N-terminal kinases

LBD	Ligand-binding domain
LH	Luteinizing hormone
LHRF	Luteinizing hormone releasing factor
LNCaP	Lymph node-cancer of the prostate cell line
LS	Low spin state
MAPKs	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MDM2	Mouse DOUBLE minute 2
NFκB	Nuclear Factor KAPPA B
NO	Nitrogen monoxide
NTD	Amino-terminal domain
P/CAF	p300/CPB-associated factor
PAP	Prostatic acid phosphatase
PC3	Lumbar cancer of the prostate cell line
PC3^{AR+}	PC3 cells expressing AR (transiently transfected)
PCa	Prostate cancer
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue gene
SAPKs	Stress-activated protein kinases
SEM	Standard error of mean
SGRM	Selective GR modulators
SHBG	Sex hormone binding globulin
STAT5	Signal transducer and activator of transcription 5
T	Testosterone
TF	Transcription factor

TNF	Tumour Necrosis factor
TNF-R	Tumour necrosis factor receptor
TRADD	Tumour necrosis factor receptor type 1-associated DEATH domain protein
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRAIL-R	Tumour necrosis factor-related apoptosis-inducing ligand receptor
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

This chapter presents a summary of the socioeconomic and medical significance of prostate cancer and the role of steroid receptors in prostate tumorigenesis as well as the motivation for *Salsola tuberculatiformis* Bostchantzev extract and Compound A as the basis in the development of potential treatment. This leads to the formulation of the aims and objectives of the study in relation to this issue in the global and South African settings. An overview of the structure of the thesis is included.

1.1 BACKGROUND

Cancer represents a growing social and economic burden in both developed and developing countries. In 2012 global cancer statistics estimated 14.1 million new cases and 8.2 million deaths [1,2]. Developing countries comprise ±82% of the world's population and despite this they only account for 57% of cases and 65% of cancer deaths. This is due to several factors including population demographics and acquisition bias. However, the burden of cancer will continue to shift to less developed countries due to growth and ageing of the population and increasing prevalence of known risk factors [2]. In South Africa, more than 100 000 people are diagnosed with cancer every year with only 60% surviving the diagnosis (CANSA, 2010).

The incidence of prostate cancer is on the rise globally in almost all countries, with prostate cancer being predicted to be the most common cancer in men in fifteen years [1,2]. Currently, the non-localised disease is without any curative therapy [3]. In 2012, more than 1.1 million cases of prostate cancer (PCa) were recorded accounting for ±8 % of all new cases of cancer worldwide (15 % of all cancers in men) [4].

The most effective treatment for disseminated prostate cancer is hormone therapy, in particular chemical castration through androgen ablation therapy [5]. This therapy is effective because most prostate cancers are dependent on androgens at

initial diagnosis. The androgen receptor (AR) acts as an oncogene promoting carcinoma development. Thus the endocrine therapy of prostate cancer aims to reduce serum androgens and inhibit the AR [6,7]. The likelihood of recovery is highest if treated early but prostate cancer can persist and continue to grow slowly following initial therapy and there is a significant probability (approximately 20-30%) of it reoccurring within the first 5 years following treatment [Prostate Cancer Foundation, 2014]. These re-occurrences are accompanied by functional changes in which the AR activates genomic and non-genomic signalling pathways that can be activated in low androgen ligand environments and lead to a resistance to hormone manipulations. Treatment subsequently becomes palliative. This progression is known as castration resistant prostate cancer (CRPC). Despite this loss of sensitivity to Androgen Ablation Therapy (AAT), the AR continues to be expressed throughout prostate cancer progression and AR-negative prostate cancer cells do not have a significant growth or survival advantage [6].

Glucocorticoid receptor (GR) mediated pathways play an opposing role in prostate tumorigenesis and result in tumour suppression. Glucocorticoids are therefore a part of the standard treatment for prostate cancer [6]. The AR and GR are closely related transcription factors (TF) that belong to a superfamily of nuclear hormone receptors. Once these receptors bind to their cognate steroid ligands, the AR and GR dissociate from their cytoplasmic chaperone proteins, form homodimers and translocate to the nucleus where they bind to palindromic hormone response elements (HRE) to activate gene expression. This is known as transactivation [7]. In addition to the genomic, DNA-binding transactivation there is a negative regulation of gene expression, referred to as transrepression that does not require DNA-binding but is mediated through cross-talk with other TFs [5–7]. The GR also activates other mechanisms for indirect gene repression by blocking mitogen-activated protein kinases [6]. The GR transrepression of other TFs, including NFκB and AP-1, are critical for the anti-inflammatory activity of glucocorticoids and this mechanism was recently shown to be vital for its tumor suppressor effects [6]. The AR has also been shown to interact and modulate some TFs, including NFκBβ and AP-1[7], although the mechanisms have yet to be fully elucidated. Thus the opposing roles of the AR and GR in prostate cancer suggest that a dual steroid receptor modulator that

inhibits androgen and glucocorticoid transactivation while enhancing GR transrepression would be the ideal treatment [7].

The steroid modulation of the African shrub known as *Salsola tuberculatiformis* Botschantzev, locally known as Gannabos, was first postulated when Basson *et al.* identified it as the agent responsible for the "Grootlamsiekte" syndrome that occurs in Karakul sheep. The syndrome is characterised by a prolonged gestation period resulting in fetal post-maturity rendering the lamb's coat overgrown and economically worthless [8,9]. Basson *et al.* also described additional biological effects of this plant such as its ability to inhibit the estrus cycle in rats and its use as a contraceptive by Bushmen [8,9]. These finding initiated investigations into the active components of the plant. It was discovered that the active contraceptive properties could be extracted with methanol and Swart *et al.* developed a bioassay based on the terminal enzyme in adrenal corticosteroidogenesis, cytochrome P450-dependent 11β-hydroxylase, being inhibited by the active component. The isolated active fractions were, however, extremely labile and only partial structures of the active components could be obtained. These structures indicated the presence of synephrine and a highly reactive aziridine and this information led to the synthesis of a more stable analogue, 2-(4-acetoxyphenyl) 2-chloro-N-methylethylammonium-chloride or Compound A (CpdA) [8]. It was shown that this synthetic compound mimicked the properties of its natural counterpart and, in addition, was stabilised by steroid-binding globulins in plasma thus enhancing its biological activity in vivo [8]. Further investigation of the properties of CpdA by Yemelyanov *et al.* revealed that CpdA acted as a dual steroid receptor modulator that interacted with the GR, the AR and multiple TFs, including NFκB and AP-1, resulting in translocation but inhibiting transactivation of GR and AR while simultaneously enhancing transrepression of the GR. They also showed that, due to opposing roles of these receptors in PCa, CpdA initiated apoptosis in various PCa cell-lines with differing steroid receptor status [7].

Since CpdA is a synthetic chemical and based upon the partial structures identified in the active *S.tuberculatiformis* fraction, it may be argued that the original extracts may demonstrate additional or alternative therapeutic activity. Elements characteristic of the active *S.tuberculatiformis* compound(s) that potentially possess alternate, enhanced or complimentary benefits in PCa prevention or treatment or

other medical research may have been lost in CpdA being synthesized as the lead compound.

1.2 AIMS

This study set out to determine if the biological properties of CpdA, in which the compound acts as a dual steroid receptor modulator could be reproduced using the original *S.tuberculatiformis* crude extract. The study was carried out with the following aims:

1. To extract the active components from *S.tuberculatiformis* with methanol and determine the biological activity in both the crude extract of *S.tuberculatiformis* and CpdA

2. To demonstrate that the *S.tuberculatiformis* extract interacts with the androgen and glucocorticoid receptors resulting in nuclear translocation.

3. To determine the potency of the *S.tuberculatiformis* crude extract and CpdA in PCa cell models which express the relevant steroid receptors.

4. To demonstrate that the cell-death caused by the *S.tuberculatiformis* crude extract in PCa is due to apoptosis.

1.3 THESIS OVERVIEW

In the current study, the burden of cancer, with specific reference to PCa prevalence, incidence and epidemiology, is discussed in Chapter Two. It further describes the anatomy and physiology of the normal prostate and the role of steroid signaling in prostatic maintenance and development. The manipulation of these mechanisms in prostate cancer development and progression, with specific emphasis on apoptotic pathways is also discussed. The mechanisms of action of nuclear hormone receptors are described in depth with specific reference to the AR and GR and their roles in prostate cancer development and treatment. These are discussed with reference to the current literature and accepted knowledge base for *S.tuberculatiformis* and CpdA, highlighting the therapeutic potential for these agents and the need for further research.

Chapter Three presents the methodology used in this study with particular emphasis on the framework of the techniques as these were unfamiliar methods within our group which were acquired and subsequently established as novel protocols in our laboratory. This included immunofluorescence to demonstrate receptor nuclear translocation in PCa cell models in the presence of CpdA and *S.tuberculatiformis*. In addition, cell viability and caspase assays were optimised to demonstrate the potency and apoptotic effects of *S.tuberculatiformis* and CpdA, respectively. The methanol extraction and determination of the biological activity by cytochrome P450-dependent 11β-hydroxylase difference spectral assay are also presented together with techniques applied in assays using PCa cell models expressing steroid receptors.

In Chapter Four the results of these investigations are presented together with statistical analyses. In the final chapter these results are discussed critically in the context of the current body of information, with reference to the direction of future studies and research, indicating the potential of *S.tuberculatiformis* as a novel therapeutic agent for PCa, capitalising on the selective steroid modulation demonstrated in this and other studies.

CHAPTER 2

LITERATURE REVIEW:

PROSTATE CANCER, *SALSOLA TUBERCULATIFORMIS* AND COMPOUND A

This chapter presents an overview of carcinogenesis with specific emphasis on mechanisms for the initiation and progression of PCa as well as the role of steroids and steroid receptors in health and disease. Additionally pathways for apoptosis are presented, highlighting therapeutic targets for treatment interventions in PCa focusing on selective steroid receptor modulation. This information is then applied to the present state of knowledge on the mechanisms of action of the novel PCa chemotherapeutic agents CpdA and *S.tuberculatiformis* crude extract as a background to this study.

2.1 PROSTATE CANCER

Cancer is thought to be one of the oldest diseases in existence with the earliest evidence of it being recorded ± 1600 B.C [10]. Cancer refers to a group of diseases characterised by the formation of abnormal cells that grow and spread uncontrollably. This results in the invasion of other organs within the body, a process known as metastasis, and is the major cause of death from cancer [4]. Cancer development has been divided into three phases: initiation, promotion and progression (Figure 2.1). Initiation involves the formation of abnormal cells through the malfunction of, or damage to, genes that control cell growth, division and apoptosis. Promotion is associated with the uncontrolled growth of abnormal cells resulting in tumour formation within an organ. Progression refers to the metastasis of the tumour and patient survival is significantly reduced at this stage.
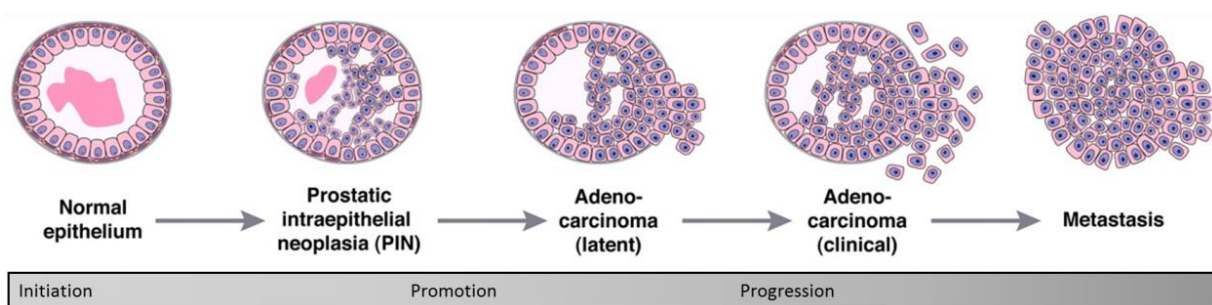
Figure 2.1 Illustration of the progression of PCa from normal epithelium to metastasis. The three stages (initiation, promotion and progression) of cancer development are shown along with the clinical evolution of a PCa tumour. Adapted from Abate-Shen & Shen (2000) [11].

Since its discovery and consequential investigation there have been great strides in understanding and treating this disease. However, the developed therapies are relatively inadequate and cancer is still a leading cause of death today. Cancer may be treated with surgery, radiation, immunotherapy, hormones and chemotherapy [4,12]. All these therapeutic strategies are invasive and associated with significant side-effects. A specific combination of therapies is chosen depending on the characteristics and site of the cancer at diagnosis. The best treatment option for metastatic cancer is chemotherapy as it is a drug treatment that targets cell growth and proliferation. These powerful drugs circulate in the bloodstream and target the cells that are actively growing. Since cancer cells divide faster than normal cells they are more susceptible but damage to healthy cells is unavoidable. Owing to toxicity ,which is often severe, there are extensive drug-related side-effects and this treatment is therefore used sparingly or in conjunction with other targeted treatments [12]. Chemotherapy is thus primarily used to remove recurrent or metastatic cancer and as a palliative therapy. New therapies need to be developed to prevent or improve the treatment of cancers prior to metastasis thus improving therapeutic approaches and disease related mortality and morbidity.

2.1.1 EPIDEMIOLOGY

PCa is the second most common cancer, as well as one of the leading causes of cancer-related deaths, in men worldwide (World Cancer Research Fund International, 2012). Global statistics for PCa incidence and mortality are significantly varied due to availability of resources, access to diagnostic facilities and erratic or incomplete records. Despite this, trends have become apparent. The incidence rate

is lowest in Asia and highest in the USA with the current lifetime risk of PCa within the USA estimated to be 1 in 6 with a risk of death between 8% and 26%, depending on the staging and degree of differentiation of the tumour at diagnosis [13,14]. This disparity indicates that lifestyle and genetics could have an influence on PCa development and progression. This observation is further supported by the variation found between African-American men (AAM) and Caucasian-American men (CAM). The incidence rate in AAM is 1.6-1.9 times higher than in CAM with a resulting mortality rate that is 2-3 times higher in AAM [15–20]. AAM were also younger at diagnosis, had higher grade and stage tumours as well as the worst prognosis, all of which could be attributed to a greater biological aggressiveness [19,21]. Overall, a weak but consistent trend between higher circulating testosterone (T) and/or estrogens (E) and high-risk ethnic/racial groups has been observed by several studies. Ross *et al* found higher T, free T, and estrone in 50 healthy young AAM when compared with 50 young CAM but later ascertained that serum T levels in Japanese men were not lower than those of the United States whites and blacks [22,23]. De Jong *et al.* found 71% higher circulating total T levels in Caucasian-Dutch men than in Japanese men. These studies, as well as others, reveal an association between increased risk and increased ratio of T to 5α-dihydrotestosterone (DHT) [24]. This association is endorsed by the human prostate's dependency on androgens for normal maintenance. Thus it is logical to presume that prostate malignancies develop under abnormal androgenic stimulation and that an increased ratio would be easier to manipulate. This theory is further supported by the fact that eunuchs do not develop PCa and a higher incidence of PCa is observed in men who used androgens as anabolic agents or therapeutics [1,25]. PCa therefore seems to arise from the hormonal environment of the ageing human male, which is characterised by a steady decline in circulating androgens and a reduced production of DHT in the prostatic epithelium, but the onset/progression is heavily influenced by lifestyle and genetics.

In South Africa, the initial incidence of PCa was low with more Caucasian-Africans presenting with PCa than Black-Africans. However, owing to possible under-diagnosis/reporting, poor access to screening facilities and lack of an up-to-date cancer registry Heyns *et al.* conducted a study to compare the presenting features and management of PCa among different racial groups in the Western

Cape [26]. They found that during the period of 1995 to 2005 black men in Western Cape Province presented with higher grade and stage PCa and higher serum PSA levels than white or coloured men. Black men also received potentially curative treatment less often and had a significantly shorter duration of follow-up than white or coloured men, possibly owing to earlier deaths from PCa. This demonstrated that in regions where screening is not readily available, and men with PCa present because of cancer-related symptoms, the disease is locally advanced or metastatic in most cases and is therefore incurable. Although this study was strongly influenced by ascertainment bias and socioeconomic factors there remains a strong suggestion that men of black ancestry have a biological predisposition to this disease. This trend could account for PCa being the leading cause of cancer deaths among South African men resulting in South Africa being amongst the countries with the highest PCa mortality rates with 1 in every 23 South African men being diagnosed in their lifetimes having a ±60% chance of surviving the diagnosis (CANSA, 2011)., In South Africa PCa thus remains a very relevant threat in health-care and awareness, education and treatment development need to be further investigated. In order to better identify therapeutic targets in PCa it is important to understand the integrated mechanisms for signalling in prostate development and maintenance in health and disease.

2.1.2 HEALTHY PROSTATE

The prostate is not essential for life but plays a pivotal role in male reproduction. It forms part of the male accessory sex glands which also include the seminal vesicles and bulbourtethral glands [27]. The secretion from each of these glands is added to the testicular fluid and constitutes a substantial part of the semen. In 1861 Sir Henry Thomson stated that the word 'prostate' originated as an anatomical term that refers to this organ "standing before or anterior to the bladder and vesiculae seminales in the supine position of the subject" [28]. The prostate is often described as resembling a walnut in size and appearance. It is comprised primarily of exocrine glandular tissue enclosed by a capsule, consisting of connective and fibrous tissue, and is located at the base of the bladder surrounding the urethra and in front of the rectum [29,30]. The seminal vesicle duct and vas deferens merge above the prostate to form the ejaculatory ducts which transverse the prostate and empty into the

urethra at the verumontanum (a 70° degree turn in the urethra). Below this site excretory ducts from the prostate enter the urethra and contribute prostatic secretions to the seminal fluid during ejaculation [27,29].

The uniform structure of the prostate can be divided into four distinct regions that differ structurally and functionally. These regions or zones are known as the transition zone, central zone, peripheral zone and anterior fibromuscular stroma (Figure 2.2) [31]. The transition zone surrounds the urethra between the bladder neck and verumontanum and encompasses ± 5-10% of the prostate. It is comprised of mostly glandular tissue (± 75%) and, combined with advancing age and altered hormonal activity, is where benign prostatic hyperplasia (BPH), a non-cancerous prostate condition, primarily originates [31]. BPH causes a dramatic enlargement of the transition zone and results in lower urinary tract symptoms (Prostate Cancer Foundation, 2016). The central zone contains the ejaculatory ducts and is located between the transition and peripheral zones. It accounts for ± 20% of the prostate and possesses the least amount of glandular tissue (± 20%) [31].]. The peripheral zone is the largest, incorporating ± 70% of the prostrate, the majority of which is comprised of glandular tissue (±70%). This zone is made up of the peripheral sections of the prostate gland and is closest to the rectum [31]. Approximately 75% of PCa arises within this zone. The anterior fibromuscular stroma comprises ± 5% of the prostate and is devoid of glandular components but is instead composed of smooth muscle and fibrous tissue [31]. There is no clearly defined anatomical capsule that surrounds the prostate –instead, the smooth muscle of this zone gradually extends into fibrous tissue that subsequently ends in loose connective and adipose tissue.
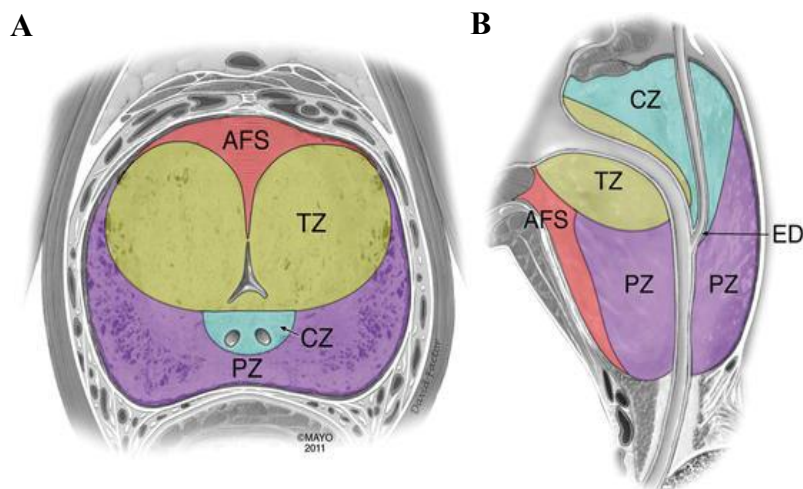
Figure 2.2 McNeal's zonal anatomy of the prostate gland. (a) Transaxial and (b) sagittal sections. AFS anterior fibromuscular stroma; CZ central zone; ED ejaculatory duct; TZ transition zone; PZ peripheral zone. (Mayo Foundation for Medical Education and Research, with permission. All rights reserved) [31]

The principle function of the prostate is to produce and excrete a fluid that makes up ± 20% of the seminal fluid during ejaculation. The prostatic secretions are a milky white alkaline mixture of simple sugars, proteins, enzymes and minerals [32]. While this secretion is not essential for fertilisation it enhances the *in vivo* health and fertility of the sperm. The simple sugars provide a source of energy for the sperm. The slightly alkaline pH neutralises the acidic environment of the vagina while specific enzymes break down proteins in an effort to free the sperm cells from the viscous semen after ejaculation [32]. Other components such as spermine and prostaglandins aid sperm mobility and uterus stimulation, respectively, in order to assist in sperm transport [32]. Specific proteins coat the sperm and prevent premature activation of factors required for egg penetration while antiagglutinin factors prevent sperm from clumping together. The prostate gland also acts as a valve preventing urine flow during ejaculation as well as using its smooth muscle to expel semen from the body [29,32].

Therefore, the prostate is a complex tubulo-alveolar gland composed of an epithelial parenchyma embedded within a stromal tissue matrix [30]. It consists of three main cell types: epithelium-basal cells, secretory luminal or glandular cells and neuroendocrine cells (Figure 2.3A) [30]. The epithelium-basal cells are undifferentiated cells that lack secretory activity and rest upon the basement membrane. These cells express low/undetectable levels of the AR and, as a result, are independent of androgens for their growth and survival [33,34]. Alternately the oestrogen receptor (ER), progesterone receptor (PR) and GR are expressed by the epithelium-basal cells [33,34]. The secretory luminal cells constitute the exocrine compartment and is the major cell type in the epithelium. These cells are terminally differentiated and are dependent on androgens for their survival. These cells are also responsible for the secretion of prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) into the glandular lumen and it is within these cells that the majority of PCa arises [33]. The neuroendocrine cells are a small number of post-mitotic, terminally differentiated, androgen-insensitive cells that are dispersed among

the secretory epithelium [35]. The stroma of the prostate is comprised of myofibroblasts, fibroblasts and smooth muscle cells. It expresses the AR and the ER as well as the 5α-reductase isozymes and supplies essential nutrients and growth factors that are responsible for the direction of epithelial cell development, maintenance and differentiation. The interactions between the stroma and epithelial cells are pivotal for normal prostate functions, growth and survival [30].
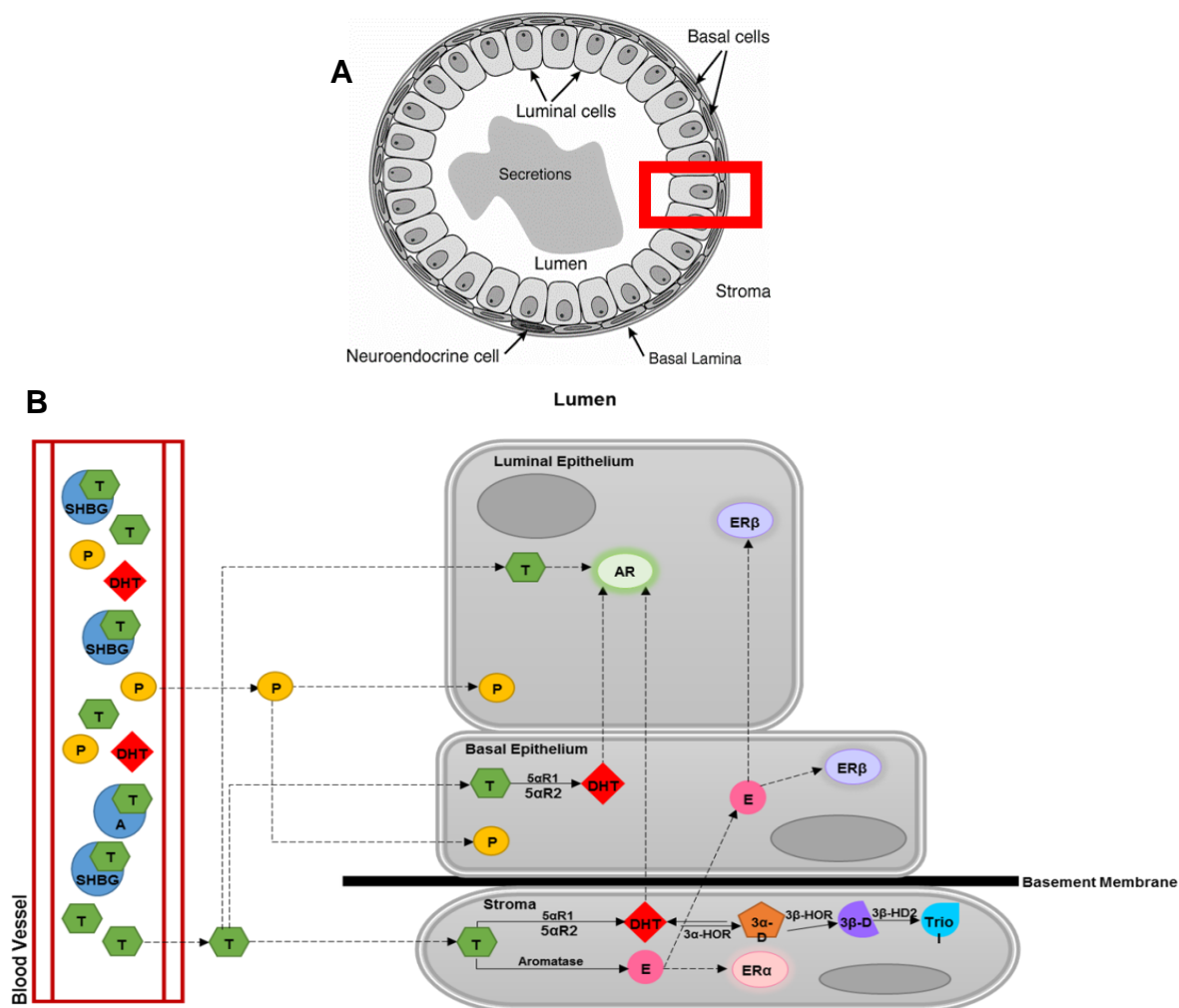
Figure 2.3 Overview of the prostate histology and endocrinology. A. The histological structure of the prostate identifying the four cell types; luminal, basal, neuroendocrine and stromal cells. Reproduced from Abate-Shen C & Shen MM (2016) [380]. **B.** Enlarged section (red outline) of the prostate illustrating the endocrinology. T, testosterone; SHBG, sex hormone binding globulin; A, albumin; DHT, 5α-dihydrotestosterone; P, prolactin; E, estradiol; 3αD, 3α,17β-androstenediol ; 3βD, 3β,17β-androstenediol ; 5αR1/2, 5α-reductase Type1/2; 3α-HOR, 3α-hydroxysteroid oxido-reductase; 3β-HOR, 3β-hydroxysteroid oxido-reductase; 3β-HD2, Type II 3β-hydroxysteroid dehydrogenase; AR, androgen receptor; ERα/β, oestrogen receptor α/β . Adapted from How CKM & Habib FK (2010) [36]

Endocrine stimulation is an essential requirement for prostate development, maintenance and function. Both normal and aberrant prostate cellular function is governed mainly by androgens although other hormones still have a synergistic or antagonistic influence (Figure 2.3B). In 1941, Huggins and Hodges first demonstrated the reduction in prostatic size post castration [25]. The main circulating androgen in men is T, mainly produced by the Leydig cells in the testes, although the adrenal gland also contributes (± 5%) to T production [32]. The release of androgens is controlled through the gonadotrophin luteinizing hormone (LH) and luteinizing hormone releasing factor (LHRF) within the hypothalamic pituitary gonadal axis through feedback mechanisms. Once in circulation most of the T is bound to serum proteins, mainly sex hormone binding globulin (SHBG) and albumin, resulting in only an estimated 2-3% of the T existing in the bioavailable free form [37]. This is important for regulating the amount of free T available for uptake in target tissues. Free T diffuses into the prostate where the majority (90%) of it is irreversibly converted by the enzyme 5α-reductase to a more potent intracellular androgen, 5α-dihydrotestosterone (DHT) [38,39]. There are two isoforms of 5α-reductase, Type 1 and 2, with type 1 being present in most tissues while type 2 is the predominant isoenzyme in the prostate [40,41]. The 5α-reduction occurs predominately in the stroma after which DHT is transported into the epithelium [39,42]. However, the epithelium can also produce some DHT (±10%) but only in the basal cells [39]. Both DHT and T bind to the AR which is responsible for cell proliferation, migration and inflammatory responses [43]. It is by regulating the effect of the androgens and the AR that the prostate maintains its function and development.

Although androgens are the primary hormone for the prostate, other hormones also exhibit important regulatory effects (Figure 2.3B). The pituitary hormone, prolactin, enhances the uptake of androgens into the prostatic epithelium allowing for easier movement of testosterone and DHT into the basal and luminal cells [44–48]. Estrogen (E) also influences prostatic function although the levels of ER in the prostate are relatively low [49,50]. E is produced when the enzyme aromatase irreversibly converts DHT into estradiol [51,52]. E appears to function in a synergistic fashion with androgens although they have opposing effects on the different cell types within the prostate [50,53–55]. The major target of E in the prostate is the stromal compartment where it stimulates cell growth [50,55,56]. However, E causes the

13

epithelium to undergo apoptosis resulting in a regression of this compartment. Thus, the prostate is influenced by a variety of sex steroids and hormones but androgens are essential for normal prostate development, maintenance and function. The different cellular compartments of the prostate also have a synergistic relationship allowing for important interactions to occur. It is when these hormones and relationships undergo alterations that abnormal prostate function can occur resulting in various diseases such as BPH or PCa.

2.1.2 PCA DEVELOPMENT

Initially, most PCas are androgen dependent. This means that the androgens, T and DHT, are required for growth and survival of PCa cells [25].]. In 1966 Huggins won the Nobel Prize for his work on hormonal treatment of PCa [57]. As a result, androgen ablation has become the cornerstone therapy in clinical approaches to the treatment of PCa [25,57]. If PCa is localised, the treatment of choice is prostatectomy and/or irradiation but if the PCa is unconfined then hormone deprivation is the front line treatment. Hormone deprivation can be accomplished by surgical (orchiectomy) or medical (LH-releasing hormone agonists, anti-androgens) castration and it is this treatment that is known as androgen ablation therapy (AAT). Despite this treatment most tumours eventually become refractory to AAT within 5 years leading to castration resistant PCa (CRPC) which is characterised by progression and metastasis, primarily in bone and lymph nodes, and patient survival is significantly reduced. The treatment of CRPC is limited and palliative with little to no increase in patient survival [48,58–62].

There are several pathways by which CRPC can develop. Chen *et al.* highlighted the critical role that the AR plays in the development of CRPC by demonstrating that the AR gene is consistently up-regulated during tumour progression in different experimental models of CRPC [62]. The mechanisms of CRPC development can be broadly divided into those pathways involving the AR and those that bypass the AR (Figure 2.4). These pathways are not mutually exclusive and often overlap in CRPC but they provide insights into the mechanisms by which cancer cells subvert normal growth control and escape attempts of treatment. They also provide possible treatment targets for the development of new therapeutic strategies.

During androgen-dependent progression, PCa cells depend primarily on the AR for growth and survival. When the AR is inactive, it is bound to heat-shock proteins in the cytoplasm of prostate cells. DHT binds to the AR, dissociating it from these heat-shock proteins. The DHT-AR complex translocates into the nucleus, dimerizes, and binds to the androgen-response elements (ARE), thereby activating genes involved in cell growth. During androgen-independent progression, PCa relies on various cellular pathways, some involving the AR and others bypassing it. Illustrated in Figure 2.4 are the 7 most documented mechanisms by which PCa progresses to CRPC. These pathways include the dysregulation of the AR caused by a mutation that would allow the AR to be activated by various ligands (3) or by deregulated growth factors and cytokines that can also activate the AR, usually with the aid of AR co-activators (5). The AR may also be amplified and therefore may be activated even by reduced DHT levels (2). Pathways that bypass the AR include the loss of PTEN which reverses the inhibition of the phosphatidylinositol 3-kinase (PI3-K)–Akt pathway, permitting activated Akt to phosphorylate the pro-apoptotic protein, Bad. This activation results in the release of the anti-apoptotic protein, Bcl-2, which eventually leads to cell survival (6). In addition, androgen-independent cells may over-express Bcl-2. PCa cells may furthermore also develop neuroendocrine-like behaviour and secrete neuropeptides that induce the growth of adjacent cells thus promoting the survival of the surrounding tumour (1). Through these mechanisms PCa may survive therapeutic interventions by manipulating AR-mediated cell survival [63,64].
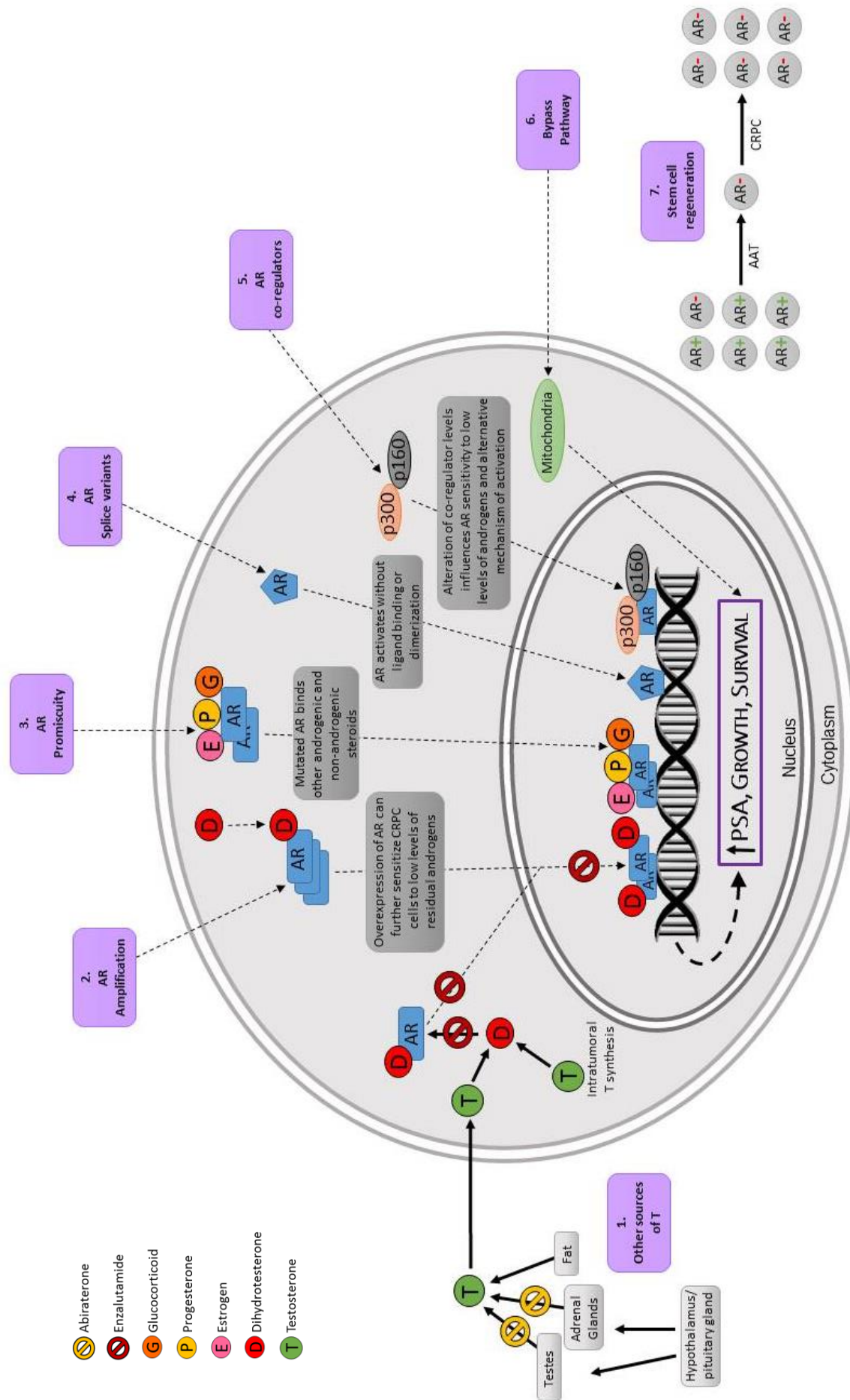
Figure 2.4 Androgen-Dependent and Androgen-Independent Progression of PCa. Reproduced from Debes & Tindall (2004) [64].

Four distinct pathways have been proven to result in CRPC: the hypersensitivity, promiscuous receptor, outlaw and co-factor pathways [reviewed in 64].

Hypersensitivity refers to the ability of PCa cells to develop enabling cells to utilise very low concentrations of androgens to maintain growth. Normal total testosterone levels in adulthood are between 240-950 ng/dL. Post castration T decreases more than 90% to 20-50 ng/dL [62,63,65,66]. In CRPC even these low levels may be sufficient for androgen stimulation. Consequently these cells are not androgen-independent but rather castration resistant and androgen sensitive. This hypersensitivity can be achieved through several mechanisms. One such mechanism is the increased expression of the AR which allows enhanced ligand binding. This mechanism is supported by multiple studies that have shown increased expression of the AR in CRPC [62,67–72]. This increase is likely associated with AR gene amplification as a result of mutations and/or selective environmental pressure. Another mechanism is increased sensitivity of the AR to androgens. Gregory *et al.* demonstrated that recurrent PCa showed increased sensitivity to the growth promoting effects of DHT [65]. The concentration of DHT required for androgen-independent cells to exhibit growth was 10^4-fold lower than that required for the androgen-sensitive LNCaP cells. A third method of hypersensitivity is observed when PCa cells increase their intracellular production of androgens by increasing the 5α-reductase activity within the PCa cell which in turn increases the rate of T conversion to DHT [72–74].

The promiscuous pathway is characterised by mutations in the AR that broaden the specificity of the AR to include non-androgenic steroids and anti-androgens that are naturally present within the circulation [75–81]. These mutations are often clustered within the ligand-binding domain.

The outlaw pathway refers to receptor mutations which allow non-steroid molecules to bind and activate the AR as ligands as well as downstream signalling of the AR by ligand-independent mechanisms [64,74,82,83]. For example dysregulated growth factors (including insulin-like growth factor, keratinocyte growth factor, epidermal growth factor and cytokines) that activate the AR and/or AR-dependent genes through dysregulated signal transduction pathways (over-expression of the receptor tyrosine kinase HER-2/neu) [84–86]. This results in proliferation of the PCa cells independent of androgen control. The activation of outlaw pathways highlights

17

the importance of tumour-microenvironment interactions in the development of CRPC, just as Paget identified that a "fertile soil" was necessary for the successful growth of cancer metastases [87–89].

The AR is influenced by a large and varied number of co-activators and co-repressors that are involved in the regulation of AR-dependent transcription [90]. These function as signalling intermediates between the AR and transcriptional machinery. In the co-factor pathway there are changes in the balance between co-activators and co-repressors that are involved in AR driven transcription which, in turn, influences AR activation [74,91–94]. An increase in co-activator proteins of the AR has been shown in CRPC resulting in the enhancement of the receptor reactivity to alternate ligands (outlaw and promiscuous pathways) and sensitised the receptor to stimulation by ligands present at low levels (hypersensitivity pathway) as well as allowing ligand independent activation [91,95–99]. These findings highlight the fact that the progression to CRPC involves multiple mechanisms. Furthermore, these pathways emphasise that the progression of PCa to CRPC is not necessarily androgen resistance but rather androgen manipulation offering a plausible mechanism as to the manner in which the androgen blockade in CRPC often remains therapeutically useful.

Pathways which bypass the AR include apoptotic modulation and neuroendocrine differentiation. The stem cell model of PCa offers an additional attractive postulate for AR independent modulation. Bypass pathways avoid the AR completely and PCa cells subsequently develop the ability to survive independent of AR activation. This survival is achieved either through modulation of apoptosis and dysregulation of apoptotic genes or through neuroendocrine differentiation of the PCa cells. Apoptotic modulation is the best known bypass mechanism as CRPC cells regularly up-regulate anti-apoptotic molecules, such as Akt and Bcl-2 [100–103]. When the tumour suppressor gene, phosphatase and tensin homologue (PTEN), is inactivated its inhibitory effect on Akt is lifted. The Akt pathway inactivates various pro-apoptotic proteins but one of its primary targets is the anti-apoptotic protein, Bcl-2 which frees it from the bound inhibitor Bad, resulting in cell survival and loss of PTEN [64].]. Over-expression of Bcl-2 is common in a number of cancers including CRPC. In the neuroendocrine differentiation mechanism PCa cells take on neuroendocrine characteristics favouring secretion of neuropeptides which increased

growth and proliferation [64].

The stem cell model of PCa postulates that not all cells within the tumour are tumorigenic but only a small subset of cells which are the PCa stem cells [101,104]. These cells are not affected by androgen depletion but differentiate into androgen-dependent and androgen-independent cells [104]. In the presence of AAT the androgen-independent cells develop a survival advantage in the heterogenic phenotype which is observed clinically and leads to CRPC [60].

PCa and its subsequent development to CRPC is a complicated, multi-step progression. The above mechanisms are by no means the only possibility for alternative signalling but by beginning to understand the survival mechanisms activated in PCa, new treatment targets can be identified. It is clear, however, that steroid hormone receptors play a pivotal role in regulating PCa and remain a primary therapeutic target.

2.1.2.1 PCa and Apoptosis

In 1972, Kerr *et al.* coined the term apoptosis as "a general mechanism of controlled cell deletion, which is complimentary to mitosis in the regulation of animal populations" [105]. Apoptosis, or programmed cell death, is fundamental in normal physiology, maintaining healthy cell populations and controlling excessive proliferation through elimination of old, mutated, damaged or infected cells. The signal transduction pathways of apoptosis ultimately all lead to the activation of the caspase cascade pathway. Caspases are a family of intracellular cysteine proteinases that affect cell death through a cascade of proteolytic cleavages which degrade cellular targets leading to apoptosis. The caspase cascade pathway is controlled by a balance between pro-and anti-apoptotic signalling. Activation of pro-proliferative pathways and inhibition of pro-apoptotic pathways allows uncontrolled cellular growth which is a hallmark of cancer. As a result apoptosis and apoptotic signal transduction pathways have become one of the main targets for novel PCa therapy strategies [105–108]. The direct apoptotic signalling pathways can be divided into extrinsic and intrinsic pathways although other signalling pathways also have a strong influence on regulation [109–111].

The intrinsic pathway (Figure 2.5) is activated by cell death signals that are generated from the interior of the cell by biological or metabolic disturbances that trigger multiple apoptotic mechanisms [109]. These death signals are mediated by the Bcl-2 family which is a group of highly conserved proteins that serve either as anti-apoptotic or pro-apoptotic molecules [112]. The anti-apoptotic and the pro-apoptotic proteins form a complex network that regulate cell fate and determine the susceptibility of cells to death signals.

In a normal cell the pro-apoptotic molecules, such as Bax and Bak, are antagonized by anti-apoptotic proteins such as Bcl-2. Initiation of the death signal within the cell can be triggered by a variety of incidents including anticancer drugs, DNA damaging agents, kinase inhibitors, hypoxia, growth factor withdrawal, and UV or ionizing radiation. In response to these and other triggers, the pro-apoptotic molecules are activated by transcriptional up-regulation (Bax, Noxa), subcellular relocalisation (Bim, Bmf), dephosphorylation (Bad), or proteolysis (Bid). These activated pro-apoptotic molecules inactivate anti-apoptotic molecules and subsequently Bax and Bak insert into the mitochondrial membrane. This insertion causes increased permeability of the outer mitochondrial membrane and enables the release of mitochondrial factors such as cytochrome c. In the cytoplasm, cytochrome c and the adaptor protein Apaf-1 form a complex that activates caspase-9, which in turn triggers the caspase cascade pathway resulting in apoptosis [113,114]. Activation of the caspase cascade pathway can be blocked by inhibitors of apoptosis proteins (IAPs) which are up-regulated in response to survival signals [109–112].

In most cancers, including PCa, Bcl-2 expression is high and, it is expressed in 70% of androgen-independent tumours in PCa [100,115]. Thus, it appears the expression levels of Bcl-2 correlate to the progression of PCa and that over-expression of Bcl-2 may enable PCa cells to survive in an androgen-deprived environment. Bcl-2 also increases the resistance of PCa cells to radio- and chemotherapy [116–120]. Even less is known about Bcl-xL, another anti-apoptotic protein, in PCa progression; however, there is emerging evidence that, in many aspects, it behaves in a manner similar to Bcl-2 [121,122].]. Pro-apoptotic Bcl-2 family members are also important in regulating apoptosis in PCa. More than 80% of PC-3 cells over-expressing Bax and Bak undergo apoptosis [123,124]. Additionally, over expression of Bax in AR-positive, but not in AR-negative PCa cells, induces

apoptosis which is lost when AR is knocked down indicating AR is required for the signalling mechanism [125]. These experiments suggest a role for the pro-apoptotic members of the Bcl-2 family in regulating apoptosis in PCa. Currently, studies suggest a significant role for IAPs in regulating apoptosis in PCa cells with implications in both cancer progression and carcinogenesis. High expression of IAP, including cIAP1, cIAP2, and XIAP, and Survivin has been linked to bad clinical prognosis for cancer patients [126–129]. Survivin expression increases during PCa progression −from normal prostate through primary low-grade to high-grade PCa with the highest expression in lymph node metastases [127–129]. There is also evidence for a role of IAPs, other than Survivin, in regulating cell death in PCa [130]. These studies thus all support the role of IAPs in PCa progression to CRPC.
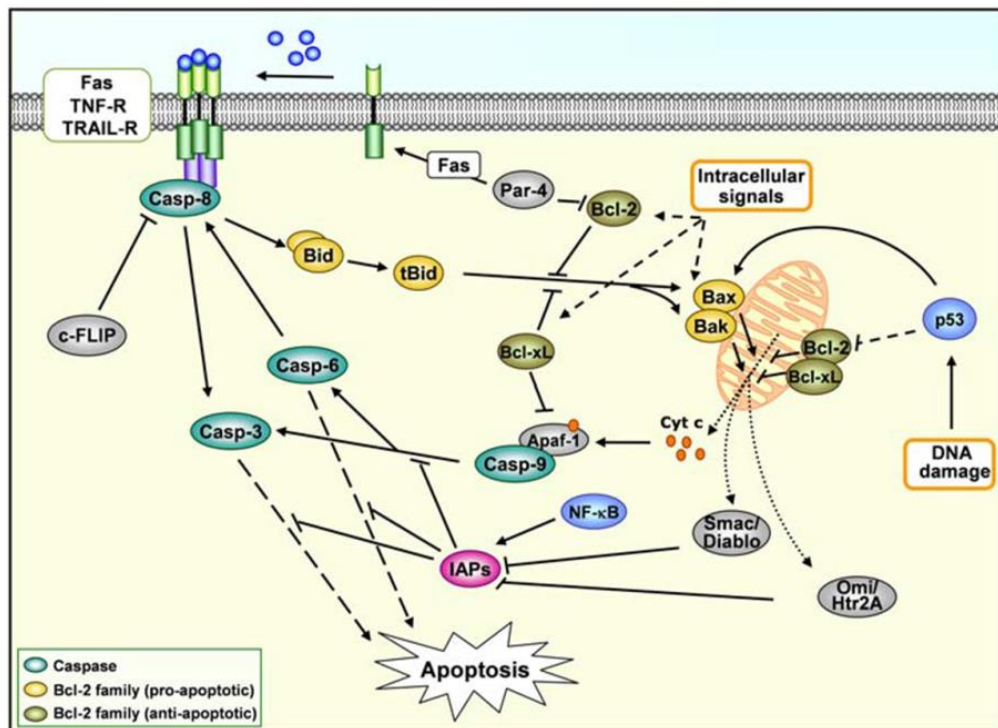
Figure 2.5 Extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is initiated by the binding of the TNF family members to their corresponding TNF receptors triggering their trimerization and activation. The intrinsic pathway is initiated by signals generated inside the cell that alter the balance between the pro- and anti-apoptotic members of the Bcl-2 family. The two pathways are interlinked and their cross-talk is mediated by Caspase-8 and -6..Reproduced from Lorenzo PI, Arnoldussen YJ & Saatcioglu S. (2007)[131].

The extrinsic pathway (Figure 2.5) is stimulated by extracellular stress signals

that are sensed and propagated via cell surface receptors referred to as death receptors. These death receptors include the Fas/CD95/Apo-1 receptor which binds the Fas ligand (FasL, Apo-1L or CD95L), the tumour necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) that interacts with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which possesses potent anti-tumour activity, and the tumour necrosis factor receptor (TNF-R) which exists as two different functional receptors, TNF-R1 and TNF-R2, that both bind tumour necrosis factor (TNF) [111]. TNF is a multi-functional cytokine involved in apoptosis, cell survival, inflammation, and immunity. These receptors belong to the TNF receptor (TNF-R) family and their combined functions include the ability to trigger proliferation, survival, differentiation, or cell death depending on the cell type and kind of signal [111]. The TNF-R family is activated by structurally related ligands that belong to the TNF family and upon binding of the ligand the receptors undergo trimerization and recruit adaptor proteins, such as FADD and TRADD. These adaptor proteins bind to an intracellular region on the receptor known as the death domain (DD). This interaction is essential for the efficient recruitment of procaspase-8, thus forming the death inducing signalling complex (DISC). Several procaspase-8 molecules must aggregate in order for autocatalytic activation to occur. Once activated the procaspase-8 molecule initiates the caspase cascade pathway [109,110,113].

In cancer cells, death receptors are often down-regulated or altogether absent [132]. TNF-R signalling can be inhibited by proteins that associate with their cytoplasmic domain. One such common protein is FLICE inhibitory protein (FLIP) which is often highly expressed in many cancers [133]. Androgens can induce the expression of c-FLIP, which has a cluster of 4 AREs within its promoter region, thereby protecting the cell from apoptosis [134]. Several studies indicate that the different TNF-R pathways can be manipulated in a variety of ways in order to induce cell survival.

In PCa Fas and FasL are elevated initially but rapidly decrease or become lost through mutations desensitising the cells to Fas-mediated apoptosis [135–140]. Chopra *et al.* compared TNF-dependent apoptosis in PCa cell lines and determined that the androgen-sensitive LNCaP cells were sensitive to TNF-mediated apoptosis, while the androgen-independent PC-3 and normal prostate cells were resistant [141,142]. This suggests that the resistance to TNF-R mediated apoptosis is a factor in

the development of CRPC. Apart from inducing apoptosis TNF can also promote cell survival as shown in PCa cells in vitro. TNF can activate NFκB-dependent pro-survival pathways, resulting in an increase of Bcl-2, through a mechanism that involves the activation of PI3K/AKT (Figure 2.6) [143,144]. In addition, PCa patients experiencing a relapse after AAT have increased serum TNF levels compared to untreated patients or patients in remission and this increase in TNF levels correlates with a higher mortality rate [145]. Conversely, TRAIL possesses potent anti-tumour activity as well as the ability to primarily kill cancer cells while having no effect on normal cells [132]. However, certain PCa cell models are resistant to TRAIL induced apoptosis and studies have revealed a link to the involvement of AKT signalling [146,147].

Cross talk between the extrinsic and intrinsic apoptotic pathways (Figure 2.5) may amplify the apoptotic response and is mediated by a pro-apoptotic Bcl-2 family member known as Bid. Caspase-8, which is up-regulated through the extrinsic pathway, mediates cleavage of Bid and this increases its activity and results in translocation to the mitochondria, where it acts together with the pro-apoptotic proteins, Bax and Bak, to induce cytochrome c release, activating the intrinsic pathway [109,110]. In addition, a mitochondria-mediated amplification of caspase-8 activity through caspase-6 has also been described [148].

2.1.2.1.1 Additional Signal-Transduction Pathways:

As mentioned above, other signal transduction pathways can indirectly influence the apoptotic pathways. There are four well-defined signalling-transduction pathways that have an effect on cell survival. They are the p53, mitogen-activated protein kinases (MAPKs), AKT/PTEN/mTOR and NFκB pathways. All these pathways have been shown to be dysregulated in cancer to promote cancer cell survival.

1. p53:

p53 is one of the most studied tumour-suppressor proteins and has the ability to signal through a variety of growth inhibitory pathways including induction of cell cycle arrest, senescence, differentiation and apoptosis. This broad range of activity establishes p53 as a key regulator of cell growth which is substantiated by the loss or alterations of its function observed in most human cancers [reviewed in 149–151].

At rest p53 is maintained at low levels by continuous ubiquitination and subsequent degradation. The activation of p53 is achieved by the inhibition of its degradation, which results in its accumulation in the nucleus where it activates the transcription of many different target genes [152]. p53-dependent transcription is a highly regulated event influenced by a range of post-translational covalent modifications of p53 [reviewed in 149,153]. A large number of p53 transcriptional targets are involved in apoptosis but the BH-3 domain protein PUMA seems to be the most critical one in this regard [154,155]. p53 also has transcription-independent pro-apoptotic properties through interaction with members of the Bcl-2 family (Figure 2.5 and 2.6) [156].

p53 mutations are present in most cancers. In PCa, the reported frequency of mutations in p53 is highly variable. Overall, it appears that clinically early stage confined tumours have few or no mutations in p53, whereas the metastatic and CRPC harbour a high frequency of mutations [157–160]. An indication that p53 is more important for the progression than for the initiation of PCa [158,161].   Androgens have been shown to negatively influence p53 expression [162,163]. In agreement with these findings, androgen deprivation caused an increase in p53 in the xenograft model CWR22, which correlated with a decrease in cellular proliferation [164]. Conversely, p53 can affect androgen signalling as physiological levels of p53 are necessary and have a protective effect on AR but overexpression of p53 blocks androgen signalling [165,166]. These findings indicate that androgen-dependent growth of PCa depends on a balance between AR and p53 and if disrupted it may result in PCa progression [166].

One of the major regulators of p53 is the ubiquitin E3 ligase MDM2 that targets p53 for proteasomal degradation. In normal cells, MDM2 is weakly expressed, allowing p53 activation after injury or DNA damage. However, in human cancers, including PCa, elevated expression of MDM2 has been observed that is associated with poor prognosis [167–169]. Interestingly, MDM2 is a target of AKT, one of the main pro-survival pathways activated in PCa progression. AKT-induced phosphorylation of MDM2 promotes its stabilization, decreasing the levels of p53 and consequently inhibits apoptosis [169].

One of the first p53 target genes which were identified was p21_Waf1/Cip1 (a

cyclin-dependent kinase inhibitor). Deletion of p21 eliminates the ability of p53 to induce G1 cell cycle arrest in many cell systems [150]. In addition to p53, p21 can be regulated by different factors, among them androgens [170]. Consistent with this, in a variety of cancers, p21 expression does not correlate with p53 status [158]. In PCa, clinical studies suggest that increased p21 expression levels are a negative prognostic factor as p21 over-expression may correlate with resistance to radiation therapy for local recurrence after radical prostatectomy and with the development of CRPC [158,171,172].

2. AKT/PTEN/mTOR

The PI3K/AKT signal-transduction pathway (Figure 2.6) has an important role in carcinogenesis due to its ability to both induce cell proliferation and inhibit apoptosis [173,174]. The AKT serine/threonine kinase family [also called protein kinase B (PKB)] has three highly conserved members, AKT1, AKT2, and AKT3. Activation of AKT requires the anchoring of the protein to the membrane and its phosphorylation at two different sites. In this process, two different kinases are involved, PDK1 and mTOR-Rictor complex [175]. Fully active AKT dissociates from the plasma membrane and targets substrates located in the cytoplasm and nucleus resulting in cell survival and proliferation [176].

Studies in PCa cell lines reveal that AKT activity contributes to PCa progression by dramatically accelerating prostate tumour growth [177]. AKT activity increases with increasing malignancy and correlates with the aggressiveness of the tumour [178–180]. The main negative regulator of AKT activation, the dual-specificity phosphatase PTEN (also called MMAC1), was initially identified as a mutation hot spot in cancers [74]. The overexpression of PTEN inhibits phosphorylation and activation of AKT by blocking its anchoring to the membrane [181,182].]. In recent years it has become clear that the loss of PTEN and subsequent activation of AKT is a critical event in PCa progression and in highly correlated with advanced PCa stage [183].

Cells lacking PTEN or harbouring constitutively active AKT present high levels of mTOR activity which results in increased cell growth [183]. Silencing mTOR in PC-3 cells induces cell cycle arrest and apoptosis [184].

The AKT pathway has many other downstream substrates −among them IKK kinase, an activator of the NFKB pathway [185,186]. The inhibition of AKT in PCa cell lines activates the apoptotic process through inactivation of NFKB signalling [187,188]. Apart from the pro-proliferative effects of AKT through mTOR or NFKB activation, this pathway also has anti-apoptotic effects through phosphorylation and inactivation of other pro-apoptotic proteins, such as Caspase-9, BAD, and FOXO transcription factors [169,179,189]
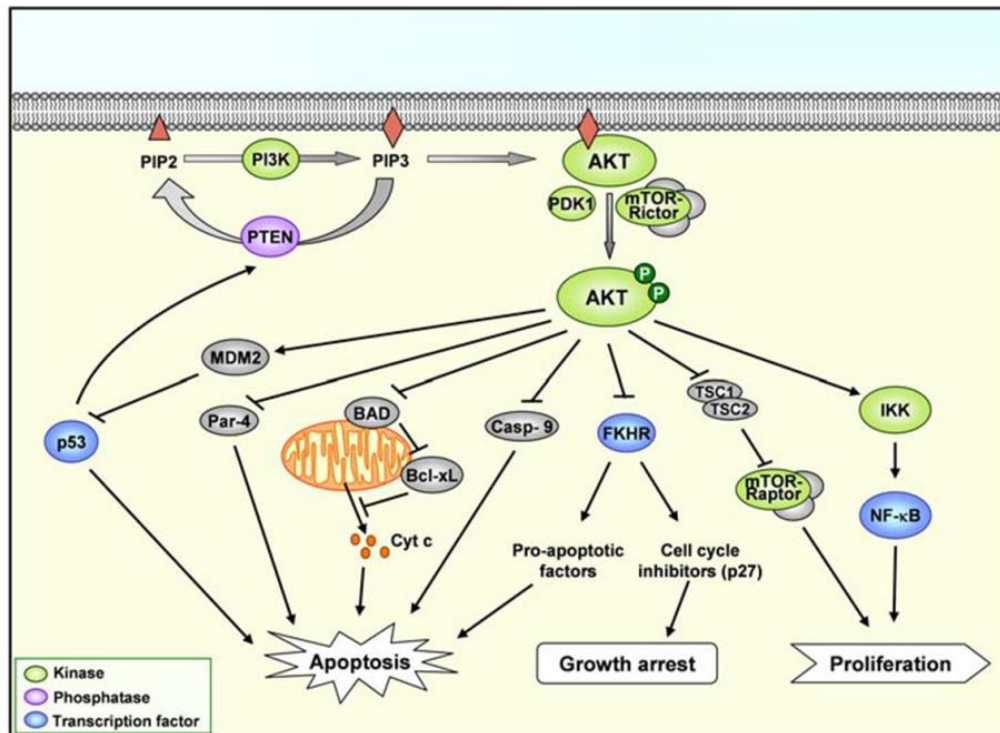
Figure 2.6 AKT/PTEN/mTOR pathways. The phosphorylation of the phosphatidylinositol phosphate PIP2 to PIP3 by phosphatidylinositol 3-kinase (PI3K) results in the phosphorylation and activation of AKT. Fully active AKT inhibits apoptosis through phosphorylation and inhibition of different pro-apoptotic proteins such as BAD, Par-4, Caspase-9, or by activating MDM2-dependent p53 degradation. Dephosphorylation of PIP3 by PTEN inhibits AKT recruitment to the membrane, thus blocking its activation. PIP2, phosphatidylinositol phosphate PtdIns(4,5)P2; PIP3, PtdIns(3,4,5)P3; PDK1, phosphoinositide dependent protein kinase 1; mTOR, mammalian target of rapamycin; TSC1, tuberous sclerosis complex 1; TSC2 tuberin; MDM2, mouse double minute 2; Par-4, prostate apoptosis response-4 protein; NFκB, nuclear factor κB; PTEN, phosphatase with tensin homology, which is deleted on chromosome 10. Reproduced from Lorenzo PI, Arnoldussen YJ & Saatcioglu S. (2007) [131].

3. Mitogen-activated Protein Kinases (MAPKs)

MAPKs are important mediators of signal transduction. They play a key role in the regulation of many cellular processes, including apoptosis [190]. The MAPKs are serine/threonine kinases that are phosphorylated and activated by stimuli such as cytokines, growth factors, and environmental stress as a result of a cascade of three protein kinases acting in series. MAPK is activated by a MAPK kinase (MAPKK), which in turn is activated by a MAPKK kinase (MAPKKK) (Figure 2.7) [190]. The three best studied MAPK pathways result in the activation of the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 MAPKs [191]. The ERK pathway is mainly involved in growth, differentiation, and development, while JNK and p38 MAPK pathways are involved mainly in inflammation, apoptosis, growth, and differentiation [192]. All three of the pathways appear to be involved in PCa proliferation but the JNK pathway seems to be involved in apoptosis as well.

In PCa the effect of activated ERK is hypothesised to either reduce apoptosis or to induce cell proliferation. Staining for active ERK in epithelial cells increases progressively from normal prostate tissue to BPH to PCa [193,194]. While loss of activation of ERK has been observed in late-stage CRPC and metastatic disease, elevated levels of active ERK in high-grade and advanced stages of prostate tumours have been linked to increased cell proliferation that directly translates to poor prognosis [195,196].

The p38 MAPK signalling pathway and its possible role in PCa is at present not clear. However, overexpression of p38 MAPK has been found in BPH and PCa, and correlates with enhanced cell proliferation [197].

The biological effect of JNK signalling in cells, including PCa cells, is determined by the cell type and the size and duration of the stimulus [162]. Discrepancies in JNK activation and function have been observed in PCa studies [198–200]. Different studies have correlated apoptosis in PCa cell models to JNK activation. In PC-3 and DU145 cells, JNK is strongly activated and linked to cell death under stressful stimuli including cytotoxic drugs [198,199]. An interaction between JNK and AR pathways has been reported in PCa cells with a negative cross talk occurring between the AR and activator protein-1 (AP-1), a downstream target of

JNK, resulting in suppression of JNK-mediated apoptosis.
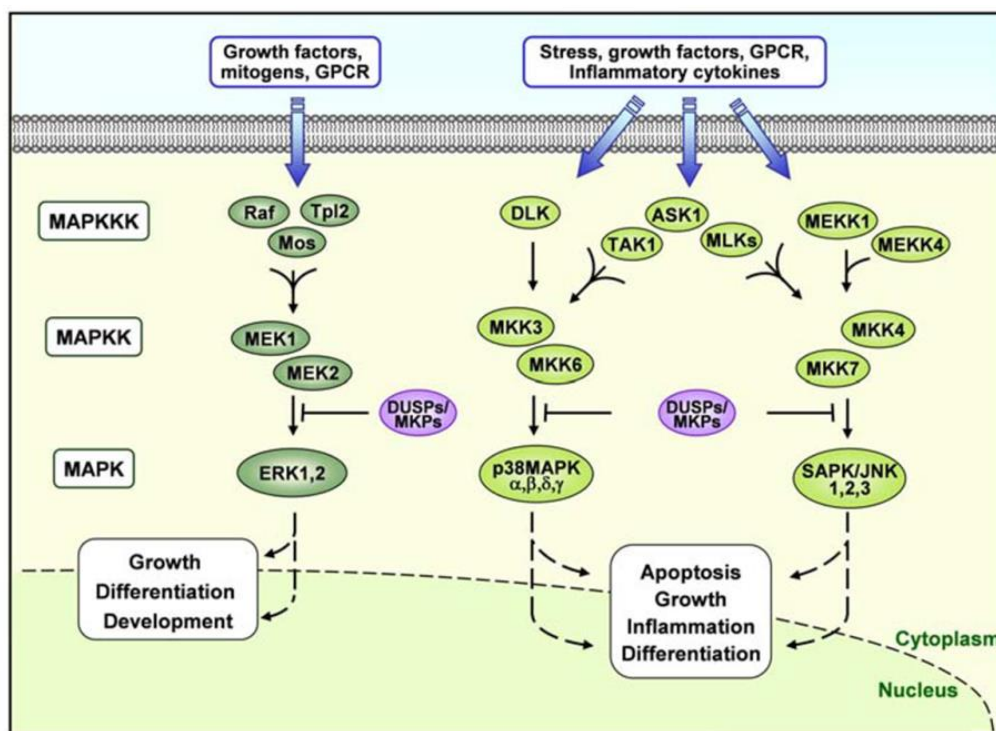
Figure 2.7 Mitogen-activated protein kinase (MAPK) pathways. The activation of the three main MAPK signaling pathways ultimately results in the activation of the extracellular signal regulated kinases (ERK), p38 MAPKs, and c-Jun N terminal kinase/stress-activated kinases (JNK/SAPK). Activated MAPKs exert their action through the phosphorylation of different effectors both in the cytoplasm and in the nucleus. Tpl2, tumor progression locus 2; DLK, dual leucine zipper kinase; TAK1, TGF-β1-associated kinase 1; ASK, apoptosis signal- regulating kinase; MLK, mixed lineage kinase; MEKK, MAPK/ERK kinase; MEK, MAPK/ERK kinase; MKK, MAPK kinase. Reproduced from Lorenzo PI, Arnoldussen YJ & Saatcioglu S. (2007) [131].

4. NFκB

NFκB is a cell survival factor and can be activated by a wide variety of stimuli. Under normal conditions, NFκB is required for normal development as well as immune and inflammatory responses to infection, and cellular stress and injury [201,202]. The NFKB family comprises five members, namely, NFκB1 (p105-p50), NFκB2 (p100-p52), c-Rel, Rel A, and Rel B [reviewed in203]. In a resting state, NFKB dimers are bound to specific inhibitors of NFκB (IBs) in the cytoplasm. In response to a variety of stimuli, the activation of the IB kinase (IKK) complex causes IB phosphorylation, which leads to nuclear translocation of NFκB dimers that regulate

transcription of target genes (Figure 2.8). Once in the nucleus, the NFKB dimers are subjected to further regulation through phosphorylation and/or acetylation [203]. Interestingly, NFκB can activate the expression of its own inhibitor IB, thereby providing a negative feedback loop on NFκB activation.

Since NFκB is involved in the regulation of apoptosis, cell proliferation, differentiation, and tumorigenesis it is not surprising to find that NFKB dependent gene expression is dysfunctional in various diseases, including cancer, which suggests that NFκB inhibitors could be used as therapeutic agents [203–206]. However, due to the large variety of NFκB effects in the normal physiology, NFκB inhibitors would be expected to have significant side effects including immuno-suppression which would limit the therapeutic application [205].

There is growing evidence that NFκB proteins play a role in the initiation and/or progression of PCa [reviewed in 204]. There is a correlation between NFκB activation and the grade of PCa [180,204,207]. Supporting this possible role of NFκB activation in PCa progression, in vitro studies using the PC-3 cell line demonstrated that increased NFκB activity contributed directly to cancer cell invasion [208,209]. Additionally NFκB activation protected PCa cells from apoptosis as well as contributed to PCa cell survival after AAT [204]. Blockade of NFκB activity is therefore associated with tumour suppression [210,211]. Surprisingly, several reports indicate a proapoptotic role for NFκB in the LNCaP cell model. NFκB activation is required for 2-methoxyestradiol-mediated p53 induction and apoptosis [211]. It is not clear if this proapoptotic role of NFκB is due to the androgen dependence of these cells or to the low basal levels of NFκB activity. It is also possible that it depends on other cellular changes such as PTEN or p53 status [204].
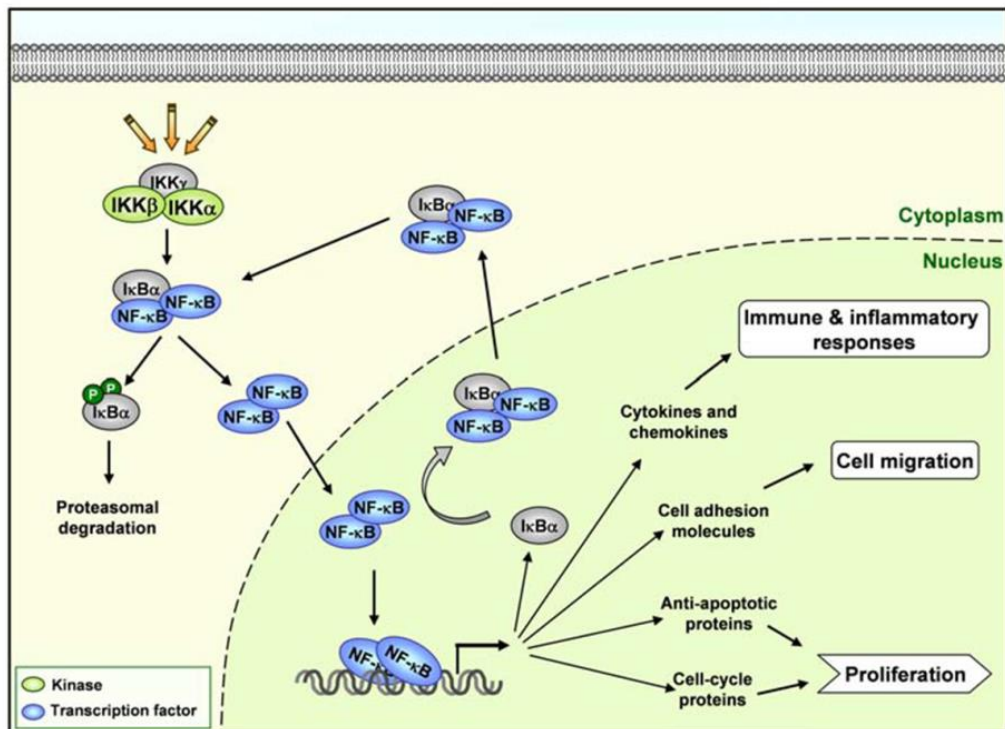
Figure 2.8 NFκB pathways. Under non-stimulated conditions, NFκB dimers are sequestered in the cytoplasm through their assembly with the specific inhibitory factor IκBα. Once activated, NFκB regulates the transcription of diverse anti-apoptotic and cell cycle regulatory genes that lead to apoptosis evasion and cell proliferation. Reproduced from Lorenzo PI, Arnoldussen YJ & Saatcioglu S. (2007) [280].

In summary, a number of signalling pathways are affected during prostate carcinogenesis. These mechanisms allow the PCa to evade the normal cell control mechanisms, ultimately leading to apoptosis-resistance and advanced CRPC. The AR and GR play important roles in this signalling.

2.1.3 NUCLEAR HORMONE RECEPTOR SIGNALLING PATHWAY

The GR and AR are closely related transcription factors (TF) from a family of nuclear hormone receptors. They are also known as NR3C1 and NR3C4 (nuclear receptor subfamily, group C, members 1 and 4) respectively [212–214]. Nuclear receptors have the same general structure (Figure 2.9) in that they are made up of modular proteins divided into five functional domains: a variable amino-terminal regulatory domain (NTD), a DNA-binding domain (DBD), a ligand-binding domain (LBD) and a small hinge region (H) containing a nuclear localisation signal and important sites for phosphorylation, acetylation and degradation [86,215–220]. In the NTD and H regions there are significant differences between the nuclear receptors allowing for variations in homeostatic control and signalling. In contrast, the sequences of the DBD and LBD regions are highly conserved resulting in considerable functional specificity [218]. In addition to these domains, nuclear receptors possess two transcriptional activation domains: activation function 1 (AF-1) located in the NTD and activation function 2 (AF-2) located in the LBD. AF-1 works in a ligand-independent manner and is responsible for optimal transcriptional activity and ligand binding. AF-2 works in a ligand-dependent manner and allows for the binding of cofactors important for inducing transcriptional activity [221–223].
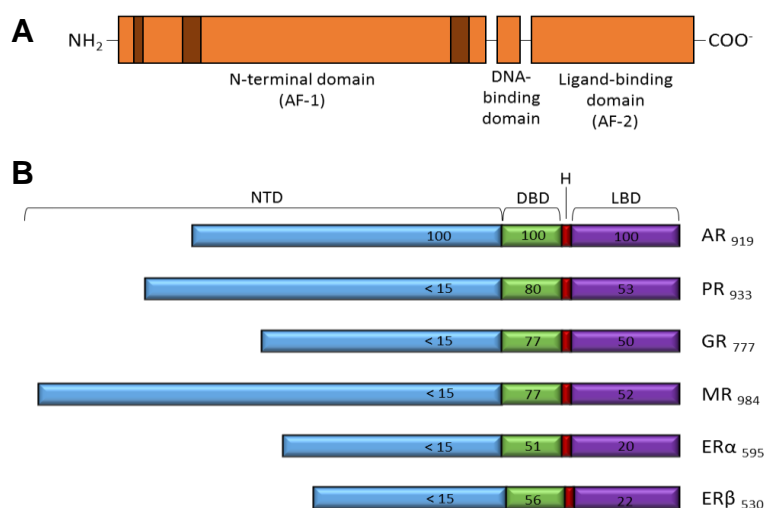
Figure 2.9 Structure of the nuclear family receptors. A) Schematic representation of the general structure and functional domains of nuclear receptors. Reproduced from Africander, 2010[221]. **B)** Individual nuclear receptor structures and there domain similarity. Activation function 1; AF-1, activation function 2; AF-2; NTD, N-terminal regulatory domain; DBD, DNA-binding domain; LBD, ligand-binding domain; H, hinge region. Reproduced from Li & Al-Azzawi, (2009) [217].

In the absence of ligands nuclear receptors are bound to heat shock proteins (HSPs) and other chaperone proteins which keep the receptor in the cytoplasm in a resting, unbound, state. The LBD allows high affinity binding of the ligand to the receptor which when bound generates a conformational change within the receptor that results in dissociation of the HSPs and chaperone proteins. This activation of the receptor is regulated by the interaction of co-regulators and receptor co-regulator phosphorylation in response to growth factors. Phosphorylation sensitises the receptors response to low levels of ligands and recruits nuclear co-activators required for chromatin remodelling [224,225]. It is the NTD receptor region that mediates the majority of transcriptional activity and is the most active co-regulator interaction surface [226,227].These interactions result in receptor translocation from the cytoplasm to the nucleus and homodimer formation. Various models of homodimerisation have been described but the most well characterised interaction is the DBD-mediated dimerisation which is essential for receptor-DNA complex formation and regulation of transcription [228]. Additional receptor interactions have been reported to result from intra- or inter- molecular interactions between the amino and carboxyl termini of the receptor, known as the N/C interactions [228–230]. Studies show that N/C interaction is dependent on the AF-2 domain and that it facilitates ligand retention by the nuclear receptors [231,232]. It is only following these events that the receptor homodimer can bind to hormone-response elements (HREs) located in the promotor regions of target genes and actively engage essential co-factors as well as assemble the transcriptional machinery required to regulate the expression of the target genes [225,233,234].

Two zinc finger motifs are contained in the cysteine-rich DBD along with a short C-terminal extension that forms part of the hinge [235,236]. These zinc fingers are responsible for the recognition and stabilisation of the hormone receptor-DNA complex. The first zinc finger assists the binding of the receptor in the major groove of DNA by recognising specific base pairs within the HRE [237]. Many genes contain HREs in their promotors that are not specific to a singular nuclear hormone receptor due to the highly conserved DBDs that recognise virtually identical DNA sequences. For example all nuclear hormone receptors, with the exception of the ER, bind to the three-nucleotide-spaced repeated palindromic binding motif TGTTCT [213]. The conserved amino acids in the second zinc finger mediate dimerization of the

receptors and stabilise the receptor-DNA complex [236,238,239]. Once the receptor homodimer has bound to the HRE transcription occurs. However, transcriptional activity is also dependent on an array of co-regulators and co-regulator complexes that are recruited upon receptor binding [225]. These co-regulators are often chromatin remodelling complexes, since they contain histone acetyltransferase (HAT) or deacetylase activity, which loosen or tighten, respectively, the chromatin structure and assist the binding of transcriptional machinery to the DNA. This stimulates (co-activator) or represses (co-repressor) gene expression [43]. Nuclear hormone receptors play a pivotal role in regulating gene expression and thereby determining cellular mechanisms and functions as evidenced by the AR and GR activity in PCa.
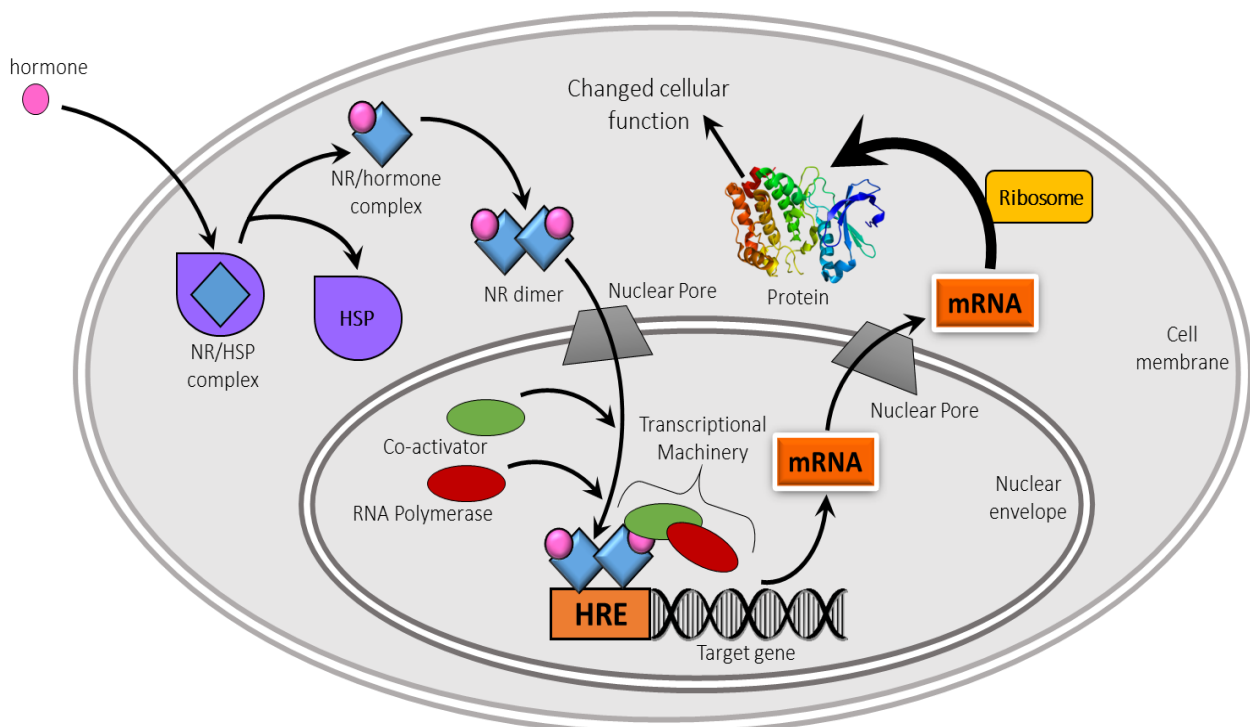
Figure 2.10 Nuclear receptor mechanism of action. Steroid receptors are synthesized in inactive forms that are associated with HSP complexes in the cytoplasm and/or nucleus. Hormone binding causes dissociation of steroid receptors from HSP complexes and the receptors to undergo dimerisation where upon they translocate into the nucleus. Various co-regulators and transcriptional machinery are then recruited so the homodimer can bind to specific response elements in target genes initiating transcription.

2.1.3.1 AR and PCa

Androgens, mediated by the AR, are the main hormonal regulators in the prostate. The AR activates genes responsible for maintaining the balance between cell proliferation, apoptosis and cell differentiation [65,72,240]. As previously described in 2.1.3, the AR is expressed, to some degree, in nearly all primary PCa and plays a critical role in the progression of PCa [241–243]. It is through manipulation of the AR pathway that most PCa circumvents the normal mechanisms of proliferation regulation, leading to uncontrolled growth, and bypassing apoptosis. Various studies, in both humans and animal models, suggest a relationship between the cellular AR expression and the level of malignancy and subsequent disease progression in both primary and metastatic PCa [244,245]. Huggins and Hodges provided the first *in vivo* evidence that targeting androgen/AR signalling via AAT could suppress PCa growth and progression [25]. Hence the AR remains a natural target for the development of current and future treatments for PCa.

AR and cell proliferation

Since early studies demonstrating the benefits of AAT in PCa, the AR has been a target to reduce tumour growth. However, it has been established that the AR plays differential roles in cell proliferation. Multiple studies have demonstrated that the AR can act as a stimulator to promote PCa cell growth as well as a suppressor [246–249]. This contrasting AR function is dependent on the different PCa cell types and tumour microenvironments. There are stem/progenitor cells and 3 types of epithelial cells in the normal prostate: (1) luminal cells, (2) intermediate cells and (3) basal cells. PCa can develop from any of these cells and so is often a mix of cells in various stages of differentiation which results in differing cellular mechanisms [250,251]. Bonkhoff *et al.* established that AAT resulted in the majority of the luminal epithelial cells being eliminated and that it had little effect on the basal cells. Therefore, AAT may only affect specific cell types which may partially explain why AAT would eventually fail [252]. These observations have led to numerous studies of the different PCa cell types and the effect that the AR has on them. It has been shown that the AR promotes PCa growth when derived from luminal and stromal cells but suppresses the PCa growth when derived from stem, basal and intermediate cells (as summarised in Table) [248,253–261].

34

Table 2.1 Summary of the sensitivity of androgens on the different PCa types. The androgen/AR signal exhibits different roles in prostate cancer progress and can either suppress or promote PCa growth depending on the PCa cell type.

Cell Types	Marker	AR roles in growth	Cell amount after AAT	Reference
Stem/Progenitor cells	CD133, CK5+, CK8-, AR-	Suppressor	Increase	Lee *et al.* (2012) [258]
Basal cells	CK5+, CK8-, AR-			Niu *et al.* (2008) [247]
Intermediate cells	CK5+, CK8+, AR-			Litvinov *et al.* (2004) [256] Litvinov *et al.* (2006) [257]
Luminal cells	CK5-, CK8+, AR+	Promoter	Decrease	Niu *et al.* (2008) [247] Eder *et al.* (2000) [253] Haag *et al.* (2005) [254] Liao *et al.* (2005) [255]
Stromal cells (Smooth muscle cells, fibroblasts)	A-SMA, Vimentin, Calponin			Niu *et al.* (2008) [247] Halin *et al.* (2007) [259] Gleave *et al.* (1991) [260] Lai *et al.* (2012) [258]

AR and Apoptosis:

In addition to affecting cell proliferation, the AR also modulates apoptotic pathways allowing for PCa survival. In the normal prostate, tissue homeostasis is maintained through controlled cell death which is a natural barrier against the development of cancer [106,262]. Importantly, studies have indicated that the AR has an essential influence on PCa cell death via distinct apoptotic mechanisms with accumulating evidence suggests that targeting androgens and AR-mediated cell death signalling could alter PCa progression [163,201,202,263–266]. The AR appears to modulate apoptosis in both positive and negative ways and can either promote or suppress apoptosis (Figure 2.11) through the adjustment of the different apoptotic signalling pathways determined again by the tumour microenvironment [267–270].

AR negative roles in PCa cell apoptosis (Anti-Apoptotic):

There are many mechanisms by which AR protects cells from apoptosis. Protection is executed through the manipulation and deregulation of the AR which is one of the regulators for the apoptotic pathways, as previously described. It is through these mechanisms that PCa progresses to CRPC and they have therefore become targets for the development of alternate and novel therapeutic strategies.

One of the most important contributors to AR cell preservation is the cyclin-dependent kinase inhibitor p21 (WAF1, CIP1, SDI1, or CAP20) that protects against

p53-mediated apoptosis [271]. An apoptosis-resistant phenotype may arise from over expression of p21 which induces cell cycle arrest [272]. Lu *et al.* showed that AR, by stimulating p21 mRNA and protein levels, acted as an inhibitor of apoptosis and thereby promoted PCa LNCaP cell growth [170]. They also found that androgens could enhance the p21 gene expression at the transcriptional level through a functional ARE located at À200bp of the p21 gene proximal to the promoter region which led to the inhibition of apoptosis. Another possible mechanism whereby apoptosis is suppressed is through direct down regulation of the p53 by the AR [163].

Rokhlin *et al.* found that the AR promotes cell survival through inhibition of p53 expression and caspase-2 activation which suppresses the TNF-R family. They also showed that this suppression could subsequently be enhanced by p53 knockdown [163]. Other studies have shown that androgens can block apoptosis induced by Fas activation and TNFα [273]. Additionally, Kadowaki *et al.* found that AR-positive LNCaP cells were resistant to TRAIL-induced apoptosis, while the AR-negative PC-3 cells were sensitive, supporting an AR protection mechanism [274].

Sun *et al.* showed the AR functioned as an anti-apoptotic element in LNCaP cells by activating PI3K and AKT1/AKT2 [275,276] The AR also suppressed apoptosis by inhibiting the pro-apoptotic molecule Bad, the Bcl-2-associated death protein. Apoptosis is inhibited when the AR activates MAPK and increases the phosphorylation of ERK-1 and ERK-2 which inactivate Bad [277,278].

The AR also protects PCa cells from apoptosis in an androgen-independent manner. Diallo *et al.* found that AR-negative PC-3 cells were more sensitive to the anti-cancer drug effect via inducing caspase-dependent apoptosis than PC-3 cells stably transfected with AR in the absence of androgen [279]. Other studies showed that the down-regulation of AR induced PCa apoptotic death upon addition of a proteasome inhibitor and the chemotherapeutic agent, docetaxel [267,280]. Liao *et al.* showed that apoptotic death in PCa cells was achieved by knockdown of AR via siRNA [255]. They also found both cisplatin and the proteasome inhibitor induced caspase-3 associated cell death in AR-negative PC-3 cells while non-caspase-3 associated cell death was observed in AR-positive PC-3_AR and LNCaP cells. In addition, they found that co-treatment with Bortezomib and the AR antagonist Casodex caused significant decrease in AR expression and led to increased

caspase-3 activity in LNCaP and PC-3_AR cells, suggesting AR might suppress caspase-3 expression [267].

AR positive roles in PCa cell apoptosis (Pro-Apoptotic)

In contrast to the dominant role of AR in cell proliferation, positive roles promoting apoptosis have been described (Figure 2.11) [125,268,281]. Godfrey *et al* reported that in androgen-independent PCa cells, the AR could promote stress-induced cell death independent of its transcription activity [268]. Similar results supporting this finding were reported in 2012 by Lin *et al.* [281]. They demonstrated that DNA damage and AR proteasomal degradation were induced by UV. Up-regulation of PIRH2 and p53 as well as down-regulation of p21 resulted from AR degradation of the N-terminal domain resulting in apoptotic cell death. This suggested that AR could promote a response to radiotherapy through the PIRH2-p53-p21 axis. However, other studies have reported conflicting results, showing that AR could protect PCa from irradiation-induced apoptosis [273].

In addition to intracellular AR functions in apoptosis, it has been suggested that membrane AR can induce apoptosis in PCa cells as well [282,283]. One study showed that membrane AR activation resulted in down-regulation of PI-3K/Akt/NFκB activity which induced apoptotic responses via Bad, FasL and caspase-3 in PCa [283]. Other studies demonstrated that truncated PolyQ expression of AR gene promoted apoptosis through up-regulation of Bax and JNK suggesting that the anti-death/pro-death function of AR is mediated by transcriptional regulation of apoptosis associated genes [281,284,285].
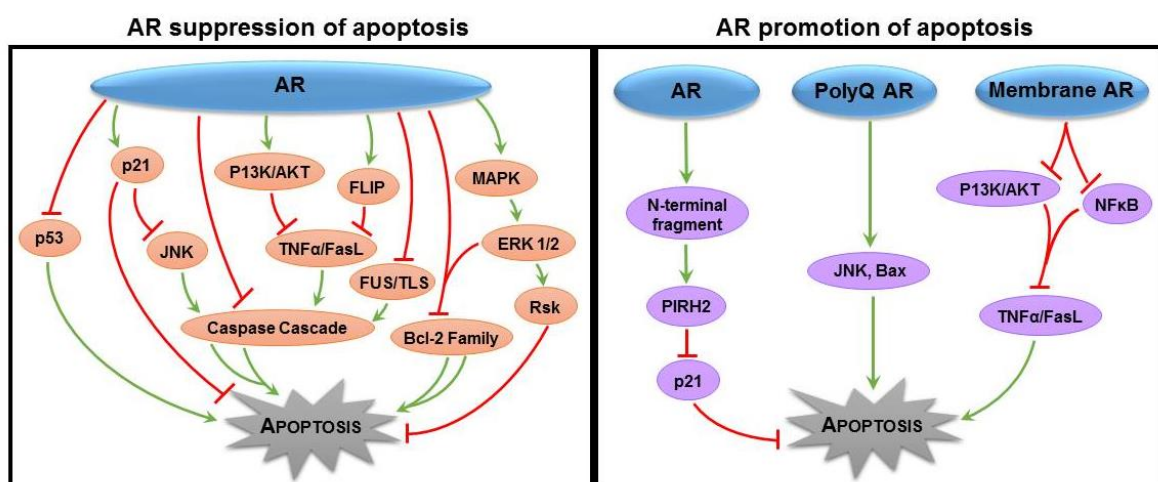
Figure 2.11 Major roles of AR in apoptosis is as a suppressor. In contrast to the main negative roles of AR in apoptosis, under specific circumstances (exposing to UV for example), AR may induce apoptosis. Reproduced from Wen *et al.* (2014) [286].

Since both positive and negative roles of AR in PCa cell apoptosis have been reported, it is still arguable when and under what circumstances AR selectively plays its roles. It is known that AR function is regulated by multiple signalling pathways and depending on different AR associated protein complexes, determined by cell micro-environments and cell type's −the AR may induce or suppress cell apoptosis. The anti-apoptosis and pro-survival function of AR is mediated mainly by transcriptional regulation of its target genes. In contrast, the pro-apoptosis function of AR is mainly transcription-independent, or mediated by membrane AR.

Challenges to current AAT

PCa progression is not dependent entirely on any single pathway and none of the mechanisms described contribute to PCa growth and metastasis equally. Apoptosis plays a dominant role in controlling the PCa proliferation and survival. This dual AR function of apoptosis and proliferation may offer a plausible explanation, in part, as to why ATT is ineffective in CRPC. Thus the AR is one of the most important mechanisms that regulate cell death induction while at the same time promoting resistance.

2.1.3.2 The GR and PCa

Glucocorticoids are involved in extensive cellular modulation and are major regulators of growth, energy production, metabolic processes, immune and cardiovascular function and reproduction as well as playing an important role in activating mechanisms that aid in the recovery from stress and maintain homeostasis. These effects are carried out through both positive and negative gene manipulations mediated through the GR.

There are two subsets of GR; GRα and GRβ. GRα is the receptor that binds glucocorticoids while GRβ does not. GRβ has inherent GRα-independent transcriptional activity and exerts a dominantly negative effect on GRα-mediated transcription [287,288]. The GRα utilises several mechanisms to manipulate cellular responses, the most prominent of which is through nuclear signalling where it acts as a TF that binds to glucocorticoid response elements (GREs) in the promotor regions of target genes and results in stimulation or suppression of the transcription of these genes, known as transactivation or transrepression, respectively (Figure 2.12). Transrepression also occurs when GRα- activated monomers interact with other TFs such as Activator Protein-1 (AP-1), nuclear factor-kB (NFκB), CRE-binding protein (CREB), signal transducer and activator of transcription 5 (STAT5), which, via protein–protein interactions, interfere with the transcription activity of these TFs and down-regulate their target genes. GR-mediated transactivation may be inhibited by inflammatory mediators such as TNF-α and growth signals such as Insulin-like Growth Factor-1 (IGF-1) which activates the transcription factors AP-1 and NFκB [289–291]. The activated GRα monomer can also interact with many other co-activators (p160, p300/CREB binding protein (CPB) and p300/CPB-associated factor (P/CAF)) and co-repressors. The GRα is governed by post-translational modifications of the receptor such as ubiquitination, sumoylation, acetylation and phosphorylation which determine whether the GR is down- or up-regulated [292–295]. Many of these control mechanisms are altered in autoimmune diseases and cancer. In the context of cancer, glucocorticoids are a standard treatment in many cancers as they exhibit an effect on the regulation of inflammation and apoptosis. Recent developments of GR-specific ligands, suggest that novel selective GR modulators (SGRMs) can trigger the expression of only a desirable subset of genes, enhancing their therapeutic effects while decreasing their side-effects and offering an attractive target for drug

development [296].
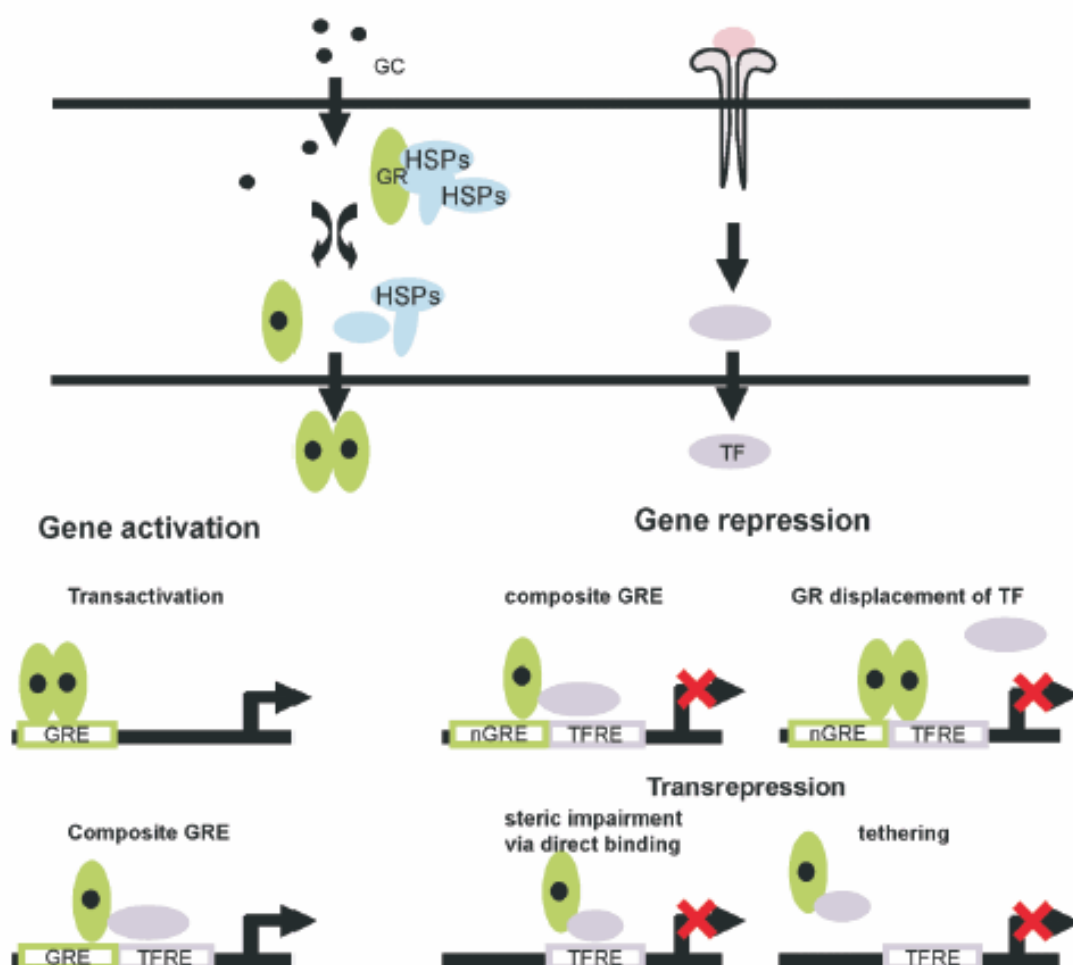
Figure 2.12 GR signal transduction pathway. Glucocorticoids, such as cortisol, diffuse into the cell where ligand binding induces molecular rearrangement of the GR complex and nuclear translocation resulting in mechanisms that either activate (transactivation) or inhibit (transrepression) of transcription. Reproduced from Labeur M & Holsboer F (2010) [11].

Despite the wide use of glucocorticoids as the treatment of PCa, it is only recently that the changes of GR expression during prostate tumorigenesis and its role in PCa have begun to be understood. In 2007, Yemelyanov *et al.* showed that GR signalling in early stage PCa exhibited anti-tumour effects on proliferation, differentiation and transformation in PCa cell models expressing the GR [6]. However loss of GR expression occurs in PCa progression and the GR is decreased or absent in 70--85% of PCa when compared to the normal prostate or BPH [6]. This early change in GR levels mirrors the changes in ERβ expression, another PCa inhibitor, reported by Fixemer *et al.* yet other steroid hormone receptor expression, such as AR and PR, remain constant [297]. Thus the GR can act as an anti-tumour agent in

early PCa when the GR is still expressed, favouring the use of glucocorticoids as treatment in the early stages of tumour development. The mechanism utilised by the GR to affect its anti-cancer effects appear to be exerted through transrepression which in most cases is mediated by direct interaction of the GR with other TFs or by crosstalk between the GR and other signalling pathways [214,290,298–300]. One of the most important interactions in this regard is with the MAPK signalling pathway. This pathway plays a vital role in the proliferation and survival of PCa [301,302]. Yemelyanov *et al.* found that activation of GR signalling blocked the activity of four major MAPKs: p38, JNK/SAPK, Mek1/2 and Erk1/2 [6]. This blockade appeared to involve increased expression of MKP1, a primary glucocorticoid-responsive gene, as well as an additional level of MAPK regulation via the post-transcriptional decrease of MAPK total protein levels [298,303]. It was further shown that numerous TFs had down-regulated activity upon GR activation many of which are recognised MAPK targets, indicating that this could be a consequence of the GR-mediated MAPK blockade [6].

Although this shows that the GR can be used as an anti-cancer agent it is applicable primarily to early-stage disease and an evaluation of the GR expression and glucocorticoid treatment in CRPC and PCa metastatic lesions still requires investigation.

Yano *et al.* demonstrated that the effects of glucocorticoid-activated GR in CRPC resulted in suppression of two major angiogenic factors, VEGF and IL-8 [304]. These two angiogenesis-related TFs mediate gene expressions that correlate with tumour metastasis and poor prognosis in PCa [305–308]. These results provide evidence that the therapeutic effect of glucocorticoids in CRPC can be directly attributed to the inhibition of angiogenesis by a reduction in both VEGF and IL-8. A recent study, supporting this, showed that dexamethasone (DEX) could directly suppress the in vitro growth of DU145 and PC-3, both of which express GRs [296]. The clinical use of glucocorticoids as an angiogenesis inhibitor in combination with anticancer agents, such as docetaxel, may enhance their therapeutic effect on CRPC.

GR as a regulator of the AR pathway

Glucocorticoid treatment is mainly used to help patients manage pain and adverse side-effects of chemotherapy [309,310]. However, glucocorticoids have also been shown to act as a ligand for the mutated AR and alter IL-6 signalling resulting in the promotion of PCa cell proliferation [79,311Additionally, the AR and GR bind to similar HRE domains and this has led to a degree of overlap. It has been determined that approximately half of the AR binding site overlaps with that of the GR in PCa cells expressing both receptors and this has revealed that the AR and GR are capable of binding to the same DNA sites [312–314]. Gene expression schemes of Dex-induced GR and DHT-induced AR are highly overlapping and the shared AR- and GR-binding events are associated with genes regulated by both androgens and glucocorticoids [313]. This led Sahu *et al.* to suggest that the AR and GR occupied by their cognate ligands are capable of using the same cis-elements to regulate transcription and that glucocorticoid-activated GR can take over the regulation of AR target genes [313].

Studies have furthermore demonstrated that activation of GR signalling can maintain an active AR under androgen deprived conditions in PCa. This is despite the previous reports on the tumour suppressive activities of the GR in PCa cells. GR should, therefore, be considered as a partial agonist/antagonist for the AR, in that liganded GR is an anti-AR in the presence of androgens but an AR agonist in the absence of androgen. [312,313,315,316]. The apparent interplay between AR and GR signalling is of potential clinical importance as about 30% of PCa cells express GR with increased GR protein expression detected in PCa after androgen-deprivation therapies and in CRPC tissue [7,317]. Unlike the AR, GR function is indispensable for life, which presents an inherent challenge in the establishment of GR signalling blockades aimed at treating or investigating CRPC and emphasises the need for selective blockades [318].
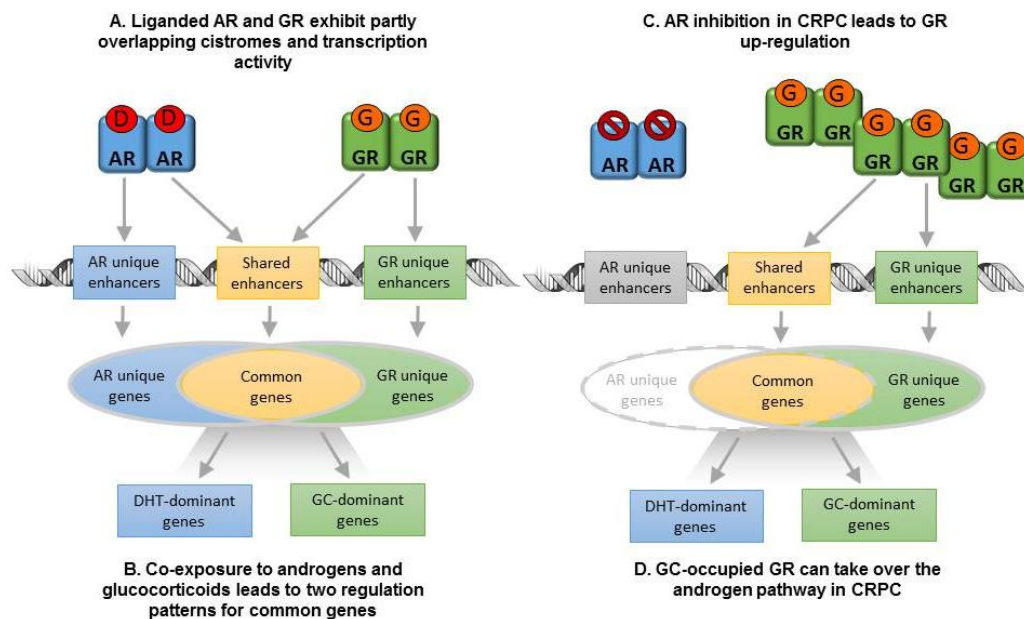
Figure 2.13 Interplay of AR and GR pathways in prostate cancer cells. Dex, dexamethasone; 🚫 anti-androgen (e.g., enzalutamide).Reproduced from Phihlajamaa *et al.* (2015) [319].

Side-effects of GR treatment:

The effects of glucocorticoid treatment can be summarised as anti-inflammatory/immuno-suppressive, metabolic, and toxic. Glucocorticoids are associated with multiple side-effects that develop in different organs and tissues during chronic treatment. The list of undesirable effects includes changes in glucose and fat metabolism, Cushing's syndrome, diabetes, glaucoma, osteoporosis, muscle and skin atrophy, decreased wound healing, changes in mood and cognition, growth retardation and infertility. Many of these effects are severe and sometimes irreversible. In cancer treatment the anti-inflammatory and immuno-suppressive effects are desired while the metabolic and toxic effects are associated with adverse side-effects [320]. The anti-inflammatory proteins are produced through the induction of the GR and the resulting transactivation of their target genes. On the contrary, the majority of the pro-inflammatory genes that encode cytokines, chemokines, and adhesion molecules, are regulated by the transcription factors NFκB and AP-1 [Reviewed in 321]. In these cases, the GR exerts its anti-inflammatory effects by interacting as a monomer with the subunits of respective TFs, inhibiting their activity and repressing the expression of the respective pro-inflammatory proteins. Based on

the large number of genes that are regulated in this manner, it has been hypothesised that the transrepression mechanism is sufficient to achieve anti-inflammatory effects [322–324].

Moreover, a number of side effects seem to be mediated predominantly via the transactivation mechanism [325–327]. Therefore, the identification of novel selective GR ligands, which cause a receptor conformation that prefers a GR-protein interaction, and not a GR-DNA binding-dependent mechanism, would be of therapeutic benefit. Such partial agonists may have widely similar anti-inflammatory effects and, while they should clearly induce fewer side effects as the DNA-independent GR mechanism alone, are sufficient for anti-inflammatory actions. Another side effect often neglected in studies is the so-called acquired resistance. Although most patients suffering from chronic inflammatory diseases have a good response to GC treatment at the start of therapy, a subpopulation of patients will over time inevitably become unresponsive to GC treatments. This may be ascribed to a physiological feedback mechanism −a homologous down-regulation of GR whereby glucocorticoids inhibit their own receptor levels. Thus, for optimal therapeutic value, the novel selective GR ligands should not only inhibit transactivation and up-regulate transrepression but also not trigger the regulatory feedback mechanism.

Differential targeting of the AR and GR in PCa

Given that AR and GR play opposing roles in PCa tumorigenesis, Yemelyanov *et al.* proposed that dual steroid receptor modulators that act as anti-androgens and simultaneously promote transrepression by the GR would make an ideal therapy for PCa [7]. They confirmed the differential modulatory effect using several PCa cell lines with different AR/GR status including a modified LNCaP cell line that expressed both receptors. They found that differential targeting of the AR and GR induces endoplasmic reticulum stress (ERS) and apoptosis in multiple PCa cell lines. Surprisingly, the PCa cells had a high expression of nuclear AR and GR following androgen ablation or chemotherapy. This was an unexpected result, as GR expression is strongly decreased in primary PCa in non-treated patients [6,328].]. However, since both AR and GR are widely expressed in treated human PCa this only strengthened the hypothesis of the proposed PCa treatment strategy by

44

differential targeting of the AR and GR. A number of compounds have been found to have a dual regulatory effect on the AR and GR in PCa. Among these is 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride (Compound A) which is a synthetic analogue of a hydroxyphenyl aziridine precursor found in the African shrub *Salsola tuberculatiformis* Botsch which is the subject of this study [8,43].

2.2 SALSOLA TUBERCULATIFORMIS BOSTCHANTZEV

The African shrub known as Salsola tuberculatiformis Botschantzev, locally known as Gannabos (Figure 2.14), is a plant that belongs to the family Chenopodiaceae which grows in the arid areas of South Africa, Namibia and Angola [329]. It belongs to the genus *Salsola* of which 69 different species are found in Southern Africa. *Salsola tuberculatiformis* Botsch occurs in a wide range of dry habitats and brackish soil and is extremely drought resistant. This plant genus has long been associated with medicinal qualities as documented by Ploss *et al.* in 1906 who reported the use of aqueous extracts from *Salsola* species as a contraceptive in Algeria [8,330].

Figure 2.14 Images of the African shrub, *S.tuberculatiformis*. Reproduced from Swart et al. (2003) [8].

Later, this same plant was revealed as the responsible agent for "Grootlamsiekte" or "Laat Lammetjie" syndrome that occurs in Karakul sheep which are farmed for the Swakara or Karakul pelt industry [9]. This syndrome is characterised by a prolonged gestation period of ± 213 days (normal gestation, 149 days) that results in foetal post-maturity rendering the lamb's coat overgrown and economically worthless [331 De Lange *et al.* established that this prolonged gestation was due to the ingestion of *S.tuberculatiformis* by the sheep and these findings initiated an investigation into the biological properties and active components of the plant [9,331 Basson *et al.* uncovered that *S.tuberculatiformis* possessed the ability to inhibit the estrus cycle in female rats in a dose dependent and reversible fashion. This supported the previously reported historical and traditional use of the plant infusion as a contraceptive [9]. In 1973, Liggins *et al.* showed that a surge in foetal cortisol, from the foetal adrenal, is the primary trigger to initiate parturition in karakul

sheep and this led to the hypothesis that ingestion of *S.tuberculatiformis* interfered with steroidogenesis resulting in inhibition of this "trigger"" [332]. This hypothesis was further supported by the atrophy of the adreno-hypophyseal axis, increased size of female genitalia and mild testicular atrophy that have been noted at autopsy in karakul sheep which would suggest a degree of androgen blockade or anti-androgen activity [333].

In 2003, Swart *et al.* published a review on the active properties of the plant [8]. His group were unable to reproduce the prolonged gestation observed by Basson *et al.* in female rats but instead rats fed with the *S.tuberculatiformis* extract underwent either foetal reabsorption in early pregnancy, or induced abortion in late pregnancy [8,9]. The group therefore decided to concentrate their efforts on the contraceptive properties as a possible bioassay for the active substances in the plant. Using the method described by Zarrow *et al.* the contraceptive properties could be extracted with methanol [334]. However, fractionation of the extract using active solvent partitioning and chromatographic procedures was associated with autocatalytic decomposition and polymerisation of the active compounds. The contraceptive activity could, however, be removed using trimethylammonium acetylhydrazide (Girard-T reagent) which isolates aldehydes and ketones. The derivatives isolated in this fashion were isolated by solvent partitioning and decomposed under acidic conditions to yield three substances (Figure 2.15) identified 1a, (1-(4-hydroxyphenyl)-ethanone; 1b, 1-(4-hydroxy-3-methoxyphenyl)-ethanone, and 1c 4-hydroxybenxaldehyde. Only the ketones 1a and 1b showed biological activity in rats but neither could account for the contraceptive activity at the levels found in the crude extract. Owing to this, in 1975 Swart *et al.* began a re-evaluation of the methodology as the extremely labile character of the active compounds required a more rapid and more sensitive biochemical micro assay [8]. Previously, Williamson and O'Donnell had described a bipyridyl derivative, metyrapone, with structural similarity to 1a and 1 b, that inhibited the terminal step in mammalian corticosteroidogenesis by binding to cytochrome P450-dependent 11β-hydroxylase (CYP11B1) [335]. Based on this work Swart *et al.* were able to develop a micro-bioassay based on the spectral and catalytic properties of CYP11B1 [336–338]. The unique spectral properties of the cytochrome P450-dependent hydroxylases allows for the analysis of enzyme-substrate and enzyme-inhibitor difference

spectroscopy. This assay provided a rapid and sensitive screening method for the biologically active extracts of *S.tuberculatiformis* [8].
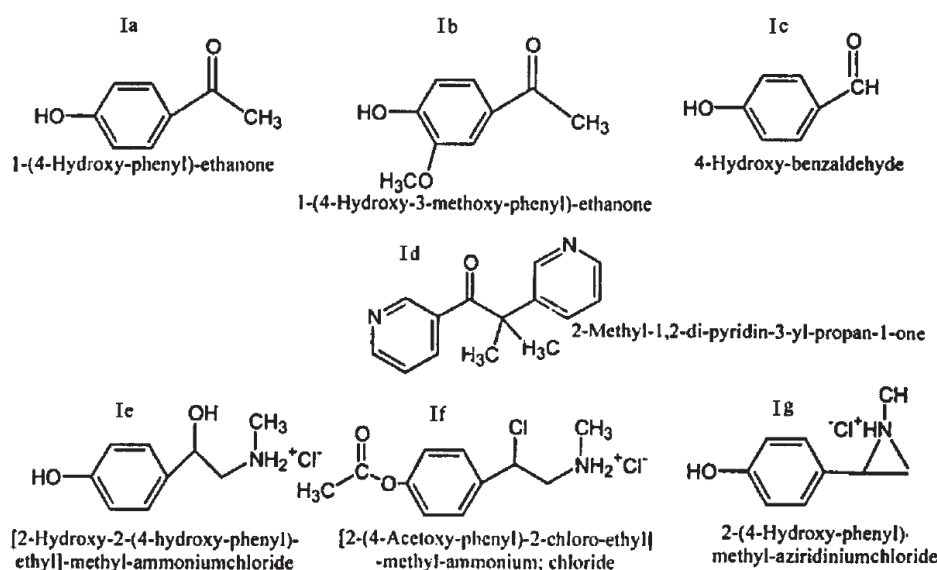
Figure 2.15 Structure of isolated compounds from S.tuberculatiformis and metyrapone, the bipyridyl derivative described by Williamson and O'Donnell (1969) [335]**.** Reproduced from Swart *et al.* (2003) [8].

Although Ia and Ib inhibited CYP11B1 it was clear that the shrub contained substances that were far more potent and two active fractions, S1 and S2, were subsequently obtained through methanol extraction, liquid ion exchange, ultra-filtration and high performance liquid chromatography (HPLC). Both fractions, although extremely labile, inhibited sheep adrenal CYP11B1. S1 decomposed rapidly when isolated and was unsuitable for any further biological work [337]. S2 could be maintained but only in a strictly controlled environment where it was protected against factors such as light, oxygen and a pH > 4. The instability of S2 prevented sufficient amounts to be prepared for structure elucidation of the active substances through gas chromatography or mass spectrometry. Acetylation of S2 followed by GC and fast atom bombardment mass spectrometry (FABMS) of the acetylated product was, however, successful and confirmed that S2 contained an aziridine as well as synephrine [339]. The decomposition of S2 is shown in Figure 2.16. In a neutral or alkaline aqueous medium S2 spontaneously decomposed to synephrine that was always present in S2 in varying amounts. Acetylation of S2 yielded the aziridine (acetic acid 4-(1-methyl-aziridin-2-yl)-phenyl ester) as well as an acetylated synephrine (Figure 2.16). This decomposition pattern was later confirmed

with electrospray mass spectrometry (ESMS) studies [340]. The isolated synephrine had no biological activity [8].
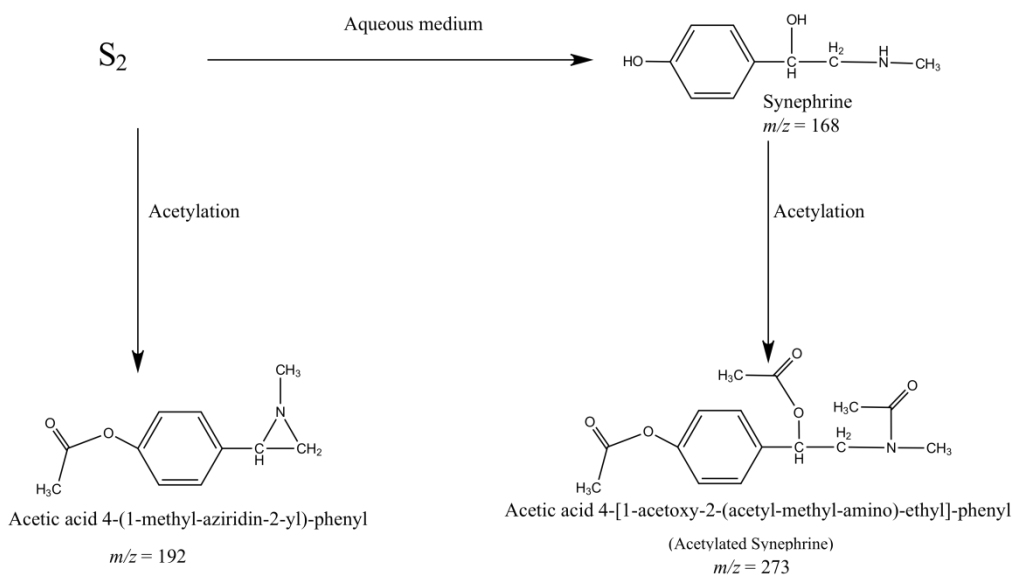
Figure 2.16 Decomposition and acetylation of S2

Aziridines are known in nature as mitomycins. Mitomycins are highly reactive N tricyclic compounds with potent antibacterial and anti-cancer properties having a broad activity against a range of tumours [341,342]. Mitomycin C, the most potent mitomycin, has been used medicinally since the 1970's for its activity against breast, stomach, oesophagus and bladder cancers [342,343]. The reactivity of the nitrogen-containing three membered aziridine ring compounds has often necessitated the use of more stable precursors in clinical studies. The precursor would have the basic pre-aziridine structure with the substitution of a good leaving group such as Cl⁻. Once ingested by or injected into the animal, cyclisation occurs to yield the active aziridine *in vivo* (Figure 2.17). It was hypothesised that the labile nature of the active S2 could be ascribed to the aziridine, 2-(4-hydroxyphenyl)-1-methyl-aziridiniumchloride, which originated from a labile hydroxyphenyl precursor present in S2 [8]. They therefore designed a more stable precursor that would cyclise, with the loss of chlorine, to form the reactive aziridine in vivo. The precursor, 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, dubbed Compound A, which was synthesised by a Ph.D. candidate, de Kock, in the laboratory (Figure 2.17) [344]. The structure of the precursor and its aziridine was confirmed by EI and FABMS and subsequently investigated as a model for the biological effects of *Salsola tuberculatiformis* Botschantzev [345,346].
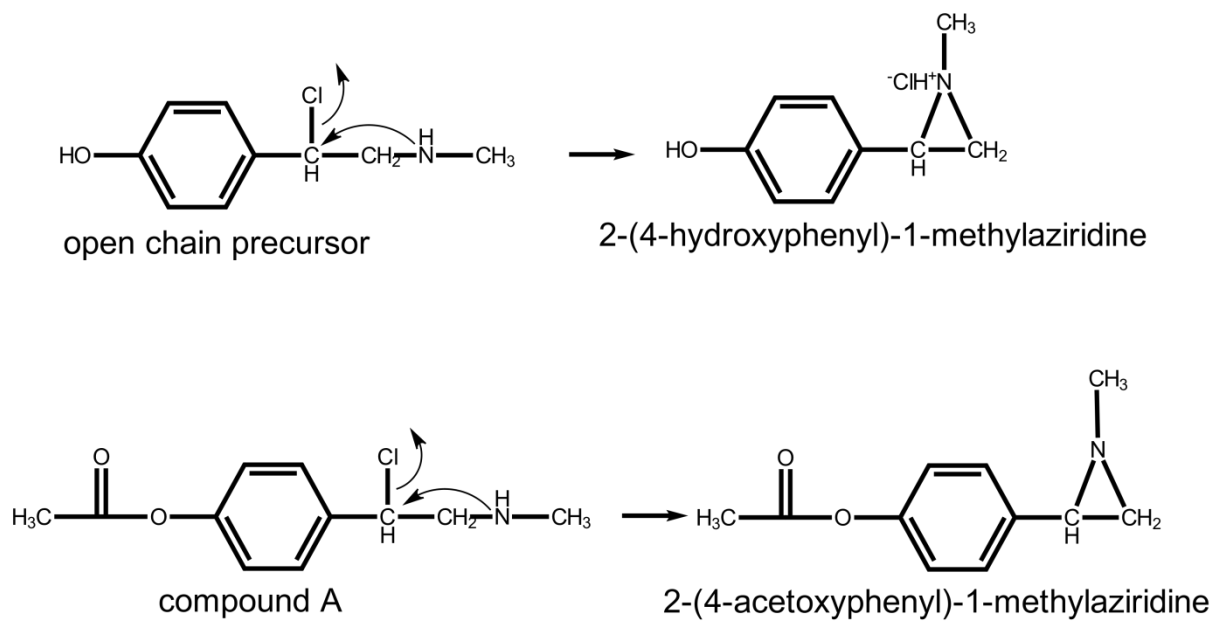
Figure 2.17 A) The cyclisation of aziridines and B) the chemical structure of CpdA. Adapted from Swart *et al.* (2003) [8].

2.3 COMPOUND A

CpdA was synthesised as an analogue of the active component in *S.tuberculatiformis* and would be expected to mimic the biological properties already exhibited by the *S.tuberculatiformis* S2 fraction. This was investigated by Louw *et al.* in two ways [346,347]. Firstly, they studied the contraceptive properties. The administration of dried *S.tuberculatiformis* and CpdA to female Wistar rats resulted in a prolonged di-estrus cycle, an effect which was reversible [346]. Secondly, they determined the influence of CpdA on CYP11B1 through the micro-bioassay developed by Swart *et al.* This assay showed that CpdA inhibited the binding of the natural substrate, deoxycorticosterone (DOC), to its enzyme, CYP11B1, comparable to S2 [347]. Further investigation revealed that CpdA produced a mixed inhibition of CYP11B1 with a stronger competitive than non-competitive element as well as inhibiting the 11β-hydroxylase conversion of DOC. Comparison with other known inhibitors of CYP11B1, such as ketoconazole and metyropone, indicated that CpdA had a lower inhibitory capacity than other inhibitors [8,348,349]. These results indicated that CpdA did mimic properties of *S.tuberculatiformis* and led to further studies using CpdA.

Louw *et al.* elucidated the possible mechanism of action of CpdA by demonstrating that CpdA interacted with steroidogenic enzymes and steroid binding globulins in plasma, displacing the endogenous steroids and affecting the bioavailability of these hormones in different target tissues. This caused a series of studies focused on CpdA and its interactions in the steroid pathways and possible therapeutic implications [350,351]. In 2008, Yemelyanov *et al.* showed that CpdA acted as a differential dual steroid receptor modulator that had the potential to be used as a novel PCa therapy [5]. They determined that CpdA could bind to the AR and GR resulting in nuclear translocation, although CpdA induced weaker translocation of the AR than the natural ligand DHT. They also showed, through virtual docking, that CpdA was able to compete with steroids for binding to the AR and GR but CpdA exerted no significant effect on other steroid hormone receptors such as mineralocorticoids, progesterone, or oestrogen receptors [5,352,353].

Yemelyanov *et al.* demonstrated more biological properties of CpdA such as its ability to inhibit transactivation of both the AR and GR and enhancement of GR

51

transrepression [5]. As mentioned previously, in addition to DNA-binding dependent gene activation, there are DNA-binding independent mechanisms of gene regulation, mostly by GR-mediated repressor interactions or cross-talk between GR and other TF's including NFκB, AP-1, p53, Ets-1, Elk-1, SRF, and NFAT. GR transrepression is central to the anti-tumour and anti-inflammatory effects of glucocorticoids. Yemelyanov *et al.* also showed that CpdA and glucocorticoids had very similar transrepression profiles as they suppressed many pro-proliferative and anti-apoptotic transcription factors [5]. In PCa cell lines CpdA significantly decreased or delayed nuclear transport of NFκB and AP-1 proteins and it was suggested that cytoplasmic retention of NFκB and AP-1 could be the leading mechanism underlying CpdA inhibition of these factors. CpdA was also shown to have anti-inflammatory effects which are believed to be due to the blocking of inflammatory genes through GR mediated transrepression and the down regulation of TF's including NFκB, T-bet and STAT6 as well as inhibition of expression of numerous pro-inflammatory cytokines and chemokines. The exact effects were shown to be dependent on the cell milieu. In PCa cell models which differentially expressed either AR or GR they showed strong cytostatic and apoptotic effects particularly in highly malignant PCa cell lines (PC3 and DUI45) with long term exposure to CpdA (4-8 days) [5]. In summary, CpdA interacted with the GR and AR while enhancing GR transrepression as well as inducing apoptosis in different PCa cell lines acting as a selective steroid modulator.

Other studies suggested that CpdA's selective steroid modulation could be due to dimerisation of the GR. It was only in the PCa cells stimulated with DEX that significant GR dimer formation was detected and it was thus deduced that CpdA-induced GR monomer formation was the mechanism by which CpdA enhanced transrepression but suppressed transactivation [354]It is, therefore, not surprising that CpdA and DEX not only differ at the level of dissociating transactivation and transrepression, but also interfering differentially with the signal transduction pathway and the activation of NFκB, in the same cell type [354,355]. An encouraging finding was that CpdA does not affect a ligand-induced GR down-regulation in primary rheumatoid arthritis and therefore does not demonstrate acquired resistance [355,356].

Although the list of reports on non-steroidal dissociated GR ligands, such as CpdA, is still growing not all studies contain sufficient data addressing side-effects.

CpdA appears to have little or no effect on GR mediated transactivation of important metabolic pathways which is why the common adverse side-effects of glucocorticoids have not been observed with CpdA. In animal models, CpdA did not lead to transactivation of important metabolic pathways and as a result did not induce hyperglycaemia and hyperinsulinemia after systemic chronic animal treatment. CpdA did not induce muscle wasting when it was used systemically in mdx mice or induce skin atrophy in rats and mice when applied topically [352,353]. More interestingly, CpdA did not affect the hypothalamopituitary axis significantly when assessed by corticosterone and ACTH serum levels [352,353]. It is now recognised that the adverse side-effects of glucocorticoids are mediated via transactivation and the anticancer and anti-inflammatory effects via transrepression making novel selective GR modulators like CpdA clinically more beneficial with less adverse side-effects [355].

Despite all the demonstrated and expected advantages for CpdA, side-effects arising during chronic *in vivo* treatment remain to be comprehensively analysed and there are some facts which have been noted which may be significant. CpdA has a relatively narrow therapeutic window and when used at high doses (~15 mg/kg) for longer periods it gradually induced apoptosis. This would also be expected to be experienced in healthy cells. The apoptosis may be induced by GR-independent metabolites of the compound and has been shown to be a dose dependent toxicity which could limit clinical development and would certainly influence dosing and the therapeutic window. CpdA, like its parent compound in *S.tuberculatiformis,* is an unstable molecule that readily decomposes into a cyclic aziridine structure when subjected to light, humidity, and a pH > 8. In vivo studies have always had to be carried out under well-controlled conditions to circumvent this problem and synthesis of more stable compounds using CpdA as a prototype are in progress [352,353].

In the review by Lesovaya *et al.* in 2015 ten years of collaborative research on CpdA was summarised [353]. It emphasised the unusual biological properties of CpdA and highlighted its action as a dual steroid receptor ligand for the AR and the GR. CpdA exhibited anti-inflammatory and anti-androgen effects and selective glucocorticoid action. These properties are expected to translate into lower glucocorticoid-like adverse side-effects and reduce the likelihood resistance. Thus, CpdA and its prospective derivatives, are attractive candidates for clinical

applications [353]. The selective steroid modulation of CpdA is a mechanism of action that, in light of the previously discussed mechanisms of PCa progression, is clearly suited to novel chemotherapy in PCa. However, the difficulty in isolating and maintaining the active compounds of Salsola *tuberculatiformis* Botsch led to the research on the plant being abandoned for the synthetic derivative. Since the main body of research to date has been performed with the synthetic CpdA analogue and not the original plant extract it remains unclear to what extent these potential benefits are shared by the *S.tuberculatiformis* crude extract and whether other or alternate compounds from *S.tuberculatiformis* would offer additional or complementary benefits. The aim of this investigation was a preliminary study in the comparative characterisation of the modulatory effects of CpdA and a crude *Salsola tuberculatiformis* Botsch extract on the AR and GR in PCa cells.

2.4 PROJECT OBJECTIVES

This study assessed the biological properties of the *S.tuberculatiformis* crude extract with specific focus on its ability to mimic known properties of CpdA and on its effects on PCa. The specific aims which were investigated are as follows:

1. The preparation of a methanol extract of *S.tuberculatiformis* and confirmation of biological activity using the CYP11B spectral assay.

2. To investigate the effect of the *S.tuberculatiformis* crude extract on the androgen and glucocorticoid receptors in prostate cancer cell models using immunofluorescent analytical techniques.

3. To determine the anti-proliferative effect in different prostate cancer cell models by firstly determining the potencies of CpdA and *S.tuberculatiformis* using viability assays followed by the assessment of apoptotic effects via caspase-3/7.

CHAPTER 3

METHODOLOGY

This chapter presents the protocols used in this study and this includes the sourcing of the general reagents, steroids, antibodies and plasmids as well as a description of the techniques used in cell culture. Protocols for immunofluorescence and the activity and apoptosis assays were developed for the purpose of this study as these had not previously been established as standard techniques used in our laboratory. Additionally methods for *S.tuberculatiformis* extraction and sourcing and the synthesis of CpdA are described.

3.1 GENERAL REAGENTS

3.1.1 GENERAL REAGENTS

Penicillin-streptomycin, trypsin-EDTA and foetal calf serum were purchased from Gibco-BRL (Gaithersburg, USA). Corning® CellBIND® Surface plates were purchased from Corning® Life Sciences (NY, USA) and 8-well Lab-Tek® Chamber Slides, DAKO were purchased from Sigma-Aldrich (St. Louis, USA). Transparent 96-well microplates were purchased from Greiner Bio-One International. Ham's F12K and RPMI-1640 medium were also purchased from Sigma-Aldrich (St. Louis, USA). X-tremeGENE HP DNA Transfection Reagent was purchased from Roche Diagnostics (Mannheim, Germany). Molecular grade agarose D1 LE was purchased from Whitehead Scientific, Brackenfell, RSA and GR Green stain was obtained from Excellgen, Inc., Rockville, MD, USA. 1 kb DNA ladder was purchased from Promega. Staurosporine, TNFα and Interleukin-1 were all purchased from Sigma-Aldrich (St. Louis, USA). DAKO mounting medium and Hoescht stain were purchased from Aligent Technologies (Santa Clara, USA). The Wizard® Plasmid isolation kit, the Luciferase Assay System, the CellTiter-Glo® Luminescent Cell Viability Assay and the Caspase-Glo® 3/7 Assay were purchased from Promega Biotech (Madison, WI, USA). The NucleoBond® Xtra Plasmid DNA Purification kit was purchased from Clontech Laboratories (Mountain View, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL., USA). Trypan blue stain (0.4%) and cell count plates were purchased from Invitrogen (Eugene, USA). All

other chemicals and reagents were sourced from reputable scientific supplies companies.

3.1.2 STEROIDS

Fluocinolone acetonide (6α, 9α-Difluoro-16α-hydroxyprednisolone 16, 17-acetonide; FA), dihydrotestosterone (5α-androstan-17β-ol-3-one; DHT) and 11-deoxycorticosterone (21-Hydroxy-4-pregnene-3, 20-dione; DOC) were purchased from Sigma-Aldrich (St. Louis, USA). All steroids were dissolved in absolute ethanol and added to the culturing medium in a final concentration of no more than 0.1% ethanol

3.1.3 ANTIBODIES

The polyclonal rabbit anti-AR(C-19) and anti-GR (H-250) IgG were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). The fluorescent secondary anti-rabbit antibody AlexaFluro®568 (A10042) was purchased from Life Technologies (Thermo Scientific).

3.1.4 PLASMID CONSTRUCTS

The plasmid expressing the human AR, pSVARo was obtained from Prof. F. Claessens (University of Leuven, Belgium). Prior to experiments, cells from cryogenic storage were routinely and aseptically streaked onto 1.5% Luria Bertani (LB)-agar plates containing 100µg/mL ampicillin. Plates were incubated at 37°C until well defined single colonies of ± 2 mm in diameter had formed. Single colonies were inoculated in 5 mL LB media, with antibiotics, and incubated at 37°C at 235 RPM for 8 hours as initial cultures. This initial culture was used to inoculate a larger (100-300ml) culture which was incubated at 37°C at 235 RPM for 12-16 hours. Plasmid DNA was extracted using a NucleoBond® Xtra Plasmid DNA Purification kit following procedure according to the manufacturer's protocol said in 3.1.1. The identity of the constructs was verified by restriction enzyme digestion and subsequent agarose gel electrophoresis. The purity and concentration of the isolated DNA was determined by calculating the absorbance of the preparation at 260 and 280 nm.

3.1.4.1 Agarose Gel Electrophoresis

Agarose gel electrophoreses of purified plasmids was performed with 1% molecular grade agarose D1 LE in TAE buffer. GR Green stain (0.01% solution) was added to the agarose-TAE mixture prior to gel formation. DNA samples were mixed in a ratio of 1:2 with Ficoll Orange loading dye (0.1% Orange G, 20% Ficoll, 10 mM EDTA). Electrophoresis was initially carried out at 75 V until DNA samples entered into the gel matrix upon which an increase to 110 V was applied. In all cases a 1 kb DNA ladder was used as a molecular size marker. Following electrophoreses, agarose gels were visualised with a UV transilluminator and analysed with E-Capt, version 12.9 for Windows (Vilber Lourmat, Marne-la-Vallée, France).

3.1.5 TISSUE CULTURE

Three PCa cell lines were included in this study, LNCaP, PC-3 and PC-3 transfected with AR. LNCaP cells are a widely used cell model representing an androgen dependent PCa model with low metastatic potential and express the AR, while PC-3 cells represent a model for androgen independent PCa with a high metastatic potential expressing the GR. PC-3 transfected with the AR express both the AR and the GR. LNCaP cells were originally derived from the left supraclavicular lymph node of a 50-year-old Caucasian male with metastatic PCa. The cells contain a ligand-binding domain-mutated version of the AR (T877A) [77]. PC-3 was originally derived from bone metastasis of grade 4 PCa in a 62 year old Caucasian male. Both PC3 and LNCaP cells were purchased from the European Collection of Cell Cultures (ECACC).

LNCaP cells were cultured in RPMI-1640 media supplemented with 10% foetal calf serum (FCS), 1.5 g NaHCO3/L (pH 7), 2.5 g D-(+)-Glucose, 1% HEPES, 1% sodium pyruvate and 1% penicillin-streptomycin and at all stages cultured using Corning® CELLBIND® surface plates. PC3 cells were cultured in Ham's F12K media supplemented with 10% foetal calf serum (FCS), 1.5 g NaHCO3/L (pH 7), 2.5 g D-(+)-Glucose, 1% HEPES, 1% sodium pyruvate and 1% penicillin-streptomycin. Cells were seeded at a density of 100 000 cells/ml and all cell lines were cultured at 37°C, in an atmosphere of 90% humidity and 5% CO_2. All cells were screened for mycoplasma infection and were mycoplasma free and were passaged for fewer than 6 months from the time of resuscitation. Both LNCaP and PC3 cells were plated in

96-well plates at 100 000 cells/ml. After 24 hours the PC3 cells were transfected with the pSVARo plasmid using X-tremeGENE HP DNA transfection reagent. After a concurrent 24 hour period the PC3 and LNCaP cells were treated for 16 hours with FA, DHT, CpdA, *S.tuberculatiformis* extract, control and vehicle.

3.1.6 S.TUBERCULATIFORMIS EXTRACT

Based on the techniques of Zarrow *et al.,* it was confirmed that the contraceptive properties of *S.tuberculatiformis* could be extracted with methanol. The following extraction method was developed [9,334]. Owing to the labile nature of the *S.tuberculatiformis* extract, it was protected from light and O_2 at all stages of preparation. The non-polar compounds were first removed by soxhlet extraction of 20 grams of ground *S.tuberculatiformis* with 200ml of dichloromethane for 8 hours. Following this preliminary extraction, the bio-active polar compounds were obtained by further soxhlet extraction with 200ml of methanol for 8 hours. The methanol was removed by solvent evaporation. The resultant extract was resuspended in 50ml of analytical grade water and centrifuged at 10 000xg for 5 minutes before filtration through a 0.45μm pore size filter. The *S.tuberculatiformis* extract was aliquoted into 1.5ml eppendorf tubes and stored at - 80˚C, protected from light

In order to determine the amount of plant material extracted from 20 grams of *S.tuberculatiformis,* the concentration of the resuspended extract was determined by pipetting 1ml into a vial and weighing it on an analytical scale. The vial was then placed in the oven for approximately 2 days or until the extract was dry and all water had evaporated. Once dried, the vial was weighed and the mass of extracted plant material per mL determined. The calculated concentration was thus assumed for all stored aliquots used in subsequent studies.

3.1.7 COMPOUND A (2-(4-ACETOXYPHENYL)-2-CHLORO-N-METHYL-ETHYLAMMONIUM CHLORIDE)

CpdA was acquired from Ms. Liezel Swart, a fellow student, who synthesized the compound in the department of Biochemistry at the University of Stellenbosch. Chromatography was performed for verification.

3.2 P450 DIFFERENCE SPECTRAL ASSAY

The P450 difference spectral assay, developed by Swart et al, was used to confirm inhibition of CYP11B1as an indicator of the biological activity in *S.tuberculatiformis* crude extract and CpdA [8,336].

The cytochrome P450 enzymes are a group of haemoproteins containing between 400 and 500 amino acids, depending on the type of P450, and a single haem prosthetic group [357,358]. The distal ligand of the haem moiety is a thiolate anion from a cysteine residue (Figure 3.1) [359]. It is the iron contained in the haem group which gives cytochrome P450 enzymes their unique spectral properties.
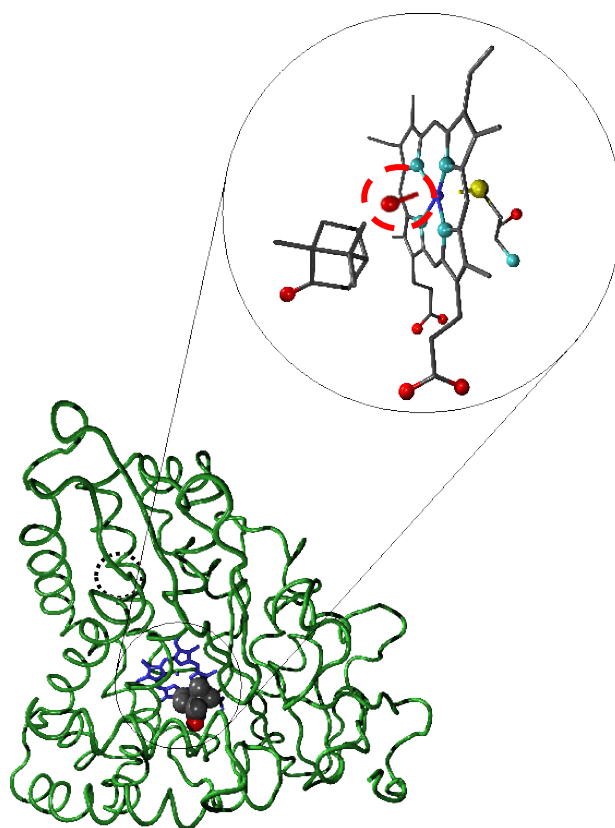
Figure 3.1: Representation of a P450 enzyme bound camphor (P450CAM). The enlarged active site region shows the camphor substrate, haem moiety and cysteine residue which forms the distal haem ligand. *Dotted circle* indicates the water molecule that forms the sixth axial ligand of the haem iron (Exists only in the substrate free form). In the representation of the full enzyme the protein backbone is shown in green, the haem moiety in blue and the substrate is coloured according to atomic species. Red; Oxygen, Grey; Carbon, Light Blue; Nitrogen, Yellow; Sulphur, Dark Blue; Iron. Reproduced from Segall (1997)[360].

Fe^{3+}, in the haem system, can exist in two spin states; a high spin (HS) and a low spin (LS). Intermediary spin states are possible but not normally found in biological systems. These two spin states are characterised by distinct traits, as follows [361]:

High Spin State [s=5/2]:

- The five 3d electrons are maximally unpaired

- Five-fold coordinated Fe^{3+} is found in the HS state

- Ionic radius is larger in the HS

- The Fe^{3+} moves out of the plane of the porphyrin ring because the central cavity is too small

Low Spin State [s = 1/2]:

- The five 3d electrons are maximally paired

- Six-fold coordinated Fe^{3+} is found in the LS state

- Ionic radius is smaller in the LS

The HS and LS states are not independent, but exist in equilibrium. The differences in the absorption and extinction coefficients between the two bands at 390nm and 420nm allow the equilibrium constant between the spin states and hence the fraction of HS character to be determined. The equilibrium between these states may be affected by many factors such as changes in the conformation of the enzyme which alter the nature of the binding of a ligand.

The nature of the axial haem ligands has an important effect on the LS-HS balance: A strong axial field will bring about a relatively large d-orbital splitting, favouring the LS state. For example, when comparing the UV absorption spectra of CO complexes with haemoglobin (histidine axial ligand) and P450 (cysteine ligand), the axial ligand field strength due to histidine is much stronger than that due to cysteine. Correspondingly the UV absorption band due to the d $\rightarrow$ d transition in CO-haemoglobin occurs at 420nm, while that due to CO-P450 occurs at 450nm. Hence the name P450 enzymes [362,363].

Owing to these spectral properties there are three types of ligand binding which may be identified by changes in absorbance in the Soret region (Figure 3.2):

1. **Type I spectrum [364–366]:**

   The spectrum is associated with the binding of a substrate. It is characterised by a minimum in the Soret absorption band at 420nm and a maximum at 390nm with an isobestic point at 407nm. This indicates a change from LS to HS state of the ferric iron. X-ray crystallography of substrate-bound complexes of the P450 enzyme show that the iron-ligated water molecule is displaced in these cases, changing the Fe^{3+} from a 6-fold to a 5-fold coordination state. The Fe^{3+} ion is also seen to move out of the plane of the haem.

2. **Type II spectrum [367–369]:**

   The spectrum is characterised by a maximum absorption between 425nm and 435nm and a minimum at 390nm with an isobestic point at approximately 419nm. This represents an increase in the LS character of the Fe^{3+}. This spectral change is associated with the interaction of inhibitors which bind directly to the Fe^{3+}, replacing the water molecule as the sixth axial ligand.

3. **Reverse type I spectrum:**

   The spectrum is a `mirror image' of the type I spectral change, with a maximum at 420nm and a minimum at 390nm. This is similar to a type II change, except that there is no shift in the location of the maxima. The exact nature of this interaction is not known and it may be that the ligand binds to a different site and not in the active pocket of the enzyme.
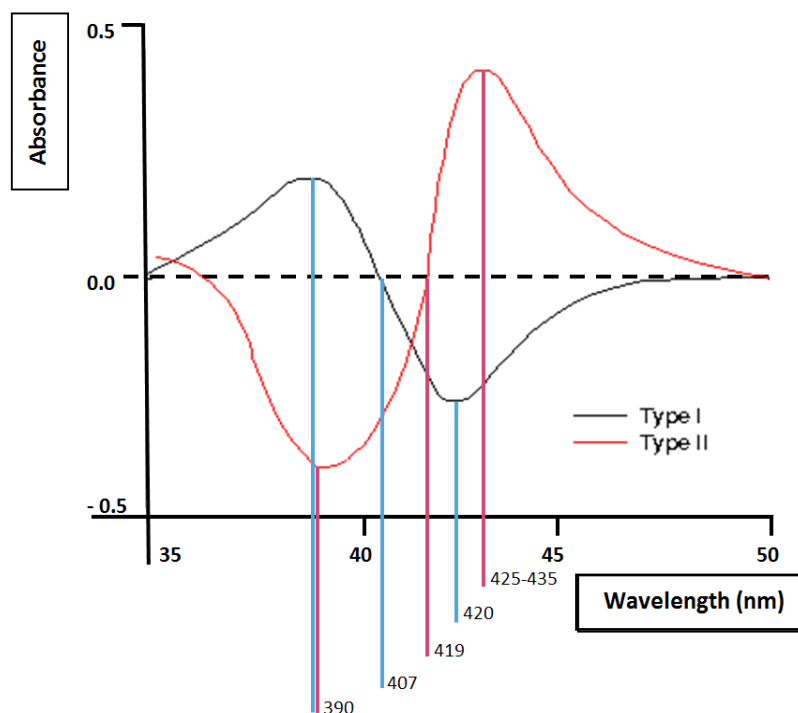
Figure 3.2 Difference spectra obtained from the addition of a substrate or inhibitor to a cytochrome P450 enzyme. The black and red lines represent the type I and type II spectra, respectively. Indicated on the graph is the wavelength of the maxima, minima and isobestic points of the type 1 (blue lines) and type 2 (red lines) difference spectra. Reproduced and adapted from Omura (1993) [370].

Substrate analogues may be similar to the natural substrates. However, structural differences between these analogues and natural substrates result in differences in binding to the active site. The crystal structures of complexes of substrate analogues with P450CAM show that the Fe^{3+}-coordinated water molecule is retained and a high fraction of the LS character of the Fe^{3+} remains [365,371,372]. The stabilisation of the LS state is believed to be primarily due to the bound water molecule [366]. The redox potentials of the substrate analogue-bound complexes fall between those of the substrate-free and substrate-bound systems. Indeed, a linear free energy relationship between the redox potential and spin state has been found [373].

Many classes of molecules may inhibit the action of a P450 enzyme. For example, small molecules such as CO and NO, which compete with O_2 in binding to the reduced haem iron, metal ions (e.g. CO^{2+}) and mechanism based inhibitors such as chloramphenicol [362,374,375]. This investigation was concerned with molecules which compete directly with the substrate molecule binding in the active site. These ligands all contain an electron negative atom which coordinates directly with the haem Fe^{3+}, displacing a water molecule as the sixth axial ligand, as demonstrated

63

by crystal structures of P450$_{CAM}$ complexed with inhibitors such as metyrapone and phenylimidazole [367]. The UV absorption spectra of the enzymes display a type II shift on binding of such molecules, indicating an increase in the LS character of the Fe^{3+} [374,376].

Thus using these unique spectral properties the binding of a natural substrate can be assayed. In addition, the enzyme/substrate interaction (complex formation) can also be assessed by assaying the degree to which a compound may either inhibit the natural substrate from binding or either may itself bind to the enzyme in the absence of substrate. This technique was applied to both CpdA and the *S.tuberculatiformis* crude extract to demonstrate biological activity using the spectral properties of sheep adrenal CYP11B1.

3.2.1 PREPARATION OF A CYP11B1 CONTAINING MITOCHONDRIAL ACETONE POWDER FROM OVINE ADRENALS:

In order to obtain a CYP11B1 containing preparation, fresh ovine adrenal glands were harvested perimortem from Tomis Abattoir & Fresh Meats Wholesalers (Hermon, South Africa) and stored at - 20˚C. P450-enriched adrenal mitochondrial acetone powder from ovine adrenals was prepared as previously published [377]. In order to avoid degradation, the mitochondrial isolation and all subsequent work with the preparation was performed at 4˚C. The adrenal glands were decapsulated and washed in 0.15M KCl solution (pH 7.4) before being re-suspended in buffer A (10mM Tris-HCl, 1mM EDTA, 0.25M sucrose, pH 7.4) in a 1:3 ratio. The decapsulated adrenals (100 g) were firstly homogenised with a Waring-Blender after which the resulting adrenal homogenate was homogenised with a Potter-Elvejhem glass homogeniser. The homogenate was subsequently centrifuged at 1000 xg for 20 minutes to remove cellular debris and fat cell. The supernatant was centrifuged at 12 000 xg for 15 minute, to yield the P450-enriched mitochondrial pellet. This P450-enriched mitochondrial pellet was washed three times by resuspension and homogenising with a Potter-Elvejhem glass homogeniser. The first resuspension was in 200ml of Buffer B (10 mM Tris-HCl buffer, containing 1mM EDTA, 0.25 M sucrose and 1% (m/v) BSA, pH 7.4) followed by two resuspensions in Buffer C (10mM Tris-HCl, 1mM EDTA, 0.25M sucrose, 1% BSA, pH 7.4). After each wash step the resuspended pellet was centrifuged at 12 000xg for 15 minutes. The

64

mitochondrial pellet was frozen with liquid nitrogen and lyophilized overnight. The dried pellet was resuspended in 100 mL dry acetone and homogenised with the Potter-Elvejhem glass homogeniser in order to remove residual lipids. The homogenate was subsequently centrifuged for 15 minutes at 12 000 xg. The supernatant was decanted and the mitochondrial acetone pellet lyophilized overnight. The lyophilized powder was stored at -20°C.

Verification of CYP11B1 activity was performed using a P450 spectral assay. The mitochondrial powder was resuspended and homogenised, with a Potter Elvejhem homogeniser, in phosphate buffer (0.1M phosphate buffer, 10% ethylene glycol, pH 7.4) at a concentration of 2mg/ml. The mitochondrial homogenate was rested on ice for 10-15 minutes before sonication. Sonification was carried out for 5 mins at 60 Watts. However, in order to avoid over-heating of the mitochondrial preparation and loss of enzyme activity one minute intervals were included between each consecutive minute of sonication. CO was bubbled through the mitochondrial sonicate to saturate the binding sites of the heme iron. Equal volumes (1.5 mL) of the mitochondrial preparation were subsequently divided into two optically matched 1.5 quartz cuvettes and a corrected baseline was recorded between 400-500 nm. Solid sodium dithionite (1 – 2 mg) was subsequently added to the sample cuvette and the contents inverted to dissolve the sodium dithionite, reducing the heme-iron from the ferric to the ferrous state. A difference spectrum was recorded between 400 - 500 nm. The P-450 content was calculated using an extinction coefficient of 91 $cm^{-1}mM^{-1}$ and a path length of 1cm [357].

3.2.3 CYP11B1 DIFFERENCE SPECTRA

Mitochondrial powder, re-suspended in phosphate buffer (2 mg/mL), was homogenised and sonicated for 5 minutes as described above (3.2.1). A substrate-induced difference spectrum was obtained by pipetting an equal volume (1496μL) of the mitochondrial suspension into two optically matched cuvettes and a corrected baseline was recorded between 360-500nm. DOC (4 μl, 1.2 mM) was added to the sample cuvette resulting in a final concentration of 3.2μM. An equal volume of absolute ethanol was added to the reference cuvette. The content of both cuvettes were inverted gently to ensure uniformity and a difference spectrum recorded between 360-500 nm.

The influence of the compounds on the substrate-induced difference spectrum was subsequently assayed to determine whether the compounds were able to inhibit the substrate from binding to CYP11B1. The mitochondrial suspension (3480 µL) was mixed with CpdA (20 µL, final concentration) or extract (20 µL, final concentration) to obtain a final volume of 3500 µL. An equal volume (1496µL) was pipetted into two optically matched cuvettes and a corrected baseline was recorded between 360-500nm. DOC (4 µL, final concentration, 1.2 mM) was added to the sample cuvette resulting in a final concentration of 3.2µM. An equal volume of absolute ethanol was added to the reference cuvette. The content of both cuvettes were inverted gently to ensure uniformity and a difference spectrum recorded between 360-500 nm. The inhibition of the compounds on the substrate-induced difference spectrum was indicated by a decrease in amplitude, i.e. decreased maximum absorbance at 390 nm and an increase in minimum absorbance at 420 nm.

From this difference spectrum the percent inhibition can be calculated owing to the discrepancy in minima between the samples.

Percent Inhibition (%) =

$$100 - \left[\frac{\text{CS OD dist (A390} - \text{A420)}}{\text{S OD dist. (A390} - \text{A420)}} \times 100 \right]$$

Where CS is substrate plus CpdA or extract and S is substrate

3.3 AR AND GR NUCLEAR TRANSLOCATION

Immunofluorescence or cell imaging techniques rely on the use of antibodies to label a specific target antigen with a fluorescent dye (also called fluorophores or fluorochromes). Antibodies that are chemically conjugated to fluorophores are commonly used in this technique. The fluorophore allows visualization of the target distribution in the sample under a fluorescent microscope (e.g. confocal microscopes) (Figure 3.3). There are two immunofluorescence methods depending on whether the fluorophore is conjugated to the primary or the secondary antibody. The direct method uses a single antibody directed against the target of interest and in this method the primary antibody is conjugated to the fluorophore. The indirect method uses two antibodies, the primary antibody is unconjugated and a fluorophore-conjugated secondary antibody directed against the primary antibody is used for detection. In this study the indirect method was used to determine if *S.tuberculatiformis* and CpdA induced nuclear translocation of the AR and GR.

3.3.1 IMMUNOFLUORESCENCE

LNCaP and PC-3 cells were cultured as previously described in 3.1.5. LNCaP cells were plated into an 8-well Chamber Slide at a seeding density of 4.5×10^4 cells per well (300µl per well) and PC-3 cells were plated into an 8-well Chamber Slide at a seeding density of 3.0×10^4 cells per well (300µl per well). After 48 hours LNCaP, PC3 and AR transfected PC3 cells were incubated with test compounds, 1 µM FA, 1 µM DHT, 10 µM CpdA, 0.03 mg/ml *S.tuberculatiformis* extract and vehicle (0.1% EtOH). Relevant controls were included; transfection control, without test compound, positive and negative controls.

After 16 hours of exposure to the relevant compounds the cells were fixed with 4% paraformaldehyde in PBS (0.01M solution, pH 7.4) for 10 minutes at room temperature. The cells were washed 3 times with PBS and were subsequently permeabilised with PBS containing 0.1% Triton X-100 for 10 minutes at room temperature. The wash step was repeated before blocking with 1% BSA in PBS buffer containing 0.1% Tween 20 (PBST) for 1 hour at room temperature. The primary antibodies (anti-AR IgG and anti-GR IgG) were diluted according to the manufacturer's specifications in PBST containing 1% BSA and added to the cells after which the cells were incubated overnight in a humidified chamber at 4˚C. The

cells were subsequently washed and incubated in the dark with a fluorescent labelled secondary antibody in PBST (1% BSA) for 1 hour at room temperature (Figure 3.3). The wash step was repeated prior to incubation with 0.1µg/ml Hoescht DNA stain for 3 minutes at room temperature. Cells were rinsed with PBS before applying a drop of fluorescent mounting medium and stored at -20˚C protected from light, this preserved the fluorescence of the samples and allows the sample to last for a prolonged period (± 1 year). The fluorescence was measured with a confocal microscope and the images were analysed with ZEN lite (ZEISS Microscopy).

Spectral Properties of DyLight Fluorescent Dyes					
Emission	**Fluor**	**Ex/Em†**	**See Spectra**	**ε††**	**Spectrally Similar Dyes**
Blue	DyLight 350	353/432		15K	Alexa Fluor* 350, AMCA
Blue	DyLight 405	400/420		30K	Alexa Fluor 405, Cascade Blue*
Green	DyLight 488	493/518		70K	Alexa Fluor 488, fluorescein, FITC
Yellow	DyLight 550	562/576		150K	Alexa Fluor 546, Alexa Fluor 555, Cy3*,TRITC
Red	DyLight 594	593/618		80K	Alexa Fluor 594, Texas Red*
Red	DyLight 633	638/658		170K	Alexa Fluor 633
Red	DyLight 650	652/672		250K	Alexa Fluor 647, Cy5*
Near IR	DyLight 680	692/712		140K	Alexa Fluor 680, Cy5.5*
Near IR	DyLight 750	752/778		210K	Alexa Fluor 750
Infrared	DyLight 800	777/794		270K	IRDye* 800

†Excitation and emission maxima in nanometers (+/- 4nm) in phosphate-buffered saline (PBS)
††Molar extinction coefficient (M^{-1} cm^{-1}) at the absorption maximum

Figure 3.3 Range of fluorescent secondary antibodies available. The bold box represents the secondary antibody used in this experiment.

3.4 CELL VIABILITY AND APOPTOSIS ASSAYS

3.4.1 CELLTITER-GLO® LUMINESCENT CELL VIABILITY ASSAY

The CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the potency and IC_{50} of both CpdA and the *S.tuberculatiformis* extract. The Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on the quantification of ATP present in the cells, which signals the presence of metabolically active cells. This assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable "glow-type" luminescent signal with a half-life of greater than five hours. The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture in agreement with previous reports (Figure 3.3A). The mechanism for this luminescence is illustrated in Figure 3.3B

LNCaP and PC-3 cells were cultured as previously described and plated in 96-well plates at a density of 100 000 cells/ml. After incubating test compounds for 16 hours the cells were equilibrated at room temperature for approximately 30 minutes. The CellTiter-Glo® Luminescent Cell Viability Assay was carried out in accordance with the manufacturer's protocol. 100ul of CellTiter-Glo® reagent was added per well and incubated in the dark at room temperature for 30 minutes. Luminescence was measured on a luminometer or spectrophotometer.

A

Cell number correlates with luminescent output.

$r^2 = 0.9999$

(Y-axis: Luminescence (RLU), ranging from 0 to 7,000,000)
(X-axis: Cells/Well, ranging from 0 to 20,000)

B

Light

Oxyluciferin

Ultra-Glo™ Luciferase O_2

Mg^{2+} ATP

Luciferin   COOH

VIABLE

The cell is a source of ATP in the Luciferase Reaction, so the luminescence produced is proportional to the number of viable cells

CellTiter-Glo® 2.0 Assay is lytic.

ATP

NADH

Figure 3.4: A) The direct correlation between cell number and Luminescence and B) the generation of luminescence in the CellTiter-Glo® Luminescent Cell Viability Assay

3.4.2 APOPTOSIS ASSAY

The ability of CpdA and *S.tuberculatiformis* extract to trigger apoptosis was measured using the Caspase-Glo3/7 assay. A key feature of caspases in the cell is that they are present as inactive precursors, termed pro-caspases, which remain inactive until a specific biochemical change causes their activation. Caspase-3 has a typical role in apoptosis, where it catalyses chromatin condensation and DNA fragmentation. Caspase-3 shares many of the typical characteristics common to all currently-known caspases. Its active site contains a cysteine residue (Cys-163) and histidine residue (His-121) that stabilize the peptide bond cleavage of a protein sequence to the carboxy-terminal side of an aspartic acid when it is part of a particular 4-amino acid sequence. This specificity allows caspases to be highly selective, with a 20,000-fold preference for aspartic acid over glutamic acid. Each pro-caspase has an N-terminal large subunit of approximately 20 kDa followed by a smaller subunit of approximately 10 kDa. These subunits are called p20 and p10, respectively.

Caspase-3 is activated in the apoptotic cell both by extrinsic and intrinsic pathways as previously discussed. The precursor of caspase-3 is required because if unregulated, caspase activity would kill cells indiscriminately. As an executioner caspase, the pro-caspase-3 has virtually no activity until it is cleaved by an initiator caspase after apoptotic signalling events have occurred (Figure 3.5).
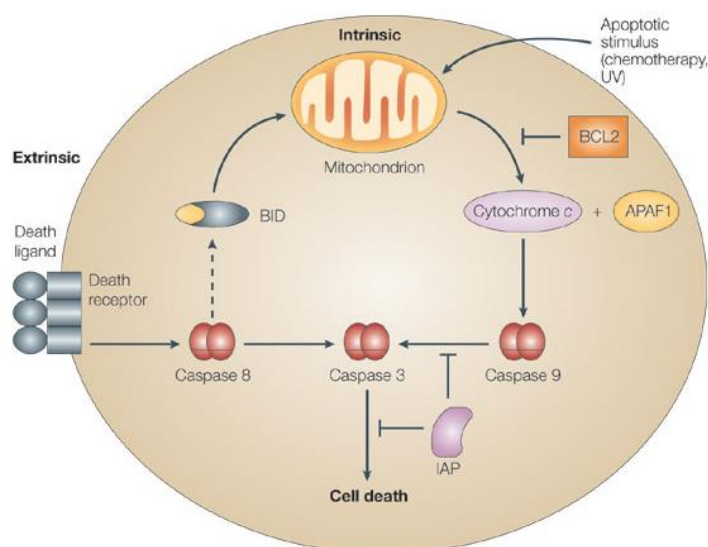
Figure 3.5: Basic schematic of the caspase cascade pathway

71

LNCaP and PC-3 cells were cultured as previously described in 3.1.5 and plated in 12-well plates at a density of 100 000 cells/ml. After the 16 hour-incubation period with test compounds the cells were washed with PBS (0.01M solution) before adding passive lysis buffer (100 µL/ well) and freezing the cells at -80°C for 3-24 hours. The Caspase-Glo® 3/7 Assay was carried out in accordance with the manufacturer's protocol. The samples were thawed at room temperature for approximately 30 minutes. Lysate (25µl per a well) was then added to a 96-well plate followed by Caspase-Glo® 3/7 reagent (25µl per a well; 1:1 ratio). The plate was incubated, in the dark, at room temperature for approximately 30 minutes to 3 hours. The luminescence was read on a luminometer or spectrophotometer. The subsequent data was normalised with the protein concentration of each well.

2.2.2 Protein determination

Protein concentrations were determined using the Pierce BCA method according to the manufactures instructions. Each thawed lysed sample, 10 µL, was placed in a 96 well transparent microplate together with a range of bovine serum albumin (BSA) standards prepared in passive lysis buffer (Table 3.1.). A BCA working reagent was prepared by mixing Reagent A and Reagent B in a 50:1 ratio and 200 µl of the working reagent was pipette into each well. Samples were incubated at 37°C for 30 minutes. Thereafter, the absorbance at 540 nm was measured on a Spark™ 10M multimode microplate reader. The average absorbance measurement of the blank (passive lysis buffer) replicates was subtracted from the measurements of all other individual standard and unknown sample replicates. A standard curve was generated by plotting the corrected absorbance for each BSA standard versus concentration. The generated standard curve was subsequently used to determine the protein concentration for each unknown sample.

Table 3.1 Preparation of standards from BCA protein determination.

	Lysis buffer volume (µl)	Volume and Source of BSA (µl)	Final [BSA] (µg/ml)
A	0	300 of Stock (2 mg/ml)	2000
B	125	375 of Stock (2 mg/ml)	1500
C	325	325 of Stock (2 mg/ml)	1000
D	175	175 of B dilution	750
E	325	325 of C dilution	500
F	325	325 of E dilution	250
G	325	325 of F dilution	125
H	400	100 of G dilution	25
I	400	0	0 = blank

3.5 STATISTICAL ANALYSIS

The Graph Pad Prism® software (version 6) was used for data manipulations, graphical representations and statistical analysis. Non-linear regression and sigmoidal dose response were used in cell viability experiments. The effect of S.tuberculatiformis extracts and CpdA on cell viability and growth were analysed using one-way ANOVA followed by Bartlett's multiple comparisons test. All compound treatments were compared to the control. Statistically significant differences are indicated by *, ** or *** for $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

CHAPTER 4

RESULTS

In this chapter the results of the study are presented. Biological activity of the *S.tuberculatiformis* extracts and CpdA is demonstrated followed by evidence for nuclear translocation through cell imaging for both the *S.tuberculatiformis* extract and CpdA. Both the *S.tuberculatiformis* extract and CpdA caused nuclear translocation in the AR and the GR receptors but the effect of CpdA on translocation of the GR receptors was weaker than that of *S.tuberculatiformis* and FA. Anti-proliferative activity was evaluated by cell viability and apoptosis assays for CpdA and *S.tuberculatiformis* extracts, both individually and comparatively. Both demonstrated reduction in cell growth with CpdA showing greater potency. Neither CpdA nor *S.tuberculatiformis* extracts were able to induce apoptosis.

4.1 BIOLOGICAL ACTIVITY

A method for screening for biologically active components in *S.tuberculatiformis* extract was previously developed in this laboratory [8,336]. Despite the practical difficulties presented by the exceptionally labile nature of the extract this bioassay which is based on the spectral properties of CYP11B1, has proven to be a rapid and sensitive evaluation of biological activity. It was through this screening method that Swart *et al.* hypothesised that the active component in *S.tuberculatiformis* originated from a labile aziridine precursor and this resulted in the synthesis of CpdA [8].   The bioassay was used to determine the effects of CpdA and *S.tuberculatiformis* extract on the spectral properties of CYP11B1 that had been partially purified from ovine adrenals. This bioassay showed that the compounds, CpdA and *S.tuberculatiformis* extract, possessed biological activity and could therefore be used in further experiments. Due to their labile nature, screening for biological activity occurred prior to all experiments to ensure that the *S.tuberculatiformis* crude extract and CpdA were active.

4.1.1 CYTOCHROME P450 ACTIVITY ASSAY

A P450 activity assay was first performed on the mitochondrial preparation. This was to ensure that the mitochondrial-bound P450 enzymes were pure, intact and active as well as to determine the concentration of the P450. The mitochondrial suspension possessed active P450 enzymes. As shown in Figure 4.1, a maximum peak at 450nm and a deep trough at ± 408nm indicated that the mitochondrial suspension was free from cytochrome P420, the inactive form of P450, characterised by a peak at 420nm. The slight shoulder observed around the isobestic point (± 425nm) can be attributed to the presence of cytochrome b5. The CO-induced difference spectrum of P450-enriched mitochondrial powder indicated that it could be used to screen for biological activity of the *S.tuberculatiformis* extract and CpdA. The P450 concentration of a 2 mg/mL mitochondrial preparation was calculated at 0.632 nmol P450/ mg protein using a millimolar extinction coefficient of 91[338]. Thus ±100g of adrenal tissue yields ±2g of mitochondrial powder and a total of ±1264 nmol P450.
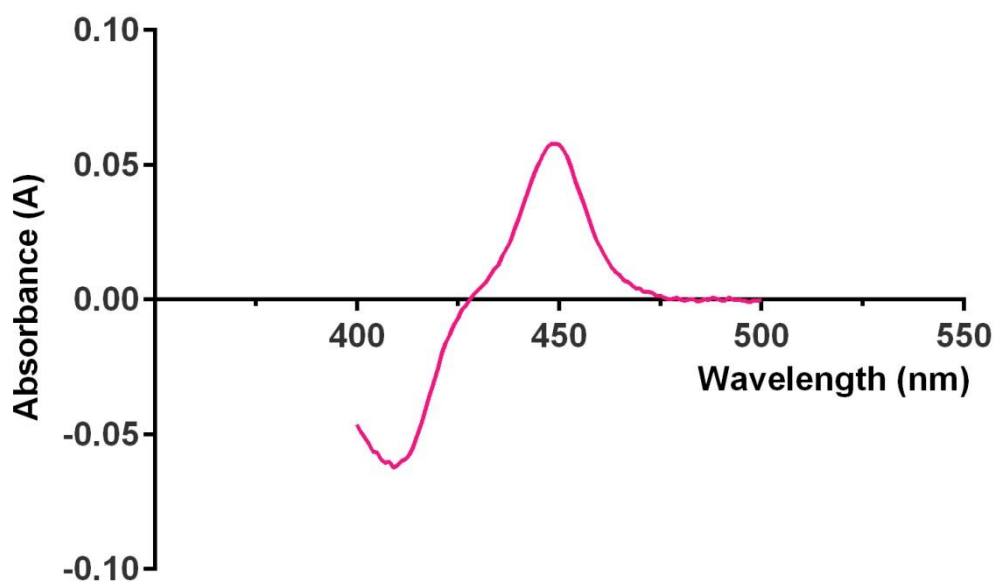
Figure 4.1 Sodium dithionite-reduced carbon monoxide-induced difference spectrum of a crude mitochondrial suspension. Mitochondrial preparation in 0.1M phosphate buffer, 10% ethylene glycol, pH 7.4, 2mg/ml; [P450], 0.632nmol P450/mg protein.

4.1.2 SUBSTRATE-INDUCED DIFFERENCE SPECTRA

The influence of CpdA (dissolved in H_2O) and the *S.tuberculatiformis* crude extract on a DOC-induced difference spectrum is shown in Figure 4.2. DOC is the natural substrate for cytochrome CYP11B1 and elicits a Type 1 difference spectrum when incubated with the mitochondrial powder isolated from ovine adrenals. The DOC-induced Type I difference spectrum exhibited an absorbance maximum at 390 nm and an absorbance minimum at 420 nm. Other compounds, that are not substrates for CYP11B1, may also bind to the enzyme and elicit a difference spectrum indicating their effect on the enzyme [378].

Figure 4.2 shows that CpdA and the *S.tuberculatiformis* extract inhibited the Type I DOC-induced difference spectra as a shift in absorbance is observed. The minimum absorbance at 420nm increased while the maximum absorbance at 390nm decreased shift. Both CpdA and *S.tuberculatiformis* inhibited the DOC-induced type I spectra by 49.2% and 61.2%, respectively (Table 4.1).

Table 4.1 Inhibition of CpdA and *S.tuberculatiformis* extract on DOC-induced spectrum.

	DOC	CpdA	*S.tuberculatiformis*
420nm	-0.0132	0.0001	0.0047
390 nm	0.0062	0.0100	0.0122
OD	0.0194	0.0098	0.0075
% Inhibition	n/a	49.216	61.155
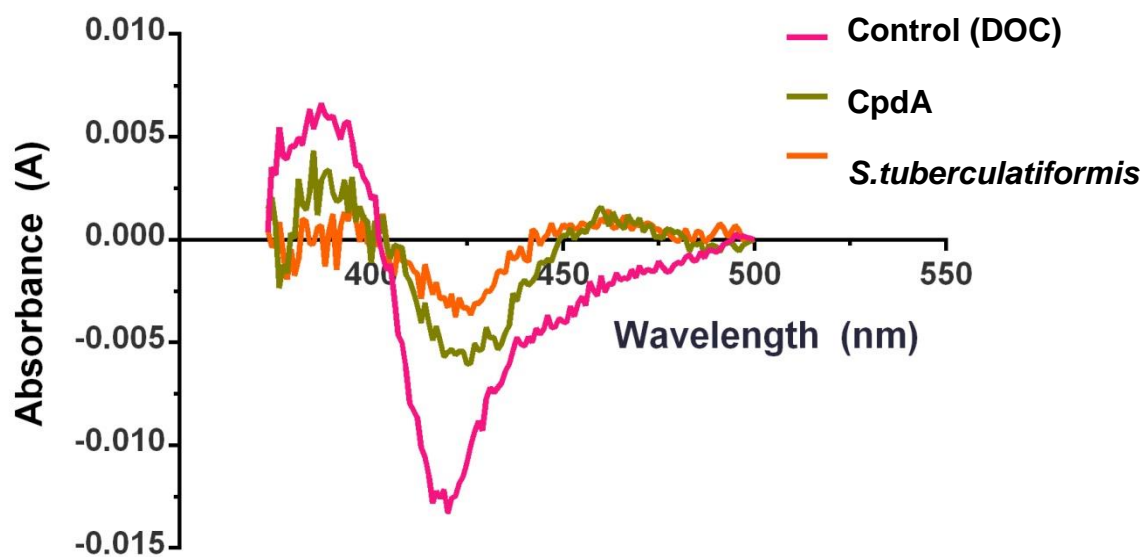
Figure 4.2 Influence of CpdA and the S.tuberculatiformis extract on the DOC-induced type I difference spectrum. Mitochondrial preparation in 0.1M phosphate buffer, 10% ethylene glycol, pH 7.4, 2mg/ml; ([P450], 0.632 P450/mg protein; [CpdA], 0.0057 mg/ml; [*S.tuberculatiformis* extract], 0.17 mg/ml.

4.2 NUCLEAR RECEPTOR TRANSLOCATION

Yemelyanov *et al.* investigated the effect of CpdA on the function of steroid hormone receptors, specifically androgen and glucocorticoid receptors, in a set of prostate cancer cell-lines with different AR/GR status [5]. This, along with virtual docking, showed that CpdA interacted with the receptors and induced translocation. We therefore set out to determine if the *S.tuberculatiformis* extract, like its synthetic analogue CpdA, would also interact with these receptors and induce translocation in cell-lines expressing the AR or and/or the GR . However, owing to the unrefined nature of the extract virtual docking was not a feasible option. Thus immunofluorescence was used in the different cell-lines to show translocation and inferred interaction of the *S.tuberculatiformis* extract with the androgen and glucocorticoid receptors.

4.2.1 IMMUNOFLUORESCENCE OF THE ANDROGEN RECEPTOR

For the androgen receptor two PCa cell-lines were utilised: androgen-sensitive LNCaP cells, that possess a mutated promiscuous AR, (AR^+/GR^-) and the highly malignant androgen-independent PC3 cells that had been transiently transfected with a wild type AR (AR^+/GR^+). The AR/GR status of these cell-lines has previously been published making them ideal for steroid hormone receptor investigations. The $PC3^{AR+}$ and LNCaP cells were cultured as described previously and treated for 16 hours with a vehicle, 1µM DHT, 10µM CpdA and 0.03mg/ml *S.tuberculatiformis* extract.

The immunostaining revealed that, similar to the natural ligand DHT, both CpdA and the *S.tuberculatiformis* extract induced significant AR nuclear translocation. Results showed that the *S.tuberculatiformis* extract and CpdA induced translocation in both LNCaP (Figure 4.3A) and $PC3^{AR+}$ (Figure 4.3B) cells by the high accumulation of receptor (red) fluorescence within the area that was distinguished as the nucleus (blue). Both cell lines showed similar staining and demonstrated that both *S.tuberculatiformis* extract and CpdA interacted with the wild type AR and the mutated AR. A low level of translocation in LNCaP cells was also demonstrated in the negative control possibly due to the promiscuous nature of the mutated AR.

A

B
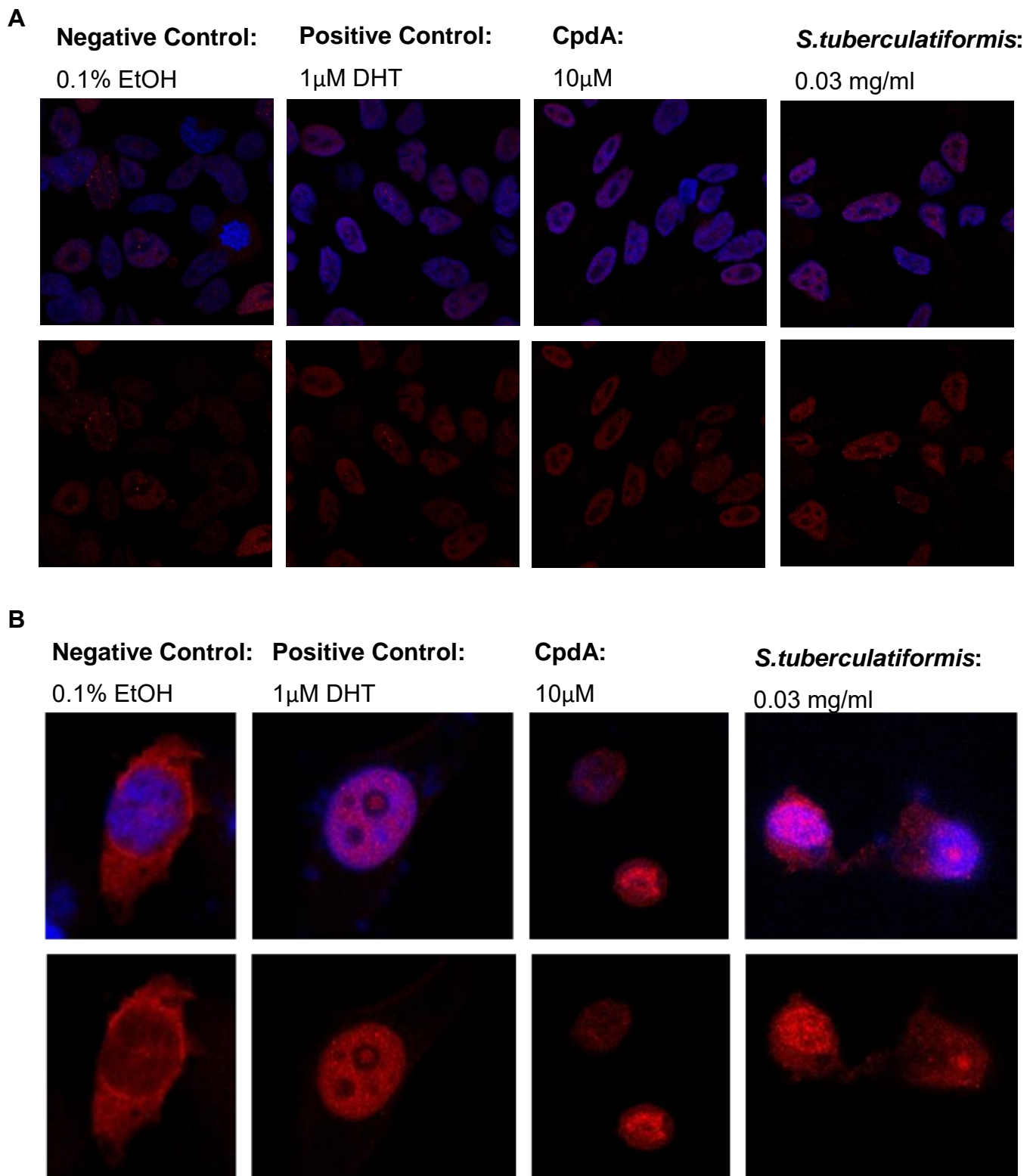
Figure 4.3 Immunofluorescence analyses of AR Translocation. A) PC3AR+ cells and B) LNCaP cells. Bottom panels: AR receptor identification, nucleus fluoresced with Alexa Flour 568 secondary antibody bound to polyclonal rabbit anti-AR (C-19) antibody; Top panels: nucleus fluoresced with Hoechst stain; Cells were exposed to vehicle control, DHT, CpdA, S.tuberculatiformis extract for 16 hours. Fluorescence was measured with a confocal microscope and the images were analysed with ZEN from ZEISS Microscopy.

4.2.2 IMMUNOFLUORESCENCE OF THE GLUCOCORTICOID RECEPTOR

The same strategy was used to study the effects of the *S.tuberculatiformis* extract on GR localisation in parental PC3 (AR$^-$/GR$^+$) PCa cells. The PC3 cells were cultured as previously described and treated for 16 hours with a vehicle, 1µM FA, 10µM CpdA and 0.03mg/ml *S.tuberculatiformis* extract.

The immunostaining (Figure 4.4) revealed that, similar to the GR agonist FA, the *S.tuberculatiformis* extract induced GR nuclear translocation. Interestingly, CpdA also induced GR nuclear translocation but to a significantly lesser extent than either the glucocorticoid FA or the *S.tuberculatiformis* extract. Results showed that the *S.tuberculatiformis* extract induced translocation in PC3 cells by the high accumulation of receptor (red) fluorescence within the area that was distinguished as the nucleus (blue).

Thus it was demonstrated that the *S.tuberculatiformis* extract and CpdA interacted with the GR resulting in nuclear translocation of the receptor but CpdA's effect appeared weaker than either the synthetic glucocorticoid FA or the *S.tuberculatiformis* extract.

Negative Control: **Positive Control:**   **CpdA:**   *S.tuberculatiformis*:

0.1% EtOH   1μM FA   10μM   0.03 mg/ml
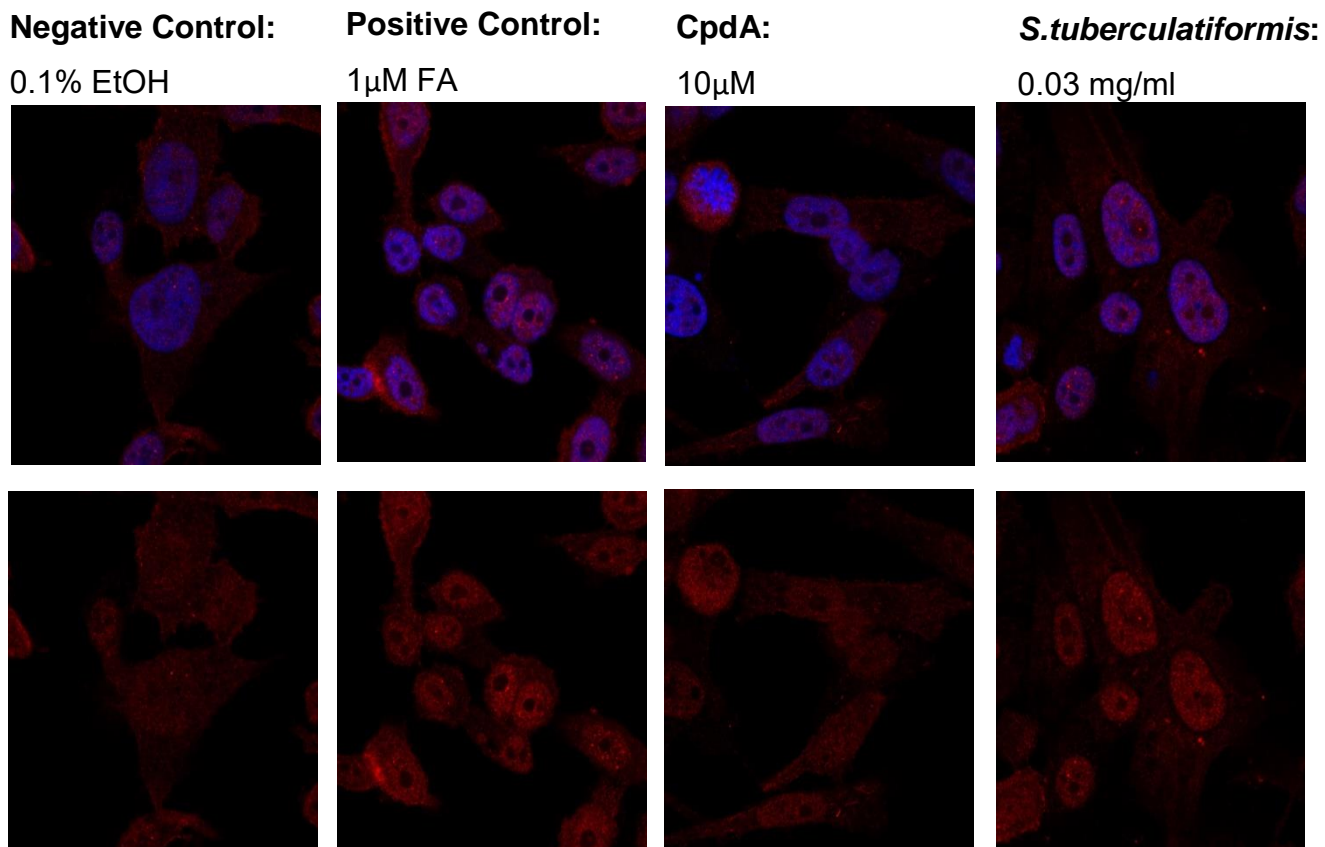
Figure 4.4 Immunofluorescence analyses of GR Translocation in PC3 cells. Bottom panels: GR receptor identification, nucleus fluoresced with Alexa Flour 568 secondary antibody bound to polyclonal rabbit anti-GR (H-250) antibody; Top panels: nucleus fluoresced with Hoechst stain. Cells were exposed to vehicle control, DHT, CpdA, S.tuberculatiformis extract for 16 hours. Fluorescence was measured with a confocal microscope and the images were analysed with ZEN from ZEISS Microscopy.

4.3 ANTI-PROLIFERATIVE PROPERTIES

It has been shown that AR blockade in combination with activation of GR signalling, specifically GR-mediated transrepression, results in the inhibition of PCa cell growth. Since Yemelyanov *et al.* reported the dual-receptor modulating properties of CpdA, in terms of the AR and GR, as well as the resultant anti-proliferative effect of CpdA on PCa the effect of *S.Tuberculatiformis* proliferation was therefore investigated [5]. They showed that CpdA inhibited cell growth and induced apoptosis in various PCa cell-lines. Since CpdA is a synthetic analogue of active compounds in the *S.tuberculatiformis* extract, the biological properties of CpdA should be mimicked by the crude extract. The immunofluorescence assays from this study confirmed that the *S.tuberculatiformis* extract interacted with the AR and GR producing translocation supporting the potential modulatory effect. The next step in this study was to analyse the effect of *S.tuberculatiformis* extract on the proliferation of PCa cells.

4.3.1 CELL VIABILITY ASSAY

In order to determine the proliferative and apoptotic effects of CpdA and the *S.tuberculatiformis* extract it was necessary to firstly determine the effective dosage. The CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the IC_{50} in the three cell models, LNCaP, PC3 and PC3^{AR+}, for CpdA and the *S.tuberculatiformis* extract. The cells were treated with increasing concentrations of CpdA (0.003 – 0.019 mg/ml) and the extract (0.031 - 1 mg/mL) and incubated for 16 hours prior to measurement of ATP production. The resulting data was fitted to sigmoidal dose-response curves and analysed to determine the potency (IC_{50}) of each compound on the different cell models (Figure 4.5A and 4.6A).
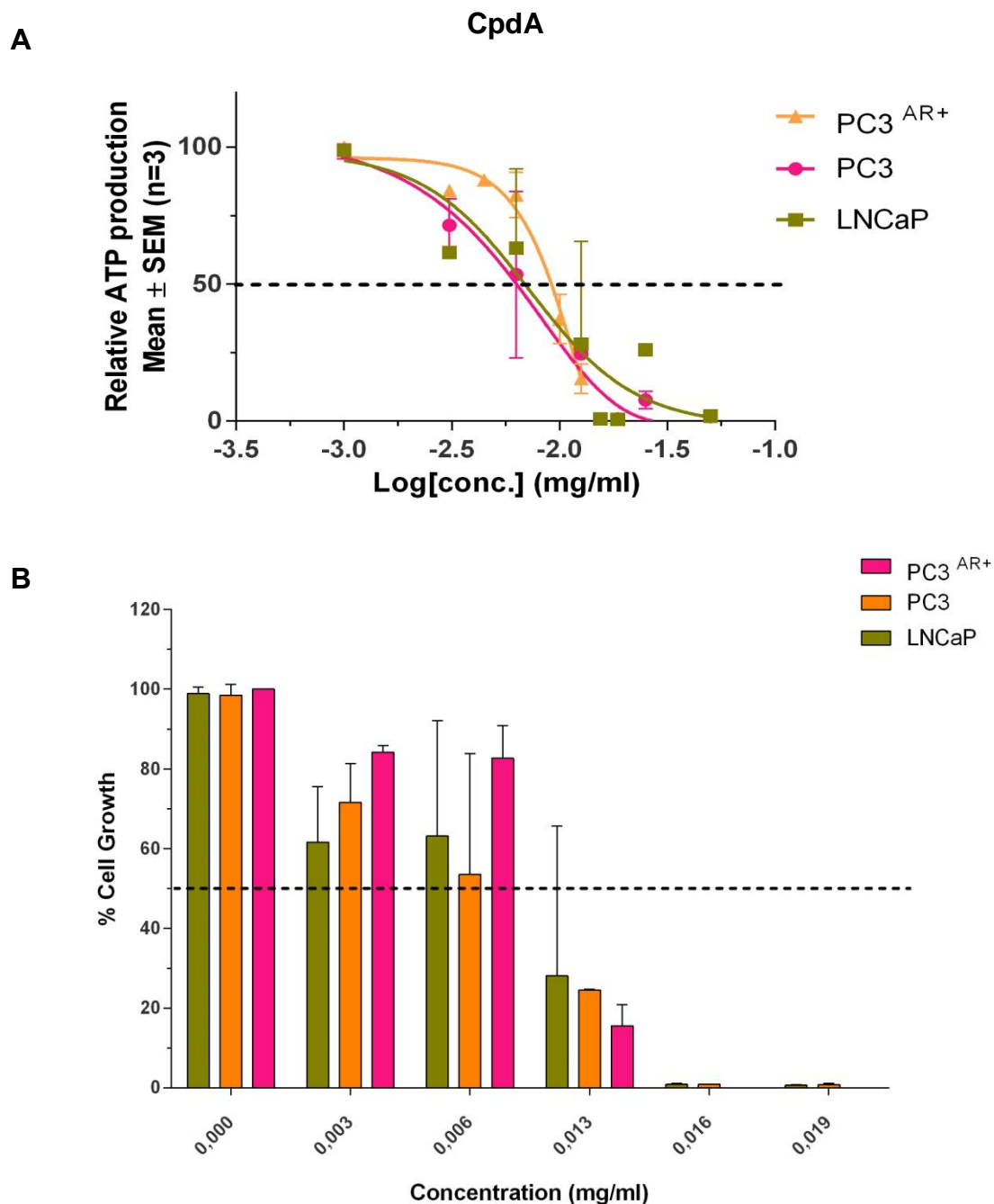
Figure 4.5 Dose-dependent curve and cell growth inhibition of LNCaP, PC3 and PC3AR+ cells for CpdA. A) The potency (IC_{50}) of CpdA and **B)** the effect on cell growth. Cells were incubated for 16 hours with CpdA, (0.003 – 0.019 mg/ml) and the subsequent data was fitted to sigmoidal dose-response curves. Results are shown as means ±SEM of three independent experiments performed in triplicate.

A

S.tuberculatiformis

B
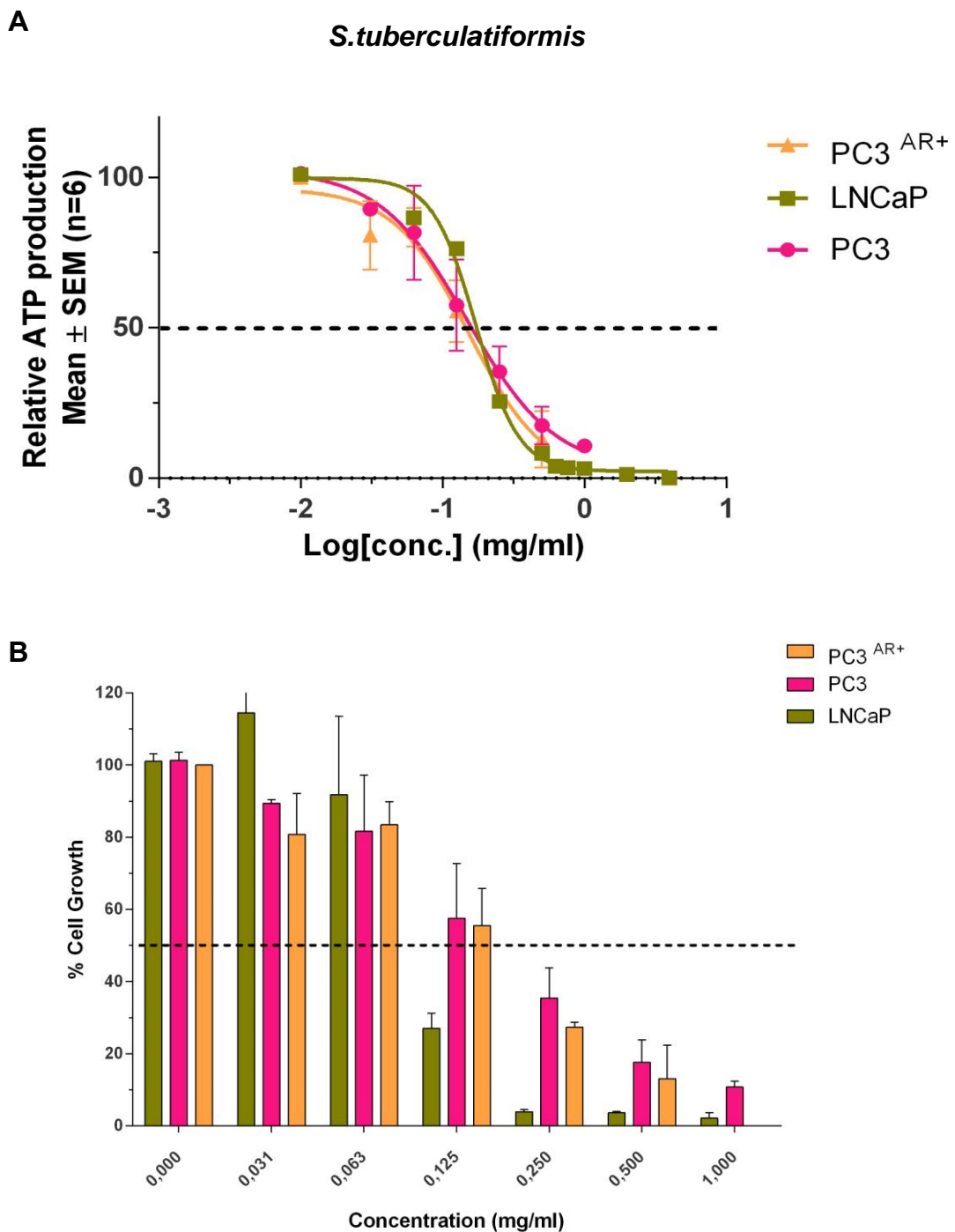
Figure 4.6 Dose-dependent curve and cell growth inhibition of LNCaP, PC3 and PC3AR+ cells for *S.tuberculatiformis*. A) The potency (IC$_{50}$) of *S.tuberculatiformis* and **B)** the effect on cell growth. Cells were incubated for 16 hours with *S.tuberculatiformis*, (0.031 - 1 mg/mL), and the subsequent data was fitted to sigmoidal dose-response curves. Results are shown as means ±SEM of three independent experiments performed in triplicate.

The results obtained in this study showed that treatment with both CpdA and *S.tuberculatiformis* extract resulted in decreased cell growth (Figures 4.5B and 4.6B)*.* An IC_{50} of ±0.01 mg/ml (Table 4.2) for CpdA was obtained regardless of the cell type used (p>0.05) (Figure 4.5A). This indicated that the potency of CpdA was unaffected by the presence of the AR and GR as PC3 (AR^-/GR^+) and $PC3^{AR+}$ (AR^+/GR^+) cells were as sensitive to CpdA as the LNCaP (AR^+/GR^-) cells. It can be hypothesised that the CpdA mechanism of action for cell death was independent of these steroid receptors. In contrast, the IC_{50} (Table 4.2) for the *S.tuberculatiformis* extract was influenced by the presence of the AR as PC3 (AR^-/GR^+) cells had an IC_{50} of 0.14 mg/ml while the LNCaP (AR^+/GR^-) and $PC3^{AR+}$ (AR^+/GR^+) cells produced an IC_{50} of 0.20 mg/ml and 0.23 mg/ml, respectively (Figure 4.6A). There was a significant (p<0.001) difference (Figure 4.7) in the IC_{50} obtained with the S.tuberculatiformis extract between the cell lines with the IC_{50} being 1.4-1.6 fold higher in the two cell-lines containing the AR, LNCaP and $PC3^{AR+}$ than in the PC3 cells. The data indicates that the presence of the AR decreased the potency of the *S.tuberculatiformis* extract, which suggests that the AR interferes with the *S.tuberculatiformis* compounds, or with the extract, resulting in a pro-survival effect. It appeared that CpdA was more potent than the *S.tuberculatiformis* extract which could be expected as the *S.tuberculatiformis* extract is a crude extract and its active components are diluted in the impure extract influencing the potency. However, the difference in receptor susceptibility indicated that CpdA and the *S.tuberculatiformis* extract possess different sensitivities.

Table 4.2 IC$_{50}$ of CpdA in PCa cell models, LNCaP, PC3 and PC3^{AR+} generated from sigmodal dose-response curves.

IC$_{50}$ (mg/ml) – Potency

		CpdA	*S.tuberculatiformis*
LNCaP	■	0.01002	0.2040
PC3^{AR+}	▲	0.00938	0.2279
PC3	●	0.00921	0.1437
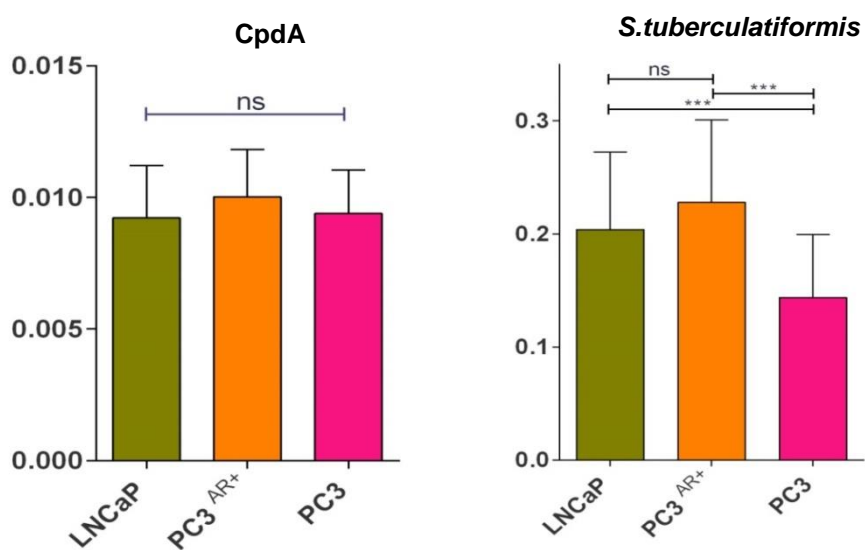
Fig 4.7 Comparative analysis of IC50 value CpdA and S.tuberculatiformis in LNCaP, PC3^{AR+} and PC3. Treatments were analysed using one-way ANOVA. Not significant, ns; $p < 0.001$, ***.

4.3.2 APOPTOSIS ASSAY

The Caspase-3 assay was used in order to determine if cell death, which resulted from CpdA and the *S.tuberculatiformis* extract treatment, could be attributed to apoptosis. The Caspase-Glo3/7 assay was carried out to determine if caspase-3 was upregulated in the two cell models, PC3 and PC3^{AR+} treated with CpdA and the S.tuberculatiformis extract. The cells were treated with a range of concentrations, CpdA, 0.0025 - 0.01 mg/mL and the *S*.tuberculatiformis extract, 0.075 – 2 mg/mL, which spanned concentrations below the IC50. TNFα, 0.2 μM, FA 1 μM, and DHT, 1 μM were included as controls. Cells were exposed for 16 hours prior to measurement of luminescence production. The resulting data was calculated relative to the untreated cells and are expressed as the fold change over the vehicle control.

Results obtained showed that neither treatment with CpdA nor the *S.tuberculatiformis* extract resulted in increased caspase-3 production, implying that the cell death demonstrated was not initiated via the caspase cascade (Figures 4.8A and 4.8B)*.* This result was comparable in both cell models and was therefore assumed to be unaffected by the AR/GR expression in the cell models. Not only was there no significant difference in caspase production by CpdA or the extract in the PC3 or in the PC3^{AR+} cells but there was also no significant difference between the two cell-lines (Figure 4.9) . Although this showed that apoptosis was not initiated via the caspase cascade it does not exclude other mechanisms of apoptosis induction such as through the extrinsic pathway and Bcl-2 family. Apoptosis cannot therefore be fully excluded, but no activation of the caspase pathway could be shown in this investigation.
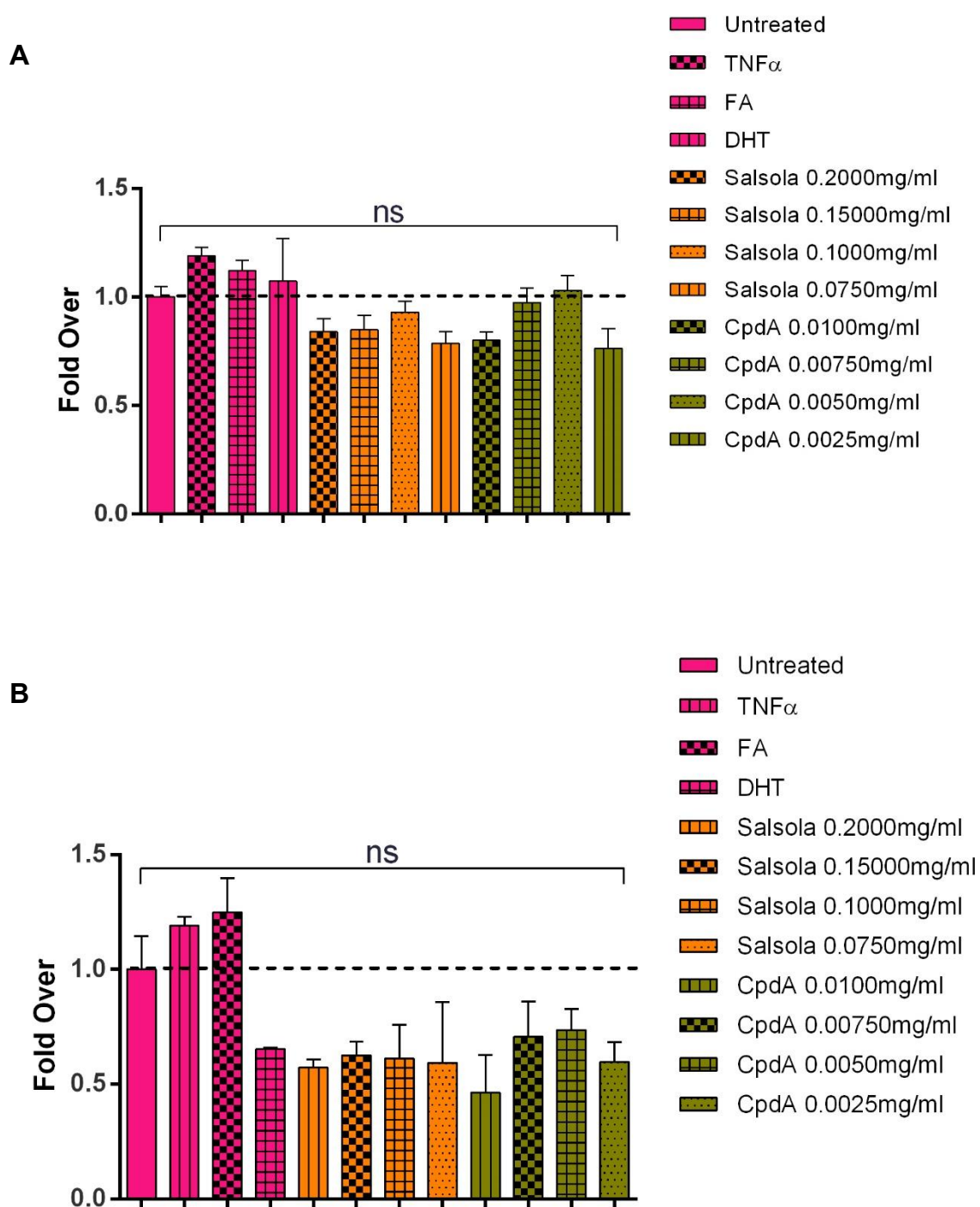
Figure 4.8 Analyses of induction of caspase 3 expression A) in parental PC3 cells (AR-) and B) **in transfected PC3^AR+ cells (AR+).** Cells were exposed to compounds for 16 hrs, [CpdA], 0.0025 - .01 mg/mL; extract, 0.075 – 2 mg/mL; TNFα, 0.2 μM, FA 1 μM; and DHT, 1 μM. Caspase expression was calculated relative to the negative control (untreated) and is expressed as the fold change over this control. Data was analysed using one-way ANOVA followed by Bartlett's multiple comparisons test and are shown as means ±SEM of three independent experiments performed in triplicate.
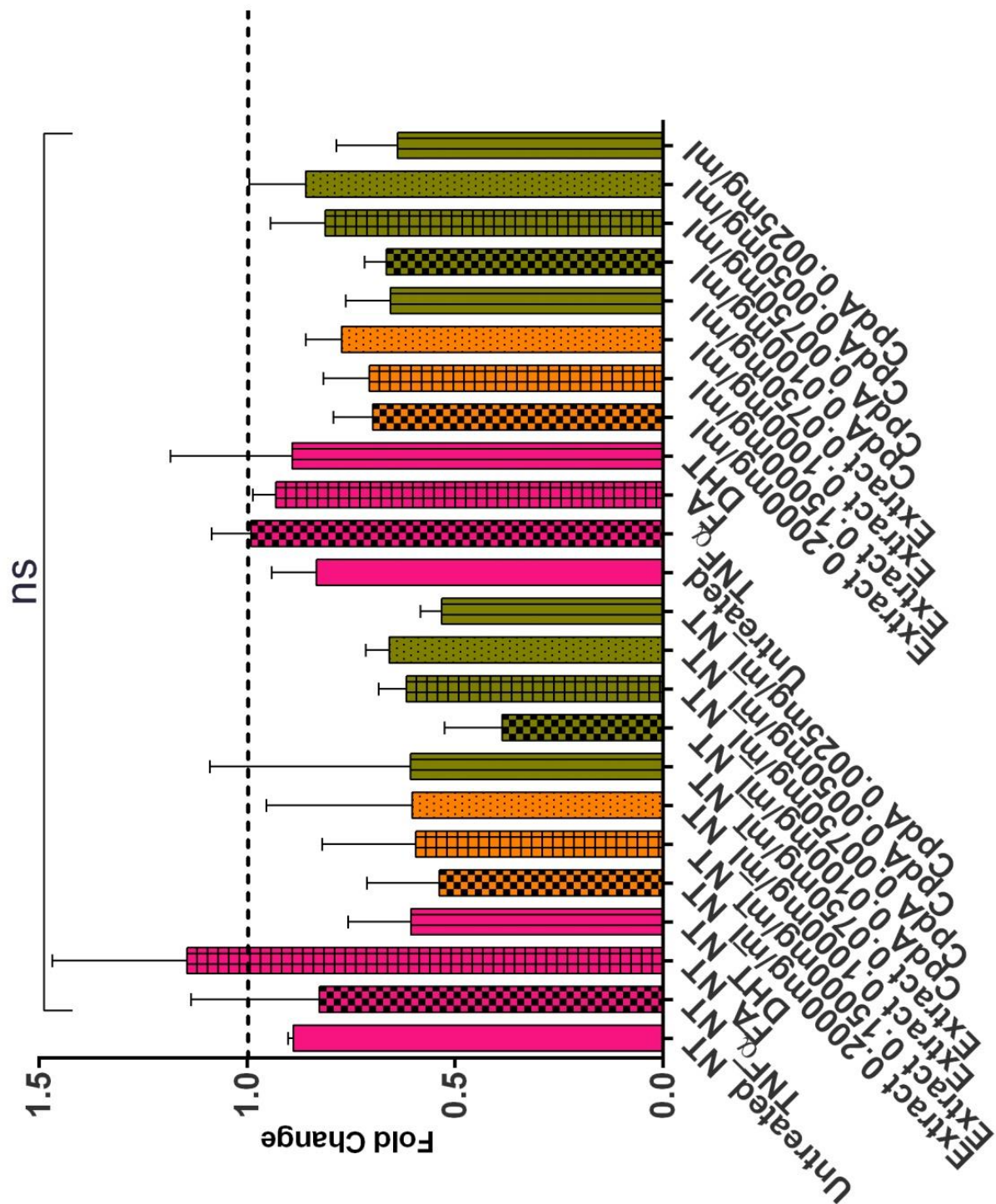
Figure 4.9 Analyses of induction of caspase 3 expression in both parental PC3 cells (AR-) and transfected PC3^{AR+} cells (AR+). Cells were exposed to compounds for 16 hrs, [CpdA], 0.0025 - .01 mg/mL; extract, 0.075 – 2 mg/mL; TNFα, 0.2 μM, FA 1 μM; and DHT, 1 μM. Caspase expression was calculated relative to the negative control (untreated) and is expressed as the fold change over this control. Data was analysed using one-way ANOVA followed by Bartlett's multiple comparisons test and are shown as means ±SEM of three independent experiments performed in triplicate. NT, not transfected.

CHAPTER 5

DISCUSSION

This chapter summarises the major findings in relation to the research aims and objectives. In light of these findings, recommendations are presented for possible future research. In light of these findings potential avenues for future studies are discussed.

5.1 INTRODUCTION

The role of androgens in PCa is now a well-developed concept and many of the mechanisms for the role of androgens in PCa proliferation and progression are becoming clearer. AAT has been the first line treatment for PCa since Huggins and Hodge demonstrated that PCa was dependent on androgens for growth and survival [25]. However, it has become apparent that this treatment is "a Band-Aid for a bullet wound" as eventually, within 5 years, most tumours become CRPC. Despite this the AR remains a critical factor in PCa initiation and progression. The treatment for CRPC is limited and palliative with little to no increase in patient survival [48,58–61,379]. Docetaxel was the first chemotherapeutic agent that, combined with prednisone (a synthetic corticosteroid drug), resulted in increased survival [380]. Docetaxel is a taxane and these chemicals initiate the apoptotic process by binding to β-tubulin and promoting its polymerization. Since its discovery several other compounds have also shown increased survival such as abiraterone and enzalutamide [381,382]. However, these treatments are accompanied by severe toxicity as well as other adverse side-affects, and patients may develop resistance to clinical approaches. Since androgens and the AR remain important even in progression, therapies which target the AR remain of interest even as the PCa becomes castration resistant. Indeed, selective steroid modulation appears to present an ideal target for management of both early and late-stage disease and this is one of the reasons the research on CpdA has been, and remains, promising. However, its clinical application is hampered by restricting factors in respect to limitations due to its labile nature, toxicity and narrow therapeutic window.

Owing to the difficulty in isolating and maintaining the active compounds of *S.tuberculatiformis* and the initial assumption that CpdA was an accurate model for the active compounds of *S.tuberculatiformis*, *S.tuberculatiformis* bioactive compounds have not been as extensively characterised as CpdA. It is difficult to draw exact comparisons of the mechanisms and effects between CpdA and the *S.tuberculatiformis* extract since most of the knowledge is based on the results of CpdA alone. CpdA is a synthetic analogue based only on partial structures obtained from a single fraction of *S.tuberculatiformis*. It is plausible that there may be significant differences between the bioactive compound(s) and CpdA thus presenting further opportunities for the extraction of alternative compound(s), perhaps less labile, with equal or additional therapeutic potential. CpdA has been shown to mimic certain biological properties of the *S.tuberculatiformis* extract but since it is not an identical compound it cannot be expected to show identical activity. Thus further investigation of the *S.tuberculatiformis* extract must begin by determining if compounds possess the same or similar characteristics as CpdA that have been proven therapeutically beneficial. This study was undertaken as a preliminary investigation to determine if *S.tuberculatiformis* extract mimics CpdA as a selective steroid receptor modulator with anti-proliferative properties.

5.2 *S.TUBERCULATIFORMIS* EXTRACT INHIBITS CYP11B1 SUBSTRATE BINDING

CYP11B1 is an enzyme located in the zona faciculata in the adrenal and is the terminal enzyme in glucocorticoid biosynthesis. It exists in 2 isoforms CYP11B1 and CYP11B2, with the latter expressed in the z glomerulosa only. CYP11B1 is a mitochondrial cytochrome P450 localised to the inner membrane of the adrenal cortex mitochondria where is converts DOC to corticosterone and deoxycortisol to the potent glucocorticoid, cortisol (Figure 5.1) [383]. CYP11B1 was identified as a key enzyme in an investigation into the syndrome of prolonged gestation and foetal post-maturity induced in Karakul sheep by the consumption of *S.tuberculatiformis* [9]. The termination of gestation is characterised by the rise in foetal cortisol which is required to initiate birth in sheep with CYP11B1 playing a critical role. The inhibition of CYP11B1 was therefore used as a bioassay to screen the *S.tuberculatiformis extract* for biologically active compounds that could lead to the extension of gestation in sheep. Although the active compounds in S. tuberculatiformis could not be unequivocally identified, a synthetic analogue, CpdA, was synthesised that had the same effect on the CYP11B1 enzyme system as the *S.tuberculatiformis* extract. In this investigation the first goal was to indicate the biological activity of a freshly prepared *S.tuberculatiformis* extract and CpdA with the CYP11B1 spectral assay.

Figure 4.2 clearly shows that CpdA and the *S.tuberculatiformis extract* were biologically active as they both inhibited the DOC-induced type I difference spectrum of CYP11B1. It is interesting to note that CpdA in solution, even when protected from light, oxygen and inconsistent temperatures, could not be stored in its active form as long as the *S.tuberculatiformis extract*. After 2-3 weeks CpdA lost its biological activity completely while the *S.tuberculatiformis extract* maintained activity for longer periods with loss of biological activity occurring gradually and only after ±1 month. This suggests that stabilising factors within the crude extract may preserve the biological activity and enhance therapeutic benefits. One explanation of this is based on observations by Louw *et al.* who demonstrated that both CBG and SHBG may be involved in the stabilization of CpdA in plasma suggesting a mechanism whereby the *S.tuberculatiformis extract*, which is highly reactive and labile *in vitro*, may be biologically active *in vivo* due to stabilizing factors [350].
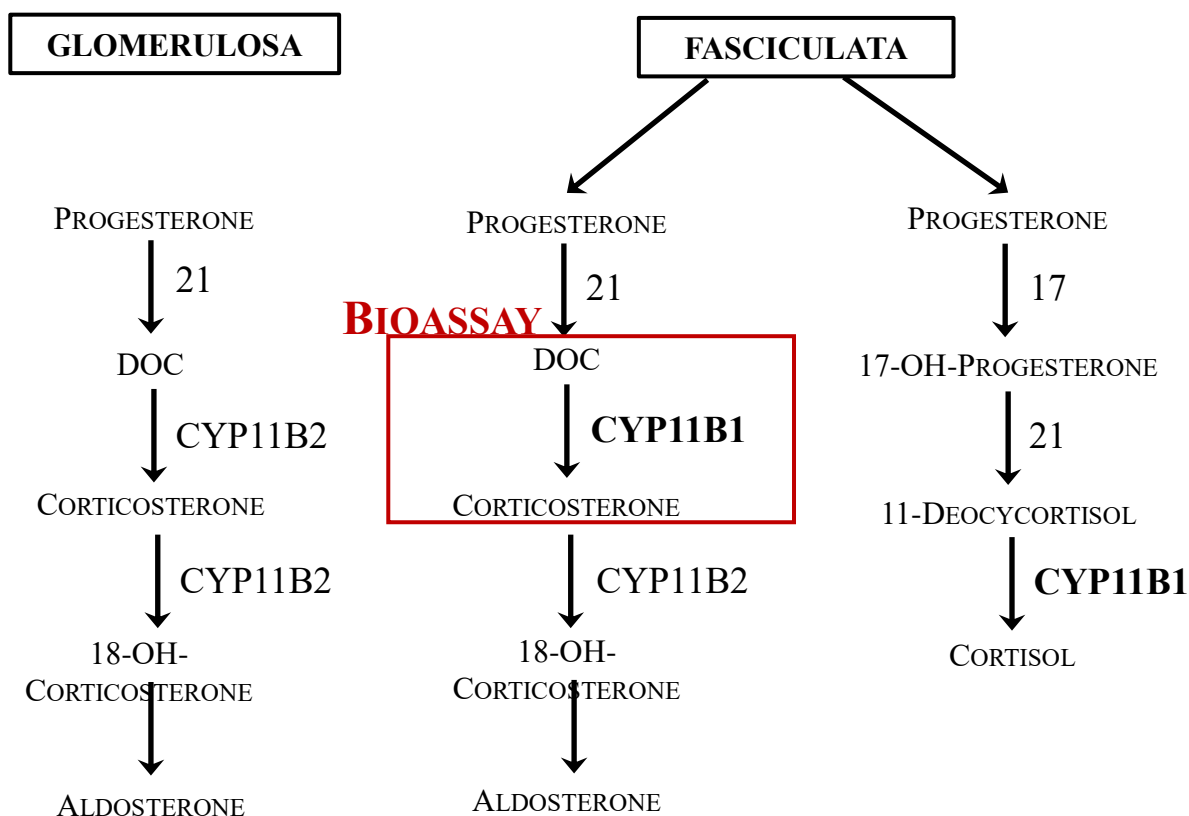
Figure 5.1 Corticosteroid pathways. Sequential dehydrogenation and hydroxylation reactions to form DOC. DOC is converted to corticosterone by CYP11B1. Reproduced and adapted from Petrelli and Stewart (1998) [384].

Yemelyanov *et al.* have determined additional biological activities of CpdA which have not been previously shown in *S.tuberculatiformis*, namely the induction of steroid receptor translocation, the inhibition of steroid receptor transactivation, the induction of GR transactivation via the inhibition of various transcription factors including AP-1 and NFκB, as well as anti-proliferative activity and increased apoptosis which correlated with an increase in caspase- [5]. They showed that the cytostatic effect of CpdA is receptor dependent and, using virtual docking analysis, that CpdA shares binding cavities in the AR and GR ligand binding domains. Thus in these studies, CpdA acts as a competitive agonist, displacing steroids from their receptors and increasing free steroids as a consequence. Louw *et al.* demonstrated that the percentage of free plasma corticosterone increased during treatment with both S. tuberculatiformis and CpdA but, on termination of the treatment, the percentage of free corticosterone returned to control levels. These changes were associated with a decrease in CBG-bound corticosterone which also, after treatment, increased to control values [346]. These studies support a similar mechanism of action for *S.tuberculatiformis* extract and CpdA.

5.3 LNCaP and PC3 cell-lines

LNCaP and PC3 PCa cell lines were used in this study. LNCaP cells were initially derived from a supraclavicular lymph node adenocarcinoma metastasis which is reflected in their name −lymph node-cancer of the prostate (LNCaP). This cell line is relatively slow growing with a doubling time of 60-72 hours. Most cell models derived from metastatic deposits are androgen independent (AI) as metastasis is usually a feature of progression to CRPC. There has been a paucity of androgen dependent (AD) cell-lines which has impeded research into early cancer initiation and proliferation. LNCaP cells are androgen sensitive and express the AR which makes them suitable for studying androgen sensitive diseases. However this cell line contains a T877A mutation in the LBD of the AR resulting in promiscuous binding to a range of steroid compounds [78,385]. This mutant AR exhibits strong AR transcriptional activity and confers increased cell growth and/or abrogated apoptotic signals in PCa cells in response to androgen treatment [385]. AR mutations have been reported in a subset of metastatic PCa and some of these mutations, such as the one exhibited in LNCaP cells, are associated with stimulation of the cells by other steroids and anti-androgens, causing concern over the use of drugs such as finasteride for the treatment of PCa [386]. LNCaP cells express low levels of 5α-reductase, in comparison to the 17βHSD isoforms, while not expressing the GR and contain a frameshift mutation in the PTEN gene that causes a down-regulation of PTEN resulting in constitutive activation of either P13-kinase or Akt, which can induce cellular transformation [387]. On the other hand, LNCaP cells produce PSA and PSMA and still possess the p53 protein, albeit it contains a silent mutation, and these are often not present in late-stage disease [387,388]. This makes the LNCaP cell-line currently the best candidate for early stage PCa research.

PC3 cells were derived from a lumbar bone metastasis of a grade IV prostatic adenocarcinoma from 62-year-old Caucasian male. In contrast to LNCaP cells, this cell model has a high growth rate, with a doubling time of ~25 hours and expresses the wild type GR while no AR is present [257,389]. Thus PC3 cells are considered an AI cell-line. In addition to the lack of an AR, PC3 cells also do not produce PSA and PSMA, and has been shown to possess 5α-reductase activity [389,390]. PC3 cells have a deletion mutation of the p53 gene as well as exhibiting homozygous deletions of

the PTEN gene or parts thereof [389,391]. This makes PC-3 cells a highly aggressive form of PCa that is resistant to most forms of current PCa therapy. This can be shown through the inability of pro-apoptotic initiators such as TNF-α and staurosporine, to affect these cells. Therefore, unlike LNCaP cells, PC3 cells are considered to be a CRPC model and a representative of late stage PCa.

By using both cell lines it is possible to study the effects of compounds on different stages of PCa (Table 5.1). PC3 cells are useful in investigating the biochemical changes in advanced PCa and in assessing their response to chemotherapeutic agents while LNCaP cells represent the earlier stage in which interventions preventing the progression of PCa to CRPC can assessed. It is also possible, by using both cell lines, to demonstrate selective steroid receptor activity.

Table 5.1 Summary of LNCaP and PC3 cell-lines and their differing phenotypes

Cell-Lines	AR	Androgen Sensitivity	GR	5α-reductase	PSA	PSMA	PTEN	p53	Stage
LNCaP	+	AS	-	+	+	+	+	+	Early
PC3	-	AI	+	+	-	-	-	-	Late

5.4 S.TUBERCULATIFORMIS EXTRACT TRANSLOCATES THE AR AND GR

The ability of the *S.tuberculatiformis* extract to interact with the AR and the GR was investigated in LNCaP, PC3 and transiently transfected PC3[AR+] to determine if nuclear translocation of these receptors occurs. This was achieved using immunofluorescence to indicate receptor translocation in the different cell models. The results showed that both the *S.tuberculatiformis extract* and CpdA interacted with the AR and GR causing translocation (Figures 4.3 and 4.4).

Yemelyanov *et al.* produced similar results with CpdA (Figure 5.2) using LNCaP cells that had been stably transfected with a wild type GR [6]. Their results showed that CpdA translocated both the AR and the GR. The AR was affected with the same intensity as the natural ligand DHT but CpdA appeared to have a weaker effect on the GR when compared to the agonist FA. Our results confirmed this effect of CpdA including the reduced translocation of the GR. However, the intensity of the signal obtained with the *S.tuberculatiformis* extract and CpdA was similar, suggesting their ability to translocate the AR was comparable. Interestingly, although both CpdA and *S.tuberculatiformis* extract also caused translocation of the GR, the intensity obtained with the *S.tuberculatiformis* extract was greater, similar to that of the FA agonist, suggesting the extract enhances translocation to the GR more so than CpdA. This suggests that S.tuberculatiformis extract has a greater translocation ability in terms of the GR than CpdA and a comparable translocation ability in terms of the AR. In order to confirm this we transiently transfected PC3 cells with the wild type AR to determine whether the translocation induced by CpdA and the *S.tuberculatiformis* extract would be similar. LNCaP cells possess a mutated AR, as previously discussed, which broadens their range of ligands which could influence the intensity of the signalling. Our results showed that both CpdA and *S.tuberculatiformis* extract remained comparable, indicated by the intensities achieved in the translocation of the wild type AR. The effect was weaker than that observed with mutated AR which could be attributed to the promiscuity of the mutated receptor. The question, however, remains as to whether the increased activity on the GR for *S.tuberculatiformis extract* will translate to increased benefit clinically.
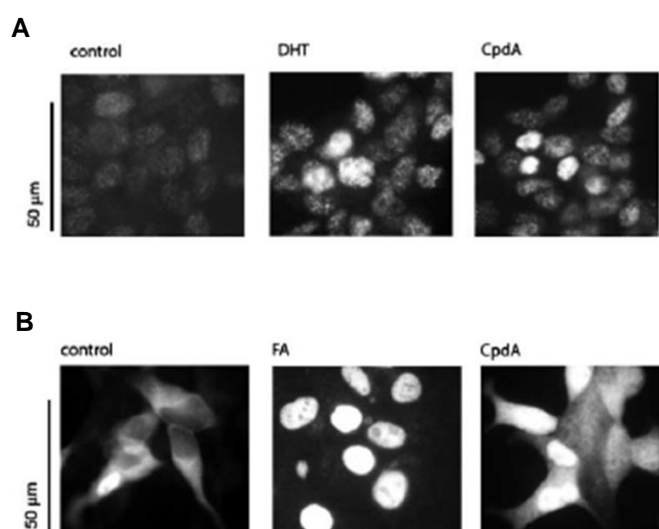
Figure 5.2 CpdA induced A) AR translocation and B) GR translocation. Immunofluorescent of nuclear translocation of AR (A) and GR (B) in cells treated for 16 h with DHT (10^{-6} mol/L), FA (10^{-6} mol/L), and CpdA (10^{-5} mol/L). Reproduced from Yemelyanov *et al.* (2008) [5].

The AR and GR play opposing roles in PCa progression and have long since been involved in PCa therapies. Compounds that can interact selectively with both receptors, favouring AR blockade and GR transrepression, provide a promising treatment mechanism that is anti-proliferative with reduced side effects, particularly those which are glucocorticoid mediated. Yemelyanov *et al.* have shown that CpdA inhibits transactivation of AR- and GR-mediated genes together with enhancement of GR transrepression due to the translocation of the receptors [5]. GR transrepression is associated with anti-inflammatory responses that impede PCa progression. Enhanced GR transrepression would therefore appear to be a beneficial factor in compound potency and anti-proliferation and anti-inflammatory effects and results obtained in this study indicate that the *S.tuberculatiformis* extract is as effective as CpdA in this regard, if not more so.

Inhibition of AR-mediated transactivation holds potential for early PCa therapy as the AR stimulates proliferation by blocking apoptosis through the P13k/Akt pathway. This pathway is constitutively active in most PCas as the gene encoding PTEN, the inhibitory protein of this pathway is often found to have been mutated or deleted altogether. Thus inhibition of the AR "switches-off" this pathway in PCa allowing pro-apoptotic proteins, such as TNFα, to initiate apoptosis. Yemelyanov *et al.* have demonstrated this mechanism for the activation of the AR by CpdA [352].

Owing to the unavailability of ARE.luc and GRE.luc plasmids in our laboratory these experiments were not conducted with *S.tuberculatiformis*. However, the comparable intensities of translocation obtained with CpdA and *S.tuberculatiformis* extract suggest this effect is shared by the two compounds and that *S.tuberculatiformis* extract inhibits transactivation of the AR and the GR while enhancing GR transrepression. Pending confirmation there remains potential for the enhanced GR response to translate to improved clinical efficacy for *S.tuberculatiformis* extract over CpdA. Further clarification is, however, still required.

The implications for CRPC are less impressive as often PCa becomes CRPC through mutations, co-regulators or by bypassing the AR entirely. AR blockade is then clinically irrelevant. PC3 cells have bypassed the AR and therefore the inhibition of the AR in this cell line would be expected to have no beneficial effect. Indeed, several studies have shown that the AR may have an inhibitory effect on advanced CRPC growth, implying that the AR blockade in these circumstances would be counterproductive [392–394]. However an interesting study was recently published by Schweizer *et al*. [395]. In this investigation a small cohort of men with CRPC was studied and it was postulated that if CRPC were to be flooded with T, apoptosis may be initiated due to hormonal shock. The cells could also react by down regulating the AR allowing the PCa cells to once more respond to AAT. Although small, the study yielded promising results. PSA reductions were seen in all of the 10 subjects, including four in whom PSA levels remained unchanged during the trial but were reduced when T-blocking drugs were recommenced after the T-shock treatment. These results suggest that pulsed high dose T therapy may have the potential to reverse the resistance to AAT that characterises CRPC which would render agents acting through an androgen blockade therapeutically beneficial once more [395 This mechanism could certainly be postulated to enhance or extend the therapeutic effect of T blocking drugs including *S.tuberculatiformis* and CpdA in CRPC.

Additionally, studies have demonstrated that activation of GR signalling can maintain an active AR pathway under androgen-deprived conditions in PCa, despite the previous reports on the tumour-suppressive activities of the GR. It appears the liganded GR acts as an anti-AR in the presence of androgens but as an AR agonist in the absence of androgen [79,311,312,315,316]. Thus the inhibition of the GR in CRPC, such as that represented by PC3, could be beneficial by sensitising these aggressive

cells to pro-apoptotic treatments through blocking of the constitutively active P13k/Akt pathway. Furthermore, restitution of the androgen sensitivity potentially will restore the GR to anti-AR activity.
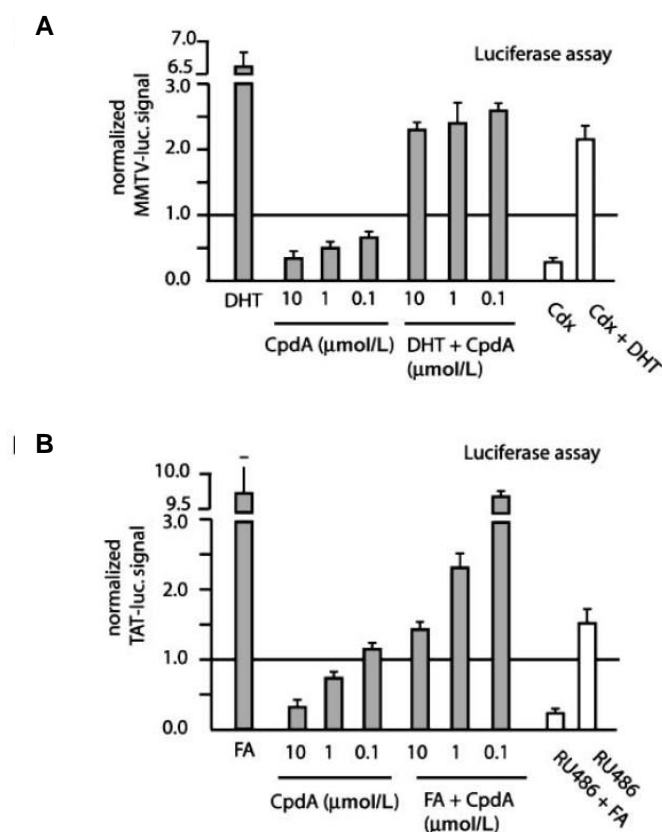
Figure 5.3 CpdA-induced inhibition of transcription activity of A) AR and B) GR. For luciferase reporter assay, cells were transfected with MMTV-Firefly luciferase (A) or TAT-Firefly luciferase (B) reporters and Renilla luciferase reference reporter. Firefly luciferase activity was normalized against Renilla luciferase activity to equalize for transfection efficacy. LNCaP-GR sells were treated with CpdA (0.1–10 µmol/L), DHT (10 µmol/L), FA (10 µmol/L), AR antagonist Casodex (Cdx; 100 µmol/L), and GR antagonist RU486 (100 µmol/L) for 32 h. Columns, mean results of one representative experiment (three wells per experimental group); bars, SD. Reproduced from Yemelyanov *et al.* (2008) [5].

It is evident that CpdA has shown promise as a novel therapeutic agent for PCa therapy with the *S.tuberculatiformis* extract appearing to mimic many of these mechanisms and sharing many of the proven properties of CpdA while it may possess additional benefits as evidenced by the greater translocation induction of the GR receptor. It can be hypothesised that extracts of *S.tuberculatiformis* could

provide improved therapeutic options and warrant further studies into the active fractions and potential stabilizing cofactors within the crude extract.

5.5 S.TUBERCULATIFORMIS EXTRACT INHIBITS PCA CELL GROWTH

The ability of *S.tuberculatiformis extract to* inhibit cell growth and cause cell death in PCa cell lines was investigated in LNCaP and PC3 cells, both the parental line and the transiently transfected PC3^{AR+}. These studies were performed to demonstrate the anti-proliferative potential and to determine the relative potencies and efficacies of the two compounds. Inhibition of cell growth was demonstrated using cell viability assays and showed the effects of *S.tuberculatiformis extract* and CpdA on PCa cells with specific AR/GR status which correlate to the stage of PCa progression. Finally, apoptotic assays were performed using the Caspase-3/7 assay to determine if the cell death could be ascribed to an apoptotic mechanism resulting in initiation of the caspase cascade.

CpdA was shown to possess an IC$_{50}$ of 0.01 mg/ml in all the cell-lines. Thus the potency of CpdA was unaffected by the steroid receptor expressed in the cell, also supporting therapeutic benefits in early and late-stage disease (Table 4.2, Figure 4.5). This could be explained in several ways. Anti-proliferative effects may be triggered through the GR and the AR or may include additional pathways that are independent of the AR and the GR. Figure 4.5B shows the effect of different concentrations of CpdA on cell growth of the three cell models. Notable changes in cell growth were demonstrated between 0.016-0.013 mg/ml and 0.013-0.006 mg/ml demonstrating a dose dependent effect but suggesting a relatively narrow therapeutic range separating ineffective dose and possible toxicity. Another observation with this experiment was marked intra-study variability, evident in the error bars (Figure 4.5), with less variability seen in the PC3 cell lines. This is probably due to the fact that obtaining tight data with cell growth assays – reproducible from one experiment to the next - is extremely difficult. In addition LNCaP cells do not always adhere so well and so are often lost when media is replenished/supplemented whereas PC3 cells firmly adhere and this could result in the discrepancies of the error bar with the LNCaP cells.

101

In a similar study by Yemelyanov *et al.* CpdA was found to inhibit PCa cell growth [5]. The growth of PC3, DU145, and parental LNCaP cells, when exposed to CpdA, was significantly impeded (reduced 50- to 100-fold) with an effective concentration range of 10^{-5} to 10^{-6} mol/L (0.02 - 0.0026 mg/ml) (Figure 5.3). This finding is congruent with the results presented in the present study on the effects of SF and CpdA on cell growth although their reported IC_{50} values varied between the different cell models. The experiment by Yemelyanov *et al.* was carried out over several days, and as such, effects on proliferation were assayed using repeat doses. The present investigation, in contrast, was performed by increasing the strength of single doses with a shorter incubation of 16 hours assaying effects on cell growth in the log phase perhaps. In this study the IC_{50} for CpdA did, however, fall within the range previously reported. Yemelyanov *et al.* also treated their cell-lines with CpdA at a relatively high dose (0.026mg/ml - 10^{-5}M) for 1-12 days showing that, in comparison to the untreated cells, CpdA treated cell-lines had inhibited growth which was also demonstrated in this study (Figure 5.4 and 4.5). Thus it appears clear that CpdA, at these concentrations, causes inhibition of PCa cell growth.
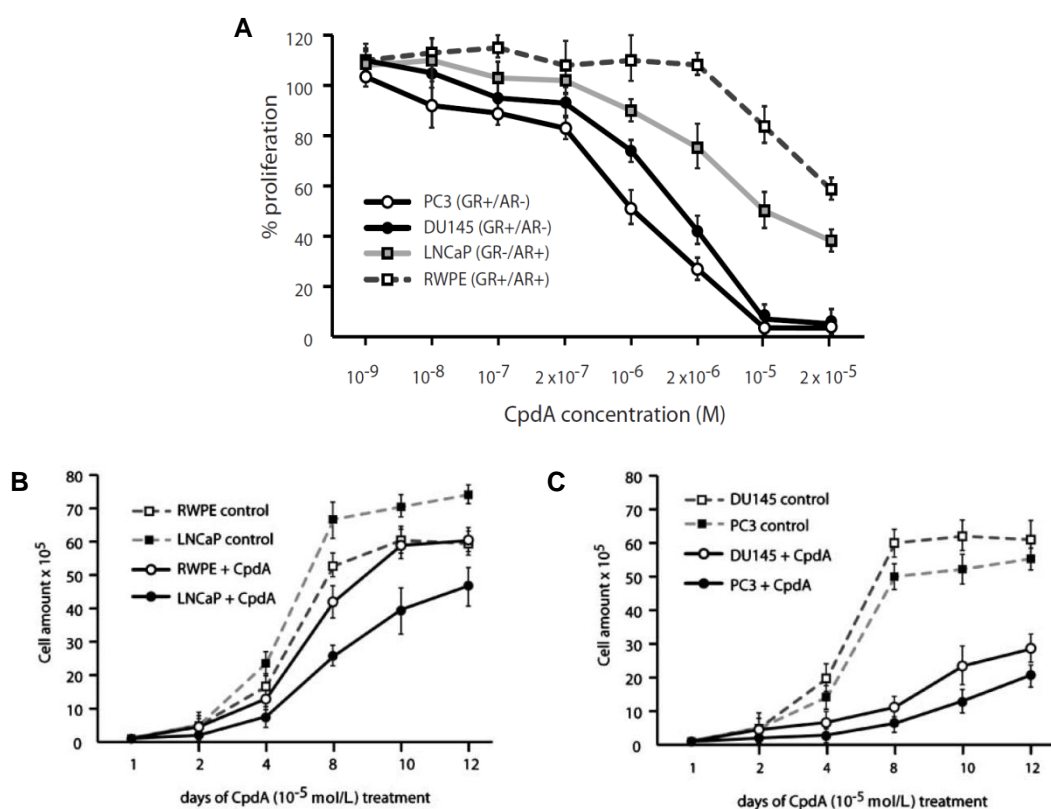
Figure 5.4 CpdA induced inhibition of cell growth in PCa cell models. A) Dose-response effect of CpdA on PCa and normal prostate cell growth. RWPE-1 (Healthy prostate cell-line) and different PCa cell-lines were treated with increasing concentrations of CpdA (10^{-9}-2×10^{-5} M) for 5 days. Cells were counted using a haemocytometer. The average number of cells/well (+/- S.D.) is presented. Results of one representative experiment are shown. B) and C) show the effect of CpdA on the growth of prostate cells in monolayer. RWPE-1 and PC3, DU145, and LNCaP cells were treated with 0.01% DMSO (control) or CpdA (10^{-5} mol/L) for 1 to 12 days. Points, average number of cells per well for one representative experiment; bars, SD. Reproduced from Yemelyanov *et al.* (2008 [5].

In our study the same experiment on cell growth was also carried out for the *S.tuberculatiformis* crude extract. Interestingly, the *S.tuberculatiformis* extract yielded data showing less intra-study variation and the presence of *S.tuberculatiformis* extract resulted in growth differences between the cell lines. LNCaP cells were most sensitive between 0.125-0.063 mg/ml limiting its dosage range (Figure 4.6B). This could be due to the mutated AR receptor indicating interference of AR in *S.tuberculatiformis* extract mechanisms. Importantly, unlike CpdA, the potency of the *S.tuberculatiformis* extract was affected by the steroid hormone status of the different cells. Parental PC3 cells had a significantly lower IC_{50} than LNCaP and PC3^{AR+} cells (Figure 4.7). PC3 cells exhibited an IC_{50} of 0.14 mg/ml while LNCaP and PC3^{AR+} cells had IC_{50}'s of 0.2 and 0.22 mg/ml, respectively. The presence of AR apparently decreased *S.tuberculatiformis extract p*otency which further indicated interference of AR in the *S.tuberculatiformis* extract mechanism. This effect was not observed with CpdA.

Another possibility for this observation is suggested by several studies that have shown that AR up-regulation in PC3 cells resulted in an inhibitory effect. Litvinov et al. transfected PC3 cells with a functional AR under a strong promoter and found overexpression of AR in PC-3 cells led to suppression of cell growth suggesting AR might play a negative role for basal intermediate-like tumour cell growth [248,257]. Another study supporting this hypothesis showed that Lenti-AR transduced PC3 and DU145 cell-lines expressed transcriptionally functional AR protein at appropriate physiological levels. Expression and engagement of AR protein in PC3-Lenti-AR cells resulted in transactivation of p21 and subsequent growth inhibition of these cells in culture and in mouse xenografts. Such growth inhibition was not observed in DU145-Lenti-AR cells. These findings suggest that the observed difference in IC_{50} between parental PC3 cells and PC3^{AR+} may be due to

S.tuberculatiformis extract inhibition of the AR resulting in its inhibitory effect being blocked thus desensitising the cells to the *S.tuberculatiformis* extract*.*

Overall, both CpdA and *S.tuberculatiformis* extract led to cell death at higher concentrations, but the *S.tuberculatiformis* extract appeared to have a dose dependent effect on cell growth. The effects of the *S.tuberculatiformis* extract on cell growth was reduced by the presence of the AR and GR receptor, observed in cell growth only declining in PC3 and PC3AR+ at higher concentrations that in LNCaP cells, while CpdA was unaffected by the AR/GR status. This would potential imply that CRPC may be a more sensitive target for the *S.tuberculatiformis* extract in early disease.

The results from the cell viability experiments suggest that the *S.tuberculatiformis extract* and CpdA have overlapping but not identical mechanisms initiating cell growth inhibition and again support the possibility that alternate compounds derived from *S.tuberculatiformis extract* might have the potential to improve those therapeutic benefits demonstrated by CpdA.

5.5.1 S.TUBERCULATIFORMIS EXTRACT AND CPDA DO NOT INDUCE APOPTOSIS

Cell death occurs either due to external injury or trauma which precipitates necrosis directly related to the injury or due to initiation of programmed cell death (PCD) or apoptosis. Whereas apoptosis is an inherent, controlled cellular death program, the conceptual counterpart, necrosis, is a more chaotic way of dying, which results from circumstances outside the cell, and is characterized by cellular edema and disruption of the plasma membrane, leading to release of the cellular components and inflammatory tissue response [105,396]. PCD or apoptosis is the natural pathway whereby the body kills off mutated or damaged cells. Therapeutically the best mechanism for chemotherapeutic drugs is to initiate a pathway that results in apoptosis, particularly if this can be directed to specific cell populations which are diseased. In this study a caspase 3/7 apoptosis assay was used to determine if the *S.tuberculatiformis extract* and CpdA initiated apoptosis as part of their anti-proliferative properties. Caspase 3 has been called the "henchman that goes around and executes the cell" because it is the final caspase in the cascade and once activated apoptosis is guaranteed [397]. This experiment was

carried out in PC3 and PC3^{AR+} as the LNCaP exhibited unpredictable and varied results in the cell viability assay.

In our study neither CpdA nor *S.tuberculatiformis* extract exhibited a significant increase in caspase-3 activity, regardless of steroid receptor status. It was therefore not possible to demonstrate apoptosis as part of their mechanisms of action (Figure 4.8A and 4.8B). There was also no significant difference between CpdA and the *S.tuberculatiformis* extract on caspase activity (Figure 4.9). As far as CpdA is concerned this was in contrast to the findings of Yemelyanov *et al* [5]. However, as mentioned previously, our cells were exposed to CpdA and *S.tuberculatiformis extract* for 16 hours only and it may be necessary to expose cells to longer incubation periods. Yemelyanov *et al.* treated PCa cells for 3-6 days with CpdA (10^{-5} mol/L) and observed increased activity of several caspases including caspase-3 (Figure 5.5). However, the induced caspase activity did not necessarily result in apoptosis. When compared to the positive control, TNFα, the CpdA caspase-induced activity was significantly lower, specifically caspase-3. Despite this, CpdA treatment did sensitize the PCa cells to TNFα induced apoptosis. This is probably due to the ability of CpdA to inhibit the Akt pathway through GR and AR activation lifting the constitutive inhibition on TNFα.
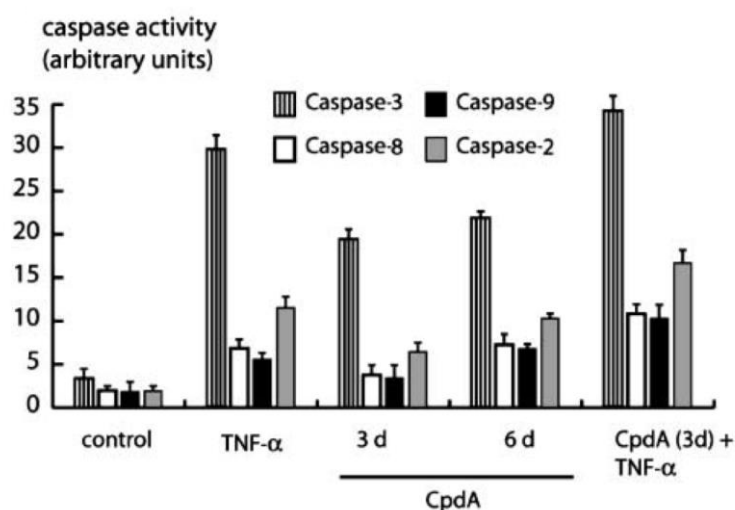
Figure 5.5 CpdA-induced apoptosis through Caspase Cascade Initiation. Evaluation of caspase activity after CpdA treatment by ApoAlert Caspase Assay. DU145 cells were pretreated with 0.01% DMSO (control) and CpdA (10^{-5} mol/L) for 3 and 6 d. TNF-a (10 ng/mL) was applied for 24 h. Columns, average caspase activity per experimental group in one representative experiment; bars, SD. Reproduced from Yemelyanov *et al.* (2008) [5].

Additionally, Yemelyanov *et al.* performed this caspase assay in DU145 cells [5]. DU145 and PC3 cells are aggressive PCa cell-lines resistant to multiple pro-apoptotic stimuli [398]. DU145 cells have distinct metabolic differences to PC3 cells that result in different sensitivities, such as DU145 being sensitive to TNFα apoptotic induction while PC3 cells remains resistant to it [399]. There are two genetic differences of note here as PC3 cells have a double homologous deletion mutation in the PTEN gene and so cannot express PTEN and they have a knock-out p51 gene while DU145 cells have a single deletion mutation in the PTEN gene, and can therefore still express it, and these cells also express a mutated p51 [400,401]. Therefore CpdA and the *S.tuberculatiformis* extract may not have induced apoptosis in PC3 cells because p51 stimulates PTEN activity which in turn inhibits the P13k/Akt pathway which is responsible for inhibition of TNFα induced apoptosis [ ]. Since DU145 cells still possess these proteins CpdA, and by extension possibly the *S.tuberculatiformis* extract may still enhance pro-apoptotic stimuli.

Interestingly, Yemelyanov *et al.* also showed that CpdA's cytostatic effects were greatly influenced by the GR. They determined this in two ways. First they compared the effect of CpdA in the stably transfected LNCaP[GR+] cells and parental LNCaP cells. LNCaP[GR+] cells were significantly more sensitive to growth inhibition by CpdA than the parental LNCaP cells (Figure 5.5). Secondly, they inhibited GR expression, 70% - 80% in PC3 and LNCaP-GR cells using the siRNA approach (Figure 5.6). This GR blockage resulted in a drastic loss of sensitivity to CpdA induced cell death. These finding correlate with the sensitivity exhibited by the *S.tuberculatiformis extract* in the cell viability assay where the parental PC3 (GR+) cells were more sensitive to the S.tuberculatiformis extract induced cell death. The same results were not seen in the CpdA cell viability assay, perhaps due to the short treatment incubation period. This could indicate that CpdA's effects required prolonged exposure whereas the effect of the *S.tuberculatiformis* extract was observed in a shorter incubation period. Importantly, Yemelyanov *et al.* also showed that RWPE-1 cells (normal prostate cells) were resistant to the growth inhibitory effects of CpdA, making CpdA an ideal candidate for PCa therapy. We did not test the S.tuberculatiformis extract on healthy prostate cancer cells, but our results exhibit similar sensitivities which may translate into similar selectivity, particularly as

106

Yemelyanov *et al.* have proposed that this selectivity is due to the AR and GR mediated signalling.
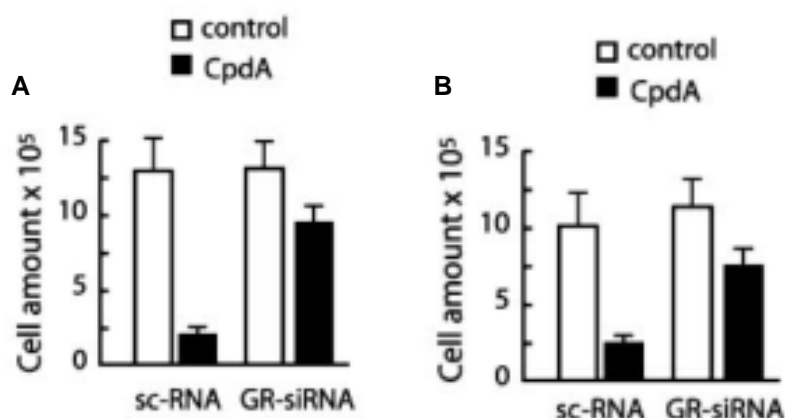
Figure 5.6 The inhibitory effects of CpdA are dependent on the steroid receptor expression in the cells, specifically the GR. Down-regulation of GR expression by siRNA decreases CpdA effect on PC cell growth. LNCaP-GR (A) and PC3 (B) cells were transfected with siRNA against GR (GR-siRNA) or scrambled siRNA (sc-RNA) as a negative control. Cells were treated with 0.01% DMSO (control) or CpdA (10^{-5} mol/L) for 8 d. Bar graphs represent quantification of CpdA effect on LNCaP (A) and PC3 (B) treated with GR-siRNA and sc-RNA. Columns, average number of cells per well for one representative experiment; bars, SD. Reproduced from Yemelyanov *et al.* (2008) [5].

The hypothesis put forward by Yemelyanov *et al.* is that the remarkable cytostatic potential of CpdA in PCa cells is the result of the inhibition of numerous pro-proliferative and anti-apoptotic TFs, such as factors from the Ets family, NFκB, and AP-1 combined with the blocked defence response of cancer cells mediated via gene activation by steroid hormone receptors [5,7]. Our results indicate that the *S.tuberculatiformis* extract could function under a similar mechanism and perhaps more efficiently. Although caspase activity was not observed in our study apoptosis is not excluded. As discussed the absence of caspase activity could be due to the shorter exposure or the choice of cell models. Further study into different cell lines is needed to demonstrate this observation.

A final consideration regarding apoptosis is that, although caspase-3 could not be shown to be involved, the *S.tuberculatiformis extract* and CpdA may also produce cell death effects through PCD pathways that are independent of caspases. In the past, PCD was considered to be the same as apoptosis, a process of cell death characterized by changes in cell morphology including cell shrinkage and condensation of chromatin leading to disintegration of the cell into ''apoptotic

bodies'' or small fragments that are removed by phagocytosis [402]. Once the apoptotic pathways are triggered, the caspase cascade is activated to execute the cell in a controlled fashion [397]. More recently it has become evident that the distinction between apoptosis and necrosis is an over simplification of the complex processes which protect the organism against unwanted, damaged and potentially harmful cells. Although caspases may be necessary for the classical apoptotic process, caspase activation is not the only determinant of life and death in PCD [403–407]. Xiang et al described one of the first examples of caspase-independent PCD. They showed that inhibition of caspase activities in the human leukemic cell line, Jurkat, did not inhibit Bax-induced cell death itself but only changed the morphology of the dying cells [408]. More evidence that PCD can occur in the absence of caspases is accumulating, and other, non-caspase proteases have been found to be able to trigger PCD [409–415]. In addition, in a study by Cauwels *et al.*, it was shown that caspase inhibition did not alleviate but rather exacerbated tumour necrosis factor (TNFα)-induced toxicity in mice, demonstrating caspase-independent PCD *in vivo* [416,417]. Thus the various forms of caspase-independent cell death cannot easily be classified as ''apoptosis'' or ''necrosis,'' and alternative types of PCD are now being described [403,404,406,409,418–420]. They occur under physiological circumstances and can also be induced by TNFα or chemotherapeutic drugs [421]. Not only caspases, but also other proteases including calpains, cathepsins, endonucleases, can initiate programmed cell death, and they can be directed by various intracellular organelles, including mitochondria, lysosomes, and the endoplasmic reticulum, alone or in collaboration with each other. Several models of caspase-independent cell death have been described but it is clear that they may overlap and several characteristics may be displayed at the same time (Figure 5.8). It has therefore been postulated that the dominant cell death phenotype is determined by the relative speed of the available death programs, and only the fastest and most effective pathway is usually evident [422]. Thus *S.tuberculatiformis* extract and CpdA could be affecting cell death through these pathways but this would need further clarification and investigation.
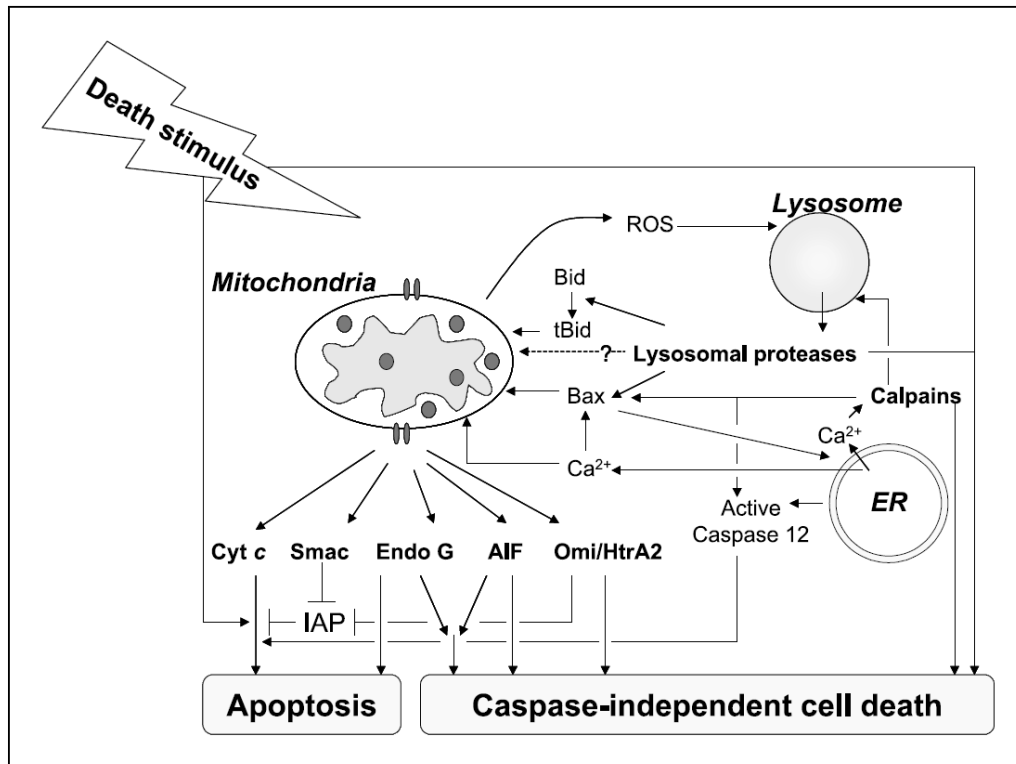
Figure 5.7. Cross-talk between cellular organelles resulting in apoptosis or caspase-independent cell death. Upon a lethal stimulus, a cell has access to different death programs. As depicted here, the signals from the different organelles are linked and may act both upstream and downstream of each other. Reproduced from Broker *et al.* (2005) [423].

Overall, the *S.tuberculatiformis extract* and CpdA both induced cell death in PCa cells making them possible therapeutic options for PCa. Based on our study the mechanism by which cell death was achieved is uncertain but these compounds continue to show great promise for applications in the treatment of advanced PCa.

109

5.6 CONCLUSIONS

Africa has many health challenges with significant health, social and economic implications. PCa can be considered one of the pivotal diseases. It is set to become the leading cancer in men and there is clear evidence, supported by the studies by Heyns *et al.* and others, that people of African descent have increased risk and present with more aggressive and more advanced disease with reduced chance of recovery on the currently available therapies. As a consequence there is a significant need, as well as a large gap, between the current situation and the need for more effective interventions and therapies. This places research into novel treatment strategies and new chemotherapeutic agents for PCa as a medical priority and drugs derived from our indigenous flora are an attractive alternative with implications for cost effective development.

The first aim of this study was to produce an *S.tuberculatiformis* extract with confirmed biological activity. This was achieved successfully through methanol extraction, with the biological activity verified using the bioassay developed by Swart *et al* [8,336]. Subsequently this extract could be used for the comparative evaluation against CpdA. As the selective steroid modulation is one of the characteristics of CpdA with the most exciting therapeutic applications, the initial aim was to prove steroid receptor translocation as a marker for potential modulatory bioactivity in the S.tuberculatiformis extract. Lastly, and with particular reference to the anticancer effects, the effects on cell growth and apoptosis were investigated and compared between the S.tuberculatiformis extract and CpdA.

The results showed that both the extract and CpdA interacted with the GR and AR causing nuclear translocation of both receptors. Furthermore, the effect of the *S.tuberculatiformis* extract on GR translocation was stronger than that exhibited by CpdA. This is the first indication that the two compounds are not biologically identical and supports the conjecture that there may be alternate or differing clinical effects that could affect the therapeutic potential. Although it is too early to speculate that the advantage lies with the crude extract, it is the GR-mediated transrepression that controls the anti-inflammatory response and therefore a stronger effect on the GR would lend support for the extract's improved potential over CpdA. Lastly, in terms of cell viability, both CpdA and the S.tuberculatiformis extract demonstrated

comparable effects on growth inhibition which is central to the expected benefit in PCa therapy. However, further investigation failed to demonstrate caspase cascade-induced apoptosis. It is possible that the mechanism for cell death as the extrinsic pathway and the Bl-2 family may be involved. Further clarification and exploration is needed to resolve this. Despite this the effect on cell growth remains supportive of anti-cancer potential, at least as potent as CpdA if not more.

Thus CpdA appears to be a good biological mimic of *S.tuberculatiformis* extract activity but the crude extract has exhibited early indications of possible additional beneficial properties. Future studies need to revisit the isolation of active compounds and possible co-factors to better decide on the development of novel lead compounds that could be applied therapeutically especially in the areas of cancer chemotherapy and autoimmune conditions where selective steroid modification has proven therapeutic

5.7 FUTURE RESEARCH

This study is only the beginning of the work needed to fully characterise the *S.tuberculatiformis* extract and many more biological characteristics need to be demonstrated and proven. Mechanisms of action must be further elucidated and related to biological functions to direct future research.

For the crude extract transactivation and transrepression need to be demonstrated to prove selective steroid modulation and anti-inflammatory effects. Apoptosis must be revisited using extended incubation periods and different cell lines to further determine the mechanisms of cell death. Active fractions from the crude extract must be identified and isolated with cofactors, if these are found to significantly affect stability or potency, and from there, more detailed studies to examine specific receptor properties such as virtual docking for the AR, GR and other steroid receptors.

The potential therapeutic application of *S.tuberculatiformis* in diseases other than PCa, specifically other hormone dependent cancers and inflammatory diseases, needs to be investigated. This would also be influenced by the effects on other steroid receptors. Finally, in terms of clinical applications, it will become important to investigate *S.tuberculatiformis* as part of multidrug therapies focusing on enhancing potency of therapy through multiple simultaneous targets.

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