



# **Targeting the Androgen Receptor as a Therapeutic Strategy for Prostate Cancer**

A thesis submitted to the University of Adelaide in the fulfilment of the requirements for  
the degree of Doctor of Philosophy

by

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## ERRATA

- Page *vii*: replace '2003' with '2006'.
- Chapter 1, figure 1.1A: image obtained from the following website - [www.biotherapy-clinic.com](http://www.biotherapy-clinic.com).
- Chapter 2, pg37: 1M Tris (pH 8.8 and 6.8) were made up using HCl.
- Chapter 2, pg74, 7.2.2, line 6: missing text following '5% and 10%' should read: '5% and 10% FCS, respectively'.
- Chapter 2, pg33 (and throughout thesis): replace 'C42B' with 'C4-2B'.
- Chapter 4, pg 76 (figure 4.2 and results): the text states that maximal effects on cell proliferation and survival were observed with 60nM and 120nM 17-AAG, respectively in PC-3 cells. As the highest dose used in this experiment was 120nM 17-AAG, this should read: the effect observed with 120nM 17-AAG was an increase in the percentage of dead cells to approximately 25%.
- Chapter 6, figure 6.15: replace 'nm' with 'nM'.
- Chapter 7, pg167: A xenograft model is a model in which cells, tissues or organs are grafted from one species to another. In order to avoid immunological rejection of the implant the host is immunocompromised. Specifically, in this thesis human LNCaP prostate cancer cells were implanted beneath the skin of nude (*nu/nu*) mice.
- Chapter 7, pg169: ECM gel – from Engelbreth-Holm-Swarm mouse sarcoma (*Sigma-Aldrich, Saint Louis, MO, USA*). ECM gel contains laminin, collagen type IV, heparan sulphate proteoglycan, entactin and other minor components.
- Chapter 7, pg 170: '4 $\mu$ M' should read '4 $\mu$ m'.
- Chapter 7, pg 170: 'H&E' should read 'haematoxylin and eosin (H&E)'.
- Chapter 7, pg171: Three separate cores from each xenograft tumour were selected for inclusion into the tissue microarray, this process was guided by use of haematoxylin and eosin stained sections. Following immunostaining of the tissue microarrays, 10 images of

each core (40X magnification) were randomly captured and analysed using video image analysis software as described on page 174.

- Chapter 7, pg 171: 'testies' should read 'testes'.
- Chapter 7, figure 7.3 legend: '100ml' should read '100 $\mu$ l'.
- Chapter 7, pg 176: In the first cohort of mice inoculated with LNCaP cells (n=30), the take rate was 18/30 (60%). In the second cohort (n=30), the take rate was 19/30 (64%).
- Chapter 7, pg 171 (and results): For immunohistochemical staining a negative control slide was included for each run, where the primary antibody was replaced with serum only. In each case the control slide showed no positive staining (data not shown).
- Chapter 8, pg195, 8.4.1, 2<sup>nd</sup> sentence: should read 'Moreover, there remain a number of chemotherapeutic or chemopreventive agents under investigation for the treatment of various cancers, which have recently demonstrated AR-targeting activities in prostate cancer cells.'

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## Abstract

Prostate cancer is the second leading cause of cancer related deaths in Australian men. Due to the dependence of prostate cancer cells on androgens for survival, androgen ablation therapy (AAT) is the standard therapy for men who have failed treatment for localised prostate cancer. Although initially responsive, most tumours relapse and are typically unresponsive to chemotherapeutic drugs. Recent evidence indicates that the intracellular mediator of androgens, the androgen receptor (AR) continues to play an integral role in maintaining tumour growth in an androgen-depleted milieu following failure of AAT. Therefore treatment strategies aimed at the activity of the AR itself either alone or in addition to reducing the levels of ligand may be more effective in inhibiting the growth of prostate cancer cells.

The objectives of this thesis were to characterise the effects of AR-targeting agents on the growth of prostate cancer cells and to determine whether combining these agents to target the AR at more than one level in the signalling pathway would provide a more complete block of androgen signalling and prostate cancer cell growth. Four agents were analysed for their ability to reduce the levels and/or activity of the AR: the histone deacetylase inhibitor SAHA, the Hsp90 inhibitor 17-AAG, the AR antagonist bicalutamide and AR-specific antisense oligonucleotides. *In vitro* culture of LNCaP human prostate carcinoma cells with each of these agents resulted in reduced AR protein levels and/or activity, inhibition of cellular proliferation and induction of cell death in a dose-dependent manner.

Combinations of low, sub-effective doses of the above agents were tested for additive or synergistic effects on prostate cancer cell growth. Combination treatment with SAHA and bicalutamide, 17-AAG and bicalutamide, or 17-AAG and SAHA, synergistically inhibited proliferation and induced caspase-dependent cell death in LNCaP prostate

cancer cells. The results of this thesis indicate that AR-targeting therapies are effective in suppressing the growth of prostate cancer cells. Furthermore when used in combination, the efficacy of these agents is markedly increased, even at low doses that individually have little effect. The results of this thesis provide a basis for clinical trials of AR-targeting strategies for prostate cancer.

## **Declaration**

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis being made available in the University Library.

Deborah Lydia Marrocco

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## List of Abbreviations

AAT	androgen ablation therapy
ANOVA	analysis of variance
AR	androgen receptor
ARE	androgen response element
AWS	androgen withdrawal syndrome
CAB	combined androgen blockade
CML	chronic myeloid leukaemia
DAB	diaminobenzidine
DBD	DNA binding domain
DES	diethylstilbestrol
DHT	5 $\alpha$ -dihydrotestosterone
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
FCS	foetal calf serum
FSH	follicle stimulating hormone
GA	geldanamycin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HPG	hypothalamic-pituitary-gonadal
Hsp90	heat shock protein 90
IAA	intermittent androgen ablation
IGF-1	insulin-like growth factor 1
IL-6	interleukin 6
KGF	keratinocyte growth factor
KLK2	kallikrein 2
LBD	ligand binding domain
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
MAPK	mitogen-activated protein kinase
MIOD	mean integrated optical density
NLS	nuclear localisation sequence
NTD	N-terminal domain
PIN	prostatic intraepithelial neoplasia
PI3-kinase	phosphatidylinositol 3-kinase
PSA	prostate specific antigen
SAHA	suberoylanilide hydroxamic acid
s.c.	subcutaneously
SHBG	steroid hormone binding globulin
siRNA	small interfering RNA
TMA	tissue microarray
TPR	tetratricopeptide repeat
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
TUNEL	TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labelling
UGM	urogenital sinus mesenchyme
VIA	video image analysis
wtAR	wild-type androgen receptor
17-AAG	17-allylamino-demethoxygeldanamycin

## Publications Arising From This Thesis

### *Articles Submitted to Scientific Journals*

*Suberoylanilide hydroxamic acid (SAHA; vorinostat) represses androgen receptor expression and acts synergistically with an androgen receptor antagonist to inhibit prostate cancer cell proliferation.*

Deborah L. Marrocco, Wayne D. Tilley, Tina Bianco-Miotto, Andreas Evdokiou, Howard I. Scher, Richard A. Rifkind, Paul A. Marks, Victoria M. Richon and Lisa M. Butler. *Mol Cancer Ther* (In press 2006)

### *Articles in Preparation*

*Synergistic activity of 17-AAG and bicalutamide on LNCaP prostate cancer cell growth.*

Deborah L. Marrocco, Lisa M. Butler, Grant Buchanan, Joanna Gillis, Willis R. Marshall and Wayne D. Tilley

*Histone deacetylase inhibitors in the treatment of prostate cancer.*

Deborah L. Marrocco, Paul A. Marks, Wayne D. Tilley and Lisa M. Butler

### *Abstracts Published in the Proceedings of Scientific Meetings*

JL Gillis, **DL Marrocco**, LM Butler, G Buchanan, WD Tilley. *The effect of 17-AAG on androgen receptor function in prostate cancer cells.* The Australian Society for Medical Research Annual Scientific Meeting, Adelaide, Australia, June 2004.

**DL Marrocco**, LM Butler and WD Tilley. *Targeting the androgen receptor for the treatment of prostate cancer.* The Australian Society for Medical Research Annual Scientific Meeting, Adelaide, Australia, June 2005.

**Deborah L. Marrocco**, Wayne W. Tilley, Andreas Evdokiou, Howard I. Scher, Richard A. Rifkind, Paul A. Marks, Victoria M. Richon and Lisa M. Butler. *The histone deacetylase inhibitor SAHA represses androgen receptor expression and acts synergistically with bicalutamide to inhibit prostate cancer cell proliferation.* The Australian Society for Medical Research National Scientific Conference, Couran Cove, Queensland, Australia, November 2005.

**Marrocco DL**, Tilley WD, Scher HI, Marks PA, Richon VM and Butler LM. *The histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) represses androgen receptor expression and acts synergistically with an antiandrogen to inhibit prostate cancer cell proliferation.* Australian Society for Medical Research 44th National Scientific Conference, Couran Cove, QLD, November 2005.

Butler LM, **Marrocco DL**, Evdokiou A, Scher HI, Marks PA, Richon VM and Tilley WD. *Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, represses androgen receptor expression and acts synergistically with an androgen receptor antagonist to inhibit prostate cancer cell proliferation.* 49th Annual



Scientific Meeting of the Endocrine Society of Australia, Gold Coast, QLD, August 2006.

LM Butler, **DL Marrocco**, T Bianco-Miotto, A Evdokiou, HI Scher, PA Marks, VM Richon and WD Tilley. *Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, represses androgen receptor expression and acts synergistically with bicalutamide to induce death of prostate cancer cells.* AACR Special Conference in Cancer Research: Innovations in Prostate Cancer Research, San Francisco, California, December 2006.

# **Chapter 1**

## **Introduction**

## 1.1 Background

---

The prostate is the leading site for cancer development in men (Jemal *et al.* 2006). The incidence of prostate cancer is rising rapidly in most countries, including low-risk populations, due in part to the widespread screening for the serum marker prostate specific antigen (PSA) (Hsing *et al.* 2000). Australia has the second highest incidence of prostate cancer worldwide, following North America, with one in eleven Australian men diagnosed in their lifetime. Prostate cancer is responsible for 14% of cancer related deaths in Australia, second only to lung cancer (23%) (Australian Bureau of Statistics, Causes of Death Australia, 2003).

Factors predisposing to the development of prostate cancer remain unclear. The only established risk factors are age, ethnicity or country of residence and family history; however, additional risk factors may include environmental and dietary factors (Prentice and Sheppard 1990; Sonn *et al.* 2005; Wolk 2005). Interestingly, an international autopsy study (Breslow *et al.* 1977) demonstrated equal frequency of microscopic prostate cancer between different geographical areas, suggesting that exogenous factors may be involved in the transition from latent to clinically relevant prostate cancer.

Since the implementation of routine testing for PSA, prostate cancer is increasingly diagnosed at early stages with increased likelihood that the tumour is organ-confined and amenable to cure by surgery or radiotherapy (Scher and Fossa 1995). However, 20-30% of patients with clinically localised disease will relapse following definitive treatment, necessitating systemic therapies for metastatic disease. Since the pioneering studies of Huggins, Stevens and Hodges in the 1940's (Huggins 1941), orchidectomy or, more typically, medical forms of androgen ablation, has been the mainstay of systemic management of prostate cancer. While effective initially, androgen ablation therapy (AAT) eventually fails in most men.

This chapter will discuss the involvement of the androgen signalling axis in the normal prostate and prostate cancer, and in particular the contribution of alterations in androgen signalling to the failure of androgen ablation therapies.

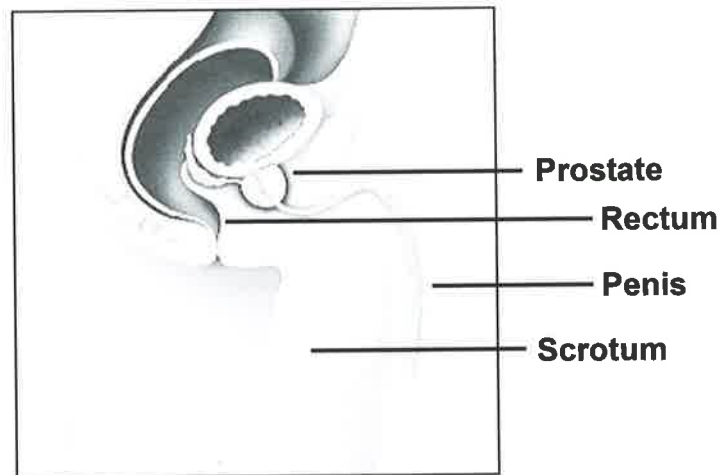
## **1.2 The Prostate Gland**

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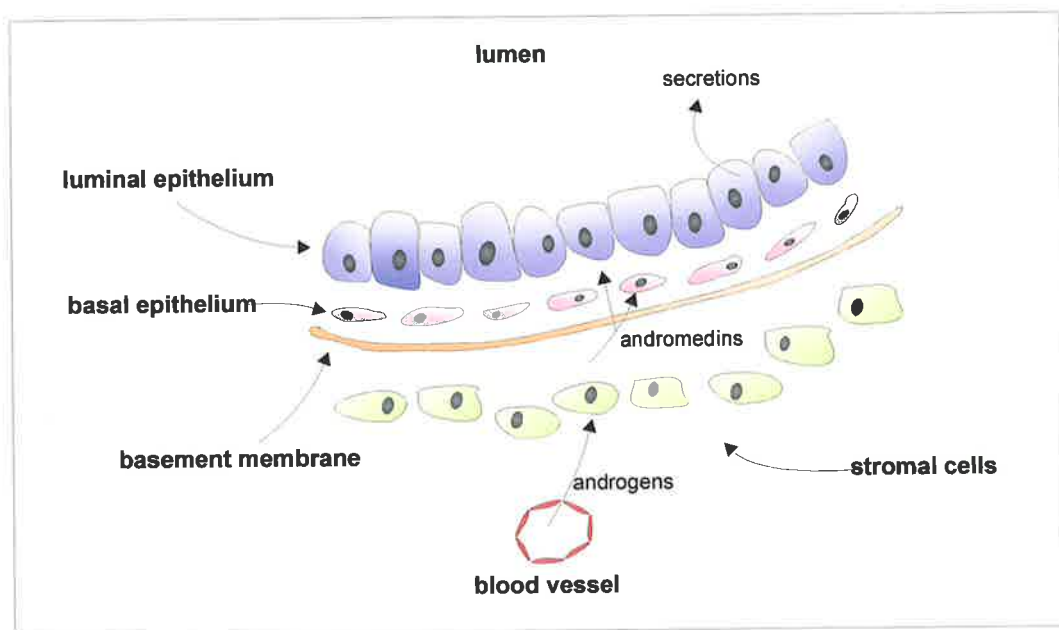
The prostate gland is situated below the bladder and seminal vesicles (Figure 1.1A). Its main function is the production of secretory proteins that make up the seminal fluid. Circulating androgens are necessary to maintain the integrity of prostate tissue and continued production of secretory proteins (Cunha 1994; Kokontis and Liao 1999). Production of androgens by the testes is regulated through the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus releases luteinizing hormone-releasing hormone (LHRH), which results in production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary. LH induces production of testosterone by Leydig cells in the testes, which then forms a feedback loop, acting on the pituitary to inhibit the production of LH and LHRH. Once secreted from the testes, testosterone is transported in the blood to the prostate and other target tissues where it activates the mediator of androgen signalling, the androgen receptor (AR).

Prostate development is influenced by testicular androgens acting on ARs in the urogenital sinus mesenchyme (UGM) during embryogenesis, inducing epithelial budding and differentiation of cells into luminal and basal cells (Cunha *et al.* 1987). The UGM differentiates into smooth muscle cells and fibroblasts (Hayward *et al.* 1996a; Hayward *et al.* 1996b; Hayward *et al.* 1996c). The adult prostate is comprised of epithelial ducts (reviewed in Cunha *et al.* 1987) that are divided into epithelial and stromal cell compartments. The stromal compartment of the prostate contains a variety of cell types, including fibroblasts, lymphocytes, macrophages, endothelial cells and smooth muscle

**A**



**B**



**Figure 1.1 - The anatomy of the prostate.** A) Location of the prostate gland. The prostate is the size of a walnut and is located beneath the bladder, surrounding the urethra and attached to the seminal vesicles. B) Cellular organisation of the normal prostate. Stromal cells are stimulated by circulating androgens to produce growth factors that act to promote growth and survival of epithelial cells. Basal epithelial cells give rise to the differentiated luminal epithelium, which when stimulated by androgens produces secretions such as PSA and kallikrein 2. The stromal and epithelial compartments are separated by a basement membrane.

cells (Litvinov *et al.* 2003). Separated from the stromal cells by a basement membrane, the epithelial cell compartment is comprised of three major cell types (Figure 1.1B), luminal cells, basal cells and neuroendocrine cells, thought to be progenitors derived from the same precursor stem cells located within the basal cell layer (Foster and Ke 1997). Basal cells themselves are thought to represent an intermediate between the stem cell population and the fully differentiated luminal epithelium (Litvinov *et al.* 2003). This cellular compartment is less differentiated than the luminal cells, with a greater proliferative capacity (Bonkhoff *et al.* 1994; Bonkhoff and Remberger 1998). Luminal epithelial cells are secretory cells and as their name suggests are located closest to the prostate lumen.

Circulating testosterone diffuses across the basement membrane into the epithelial cell compartment. Conversion of testosterone to DHT by the enzyme 5 $\alpha$ -reductase occurs both within basal and luminal epithelial cells (Bonkhoff *et al.* 1996). In luminal epithelial cells, activation of AR signalling by DHT (refer to section 1.5) induces the expression of prostate differentiation markers such as PSA and kallikrein 2 (Schuur *et al.* 1996; Mitchell *et al.* 2000; Watt *et al.* 2001; Jain *et al.* 2002; Zelivianski *et al.* 2002). Stromal cells influence epithelial cell differentiation and survival by producing a variety of growth factors following androgenic stimulation (Cunha *et al.* 1987). These growth factors, termed andromedins (androgen-induced stromal peptide growth factors) (Lu *et al.* 1999; Planz *et al.* 1999; Litvinov *et al.* 2003), bind to their receptors in the membranes of the epithelial cells resulting in repression of apoptotic pathways (Martikainen *et al.* 1990). In this way epithelial cells are indirectly stimulated by androgens for growth and survival via paracrine factors derived from stromal cells. Therefore, although androgens are able to act directly on ARs within luminal epithelial cells to induce the production of

androgen-dependent genes such as PSA and kallikrein 2, direct androgenic stimulation is not necessarily responsible for their growth and survival (Litvinov *et al.* 2003).

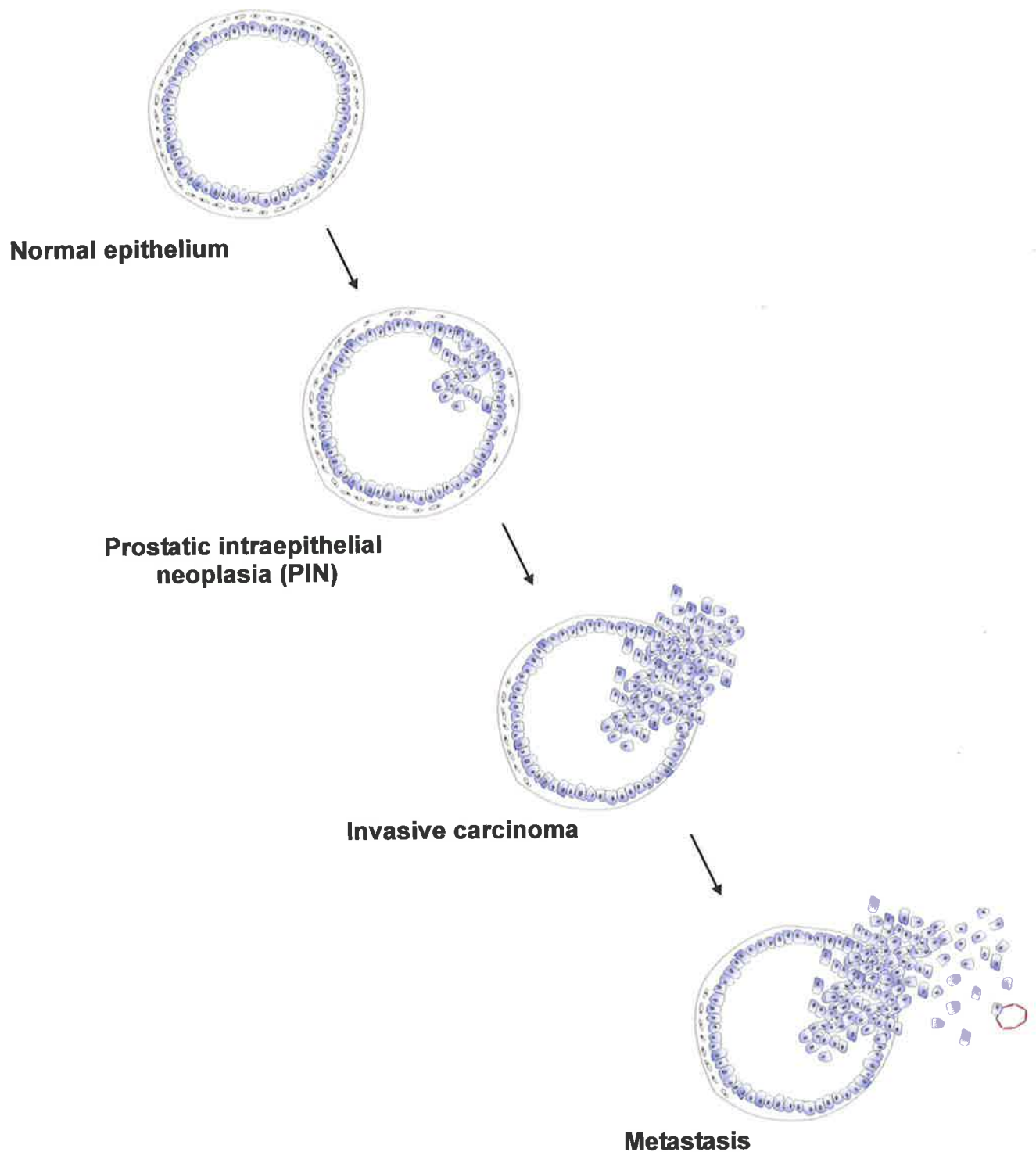
### **1.3 Prostate Cancer Development**

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Figure 1.2 depicts the progression of prostate cancer from the normal prostate gland through to metastatic disease. Most prostate cancers are adenocarcinomas, derived from epithelial cells, and are relatively slow growing tumours. Although the molecular alterations associated with development of prostate cancer are poorly defined, development of prostate cancer has been associated with loss of specific chromosome regions and tumour suppressor genes (for reviews see Ruijter *et al.* 1999; Abate-Shen and Shen 2000). Prostatic intraepithelial neoplasia (PIN) is thought to be a precursor of early invasive carcinoma (McNeal and Bostwick 1986), associated with enlarged nuclei and prominent nucleoli and metaplasia within the confines of the basement membrane (Bostwick *et al.* 1993). Prostate cancer is characterised by metaplasia and disorganisation of the glandular structures with loss of the basal cell layer. Invasion and penetration of the prostatic capsule follows, allowing local invasion of the peri-prostatic tissue. Whereas prostate cancer metastases occur primarily in the bone and lymph nodes, they can occur in other organs such as lung, liver and adrenal glands (Bubendorf *et al.* 2000).

#### ***1.3.1 Involvement of the AR in prostate cancer development***

It is well established that androgen signalling is involved in development and maintenance of the normal prostate. There is also evidence to support the involvement of androgen signalling in the initiation of prostate cancer. High levels of plasma androgens have been associated with an increased risk of prostate cancer development (Hamalainen *et al.* 1984; Dorgan *et al.* 1996; Gann *et al.* 1996), suggesting that excess androgens or increased signalling by the AR is involved in the development of prostate cancer.



**Figure 1.2 - Progression of prostate cancer.** The normal prostate gland is a well organised structure consisting of epithelial cells surrounded by a basement membrane. Prostatic intraepithelial neoplasia (PIN) is associated with increased proliferation of luminal epithelium and some loss of basal cells. Continued proliferation and invasion of the basement membrane occurs in invasive carcinoma, with greater loss of the basal cell layer. Metastasis occurs when prostate cancer cells break through the prostatic capsule and move via the blood stream or lymph nodes to distant sites.



Consistent with this hypothesis, the AR contains a polyglutamine tract ranging from 6-39 repeats (Buchanan *et al.* 2004) that is inversely correlated with AR transcriptional activity (Chamberlain *et al.* 1994; Kazemi-Esfarjani *et al.* 1995). A more active AR, i.e. with shorter polyglutamine tract, has been associated with increased risk of prostate cancer development, onset at a younger age and increased risk of diagnosis at an advanced stage (Hardy *et al.* 1996; Giovannucci *et al.* 1997; Stanford *et al.* 1997). Critically, a recent study Han *et al.* (2005) demonstrated for the first time the potential for the AR to act as an oncogene. Prostate-specific expression of an AR variant, containing a mutation in the amino-terminal domain that results in increased basal activity and increased activation in response to co-activators, resulted in development of PIN and progression to prostate cancer in 100% of mice. This evidence suggests that aberrant androgen signalling may be involved early in the development of prostate cancer. Although the role of androgen signalling in the development of prostate cancer is not clearly defined, AR expression at all stages of clinical prostate cancer progression has been well documented (Sadi and Barrack 1993; Pertschuk *et al.* 1994; Tilley *et al.* 1994; Pertschuk *et al.* 1995; Takeda *et al.* 1996; Prins *et al.* 1998; Sweat *et al.* 1999a; Sweat *et al.* 1999b; Henshall *et al.* 2001). As discussed above (section 1.2), whereas in the normal prostate paracrine signalling maintains survival of epithelial cells through andromedin secretion by stromal cells, during malignant progression there is a switch from paracrine to autocrine androgen action that allows AR signalling to stimulate proliferation and survival of tumour cells directly (Gao *et al.* 2001). Resultant activation of AR-regulated genes, including angiogenic factors, cell adhesion molecules and cell cycle regulators in this context can promote tumour progression (Lu *et al.* 1997; Ruohola *et al.* 1999). This switch in signalling favours AR-mediated growth and survival of prostate cancer cells and therefore forms the basis for current androgen ablation therapies for prostate cancer.

## **1.4 Current Treatment Strategies**

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Due to widespread PSA testing prostate cancer is diagnosed more frequently in younger men and at earlier stages of disease progression, allowing several therapeutic options (Figure 1.3).

### ***1.4.1 Localised disease***

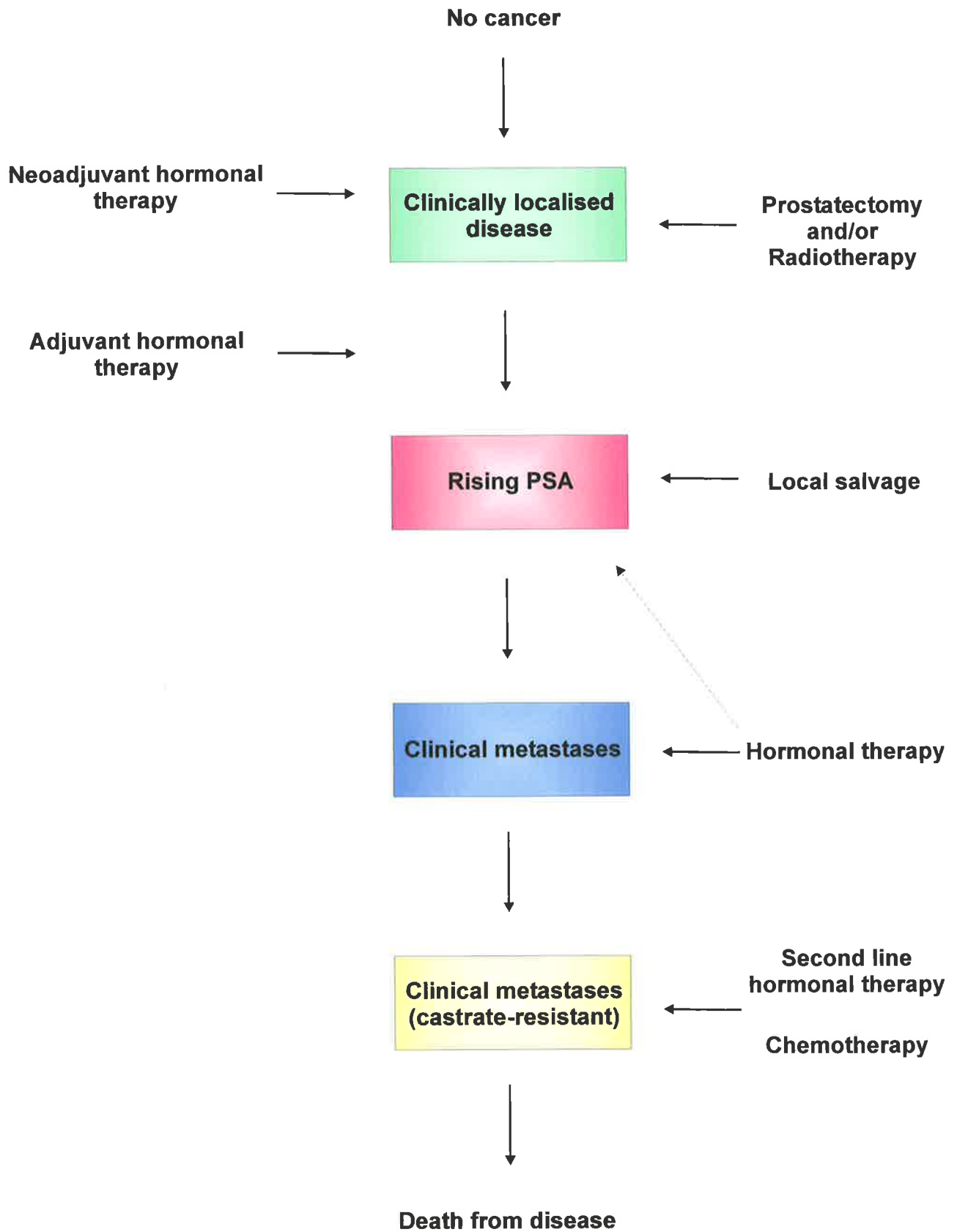
For patients with localised disease, treatment may be delayed and a conservative approach taken, where tumour growth is monitored closely. This approach is usually used for those patients that are assessed as having clinically insignificant disease which is unlikely to become life-threatening.

#### ***1.4.1.1 Prostatectomy & Radiotherapy***

For clinically localised prostate cancer, radical prostatectomy and radiotherapy offer the greatest chance for a cure. Although potentially curative, a percentage of patients will relapse with biochemical recurrence, indicated by a rise in serum PSA. The relapse rate for radiotherapy at 5 years is 30-70%, depending on the doses used (Soloway and Roach 2005), while up to 38% of patients relapse following prostatectomy (Wheeler *et al.* 1998; Amling *et al.* 2001; Han *et al.* 2001b; Hull *et al.* 2002). The rise in PSA levels can reveal local recurrence and/or metastatic spread.

#### ***1.4.1.2 Hormonal Therapy***

The dependence of prostate cancer cells on androgen signalling for growth and survival is exploited during hormonal therapy for prostate cancer. Rather than surgical removal of the tumour or attempting to eradicate tumour cells by radiotherapy, hormonal manipulation inhibits the production of androgens or blocks the interaction of androgens with the AR, thereby inducing prostate cancer cell death. Although traditionally used to treat metastatic disease, hormonal therapy is now being used to treat earlier stages of prostate cancer. Administered in the neoadjuvant setting (prior to localised therapy), the



**Figure 1.3 - Therapeutic options during progression of prostate cancer.** Depending on stage of progression, several therapeutic options may be available for patients with prostate cancer. For clinically localised disease treatment options include prostatectomy or radiotherapy. These primary therapies can be administered with neoadjuvant or adjuvant hormonal therapy. Following failure of localised treatment (indicated by a rise in PSA) local salvage can be administered. Hormonal therapy is the next therapeutic option for advanced, metastatic disease. Tumour progression following hormonal therapy is treated with second line hormonal therapy or chemotherapy.

aim is to induce tumour shrinkage, which will facilitate complete tumour excision by prostatectomy. Studies to date have not demonstrated a clear survival benefit of neoadjuvant hormonal therapy (Soloway 1995; Van Poppel *et al.* 1995; Scolieri *et al.* 2000). Administered in the adjuvant setting (in conjunction with localised therapy), the aim is to enhance the effects of localised therapy and eradicate tumour cells that may have spread beyond the confines of the prostate. In contrast to neoadjuvant hormonal therapy, adjuvant hormonal therapy has demonstrated a survival benefit (Bolla *et al.* 1997; Pilepich *et al.* 1997; Messing *et al.* 1999).

#### ***1.4.2 Local relapse and metastatic disease***

While local recurrence can be managed by salvage radiotherapy or prostatectomy (Schild *et al.* 1994; Haab *et al.* 1995; Crane *et al.* 1997; Garg *et al.* 1998; Nudell *et al.* 1999; Moul 2006), the high relapse rate suggests that micro metastases existed at the time of treatment (Soloway and Roach 2005); for these patients, systemic hormonal therapy is administered, however once bony metastasis develops, AAT is considered essentially palliative.

##### *1.4.2.1 Surgical Castration*

The beneficial effects of castration were first demonstrated in the 1940's by Huggins, Stevens and Hodges (Huggins 1941). By removing the main source of testosterone in the body, castration rapidly decreases circulating testosterone by 90-95% within a few hours, with an initial response in nearly all patients (Maatman *et al.* 1985). Surgical castration is relatively inexpensive, permanent and safe.

##### *1.4.2.2 LHRH Analogues*

An alternative to surgical castration is hormonal suppression of the HPG axis (refer to section 1.2). While this was initially achieved using exogenous estrogens, it is now more commonly achieved with LHRH analogues. Secretion of LHRH from the hypothalamus

occurs in pulses in order to prevent LHRH receptor desensitisation. Continuous administration of LHRH agonists, such as leuprolide (Ahmed *et al.* 1983), goserelin acetate (Ahmann *et al.* 1987) and buserelin (Borgmann *et al.* 1982), results in receptor desensitisation, thereby causing a down-regulation of pituitary LHRH receptors and suppression of serum testosterone by as early as 2 weeks (Labrie *et al.* 1980). Treatment with LHRH agonists initially results in a surge in LH release prior to receptor down regulation, causing an increase in testosterone levels and a transient increase in prostate cancer growth (Schulze and Senge 1990). To minimise this early side-effect antiandrogens are usually administered at the beginning of LHRH therapy. More recently LHRH antagonists have been developed, which include aborelix (Cook and Sheridan 2000; Garnick and Campion 2000) and cetorelix (Reissmann *et al.* 2000). These agents achieve castrate levels of testosterone faster than LHRH agonists, without the early rise in serum testosterone (Cook and Sheridan 2000; Pechstein *et al.* 2000). Adverse effects associated with LHRH analogue treatment include hot flushes, loss of libido, lethargy and impotence. No significant differences in time to disease progression or survival rates have been observed when comparing LHRH agonist treatment with surgical castration or the oral estrogen diethylstilbestrol (DES) (Peeling 1989; Kaisary *et al.* 1991; Waymont *et al.* 1992; Seidenfeld *et al.* 2000).

#### *1.4.2.3 Antiandrogens*

Antiandrogens (also known as AR-antagonists) exert their effects by competing with DHT for AR binding, thereby preventing AR-mediated transcriptional activity. Antiandrogens can be divided into two categories; steroidal antiandrogens (cyproterone acetate) and non-steroidal antiandrogens (flutamide, bicalutamide, nilutamide). Steroidal antiandrogens have progestational properties, inhibiting LHRH release from the pituitary thereby decreasing circulating testosterone, as well as directly binding to AR (Reid *et al.*

1999). Non-steroidal antiandrogens do not have progestational properties and therefore do not decrease levels of circulating testosterone (Reid *et al.* 1999). Antiandrogen treatment results in overall survival rates similar to those observed with surgical castration (Seidenfeld *et al.* 2000), however there have been no studies to directly compare the different antiandrogens (Seidenfeld *et al.* 2000).

Although tumour growth can be inhibited by the use of antiandrogens, a phenomenon known as the antiandrogen withdrawal syndrome (AWS) can occur after prolonged treatment. First reported in 1993, the AWS was observed in patients with biochemical (PSA) relapse during treatment with combined LHRH agonist and flutamide. A paradoxical decrease in PSA levels and/or clinical improvement was observed when the antiandrogen treatment was withdrawn (Scher and Kelly 1993). The observed median duration of response was reported to be 5 months, with some patients maintaining a response for more than 2 years. Although initially observed following treatment with flutamide (Scher and Kelly 1993; Schellhammer *et al.* 1997), antiandrogen withdrawal was later reported following treatment with bicalutamide (Small and Carroll 1994; Nieh 1995; Schellhammer *et al.* 1997), nilutamide (Gomella *et al.* 1997; Huan *et al.* 1997), estrogens and progestational agents (Scher and Kolvenbag 1997).

Following failure and relapse after antiandrogen therapy, second line antiandrogens may be the next option. Responses have been demonstrated with second line treatment using nilutamide (Kassouf *et al.* 2003), flutamide, bicalutamide and megestrol acetate (Small and Vogelzang 1997). Relapse rates following hormonal therapy vary depending on the stage of disease at time of treatment. For patients treated with early intervention at the time of biochemical recurrence, hormonal therapy demonstrates some improvement in survival (Messing *et al.* 1999). In contrast, treatment of bony metastasis by surgical or

chemical castration is considered a palliative approach as it does not increase survival (Sharifi *et al.* 2005)

#### *1.4.2.4 Combined Androgen Blockade*

The rationale for combined androgen blockade (CAB) is based on inhibition of total androgen production, which includes testicular androgens in addition to peripheral androgen production by the adrenal glands. While LHRH analogues are effective inhibitors of testicular androgen production, approximately 5% of serum testosterone is produced by the adrenal glands under independent control (Gommersall *et al.* 2002). Therefore treatment with LHRH analogues is combined with antiandrogen to block the interaction of the AR with adrenal-derived testosterone. While CAB has been shown to marginally prolong time to tumour recurrence (Crawford 1989; Denis and Murphy 1993; Janknegt 1993), it also results in increased side-effects and treatment costs and therefore combined androgen blockade is not readily used.

#### *1.4.2.5 Intermittent Androgen Ablation*

Another strategy that has been incorporated into hormonal therapy to treat advanced prostate cancer is intermittent androgen ablation (IAA). The rationale behind IAA is that prolonged androgen ablation selects for a subset of resistant tumour cells (Isaacs and Coffey 1981) that are able to maintain proliferation and survival in the androgen ablated environment (Craft *et al.* 1999a). It has been proposed that administering androgen ablation in an intermittent fashion limits or delays the selection of therapy-resistant clones (Theyer and Hamilton 1998). *In vivo* xenograft studies have shown that disease progression can be delayed up to 3-fold with IAA therapy (Gleave *et al.* 1998a). Although controversial, preliminary clinical data suggests that the therapy-responsive phase of prostate cancer can be maintained during IAA therapy with no significant risk (Gleave *et al.* 1998a; Rambeaud 1999). Whether these results translate to increased

survival in long-term clinical trials is still to be determined. Favourable aspects of this mode of treatment include an improvement in quality of life as well as a reduced cost of therapy.

### ***1.4.3 Failure of hormonal therapy***

Although hormonal therapy is initially effective with a response rate as high as 80%, in metastatic disease the tumour inevitably becomes resistant to therapy and re-grows within 4-5 years (Goodin *et al.* 2002), after which survival time is estimated at about 16 months (Martel *et al.* 2003). Relapsed prostate cancer has previously been termed ‘hormone-refractory’ or ‘androgen-independent’ since it was thought that the tumour had escaped hormonal control and thus tumour progression could no longer be controlled by hormonal intervention. It is now evident that prostate tumours relapsed on hormonal therapy actually maintain responsiveness to AR signalling. For this reason the term ‘castrate-resistant’ will be used hereafter to describe prostate cancers that have relapsed during hormonal therapy. At present there is no cure for castrate-resistant prostate cancer, however various therapeutic options include antiandrogen therapy or cytotoxic chemotherapy (Goodin *et al.* 2002; Martel *et al.* 2003).

#### ***1.4.3.1 Chemotherapy***

In the 1990’s trials using mitoxantrone combined with corticosteroids provided the standard primary care for men advancing on hormonal therapies (Tannock *et al.* 1996; Kantoff *et al.* 1999). Since then two trials using taxane-based chemotherapy have for the first time shown survival benefits compared to the standard mitoxantrone plus prednisone where median survival is 15.6-16.4 months (Petrylak 2005). Treatment with docetaxel plus estramustine or docetaxel plus prednisone showed median survival of 17.5 months (Petrylak *et al.* 2004) and 18.9 months (Tannock *et al.* 2004), respectively. Although promising, the survival benefit is only modest and there remains a need to further



improve survival and quality of life of patients with castrate-resistant disease. Emerging therapeutic options include immune based therapy and gene therapy (for reviews see Isaacs 2005; Desai *et al.* 2006).

## **1.5 Androgen Receptor Biology**

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The AR is a ligand-dependent transcription factor belonging to the steroid receptor superfamily (Tsai and O'Malley 1994). It is expressed in nearly all tissues, with the exception of the spleen and bone marrow (Lindzey *et al.* 1994; Taplin *et al.* 1995) and is located on the X-chromosome. When translated the AR is a 918 amino acid protein with a molecular weight between 100-110kd. The AR consists of four functional domains, which include an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region and a ligand-binding domain (LBD). The NTD is involved in regulating transcriptional activity of the receptor, while the DBD is made up of 68 amino acids, forming two zinc fingers that are involved in recognition and binding to specific androgen response elements (AREs) located in the promoter region of androgen-regulated genes (Schoenmakers *et al.* 1999). The hinge region of the receptor contains a lysine-rich nuclear localisation sequence (NLS) at amino acids 617-633 that targets the AR for nuclear import upon ligand binding (Zhou *et al.* 1994). The LBD is a 253 amino acid hydrophobic domain involved in regulation of ligand binding (Matias *et al.* 2000). In addition, sequences contained within the LBD may also be involved in heat shock protein association, receptor phosphorylation and dimerization (Prins 2000).

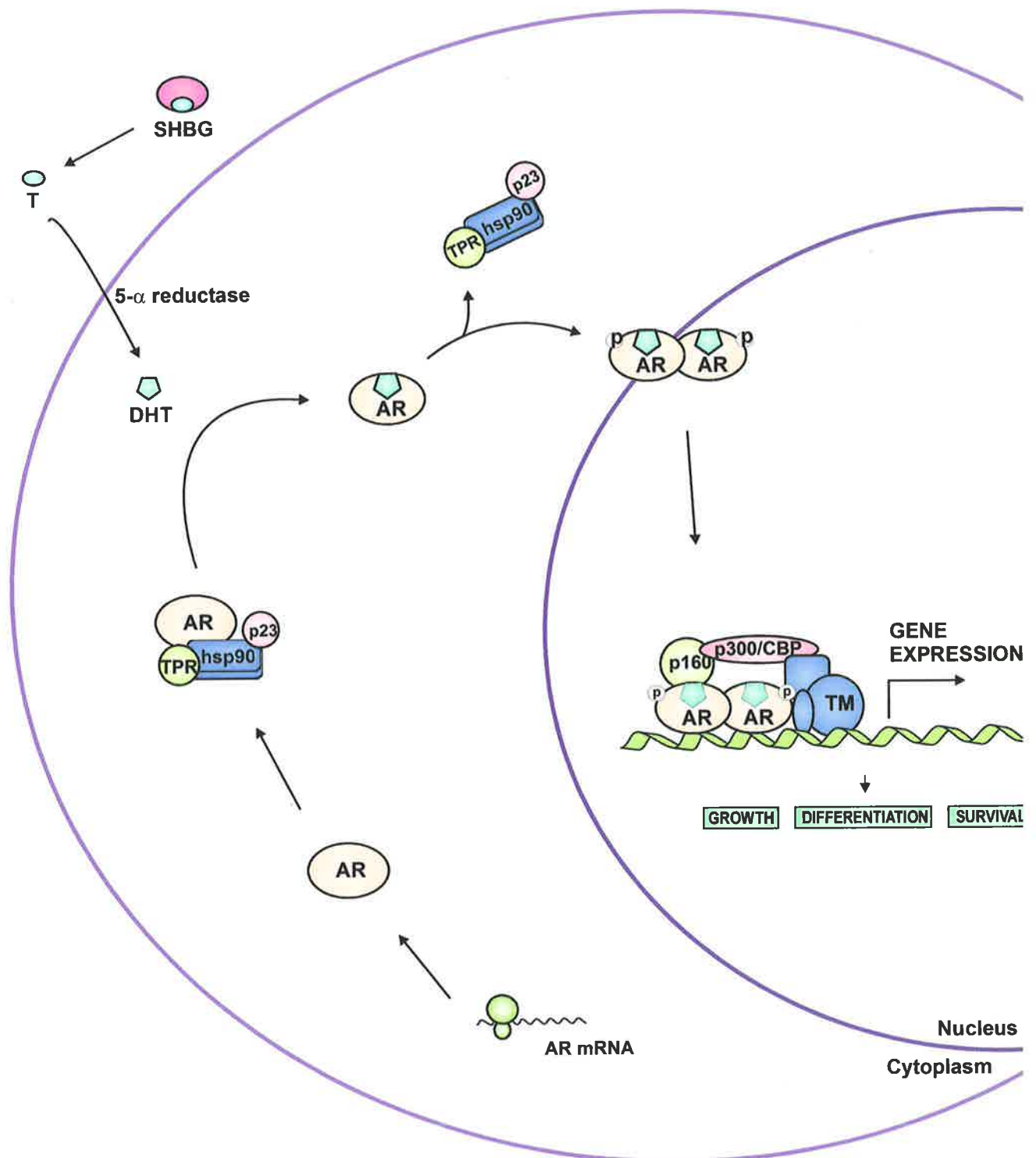
### **1.5.1 Activation of AR Signalling**

Signal transduction through the androgen signalling axis begins when testosterone dissociates from its serum transport proteins, steroid hormone binding globulin (SHBG) or albumin (Siiteri *et al.* 1982), and diffuses from the blood into the cell cytoplasm

(Figure 1.4). In prostate cells testosterone is converted by the enzyme 5 $\alpha$ -reductase to the more potent androgen 5 $\alpha$ -dihydrotestosterone (DHT). When it is not bound to ligand, the AR is maintained in a configuration with high ligand binding affinity by a molecular chaperone heterocomplex composed of heat shock proteins, co-chaperones and tetratricopeptide repeat (TPR)-containing proteins (Pratt and Toft 1997). Ligand binding alters the conformation of the receptor, exposing the nuclear localisation sequence and DBD, resulting in dissociation of the chaperone complex, phosphorylation and dimerization of the receptor (Ikonen *et al.* 1994; Zhou *et al.* 1994; Trapman and Brinkmann 1996; Culig *et al.* 1998) followed by translocation into the nucleus (Georget *et al.* 1997; Pratt and Toft 1997; Richter and Buchner 2001), where the receptor homodimer binds to specific androgen response elements in the promoter of target genes, inducing co-regulator recruitment and activating gene transcription (Montgomery *et al.* 2001). The androgen receptor is able to shuttle back and forth from the cytoplasm undergoing multiple rounds of recycling (Tyagi *et al.* 2000). AR signalling in the prostate affects a variety of processes including differentiation, morphogenesis, angiogenesis, proliferation and apoptosis (O'Malley 1990; Kokontis and Liao 1999; Roy *et al.* 1999; Stewart *et al.* 2001). In contrast to direct activation of the AR through ligand binding, non-genomic effects of androgen signalling have also been reported, although the mechanisms and physiological consequences of activation via these signalling pathways are not completely understood (reviewed in (Heinlein and Chang 2002b)).

### **1.6 Mechanisms Associated with Relapse Following Androgen Ablation Therapy**

Although hormonal therapy for prostate cancer inevitably fails, recent evidence suggests, contrary to the previous dogma of a hormone refractory state, tumours invariably retain a requirement for AR signalling. Tumour cells appear to acquire mechanisms that allow the



**Figure 1.4 - Androgen signalling axis.** The androgen receptor (AR) is activated by serum testosterone (T) that dissociates from steroid hormone binding globulin (SHBG) and diffuses into the cytoplasm of cells where it is converted by the enzyme 5 $\alpha$ -reductase to 5 $\alpha$ -dihydrotestosterone (DHT). The AR is present in a chaperone heterocomplex, maintained in a conformation with high ligand-binding affinity. The AR binds DHT and dissociates from its chaperone complex, dimerises, is phosphorylated and translocates to the nucleus. Once nuclear the AR homodimer binds to specific androgen responsive elements in the promoter of target genes and activates gene transcription. AR activity is results in the transcription of genes associated with growth differentiation and survival.

AR to maintain signalling despite therapeutic intervention. Consistent with this view, the AR is expressed in most castrate-resistant tumours, and expression of AR-regulated genes such as PSA and kallikrein 2 are increased (Hobisch *et al.* 1995; Bentel and Tilley 1996; Culig *et al.* 1998; Gregory *et al.* 1998; Koivisto *et al.* 1998; Bubendorf *et al.* 1999; Koivisto and Helin 1999). An increasing number of studies have indicated that a range of mechanisms could facilitate continued AR signalling in prostate cancer cells, including an increase in AR levels, AR gene mutations, alterations in co-factor expression and activation of the AR through growth factor signalling pathways.

### ***1.6.1 Increased AR levels***

Increased AR levels can result from gene amplification or increased protein stability. AR gene amplification has been reported in up to 31% of recurrent prostate cancers, but is observed less frequently in untreated tumours (Koivisto *et al.* 1995; Visakorpi *et al.* 1995; Palmberg *et al.* 1997; Bubendorf *et al.* 1999; Linja *et al.* 2001), suggesting a role for gene amplification in failure of hormonal therapy. Continued growth of CWR22 xenograft tumours following castration has been associated with an increase in AR expression and stability (Gregory *et al.* 2001b). This reduces the concentration of DHT required for growth stimulation of prostate cancer cells (Gregory *et al.* 2001b), providing a means for maintenance of AR signalling in the castrate environment. A recent study by (Chen *et al.* 2004) demonstrated that over-expression of the AR in prostate cancer cells was sufficient to overcome bicalutamide therapy, converting bicalutamide from an antagonist to an agonist, thereby providing a mechanism for castrate-resistant tumour growth.

### ***1.6.2 Gain-of-function mutations***

AR gene mutations have been associated with diseases such as androgen insensitivity syndrome, spinal and bulbar muscular atrophy and prostate cancer (Brown 1995;

McPhaul 2002). The site of the mutation determines the functional consequences on AR activity, and ultimately the phenotypic outcomes. Unlike mutations found in patients with androgen insensitivity syndrome that result in loss of AR activity, mutations identified in prostate cancer patients usually result in reduced ligand specificity and/or increased AR activity. These gain-of-function AR gene mutations have been identified at varying frequencies in up to 50% of metastatic lesions from patients with hormone refractory prostate cancer (Taplin *et al.* 1995; Tilley *et al.* 1996; Hyytinen *et al.* 2002).

#### 1.6.2.1 Ligand binding domain mutations

Mutations in the AR locate to several discrete regions (Buchanan *et al.* 2001a), the most well characterised being the LBD. Mutations within the LBD alter the conformation of the ligand-binding pocket, so as to accommodate larger, non-classical ligands, including antiandrogens (Taplin *et al.* 1995; Taplin *et al.* 1999; Han *et al.* 2001a; Hara *et al.* 2003a), thereby providing a growth advantage in the castrate environment. In clinical samples 31% of patients treated with combined androgen blockade with flutamide had mutations in the AR at codon 875 in the LBD allowing the AR to be activated by flutamide at concentrations of <100nM (Taplin *et al.* 1999). This has clinical implications as serum concentrations of flutamide during treatment reach the  $\mu\text{M}$  range (Belanger *et al.* 1988). A novel mutation was detected at codon 741 in the LBD in prostate cancer cells following antiandrogen withdrawal of bicalutamide (Hara *et al.* 2003a), which may be the mechanism responsible for the switch of bicalutamide from antagonist to agonist of the AR. Interestingly in a cohort of patients treated with castration plus bicalutamide (Haapala *et al.* 2001) a high frequency of AR gene mutations (36%) was observed, but none of the mutations were at position 875; however, one mutation was in codon 741. Collectively, these findings suggest that a particular therapy selects for a type of mutation that will allow the cells to grow in that particular hormonal milieu.

### *1.6.2.2 N-terminal domain mutations*

In addition to LBD mutations, NTD mutations have been identified in prostate cancer, however this class of mutation is less well characterised. Using the TRAMP mouse model of prostate cancer, Han *et al.* (2001a) demonstrated the involvement of NTD mutations in the development of castrate-resistant prostate cancer. 78% of the mutations found in tumours that relapsed following castration located to the NTD of the receptor, with the remaining locating within the LBD (Han *et al.* 2001a). The consequences of the NTD mutations on the AR were increased activation by classical and non-classical ligands, as well as alterations in co-factor interactions with the receptor, providing a growth advantage in the castrate environment (Han *et al.* 2001a). Subsequent analysis of one of these mutations, an E231G substitution, demonstrated an increase in basal levels of activity and increased response to AR co-activators. Additionally, enforced expression of this mutant in the mouse prostate resulted in the development of prostate cancer in 100% of mice (Han *et al.* 2005). N-terminal domain mutations have also been identified in human prostate cancer that had relapsed following castration plus estramustine phosphate (Hyytinen *et al.* 2002). The mutations identified were located to a region between amino acids 514-533 in the NTD (Hyytinen *et al.* 2002).

The co-location and functional homogeneity of AR gene mutations associated with escape from different hormonal manipulations suggests that the therapy itself selects for specific mutations that facilitate continued AR signalling (Taplin *et al.* 1999; Hyytinen *et al.* 2002).

### *1.6.3 Alterations in cofactor expression*

Co-regulators are essential components of the AR transcriptional complex with a number of putative co-activators and co-repressors being identified (for reviews see McKenna *et al.* 1999; Robyr *et al.* 2000; Heinlein and Chang 2002a). Aberrations in co-regulator

expression or co-regulator/AR mutations that inhibit normal AR signalling may play a role in the progression of prostate cancer due to deregulation of the AR signalling axis by altering transactivation capacity of the AR in response to low levels of DHT or physiological levels of non-classical ligands (Buchanan *et al.* 2001b). Over-expression of several AR co-activators has been noted during prostate cancer progression, including ARA24, PIAS1 (Li *et al.* 2002), Cdc25B (Ngan *et al.* 2003) and RAC3 (Gnanapragasam *et al.* 2001). Levels of Cdc25B and RAC3 have been correlated with tumour grade and stage (Gnanapragasam *et al.* 2001; Ngan *et al.* 2003), suggesting a role for AR co-activator up-regulation in the progression of prostate cancer. In support of this hypothesis, an increase in expression of ARA55 and SRC1 (Fujimoto *et al.* 2001), SRC1, TIF2 (Gregory *et al.* 2001a) and Tip60 (Halkidou *et al.* 2003) has been demonstrated following AAT. In addition, over-expression of the AR co-activators ARA70, ARA55 and ARA54 can increase AR activation by flutamide and  $\Delta$ 5-androstenediol (Miyamoto *et al.* 1998; Yeh *et al.* 1999a) and over-expression of the AR co-activator CBP results in increased AR activation by bicalutamide and flutamide (Comuzzi *et al.* 2003). In addition, over-expression of TIF2 results in increased AR activation by physiological concentrations of non-classical ligands such as estradiol, progesterone, DHEA and androstenedione (Gregory *et al.* 2001a). Whereas at a molecular level the interaction between TIF2 and the AR is weak, over-expression of TIF2 enhances the interaction resulting in an increase AR activity (Gregory *et al.* 2001a).

AR co-repressor molecules have also been implicated in the progression of prostate cancer. For example, the tumour suppressor protein LATS2 is recruited to the promoter of AR target genes and interacts with the AR to repress ligand-dependent activation (Powzaniuk *et al.* 2004). Powzaniuk *et al.* (2004) demonstrated that recruitment of this co-repressor resulted in decreased PSA production, and that its expression was

significantly reduced in 6 of 7 prostate tumour samples tested, compared to normal prostate tissue.

The ratio of co-repressors to co-activators is therefore likely to influence AR signalling in castrate-resistant prostate cancer (Scher *et al.* 2004) and alterations in the ratio may be a mechanism whereby AR signalling is maintained in the castrate environment.

#### ***1.6.4 Activation via alternate signalling pathways***

Activation of the AR in the absence of ligand can occur through stimulation of growth factor or cytokine signalling pathways (reviewed in Feldman and Feldman 2001). For example signalling through insulin-like growth factor-I (IGF-I), keratinocyte growth factor (KGF) and epidermal growth factor (EGF) pathways can induce AR activation, an effect that can be blocked by bicalutamide (Culig *et al.* 1994). Additionally interleukin-6 (IL-6) signalling via the MAP kinase pathway can stimulate PSA secretion in an androgen-independent manner (Abreu-Martin *et al.* 1999). Although ligand-independent activation of the AR is yet to be demonstrated *in vivo*, circulating levels of IL-6 are increased in patients with castrate-resistant disease (Adler *et al.* 1999; Drachenberg *et al.* 1999), consistent with the potential for cytokine activation of the AR. Receptor tyrosine kinases such as Her2/neu have also been reported to increase AR activity in the absence of androgens (Craft *et al.* 1999b; Yeh *et al.* 1999b). Her2/neu is over-expressed in tumours following AAT compared to untreated tumours (Signoretti *et al.* 2000; Osman *et al.* 2001). Signalling through this pathway increases AR transactivation through activation of MAP kinase and Akt signalling pathways that result in AR phosphorylation and ligand-independent activation (Craft *et al.* 1999b; Mellinghoff *et al.* 2004), providing an alternate mechanism for maintaining AR signalling that may allow the continued proliferation of prostate cancer cells during periods of therapeutic intervention.



### ***1.6.5 Persistence of intratumoural androgens***

More recently it has been found that while serum testosterone and DHT are reduced following AAT, intraprostatic testosterone is maintained at levels similar to benign prostate tissue (Mohler *et al.* 2004; Nishiyama *et al.* 2004; Titus *et al.* 2005). Furthermore, although intratumoural levels of DHT are significantly reduced following AAT, the remaining levels are sufficient to induce AR activation (Mohler *et al.* 2004; Titus *et al.* 2005). As prostate cancer cells retain 5 $\alpha$ -reductase enzymes (Bonkhoff *et al.* 1996), persistence of intratumoural androgens has been suggested to occur by conversion of adrenal androgens to testosterone and DHT within prostate tumour cells (Titus *et al.* 2005). In support of this hypothesis, a study by Holzbeierlein *et al.* (2004) found an increase in levels of enzymes involved in steroid biosynthesis, which were associated with castrate-resistant progression. Moreover Stanbrough *et al.* (2006) reported an increase in the expression of genes involved in androgen metabolism in androgen-independent bone metastases compared to primary prostate cancer, providing a means for the conversion of adrenal androgens in metastatic deposits.

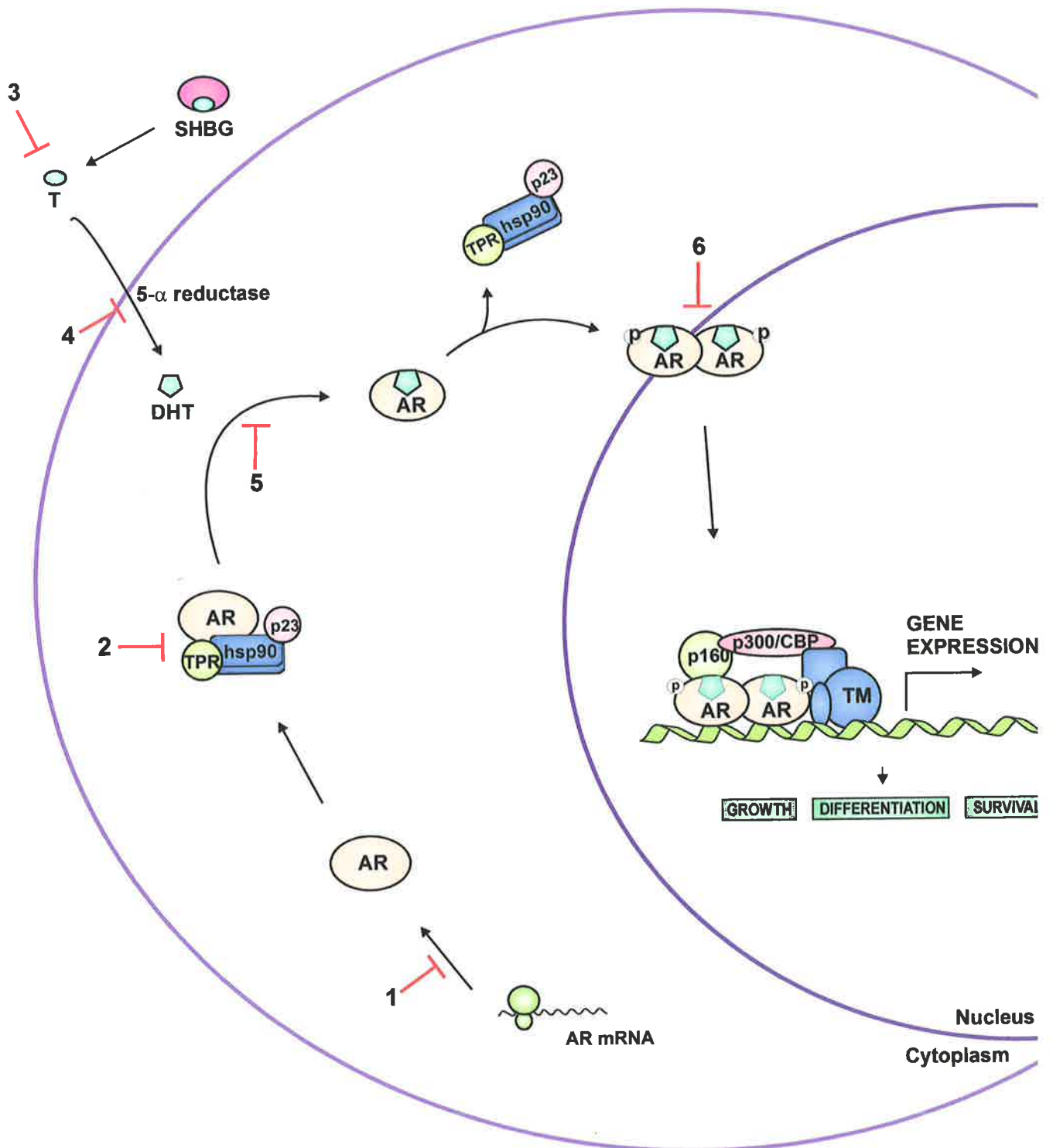
### **1.7 AR-Targeted Strategies**

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The evidence presented above demonstrates that AAT fails not necessarily due to the selection of tumour cells with mechanisms that allow growth independent of AR signalling, but rather that tumour cells acquire the ability to maintain AR signalling in a castrate environment with low levels of ligand. The fact that continued AR signalling is associated with tumour relapse and that secondary hormonal manipulations can induce a further biochemical (PSA) response, implicates the AR signalling axis as a viable therapeutic target in castrate-resistant disease. Consequently, treatments for castrate-

resistant prostate cancer might be more effective if they target the mediator of androgen signalling, the AR itself.

Inhibition of this pathway by decreasing AR transcription/translation, stability, maturation, DNA-binding or transactivation are all potential points to target AR signalling (Figure 1.5). At the time this thesis was initiated there were few studies in the literature reporting the potential therapeutic benefit of targeting the AR in prostate cancer. Two studies showed that AR antisense oligonucleotides decreased AR protein levels and inhibited growth of LNCaP prostate cancer cells both *in vitro* and *in vivo* (Eder *et al.* 2000; Eder *et al.* 2002). In another study, microinjection of monoclonal anti-AR antibodies were shown to inhibit the proliferation of androgen-dependent as well as androgen-independent prostate cancer cells (Zegarra-Moro *et al.* 2002). In a less targeted strategy, Solit *et al.* (2002) demonstrated that the Hsp90 inhibitor 17-AAG reduced AR protein levels and inhibited the growth of prostate cancer cells and xenografts. These studies were consistent with the hypothesis that targeting AR signalling is effective at inhibiting the growth of prostate cancer cells, therefore providing support for further research into the identification and/or characterisation of agents that target the AR as potential therapeutic strategies for the treatment of castrate-resistant prostate cancer. Further support can be drawn from the development of targeted therapy in other malignancies, where it has been shown that specific targeting of critical growth regulatory pathways involved in tumour growth has been very successful (Sawyers 2004). One such example is Bcr-abl tyrosine kinase mutations in chronic myeloid leukaemia (CML). Constitutive activity of Bcr-abl, most commonly due to activating mutations, is responsible for disease progression in CML (Sawyers 2004). Clinical responses to Bcr-abl targeted therapy are seen in tumours that are dependent on this signalling pathway (Sawyers 2004). Development of resistance to targeted therapy is



**Figure 1.5 - Targeting the androgen signalling axis.** Possible therapeutic targets within the androgen signalling axis include 1) translation of AR mRNA to protein, 2) maturation and stability of the AR, 3) testosterone production, 4) conversion of testosterone to the more potent androgen DHT, 5) AR-ligand binding and 6) binding to and activating gene transcription.

associated with reactivation of Bcr-abl signalling by mutations that alter the prohibit drug interaction (Gorre *et al.* 2001; Shah *et al.* 2002); however subsequent treatment with alternate targeted small molecule inhibitors is able to induce a response (Shah *et al.* 2004). These studies provide proof of concept that targeting a single pathway responsible for the growth and survival of tumour cells can be an effective therapeutic strategy.

AR-targeting agents most likely to be of clinical use in the short-term are those that will not require the development of specialised delivery technology. For example the development of AR-targeting siRNA would also involve optimisation of delivery methods. Antisense oligonucleotides provide a similar specific knockdown to siRNA and can be administered *in vivo* without the use of a delivery agent and therefore could be more rapidly evaluated in clinical trials. In addition, agents already in clinical trials, such as Hsp90 inhibitors, may provide an advantage in that they are further developed clinically and target more than one signalling pathway, which may increase the inhibitory effect on tumour growth.

## **1.8 Summary**

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Androgen signalling is a key regulatory pathway involved in the growth and development of the normal prostate, prostate cancer initiation, progression, and continued growth in a castrate-resistant state following failure of AAT. Established androgen ablation strategies aim to inhibit growth of tumour cells by i) depriving the tumour cells of androgens, or ii) preventing androgens from binding to the AR. However, it has become evident that therapy-resistant prostate cancer occurs when the AR continues to signal despite reducing the availability of androgens. The emerging data suggests that AR signalling is involved in the progression of castrate-resistant prostate cancer and is therefore a viable therapeutic target. There has been relatively little research into agents that directly target

AR signalling at levels other than ligand bioavailability. Therefore characterisation and development of agents that are able to target the androgen signalling axis has the potential to provide new therapeutic options for prostate cancer. In addition, targeting androgen signalling at more than one point in the signalling axis may provide a more complete blockade and possibly a better therapeutic outcome.

### **1.9 Objectives of this Thesis**

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The overall objective of this thesis was to characterise a series of putative AR-targeting agents, and assess their ability to inhibit prostate cancer cell proliferation and survival.

This was achieved by addressing the following specific aims:

1. Characterise the effects of AR-targeting agents on AR protein levels and/or activity.
2. Characterise the effects of AR-targeting agents on prostate cancer cell proliferation and survival.
3. Determine whether simultaneous targeting of AR levels and activity is more effective than each agent alone at inducing arrest of prostate cancer cell growth and induction of cell death.

## **Chapter 2**

### **General Materials and Methods**

## 2.1 Materials

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The suppliers of all materials used throughout this thesis are indicated below.

### 2.1.1 Chemicals, solutions and general reagents

*AGFA (Mortsel, Belgium)*: developer (parts A and B), rapid fixer

*Ajax Chemicals (Sydney, NSW, Australia)*: acetone, citric acid, ethanol, formaldehyde, methanol, propylene glycol, xylene

*Ambion (Austin, TX, USA)*: nuclease-free water, 3M NaAc

*Amersham Biosciences (Buckinghamshire, England)*: ECL<sup>TM</sup> chemiluminescence detection kit, Hybond<sup>TM</sup>-C Extra nitrocellulose transfer membrane, hyperfilm ECL<sup>TM</sup>

*Asia Pacific Specialty Chemicals (Seven Hills, NSW, Australia)*: hydrogen peroxide

*Australian Biostain (Traralgon, Vic, Australia)*: Lillie-Mayer haematoxylin

*Baxter Healthcare (Old Toongabbie, NSW, Australia)*: sterile sodium chloride solution, sterile water

*BioRad (Hercules, CA, USA)*: 40% Acrylamide/Bis solution, protein assay (dye reagent concentrate), SYBR green

*BDH Laboratory Supplies (Kilsyth, Victoria, Australia)*: DMSO, NP-40, paraformaldehyde, sucrose

*Calbiochem (Merck Pty Ltd)*: zDEVD-AFC caspase fluorogenic substrate, z-VAD-fmk caspase inhibitor

*Chemsupply (Gilman, SA, Australia)*: DPX mounting medium

*Cytosystems (Castle Hill, NSW, Australia)*: MultiSer<sup>TM</sup> Foetal Calf Serum (FCS)

*DAKO (Botany, NSW, Australia)*: fluorescent mounting medium, HRP conjugated streptavidin

*Diploma (Melbourne, Victoria, Australia)*: skim milk powder

*Fronine Laboratory Supplies (Riverstone, NSW, Australia):* 10% neutral buffered formalin

*Hopkin and Williams (Chadwell Heath, Essex, England):* trypan blue

*Invitrogen (Carlsbad, CA, USA):* Lipofectamine<sup>TM</sup>2000, Lipofectin<sup>TM</sup>, Oligofectamine<sup>TM</sup>, SeeBlue Plus 2<sup>TM</sup> pre-stained protein standard, Superscript III cDNA synthesis kit, Trizol

*JRH Biosciences (Lenexa, KS, USA):* phenol red free RPMI medium 1640 (containing L-Glutamine), RPMI medium (containing L-glutamine), 10x Trypsin

*Johnson & Johnson Medical (North Ryde, NSW, Australia):* silk sutures

*Lyppard (Thebarton, SA, Australia):* halothane

*Promega (Madison, WI, USA):* luciferase reporter kit: passive lysis buffer, luciferase assay reagent

*Qiagen (Doncaster, Vic, Australia):* RNase

*Roche Applied Science (Castle Hill, NSW, Australia):* complete protease inhibitor cocktail tablets, in situ cell death detection kit AP, TUNEL dilution buffer, TUNEL POD

*Sciencelab (Houston, Texas, USA):* Igepal CA-630

*Sigma (St Louis, MO, USA):* Accustain Eosin Y solution, ammonium persulphate (APS), bovine serum albumin (BSA), bromophenol blue, CHAPS, cobalt chloride, DAB, DOC, donkey serum, DTT, Extracellular matrix (ECM) gel, EDTA, glycerol, glycine, goat serum, hepes, isopropanol (molecular biology grade), magnesium sulphate, NaCl, propidium iodide, protease inhibitor cocktail, rabbit serum, SDS, sodium bicarbonate, sodium cacodylate, sodium chloride, temed, Tris base, Tris-Cl, Tris-HCl, 1% Triton X-100, Tween 20

### **2.1.2 Oligonucleotide primers**

Oligonucleotide primers were obtained from Geneworks (*Hindmarsh, SA, Australia*).



L32 forward primer:	5'-TTCCTGGTCCACAACGTCAAG-3'
L32 reverse primer:	5'-TTGTGAGCGATCTCGGCAC-3'
AR forward primer:	5'-CCTGGCTTCCGCAACTTACAC-3'
AR reverse primer:	5'-GGACTTGTGCATGCGGTACTION-3'
PSA forward primer:	5'-ACCAGAGGAGTTCTTGACCCCAAA-3'
PSA reverse primer:	5'-CCCCAGAATCACCCGAGCAG-3'
KLK2 forward primer:	5'-GGTGGCTGTGTACAGTCATGGAT-3'
KLK2 reverse primer:	5'-TGTCTTCAGGCTCAAACAGGTTG-3'

### **2.1.3 Antibodies**

*Affinity Bioreagent (Golden, CO, USA):* mouse polyclonal anti-calnexin

*DAKO (Botany, NSW, Australia):* mouse monoclonal Ki-67, rabbit anti-mouse HRP conjugated IgG, goat anti-rabbit HRP conjugated IgG

*Cell Signalling technology (Danvers, MA, USA):* rabbit polyclonal anti-Akt

*Chiron (Clayton, Victoria, Australia):* rabbit polyclonal (U407) raised against a peptide corresponding to amino acids 200-220 of the AR (Wilson and McPhaul 1996)

*Chemicon International (Temecula, CA, USA):* donkey anti-sheep/goat HRP conjugated IgG

*Labvision Corporation (Fremont, CA, USA):* mouse monoclonal anti-P21<sup>WAF1</sup> Ab3 (Clone DCS-60.2), mouse monoclonal anti-c-erbB2 A-15 (Clone 3B5).

*Santa Cruz (Santa Cruz, CA, USA):* goat polyclonal anti-actin (I19), rabbit polyclonal anti-AR (C19), rabbit polyclonal anti-AR (N20), goat polyclonal anti-PSA (C19), mouse monoclonal anti-cyclin D1 (A-12), rabbit polyclonal anti-Raf-1 (C20)

### **2.1.4 Cell lines**

*American Type Culture Collection (Rockville, MD, USA):*

The LNCaP cell line, derived from a lymph node metastasis from 50 year old patient with prostate cancer was maintained in RPMI medium containing 10% FCS. The PC-3 cell

line, derived from a bone metastasis from a 62 year old patient with prostate cancer was maintained in RPMI medium containing 5% FCS.

*Other cell lines:*

The C42B cell line was provided by Dr Gerhard A. Coetzee (University of Southern California, USA), derived from LNCaP cells that were serially passaged in castrated mice (Thalmann *et al.* 1994)

**2.1.5 Hormones and drugs**

17-(allylamino)demethoxygeldanamycin (17-AAG) was provided by Professor Neal Rosen (Memorial Sloan-Kettering Cancer Centre, New York).

Suberoylanilide hydroxamic acid (SAHA; Vorinostat) was provided by Dr Victoria Richon (Aton Pharma, Tarrytown, New York).

*Sigma (St Louis, MO, USA):* 5 $\alpha$ -dihydrotestosterone, testosterone

*AstraZeneca Pharmaceuticals (North Ryde, NSW, Australia):* Bicalutamide (Casodex)

**2.1.6 Antisense oligonucleotides**

Phosphorothioate oligonucleotides purified by reverse-phase high performance liquid chromatography were purchased from Geneworks (*Hindmarsh, SA, Australia*).

Antisense-750: 5'GTC GTC GTC GTC GTC3'

Mismatch-750: 5'TTG CAG CTG ATG CTA3'

Antisense-31: 5'GTG CAA TCA TTT CTG3'

Mismatch-31: 5'GTA CAA TCC TTT GTG3'

Antisense-AUG: 5'CTG CAC TTC CAT CCT3'

Mismatch-AUG: 5'TTG CAA TAC CCT TCT3'

**2.1.7 Mice**

Male Balb/c nude mice (*nu/nu*) 5-6 weeks of age were obtained from Perth Animal Resource Centre (*Perth, WA, Australia*) and housed in a barrier facility at the Institute of

Medical and Veterinary Science (IMVS) animal house, on a 12 hr light/dark cycle. Animals were housed in filter top cages with food and water *ad libitum*. Animal ethics approval was obtained from the University of Adelaide and IMVS ethics committee prior to initiation of animal studies.

### **2.1.8 Equipment**

*Perkin Elmer Life Sciences (Wellesley, MA, USA):* LS50 spectrofluorometer

*Sakura Finetek (CA, USA):* Tissue-Tek VIP 5 vacuum infiltration processor

*Eppendorf (Hamburg, Germany):* Eppendorf centrifuge 5415R, Eppendorf centrifuge 5810

*Richard-Allan Scientific (Kalamazoo, MI, USA):* MICROM HM 325 microtome

*Chemicon International (Temecula, CA, USA):* ATA100 tissue microarrayer

*Olympus America Inc.:* Olympus Bx50 microscope with Olympus U-PMTVC solid state camera, Olympus IX71 fluorescent microscope and camera, Olympus CK40 inverted microscope

*Beckman Coulter (Gladesville, NSW, Australia):* DU 530 spectrophotometer (Life Science)

*Bio-Rad (Hercules, CA, USA):* Bio-Rad iCycler thermal cycler

*BMG Labtechnologies Pty Ltd (Mornington, Vic, Australia):* LUMIstar Galaxy plate reader

### **2.1.9 Software**

*Bio-Rad (Hercules, CA, USA):* Bio-Rad IQ5 optical system software version 1.0

*Leading Edge (Adelaide, SA, Australia):* VideoPro 32 video image analysis software

*Olympus America Inc.:* Olympus DP controller and image manager software

*SPSS Inc (Chicago, IL, USA):* SPSS statistical analysis software version 13

## 2.2 Buffers and Solutions

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All solutions were made up using RO water and stored at room temperature unless otherwise stated.

### *10% APS (ammonium persulphate):*

APS                    1g  
Water                 10mL  
(stored at -20°C)

### *Caspase assay buffer:*

Hepes                 2.4g  
Sucrose              20g  
CHAPS                0.2g  
Made up to 200mL with water, pH to 7.4  
(stored at 4°C)

### *Caspase lysis buffer:*

1M EDTA (pH 7.4)   50µL  
1M Tris-HCl (pH 7.6) 50µL  
10% NP-40           500µL  
Water                 9.4mL

### *Citrate buffer:*

Citric acid           1.05g  
Water                 500mL  
pH to 6.5

### *0.01M Cobalt Cl<sub>2</sub>:*

Cobalt Cl<sub>2</sub>            0.12g  
Water                 50mL

### *0.7M Na Cacodylate:*

Na Cacodylate       7.49g  
Water                 50mL

### *Dextran coated charcoal in Tris/EDTA buffer*

0.5% Charcoal       5g  
55mM Dextran       0.5g  
20% Glycerol        100mL  
Made up to 1L with Tris/EDTA buffer and allowed to mix overnight on rotation

### *4% paraformaldehyde:*

Paraformaldehyde   2g  
hot PBS               40mL  
pH to 7.5  
(stored at 4°C for up to 2 weeks)

*Propidium iodide solution:*  
50µg/mL propidium iodide in 0.1% Tx-100

*6x protein loading dye:*  
4x Tris-Cl/SDS      7mL  
glycerol              3mL  
SDS                    1g  
DTT                    0.93g  
Bromophenol blue   1.2mg

*Ripa lysis buffer:*  
10mM Tris (pH 7.4)   0.1211g  
150mM NaCl          0.8766g  
1mM EDTA            0.0292g  
1% Triton X-100      1mL  
Made up to 100mL with water  
Inhibitor tablet added immediately prior to use (1 tablet per 50mL),  
(stored at 4°C)

*10x Running buffer:*  
Tris                    75.75g  
Glycine                360g  
SDS                    25g

*1x Running buffer:*  
10x Running buffer   250mL  
Water                  2.25L

*20% SDS:*  
SDS                    20g  
Water                  100mL

*Separating gel (7.5%):*  
Water                  4.3mL  
1M Tris pH 8.8        3.75mL  
Acrylamide/Bis        1.9mL  
20% SDS                50uL  
10% APS                50uL  
Temed                  5uL

*Separating gel (12%):*  
Water                  3.2mL  
1M Tris pH 8.8        3.75mL  
Acrylamide/Bis        3mL  
20% SDS                50uL  
10% APS                50uL  
Temed                  5uL

*Stacking gel:*  
Water                  3.7mL

1M Tris pH 6.8	625uL
Acrylamide/Bis	500uL
20% SDS	25uL
10% APS	25uL
Temed	5uL

*TBS + Tween 20 (TBST):*

Tween 20	5mL
TBS	2.5L

*10x Transfer buffer:*

25mM Tris	75.75g
Glycine	360g
Water	2.5L

(stored at 4°C)

*1x Transfer buffer:*

Methanol	800mL
10x Transfer buffer	400mL
Water	2.8L

(stored at 4°C)

*1M Tris pH 8.8:*

Tris	60.55g,
Water	500mL

pH to 8.8

*1M Tris pH 6.8:*

Tris	12.11g
Water	100mL

pH to 6.8

*10x Tris buffered saline (TBS):*

0.5M Tris	75.75g
1.5M NaCl	109.5g
Water	2.5L

pH to 7.4

*4x Tris-Cl/SDS:*

Tris-Cl	3.025g
SDS	0.2g

Made up Tris-Cl to 20mL with water and pH to 6.8 prior to addition of SDS

Following addition of SDS made up to 50mL with water

*0.15M Tris:*

Tris	0.91g
Water	50mL

*Tris/EDTA buffer:*

0.01M Tris	1.211g
------------	--------

1.5mM EDTA            0.588g  
0.01M Na Molybdate 2.42g  
10% Glycerol        100mL  
Water                    900mL  
pH to 7.4  
Made up to 1L

*Trypan blue:*  
0.01% trypan blue dissolved in sterile saline

*TUNEL buffer:*  
0.15M Tris              1mL  
0.7M Na Cacodylate 1mL  
0.01M Cobalt Cl<sub>2</sub>    500μL  
10% BSA                50μL  
Water                    2.45mL

*0.1% Tx-100 in 0.1% Na Citrate:*  
Tx-100                  0.1mL  
Na Citrate              0.1g  
Water                    100mL

## 2.3 Methods

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Experimental procedures included in two or more chapters of this thesis are described in this section. More specific experimental procedures are described in each relevant chapter.

### 2.3.1 Cell culture

Cell lines were maintained in RPMI medium 1640 containing 5% (PC3, C42B cells) or 10% FCS (LNCaP cells) at 37°C in 5% CO<sub>2</sub>. All procedures using cell lines were performed under aseptic conditions in a laminar flow cabinet.

#### 2.3.1.1 Cell passaging

At regular intervals, when cells were almost confluent medium was aspirated from flasks and the cells were washed with ~5mL sterile phosphate buffered saline (PBS). PBS was aspirated and 5mL 1x trypsin was added and incubated for 5min at 37°C. Once cells were detached from the flask, RPMI medium was added and the cell suspension was centrifuged for 5min at 1500rpm. The supernatant was aspirated and the cell pellet was resuspended in 10mL medium prior to cell counting using a haemocytometer.  $5 \times 10^6$  cells were passaged into fresh medium or cells were seeded at the specified density into the appropriate tissue culture dish as indicated for each experiment.

#### 2.3.1.2 Freezing of cell lines

Cells were trypsinised as described above and resuspended in RPMI medium containing 10% FCS. 500µL of freezing medium comprising 40% DMSO, 40% FCS and 20% RPMI medium was added to 500µL of cell suspension, on ice. Cell suspension was frozen overnight in a cell freezing rack containing isopropanol at -70°C, followed by long-term storage in liquid nitrogen.



### *2.3.1.3 Thawing of cell lines*

Stored vials containing frozen cell lines were thawed at 37°C and added to 25mL RPMI medium containing 10% FCS. Cell suspension was centrifuged for 5min at 1500rpm. Supernatant was removed and the cells were resuspended in fresh RPMI medium and seeded into tissue culture flasks.

### *2.3.1.4 Charcoal stripping of Foetal Calf Serum*

Foetal Calf Serum was thawed in a water bath at 37°C overnight. Dextran coated charcoal (DCC) was centrifuged for 30 min at 4000rpm and supernatant was discarded. 50mL of FCS was placed into 50mL tubes containing the charcoal pellet and incubated for 2 hr at room temperature on rotation. The charcoal and FCS mixture was centrifuged at 4000rpm for 30min. The FCS supernatant was poured into tubes containing fresh charcoal pellets and incubated for 2 hr at room temperature on rotation. The charcoal and FCS mixture was centrifuged at 4000rpm for 30min. The charcoal stripped serum was filter sterilised and stored at -20°C.

### *2.3.2 Growth curves*

Cells were seeded into 24 well dishes at a density of  $2.5 \times 10^4$  cells per well in 1mL of RPMI medium containing 5 or 10% FCS<sup>1</sup>. Cells were allowed to attach for 24 (PC-3, C42B) or 48 hr (LNCaP) and then treated with drug as indicated. Following treatment PC-3 and C42B cells were harvested daily, while LNCaP cells were harvested on alternate days for cell counting. On counting days, the culture medium in each well was aspirated into 10mL falcon tubes and the cells were washed with 1mL PBS per well, which was also aspirated into corresponding tubes. 0.5mL of 1x trypsin was added to each well for 5 minutes. 1mL of medium was added and the content of each well was aspirated into its corresponding tube. Tubes were centrifuged at 1500rpm for 5 minutes

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<sup>1</sup> Cells were cultured in medium containing either normal or charcoal stripped FCS as indicated for each experiment.

and the supernatant was aspirated and discarded. The cell pellet was resuspended in 0.5mL of medium and 100 $\mu$ L of each sample was placed into wells of a 96 well plate with 100 $\mu$ L of Trypan blue per well. The cell suspension was loaded onto a haemocytometer and the cells contained within 8 large squares were counted.

### **2.3.3 Western blots**

#### *2.3.3.1 Preparation of lysates*

Following specified drug treatment, the medium was aspirated from the culture dishes, the cells were gently rinsed with ice cold PBS, and RIPA buffer was added on ice. Cells were scraped into RIPA buffer using a cell scraper and syringed using a 21G needle and 1mL syringe. Cells were spun for 10min at 1000rpm at 4°C. The supernatant was placed into clean eppendorf tubes and 20 $\mu$ L was used for Bradford protein estimation. Protein concentrations were determined using a spectrophotometer. Each protein sample was prepared by adding 10 $\mu$ L to 2mL of Bradford protein dye (diluted 1:5) in duplicate. Standard protein dilutions were prepared using known standards of BSA diluted in water. Samples were read using a wavelength of 595nm.

#### *2.3.3.2 Gel electrophoresis*

Appropriate amounts of cell lysates, as determined by Bradford protein estimation was added to loading dye (1/6 the volume of the lysate). The mixture was boiled for 5 minutes at 90°C prior to gel loading. Protein marker was added to lane 1 and 20 $\mu$ g lysate was added to each lane of SDS gels. SDS gels were prepared by adding stacking gel mixture onto 7.5 or 12% SDS separating gel mixture. Proteins were electrophoresed through the stacking layer at 15mA (per gel) for 20 minutes, and resolved through the separating gel at 25mA (per gel) using 1x running buffer. Protein was then transferred to nitrocellulose membrane using 1x transfer buffer for 1 and a half hours at 250mA.

### 2.3.3.3 Protein detection

Following transfer, membranes were blocked in 3% skim milk powder in TBST for 60 minutes with agitation (or overnight at 4°C). Membranes were probed with the appropriate antibody (refer to table 2.3.3) for 1hr in 1% skim milk powder and rinsed three times in TBST for 10 minutes. The appropriate secondary antibody was added for half an hour in 1% skim milk powder at room temperature, followed by 3x 10 minute washes in TBST. Protein was detected using a chemiluminescent detection kit (ECL™) for 1min, followed by exposure to hyperfilm ECL™. Films were developed in a dark room by first immersing in developing solution followed by fixing solution.

**Table 2.3.3 – Antibody dilutions**

Primary Antibody	Dilution	Secondary Antibody	Dilution
Actin	1/1000	Donkey anti-sheep/goat	1/1000
Akt	1/1000	Goat anti-rabbit	1/1000
AR-C19/AR-N20	1/500	Goat anti-rabbit	1/1000
Calnexin	1/4000	Rabbit anti-mouse	1/1000
Cyclin D1	1/500	Rabbit anti-mouse	1/1000
Her2	1/200	Rabbit anti-mouse	1/1000
p21	1/500	Rabbit anti-mouse	1/1000
PSA	1/1000	Donkey anti-sheep/goat	1/1000
Raf-1	1/1000	Goat anti-rabbit	1/1000

### 2.3.4 Transactivation assays

All transactivation assays described in this thesis were performed by Joanna Gillis or Michelle Newman.

#### 2.3.4.1 Construction of AR variant expression plasmids

Creation of AR variant expression plasmids are described in (Buchanan *et al.* 2001c). The pCMV-AR-A746T expression vector was supplied by Dr Nancy L. Weigel (Department of Molecular Cellular Biology, Baylor College of Medicine, Houston, Texas) (James *et al.* 2002). The ARR3-tk-luciferase reporter construct was provided by

Dr Marco Marcelli (Baylor College of Medicine, Houston, Texas). For a detailed description of expression vectors refer to appendix 1.

#### *2.3.4.2 Transactivation activity*

PC-3 cells ( $1.5 \times 10^4$  cells per well) were transfected with pCMV3.1-AR (2.5ng/well) and 100ng/well of the ARR3-tk-luciferase plasmid using LipofectAMINE™ 2000 (Buchanan *et al.* 2001c). After 3-4 hours, the transfection mix was replaced with phenol red-free RMPI supplemented with 5% charcoal stripped FCS containing 1nM DHT and specified drug treatment. Following 36 hours, cells were lysed with passive lysis buffer according to the manufacturer's specifications, and assayed for reporter gene activity using a Luciferase Reporter Gene Assay and a plate reading luminometer.

## **Chapter 3**

### **Targeting the Androgen Receptor with Antisense**

#### **Oligonucleotides**



### 3.1 Introduction

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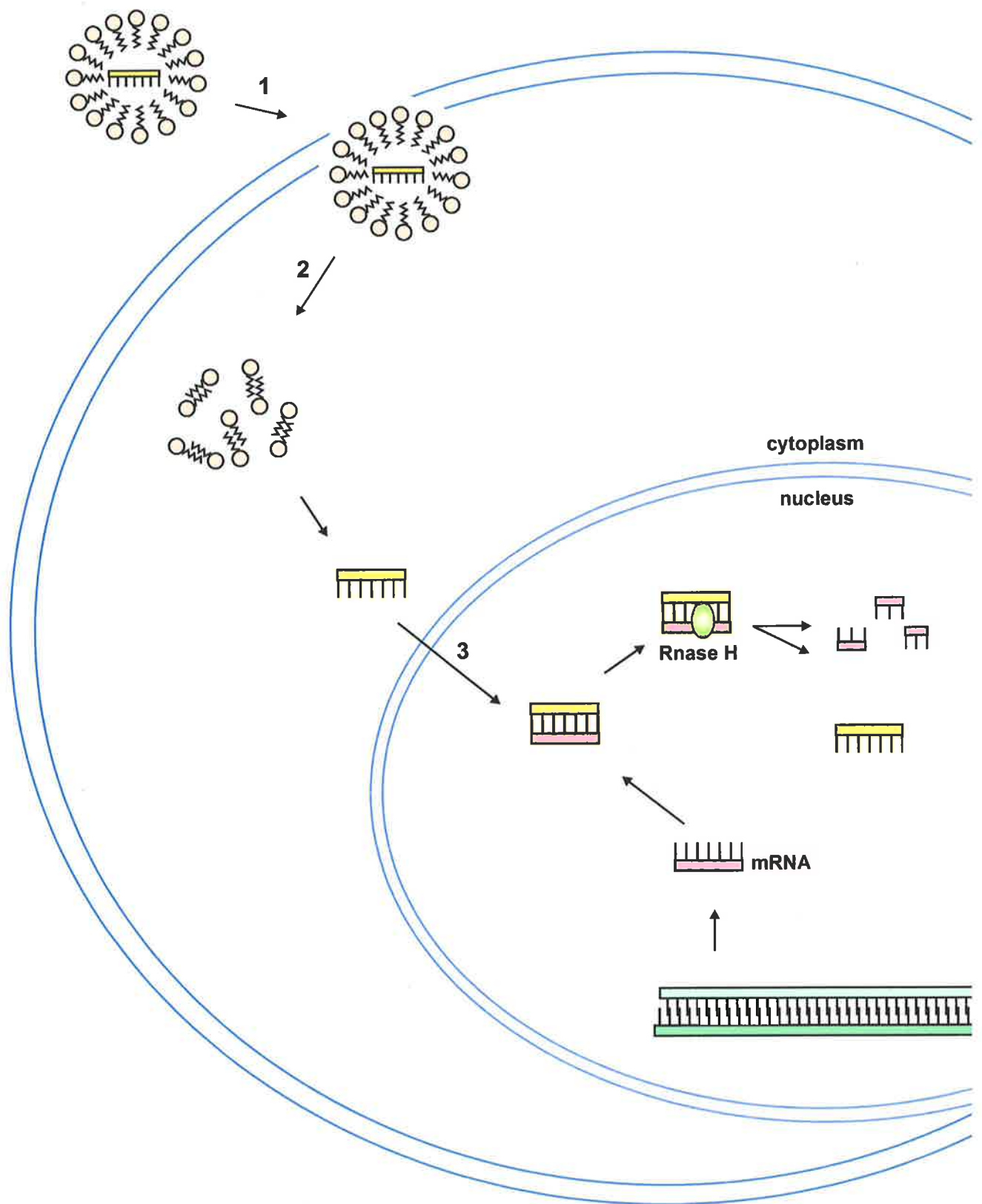
As resistance to current forms of AAT is associated with continued AR activity in prostate cancer cells, specific inhibition of AR signalling may be a more effective treatment for prostate cancer. One way to achieve this is to inhibit AR protein expression in prostate cancer cells using antisense oligonucleotides. Antisense oligonucleotides were first used to inhibit gene expression in the late 1970's (Stephenson and Zamecnik 1978; Zamecnik and Stephenson 1978). Since that time, antisense technology has been used as a powerful research tool to delineate the role of specific cellular proteins. In more recent years, antisense technologies have been increasingly applied in a clinical setting. One of the advantages of antisense oligonucleotides as therapeutic agents is that they can be designed to specifically target mRNA sequences, and are cheaper to produce than conventional protein-targeting drugs. The only antisense agent currently approved for clinical use is Vivatrene<sup>TM</sup>, targeted against cytomegalovirus mRNA, used to treat cytomegalovirus-induced retinitis in patients with AIDS (Marwick 1998). Additionally, antisense oligonucleotides for Bcl-2, H-ras, c-MYC, protein kinase C and Raf-1 are in phase I and II clinical trials for the treatment of various forms of cancer (Cunningham *et al.* 2001; Tolcher *et al.* 2002; Alberts *et al.* 2004; Villalona-Calero *et al.* 2004; Devi *et al.* 2005; Tolcher *et al.* 2005; Vansteenkiste *et al.* 2005). In the current study AR-antisense oligonucleotides were investigated as a means of reducing AR levels and inhibiting prostate cancer cell growth.

#### 3.1.1 Antisense Mechanism of Action

Antisense oligonucleotides are single-stranded sequences of nucleic acid typically 15-25 bases in length. Oligonucleotides are generally longer than a minimum of 13 bases to minimise the likelihood of hybridisation with more than one target, based on the estimate

that an RNA sequence larger than 13 bases will only occur once in the human genome (Jansen and Zangemeister-Wittke 2002). Conversely, oligonucleotides larger than 25-30 bases in length pose a problem for cellular uptake due to their size. Therefore, the optimal size is regarded as 15-25 bases for specificity and efficacy. Antisense oligonucleotides bind to their target mRNA by Watson-Crick base pairing and inhibit translation from mRNA to protein. There are two major classes of antisense oligonucleotides, cleavers and blockers, grouped according to their mechanism of action. Antisense oligonucleotides belonging to the class of cleavers inhibit translation by recruitment of RNase H, a ubiquitous enzyme that is usually involved in DNA replication processes. While the exact mechanism involved in RNase H recruitment is unknown, RNase H recognises oligonucleotides as short as tetramers with DNA-like properties, which leads to activation of its endonuclease activity, resulting in cleavage of the RNA strand in an RNA/DNA duplex (Donis-Keller 1979). Blockers are unable to recruit RNase H and exert their activity simply by steric hindrance of translation. However, RNase H cleavage is thought to be the most important mechanism of antisense activity, and all oligonucleotides currently in clinical trials belong to the class of cleavers.

In order to exert their activity, antisense oligonucleotides must overcome several cellular barriers (Shi and Hoekstra 2004) (Figure 3.1). The first barrier to effective antisense inhibition is crossing the cell membrane. As oligonucleotides have a negative charge this prohibits interaction with the negatively charged cell surface, allowing only a small fraction of free oligonucleotide to permeate the cell membrane. This barrier can be overcome using carrier molecules, which facilitate entry of oligonucleotides into the cell by endocytosis. Although there are several delivery methods available for the transfection of oligonucleotides, cationic lipids are most commonly used in cell culture. Cationic lipids are composed of a charged polar head connected to a hydrophobic tail



**Figure 3.1- Mechanism of antisense oligonucleotide-induced degradation of target mRNA.**

1) Liposome enclosed oligonucleotides enter the cell by endocytosis. 2) Once internalised cationic lipids form dimers and facilitate entry of antisense oligonucleotides into the cytoplasm. 3) Free oligonucleotide translocates through the nuclear pore into the nucleus where it binds to target mRNA by Watson-Crick base-pairing, inducing RNase H degradation of the mRNA strand.



(Audouy and Hoekstra 2001). The lipids encapsulate the oligonucleotide, giving it an overall positive charge so that it is able to interact with the negatively charged cell membrane (Lappalainen *et al.* 1994; Zelphati and Szoka 1996b; Fimmel *et al.* 2000) where endocytosis occurs (Zelphati and Szoka 1996a, Zelphati and Szoka 1996b). Once inside the cell, the oligonucleotides are trapped in endosomes, which represent the second barrier to effective antisense activity. Endosomal escape is essential for antisense oligonucleotides to travel to the nucleus and exert their effect on target mRNA. It is also the carrier molecules that are able to assist in breakdown of the endosome by forming charge-neutral ion pairs, thus releasing the oligonucleotides into the cytoplasm (Zelphati and Szoka 1996b; Marcusson *et al.* 1998). The oligonucleotides then rapidly move into the nucleus (Chin *et al.* 1990; Leonetti *et al.* 1991), while the lipid remains in the cytoplasm (Zelphati and Szoka 1996b). The third barrier to antisense activity is movement from the cytoplasm to the nucleus, at which point the oligonucleotides are subject to nuclease degradation. If the oligonucleotides are able to evade degradation then entry into the nucleus occurs through nuclear pores (Shoeman *et al.* 1997; Hartig *et al.* 1998) where the oligonucleotides bind to target mRNA inducing RNase H activity. Chemical modification of oligonucleotides can improve their stability, while retaining the ability to recruit RNase H, as well as improving affinity for target mRNA and reducing toxicity *in vivo*. The original phosphodiester backbone of the oligonucleotide is very unstable, although it is able to activate RNase H. These problems have led to the development of new generation oligonucleotide modifications (reviewed in Kurreck 2003a, Kurreck 2003b). The class of antisense oligonucleotides almost exclusively used in clinical trials to date are those with a phosphorothioate backbone, where one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by a sulphur. Clinical

use of this class continues because of its increased stability in serum and uptake into cells as well as its ability to induce RNase H activity.

The aim of the current study was to optimise an AR-antisense oligonucleotide for use as an AR-targeting agent, and to assess the effect of specific AR-targeting on proliferation and survival of LNCaP prostate cancer cells.

### 3.2 Methods

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For the purposes of these studies, three different antisense oligonucleotides were chosen, which are targeted to different regions of the AR. These oligonucleotides were previously reported to decrease AR expression, and are listed in table 3.2.

**Table 3.2:** Oligonucleotide sequences

Oligonucleotide Sequence (5'→3')	Mismatch control sequence (5'→3')	Reference
CTGCACTTCCATCCT	TTGCAATACCCTTCT	Birrell <i>et al.</i> (1995)
GTCGTCGTCGTCGTC	TTGCAGCTGATGCTA	Eder <i>et al.</i> (2000)
GTGCAATCATTCTG	GTACAATCCTTTGTG	Hamy <i>et al.</i> (2003)

The first 15bp oligonucleotide was identified by Hamy *et al.* (2003) in a screen of 46 other oligonucleotides for their ability to decrease AR transactivation activity. In this study an oligonucleotide designated oligonucleotide number 31, targeted against the N-terminal domain of the AR was determined to be superior to all other oligonucleotides tested. This oligonucleotide decreased AR activity at concentrations as low as 1nM. The second oligonucleotide chosen for the current study is directed against the AR N-terminal polyglutamine region of the AR, and was designated oligonucleotide number 750 (Eder *et al.* 2000; Eder *et al.* 2002). Although other proteins are known to have polyglutamine repeats the number of repeat sequences differ and range from 6-9 repeats (c-jun, TFIID, HDAC2) compared with 22 repeats in the AR. In addition, Eder *et al.* (2000) showed no significant decrease in other proteins containing a polyglutamine repeat region following transfection of LNCaP prostate cancer cells with this oligonucleotide. The third oligonucleotide chosen has been used previously in our laboratory to decrease AR expression in breast cancer cells (Birrell *et al.* 1995). This oligonucleotide is targeted against the initiation methionine codon (AUG) of the AR gene. Therefore the three antisense oligonucleotides chosen for use in these studies were designated antisense-31

(as-31), antisense-750 (as-750) and antisense-AUG (as-AUG). For preliminary studies, only one mismatch oligonucleotide was used as a control, mismatch-750 (mm-750), which is the specific mismatch control for as-750 (Eder *et al.* 2000). For subsequent experiments appropriate mismatch oligonucleotides were used, as indicated.

### 3.2.1 Oligonucleotide transfection

**Table 3.2.1:** Oligonucleotide transfection volumes and cell densities for different experimental formats

	Seeding density	Oligonucleotide (20 $\mu$ M stock) + prf-RMPI	Transfection reagent + prf-RMPI	prf-RMPI overlay	Total transfection volume	RPMI + 10% FCS added
<b>8-well chamber slides</b>	1x10 <sup>4</sup> per well	2.5 $\mu$ L + 40 $\mu$ L	2 $\mu$ L + 5.5 $\mu$ L	200 $\mu$ L	250 $\mu$ L	125 $\mu$ L
<b>6-well plates</b>	1x10 <sup>5</sup> per well	25 $\mu$ L + 437.5 $\mu$ L	10 $\mu$ L + 27.5 $\mu$ L	2000 $\mu$ L	2500 $\mu$ L	1250 $\mu$ L
<b>24-well plates</b>	2.5x10 <sup>4</sup> per well	6.25 $\mu$ L + 100 $\mu$ L	5 $\mu$ L + 13.75 $\mu$ L	500 $\mu$ L	625 $\mu$ L	312.5 $\mu$ L

Amounts are calculated per well. Abbreviations: phenol red free RPMI (prf-RMPI)

It is recommended by the manufacturers of Oligofectamine that cells should be 30-40% confluent to achieve optimal transfection efficiency. Preliminary experiments determined that the optimal seeding density of LNCaP cells in 6-well culture dishes was 1x10<sup>5</sup> cells per well. Further experiments determined optimal seeding density to be 1x10<sup>4</sup> and 2.5x10<sup>4</sup> cells per well for chamber slides and 24 well plates, respectively. As recommended by the manufacturers of Oligofectamine reagent, which has been specifically developed for transfection of oligonucleotides, a concentration of up to 200nM oligonucleotide was transfected into LNCaP cells. Further optimisation of conditions for transfection of LNCaP cells with antisense oligonucleotides led to an

increase in transfection added to the cells. For each experimental format the volume of transfection mix was adjusted, and is shown in table 3.2.1.

LNCaP cells were seeded at the appropriate density into culture dishes in RPMI medium containing 10% FCS and allowed to attach for 48 hr. For each well 20 $\mu$ M oligonucleotide stock solution was diluted into phenol red free RPMI to give a final concentration of 200nM (refer to table 3.2.1), and incubated at room temperature for 5 minutes. Transfection reagent was diluted in phenol red free RPMI and incubated at room temperature for 5 minutes. Diluted oligonucleotide was combined with diluted transfection reagent and incubated for 20 minutes at room temperature. Culture medium was removed and the cells were overlaid with medium and oligonucleotide-lipid complexes (refer to table 3.2.1). Cells were incubated for 5 hours at 37°C, followed by the addition of RPMI containing 10% FCS. Cells were harvested at indicated times following the 5 hr transfection period.

### ***3.2.2 Cell fixation***

At time points of 12, 24, or 48 hr following transfection in 8-well chamber slides, LNCaP cells were fixed with cold 4% paraformaldehyde for 10 minutes at room temperature. The cells were washed with cold PBS, rinsed in methanol and acetone prior to staining with Hoechst (33342) DNA stain for 15 minutes at room temperature in the dark. Slides were rinsed in PBS and then in distilled water before they were mounted using DAKO fluorescent mounting medium. The slides were stored at 4°C until they were analysed for intracellular localisation of oligonucleotides using a fluorescent microscope (at 60X magnification).

### ***3.2.3 Titration of oligonucleotides***

When more than one concentration of oligonucleotide was used the concentration of Lipofectin was maintained in order to rule out any effects of titrating out the Lipofectin

(Reed *et al.* 2005). For these experiments the volume of 20 $\mu$ M oligonucleotide stock was adjusted to give 50 and 100nM doses, while the total volume was made up with phenol red free RPMI.

#### **3.2.4 Immunoblot**

The effect of antisense oligonucleotides on protein expression in LNCaP cells was determined by culturing LNCaP cells with 50, 100 or 200nM antisense oligonucleotide, mismatch control or transfection reagent only, for 12, 24 or 48 hr in 6-well culture dishes. AR Steady-state protein levels were analysed by immunoblot using the AR-C19 antibody as described in chapter 2.

#### **3.2.5 Growth curves**

The effect of as-AUG and mismatch control on LNCaP cell proliferation was assessed by transfecting LNCaP cells with 50, 100 or 200nM antisense or mismatch control in 24-well culture dishes. Cell viability was determined using Trypan blue dye exclusion as described in chapter 2.

#### **3.2.6 Statistical analysis**

Statistical differences between treatment groups and controls were determined by One-way ANOVA with Dunnett post-hoc test, using SPSS statistical analysis software. A p-value of <0.05 was considered statistically significant.

### 3.3 Results

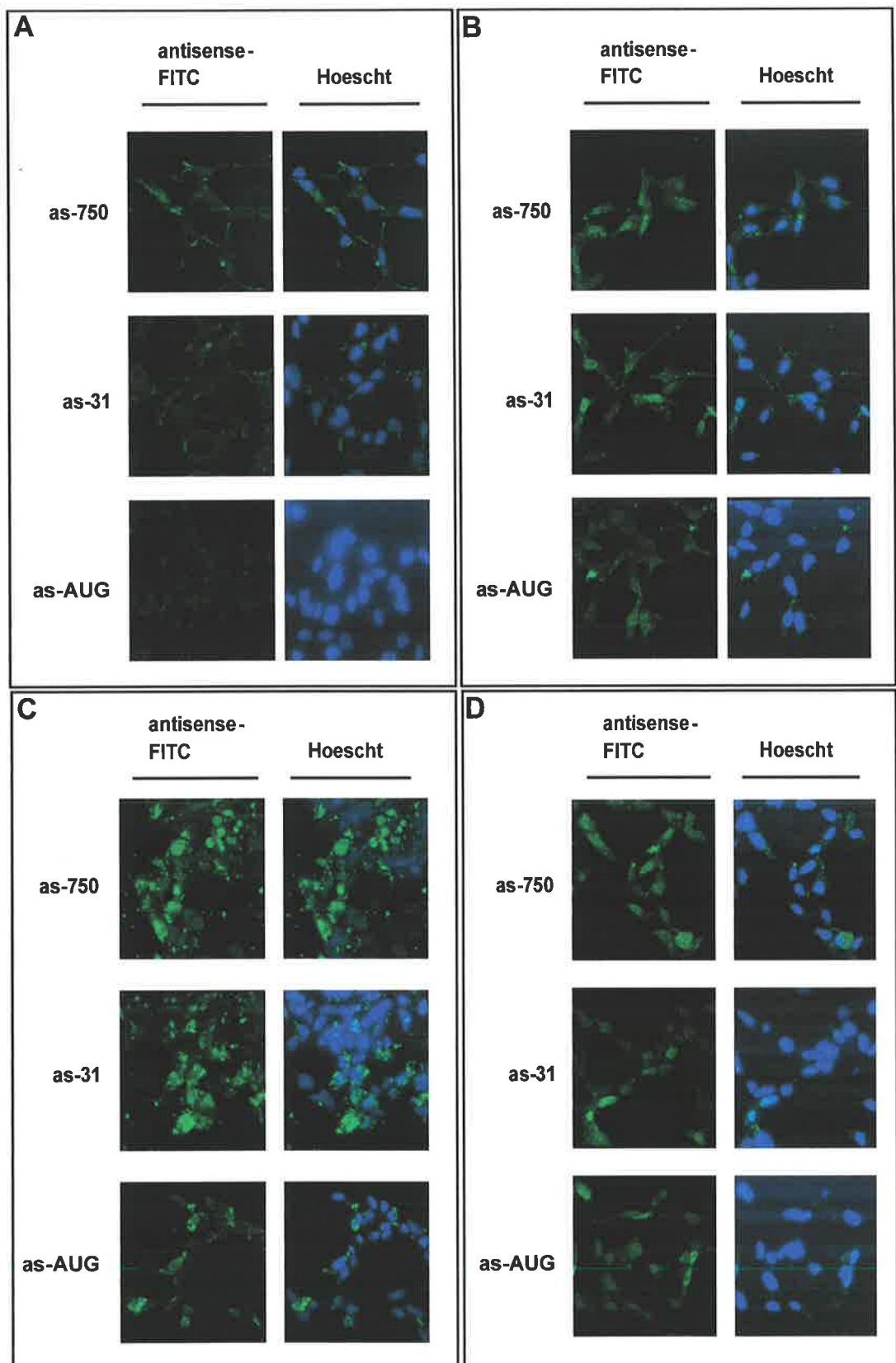
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#### *3.3.1 Comparison of antisense oligonucleotides and transfection reagents*

A comparison of three transfection reagents was performed to determine which would be optimal for transfecting LNCaP cells with AR antisense oligonucleotides. Oligofectamine, Lipofectamine2000 and Lipofectin were compared in the following experiments.

##### *3.3.1.1 Intracellular accumulation of antisense oligonucleotides*

To assess the intracellular accumulation of antisense oligonucleotides following transfection of LNCaP cells, FITC-tagged oligonucleotides were used. The cells were also stained with Hoechst in order to distinguish between nuclear and cytoplasmic localisation of the oligonucleotides. For these experiments LNCaP cells were fixed at 12, 24 and 48 hr post-transfection. Representative images from 12 hr time points are shown as there was no difference in levels of fluorescence observed between the three time points (Figure 3.2). Intracellular accumulation of each oligonucleotide transfected using all reagents (Figure 3.2B-D) was greater than in cells incubated with naked oligonucleotides, in the absence of a transfection reagent (Figure 3.2A). While there appeared to be no difference in uptake when comparing between oligonucleotides, there were differences in uptake observed when comparing between transfection reagents. Transfection of oligonucleotides using Lipofectamine2000 (Figure 3.2C) resulted in a greater amount of cellular uptake compared to Oligofectamine and Lipofectin (Figure 3.2B,D). LNCaP cells transfected using Lipofectin (Figure 3.2C) had greater intracellular accumulation of oligonucleotides compared to LNCaP cells transfected using Oligofectamine (Figure 3.2B).



**Figure 3.2 - Intracellular accumulation of AR-antisense oligonucleotides.** LNCaP cells transfected with FITC-labelled oligonucleotides were visualised at 60X magnification using a fluorescent microscope. Hoescht DNA staining was used to differentiate between nuclear and cytoplasmic localisation. (A) LNCaP cells treated with naked oligonucleotides. (B) LNCaP cells transfected using Oligofectamine. (C) LNCaP cells transfected using Lipofectamine2000. (D) LNCaP cells transfected using Lipofectin. Images were taken 12 hr post-transfection, and are representative of two separate experiments.



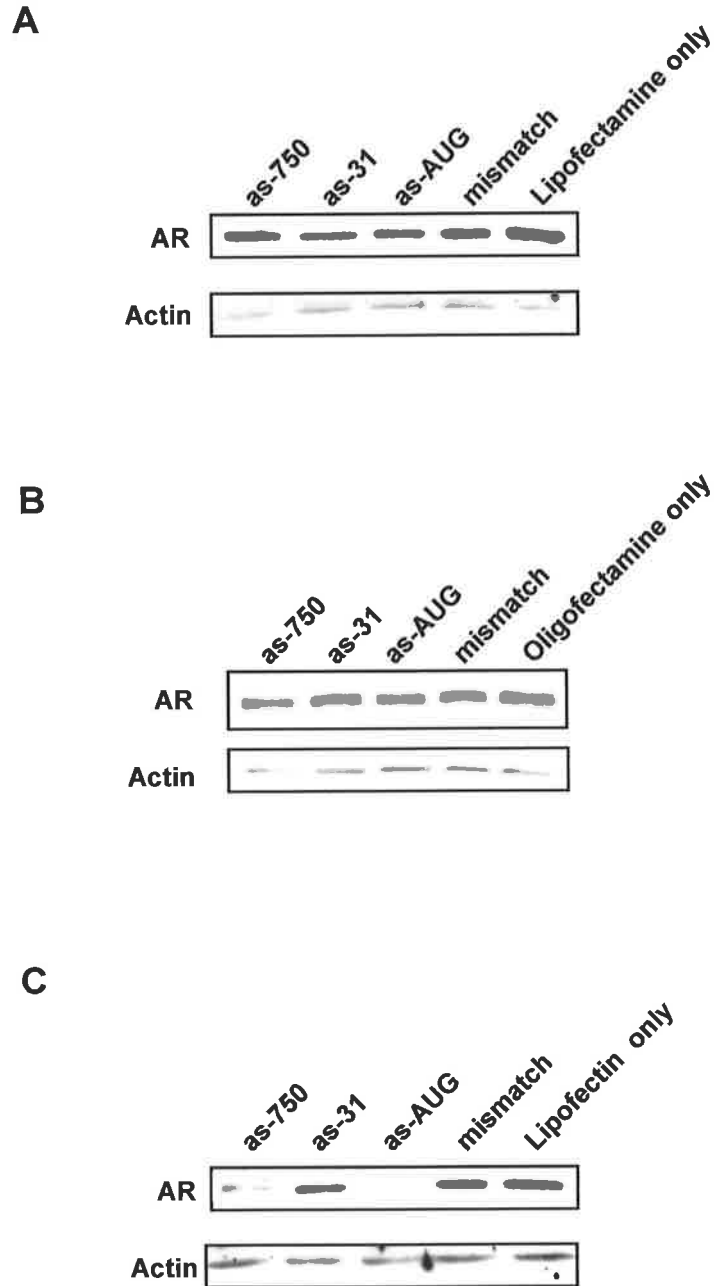
### *3.3.1.2 Effect of antisense oligonucleotides on AR steady-state protein levels*

To determine whether target reduction was consistent with the observed intracellular accumulation, lysates from LNCaP cells were transfected with one of three AR-antisense oligonucleotides or a corresponding mismatch control, in the presence or absence of one of three transfection reagents, and were analysed by immunoblot (Figure 3.3). Transfection of LNCaP cells using Lipofectamine2000 resulted in a modest decrease in AR steady-state protein levels by as-31 and as-AUG compared to the mismatch control and Lipofectamine2000-only treated cells (Figure 3.3A). There were no observable differences in AR steady-state protein levels in LNCaP cells transfected using Oligofectamine (Figure 3.3B). A noticeable reduction in AR steady-state protein was also observed in LNCaP cells transfected with as-750 using Lipofectin (Figure 3.3C). Under these experimental conditions, in LNCaP cells, the most effective knockdown of AR protein was achieved with as-AUG transfected using Lipofectin reagent (Figure 3.3C). Therefore as-AUG and the appropriate mismatch control (mm-AUG) were used with Lipofectin reagent in all subsequent experiments.

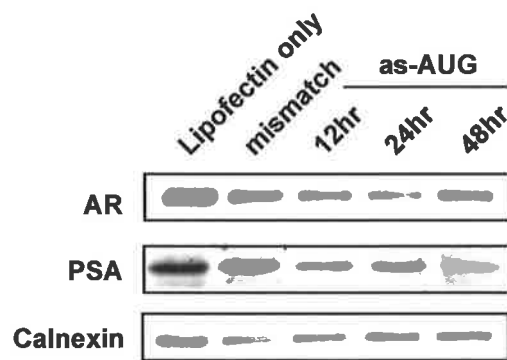
### *3.3.2 Effect of the as-AUG in LNCaP cells*

#### *3.3.2.1 as-AUG decreases AR protein for at least 48 hr in LNCaP cells*

Transfection of LNCaP cells with as-AUG reduced AR steady-state protein levels for up to 48 hr, compared to mm-AUG and Lipofectin-only controls (Figure 3.4). AR steady-state protein levels were not reduced to the same extent as observed in the previous experiment where AR steady-state protein levels were analysed at 8 hr post-transfection (Figure 3.3C), suggesting that the greatest knockdown of protein occurs up to 8 hr following transfection. There was a corresponding reduction in PSA steady-state levels following transfection of as-AUG for at least 48 hr, which was not observed in mismatch



**Figure 3.3 – Expression of AR in LNCaP cells transfected with AR-antisense oligonucleotides.** Lysates from LNCaP cells treated with transfection reagent only, mismatch control, or 200nM antisense oligonucleotide for 8 hr were analysed for expression of AR by immunoblot. LNCaP cells were transfected with (A) Lipofectamine2000, (B) Oligofectamine, or (C) Lipofectin. Actin was used as a loading control and is shown in the lower panels. Images are representative of three separate experiments.



**Figure 3.4 – Reductions in AR and PSA expression in LNCaP cells transfected with as-AUG.** Lysates from LNCaP cells treated with Lipofectin only (12hr); mismatch control (12hr), or 200nM as-AUG (12, 24 or 48 hr) were analysed for expression of AR and PSA by immunoblot. Calnexin was used as a loading control and is shown in the lower panel.

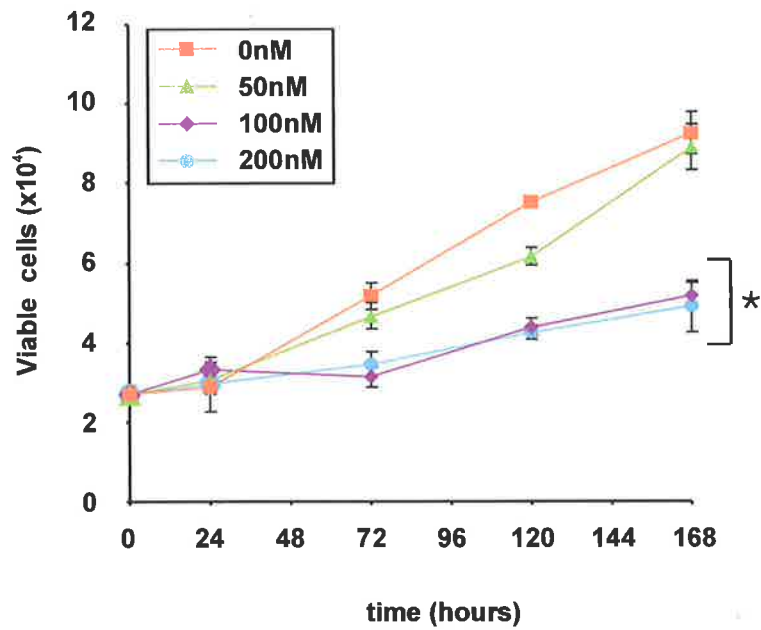
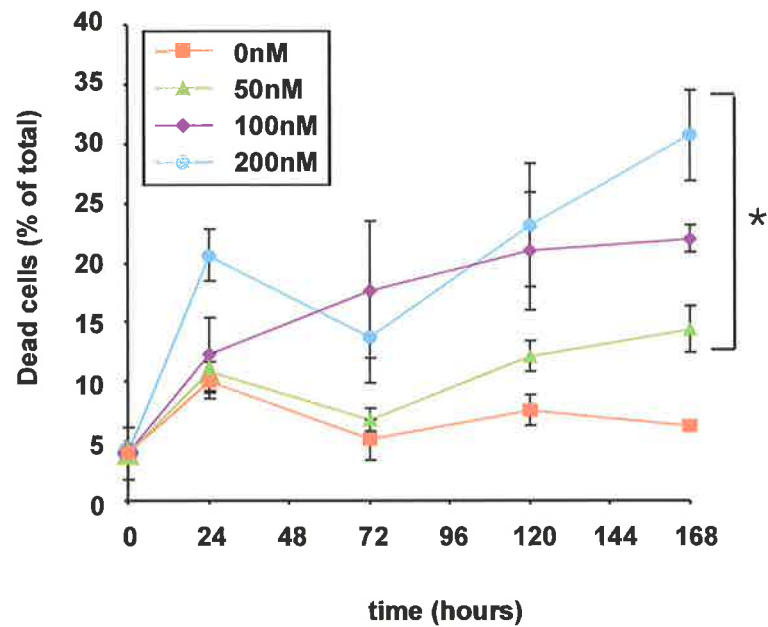
or Lipofectin–only treated cells.

### *3.3.2.2 as-AUG inhibits proliferation and induces LNCaP cells death*

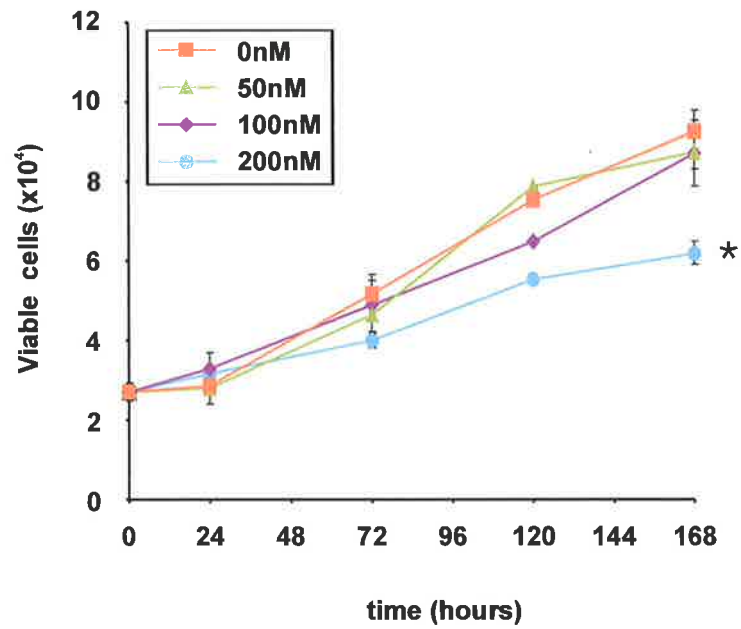
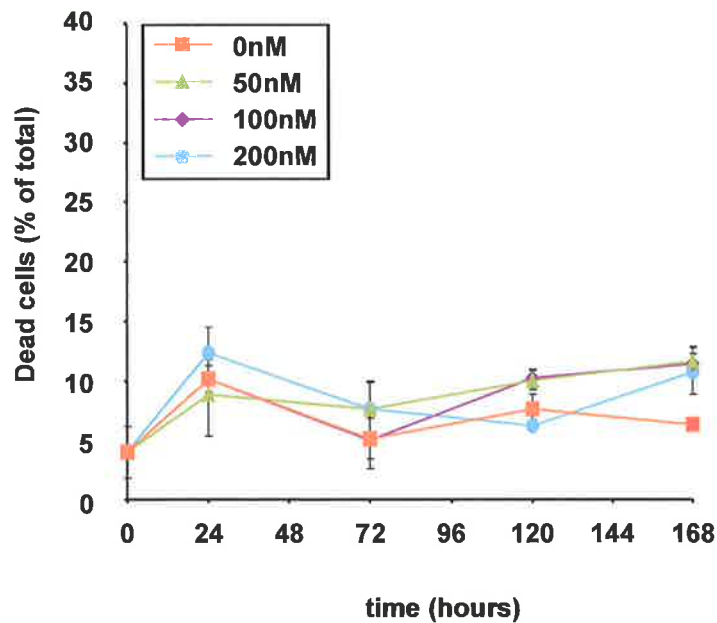
Transfection of LNCaP cells with either 100nM or 200nM as-AUG significantly suppressed LNCaP cell proliferation over the 7-day treatment period (Figure 3.5A) and significantly induced cell death at all concentrations, to a maximum of approximately 30% at 7 days (Figure 3.5B). In contrast, transfection of LNCaP cells with 50 or 100nM of the control mm-AUG had no effect on cell proliferation compared to control cells (Figure 3.6A). However, transfection of LNCaP cells with 200nM mm-AUG inhibited proliferation by approximately 30% ( $p < 0.05$ ) (Figure 3.6A), indicating that there may be some non-specific growth inhibitory effects associated with oligonucleotide transfection at the higher concentrations. There was no significant effect of mm-AUG on LNCaP cell death at any concentration (Figure 3.6B).

### *3.3.2.3 Dose-dependent effect of as-AUG on AR expression in LNCaP cells*

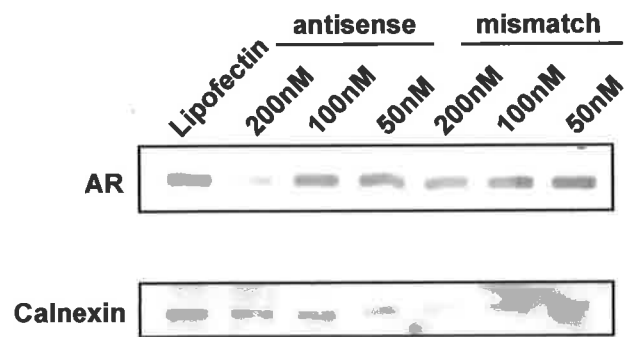
In order to determine whether the effects of as-AUG on LNCaP cell proliferation and survival were associated with decreases in AR protein, AR steady-state levels were assessed by immunoblot 12 hr following transfection with 50, 100 and 200nM as-AUG (Figure 3.7). A reduction in AR steady-state protein levels was observed in LNCaP cells transfected with 200nM as-AUG. There was no detectable reduction in AR steady-state protein at 12 hr post-transfection with either 50 or 100nM as-AUG (Figure 3.7). AR steady-state protein levels in LNCaP cells were not reduced 12 hr following transfection with 50, 100 or 200nM mm-AUG.

**A****B**

**Figure 3.5 - Inhibition of LNCaP prostate cancer cell growth by as-AUG.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with antisense oligonucleotide (0, 50, 100 or 200nM) for up to seven days. (A) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  as-AUG *versus* control.

**A****B**

**Figure 3.6 - Effect of mismatch control oligonucleotide on LNCaP cell growth.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with mismatch oligonucleotide (50, 100 or 200nM) for up to seven days. (A) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  mm-AUG *versus* control.



**Figure 3.7 - AR expression in LNCaP cells following treatment with increasing concentrations of AUG-antisense oligonucleotide.** Lysates from cells treated with 50, 100 or 200nM as-AUG or mismatch control were analysed by immunoblot for expression of AR 12 hr post-transfection. Calnexin was used as a loading control and is shown in the lower panel.

### 3.4 Discussion

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In this study, antisense oligonucleotides targeting different regions of the AR mRNA were compared for their ability to reduce AR protein levels in LNCaP cells. The most effective antisense oligonucleotide in inhibiting AR expression is directed against the initiation methionine codon of the AR mRNA. Transfection of this oligonucleotide (as-AUG) into LNCaP cells resulted in knockdown of AR for up to 48 hr, inhibition of LNCaP cell proliferation and induction of cell death. The lack of an antisense effect of as-750 and as-31 on AR protein levels was unexpected as the sequences were derived from previously published studies in LNCaP cells (Eder *et al.* 2000; Hamy *et al.* 2003). The differences in transfection methods and concentrations of oligonucleotide used may account for the discrepancies observed between studies. Eder *et al.* (2000) used electroporation rather than cationic lipid transfection, thereby overcoming the difficulty in cellular uptake and release from lipid complexes. Hamy *et al.* (2003) used the cationic lipid Effectene, which may have been more effective at transfecting antisense oligonucleotides into LNCaP cells.

The results of the current study highlight some key issues associated with the use of antisense oligonucleotides. Firstly, the delivery of oligonucleotides must be optimal to achieve reductions in target protein, which includes intracellular accumulation and release of oligonucleotides into the cytoplasm. Determinants of intracellular delivery include oligonucleotide length, overall charge and carrier molecules (Tonkinson and Stein 1994). There was not a noticeable difference in intracellular accumulation between the different oligonucleotides. The greatest differences, in terms of intracellular accumulation and antisense effect, appeared to be related to the transfection reagent used. While there was a greater amount of intracellular accumulation in cells transfected using Lipofectamine2000, this did not correlate with an increase in antisense effect in these



cells. A superior antisense effect was observed in LNCaP cells transfected using Lipofectin. This observation is consistent with other studies comparing various cationic lipids including Cytofectin, Superfect, Oligofectamine, Lipofectamine2000, Lipofectamine, Polyfect, JetPEI and Fugene-6 (Vickers *et al.* 2003; Reed *et al.* 2005). It was found that the most efficient transfection reagent was Lipofectin (Vickers *et al.* 2003; Reed *et al.* 2005). Reed *et al.* (2005) demonstrated higher intracellular localisation of oligonucleotides transfected with Cytofectin and Lipofectamine, but this did not translate to increased target reduction. In a study comparing cationic lipids and other delivery methods such as virus capsoids and nanoparticles, it was found that transfection using Lipofectin resulted in the greatest antisense effect regardless of intracellular accumulation (Weyermann *et al.* 2004). This suggests that although Lipofectin may not be superior when assessing the amount of intracellular accumulation, it is the most effective in terms of target reduction. While it has been said that cellular uptake is the greatest limiting factor of oligonucleotide effect (Gewirtz *et al.* 1998), the results of the current study support the notion that release from carrier molecules is just as important (Akhtar and Juliano 1992).

A second issue highlighted by the current study was the appropriate use of control oligonucleotides, not only for target reduction, but also for experiments assessing other biological processes such as cell proliferation. There are three different kinds of control sequences that are widely used. Firstly, mismatch controls are oligonucleotides in which the sequence remains essentially the same as the antisense sequence, but approximately 5 base pair mismatches are inserted. Secondly, scrambled sequences contain the same composition of bases as the antisense in a random order, and thirdly, inverse sequences, also known as sense oligonucleotides, are simply the antisense sequence in reverse. It has been suggested that scrambled or inverse oligonucleotides are superior to mismatch

controls, as the sequences are completely different to the antisense sequence and therefore there is no possibility of low-affinity binding to the target mRNA, whereas a scrambled sequence may bind to the target mRNA, albeit with less affinity than the antisense sequence. In addition, the chemical nature and molecular weight of antisense oligonucleotides are important factors in their cellular uptake and activity (Dias and Stein 2002). Therefore scrambled or inverse sequences may be better controls in terms of cellular uptake and intracellular localisation as they are composed of the same bases as the antisense sequence. The mismatch control oligonucleotide used in the current study could account for some of the non-specific effects observed, possibly by low-affinity hybridisation to AR mRNA. Alternatively, binding of mm-AUG to other mRNA sequences could account for some of the antiproliferative effects observed. A BLAST search against the public database (Genbank) revealed several possible matches. Future experiments should compare an inverse or scrambled control oligonucleotide with mismatch controls, and ensure that the sequence does not bind to another mRNA sequence.

In the current study effective knockdown of AR and a corresponding decrease in the levels of the AR-regulated gene, PSA were observed. The reduction in PSA was maintained for a greater time than the reduction in AR. Moreover, LNCaP cell proliferation and survival was inhibited for up to 7 days following a single dose of as-AUG, suggesting that an initial decrease in AR levels may have prolonged effects on AR activity, prostate cancer cell proliferation and survival. Interestingly, transfection with 100nM as-AUG inhibited LNCaP cell proliferation to the same extent as observed with 200nM as-AUG. This result was surprising as the decrease in AR protein does not appear to correlate with growth inhibitory effects of as-AUG at lower concentrations. It is possible that there was a relatively small decrease in AR in LNCaP cells treated with

100nM as-AUG that was not detected by immunoblot. Alternatively, the duration of AR knockdown at this dose may not have been long enough to be detected at the 8 hr time point, but was sufficient to have an inhibitory effect on LNCaP cell proliferation. Taken together the results suggest that a modest target reduction by antisense oligonucleotides can have significant biological effects (Kurreck 2004). Several studies have now demonstrated significant AR reductions in LNCaP prostate cancer cells following transfection with AR-antisense oligonucleotides (Eder *et al.* 2000; Eder *et al.* 2002; Hamy *et al.* 2003; Ko *et al.* 2004). Moreover, dose-dependent reductions in AR protein levels have been noted in the xenograft tumours and normal prostates of mice injected with AR-antisense oligonucleotides (Eder *et al.* 2002; Ko *et al.* 2004). These studies have also demonstrated inhibitory effects of AR-antisense oligonucleotides on proliferation and survival of LNCaP cells (Eder *et al.* 2000; Hamy *et al.* 2003), as well as LNCaP and LAPC-4 prostate cancer xenografts (Eder *et al.* 2002; Ko *et al.* 2004). The ability of AR-antisense oligonucleotides to reduce AR protein levels thereby inducing significant inhibitory effects on prostate cancer cell proliferation and survival makes them an attractive alternative to current forms of AAT that exert inhibitory effects through reductions in ligand bioavailability.

Antisense oligonucleotides were chosen for the purpose of specific AR-targeting in the current study, rather than siRNA. Currently, there is much debate in the literature regarding whether antisense is superior to siRNA or vice versa. Unlike antisense oligonucleotides, which are short single strands of DNA, RNA interference requires introduction of longer double stranded RNA into cells where it is cut, by the enzyme DICER, into smaller fragments known as small interfering RNAs (siRNA). The siRNA is then unwound and the antisense strand binds to target mRNA inducing endonuclease activity (Caplen 2003). Several studies have attempted to determine which of the two

strategies are more effective at inhibiting protein translation of target mRNA (Bertrand *et al.* 2002; Grunweller *et al.* 2003; Miyagishi *et al.* 2003). The main limitations of these studies is that they usually use siRNA and antisense molecules designed to hybridise to the same region of the target mRNA; however it is possible that each agent will be more effective at targeting different sites (Jason *et al.* 2004) and therefore these studies may be misleading.

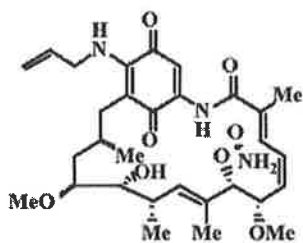
While there are advantages and disadvantages to both antisense and siRNA technology, antisense is currently more advanced clinically because of its relative ease of delivery *in vivo*, greater stability and lower cost to produce compared to siRNA (Thompson 2002; Jason *et al.* 2004). In addition, antisense therapeutics in clinical trials for treatment of cancer lack many of the toxic side-effects and are better tolerated than conventional chemotherapeutic agents (Gleave *et al.* 2002). The main limitations are non-specific backbone related issues, which include complement activation, thrombocytopenia hypotension, tachycardia, and hemodynamic changes at high doses (Shi and Hoekstra 2004; Chi 2005). Nevertheless, current antisense oligonucleotides in clinical trials are demonstrating acceptable toxicity profiles. For example Oblimersen (Genasense, G3139 Genta) is the first oligonucleotide to show proof of principle of an antisense effect in tumours, and specifically in prostate cancer. Oblimersen is an 18mer phosphorothioate oligonucleotide complementary to the first 6 codons of the open reading frame of the mRNA of the critical antiapoptotic protein, Bcl-2 (Herbst and Frankel 2004; Chi 2005). Oblimersen has been used to sensitise tumour cells to other forms of therapy for prostate cancer and is showing promising results (Gleave *et al.* 2002; Chi 2005), leading the way for antisense therapeutics in prostate cancer.

In summary, the current study demonstrates that knockdown of AR protein by as-AUG can reduce the expression of AR and PSA, inhibit prostate cancer cell proliferation and

induce cell death. This provides a basis for further study of AR-targeting antisense oligonucleotides in treatment of prostate cancer. In particular, drawing from studies of Bcl-2 targeting antisense in prostate cancer, combining AR-targeting antisense with other prostate cancer therapeutics may enhance the inhibitory effects on prostate cancer cell proliferation and survival. Preclinical studies in a mouse model will also be important to determine whether *in vitro* and *in vivo* uptake and efficacy of AR-antisense oligonucleotides differ substantially.

## Chapter 4

# 17-allylamino-demethoxygeldanamycin (17-AAG) Reduces AR Protein and Inhibits Prostate Cancer Cell Proliferation and Survival



## 4.1 Introduction

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The studies described in the previous chapter investigated the feasibility of targeting the AR in prostate cancer cells using AR-antisense oligonucleotides. Whereas the findings support the potential for antisense oligonucleotides to target the AR, and recent clinical trials using antisense oligonucleotides in a range of diseases have been promising, difficulties associated with optimising oligonucleotide stability and delivery *in vivo* have precluded their widespread clinical use to date. Consequently, there is a need to investigate alternative strategies to target the AR in prostate cancer cells. One class of molecular agents with the potential to block AR signalling, which are already approved for clinical applications, are the inhibitors of the molecular chaperone protein, Hsp90. Hsp90 inhibitors have exhibited considerable potential for inhibiting the growth of solid tumours including ovarian, breast and prostate cancer (Solit *et al.* 2002; Beliakoff *et al.* 2003; Chung *et al.* 2003; Bull *et al.* 2004; Banerji *et al.* 2005b; Kang *et al.* 2006). As the functional maturation of the AR is dependent on Hsp90, inhibitors of this molecule are likely to have profound effects on AR function and/or stability and therefore the potential to inhibit AR dependent prostate cancer cell growth. However, only a limited number of studies have examined the activity of Hsp90 inhibitors in prostate cancer and, more specifically, the mechanism of growth inhibition. Therefore, the objective of the current study was to investigate Hsp90 inhibitors as putative AR-targeting agents in prostate cancer cells.

### 4.1.1 Hsp90

Hsp90 is an abundant molecular chaperone protein comprising approximately 1-2% of the total protein content within a cell (Maloney *et al.* 2003). Hsp90, together with multiple co-chaperone proteins, is involved in folding and stabilisation of a diverse array

of client proteins (Pratt and Toft 1997; Neckers and Ivy 2003). Client proteins previously studied in detail include the receptor tyrosine kinase Her2/neu/ERBB2, the serine/threonine kinase, Akt, and steroid hormone receptors (reviewed in Maloney *et al.* 2003).

Hsp90 contains an ATP/ADP binding site in the N-terminal domain of the molecule. Hsp90-bound ATP is hydrolysed to ADP, and this switch from an ATP bound to an ADP bound conformation is essential to Hsp90 chaperone activity. Cycling between the ATP and ADP bound states determines the composition of the chaperone complex and therefore the function and stability of the client protein (Grenert *et al.* 1997; Obermann *et al.* 1998; Panaretou *et al.* 1998).

It has been suggested that Hsp90 plays a role in cancer progression by stabilising key signalling molecules, including oncogenic proteins involved in proliferation and survival of cancer cells, such as p53, receptor tyrosine kinases, serine threonine kinases and steroid receptors (Neckers and Lee 2003; Whitesell and Lindquist 2005; Calderwood *et al.* 2006) (for reviews see Neckers *et al.* 1999; Maloney *et al.* 2003). Consistent with a critical role in cancer cell proliferation and survival, Hsp90 is over-expressed in various cancers (Ferrarini *et al.* 1992; Jameel *et al.* 1992; Yufu *et al.* 1992; Gabai *et al.* 1995; Yano *et al.* 1996), and is inversely correlated with patient survival (Jameel *et al.* 1992; Yano *et al.* 1996). Hsp90 itself has therefore been proposed as a potential target for cancer therapy.

#### **4.1.2 Hsp90 inhibitors**

A number of bacterial-derived antibiotics have been identified as Hsp90 inhibitors, including radicicol and the ansamycin antibiotics geldanamycin (GA) and herbimycin-A (Soga *et al.* 2003; Workman 2004). Hsp90 inhibitors exert their activity by binding



preferentially to the ATP binding pocket of Hsp90 (Stebbins *et al.* 1997; Schulte *et al.* 1998; Schulte and Neckers 1998; Roe *et al.* 1999). Binding of the inhibitor prevents the characteristic cycling between the ATP and ADP-bound state, thereby inhibiting the essential ATPase activity of Hsp90 and preventing its chaperone function (Prodromou *et al.* 1997; Scheibel *et al.* 1998). Due to the inhibition of client protein folding, the Hsp90-client protein complex undergoes proteasomal degradation (Schneider *et al.* 1996; Dittmar *et al.* 1997).

Whereas GA has been the most extensively investigated Hsp90 inhibitor for antitumour activity, excessive liver toxicity in mice and dogs has limited its clinical application (Supko *et al.* 1995). 17-allylamino-demethoxygeldanamycin (17-AAG) is a structural and functional derivative of GA with reduced liver toxicity (Amin *et al.* 2005; Behrsing *et al.* 2005). 17-AAG was the first Hsp90 inhibitor to be tested clinically, demonstrating acceptable toxicity profiles in phase I studies (Banerji *et al.* 2005a; Goetz *et al.* 2005; Grem *et al.* 2005; Ramanathan *et al.* 2005). Consequently, 17-AAG is now in phase II clinical trials for the treatment of solid and haematological malignancies.

While the inhibition of multiple client proteins with a single agent is an advantage of Hsp90 inhibitor therapy, it has the potential to be a disadvantage as it may increase non-specific side-effects. However, 17-AAG has demonstrated low toxicity profiles *in vivo*, which may be explained by several factors. Firstly, the relative abundance of Hsp90 in tumour cells compared with normal cells may afford some selectivity, and minimize side-effects in normal tissues. In addition, Kamal *et al.* (2003) found that a greater proportion of Hsp90 in tumour cells was present in chaperone complexes whereas in normal cells Hsp90 was mostly present in an un-complexed form. Moreover, tumour cell Hsp90 had

greater ATPase activity, consistent with active Hsp90 in these cells, and 17-AAG had a higher binding affinity for 'active' Hsp90 in these tumour cells (Kamal *et al.* 2003).

#### ***4.1.3 Hsp90 inhibitors and prostate cancer***

The growth inhibitory effects of Hsp90 inhibitors likely result from degradation of multiple Hsp90 client proteins involved in regulation of cancer cell proliferation and survival. Steroid hormone receptors, including the AR, require Hsp90 for maturation and formation of a high affinity ligand-binding state (Fang *et al.* 1996; Pratt and Toft 1997). Inhibition of Hsp90-dependent maturation of the AR has functional consequences for prostate cancer cell growth. Vanaja *et al.* (2002) demonstrated that treatment of LNCaP prostate cancer cells with GA results in cell cycle arrest at G0-G1 and G2-M phases and depletion of cells in the S-phase, proteasomal degradation of the AR, inhibition of ligand-binding activity and a dose-dependent decrease in PSA expression (Vanaja *et al.* 2002).

To date few studies have assessed the effect of 17-AAG on AR signalling in prostate cancer cells. Solit *et al.* (2002) demonstrated a decrease in expression of AR, Her2, Akt and Raf-1 in LNCaP prostate cancer cells cultured with nanomolar concentrations of 17-AAG. In addition 17-AAG had antitumour activity *in vivo* in CWR22 prostate cancer xenografts (Solit *et al.* 2002). Dose-dependent inhibition of LNCaP cell proliferation has also been demonstrated with additive growth suppressing effects when 17-AAG is used in conjunction with radiation (Enmon *et al.* 2003; Russell *et al.* 2003). While these studies demonstrate antiproliferative and proapoptotic activity of Hsp90 inhibitors in prostate cancer cells, the AR-targeting capabilities of Hsp90 inhibitors has not been explored. The objectives of the current study were to assess the activity of 17-AAG on (i) proliferation and survival of prostate cancer cell lines, (ii) AR expression in LNCaP prostate cancer cells, and (iii) AR variants identified in clinical prostate cancer.

## **4.2 Methods**

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### **4.2.1 17-AAG**

17-AAG was provided by Professor Neal Rosen (Memorial Sloan-Kettering Cancer Centre, New York). Stock solutions of 1mM 17-AAG were dissolved in 100% DMSO and stored at -20°C.

### **4.2.2 Growth curves**

The effect of 17-AAG on LNCaP and PC-3 cell proliferation was assessed by culturing LNCaP cells in the presence of 62.5, 125, 250, 500 or 1000nM 17-AAG in 0.1% DMSO, or vehicle control. Subsequently it was found that PC-3 cells were more sensitive to 17-AAG than LNCaP cells, and therefore the concentrations of 17-AAG utilised were adjusted accordingly to 10, 20, 40, 60 or 120nM. PC-3 and LNCaP cells were cultured in RPMI medium containing 5% and 10%, respectively. Cell viability was determined using Trypan blue dye exclusion as described in chapter 2.

For experiments carried out in steroid depleted conditions, phenol red-free RMPI medium containing 10% charcoal stripped FCS was utilised, as indicated. For charcoal stripping procedure refer to chapter 2.

### **4.2.3 Immunoblot**

The effect of 17-AAG on protein expression was determined by culturing LNCaP cells with 62.5, 125, 250, 500, 1000nM 17-AAG or vehicle control for 12, 24 or 48 hr. AR steady-state protein levels were analysed by immunoblot using the AR-N20 antibody as described in chapter 2.

#### ***4.2.4 Transactivation assays***

The effect of 17-AAG on AR activity was determined by culturing PC-3 cells transfected with wild-type AR or AR variants L57Q, K179R, P502L, S513G, D526G, F671I, V728M, A748T, S780N or T875A in the presence of 1nM DHT +/- 17-AAG (62.5, 125, 250, 500 or 1000nM) for 36 hr. Transactivation activity of transfected AR was measured using the ARR3-tk-luciferase assay system as described in chapter 2.

#### ***4.2.5 Statistical analysis***

For growth curves and transactivation assays statistical differences between 17-AAG treatment groups and controls were determined by One-way ANOVA with Dunnett post-hoc test, using SPSS statistical analysis software. A p-value of <0.05 was considered statistically significant.

Student's T-test was used to determine differences in transactivation activity between wtAR and AR variants, using SPSS statistical analysis software. A p-value of <0.05 was considered statistically significant.

## 4.3 Results

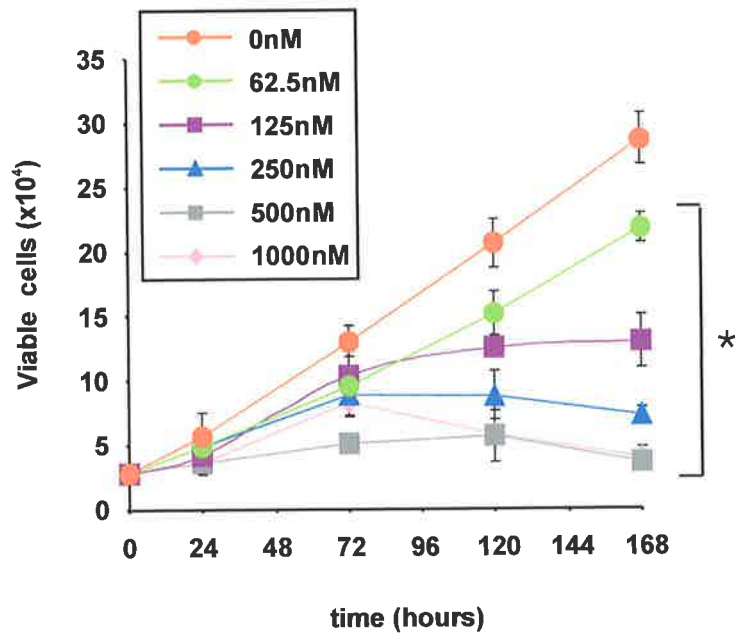
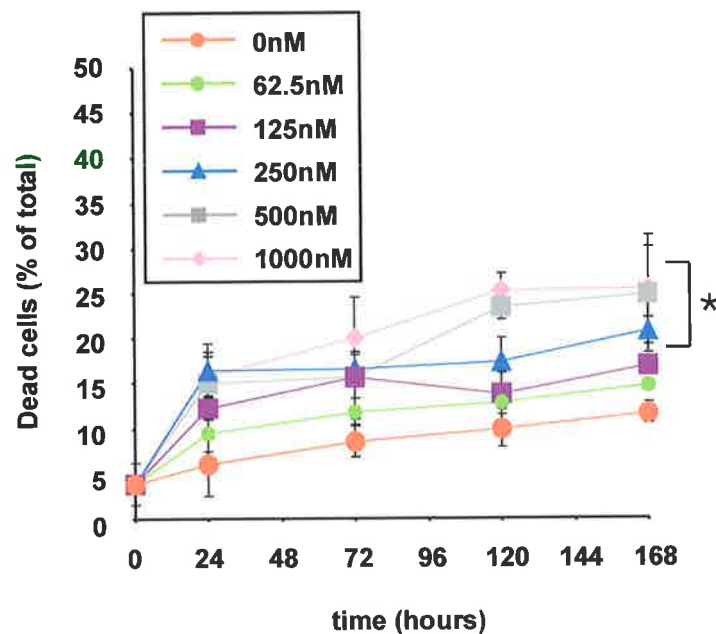
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### ***4.3.1 17-AAG inhibits proliferation of LNCaP and PC-3 prostate cancer cells***

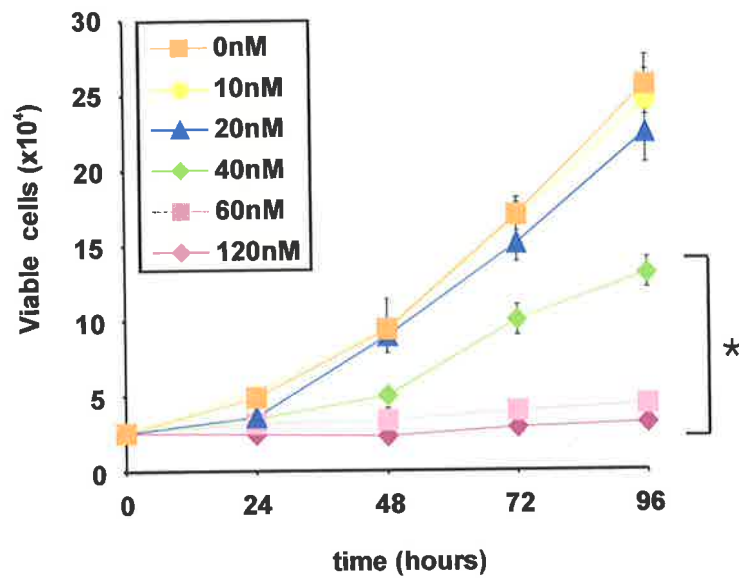
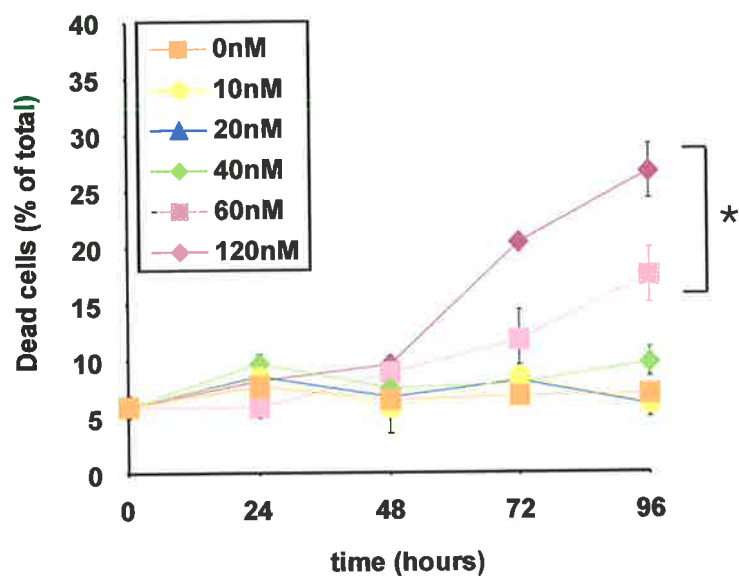
LNCaP cell proliferation was significantly inhibited in a dose-dependent manner by all concentrations of 17-AAG tested (62.5, 125, 250, 500 or 1000nM), with maximal inhibition observed using 500nM 17-AAG (Figure 4.1A). In contrast to proliferation, a higher dose of 17-AAG was required to induce cell death. A significant increase in cell death compared to controls was only observed with 250nM, 500nM and 1000nM 17-AAG (Figure 4.1B). AR-negative PC-3 prostate cancer cells were markedly more sensitive to 17-AAG than LNCaP cells (Figure 4.2). Whereas 62.5nM 17-AAG had only a minimal effect on LNCaP cell proliferation and survival (Figure 4.1A,B), PC-3 cell proliferation and cell death were significantly inhibited by 40nM and 60nM 17-AAG, respectively (Figure 4.2A,B). Maximal effects on cell proliferation and survival were observed with 60nM and 120nM 17-AAG, respectively in PC-3 cells, compared with 500nM in LNCaP cells (Figure 4.1A,B).

### ***4.3.2 17-AAG decreases expression of Hsp90 client proteins, including the AR, in LNCaP cells***

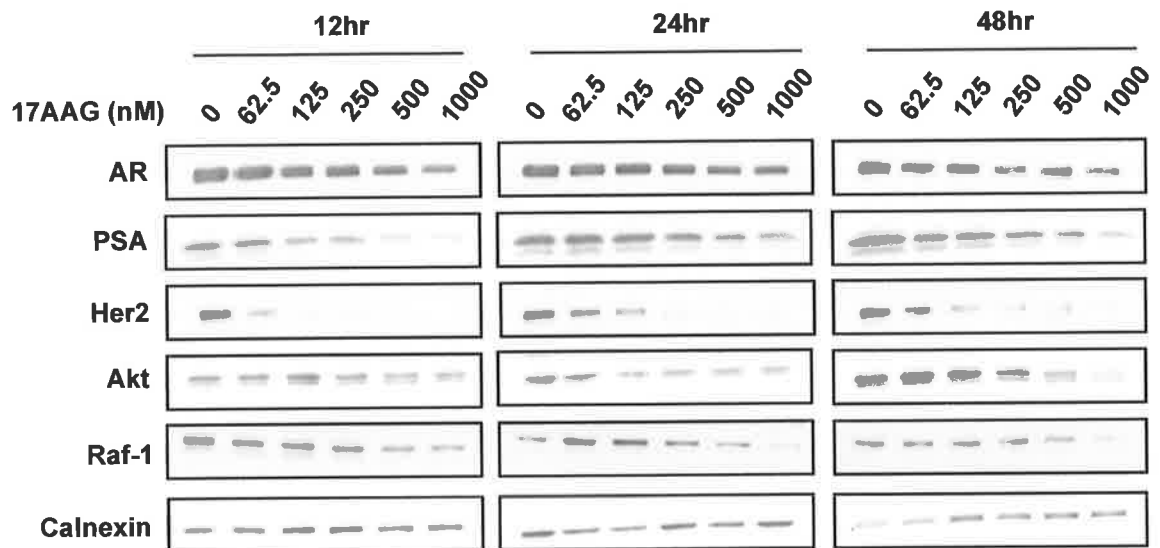
Steady-state levels of Hsp90 client proteins were determined in LNCaP cells by immunoblot analysis (Figure 4.3). Steady-state protein levels of AR decreased in a dose-dependent manner with increasing concentrations of 17-AAG, with >50% reduction being achieved at 12hr with 1000nM 17-AAG. The reduction in AR level was maintained for at least 48 hr with 250nM 17-AAG. A corresponding decrease in steady-state protein levels of the AR-regulated gene, PSA was observed in LNCaP cells with increasing concentrations of 17-AAG. Steady-state levels of the Hsp90 client protein, Her2, was also reduced in a dose-dependent manner in LNCaP cells cultured in the presence of

**A****B**

**Figure 4.1 - Inhibition of LNCaP prostate cancer cell growth by 17-AAG.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured in RPMI medium containing 10% FCS and increasing concentrations of 17-AAG (62.5, 125, 250, 500 or 1000nM) or vehicle control for up to 7 days. Cells were counted every two days using a haemocytometer. (A) Cell viability was assessed by Trypan blue dye exclusion, and (B) number of dead cells is expressed as percent of total cells counted (B). Results are presented as the mean  $\pm$  the SE of triplicate wells from an experiment. Results are representative of three separate experiments.\* ANOVA;  $p < 0.05$  17-AAG *versus* control.

**A****B**

**Figure 4.2 - Inhibition of PC-3 prostate cancer cell growth by 17-AAG.** PC-3 cells ( $2.5 \times 10^4$ ) were cultured in RPMI medium containing 5% FCS with increasing concentrations of 17-AAG (10, 20, 40, 60 or 120nM) or vehicle control for up to 4 days. Cells were counted every day using a haemocytometer. (A) Cell viability was assessed by Trypan blue dye exclusion, and (B) number of dead cells is expressed as percent of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells from an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  17-AAG *versus* control.



**Figure 4.3 - Expression of AR, PSA and hsp90 client proteins in LNCaP cells following treatment with 17-AAG.** Lysates from LNCaP cells treated with 17-AAG (62.5, 125, 250, 500 or 1000nM) in RPMI medium containing 10% FCS for 12, 24 and 48 hours were analysed by immunoblot for expression of AR, PSA, Akt, Raf-1 and Her-2. Calnexin was used as a loading control and is shown in the lower panel. Images are representative of three separate experiments.



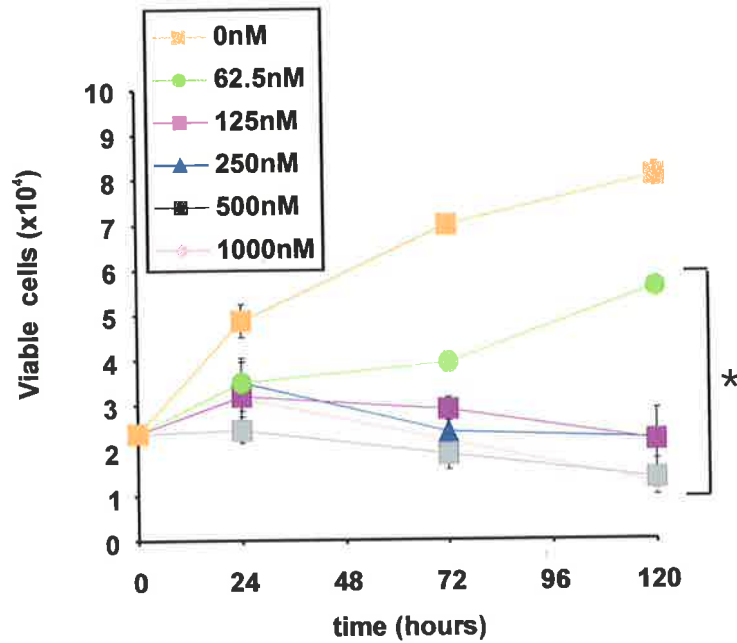
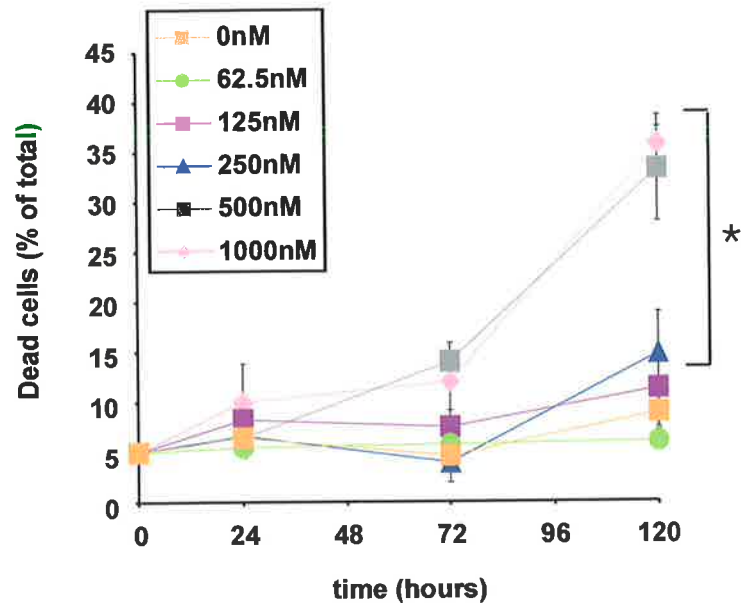
17-AAG, with maximal inhibition using 125nM at 12 hr post-treatment. The reduction in Her2 steady-state levels was maintained for at least 48 hr with 125nM 17-AAG. While steady-state protein levels of the Hsp90 client proteins Akt and Raf-1 were reduced at 12hr post-treatment with 17-AAG, the greatest reduction was observed at 24 hr post-treatment with concentrations greater than 250nM. The reduction in steady-state protein levels of Akt and Raf-1 were maintained for at least 48 hr with 500nM 17-AAG.

#### ***4.3.3 Inhibition of proliferation and induction of cell death by 17-AAG is enhanced by culture in steroid depleted medium***

The previous experiments described in 4.3.1, were performed in medium containing 10% normal serum, which contains physiological levels of testosterone (~2.6nM/L<sup>1</sup>). To determine the influence of steroids, particularly androgens, on sensitivity of LNCaP cells to 17-AAG, additional experiments were performed in medium containing 10% charcoal stripped serum (testosterone levels not detectable<sup>1</sup>). Under these conditions, proliferation of LNCaP cells was completely inhibited by 125nM 17-AAG (Figure 4.4A). This represents half the concentration of 17-AAG required to achieve an equivalent decrease in proliferation of LNCaP cells cultured in medium containing 10% normal FCS (Figure 4.1A). Culture in medium containing 10% charcoal stripped FCS resulted in a statistically significant increase of LNCaP cell death with 250nM 17-AAG, to a maximum of approximately 35% in the presence of 500nM 17-AAG (Figure 4.4B); this compared to a maximum of 25% when cultured with 500nM 17-AAG in 10% normal FCS.

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<sup>1</sup> Testosterone was routinely analysed in FCS by automated immunoassay on the Immulite 2000 system (Diagnostic Products Corporation, Los Angeles, USA).

**A****B**

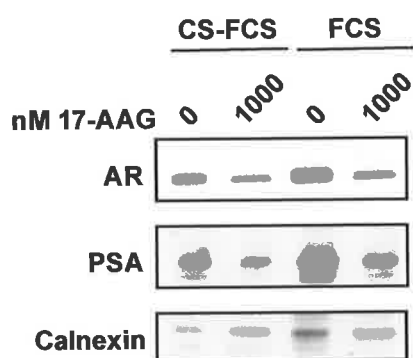
**Figure 4.4 - Effect of 17-AAG in androgen depleted medium on LNCaP cell proliferation.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured in phenol red-free RPMI medium containing 10% charcoal stripped FCS in the presence of 17-AAG (62.5, 125, 250, 500 or 1000nM) for up to five days. Cells were counted every two days using a haemocytometer. (A) Cell viability was assessed by Trypan blue dye exclusion, and (B) the number of dead cells is expressed as percent of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells from an experiment. Results are representative experiment of three separate experiments. \* ANOVA;  $p < 0.05$  17-AAG *versus* control.

#### ***4.3.4 AR protein is decreased by 17-AAG in the presence and absence of DHT***

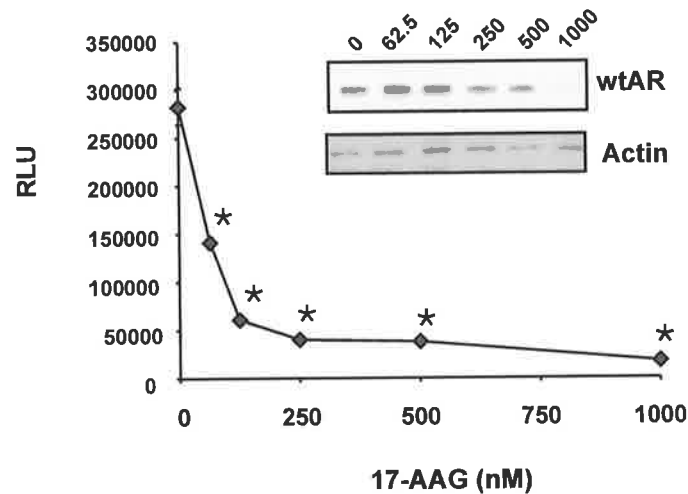
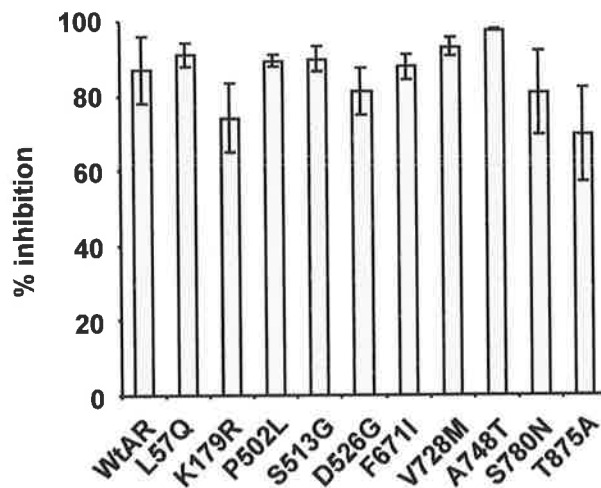
To determine whether the presence of androgens in the medium had any effect on AR steady-state protein levels, LNCaP cells were treated with a maximal dose of 17-AAG (1000nM) in 10% normal versus charcoal stripped serum. In the absence of 17-AAG, AR steady-state protein levels were lower in LNCaP cells cultured in charcoal stripped FCS compared to normal FCS (Figure 4.5). In the presence of 1000nM 17-AAG, steady-state levels of AR were reduced in LNCaP cells cultured in both 10% normal serum and 10% charcoal stripped serum. Steady-state levels of PSA were also reduced in LNCaP cells cultured with 1000nM 17-AAG in both normal and charcoal stripped serum (Figure 4.5).

#### ***4.3.5 AR variants from clinical prostate cancer are sensitive to 17-AAG***

The effect of 17-AAG on the activity and expression of wild-type AR (wtAR) and variant AR was determined using transactivation assays in transiently-transfected AR-negative PC-3 cells. The ability of wtAR to activate the androgen-responsive probasin reporter was reduced by approximately 50% with as little as 62.5nM 17-AAG (Figure 4.6A). There was no decrease in AR steady-state protein levels at this low concentration; however AR levels were reduced at concentrations of 250nM, 500nM and 1000nM. To determine the activity of 17-AAG against AR variants, PC-3 cells were transfected with one of the following AR variants in the presence of 1nM DHT: L57Q, K179R, P502L, S513G, D526G, F671I, V278M, A748T, S780N or T875A. 17-AAG dose-dependently inhibited transactivation activity of all AR variants tested (data not shown). Transactivation activity of each AR variant was inhibited by >80% using 500nM 17-AAG, with the exception of K179R, S780N and T875A, which were inhibited by approximately 70% (Figure 4.6B).

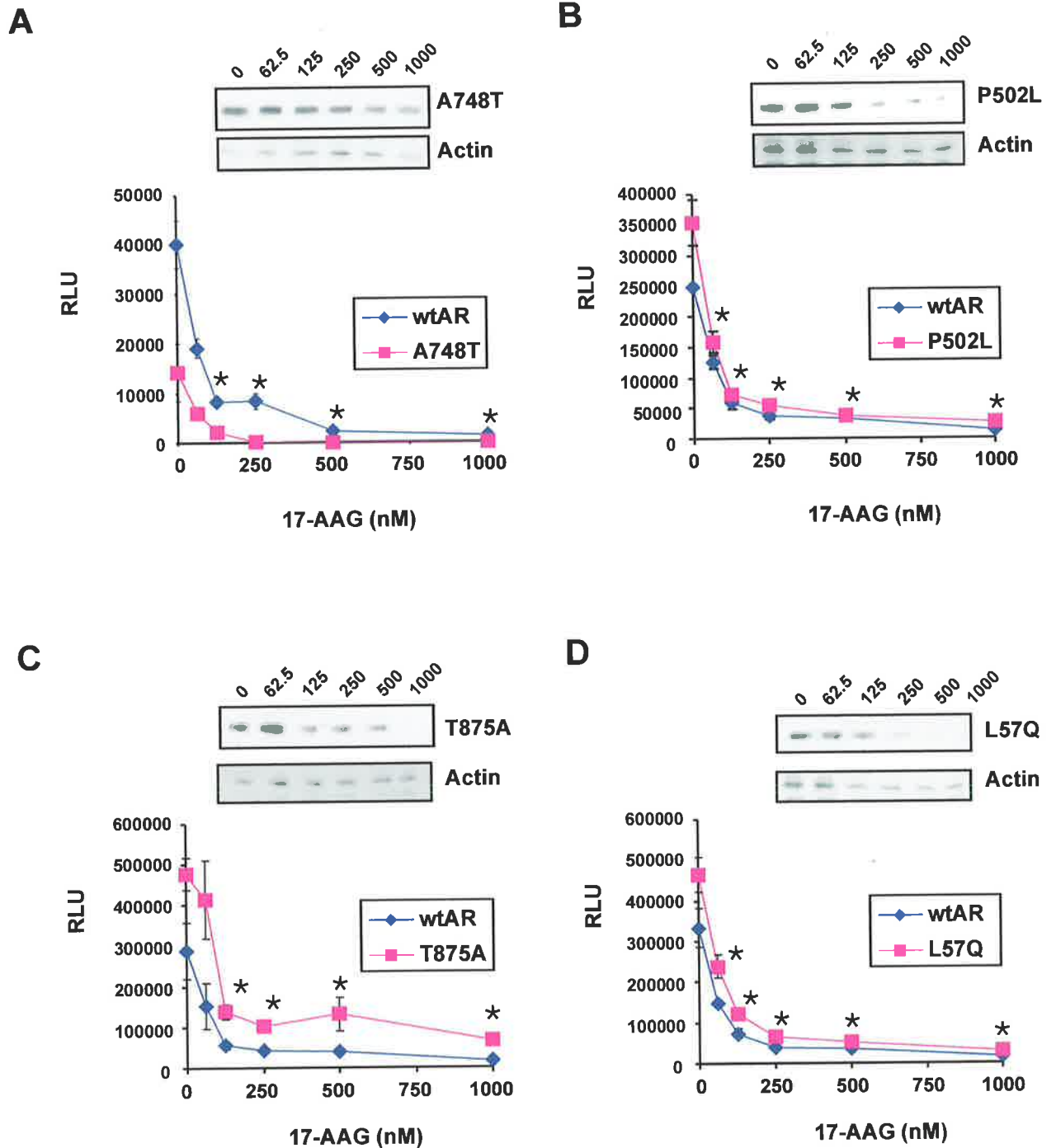


**Figure 4.5 – Effect of 17-AAG in androgen depleted medium on AR and PSA expression in LNCaP cells.** Lysates from LNCaP cells treated with or without 17-AAG (1000nM) in RMPI medium containing either 10% FCS or 10% charcoal stripped FCS (CS-FCS) for 12 hours were analysed by immunoblot for expression of AR and PSA. Calnexin was used as a loading control and is shown in the lower panel.

**A****B**

**Figure 4.6 - Inhibition of wild-type AR and AR-variant activity by 17-AAG.** PC-3 cells ( $1.5 \times 10^4$ ) were transiently transfected with wtAR (2.5ng/well), and an androgen responsive luciferase reporter (100ng/well). Four hours after transfection the cells were treated with 17-AAG (62.5, 125, 250, 500 and 1000nM) in the presence of 1nM DHT for 36 hours. Luciferase activity was read on a luminometer. (A) Activity of wtAR is plotted as relative luciferase units (RLU). Expression of AR was determined by immunoblot analysis and expression of actin was used as a loading control, shown in the lower panel. (B) Inhibition of AR-variants L57Q, K179R, P502L, S513G, D526G, F671I, V728M, A748T, S780N and T875A treated with 500nM 17-AAG + 1nM DHT is plotted as % of activity. Results are presented as the mean  $\pm$  the SE of 6 replicates in a representative experiment. Each experiment was performed at least twice. Experiments were performed by Joanna Gillis. \* ANOVA;  $p < 0.05$  17-AAG *versus* control.

Representative AR variant dose response curves showing dose-dependent inhibition of transactivation activity by 17-AAG are shown in Figure 4.7A-D. In the presence of 1nM DHT the transactivation activity of AR variants P502L, T875A, and L57Q was significantly higher compared to wtAR ( $p < 0.05$ ) (Figure 4.7B,C,D). Conversely, transactivation activity of the AR variant A748T was significantly lower than wtAR in the presence of 1nM DHT ( $p = 0.007$ ) (Figure 4.7A). AR-T875A was less sensitive to 17-AAG compared to wtAR (Figure 4.7C), whereas AR-A748T was the most sensitive to 17-AAG, with 100% inhibition achieved using 250nM 17-AAG. While a significant reduction in AR variant transactivation activity was observed with 62.5nM (P502L, L57Q) or 125nM (A748T, T875A) 17-AAG, a reduction in AR variant steady-state protein levels was only observed with 250nM for AR-P502L, AR-T875A and 500nM for AR-L57Q or for AR-A748T (Figure 4.7A-D).



**Figure 4.7 - Inhibition of wild-type AR and AR-variant activity by 17-AAG.** PC-3 cells ( $1.5 \times 10^4$ ) were transiently transfected with wtAR (2.5ng/well), and an androgen responsive luciferase reporter (100ng/well). Four hours after transfection the cells were treated with 17-AAG (62.5, 125, 250, 500 and 1000nM) in the presence of 1nM DHT for 36 hours. Luciferase activity was read on a luminometer and are plotted as relative luciferase units (RLU). Results are presented as the mean  $\pm$  the SE of 6 replicates in a representative experiment. Each experiment was performed at least three times. Experiments were performed by Michelle Newman. \* ANOVA;  $p < 0.05$  (A748T, P502L, T875A, L57Q) 17-AAG versus control.

#### 4.4 Discussion

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In the context of castrate-resistant prostate cancer, where continued androgen signalling can sustain prostate cancer cell proliferation, use of agents that can reduce AR levels or inhibit AR activity may be advantageous. In this respect Hsp90 inhibitors, including the geldanamycin derivative, 17-AAG may be useful. These agents inhibit Hsp90 and abrogate the function of a diverse range of Hsp90 client proteins, including steroid receptors (Maloney *et al.* 2003). The results of the current study demonstrate a reduction in steady-state levels of the Hsp90 client proteins Her2, Akt, Raf-1 and the AR. In addition, 17-AAG inhibited the activities of wtAR and 10 AR variants identified in clinical prostate cancer. 17-AAG also inhibited both AR-positive LNCaP and AR-negative PC-3 cell proliferation and induced cell death.

The AR-negative prostate cancer cell line, PC-3 was markedly more sensitive to growth inhibition by 17-AAG compared to LNCaP cells. One explanation for this is that Hsp90 client proteins important in controlling cell growth and survival are expressed in a cell specific manner. For example, the Hsp90 client protein Akt may contribute to the reduced sensitivity of LNCaP cells to 17-AAG compared to PC-3 cells. LNCaP cells contain higher levels of phosphorylated (active) Akt compared to PC-3 cells (Nesterov *et al.* 2001). Moreover, it has been shown that Akt signalling is constitutively active in LNCaP cells (Carson *et al.* 1999; Nesterov *et al.* 2001), which has been associated with resistance to TRAIL-induced apoptosis (Nesterov *et al.* 2001). Akt has been implicated in regulation of a survival signal transduction pathway, via PI3 kinase activation; phosphorylation of Akt inhibits apoptosis by phosphorylating and thereby inactivating proapoptotic proteins such as BAD (Datta *et al.* 1997; del Peso *et al.* 1997; Carson *et al.* 1999).



Based on the observations in the current study, an alternative hypothesis to explain the difference in sensitivity between LNCaP cells and PC-3 cells is that activation of androgen signalling protects LNCaP cells from the effects of 17-AAG. In normal and malignant prostate cells, androgens stimulate cell proliferation (Bruchovsky *et al.* 1975; Isaacs *et al.* 1992), while androgen withdrawal induces apoptosis (Kyprianou and Isaacs 1988). As LNCaP cells are AR-positive and androgen sensitive, it is possible that reduced sensitivity of LNCaP cells to 17-AAG is due to the effects of androgens on these cells. In support of this hypothesis, inhibition of cell proliferation and induction of cell death by 17-AAG were enhanced when LNCaP cells were cultured in charcoal stripped FCS compared to normal FCS. Reduced sensitivity to other therapies such as radiation (Harashima *et al.* 2005) and etoposide treatment (Berchem *et al.* 1995) has been demonstrated in LNCaP cells in the presence of DHT, consistent with protective effects of androgen signalling in these cells. In addition, (Harashima *et al.* 2005) observed greater reductions in AR and PSA expression in response to the Hsp90 inhibitor, radicicol in LNCaP cells cultured in the absence of DHT. The LNCaP cell line contains an AR variant that has increased basal activity in the absence of androgens compared to wtAR, which may contribute to the protective effect in this cell line. These observations are consistent with a previous study that demonstrated greater sensitivity of wtAR to degradation following treatment with 17-AAG compared to the LNCaP variant (Solit *et al.* 2002).

As Hsp90 has many client proteins, 17-AAG has the capability to inhibit multiple cellular pathways that impact directly or indirectly on AR signalling. Ligand-independent activation of the AR via tyrosine kinase signalling pathways has been suggested as a means of maintaining androgen signalling in the castrate environment (reviewed in

Edwards and Bartlett 2005; Gioeli 2005; Paule 2005). For example, several studies have demonstrated induction of AR transactivation activity and PSA expression upon activation or over-expression of Her2 (Yeh *et al.* 1999b; Mellinshoff *et al.* 2004; Gregory *et al.* 2005). In contrast, inhibition of Her2 signalling results in decreased AR activity and corresponding decreases in prostate cancer cell proliferation (Yeh *et al.* 1999b; Gregory *et al.* 2005). Signalling via the Her2 pathway can also be blocked by inhibiting Akt (Wen *et al.* 2000), suggesting involvement of this Hsp90 client protein in AR activation via Her2 signalling. Moreover, Her2 signalling activates the mitogen activated protein kinase (MAPK) signalling pathway (Yeh *et al.* 1999b), involving Raf-1, another Hsp90 client protein. Inhibition of Hsp90 activity would therefore have the potential to inhibit AR activation by Her2 signalling at more than one level, including degradation of Her2, Akt, Raf-1 and the AR itself (Paule 2005), thereby inhibiting activation of the AR and preventing castrate-resistant tumour progression.

Mutations that reduce the requirement of client proteins for Hsp90 may decrease the sensitivity of cells to treatment by 17-AAG, thereby limiting the effectiveness of 17-AAG and may even result in resistance to therapy. For example mutated, gain-of-function v-src, bcr-abl, c-kit and p53 have increased dependence on Hsp90 compared to their wild-type counterparts, making them more sensitive to degradation by 17-AAG (Whitesell *et al.* 1994; Blagosklonny *et al.* 1996; Schulte and Neckers 1998; An *et al.* 2000; Fumo *et al.* 2004; Grbovic *et al.* 2006). One mechanism proposed to contribute to castrate-resistant prostate cancer is gain-of-function mutations that allow activation of the AR at low levels of ligand or by non-classical ligands. It is therefore possible that an AR variant could arise with decreased dependency on Hsp90 and be selected for in response to prolonged 17-AAG treatment, which theoretically could result in therapy failure. A

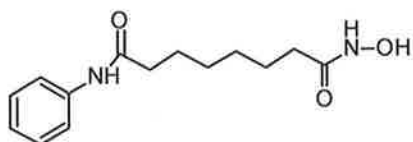
previous study demonstrated that steady-state protein levels of the AR variant A748T is not affected by treatment with GA (James *et al.* 2002), possibly due to a lack of stable interaction with Hsp90 (James *et al.* 2002). In that study, the effect of GA on AR functional activity was not investigated. The results of the current study demonstrate that 17-AAG is effective at inhibiting the transactivation activity of the A748T AR variant. Moreover, a series of AR variants identified in clinical prostate cancer with higher basal transactivation activity compared to wtAR (Buchanan *et al.* 2001a; Buchanan *et al.* 2001c), retained sensitivity to 17-AAG. This finding suggests that treatment of metastatic prostate cancer with 17-AAG would not be less effective in tumour cells expressing these AR variants. Interestingly, 17-AAG inhibited wtAR and AR variant activity at lower concentrations than were required to induce reductions in AR steady-state protein levels. These observations could indicate that 17-AAG inhibits the activity of an AR coactivator protein required for AR transactivation activity in LNCaP cells. If this is the case, identification of this putative AR coactivator may provide another target for inhibition of AR activity in prostate cancer cells.

The results of the current study suggest that AR is not the only important target of 17-AAG in prostate cancer cells, however 17-AAG inhibits not only AR protein levels, but also AR transactivation activity in LNCaP prostate cancer cells. In addition, sensitivity to 17-AAG was increased by culture in a steroid depleted environment. Clinically, an AR-positive phenotype is the most common following failure of AAT. Consequently, treatment of prostate cancer with Hsp90 inhibitors in combination with AAT may enhance the inhibitory effects on tumour growth. While 17-AAG is the first Hsp90 inhibitor to enter clinical trials, issues such as poor solubility and dose-limiting liver toxicity in patients (Banerji *et al.* 2003) has led to the development of second and third

generation Hsp90 inhibitors, including hydroquinone derivatives, pyrazoles and purine based molecules (Soga *et al.* 2001; Chiosis *et al.* 2003; Le Brazidec *et al.* 2004; Glaze *et al.* 2005; Ge *et al.* 2006; McDonald *et al.* 2006; Smith *et al.* 2006). Pre-clinical evaluation of these new generation inhibitors in conjunction with AAT may provide a therapeutic strategy with greater antiproliferative effects than Hsp90 inhibitors alone, while reducing the toxic side-effects of current Hsp90 inhibitors, such as 17-AAG.

## Chapter 5

### **The Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid (SAHA) Reduces AR Expression and Inhibits Prostate Cancer Cell Proliferation**



## 5.1 Introduction

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Whereas the previous chapter investigated the potential for the Hsp90 inhibitor, 17-AAG, to inhibit AR signalling in prostate cancer cells, another class of therapeutic agents already approved for clinical use, that are potential AR-targeting agents are the histone deacetylase (HDAC) inhibitors. HDAC inhibitors have gained considerable interest in the past decade as agents to treat both solid and hematologic malignancies (Marks *et al.* 2000; Marks *et al.* 2001; Marks and Dokmanovic 2005), and have antiproliferative and proapoptotic effects in a variety of cancer cell lines and animal models, including prostate cancer (Gleave *et al.* 1998b; Butler *et al.* 2000; Butler *et al.* 2001; Piekarz *et al.* 2001; Sandor *et al.* 2002; Kelly *et al.* 2003; Camphausen *et al.* 2004; Kuefer *et al.* 2004; Reid *et al.* 2004; Thelen *et al.* 2004; Byrd *et al.* 2005; Fronsdal and Saatcioglu 2005; Gesine Bug 2005; Kelly *et al.* 2005; Roy *et al.* 2005; Ryan *et al.* 2005; Andrea Kuendgen 2006). There are four classes of HDAC inhibitors currently in clinical trials, including short chain fatty acids such as phenylbutyrate, valproate and AN-9; hydroxamic acids such as suberoylanilide hydroxamic acid (SAHA) and LBH-589; cyclic tetrapeptides such as depsipeptide; and the benzamides such as MS-275 (Piekarz *et al.* 2001; Sandor *et al.* 2002; Kelly *et al.* 2003; Reid *et al.* 2004; Bug *et al.* 2005; Byrd *et al.* 2005; Kelly *et al.* 2005; Ryan *et al.* 2005; Kuendgen *et al.* 2006). To date there has been relatively little research into the mechanisms involved in HDAC inhibitor-induced antiproliferative or proapoptotic effects in prostate cancer cells. A previous study by Butler *et al.* (Butler *et al.* 2000) demonstrated greater sensitivity to HDAC inhibitor treatment of AR-positive compared to AR-negative prostate cancer cell lines. This observation raised the possibility that the AR signalling axis is an important target of HDAC inhibitors in prostate cancer cells and was investigated further in this chapter.

### ***5.1.1 Histone acetyltransferases and histone deacetylases***

Acetylation is a reversible covalent modification that affects a variety of cellular proteins and is regulated by the catalytic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Through their ability to modulate acetylation, HATs and HDACs are involved in regulating the activity of proteins involved in cellular processes such as transcription, signal transduction and intracellular transport (Agalioti *et al.* 2002; Polevoda and Sherman 2002; Richards and Elgin 2002).

#### *5.1.1.1 Acetylation of nuclear histones*

The best characterized targets of HATs and HDACs are nuclear histone proteins. In eukaryotes chromosomes are organized into nucleosomes, which are structures consisting of compacted DNA wrapped around histone proteins. The N-terminal tails of these histones are subject to chemical modifications such as methylation, phosphorylation, ubiquitination and acetylation (Agalioti *et al.* 2002; Richards and Elgin 2002). These modifications can alter the structure and organization of the nucleosome. Regulated by the activities of HATs and HDACs, acetylation of lysine residues within histone tails decreases their positive charge, causing repulsion between the DNA and histone tails, resulting in a more relaxed chromatin configuration, known as euchromatin. The loose configuration of euchromatin allows increased access to transcriptional complexes and is associated with transcriptionally active DNA. Conversely, deacetylation increases the positive charge of the histone tails, resulting in a ‘tightening’ of the tails around the negatively charged DNA. This DNA conformation, called heterochromatin, prevents access of transcriptional machinery and is therefore associated with transcriptionally inactive DNA.

### *5.1.1.2 Acetylation of non-histone proteins*

Acetylation of lysine residues can also occur within non-histone proteins, including transcription factors and nuclear import proteins (Polevoda and Sherman 2002). The acetylation status of a protein can affect diverse functions such as enzyme activity, DNA binding, protein stability, protein-protein interactions and peptide-receptor recognition through alterations in charge or by changing the physical properties of the acetylated protein. An example of non-histone proteins that can be acetylated are nuclear receptors. Nuclear receptor activity is modulated by co-activator and co-repressor complexes. Several of these nuclear receptor co-factors have intrinsic HAT activity which is involved in regulating the acetylation of histones during nuclear receptor transcriptional activation, in addition to regulating acetylation of the nuclear receptors themselves (Fu *et al.* 2003; Fu *et al.* 2004).

### *5.1.2 Histone deacetylase inhibitors*

HDAC inhibitors act by binding to the enzyme pocket of HDACs thereby inhibiting their enzymatic activity (Finnin *et al.* 1999), leading to a net increase in acetylation of HDAC substrate proteins, including histones. Gene profiling studies have demonstrated that both up-regulation and down-regulation of gene expression occurs in response to HDAC inhibition, which may depend on the recruitment of transcription activation versus repression complexes to the DNA (Jenuwein and Allis 2001). Interestingly, it has been reported that only a small percentage (<10%) of gene expression is significantly altered in bladder cancer, breast cancer and human lymphoid cell lines following treatment with histone deacetylase inhibitors (Van Lint *et al.* 1996; Glaser *et al.* 2003). These studies suggest that while HDAC inhibitor treatment causes a net increase in histone acetylation, the expression of only a relatively small number of genes is changed. The genes that are



most commonly altered following treatment with HDAC inhibitors are those involved in cell cycle progression, DNA synthesis and apoptosis (Richon *et al.* 2001; Glaser *et al.* 2003).

### ***5.1.3 HDAC inhibitors and prostate cancer***

In 1998, Gleave *et al.* demonstrated that treatment with the HDAC inhibitor butyrate was able to induce cell cycle arrest and inhibit prostate xenograft tumour growth. In addition, Melchior *et al.* (1999) found that the related HDAC inhibitor phenylbutyrate inhibited proliferation and induced apoptosis of LNCaP and LuCaP 23.1 cells *in vitro* and *in vivo*, with a low toxicity profile. More recently Butler *et al.* (2000) demonstrated that the hydroxamic acid-based HDAC inhibitor, SAHA, inhibited proliferation of AR-positive LNCaP cells and AR-negative PC-3 cells in a dose-dependent manner, while cell death was induced only in LNCaP cells. Inhibition of CWR22 xenograft tumour growth by SAHA was also observed at doses that were not toxic to the animals (Butler *et al.* 2000). Subsequently it was shown that the related compound pyroxamide had antiproliferative effects in LNCaP prostate cancer cells and in the CWR22 prostate cancer xenograft model (Butler *et al.* 2001).

While these studies demonstrate antiproliferative activity of HDAC inhibitors in prostate cancer cell lines, the fact that cell death is induced in AR-positive LNCaP cells and not in AR-negative PC-3 cells suggests that modulation of AR signalling may be a critical determinant of the efficacy of HDAC inhibitors in prostate cancer cells. Therefore the aims of the current study were to investigate the effects of the HDAC inhibitor SAHA on (i) prostate cancer cell proliferation and survival, and (ii) AR signalling in prostate cancer cells.

## **5.2 Methods**

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### **5.2.1 SAHA**

SAHA was provided by Dr Victoria Richon (Aton Pharma, Tarrytown, New York). Stock solutions of 100mM SAHA were dissolved in 100% DMSO and stored at -70°C. Working solutions of 10mM SAHA were dissolved in 100% DMSO and stored at -20°C.

### **5.2.2 Growth curves**

The effect of SAHA on LNCaP, PC-3 or C42B prostate cancer cell proliferation and cell death was determined by culturing the cells in the presence of SAHA (0.5, 1, 2.5, 5, 7.5 or 10µM) in 0.1% DMSO, or vehicle control for up to 7 days. Cells were counted using a haemocytometer, and cell viability was determined by Trypan blue dye exclusion, as described in chapter 2.

For growth curve experiments using the caspase inhibitor, z-VAD-fmk, LNCaP cells were cultured as described above, in the presence or absence of z-VAD-fmk (50µM) for the duration of the experiment (5 days).

PC-3 cells and C42B cells were cultured in medium containing 5% FCS, whereas LNCaP cells were routinely cultured in medium containing 10% FCS. For experiments where cells were cultured in steroid depleted conditions, phenol red free RPMI medium containing 10% charcoal stripped FCS was used, as indicated. For charcoal stripping procedure refer to chapter 2.

### **5.2.3 Cell cycle analysis**

LNCaP cells were cultured with DMSO or SAHA (2.5 or 7.5µM) for 24 hr. The cells were rinsed in PBS, fixed in 80% ethanol and stored at -20°C until analysed. Prior to analysis the cell suspension was treated with DNase-free RNase for 20 min at 37°C and stained with

propidium iodide solution. Cell cycle distribution was analysed using CellQuest software. Cell cycle analysis was performed by Dr Lisa Butler.

#### **5.2.4 Caspase assay**

DEVD-caspase activity was assayed by cleavage of zDEVD-AFC (z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin), a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly(ADP-ribose) polymerase. LNCaP cells were cultured with DMSO or SAHA (2.5 or 7.5 $\mu$ M). After 48 hr the cells were lysed in NP-40 lysis buffer, on ice for 30 minutes with shaking. Lysates were centrifuged for 5 minutes at 11,000 rpm, the supernatant was stored at  $-70^{\circ}\text{C}$  until assayed. Cell lysates were added to each assay tube containing 8 $\mu$ M of substrate in 200 $\mu$ L of fluorometric caspase assay buffer. After incubation for 4 hr at room temperature, fluorescence was quantified (Exc 400, Emis 505) in a Perkin Elmer LS50 spectrofluorometer. One unit of caspase activity was taken as one fluorescence unit (at slit widths of 10nm) per 4 hr incubation with substrate. The tetrapeptide caspase inhibitor z-VAD-fmk, was dissolved in DMSO and added to cells at a final concentration of 50 $\mu$ M, 30 minutes before addition of SAHA (2.5 or 7.5 $\mu$ M). Control cells were incubated with DMSO at the same concentration.

#### **5.2.5 Gene Profiling**

Gene expression in DMSO or SAHA (2.5 or 7.5 $\mu$ M) treated LNCaP cells was determined using the UniGEM human cDNA V2.0 array, which contained 9182 cDNA probes representing 8372 individual genes/ESTs and 192 internal controls. Following 6 hr culture with SAHA, total RNA was isolated from the cells using Trizol reagent. Poly(A)<sup>+</sup> mRNA was isolated from the total RNA using Oligotex columns. The results were analyzed using GEM Tools image and data analysis software, and a 2-fold change was considered as a

threshold for regulation of gene expression. Microarray experiments were conducted by Dr Lisa Butler.

### ***5.2.6 Analysis of AR mRNA levels in LNCaP cells***

#### *5.2.6.1 Preparation of cDNA*

LNCaP cells were cultured with SAHA (2.5 or 7.5 $\mu$ M) in 0.1% DMSO, or vehicle control for 2 hr or 6 hr. Cells were lysed in Trizol reagent, and RNA was prepared as per the manufacturer's instructions. The RNA samples were incubated with DNA-free<sup>TM</sup> to remove any contaminating genomic DNA before cDNA preparation. Each DNase-treated RNA sample was mixed with 200ng of random primers plus dNTP mix in sterile, distilled water. Following 5 minute incubation at 65°C, the samples were incubated on ice for 1 minute. First strand buffer, 0.1M DTT, RNase inhibitor and Superscript<sup>TM</sup>III was added to each sample and incubated for 1 hr at 50°C. The cDNA samples were diluted to a concentration of 100ng/ $\mu$ l.

#### *5.2.6.2 Real-time PCR*

The forward and reverse primers for L32, AR, PSA and KLK2 used for real-time PCR are listed in chapter 2. Master mix containing forward primers, reverse primers and iQ SYBR Green Supermix was made up in Ultra Pure water. Each cDNA sample (200ng) was analysed in triplicate. Real-time PCR was run using iQ5 Real-Time PCR detection system and analysed using iQ5 software. Mean Ct values for AR, PSA and KLK2 expression were normalized to RPL32 housekeeping gene. For cycloheximide experiments, LNCaP cells were pre-treated for 30 minutes with the protein synthesis inhibitor, cycloheximide (10 $\mu$ g/ml) prior to the addition of SAHA (2.5 or 7.5 $\mu$ M). Real-time PCR analysis was performed by Mr Ben Copeland.

### **5.2.7 Immunoblot**

LNCaP cells were cultured with DMSO or SAHA (2.5, 5 or 7.5 $\mu$ M) for 12, 24 or 48 hr. Immunoblot analysis was performed as described in chapter 2. AR steady-state protein levels were determined by immunoblot using the AR-N20 antibody as described in chapter 2.

### **5.2.8 Statistical analysis**

Statistical differences between SAHA treatment groups and controls were determined by One-way ANOVA with Dunnett post-hoc test, using SPSS statistical analysis software. A p-value of <0.05 was considered statistically significant. Differences in mRNA expression of AR, PSA and KLK2 were determined by real-time PCR were analysed using Student's t-test. A p-value of <0.05 was considered statistically significant.

## 5.3 Results

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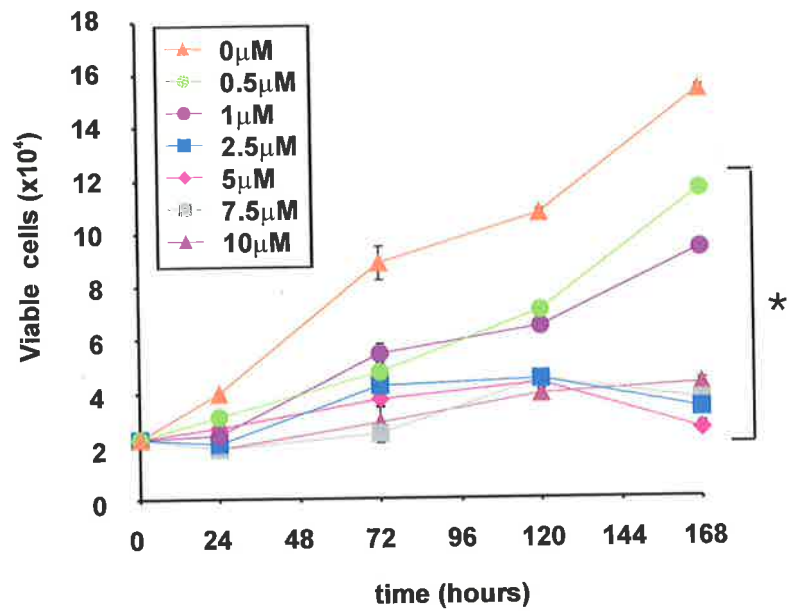
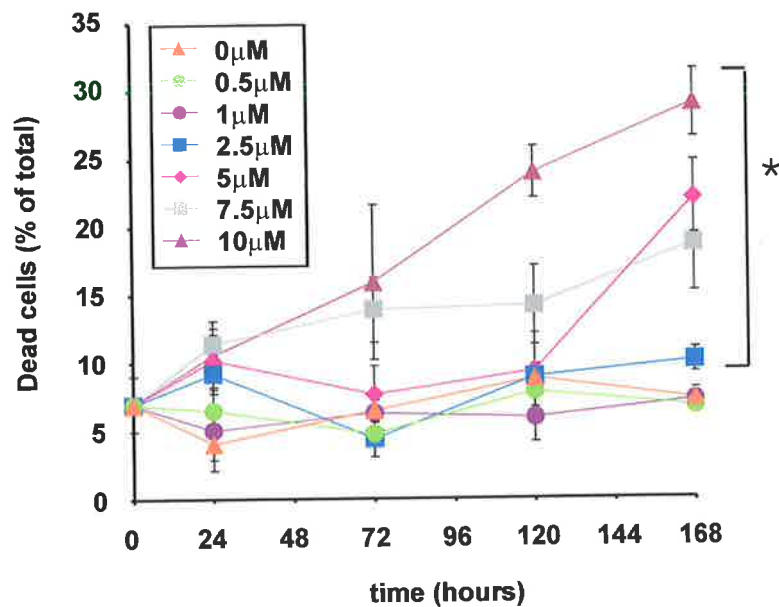
### 5.3.1 Effect of SAHA on prostate cancer cell growth

#### 5.3.1.1 SAHA suppresses the growth of LNCaP and PC-3 prostate cancer cells

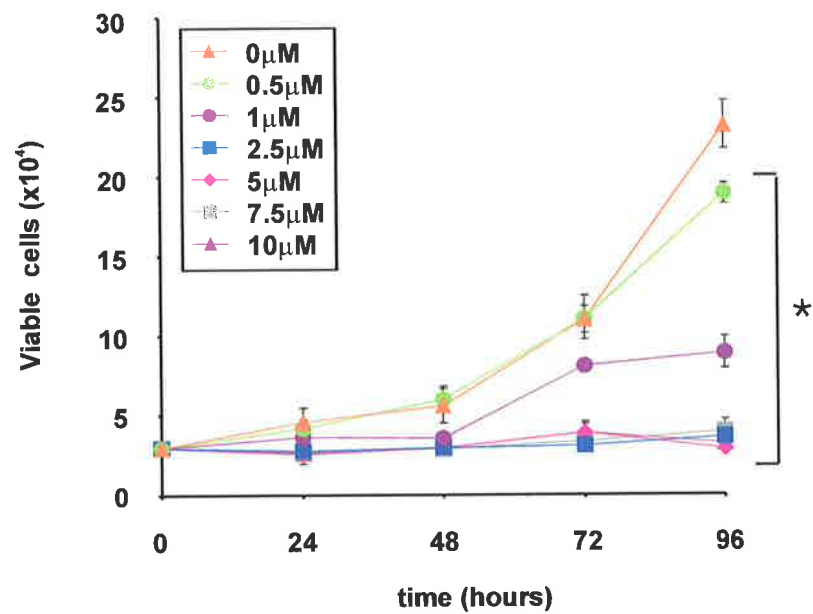
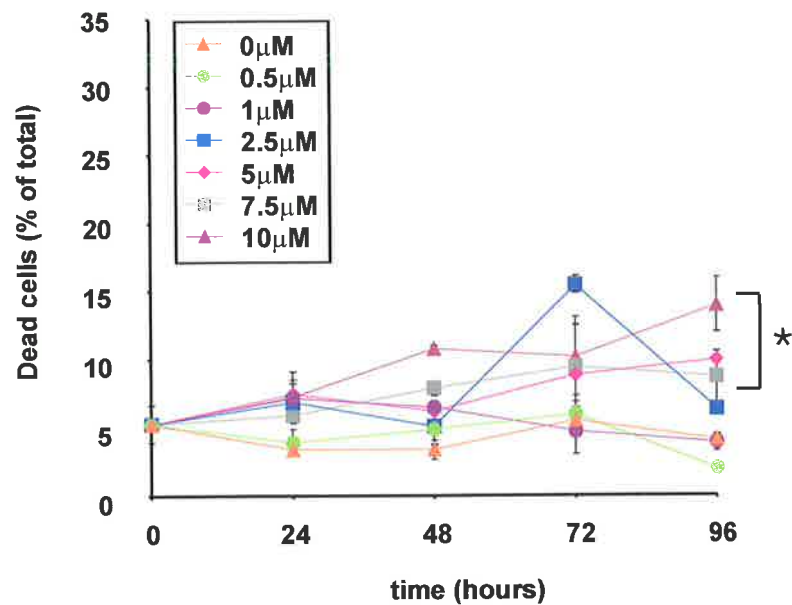
A dose-dependent inhibition of LNCaP cell growth was observed with increasing concentrations of SAHA (Figure 5.1A). While a significant growth inhibitory effect of SAHA was observed for up to 7 days with 0.5 $\mu$ M SAHA (Figure 5.1A), maximal reduction of cell number was achieved with 2.5 $\mu$ M and greater concentrations of SAHA. Minimal LNCaP cell death was observed with 0.5, 1 or 2.5 $\mu$ M of SAHA, but 5 and 7.5 $\mu$ M markedly induced cell death, with cell death reaching approximately 30% at 7 days post-treatment with 10 $\mu$ M SAHA (Figure 5.1B). Dose-dependent inhibition of PC-3 cell growth was also observed with SAHA (Figure 5.2A). Similarly to LNCaP cells, maximal reduction of cell number was achieved with 2.5 $\mu$ M SAHA. In contrast to LNCaP cells, cell death was induced to a maximum of approximately 15% in PC-3 cells following treatment with SAHA (Figure 5.2B).

#### 5.3.1.2 SAHA induces cell cycle arrest of LNCaP cells

To further investigate the mechanism of SAHA-induced suppression of cell growth, cell cycle distribution was analysed in control and SAHA-treated cells. After 24 hr culture with 2.5 $\mu$ M SAHA, 57% of LNCaP cells were arrested in the G1 phase of the cell cycle (Table 5.1). This block in cell cycle progression was observed for up to 72 hours post-treatment (data not shown). In contrast to the above findings, LNCaP cells cultured with 7.5 $\mu$ M SAHA showed a shift in distribution from S phase to the G2/M phase of the cell cycle, with no change in the proportion of cells in G1 (Table 5.1).

**A****B**

**Figure 5.1 - Inhibition of LNCaP prostate cancer cell growth by SAHA.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0, 0.5, 1, 2.5, 5, 7.5 or  $10 \mu\text{M}$ ) for up to seven days. (A) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  SAHA *versus* control.

**A****B**

**Figure 5.2 - Inhibition of PC-3 prostate cancer cell growth by SAHA.** PC-3 cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0, 0.5, 1, 2.5, 5, 7.5 or  $10 \mu\text{M}$ ) for up to four days. (A) Cells were counted every day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $P < 0.05$  SAHA *versus* control.



**Table 5.1 - Effect of SAHA on cell cycle distribution**

	<b>G1</b>	<b>G2/M</b>	<b>S</b>
0 $\mu$ M SAHA	57%	13%	30%
2.5 $\mu$ M SAHA	82%	15%	3%
7.5 $\mu$ M SAHA	53%	43%	4%

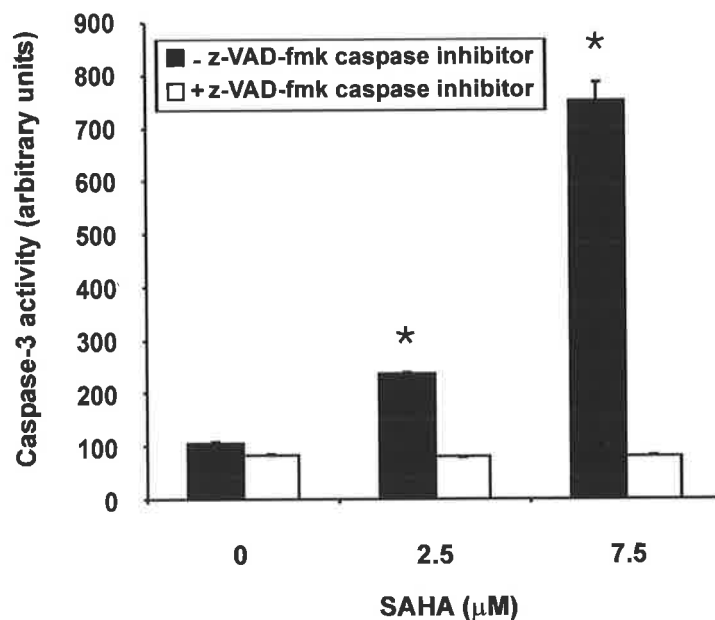
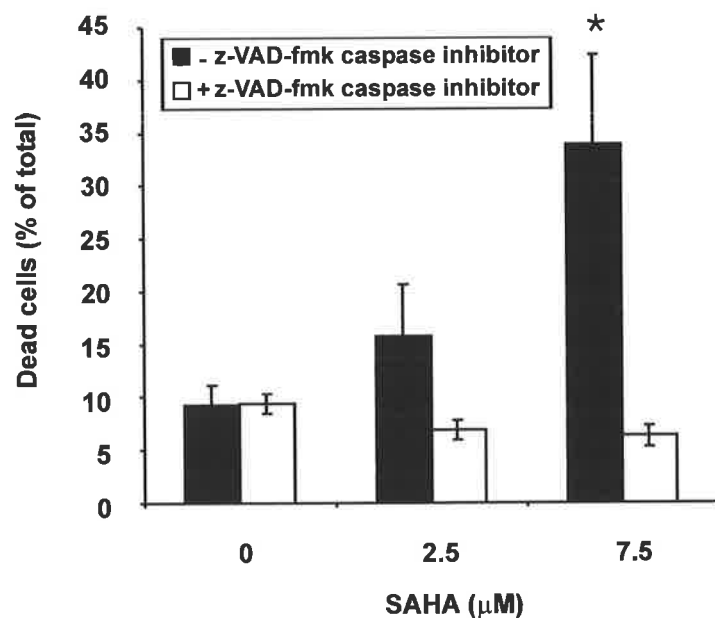
### *5.3.1.3 Cell death induced by SAHA is caspase-dependent*

Caspase-3 activity was significantly induced at 24 hr in LNCaP cells cultured with SAHA (2.5 or 7.5 $\mu$ M) (Figure 5.3A). This activity was completely blocked when cells were treated with the z-VAD-fmk pan-caspase inhibitor. To determine whether the induction of cell death by SAHA is dependent on caspase activity, LNCaP cells were cultured with SAHA (2.5 or 7.5 $\mu$ M) in the presence or absence of the z-VAD-fmk caspase inhibitor and cell viability was determined after 5 days of culture. LNCaP cell death was significantly increased to a maximum of approximately 35% following treatment with 7.5 $\mu$ M, compared to approximately 10% cell death observed in control cells. Addition of the caspase inhibitor prevented the induction of cell death by both 2.5 $\mu$ M and 7.5 $\mu$ M SAHA (Figure 5.3B), with the proportion of dead cells remaining below 10%.

### *5.3.2 Gene expression profiles in LNCaP prostate cancer cells cultured with SAHA*

#### *5.3.2.1 SAHA-induced gene expression changes in LNCaP cells*

Gene expression profiles were examined following culture of LNCaP cells with low (2.5 $\mu$ M) or high (7.5 $\mu$ M) concentrations of SAHA. LNCaP cells were cultured with SAHA for 6 hr prior to isolation of RNA. A >2-fold change in gene expression was considered significant. Approximately 1% of the total number of genes analysed were induced or repressed following culture with 2.5 or 7.5 $\mu$ M SAHA. As expected, the expression of the known SAHA target genes, cyclin D1 and thioredoxin-binding protein- 2/VDUP1, decreased and increased, respectively. For a summary of gene expression changes induced in LNCaP cells following treatment with SAHA refer to appendix 2.

**A****B**

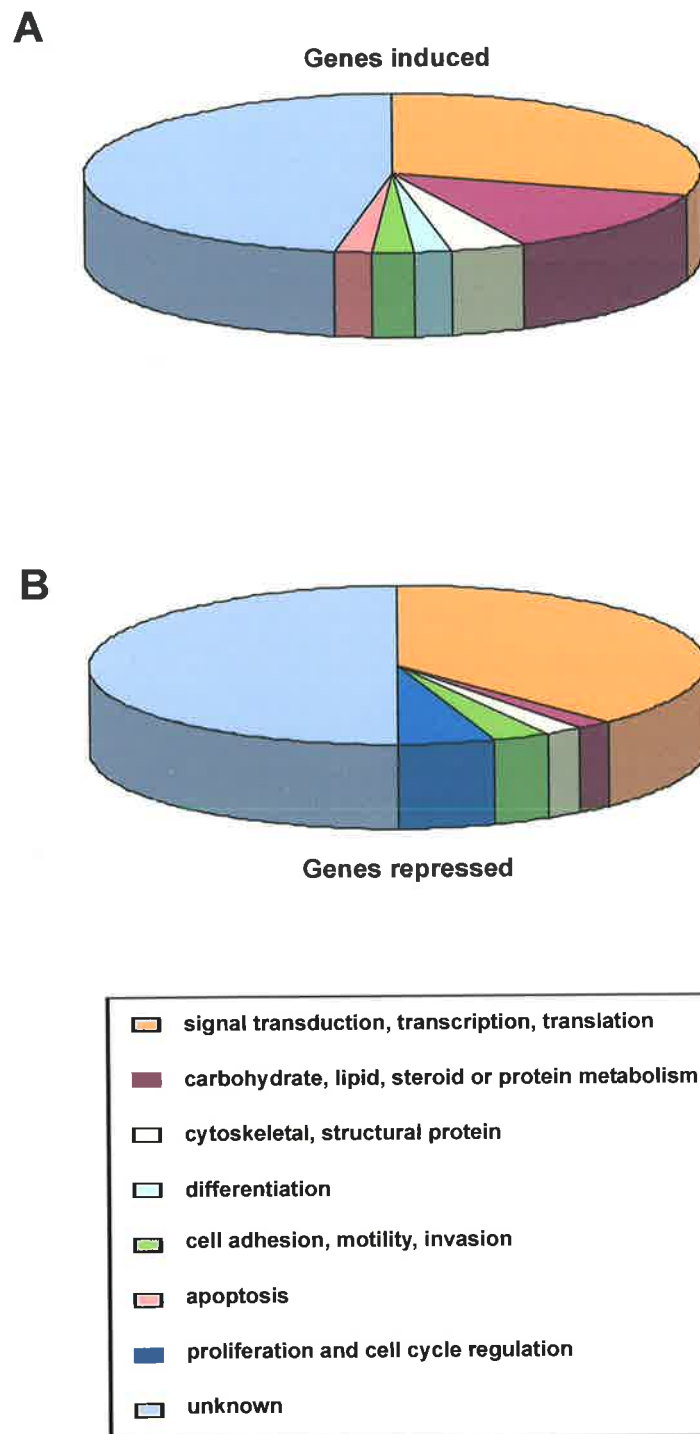
**Figure 5.3 - Caspase activity in LNCaP prostate cancer cells cultured with SAHA.**

(A) LNCaP cells ( $1.5 \times 10^4$ ) were cultured with SAHA (0, 2.5 or 7.5 μM) +/- the z-VAD-fmk caspase inhibitor (50 μM) for 48 hr and assayed for caspase-3 activity. (B) LNCaP cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0, 2.5 or 7.5 μM) +/- z-VAD-fmk (50 μM) caspase inhibitor for 5 days. Cells were counted at day 5 using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. The number of dead cells is expressed as % of total cells counted. Results are represented as the mean +/- the SE of triplicate wells. \* ANOVA;  $p < 0.05$  SAHA *versus* control.

The 1% of genes that were significantly induced or repressed following treatment with SAHA were divided into groups according to their function (Figure 5.4). Of this 1% of genes, a high percentage is as yet uncharacterised. The steady-state levels of a large proportion of genes with known function that were increased following treatment with SAHA are involved in signal transduction, transcription or translation (Figure 5.4A). A further 13% of induced genes are associated with carbohydrate, lipid, steroid or protein metabolism (Figure 5.4A), whereas 5% of the repressed genes are predominantly associated with proliferation and cell cycle regulation (Figure 5.4B).

#### 5.3.2.2 Alterations in genes associated with AR signalling

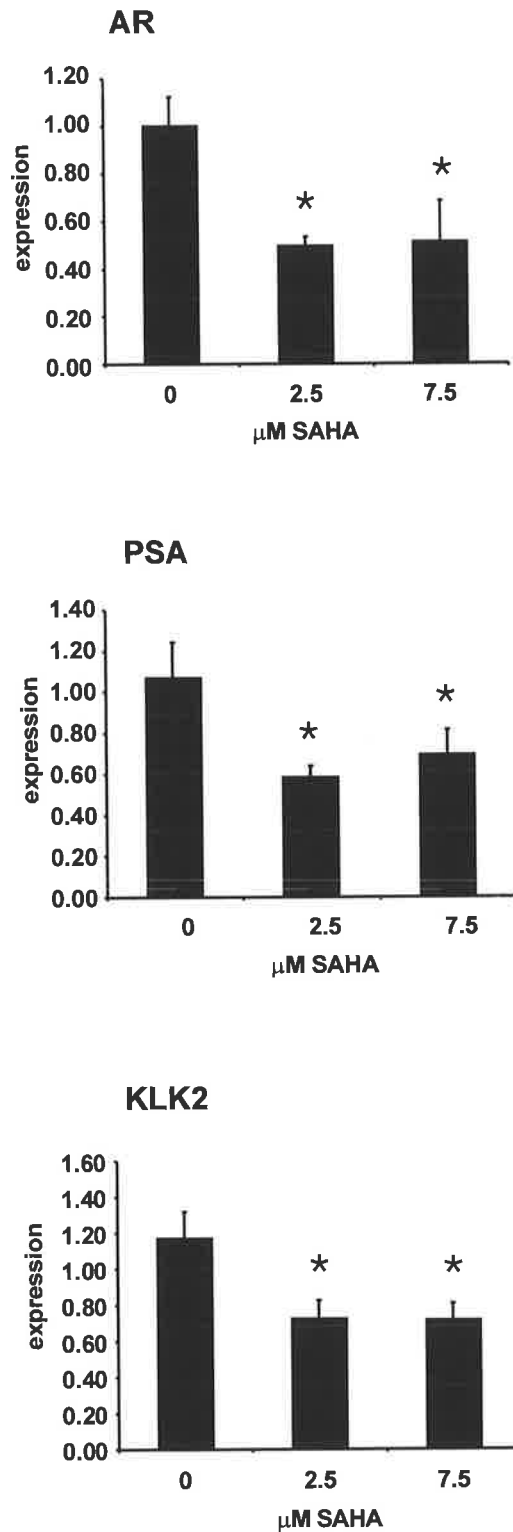
Interestingly, a subset of genes involved in AR signalling were repressed by at least 2-fold following treatment with SAHA (Table 5.2) (Sun *et al.* 1997; Lin *et al.* 1999; Pestell *et al.* 1999; Qi *et al.* 2003). Of particular interest, the steady-state mRNA levels of the AR itself and the AR-regulated genes, PSA and KLK2, were reduced in LNCaP cells cultured with SAHA compared to untreated controls. Whereas expression of AR was reduced by SAHA (2.5 and 7.5 $\mu$ M), expression of PSA and KLK2 was only decreased by 7.5 $\mu$ M SAHA. Real-time reverse transcription PCR confirmed that the levels of AR, PSA and KLK2 mRNA were significantly reduced in cells cultured with 2.5 or 7.5 $\mu$ M SAHA at 2 hr post-treatment (Figure 5.5). To determine whether the gene expression data identified by the microarray and mRNA expression analyses resulted in biologically meaningful changes in protein expression in the cell, the effects of SAHA on AR and PSA expression were evaluated by immunoblot analysis (Figure 5.6). AR steady-state protein levels were markedly reduced in a dose-dependent manner for up to 48 hr, with a corresponding decrease in the steady-state protein levels of PSA (Figure 5.6A). Protein levels of cyclin D1 and p21<sup>WAF1</sup>, which were used as controls



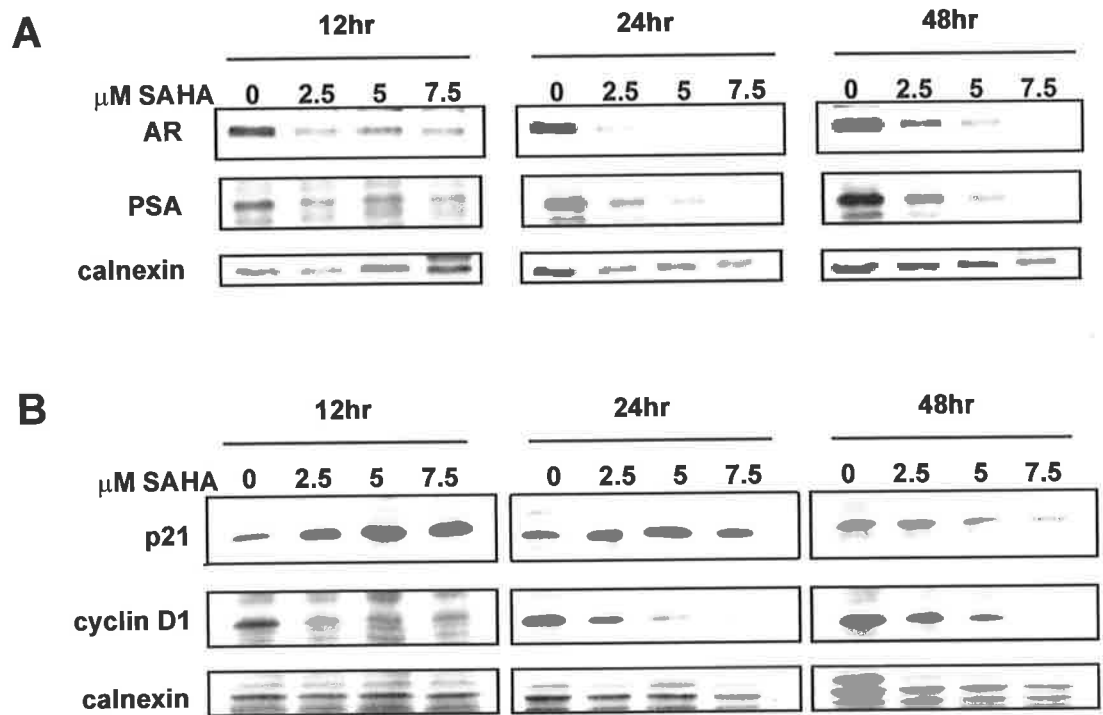
**Figure 5.4 – Summary of gene expression changes in LNCaP cells following treatment with SAHA. (A) Genes induced by SAHA (B) genes repressed by SAHA, expressed as a percentage of total number of gene alterations as determined by microarray analysis.**

**Table 5.2 : Genes involved in androgen signalling**

Genes repressed by SAHA	fold reduction	
	(2.5 $\mu$ M)	(7.5 $\mu$ M)
androgen receptor	3.6	2.5
Cyclin D1 ( <i>Pestell 1999</i> )	3.1	2.7
kallikrein 3 (PSA) ( <i>Sun 1997</i> )	-	2.6
kallikrein 2 ( <i>Sun 1997</i> )	-	2.2
NEDD4L ( <i>Qi 2003</i> )	2	-
transmembrane serine protease ( <i>Lin 1999</i> )	-	2



**Figure 5.5 – SAHA reduces steady-state mRNA levels of AR and AR-regulated genes in LNCaP cells.** Real-time PCR analysis of changes in AR, PSA and Kallikrein 2 (KLK2) mRNA steady-state levels 2 hr following treatment with 2.5 and 7.5 μM SAHA. Results are representative of two separate experiments. Real-time PCR analysis was performed by Mr Ben Copeland. \* Student's t-test;  $p < 0.05$  SAHA *versus* control.



**Figure 5.6 – Steady-state protein levels of AR, PSA, p21 and cyclinD1 in LNCaP prostate cancer cells following treatment with SAHA.** Lysates from cells treated with 0, 2.5, 5 or 7.5  $\mu\text{M}$  SAHA for 12, 24 or 48 hr were analysed by immunoblot for expression of (A) AR and PSA, and (B) cyclin D1 and p21<sup>WAF1</sup>. For each immunoblot calnexin was used as a loading control and is shown in the lower panels. Images are representative of three separate experiments.



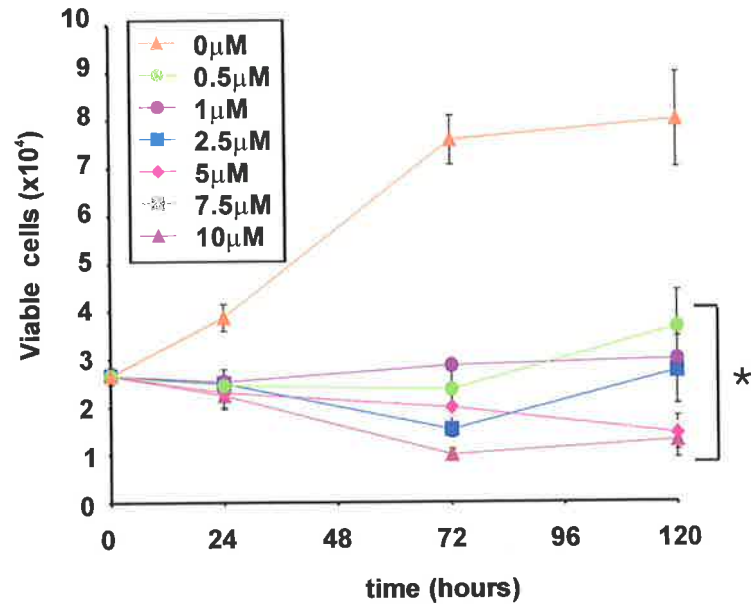
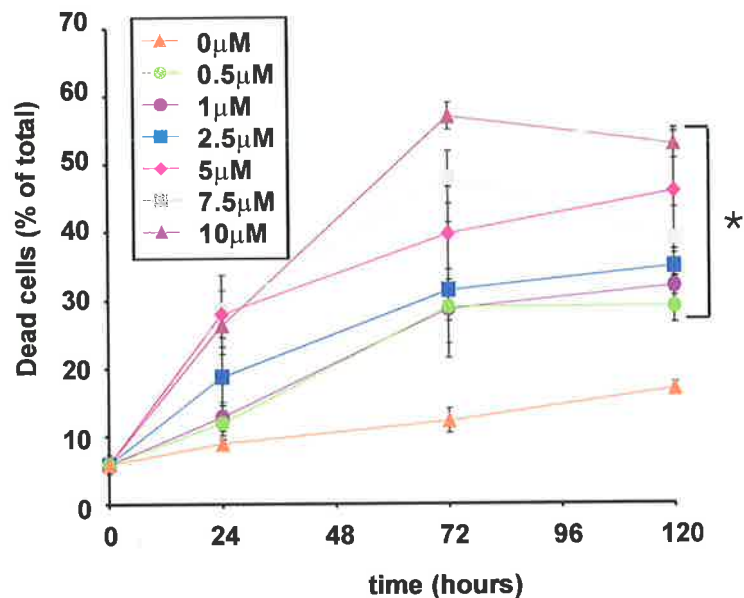
for SAHA activity, were consistently decreased and increased, respectively, in a dose-dependent manner by SAHA (Gui *et al.* 2004; Sakajiri *et al.* 2005) (Figure 5.6B).

### ***5.3.3 Activity of SAHA is enhanced by culture in steroid depleted medium***

To determine the effect of SAHA on LNCaP prostate cancer cells in the absence of steroids, LNCaP cells were cultured in medium comprising 10% charcoal stripped FCS (testosterone levels not detectable, refer to section 4.3.3) in the presence of increasing concentrations of SAHA. Compared to experiments conducted in medium containing normal serum (Figure 5.1), culture in steroid depleted medium substantially increased sensitivity of LNCaP cells to SAHA. Cell growth was completely suppressed by treatment with 0.5 $\mu$ M SAHA (Figure 5.7A), while equivalent growth inhibitory effects in normal medium required concentrations of SAHA in excess of 2.5 $\mu$ M (Figure 5.1A). Similarly there was a significant increase in the percentage of LNCaP cell death with all doses of SAHA tested, which reached a maximum of approximately 60% with 10 $\mu$ M SAHA (Figure 5.7B). This was approximately two times the percent of cell death observed in normal serum with the same concentrations of SAHA (Figure 5.1B). AR steady-state protein levels were reduced to a greater extent in charcoal stripped FCS compared to normal FCS in response to low levels of SAHA (2.5 $\mu$ M) (Figure 5.8B). These results were derived from a single experiment and are yet to be confirmed.

### ***5.3.4 SAHA inhibits proliferation of and decreases AR levels in the C42B 'androgen independent' prostate cancer cell line***

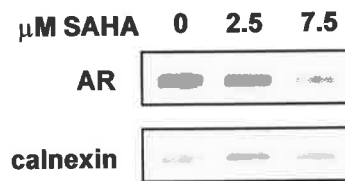
The AR-positive, androgen-independent C42B cell line is a sub-line of LNCaP cells passaged in castrated mice (Thalmann *et al.* 1994). Proliferation of C42B cells was

**A****B**

**Figure 5.7 - Effect of SAHA on LNCaP cells cultured in steroid-depleted medium.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured in medium containing charcoal stripped serum in the presence of SAHA (0, 0.5, 1, 2.5, 5, 7.5 or  $10 \mu\text{M}$ ) for up to five days. (A) Cells were counted every second day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  SAHA *versus* control.

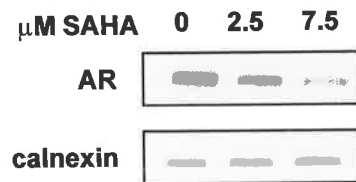
**A**

Normal serum



**B**

Stripped serum



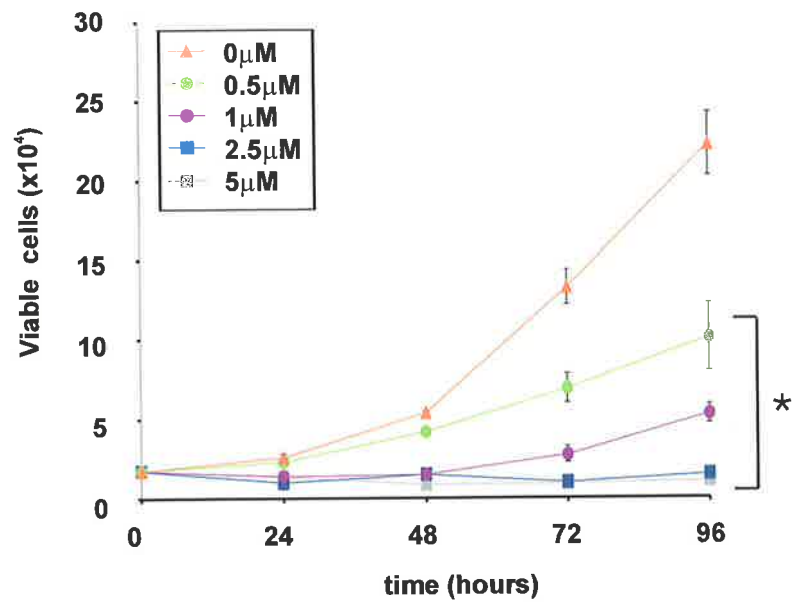
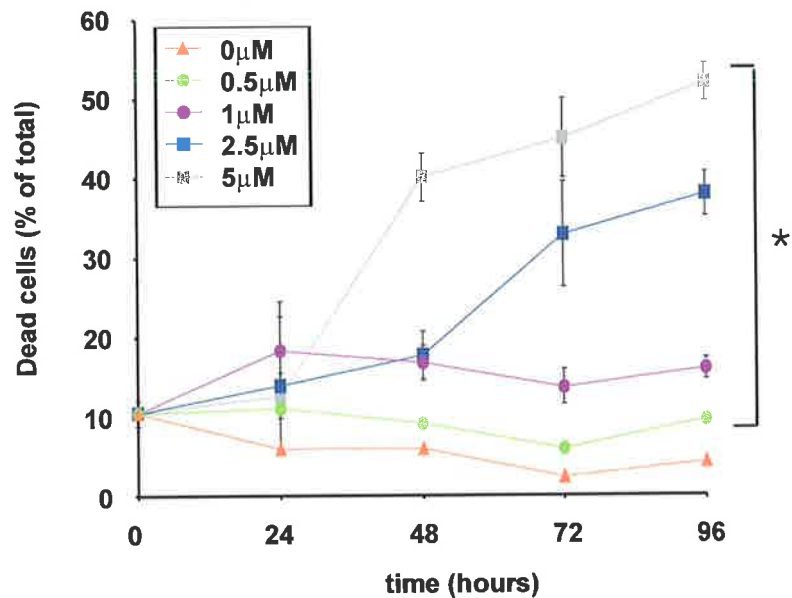
**Figure 5.8 – Effect of SAHA on AR protein levels in the absence of steroids.** Expression of AR in LNCaP cells cultured in (A) 10% normal serum or (B) 10% charcoal stripped serum. Lysates were derived from LNCaP cells treated with 0, 2.5 or 7.5 $\mu\text{M}$  SAHA for 24 hr. For each immunoblot calnexin was used as a loading control and is shown in the lower panels.

inhibited by approximately 60% with 0.5 $\mu$ M SAHA; treatment with 2.5 $\mu$ M SAHA resulted in 100% growth inhibition (Figure 5.9A). Cell death was increased in a dose-dependent manner, to a maximum of 50% with 5 $\mu$ M SAHA (Figure 5.9B). Steady-state levels of AR were reduced in C42B cells with 2.5 and 7.5 $\mu$ M SAHA at 12 and 24 hr (Figure 5.10). A maximal reduction in AR steady-state protein levels were observed at 24 hr with 7.5 $\mu$ M SAHA. A corresponding decrease in PSA steady-state protein was observed with 2.5 and 7.5 $\mu$ M SAHA compared to controls, which was maintained for at least 48 hr (Figure 5.10).

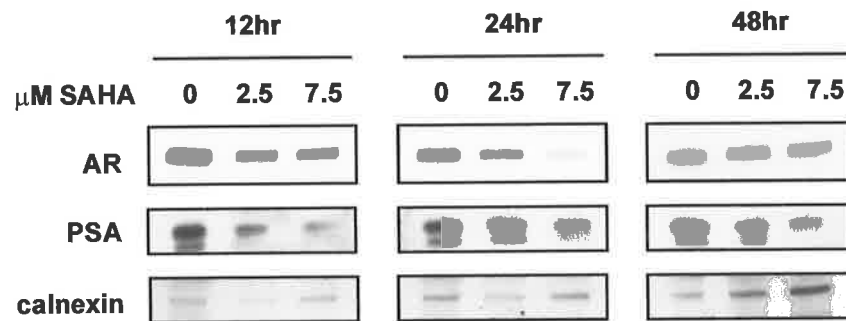
### ***5.3.5 Mechanism(s) of reduction in AR following SAHA treatment***

#### *5.3.5.1 Hsp90 client proteins are reduced in LNCaP cells following treatment with SAHA*

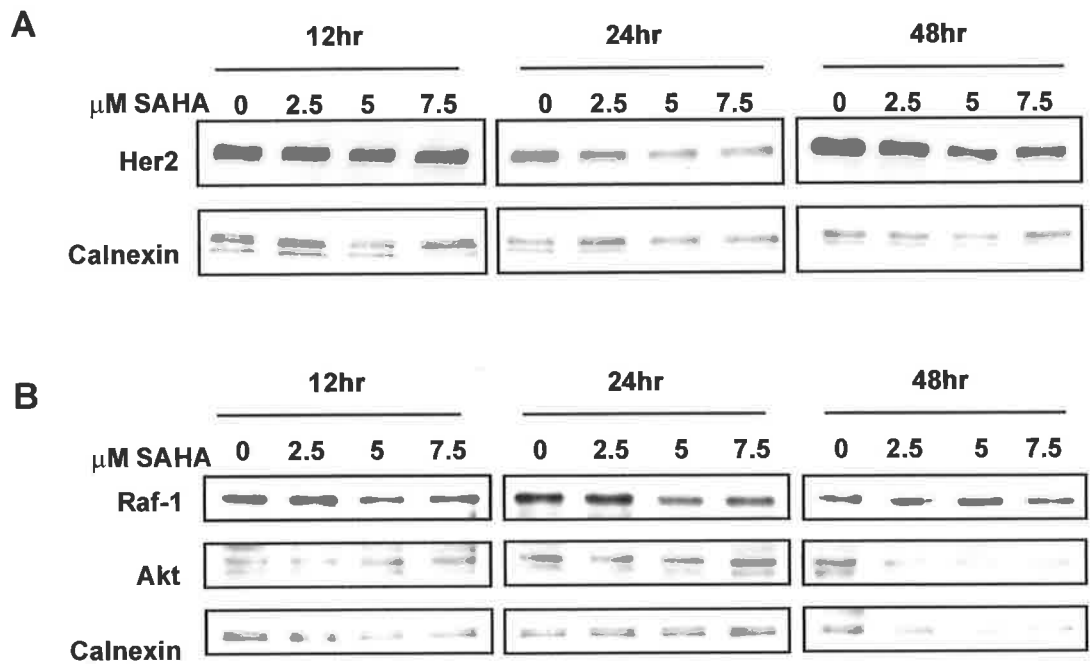
The same lysates previously analysed for expression of AR and PSA by immunoblot (Figure 5.6) were used for analysis of the Hsp90 client proteins Her2, Raf-1 and Akt in LNCaP cells following culture with SAHA. Steady-state levels of Her2, Raf-1 and Akt were reduced in LNCaP cells following treatment with 2.5, 5 and 7.5 $\mu$ M SAHA (Figure 5.11A). A maximal reduction in Her2 steady-state protein levels was observed following culture with 5 and 7.5 $\mu$ M SAHA (Figure 5.11A), which was maintained for up to 48 hr. Steady-state protein levels of Raf-1 were also reduced at 24 hr with both doses of SAHA, however the reduction returned to control levels by 48 hr post-treatment (Figure 5.11B). In contrast steady-state protein levels of Akt were unaffected 12 and 24 hr post-treatment with SAHA. However, a reduction in steady-state protein levels of Akt was observed at 48 hr (Figure 5.11B). The Hsp90 client proteins detected in these lysates were not reduced to the same extent as AR following treatment with SAHA (Figure 5.6A).

**A****B**

**Figure 5.9 - Inhibition of C42B prostate cancer cell proliferation by SAHA.** C42B cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0, 0.5, 1, 2.5 or  $5 \mu\text{M}$ ) for up to four days. (A) Cells were counted every day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  SAHA *versus* control.



**Figure 5.10 – Steady-state protein levels of AR and AR-regulated genes in C42B cells following treatment with SAHA.** Lysates from cells treated with 0, 2.5 or 7.5 $\mu\text{M}$  SAHA for 12, 24 or 48 hr were analysed for expression of AR and PSA by immunoblot. Calnexin was used as a loading control and is shown in the lower panels.



**Figure 5.11 – Steady-state protein levels of hsp90 client proteins in LNCaP prostate cancer cells following treatment with SAHA.** Lysates from cells treated with 0, 2.5, 5 or 7.5 $\mu$ M SAHA for 12, 24 or 48 hr were analysed by immunoblot for expression of (A) Her2, and (B) Raf-1 and Akt. For each immunoblot calnexin was used as a loading control and is shown in the lower panels. Images are representative of two separate experiments.

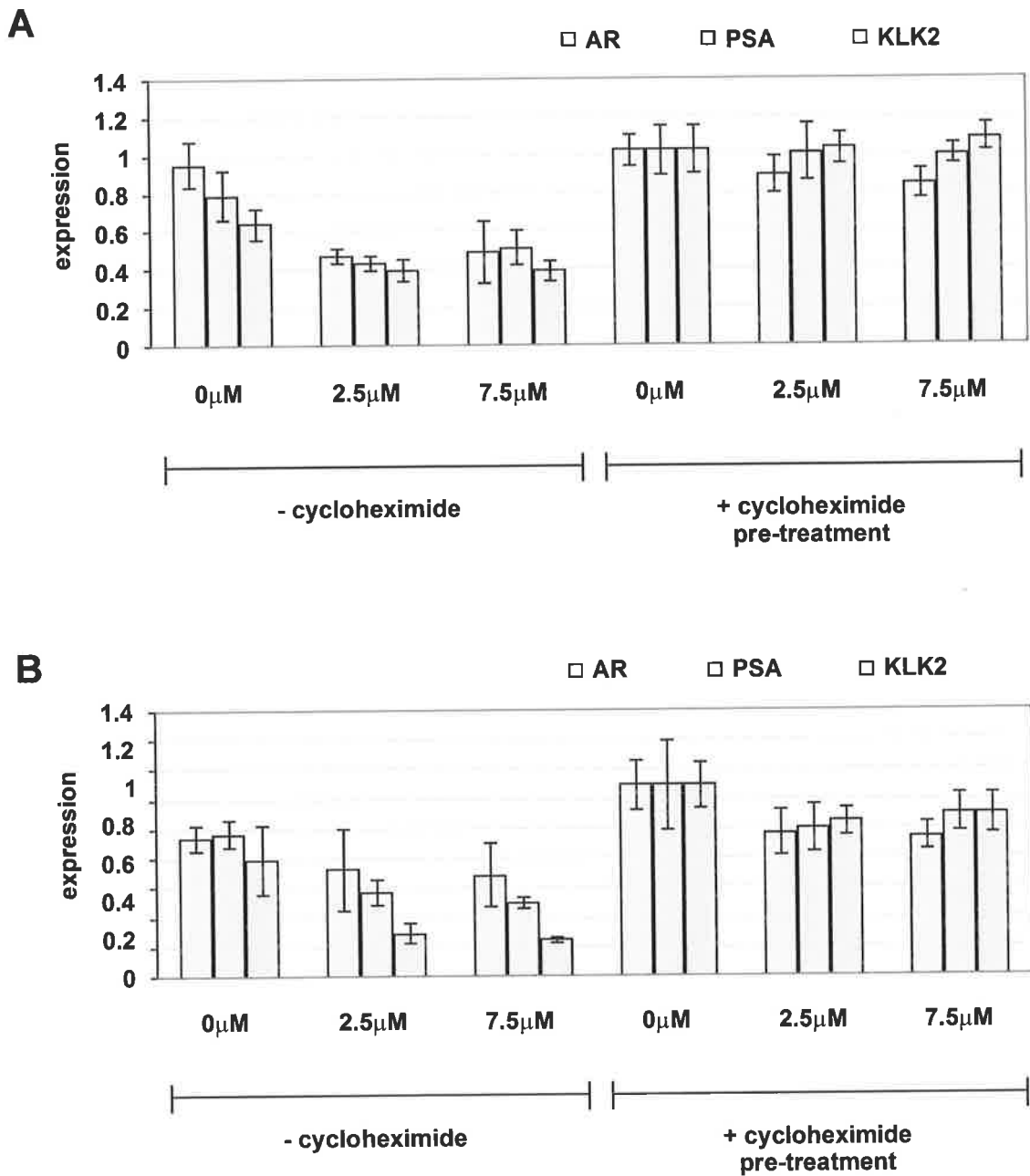
#### *5.3.5.2 SAHA-induced reduction in AR mRNA requires protein synthesis*

To determine whether the decrease in AR mRNA was a direct or indirect effect of SAHA, LNCaP cells were pre-treated with the protein synthesis inhibitor, cycloheximide for 30minutes<sup>1</sup>. Following pre-treatment with cycloheximide, LNCaP cells were cultured with SAHA (2.5 or 7.5µM) and mRNA levels were assessed using real-time PCR. Treatment with either 2.5 or 7.5µM SAHA for 2 hr resulted in an approximate 50% decrease in AR mRNA compared to controls (Figure 5.12A). A corresponding decrease in the AR-regulated genes PSA and KLK2 was observed at 2 hr, and this was maintained for up to 6 hr post-treatment with either 2.5 or 7.5µM SAHA (Figure 5.12A&B). Pre-treatment of cells with cycloheximide inhibited the reduction in AR, PSA and KLK2 (Figure 5.12A&B). In the presence of cycloheximide steady-state mRNA levels of AR, PSA and KLK2 were higher than observed in control cells. These results were acquired from a single experiment and are yet to be confirmed.

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<sup>1</sup> Cycloheximide inhibits >85% of protein synthesis in LNCaP cells within 1 hr in (Dr Lisa Butler, personal communication)





**Figure 5.12 - Changes in AR, PSA and KLK2 mRNA steady-state levels following treatment with SAHA +/- cycloheximide.** LNCaP cells pre-treated for 30 minutes +/- cycloheximide (10  $\mu$ g/ml) were cultured with SAHA (2.5 and 7.5  $\mu$ M). Expression of AR and the AR-regulated genes PSA and KLK2 are shown from cells harvested at (A) 2 hr or (B) 6 hr post-treatment. Real-time PCR was performed by Mr Ben Copeland.

## 5.4 Discussion

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The results of the current study demonstrate antiproliferative effects in LNCaP, PC-3 and C42B prostate cancer cell lines. However, cell death was induced to a greater extent in the AR-positive cell lines, LNCaP and C42B. These data suggest that a component of SAHA activity in prostate cancer cells may relate to the presence of a functional androgen signalling axis. As the majority of prostate cancers that relapse following AAT are AR-positive (Hobisch *et al.* 1995; Bentel and Tilley 1996), this would be an advantage for the treatment of castrate-resistant tumours.

Investigation of alterations in gene expression following SAHA treatment of LNCaP cells may lead to a greater understanding of the mechanisms involved in SAHA-induced cell death, and allow for enhancement of cell death in prostate cancer cells. In the current study microarray analysis was performed to further investigate the effect of SAHA on LNCaP cell gene expression. Of note, upregulation of p21, metallothionein 1L, and down regulation of importin was observed in the current study. These genes have been shown to be part of common gene alterations associated with HDAC inhibitor treatment (Glaser *et al.* 2003), providing validation of the data obtained in the current study. While previously noted alterations in gene expression were observed in the current study, an important finding was the effect of SAHA on AR mRNA and protein expression in LNCaP cells. The 1% of genes that were either induced or repressed in response to SAHA included the AR and several AR-regulated genes, such as PSA, KLK2, NEDD4L and transmembrane serine protease (Sun *et al.* 1997; Lin *et al.* 1999; Qi *et al.* 2003). The reduction in AR expression likely contributes to the effect of SAHA on LNCaP cell proliferation and survival, and may explain the greater induction of cell death by SAHA in LNCaP and C42B cells compared to the AR-negative PC-3 cells. Culture of LNCaP cells in steroid depleted medium enhanced

the inhibitory effects of SAHA on LNCaP cell proliferation and survival. Moreover, there was an enhanced reduction in AR and PSA steady-state protein levels in LNCaP cells cultured in steroid depleted medium. Taken together, these data demonstrate that a component of SAHA activity in LNCaP prostate cancer cells is through reduction of AR, and that sensitivity of SAHA can be increased by manipulation of androgen signalling resulting in enhanced inhibition of prostate cancer cell proliferation and survival.

The results of the current study are consistent with recent reports demonstrating antiproliferative and proapoptotic effects of a range of HDAC inhibitors in DU145, PC-3, LAPC-4 and LNCaP prostate cancer cells (Kuefer *et al.* 2004; Thelen *et al.* 2004; Qian *et al.* 2004; Chen *et al.* 2005; Chinnaiyan *et al.* 2005; Gediya *et al.* 2005; Rokhlin *et al.* 2006; Sonnemann *et al.* 2006). These studies reported that cell death induced by HDAC inhibitors in prostate cancer cells is mediated through a caspase-dependent pathway (Thelen *et al.* 2004; Fronsdal and Saatcioglu 2005; Sonnemann *et al.* 2006), and can be inhibited by treatment with caspase inhibitors (Sonnemann *et al.* 2006). In addition, recent publications have demonstrated a reduction in AR and PSA expression in prostate cancer cells following treatment with the HDAC inhibitors LAQ824 (Chen *et al.* 2005) and trichostatin A (TSA) (Wang *et al.* 2004; Rokhlin *et al.* 2006). The mechanism involved in HDAC inhibitor-induced AR reduction is suggested to be through inactivation of Hsp90 by acetylation (Chen *et al.* 2005). It has been established that inhibition of Hsp90 activity causes degradation of its client proteins, including the AR (Fuino *et al.* 2003; Nimmanapalli *et al.* 2003; Bali *et al.* 2005a). Several studies have shown an increase in acetylated Hsp90 following treatment with the HDAC inhibitors LAQ824 (Fuino *et al.* 2003; Nimmanapalli *et al.* 2003; Bali *et al.* 2005a; Chen *et al.* 2005), SAHA (Bali *et al.* 2005b) and depsipeptide (Yu *et al.* 2002). Acetylation of Hsp90 causes a decrease in ATP-binding (Bali *et al.* 2005a) and therefore inactivation of Hsp90 and dissociation from client proteins, leading to

their proteasomal degradation (Bali *et al.* 2005a; Kovacs *et al.* 2005). Degradation of several Hsp90 client proteins including, Bcr-Abl, Her2, Akt, c-Raf, and AR (Yu *et al.* 2002; Fuino *et al.* 2003; Nimmanapalli *et al.* 2003; Bali *et al.* 2005a; Bali *et al.* 2005b; Chen *et al.* 2005) has been observed following treatment with HDAC inhibitors. Acetylation of Hsp90 is regulated by HDAC6 and its inactivation results in an increase in Hsp90 acetylation and loss of chaperone activity (Kovacs *et al.* 2005). In HDAC6 knockout cells there is decreased chaperone activity of Hsp90, which can be restored by re-expression of HDAC6 (Murphy *et al.* 2005). Moreover, direct binding of HDAC6 to Hsp90 has been demonstrated (Bali *et al.* 2005a), and the effect of LAQ-824 can be reversed by over-expression of HDAC6.

Although the results of the current study demonstrate modest reductions in expression of the Hsp90 client proteins Her2, Akt and c-Raf, the reduction in steady-state protein levels is markedly less than observed for AR. Her2 is reportedly much more sensitive to Hsp90 inhibition than AR (Solit *et al.* 2002; Smith-Jones *et al.* 2004), suggesting that inactivation of Hsp90 may not be the primary mechanism involved. In addition, preliminary experiments conducted in the current study did not demonstrate an increase in levels of acetylated Hsp90 following treatment with SAHA (data not shown). Consistent with the hypothesis of an alternative mechanism for SAHA-induced AR reduction in LNCaP cells, a rapid reduction in AR mRNA following treatment with SAHA was observed in the current study. Addition of the protein synthesis inhibitor cycloheximide blocked the reduction in AR mRNA levels as well as the reduction of the AR-regulated genes PSA and KLK2, demonstrating the necessity for de novo protein synthesis. A hypothesis that may explain this observation is that expression of an AR-transcriptional suppressor gene is up-regulated following treatment with SAHA, which is responsible for the indirect reduction of AR levels in LNCaP cells. Interestingly, addition of cycloheximide alone resulted in an

increase in AR mRNA levels compared to control levels. By inhibiting protein synthesis, cycloheximide may reduce the levels of the aforementioned putative AR suppressor protein, thereby resulting in the observed increase in steady-state AR mRNA levels. A balance of this putative suppressor protein might exist in prostate cancer cells, which may contribute to the regulation of AR activity. This mechanism of AR regulation is potentially a novel target for inhibiting AR activity, and future studies aimed at identification and specific inhibition of this protein could lead to the development of novel AR-targeting drugs. The results presented in this chapter are consistent with a study by Rokhlin *et al.* (2006) who demonstrated a reduction in AR mRNA following treatment of LNCaP cells with the HDAC inhibitor TSA. They demonstrated a reversal of TSA-induced AR reduction by culturing cells with the inhibitor of transcription, actinomycin D, indicative of indirect inhibition of AR gene expression through transcription of another gene. Taken together these data indicate that HDAC inhibitors reduce AR expression indirectly through transcription of a gene that may be involved in regulating AR transcription. Consistent with this hypothesis, Wang *et al.* (2004) suggest that an AR transcriptional suppressor complex is increased following treatment with TSA and acts to inhibit transcription of the AR gene. In addition, they demonstrate that this suppressor complex is lost in ‘androgen-independent’ cell lines, but can be restored by TSA treatment.

This observation, together with the fact that HDAC inhibitors reduce AR expression in prostate cancer cells suggests that these agents would be effective in treating castrate-resistant prostate cancer. The so-called ‘androgen-independent’ C42B cell line was used as a model to further investigate the effect of SAHA for the treatment of castrate-resistant prostate cancer. C42B prostate cancer cells were derived from serially passaging LNCaP cells in castrated athymic mice (Thalmann *et al.* 1994) resulting in a cell line that is unresponsive to hormonal manipulation, however contains a functional AR (Denmeade *et*

*al.* 2003). This cell line expresses the androgen regulated genes PSA and KLK2 in the absence of androgens, while an increase in expression of these genes is observed upon addition of androgens (Denmeade *et al.* 2003), indicative of an active androgen signalling axis. Results of the current study demonstrate reduced proliferation and increased cell death, as well as a reduction in AR and PSA steady-state protein levels in C42B cells cultured with SAHA. While it is not possible from these studies to attribute the activities of SAHA in C42B cells to its inhibition of AR expression, it is tempting to speculate that a reduction in AR levels in these cells induces growth arrest and cell death. There are no studies currently in the literature that have attempted to specifically knockdown AR in C42B cells in an attempt to determine whether they require AR signalling for survival, however there are studies that have addressed this question in other AR-positive, androgen-independent cell lines. For example specific inhibition of AR protein expression in LNCaP-derived androgen-independent cell lines by antisense oligonucleotides, AR-antibodies or hammerhead ribozymes results in growth arrest and apoptosis in these cells (Eder *et al.* 2000; Zegarra-Moro *et al.* 2002). These studies demonstrate that while the cells are able to grow in an androgen depleted environment, they still require AR signalling for survival. This requirement may be common to C42B cells as they are also derived from LNCaP cells by prolonged passage in an androgen depleted environment. Taken together, these data provide preliminary evidence that SAHA may be effective against castrate-resistant prostate cancer.

In summary, the results of this chapter demonstrate that SAHA markedly reduces AR mRNA and protein levels in LNCaP prostate cancer cells and results in cell cycle arrest and apoptosis. While the microarray and real-time RT-PCR analyses indicate that expression of AR is reduced by SAHA at the level of mRNA, acetylation of non-histone proteins by SAHA, such as the chaperone protein Hsp90, could also influence steady-state AR protein

levels. Taken together, the results suggest that inhibition of androgen signalling is an important component of SAHA activity in prostate cancer cells, and that androgen withdrawal or blockade may sensitise prostate cancer cells to undergo cell death in response to histone deacetylase inhibitors. Given that the majority of clinical prostate tumours express the AR, including those that fail hormonal therapy, further investigation into the use of SAHA for the treatment of prostate cancer is warranted, particularly in the context of combination therapy with conventional AAT. The fact that SAHA can target more than one pathway, including AR signalling, makes it attractive for use as targeting multiple signalling pathways in cancer may lead to a better therapeutic outcome. Although this strategy of targeting multiple signalling pathways has the potential to increase side-effects, clinical trials have demonstrated acceptable toxicity profiles in patients treated with SAHA (Kelly *et al.* 2003; Kelly *et al.* 2005).

## **Chapter 6**

### **Combination of AR-targeting Agents Synergistically Inhibits Proliferation and Induces Death in LNCaP Cells**



## 6.1 Introduction

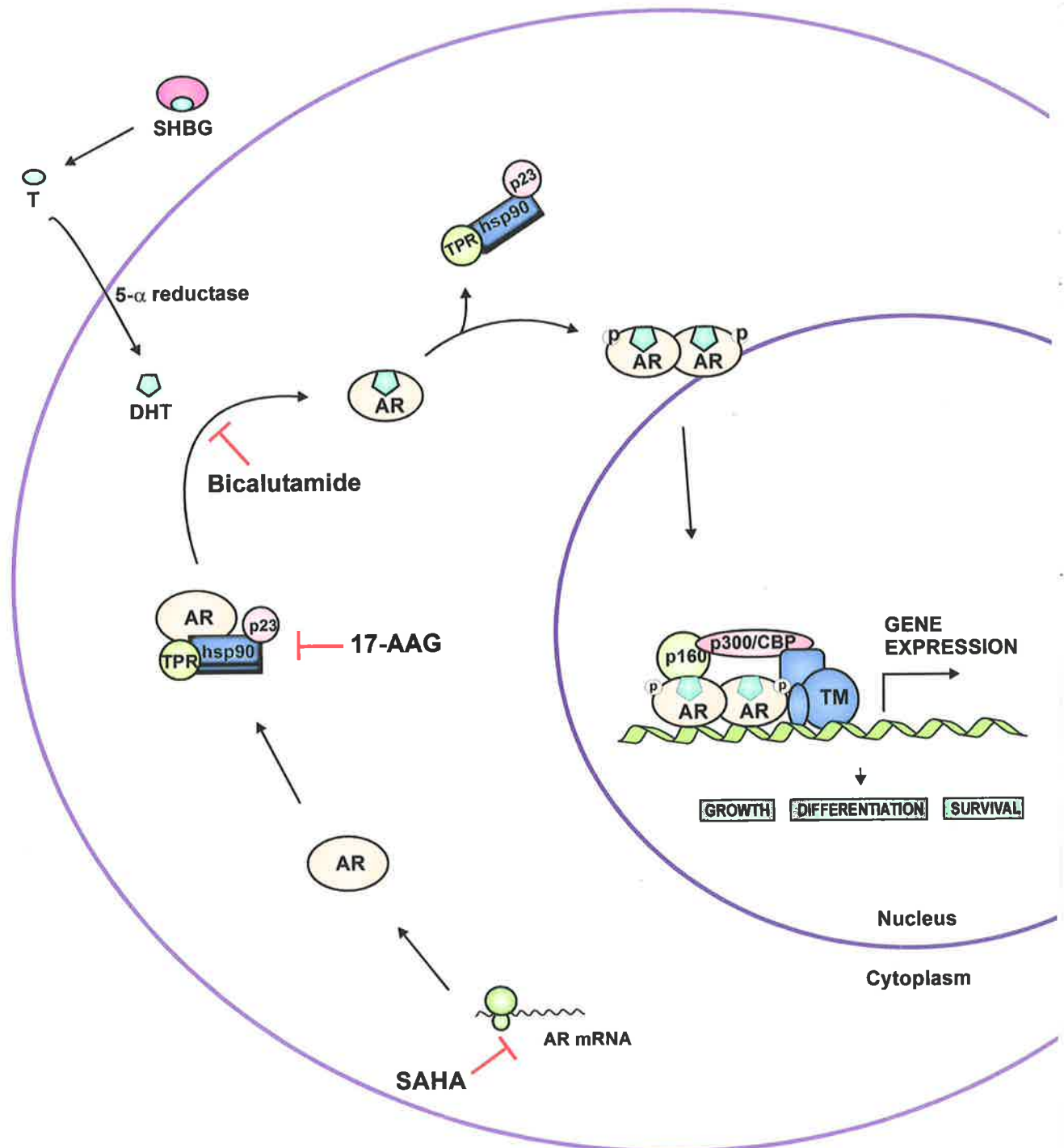
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The studies described in chapters 4 and 5 revealed enhanced activity of 17-AAG and SAHA when LNCaP cells were cultured in a steroid depleted environment (refer to sections 4.3.3 and 5.3.3). These results suggested that prostate cancer cells could be sensitised to other anticancer agents with AR-targeting activities by reducing ligand-bioavailability, leading to the hypothesis that simultaneous targeting of the AR at different sites in the signalling pathway may enhance the antiproliferative and proapoptotic effects observed. There are several points in the androgen signalling axis that can be inhibited by chemical agents (refer to figure 1.5). The studies outlined in chapters 3-5 have described anticancer agents that also inhibit AR activity through different mechanisms and at different points in the AR signalling pathway. A combination of these agents with specific AR-targeting agents, such as the AR-antagonist, bicalutamide, may increase the efficacy of the drugs and potentially minimise their side-effects. In addition, targeting multiple points in the androgen signalling axis may prevent the outgrowth of a resistant population of cells with mechanisms that allow AR signalling despite therapy (as described in section 1.6). The current study therefore investigated the effects of combinatorial strategies on prostate cancer cell growth using the Hsp90 inhibitor 17-AAG the HDAC inhibitor SAHA or the AR-antagonist bicalutamide.

Bicalutamide binds to the AR, preventing its activation and leading to inhibition of prostate cancer cell proliferation and survival (Furr and Tucker 1996). Whereas bicalutamide therapy has been associated with significant increases in quality of life compared to castration, patients with non metastatic, locally advanced prostate cancer treated with bicalutamide monotherapy show no statistical difference in overall survival

or time to progression when compared with castration (Iversen *et al.* 1998; Iversen *et al.* 2000). High dose bicalutamide therapy however, is linked to cardiovascular toxicity (Wirth *et al.* 2004). Combining bicalutamide with other agents at lower doses than are currently used may reduce the cardiovascular side-effects observed at higher doses. In addition, prolonged therapy with bicalutamide has been associated with antiandrogen withdrawal syndrome (Schellhammer *et al.* 1997). Mutations in the AR identified in prostate cancer cells following prolonged bicalutamide exposure result in promiscuous activation of the AR by non-classical ligands, including bicalutamide itself (Hara *et al.* 2003a; Bohl *et al.* 2005; Yoshida *et al.* 2005). Simultaneous targeting of the AR with bicalutamide and another agent with AR-targeting activity may prevent tumour relapse following prolonged bicalutamide monotherapy.

The specific aim of this chapter was to determine whether targeting the AR at multiple points in the signalling pathway (Figure 6.1), using agents that target both the ligand and the receptor would have enhanced effects of prostate cancer cell proliferation and survival. The three combinatorial strategies described in this chapter are: bicalutamide and SAHA, bicalutamide and 17-AAG or SAHA and 17-AAG.



**Figure 6.1 - Targeting the AR at different points in the androgen signalling axis.** Signalling through the androgen receptor can be inhibited at multiple stages in the androgen signalling axis. Agents used to inhibit AR signalling in this thesis are shown. The combination AR-targeting strategies employed in the current studies are 1) Bicalutamide + SAHA, 2) Bicalutamide + 17-AAG, and 3) SAHA + 17-AAG.

## **6.2 Methods**

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### **6.2.1 Drugs**

Bicalutamide (AstraZeneca Pharmaceuticals) was dissolved in 100% DMSO and stored as  $10^{-2}$  stock solution at  $-20^{\circ}\text{C}$ .

17-AAG was provided by Professor Neal Rosen (Memorial Sloan-Kettering Cancer Centre, New York). Stock solutions of 1mM 17-AAG were dissolved in 100% DMSO and stored at  $-20^{\circ}\text{C}$ .

SAHA was provided by Dr Victoria Richon (Aton Pharma, Tarrytown, New York). Stock solutions of 100mM SAHA were dissolved in 100% DMSO and stored at  $-70^{\circ}\text{C}$ . Working solutions of 10mM SAHA were dissolved in 100% DMSO and stored at  $-20^{\circ}\text{C}$ .

### **6.2.2 Growth curves**

The effect of AR-targeting agents alone or in combination on LNCaP and PC-3 cell proliferation was assessed by generating growth curves. LNCaP and PC-3 cells were cultured in the presence of the indicated agent(s), or appropriate vehicle controls for 7 or 4 days, respectively. Cells were counted using a haemocytometer and cell viability was determined using Trypan blue dye exclusion as described in chapter 2.

For experiments using the caspase inhibitor, z-VAD-fmk, LNCaP cells were cultured as described above, in the presence or absence of z-VAD-fmk ( $50\mu\text{M}$ ) for the duration of the experiment (5 days).

### **6.2.3 Immunoblot**

The effect of AR-targeting agents on protein expression in LNCaP cells was determined by culturing LNCaP cells in the presence of the indicated agents(s), or appropriate

vehicle controls for 12 or 24 hr. AR steady-state protein levels were analysed by immunoblot using the AR-N20 antibody as described in chapter 2.

#### **6.2.4 Transactivation assays**

LNCaP cells were transfected with a luciferase reporter construct and cultured in the presence of 1nM DHT with 250nM bicalutamide + 62.5nM 17-AAG or 500nM bicalutamide + 125nM 17-AAG. Transactivation activity of endogenous LNCaP AR was measured as described in chapter 2.

#### **6.2.5 Statistical analysis**

##### *6.2.5.1 Isobole analysis*

Following treatment of LNCaP cells with the various combinations of agents, analysis of the drug interaction was performed. To determine whether the drug combination was antagonistic, additive or synergistic, growth curve values were analysed using the isobole equation:  $A_c/A_e + B_c/B_e = D$  (Berenbaum 1981).

$A_c$  and  $B_c$  represent the concentration of drug A and drug B used in the combination, and  $A_e$  and  $B_e$  represent the concentration of drug A and B that produced the same magnitude of effect when administered alone. If  $D$ , the combination index, is less than 1 then the drugs are considered to act synergistically. If the combination index is greater than or equal to one, the drugs act in an antagonistic or additive manner, respectively.

##### *6.2.5.2 Analysis of variance*

Statistical differences between treatment groups and controls were determined by One-way ANOVA with Dunnett post-hoc test, using SPSS statistical analysis software. A p-value of  $<0.05$  was considered statistically significant.

## 6.3 Results

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Sub-optimal doses of each agent were chosen for subsequent combination experiments. Two minimally active doses were chosen from previously generated growth curves (chapters 4 or 5), or the growth curves described below.

### ***6.3.1 Bicalutamide dose response in LNCaP and PC-3 cells***

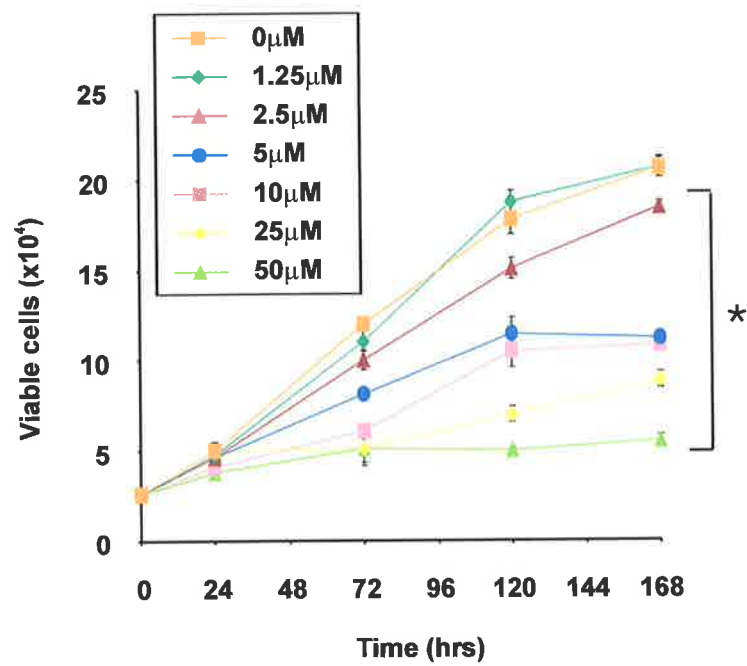
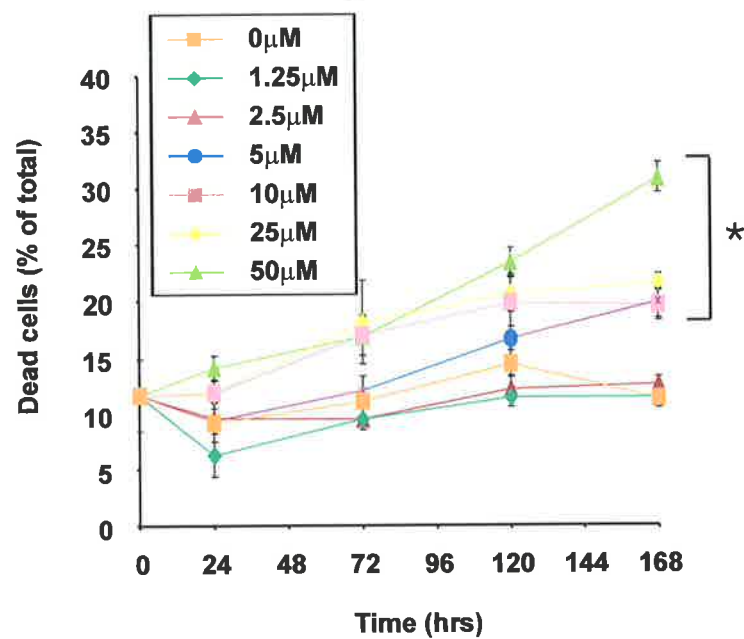
LNCaP cells were cultured with increasing concentrations of bicalutamide (0, 1.25, 2.5, 5, 10, 25 or 50 $\mu$ M) for up to 7 days. LNCaP cell proliferation was inhibited in a dose-dependent manner by bicalutamide, with complete inhibition of cell growth observed using 50 $\mu$ M bicalutamide (Figure 6.2A). LNCaP cell death was significantly induced using 5 $\mu$ M bicalutamide, with maximal induction of cell death to approximately 30% with 50 $\mu$ M bicalutamide (Figure 6.2B). The sub-effective doses of bicalutamide, which minimally inhibited proliferation and did not induce cell death in LNCaP cells were determined to be 1.25 and 2.5 $\mu$ M.

Culture of AR-negative PC-3 cells with up to 50 $\mu$ M bicalutamide had no effect on PC-3 cell proliferation (Figure 6.3A) or cell death (Figure 6.3B). Immunoblot analysis of LNCaP versus PC-3 whole cell lysates confirmed the lack of AR expression in PC-3 cells (Figure 6.4).

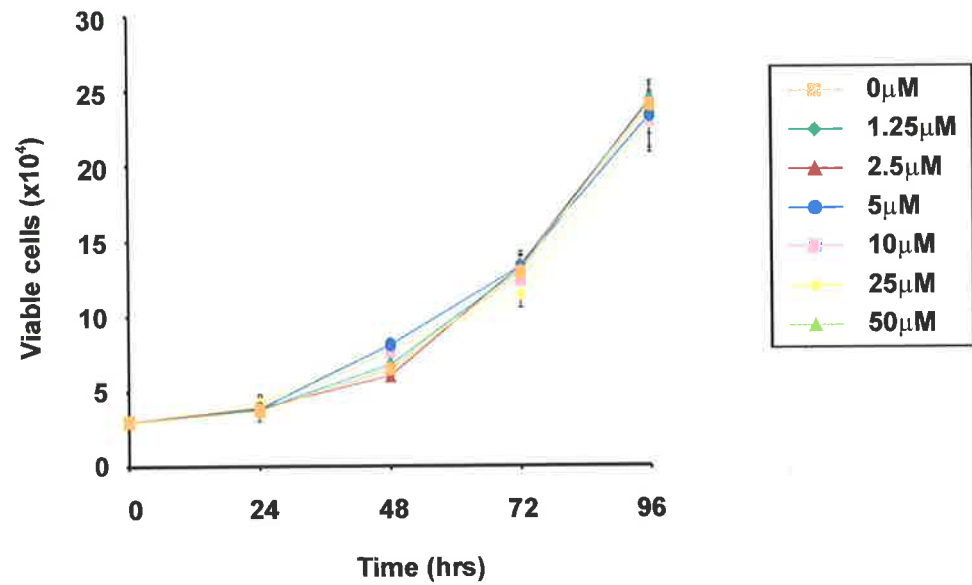
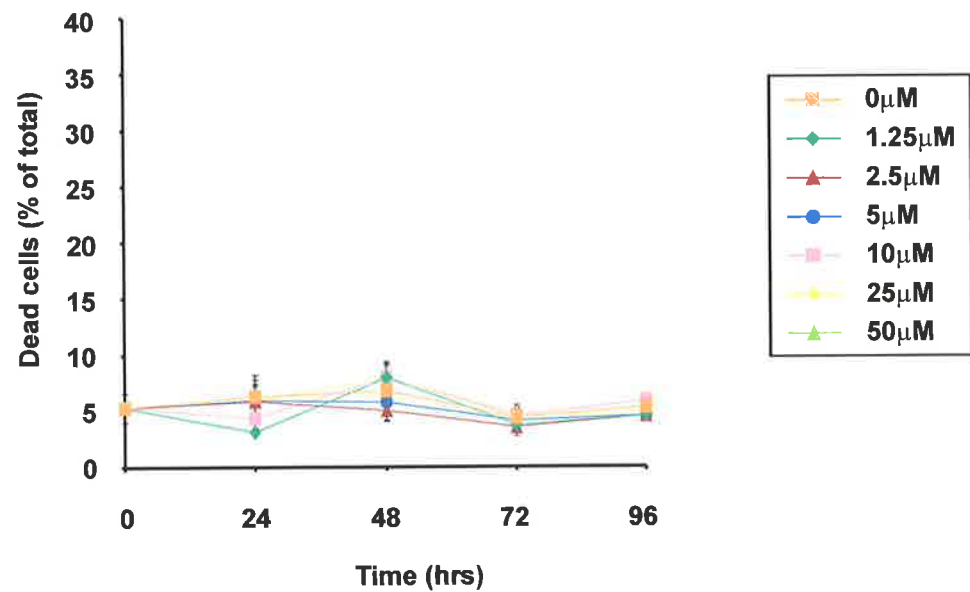
### ***6.3.2 Combination of SAHA and bicalutamide***

The sub-effective doses of SAHA were determined to be 0.5 and 1 $\mu$ M (refer to figure 5.1 and 5.2) and for bicalutamide 1.25 and 2.5 $\mu$ M (refer to figure 6.2 and 6.3).

#### ***6.3.2.1 Effect of SAHA and bicalutamide on cell viability in LNCaP cells***

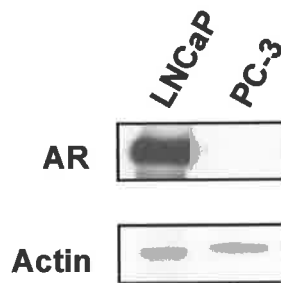
**A****B**

**Figure 6.2 - Inhibition of LNCaP prostate cancer cell growth by bicalutamide.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with bicalutamide (0, 1.25, 2.5, 5, 10, 25 or  $50 \mu\text{M}$ ) for up to seven days. (A) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  bicalutamide *versus* control.

**A****B**

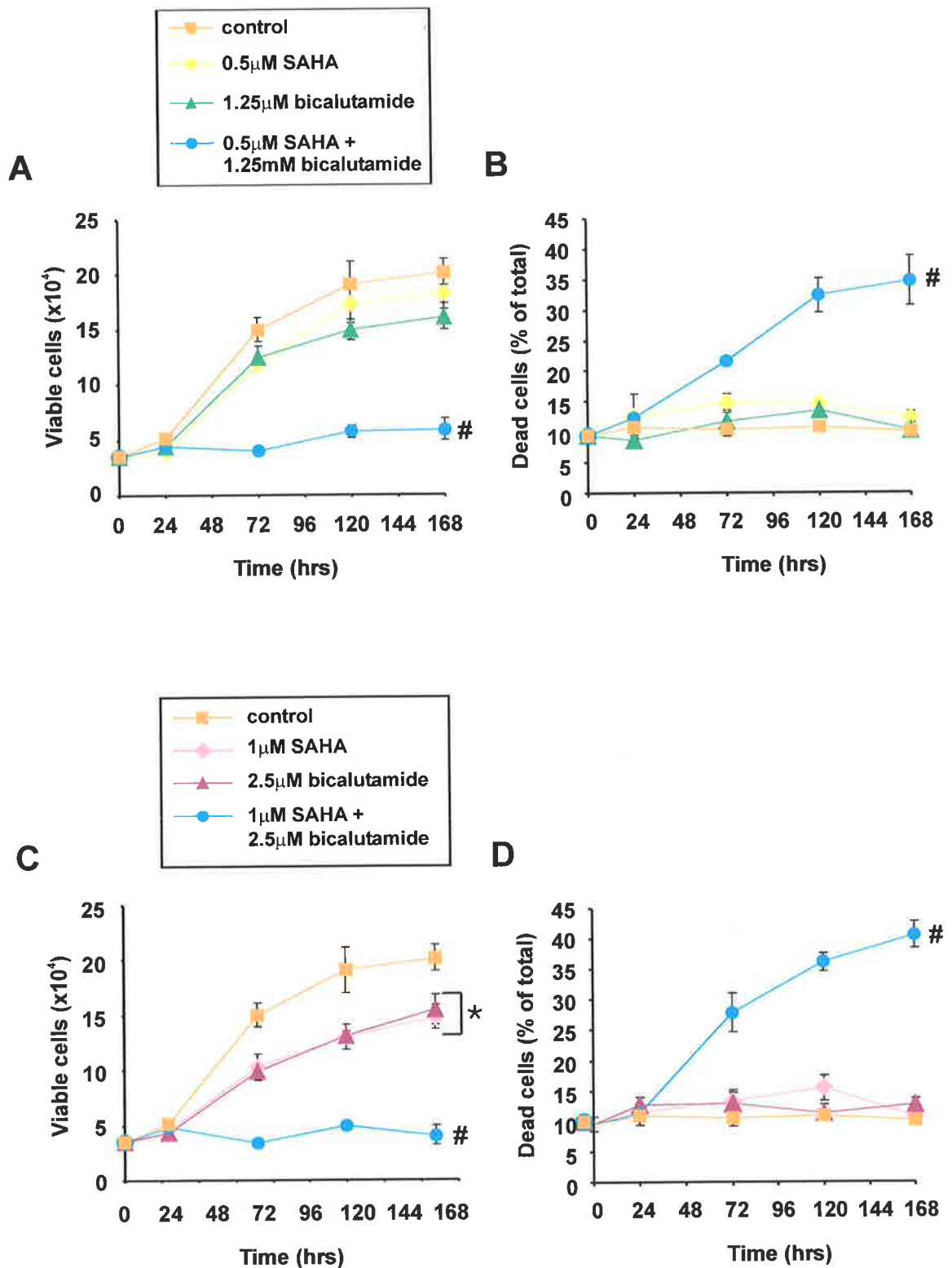
**Figure 6.3 - Inhibition of PC-3 prostate cancer cell growth by bicalutamide.** PC-3 cells ( $2.5 \times 10^4$ ) were cultured with bicalutamide (0, 1.25, 2.5, 5, 10, 25 or  $50 \mu\text{M}$ ) for up to four days. (A) Cells were counted every day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments.





**Figure 6.4 – Expression of AR in LNCaP versus PC-3 prostate cancer cells.** Lysates from untreated LNCaP or PC-3 cells were analysed by immunoblot for expression of AR. Actin was used as a loading control and is shown in the lower panel.

To determine the effect of simultaneous treatment with sub-effective doses of bicalutamide and SAHA on LNCaP cell proliferation, LNCaP cells were cultured in the absence or presence of SAHA (0.5 or 1 $\mu$ M) and/or bicalutamide (1.25 or 2.5 $\mu$ M) for up to 7 days. Bicalutamide (1.25 $\mu$ M) or SAHA (0.5 $\mu$ M) alone had no significant effects on LNCaP cell proliferation (Figure 6.5A), and did not increase the percentage of dead cells compared to control cells (Figure 6.5B). Culture with 1 $\mu$ M SAHA or 2.5 $\mu$ M bicalutamide alone inhibited proliferation of LNCaP cells by approximately 30% (Figure 6.5C), with no increase in the percentage of dead cells above that of control cells (Figure 6.5D). However, both combinations of 0.5 $\mu$ M SAHA + 1.25 $\mu$ M bicalutamide and 1 $\mu$ M SAHA + 2.5 $\mu$ M bicalutamide completely inhibited LNCaP cell proliferation compared to control cells (Figure 6.5A&C). Cell death was increased to approximately 35% with the lower sub-effective doses (Figure 6.5B) versus approximately 45% with the higher sub-effective doses (Figure 6.5D). The enhanced action of SAHA and bicalutamide on LNCaP cells was synergistic according to the isobole equation. The isobole equation (refer to section 6.2.5) determines the combination index by calculating a D value that indicates whether the observed effect is, synergistic (<1), antagonistic (>1) or additive (=1). The D values for cell proliferation and cell death treated with 0.5 $\mu$ M SAHA and 1.25 $\mu$ M bicalutamide were 0.2 and 0.8 respectively (Figure 6.5A&B). The D value for both cell proliferation and death treated with 1 $\mu$ M SAHA and 2.5 $\mu$ M bicalutamide was 0.054 (Figure 6.5C&D). An equivalent effect on cell viability was only observed with 50 $\mu$ M bicalutamide or 7.5 $\mu$ M SAHA when these agents were used individually (Figure 6.2 and 5.1).



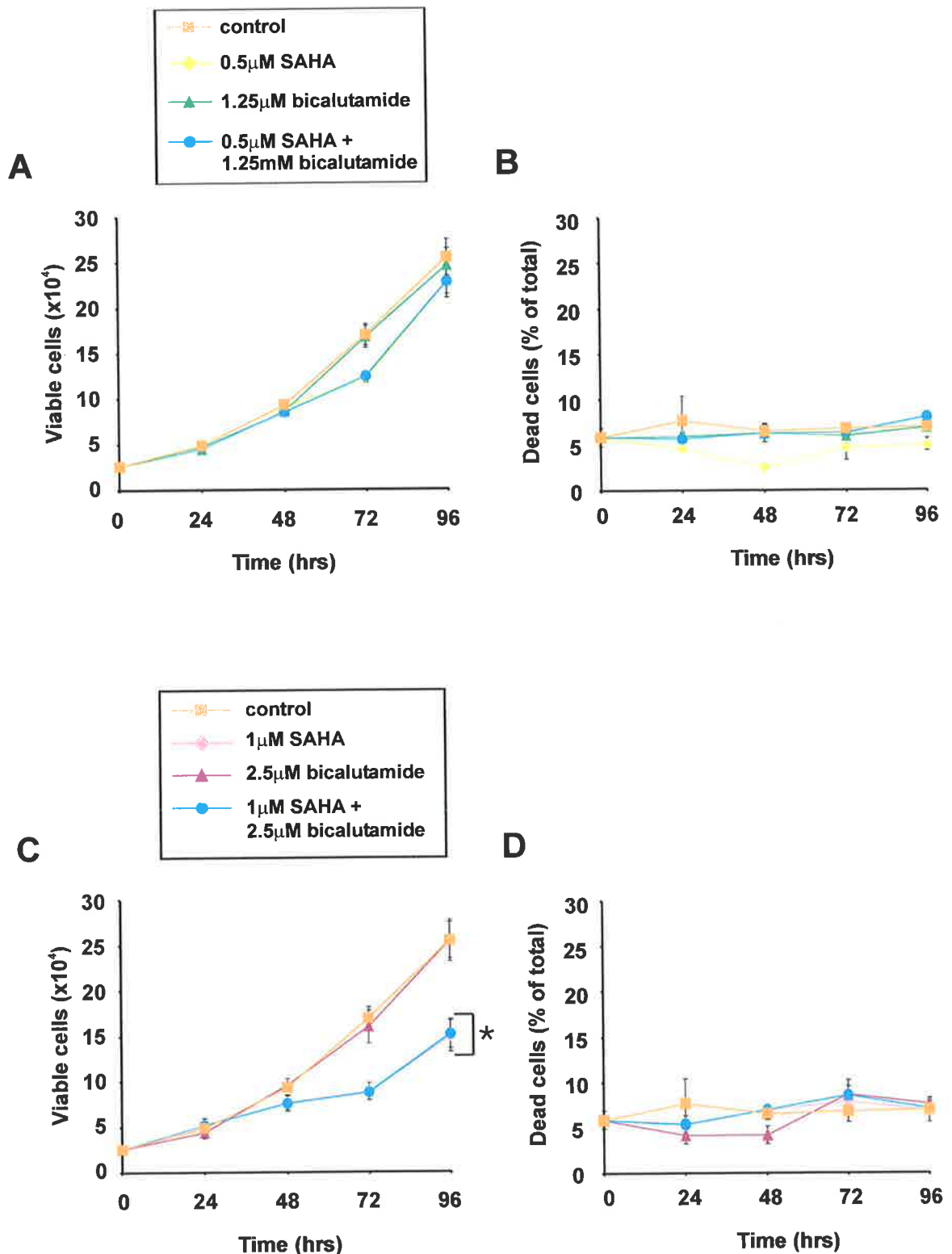
**Figure 6.5 - Inhibition of LNCaP prostate cancer cell growth by SAHA and bicalutamide.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0.5 or  $1 \mu\text{M}$ ) +/- bicalutamide (1.25 or  $2.5 \mu\text{M}$ ) for up to seven days. (A,C) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B,D) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of three separate experiments. # Combination index; indicates synergistic effects of SAHA and bicalutamide. \* ANOVA;  $p < 0.05$  SAHA or bicalutamide *versus* control.

### *6.3.2.2 Effect of SAHA and bicalutamide on cell viability in PC-3 cells*

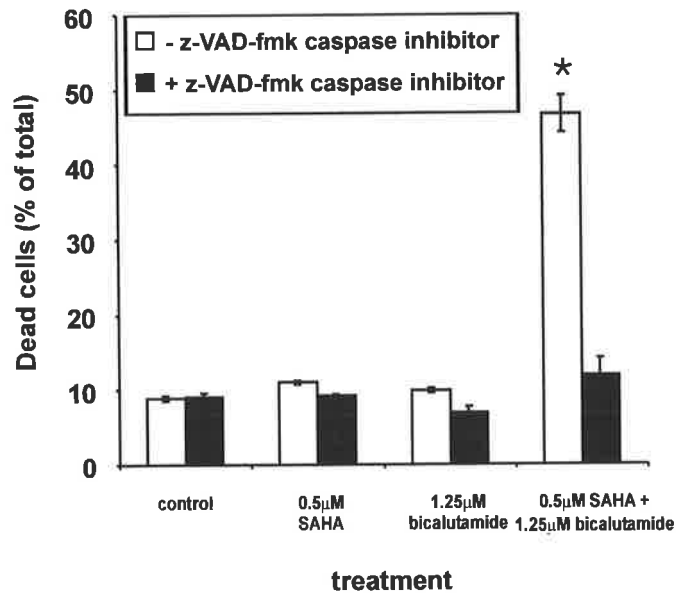
The same sub-effective doses of SAHA and bicalutamide were used to assess the effect of this combination on PC-3 cell proliferation. Culture of PC-3 cells with the lower doses of SAHA (0.5 $\mu$ M) and bicalutamide (1.25 $\mu$ M), either alone or in combination, had no effect on PC-3 cell viability (Figure 6.6A&B). Culture of PC-3 cells with 1 $\mu$ M SAHA significantly inhibited PC-3 cell proliferation by approximately 40% compared to control cells, while bicalutamide (2.5 $\mu$ M) had no effect on cell growth (Figure 6.6C&D). Combining 1 $\mu$ M SAHA with 2.5 $\mu$ M bicalutamide inhibited proliferation of PC-3 cells to the same extent as 1 $\mu$ M SAHA alone. The combinations of SAHA and bicalutamide therefore did not have an additive or synergistic effect on PC-3 prostate cancer cell proliferation or survival (Figure 6.6D).

### *6.3.2.3 Cell death induced by SAHA and bicalutamide is caspase-dependent*

For subsequent combination experiments the low doses of 0.5 $\mu$ M SAHA and 1.25 $\mu$ M bicalutamide were used. To determine whether cell death induced by the combination of SAHA and bicalutamide in LNCaP cells was a caspase-dependent event, LNCaP cells were cultured in the presence or absence of SAHA and bicalutamide +/- the z-VAD-fmk pan-caspase inhibitor. Cells were counted at day 5 post-treatment and cell viability was assessed. LNCaP cells cultured in the absence of the caspase inhibitor were unaffected by SAHA or bicalutamide alone, however a synergistic increase in cell death (45%) was observed when the two agents were combined (Figure 6.7). The addition of the caspase inhibitor prevented the synergistic induction of cell death by the combination of SAHA and bicalutamide, with the percentage of dead cells (10%) similar to the vehicle-treated controls (Figure 6.7).



**Figure 6.6 - Inhibition of PC-3 prostate cancer cell growth by SAHA and bicalutamide.** PC-3 cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0.5 or  $1 \mu\text{M}$ ) +/- bicalutamide (1.25 or  $2.5 \mu\text{M}$ ) for up to four days. (A,C) Cells were counted every day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B,D) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  bicalutamide and/or SAHA *versus* control.



**Figure 6.7 – Induction of LNCaP prostate cancer cell death by SAHA and bicalutamide is caspase dependent.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with SAHA (0.5µM) +/- bicalutamide (1.25µM) in the presence or absence of z-VAD-fmk caspase inhibitor (50µM) for 5 days. Cells were counted using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of two separate experiments. \* ANOVA;  $p < 0.05$  SAHA and/or bicalutamide (+/- z-VAD-fmk) *versus* control.

#### *6.3.2.4 Protein expression changes in LNCaP cells following treatment with SAHA and bicalutamide*

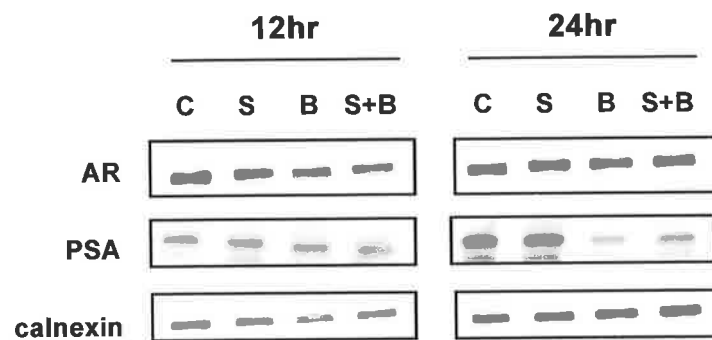
LNCaP cells were cultured in the absence or presence of SAHA +/- bicalutamide for 12 and 24 hr, and cell lysates were analysed by immunoblot. There was no difference in AR steady-state protein levels in LNCaP cells treated with SAHA alone or in combination with bicalutamide compared to vehicle-treated control cells (Figure 6.8). While there was a modest decrease in PSA expression at 24 hr post-treatment with bicalutamide, this was not enhanced with the addition of SAHA (Figure 6.8). Calnexin was used as a loading control and is shown in the lower panels.

#### *6.3.2.5 Effect of SAHA and bicalutamide in LNCaP cells is attenuated by DHT*

To determine whether the effect of combined SAHA and bicalutamide on LNCaP cell death was dependent on AR signalling, LNCaP cells were cultured with SAHA +/- bicalutamide in the presence or absence of 10nM DHT for 5 days. The percentage of LNCaP cell death in the presence of SAHA or bicalutamide alone was similar to that of control cells, approximately 10% (Figure 6.9). Culture with both SAHA and bicalutamide significantly induced cell death to approximately 45%. While the addition of 10nM DHT did not significantly alter the levels of cell death in the presence of SAHA or bicalutamide alone, culture of LNCaP cells in the presence of excess DHT completely inhibited the synergistic induction of cell death by the combination of the two agents compared to control cells (Figure 6.9).

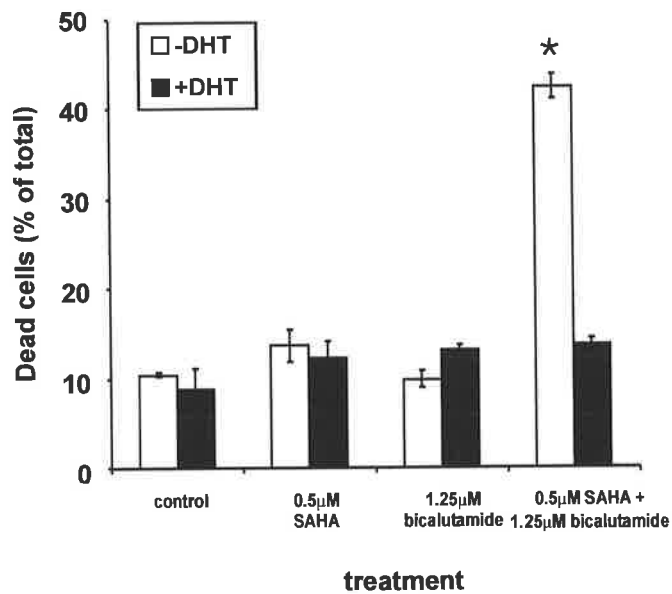
#### *6.3.2.6 Effect of sequential addition of SAHA and bicalutamide on LNCaP cells*

To determine whether both SAHA and bicalutamide were required to induce the synergistic effects, or whether one agent sensitised cells to the other agent, LNCaP cells



**Figure 6.8 – Protein expression changes in LNCaP cells following treatment with SAHA and bicalutamide.** Lysates from LNCaP cells cultured with 0.5 $\mu$ M SAHA (S), 1.25 $\mu$ M bicalutamide (B), 0.5 $\mu$ M SAHA and 1.25 $\mu$ M bicalutamide (S+B) or control cells (C) were analysed by immunoblot for expression of AR and PSA. Calnexin was used as a loading control and is shown in the lower panels. Images are representative of three separate experiments.





**Figure 6.9 – LNCaP prostate cancer cell death induced by SAHA and bicalutamide is reversed by DHT.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with SAHA ( $0.5 \mu\text{M}$ ) +/- bicalutamide ( $1.25 \mu\text{M}$ ) in the presence or absence of  $10 \text{ nM}$  DHT for five days. Cells were counted using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  SAHA and/or bicalutamide (+/- DHT) *versus* control.

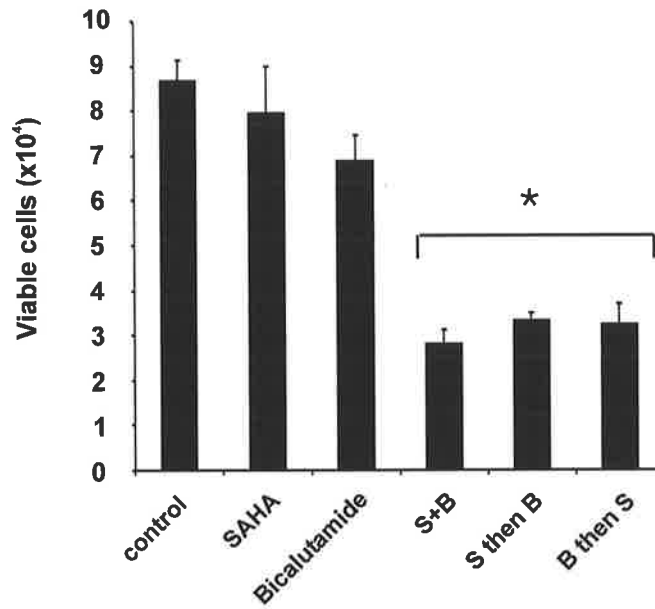
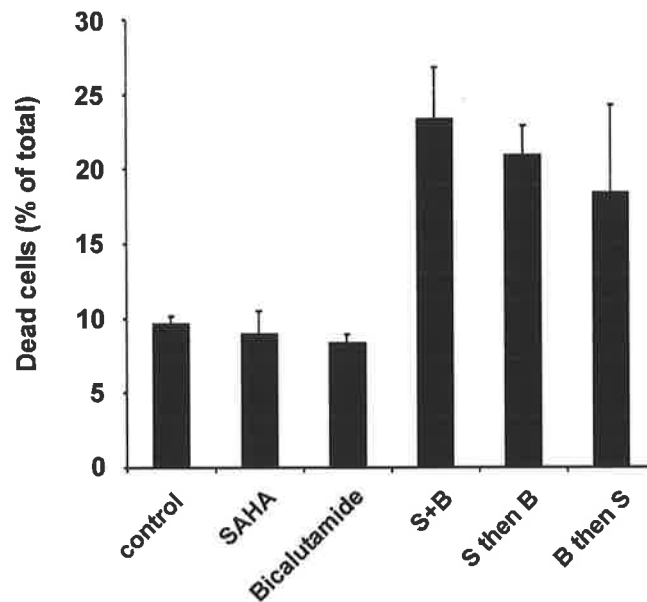
were pre-treated for 24 hr with either SAHA or bicalutamide compared to simultaneous treatment with SAHA plus bicalutamide, for 5 days. Cell number was significantly reduced in LNCaP cells treated with SAHA and bicalutamide compared to control cells (Figure 6.10A). There was no significant difference in cell number when comparing between SAHA pre-treated cells, bicalutamide pre-treated cells or simultaneously treated cells (Figure 6.10A). LNCaP cell death was increased to approximately 20-25% with combined SAHA and bicalutamide, regardless of pre-treatment (Figure 6.10B).

### ***6.3.3 Combination of 17-AAG and bicalutamide***

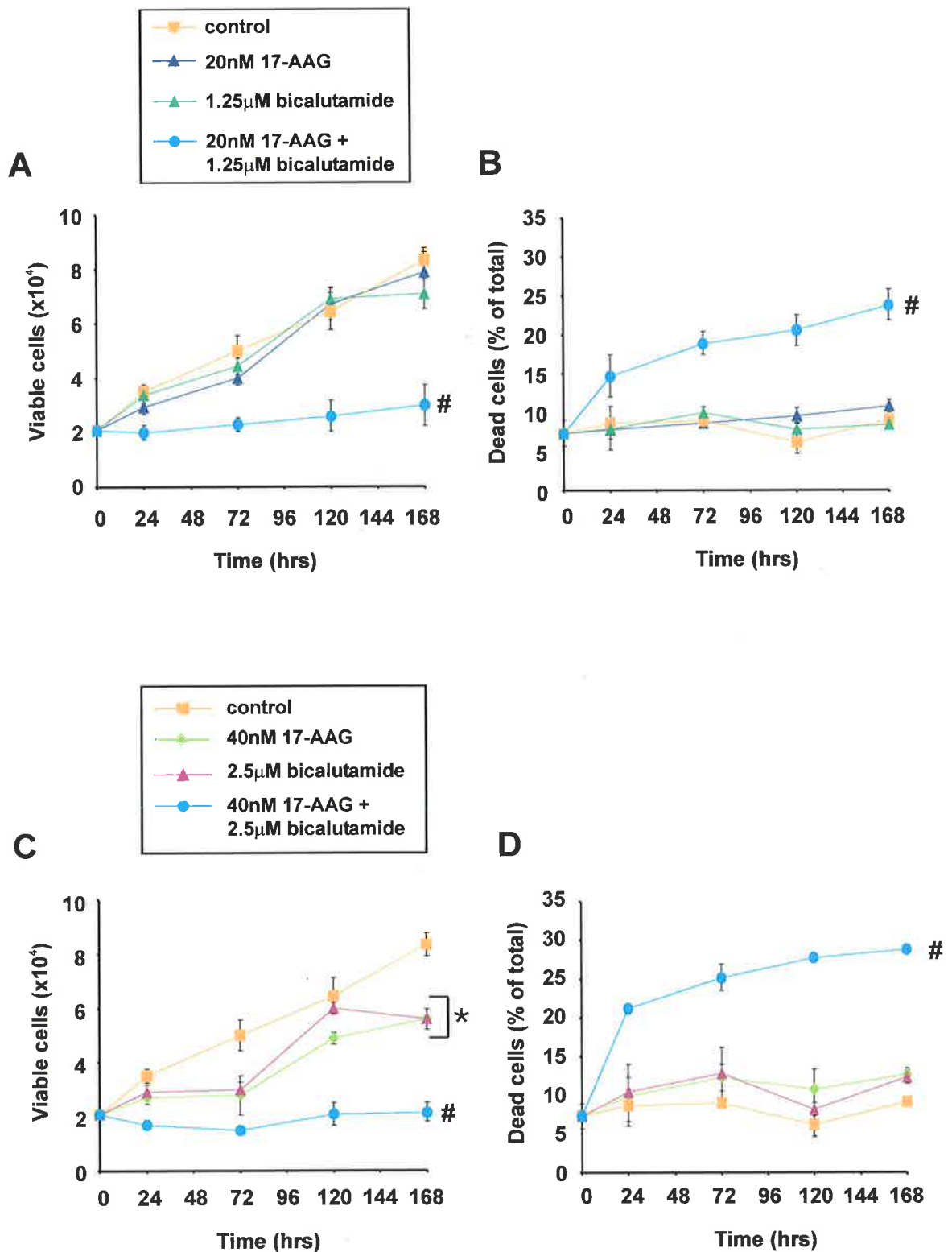
Sub-effective doses of 20nM and 40nM 17-AAG were chosen from growth curves previously generated in LNCaP and PC-3 cells (refer to figure 4.1 and 4.2). The same doses of bicalutamide used in conjunction with SAHA were also used in these experiments, being 1.25 and 2.5 $\mu$ M.

#### ***6.3.3.1 Effect of 17-AAG and bicalutamide on LNCaP cell viability***

Culture of LNCaP prostate cancer cells with low, sub-effective doses of 17-AAG (20nM) or bicalutamide (1.25 $\mu$ M) alone had minimal effects on LNCaP cell growth compared with vehicle-treated controls (Figure 6.11A), and did not induce cell death (Figure 6.11B). Culture of LNCaP cells with 20nM 17-AAG and 1.25 $\mu$ M bicalutamide completely suppressed proliferation (Figure 6.11A), and induced cell death to approximately 25% (Figure 6.11B). Culture with 40nM 17-AAG or 2.5 $\mu$ M bicalutamide alone significantly inhibited proliferation of LNCaP cells by approximately 30% compared to control cells (Figure 6.11C). When combined these agents caused complete suppression of LNCaP cell growth over the 7-day period and induction of cell death to approximately 30% (Figure 6.11D). The action of 17-AAG and bicalutamide on LNCaP

**A****B**

**Figure 6.10 - Inhibition of LNCaP prostate cancer cell growth by SAHA and bicalutamide.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with SAHA ( $0.5 \mu\text{M}$ ) and bicalutamide ( $1.25 \mu\text{M}$ ) for five days. Cells were either treated with SAHA and bicalutamide simultaneously (S+B), pre-treated with SAHA for 24 hr (S then B) or pre-treated with bicalutamide for 24 hr (B then S). (A) Cells were counted using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean  $\pm$  the SE of triplicate wells in an experiment. \* ANOVA;  $p < 0.05$  SAHA and/or bicalutamide *versus* control.



**Figure 6.11 - Inhibition of LNCaP prostate cancer cell growth by 17-AAG and bicalutamide.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with 17-AAG (20 or 40nM) +/- bicalutamide (1.25 or 2.5μM) for up to seven days. (A,C) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B,D) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of three separate experiments. # Combination index; indicates synergistic effects of 17-AAG and bicalutamide. \* ANOVA;  $p < 0.05$  17-AAG or bicalutamide *versus* control.

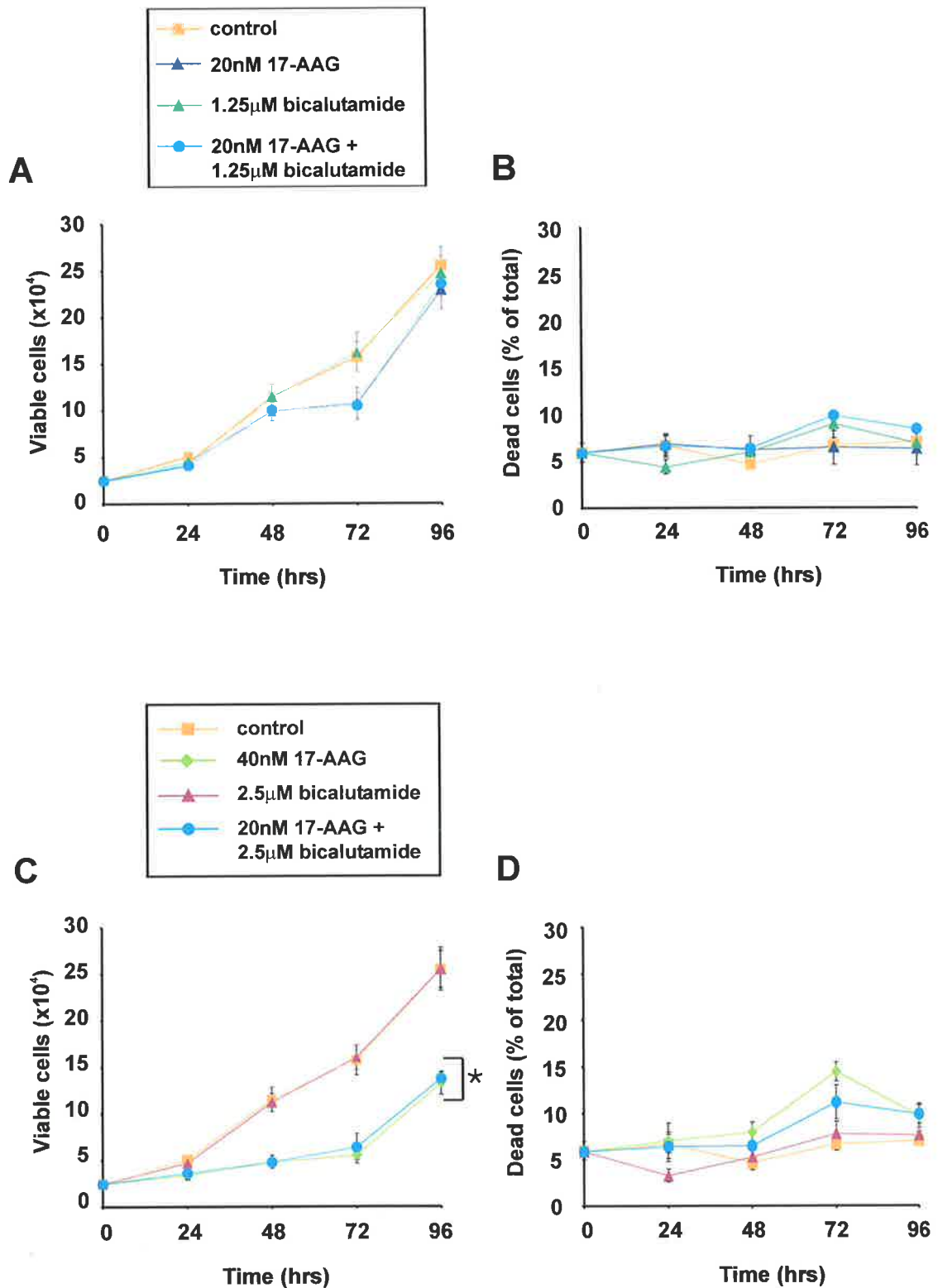
cell proliferation and death was synergistic according to the isobole equation, with a D value of 0.065 when cells were cultured with 20nM 17-AAG and 1.25 $\mu$ M bicalutamide (Figure 6.11A&B) or 0.052 when cells were culture with 40nM 17-AAG and 2.5 $\mu$ M bicalutamide (Figure 6.11C&D). An equivalent effect on cell viability was observed when the two agents were used individually (50 $\mu$ M bicalutamide or 500nM 17-AAG) (Figure 6.2 and 4.1).

#### *6.3.3.2 Effect of 17-AAG and bicalutamide on PC-3 cell viability*

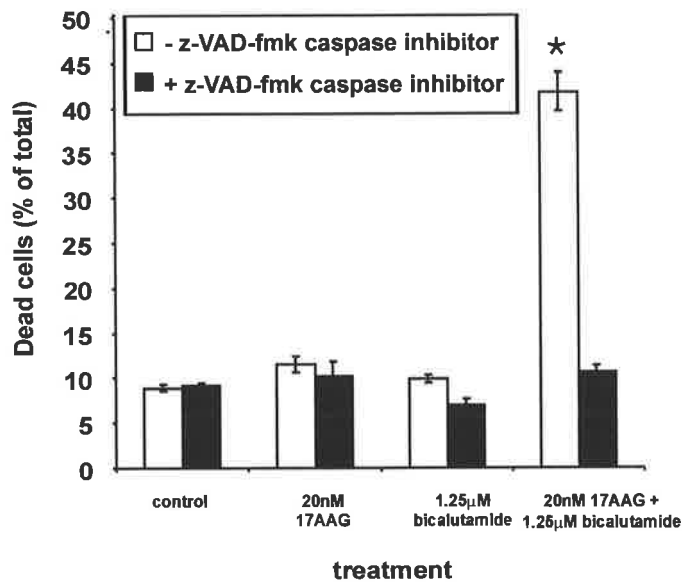
The same sub-effective doses of 17-AAG and bicalutamide were used to assess the effect of combining 17-AAG and bicalutamide on PC-3 cells. Culture of PC-3 cells in the presence of 20nM 17-AAG or 1.25 $\mu$ M bicalutamide alone had no effect on PC-3 cell proliferation or cell death (Figure 6.12A&B). Culture with 40nM 17-AAG significantly inhibited PC-3 cell proliferation by approximately 40%, with no significant effect on cell death, while combined 17-AAG (40nM) and bicalutamide (2.5 $\mu$ M) inhibited proliferation to the same extent as 40nM 17-AAG alone (Figure 6.12C). There was no synergistic effect of combined 17-AAG and bicalutamide in PC-3 cells (Figure 6.12C&D).

#### *6.3.3.3 Cell death induced by 17-AAG and bicalutamide is caspase-dependent*

For all subsequent experiments the lower sub-effective doses of 17-AAG (20nM) and bicalutamide (1.25 $\mu$ M) were used. To determine whether cell death induced by 17-AAG and bicalutamide in LNCaP cells was a caspase-dependent event, LNCaP cells were cultured with 17-AAG and/or bicalutamide +/- the z-VAD-fmk pan-caspase inhibitor for 5 days. Similar to control cells, approximately 10% cell death was observed when LNCaP cells were cultured with 17-AAG or bicalutamide in the absence of the caspase inhibitor (Figure 6.13). Culture of LNCaP cells with 17-AAG and bicalutamide



**Figure 6.12 - Inhibition of PC-3 prostate cancer cell growth by 17-AAG and bicalutamide.** PC-3 cells ( $2.5 \times 10^4$ ) were cultured with 17-AAG (20 or 40nM) +/- bicalutamide (1.25 or 2.5µM) for up to four days. (A,C) Cells were counted every day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B,D) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  17-AAG or bicalutamide *versus* control.



**Figure 6.13 – Induction of LNCaP prostate cancer cell death by 17-AAG and bicalutamide is caspase dependent.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with 17-AAG (20nM) +/- bicalutamide (1.25µM) in the presence or absence of z-VAD-fmk caspase inhibitor (50µM) for 5 days. Cells were counted using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of two separate experiments. \* ANOVA;  $p < 0.05$  17-AAG and/or bicalutamide (+/- z-VAD-fmk) *versus* control.

significantly increased cell death to approximately 45% compared to control cells. The addition of the caspase inhibitor prevented the induction of cell death observed with 17-AAG and bicalutamide (Figure 6.13).

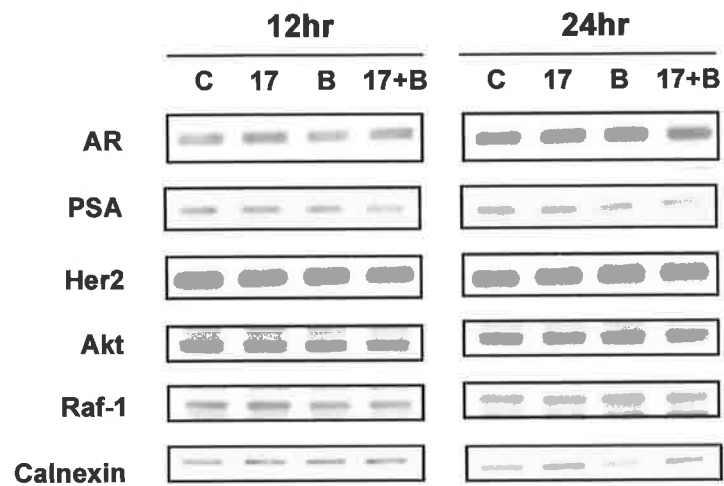
#### *6.3.3.4 Protein expression changes in LNCaP cells following treatment with 17-AAG and bicalutamide*

LNCaP cells were cultured with 17-AAG +/- bicalutamide and cell lysates were analysed by immunoblot. There was no difference in AR steady-state protein levels at 12 or 24 hr following treatment with 17-AAG +/- bicalutamide (Figure 6.14). Culture with 17-AAG and bicalutamide resulted in a modest decrease in steady-state protein levels of the androgen-regulated gene, PSA at 24 hr post-treatment. As 17-AAG inhibits the activity of Hsp90 the steady-state levels of the Hsp90, client proteins Her2, Akt and Raf-1 were also analysed in these cells. Steady-state levels of Hsp90 client proteins were unaffected in LNCaP cells treated with 17-AAG alone or in combination with bicalutamide (Figure 6.14). Calnexin was used as a loading control and is shown in the lower panels.

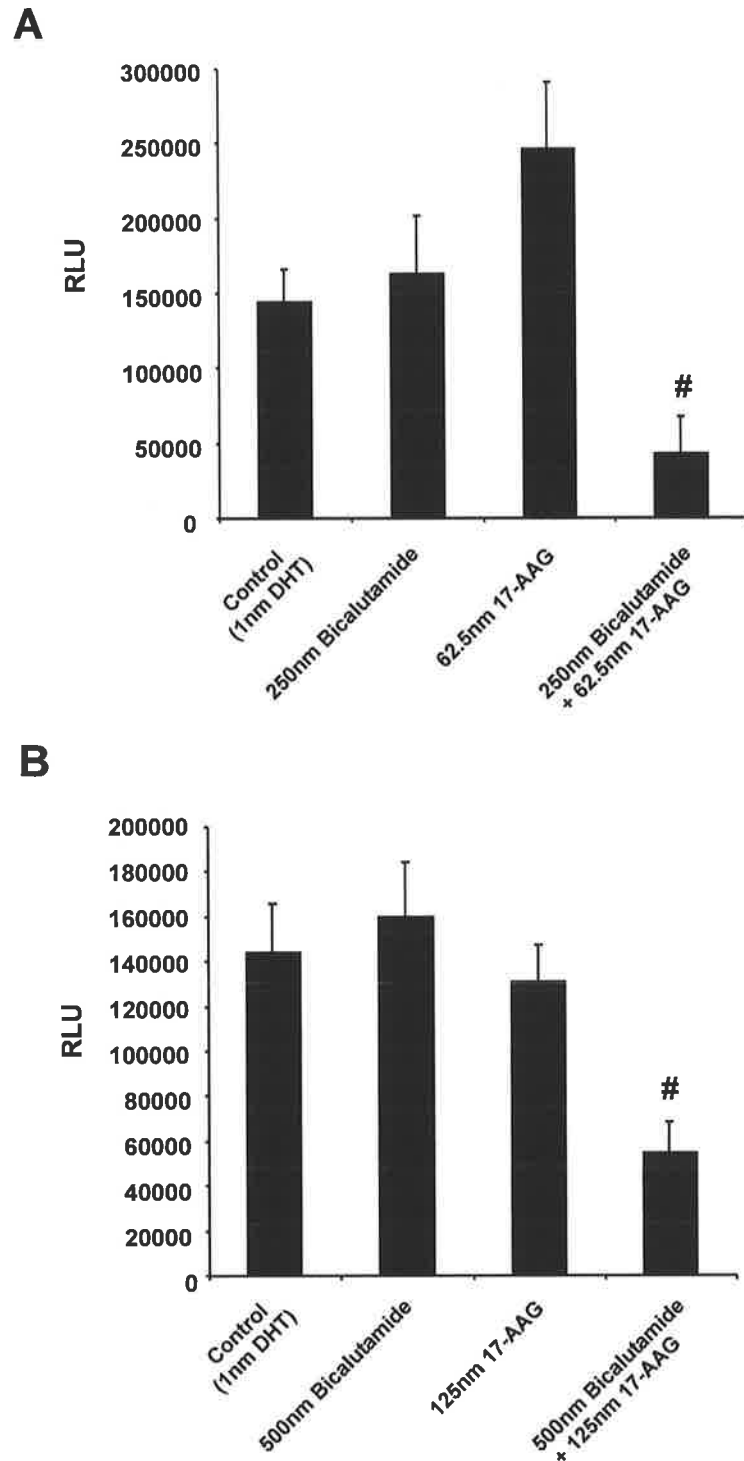
#### *6.3.3.5 17-AAG and bicalutamide synergistically inhibits AR transactivation activity*

While treatment with 17-AAG reduces AR steady-state protein levels, a greater proportion of its activity appears to occur through inhibiting AR transactivation activity (refer to section 4.3.5). Transactivation assays in LNCaP cells were therefore used to determine whether the synergistic activity of 17-AAG and bicalutamide in LNCaP cells was due synergistic inhibition of endogenous AR activity. Two minimally active doses were chosen to use in the following combination experiments. Minimally active doses of 17-AAG (62.5 and 125nM) and bicalutamide (250 and 500nM) in this system were





**Figure 6.14 – Protein expression changes in LNCaP cells following treatment with 17-AAG and bicalutamide.** Lysates from LNCaP cells cultured with 20nM 17-AAG (17), 1.25 $\mu$ M bicalutamide (B), 20nM 17-AAG and 1.25 $\mu$ M bicalutamide (17+B) or control cells (C) were analysed by immunoblot for expression of AR, PSA, Her2, Akt and Raf-1. Calnexin was used as a loading control and is shown in the lower panels. Images are representative of three separate experiments.



**Figure 6.15 - Inhibition of endogenous AR transactivation activity by 17-AAG and bicalutamide in LNCaP prostate cancer cells.** LNCaP cells ( $1.5 \times 10^4$ ) were transiently transfected with an androgen responsive luciferase reporter (100ng/well). Four hours after transfection the cells were treated with (A) 62.5nM 17-AAG + 250nM bicalutamide, or (B) 125nM 17-AAG + 500nM bicalutamide, in the presence of 1nM DHT. Luciferase activity was read on a luminometer and activity of endogenous AR is plotted as relative luciferase units (RLU). Results are presented as the mean  $\pm$  the SE of 6 replicates in an experiment. # Combination index; indicates synergistic effects of 17-AAG and bicalutamide.

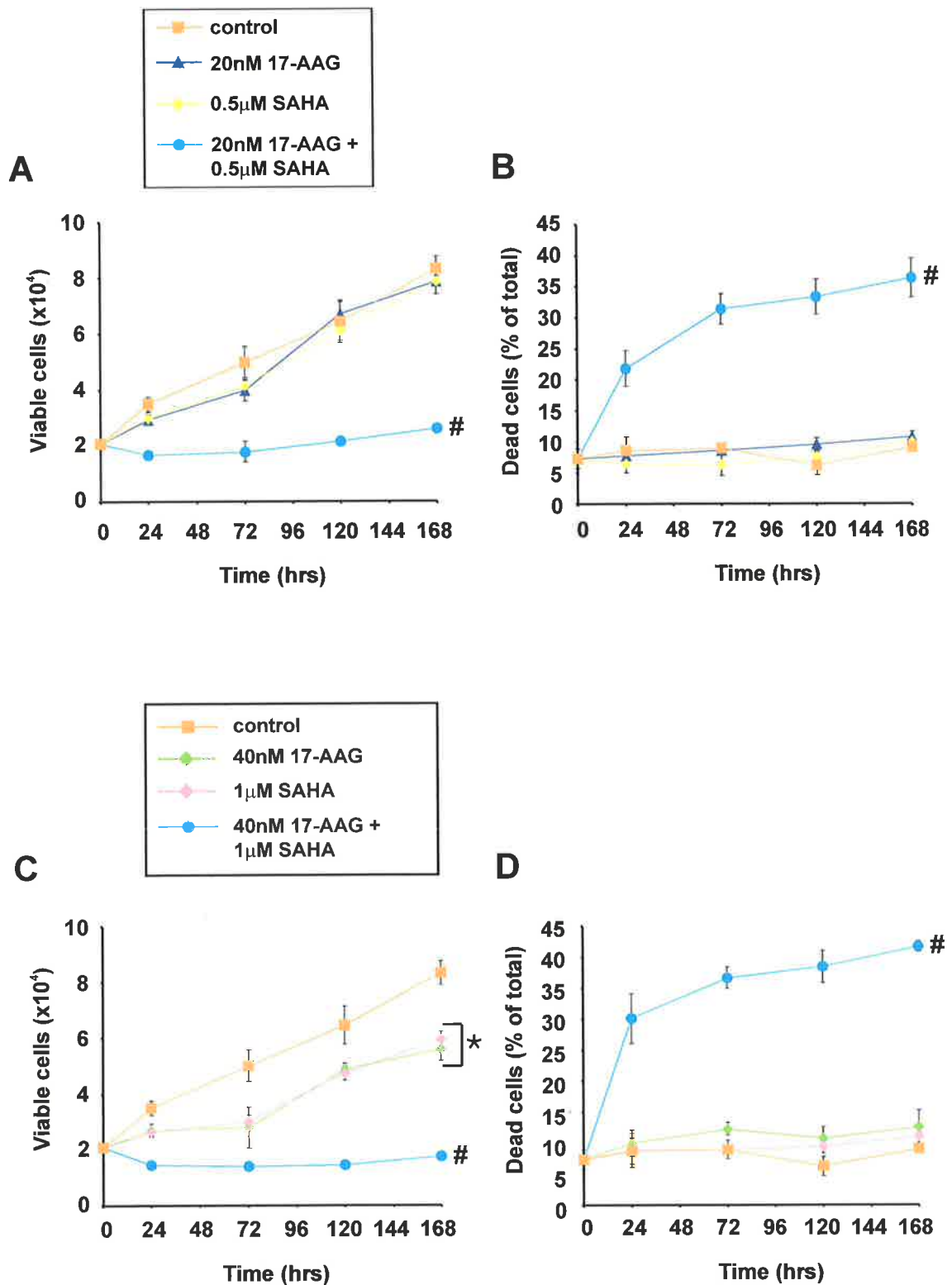
determined by dose-response experiments (data not shown). LNCaP cells were cultured in the presence of 1nM DHT, with 17-AAG +/- bicalutamide, and transactivation activity of the endogenous LNCaP AR was assessed 36 hr post-treatment. Culture of LNCaP cells with 17-AAG (62.5 and 125nM) or bicalutamide (250 and 500nM) alone had no significant effect on AR transactivation (Figure 6.15A&B). When 17-AAG and bicalutamide were combined, the inhibition of endogenous AR transactivation activity was enhanced. This effect was observed at both concentrations of 17-AAG and bicalutamide (Figure 6.15A,B). The inhibition of AR transactivation activity was synergistic according to the isobole equation, with D values of 0.2 for the lower doses, and 0.4 for the higher doses, respectively. Equivalent effects on endogenous AR transactivation activity were observed when LNCaP cells were cultured with 250nM 17-AAG or 1.25 $\mu$ M bicalutamide (data not shown).

#### ***6.3.4 Combination of 17-AAG and SAHA***

Sub-effective doses of 20nM and 40nM 17-AAG were chosen from growth curves previously generated in LNCaP and PC-3 cells (refer to figure 4.1 and 4.2). Doses of 0.5 and 1 $\mu$ M SAHA were also chosen for use in the following experiments (refer to figure 5.1 and 5.2).

##### ***6.3.4.1 Effect of 17-AAG and SAHA on LNCaP cell viability***

Culture of LNCaP prostate cancer cells with low, sub-effective doses of 17-AAG (20nM) or SAHA (0.5 $\mu$ M) alone had minimal effects on LNCaP cell growth (Figure 6.16A) and did not induce cell death compared to vehicle-treated controls (Figure 6.16B). Culture with 1 $\mu$ M SAHA or 40nM 17-AAG alone significantly inhibited proliferation of LNCaP cells by approximately 30%, with no increase in cell death compared to control cells



**Figure 6.16 - Inhibition of LNCaP prostate cancer cell growth by 17-AAG and SAHA.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with 17-AAG (20 or 40nM) +/- SAHA (0.5 or 1μM) for up to seven days. (A,C) Cells were counted every two days using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B,D) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of three separate experiments. # Combination index; indicates synergistic effects of 17-AAG and SAHA. \* ANOVA;  $p < 0.05$  17-AAG or SAHA *versus* control.

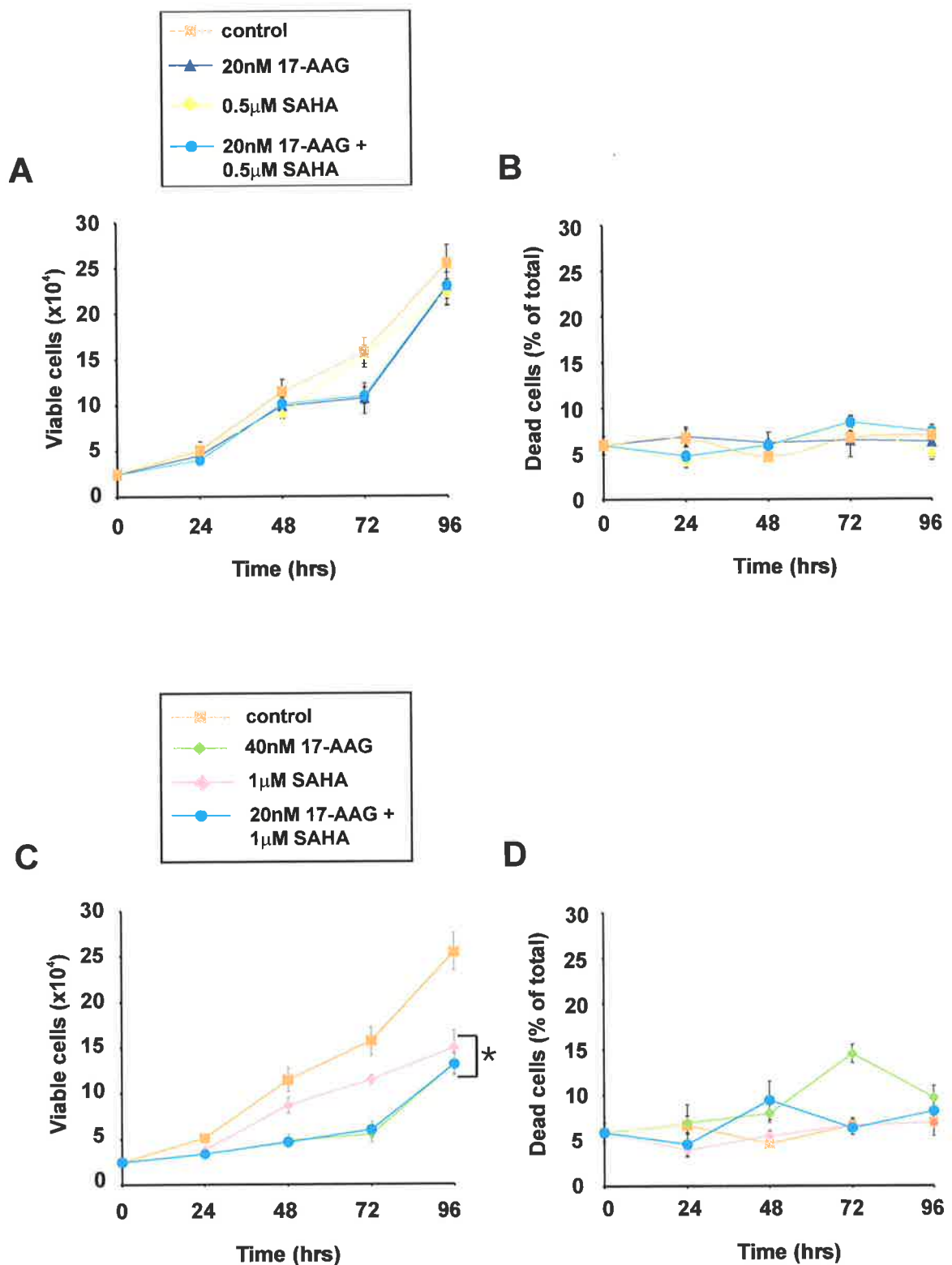
(Figure 6.16C). When combined these agents caused complete suppression of LNCaP cell growth over the 7-day period and induction of cell death. Cell death was induced to approximately 35% (20nM 17-AAG and 0.5 $\mu$ M SAHA) (Figure 6.16B), and approximately 40% (40nM 17-AAG and 1 $\mu$ M SAHA), compared to control cells (Figure 6.16D). The action of 17-AAG and SAHA on LNCaP cell proliferation and cell death was synergistic, with a D value of 0.065 and 0.14 when cells were cultured with 20nM 17-AAG and 0.5 $\mu$ M SAHA (Figure 6.16A&B) or 40nM 17-AAG and 1 $\mu$ M bicalutamide, respectively (Figure 6.16C&D). An equivalent effect on cell viability was observed with 7.5 $\mu$ M SAHA or 500nM 17-AAG when the agents were used individually (Figure 5.1 and 4.1).

#### *6.3.4.2 Effect of 17-AAG and SAHA on PC-3 cell viability*

Culture of PC-3 cells with the lower doses of SAHA (0.5 $\mu$ M) and 17-AAG (20nM) alone or in combination had no effect on PC-3 cell viability (Figure 6.17A&B). Culture of PC-3 cells with either 1 $\mu$ M SAHA or 40nM 17-AAG alone significantly inhibited PC-3 cell proliferation by approximately 40% compared to control cells (Figure 6.17C), with no significant effect on cell death (Figure 6.17D). Combining 1 $\mu$ M SAHA with 40nM 17-AAG inhibited proliferation of PC-3 cells to the same extent as cells cultured with 17-AAG alone (Figure 6.17C). The combinations of SAHA and 17-AAG therefore did not have an additive or synergistic effect in PC-3 prostate cancer cells.

#### *6.3.4.2 Cell death induced by 17-AAG and SAHA is caspase-dependent*

For subsequent combination experiments the lower doses of 0.5 $\mu$ M SAHA and 20nM 17-AAG were used. To determine whether cell death induced by 17-AAG and SAHA in LNCaP cells was a caspase-dependent event, LNCaP cells were cultured with 17-AAG

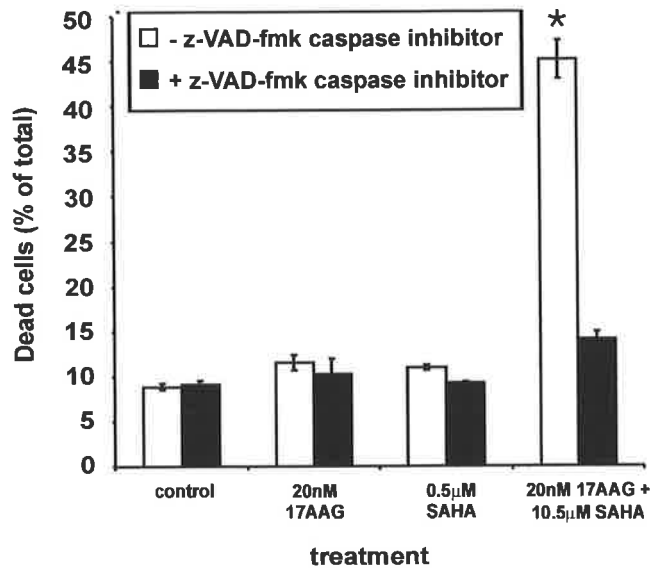


**Figure 6.17 - Inhibition of PC-3 prostate cancer cell growth by 17-AAG and SAHA.** PC-3 cells ( $2.5 \times 10^4$ ) were cultured with 17-AAG (20 or 40nM) +/- SAHA (0.5 or 1μM) for up to four days. (A,C) Cells were counted every day using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. (B,D) The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of three separate experiments. \* ANOVA;  $p < 0.05$  17-AAG and/or SAHA *versus* control.

and SAHA +/- the z-VAD-fmk pan-caspase inhibitor for 5 days. There was no increase in cell death when LNCaP cells were cultured with 17-AAG or SAHA alone, however a synergistic induction of 45% cell death was observed when the two agents were combined (Figure 6.18). The addition of the caspase inhibitor prevented the synergistic effect of 17-AAG and SAHA on LNCaP cell death.

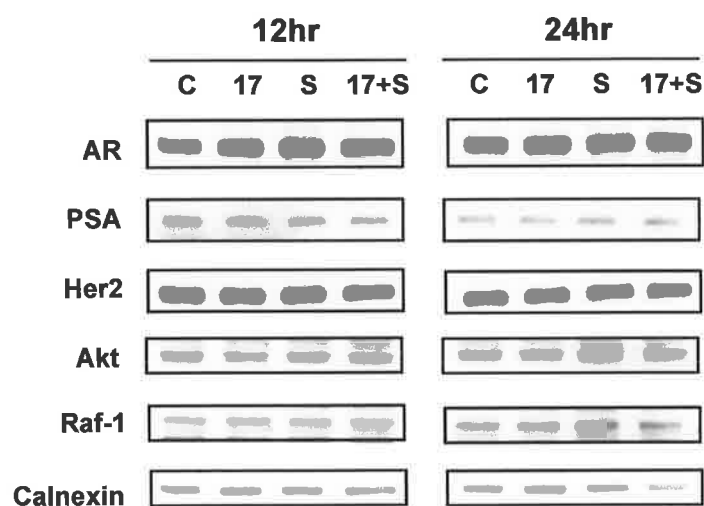
#### *6.3.4.3 Protein expression changes in LNCaP cells following treatment with 17-AAG and SAHA*

No difference in AR or PSA steady-state protein levels 12 or 24 hr following treatment with 17-AAG and SAHA, either alone or in combination, was observed (Figure 6.19). As 17-AAG and SAHA inhibit the activity of Hsp90, the steady-state levels of Hsp90 client proteins Her2, Akt and Raf-1 were also analysed by immunoblot. Steady-state protein levels of Hsp90 client proteins were unaffected in LNCaP cells treated with 17-AAG alone or in combination with SAHA (Figure 6.19). Calnexin was used as a loading control and is shown in the lower panels.



**Figure 6.18 – Induction of LNCaP prostate cancer cell death by 17-AAG and SAHA is caspase dependent.** LNCaP cells ( $2.5 \times 10^4$ ) were cultured with 17-AAG (20nM) +/- SAHA (0.5µM) in the presence or absence of z-VAD-fmk caspase inhibitor (50µM) for 5 days. Cells were counted using a haemocytometer and cell viability was assessed by Trypan blue dye exclusion. The number of dead cells is expressed as % of total cells counted. Results are presented as the mean +/- the SE of triplicate wells in an experiment. Results are representative of two separate experiments. \* ANOVA;  $p < 0.05$  17-AAG and/or SAHA (+/- z-VAD-fmk) *versus* control.





**Figure 6.19 – Protein expression changes in LNCaP cells following treatment with 17-AAG and SAHA.** Lysates from LNCaP cells cultured with 20nM 17-AAG (17), 0.5μM SAHA (S), 20nM 17-AAG and 0.5μM SAHA (17+S) or control cells (C) were analysed by immunoblot for expression of AR, PSA, Her2, Raf-1 and Akt. Calnexin was used as a loading control and is shown in the lower panels. Images are representative of three separate experiments.

## 6.4 Discussion

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The results described in this chapter demonstrate synergistic inhibition of LNCaP prostate cancer cell growth and survival using a combination of agents with AR-targeting activities. That androgen deprivation markedly enhanced sensitivity of LNCaP cells to 17-AAG (refer to figure 4.4) and SAHA (refer to figure 5.7) suggested that Hsp90 and HDAC inhibitors in combination with AAT may more effectively inhibit prostate cancer cell proliferation and survival. Indeed, in the current study, synergistic effects of SAHA and bicalutamide were observed on LNCaP cell proliferation and caspase-dependent cell death. This combination did not have a synergistic effect on proliferation or death of AR-negative PC-3 prostate cancer cells, even though SAHA inhibits PC-3 cell proliferation at higher concentrations. These results are consistent with the hypothesis that the synergistic effect of SAHA and bicalutamide acts through inhibition of AR signalling in prostate cancer cells. Moreover, the synergistic induction of LNCaP cell death by SAHA and bicalutamide was attenuated with addition of excess DHT to the culture medium, indicating that binding of bicalutamide to the AR is necessary for the observed synergy.

Similar to that observed with SAHA, a combination of 17-AAG and bicalutamide at sub-effective doses synergistically inhibited LNCaP cell proliferation and survival. The synergistic effects observed in LNCaP cells were not recapitulated in AR-negative PC-3 cells, despite the fact that PC-3 cells are more sensitive to 17-AAG compared to LNCaP cells (refer to figures 4.1 and 4.2), consistent with the requirement for an intact androgen signalling axis. More direct evidence for an effect of this combination on AR signalling was given by experiments showing synergistic inhibition of endogenous AR transactivation activity by combination treatment with 17-AAG and bicalutamide. In addition, there was no observable reduction in protein levels of the Hsp90 client proteins

Her2, Akt and Raf-1, suggesting that these pathways may not have been significantly affected by the low doses of 17-AAG used. Taken together, these studies demonstrated effective AR-targeting by combination 17-AAG and bicalutamide in LNCaP prostate cancer cells.

The pleiotropic effects of HDAC inhibitors and Hsp90 inhibitors on multiple signalling pathways potentially lend these agents to use in combination with other targeted drugs. HDAC inhibitors have demonstrated synergistic effects in prostate cancer cell lines in combination with radiotherapy (Goh *et al.* 2001; Camphausen *et al.* 2004; Chinnaiyan *et al.* 2005; Jung *et al.* 2005), inhibitors of angiogenesis (Qian *et al.* 2004) and TRAIL (Taghiyev *et al.* 2005; Vanoosten *et al.* 2005; Lakshmikanthan *et al.* 2006; Sonnemann *et al.* 2006). Previous reports have demonstrated enhanced effects on cancer cell growth when 17-AAG was combined with other anti-cancer agents, including imatinib and PI3-kinase inhibitors (Radujkovic *et al.* 2005; Yao *et al.* 2005; Ma *et al.* 2006; Paduano *et al.* 2006; Premkumar *et al.* 2006a; Premkumar *et al.* 2006b). In prostate cancer however there have been few studies combining Hsp90 inhibitors with other forms of therapy. Recent studies have demonstrated enhanced cell killing when 17-AAG was used in conjunction with radiation (Enmon *et al.* 2003; Russell *et al.* 2003; Harashima *et al.* 2005). In addition, synergistic growth inhibitory effects have been demonstrated in prostate cancer cells when combining Hsp90 inhibitors with survivin siRNA (Paduano *et al.* 2006) and TRAIL (Ma *et al.* 2006).

The studies described in this chapter also demonstrated synergistic inhibition of LNCaP cell proliferation and induction of caspase-dependent cell death induced by 17-AAG and SAHA. The histone deacetylase inhibitors depsipeptide and LAQ824 have been shown to cause Hsp90 acetylation, thereby inhibiting normal protein-protein interactions and

leading to degradation of client proteins (including the AR), in a similar manner to that seen following treatment with the specific Hsp90 inhibitor 17-AAG (Yu *et al.* 2002; Bali *et al.* 2005a; Kovacs *et al.* 2005). This mechanism may explain the synergistic activity of histone deacetylase inhibitors and the Hsp90 inhibitor 17-AAG on inhibition of cell growth (Ferrarini *et al.* 1992; Workman 2004; Murphy *et al.* 2005). However, the current studies do not support a major role of Hsp90 inhibition in SAHA activity in LNCaP prostate cancer cells (refer to chapter 5), suggesting that inactivation of Hsp90 may contribute to, but may not be a major mechanism involved in the synergistic effects of 17-AAG and SAHA in these cells. Alternatively, a reduction in AR mRNA levels simultaneous to destabilisation of AR-Hsp90 heterocomplexes in LNCaP cells may reduce AR levels and/or activity synergistically while targeting two separate points in the AR signalling pathway. During the course of this thesis other studies have investigated the effects of combining 17-AAG and HDAC inhibitors in leukaemia cells (Rahmani *et al.* 2003; George *et al.* 2005). Co-treatment of leukaemia cells with the HDAC inhibitor LBH589 and 17-AAG resulted in synergistic cytotoxic effects, and induction of apoptosis in imatinib-refractory leukaemia cells (George *et al.* 2005). In addition, this combination was significantly less toxic against normal bone marrow progenitor cells, suggesting some selectivity for malignant cells.

Immunoblot analyses in the current study showed no significant reductions in AR levels, suggesting that a greater effect on AR activity as opposed to AR protein levels may be the primary mechanism responsible for the synergistic inhibitory effects on proliferation and survival of LNCaP cells. Although there were no observable reductions in AR steady-state levels, a common feature of each combination appears to be the requirement for AR signalling in LNCaP prostate cancer cells, as none of the combinatorial strategies,

at either the lower or higher sub-effective doses, had any additive or synergistic effect on AR-negative PC-3 cells. However, the synergistic effects of the combination strategies described in the current study may not occur solely through inhibition of androgen signalling in LNCaP cells as both 17-AAG and SAHA have multiple targets. It may be that targeting of the androgen signalling axis sensitises prostate cancer cells to other targets of SAHA and 17-AAG. This may contribute to the inhibition of LNCaP cell proliferation and survival, and may therefore be collectively responsible for the dramatic effects observed with combinations of these agents at such ineffective doses. An example of an additional target in LNCaP prostate cancer cells is the Akt signalling pathway, which is more active in LNCaP cells compared to PC-3 cells and is involved in survival of LNCaP cells and resistance to therapeutic intervention (refer to section 4.4). Combinatorial targeting of Akt signalling by 17-AAG and SAHA, through inactivation of Hsp90 may contribute to the synergistic effects observed in LNCaP cells.

Several studies have now demonstrated effective knockdown of AR mRNA and a decrease in AR protein following treatment with AR-targeted antisense or siRNA (Eder *et al.* 2000; Eder *et al.* 2002; Hamy *et al.* 2003; Haag *et al.* 2005), with a corresponding decrease in proliferation and increase in death of prostate cancer cell lines and xenografts. These studies however, have not attempted to assess the effect of the specific AR-targeted antisense oligonucleotides or siRNA in combination with other forms of therapy. Preliminary experiments combining AR-antisense oligonucleotides with other AR-targeting agents in the current study were promising (data not shown), however due to time constraints further investigation of these combinations could not be completed. Combining AR antisense oligonucleotides with other therapeutic agents remains a promising avenue for future studies, exemplified by the clinical efficacy of the Bcl-2

antisense oligonucleotide, Oblimersen, in combination with docetaxel in patients with castrate-resistant prostate cancer (Tolcher *et al.* 2004; Tolcher *et al.* 2005).

The results of the current studies demonstrate that treatment with SAHA, bicalutamide or 17-AAG in combination, even at doses that are ineffective when used alone, act synergistically to inhibit LNCaP cell proliferation and survival. While the results of the current studies suggest that the synergy observed occurs through inhibition of AR signalling, 17-AAG and SAHA have multiple targets and the synergistic effects observed at low doses may therefore act partially through alternate mechanisms following sensitisation of prostate cancer cells by inhibition of AR signalling. The studies presented in this chapter provide proof of principle that combination AR-targeting with other therapies that have AR-targeting activities may be a better strategy to more completely inhibit prostate cancer cell proliferation and survival. Moreover, these strategies have the potential to prevent the outgrowth of a resistant population of cells with mechanisms to overcome therapeutic intervention. Elucidation of the most effective combinations in a preclinical model of prostate cancer will be important to determine *in vivo* efficacy, facilitating progression of the most suitable combination(s) to clinical evaluation.

## **Chapter 7**

# **The LNCaP Xenograft Model is Suitable for the Evaluation of AR-targeting Strategies *in Vivo***



## 7.1 Introduction

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In this chapter the LNCaP xenograft model was established and characterised as a potential model to evaluate AR-targeting strategies *in vivo*. Prostate cancer xenograft models consist of human prostate cancer cells implanted beneath the skin of nude (*nu/nu*) mice. Nude mice lack a thymus and are therefore immunodeficient and accept xenografts of foreign tissues, allowing the study of malignant cell growth in an *in vivo* environment. In addition the tumours are visible beneath the skin making it easy to monitor and measure tumour growth. Several series of prostate cancer xenograft models have been characterised; CWR, LAPC, LNCaP, LuCaP, MDA-Pca and the PC series. Most of these models now have more than one sub-line, usually recapitulating ‘androgen-dependent’ and ‘androgen-independent’ stages of tumour growth (van Weerden and Romijn 2000). There are some disadvantages associated with the use of xenograft models. For example, due to the immunodeficient background of the host, it is not known whether the processes involved in tumour growth can be extrapolated to immune-competent hosts (Stearns *et al.* 1998). In addition, most xenograft tumours are derived from late stage disease and therefore do not represent the entire spectrum of tumour types found *in vivo* (Stearns *et al.* 1998). Nonetheless, xenograft models have proven useful for assessing the efficacy of therapeutic agents *in vivo*. Alterations in gene expression (eg AR) that are integral to tumour growth and progression can be determined during treatment allowing a preclinical evaluation of novel forms of therapy. Prostate cancer xenograft models have been used in the development of therapeutics such as antisense oligonucleotides (Iversen *et al.* 2003; Miyake *et al.* 2005), Hsp90 inhibitors (Solit *et al.* 2002) and histone deacetylase inhibitors (Butler *et al.* 2000), where efficacy in terms of target reduction and inhibition of tumour proliferation can be monitored.



### **7.1.1 The LNCaP xenograft model**

The *in vivo* model chosen for use in the current studies is the LNCaP xenograft model. LNCaP cells are derived from a lymph node metastasis of prostate cancer (Horoszewicz *et al.* 1983) and express an AR variant containing a ligand-binding domain mutation (T875A), which broadens ligand specificity (Veldscholte *et al.* 1992a; Veldscholte *et al.* 1992b). LNCaP cells are androgen sensitive and when stimulated by androgens, express androgen regulated genes such as PSA (Zhu *et al.* 2003) and KLK2 (Mitchell *et al.* 2000). When grown subcutaneously (s.c.) in nude mice, the LNCaP xenograft recapitulates several important features of prostate cancer in humans. LNCaP xenograft tumours express AR, are androgen sensitive and secrete PSA proportional to tumour volume (Horoszewicz *et al.* 1983; Lim *et al.* 1993; Thalmann *et al.* 1994; Stearns *et al.* 1998). The LNCaP xenograft model has been used previously as a preclinical model for prostate cancer to test agents for their ability to delay tumour progression (Gleave *et al.* 1999; Ruggeri *et al.* 2003). In contrast to other xenografts that must be serially passaged in mice, a major advantage of this model is that it can be propagated both as a cell line *in vitro* and a xenograft *in vivo*, allowing direct comparison of *in vitro* and *in vivo* effects. One drawback of the LNCaP model is its limited metastatic potential when grown s.c. To circumvent this, several LNCaP sub-lines with increased metastatic potential have now been developed allowing for study of various stages of disease progression (Thalmann *et al.* 1994; Wu *et al.* 1994).

The objective of the current study was to establish the LNCaP xenograft model in the laboratory for future use in testing the efficacy of AR-targeting therapies *in vivo*. Specifically, tumour growth was characterised in intact versus castrated mice using markers such as tumour volume, AR and PSA expression.

## 7.2 Methods

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### 7.2.1 Inoculation of nude mice with LNCaP cells

LNCaP cells were trypsinised and resuspended in 10mL RPMI medium containing 10% FCS. Large clumps were removed and the cells were counted using a haemocytometer. Enough cell suspension for inoculation with  $1 \times 10^6$  cells per mouse was separated and centrifuged for 5 minutes at 1500rpm. The cells were resuspended in extracellular matrix (ECM) gel, 100 $\mu$ l for every  $1 \times 10^6$  cells. 100 $\mu$ l of cell suspension was injected subcutaneously on the right flank of nude mice 6-7 weeks of age.

### 7.2.2 Castration

When tumours reached approximately 250mm<sup>3</sup> mice were randomised to castration or sham-castration treatment groups. Mice were anaesthetised in a halothane chamber prior to surgery. Once the mice were fully anaesthetised an incision was made in the scrotal sac between the penis and the anus. Another incision was made to cut through the connective tissue of the scrotal sac. Each testis was removed by tying suture material around and excising the testis, vas deferens and associated fat. The remaining tissue was gently pushed back inside the scrotal sac and the incision was closed using 1-2 sutures. For sham-castrated mice an incision was made in the scrotal sac and closed with a single suture.

### 7.2.3 Testosterone injections

Initial attempts to establish LNCaP xenograft tumours resulted in inconsistent tumour take rate and tumour growth and insensitivity to castration. As athymic (*nu/nu*) mice have significantly less circulating levels of testosterone compared to their heterozygous littermates (Rebar *et al.* 1982), testosterone injections were administered (2mg/kg) via

intraperitoneal injections 5x weekly, beginning 2 days prior to inoculation with LNCaP tumour cells.

#### ***7.2.4 Calculation of tumour volume***

The mice were monitored until they developed palpable tumours. Tumour growth was then measured twice a week using calipers. The length and width of the tumours were measured and tumour volume was calculated using the formula; length x width<sup>2</sup> x  $\pi/6$ . When over 50% of tumours reached a volume of approximately 1000mm<sup>3</sup>, the mice were sacrificed by carbon dioxide inhalation and the tumours were removed, however a small group of mice were sacrificed at a shorter time point of 3-days post castration. The tumours were divided into smaller segments; approximately 2/3 of the tumour was fixed in formalin, while the remaining 1/3 was snap frozen in liquid nitrogen for further analysis.

#### ***7.2.5 Haematoxylin and Eosin staining***

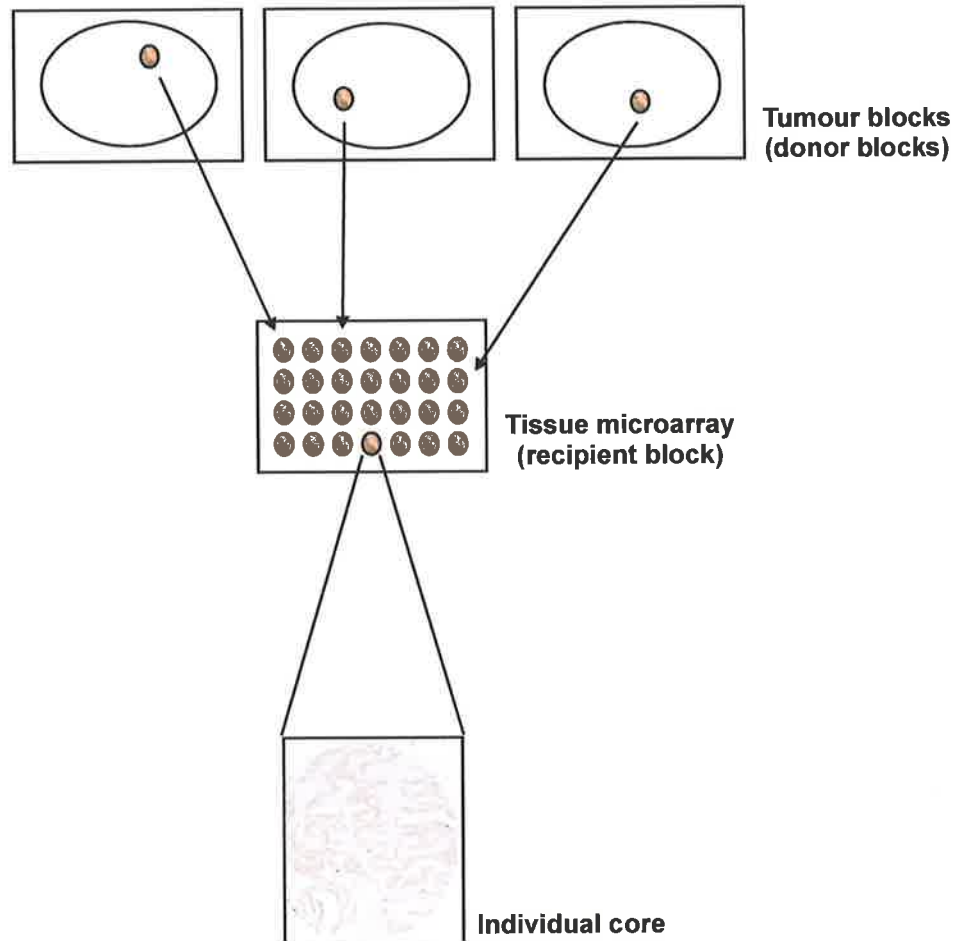
Tissue sections 4 $\mu$ M in thickness were cut from paraffin blocks using a microtome and placed onto glass slides. The slides were dewaxed in xylene, and rehydrated in ethanol washes. The slides were rinsed in running tap water and stained in Lillie Mayer's haematoxylin, then dipped in acid alcohol and rinsed in running tap water. The sections were blued in Scott's tap water and rinsed in running tap water prior to counterstaining with eosin. Following rinses in running tap water, the sections were dehydrated in ethanol for and cleared in xylene before they were cover slipped using DPX mounting medium. The H&E stained sections were used as a guide in the construction of tumour tissue microarrays.

### ***7.2.6 Construction of tissue microarrays***

Tissue microarrays were constructed from paraffin blocks of LNCaP xenograft tumours. A standard plastic cassette, containing the paraffin embedded donor block was placed into the donor block holder of the tissue microarrayer. A standard plastic cassette, containing a blank paraffin block (the recipient block) was placed into the recipient block holder and the height of the block was adjusted to ensure the donor needle was in a position at the top surface of the recipient block. Needles (2mm in diameter) were aligned to the grid pattern chosen for the tissue microarray (see appendix 3). The recipient needle was used to punch the recipient hole in the blank paraffin block. The area of tissue core to be selected was chosen from H&E stained reference slides. The donor needle was then used to core through the donor block at the selected position of the tumour sample, which was subsequently placed into the hole in the recipient block. This process was repeated until all desired tumour samples were collated into the tissue microarray (Figure 7.1). For each tumour sample three separate cores were placed into the array block. Cores of human prostate tissue and mouse testies were also collated into the microarray, serving both as orientation guides and positive controls for immunohistochemistry. During staining, several of the cores were washed off the tissue microarray slide and could therefore not be included in analyses. The number of samples analysed for each antibody is stated in the corresponding figures.

### ***7.2.7 Immunohistochemistry***

The following immunostaining protocol was used for detection of AR, PSA and Ki-67. Table 7.2.7 lists the concentrations of primary and secondary antibodies and the blocking solution used for each antibody. Antibody dilutions listed in table 7.2.7 were previously optimised in our laboratory.



**Figure 7.1 - Construction of tissue microarrays.** Tissue blocks containing LNCaP xenograft tumours were used as donor blocks to construct an LNCaP xenograft tumour tissue microarray. A total of three 2mm cores from each donor block were assembled into a recipient block, making up the tissue microarray. Also included in the tissue microarray were cores of human prostate tissue and mouse testes, which assisted in orientation of the microarray.

**Table 7.2.7 – Concentrations of antibodies used for immunohistochemistry**

Primary Ab	Primary Ab concentration	Secondary Ab	Secondary Ab concentration	Blocking solution
AR-U407	1:500	Goat anti-rabbit	1:400	5% goat serum
Ki-67	1:400	Rabbit anti-mouse	1:400	5% goat serum
PSA	1:3000	Goat anti-rabbit	1:400	5% goat serum

Abbreviations: Ab = antibody, NB: For negative control slides primary antibody was replaced by PBS. Blocking solution was made up in PBS.

Tissue sections 4 $\mu$ M in thickness were cut from paraffin blocks using a microtome and placed on glass slides, which were then baked at  $\sim$ 50 $^{\circ}$  for 2hr prior to staining. The slides were de-waxed in xylene and re-hydrated in ethanol washes and PBS. The slides were treated with 1% H<sub>2</sub>O<sub>2</sub> and boiled in 10mM citrate buffer (pH 6.5) for 20 minutes. Following PBS washes, 5% block was added and the sections were incubated for 1 hour at room temperature in a humid chamber. The blocking solution was removed and primary antibody was added overnight in a humid chamber at 4 $^{\circ}$ C. Following overnight incubation the slides were washed in PBS and the secondary antibody was added to the sections and incubated at room temperature for 1 hr in a humid chamber. The sections were washed in PBS followed by incubation with Streptavidin-horseradish peroxidase reagent (1:500 in PBS) at room temperature for 1 hour in a humid chamber. The slides were incubated with DAB/H<sub>2</sub>O<sub>2</sub>, followed washes in PBS. The sections were counterstained with Lillie Mayer's haematoxylin and rinsed in tap water. The slides were dehydrated and cleared in ethanol and xylene, and cover slipped using DPX mounting medium.

### **7.2.8 TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labelling (TUNEL) staining**

Tissue sections 4 $\mu$ M in thickness were cut from paraffin blocks using a microtome and placed on glass slides, which were then baked at  $\sim$ 50 $^{\circ}$  for 2hr prior to staining. The slides

were re-hydrated ethanol washes. Following washes in distilled water and PBS the slides were placed in 1% H<sub>2</sub>O<sub>2</sub>. The slides were washed in PBS and then immersed in 0.1% Triton-X in 0.1% sodium citrate buffer (pH 6.5). The slides were rinsed twice in PBS prior to the addition of TUNEL buffer for 10 minutes at room temperature. TUNEL reaction mix from the TUNEL staining kit was added and incubated in a humid box at 37°C for 3 hr. The slides were rinsed in PBS and 100µL of converter POD was added to each slide and incubated for 30 minutes at 37°C. Slides were rinsed in PBS and then incubated with DAB/H<sub>2</sub>O<sub>2</sub>, followed by washes in PBS. The sections were counterstained with Lillie Mayer's haematoxylin and rinsed in tap water. The sections were then dehydrated in ethanol and cleared in xylene, and cover slipped using DPX mounting medium.

TUNEL staining of LNCaP xenograft tumours resulted in high background staining; therefore three alterations to the protocol were made in separate experiments in an attempt to reduce the background staining. (i) Incubation time with DAB was reduced from 15 minutes to 10 and 5 minutes. (ii) The slides were boiled in citrate buffer for 20 minutes rather than incubation at room temperature for 8 minutes. (iii) TUNEL reaction mix was titrated at dilutions of 1:5, 1:10, 1:25 and 1:50.

A positive control, section from a rat mammary adenocarcinoma (Gibson *et al.* 2003), was also included in these experiments, which has previously shown appropriate TUNEL staining.

## ***7.2.9 Quantitation of immunohistochemical staining***

### *7.2.9.1 Measurement of AR and PSA concentration by video image analysis (VIA)*

The area and absorbance of DAB deposition was measured for AR and PSA in LNCaP tumour cells using automated image analysis system as reported previously (Tilley *et al.*

1994; Ricciardelli *et al.* 1997). Images of the immunostained LNCaP xenograft sections were taken at 40X magnification. The images of LNCaP xenograft tissues also contained stromal cells, which were edited out in order to measure only epithelial cell staining. The absorbance of DAB deposition for each image was determined and expressed as % positivity or mean integrated optical density (MIOD). % positivity is a calculation of the positively stained area divided by the total area. MIOD is a calculation of integrated optical density divided by total area. This measurement takes into account the optical density and is a measurement of the concentration of DAB deposition.

The intensity of tissue staining was also assessed by eye to ensure correlation between the intensity of staining measured by VIA.

#### *7.2.9.2 Manual scoring of Ki-67*

Images of Ki-67 stained LNCaP xenograft sections were taken at 40X magnification. The positive nuclei were counted and expressed as a percentage of total nuclei per image in a total of 5 images per core. A value for % positivity was derived by calculating the average percent of positive cells in the 5 images per core, therefore giving an average of 15 images per tumour.

#### *7.2.10 Statistical analysis*

Differences in immunohistochemical staining intensity between treatment groups was analysed by non-parametric Mann-Whitney U Tests using SPSS statistical analyses software. A p-value of <0.05 was considered statistically significant.



## 7.3 Results

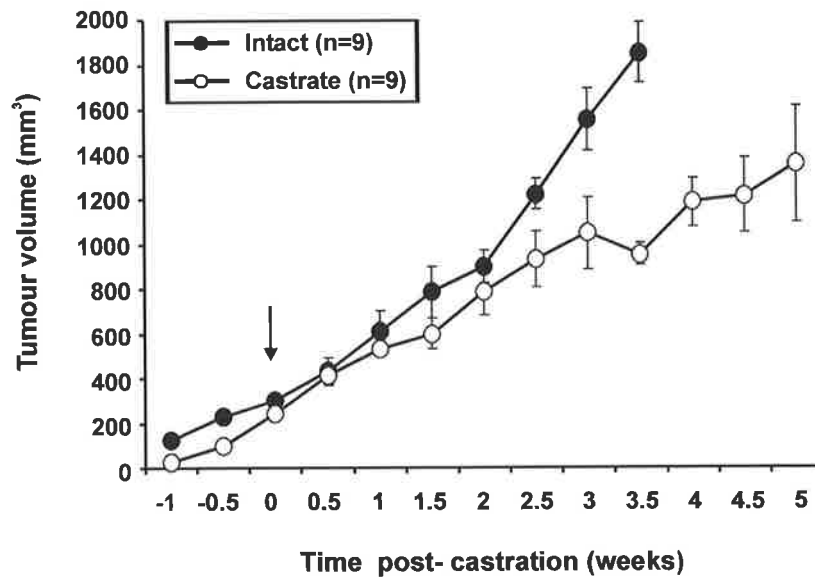
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### 7.3.1 Tumour growth

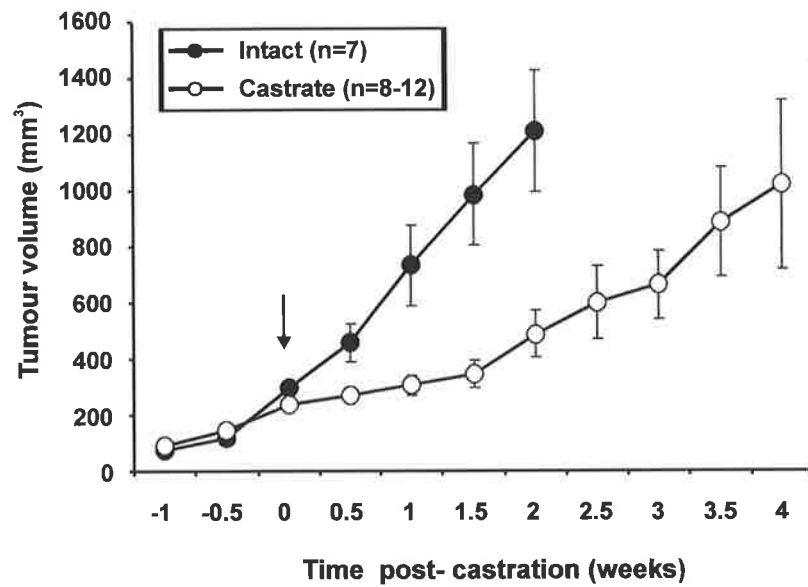
An initial cohort of 30 mice were inoculated with LNCaP cells and after tumours reached a volume of approximately 250mm<sup>3</sup> the mice were randomised to sham-castration or castration treatment groups (Figure 7.2). The first cohort of mice inoculated with LNCaP cells had a poor take rate, and showed no difference in tumour growth rate between intact and castrated groups of mice until 2-3 weeks post castration where tumours from castrated mice grew more slowly compared to tumours from intact mice. A second cohort of mice was also inoculated with LNCaP cells, however this group received testosterone injections, which resulted in increased consistency of tumour growth rate and increased sensitivity to castration (Figure 7.3). In this group, tumours derived from intact mice grew to approximately 1000mm<sup>3</sup> in 1.5-2 weeks following surgery, while castration delayed tumour progression for up to 2 weeks. All subsequent analyses were performed on tumours derived from mice in the second cohort (Figure 7.3).

### 7.3.2 AR levels are increased in LNCaP xenograft tumours following castration

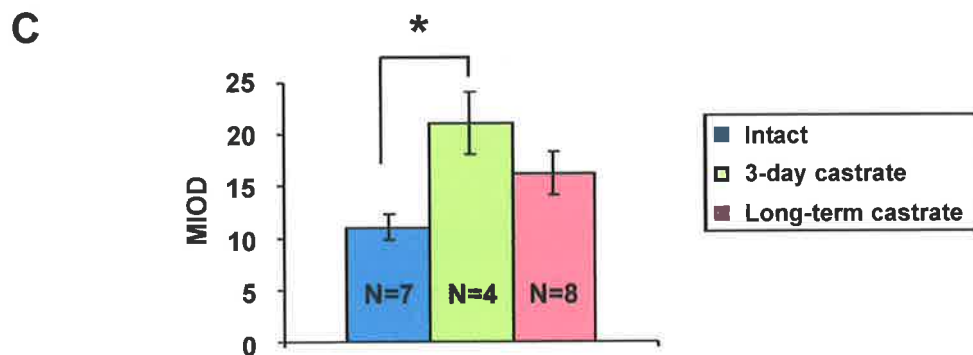
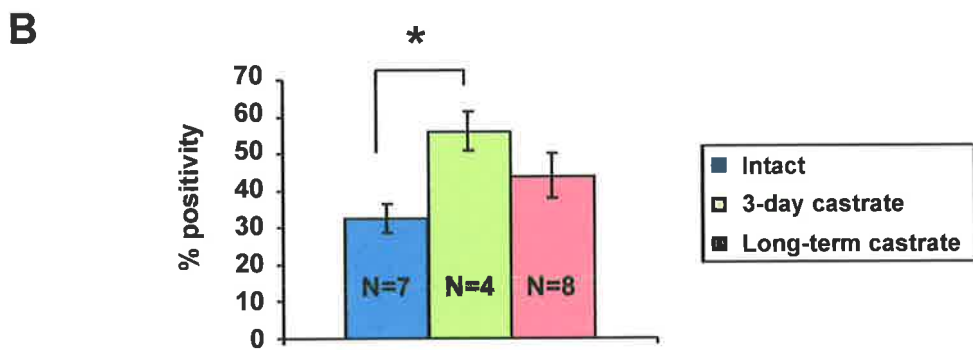
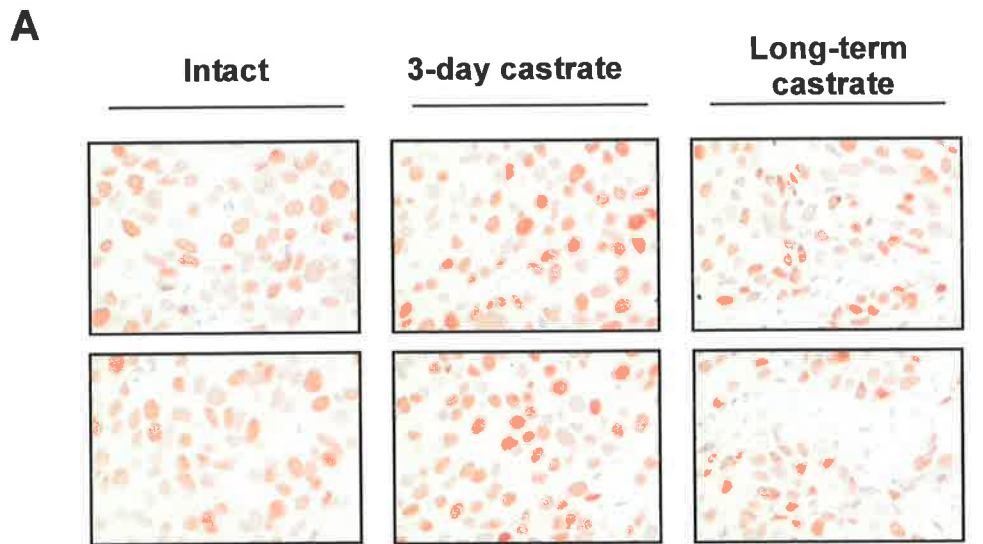
To visualise AR levels in LNCaP xenograft tumours, sections from a tissue microarray compiled from individual paraffin embedded tumours were sectioned and stained using the anti-AR U407 antibody, which is routinely used in our laboratory. Representative images of anti-AR U407 stained sections derived from intact, 3-day castrate or long-term castrate mice (i.e. tumours that grew to approximately 1000mm<sup>3</sup> following castration) are shown in figure 7.4A. Anti-AR U407 stained tissue sections show moderate-strong stained nuclei with weak cytoplasmic staining and negative stromal staining. Quantitation of staining intensity demonstrated a difference in AR staining between treatment groups (Figure 7.4B,C). While there was a trend towards an increase in AR positivity



**Figure 7.2 - Growth of LNCaP xenograft tumours in intact versus castrated mice (cohort 1)** Nude mice were inoculated subcutaneously on their right flank with  $1 \times 10^6$  LNCaP cells suspended in 100mL ECM gel. Once tumours reached a volume of approximately  $250 \text{ mm}^3$  the mice were either castrated or sham castrated, as indicated by the arrow.



**Figure 7.3 - Growth of LNCaP xenograft tumours in intact versus castrated mice (cohort 2)** Nude mice supplemented with testosterone (2mg/kg, 5x weekly) were inoculated subcutaneously on their right flank with  $1 \times 10^6$  LNCaP cells suspended in 100mL ECM gel. Once tumours reached a volume of approximately  $250 \text{ mm}^3$  the mice were either castrated or sham castrated, as indicated by the arrow.



**Figure 7.4 - Immunostaining of LNCaP xenograft tumours using anti-AR U407 antibody.** (A) LNCaP tumour sections derived from intact, 3-day castrate or long-term castrate mice were stained for AR immunoreactivity using the anti-AR U407 antibody. Images captured at 40X magnification were analysed by video image analysis to quantify AR levels. (B, C) % positivity and mean integrated optical density (MIOD) readings from tissue microarrays. \* Mann-Whitney U test;  $p < 0.05$  3-day castrate *versus* control.

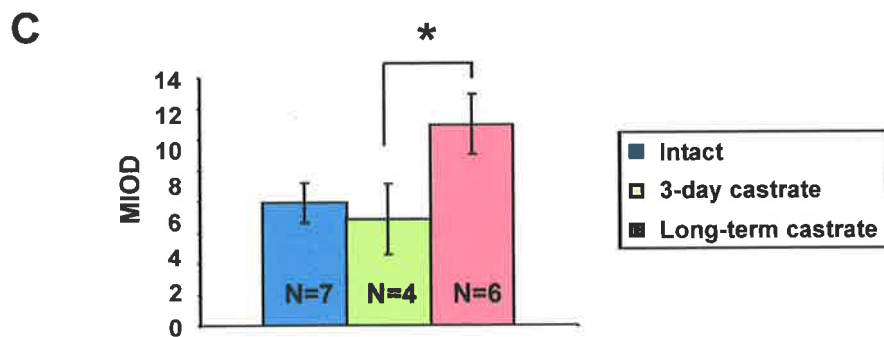
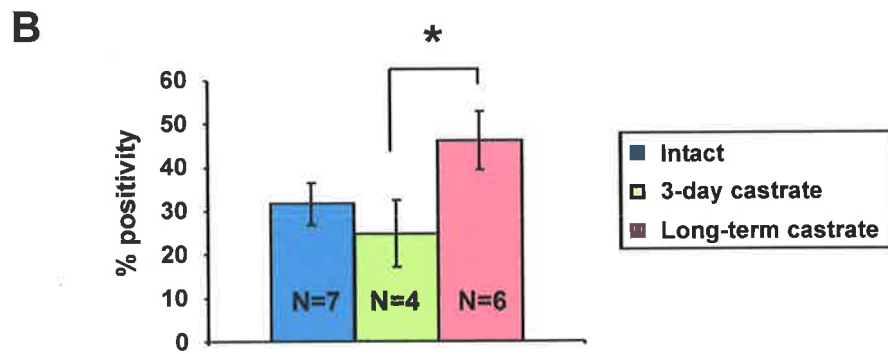
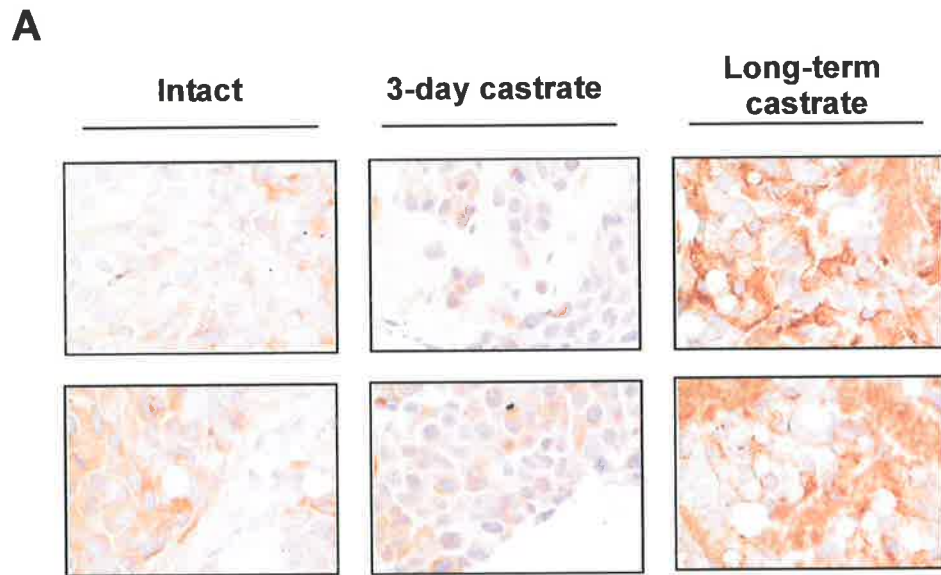
and MIOD in tumours from long-term castrate mice compared to intact mice, statistical significance was only observed when comparing AR levels in tumours from 3-day castrate mice compared to tumours from intact mice ( $p=0.023$ ).

### ***7.3.3 Intratumoural PSA is increased following castration***

As a marker of AR activity, intratumoral PSA staining was analysed in LNCaP xenograft tumours. Representative images from anti-PSA stained tumours are shown in figure 7.5A. While there was no difference in % positivity or MIOD between tumours from intact compared to 3-day castrate mice, levels of PSA staining were higher in tumours from long-term castrate mice compared to the other two groups. Statistically significant differences were observed when comparing tumours from long-term castrate mice with tumours from 3-day castrate mice ( $p=0.05$ ).

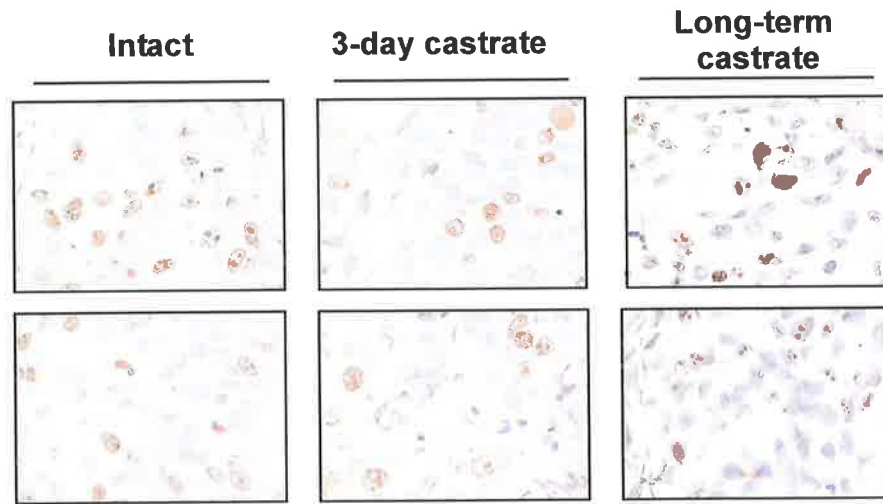
### ***7.3.4 Reduced levels of the proliferation marker, Ki-67, following castration***

Ki-67 is an antigen that is present only in the nuclei of cycling cells and is therefore used as a marker of proliferation. Representative images of the anti-Ki-67 stained LNCaP xenograft tumour sections are shown in figure 7.6A. Analysis of Ki-67 staining was carried out using a manual scoring system, where the number of positive nuclei in an image was expressed as a percentage of the total positive nuclei. Ki-67 positivity decreased with increasing time post-castration (Figure 7.6B). At 3-days post-castration Ki-67 positivity was lower than levels observed in tumours from the intact group, while positivity in tumours derived from long-term castrate mice was lower compared to the other two treatment groups, indicative of a lower proliferation rate. Statistically significant differences were observed when comparing tumours from intact mice versus tumours from long-term castrate mice ( $p=0.011$ ).

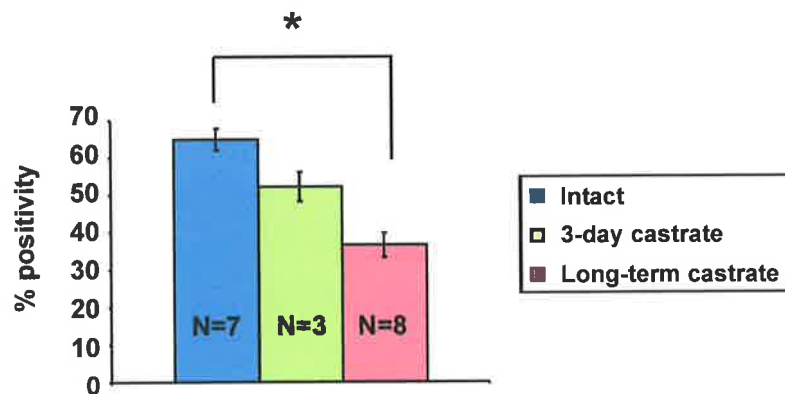


**Figure 7.5 - Immunostaining of LNCaP xenograft tumours using anti-PSA antibody.** (A) LNCaP xenograft tumour sections derived from intact, 3-day castrate or long-term castrate mice were assessed for PSA immunoreactivity using anti-PSA antibody. Images captured at 40X magnification were analysed by video image analysis to quantify PSA levels. (B, C) % positivity and mean integrated optical density readings from tissue microarrays. \* Mann-Whitney U test;  $p < 0.05$  3-day castrate *versus* long-term castrate.

**A**



**B**



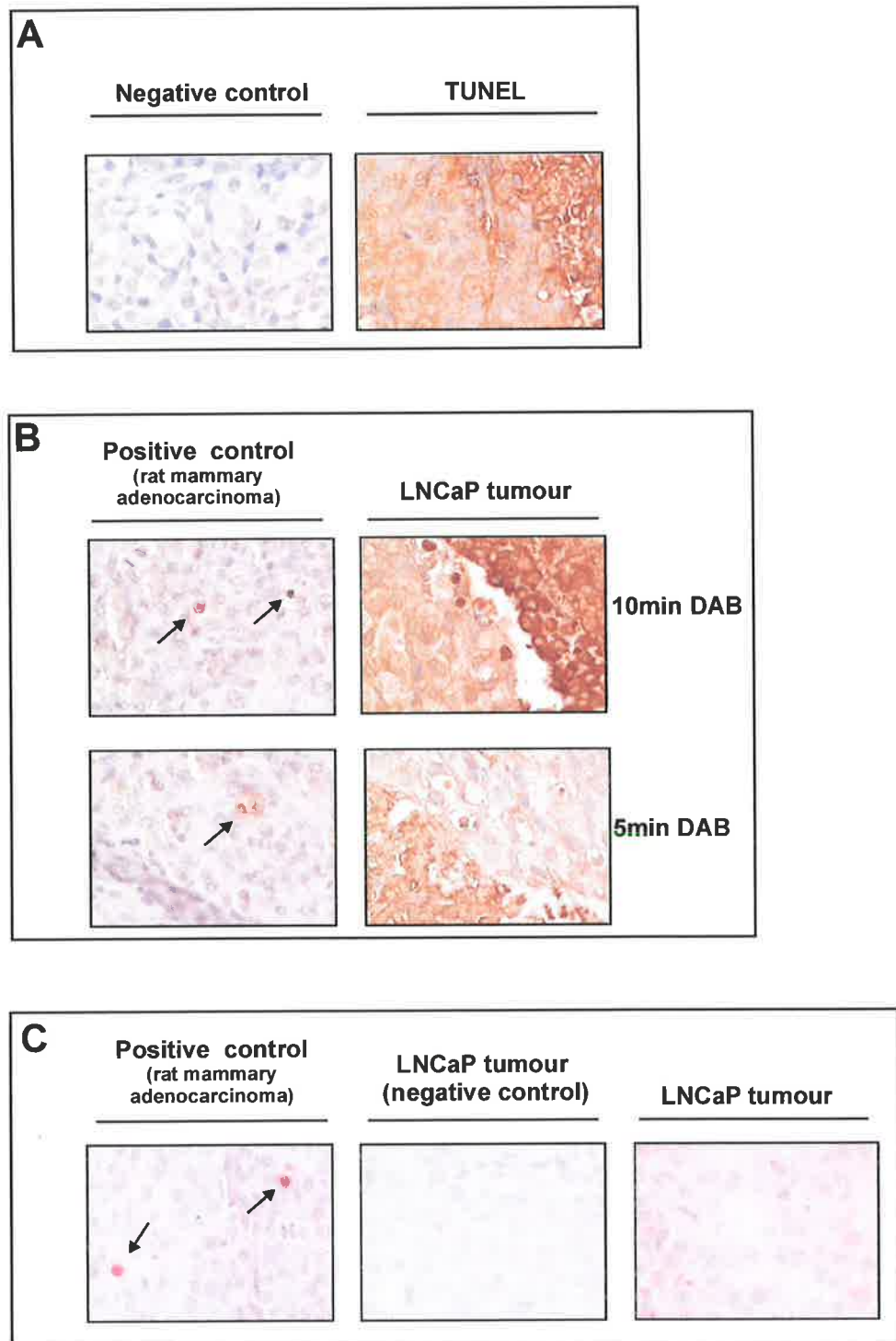
**Figure 7.6 - Immunostaining of LNCaP xenograft tumours using anti-Ki-67 antibody.**

(A) LNCaP xenograft tumour sections derived from intact, 3-day castrate or long-term castrate mice were assessed for Ki-67 immunoreactivity using anti-Ki-67 antibody. Images captured at 40X magnification were manually scored for percent positive nuclei. (B) An average percentage was calculated from five separate images per core to give % positivity of Ki-67 staining. \* Mann-Whitney U test;  $p < 0.05$  long-term castrate *versus* intact.

### ***7.3.5 TUNEL staining of LNCaP xenograft tumours***

TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labelling (TUNEL) was used to detect apoptotic cells in LNCaP xenograft tumours. Immunostaining with this method was associated with high background staining in the LNCaP xenograft tissue sections (Figure 7.7A). Several methods were investigated to decrease the background staining, including incubation with DAB for shorter time periods, and detection of apoptotic cells using fast red reagent instead of DAB. Although reducing DAB incubation time reduced background staining (Figure 7.7B), this also reduced the intensity of positively stained cells, making it difficult to clearly identify apoptotic cells. Apoptotic cells were clearly visible in tissue sections from a rat mammary adenocarcinoma model, which was used as a positive control (Figure 7.7B). Detection of apoptotic cells using fast red reagent also resulted in high background staining of the LNCaP tumours (Figure 7.7C), however this background staining was not seen in the rat mammary adenocarcinoma, which showed clearly identifiable positively stained apoptotic cells (Figure 7.7C).





**Figure 7.7 - TUNEL staining of LNCaP xenograft tumours.** (A) LNCaP tumour sections were stained using TUNEL staining kit. For negative control slide TUNEL reaction mix was omitted. (B) Tissue sections from a rat mammary adenocarcinoma were used as a positive control for TUNEL staining. DAB incubation time was reduced from 15 min to 10 and 5 min (C) Detection of TUNEL positive cells using fast red reagent. For the negative control TUNEL reaction mix was omitted. Images were taken at 40X magnification. Arrows indicate TUNEL positive cells.

## 7.4 Discussion

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Consistent with previous findings, the results of the current study demonstrate retarded tumour growth following castration in the LNCaP xenograft model (Nicholson *et al.* 2004). The growth characteristics of the LNCaP xenograft model differ from other prostate cancer xenografts. For example LuCaP 23.1 xenograft tumour growth is arrested following castration and re-grows up to 10-14 weeks post-castration (Bladou *et al.* 1996; Fina *et al.* 2005), while the most well characterised prostate cancer xenograft, the CWR22 model regresses markedly following castration and begins to regrow from 3-10 months later (Nagabhushan *et al.* 1996).

Tissue microarrays (TMAs) were used in the current studies to assess molecular markers in the LNCaP xenograft tumours. Tissue microarray technology was developed in 1998 (Kononen *et al.* 1998) making analysis of tissue sections much faster as hundreds of samples can be stained and analysed simultaneously. In addition, individual tumour blocks can be used multiple times in constructing TMAs, increasing the amount of information that can be obtained from a single tissue block. One concern with TMAs is that small cores may not be representative of the entire tissue block from which it was taken. This becomes a greater concern when the tissues being collated into a microarray have a high level of heterogeneity. However, several studies have now demonstrated concordance of results between whole tissue section analyses versus tissue microarray analyses, even for heterogenous tumour samples (reviewed in Simon and Sauter 2002). Arraying 2-3 cores from each sample was found to be more representative of the entire block, while the size of the cores did not seem to make much difference (Simon and Sauter 2002). In the current study, triplicate cores from each LNCaP xenograft tumour

were arrayed into a single paraffin block, to increase likelihood of capturing representative samples of each tumour.

The results of the current study demonstrating an increase in AR levels in LNCaP xenograft tumours that continue to proliferate following long-term castration are consistent with previous studies that have shown an increase in AR mRNA and protein in castrate-resistant CWR22, LAPC4, LAPC9, LNCaP, LuCaP23, LuCaP35, LuCaP41, and MDA-Pca xenografts compared with their intact counterparts (Kim *et al.* 2002; Hara *et al.* 2003b; Chen *et al.* 2004). In the current study it was found that the intensity of PSA staining was higher in tumours derived from long-term castrate mice compared to those from intact mice, indicative of active AR signalling in these tumours. In LuCaP23.1 xenograft tumours, increased expression of AR correlated with tumour size (Fina *et al.* 2005). In castrate-resistant tumours, the AR may acquire the ability to signal via alternate activation pathways, which would lead to continued tumour growth and an increase in PSA expression. It has recently been demonstrated that increased expression of the AR is sufficient for castrate-resistant tumour growth to occur in xenograft tumours, which can be inhibited by AR-siRNA (Chen *et al.* 2004). Taken together these results implicate increased expression of the AR as a mechanism for castrate-resistant regrowth in LNCaP xenograft tumours.

Ki-67 positivity in LNCaP xenograft tumours was significantly decreased in castrate-resistant tumours compared to intact controls. The results are consistent with a slower rate of tumour proliferation in these mice. Ki-67 positivity in tumours from 3-day castrate mice is higher than observed in the long-term castrate group but lower than intact controls, consistent with slowing of tumour growth rate at 3-days post castration. These results are consistent with Kim *et al.* (2002) who reported greater levels of Ki-67 staining

in CWR22 xenograft tumours grown in intact mice compared with tumours that were grown in castrate mice that have become castrate-resistant. Moreover, at short intervals post-castration, intermediate levels of Ki-67 staining were noted (Kim *et al.* 2002). It was not possible to analyse apoptosis in the current studies due to the high levels of background observed with the TUNEL staining method. Further analysis of apoptosis was not feasible for this thesis due to time constraints; however future studies should aim to optimise an alternate marker of apoptosis, such as activated caspase-3 (Hu *et al.* 2000; Gown and Willingham 2002).

In summary, the LNCaP xenograft model was established in the current study as a model for assessing the effect of AR-targeting strategies on tumour growth. Castrate-resistant tumour growth in the LNCaP xenograft model is associated with an increase in AR and PSA expression. The model is consistent with therapy resistant progression of human prostate cancer, which has been associated with increased levels and/or activity of the AR, making it ideal to study AR-targeting agents in an attempt to prohibit or delay tumour progression. Future studies using AR-targeting strategies in the LNCaP xenograft model should aim to optimise dosages and treatment protocols (ie, simultaneous or sequential therapy) in assessing the effects of combinatorial AR-targeting strategies on tumour proliferation and apoptosis.

## **Chapter 8**

### **General Discussion**

### ***8.1 AR-targeting in prostate cancer***

Androgen ablation therapy has been the mainstay of treatment for patients with advanced prostate cancer since the 1940's when Huggins, Stevens and Hodges first demonstrated the beneficial effects of castration (Huggins 1941). Although initially successful, almost all patients will relapse with castrate-resistant tumour progression, with a median survival of 12 months (Trachtenberg *et al.* 2002). Disease relapse occurs not necessarily because tumour cells bypass their requirement for androgen signalling, but rather that mechanisms are acquired that allow continued AR activation and tumour growth in the castrate environment (reviewed in Buchanan *et al.* 2001b). The AR itself is therefore a potential therapeutic target for the treatment of castrate-resistant prostate cancer.

Since the initiation of this thesis, a series of studies have provided further evidence for the involvement of the AR in the initiation and progression of prostate cancer to a castrate-resistant state following treatment with AAT. This in turn has provided support for the development of AR-targeting strategies to treat castrate-resistant prostate cancer. A study by (Chen *et al.* 2004) assessed the molecular mechanisms associated with the progression of seven prostate cancer xenograft models from an androgen sensitive to an androgen insensitive state. This study found that the only consistent alteration between so-called 'androgen sensitive' and 'androgen insensitive' states was an increase in expression of AR mRNA. In addition, over-expression of the AR in LNCaP cells was sufficient to overcome growth inhibition associated with low levels of ligand, and to overcome the inhibitory effects of bicalutamide (Chen *et al.* 2004). Conversely, LAPC4 and LNCaP xenograft tumour growth rate was significantly slowed following a reduction in AR levels by a lentiviral vector expressing short-hairpin RNA directed against the AR (Chen *et al.* 2004). This study provided direct evidence that the over-expression of the AR was sufficient to overcome the growth inhibitory effects of castration or treatment

with the AR-antagonist, bicalutamide. Critical evidence for a role of AR in the initiation of prostate cancer was provided by Han *et al.* (2005) who demonstrated the oncogenicity of an AR variant. In that study, enforced prostatic expression of the gain-of-function AR-E231G variant resulted in prostate tumour formation and lung metastases in 100% of mice. This report demonstrated for the first time the involvement of the AR in the initiation of prostate cancer, in addition to the processes of invasion and metastasis, thereby raising the possibility that the AR may also be a viable target for the prevention of prostate cancer.

A number of groups have developed molecular agents designed to block AR expression or function, including siRNA (Caplen *et al.* 2002; Haag *et al.* 2005; Liao *et al.* 2005; Yang *et al.* 2005), antisense oligonucleotides (Eder *et al.* 2000; Eder *et al.* 2002; Hamy *et al.* 2003; Ko *et al.* 2004), hammerhead ribozymes, anti-AR antibodies (Zegarra-Moro *et al.* 2002), and dominant negative androgen receptors (Butler *et al.* 2006). Reduction of AR expression and/or activity using these strategies results in inhibition of prostate cancer cell proliferation and survival both *in vitro* and *in vivo*. In addition, inhibiting AR expression is an effective strategy for inhibiting the growth of so-called ‘androgen-independent’ LNCaP cell sub-lines that express the AR (e.g. LNCaP-abl, LNCaP-Rf and LNCaP-C4) (Zegarra-Moro *et al.* 2002; Hamy *et al.* 2003; Haag *et al.* 2005; Yang *et al.* 2005). Importantly, inhibiting AR expression not only inhibits cell proliferation, but sustained suppression of AR levels results in apoptosis of prostate cancer cells (Liao *et al.* 2005; Yang *et al.* 2005).

## **8.2 Major findings of this thesis**

The results presented in this thesis describe the use of several novel AR-targeting strategies in prostate cancer cells. A reduction in AR expression at the mRNA and protein

levels, and inhibition of AR activity was achieved using the AR-antisense oligonucleotide as-AUG, the Hsp90 inhibitor 17-AAG and the histone deacetylase inhibitor SAHA. Culture of prostate cancer cells with each of these agents inhibited proliferation and induced cell death in a dose-dependent manner. While as-AUG markedly reduced AR expression, the effect was maintained for a relatively short period of time, 12 hr, compared to SAHA treatment which caused a reduction in AR steady-state protein levels for at least 48 hr. Unlike as-AUG or SAHA, 17-AAG had a less marked effect on AR protein levels; however, 17-AAG at low nanomolar concentrations that had little effect on AR protein levels effectively inhibited transactivation activity of both wtAR and 10 AR variants.

A reduction in AR levels may be one of the mechanisms responsible for SAHA-induced cell death in AR-positive prostate cancer cells. Therefore, unlike 17-AAG which inhibits AR to a greater extent at the level of transactivation, SAHA exerts its effects on AR signalling via a reduction of AR mRNA levels. In the context of castrate-resistant prostate cancer it may be important to determine whether SAHA retains its ability to reduce levels of AR variants, both alone and in combination with other AR-targeting agents. The current studies in LNCaP cells containing the AR variant T875A suggest that it would be effective against castrate-resistant prostate cancers harbouring AR variants.

Enhanced inhibition of proliferation and induction of cell death in response to both 17-AAG and SAHA was observed in the absence of steroids, suggesting that reduction of AR signalling by reducing ligand bioavailability sensitises prostate cancer cells to other forms of therapy. Moreover, all three combinations (i.e. SAHA and bicalutamide, 17-AAG and bicalutamide or SAHA and 17-AAG) synergistically inhibited LNCaP cell proliferation and survival, at concentrations that were not efficacious when the drugs were used alone. Conversely, the drug combinations had no effect in AR-negative PC-3



cells. This difference in response between AR-positive and AR-negative cell lines was not surprising for combinations that included the specific AR-antagonist bicalutamide, as PC-3 cells are AR-negative. However, as both SAHA and 17-AAG have multiple targets within a cell, the lack of effect of combined SAHA and 17-AAG in PC-3 cells was unexpected. Collectively, these results suggest that targeting of the AR in prostate cancer cells appears to be necessary for the synergistic effects of the combination treatments. However, targeting of the AR may not be entirely responsible for the dramatic antiproliferative and proapoptotic effects observed. Instead, AR-targeting may result in sensitisation of prostate cancer cells to the effects of 17-AAG and SAHA on other signalling pathways. Further study to elucidate the alternate mechanisms involved may reveal other important targets for prostate cancer therapy.

These promising results of combinatorial AR-targeting presented in this thesis suggest that this strategy may be useful for the treatment of castrate-resistant prostate cancer. Moreover, the concentrations of SAHA, 17-AAG and bicalutamide used are achievable in the serum of patients (Soloway *et al.* 1996; Kelly *et al.* 2003; Ramanathan *et al.* 2005). These findings support the use of SAHA or 17-AAG in combination with AAT, which may have greater efficacy in clinical prostate cancer. In addition, combining agents that target multiple points in the androgen signalling pathway may prevent the outgrowth of a resistant population of cells that harbour mechanisms to overcome therapeutic intervention (refer to section 1.6).

### ***8.3 Potential side-effects of systemic AR-targeting***

A concern with completely blocking AR signalling as a therapeutic strategy for prostate cancer is the side-effects that may occur in peripheral androgen-dependent tissues such as bone, brain and muscle. Complete ablation of AR activity is not life-threatening, which is

exemplified by men with complete androgen insensitivity syndrome. It has therefore been stated that inhibition of AR signalling as a therapeutic goal should only have lethal effects in male accessory sex organs (Litvinov *et al.* 2003). While the effects of systemic AR-targeting may not be life-threatening, there are important physical and psychological issues that should be addressed when assessing the efficacy of systemic AR-ablation. The side-effects currently associated with prolonged AAT include osteoporosis, anaemia, sarcopenia, depression, cognitive decline and sexual dysfunction (reviewed in (Holzbeierlein *et al.* 2003; Holzbeierlein *et al.* 2004). Implementation of AR-targeting strategies could have similar side-effects, possibly to a greater degree due to the complete block of AR signalling in peripheral tissues. Although the incidence of prostate cancer rises sharply with age, prostate cancer is increasingly diagnosed in younger men (Merrill and Morris 2002). If AR-targeting therapies have to be administered in younger men, for longer periods of time, quality of life could have a greater bearing on the decision to undergo therapy.

Both 17-AAG and SAHA demonstrate acceptable toxicity profiles *in vivo* (Kelly *et al.* 2003; Banerji *et al.* 2005a). While 17-AAG is thought to bind with greater affinity to 'active' Hsp90 within tumour cells (refer to section 4.1.2), the mechanism of SAHA selectivity for tumour cells is not well understood. Whereas histone acetylation occurs in both transformed and normal cells, the inhibitory effects of HDAC inhibitors on proliferation and survival appear to be tumour cell specific (Brinkmann *et al.* 2001; Fournel *et al.* 2002). Although both 17-AAG and SAHA demonstrate some tumour cell selectivity, suggesting that significantly lower doses used in combination could minimise toxicity, a critical issue yet to be addressed is whether the combinations of these agents will have synergistic antiproliferative and proapoptotic effects in normal cells *in vivo*.

An alternative to AR-targeting using systemic therapy would be the development of prostate-targeted therapies that could potentially reduce toxicity to peripheral tissues. For example the use of viral vectors with prostate-specific promoters to target gene therapy to prostate tissue would result in expression of gene therapy solely in target tissue thereby abrogating systemic side-effects. Gene therapy strategies could be used to increase expression of an AR suppressor protein, or viral delivery of AR-targeted antisense oligonucleotides or siRNA used to inhibit AR expression in prostate cancer cells (Galanis *et al.* 2001; Lu 2001). A combination of these prostate-targeted strategies with a systemic agent, such as bicalutamide, may act synergistically to inhibit proliferation and induce apoptosis in prostate tumour cells while sparing peripheral androgen-regulated tissue. Combination strategies that include gene therapy administered locally in the prostate will be useful only if tumour cells have not spread beyond the confines of the prostate. If metastases are already present, then alternative strategies would be necessary. In these cases specific targeting of therapy to metastatic sites may be effective. Specific targeting of gene therapy is currently under investigation for the treatment of metastatic prostate cancer to bone (Koeneman *et al.* 2000). While there are several clinical trials underway for prostate cancer gene therapy (Lu 2001), none of these strategies are directed against the AR or critical components of the androgen signalling axis. This is an area of research that warrants further development as a strategy to treat castrate-resistant prostate cancer.

#### **8.4 Future studies**

##### *8.4.1 Further investigation of the AR-targeting strategies tested in this thesis*

The current studies demonstrating synergistic activities of 17-AAG, SAHA and bicalutamide combinations were assessed in LNCaP and PC-3 prostate cancer cells. Further studies should aim to assess the effects of combination AR-targeting strategies in

a panel of prostate cancer cell lines including AR-positive ‘androgen-independent’ cell lines to ensure that the enhanced activity of the combination therapies observed in LNCaP cells is consistent across a range of prostate cancer cell lines. Moreover, evaluation of the effects of combination AR-targeting in normal prostate cells may be useful in predicting toxicity in normal cells and tissues. Testing of these AR-targeting strategies in an *in vivo* prostate cancer model, such as the LNCaP xenograft model described in chapter 7, will allow investigation of optimal dosage, schedule and route of administration, facilitating identification of the most efficacious regimens that should be evaluated clinically. Moreover, strategies regarding the timing and/or sequential administration of AR-targeting agents should be investigated.

Investigation into the mechanism(s) of SAHA-mediated AR reduction in prostate cancer cells should also be pursued. The findings of the current study suggest that the effect occurs predominantly at the transcriptional level, possibly due to induction of an AR suppressor protein. Identification of this putative AR-suppressor protein may enable the design of novel therapeutic agents that target AR signalling more specifically in prostate cancer cells.

#### *8.4.1 Investigation of alternate AR-targeting strategies*

While four AR-targeting agents were used in the current studies, alternate forms of AAT, such as the 5 $\alpha$ -reductase inhibitors finasteride and dutasteride, which inhibit the conversion of testosterone to DHT, may be a useful component of combination AR-targeting strategies. Moreover, there remain a number of chemotherapeutic or chemopreventive agents under investigation for the treatment of various cancer, which have recently demonstrated AR-targeting activities in prostate cancer cells. Therefore a range of opportunities exist for further combinatorial AR-targeting strategies warranting

further investigation. Chemotherapeutic agents currently used for the treatment of advanced prostate cancer such as cisplatin and the taxanes have been reported to bind to Hsp90, with potential for downstream effects on AR signalling. Cisplatin binds to the C-terminal end of the Hsp90 molecule, unlike Hsp90 inhibitors such as GA and 17-AAG, which bind to the N-terminal portion of Hsp90 (Caplan *et al.* 2003). Rosenhagen *et al.* (2003) demonstrated a reduction of both GR and AR transactivation activity in neuroblastoma cells following treatment with cisplatin at micromolar concentrations. Resultant proteasomal degradation of glucocorticoid receptor was observed similar to that induced by GA (Rosenhagen *et al.* 2003). Interestingly, other Hsp90 client proteins such as Raf-1 were unaffected by treatment with cisplatin, suggesting a mechanism of Hsp90 inhibition that may specifically disrupt the interaction between Hsp90 and steroid receptors (Rosenhagen *et al.* 2003). Another class of chemotherapeutic agents with potential AR-targeting capacity are the taxanes, docetaxel (Taxotere) and paclitaxel (Taxol). Paclitaxel also reportedly binds to Hsp90 (Byrd *et al.* 1999). Furthermore, treatment with docetaxel results in ubiquitination and proteasomal degradation of Hsp90 in endothelial cells (Murtagh *et al.* 2006). This reduction in Hsp90 would obviously impact on its client proteins, including the AR.

Several chemopreventive agents have recently been shown to have inhibitory effects on AR signalling in prostate cancer cells. For example non-steroidal anti-inflammatory drugs such as celecoxib, exisulind and nimesulide reduce both AR mRNA and protein expression in prostate cancer cell lines at low micromolar concentrations (Lim *et al.* 2003; Pan *et al.* 2003; Narayanan *et al.* 2004). The reduction in AR expression is suggested to be through increased expression and activation of c-Jun, which inhibits the expression of AR and AR-regulated genes (Pan *et al.* 2003). Nutritional compounds such as antioxidants may also contribute to the growing list of potential AR-targeting

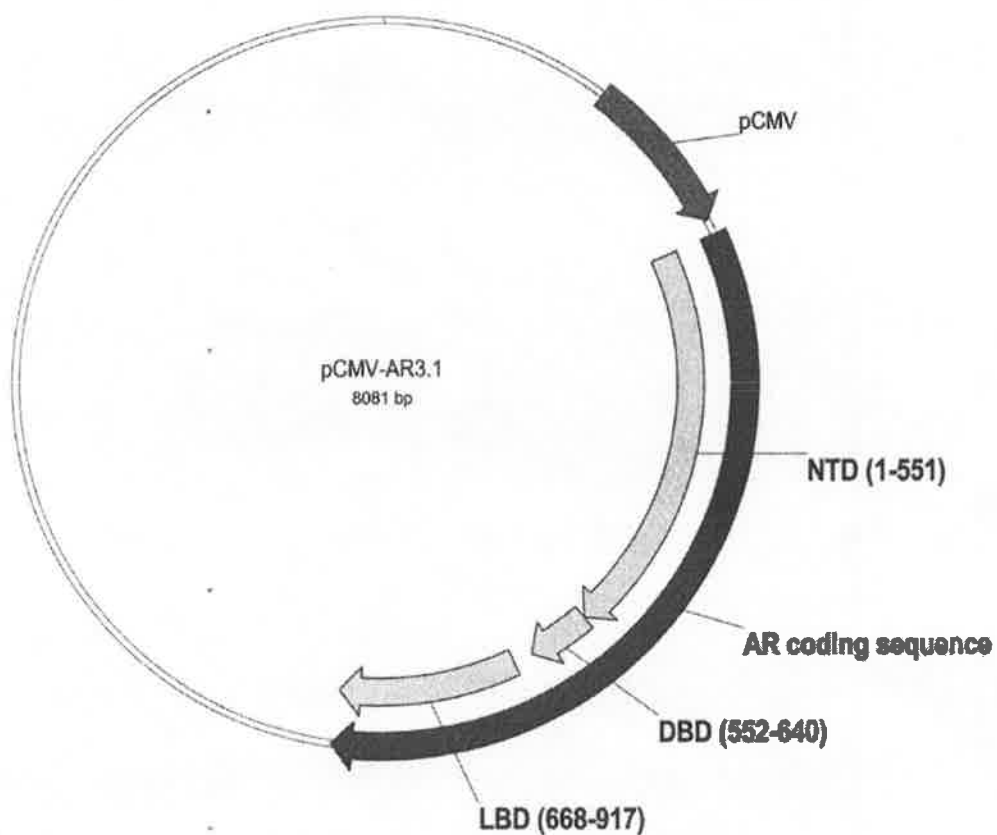
molecules. For example selenium and its derivatives reportedly inhibit AR expression and signalling in prostate cancer cell lines (Cho *et al.* 2004; Dong *et al.* 2004; Chun *et al.* 2006; Husbeck *et al.* 2006). A study by Chun *et al.* (2006) revealed that treatment with selenium resulted in an increase in the recruitment of AR co-repressors to the PSA promoter, which contributed to the inhibition of AR signalling in prostate cancer cells.

### **8.5 Summary & Conclusions**

As prostate cancer progresses despite AAT, and there is strong evidence that this progression occurs due to continued AR signalling in the castrate environment, combination AR-targeting strategies is a rational step in the development of therapeutic intervention for advanced prostate cancer. The studies described in this thesis, demonstrating AR-targeting activities of antisense oligonucleotides, 17-AAG and SAHA, with antiproliferative and proapoptotic effects in prostate cancer cells provides proof of principle that AR-targeting strategies may be a more effective strategy. Importantly, these studies demonstrate that the inhibitory effects of these agents on proliferation and survival in LNCaP cells are synergistically enhanced when two AR-targeting agents are combined *in vitro*. Given that the majority of clinical prostate tumours express the AR, including those that fail hormonal therapy, further investigation into the use of agents that have AR-targeting activities for the treatment of prostate cancer in appropriate pre-clinical models is warranted, particularly in the context of combination therapy with conventional androgen ablation therapies.

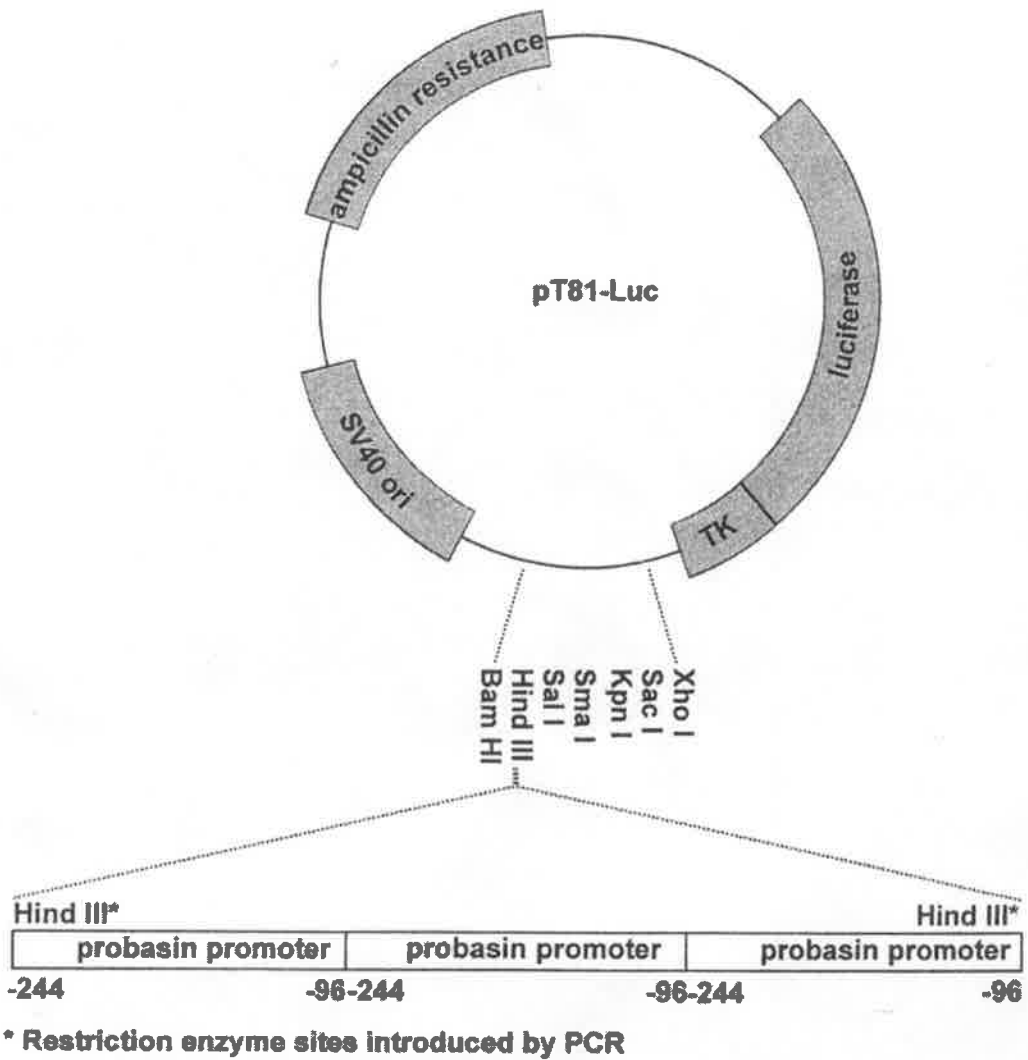
**pCMV3.1-AR:**

contains the entire coding region of human wtAR cDNA or AR variants cloned into the Eco RI restriction sites of the pCMV3.1 expression vector, under the control of the cytomegalovirus promoter.



**ARR3-tk-luciferase reporter construct:**

contains three copies of the androgen responsive minimal rat probasin promoter sequence (nucleotides -244 to -96) linked in tandem in front of the thymidine kinase enhancer element and firefly (*Photinus pyralis*) luciferase reporter gene of the T81-luc vector.





**Supplementary data - Table i: Genes upregulated by SAHA in LNCaP cells**

accession	Gene	fold induction		functional group
		2.5 $\mu$ M	7.5 $\mu$ M	
Z46973	Phosphoinositide-3-kinase	-	7	signal transduction, transcription and translation
NM_000111	down-regulated in adenoma	-	6.4	cytoskeleton/structural protein
Y00695	motilin	-	5.5	signal transduction, transcription and translation
U60873	trophoblast-derived non-coding RNA	2.4	4.6	unknown
NM_006472	thioredoxin binding-protein 2	2.4	3.5	differentiation
F26137	metallothioneine 1L	-	3.2	metal binding
NM_005794	alcohol dehydrogenase family member	2.8	3.1	other enzyme
AW663903	insulin induced gene 1	2.4	3	carbohydrate, lipid, steroid or protein metabolism
U03494	CP-2	-	2.9	signal transduction, transcription and translation
NM_003373	vinculin	-	2.6	cytoskeleton/structural protein
NM_006621	S-adenosylhomocysteine hydrolase-like	-	2.6	carbohydrate, lipid, steroid or protein metabolism
AA663562	kinesin family member 3A	-	2.5	transporter
AA001277	chaperone containing TCP-1 (subunit 8)	-	2.4	signal transduction, transcription and translation
AI245471	tissue inhibitor of metalloproteinase 3	-	2.3	cell adhesion, motility, invasion
AA780042	EST (AA780042)	-	2.3	unknown
AI802267	regulator of G-protein signaling	-	2.2	signal transduction, transcription and translation
NM_005345	heat shock 70 kDa protein	-	2.2	signal transduction, transcription and translation
NM_004760	DRAK1	-	2.2	apoptosis
AW090833	aldolase C	-	2.2	carbohydrate, lipid, steroid or protein metabolism
AL050337	Interferon gamma-receptor 1	2	2.1	signal transduction, transcription and translation
AA194950	RNA helicase-related protein	2	2.1	signal transduction, transcription and translation
AI133613	neuroserpin	-	2.1	other enzyme
AI816088	stimulator of Fe transport	-	2.1	carbohydrate, lipid, steroid or protein metabolism
NM_001656	ADP-ribosylation factor domain protein	-	2.1	transporter
AF054828	human homolog of C.elegans sur-8	-	2.1	signal transduction, transcription and translation
NM_001071	thymidylate synthetase	-	2	DNA synthesis
AB007898	praja-2 RING-H2 motif containing	-	2	unknown
AF099082	xenotropic & polytropic retrovirus factor	-	2	unknown
L06237	microtubule-associated protein 1B	-	2	signal transduction, transcription and translation
NM_001614	actin, gamma 1	-	2	cytoskeleton/structural protein
NM_002310	leukemia inhibitory factor receptor	-	2	signal transduction, transcription and translation
D87440	KIAA0252 protein	-	2	unknown
N39944	Activating transcription factor 3	-	2	signal transduction, transcription and translation
AF120265	tetraspan NET-6 protein	-	2	signal transduction, transcription and translation
AI085335	v-jun oncogene homolog	2.9	-	signal transduction, transcription and translation
AI253609	ribosomal protein S27	2.4	-	signal transduction, transcription and translation
NM_002218	inter-alpha inhibitor H4	2.3	-	cell adhesion, motility, invasion
NM_016126.1	HSPCO34 protein	2.2	-	unknown
S78825	Inhibitor of DNA binding 1	2.1	-	signal transduction, transcription and translation
AI885178	RP/EB family member 3	2.1	-	cytoskeleton/structural protein
AI191485	Inhibitor of DNA binding 2	2.1	-	signal transduction, transcription and translation
AW162336	Ubiquinone-binding protein	2.1	-	unknown
AW090833	aldolase C, fructose-bisphosphate	2.1	-	carbohydrate, lipid, steroid or protein metabolism
F27891	cytochrome c oxidase subunit Via	2	-	carbohydrate, lipid, steroid or protein metabolism
AC002550	human chromosome 16 BAC clone	2	-	unknown
AW248089	chromosome 14 ORF 2	2	-	unknown

**Supplementary data - Table ii: Genes downregulated by SAHA in LNCaP cells**

accession	Gene	fold reduction		functional group
		2.5µM	7.5µM	
AB007926	disrupted in schizophrenia-1	-	6.8	unknown
NM_007351	multimerin	-	5.9	signal transduction, transcription and translation
AI684143	EST (AI684143)	-	5.7	unknown
U39840	hepatocyte nuclear factor 3 alpha	3.4	5.6	signal transduction, transcription and translation
U58855	guanylate cyclase 1	5.5	5.5	other enzyme
AA114849	EST (AA114849)	-	5.2	unknown
AI424897	MD-2 protein	-	4.8	signal transduction, transcription and translation
AA766331	myosin ld	-	4.6	other enzyme
NM_003328	TXK tyrosine kinase	-	4.6	signal transduction, transcription and translation
AB002365	BNIP2 motif containing molecule	-	4.1	unknown
AW016900	chrom. 19 cosmid F23858	-	3.7	unknown
-	Incye EST (123829)	-	3.5	unknown
O14734	peroxisomal acyl CoA thioesterase	-	2.8	other enzyme
XM_498913.1	hypothetical protein XP-498913	-	2.8	unknown
X59798	cyclin D1	3.1	2.7	proliferation and cell cycle regulation
M30646	erythrocyte membrane protein	-	2.7	cytoskeleton, structural proteins
AJ224481	dyskeratosis congenita 1	2.1	2.6	unknown
M26663	prostate-specific antigen	-	2.6	other enzyme
DB7716	WD-repeat protein 43	2.6	2.6	signal transduction, transcription and translation
L29496	androgen receptor	3.6	2.5	signal transduction, transcription and translation
D50922	Kelch-like ECH-associated protein 1	-	2.4	unknown
AI571494	Incye EST (391692)	-	2.3	unknown
AF025409	zinc transporter 4	-	2.3	transporter
AW139507	PAG1	-	2.3	signal transduction, transcription and translation
U49436	eukaryotic translation initiation factor 5	2.4	2.2	signal transduction, transcription and translation
AF016050	neuropilin 1	2.4	2.2	signal transduction, transcription and translation
S39329	kallikrein 2	-	2.2	other enzyme
NM_004966	heterogeneous nuclear ribonucleoprotein F	-	2.2	signal transduction, transcription and translation
AB011119	leucine carboxyl methyltransferase 2	-	2.2	proliferation and cell cycle regulation
AF035300	cadherin 22	-	2.2	cell adhesion, motility and invasion
X56687	Upstream binding transcription factor, RNA pol I	3.2	2.2	signal transduction, transcription and translation
NM_002731	Protein kinase, cAMP-dependent	2.6	2.1	signal transduction, transcription and translation
NM_006196	poly(rC)-binding protein 1	-	2.1	signal transduction, transcription and translation
D00015	prion protein (p27-30)	-	2.1	unknown
AW418539	selenophosphate synthetase	-	2.1	other enzyme
AA421326	hypothetical protein FLJ21918	-	2.1	signal transduction, transcription and translation
U74612	forkhead box M1	2.4	2.1	signal transduction, transcription and translation
AF038966	secretory carrier membrane protein 1	-	2	transporter
AB018274	La ribonucleoprotein domain family 1 (LARP)	2.4	2	transporter
M30607	Y-linked zinc finger protein	-	2	signal transduction, transcription and translation
AI017452	G protein-coupled receptor 65	-	2	signal transduction, transcription and translation
AA903507	CGI-96 protein	-	2	other enzyme
D38552	PPWD1	-	2	metal binding
NM_001141	arachidonate 15-lipoxygenase	-	2	metal binding
AC004969	PAC clone DJ1121E10	-	2	signal transduction, transcription and translation
NM_002731	protein kinase, cAMP-dep, catalytic	-	2	signal transduction, transcription and translation
U75329	transmembrane serine protease	-	2	other enzyme
AA493890	putative translation initiation factor	-	2	signal transduction, transcription and translation
X12492	nuclear factor I/C	3.5	-	signal transduction, transcription and translation
AB011167	PGC-1 related coactivator	3.3	-	signal transduction, transcription and translation
NM_001280	RNA binding protein	3.3	-	signal transduction, transcription and translation
AI394286	desmocollin 2	3.1	-	cell adhesion, motility and invasion
AA921756	NADPH-cytochrome P450 reductase	2.8	-	other enzyme
O14863	solute carrier family 30, member 4	2.6	-	transporter
Z30425	nuclear receptor subfamily 1, group I, member 3	2.6	-	signal transduction, transcription and translation
NM_001147	angiopoietin 2	2.4	-	differentiation
L38951	karyopherin (importin)	2.4	-	transporter
U66618	SWI/SNF related regulator of chromatin	2.4	-	signal transduction, transcription and translation
AW298572	transcriptional adaptor 2 (ADA2)	2.3	-	signal transduction, transcription and translation
AF019039	karyopherin beta 2b, transportin	2.3	-	transporter
D63487	KIAA0153 protein	2.2	-	unknown
AI435383	chromosome 3p21.1 gene sequence	2.2	-	unknown
Z23064	RNA binding motif protein	2.2	-	unknown
NM_004554	nuclear factor of activated T-cells	2.2	-	signal transduction, transcription and translation
NM_004817	tight junction protein 2 (zona occludens 2)	2.1	-	cell adhesion, motility and invasion
AF007140	interleukin enhancer binding factor 3	2.1	-	signal transduction, transcription and translation
M82919	GABA A receptor	2.1	-	signal transduction, transcription and translation
DB0004	KIAA0182 protein	2.1	-	unknown
AF016052	zinc finger protein 24 (KOX 17)	2.1	-	unknown
X59303	valyl-tRNA synthetase 2	2.1	-	carbohydrate, lipid, steroid or protein metabolism
NM_002731	protein kinase, cAMP-dependent	2.1	-	signal transduction, transcription and translation

### Tissue Microarray Layout:

Cores from tumours derived from mice numbered 61-90 were assembled in a tissue microarray using 2mm cores. Mouse testis samples were inserted for orientation. Human prostate samples were randomly inserted and used as positive controls for immunohistochemistry.

	1	2	3	4	5	6	7	8	9
<b>A</b>	Testis	Testis	87a	87b	prostate	87c	75a	75b	75c
<b>B</b>	blank	79a	79b	79c	80a	80b	80c	65a	65b
<b>C</b>	Testis	65c	67a	67b	67c	72a	72b	72c	76a
<b>D</b>	76b	76c	84a	84b	84c	77a	77b	77c	prostate
<b>E</b>	78a	78b	78c	82a	82b	82c	81a	81b	81c
<b>F</b>	86a	86b	86c	88a	88b	88c	89a	89b	89c
<b>G</b>	66a	68b	prostate	66c	74a	74b	74c	83a	83b
<b>H</b>	83c	70a	70b	70c					

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