DOCTORAL THESIS

Quinazoline-based Alpha1-Adrenoceptor Antagonists and Prostate Cancer

Forbes, Amanda

Award date:
2016

BOND UNIVERSITY

FACULTY OF HEALTH SCIENCES & MEDICINE

PhD Thesis

By

Amanda Lynn Forbes

QUINAZOLINE-BASED ALPHA1-ADRENOCEPTOR ANTAGONISTS AND PROSTATE CANCER

Submitted in the fulfilment of the requirements of the degree of Doctor of Philosophy by Research

December 2015

ABSTRACT

Early stage prostate cancer is highly manageable using definitive radical prostatectomy and/ or radiotherapy techniques. Unfortunately, for some men, transition to castrate-resistant prostate cancer is both inevitable and incurable with few life-extending therapies available. Therefore, there is an urgent need for novel agents to improve the oncological and survival outcomes for these last-resort patients. One such modality may be α1-adrenoceptor antagonists. Clinically, some of these drugs reportedly increase benign and cancerous prostatic apoptosis. *In vitro* studies indicate that this anticancer effect occurs via α1-adrenoceptor independent mechanisms. However, the cytotoxic profile of these drugs have yet to be fully characterised, including whether these agents may be useful in improving anticancer treatment efficacy. To address the gaps in literature, the relative cytotoxic potencies and underlying cell death mechanisms (apoptosis and autophagy) were determined for six α1-adrenoceptor antagonists on castrate-sensitive and castrate-resistant prostate cancer cells. Molecular mechanisms were explored using immunoassays. The effects of these drugs were also investigated on normoxic or hypoxic irradiated prostate cancer cells to mimic outer and inner portions of a solid tumour. In an adjunct study, comparisons between the cytotoxic profile of doxazosin and the chemotherapeutic mitomycin c were made in an *in vitro* model of bladder cancer intravesical therapy. Overall, prazosin and doxazosin were found to be equipotent and were the most potent of all investigated drugs by inducing apoptosis and/or autophagy in a cell type-dependent manner. This cytotoxic effect was attributed to decreased mTOR/p70S6K signalling coupled with increases in p27 and p38 mitogen-activated protein kinase. Prazosin was also found to selectively radiosensitise hypoxic prostate cancer cells. This effect was characterised by increased reactive oxygen species and suppression of HIF-1α accumulation, further implicating mTOR-signalling as an underlying cytotoxic mechanism. Exploration of additional novel uses of these drugs revealed that doxazosin was 6-times more toxic than mitomycin C on bladder cancer cells in modelling of intravesical therapy. Taken together, these findings indicate that prazosin/doxazosin have potent cytotoxic actions in prostate cancer cells that are characterised by induction of apoptosis and autophagy, possibly by inhibition of the mTOR-signalling cascade. This is the first report of radiosensitising effects of these

drugs in prostate cancer cells, suggesting that these agents may have novel clinical benefits for patients undergoing radiotherapy. Likewise, the preliminarily findings of this thesis suggest that these drugs may be a novel alternative intravesical treatment option for bladder cancer and warrants further investigation.

DECLARATION

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy. This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

23 December 2015

Amanda Lynn Forbes | Date

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the following people for their guidance and support throughout my PhD.

Firstly, I would like thank my supervisors Dr Catherine McDermott, Prof Russ Chess-Williams and Dr Shailendra Anoopkumar-Dukie for giving me the opportunity to undertake this PhD project. It was an absolute privilege and pleasure to be their student, and I will be forever grateful for their encouragement and support during my candidature.

Thank you to the Bond University Faculty of Health Sciences and Medicine staff and students, particularly those in the Centre for Urology Research team, who made this work possible. I feel incredibly blessed to have the opportunity to work aside such friendly colleagues, and I will never forget the joys we have shared in the lab. I would like to especially acknowledge and thank Kylie and Stefanie for making this journey such a wonderful experience.

A special thank you goes to Prof David Christie and fellow staff at Genesis Cancer Care (Southport, QLD). Not only were they a pleasure to work with, they have been incredibly accommodating and patient over the many weeks (months) of data collection.

Lastly, I would like to dedicate this thesis to my parents Darren and Kathy Forbes, and especially to my future-husband Mick. Without their emotional, financial and physical support, this work would have not been possible. They have lead by example in teaching me resilience and the meaning of and hard work, and for that, I am forever grateful. I am eternally indebted to Mick for his unconditional support and endless patience – from countless trips driving me to and from Bond University, to pretending to understand my scientific tangents. I couldn't imagine a better companion to support me during my challenges and celebrate my successes.

PUBLICATIONS

JOURNAL ARTICLES AS A RESULT OF THIS THESIS:

Forbes A, McDermott C, Anoopkumar-Dukie, Chess-Williams R (2015). Relative cytotoxic potencies and cell death mechanisms of alpha1-adrenoceptor antagonists in prostate cancer cell lines. The Prostate. *In press*. Manuscript acceptance number: PROS-15-534

ABSTRACTS AS A RESULT OF THIS THESIS:

Forbes A, McDermott C, Anoopkumar-Dukie S, Christie D Chess-Williams R. (2015). The alpha1-adrenoceptor antagonist prazosin radiosensitises hypoxic-prostate cancer cells. 237 Abstract available at: http://www.ics.org/Abstracts/Publish/241/000237.pdf

Forbes A, McDermott C, Anoopkumar-Dukie, Chess-Williams R. (2015). Relative cytotoxic potencies of alpha1-adrenoceptor antagonist in human stromal and epithelial cell lines. International Continence Society, Montreal 2015.

A. Forbes, C. McDermott. S. Anoopkumar-Dukie, D. Christie, R. Chess-Williams. (2014) The α1-adrenoceptor antagonist prazosin enhances sensitivity of hypoxic prostate cancer cells to irradiation. P565 Abstract available at : http://www.asceptasm.com/wp-content/uploads/2014/01/ASCEPT-Poster-Program-Abstracts-FINAL1.pdf

Amanda Forbes, Russ Chess-Williams, Shailendra Anoopkumar-Dukie, Catherine McDermott (2014) RELATIVE CYTOTOXIC POTENCY AND CELL DEATH MECHANISMS OF α1-ADRENOCEPTOR ANTAGONISTS ON PROSTATE CANCER CELL LINES Asia-Pacific Journal of Clinical OncologyVolume 10, Issue Supplement S8, 216 Abstract available at: https://www.cosa.org.au/events/past-annual-scientific-meetings.aspx

A Forbes, R Chess-Williams, S Anoopkumar-Dukie, C McDermott (2013) Quinazoline-based alpha1-adrenoceptor antagonist doxazosin induces human bladder cancer cytotoxicity in vitro: potential for novel intravesical therapy? 5th National Symposium on Advances in Urogenital and Gut Research, Melbourne 2013.

TABLE OF CONTENTS

ABBREVIATIONS

ADR	Adrenoreceptor
ADAMTS	A disinegrin and metalloproteinase with throbospondin motifs
AR	Androgen receptor
ASTRO	American Society for Radiation Oncology
AUA	American Urological Association
BPH	Benign prostatic hyperplasia
Cited2	Cbp/p300-interacting transactivator with Gly/Asp-rich arboxy-terminal domain 2
DCF-DA	Dichloro-dihydro-fluorsecein diacetate
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular-related kinase
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FDA	Food and drug administration (USA)
HGPIN	High-grade prostatic neoplasia
HIF	Hypoxia-inducible factor
LBD	Ligand-binding domain
LC3	Ligand binding domain
LHRH	Lutenising hormone-releasing hormone
LUTS	Lower urinary tract symptoms
GAG	Glycosaminoglygans
Gy	Gray
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian (mechanistic) target of rapamycin
NFAT	Nuclear factor of activated T-cells
PBS	Phosphate buffered saline
PI3P	Phosphatidylinositol 3-phosphate
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PKC	Protein kinase C
PSA	Prostate specific antigen
PDGF	Platelet-derived growth factor
pVHL	Von Hippel-Lindau
ROS	Reactive oxygen species
RP	Radical prostatectomy
TIMP	Tissue inhibitor of metalloproteinase

TGA	Therapeutic Goods Administration (Australia)
TGF	Tumour growth factor
TRP	Transient receptor potential channels
TSC	Tuberous sclerosis protein
TNF	Tumour necrosis factor
TURP	Transurethral resection of the prostate
TURBT	Transurethral resection of the bladder tumour
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

CHAPTER 1:
INTRODUCTION

1.1 HUMAN PROSTATE: ANATOMY AND FUNCTION

The prostate is an exocrine gland of the male reproductive system, which sits anterior to the rectum and inferior to the urinary bladder, surrounding the urethra. The prostate is considered to be comprised of three zones (peripheral, translational and central) and one fibromuscular zone referred to as the "stroma" (Fine and Reuter, 2012).

Briefly, the primary function of the prostate is to secrete alkaline fluid, which makes up a portion of semen. Prostatic fluid consists of several enzymes, including the prostate specific antigen (PSA), a protease secreted by prostatic epithelial cells that acts to reduce the viscosity of semen to improve sperm motility (National Health and Medical Research Council, 2013). Additionally, the prostate contracts to prevent urine from escaping the urinary bladder or entering the seminal vesicles during ejaculation or urination (U.S. National Library of Medicine, 2012).

1.2 PROSTATE CANCER

INCIDENCE

Prostate cancer is the most commonly diagnosed male cancer in the world and accounts for approximately 30% of all newly diagnosed cancers in Australia (Bray et al., 2013, Australian Institute of Health and Welfare, 2014) (**Figure 1.1**). The mean age at diagnosis in Australia is 68.2 years old (**Figure 1.2**). As shown in **Figure 1.3**, the age-standardised incidence rate of prostate cancer has increased between 1982 and 2007, with estimates of approximately 18,140 new cases in 2016 (Australian Institute of Health and Welfare, 2012). Of note, prostate cancer incidence appeared to increase drastically in the early 1990's with the introduction of PSA testing. As a direct result of PSA testing, more prostate cancers were detected and diagnosed earlier, giving a false sense of increased incidence when in fact these men would have been diagnosed at a later time upon the presentation of symptomatic disease in the pre-PSA era. It is currently estimated that 1 in 7 Australian men are at risk for developing prostate cancer before the age of 85 (Australian Institute of Health and Welfare, 2013,

Australian Institute of Health and Welfare, 2014). Interestingly, a recent retrospective review of autopsy studies identified a significantly higher incidence of prostate cancer than previously thought (Bell et al., 2015). Overall, it was reported that approximately 59% of men had prostate cancer by the time they reached their eighth-decade of life. The disparity between reported incidence by the Australian Institute of Health and Welfare (AIHW) statistics and the recent findings is likely due to the characteristic slow growth of early stage prostate cancer tumours that are often asymptomatic for many years. In essence, the AIHW statistics represent the risk of being diagnosed, not necessarily the risk of developing prostate cancer.

There are regional and ethnicity differences amongst prostate cancer incidence amongst Australian men. Aboriginals and Torres Straight Islanders are less likely than other Australian men to be diagnosed with prostate cancer. Interestingly, men living in moderately remote areas are at a lower risk, whereas men residing in very remote areas are at a greater risk for being diagnosed with prostate cancer than those living in other locations (Australian Institute of Health and Welfare, 2013). One study reported in 2005 that men residing in regional or remote areas were 16% less likely to undergo PSA testing compared to the rest of the Australia (Coory and Baade, 2005). In a subsequent study conducted in 2011, the same authors reported that there was no significant improvement in prostate cancer diagnosis between men in rural compared to men residing in urban areas (Baade et al., 2011). These findings indicate that despite technological advances and improved access to healthcare, there are unidentified factors that contribute to the regional inequalities in prostate cancer diagnosis with in Australia that remain to be explored further. While only speculative, differences in prostate cancer incidence amongst geographical locations in Australia may be in part to regional differences in diet, social norms and access to healthcare (discussed in more detail below).

Inequalities in prostate cancer incidence also exist between men of other ethnic backgrounds. One recent retrospective chart review evaluated prostate cancer incidence amongst American men with Asian-Pacific background (Chao et al., 2016). The authors reported a greater incidence of advanced prostate cancers amongst Asian Indian/Pakistani, Filipino, Hawaiian and Pacific Islanders (odds ratio = 1.37, 1.38, 1.70 and 1.90, respectively) compared to non-Hispanic white males. While data on

diet and social norms of these men is not available, it can be speculated that many of these men would have been subject to westernised diet influences controlling for this typical confounding factor. This suggesting that genetic variations between ethnicities may play a significant role the apparent disparity in prostate cancer incidence amongst Asian-Pacific men compared their White male counterparts.

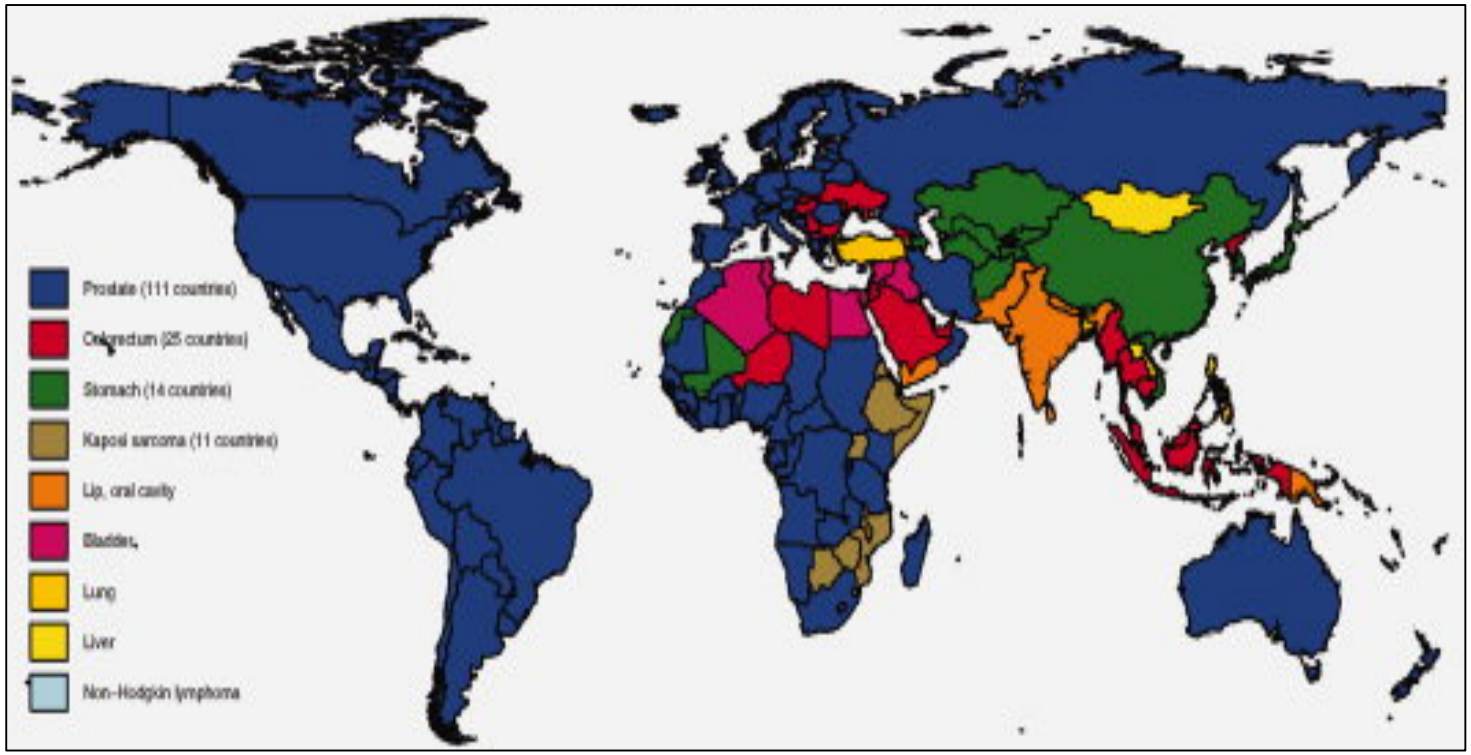


Figure 1.1. Global map illustrating the most prevalent male cancers across 184 countries. The top nine male cancers in the world are: prostate (blue, 111 countries), colorectal (red, 25 countries), stomach (green, 14 countries), Kaposi sarcoma (brown, 11 countries), lip and oral cavity (orange), bladder (pink), lung (gold), liver (yellow) and non-Hodgkin's lymphoma (light blue) (Bray et al., 2013). Image reprinted with permission from copyright holder.

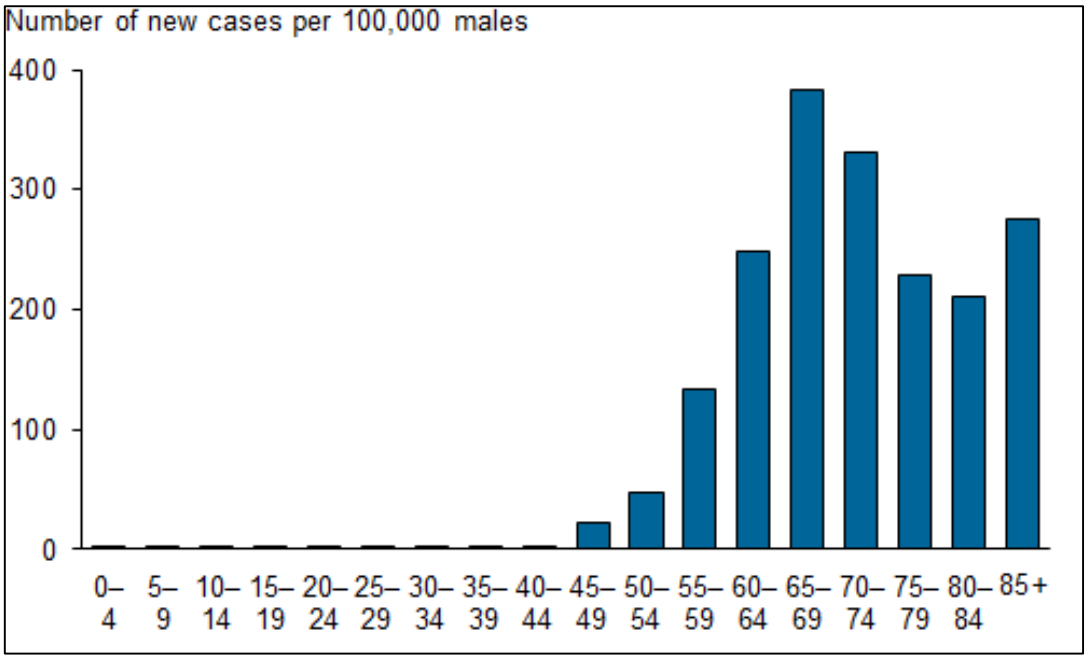


Figure 1.2. Incidence of prostate cancer in Australia according to age groups (Australian Institute of Health and Welfare, 2015)

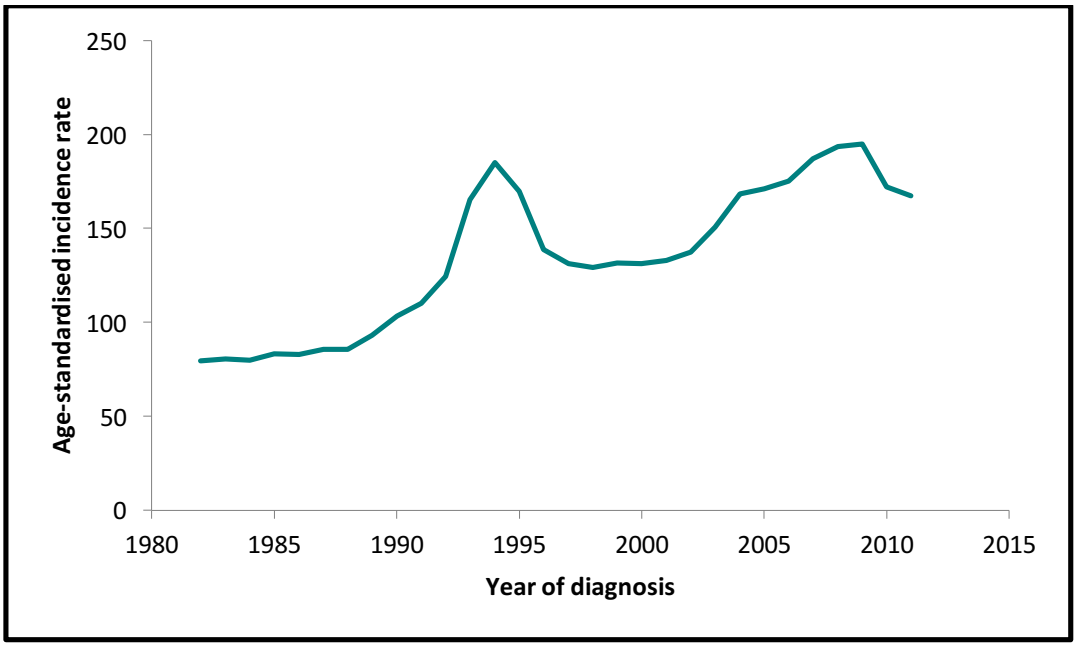


Figure 1.3. Age standardised prostate cancer incidence (number of new cases) per 100,000 Australian males between years 1983 – 2011. (Australian Institute of Health and Welfare, 2015).

MORTALITY

Prostate cancer is the second most common cause of cancer-specific death, and the fourth leading cause of death amongst Australian men (Australian Institute of Health and Welfare, 2013, Australian Institute of Health and Welfare, 2014). Over more than two decades, between 1968 and 2012, prostate cancer mortality decreased by a modest ~8% (36 deaths to 28 deaths per 100,000 males, respectively) (**Figure 1.4**). Importantly, this downward trend is expected to continue, with an estimated 26 deaths per 100,000 Australian men in 2020 (Australian Institute of Health and Welfare, 2013). However, the risk of cancer-specific mortality differs between ethnicity and regional demographics. For example, in the United States, African-American men are twice as likely to die as a result of prostate cancer compared to Caucasians. Furthermore, mortality rates are ten-times greater for African-Americans than men residing in Asian countries (Higgins, 1975, Newman, 1996). In Australia, a recent retrospective study identified a higher 5-year mortality rate amongst aboriginal men compared to non-aboriginal men (17.5% vs. 11.4%, respectively), with aboriginal men nearly 50% more likely to die from the disease. This difference is likely attributed to differences in treatment choice, with one-third of aboriginal men choosing to forgo treatment for local prostate cancer (Rodger et al., 2015).

ECONOMIC BURDEN

Between 2008 and 2009, the economic burden of prostate cancer has tripled since 1994, and costs Australians over $349 million dollars annually (Australian Institute of Health and Welfare, 2013, Marks et al., 2010). However, the actual cost to the Australian community are likely to be significantly greater as the majority of estimates do not incorporate the costly end-of-life treatments, which include more frequent hospitalisations, palliative care and hospice (Roehrborn and Black, 2011). Furthermore, as prostate cancer survival rates are increasing, more men are likely to require long term follow-up and/or maintenance therapy which represents additional economic burden.

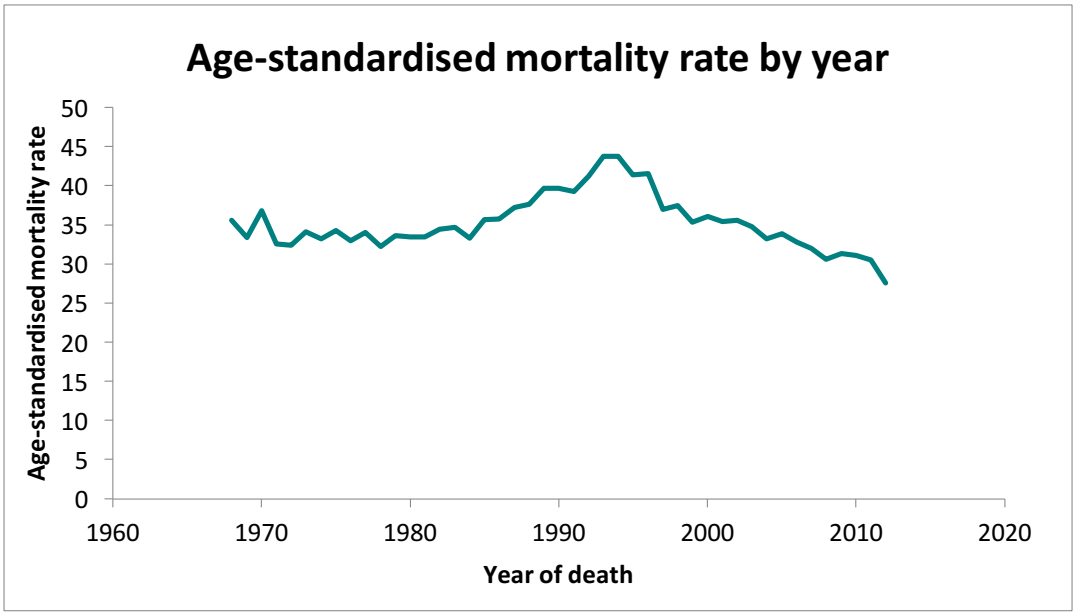


Figure 1.4. Prostate cancer-specific mortality in Australia between years 1968 – 2012 (Australian Institute of Health and Welfare, 2015).

RISK FACTORS

The most common factors associated with increased risk of prostate cancer include positive family history, African American decent, high basal levels of PSA and, most importantly, advancing age. One study evaluated the prostates of men who underwent a radical cysto-prostatectomy for advanced bladder cancer, with no prior suspicions of prostate cancer (Pignot et al., 2015). Overall, 21.4% of men (518 of 2424) aged between 41.5 and 95.6 years were found to have prostate cancer in their tissue specimen, which was strongly correlated with age increasing age. Both prostate cancer incidence (percent of evaluated prostate glands) and percent of cancers with Gleason score ≥ 7 was found to increase with age, from 15% and 0% amongst 50-59 age group to 31% and 37% in men 75 years or older, respectively.

Other risk factors linked to prostate cancer incidence may include hypertension and metabolic syndrome (Bhindi et al., 2015). To date, no clear association has been made between prostate cancer incidence and typical independent risk factors for cancer such as history of tobacco smoking, weight gain/ obesity (Ho et al., 2014, Ohwaki et al., 2015).

PROGNOSTIC FACTORS

There are no currently defined clinical predictors of disease progression and overall survival, although there is strong evidence to suggest that high Gleason scores (≥ 8 – 10) are correlated with poorer survival outcomes (Rusthoven et al., 2014). One study identified significant differences between Gleason scores (7 vs. 8, 8 vs. 9, and 9 vs. 10) in predicting cancer-specific survival, suggesting that there is a relative stepwise decrease in survival at 4 years with increasing Gleason scores (Rusthoven et al., 2014).

Interestingly, life style factors, such as cigarette smoking, and those leading to obesity and hypertension, may also be linked to disease progression and poor survival outcomes. While not established predictors of prostate cancer diagnosis, cigarette smoke and obesity are positively correlated with increased disease aggression, shorter duration to biochemical recurrence and higher mortality (Ho et al., 2014, Moreira et al., 2014, Moller et al., 2015). Furthermore, patients with uncontrolled and untreated hypertension had an increased risk for biochemical recurrence after RP compared to those who received treatment for controlled or uncontrolled hypertension (Ohwaki et al., 2015, Asmar et al., 2013).

DIETARY FACTORS

The protective or adverse effect of the consumption of fatty acids, vitamins, calcium, fruits and vegetables on prostate cancer risk remains unclear (Lin et al., 2015). However, a previous literature review of meta-analyses and clinical trials identify associations between dietary patterns and prostate cancer risk (Lin et al., 2015). Retrospective studies identified a higher risk amongst individuals consuming a Westernised diet, whereas Asian and Mediterranean diets were associated with reduced prostate cancer risk (Ambrosini et al., 2008, Kapiszewska, 2006). A growing body of evidence including pre-clinical, retrospective and prospective clinical studies indicates a negative correlation between number of cups of coffee/day and risk, recurrence, and fatality of prostate cancer (Lu et al., 2014). Of particular interest, one

study identified an inverse correlation between solar radiation and prostate cancer incidence amongst Australian men (Loke et al., 2011). These findings have been attributed to increased vitamin D production as a result of greater sun exposure, which was been postulated to have a protective effect against prostate cancer. However, the evidence between vitamin D and risk of prostate cancer is mixed, with pre-clinical and retrospective studies identifying a higher and lower risk (Schenk et al., 2014), whereas clinical trials have determined no benefit of vitamin D supplementation.

CARCINOGENESIS

The development of prostate cancer is a lengthy process that typically unfolds over the course of ten years or more. The development of pre-neoplastic lesions known as high-grade prostatic intraepithelial neoplasia (HGPIN) (Sakr et al., 1994) is the only known pre-cursor to prostate cancer. Similar to prostate cancers, HGPIN is most commonly found in the peripheral zone of the prostate, distantly followed by the transition and central zones. Evidence suggests HGPIN contains similar genetic and molecular abnormalities as early invasive prostate cancers; HGPIN incidence increases with age and is more common amongst African American men (Sakr et al., 1996), and HGPIN is frequently identified within surgically removed prostates for prostate cancer treatment.

Clinical significance of HGPIN diagnosis and is still unclear, however identification of unilateral or bilateral multifoci HGPIN is associated with a 48.1 and 57.8% risk for being diagnosed with prostate cancer five years following initial biopsy (Lee et al., 2010b). Unfortunately, there are no current clinical parameters that may help identify which men with HGPIN histological findings have the highest risk developing prostate cancer (Clouston and Bolton, 2012a, Clouston and Bolton, 2012b).

Chromosomal or molecular abnormalities may tip the scale from pre-neoplastic HGPIN to invasive carcinoma of the prostate (Abate-Shen and Shen, 2000). Most commonly, the loss of one or more regions on chromosomes 8p, 10q and 13q, specifically areas which code for tumour suppressors genes may play a pivotal role in the development of prostate cancer. For example, loss of functional PTEN (10q) and

Rb (13q) (retinoblastoma) tumour suppressor proteins, which negatively regulate proliferation, is frequently absent from prostate cancer tissues and cell lines (Abate-Shen and Shen, 2000, van Bokhoven et al., 2003), leading to uncontrolled proliferation and tumour growth.

PROSTATE CANCER TREATMENT

An overview of typical progression of prostate cancer through various treatment modalities is show in **Figure 1.10** at the end of this section.

SCREENING

Today, there is no population-wide screening protocol for early detection of prostate cancer in men (Heidenreich et al., 2011). A growing number of clinical studies indicate screening is positively correlated with reduced incidence of mortality compared to standard care (Schroder et al., 2009, Schroder et al., 2012). However, urological societies have concluded the economic, physical, and emotional burden from intensive screening regimen and high incidence of false-positives offsets the marginal increase in diagnosis and survival rates (Heidenreich et al., 2011).

DIAGNOSIS

Prostate cancer is diagnosed using a multi-study approach employing the use of digital rectal examination (DRE), prostate specific antigen (PSA) and transrectal-ultrasound (TRUS) guided biopsies (Heidenreich et al., 2011). Relative serum PSA levels are considered the "gold standard" for detection and are strongly correlated with the increased risk of prostate cancer (Heidenreich et al., 2011). In contrast to previous practice, PSA kinetics known as PSA "velocity" or "doubling time" have not been shown to have significant predictive benefits in identify men at risk of prostate cancer compared to PSA levels alone (O'Brien et al., 2009, Heidenreich et al., 2011).

The current recommended PSA threshold is ≥ 4.0 ng/ml (Heidenreich et al., 2008, Heidenreich et al., 2011). Nearly all prostate cancers are detected by PSA serum levels, with half of cases utilizing both DRE and PSA testing. In contrast, only 5% of

prostate cancers are detected by DRE alone (Baade et al., 2012a). In the presence of inconclusive DRE or PSA levels, the patient risk factors are taken into consideration to determine if further work up is required. Where indicated, patients undergo diagnostic TRUS or transperineal laterally-directed biopsy for histopathological examination (Heidenreich et al., 2011).

Of particular note, the use of PSA testing is not without controversy. As mentioned previously, there was a dramatic increase in prostate cancer incidence in with the introduction of PSA testing in the early 1990's leading to earlier detection, particularly in men with asymptomatic disease. This poses the issue of over-diagnosis and thus potential over-treatment of asymptomatic cancers, many of which would passively exist without life-limiting consequences. For example, one recent Australian study found that nearly three-quarters (73%) of Australian men diagnosed with prostate cancer presented to their health care provider with other non-cancer related concern (Baade et al., 2012a). Over-treatment as a result of over-diagnosis is problematic in that it subjects men to unnecessary treatment that may impact quality of life. On the contrary, PSA testing may. An Australian conducted review of PSA testing (± DRE) in asymptomatic men concluded there is no evidence to suggest a benefit or negative impact relating to cancer-specific and all cause-mortality, compared to no PSA testing. On the contrary, PSA testing was associated with reduced occurrence of metastatic disease at diagnosis (National Health and Medical Research Council, 2013), providing an opportunity to treat early disease where curative treatments, such as surgery, are most effective.

STAGING

Prostate cancer is staged using the common tumour, node, and metastasis classifications outlined by the Union International Contra Cancer 2002 guidelines (Wittekind et al., 2002). Additionally, a Gleason score is given as measure of disease aggression and prognosis (Xu and Zhou, 2014). The Gleason score is on a scale ranging from 2 to 10, with 10 being the most aggressive, and is obtained by summation of the two most common grades (1-5) amongst biopsy specimens.

The combination of DRE, PSA levels, magnetic resonance imaging, tumour grade and number of positive foci are used to for local staging (T-staging). In Australia, early stage or local disease is defined as T1-T2; whereas locally advanced or high-risk prostate cancer is defined as diagnosis of clinical stage of T3 – T4 cancer and/or early-stage (T1-T2) with serum PSA levels > 20 ng/ml (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010).

Prostate cancers with moderate to high Gleason scores are at greater risk of involving surrounding or distant lymph nodes. Accurate lymph node staging (N-staging) via extended lymphadenectomy is primarily reserved for men who are considering curative treatment options. Staging for skeletal metastasis (M-staging) is indicated for men with PSA levels greater than 20 ng/ml and tumours with minimal differentiation (Heidenreich et al., 2008).

LOCAL TREATMENT

Local treatment strategies are determined on an individual basis taking into account disease stage and life expectancy. For patients with less than 10 years of life expectancy or low stage cancer, are often recommended for active surveillance. Active surveillance (or watchful waiting) involves careful follow-up in which treatment is postponed until disease advancement may impede the patient's quality of life (Heidenreich et al., 2008, Heidenreich et al., 2011).

Patients that have 10 years or greater life expectancy with organ-confined disease are commonly recommended for radical prostatectomy (RP), external-beam radiation therapy, or brachytherapy (Heidenreich et al., 2011). However, for Australian men, initial treatment choice is impacted by several factors such as age, proximity to treatment centre, and socioeconomic status (Baade et al., 2012b).

Surgical treatment

Radical prostatectomy (RP) is the only treatment option with significant long-term survival benefits compared to other more conservative therapies (Bill-Axelson et al.,

2011), including radiation therapy (Shao et al., 2013) for men with early-stage disease. While RP best serves men with organ-confined disease, those with locally advance disease may also benefit from RP (Heidenreich et al., 2008), with one study identifying survival rates of 95% and 90% at 5 and 10 years, respectively (Ward et al., 2005). However, this delicate procedure carries the risk of impotence and incontinence, which may adversely affect quality of life (Barry et al., 2012).

Radiation therapy

External beam radiation therapy

Radiation therapy (dose of ≥ 72-81 Gy) is considered a curative treatment option for patients with a life expectancy of greater than 5-10 years who are diagnosed with local/ early-stage (T1-T2) or locally advanced disease (clinical stage T3-T4), as well as those who are unfit for surgery (Heidenreich et al., 2008). In Australia, limited field radiotherapy is the primary treatment used to minimise toxicity. However, whole pelvic radiotherapy, including para-aortic nodes, may be indicated in some patients with high-risk disease. In contrast, 3D conformal radiotherapy allows the radiation field to be shaped to the prostate by CT imaging, therefore allowing highly-targeted radiation and minimise toxicity to the surrounding tissues or organs (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010).

In Australia, men with locally advanced disease are recommended to receive dose-escalation of ≥ 74 Gy, if tolerable, using 3D conformal techniques (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010). Although the clinical or survival benefit of radiotherapy alone for this group remains unclear, there is a marked improvement in overall survival when radiotherapy was combined with hormonal therapy (Widmark et al., 2009). Furthermore, the combination of radiotherapy with neo-adjuvant and long term adjuvant hormonal modulation (2 years) significantly improved 5-year biochemical disease free survival in men with locally advanced disease (Heidenreich et al., 2008).

Post-operative adjuvant radiotherapy of the tumour bed, particularly in the case of positive surgical margins, is well known to have important oncological and survival

benefits for locally advanced prostate cancer. Three separate prospective randiomised clinical trials (SWOG8794, EORTC2291 and ARO 92-02) evaluated the clinical effect of adjuvant radiotherapy following RP for men with locally advanced disease (Heidenreich et al., 2008, Swanson et al., 2008, Wiegel et al., 2009). All three trials reported improved time to PSA-progression free survival, with two of the three trials demonstrating improved locoregional (i.e. tumour site and associated areas) treatment response and radiographic disease-free progression in RP patients who received adjuvant radiotherapy compared to the observation groups. Furthermore, the data from the SWOG8794 trial demonstrated marked improvement in overall survival amongst the adjuvant radiotherapy treatment arm (74% compared to 66% at 12 years follow up) (Swanson et al., 2008). As a result of these three trials, the American Urological Association (AUA) and ASTRO strongly recommend the use of adjuvant radiotherapy in men with high-risk pathological findings, including seminal vesicle involvement, following RP to reduce the risk of biochemical relapse and potentially improve survival outcomes (Thompson et al., 2013).

Brachytherapy

Men with early stage, intermediate risk prostate cancer with baseline good prostate-specific lower urinary tract symptoms (LUTS) may be candidates for brachytherapy (Heidenreich et al., 2008). Brachytherapy is the permanent or temporary implantation of a radioactive "seed" in the prostate gland to deliver high doses of radiation to the cancer, while minimising toxicity to adjacent tissues and organs (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010)). On one hand, permanent transperineum brachytherapy involves the surgical placement of multiple radioisotope (Iodine-125). Where as a temporary implant brachytherapy involves the placement of radioactive compounds (usually Iridium-92) into the prostate via the urethra (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010).

In locally advanced disease, there is little clinical evidence demonstrating superiority of brachytherapy over surgery or external beam radiotherapy with regards to oncological or survival parameters (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010). However, in some cases the

combination of low-dose radiotherapy and high-dose brachytherapy may provide clinical benefit.

Androgen-deprivation therapy

The use of androgen-deprivation therapy (ADT) is indicated in early-stage hormone-responsive with symptomatic, locally advanced (clinical stage T3-4), or metastatic disease (Grimm et al., 2002, Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010). Additionally, ADT is also indicated for men who by preference or due to comorbidities, choose to forgo curative treatment options, and opt for pharmacological management of their disease (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010). As its name suggests, ADT capitalises on the androgen-dependence of prostate cancer growth by suppressing testosterone production to castrate levels (< 50 ng/dl) or by inhibiting the survival-promoting actions of the androgen receptor (described in detail below).

Androgen-dependence of prostate cell growth and proliferation

As shown in **Figure 1.5**, the main circulatory androgen testosterone is primarily synthesised (~95%) by the testes and regulated by gonadotropin-releasing hormone (GnRH)-mediated pituitary stimulation and subsequent lutenising hormone (LH) release (Feldman and Feldman, 2001, Debes and Tindall, 2002, Chen et al., 2009, Lamb and Neal, 2013). LH acts on the Leydig cells of the testes to induce synthesis of testosterone. In contrast, approximately five percent of endogenous testosterone is produced by the adrenal glands in response to the corticosteroid-adrenocorticotropic hormone pathway (Lamb and Neal, 2013). Non-malignant and malignant prostate cell proliferation is regulated by testosterone-androgen receptor (AR) interactions. In prostate cells, testosterone crosses the cell membrane and is enzymatically converted to dihydrotestosterone (DHT) by 5α-reductase. DHT binds to cytosolic AR triggering dimerization and activation by phosphorylation (Feldman and Feldman, 2001). The activated AR translocates to the nucleus where it binds to androgen response elements in the promoter region of DNA stimulating transcription of target genes to induce protein synthesis (Geller et al., 1987), PSA secretion, cell proliferation and survival (Feldman and Feldman, 2001). In the context of prostate cancer, blocking of androgen-mediated AR signalling through suppression of androgen synthesis or direct inhibitory actions on AR, ADT indirectly triggers prostate cancer cell death (Westin et al., 1995, Kimura et al., 2001), leading to rapid decline in both PSA (< 0.5 ng/mL) and tumour growth (Choueiri et al., 2009).

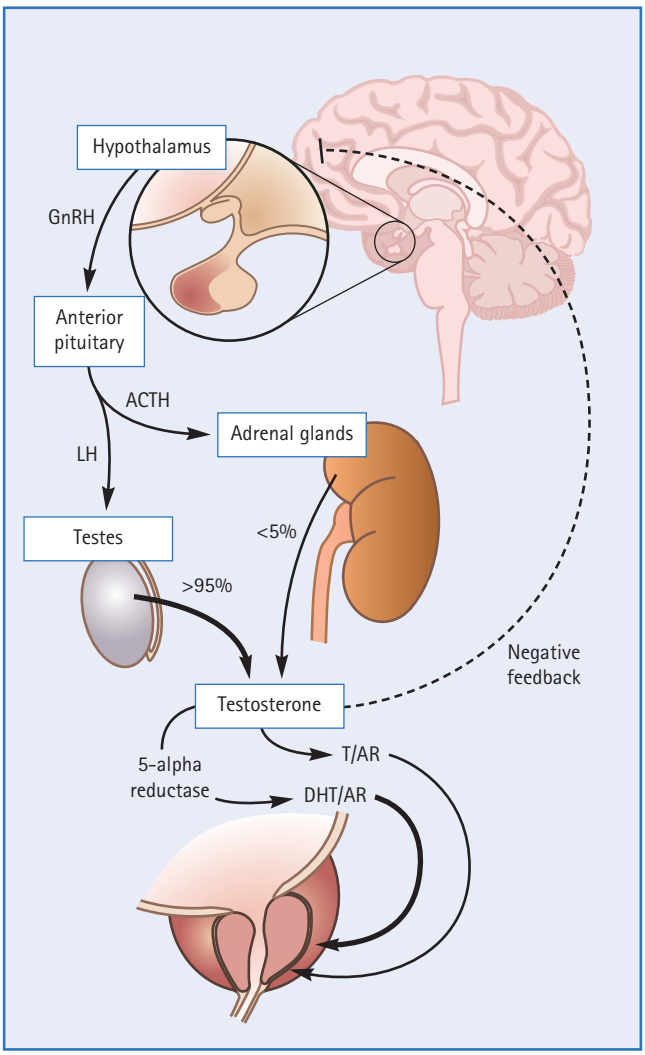


Figure 1.5. Testosterone biosynthesis via hypothalamo-pituitary- gonadal axis (Lamb and Neal, 2013). Image reprinted with permission from copyright holder.

Clinical use of ADT

In addition to surgical castration (orchiectomy), ADT is achieved centrally and/or peripherally through GnRH agonists (referred to as lutenising hormone releasing hormone [LHRH] agonist) or anti-androgens, respectively (Heidenreich et al., 2008). While highly effective, prevalence of orchiectomy has significantly dropped in Westernised nations primarily due to the irreversible nature of the procedure

(Connolly et al., 2012). As such, androgen-deprivation is frequently achieved using the LHRH agonists including leuprolide and goserelin, which act to suppress the gonadal-testosterone synthesis axis. In contrast, anti-androgens, including bicalutamide, flutamide, nilutamide and cyproterone acetate are competitive antagonists of AR, preventing AR-mediated transcription of survival genes. While LHRH agonists are available to Australian men as a monotherapy, LHRH agonists are commonly prescribed in combination with anti-androgens to target both production and AR activity to provide a total androgen-blockade. Furthermore, anti-androgens mitigate the tumour promoting effects of the characteristic LHRH agonist-induced "flare" of testosterone production as a result of adrenal compensation (Thompson, 2001). Unlike LHRH, prescribing limitations exist of available anti-androgens (nilutamide, bicalutamide and flutamide), in that all but cyproterone acetate, are only available for treatment of locally advanced or metastatic disease when prescribed in conjunction with LHRH analogues (Australian Cancer Network Management of Metastatic Prostate Cancer Working Party, 2010).

While ADT may be used as a primary treatment modality for locally advanced or metastatic prostate cancer with contraindications or due to treatment preference, ADT is particularly effective in conjunction with definitive (curative) treatments such as RP and/ or radiotherapy for locally advanced or high-risk prostate cancer. As briefly touched on previously, combination ADT and radiotherapy has been shown to have significant survival benefits in men with locally advanced disease (Widmark et al., 2009). Similarly, short course neoadjuvant and adjuvant ADT with radiation therapy provided a survival benefit over radiotherapy alone in men with either intermediate- (Jones et al., 2011) or high-risk disease (Koie et al., 2014). Survival benefits of androgen-deprivation is not just limited to radiotherapy, as results from a small scale clinical study found that three-year overall survival was not different between groups receiving neo-adjuvant ADT prior to definitive RP or radiotherapy (Koie et al., 2014).

Controversy exists with regard to the timing of ADT in locally advanced disease. While generally considered safe, continual use of ADT predisposes men to a plethora of quality of life-limiting effects affecting such as cardiovascular disease, hypertension, weight gain and obesity, metabolic syndrome, diabetes, osteoporosis and decline in cognitive abilities (Mohler, 2014). It is well documented that many

prostate cancers progress slowly; therefore one must wonder if subjecting elderly men to the adverse side effects of ADT pays off with significant survival benefit. The choice between immediate or delayed ADT is more clear-cut in the context of metastatic disease, particularly in symptomatic cases, as immediate induction of ADT provides significant improvement in relative risk of prostate-cancer specific mortality (17% reduction) compared to delayed ADT regimens (Loblaw et al., 2007, Mohler, 2014). However, in some circumstances, ADT has been reported to negatively impact non-cancer-specific mortality (15% increase), and consequently fail to provide an overall survival benefit (Loblaw et al., 2007, Mohler, 2014). To balance the therapeutic benefits and quality of life-limiting side effects, many oncologists successfully employ intermittent ADT via employing immediate, yet, short term androgen-deprivation to drive disease to remission, and using subsequent cycles of androgen-deprivation upon presence of biochemical recurrence or clinically measurable disease. Two separate systematic reviews of available clinical studies, inclusive of both phase II and III trials, uncovered similar oncological and survival benefits between continual and intermittent ADT, with the side effect profile favoring intermittent treatment protocols (Abrahamsson, 2010, Magnan et al., 2015). This suggests, that intermittent ADT may be a viable alternative to classical continuous androgen-deprivation treatment regimens, with improved tolerability and quality of life parameters, particularly in sexual potency. Additionally, reduction in adverse side effects associated with intermittent treatment may improve non-cancer- and cancer-specific overall survival outcomes. However this remains to be fully elucidated in a randomised prospective clinical trial.

BIOCHEMICAL RECURRENCE

Generally, a consecutive rise in PSA following definitive local therapy such as radiotherapy or RP is considered to be biochemical recurrence of prostate cancer. However, debate exists surrounding the precise PSA threshold to be considered recurrence following curative treatments. A review of the literature suggests a PSA level > 0.2 – 0.6 ng/mL above nadair in men with or without intact prostates may be clinically appropriate (Stephenson et al., 2006). However, several independent institutions have suggested the use of tailored definitions of biochemical recurrence based on clinicopathologic stratification of prostate cancers into low-, intermediate-

and high-risk groups (Taplin, 2003, Morgan et al., 2014). Importantly, the American Society for Therapeutic Radiology and Oncology (ASTRO) has made it clear in their guidelines for the interpretation of PSA rise following radiotherapy that an increase in PSA alone does not necessarily translate to clinical recurrence, therefore does not justify additional treatment in every context. The ASTRO are also point out that there are no current definition of biochemical failure which has been linked to better or worse treatment or survival outcomes (1997).

Unfortunately, an estimated one-fifth of men will experience disease recurrence following curative treatment modalities. In the context of RP, an estimated 19% of men will experience biochemical treatment failure (Freedland et al., 2005). Similarly, between 23 - 34%, of men receiving definitive radiotherapy will experience a rise in PSA levels, however, time to biochemical failure is less clear (Kupelian et al., 2002, Zumsteg et al., 2015). Of these men, an estimated 34% will develop appreciable clinical metastasis (Pound et al., 1999). However, it appears that the time to from biochemical relapse (PSA rise) to measureable disease progression is somewhat worse for those treated with definitive radiotherapy; with an estimated 5.4 years to distant metastasis compared to 8 years in RP treated tumours (Zumsteg et al., 2015, Pound et al., 1999).

Depending on several factors, including but not limited to; comorbidities, prior treatment history, extent of local or distant metastasis, Gleason score or PSA levels at diagnosis, patients may undergo salvage RP or radiotherapy, induction of first-line or second-generation ADT, or a combination of one or more therapies. Retrospective and clinical studies report better prognoses following salvage RP or radiotherapy, with these salvage treatments favoring lower Gleason score and PSA serum levels at biochemical treatment failure (Chade et al., 2011, Trock et al., 2008). Specifically, a moderately sized multi-institute clinical study identified a significant three-fold increase in cancer-specific survival, as well as an improved overall survival benefit when salvage external beam radiotherapy was employed following RP failure, particularly within two years of biochemical relapse. Furthermore, PSA doubling time was a prognostic marker for prostate cancer-specific survival, and survival benefit was only observed in men with PSA doubling times less than six months (Trock et al., 2008). Further biochemical failure following local salvage therapies is often treated

with ADT. In the event of disease progression during first-generation ADT (bicalutamide, flutamide, cyproterone acetate, etc.), the disease is considered to have transition to the incurable and often fatal variant known as castrate-resistant prostate cancer.

TRANSITION TO CASTRATION-RESISTANT DISEASE

While the use of ADT is very effective for most men in reducing prostate tumour growth, there exists a caveat. Unfortunately, for many men disease progression during or after ADT is inevitable, facilitated by restoration of AR survival signaling through various mechanisms described below (Yuan and Balk, 2009). ADT failure indicates transition from castration-sensitive to castration-resistant prostate cancer (Mottet et al., 2011). Castration-resistant prostate cancer was previously, however, incorrectly referred to as androgen-insensitive, androgen-independent or hormone-refractory. It is now known that despite failure of anti-androgens, AR signaling continues to promote prostate cancer growth and survival (described in further detail below).

Castration-resistant prostate cancer (CRPC) is commonly defined by rising PSA levels, despite castrate-levels of testosterone, in the presence of either symptomatic disease or radiographic evidence of disease progression (Scher et al., 2008, Saad and Hotte, 2010). It remains to be elucidated whether the transition to CRPC is a natural progression of the disease or provoked by ADT. Unfortunately, it appears the use of ADT is a double-edge sword. Firstly, it reduces testosterone to castrate levels, rapidly inducing tumour regression and palliation of symptoms. However, ADT may select for prostate cancer cells containing mutations that allow these cells to evade therapy and continue to proliferate under low levels of testosterone (Steinkamp et al., 2009, Chen et al., 2009). In contrast to what was previously known, true androgen-independence is rare and most CRPCs maintain AR signaling activity despite ADT failure (Attard et al., 2009, Wang et al., 2009).

Several mechanisms for the establishment of CRPC have been described including AR overexpression, hypersensitivity, up-regulation of alternate survival mechanisms or intratumoural androgen production (Tilley et al., 1996, Feldman and Feldman,

2001, Steinkamp et al., 2009, Yuan and Balk, 2009, Eikenberry et al., 2010, Sun et al., 2010). One pre-clinical study evaluating the impact of testosterone (and DHT) on prostate cancer cell growth through mathematical modeling demonstrated that in fact low levels of circulating testosterone (or DHT) selects for cells containing aberrant AR expression, and therefore may contribute to ADT failure and disease progression (Eikenberry et al., 2010).

Of particular importance are AR mutations in the ligand-binding domain (LBD) and splice variants devoid of LBD. In the first instance, LBD mutations may lead to "promiscuous" or decreased specificity for other ligands, including anti-androgens. This promiscuous agonistic activity has been demonstrated clinically in men experiencing rising PSA levels despite receiving anti-androgen therapy (Small and Srinivas, 1995, Culig et al., 1999). In this case, PSA levels soon decline following drug withdrawal, suggesting a switch from antagonist to agonist in these prostate cancers. This observation is known as "anti-androgen withdrawal syndrome". More recently, truncated AR splice variants lacking a LBD were found to be constitutively active, participate in survival signaling, and is unaffected by ADT (Sun et al., 2010). These LBD-lacking AR splice variants have also been isolated clinically and correlated to risk of progression (Zhang et al., 2011)

More recently, intratumoural synthesis of androgens (primarily DHT) has been shown to be a novel mechanism for evading central and/or peripheral androgen-deprivation. Previous literature suggest in response to ADT, prostate tumour cells express a gain-of-function mutation in 3beta-hydroxysteroid dehydrogenase type 1 (3βHSD1) either by selective pressure of ADT or through adaptation mechanisms. The function of 3βHSD1 is to catalyse the synthesis of DHT from dehydropiandrosterone (an adrenal-synthesised steroid) (Chang et al., 2013) thereby driving AR-signaling in the absence of circulating testosterone.

Second-generation anti-androgens

As previously mentioned, prostate cancer is typically considered to be castration-resistant (CRPC) following first generation ADT failure. It is now known that ligand-dependent AR signaling remains an important aspect of CRPC progression so may still be responsive to further hormonal manipulation. The new second generation ADT agents, enzalutamide and abiraterone acetate may be useful as the primary treatment for CRPC. Previously in the United States and Australia, these drugs were only approved as a salvage therapy following chemotherapy-treatment failure. However, the FDA recently expanded both enzalutamide and abiraterone acetate treatment indications to include chemotherapy-naïve men with castrate-resistant disease (Ning et al., 2015, D'Amico, 2014). Following suit, the TGA has made similar changes to the abiraterone acetate treatment indications in Australia, although no modification to the enzalutamide treatment indications have been made to date.(Australian Therapeutic Goods Administration, 2014)

Enzalutamide

Enzalutamide (Xtandi®, previous known as MDV3100) has been designed to overcome the low binding affinity and agonistic activity of the widely used AR receptor antagonists (**Figure 1.6**). Unlike first generation AR antagonists such as bicalumatide, enzalutamide targets AR signaling by directly preventing AR nuclear translocation and subsequently, transcription of survival promoting genes (Tran et al., 2009). This novel mechanism has shown significant efficacy *in vitro*, *in vivo*, and in clinical settings with few reported side effects (Tran et al., 2009, Scher et al., 2010).

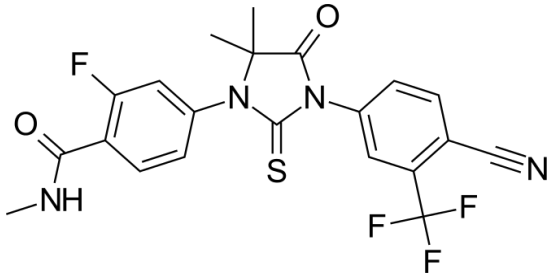


Figure 1.6. Chemical structure of enzalutamide

Of particular importance, the PREVAIL trial uncovered a significant delay in disease progression, and subsequently, initiation of chemotherapy amongst chemo-naïve men treated with enzalutamide compared to placebo (Beer et al., 2014). In this trial, it was found that duration to initiation of chemotherapeutics was 28.0 months compared to 10.8 months for enzalutamide versus placebo treatment arm. Importantly, enzalutamide treatment reduced the risk of death by nearly 30%, and improved overall survival by 2.2 months compared to placebo. However, whether enzalutamide may be beneficial as a first-line therapy in ADT-naïve men remains to be fully explored. A recent and ongoing phase II clinical trial, investigated PSA response as a result of enzalutamide mono-therapy for up to 25 weeks (Tombal et al., 2014). The preliminary findings of this trial reported a >80 % reduction in PSA levels for 62 of 67 enrolled subjects (92.5%), with few adverse side-effects. To date, no further clinical trials are planned.

Abiratrone acetate

Abiraterone acetate is a highly selective and irreversible inhibitor of CYP17, which is an important rate-limiting enzyme for the production of estrogen and testosterone (**Figure 1.7**). In chemotherapy naive men with metastatic CRPC disease, abiraterone plus demexathasone resulted in ≥ 50% reduction in PSA levels for the majority of patients with a mean of 225 days until PSA progression (Attard et al., 2009). In a related, more recent clinical trial, radiographic progression free survival time was two-fold greater (16.5 months) in the abiraterone-prednisone group compared to placebo (8.5 months) (Ryan et al., 2013). Like enalutamide, abiraterone was

previously only approved for use following docetaxel-failure; however, both the US FDA and the Australian TGA have revised the treatment indications to include men who have not received prior chemotherapy.

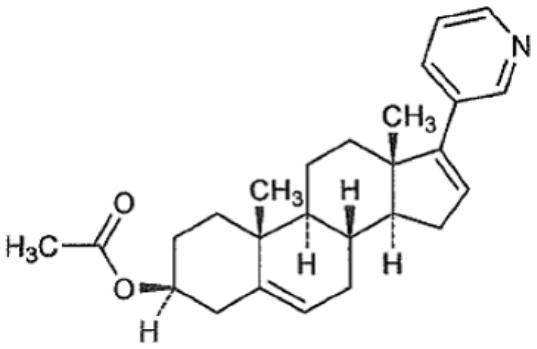


Figure 1.7. Chemical structure of abiraterone acetate

Chemotherapy

Chemotherapy is indicated in patients with recurrent prostate cancers with extensive asymptomatic or symptomatic metastases that are no longer responsive to hormonal manipulation (Heidenreich et al., 2008). For years, chemotherapy has been employed only as a palliative measure. Following the TAX327 clinical trial in 2004, docetaxel (75 mg/m^2 every 3 weeks) became the first chemotherapeutic to improve overall survival and quality of life for men with CRPC (Tannock et al., 2004). In mid-2005, docetaxel-prednisone was approved by the TGA as first-line treatment for CRPC, and subsequently added to the pharmaceutical benefits scheme.

Docetaxel is a semi-synthetic derivative of paclitaxel and is a member of the taxane class of chemotherapeutic agents. The chemical structure of docetaxel is shown in **Figure 1.8**. Similar to other taxanes, the anti-neoplastic activity of docetaxel is achieved primarily through microtubular stabilization and disruption of essential cellular activities. Microtubules are cytoskeletal fibers comprised of intertwined α and β tubulin subunits, which undergo polarization and depolarization in regulating important cellular processes such as mitosis and cell cycle progression. Taxanes disrupt microtubule dynamics by binding the β-tubulin subunit resulting in

stabilization and increased polymerization. This leads to inhibition of mitosis by inducing G_2/M phase cell cycle arrest and subsequently triggering apoptotic cell death (Mackler and Pienta, 2005, Perez, 2009). In addition, it is thought that that docetaxel exerts a pro-apoptotic effect by inhibiting Bcl-2. Briefly, Bcl-2 is a member of the pro-survival class of proteins involved in the regulation of apoptosis. Docetaxel-induced stabilization of microtubules induces inactivation of Bcl-2, thereby preventing interaction with pro-apoptotic proteins, such as Bax. Free pro-apoptotic proteins induce the activation of the caspase cascade and downstream apoptosis (Mackler and Pienta, 2005). Recent findings suggest that taxanes may also target AR-mediated survival signaling (Zhu et al., 2010, Darshan et al., 2011). As previously mentioned, AR translocation and binding to androgen response elements in the nucleus are important events in prostate cancer survival. *In vitro* exposure to paclitaxel inhibited androgen-sensitive and –insensitive AR nuclear translocation and DNA-binding activity, which was dependent on microtubule stabilization (Zhu et al., 2010, Darshan et al., 2011).

Prior to the adoption of docetaxel, mitoxantrone was the cytotoxic agent of choice for late stage metastatic CRPC. However, mitoxantrone provided little survival benefit and was considered primarily a palliative measure (Denmeade and Isaacs, 2002). In the early 1990s, clinical trials of single-agent docetaxel demonstrated an overall mean survival of 27 months in chemotherapy-naïve patients (Picus and Schultz, 1999). Later, a head-to-head clinical trial (TAX327) demonstrated superior efficacy of docetaxel over mitoxantrone, providing men with a 3-month survival benefit and improved quality of life (Tannock et al., 2004). However, the survival benefit is not without side effects. As reported by Tannock et al. (2004), the most prevalent side effects affecting 10% of patients or more included neutropenia, hypersensitivity, neuropathy, nail toxicities, and fluid retention.

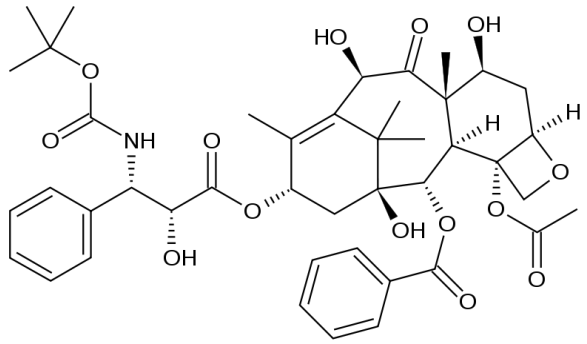


Figure 1.8. Chemical structure of the taxane-based docetaxel

Sequencing of enzalutamide, abiraterone acetate and docetaxel for CRPC

Interestingly, several clinical studies suggest the treatment sequence of enzalutamide, abiraterone, docetaxel and cabazataxel may impact duration of disease response in furture treatments. For example, prior treatment with abiraterone acetate may impair docetaxel efficacy. A small, single institution study reported a significantly higher risk of docetaxel treatment failure (PSA and radiographic) amongst men previously treated with abiraterone acetate, compared to those who did not receive prior abiraterone acetate treatment (Schweizer et al., 2014). In contrast, a separate small scale study evaluating the efficacy of either enzalutamide or docetaxel following abiraterone acetate-treatment failure, found than both docetaxel and enzalutamide had similar clinical activity post-abiraterone (Suzman et al., 2014). Furthermore, the anti-neoplastic effects of enzalutamide in CRPC settings was diminished following prior treatment with abiraterone or docetaxel, and prior treatment with both abiraterone and docetaxel further suppressed disease response to enzalutamide (as measured by PSA kinetics, PSA-progression free survival) (Cheng et al., 2015). Unfortunately, no large scale randomised clinical trial has yet to report on appropriate sequencing of these drugs for the management of CRPC.

TREATMENT OF DOCETAXEL-RESISTANT TUMOURS

Disease progression as a result of docetaxel-based treatment failure may occur within 6-8 months (Mottet et al., 2011) and is commonly attributed to intrinsic or acquired drug resistance (Zhang et al., 2015b). Following occurrence of docetaxel-resistance, second-line chemotherapy regimens are limited. The taxane-based chemotherapeutic agent cabazitaxel (Jevanta ®) is currently the prescribed first-line therapy in docetaxel refractory CRPC in Australia. The chemical structure of cabazitaxel is shown below in **Figure 1.9**. In clinical trials, cabazitaxel demonstrated modest superiority to mitoxatrone, the commonly used second line docetaxel-refractory CRPC, and improved survival by 2.4 months (de Bono et al., 2010). The first-line efficacy of cabazitaxel compared to docetaxel in men with metastatic CRPC remains to be established. However, a large multi-national clinical trial is underway to demonstrate superiority of cabazitaxel over docetaxel for first-line CRPC therapy (Sanofi, 2011).

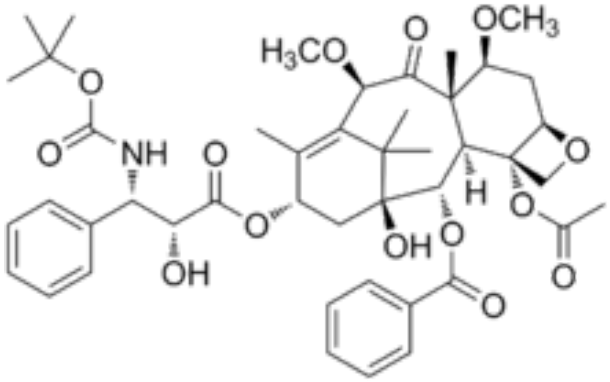


Figure 1.9. Chemical structure of the docetaxel analogue cabazitaxel

While the anti-cancer mechanisms of cabazitaxel remain to be fully investigated, early studies suggest a combination of tubulin-binding and efflux-resistance underlie cabazitaxel-mediated toxicity. Unlike docetaxel and paclitaxel, cabazitaxel has poor affinity for P-gp efflux pumps (Paller and Antonarakis, 2011). As a result, cabazitaxel is able to escape efflux-mediated cancer cell survival mechanisms and maintain cytotoxic intracellular concentrations for up to 96 h (Vrignaud et al., 2013). Furthermore, cabazitaxel is able to cross the blood-brain barrier; however, the clinical

implications of this unique property have yet to be fully investigated (Paller and Antonarakis, 2011).

As a monotherapy, abiraterone acetate and enzalutamide provide modest increases in progression free and overall survival compared to placebo in men with docetaxel-refractory CRPC. Specifically, abiraterone-prednisone treatment extended progression-free and overall survival by 2 and 3.9 months (de Bono et al., 2011). Enzalutamide was slightly better than abiraterone, with an observed 3.3 and 4.8 month increase in disease-free and overall survival compared to placebo, respectively (Scher et al., 2012). More recently, enzalutamide following sequential docetaxel and abiraterone treatment failure was evaluated in a small-scale trial. Response to enzalutamide following docetaxel and abiraterone was only modest, with a median survival of 7.1 months from initiation of treatment (Schrader et al., 2013).

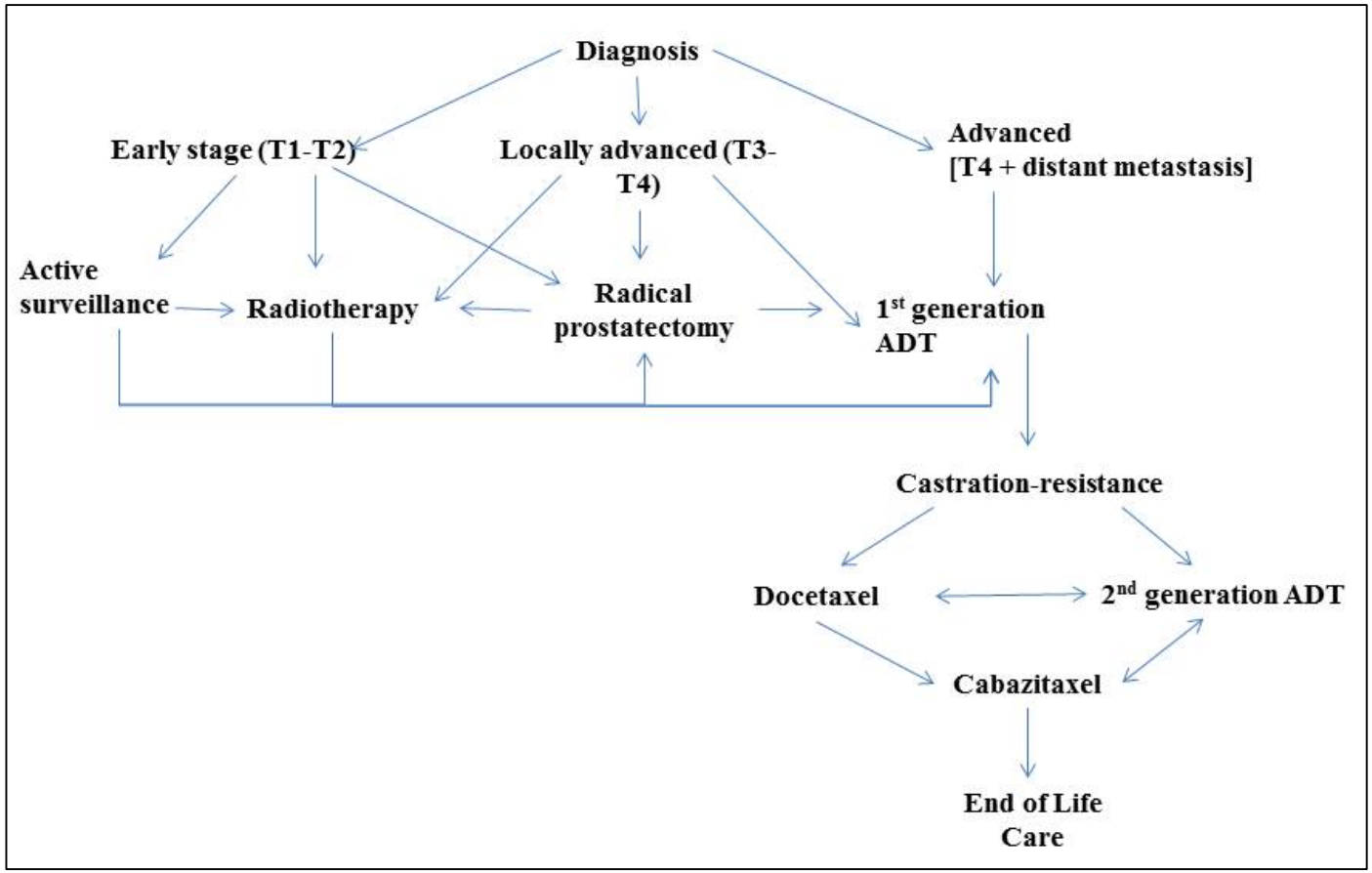


Figure 1.10. Flow diagram of the natural progression of prostate cancer through available treatment modalities in Australia

1.3 ALPHA1-ADRENOCEPTORS

Adrenoceptors (also known as adrenergic receptors) are members of the G protein-coupled receptor (GPCR) superfamily. Adrenoceptors (ADRs) are stimulated by endogenous catecholamines such as epinephrine and norepinephrine, and exogenous agonists such as phenylephrine (Alexander et al., 2011). As illustrated in **Figure 1.11**, the ADR family can be broken down into α and beta subtypes with several homologous isoforms including α-1 (A, B, and D), -2 (A, B, and C), and β-1, 2, and 3 (Cotecchia, 2010). Of note, pharmacological studies have identified a functional variant of the α1A-subtype known as α1L, due to its low affinity towards α1-ADR antagonists, prazosin and RS17053 (Davis et al., 2015). Despite this, the agonist noradrenaline was found to not discriminate between the α1A- and α1L-subtypes as the affinities of these drugs are reportedly similar between subtypes (Ford et al., 1997).

While all adrenergic receptors play an important role in regulating human tissue homeostasis, the focus of this review will primarily cover α1-ADRs as these receptors pertain specifically to the topic of this study. α1-ADR are commonly found in many human tissues such as neural, cardiac, vascular, renal, urinary, and prostate and are known to modulated many functions including neurotransmission, cardiac homeostasis, vasoconstriction and smooth muscle contraction (Cotecchia, 2010, Andersson, 2002).

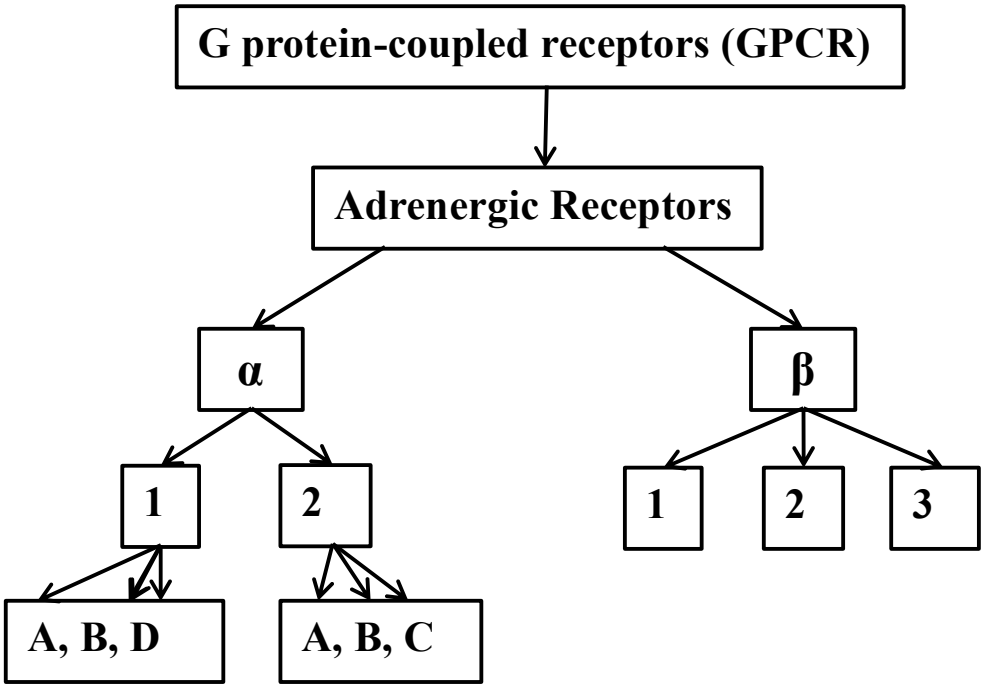


Figure 1.11. Adrenergic receptor subtype classification

ALPHA$_1$-ADR INNERVATION OF THE HUMAN PROSTATE

In the human prostate, differences exist in the localisation and expression of the three α1-ADR isoforms. The α1A-ADR isoform (previously identified as α1C) makes up approximately 70% of the prostatic α1-ADRs, which are primarily found in the stromal region (Price et al., 1993). The α1D-ADR isoform is also found in the prostate stroma as well as the innervating prostatic blood vessels (Walden et al., 1999). In contrast, α1B-ADR is localised to the glandular epithelium (Walden et al., 1999). Prior evidence suggests that the distribution of the three isoforms change with advancing chronological age and subsequently the onset of prostatic hyperplasias (White et al., 2013). One study reported increased a six-fold increase in α1-ADR mRNA expression in the hyperplastic prostate, specifically α1A and α1D subtype mRNA (Nasu et al., 1996). In contrast, the overall receptor expression and localisation remained unchanged between the normal and hyperplastic prostate (Walden et al., 1999). The α1L-subtype has also been reported to be an important mediator of prostatic contraction suggesting this phenotypic α1A-varient may be important for therapeutic BPH interventions (Morishima et al.,

2010). Overall, it is unknown whether the altered isoform mRNA levels and receptor isoform localisation contributes to BPH pathogenesis or occurs secondary to the development of hyperplasia.

Similar to BPH, receptor localisation and expression appears to be altered in prostate cancer tissues. Unlike normal prostate epithelium which expresses few α1-ADRs, prostate cancer epithelia have been reported to express functional α1A-ADR (Thebault et al., 2003, Jensen et al., 2009), as well as increased mRNA levels of α1B and α1D isoforms (Tseng-Crank et al., 1995). It remains unclear whether α1-ADRs have a role in promoting prostate carcinogenesis remains unclear. However, α1-ADRs have been identified to play a role in cellular proliferation (Thebault et al., 2003, Munaron et al., 2004, Thebault et al., 2006) and therefore may be exploited by neoplasms. In contrast, the expression of alpha1L in prostate cancer has yet to be reported.

ALPHA1-ADR-MEDIATED CELLULAR PROLIFERATION

Signal transduction by activated α1-ADR begins with the activation of receptor-coupled G proteins. α1-ADRs primarily interact with $G_{q/11}$. However, α1-ADRs demonstrate subtype-dependent selectivity in G protein-coupling. The G proteins disassociate from the membrane bound receptor where it activates phospholipase C (PLC) and RhoA/Rho kinase pathways. PLC indirectly triggers release of intracellular stores of Ca^{2+} mediated by inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The release of intracellular Ca^{2+}, activates ion channels, promoting influx of extracellular Ca^{2+}. Activation of α1-ADRs by their agonists also results in the opening of receptor-operated calcium channels within the plasma membrane and subsequent influx of Ca^{2+} ions. In addition to mediating Ca2+ release, DAG activates protein kinase C (PKC), which participates in signaling cascades with many important downstream regulators of cell survival and proliferation (Graham et al., 1996, McFadzean and Gibson, 2002).

Calcium signaling appears to play a central role in carrying out α1-ADR-regulated cellular proliferation (Thebault et al., 2003, Munaron et al., 2004, Thebault et al., 2006). Calcium serves as an ubiquitous inhibitor or activator of various cytosolic targets, including calmodulin (Munaron et al., 2004). Calmodulin (also known as calcium-

modulated protein) is a calcium-binding protein, which acts as a secondary messenger promoting activation of enzymes and kinases. Ca^{2+}:calmodulin complex has been shown to promote PKC-mediated activation of epidermal growth factor receptor (EGFR) and subsequently the mitogen-activated protein kinase (MAPK) extracellular regulated kinase (ERK) 1/ 2 (Tebar et al., 2002). ERK1/2 activation is well known to contribute to cellular proliferation and/ or death in a cell type- and stimulus-specific manner. Furthermore, aberrant ERK1/2 activity is frequently linked to development of many cancers (Zelivianski et al., 2003, Carey et al., 2007) including prostate (Price et al., 1999), which may be mediated by α1-ADRs (Graham et al., 1996).

In human prostate cancer cells, chronic α1-ADR stimulation by phenylephrine (PE) or norepinephrine (NE) resulted in increased proliferation (Thebault et al., 2003, Liou et al., 2009) triggered by PLC/ DAG-mediated influx of extracellular Ca^{2+}, with no reported role for IP_3-mediated release of intracellular stores of Ca^{2+} (Thebault et al., 2006). Mechanisms underlying α1-ADR-mediated proliferation have been suggested to involve upregulation of membrane-bound transient receptor potential canonical 6 (TRPC6) expression, Ca^{2+}/calmodulin/calcineurin-mediated activation of NFAT (nuclear factor of activated T-cells), and modulation of cell cycle regulator expression (Thebault et al., 2006). In a related study, TRPC6-mediated Ca^{2+} entry was strongly correlated with androgen-sensitive prostate cancer survival and proliferation (Lehen'kyi et al., 2007), which may be regulated by upstream α1-ADR activation. Interestingly, AR may regulate TRPC6 expression and subsequently Ca^{2+} influx-mediated proliferation (Lehen'kyi et al., 2007). Genetic knock down of AR reduced protein levels of TRPC6 and resulted in decreased Ca2+ intracellular influx (Lehen'kyi et al., 2007). From these findings, one can conclude that α1-ADRs contribute, to some degree, to prostate cancer proliferation *in vitro*.

1.4 ALPHA1-ADR ANTAGONISTS

Alpha1-ADR antagonists (also referred to as 'blockers') are commonly used in clinical practice to treat hypertension and more recently, the urodynamic symptoms associated with benign prostate hyperplasia (BPH). In BPH, α_1-ADR antagonists block receptor activation to relax the prostatic smooth muscle thereby improving rate of urine flow and other associated lower-urinary tract symptoms (LUTS) (Jepsen and Bruskewitz, 1998, Gillenwater et al., 1995).

There are regional differences in the commonly prescribed α1-ADR antagonists for BPH. In the United States, the non-selective doxazosin and terazosin are the most commonly prescribed α1-blockers due to their relatively long half-life (Vincent et al., 1983, Sonders, 1986) and clinically significant improvement in BPH-related LUTS. Furthermore, these drugs have been associated with fewer adverse drug-related cardiovascular side effects, compared to prazosin (Lepor et al., 2012). However, in Australia, the short acting and non-selective prazosin is clinically favored over other α1-blockers primarily due to the rapid mitigation of LUTS. At the present time, the TGA has yet to approve definitive use of this drug for treatment of BPH and instead is only indicated for pre-operative treatment only (Australian Therapeutic Goods Administration, 2015). The highly selective tamulosin, also offer significant reduction in BPH-related LUTS symptoms, however, at a cost of ejaculatory dysfunction making this α1-ADR antagonists undesirable for some men (Lepor et al., 2012). Depending on impact to quality of life and affordability, α1-ADR antagonists may be combined with 5α-reductase inhibitors, such as finasteride and dutasteride (Woo et al., 2011). These drugs act to reduce prostatic volume and prevent disease progression via inhibition of the conversion of testosterone to DHT (as shown previously in **Figure 1.5**). Together, these drugs have been reported to provide long-term synergistic improvement BPH-associated LUTS, and in turn, delay surgical interventions (McConnell et al., 2003, Roehrborn et al., 2010).

In the late 1990s, monotherapy with α1-ADR antagonists were suggested to provide long-term clinical benefits that could not be explained solely by acute prostatic relaxation (McConnell et al., 1998, Lukacs et al., 1999, Michel et al., 2000). In further support, a more recent study uncovered a large proportion of men (70 %), which

experienced continued improvement of BPH-associated LUTS following discontinuation of α1-ADR antagonists (Yokoyama et al., 2007).

Novel Anti-tumour activity of α1-ADR antagonists

Over the last fifteen years, quinazoline-based α1-ADR antagonists such as doxazosin and prazosin have demonstrated significant potential in either preventing or treating prostate cancer. Clinically relevant doses (nM range) of quinazoline α1-ADR antagonist-induced cell death were identified to primarily target cancerous cells (Benning and Kyprianou, 2002) with minimal toxicity in non-cancerous cells (Chon et al., 1999, Hui et al., 2008, Benning and Kyprianou, 2002). Potential therapeutic benefit is further exemplified *in vivo* models demonstrating prostate cancer regression (Kyprianou and Benning, 2000), reduced tumour vascularity (Pan et al., 2003) and decreased incidence of prostate and renal cancer metastasis (Chiang et al., 2005, Sakamoto et al., 2011). While α1-ADR activity might contribute to prostate cancer proliferation, the cytotoxic effects of these antagonists are known to occur independent of α1-ADR antagonism in pre-clinical models (Benning and Kyprianou, 2002). The potential anticancer actions of α1-ADR antagonism are further explored later in this thesis (Chapter 7).

In clinical settings, retrospective studies conducted by Harris et al. and Martin et al. showed a reduced incidence of prostate (Harris et al., 2007) and bladder cancer (Martin et al., 2008), respectively, in men treated with quinazoline-based α1-ADR antagonist. Interestingly, the non-quinazoline naftopidil has also been retrospectively reported to decrease prostate cancer incidence and increased prostate cancer apoptosis (Yamada et al., 2013). Likewise, men treated with terazosin displayed a similar increase in apoptotic cells, as well as a reduction in tumour vascularity in both bladder and prostate cancer tissue specimens (Keledjian et al., 2001, Tahmatzopoulos et al., 2005). However, the mechanisms contributing to the anticancer effects of these drugs have yet to be fully characterised. Furthermore, the cell death (autophagy) and molecular mechanisms contributing α1-ADR antagonist-mediated cytotoxicity remains to be fully explored. Elucidation of these mechanisms may provide insight as to whether these drugs may be useful alone or concurrently with anticancer therapies to mitigate prostate cancer or improve treatment efficacy.

SUMMARY

Prostate cancer is a significant global public health concern, and in Australia, 1 in 7 men will be diagnosed with prostate cancer before the age of 85. While early-stage prostate cancer is highly manageable and even curable, few treatment options exist for advanced disease. Despite recent addition of cabazitaxel, enzalutamide and abiraterone acetate, these agents have shown only modest benefit and many men experience disease progression within months. The ferocity of prostate cancer is further exemplified with the occurrence of treatment-dependent selection for malignant cells containing survival-enhancing mutations. Therefore, new and novel agents are needed to continue to "out-smart" prostate cancer thereby increasing overall survival and quality of life.

Pharmacological treatment with α1-ADR antagonists are by far the most common treatment option used to improve BPH- associated LUTS. Additionally, men undergoing radiotherapy for prostate cancer are often prescribed α1-antagonists either prophylactically or concurrently to reduce treatment-induced LUTS. Of particular interest, some of these drugs possess novel-anti cancer effects, particularly amongst prostate cancers, in pre-clinical and in retrospective clinical settings. While monotherapy with α1-ADR antagonist at safe doses are unlikely to be effective against prostate cancer, treatment with these drugs may reduce the incidence of prostate cancer (Harris et al., 2007) and delay disease progression, particularly in the transition from the castrate-sensitive to castrate-resistant forms. Furthermore, these agents, when combined with current prostate cancer treatments, may have synergistic actions and in turn improve treatment efficacy, or restore sensitivity to existing treatment in resistant prostate cancers.

This study investigated the relative cytotoxic potencies, cell death mechanisms, molecular mechanisms of various α1-ADR antagonists on prostate cancer cell lines. Furthermore, the potential synergistic activity of these antagonists in combination with radiation therapy and chemotherapy was investigated. The findings from this thesis will provide important insight into the development of novel therapies for men diagnosed with prostate cancer.

1.5 AIMS

The overall of aim of this thesis was to investigate the cytotoxic effects of various α1-ADR antagonists on *in vitro* models of castrate-sensitive and castrate-resistant prostate cancer cells.

Using castrate-sensitive (AR-positive) LNCaP and castrate-resistant (AR-negative) PC-3 cells, the specific aims were to:

1. Determine the relative potency and cell death mechanisms (autophagy and apoptosis) of various α1-ADR antagonists (Chapter 3).

2. Uncover the underlying molecular mechanisms contributing to α1-ADR antagonist-mediated prostate cancer cytotoxicity (Chapter 4).

3. Investigate potential radiosensitising actions of α1-ADR antagonist in hypoxic and normoxic conditions and the underlying mechanisms underlying this (Chapter 5).

4. Further explore novel uses of α1-ADR antagonists in treatment of urogenital cancers.

CHAPTER 2:
GENERAL METHODS

CELL LINES

All cells used were obtained from the American Tissue Culture Collection (ATCC, Mannassas, VA, USA) and were grown and maintained according to ATCC guidelines.

HUMAN PROSTATE CANCER LNCAP CELLS

The LNCaP prostate carcinoma cell line was initiated from lymph node metastases in a 50-year old Caucasian male. LNCaP cells express functional androgen receptor (AR). However molecular characterisation studies have revealed a missense mutation at locus T877, causing binding promiscuity of AR to other steroid ligands including bicalutamide. LNCaP cells express normal p53 and secrete PSA (van Bokhoven et al., 2003). The LNCaP cells were used as a model of early, castration-sensitive prostate cancer (**Figure 2.1**).

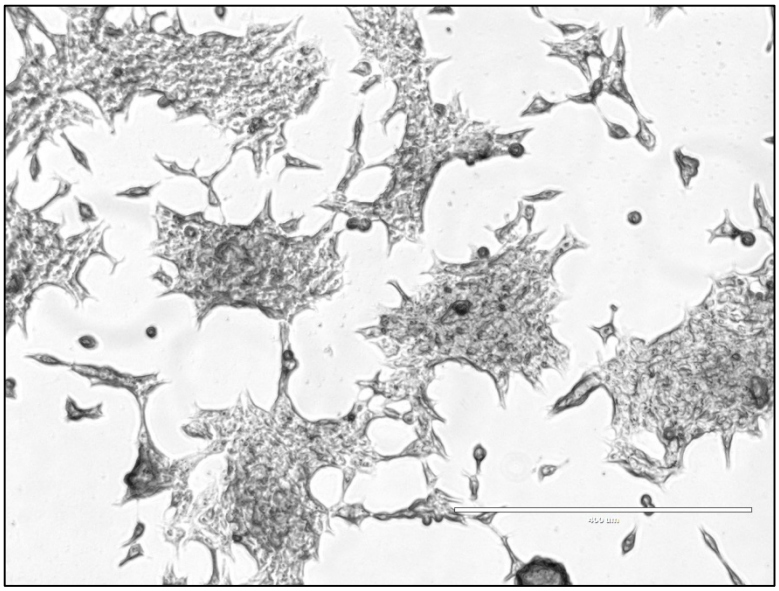


Figure 2.1. Morphology and growth pattern of human androgen receptor-positive (castrate-sensitive) LNCaP prostate cancer cell line. Cells were fixed and stained with crystal violet as described below. Images were taken using an Evos ® Cell Imaging Systems (Thermo Fischer Scientific). Length of calibration bar is equal to 400 μm.

HUMAN PROSTATE CANCER PC-3 CELLS

The PC-3 prostate carcinoma cell line was isolated from bone metastasis of grade IV prostate cancer in a 62-year old Caucasian male (Kaighn et al., 1979). PC-3 cells are androgen-insensitive as this cell line does not express AR, and therefore were considered to be a model of CRPC (**Figure 2.2**). Additionally, these cells express non-functional truncated p53 (van Bokhoven et al., 2003).

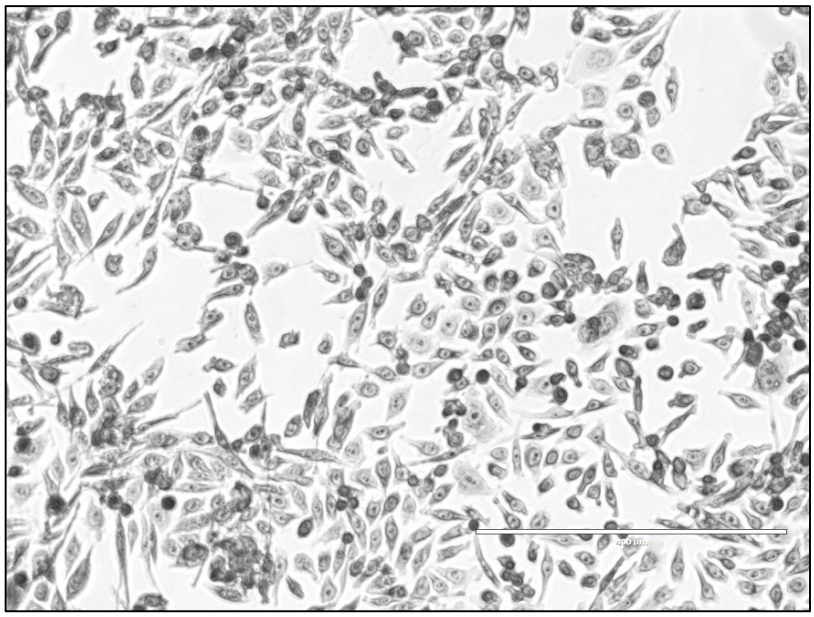


Figure 2.2. Morphology and growth pattern of human AR-negative (castrate-resistant) PC-3 prostate cancer cells. PC-3 cells were fixed and stained with crystal violet, as described below. Images were captured using an Evos® Cell Imaging System (Thermo-Fischer Scientific). Length of calibration bar is equal to 400 μm.

HUMAN PROSTATE MYOFIBROBLAST WYMP-1 STROMAL CELLS

The non-cancerous stromal AR-positive WYMP-1 cells were isolated from the normal prostate of a 54 year-old Caucasian male and were immortalised by plasmid delivery of viral SV40 DNA sequence (Bello et al., 1997, Webber et al., 1999). To date, α1-ADR subtype expression profile remains unknown. WPMY-1 cells were used to model

effects of prostate cancer-stromal interactions via indirect 2D co-cultures as described in the relevant sections of Chapter 6.

HUMAN BLADDER CANCER T24 CELLS

The human bladder transitional (urothelial) cell carcinoma T24 cell line was used briefly throughout Chapter 6. The reader is referred to the relevant methods section in Chapter 6 for further details.

CELL CULTURE

CHEMICALS AND SOLUTIONS

Chemicals and solutions that were routinely used for growth and maintenance of cultured cells are detailed in **Table 2.1.**

GROWTH MEDIUM AND MAINTENANCE

LNCaP cells were grown in RPMI 1640 culture medium containing L-glutamine and phenol red, and supplemented with 10 % foetal bovine serum (FBS), 10 mM HEPES, 1500 mg/L sodium bicarbonate, and 1% gentamicin solution. PC-3 cells were grown in Ham's F-12K culture medium containing L-glutamine and phenol red, and supplemented with 10% FBS and 1% gentamicin solution.

For the relevant sections of this thesis (Chapter 6), WPMY-1 cells were grown in high glucose Dulbeccos's Modified Eagle's Media culture medium containing phenol red, L-glutamine, sodium pyruvate and sodium bicarbonate, and supplemented with 5 % FBS and 1% gentamicin solution. T24 cells were grown in McCoy's A5 modified growth medium containing L-glutamine and sodium bicarbonate and supplemented with 10 % FBS and 1% gentamicin solution.

All cells were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. For all experiments, cells were harvested upon reaching 80-90% confluency. Only cells

≤ 30 passages were used. Unless stated otherwise, all experiments were conducted in respective complete culture medium.

Table 2.1. Cell culture chemicals and solutions

Solution / Chemical	Use	Working conc.	Supplier	Cat. No.
RPMI-1640 culture medium	LNCaP cell culture	-	Life Technologies	11875
Ham's F12 Kaighn's (F12-K) culture medium	PC-3 cell culture	-	Life Technologies	21127
Dulbecco's Modified Eagle's Medium (DMEM)	WPMY-1 cell culture	-	Sigma-Aldrich	D6429
McCoy's A5 modified medium	T24 cell culture	-	Sigma-Aldrich	M9309
Poly-L-Lysine	Coat LNCaP tissue flasks and plates	0.10%	Sigma-Aldrich	P4707
Gentamicin (50 mg/ml)	Antibiotic	1%	Life Technologies	15750
Fetal Bovine Serum (FBS)	Supplement culture medium	5 – 10%	Bovogen	SFBS-F
HEPES	Supplement LNCaP culture medium	100 mM	Life Technologies	15630
Sodium bicarbonate	Supplement LNCaP culture medium	100 mM	Sigma-Aldrich	25080
Trypsin-EDTA (0.25%)	Detachment of cells	0.25%	Life Technologies	25200
Dulbecco's phosphate buffered saline (DPBS) (10X)	Sub-culturing of WPMY-1 cells	1X	Sigma-Aldrich	D8537
Phosphate Buffered Saline (PBS) (10X)	Sub-culturing LNCaP, PC-3 and T24 cells	1X (diluted in sterile dH20)	Sigma-Aldrich	P5493
DMSO	Cryo-preservation freeze medium	5%	Sigma-Aldrich	D2438

SUB-CULTURING TECHNIQUE

For all work with cells, strict aseptic technique was adhered to. Briefly, spent culture medium was removed and the cell monolayer was washed with 1X phosphate buffered saline (PBS) (LNCaP and PC-3). Cells were incubated at 37°C for 1-5 minutes with 0.25% trypsin-EDTA for detachment from tissue-culture flask. Trypsin was inactivated by the addition of culture medium containing FBS (> 5%), and trypsin was removed by centrifugation of cells. Cells were then re-suspended in desired amount of culture medium to achieve a split ratio between 1:6 and 1:3.

Prior to seeding of LNCaP cells, tissue-culture flasks and experimental plates or petri dishes were pre-coated with 0.1% poly-L-lysine for 15 minutes, rinsed with sterile deionized H_20, and allowed to dry overnight in the tissue culture hood. Spare poly-l-lysine coated flasks/plates were stored at 4◦C for up to 7 days.

CRYOPRESERVATION AND RESTORATION

Stocks of LNCaP, PC-3, WYMP-1 and T24 cells were generated and preserved in liquid nitrogen. For LNCaP and PC-3 cell lines, freeze medium contained 5% DMSO dissolved in respective complete culture medium, whereas WPMY-1 and T24 freeze medium consisted of cell line-specific culture medium supplemented with 15% or 10% FBS, respectively. Cells were detached and washed as previously described; however, after centrifugation, cells were re-suspended in freeze medium at a density of approximately $2.0x10^6$/mL. One mL of cell suspension was aliquoted in cryopreservation vials, and was cooled at approximately -1°C per minute using a Mr. Frosty (Nalgene®) in a -80°C freezer. After 24 h, vials were transferred to liquid nitrogen for indefinite storage. After 24 h, one vial of cells was restored to check viability and sterility.

Restoration of cryopreserved cell lines was accomplished by rapidly thawing cells in a 37°C water bath upon removal from liquid nitrogen storage. Freeze medium containing DMSO was removed by centrifugation, and the cell pellet was re-suspended in fresh

complete growth medium and seeded in a T-25 flask. Cells were monitored visually for attachment, growth patterns and sterility.

TRYPAN BLUE-EXCLUSION ASSAY

Cell counts were conducted using the trypan blue-exclusion assay. Trypan blue is a dye that is impermeable to living health cells, but is able to permeate the cell membranes of dead cells. Living cells appear clear with a dark blue border and dead cells appear dark blue. Based on this principle, cell counts and cell viability were determined using 0.4% trypan blue mixed 1:1 with cells in suspension. Cells were either counted manually using a haemocytometer, or by the automated Countess® cell counter (Life Technologies). For all experiments, cell viability was ≥ 95%.

RESAZURIN REDUCTION ASSAY

Resazurin is a stable, non-toxic, and non-fluorescent blue dye. In the presence of cellular metabolic activity, resazurin is reduced to form resorufin, a pink highly fluorescent product. The rate of reduction of resazurin to resorufin directly correlates with cell number, as shown in **Figure 2.3**, and is a reliable indicator of cellular viability in the presence of cytotoxic chemicals (Anoopkumar-Dukie et al., 2005a, Czekanska, 2011). The resazurin reduction assay was used throughout this study as an index of cell viability or survival.

A resazurin stock solution (440 μM) was made from dry powder in sterile PBS then sterile filtered using a 0.2 μm pore syringe filter. Aliquots of stock solution were stored at 4◦C for up to 6 months. Immediately prior to use, resazurin stock solution was diluted 1:10 in complete culture medium for a final concentration of 44 μM.

The resazurin reduction assay was carried out as previously described (Anoopkumar-Dukie et al., 2005a). Briefly, cells were seeded as a monolayer at sub-confluent

densities to allow for exponential growth throughout the duration of the experiments. Cells were incubated for 24 h (PC-3, WPMY-1 and T24) or 48 h (LNCaP) to allow for adhesion prior to treatment. Following desired incubation or treatment time, spent culture medium above the cells was removed and replaced with culture medium containing 44 μM resazurin and incubated at 37°C between 0.5 – 4 h depending on cell type and density. After an appropriate incubation time, fluorescence (excitation: 530 nm; emission: 590 nm) was measured using a Modulous Microplate multimode reader (Promega). Unless stated otherwise, the resazurin reduction assay was conducted in triplicate over three or more independent experiments.

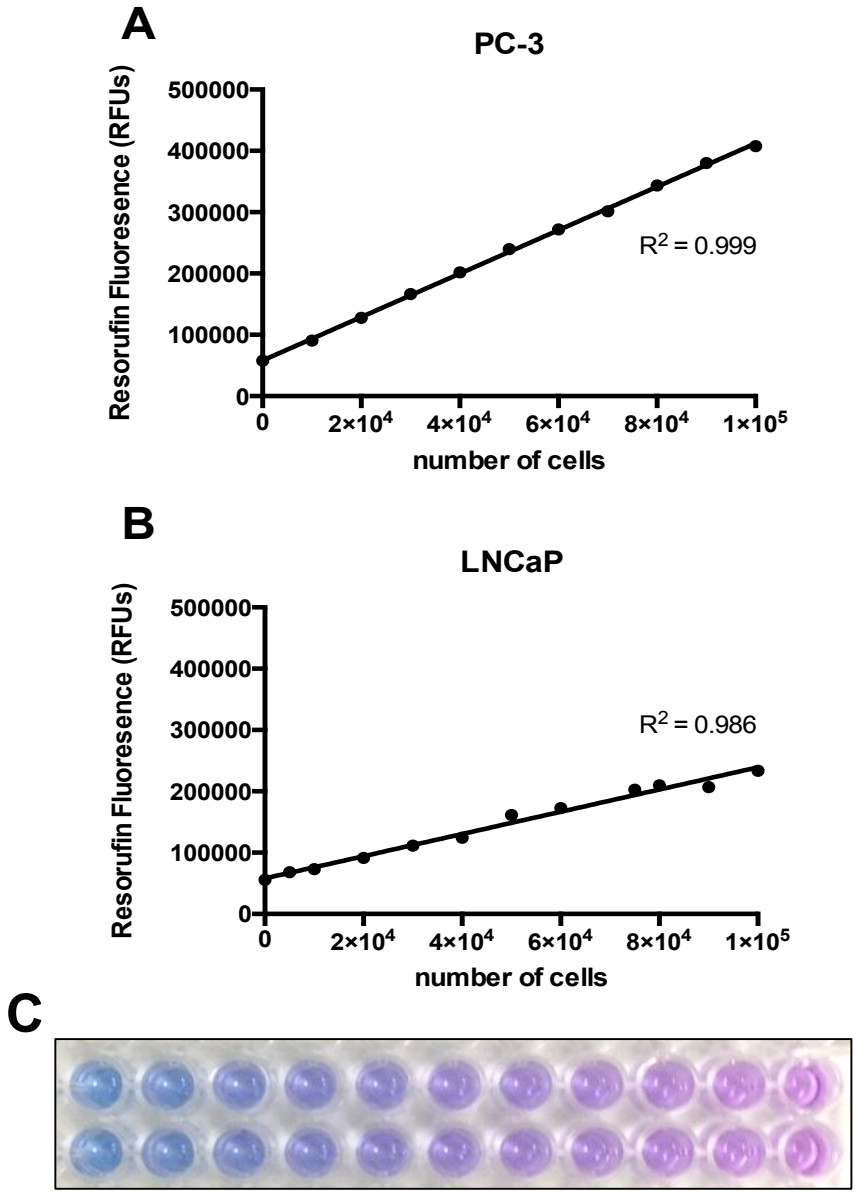


Figure 2.3. Linear relationship between PC-3 [A] and LNCaP [B] cell number and resazurin reduction to the fluorescent by product resorufin. [C] A representative image of cell density-dependent reduction of resazurin to resorufin (cell density increases left to right).

DRUGS AND STOCK SOLUTIONS

The drugs commonly used throughout this study are listed below in **Table 2.2**. Stock solutions were made up aseptically from dry powder dissolved in DMSO (≤ 0.2% in cell culture), except for prazosin where drug was made up in sterile deionized H_20 (dH_2O). No appreciable cytotoxicity was observed with DMSO concentrations used throughout this thesis. To ensure DMSO had no confounding effects, all experiments using drugs dissolved in DMSO was matched with an appropriate DMSO vehicle control.

Immediately prior to treatment, 2X working concentrations were made in pre-warmed complete culture medium and diluted 1:2 in cell culture medium on the plate to yield final concentrations of 0.01 – 100 μM.

Table 2.2. Commonly used drugs and their stock concentrations, storage conditions and supplier details.

Drug / Reagent	Actions	Stock (mM)	Solvent/ Vehicle	Storage	Supplier	Cat. No.
Alfuzosin HCl	Non-selective α1-ADR antagonist	100	DMSO	< -20◦C; 30 days	Selleck Chemicals	S1409
Doxazosin mesylate	Non-selective α1-ADR antagonist	50	DMSO	< -20◦C; 30 days	Sigma-Aldrich	D9815
Prazosin HCl	Non-selective α1-ADR antagonist	1	dH_2O	2-8◦C; 7 days	Sigma-Aldrich	P7791
Silodosin	Selective α1A-ADR antagonist	100	DMSO	< -20◦C; 30 days	LKT Laboratories	S3346
Terazosin HCl	Non-selective α1-ADR antagonist	100	DMSO	< -20◦C; 30 days	Selleck Chemicals	S2059
Tamsulosin HCl	Selective α1A/D-ADR antagonist	50	DMSO	< -20◦C; 30 days	Sigma-Aldrich	T1330
Dimethyl sulfoxide (DMSO)	Solvent; Freeze medium	N/A	N/A	21◦C	Sigma-Aldrich	D2438

CRYSTAL VIOLET

Staining with crystal violet was conducted to examine the morphology of the prostate cancer cells. Traditional methodology for fixing and staining cells was used but in a simplified form. After the desired incubation or treatment time, culture medium above the cells was removed and replaced with methanol (100 %) containing 0.5% w/v crystal violet. Cells were incubated on ice for 15 minutes prior to rinsing gently with water.

Fixed cells were allowed to air dry completely before being examined under phase contrast microscopy using an Evos ® Cell Imaging System (Thermo-Fischer Scientific).

ANALYSIS

Statistical testing was conducted throughout this thesis, which is detailed in the relevant sections of each chapter. In general, GraphPad Prism (version 6 for Mac OS X, San Diego, USA) was used to generate all graphical representations and statistical analyses. A p-value of < 0.05 was considered statistically significant.

CHAPTER 3:
RELATIVE CYTOTOXIC POTENCIES AND CELL DEATH MECHANISMS OF ALPHA1-ADR ANTAGONISTS

3.1 BACKGROUND

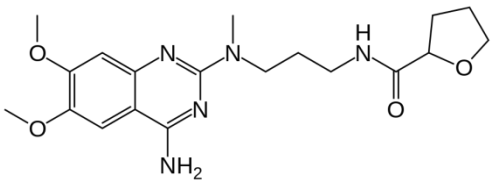
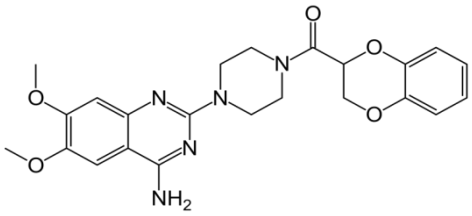
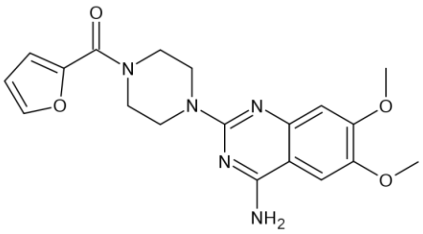
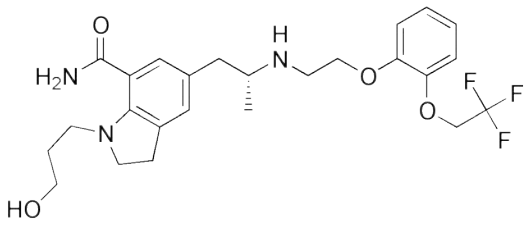
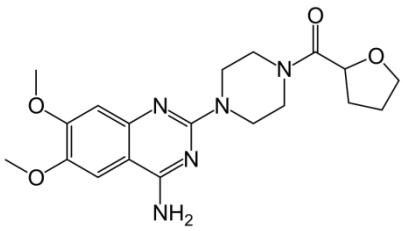
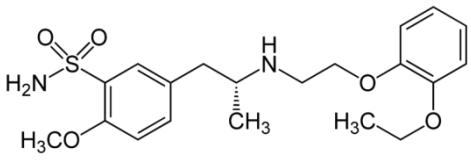
ANTICANCER EFFECTS OF ALPHA1-ADR ANTAGONISTS

Alpha1-ADR antagonists have generated significant interest amongst researchers for their novel urogenital (Hui et al., 2008, Gan et al., 2008, Sakamoto et al., 2011, Gotoh et al., 2012) anticancer activity *in vitro* and *in vivo*, particularly against prostate cancer (Kyprianou and Benning et al., 2000, Chaing et al., 2005, Lin et al., 2007). Doxazosin was first of the α1-ADR antagonists to be identified by as having cytotoxic properties. In 1997, Yang et *al.* demonstrated decreased weight accompanied by prostatic cytotoxicity in a BPH murine model (Yang et al., 1997). Shortly after, similar findings were uncovered in human BPH specimens (Kyprianou et al., 1998, Chon et al., 1999) and human vascular (Hu et al., 1998) and bladder (Austin et al., 2004) smooth muscle cells (SMCs). Later, doxazosin-induced cytotoxicity was reported to extend to malignant cells including prostatic (Kyprianou and Benning, 2000, Partin et al., 2003), cervical (Gan et al., 2008), breast (Hui et al., 2008), and renal (Sakamoto et al., 2011). Likewise, other quinazoline-based α1-ADR antagonists such as prazosin and terazosin, were also found to possess cytotoxic actions (Kyprianou and Benning, 2000, Partin et al., 2003, Lin et al., 2007).

In contrast, the non-quinazoline antagonist, tamsulosin, reportedly lacks the cytotoxic capacity of its quinazoline-containing antagonist cousins. The inability of tamsulosin to induce cancer cell cytotoxicity (Benning and Kyprianou 2002) has since been generalised to all non-quinazoline-based antagonists, giving rise to the notion that antagonist-induced toxicity occurs via quinazoline-dependent mechanisms (Kyprianou and Benning, 2000, Benning and Kyprianou, 2002, Partin et al., 2003). However, little is known with regards to the anticancer potential of other α1-ADR antagonists such as, alfuzosin and the non-quinazoline silodosin. The comparisons of the chemical structures of investigated α1-ADR antagonists are shown in **Table 3.1**.

Recent findings challenge the central dogma to α1-ADR antagonists-induced toxicity, indicating that quinazoline-dependency and receptor antagonism-independent mechanisms are not absolute. The non-quinazoline antagonists labedipinediol-A (Liou

et al., 2009) and naftopidil (Gotoh et al., 2012) displayed comparable cytotoxicity to doxazosin or prazosin against prostate cancer cells. Furthermore, irreversible inhibition of α1-ADRs partially blocked labedipinediol mediated toxicity, suggesting receptor antagonism-dependent and independent mechanisms (Liou et al., 2009).

Table 3.1. Chemical structures of commonly used α1-subtype selective ADR antagonists	
Alfuzosin	
Doxazosin	
Prazosin	
Silodosin	
Terazosin	
Tamsulosin	

Apoptosis

Apoptosis, or type I programmed cell death, was first described by Kerr and colleagues in 1972 and is characterised by several morphological and biochemical changes including cell shrinking, membrane blebbing, and DNA fragmentation (Kerr et al., 1972). Apoptosis occurs in multicellular organisms as a mechanism for homeostasis and elimination of neoplastic cells. Anomalies in apoptotic machinery prevent cells from being eliminated in response to apoptotic stimuli and are commonly associated with the development of carcinomas (Kerr et al., 1994).

Apoptosis can occur via intrinsic or extrinsic mechanisms, as shown in **Figure 3.1**. The intrinsic or "mitochondrial-dependent" apoptotic pathway is initiated in response to intracellular stimuli such as DNA damage or endoplasmic reticulum stress. This triggers activation of pro-apoptotic members of the Bcl-2 protein superfamily such as Bad, Bax, Bim, and Bid. The apoptotic signal is negatively regulated by pro-survival proteins including Bcl-2 and Bcl-xL, which bind to inhibit pro-apoptotic protein activity. Free, unbound, pro-apoptotic proteins stimulate mitochondrial cytochrome c release and formation of the apoptosome. The apoptosome consists of cytochorome c and apoptosis protease activating factor 1 (Apaf-1), which activates caspase-9. Caspase-3 becomes activated by caspase-9 and participates in activation of downstream effector caspases. In contrast, the extrinsic apoptotic pathway begins with ligand-mediated activation of membrane bound death receptors. Upon activation, the receptor recruits several components of the death-inducing signaling complex (DISC). The inactivated form of Caspase-8, pro-caspase-8, becomes activated by DISC-mediated cleavage. Caspase-8 activates caspase-3, triggering activation of the caspase cascade and ensuing apoptotic cell death (Taylor et al., 2008).

The role of apoptosis in prostate cancer

As mentioned previously, apoptosis is an important mechanism regulating tissue homeostasis and loss of normal apoptotic signaling mechanisms are known to contribute to carcinogenesis. Cancer cells maintain functional apoptotic machinery but employ numerous strategies to evade traditional apoptotic signals including contact-inhibition and detachment. Anticancer therapies such as ADT, chemotherapy and

radiotherapy exploit the residual apoptotic mechanisms leading to cancer cell death and tumour regression. However, neoplasms eventually acquire further anti-apoptotic mechanisms, presumably through treatment-dependent selection, which are responsible for disease recurrence or progression. For example, altered expression of the anti- and pro-apoptotic proteins, such as Bcl-2 and Bax, are partially responsible for ADT failure (Raffo et al., 1995, Yang et al., 2003), radioresistance (Khor et al., 2007) and docetaxel-resistance (DiPaola and Aisner, 1999, Lebedeva et al., 2000).

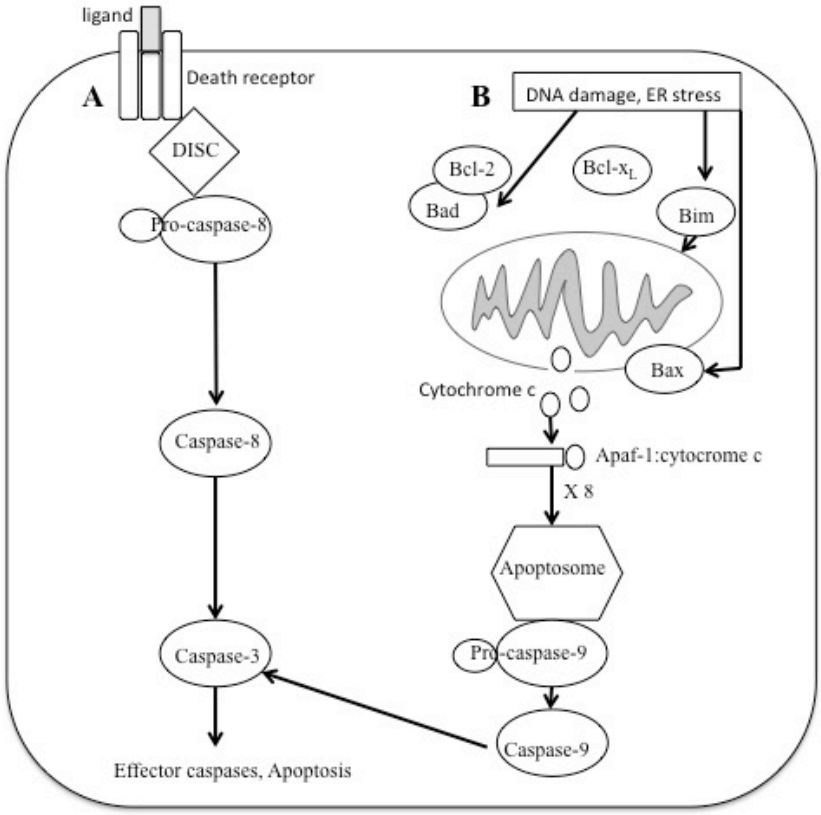


Figure 3.1. Extrinsic (A) and Intrinsic (B) apoptotic pathways.

Apoptotic effect of α1-ADR antagonists in prostate cancer

Exposure of androgen receptor (AR)-positive and AR-negative prostate cancer cell lines to cytotoxic concentrations of doxazosin, prazosin, or terazosin has been reported to trigger apoptotic cell death *in vitro* (Partin et al., 2003, Lin et al., 2007, Kyprianou et al., 2000, Garrison and Kyprianou, 2006). Interestingly, these effects were also observed in rodent models (Papadopoulos et al., 2013, Chiang et al., 2005) and in human tissue specimens from resected prostates (Kyprianou et al., 1998, Keledjian et al., 2001).

Consistent with findings of apoptotic activity *in vitro*, the non-quinazoline naftopidil was also reported to have pro-apoptotic effects in prostate cancer specimens from men treated with this drug (Yamada et al., 2013).

In addition to targeting the prostate cancer cells directly, the apoptotic effect of α1-antagonists is also seen amongst endothelial cells *in vitro* (Keledjian et al., 2005). In a previous study, doxazosin was able to induced apoptosis in human umbilical vein endothelial cells (HUVEC), and as a result prevented cell migration, invasion, adhesion and overall endothelial tube formation in response to angiogenic factors (Keledjian et al., 2005). This endothelial cell apoptotic effect has also been observed *in vivo* and in clinical settings, where it has been reported that treatment with quinazoline-based doxazosin and terazosin reduced tumour vascularity in mice (Pan et al., 2003) and humans (Keledjian et al., 2001, Tahmatzopoulos et al., 2005), respectively.

Quinazoline-based α1-ADR antagonist-induced prostate cancer apoptosis has been reported to primarily involve transforming growth factor beta (TGF-β) and Smad signaling, activation of IκBα, and subsequent inhibition of the survival transcription factor NfκB (Partin et al., 2003). However, a growing body of evidence suggests apoptotic cell death may occur by other intrinsic (Lin et al., 2007) and extrinsic mechanisms (Partin et al., 2003, Garrison and Kyprianou, 2006) including, mitochondrial-dependent activation of caspase cascade (Lin et al., 2007) and Fas-associated death domain (FADD) activation (Garrison and Kyprianou, 2006). Additional signaling mechanisms underlying α1-ADR antagonist-induced prostate cancer apoptosis will be detailed further in Chapter 4.

AUTOPHAGY

Macrophagy (herein referred to as 'Autophagy') is a catabolic process in which a cell triggers "self-eating" to recycle unneeded organelles and other cellular components. The induction of autophagy is most commonly viewed as a survival mechanism in the presence of various stimuli including nutrient starvation, hypoxia, and metabolic stress (Dalby et al., 2010). In a growth state, autophagy is typically inhibited. Binding of growth factors to their respective receptors triggers activation of phosphoinositide 3-kinase PI3K / Akt / mammalian target of rapamycin (mTOR) and subsequently inhibits autophagy by negative regulation of Ulk complex. In the presence of autophagic stimuli, mTOR is inhibited, freeing Ulk complex to initiate autophagy. As shown in **Figure 3.2**, autophagy is carried out in five distinct stages: membrane isolation, elongation, completion (sequestration), maturation (fusion), and degradation (Kondo et al., 2005). Activated by Ulk complex, PI3K associates with beclin-1 to convert phosphatidylinositol to phosphatidylinositol 3-phosphate (PI3P), which functions to recruit other autophagy-related proteins to the phagopore. The conjugation of autophagy-related proteins, Atg5 and Atg12 in the phagopore recruits microtubule-associate light chain 3 (LC3). Atg5-Atg12 facilitates the conversion of LC3 from isoform I to II, a hallmark of late stage autophagy. In the final stages, the autophagosome, containing sequestered cellular components, matures via H^+ -ATPase acidification and fusion with lysosomes forming an autolysosome. In the final stage, degradation is carried out by lysosomal hydrolases and resulting macromolecules are released into the cytosol (Kondo et al., 2005).

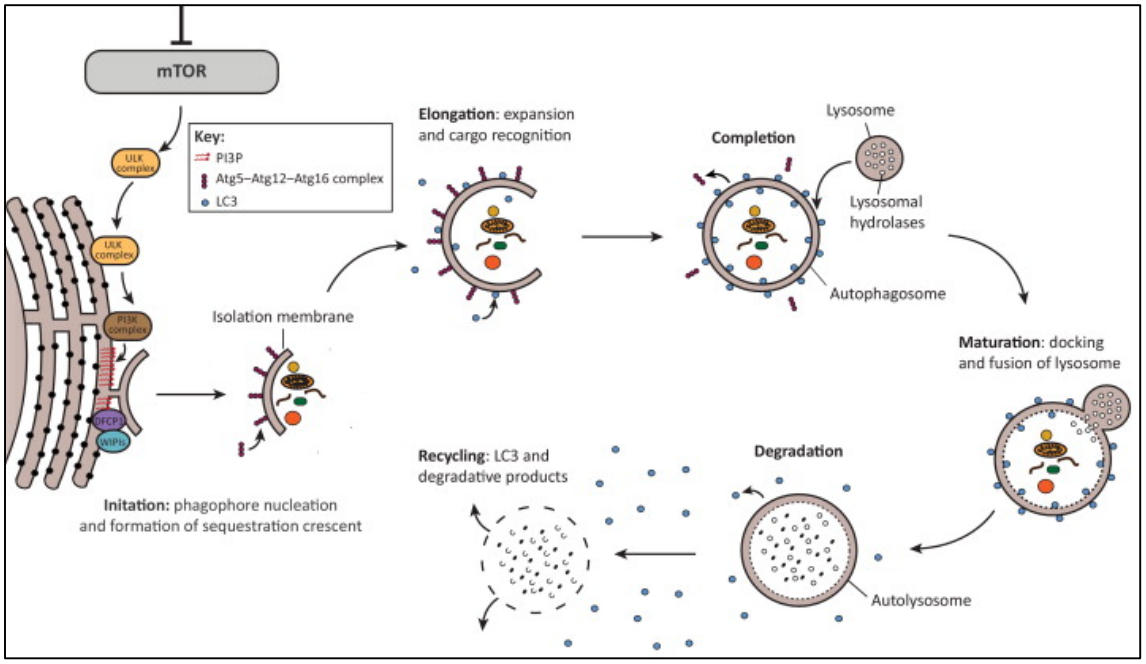


Figure 3.2. The autophagy pathway (Greenfield and Jones, 2013). Figure reprinted with permission from copyright holder.

Autophagic (Type II) programmed cell death

In recent years, autophagy has been identified to play a role in a type of programmed cell death known as Type II or autophagic cell death. This notion remains controversial amongst researchers, questioning whether autophagy occurs secondary to other forms of programmed cell death, either as a preventative or adaptive measure to cytotoxic stimuli, rather than driving programmed cell death (Shen et al., 2012, Shimizu et al., 2014). Despite the critics, it has been proposed that the primary hallmark of autophagic cell death is whether inhibition of autophagy by pharmacological or genetic knockdown is able to protect against cell death (Shen and Codogno, 2011, Shimizu et al., 2014).

Role of autophagy in cancer

Cancer is well characterised to involve up-regulation of autophagy mechanisms to provide a survival advantage in harsh environmental conditions not well suited to normal non-cancerous cells. Modulation of autophagy to enhance sensitivity to cytotoxic agents or radiotherapy or to overcome treatment resistance has become of significant interest to the scientific community. Of particular importance, ADT treatment failure may in part be due to the up-regulation of autophagic mechanisms to

maintain survival signaling and to protect cells in the absence of androgens (Li et al., 2008a, Boutin et al., 2013). Likewise, the development of chemoresistance may be an incidental by-product of autophagy-mediated "housekeeping", by eliminating treatment-induced damaged organelles to mitigating intracellular stress (Mohammad et al., 2015) and evading apoptosis. Together, these concepts set the stage for an interesting, yet difficult scenario. While ADT is particularly effective in treating early stage prostate cancer, androgen-deprivation drives the transition from androgen/AR- to autophagy-mediated survival signaling (Boutin et al., 2013), eventually leading to ADT-failure and disease progression. The ADT-mediated acquisition of autophagic survival mechanisms may confer resistance to chemotherapeutics, which are often the first line treatment for hormone-refractory disease.

In current medicine, many therapies have been employed to induce autophagic cell death in cancer cells including tamoxifen and γ-irradiation. Likewise, autophagy inhibitors also have shown efficacy in various cancers where autophagy provides a survival advantage, such as treatment with the autophagy inhibitor 3-methyladenine (3-MA), in breast, colon, cervical, and prostate cancers (Kondo et al., 2005). In prostate cancer, autophagy is reported to have a cell-type dependent paradoxical effect; contributing to both survival (Li et al., 2008a, Wu et al., 2010b, Bennett et al., 2013, Shin et al., 2013, Pickard et al., 2015) and cell death in response to various toxic stimuli including chemotherapeutics (Pickard et al., 2015).

Autophagy and α1-ADR antagonists

The role of autophagy in quinazoline-based α1-ADR antagonist-mediated toxicity, particularly in prostate cancer cells, remains to be fully explored. Autophagic response to α1-ADR antagonists was first reported by Yang et al (2011). Prazosin was found to induce autophagy in rodent cardiac cells, which was evidenced by autophagic vacuole formation and LC3 conversion (Yang et al., 2011b). However, prazosin induced cell death was not dependent on autophagic machinery, but was suggested to involve cross talk with apoptotic pathways (Yang et al., 2011b). More recently, a potential role for autophagy in doxazosin-induced prostate cancer cell death was uncovered (Pavithran and Thompson, 2012). Using transmission electron microscopy, the authors reported that toxic doses of doxazosin triggered acidic vacuole formation and lipofuscin

accumulation (Pavithran and Thompson, 2012). While these findings point to the occurrence of autophagy, its contribution to programmed cell death in response to antagonist-induced toxicity is unclear and further investigation is required as suggested by Klionsky et al. (2012).

SUMMARY

Few studies have evaluated the relative cytotoxic potency and cell death mechanism of various α1-antagonists, including quinazoline- and non-quinazoline-based antagonists in prostate cancer cells. Additionally, there is a gap in knowledge with regards to how α1-ADR antagonist potencies vary between prostate cancer cell lines and with duration of exposure.

3.2 AIMS

The overall aim of this study was to investigate the relative cytotoxic potency and cell death mechanisms (apoptosis and autophagy) of quinazoline- and non-quinazoline based α1-ADR antagonists in cellular models of castration-sensitive and castration-resistant prostate cancer.

Using AR-positive (castration-sensitive) LNCaP and AR-negative (castration-resistant) PC-3 cells, the specific aims of this study were to:

1. Determine the relative cytotoxic potencies of quinazoline- and non-quinazoline-based α1-ADR antagonists following continuous 24, 48 and 72 h exposures.

2. Investigate the underlying cell death mechanisms of these α1-ADR antagonists.

3. Determine whether cell type-dependent differences exist in the cytotoxic potencies and cell death mechanisms.

3.3 MATERIALS AND METHODS

CHEMICALS AND SOLUTIONS

Stock solutions of the autophagy inhibitor 3-MA (10 mM) were made up fresh daily from powder dissolved in complete culture medium containing 0.1% DMSO. 3-MA stock solution was diluted 1:2 in cell culture medium for a final concentration of 5 mM. All other chemicals and solutions (including α1-ADR antagonists) are detailed in Chapter 2 (General Methods).

CELL LINES AND TREATMENT

Human prostate cancer cell lines LNCaP and PC-3 were seeded in 96-well plates at a density of $5.0x10^3$ and $3.0x10^3$ trypan blue-excluding cells per well, respectively. Cells were incubated for 24 (PC-3) or 48 h (LNCaP) for adhesion. Prior to treatment, culture medium above the cells was renewed. Cells were treated continuously with alfuzosin, terazosin, silodosin, doxazosin, prazosin (0.01 – 100 μM) or appropriate vehicle control and for 24, 48, or 72 h. The concentrations chosen range from therapeutic to supratherapeutic. The therapeutic concentrations of investigated drugs are detailed below in **Table 3.2**.

Table 3.2. Therapeutic concentrations of investigated α1-ADR antagonists

Drug	Concentration in human plasma [C_{max}]	Reference
Alfuzosin	41 nM	(Ahtoy et al., 2002)
Doxazosin	33 nM	(Vincent et al., 1983)
Prazosin	52.2 – 391 nM	(Jaillon, 1980)
Silodosin	97 nM	(Zhao et al., 2009)
Tamsulosin	37.2 nM	(Korstanje et al., 2011)
Terazosin	95 - 120 nM	(McNeil et al., 1991)

CYTOTOXIC POTENCY OF α1-ADR ANTAGONISTS

The resazurin reduction assay was used as a measurement of cell viability and was conducted as detailed previously in Chapter 2 (General Methods) following desired treatment time.

To ensure observed changes in cell viability were in fact due to cytotoxicity and not to change in cellular metabolic activity, the quantitative DNA-based proliferation assay CyQuant NF® (Life Technologies) was used according to the manufacturer's instructions. Briefly, culture medium above the cells was removed and replaced with 100 μL of CyQuant dye reagent and incubated at 37°C for 60 minutes. Fluorescence (excitation: 485 nm; emission 530 nm) was read using Modulous Microplate multimode plate reader (Promega). All CyQuant NF® experiments were conducted in duplicate over three independent experiments.

Using the resazurin data, the concentration that resulted in 50% of maximal reduction in cell viability (IC_{50}) was determined for all cell lines, drugs, and time points.

CASPASE-3 ACTIVITY ASSAY

Apoptotic programmed cell death is carried out by activation of the caspase cascade initiated rom either the intrinsic (mitochondrial) or extrinsic (death receptor) pathways. Caspase-3 is the "universal" caspase that is activated by both intrinsic and extrinsic pathways, making it a good marker for apoptosis.

Following 24 h treatment with alfuzosin, terazosin, tamsulosin, silodosin (30 μM), doxazosin, prazosin (10 and 30 μM), or appropriate vehicle control, apoptosis was measured using the commercially available caspase-3 fluorescence assay kit (Cat. No. 10009135, Cayman Chemicals) according to manufacturer's instructions. Briefly, immediately following treatment, plates containing cells were centrifuged at 800 x g for 5 minutes prior to and after washing of adherent cells with assay buffer. The assay buffer was aspirated, and cells were lysed for 30 minutes on an orbital shaker using kit provided lysis buffer. Samples were transferred to a clean 96-well black plate prior to addition of caspase-3 substrate solution, and incubated for 30 minutes at 37°C.

Fluorescence was read (excitation 485 nm; emission 530 nm) using a Modulus Microplate multimode plate reader (Promega).

AUTOPHAGY

Changes in autophagy can be measured in numerous ways (Klionsky et al., 2012), with many assays utilising fluorometric stains of autophagic vesicles as a marker of autophagic activity. One such assay, the Cyto-ID Autophagy Detection kit (Cat. No. ENZ-51031, Enzo Life Sciences) has previously been used detect changes in vesicle formation specific to autophagy and correlates with the LC3-II puncta localised in the autophagosome (Guo et al., 2015). In this study, the commercially available Cyto-ID Autophagy Detection kit was used to detect changes in autophagic vesicle formation following 24 h treatment with alfuzosin, terazosin, tamsulosin, silodosin (30 μM), doxazosin, prazosin (10 and 30 μM), or appropriate vehicle control. Increase in autophagy-related vesicles was measured using Cyto-ID® Autophagy Detection kit according to the manufacturer's instructions. Briefly, cells were washed once with the buffer solution provided in kit, which was supplemented with 5% FBS to prevent cell detachment during washes. Cells were stained for 30 minutes at 37°C with the dual reagent staining solution consisting of Cyto-ID® reagent and the nuclear counterstain Hoechst 33342 diluted 1:1000 in phenol-red free culture medium containing 5% FBS. Cells were washed twice and autophagic vesicle formation was visualised by fluorescence microscopy using an Evos® Cell Imaging System (Thermo-Fischer Scientific). The Cyto-ID Autophagy Detection kit was conducted in duplicate for each condition over three or more independent experiments.

Rapamycin, an inducer of autophagy, was used as a positive control. Cells were treated with 100 nM of rapamycin for 24 h, which has been previously identified to be a sufficient concentration and timeframe to activate autophagic machinery in PC-3 cells (Sarbassov et al., 2006). As shown in **Figure 3.3** on the following page, the Cyto-ID® Autophagy Detection kit is highly sensitive in visualising changes in rapamycin-induced autophagic activity.

The pharmacological inhibitor of autophagy, 3-methyladenine (3-MA), was used to

establish the role of autophagy in α_1-ADR antagonist-induced cytotoxicity in LNCaP and PC-3 cells. 3-MA inhibits autophagy by targeting class III PI3K to block the formation of PI3P, which is essential for recruitment of Atg proteins and initiation of autophagy (Wu et al., 2010a). A concentration of 5 mM has previously been shown to inhibit autophagy in prostate cancer cells (Pickard et al., 2015) and was found to suppress 100 nM rapamycin-induced autophagy in the present study (data not shown). Cells were pre-treated with 3-MA (5 mM) for 30 minutes prior to co-treatment with 3-MA and doxazosin or prazosin for 24 h. Final concentration was 5 mM of 3-MA and 10 - 30 μM prazosin and doxazosin. Following treatment, either resazurin reduction (described in Chapter 2) or caspase-3 activity (as described previously) assay was conducted to determine changes cell survival or apoptotic cell death, respectively.

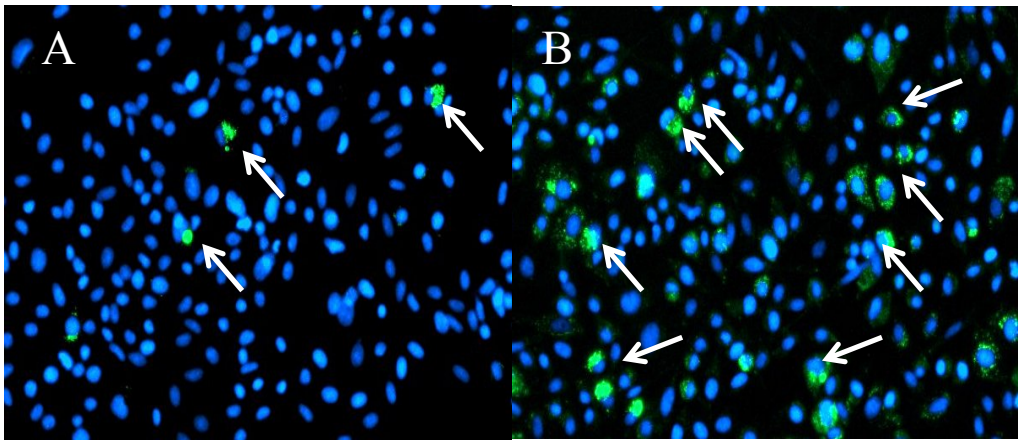


Figure 3.3. Cyto-ID® detection of rapamycin-induced autophagy. Basal (A) and rapamycin-induced (100 nM, 24 h) (B) autophagy in PC-3 cells was determined using CytoID® Autophagy detection kit consisting of an autophagic vesicle (green) and nuclear Hoechst 33342 (blue) stains, visualised by fluorescence microscopy (Evos ® Cell Imaging System, Thermo-Fischer Scientific). Arrows indicate cells with autophagic activity.

STATISTICAL ANALYSIS

Statistical analyses and graphical representation were generated using GraphPad Prism (version 6 for Mac OSX). Unless indicated otherwise, all conditions were assayed in

triplicate over three or more independent experiments. The relative IC_{50} (50% of maximal effect) was determined by non-linear regression of dose-response curve for each α1-ADR antagonist. For comparisons between control and treated cells, a one-way ANOVA with Dunnett's post hoc test was conducted. Where two or more conditions were compared, a two-way ANOVA was conducted with a Tukey-Kramer post-hoc test. Statistical significance was defined as $P < 0.05$.

3.4 RESULTS

α1-ADR ANTAGONIST CYTOTOXICITY

The cytotoxic potential of several α1-ADR antagonists was examined in AR-positive LNCaP and AR-negative PC-3 prostate cancer cell lines. Prostate cancer cells were treated continuously with alfuzosin, doxazosin, prazosin, silodosin, tamsulosin or terazosin for 24 – 72 h. Following treatment, cell survival (survival) was determined using a resazurin reduction assay.

Of the α1-ADR antagonists tested, doxazosin and prazosin were found to have the greatest cytotoxic effect at all time points and in both cell lines (**Figure 3.4**). At concentrations greater than 10 μM, these drugs significantly reduced cell survival in a time-dependent manner, with 72 h exposure completely suppressing PC-3 and LNCaP cell survival ($P<0.001$). In contrast, the highest concentration (100 μM) of terazosin, silodosin and alfuzosin, resulted in a modest time-dependent reduction of cell survival, with 72 h exposure decreasing PC-3 survival by 42.2%, 25.5%, 28.7% and LNCaP survival by 30.0%, 26.7%, 14.0%, respectively ($P\leq 0.01$ for all drugs, **Figure 3.4**). In contrast, tamsulosin treatment failed to a have a statistically significant effect on cell survival at all time points.

To ascertain that the observed findings were in fact due to a reduction in cell survival and not to decreased cellular metabolic activity, cellular DNA was quantified using the CyQuant® NF cell proliferation assay (Life Technologies) following treatment of PC-3 cells with prazosin and doxazosin at all time points. As shown in **Figure 3.5**, resazurin reduction was generally consistent with CyQuant® NF findings, except for prazosin, where resazurin reduction significantly underestimated PC-3 toxicity following 24 and 72 h exposure at lower concentrations (1 and 10 μM, and 10 μM, respectively). Overall, resazurin reduction was considered an effective assay for the measurement of α1-ADR antagonist-induced cytotoxicity and resazurin data was subsequently used to determine the relative cytotoxic potencies of these drugs.

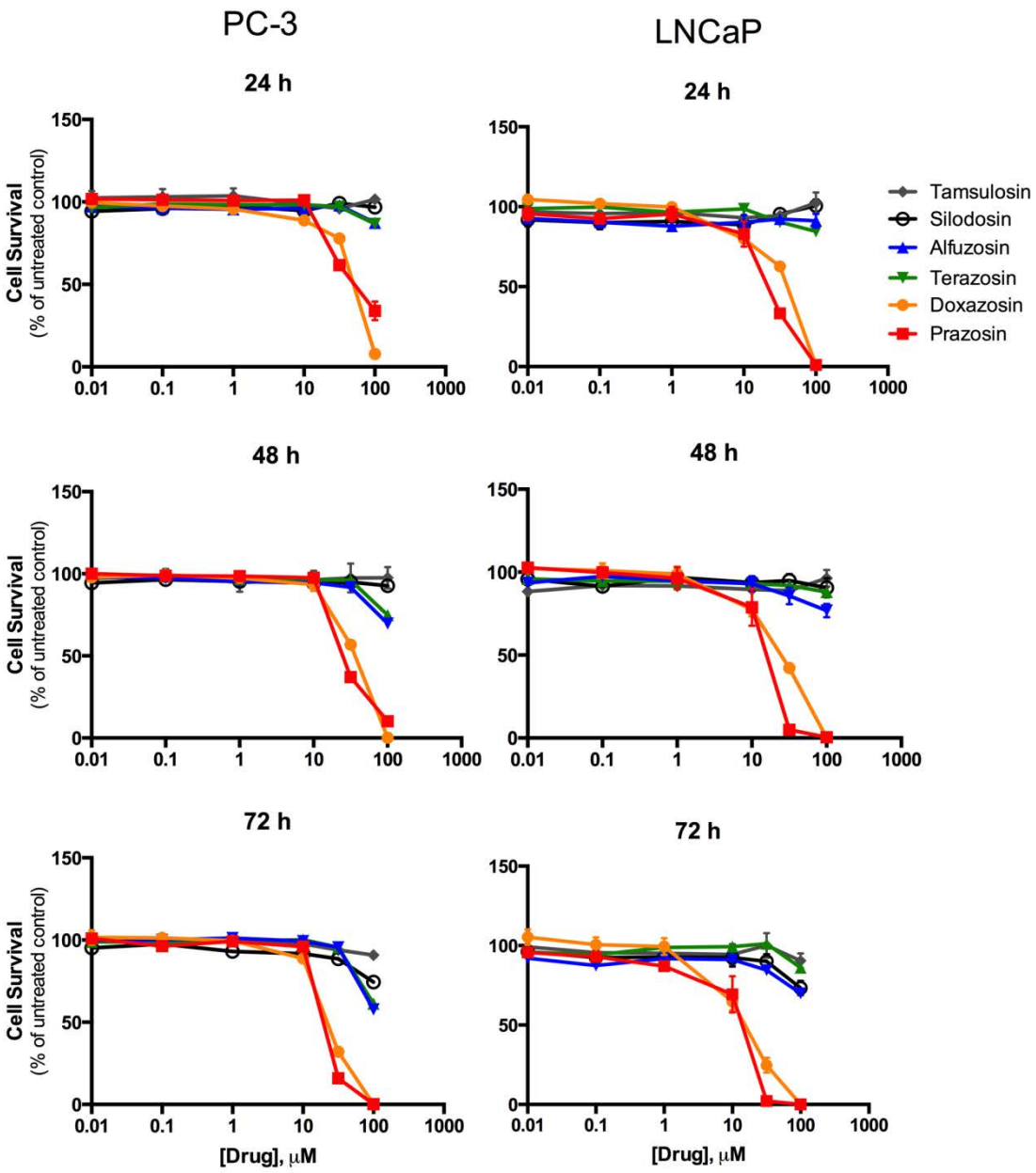


Figure 3.4. Relative cytotoxic potency of α1-ADR antagonists in PC-3 (left column) and LNCaP (right column). Cell survival was determined using the resazurin reduction assay as previously described. Data are expressed as the percentage of untreated control (mean ±SEM, n≥5). Fix!

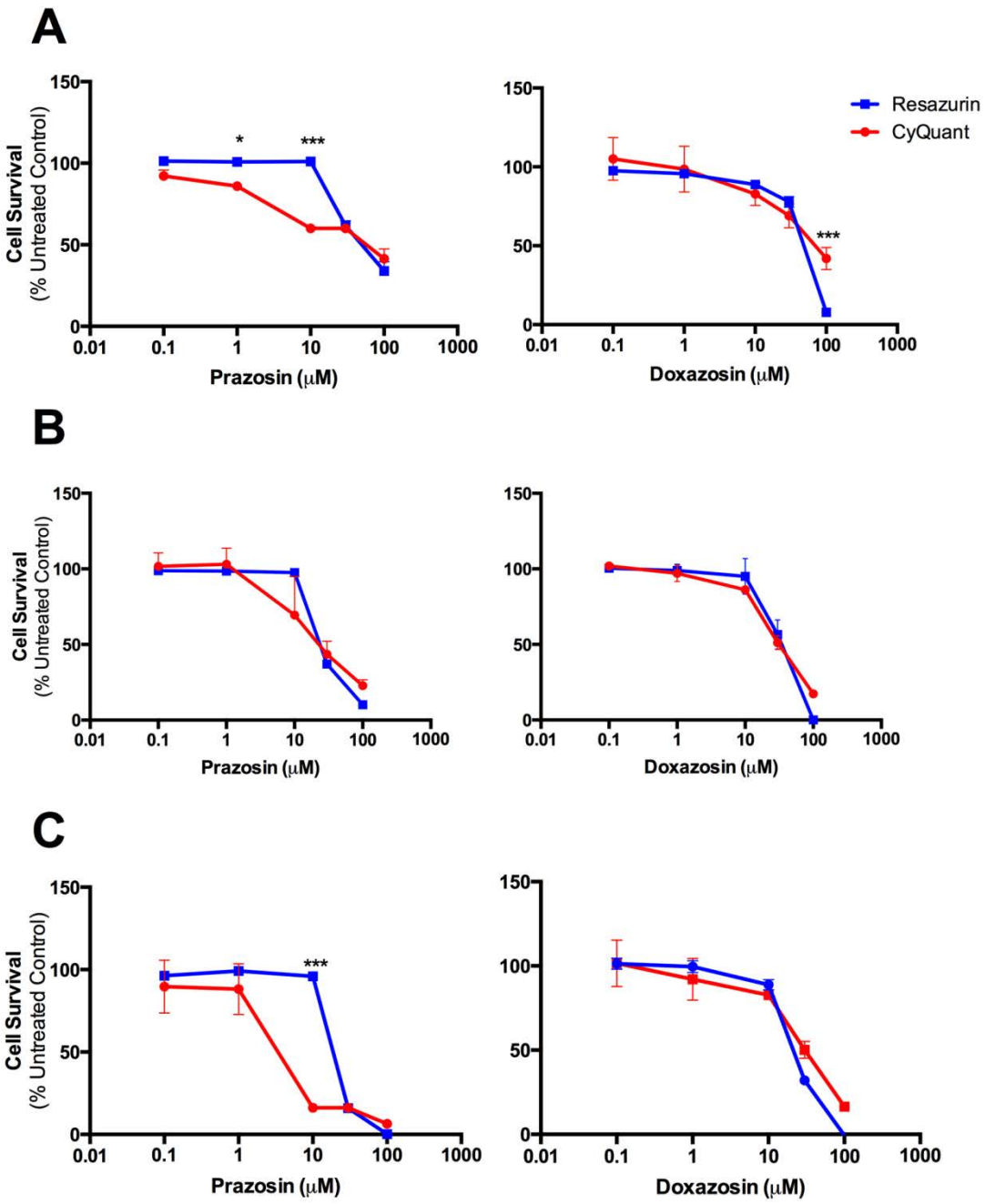


Figure 3.5. Comparison of resazurin reduction and CyQuant NF proliferation assays in human prostate cancer PC-3 cells following treatment with prazosin (left) or doxazosin (right) for 24 (A), 48 (B) or 72 h (C). Results are expressed as percent of untreated control (mean ± SEM, n=3). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. *P<0.05 and ***P<0.001 vs. CyQuant® NF.

CYTOTOXIC POTENCY OF A1-ADR ANTAGONISTS

The concentration which resulted in a 50% reduction in cell survival (IC_{50}) was used to determine relative cytotoxic potency of the investigated α1-ADR antagonists. As shown in **Table 3.3**, prazosin was found to be the most potent, closely followed by doxazosin, except at 24 h where doxazosin was insignificantly more potent than prazosin in PC-3 cells. AR-positive LNCaP cells were more sensitive to doxazosin (48 h, $P < 0.05$) and prazosin (24-72 h, $P<0.001$) toxicity, compared to AR-negative PC-3 cells. At the time points investigated, alfuzosin, silodosin, terazosin and tamsulosin had IC_{50} values greater than the maximal investigated concentration (100 μM), therefore were excluded from statistical analysis. Overall, the relative cytotoxic potency in this study was found to be: doxazosin = prazosin > terazosin = silodosin = alfuzosin > tamsulosin.

Table 3.3. Comparison of doxazosin and prazosin IC_{50} values between cell lines

	Relative IC_{50} (95% CI) (μM)		
	24 h	**48 h**	**72 h**
Prazosin			
PC-3	55.3 *(47.8-64.1)*	26.8 *(25.5-28.2)* ##	21.3 *(19.5-23.3)*
LNCaP	22.0 *(18.8-25.8)* ***	14.3 *(11.6-17.5)* ***	13.0 *(8.6-19.6)* **
Doxazosin			
PC-3	46.8 *(42.5-51.5)*	34.1 *(32.0-36.4)*	23.3 *(21.0-25.7)*
LNCaP	35.4 *(30.6-41.0)*	23.4 *(20.3-27.0)* *	17.2 *(10.9-27.1)*

*Mean values (n≥5) with 95% confidence limits are shown for cells treated with drug for 24, 48 or 72h. Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01 and ***P<0.001 vs. PC-3. ## P<0.01 vs. doxazosin. Non-linear regression of alfuzosin, terazosin, silodosin and terazosin dose-response curves returned ambiguous results at all time points, and therefore were excluded from statistical analysis.*

ALPHA1-ADR ANTAGONISTS-INDUCED APOPTOSIS

Doxazosin is well documented to induce apoptosis of prostate cancer cells (Benning and Kyprianou, 2002, Partin et al., 2003); however, less is known about the apoptotic potential of equipotent prazosin in these cells. Furthermore, there have been no studies directly comparing the apoptotic potential between doxazosin and prazosin, as well as, the lesser investigated alfuzosin, terazosin and silodosin in prostate cancer cells. To investigate this, the activation of the universal marker of apoptosis, caspase-3, was used as an index of apoptotic cell death following continuous 24 h treatment with alfuzosin, terazosin (30 μM), doxazosin, prazosin (10-30 μM), tamsulosin (30 μM), or vehicle. For this study, the 24 h time-point was chosen, as activation of the apoptotic cascade is considered to be an early event in apoptosis, and would likely go undetected at longer treatment times.

As shown in **Figure 3.6**, doxazosin and prazosin treatment resulted in significant increases in caspase-3-mediated apoptosis in PC-3 cells (**Figure 3.6 A**), whereas only prazosin was able to enhance LNCaP caspase-3 activity after 24 h exposure **(Figure 3.6 B**). Overall, LNCaP cells were more sensitive to the apoptotic effect of doxazosin (30 μM, 24 h) compared to PC-3 cells. Specifically, 30 μM prazosin resulted in an approximately 6-fold increase in LNCaP caspase-3 activity compared to untreated control. In contrast, no significant change was observed in caspase-3 activation following treatment with alfuzosin, terazosin or tamsulosin (30 μM, $p > 0.05$) in either cell line (**Figure 3.6 C** and **D**).

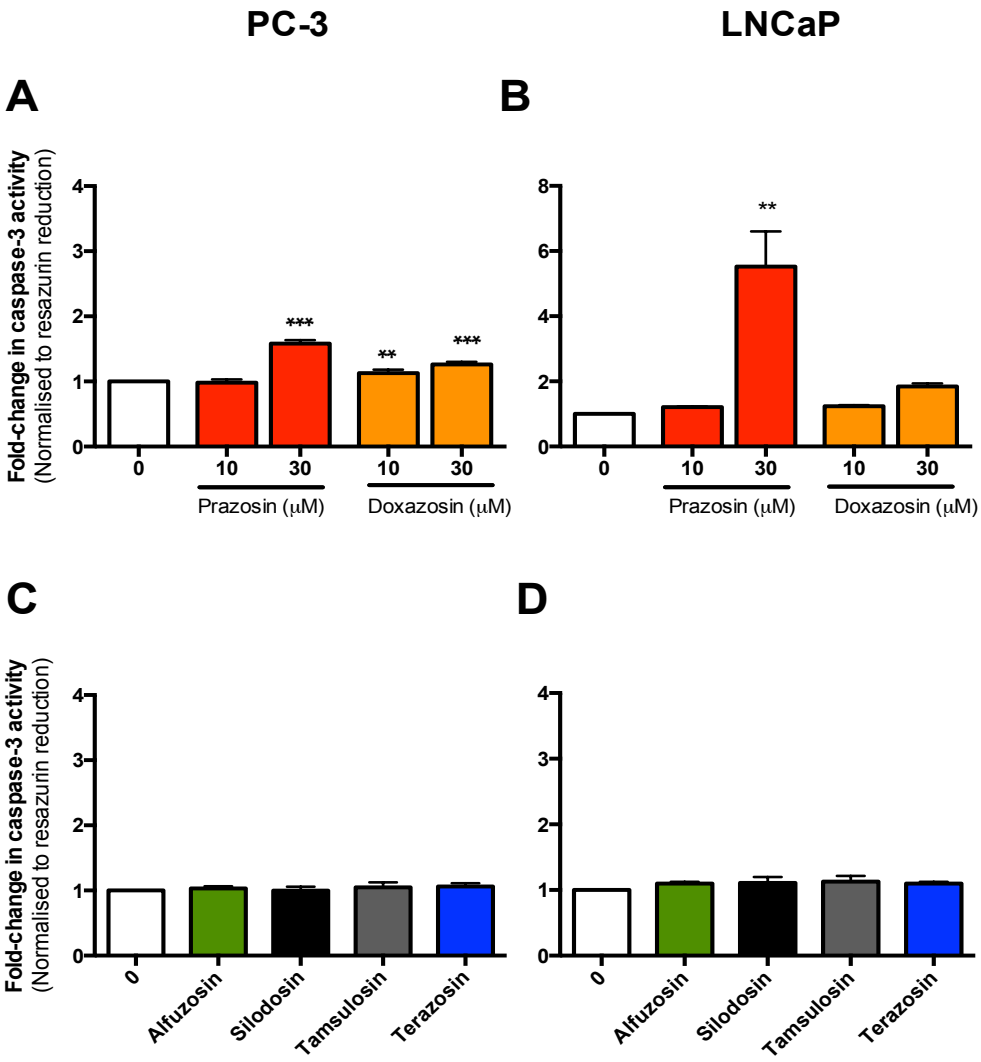


Figure 3.6. Caspase-3 activity following 24 h treatment of LNCaP (A and C) and PC-3 (B and D) with either prazosin, doxazosin at the concentrations shown (A, B), or 30 μM alfuzosin, silodosin, tamsulosin or terazosin (C and D). Results are expressed as fold-change from control (mean ± SEM, n =3). Statistical analysis was determined using a one-way ANOVA with Dunnetts' post hoc test. **P<0.01 and ***P<0.001 vs. control.

ALPHA1-ADR ANTAGONIST-INDUCED AUTOPHAGY

The contribution of autophagy to the underlying cytotoxic mechanisms of α1-ADR antagonist remained to be fully elucidated. To investigate whether α1-ADR antagonists are able to induce autophagy, cells were treated with alfuzosin, doxazosin, prazosin, silodosin, terazosin and tamsulosin; and live cells were stained with a commercially available Cyto-ID® Autophagy Detection kit (Enzo Life Sciences) as described previously (Section 3.2).

Basal autophagic vesicle formation (green) was greater in LNCaP than PC-3 cells, as shown in **Figure 3.7 A**. Qualitatively, prazosin and doxazosin were found to increase formation of autophagic vesicles in PC-3 and LNCaP cells (**Figure 3.7 B**). These findings were confirmed through quantitative measurement of Cyto-ID® (green) fluorescence intensity, which was normalised to fluorescence of the Hoechst 33342 nuclear counter stain (**Figure 3.8**) As shown in **Figure 3.8**, doxazosin-induced autophagy was found to be significantly greater than control in both cell lines ($P<0.01 – 0.001$); however, only the highest doxazosin concentration tested (30 μM) was able to significantly enhance autophagy within PC-3 cells (10 μM, $P=0.068$; 30uM $P=0.001$). In contrast to doxazosin, prazosin exposure only possessed autophagic potential in PC-3 cells, with 30 μM significantly enhancing autophagy by nearly 3-fold ($P=0.002$). However, no appreciable increases in the level of LNCaP autophagy were observed following prazosin treatment.

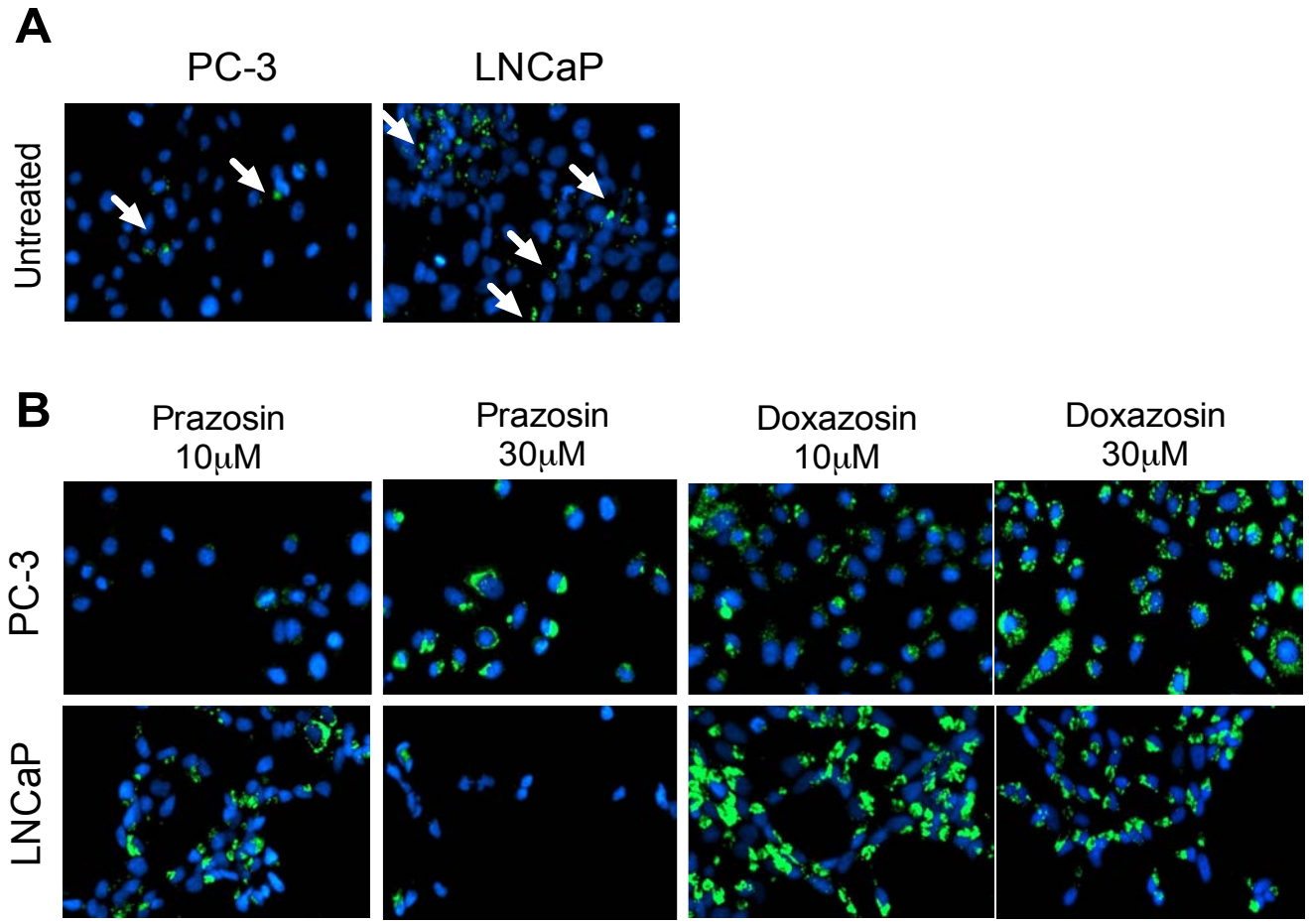


Figure 3.7. Autophagic activity of PC-3 and LNCaP cells in basal conditions (A) or following 24 h treatment with prazosin or doxazosin (10 – 30 μM) (B) was determined using a commercially available kit and visualised using an Evos Cell Imaging system (Thermo-Fischer Scientific) as described previously in this chapter (Materials and Methods section). Autophagic vesicles (green) are indicated by arrows.

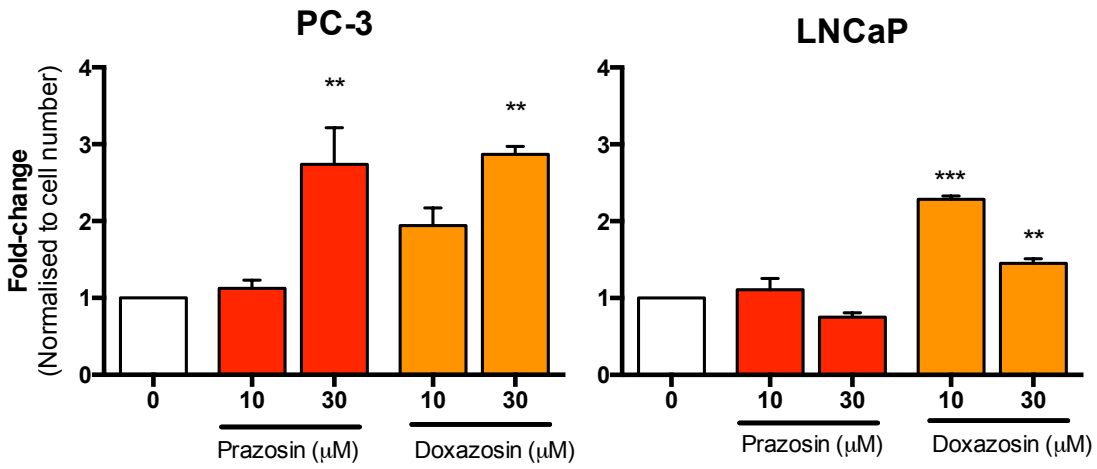


Figure 3.8. Prazosin and doxazosin (10 – 30 μM) induced autophagy was quantified by measuring Cyto-ID® (green) fluorescence (excitation: 480 nm; emission: 530 nm) using a Modulus Multimode plate reader (Promega). Cyto-ID fluorescence was normalised to the nuclear counter stain fluorescence (Hoechst 33342, excitation: 350 nm; emission: 460 nm). Data were represented as fold-change in Cyto-ID® fluorescence from untreated control (mean ± SEM, n=4). Statistical analysis was determined using a one-way ANOVA with Dunnett's post hoc test. ** P < 0.01 and *** P < 0.001 vs. respective untreated control.

As seen in **Figure 3.9 B**, no appreciable change in autophagic vesicles compared to vehicle control were observed following treatment with alfuzosin, tamsulosin or terazosin (30 μM, 24 h) in either LNCaP or PC-3 cells. Interestingly, the non-cytotoxic silodosin (30 μM) was observed to increase autophagic vesicles at 24 h. However, since silodosin was found to not possess cytotoxic actions on LNCaP cells at the time points tested (24 – 72 h), no further investigations of silodosin-induced autophagy were pursued.

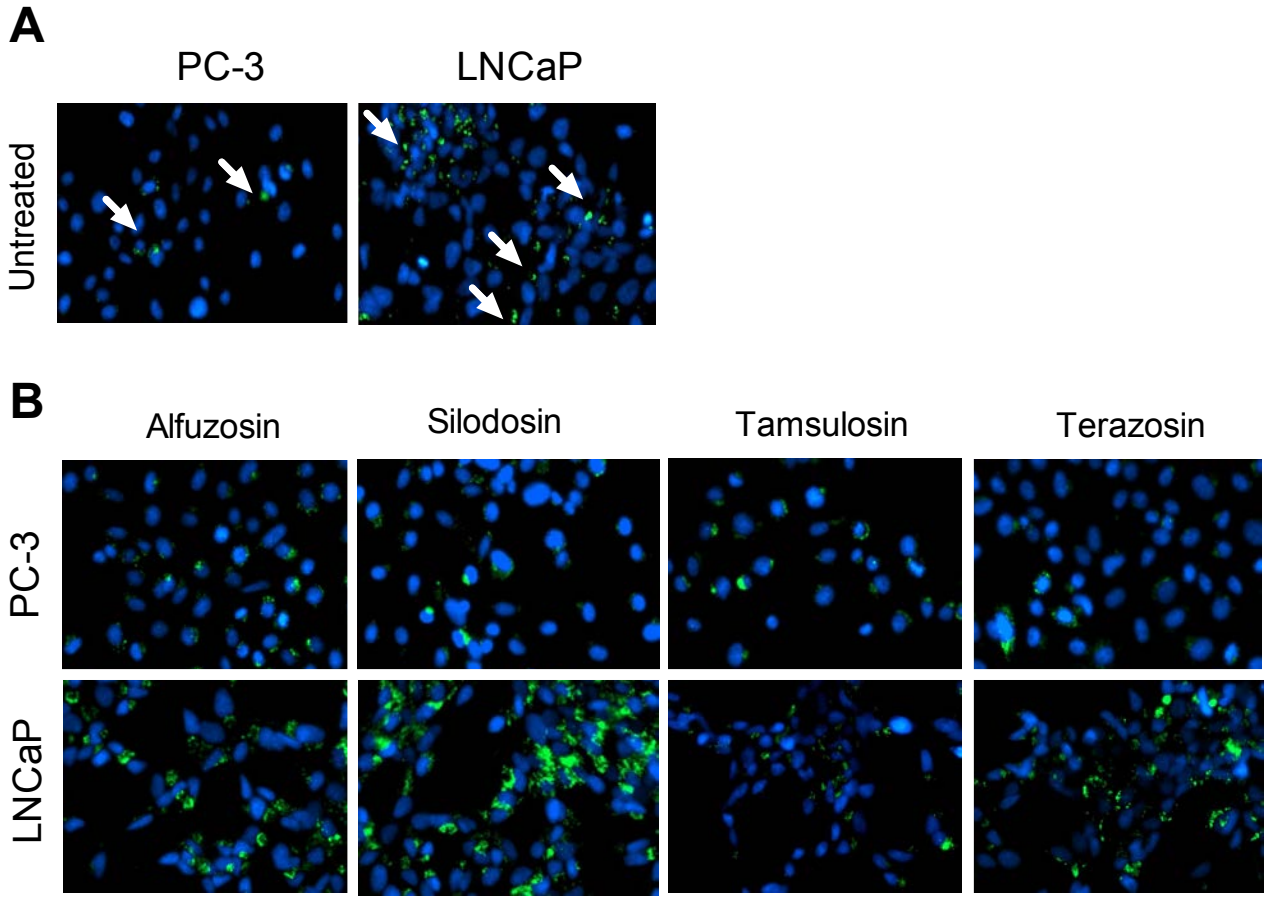


Figure 3.9. Autophagic activity of PC-3 and LNCaP cells in basal conditions (A) or following 24 h treatment with alfuzosin, silodosin, tamsulosin or terazosin (30 μM) (B) was determined using a commercially available kit and visualised using an Evos Cell Imaging system (Thermo-Fischer Scientific) as described previously in this chapter (Materials and Methods section). Autophagic vesicles (green) are indicated by arrows.

THE CONTRIBUTION OF AUTOPHAGY TO A1-ADR ANTAGONIST INDUCED CYTOTOXICITY

To determine whether doxazosin and prazosin-induced autophagy may be contributing to cell survival or to cell death, cells were pretreated for 30 minutes with the autophagy inhibitor 3-MA (5 mM) prior to concurrent treatment with doxazosin or prazosin (10 - 30 μM) for 24 h. Following treatment, cell survival and apoptosis was determined by resazurin reduction and caspase-3 activity assays. As seen in **Figure 3.10**, 5 mM 3-MA was capable of suppressing basal autophagic activity (basal activity shown in **Figure 3.11 A**), as well as the formation of autophagy-related vesicles in response to prazosin and doxazosin.

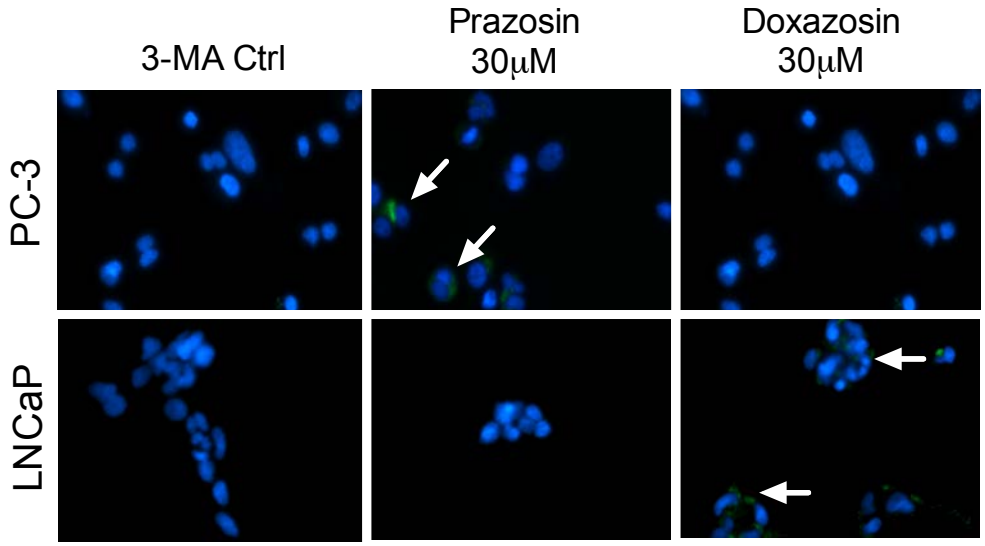


Figure 3.9. Effect of the pharmacological autophagy inhibitor 3-MA (5mM) on prazosin- and doxazosin-induced formation of autophagy-related vesicles following 24 h treatment. Inhibition of prazosin- and doxazosin-induced autophagy in PC-3 and LNCaP cells. Autophagic vesicles (and nuclei) were stained using a commercially available kit, and visualised using an Evos® Cell Imaging System (Thermo-Fischer Scientific) as described previously. Arrows indicated autophagy-related vesicles.

In the absence of α1-ADR antagonists, pharmacological inhibition of autophagy with 3-MA treatment significantly reduced LNCaP viability by approximately 41% (± 1.1) compared to untreated control ($P<0.001$) (**Figure 3.10 B**). In contrast, 3-MA-mediated

autophagy inhibition had no effect on PC-3 cell survival. In LNCaP cells, 24 h co-treatment with either doxazosin or prazosin and 3-MA did not possess an appreciable additive effect in all conditions tested. However, co-treatment with 3-MA and 30 μM prazosin in LNCaP cells had a greater effect than other combinations tested (reduced cell survival by 20% ±1.7), which corresponded to a significant increase in apoptotic caspase-3 activity compared to single-agent-treated controls (P=0.002) (**Figure 3.10 D**). Conversely, inhibition of autophagy by 3-MA was found to partially protect PC-3 cells from prazosin-induced toxicity (P=0.004) and significantly suppressed caspase-3 activity (P<0.001) (Figure **3.10 A** and **C**). Doxazosin treatment in the absence of functional autophagy machinery had no statistically significant effect in either cell line.

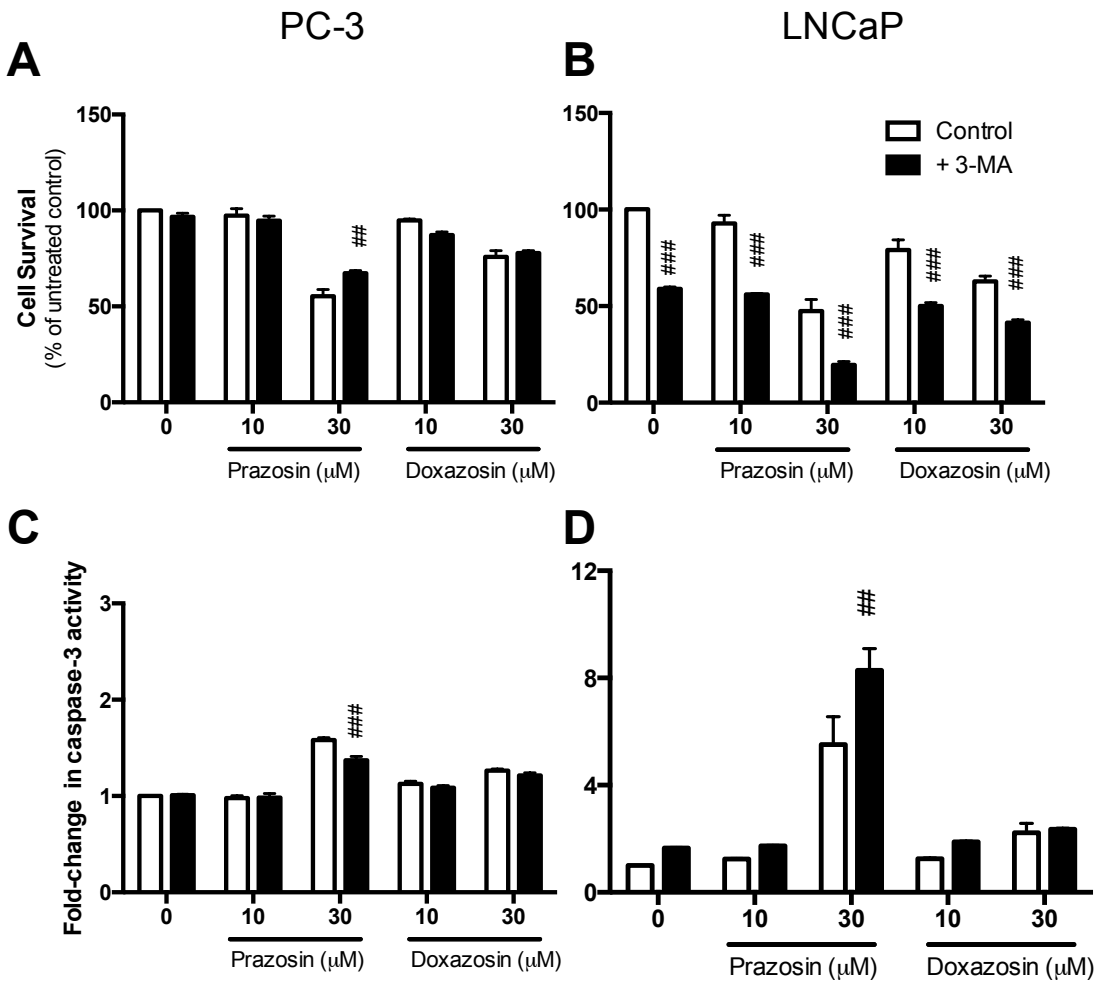


Figure 3.10. PC-3 and LNCaP cells were treated with prazosin or doxazosin (10-30 μM) in the presence or absence of the autophagy inhibitor 3-MA (5 mM) for 24 h. Cell survival (A) was determined by resazurin reduction and normalised as a percentage of untreated control. Caspase-3 activity (B) was determined using a commercially available kit and data were expressed as fold-change from control. All data are expressed as the mean ± SEM (n=3). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. ##P<0.01, ###P<0.001 vs. doxazosin or prazosin treated control.

3.5 DISCUSSION

To date, α1-ADR antagonists remain the primary treatment for men experiencing BPH-associated LUTS, which mitigate the symptoms by blocking α1-ADR-mediated prostatic contraction. Over the years, some of these drugs have been found to exert anticancer effects in pre-clinical models (Kyprianou and Benning, 2000, Benning and Kyprianou, 2002, Partin et al., 2003, Lin et al., 2007), and importantly, reported to reduce the risk of prostate cancer in clinical settings compared to unexposed men (Harris et al., 2007). The current study is the first to compare the cytotoxic potency various chemically dissimilar (quinazoline vs. non-quinazoline) antagonists in different cellular models of prostate cancer cell lines. Additionally, it was previously unknown how the cytotoxic efficacy of these antagonists changed with respect to treatment duration and AR-receptor status. The investigated α1-ADR antagonists ranged from the non-subtype-selective prazosin, doxazosin and terazosin, which possessed the greatest cytotoxicity, to the subtype-selective alfuzosin, tamsulosin and silodosin, which had little cytotoxic effect on prostate cancer cells.

AR-STATUS AND QUINAZOLINE-DEPENDENT CYTOTOXICITY

Prazosin and doxazosin were generally equipotent in all conditions tested, and these drugs possessed the greatest potency of all drugs investigated. These findings are supported by previous literature (Lin et al., 2007), which also investigated the relative cytotoxic potencies of several α1-ADR antagonists including tamsulosin, terazosin, doxazosin, prazosin and phentolamine. Consistent with the present findings, prazosin was previously found to be the most potent, closely followed by doxazosin in prostate cancer PC-3, LNCaP and DU145 cells after 48 h treatment (Lin et al., 2007). The overall relative cytotoxic potencies in the present study were found to be: prazosin = doxazosin > terazosin = alfuzosin = silodosin > tamsulosin. Overall, there was little difference in doxazosin or prazosin cytotoxic potency between prostate cancer cell lines with differing AR-status. Only prazosin was found to have significantly greater potency in AR-positive LNCaP cells compared to AR-negative PC-3 cells. In contrast, doxazosin potency was similar between both prostate cancer cell lines.

The cytotoxic potential of the investigated drugs appeared to correspond with previous reports of quinazoline-dependence (Benning and Kyprianou, 2002), with the quinazoline derivatives prazosin, doxazosin, possessing greater potency than the non-quinazolines, tamsulosin and silodosin in both cell lines. However, it appears that the quinazoline moiety does not ensure cytotoxic effects at the concentrations tested. The cytotoxic effects of 100 μM alfuzosin and terazosin were found to not be significantly different than silodosin and tamsulosin in exposures of 24 and 48 h. While the quinazoline structure might be important for doxazosin / prazosin cytotoxicity, it must be acknowledged that quinazoline-dependent cytotoxicity of prostate cancer cell lines is not absolute in the conditions tested here.

AR-STATUS AND QUINAZOLINE-DEPENDENT APOPTOSIS

The anti-cancer effect of these cytotoxic antagonists has largely been attributed to the induction of apoptotic programmed cell death (Partin et al., 2003, Walden et al., 2004, Hui et al., 2008, Garrison and Kyprianou, 2006, Romanska et al., 2010). However, no studies have directly compared the apoptotic potential of these drugs in different cellular models of prostate cancer. Consistent with the previous reports of cytotoxic and apoptotic quinazoline-dependence (Partin et al., 2003), the present findings demonstrated a significant increase in apoptotic caspase-3 activity in both cell lines following exposure to doxazosin or prazosin treatment, which occurred in a concentration-dependent manner. However, no increase in apoptotic activity could be measured following treatment with the lesser-toxic quinazolines alfuzosin and terazosin, or the non-quinazolines silodosin or tamsulosin. Similar to the cytotoxic potency data, AR-positive LNCaP cells were more sensitive to prazosin exposure and apoptotic activity was found to be greater in these cells compared to AR-negative PC-3 cells. In contrast to the present findings, terazosin has been reported to elicit apoptosis in urogenital cancer cells *in vitro*, in animal models, as well in tissue specimens from clinically treated men (Kyprianou and Benning, 2000, Pan et al., 2003, Tahmatzopoulos et al., 2005, Papadopoulos et al., 2013). Discrepancies between the previous findings *in vitro* and the present study are likely attributed to differences in treatment duration or concentrations evaluated for apoptotic effect. However, there is a large gap in the apoptotic efficacies of these antagonists between *in vitro* models and *in vivo* or in

clinical models. *In vitro*, supra-therapeutic concentrations (<10 μM) are required to induce prostate cell apoptosis. However therapeutically relevant doses (nM serum concentrations) reportedly increased apoptosis in urogenital tumours of men treated with these drugs (Tahmatzopoulos et al., 2005). While the discrepancy between apoptotic (and cytotoxic) efficacies are currently unknown, it can be inferred that chronic treatment with low concentrations of these antagonists may have a delayed apoptotic effect, possibly via antagonist accumulation in the prostate (Korstanje et al., 2011) or chronic antagonism of endogenous α1-ADR stimulation.

QUINAZOLINE-DEPENDENT AUTOPHAGIC CYTOTOXICITY

In the context of cancer, autophagy is classically associated with maintaining cellular homeostasis, thereby acting as a cytoprotective mechanism leading to treatment-resistance, and subsequent disease progression. By contrast, sustained autophagy can have cytotoxic effects in some cancers in a cell-type dependent manner (Pickard et al., 2015). In the present study, autophagy was found to play opposing roles, contributing to LNCaP survival and PC-3 toxicity in response to prazosin exposure. These findings are consistent with the cytotoxic data presented in this Chapter, which showed that the quinazoline prazosin was the most cytotoxic of the investigated antagonists. Quinazoline-based doxazosin, and to a lesser-extent terazosin, were also found to trigger autophagic vesicle formation in both cell lines. However, the quinazoline-dependence of autophagy induction in prostate cancer cells is not as clear cut as overall cytotoxicity and apoptosis. Similar to the cytotoxicity and apoptosis data, the non-toxic quinazoline alfuzosin (24 h , 30 μM) was unable to enhance autophagic activity. Furthermore, by it was found the non-quinazoline silodosin was able to significantly enhance autophagy levels in LNCaP, but not PC-3 cells. Taken together, it is proposed that the mechanisms underlying autophagic cell death in the present study are complex and may not be quinazoline-dependent. Rather autophagic cell death may be a function of cytotoxic potency and AR-status in prostate cancer cells.

AUTOPHAGY AND AR-STATUS

This is the first report of autophagy involvement in α1-ADR antagonist-induced cytotoxicity, particularly in prostate cancer cells. The paradoxical effect of autophagy induction in prostate cancer cells was also demonstrated previously (Pickard et al., 2015) in response to chemotherapeutic agents. The opposing roles are likely to be related to AR-status, with AR-signaling in LNCaP cells requiring autophagy for cell survival mechanisms. It was previous demonstrated that prostate cancer AR-signaling induced accumulation of intracellular reactive oxygen species, which in turn stimulated autophagy and subsequently promoted survival of LNCaP cells (Shi et al., 2013). Therefore, in LNCaP cells, antagonist-induced autophagy acts as an adaptive response to protect from cell death, and in turn, inhibition of autophagy enhances prazosin and doxazosin cytotoxicity and increased apoptosis. In contrast, the absence of functional autophagy machinery partially protected PC-3 cells from prazosin-induced cell death via suppression of apoptotic mechanisms. While these findings suggest that autophagy contributes to some degree to prazosin-induced PC-3 toxicity, it appears cross-talk between autophagic and apoptotic mechanisms exists to regulate α1-ADR antagonist-mediated cell death.

In conclusion, prazosin and doxazosin were found to be the most potent of the investigated antagonists and possessed the greatest apoptotic potential in both AR-positive and AR-negative LNCaP and PC-3 cells, respectively. This is the first report of autophagy involvement in prazosin-induced cytotoxicity. However, autophagy contributed differentially in a cell type-dependent manner to promote LNCaP survival and PC-3 cell death. Inhibition of autophagy partially protected and suppressed apoptotic mechanisms in PC-3 cells exposed to prazosin, giving rise to the possibility of cross-talk between these cell death pathways in regulating antagonist-induced cell death. Future investigation of longer treatment durations (>72 h) may be required to replicate the potential latent cytotoxic actions of alfuzosin, terazosin, silodosin and tamsulosin which have been reported *in vitro*, *in vivo* or clinically. Additionally, the cell signaling pathways regulating doxazosin and prazosin-induced apoptosis or autophagy remain to be fully elucidated. Some of these molecular mechanisms are further explored in the next chapter (Chapter 4). Further studies evaluating the novel autophagic activity of silodosin on LNCaP cells reported here would be of significant interest. While

insignificant and unconfirmed, silodosin appeared to have a pro-survival effect on LNCaP cells which is consistent with the survival-promoting effect of autophagy in AR-positive cells. This effect might have clinical implications for men undergoing treatment for prostate cancer.

CHAPTER 4:
MOLECULAR MECHANISMS UNDERLYING DOXAZOSIN AND PRAZOSIN CYTOTOXICITY

4.1 BACKGROUND

MOLECULAR MECHANISMS OF ALPHA1-ADR ANTAGONIST-INDUCED CELL DEATH

The molecular mechanisms underlying quinazoline-based α1-ADR antagonists-induced cell death are undoubtedly complex and remain to be fully elucidated. However, a growing body of evidence suggests that quinazoline-based prazosin, doxazosin and terazosin target prostate cancer by triggering apoptosis, anoikis, cell cycle arrest, and potentially, autophagic cell death. Together, these mechanisms act to impair tumour growth, angiogenesis and metastasis, all of which are important events associated with disease progression. **Table 4.1** provides a summary of the reported biochemical and molecular changes associated with quinazoline-based antagonist-induced cytotoxicity.

Table 4.1. Overview of molecular mechanisms contributing to α1-ADR antagonist-induced cytotoxicity

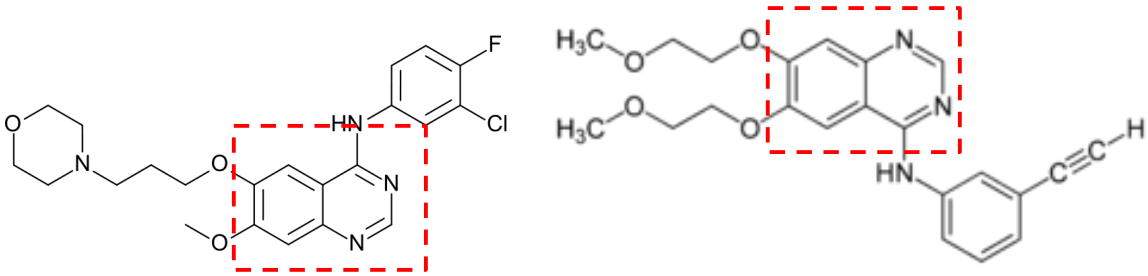
Effect	Experimental model	References
Apoptosis-associated		
Activation of TGF-β signaling	PC-3	(Partin et al., 2003)
	Prostate stromal cells	(Ilio et al., 2001)
Activation of IkBα signaling	PC-3	(Partin et al., 2003)
Decrease NFκB mRNA	MCF-7	(Hui et al., 2008)
Upregulation p21WAF-1	PC-3	(Partin et al., 2003)
Caspase-8 activation	PC-3, BPH cells	(Garrison and Kyprianou, 2006)
	786-0, Caki	(Sakamoto et al., 2011)
Increase Fas ligand expression	PC3	(Partin et al., 2003)
Fas-associated death domain	PC3, BPH cells	(Garrison and Kyprianou, 2006)
Caspase-3 activation	PC-3	(Partin et al., 2003, Walden et al., 2004)
	HUVEC	(Keledjian et al., 2005)
	HeLa	(Gan et al., 2008)
	786-0, Caki	(Sakamoto et al., 2011)
	H9C2	(Yang et al., 2011b)
	TRAMP Mice	(Chiang et al., 2005)
Increase Caspase-3 expression	Winstar rats	(Papadopoulos et al., 2013)
Cytochrome c release	HUVEC	(Liao et al., 2011)
PARP Cleavage	TRAMP Mice	(Chiang et al., 2005)
	PC3	(Lin et al., 2007)
Increase Bax expression	PC3, BPH cells	(Garrison and Kyprianou, 2006)
	TRAMP Mice	(Chiang et al., 2005)
Increase Bad expression	PC-3	(Lin et al., 2007)
	HUVEC	(Liao et al., 2011)
Increase Bid expression	PC-3	(Lin et al., 2007)
Increase Bcl-xL expression	HUVEC	(Liao et al., 2011)
Decrease Bcl-2 levels	TRAMP Mice	(Chiang et al., 2005)
Decrease MCL-1 expression	HUVEC	(Liao et al., 2011)
EGFR inhibition	MCF-7	(Hui et al., 2008)
ERK1/2 inhibition	MCF-7	(Hui et al., 2008)
Activation of p38 MAPK	H9C2	(Yang et al., 2009)
Anoikis associated		
Decrease Akt activity	PC-3	(Garrison and Kyprianou, 2006)
	DU145	(Romanska et al., 2010)
	TRAMP Mice	(Chiang et al., 2005)
Decrease FAK expression	PC-3	(Walden et al., 2004)

Effect	Experimental model	References
	HUVEC	(Keledjian et al., 2005)
EphA2 receptor activation	PC-3	(Petty et al., 2012)
Decrease integrin expression	PC-3, BPH cells	(Garrison and Kyprianou, 2006)
Angiogenesis associated		
Decrease VEGF mRNA	HUVEC	(Keledjian et al., 2005)
Inhibition of glutathione transferase	Bovine liver cytosol	(Isgor and Isgor, 2012)
Inhibition of Src kinase	Bovine liver cytosol	(Isgor and Isgor, 2012)
Inhibition of PTK activity	Bovine liver cytosol	(Isgor and Isgor, 2012)
Increase AP-2α expression	HeLa	(Gan et al., 2008)
Autophagy associated		
Vacuole formation	H9C2 PC-3	(Yang et al., 2011b) (Pavithran and Thompson, 2012)
LC3 conversion	H9C2	(Yang et al., 2011b)
Decrease mTOR activation	H9C2 SKOV-3,	(Yang et al., 2011b) (Yang et al., 2009)
Decrease Akt activity	H9C2 SKOV-3	(Yang et al., 2011b) (Yang et al., 2009)
Lipofuscin accumulation	PC 3	(Pavithran and Thompson, 2012)
Cell cycle associated		
Inhibition of CDK1	PC-3, LnCAP, DU145	(Lin et al., 2007)
Inhibition of Rb	MCF-7 Coronary artery SMC Bladder SMC	(Hui et al., 2008) (Kintscher et al., 2000a) (Austin et al., 2004)
Decrease Cyclin A expression	Bladder SMC	(Austin et al., 2004)

Apoptosis

The reader is referred to Chapter 3 for detailed overview of the apoptotic signaling transduction pathway. Quinazoline-based antagonist-induced prostate cancer apoptosis has been reported to primarily involve transforming growth factor beta (TGF-β) and Smad signaling, activation of IκBα, and subsequent inhibition of the survival transcription factor NfκB (Partin et al., 2003). However, subsequent reports indicate apoptotic cell death may occur by additional intrinsic (Lin et al., 2007) and extrinsic mechanisms (Partin et al., 2003, Garrison and Kyprianou, 2006) including, mitochondrial-dependent activation of the caspase cascade (Lin et al., 2007) and Fas-associated death domain (FADD) activation (Garrison and Kyprianou, 2006).

Doxazosin, and related quinazoline antagonists, may also induce prostate cancer apoptosis though inhibition of tyrosine kinase receptors such as EGFR. EGFR is a cell surface receptor that is stimulated by a diverse number of ligands to promote cellular proliferation and survival by downstream signaling via several pathways including MAPK (Yarden, 2001). The mitogen-activated protein kinase (MAPK) family consists of JNK, p38, and ERK1/2. These MAPKs transduce extracellular and intracellular stimuli to regulate cellular responses such as differentiation, proliferation, or cell death (Krishna and Narang, 2008). In particular, ERK1/2 is frequently associated with EGFR activation and is known to contribute to androgen-dependent and –independent prostate cancer survival mechanisms (Zelivianski et al., 2003, Carey et al., 2007). Interestingly, the quinazoline structure is common to cytotoxic antagonists (doxazosin, prazosin and terazosin) and the EGFR inhibitors gefitinib (Iressa) and erlotinib (Tarceva) (**Figure 4.1**). Similar to these EGFR inhibitors, it is postulated that quinazoline-based α1-ADR antagonists may trigger apoptosis by inhibiting aberrant EGFR activity and, subsequently, downstream ERK1/2-mediated survival-signaling (Hui et al., 2008).



Gefitinib (Iressa) **Erlotinib (Tarceva)**

Figure 4.1. Chemical structures of the EGFR inhibitors, gefitinib and erlotinib. These drugs also contain a quinazoline structure (red box) common to doxazosin, prazosin, alfuzosin and terazosin.

ANOIKIS

Anoikis is a type of apoptotic cell death in response to detachment from the extracellular matrix (ECM) (Frisch and Francis, 1994). Upon loss of ECM contact, normal endothelial or epithelial cells undergo rapid intrinsic and extrinsic apoptotic signaling resulting in cell death. Cancer cells can become anoikis-resistant and are able to freely detach from the primary tumour and migrate, leading to cancer metastases. Metastasis is the spread or colonization of cancers cells originating from the primary tumour to local or distant sites (**Figure 4.2**) (Sakamoto and Kyprianou, 2010). The development of metastasis is an important, yet devastating event in disease progression and is strongly associated with prostate cancer related-death (Bubendorf et al., 2000).

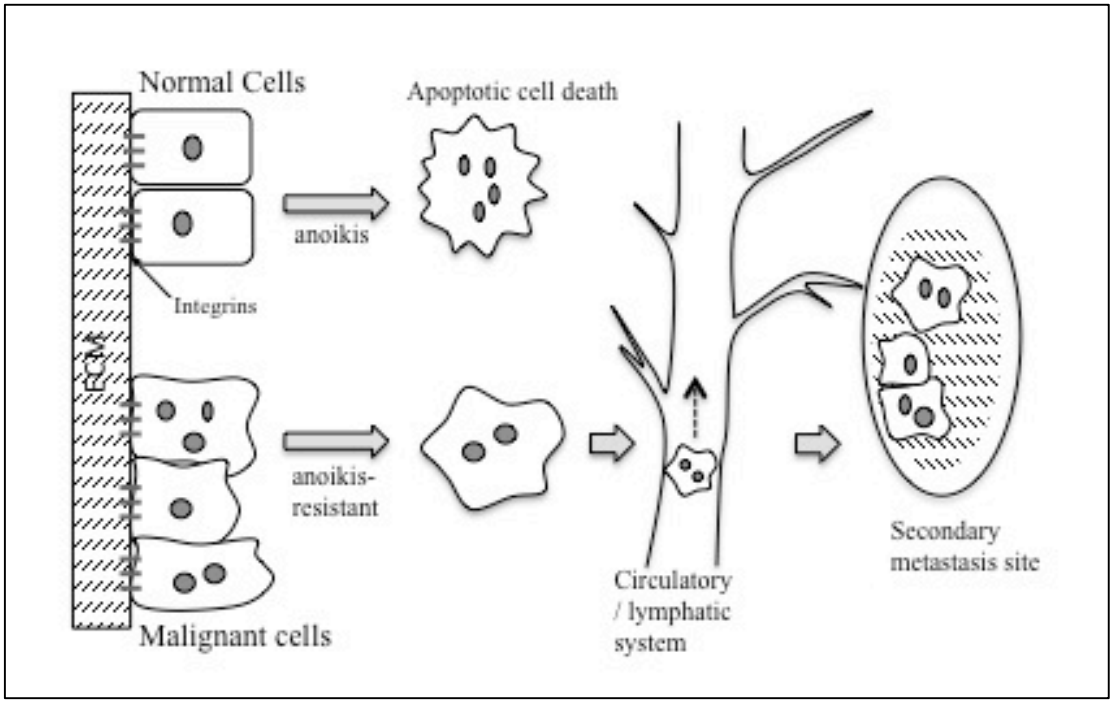


Figure 4.2. Anoikis-resistance leads to cancer metastasis. Following loss of cell-ECM interaction, normal non-cancerous cells undergo apoptotic cell death. However, cancerous cells can develop anoikis-resistance or an anchorage-independent phenotype allowing these cells to migrate and develop secondary tumours. Figure adapted from Sakamoto and Kyprianou, 2010.

Anoikis occurs via various mechanisms including loss of integrin-mediated survival signaling and activation of intrinsic and extrinsic apoptotic pathways (**Figure 4.3**).

Integrins are transmembrane receptors that play a key role in detecting cell-ECM interactions. Detachment of the cell, and thus integrins, from the ECM results in dephosphorylation (inactivation) of important survival factors such as focal adhesion kinase (FAK), P13K, Akt, MAPK, and ERK (Sakamoto and Kyprianou, 2010). Of particular interest, quinazoline-based antagonists have been shown to induce anoikis-mediated cell death in prostate cancer (Keledjian and Kyprianou, 2003) and vascular endothelial cells (Keledjian et al., 2005) thereby reducing cancer metastasis. As summarised in **Table 4.1**, quinazoline-based α1-ADR antagonist-mediated anoikis has been evidenced previously by anoikis-associated morphological changes (Walden et al., 2004), inactivation (dephosphorylation) of FAK / Akt survival signaling (Keledjian and Kyprianou, 2003, Walden et al., 2004, Garrison and Kyprianou, 2006, Romanska et al., 2010, Sakamoto et al., 2011), and downregulation of integrin expression (Garrison and Kyprianou, 2006). Additionally, inhibition of survival signaling was also paired with increased in extrinsic (Garrison and Kyprianou, 2006) and intrinsic apoptotic pathways (Keledjian and Kyprianou, 2003).

More recently, Petty and colleges (2012) identified doxazosin as a novel agonist for EphA2 receptors. In this study, direct doxazosin-EphA2 receptor interactions resulted in anoikis-like events, including cell rounding, detachment and apoptosis. In some cancers, Eph receptors acquire ligand-independent activity, possibly mediated through aberrant AKT activity (Miao et al., 2009), and in turn, promote metastatic-like behaviors, including resistance to anoikis, and increased cellular proliferation (Chen et al., 2014). This tumourigenic propensity to proliferate/metastasize can be reversed through ligand-dependent stimulation leading to suppression of downstream pro-survival signaling, including FAK and integrins, to restore tumour suppressor actions and anoikis mechanisms (Miao et al., 2000, Pasquale, 2005, Petty et al., 2012). Interestingly, these ligand-dependent tumour-suppressor actions are consistent with previously documented anoikis-related effects of these drugs, suggesting that some quinazoline-based α1-ADR antagonists may activate EphA2 and restore tumour suppressor functions in prostate cancer cells. Nevertheless, it is currently unknown if the chemically similar quinazoline, prazosin has EphA2 agonist potential.

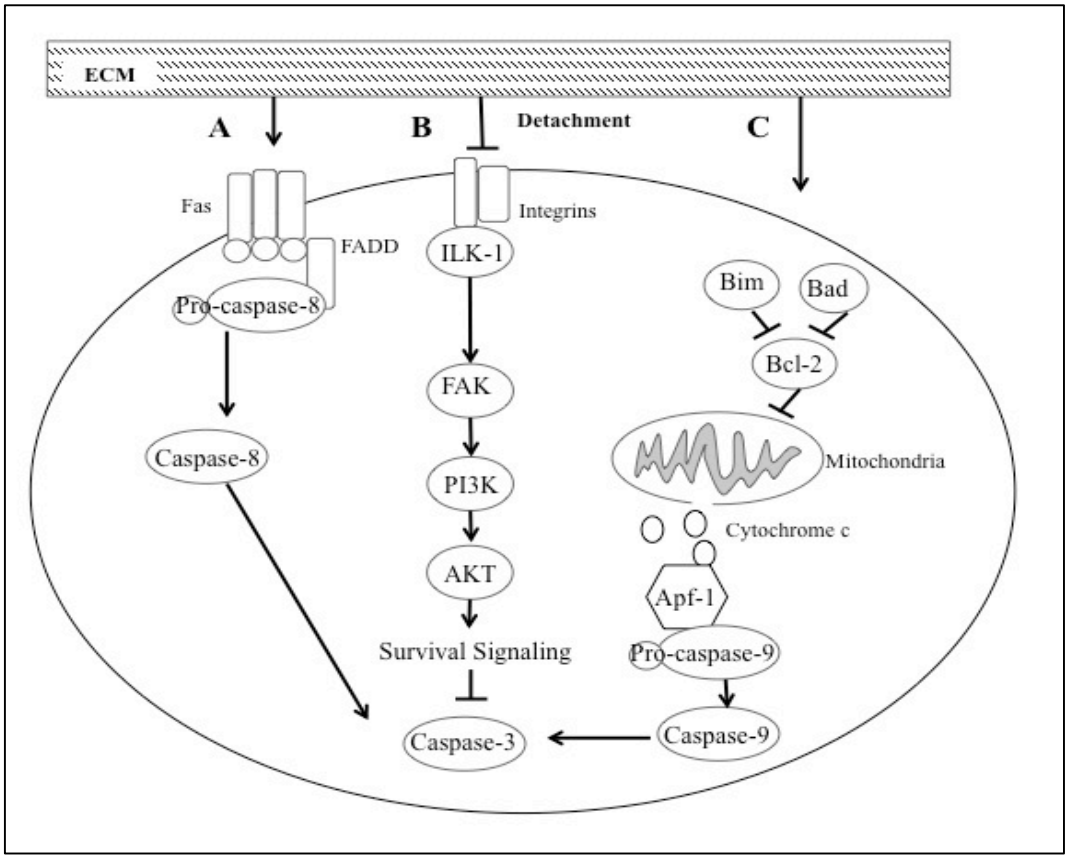


Figure 4.3. Cell signaling and apoptotic pathways following non-cancerous cell detachment from the extracellular membrane. Following detachment, the extrinsic apoptotic pathway is activated initiated by the Fas receptor (A), leading to effector caspase activation. Integrin-mediated contact with the ECM promotes inactivation of survival signaling pathways. Loss of cell-ECM interaction results in dephosphorylation (inactivation) of PI3K / Akt thereby sensitising the cell to apoptosis (B). Additionally, intrinsic apoptosis mechanisms have also been shown to be involved in anoikis cell death (C) Figure adapted from Sakamoto and Kyprianou, 2010.

AUTOPHAGY

The reader is referred to Chapter 3 for background information on autophagy. Little is currently known with regards to the molecular mechanisms underlying doxazosin, prazosin induced autophagy, particularly in prostate cancer cells. In one study using rodent cardiomyocytes, prazosin was found to decrease expression of mTORC1 and its substrate, p70S6K, which corresponded with an expressional decrease and increase in the upstream mTORC1 modulators AKT and AMPK, respectively (Yang 2011). Paired with these findings, this study demonstrated the formation of autophagy-related vesicles

in response to prazosin treatment. However, pharmacological autophagy inhibition could not confer significant cytoprotection. On the contrary, inhibition of autophagic mechanisms resulted in enhanced caspase activity. From this, the authors controversially concluded that prazosin induced autophagic cell death despite failing to demonstrate a causal role between autophagy and cytotoxicity. In this study, the involvement of autophagy in prazosin toxicity remains unclear. The absence of cytoprotection following autophagy inhibition suggests that autophagy either plays a protective role from prazosin-toxicity or cross-talks with apoptotic mechanisms to increase apoptosis to compensate for the absence of autophagic-mediated cell death.

Similar findings were reported in a separate study investigating the effects of doxazosin on human endothelial cells, indicating the doxazosin also modulates mTORC1 activity, possibly by reducing AKT activity (Park et al., 2014a). However, this study did not show a direct causal link between mTORC1 inhibition and the induction of apoptosis. Taken together, it can be inferred that these drugs have autophagy-modulating effects through their indirect actions on mTORC1. At the present time, it remains to be elucidated whether prazosin/doxazosin also have autophagy modulating effects in prostate cancer cell lines. Furthermore, it remains to be seen if autophagy actively contributes to the cytotoxic potential of these drugs.

CELL CYCLE ARREST

In eukaryotic cells the cell cycle consists of interphase and mitosis (Figure 13). Interphase is comprised of growth (G_1, G_2) and DNA replication (S) phases in preparation for mitosis (M phase). M phase is divided into four distinct phases: prophase, metaphase, anaphase, and telophase. Prophase and metaphase are characterised by condensation of chromosomes and alignment of duplicate chromosomes on in the center of the cell, respectively. The mitotic spindle-mediated separation of chromosome pairs to opposite poles of the cell occurs during anaphase. Telophase is the final stage of mitosis where the nuclear envelope reassembles and chromosomes de-condense (Santella et al., 2005). The progression of the cell cycle is regulated by cyclin-dependent kinases (CDKs). Complete activation of CDKs requires both cyclin-binding and phosphorylation by cyclin-activating kinase. Inhibition of CDK can occur via dephosphorylation of activation sites, phosphorylation of inhibitory

subunits, binding of cyclin-dependent kinase inhibitors, or disassociation of cyclins (Morgan, 1995).

Few have investigated the effects of α1-ADRs antagonists on cell cycle progression; however, it appears to be drug- and cell type-dependent. One study by Lin et al. demonstrated that prazosin triggered G_2 check point arrest in prostate cancer lines, which was possibly mediated by inactivation of the cell cycle promoter CDK1 (Lin et al., 2007). In contrast, prazosin-mediated cell cycle arrest could not be replicated in human endothelial vascular cells (Liao et al., 2011). In breast cancer cells, doxazosin caused an increase in G_0/G_1 phase cells paired with a dose-dependent decrease in the number of cells in S phase (Hui et al., 2008). Similar findings were also evidenced in human coronary (Kintscher et al., 2000a) and bladder (Austin et al., 2004) smooth muscle cells, implicating inactivation of the tumour suppressor protein retinoblastoma (Rb) (Kintscher et al., 2000a, Austin et al., 2004, Hui et al., 2008) and decreased Cyclin A expression (Austin et al., 2004). Further investigation is required to fully elucidate the dose and treatment duration effects of quinazoline-based α1-ADR antagonists on prostate cancer cell cycle.

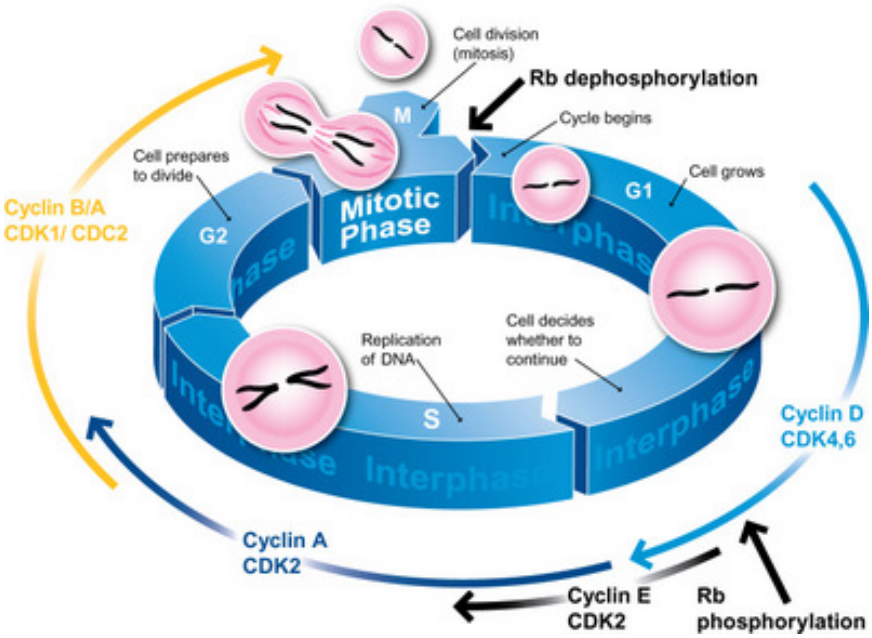


Figure 4.4. Regulation of cell cycle progression. The cell cycle consists of interphase (G1, S, and G2 phases) and mitosis (M phase). Each step in the cell cycle is primarily regulated by CDK-cyclin interactions (Santella et al., 2005). Image provide courtesy of Abcam. Image copyright © 2016 Abcam.

ANGIOGENESIS

Angiogenesis, or development of blood vessels, is a hallmark of malignant tumours. Like normal, non-malignant tissues in the human body, tumours require sufficient blood supply to grow beyond a critical size (Carmeliet and Jain, 2000). Tumours are able to recruit blood vessels by release of pro-angiogenesis regulators such as vascular endothelial growth factor (VEGF) (Carmeliet and Jain, 2000). Briefly, free VEGF binds to its receptor (VEGFR) on the membrane of vascular endothelial cells which triggers a complex signaling cascade promoting vascular endothelial proliferation, survival, and development of blood vessels (Karamysheva, 2008). Angiogenesis is a common therapeutic target for many human cancers, including advanced stage prostate cancer (Shojaei, 2012). Suppression of angiogenesis is commonly achieved through VEGF-neutralizing or VEGFR inhibiting agents such as bevacizumab (Shojaei, 2012). Despite promising *in vitro* and *in vivo* pre-clinical trials (Melnyk et al., 1999), single agent anti-angiogenic therapies have demonstrated unsatisfactory oncological and survival effects

in men with prostate cancer (Figg et al., 2001b, Reese et al., 2001). These findings prompted investigation of anti-angiogenetic agents in combination with docetaxel for men with advanced prostate cancer (Figg et al., 2001a, Ning et al., 2010), including those who had failed docetaxel therapy previously (Di Lorenzo et al., 2008). Phase II clinical trials of docetaxel plus anti-angiogenics improved progression free and overall survival (Figg et al., 2001a, Di Lorenzo et al., 2008, Ning et al., 2010). Unfortunately, a phase III clinical trial demonstrated no survival benefit with the addition of bevacizumab to docetaxel and was also associated with greater incidence of adverse events (Kelly et al., 2012). While these results may be discouraging, the use of anti-angiogenic agents in combination with docetaxel remains a viable option for late stage prostate cancer and should be investigated further.

Interestingly, the quinazoline-based drugs doxazosin and terazosin are known to impair tumour angiogenesis. Early findings demonstrated reduced tumour vascularity in prostate specimens of men exposed to terazosin for BPH or hypertension compared to men who received no treatment (Keledjian et al., 2001). Similar anti-angiogenic activities were also found in men with bladder cancer (Tahmatzopoulos et al., 2005). *In vitro* and *in vivo* studies suggest that quinazoline-based antagonists suppress tumour angiogenesis by downregulating VEGF transcription and expression levels (Pan et al., 2003). Similarly, doxazosin was found to inhibit VEGF-mediated human vascular endothelial cell invasion, migration and endothelial tube formation (Keledjian et al., 2005). Together these findings suggest a two-fold effect, targeting tumour angiogenic signals and vascular endothelial response. Therefore, these drugs may prove effective as anti-cancer therapies.

SUMMARY

The underlying cell signaling mechanisms contributing to the anti-cancer effects of α1-ADR antagonists are undoubtedly complex. Fully understanding the mechanism of action of the cytotoxic antagonist is important for exploitation of their anti-cancer potential to either prevent prostate cancer or delay disease progression. Furthermore, the exploration of the molecular mechanisms may also provide insight to the observed enhanced anti-cancer potency of these antagonists *in vivo* or clinically at therapeutic concentrations, compared to the low cytotoxic potency observed *in vitro*.

4.2 AIMS

The overall aim of this study was to further elucidate the molecular mechanisms underlying the cytotoxic actions of doxazosin and prazosin in prostate cancer cells.

The specific aims include:

1. To determine the change in expression or activation of tumour suppressor- or promotor-related proteins in response to doxazosin and prazosin exposure.
2. To determine changes in activation status of various receptor tyrosine kinases and related signalling nodes following treatment with doxazosin and prazosin.

4.3 MATERIALS AND METHODS

DRUGS AND REAGENTS

Drugs and reagents used throughout this chapter are listed below in Table 4.2. Stock solutions were made up either in sterile dH_20 or DMSO and stored according to the manufacturer's instructions.

Table 4.2. Drugs and chemicals including details of stock solutions, storage conditions

Drug / Chemical	Stock solution	Storage	Supplier	Cat. No.
Bovine serum albumin	N/A	N/A	Sigma-Adrich	A7030
Bradford's Reagent	N/A	N/A	Sigma-Adrich	B6916
Lithocholic Acid	100mM, DMSO	30 days, 20ºC	Sigma-Adrich	L6250
Protease Inhibitor cocktail	10X, solution in sterile dH20	14 days, 4ºC	Sigma-Adrich	P2714
PMSF	100 mM, DMSO	Made fresh daily	Sigma-Adrich	P7626

ASSAYS AND KITS

The following assays and kits were used thoughout this chapter (Table 4.3). All kits were stored as per the manufacture's instructions and were used well within the expiry date.

Table 4.3. Details of assays and kits used

Assay / Kit	Source	Cat. No.
Human Cell Stress Array	R&D Systems	ARY018
PathScan® RTK Signaling Antibody Array Kit (Chemiluminescent Readout)	Cell Signaling Technologies	7982
Human Phospho-EphA2 DuoSet IC ELISA	R&D Systems	DYC4056

TREATMENT OF CELLS

Human LNCaP and PC-3 prostate cancer cells were seeded at sub-confluent densities (Table 4.4) and allowed to attach for 24 or 48 h, respectively. Cells were treated with tamsulosin, prazosin, doxazosin (30 – 100 μM) or vehicle for 1 – 2 h, or overnight for 24 h. As previously demonstrated in Chapter 3, doxazosin and prazosin concentration of 30 μM was found to induce apoptosis and/ or autophagy in a cell-type dependent manner.

Table 4.4. Experimental treatment times and typical seeding conditions for PC-3 and LNCaP cell lines.

Experiment	Treatment time	Tissue Culture Vessel	Seeding Density (cells/cm^2)	
			PC-3	LNCaP
Human Cell Stress Array	24 h	T-75 Flask	$1.9x10^4$	$3.0x10^4$
Human RTK Array	24 h	T-75 Flask	$1.9x10^4$	$3.0x10^4$
Phospho-EphA2 ELISA	2 h	T-75 Flask	$3.7x10^4$	N/A
Lithocholic Acid Treatment	1, 2 and 24 h	96-well and 24-well plate	$3.2x10^4$	N/A

GENERATION OF CELLULAR LYSATE

Following treatment, cells were immediately placed on ice and cells were dislodged by scraping in ice-cold PBS. Cells were collected by centrifugation at 1500 RPM for 5 minutes at 4ºC. Assay-specific lysis buffer supplemented with 1X protease cocktail inhibitor and PMSF (1mM) was added to cells and incubated for 15 – 30 minutes, depending on assay-specific manufacture instructions. Unless specified, lysates were clarified by centrifugation at 14,000g for 10 minutes and stored at -80ºC until use.

BRADFORD'S ASSAY

Bradford's Assay was conducted as per the manufacturer's instructions, with some modifications. Briefly, clarified lysates were diluted in distilled and deionized H_20 by 1:10 – 1:20 depending on expected protein concentration and to prevent incompatibility of the lysis buffer and Bradford's reagent. BSA protein standards (0.0612 – 1 mg/ml) were made up in distilled / deionized H_20, with 1:2 serial diluations. Standards or samples (10 µL) of lysates were added to wells of a 96-well plate in triplicate. A zero standard and lysis buffer control was included in all experiments. Bradford's reagent

(200 µL) was added to wells containing standards or samples, and incubated for 5 minutes at room temperature. Absorbance was read at 595 nm using a Modulus Microplate plate reader (Promega). Blank absorbance values (dH_20/lysis buffer) were subtracted from absorbance values of samples/standards. Unknown sample protein content was interpolated from the linear standard curve equation (as seen in **Figure 4.4**) and adjusted for dilution factor.

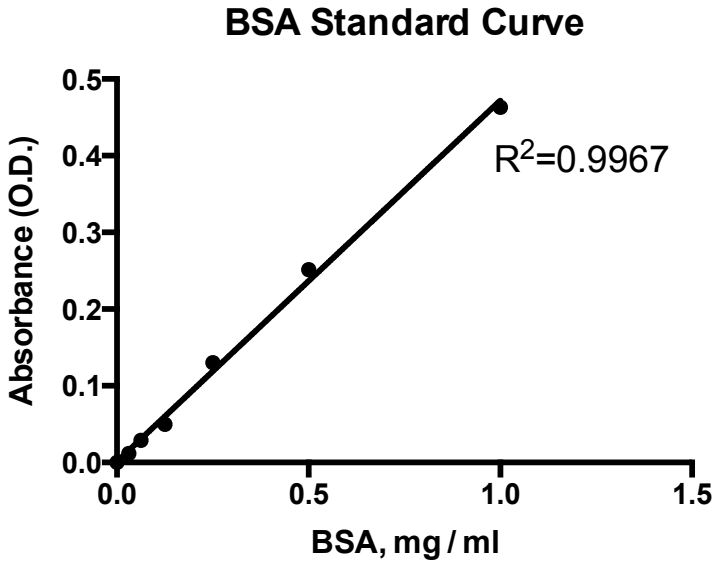


Figure 4.4. An example of a linear regression of known BSA protein standards plotted against respective Bradford's absorbance values (read at 595 nm) from which protein concentration of generated lysates was determined by interpolation.

HUMAN CELL STRESS ARRAY

Cells were treated with prazosin, doxazosin, tamsulosin (30 μM) or vehicle for 24 h. After treatment, cells were lysed as described previously (Generation of cellular lysate).

The relative expression of cell stress related-proteins following 24 h doxazosin, prazosin or tamsulosin treatment was determined using a Human Cell Stress Array kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Briefly, following blocking, membranes were incubated with samples containing 350 μg of protein overnight at 4°C on a platform rocker. Membranes were then washed and incubated with HRP-linked secondary antibody (1:2000) for 1 h at room temperature. Protein expression was visualised by chemiluminescence using a ChemiDoc SSD Camera (BioRad), and semi-quantified using ImageJ (Version 1.49 for Mac OS X) image analysis software.

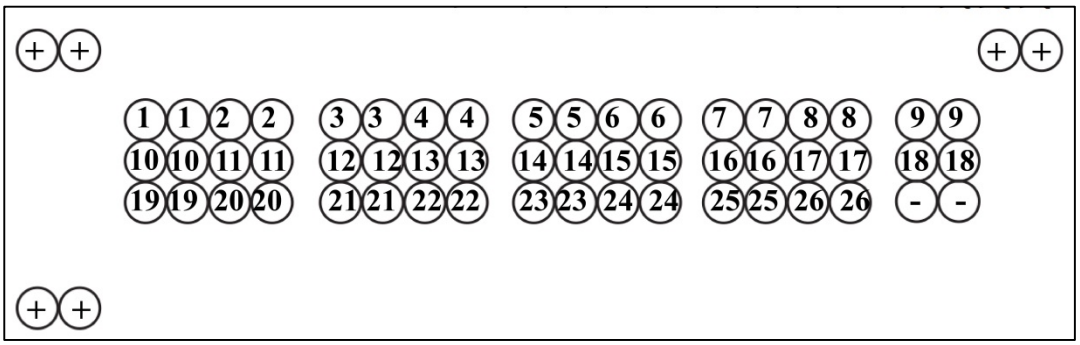


Figure 4.5. Map of cell stress-related protein targets (R&D Systems) as described in Table 4.5.

Table 4.5. Evaluated cell stress-related proteins and alternate nomenclature using the commercially available human cell stress protein array (R&D Systems).

*	Target (Alt. Nomenclature)	Phosphorylation Site
1	ADAMTS1	
2	Bcl-2	
3	Carbonic Anahydrase IX (CA9)	
4	Cited-2	
5	COX-2	
6	Cytochrome C	
7	Dkk-4	
8	FABP-1 (L-FABP)	
9	HIF-1**α**	
10	HIF-2**α** (EPAS1)	
11	Phospho-HSP27	Ser78 / Ser82
12	HSP60	
13	HSP70	
14	IDO (Indoleamine 2, 3-dioxygenase)	
15	Phospho-JNK Pan	
16	NFκB1	
17	p21/CIP1 (CDNK1A)	
18	p27 (Kip1)	
19	Phospho-p38α	Thr180 / Tyr182
20	Phospho-p53	Ser46
21	PON1	
22	PON2	
23	PON3	
24	Thioredoxin-1	
25	SIRT2 (Sirtuin 2)	
26	SOD2 (Mn-SOD)	

**Numbers correspond to location on array membrane as shown in Figure 4.5.*

HUMAN PHOSPHO-RECEPTOR TYROSINE KINASE SIGNALING ARRAY

Cells were treated with 30 µM prazosin, doxazosin, tamsulosin (treatment control) or DMSO vehicle control (0.06%) for 24 h. After treatment, cells were lysed as described previously (Generation of cellular lysate).

The relative expression of phosphorylated (activated) receptor tyrosine kinases following 24 h treatment with doxazosin, prazosin, or tamsulosin treatment was determined using a PathScan® RTK Signaling Antibody Array Kit (Cell Signaling Technologies) according to the manufacturer's instructions. Following blocking of the membranes for 15 minutes, samples containing 80 µg of protein were loaded onto the membrane and incubated for 2 h at room temperature on an orbital shaker. The membrane was washed before and after addition of the detection antibody cocktail (1 h) and HRP-linked streptavidin (30 minutes). Phosphoprotein expression was visualized by chemiluminescence using a ChemiDoc SSD Camera (BioRad), and semi-quantified using ImageJ (Version 1.49 for Mac OS X) image analysis software.

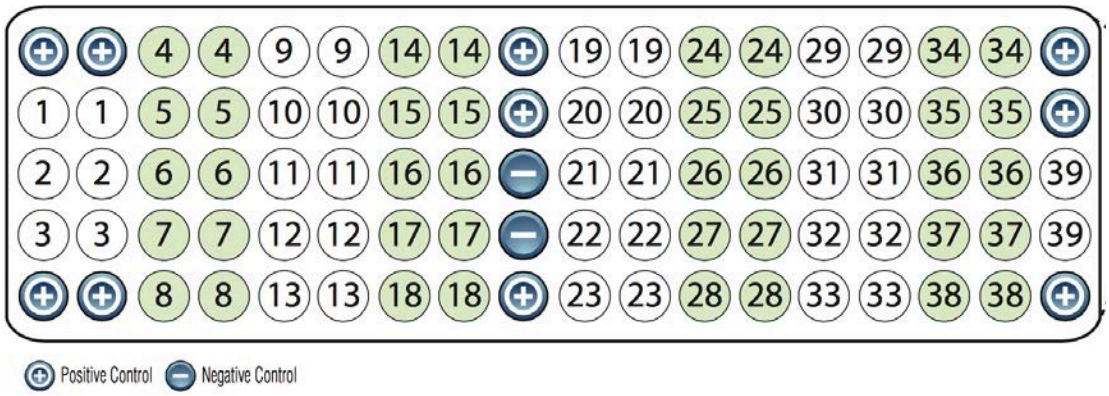


Figure 4.6. Map of RTK and related signaling node of the Human RTK signaling array (Cell Signaling Technology) listed in Table 4.6.

Table 4.6. Human RTK signaling array targets

#*	Target	Family	Phosphorylation Site
1	EGFR/ErbB1	EGFR	pan-Tyr
2	HER2/ErbB2	EGFR	pan-Tyr
3	HER3/ErbB3	EGFR	pan-Tyr
4	FGFR1	FGFR	pan-Tyr
5	FGFR3	FGFR	pan-Tyr
6	FGFR4	FGFR	pan-Tyr
7	InsR	Insulin R	pan-Tyr
8	IGF-IR	Insulin R	pan-Tyr
9	TrkA/NTRK1	NGFR	pan-Tyr
10	TrkB/NTRK2	NGFR	pan-Tyr
11	Met/HGFR	HGFR	pan-Tyr
12	Ron/MST1R	HGFR	pan-Tyr
13	Ret	Ret	pan-Tyr
14	ALK	LTK	pan-Tyr
15	PDGFR	PDGFR	pan-Tyr
16	c-Kit/SCFR	PDGFR	pan-Tyr
17	FLT3/Flk2	PDGFR	pan-Tyr
18	M-CSFR/CSF-1R	PDGFR	pan-Tyr
19	EphA1	EphR	pan-Tyr
20	EphA2	EphR	pan-Tyr
21	EphA3	EphR	pan-Tyr
22	EphB1	EphR	pan-Tyr
23	EphB3	EphR	pan-Tyr
24	EphB4	EphR	pan-Tyr
25	Tyro3/Dtk	Axl	pan-Tyr
26	Axl	Axl	pan-Tyr
27	Tie2/TEK	Tie	pan-Tyr
28	VEGFR2/KDR	VEGFR	pan-Tyr
29	Akt/PKB/Rac	Akt	Thr308
30	Akt/PKB/Rac	Akt	Ser473

#*	Target	Family	Phosphorylation Site
31	ERK 1/2 (p44/42 MAPK)	MAPK	Thr202/Tyr204
32	S6 Ribosomal Protein	RSK	Ser235/236
33	C-Able	Abl	pan-Tyr
34	IRS-1	IRS	pan-Tyr
35	Zap-70	Zap-70	pan-Tyr
36	Src	Src	pan-Tyr
37	Lck	Src	pan-Tyr
38	Stat1	Stat	Tyr701
39	Stat3	Stat	Tyr705

**Numbers correspond to location on array membrane as shown in Figure 4.5.*

PHOSPHO-EPHA2 ELISA

Human prostate cancer PC-3 cells were seeded at sub-confluent densities as detailed in **Table 4.4**. Cells were treated with 100 µM doxazosin or prazosin for 1 – 2 h. Immediately following treatment, cells were lysed as previously described (generation of cellular lysate). Prior to use, protein content of each sample was determined using Bradford's Assay.

Phosphorylated (activated) EphA2 was determined using a human phospho-EphA2 DuoSet IC ELISA kit (R&D Systems) according to the manufacture's instructions, with some modifications. The wells of a high-binding 96-well strip plate (Corning, Sigma Cat. No. CLS3590) were coated with capture antibody (8 µg/mL) overnight at room temperature. Wells were washed (0.05% Tween® 20 in PBS) before and after blocking with buffer containing 1% BSA. Lysates (200 µg of protein) were added to wells and incubated for 2 h at room temperature. Wells were again washed before and after addition of streptavidin-HRP (1:250) for 20 minutes at room temperature, followed by addition of substrate solution for 30 minutes (1:1 mixture of H_2O_2 and tetramethylbenzidine, Cat. No. DY999, R&D Systems). The colourimetric reaction was stopped by addition of the stop solution (2 N H_2SO_4, Cat. No. DY994, R&D Systems). Absorbance (450 nm) was read using a Modulus Multimode plate reader (Promega).

Samples were diluted in lysis buffer to ensure quantifiable phosphorylated EphA2 absorbance values fell within the linear portions of the standard curve. The amount of phosphorylated EphA2 in samples was determined by interpolation from known standard curve as shown in **Figure 4.7**. Fresh phospho-EphA2 standards provided in the kit were used for each independent experiment to generate a matched standard curve.

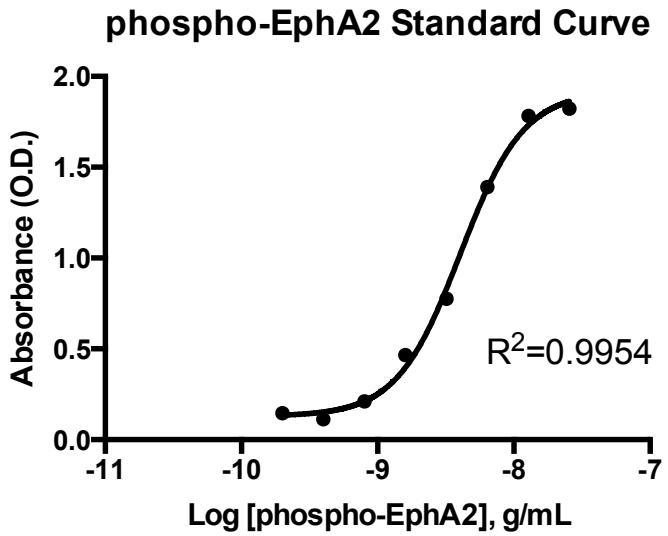


Figure 4.7. Example non-linear sigmoidal regression of known concentrations of phosphorylated EphA2 plotted against absorbance (read at 490 nm) from which sample concentrations were interpolated from the linear portion of the curve.

INHIBITION OF EPHA2 ACTIVATION

Human prostate cancer PC-3 cells were seeded at sub-confluent densities as detailed in **Table 4.4**. Cells were pre-treated with lithocholic acid (10-100 μM) for 30 minutes, prior to co-treatment with doxazosin or prazosin (10-100 μM) for 1, 2, or 24 h. Lithocholic acid (LCA) is a bile acid that has previously been shown to inhibit EphA2 receptor-ligand interactions and subsequent activation in PC-3 cells (Giorgio et al., 2011). Single agent and vehicle treatment controls were included in all experiments.

Cell viability was determined using resazurin reduction as previously described (Chapter 2, General Methods). Images were taken using an EVOS® Cell Imaging System (Thermo-Fisher Scientific)

STATISTICAL ANALYSIS

As mentioned previously, arrays were semi-quantified using ImageJ (Version 1.49 for Mac OS X) image analysis software. Array data were normalised to positive control spots for comparisons between membranes. Membrane positive controls consisted of anti-species antibodies that captured the detection antibodies. Unless indicated otherwise, all investigated conditions were assayed in duplicate over three or more independent experiments. Due to the preliminary nature of the array-based experiments, no expression or phosphorylation controls of evaluated targets were included. Where appropriate, results were expressed as mean ± standard error of the mean. Statistical significance was determined using a one- or two-way ANOVA with either Tukey's or Dunnett's post hoc test as appropriate. The statistical tests used are specified in the following Results section.

4.4 RESULTS

HUMAN CELL STRESS-RELATED PROTEINS

To further the understanding of how prazosin and doxazosin induce cell death, expressional changes in twenty-six cell stress-related proteins following 24 h treatment with these drugs (30 μM) in AR-positive LNCaP and AR-negative PC-3 cells were investigated using a commercially available array, and were semi-quantified using ImageJ analysis software (**Figure 4.8**). The experimental conditions correspond to concentration and treatment duration previously determined to induce apoptosis and/or autophagy in PC-3 and LNCaP cells. As detailed in **Table 4.7,** the stress-related proteins have been grouped together based on their documented activity as either tumours promoters or suppressors (**Figures 4.9 & 4.10**, respectively).

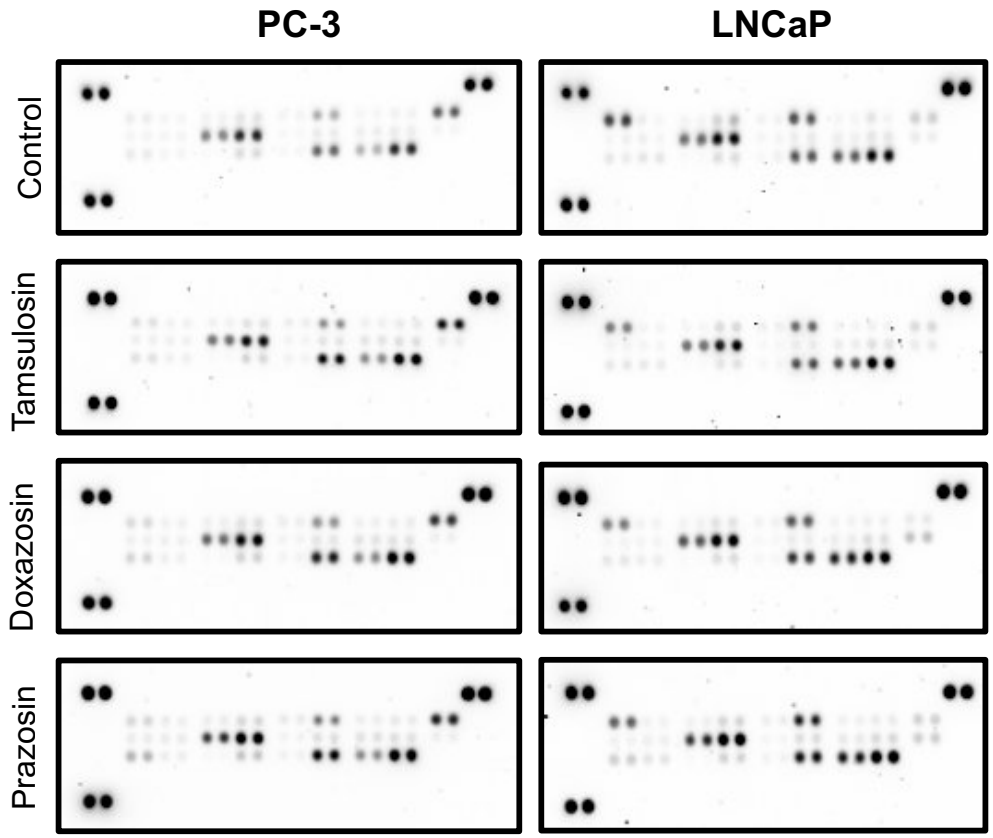


Figure 4.8. Representative images of cell stress arrays (n=3) following 24 h treatment with either tamsulosin, doxazosin, prazosin (30 µM) or vehicle control. Refer to Methods section for map of protein targets. Pixel density (signal intensity) of each spot was determined using ImageJ analysis software and normalised to positive control for standardization between membranes.

Table 4.7. Cell stress-related proteins investigated grouped into either promoters or inhibitors of tumourigenesis.

Tumour promotors		
Target	**Change (P>0.05, n=4)**	**Function / Activity**
BCL-2	–	Anti-apoptotic (Yang et al., 2003)
CA9	–	pH homeostasis, tumour microenvironment acidosis (Benej et al., 2014)
COX-2	–	Inflammatory protein, pro-survival actions (Kirschenbaum et al., 2001)
FABP-1	–	Uptake of fatty acids (Inoue et al., 2014)
HIF-1α	↓	Adaptive response to hypoxia (Masoud and Li, 2015)
HIF-2α	–	Adaptive response to hypoxia (Masoud and Li, 2015)
IDO	–	Immunomodulary (Kallberg et al., 2010)
HSP27	–	Chaperone protein; pro-survival signaling (Lianos et al., 2015)
HSP60	–	Mitochondrial chaperone; regulates permeability (Lianos et al., 2015)
HSP70	–	Chaperone protein (Lianos et al., 2015)
NFκB	–	Transcription factor with pro-survival activity
PON1	–	Cellular antioxidant (Devarajan et al., 2014)
PON2	–	Cellular antioxidant (Devarajan et al., 2014)
PON3	–	Cellular antioxidant (Devarajan et al., 2014)
SOD2	–	Antioxidant (Hempel et al., 2011)
Thioredoxin-1	–	Redox signaling; antioxidant (Watanabe et al., 2010)
Tumour suppressors		
Target	**Change (P>0.05, n=4)**	**Function / Activity**
ADAMTS1	↑↓	Anti-angiogenic (Gustavsson et al., 2009)
Cytochrome-C	–	Pro-apoptosis (Mohammad et al., 2015)
Cited-2	↓	Negative regulator of HIF-1 transcription (Koritzinsky et al., 2005)
Dkk-4	–	Inhibitor of cell invasion (Baehs et al., 2009)
Phospho-JNK	–	Cell death signal transduction (Tournier, 2013)
p21	–	Inhibitor of cell cycle progression (Dutto et al., 2015)
p27	↑	Inhibitor of cell cycle progression (Lee and Kim, 2009)
Phospho-p38α	↑	Apoptosis signal transduction (Cai et al., 2006)
Phospho-p53	–	Pro-apoptosis (Mishra et al., 2015)
SIRT2	–	Regulation of cell cycle (Inoue et al., 2007)

TUMOUR PROMOTERS

Hypoxia-inducible factor 1 alpha (HIF-1α)

In response to doxazosin or prazosin treatment, the expression of only one tumourigenesis-related protein, HIF1α, was found to be statistically different to control in LNCaP cells (**Figure 4.9**). Briefly, the hypoxia-sensing signaling molecule, HIF-1α, is stabilised in the absence of oxygen as an adaptive mechanism (Fraga et al., 2015) and is known to be overly expressed in cancers to enhance tumour growth, and in prostate cancer, promotes transition to castrate-resistant disease (Ranasinghe et al., 2013). The role of HIF-1α in tumourigenesis is discussed further in Chapter 5. Consistent with previous findings (Ranasinghe et al., 2013), the degree of HIF-1α expression was associated with metastatic potential, with the aggressive PC-3 cells possessing nearly 10-times higher levels of HIF-1α, compared to the more docile LNCaP cells. However, only LNCaP HIF1α was altered by 24 h antagonist treatment, with doxazosin possessing the greatest suppressive effect. Doxazosin (30 μM) treatment significantly reduced levels of HIF-1α ($P<0.001$) by more than 50% in LNCaP cells, but the effect was not significantly different from that of prazosin ($P>0.05$). In contrast, prazosin did not have a statistically significant effect on HIF-1α compared to control. As expected, the non-toxic tamsulosin (30 μM, 24 h) had no appreciable effect on the investigated tumour promoting proteins in either cell line at the concentration and time point investigated. While it was previously reported that doxazosin treatment suppressed NFκB levels in breast cancer cells (Hui et al., 2008), the present study suggests that neither doxazosin nor prazosin affect NFκB in prostate cancer cells following 24 hour treatment **(Figure 4.9)**.

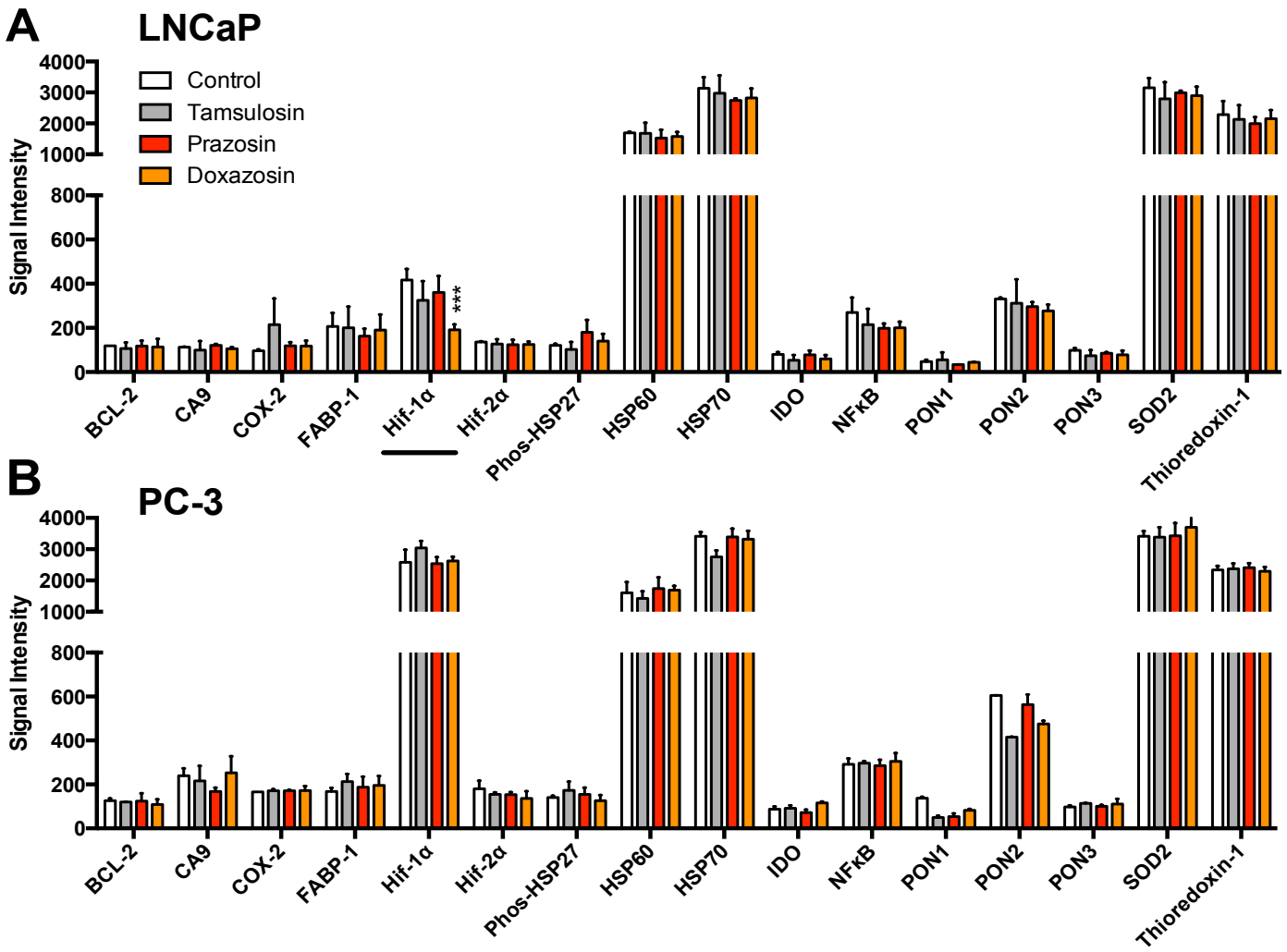


Figure 4.9. Changes in expression or phosphorylation of cell stress-related proteins with tumour promotor functions following 24 h treatment with prazosin, doxazosin, tamsulosin (30 µM) or vehicle control in LNCaP (A) and PC-3 (B) cells. Results were semiquantified using ImageJ image analysis software and were normalised to positive control. Data are represented as the mean ± SEM (n=3). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01 and **P<0.001 vs. untreated control.

TUMOUR SUPPRESSORS

Prazosin and doxazosin treatment was found to reduce the expressional or phosphorylation levels of ADAMTS1, cited-2, p27 and p38α, which occurred in a cell type- and drug-dependent manner (**Figure 4.10**). Refer to **Table 4.7** for a summary of antagonist-induced changes in tumour suppressor proteins.

A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1)

The anti-angiogenic factor ADAMTS1 is a proteinase member of the notch signaling cascade and has previously been reported to be down regulated in human prostate cancers (Gustavsson et al., 2009). In line with its tumour-suppressive actions, basal levels of ADAMTS1 were approximately 8-times higher in the less metastatic LNCaP than the aggressive PC-3 cells (**Figure 4.10 A & B**). In PC-3 cells, both doxazosin and prazosin were able to increase ADAMTS1 expression ($P<0.001$ and $P=0.056$, respectively) (**Figure 4.10 B**). However, doxazosin was found to have an insignificantly greater effect than prazosin, increasing ADAMTS1 expression by 144% ± 85.9 versus 84% ± 59.2 ($P=0.06$), respectively. Doxazosin, but not prazosin, suppressed ADAMTS1 in PC-3 cells, which was greater than that of the effect of tamsulosin (non-cytotoxic treatment control) ($P=0.001$). This indicates ADAMTS1 may participate in antagonist-mediated PC-3 cytotoxicity. No significant changes were observed in LNCaP ADAMTS1 expression. However, doxazosin and prazosin appeared to have a slight inhibitory effect on ADAMTS1 **(Figure 4.10 A)**. While statistically insignificant, doxazosin treatment had a greater inhibitory effect on ADAMTS1 compared to doxazosin or tamsulosin treatment, suppressing ADAMTS1 expression by 24% (± 13) in LNCaP cells.

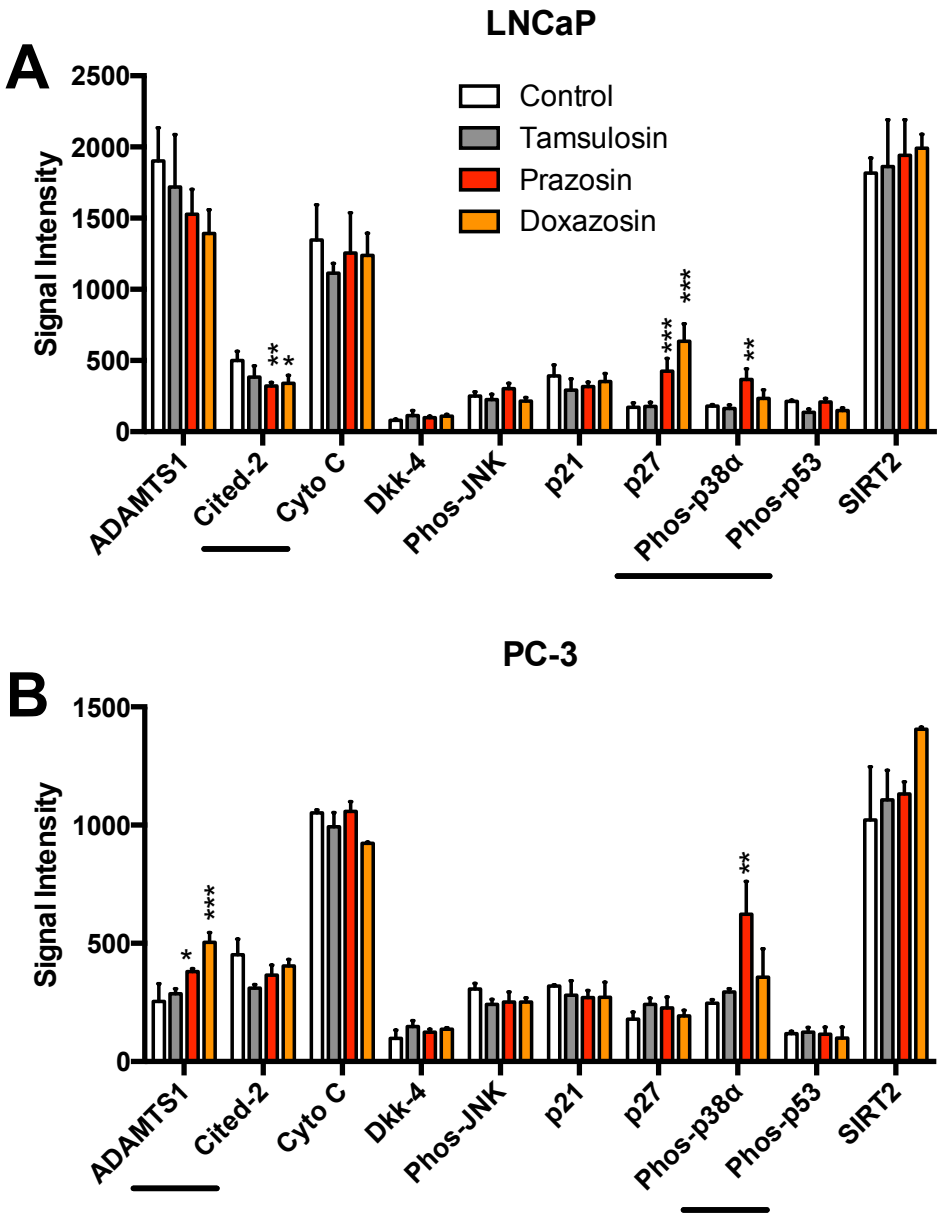


Figure 4.10. Changes in expression or phosphorylation of cell stress-related proteins with tumour suppressor functions following 24 h treatment with prazosin, doxazosin, tamsulosin (30 µM) or vehicle control in LNCaP (A) and PC-3 (B) cells. Results were semiquantified using ImageJ image analysis software and were normalised to signal intensity of positive control. Data are represented as the mean ± SEM (n=3). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01 and **P<0.001 vs. untreated control.

Cited-2

Cited-2 (Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2) acts as a tumour suppressor via negative regulation of HIF-1α activity (Koritzinsky et al., 2005). Basal expression of Cited-2 were similar in between AR-negative PC-3 and AR-positive LNCaP cell lines. Treatment with doxazosin, prazosin or tamsulosin decreased Cited-2 expression by less than 50% in both cell lines, however statistical significance was only present amongst prazosin and doxazosin treated LNCaP cells ($P<0.001$ and $P<0.01$, respectively) (**Figure 4.10**). A similar, but not significant reduction of Cited-2 was observed in PC-3 cells in response to doxazosin and prazosin treatment (**Figure 4.10 B**). Tamsulosin treatment had a significantly greater effect on Cited-2 expression than doxazosin and prazosin in PC-3 cells, whereas the effect of tamsulosin was not significantly different than doxazosin and prazosin in LNCaP cells. While unexpected, the decrease in the tumour suppressor cited-2 expression is unlikely to contribute to antagonist cytotoxicity and will be further discussed later in this chapter.

p27

Basal levels of the cell cycle inhibitor p27 were similar between PC-3 and LNCaP cells (**Figure 4.10**), and were significantly altered following treatment with doxazosin and prazosin in LNCaP cells. Consistent with previous findings (Xu et al., 2003b, Kintscher et al., 2000b), p27 expression was enhanced by approximately 1.5-fold and >2.5-fold within LNCaP cells treated with prazosin and doxazosin, respectively, compared to untreated control (**Figure 4.10 A**). Doxazosin appeared to be more effective than prazosin at up-regulating p27 regulation, however the difference between doxazosin and prazosin were not significant ($P=0.102$). Importantly, both cytotoxic drugs displayed a greater effect on p27 than tamsulosin, raising the possibility of p27 involvement in doxazosin and/or prazosin toxicity. In contrast to LNCaP cells, there were no observable expressional changes in p27 in PC-3 cells (**Figure 4.3 B**).

p38α

The mitogen-activated protein kinase (MAPK) p38α is known to participate in either cell survival or death signaling, which occurs in a stimuli- and cell type-dependent fashion. However, it is usually considered to have tumour suppressive effects by preventing malignant transformation (Igea and Nebreda, 2015). In PC-3 and LNCaP cells, activation of p38α was significantly enhanced in response to prazosin ($P<0.01$ and $P<0.001$, respectively), while doxazosin a caused a similar but insignificant enhancement of p38α phosphorylation in PC-3 and LNCaP cells ($P=0.149$ and $P=0.575$, respectively, vs. control) (**Figure 4.10**). This effect was greatest in PC-3 cells with prazosin enhancing p38α activity by 150%, in contrast to approximately 100%-increase amongst LNCaP cells. Furthermore, the effect of the cytotoxic antagonists doxazosin and prazosin on p38α activation were nearly 1.5 and 2-times greater, respectively, than tamsulosin in both cell lines, strongly suggesting involvement of p38α in underlying cytotoxic mechanisms of these drugs.

Similar to its lack of effect on levels of tumour-promoting proteins, tamsulosin (30 μM, 24 h) was found to have no effect on any of the tumour suppressor-related proteins evaluated in all tested conditions (**Figure 4.10**). In contrast to previous reports, no changes were observed in p21 (Partin et al., 2003), p53 (Shaw et al., 2004, Yang et al., 2011a, Park et al., 2014b) or cytochrome c release (Liao et al., 2011) in response to doxazosin or prazosin in either LNCaP or PC-3 cells. In contrast to LNCaP cells which express wild-type p53, a lack of change of p53 phosphorylation in PC-3 cells is expected as these cells do not express functional p53 protein (van Bokhoven et al., 2003). In PC-3 cells, the detection of phospho-p53 in likely to be an experimental artifact due to non-specific binding or limitations in the semi-quantification methodology.

RECEPTOR TYROSINE KINASES AND RELATED SIGNALING NODES

Next, whether prazosin or doxazosin (30 μM, 24 h) was able to alter the activation of receptor tyrosine kinases (RTKs) and related signaling proteins was investigated using a commercially available array (**Figure 4.11**). Pixel density (also referred to as signal intensity) was semi-quantified using ImageJ analysis software. The evaluated targets were grouped according to their role as a RTKs or a signaling node (**Figures 4.12 & 4.13**). Changes in activation (phosphorylation) following treatment with the investigated α1-ADR antagonists are summarised in **Table 4.2** on the following page. Phosphorylation levels of ALK, Axl, EphA1, EphB1, EphB3, FLT3/Flk2, Met, Ron/MST1R, Ret, PDGFR, TrkA/NTRK1, TrkB/NTRK and Tyro3/Dtk were undetectable in all tested conditions and therefore were excluded from analyses and figures.

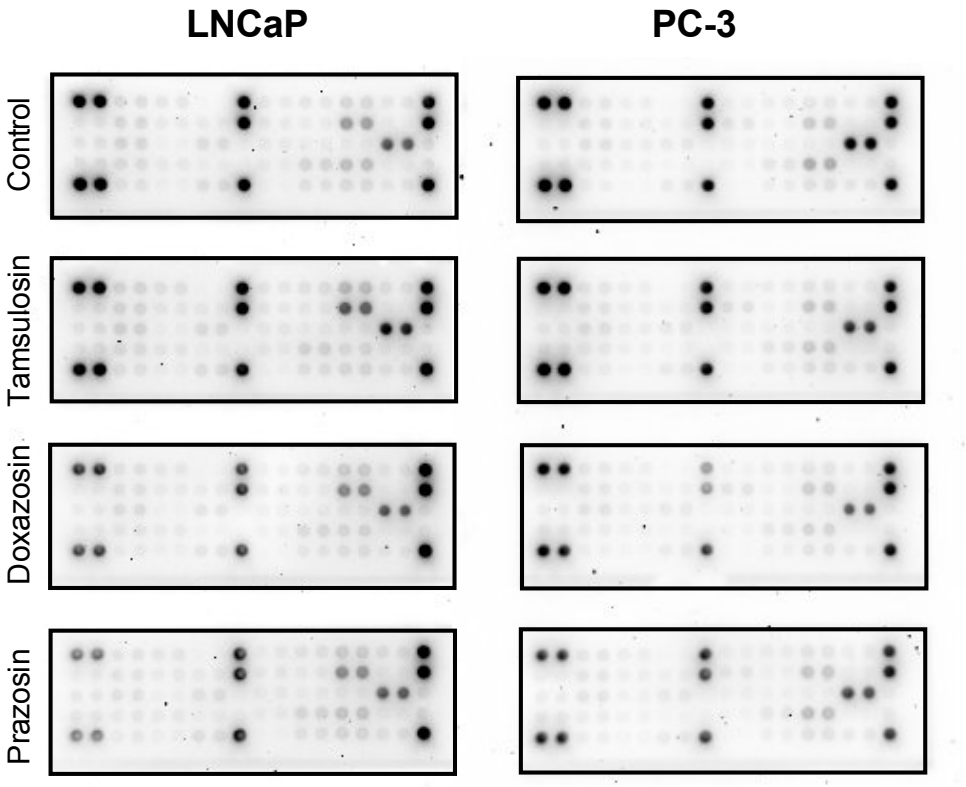


Figure 4.11. Representative images of RTK arrays following 24 h treatment with either tamsulosin, doxazosin, prazosin (30 µM) or vehicle control (n=4). Refer to Methods section for map of protein targets. Pixel density (signal intensity) of each spot was determined using ImageJ analysis software.

Table 4.2. Investigated receptor tyrosine kinases and related signaling nodes

RTK or Signaling Node	Family	Change* (P<0.05, n=3)	Function	Reference
c-Abl	Abl	–	Cell division, adhesion, differentiation, response to stress	(Zhao et al., 2014)
Akt-Ser473	Akt	↑	Survival signaling	(Lee et al., 2015)
Akt-Thr308	Akt	↑	Survival signaling	
Axl	Axl	X	Stimulates cell proliferation, involved in epithelial-to-mesenchymal transition	(Paccez et al., 2014)
Tyro3/Dtk	Axl	X	Cell survival and migration	
EGFR/ErbB1	EGFR	–	Survival signaling	(Yarden, 2001)
HER2/ErbB2	EGFR	–	Survival signaling	
HER3/ErbB3	EGFR	–	Survival signaling	
EphA1	EphR	X	Cell adhesion, proliferation, rounding and detachment	(Lisle et al., 2013)
EphA2	EphR	–	Cell adhesion, proliferation, rounding and detachment	
EphA3	EphR	–	Cell adhesion, proliferation, rounding and detachment	
EphB1	EphR	X	Cell adhesion, proliferation, rounding and detachment	
EphB3	EphR	X	Cell adhesion, proliferation, rounding and detachment	
EphB4	EphR	–	Cell adhesion, proliferation, rounding and detachment	
FGFR1	FGFR	–	Cell proliferation, migration, differentiation	(Touat et al., 2015)
FGFR3	FGFR	–	Cell proliferation, migration, differentiation	
FGFR4	FGFR	–	Cell proliferation, migration, differentiation	
Met/HGFR	HGFR	X	Cell proliferation, treatment resistance	(Gelsomino et al., 2014)
Ron/MST1R	HGFR	X	Pro-metastatic activity	(Yao et al., 2013)
IGF-IR	Insulin R	–	Glucose homeostasis	(Wu and Yu, 2014)
InsR	Insulin R	–	Glucose homeostasis	
IRS-1	IRS	–	Glucose homeostasis	(Shaw, 2011)
ALK	LTK	X	Survival signaling	(Ou and Shirai, 2016)

RTK or Signaling Node	Family	Change* (P<0.05, n=3)	Function	Reference
ERK 1/2	MAPK	–	Cell adhesion, proliferation, rounding and detachment	(Carey et al., 2007)
TrkA/NTRK1	NGFR	X	Cell survival	
TrkB/NTRK2	NGFR	X	Cell survival, differentiation	(Thiele et al., 2009)
c-Kit/SCFR	PDGFR	–	Pro-migration and invasion signals	(Liang et al., 2013)
FLT3/Flk2	PDGFR	X	Survival signaling	(Kayser and Levis, 2014)
M-CSFR	PDGFR	–	Differentiation, invasion, angiogenesis, metastasis, treatment resistance	(Chockalingam and Ghosh, 2014)
PDGFR	PDGFR	X	Survival signaling	(Ehnman and Ostman, 2014)
Ret	Ret	X	Cell proliferation	(Plaza-Menacho et al., 2014)
S6 Ribosomal Protein	RSK	↓	Substrate of p70S6K, protein synthesis	(Fenton and Gout, 2011)
Lck	Src	–	Cell proliferation, angiogenesis, invasion	(Varkaris et al., 2014)
Src	Src	↑↓	Cell proliferation, angiogenesis, invasion	
Stat1	Stat	–	Anti-proliferative, pro-apoptotic	(Thota et al., 2014)
Stat3	Stat	–	Cell survival and proliferation	(Lavecchia et al., 2011)
Tie2/TEK	Tie	–	Pro-angiogenesis activity	(Barton et al., 2014)
VEGFR2/KDR	VEGFR	–	Pro-angiogenesis activity	(Park et al., 2014a)
Zap-70	Zap-70	–	Survival signaling	(Wong and Abubakar, 2008)

Changes in phosphorylation (activation) were indicated as increase (↑), decrease (↓), not changed (–) or undetectable (X).

Statistically significant changes were observed in only three of the thirty-nine total RTKs and related signaling nodes evaluated in LNCaP and PC-3. These included: S6 ribosomal protein, Src, and Akt, which were all signaling-related proteins and not RTKs (**Figure 4.5**). None of the investigated drugs had any significant effect on the investigated RTKs (**Figure 4.12**)

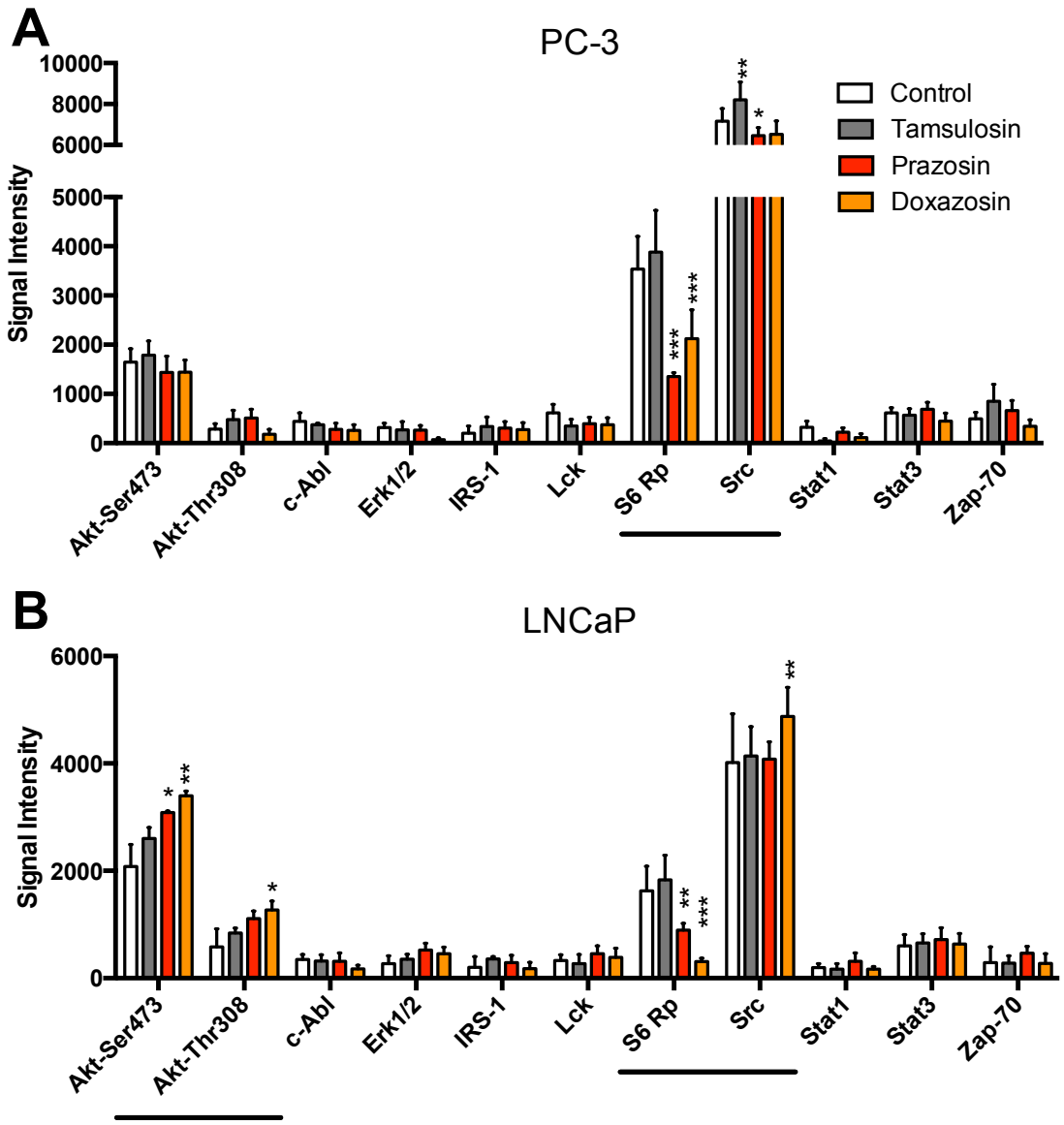


Figure 4.12. Change in phosphorylation of RTK-related signaling nodes in PC-3 (A) and LNCaP (B) cells following treatment with tamsulosin, prazosin or doxazosin (30 µM, 24 h). Data are represented as the mean ± SEM (n=4). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. * P<0.05, ** P<0.01 and *** P<0.001 vs. untreated control.

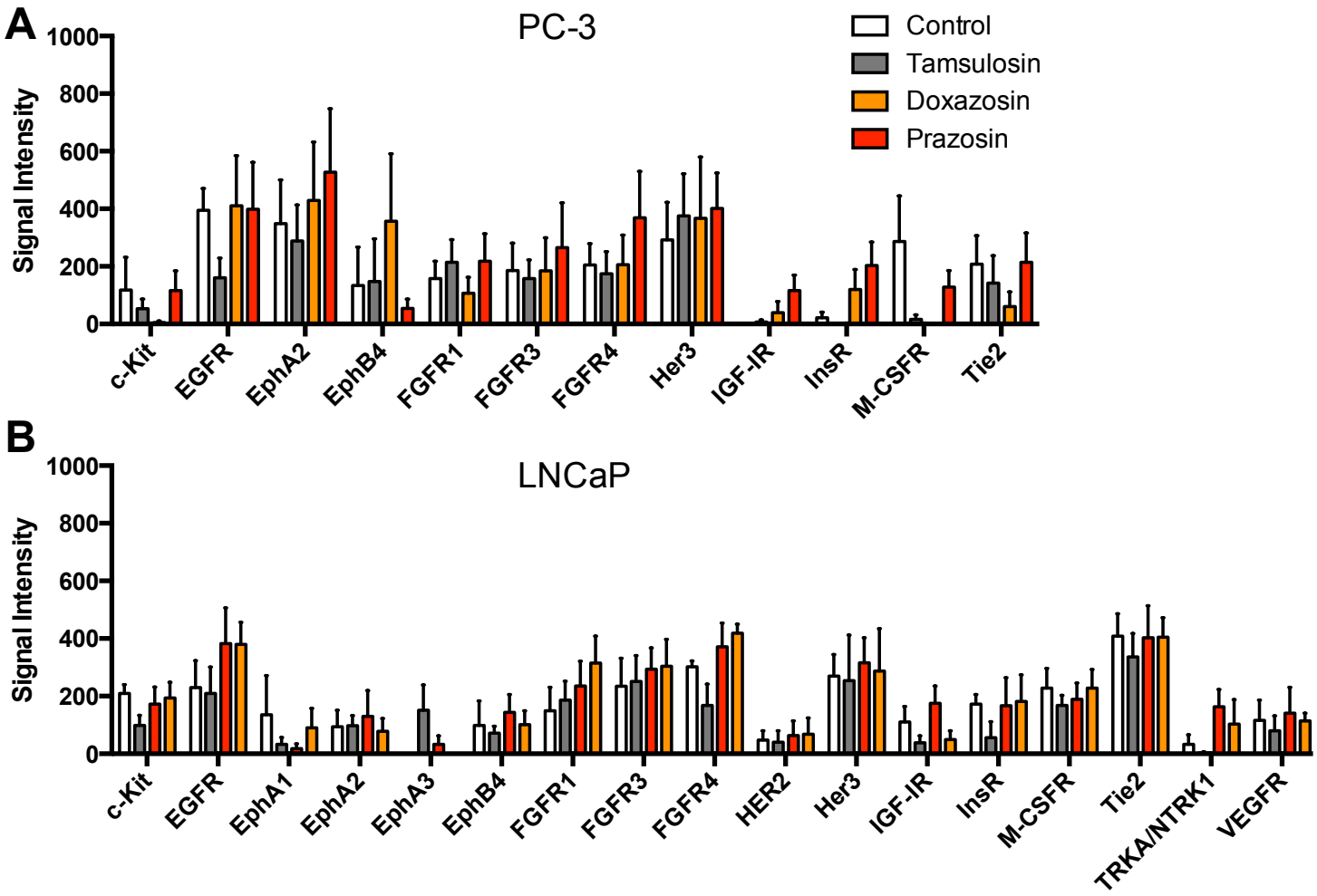


Figure 4.13. Changes in phosphorylation of RTKs in PC-3 (A) and LNCaP (B) cells following treatment with tamsulosin, prazosin or doxazosin (30 µM, 24 h). Results were semi-quantified using ImageJ image analysis software and were normalised to signal intensity of positive control. Data are represented as the mean ± SEM (n=4). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01 and **P<0.001 vs. untreated control.

Akt (Ser473 / Thr308)

Akt, also known as protein kinase B, is well documented to have two activation sites, serine 473 (Ser473) and threonine 308 (Thr308), and phosphorylation of both sites are required for full enzymatic activity (Alessi et al., 1997, Stephens et al., 1998, Chang et al., 2015). In basal conditions, Akt phosphorylation levels were similar between both cell lines. However, basal Akt appeared to exist partially phosphorylated at Ser473 site, with little phosphorylation at Thr308 (**Figure 4.12**). There were no significant changes in Akt activation in PC-3 cells following treatment with any of the antagonists (**Figure 4.12 A**). However, prazosin and tamsulosin were found to partially enhance PC-3 Akt activity via Thr308 phosphorylation, whereas doxazosin possessed a modest inhibitory effect on Thr308 activation. In contrast, all drugs enhanced LNCaP Akt activation, by increasing phosphorylation of both Ser473 and Thr308 (**Figure 4.12 B**) in a drug-dependent manner, with only doxazosin and prazosin exhibiting significance ($P<0.001$). While prazosin was able to increase phosphorylation of Ser473 by 100% and Thr308 by approximately 475% ($P<0.001$ and $P=0.136$ versus control, respectively), only doxazosin was able to significantly increase full activation of Akt ($P<0.001$ and $P=0.025$ for Ser473 and Thr308 versus control, respectively) (**Figure 4.12 B**). However, modulation of Akt activity by doxazosin or prazosin was not significantly different than the non-toxic tamsulosin, except for doxazosin on Thr308 in LNCaP cells ($P=0.007$) (**Figure 4.12 B**).

S6 ribosomal protein

The most notable change was the doxazosin and prazosin-mediated suppression of S6 ribosomal protein activation in both cell lines. Briefly, S6 ribosomal protein belongs to the ribosomal s6 kinase (RSK) family and is activated primarily by p70-RSK. Activation of S6 ribosomal protein promotes protein synthesis and cell proliferation (Fenton and Gout, 2011). In basal conditions, both cell lines had relatively high levels of S6 ribosomal protein activity, which were approximately 1.5-times greater in PC-3 cells than LNCaP. Exposure to prazosin and doxazosin significantly decreased (> 50% of control) ribosomal S6 protein kinase in both cell lines compared to untreated control

(P<0.01 for both cell lines and antagonists) (**Figure 4.5**). The drug which possessed the greatest effect was cell type-dependent, with prazosin possessing a larger effect in PC-3 cells (P=0.041 vs. doxazosin) and doxazosin amongst LNCaP cells (P=0.075 vs. prazosin (**Figure 4.12 A & B,** respectively). Tamsulosin had a minimal effect on S6 ribosomal protein activity (< 25% in both cell lines, and the suppressive effect of prazosin and doxazosin were found to be significantly greater compared to the non-toxic tamsulosin (P<0.001 between tamsulosin and prazosin or doxazosin for both cell lines), indicating potential involvement of the p70S6K/ S6 ribosomal protein signaling cascade in antagonist-induced cytotoxicity.

Src

Cellular Src kinase (c-Src, and referred to as Src), is a non-receptor tyrosine kinase which possesses a plethora of tumourigenic actions such as cell survival, proliferation, invasion and angiogenesis (Varkaris et al., 2014). The phosphorylation status of Src was nearly twice as great in the metastatic PC-3 cells, compared the more docile LNCaP cells (**Figure 4.12**). Modest, yet significant changes were observed in the phosphorylation status of the proto-oncogene Src in both cell lines, which occurred in a drug-dependent manner. Consistent with the cytotoxic actions, both prazosin and doxazosin treatment slightly suppressed Src phosphorylation in PC-3 cells (< 10%, P>0.05 for both drugs), **Figure 4.12 A**). In contrast, both doxazosin and prazosin increased Src phosphorylation in LNCaP cells, but only doxazosin had a significant pro-activation effect (P=0.007) (**Figure 4.12 B**). Interestingly, tamsulosin had no effect on the phosphorylation status of other RTK and related signaling kinases, except for Src where treatment with the non-toxic agent significantly increased Src activation by nearly 20% in PC-3 cells (P<0.002).

In contrast to previous literature, no significant changes were observed in vascular endothelial growth factor receptor 2 (VEGFR2) activity (Park et al., 2014a) or EphA2 (or related Eph receptors) (Petty et al., 2012) after 24 h treatment. Conversely, epidermal growth factor receptor (EGFR) and the downstream signaling effector ERK1/2 appear to be insignificantly enhanced in response to doxazosin and prazosin, which opposes previous reports of the inhibitory effects of antagonist treatment on these targets (Hui et al., 2008).

INVOLVEMENT OF EPHA2 IN ANTAGONIST-INDUCED CYTOTOXICITY

In the present study, 24 h treatment with doxazosin or prazosin did not alter phosphorylation status of EphA2 (**Figure 4.13**). However, a study by Petty and colleagues (2012) demonstrated transient EphA2 phosphorylation (activation)-dependent cytotoxicity of prostate cancer cells in response to acute 60-minute doxazosin treatment. While the authors did not report doxazosin-mediated EphA2 activation timecourse in PC-3 cells, significant EphA2 activation was observed within five minutes and peaked after 30 minutes of doxazosin exposure in human breast cancer MDA-231-A cells. These findings prompted further investigation of EphA2 involvement in quinazoline-based cytotoxicity; particularly whether acute prazosin treatment is able to also enhance activation of EphA2 receptors.

To investigate whether doxazosin, prazosin or tamsulosin are able to alter the phosphorylation status of EphA2, PC-3 cells were treated with these drugs for 1 – 2 h. Subsequent EphA2 activation was determined by ELISA method using a commercially available kit. Only PC-3 cells were investigated for EphA2 activity, as they have previously been shown to express high levels of EphA2, whereas LNCaP cells reportedly do not (Walker-Daniels et al., 1999). Consistent with findings of Petty et al. (2012), doxazosin was found to induce significant transient phosphorylation of EphA2 at 1 h, which was completely abolished at 2 h (**Figure 4.14**). In contrast, prazosin treatment resulted in a slight but not significant increase in EphA2 phosphorylation, whereas EphA2 activity was undetectable in tamsulosin treated PC-3 cells (data not shown).

Stimulation of EphA2 receptors are known to trigger cell rounding and detachment that was previously demonstrated in response to doxazosin treatment (Giorgio et al., 2011, Petty et al., 2012). Consistent with prior and present findings, doxazosin induced cell rounding affected more than 50% of cells at 1 h, and nearly all cells displayed a rounded morphology at 2 h, presumably via EphA2 activation (**Figure 4.16)**. Unlike doxazosin, prazosin was unable to stimulate cell rounding at the time points investigated.

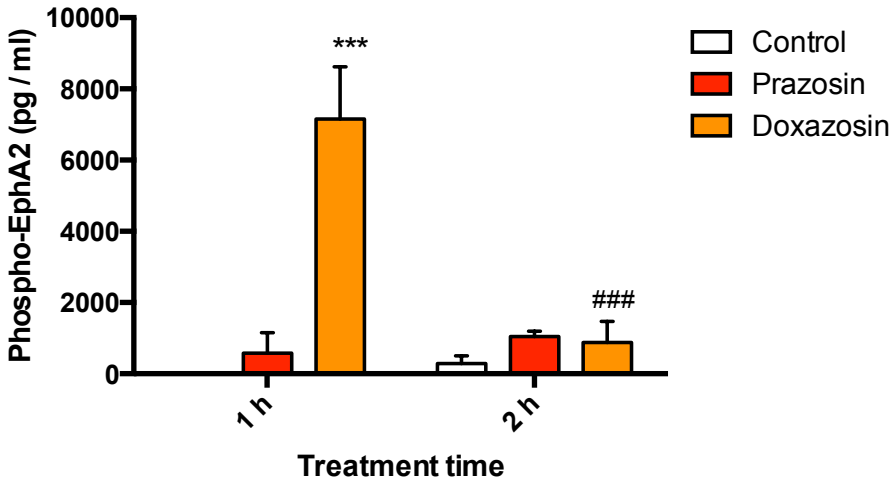


Figure 4.14. Change in phosphorylation (activation) of EphA2 in PC-3 cells following 1 – 2 h treatment with prazosin, doxazosin (100 µM) or vehicle control. EphA2 phosphorylation status was determined using a Phospho-EphA2 ELISA kit (R&D Systems). Phospho-EphA2 levels were normalised to loaded protein, and results are expressed as mean ± SEM (n=3). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. *** P<0.001 vs, control; and ### P<0.001 vs. 1 h doxazosin treatment.

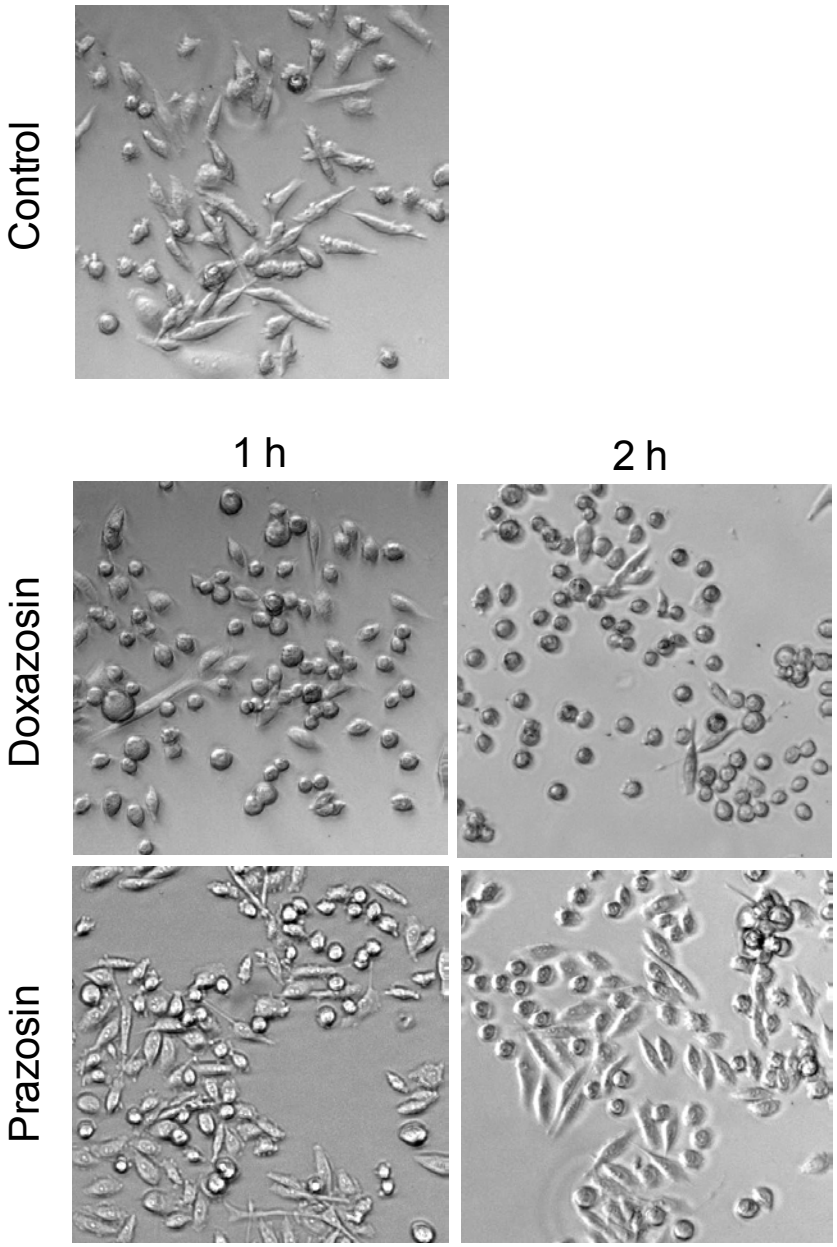


Figure 4.16. Time lapse of PC-3 cell rounding following 1- 2 h exposure to doxazosin and prazosin (100 µM). All images are representative of three independent experiments and were captured using a Evos® Cell Imaging System (Thermo-Fischer Scientific) at 100X magnification.

Next, whether inhibition of transient EphA2 activation may protect cells from doxazosin or prazosin-induced cytotoxicity was investigated. EphA2 phosphorylation was inhibited pharmacologically using lithocholic acid. Lithocholic acid (LCA) was previously identified to hinder EphA2 activation in prostate cancer cells (IC_{50} value of 48 μM) with no effect on cell survival (Giorgio et al., 2011). Consistent with previous findings (Giorgio et al., 2011), at concentrations ≤ 100 μM, LCA had no cytotoxic effect on PC-3 survival following 24 h treatment (**Figure 4.17 A**). In contrast, the highest dose of LCA investigated (300 μM), significantly reduced cell survival by 26.4% ($P<0.01$), and thus, was subsequently excluded from further testing. In these experimental conditions, doxazosin and prazosin suppressed cell viability by 18 (±5.5) and 34 (±5.7)%, respectively ($P<0.05$-0.01) (**Figure 4.17 B**). The combination of antagonist and LCA was only able to partially protect PC-3 cells from doxazosin-induced cytotoxicity (**Figure 4.17 C**) and cell rounding (**Figure 4.18**) after 24 h treatment. In contrast, LCA (10-100 μM) had no appreciable effect on cell survival (**Figure 4.17 D)** or cell rounding when combined with prazosin (data not shown).

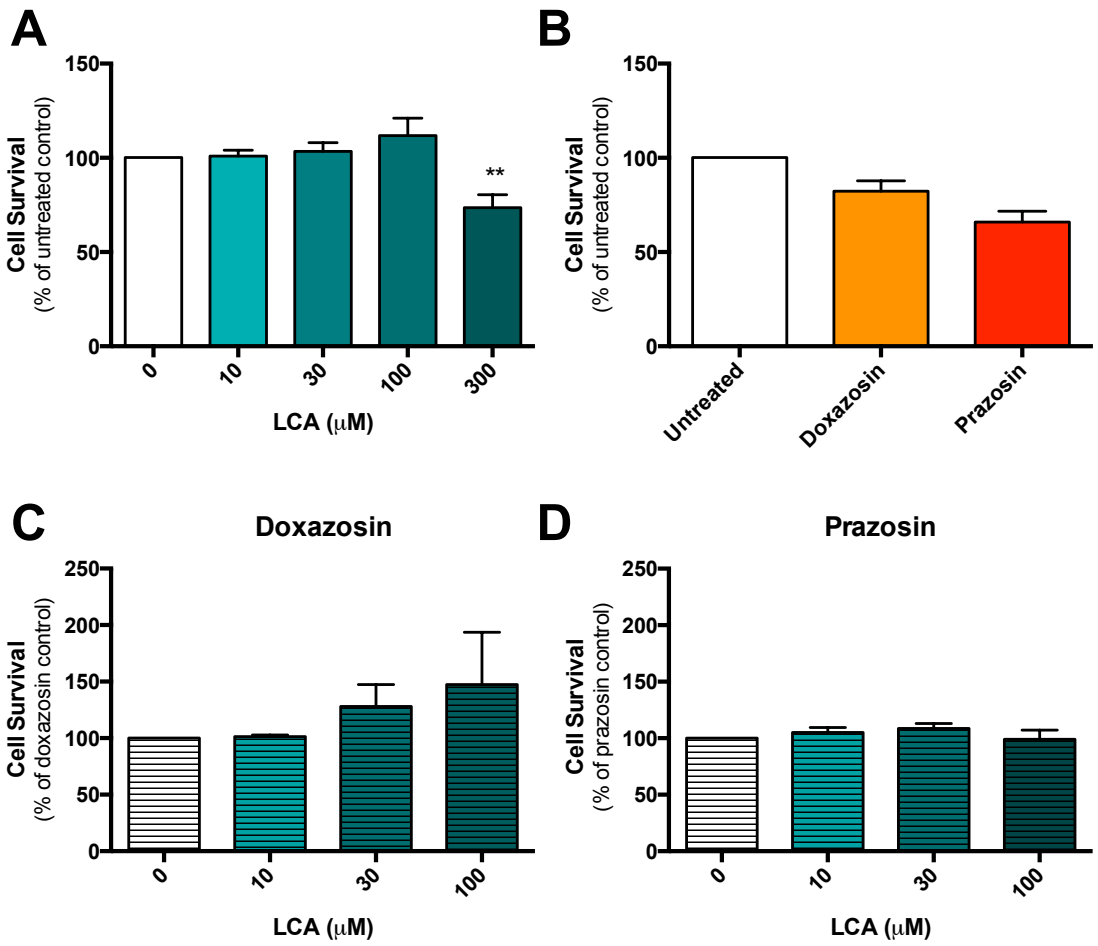


Figure 4.17. Human prostate cancer PC-3 cells were treated for 24 h with single-agent lithocholic acid (LCA, 0-300 µM) (A), doxazosin, prazosin (30 µM) (B), or combination treatment with LCA (0-100 µM) and either doxazosin (C) or prazosin (D) (30 µM for both). A resazurin reduction assay was used as an index of cell survival. Results are expressed as a percentage (mean ± SEM, n≥3) of untreated control (A,B) or of doxazosin or prazosin treated control (C and D, respectively). Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test. ** P<0.01 vs. untreated control.

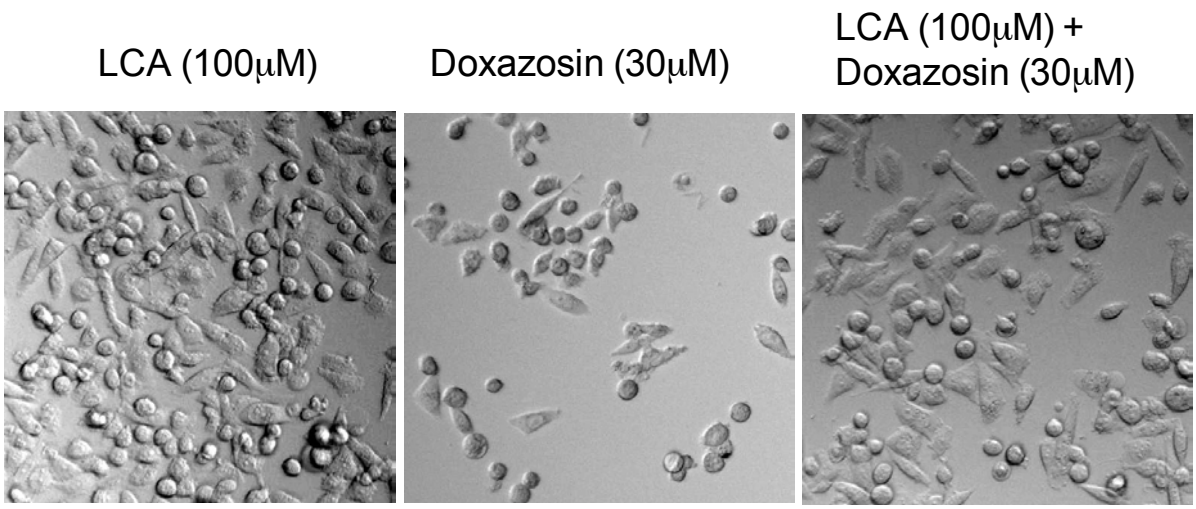


Figure 4.18. Protection from doxazosin-induced (30 μM) cell rounding of PC-3 cells by LCA (100 μM) following 24 h co-treatment. Live cell images were captured using an Evos® Cell Imaging System at 100X magnification.

4.5 DISCUSSION

In Chapter 3, which investigated the relative cytotoxic potencies and cell death mechanisms, it was demonstrated that prazosin and doxazosin possess apoptotic potential in prostate cancer cell lines. In addition, these drugs are able to increase autophagy in PC-3 and LnCAP cells with paradoxical outcomes; contributing to antagonist-induced cytotoxicity or survival mechanisms, respectively. However, the cell signaling pathways underlying antagonist-induced apoptosis or autophagy remained to be fully elucidated. The overall aim of this study was to investigate the molecular mechanisms underlying doxazosin and prazosin cytotoxicity. The findings of the present study provided evidence for the involvement of mTOR signaling, p27 cell cycle inhibition, and p38α MAPK activity in mediating antagonist-induced cytotoxicity. Additionally, EphA2 activity was further confirmed in the present study as an underlying mechanism of doxazosin-induced toxicity, but did not appear to contribute to prazosin toxicity.

MTOR SIGNALING

The mTOR family comprises the well-characterised complex 1 (mTORC1) and the lesser known complex 2 (mTORC2). While mTORC2 is undoubtedly important, the focus of this text will be to examine the involvement of mTORC1 in doxazosin- and prazosin-induced cell death. As previously reviewed in Chapter 3, autophagy is negatively regulated by mTORC1. In addition to the classical autophagy inhibition cascade via Ulk complex, mTORC1 acts on 4E-BP1, serum/glucocorticoid regulated protein kinase 1 (SGK1) and p70S6 kinase (p70S6K) (Hong et al., 2008, Laplante and Sabatini, 2009, Mori et al., 2014). Of particular interest, p70S6 kinase promotes the activation of a plethora of regulators of cell survival, including S6 ribosomal protein (rpS6) (Laplante and Sabatini, 2009). Doxazosin- and prazosin-induced autophagic response of both PC-3 and LNCaP cell lines (Chapter 3) is further confirmed by the significant inhibition of S6 ribosomal (rbS6) protein, a downstream target of mTORC1. Therefore, it can be inferred that these drugs suppress the activity of mTORC1, thereby resulting in downregulation of the mTORC1 substrates including p70S6K, and in turn,

inhibit rbS6 activity. In support of the present findings, the down-regulation of mTORC1/p70S6K signaling axis was previously shown in rodent cardiomyocytes treated with prazosin (Yang et al., 2011a). However, the precise molecular mechanism of mTORC1 inhibition by these drugs, and thus induction of autophagy, remains unclear.

Activation of the signaling kinase, Akt, inhibits autophagy via indirect activation of mTORC1. Likewise, the induction of autophagy is classically associated with suppression of Akt phosphorylation (Laplante and Sabatini, 2009). The present findings demonstrated otherwise, showing a paralleled increase in Akt phosphorylation (current chapter) and increase in autophagy activity (Chapter 3). While the present findings appear to be inconsistent, it suggests that prazosin/doxazosin-mediated mTORC1 inhibition, and subsequently induction of autophagy, occurs downstream or independently of Akt-mediated regulation of mTORC1. Prior literature is consistent with these findings, demonstrating that the induction of autophagy was associated with either inhibition or activation of Akt which was cell type specific (Sarbassov et al., 2006) In this study, Akt phosphorylation remained unchanged in PC-3 cells, suggesting prazosin- and doxazosin-induced autophagy also occurs independently of Akt in PC-3 cells.

Several Akt-dependent mechanisms are known to regulate mTOR activity, including the Ras/MEK/ERK signaling cascade, phospholipase D/phosphatidic acid signaling, and of particular interest, the energy-sensing AMPK. A previous study demonstrating prazosin-induced autophagic cytotoxicity in rodent cardiac cells, reported increased activation of AMPK, which correlated with decreased mTORC1 and p70S6K activation (Yang et al., 2011a). AMPK is regulated by two primary mechanisms, LKB1 or calcium/calmodulin-dependent protein kinase beta (CaMKKβ) (Mihaylova and Shaw, 2011, Gormand et al., 2011). In response to energy depletion or genotoxic stress, LKB1 acts on AMPK to modulate the activity of many targets, including the inhibition of mTORC1, and in turn, induction of autophagy. In contrast, AMPK is also responsive to increases in intracellular calcium through actions of CaMKKβ (Kishi et al., 2000), and has the potential to promote AMPK-dependent inhibition of mTORC1. However, direct evidence of this has yet to be reported in the literature. The LKB1/AMPK pathway is known to crosstalk with the mTORC1 signaling pathway at several points (Mihaylova

and Shaw, 2011), including the activation of tuberous sclerosis protein (TSC)1/2 and phosphorylation of raptor (Gwinn et al., 2008, Lee et al., 2010a) contributing to mTORC1 inhibition and induction of autophagy. Taken together with the previous (Chapter 3) and present findings, it is proposed that doxazosin and prazosin-induced autophagy occurs via the AMPK/mTORC1 signaling axis in PC-3 and LNCaP prostate cancer cells. However, precisely how these drugs induce AMPK activation is unclear. Early reports suggest these drugs do not affect intracellular ATP levels (Gordon et al., 1991), which suggest AMPK becomes activated by mechanisms other than energy depletion. As mentioned previously, genotoxic stress and DNA damage also promote activation of AMPK signaling activity. Interestingly, prazosin (≥10 μM) was previously associated with increased DNA double strand breaks following acute 1 h exposure (Lin et al., 2007). Likewise, doxazosin was found to directly interact with DNA to down-regulate genes associated with DNA repair after 24 h drug exposure (Arencibia et al., 2005). Taken together, this suggests that AMPK may be activated as a consequence of doxazosin/prazosin-mediated DNA interactions and damage stress.

While autophagy appears to be an important mediator of antagonist-induced PC-3 cytotoxicity, it is clear from findings in Chapter 3 that autophagy does not contribute to toxicity in LNCaP cells, and instead is primarily regulated by apoptotic mechanisms. Furthermore, it is unknown whether autophagy alone (in PC-3 cells), or the autophagy-independent effects of mTORC1 inhibition, such as suppression of p70S6K, underlie doxazosin and prazosin toxicity. In previous, but separate, studies it was found that prazosin treatment resulted in reduction of mTORC1 and p70S6K activity (Yang et al., 2011a), and direct or indirect inhibition of p70S6K had anti-proliferative effects in cancer cell lines (Doscas et al., 2014, Kyou Kwon et al., 2014). Overall, the cytotoxic effects of p70S6K inhibition are likely mediated by the loss of cell growth and survival signaling, including but not limited to loss of p70S6K-mediated cell motility and chemotaxis, suppression of EEF2 and rbS6 mediated translation and restoration of BAD (pro-apoptotic protein) activity (Ip et al., 2011, Zhang et al., 2013). In addition, p70S6K and 4EBP1 jointly promote the protein synthesis of the tumourigenesis-related protein, HIF-1α, in an oxygen-independent manner. Although supported by previous findings (Keledjian and Kyprianou, 2003, Park et al., 2014a), the modest reduction in HIF-1α levels in the present study is unlikely to underlie doxazosin/prazosin-induced toxicity

by itself, but provides further support for the doxazosin/prazosin-mediated suppression of mTORC1 signaling cascade.

P27 SIGNALING PATHWAY

In general, p27 activity is regulated by site-specific phosphorylation resulting in degradation, inhibition of nuclear translocation, or inhibition of cell cycle progression (Lee and Kim, 2009). Appropriately phosphorylated-p27 inhibits the cell cycle, through inhibition of cyclin (D, E):CDK(2, 4, 5) complex preventing hyper-phosphorylation and inactivation of the cell cycle inhibitor, retinoblastoma protein (Rb). In the presence of doxazosin and prazosin, levels of the cell cycle inhibitor, p27 (KIP1) were found to be enhanced in LNCaP cells. These findings are supported by previous literature showing increases in p27(KIP1) expression and corresponding G1/S cell cycle arrest following doxazosin and terazosin treatment (Kintscher et al., 2000b, Xu et al., 2003b). Likewise, these drugs have also been shown to prevent inhibition of the p27-regulated cell cycle inhibitor Rb in various cell lines (Kintscher et al., 2000b, Austin et al., 2004).

In further support, p27 is also known to be negatively regulated indirectly by mTORC1. The downstream substrate of mTORC1, SGK1, has been previously reported to promote instability and suppress nuclear translocation of p27 (Hong et al., 2008), permitting progression of the cell cycle. In the absence of mTORC1 signaling, as in the proposed model of doxazosin/prazosin-induced cytotoxicity, loss of SGK1 activity would allow p27 to persist, leading to cell cycle arrest. Interestingly, AMPK signaling may also impact levels of p27, however with potentially unconventional outcomes. The actions of AMPK have been reported to follow the classical pathway by increasing p27, decreasing Rb and in turn induce cell cycle arrest and toxicity (Ben Sahra et al., 2008). In contrast, AMPK reportedly promotes cytosolic accumulation of p27, via phosphorylation-site-dependent (Thr198) inhibition of nuclear translocation, promoting cell cycle progression, inhibiting apoptosis and parallel induction of autophagy (Short et al., 2008, Liang et al., 2007). However, it is unknown whether p27 is able to directly activate autophagy mechanisms via interaction with autophagy-related proteins or if it occurs passively through the AMPK/mTORC1/Ulk1 autophagy signaling axis.

p38 MAPK SIGNALING PATHWAY

The p38 MAPK protein family consists of four isoforms including the well documented alpha and beta, and the lesser known gamma and sigma. A plethora of stimuli result in p38 MAPK activation, including growth factors, cellular stress, pro-inflammatory cytokines, oxidative stress and DNA damage (Igea and Nebreda, 2015). In neoplasms, p38α is specifically known to act as either a tumour-promoter or –suppressor, which is likely to be dependent on stimuli and cell type (Igea and Nebreda, 2015). In the context of apoptosis, p38 MAPK alters activity of anti-apoptotic and pro-apoptotic Bcl-2 family to tip the scale towards cell death, as well as congruently promoting the downregulation of survival pathways (Cai et al., 2006). Specifically, p38α activity was found to promote prostate cancer cell apoptosis to various cytotoxic agents (Gao et al., 2014, Honisch et al., 2014) by facilitating the caspase activation cascade (Al-Azayzih et al., 2012). Likewise, doxazosin and prazosin were able to increase p38α phosphorylation to some degree in both prostate cancer cell lines. However, only prazosin treatment yielded a statistically significant increase p38α in both cell lines. Taken together with previous reports of quinazoline induced p38 MAPK-dependent cytotoxicity (Yang et al., 2009), it can be inferred that phosphorylation and activation of p38α isoform underlies prazosin, and potentially, doxazosin-induced apoptosis. One proposed mechanism underlying increased activation of p38α is by doxazosin/prazosin-induced DNA damage. It is known that these drugs either indirectly or directly result in DNA damage (Arencibia et al., 2005, Lin et al., 2007) and interestingly, the DNA damage response element Gadd45a and activator of p38α (Jehle et al., 2012, Salerno et al., 2012), was previously shown to be deregulated following treatment with doxazosin in LNCaP cells (Arencibia et al., 2005).

EPHA2-MEDIATED ANOIKIS

Mechanisms regulating anoikis, or detachment-induced apoptotic cell death (see current chapter Introduction for review), are commonly suppressed in many cancers, including prostate cancer, leading to increased metastatic potential. Doxazosin, but not prazosin, has been reported to restore anoikis-mediated cell death mechanisms, and has been

evidenced primarily through the suppression of focal adhesion kinase (FAK) (Walden et al., 2004, Sakamoto and Kyprianou, 2010, Sakamoto et al., 2011). In a study conducted by Petty and colleagues (2012), it was previously demonstrated that doxazosin was a small molecule agonist for the EphA2 receptor, which was found to be a significant mediator of prazosin-induced anoikis-mediated apoptotic cell death. Consistent with these previous findings, short doxazosin treatment resulted in a significant increase in EphA2 activity and subsequent cell rounding and detachment, which are known hallmarks of EphA2-mediated anoikis. Likewise, inhibition of EphA2 activity partially protected cells from doxaozin-induced cytotoxicity. Despite a similar quinazoline-based structure, the current findings suggest that prazosin toxicity occurs independently of EphA2. The difference between these drugs is likely attributed to minor, yet important, conformational differences between doxazosin and prazosin chemical structure. Surprisingly, interactions between the EphA2 receptor and doxazosin are likely to involve the methoxy groups as well as the benzodioxin and carbonyl groups (Petty et al., 2012) (**Figure 4.19**), and not the quinazoline structure as originally proposed (Benning and Kyprianou, 2002). In addition to prostate cancer, doxazosin-induced EphA2-mediated cytotoxicity and apoptosis, is also known to occur in cardiomyocytes (Jehle et al., 2012).

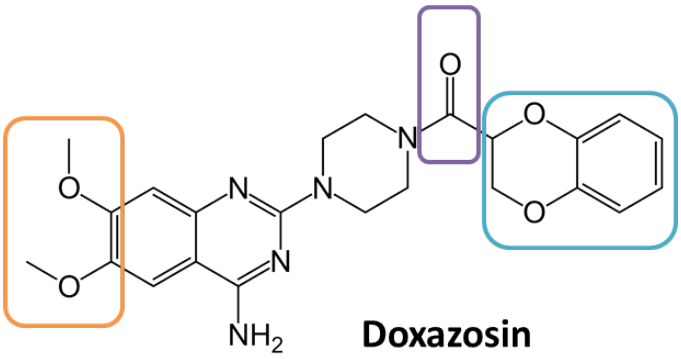


Figure 4.19. Chemical structure of doxazosin highlighting methoxy (orange), carbonyl (purple) and benzodioxin groups (blue).

CITED-2, ADAMTS1 AND SRC

In addition to doxazosin and/or prazosin responsive targets previously discussed, Cited-2, ADAMTS1 and Src were found to be altered following treatment with these drugs. While this is the first report of significant expressional changes in these target proteins in response to cytotoxic drugs investigated, the biological implications of these findings remain to be experimentally elucidated.

CITED-2

Cited-2 (Cbp/p300-interacting transactivator 2) acts to modify gene transcription via interactions with transcription factors as opposed to binding DNA directly. Activation of Cited-2 is regulated by various cytokines and growth factors such as interleukins, interferon-gamma, TGF-α/EGFR, PDGF and insulin, while TGF-beta inhibits Cited-2 activity (Chou et al., 2006, Chou and Yang, 2006, Chou et al., 2012), As its name suggests, activated Cited-2 binds p300/CBP, as well as several other transcription factors including Smad7, TFAP2, Lhx2 and HIF-1α to modify their transcriptional activity (Chou et al., 2012, Chou and Yang, 2006). The present study found doxazosin/prazosin suppressed Cited-2 expression in AR-positive LNCaP but not significantly in AR-negative PC-3 cells. In support of these findings, the negative regulator of Cited-2, TGF-β, was previously reported to be involved in doxazosin-mediated toxicity (Partin et al., 2003), providing a potential mechanism contributing to the observed inhibition of Cited-2 expression by doxazosin and prazosin. TGF-β is known to primarily function as a tumour suppressor via induction of apoptotic mechanisms; however pro-oncogenic roles of TGF-β have also been reported (Horbelt et al., 2012). While cytotoxic doxazosin/prazosin-induced TGF-β/Smad signaling and consequent suppression of Cited-2 is likely to promote apoptosis, it cannot be ascertained for certain from the current literature as TGF-β-dependence has not yet been established. It is acknowledged that tamsulosin had the most consistent suppression of Cited-2 expression. At this time,

ADAMTS1

ADAMTS1 is a member of the ADAMTS family of proteases, which is known to have opposing roles in regulating tumour vascularisation; acting to promote or inhibit angiogenesis mechanisms (Sun et al., 2015b). In general, whether ADAMTS1 has pro- or anti-angiogenic effects is understood to be influenced by the tumour microenvironment, proteolytic cleavage of thrombospondin (TSP)1 and/or TSP2, and interactions with VEGFR. Briefly, the presence or absence of heparin in the tumour microenvironment dictates whether ADAMTS1 exists as the full-length or truncated form, respectively, mediated by blocking ADAMTS1 cleavage site. Full length ADAMTS1 is reported to have pro-angiogenic actions, which has been suggested to involve heparin-dependent ADAMTS1-VEGFR interaction. Additionally, ADAMTS1-VEGFR may also have anti-proliferative effects, by inhibiting ERK1/2 survival signaling. In contrast, shortened ADAMTS1 has anti-angiogenic effects, particularly through interactions with the well-known angiogenesis inhibitors TSP 1/2. While these molecules are able to regulated angiogenesis, truncated ADAMTS1 binds and cleaves TSPs, resulting in the release of the highly potent and soluble anti-angiogenic molecule, 3TSR from the extracellular matrix (Bak and Weerapana, 2015).

Both human prostate tissues and cancer cell lines have been shown to differentially express members of the ADAMTS family. In particular, levels of ADAMTS1 are known to be inversely correlated with tumourigenic phenotype in prostate cancer. This relationship has been demonstrated in both clinical and laboratory settings. Specifically, it has been reported that levels of ADAMTS1 are decreased within prostate tumours compared to non-malignant epithelial cells (Gustavsson et al., 2009). Furthermore, the same study identified lower expression of ADAMTS1 in castrate-resistant disease compared to hormone-sensitive tumours. These clinical findings are supported by previous studies demonstrating that established *in vivo* prostate cancer cell tumours contain lower expressional levels of ADAMTS compared to primary BPH cells isolated from resected human prostates and *in vitro* prostate cancer cell lines (LNCaP, and PC-3) (Cross et al., 2005). While the precise contribution of ADAMTS to tumourigenesis has yet to be clearly defined, one study elucidated that ADAMTS regulates the morphology of tumour vasculature leading to reduced tumour growth and establishment (Gustavsson et al., 2010). Together, these prior findings are consistent

with the present study that identified lower basal levels of ADAMTS1 in the highly metastatic and castrate-resistant (AR-negative) PC-3 cell compared to the modestly tumorigenic AR-positive LNCaP cells. Interestingly, doxazosin and prazosin were found to have differential and cell type-dependent effects on ADAMTS1 expression. ADAMTS1 expression was enhanced in PC-3 cells, which is line with previous reports of decreased metastatic and angiogenic potential of prostate cancer cells treated with these drugs (Chiang et al., 2005, Park et al., 2014a). In contrast, conflicting findings were found in LNCaP cells where these cytotoxic drugs decreased expression of ADAMTS1. While the relationship is unclear, one study reported that suppression of ADAMTS1 in these cells was associated with smaller blood vessel and decreased tumour establishment (Gustavsson et al., 2010). In further support of these apparently conflicting findings, LNCaP prostate cancer cells challenged with TGF-β displayed decreased expression of ADAMTS1; whereas TGF-β had no effect on PC-3 ADAMTS1 levels (Cross et al., 2005). The authors proposed that TGF-β enhanced expression of the negative regulator of ADAMTS1, tissue inhibitor of metalloproteinase (TIMP) 3. TIMP3 has recently been reported to have important implications in tumourigenesis in an animal model, where loss of TIMP3 resulted in increased tumour growth and micro-vessel density (Adissu et al., 2015). As previously mentioned, doxazosin was found to increase TGF-β expression in prostate cancer cell lines (Partin et al., 2003), and although currently unknown, it is assumed prazosin may possess a similar effect on TGF-β. It can be inferred that in the present study doxazosin/prazosin-mediated TGF-β expression indirectly downregulates ADAMTS1 by upregulating TIMP3 in LNCaP cells. While these drugs appear to have a tumour-promoting effect on ADAMTS1 in LNCaP cells, the plethora of apoptotic mechanisms induced by doxazosin/prazosin is likely to override this effect. However, the downregulation of ADAMTS1 in these cells may also be associated with decreased tumour establishment (Gustavsson et al., 2010) and remains to be fully elucidated.

In summary, the combination of current findings in PC-3 cells and previous literature suggest that doxazosin/prazosin increase ADAMTS1 by an unknown mechanism, and subsequently contribute to reduced tumourigenesis through ADAMTS1-mediated anti-angiogenesis effects. Conversely, these drugs reduced ADAMTS expression in LNCaP cells, which is proposed to occur via TNGβ-mediated upregulation TIMP3.

Src

Src is a non-receptor tyrosine kinase that belongs to the Src family of kinases (SFK) along with Fyn, Lyn, Yes, Blk, Lck, Hck, Fgr and Yrk (Varkaris et al., 2014). The transition to a cancerous phenotype is often associated with increased activity of the SFKs, as they often sit downstream of aberrantly hyperactive RTKs that possess pro-oncogenic activity, thereby promoting survival, proliferation and metastasis (Varkaris et al., 2014). An abundant number of SFK activators have been identified, which include, but are not limited to; growth factor receptors (such as PDGF, EGFR, FGFR, IGFR and HGFR), β-adrenergic receptor, integrin/FAK, beta catenin, RANKL, ILR and the androgen receptor (Varkaris et al., 2014). It can be reasoned that the modest, yet insignificant, increase in the phosphorylation status of growth factor receptors such as EGFR and FGFRs following treatment with doxazosin and prazosin may facilitate elevated Src phosphorylation. However, this may only be true for LNCaP cells where increases in growth factor receptors and Src phosphorylation were greatest. Enhanced activity of EGFR and FGFR are known to participate in pro-oncogenic signaling, leading to increased prostate cancer survival (Yarden, 2001, Feng et al., 2012, Ojemuyiwa et al., 2014). However, due to the lack of statistically significant effects of doxazosin/prazosin on these growth factor receptors, further investigation is required to evaluate the relationship between Src and EGFR/FGFR in prostate cancer cells.

The absence of appreciable doxazosin- or prazosin-mediated suppression of EGFR, ERK1/2, NFKB or VEGFR phosphorylation observed here (Hui et al., 2008, Park et al., 2014a), may be due in part to transient activation or expression of these kinases in response to cytotoxic doxazosin/prazosin. For example, in previous studies doxazosin-mediated suppression of EGFR and ERK1/2 was assayed following an acute thirty-minute treatment, whereas the present study evaluated the sustained effects (24 h) of these drugs on various protein and RTK targets. Likewise, doxazosin-mediated EphA2 phosphorylation was found to be unchanged at 24 h (array data), which was later demonstrated to be maximally phosphorylated following 1 h treatment (ELISA data) and is consistent with previous reports of transient EphA2 activity (Petty et al., 2012). While many of proteins targets act early in the cytotoxic signalling, such as EGFR, are likely to display similar transient activation. However, it was the intention of this study to evaluate the molecular mechanisms underlying the more therapeutically relevant

treatment durations (24 h), as opposed to acute (< 24 h) antagonist treatment of prostate cancer cells. Likewise, in a preliminary study it was found that acute treatment with doxazosin or prazosin (1 h, 100 μM) is only modestly cytotoxic following 24 h drug-free recovery, suggesting that transient suppression or activation of cell death signal transduction pathways by doxazosin/prazosin does not fully regulate the cytotoxic actions of these drugs. It is acknowledged that the present findings are indeed a snapshot of protein expression and phosphorylation changes in response to 24 h exposure to doxazosin, prazosin or tamsulosin. It would be of significant interest to investigate identified protein targets in time-lapse experiments.

SUMMARY

The present study expands the current knowledge of molecular mechanisms contributing to the doxazosin and prazosin-induced autophagic and apoptotic mechanisms in AR-positive and AR-negative prostate cancer cells. These findings suggest inhibition of mTOR signaling (independent of Akt), and subsequent activation and that inhibition of the p70S6K/rbS6 axes, may play an important role in doxazosin and/or prazosin cytotoxicity following 24 h exposure. Additionally, the current findings are consistent with previous findings indicating that EphA2 activity mediates anoikis response to doxazosin, which appears to be drug-specific, with prazosin having no effect on EphA2 phosphorylation in acute or 24 h treatment settings. In addition, signaling mechanisms involving p38α MAPK and p27 also appear to contribute to doxazosin and prazosin-induced prostate cancer cell death. However, the contribution of these protein targets to doxazosin/prazosin-mediated cell death requires further investigation. Moreover, this is the first report of doxazosin and prazosin induced expressional/phosphorylational changes in ADAMTS1, Cited-2 and Src. However, their role in mediating cell survival or death in response to these drugs remain to be fully explored.

CHAPTER 5:
EFFECTS OF PRAZOSIN IN COMBINATION WITH IRRADIATION ON PROSTATE CANCER CELLS

5.1 INTRODUCTION

RADIATION THERAPY

As discussed in Chapter 1, radiotherapy is a standard treatment used to treat local or locally advanced prostate cancer by delivering lethal doses of ionising-radiation to the tumour. In most cases, the cytotoxic effect of irradiation is primarily attributed to its ability to indirectly induce DNA damage via oxygen-dependent production of free radicals **(Figure 5.1**). Second to this, when absorbed by DNA, irradiation causes direct DNA damage. In both cases, the damaged DNA will trigger cell death in the affected cell. A third cytotoxic mechanism of radiotherapy known as the radiation-induced bystander effect (RIBE) also exists. While the precise mechanism is still under investigation, it is currently defined as the phenomenon where irradiated cells indirectly induce various biological effects, such as abnormal chromosomal modification, formation of ROS and apoptosis of nearby un-irradiated cells (Hatzi et al., 2015). Interestingly, there is recent evidence that a pro-survival or "rescue" bystander effect exists, where un-irradiated cells may provide a survival benefit or "rescue" irradiated cells by pro-survival intercellular signalling. Similarly, mechanisms contributing to this rescue-bystander effect are not fully elucidated (Lam et al., 2015).

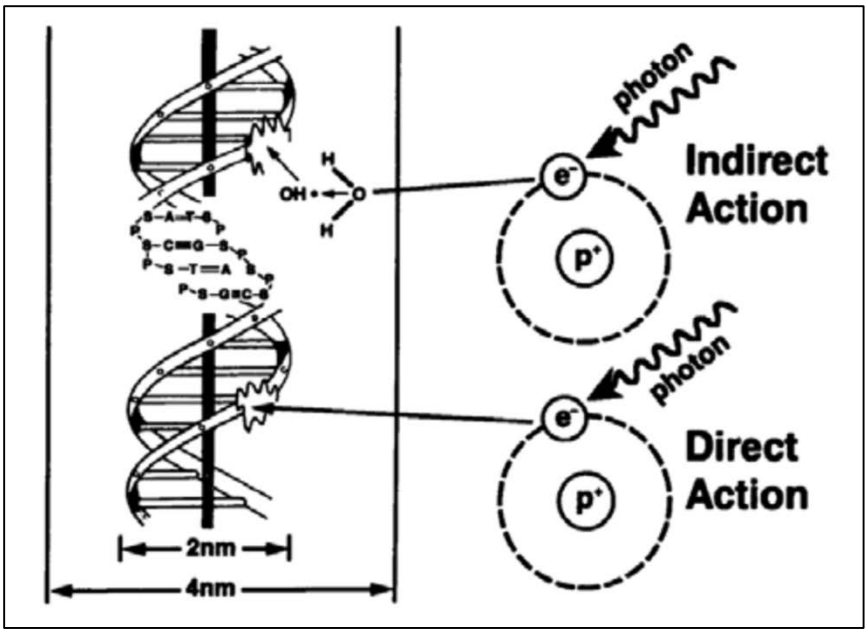


Figure 5.1. The indirect and direct cytotoxic actions of irradiation (Hall, 2006).

RADIORESISTANCE

Approximately one-third of patients will experience biochemical recurrence following definitive radiotherapy (Zumsteg et al., 2015), with approximately 30% of these patients developing clinical metastasis (Pound et al., 1999). In many cases, radiotherapy treatment failure is attributed to either innate or acquired tumour radioresistance by many mechanisms (Chang et al., 2014). However, tumour hypoxia is one of the most important drivers of radioresistance, and therefore, will be the primary focus of this literature review.

TUMOUR HYPOXIA

Hypoxia is generally defined as oxygen levels below typical physiological levels (20-70 mm Hg), ranging from 5-15 to 25- 30 mmHg (Rademakers et al., 2008); however a specific hypoxic threshold remains to be defined. Tumour hypoxia is a common feature of many tumors and occurs as a direct result of abnormal or insufficient vasculature as a consequence of rapid tumour proliferation. However, hypoxia is not only a by-product,

but also aggressively drives tumourigenesis and malignancy. Therefore, hypoxia may be the "Achilles heel" of many cancers and targeting hypoxia may be an effective treatment strategy.

Varying degrees of hypoxia exist from low, moderate to high levels, which are distributed heterogeneously throughout the tumour. Regional differences are commonly attributed to two types of hypoxia, chronic or acute/cycling hypoxia. Chronic hypoxia is stable and its existence is highly predictable, occurring just outside the limits of oxygen diffusion (approximately 70 μm, or 10 cells) from local blood supply (**Figure 5.2**). Cancer cells residing >100 μm from blood vessels are in a state of anoxia, or total lack of oxygen, and subsequently undergo necrotic cell death. In contrast, the manifestation of acute or cycling tumour hypoxia is unpredictable, but is thought to be influence by several factors including imbalanced angiogenesis and changes in blood flow, possibly via blood vessel occlusion (Harada, 2011).

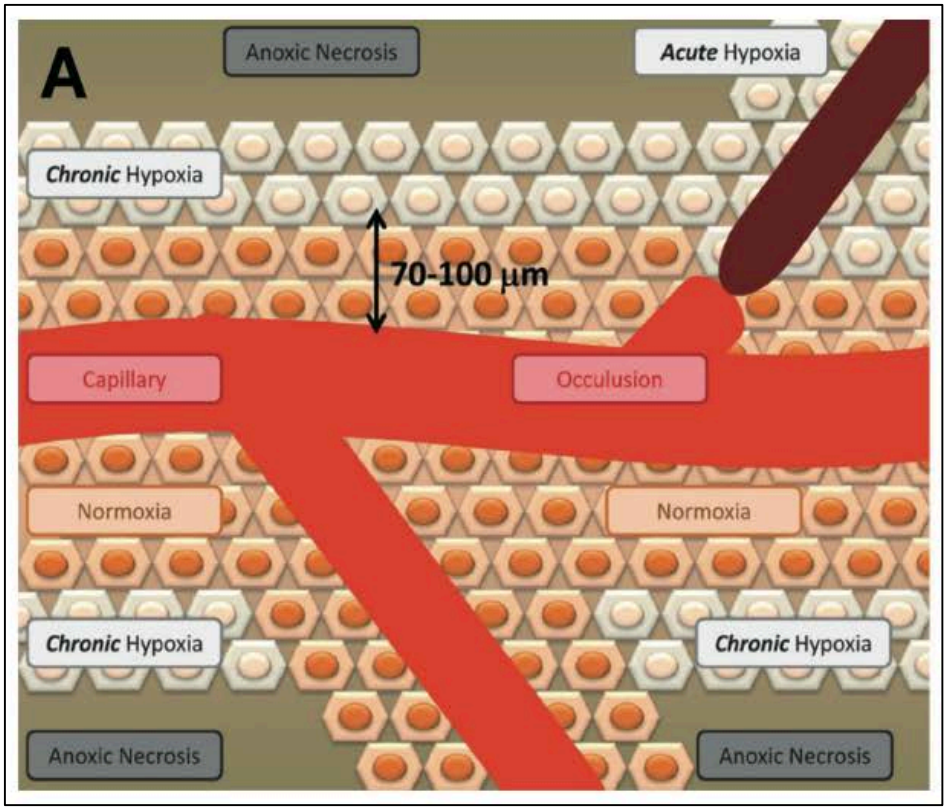


Figure 5.2. Relationship between blood vessels and tumour hypoxia. Illustration indicating locations of acute and chronic regions of hypoxia (Harada, 2011). Figure reprinted with permission from copyright holder.

CLINICAL SIGNIFICANCE OF TUMOUR HYPOXIA

While extreme hypoxia and anoxia (< 1 mmHg) is disadvantageous to tumour growth, low and moderate levels of hypoxia contribute to disease progression and treatment failure via two intertwined mechanisms. Firstly, hypoxia promotes tumourigenesis and angiogenesis, and secondly, by reducing anticancer treatment efficacy (Jans et al., 2010, Milosevic et al., 2012, Ranasinghe et al., 2013). Of particular importance, the microenvironment of cyclical (acute) hypoxic regions is believed to further promote tumourigenesis, by exacerbating pro-proliferation inflammation (Tellier et al., 2015) and enhancing factors contributing to cancer cell survival, invasion and metastasis (Dai et al., 2011). Additionally, acute hypoxia was found to have a greater effect on the metastatic potential of tumour cells, compared to chronic hypoxia (Rofstad et al., 2007).

Tumour hypoxia is well documented to be a prognostic marker for poor survival in many cancers including cervical, neck and neck, pancreatic and prostate cancer (Dhani et al., 2015). In the case of prostate cancer, localised disease can be treated successfully by surgery or radiotherapy. However, nearly 25 - 30% of men will experience recurrence. Tumour hypoxia is likely to be responsible for a significant portion of prostate cancer treatment failures. One study uncovered a greater risk for biochemical relapse in men with greater levels of tumour hypoxia at diagnosis (Milosevic et al., 2012).

Hypoxia-inducible factor 1alpha

The cell response to hypoxia is predominantly regulated by the transcription factor, hypoxia inducible factor 1 alpha (HIF-1α). HIF-1 consists of two subunits, alpha and beta. Unlike the stable beta subunit, the alpha subunit is negatively regulated by the presence of molecular oxygen, and plays a critical role in the transcriptional activity of HIF-1α (discussed below).

Oxygen-dependent regulation of HIF-1α

In normoxic conditions, oxygen promotes the destabilization of HIF-1α by the enzymatic hydroxylation of key residues by HIF-specific prolyl-hydroxylases (PHD). In turn, this triggers von Hippel-Lindau (pVHL) complex-mediated ubiquination and downstream proteosomal degradation (Huang et al., 1998) (**Figure 5.3**). Additionally, transcriptional activity of HIF-1α is negatively regulated by factor inhibiting HIF1-α (FIH-1). In normoxic conditions, FIH-1 inhibits HIF-1α by oxygen-dependent hydroxylation of key co-factor binding sites, preventing HIF-1α from binding DNA and subsequently inhibiting gene transcription (Chen et al., 2015, Masoud and Li, 2015). In hypoxic environments HIF-1α is not recognized by pVHL complex, escaping pVHL-mediated sequestration, allowing HIF1α to accumulate in the cytoplasm. Stabilised HIF-1α dimerises with HIF-1β and translocates to the nucleus where the HIF-1α and -1β binding pair interacts with various cofactors to trigger expression of its gene targets to promote cell survival, migration, homeostasis, angiogenesis and anaerobic metabolism (Dewhirst et al., 2008).

Of these targets, the angiogenic vascular endothelial growth factor (VEGF) is arguably the most documented. Hypoxia and angiogenesis are believed to participate in a positive feedback loop, where hypoxia induced HIF-1α activity promotes VEGF-mediated angiogenesis, resulting in further tumour proliferation, hypoxia, and angiogenesis. Likewise, hypoxia is considered the "angiogenic switch" enabling the transition of low-grade tumours to a more aggressive phenotype (Harada, 2011).

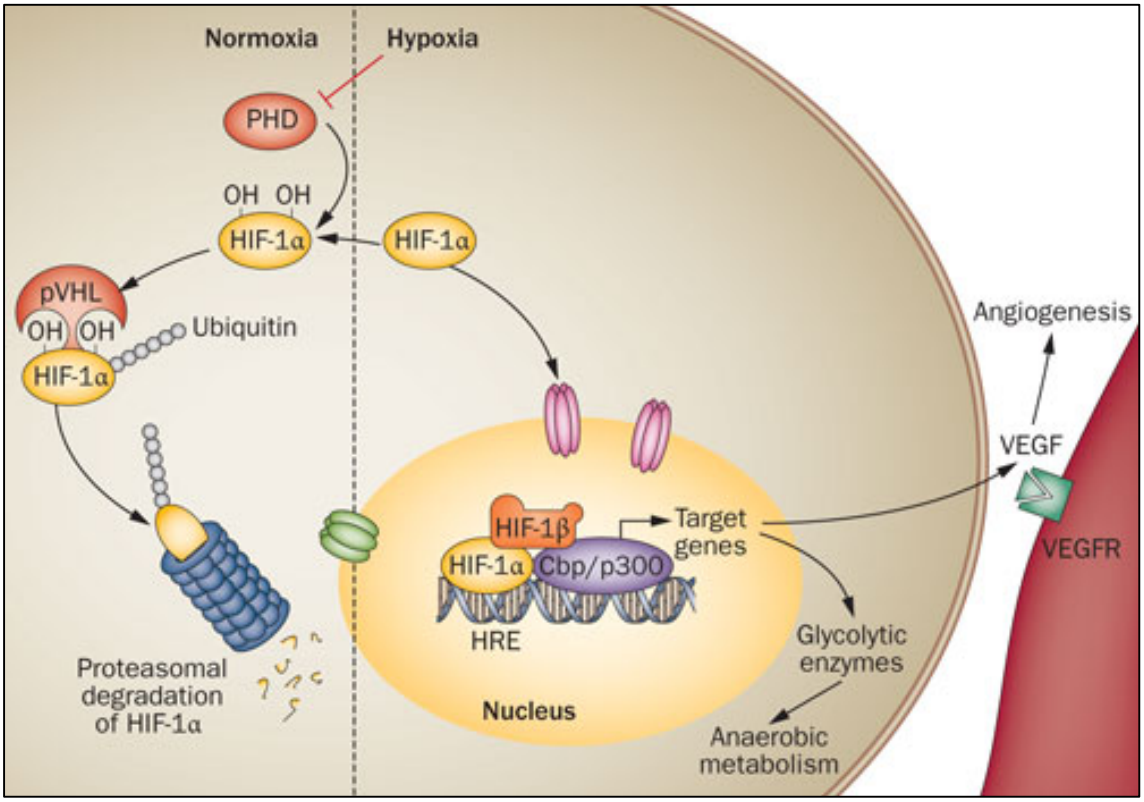


Figure 5.3. Oxygen-dependent regulation of HIF-1α (Maes et al., 2012). Figure reprinted with permission from copyright holder.

Hypoxia-independent regulation of HIF1α

Hypoxia-independent regulation of HIF-1α activity and subsequently HIF-1α-mediated tumourigenesis has also been described (Bilton and Booker, 2003). Various cytokines and growth factors including, insulin, insulin-like growth factor (IGF), EGF, platelet derived growth factor (PDGF), TGF-1β, tumour necrosis factor alpha (TNFα), and interleukin-1 beta (IL-1β) have all been shown to increase expressional levels of HIF-1α, primarily by stimulation of the PI3K/Akt/mTOR pathway (Feldser et al., 1999, Richard et al., 2000, Zhong et al., 2000, Zundel et al., 2000)

Aberrant receptor activation, either by ligand-dependent or independent mechanisms and downstream signaling also promotes the expression and transcriptional activity of HIF-1α. The signal transduction pathways involved in HIF-1α regulation include

Ras/MEK/MAPK and PI3K/Akt/mTOR cascades (Treins et al., 2002). Furthermore, activation of these pathways may co-operate with hypoxia-mediated HIF-1α stabilisation to enhance accumulation and activation of HIF-1α (Minet et al., 2000).

In prostate cancer, the androgen receptor has been implicated as a regulator of HIF-1α activation. Furthermore, correlational evidence suggests that AR-signaling may also regulate VEGF-mediated tumourigenesis via HIF1-dependent mechanisms (Boddy et al., 2005).

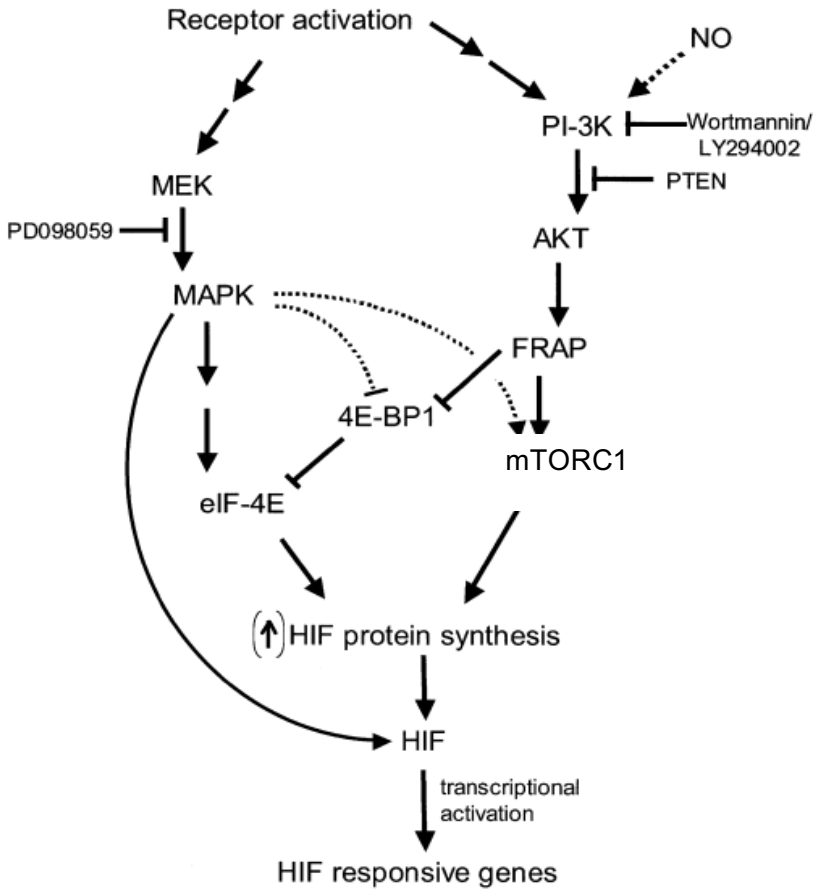


Figure 5.4. Receptor-mediated activation of HIF-1α. Figure from Bilton and Brooker, (2003), with slight modification. Original figure reprinted with permission from copyright holder.

CLINICAL SIGNIFICANCE OF HIF-1A EXPRESSION

In prostate cancer, HIF-1α level is positively correlated with Gleason score, suggesting expression of HIF-1α may enhance disease aggression, metastasis (Makarewicz et al., 2011), and in turn, poor prognostic outcomes. Additionally, HIF-1α and related regulatory proteins may predict treatment failure in men opting to receive radical prostatectomy or ADT (Jans et al., 2010). In men undergoing ADT, inhibition of HIF-1α significantly improved disease free survival by four years, and reduced incidence of developing castrate-resistant prostate cancer by 71% (Ranasinghe et al., 2013). However, it is unknown whether HIF-1α levels in these studies is driven by tumour hypoxia, occur by hypoxia-independent mechanisms, or whether both hypoxia-dependent and independent activation of HIF-1α act to promote disease progression.

HYPOXIA-MEDIATED RADIORESISTANCE

Hypoxia-mediated tumour radioresistance is a significant problem affecting the oncological and survival outcomes patients diagnosed with cancer (Milosevic et al., 2012). Ionizing radiation relies on the "oxygen enhancement effect" where well-oxygenated areas of tumours are far more radiosensitive than hypoxic areas. This effect is believed to occur through two independent pathways. Chemically, oxygen contributes to irradiation-induced accumulation of free radicals, which in turn causes irreversible damage to the DNA and ultimately cell death. In contrast, irradiation is less effective in the absence of oxygen, resulting in fewer free radicals, and in turn, less DNA damage. Furthermore, as previously discussed, a hypoxic environment alters cellular signaling pathways by increasing intracellular HIF-1α, thereby promoting adaptation and survival. As a consequence, hypoxic cancer cells are often able to repair incidental DNA damage allowing the cells to continue to proliferate. While radiotherapy is understood to promote the re-oxygenation of tumours and consequently reduce HIF-1α activity, over the long term HIF-1α remains active promoting tumour angiogenesis, proliferation, and metastasis.

RADIOSENSITISERS

To date, several radiosensitising techniques and drugs have been studied with only a handful reaching clinical trials. Strategies such as delivering oxygen to the hypoxic tumour, hypoxia-selective agents (including oxygen mimics and bio-reductive prodrugs), VEGFR and HIF-1α inhibitors, gene therapies, and enhanced radiotherapy delivery techniques are currently being investigated (Harada, 2011).

Enhanced oxygen delivery

In theory, the most direct method to overcoming hypoxia-mediated radioresistance is by increasing oxygen to the tumour. Some of these methods include hyperbaric oxygen therapy, combination of carbogen and nicotinamide, and hemoglobin modification (as reviewed by (Yoshimura et al., 2013). Unfortunately, the aforementioned therapies have shown mixed results in clinical trials and have not yet been translated into modern radiotherapy regimens (Fletcher et al., 1977, Haffty et al., 1999, Janssens et al., 2012).

Hypoxia-selective cytotoxics

Bio-reductive drugs undergo biotransformation by oxidoreductase, which yields a highly toxic metabolite. Since this process is inhibited and reversed by the presence of oxygen, the cytotoxic effects are unmasked in hypoxic regions, leaving normoxic cells unharmed. Currently, there are five classes of bio-reductive drugs, nitro(hetero)cyclic (nitroimidazole-derivatives) compounds, aromatic N-oxides, quinones and metal complexes. To date, several bioreductive drugs including the misonidazole and nimorazole (oxygen-mimics) PR-104, TH-302, EO9 and tirapazamine have been or are currently being trialed either as single agents or in combination with other anti-cancer therapies (Guise et al., 2014). While bio-reductive prodrugs and radiotherapy seem to be complementary therapies, none of the above agents, except for tirapazamine, have been clinically investigated in combination with radiotherapy to overcome hypoxia-mediated radioresistance. The outcomes of a phase III clinical trial evaluating tirapazamine in combination with cisplatin and irradiation for regionally confined cervical cancer were recently published. Unfortunately, the addition of tirapazamine did not improve

oncological outcomes, compared to cisplatin and irradiation combination therapy (DiSilvestro et al., 2014).

Angiogenesis inhibitors

Anti-angiogenetic agents such as the VEGFR inhibitor sunitinib and the anti-VEGF antibody bevacizumab were shown to have a synergistic effect when combined with irradiation in cultured cells and *in vivo* (Dings et al., 2007, Hoang et al., 2012) Specifically, it was found that xenograft ovarian, melanoma and breast cancer tumours in mice displayed a more normalised vascularity when irradiation was combined with VEGFR inhibitors compared to irradiation alone (Dings et al., 2007). However, there exists a caveat. Inhibition of angiogenetic signaling may increase tumour hypoxia over the long term (Ou et al., 2009). This suggests that the radiosensitising effects of angiogenesis inhibitors may be schedule dependent and optimisation of the anti-angiogenic agent and irradiation timing may be imperative for clinical success (Ou et al., 2009, Dings et al., 2007).

HIF-1α inhibitors

As previously reviewed, HIF-1α levels, whether existing dependent or independent of hypoxia, are directly correlated to disease aggression and metastatic potential, as well as risk for treatment failure (Dai et al., 2011, Milosevic et al., 2012). Furthermore, compounding the direct radioresistant effects of hypoxia, HIF-1α enhances radioresistance through survival and adaptive signaling mechanisms (Hennessey et al., 2013). Therefore, it can be inferred that targeting HIF-1α activity may provide significant oncological and survival benefit. The currently available HIF-1α inhibitors target each step from transcription of HIF-1α mRNA, to direct inhibition of HIF-1α expression, to suppression of HIF-1α-mediate gene transcription.

Few of the HIF-1α inhibitors, such as rapamycin (sirolimus), everolimus (previously, RAD001), topotecan and YC-1 have progressed to clinical trial as part of a combination radiation therapy regimen. However, pre-clinical studies investigating the radiosensitising effects of these agents are promising.

One of the most well investigated agents, rapamycin, is an inducer of autophagy by direct inhibition of mTOR, and also acts to inhibit of HIF-1α expression indirectly via suppression of the mTOR signaling cascade. Rapamycin was found to possess anti-angiogenic effects, presumably through suppression of HIF-1α / VEGF signaling and, in turn, enhanced radiosensitivity *in vitro* (Dai et al., 2013, Seront et al., 2013). In a separate study, treatment with rapamycin prior to radiotherapy was shown to enhance efficacy by normalising tumour vasculature, promoting perfusion, and radiosensitising rhabdomyosarcoma in rodents (Myers et al., 2012). Rapamycin and similar analogues, such as everolimus, are no stranger to clinical settings, being approved for prophylaxis of organ rejection after transplant and treatment of some cancers (Australian Therapeutic Goods Administration, 2013). However, knowledge of their efficacy to overcome hypoxia- or HIF-1α-mediated radioresistance in humans is limited. A recent phase I/II clinical trial evaluated the use of rapamycin in combination with radiotherapy for rectal cancer. Overall the investigators reported positive findings, boasting a safe and biologically active profile (Buijsen et al., 2015).

Another well investigated agent, topotecan, is a chemotherapeutic with HIF-1α targeting effects (Choi et al., 2009). In advanced solid tumours, treatment with topotecan was highly effective in suppressing both HIF-1α and VEGF expression (Kummar et al., 2011) and has also been shown to be effective when combined with radiotherapy for several cancers (Ge et al., 2013, Sun et al., 2015a, Wei et al., 2015). However, whether the improved treatment efficacy is due to topotecan-induced HIF-1α modulation or via broad inhibition of DNA topoisomerases (type I) remains to be elucidated.

The multi-target HIF-1α inhibitor YC-1 inhibits both translation and stabilisation of HIF-1α (Li et al., 2008b), however, has yet to progress to clinical trials. Strong pre-clinical data supports the anti-cancer activity of YC-1 mono-therapy (Carroll et al., 2013), as well as in combination with irradiation as a hypoxic radiosensitising agent (Harada et al., 2009, Moon et al., 2009). However, one study indicates that the use of HIF-1α inhibitor such as YC-1 to overcome hypoxia radioresistance may be schedule-dependent (Harada et al., 2009). As previously mentioned, HIF-1 α is strongly associated with angiogenesis. Therefore, inhibition of HIF-1 α would hypothetically suppress blood vessel formation, reduce perfusion, and in turn, further exacerbate

tumour hypoxia. In the context of radiotherapy, this could be detrimental to treatment efficacy. In accordance, one study demonstrated that treatment with YC-1 prior to irradiation, effectively inhibited hypoxia-induced HIF-1 α accumulation but promoted enhanced tumour hypoxia via suppression of micro-vessel density in the hours leading up to irradiation, yielding poor radiobiological outcomes. In contrast, irradiation followed by YC-1 treatment inhibited post-irradiation surge in HIF-1α activity resulting in significant radiosensitising effects (Harada et al., 2009). This paradoxical effect of HIF-1α modulation by YC-1 in radiotherapy settings will require further investigations before the full magnitude of its potential can be realised.

DOXAZOSIN, PRAZOZIN AND RADIATION THERAPY

In addition to hypertension and BPH, α1-adrenoceptor antagonists are frequently used in radiotherapy either prophylactically or concomitantly to combat the treatment-associated LUTS (Zelefsky et al., 1999). However, the effect of these drugs on solid tumours in combination with radiotherapy is largely unknown. The findings presented in Chapter 4 suggest that doxazosin and prazosin inhibit mTORC1 as evidenced by the inhibition of the downstream signaling effector rbS6. While at this time only an inference can be made, this suggests that prazosin and doxazosin may have similar actions on HIF-1α expression as the mTOR inhibitors rapamycin (sirolimus) and everolimus. A search of the literature resulted in a single report investigating the potential radiosensitising actions of doxazosin and terazosin in castrate-resistant (AR-negative) PC-3 prostate cancer cells. While the investigators reported no synergistic activity after concomitant treatment with doxazosin or terazosin plus irradiation (3 Gy), irradiation was found to have schedule-dependent radiosensitising actions when combined with doxazosin or terazosin (Cuellar et al., 2002). However, the investigators only examined immediate effect (24 h), leaving a gap in knowledge regarding long term effects of radiotherapy in the presence of these drugs. Likewise, the effect of these drugs in hypoxic conditions mimicking the biology of solid tumours is entirely unknown.

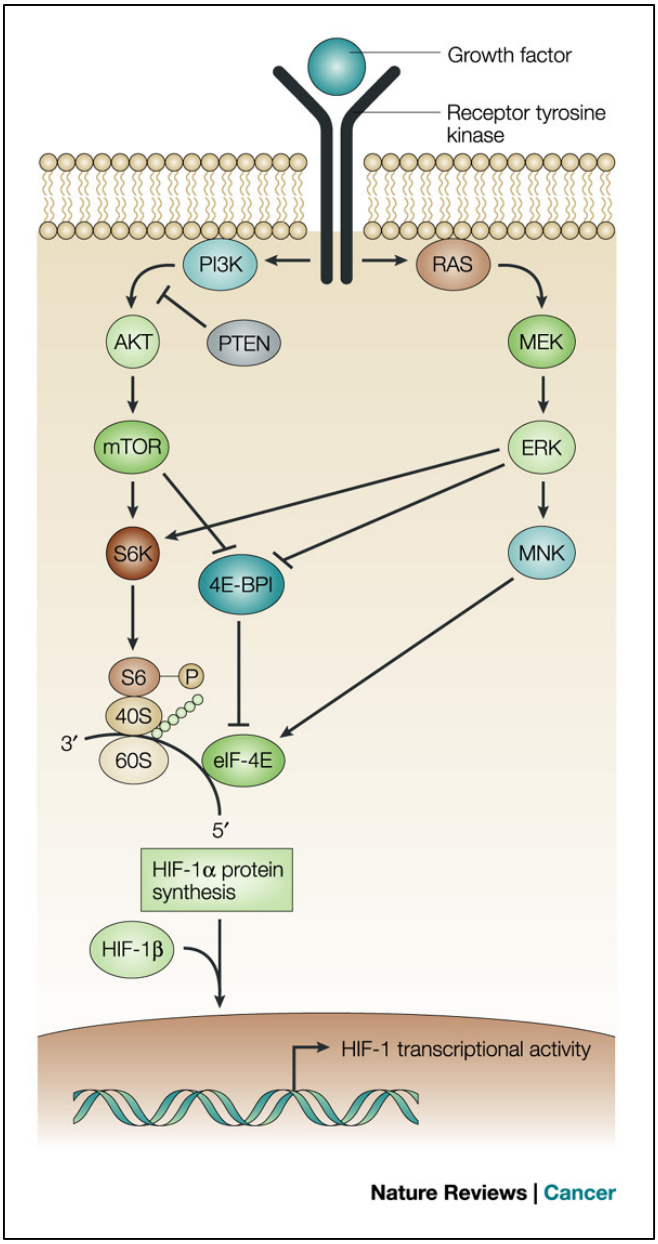


Figure 5.6. mTOR signaling cascade leading to phosphorylation of S6 and HIF-1α expression (Semenza, 2003). Figure reprinted with permission from copyright holder.

SUMMARY

It is clear from review of the current literature that tumour hypoxia is a significant problem, which contributes to treatment failure, disease progression and poor prognosis, particularly in prostate cancer. Specifically, solid tumour hypoxia poses a significant hurdle for radiotherapy-based treatments, as the low levels of oxygen hinder treatment efficacy. Compounding the issue, hypoxia-mediated HIF-1α signaling promotes tumour proliferation, adaptation and angiogenesis, further enhancing radio-resistance.

Not many of the previously investigated radiosensitising agents have reached clinical practice due to poor treatment-based side effects or insignificant oncological improvement. Therefore, there is a need for additional radiosensitising agents to overcome innate hypoxia-mediated radioresistance. Doxazosin or prazosin may make potential candidates to improve radiotherapy efficacy as they possess novel anti-cancer effects and are already used clinically for mitigation of radiotherapy-induced LUTS in men treated for prostate cancer.

5.2 AIMS

The overall aim of this study was to investigate the potential radiosensitising actions of the α1-adrenoceptor antagonist prazosin on hypoxic and normoxic AR-positive LNCaP and AR-negative PC-3 prostate cancer cell lines and the mechanisms underlying this.

The specific aims of this study were to:

1. Determine the relative radiosensitivities of LNCaP and PC-3 cells under normoxic and hypoxic conditions.

2. Evaluate the effect of prazosin on radiosensitivity of LNCaP and PC-3 cells under normoxic and hypoxic conditions.

3. Determine if prazosin effects on radiosentivity on LNCaP and PC-3 cells were mediated by ROS production, induction of HIF-1α or apoptotic or autophagic pathways.

5.3 METHODS

DRUG TREATMENT

PC-3 and LNCaP cells were seeded in glass petri dishes or glass 96-well plates at sub-confluent densities (see **Table 5.1**) to allow for uninhibited growth for the duration of treatment. Following appropriate incubation for cell adhesion (PC-3 24 h and LNCaP 48 h), PC-3 and LNCaP cells were treated with prazosin (10-100 μM), tamsulosin (100 μM) or vehicle control for 2.5 h at room temperature in a normoxic or hypoxic environment and in the presence or absence of irradiation. The acute room temperature incubation of cell cultures did not appear to have any adverse effects. However, all treatment conditions were incubated at room temperature to control for any impact on experimental outcomes. Although no additional buffers were added to the treatment culture medium, changes in pH of culture medium during treatment incubation were controlled for in all independent experiments. Refer to **Figure 5.7** for treatment timeline.

Table 5.1. Typical sub-confluent seeding densities of PC-3 and LNCaP cells used throughout this chapter.

Assay	Assay time point	Cell Line	
		PC-3	LNCaP
Resazurin reduction	3-5 days-post irradiation	$6.25x10^3/cm^2$	$6.25x10^3/cm^2$
DCF	Immediate	$3.125x10^4/cm^2$	$3.125x10^4/cm^2$
HIF-1α ELISA	Immediate	$3.125x10^4/cm^2$	$3.125x10^4/cm^2$
Caspase-3 Activity	24 h-post irradiation	$3.125x10^4/cm^2$	$3.125x10^4/cm^2$
CytoID Autophagy Detection	25 h-post irradiation	$3.125x10^4/cm^2$	$3.125x10^4/cm^2$

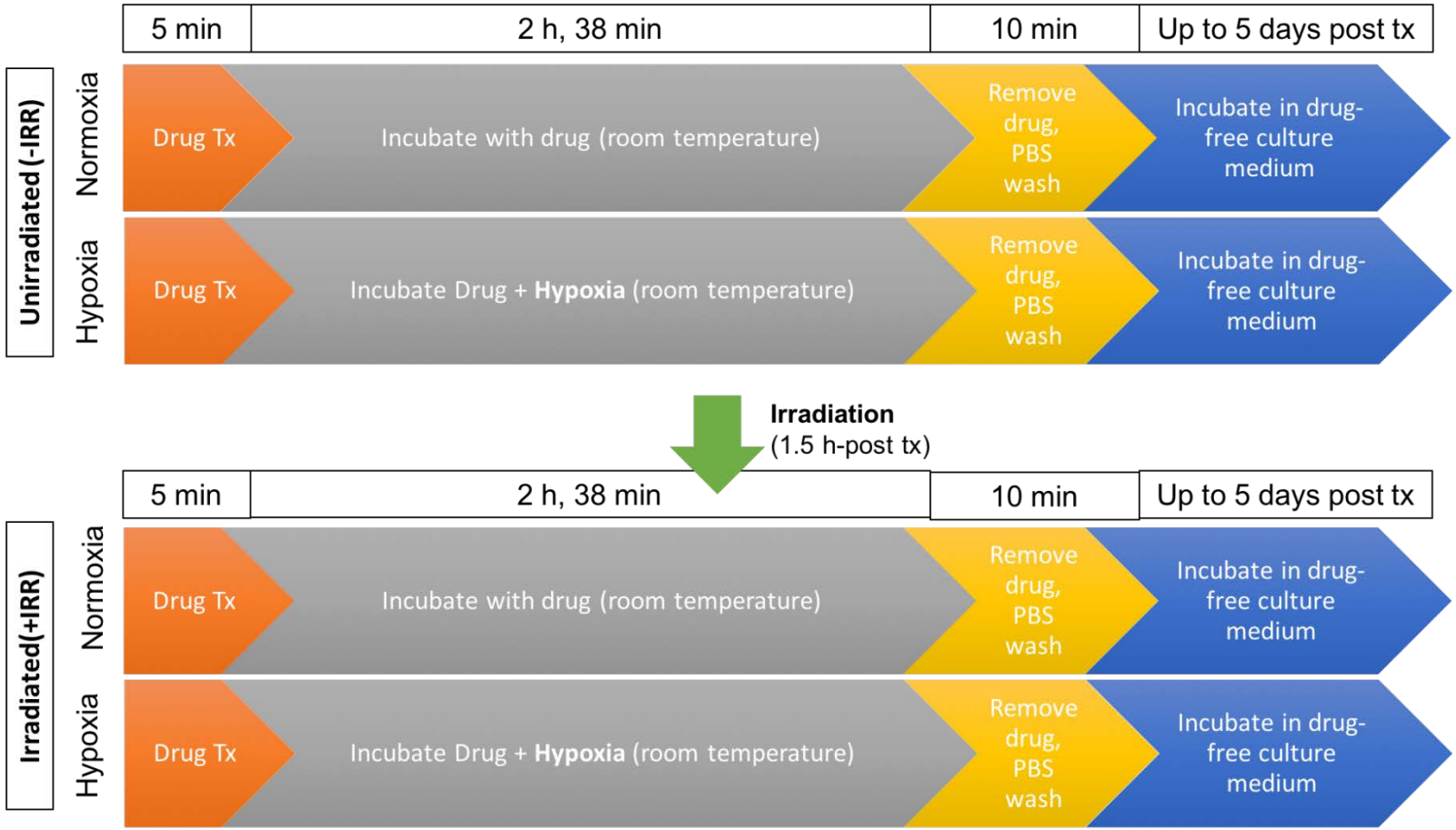


Figure 5.7. Timeline of LNCaP and PC-3 treatment for each experimental condition (unirradiated normoxia, unirradiated hypoxia, irradiated normoxia and irradiated hypoxia).

GENERATION OF HYPOXIA

For investigations involving hypoxia, cells were treated and degassed in glass vessels as opposed to plastic. Prior literature suggests plastics can act as an oxygen reservoir releasing oxygen into anoxic aqueous culture medium (Davies and Baker, 1970) and potentially confound any hypoxia-mediated effects. As shown in **Figure 5.8**, the protective effect of hypoxia may be partially lost amongst cells cultured, gassed and irradiated in plastic vessels as opposed to glass vessels.

Hypoxia (< 0.2% oxygen in culture medium) was generated as previously described (Anoopkumar-Dukie et al., 2005b). Briefly, cells seeded in glass vessels were placed in environment-controlled chambers (Billups Rothenberg, Cat. No. MIC-101) and gassed with 95% Nitrogen 5% CO_2 gas mixture at a flow rate of 10 L/min for 8 minutes, followed by 2 L/min for 30 minutes. Chambers were sealed and cells were maintained at room temperature in a hypoxic environment for an additional 2 h. Normoxic cells were also maintained at room temperature for the duration of hypoxia (2 h and 38 minutes). Refer to **Figure 5.7** for treatment timeline.

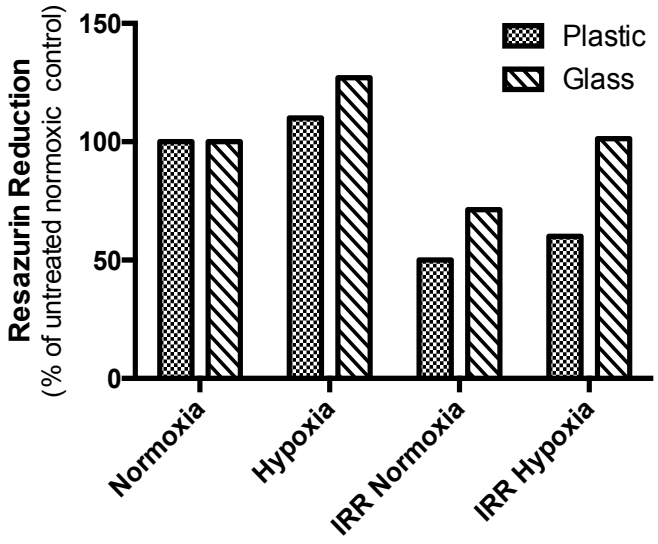


Figure 5.8. Effect of plastic (polycarbonate) or glass culture vessels on PC-3 cell proliferation 3 days following irradiation (IRR) in normoxic or hypoxic conditions. Resazurin reduction assay was used as a measure of cell proliferation. Data are expressed as mean percentage of untreated normoxic control (n=1 for plastic and n=2 for glass petri dishes).

IRRADIATION

Using a linear particle accelerator (Clinac iX Series, Varian) a radiation dose of 6.2Gy (6MV) at a dose rate of 2Gy per minute was delivered in one fraction to hypoxic or normoxic cells in environment controlled chambers (see **Figure 5.9 A & B**). The treatment field was opened to 40 cm^2 and the environment-controlled chamber containing cells was positioned atop of a piece of acrylic with a thickness of 1 cm. Cells received radiation approximately 1.5 h following initiation of drug (or vehicle control) treatment. Refer to **Figure 5.7** for treatment timeline.

RESAZURIN REDUCTION PROLIFERATION ASSAY

The resazurin reduction assay has previously been shown to be a viable alternative to the colony formation assay for determining cell viability after irradiation (Anoopkumar-Dukie et al., 2005a). Following acute 2.5 h treatment, cells were washed with phosphate buffered saline (PBS), and fresh drug-free complete culture medium was added above the cells and incubated for 3 to 5 days. For 5-day incubation, culture medium was renewed at 72 h post irradiation. After the intended incubation period, the resazurin reduction proliferation assay was conducted as previously described (Chapter 2). Changes in culture medium pH were controlled for in all experiments. Likewise, data were represented as a percent of respective untreated normoxic or hypoxic control.

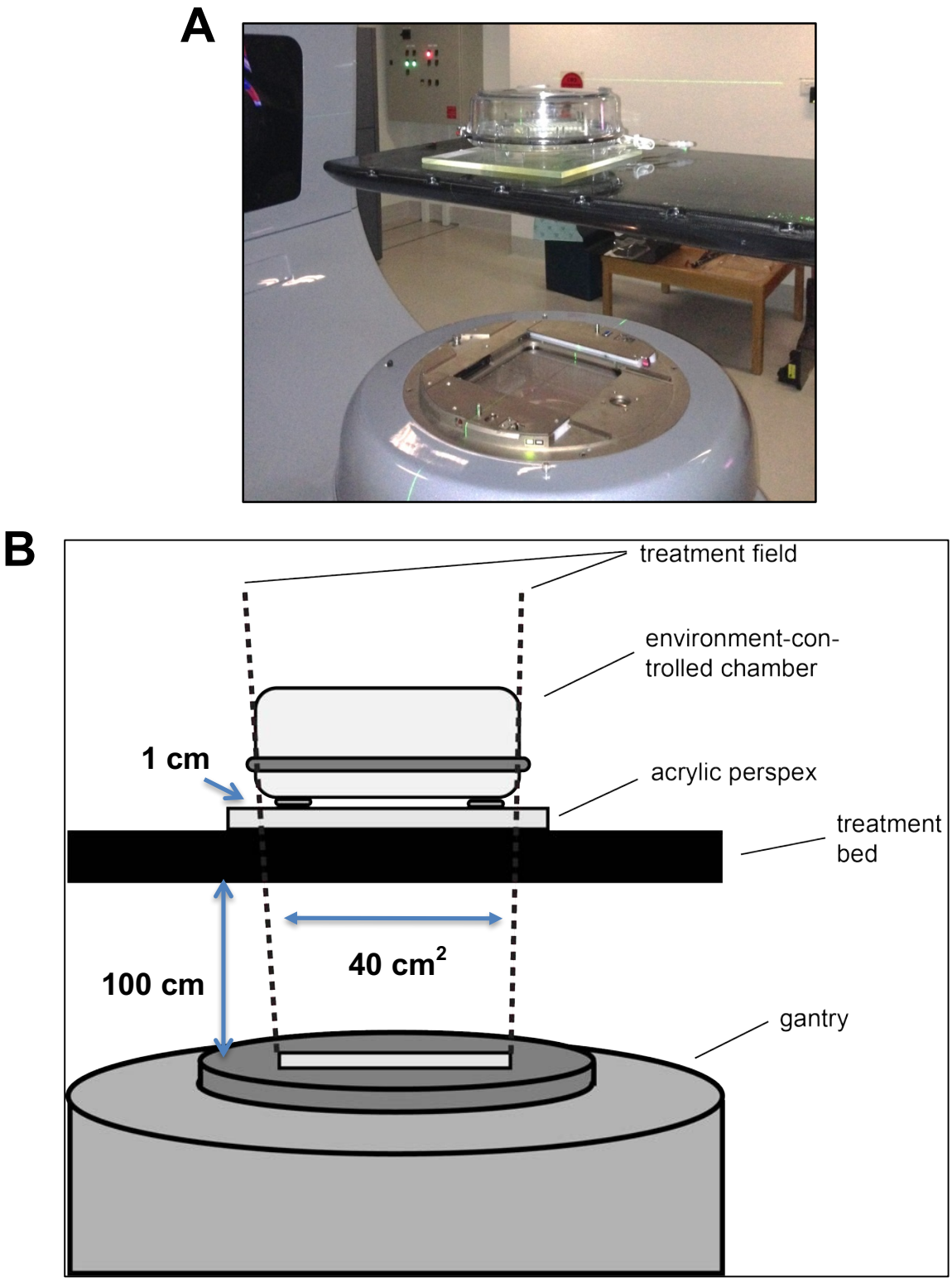


Figure 5.9. Irradiation (6.2 Gy, 6 MV) of prostate cancer PC-3 and LNCaP cells using a linear particle accelerator (Clinac iX Series, Varian). Photograph [A] and illustration [B] of positioning of environment controlled chamber with respect to gantry.

2,7-DICHLORODIHYDROFLUORECEIN DIACETATE ASSAY

The presence of intracellular reactive oxygen species (ROS) and subsequent oxidative stress is commonly measured using fluorescent probes such as 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). In its reduced form, DCFH-DA is a non-fluorescent and cell permeable product, which undergoes hydrolysis by intracellular esterases or hydroxide ion to form the byproduct, 2,7-dichlorodihydrofluorescein (DCFH). Further oxidation, presumably via the presence of intracellular ROS, results in the formation of 2,7-dichlorofluorescein (DCF) (**Figure 5.10**). Oxidation of DCFH to fluorescent DCF is known to be non-specific to the type of ROS or reactive nitrogen species present, with reports of DCFH reacting with H_20_2, superoxide, and hydroxide, ONOO- to generate DCF (**Figure 5.10**) (Chignell and Sik, 2003). Representative images of intracellular DCF fluorescence is shown in **Figure 5.11**.

The use of DCFH-DA for measuring oxidative stress in response to irradiated cells has previously been reported by various techniques (Anoopkumar-Dukie et al., 2005b), but primarily by use of a fluorescent plate reader (excitation: 498 nm; emission: 522 nm). Cells were seeded at a density of $1.0x10^4$ trypan blue-excluding cells per well and allowed to attach for 24 or 48 h (PC-3 and LNCaP, respectively). On the day of irradiation, cells were pre-loaded with 25 μM DCF-DA in fresh un-supplemented phenol red-free culture medium for 60 minutes prior to treatment. Cells were then washed once with PBS and treated with prazosin (100 μM, 2.5 h) in un-supplemented culture medium as previously described. Prazosin-treated, DCF-DA-preloaded cells were incubated in the presence or absence of oxygen and irradiation (see "Generation of Hypoxia" and "Irradiation" sections). As DCF is known to suffer from photoreduction (Chignell and Sik, 2003), cells were protected from direct light at all times during the experiment. To ensure oxidised DCF was not lost to washing, ROS formation was measured in treatment medium immediately following 2.5 h exposure using a Modulus Multimode fluorescent plate reader (Promega, excitation: 490 nm; emission: 530 nm). DCF fluorescence values were normalised to resazurin reduction obtained from parallel cell cultures. Appropriate cell-free controls were included to control for undesirable effects of prazosin on DCF fluorescence.

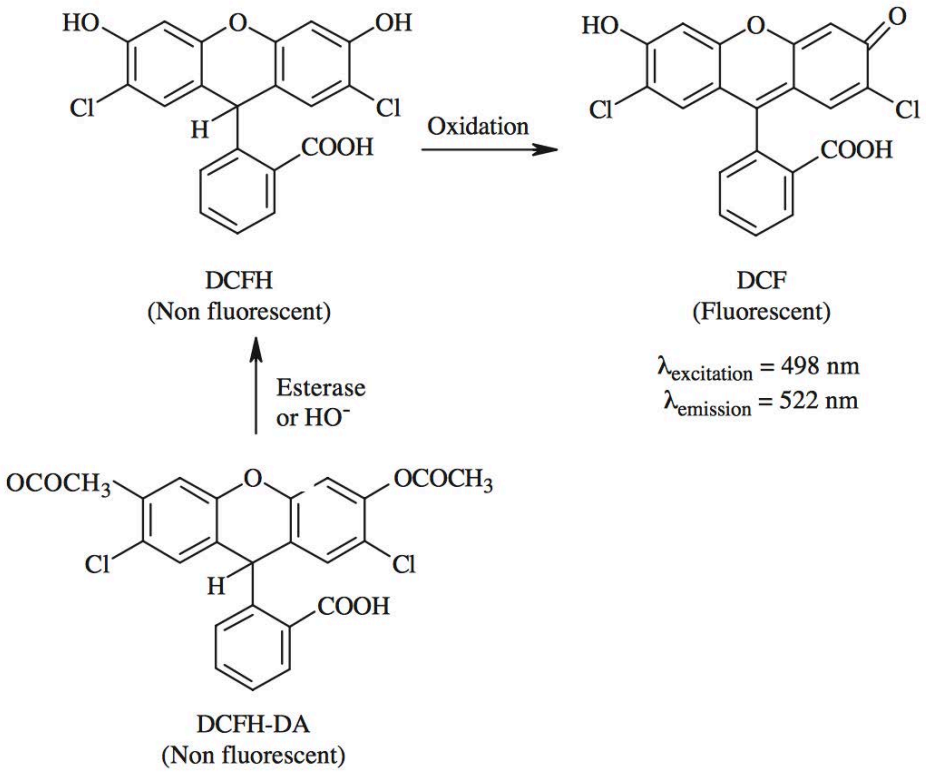


Figure 5.10. Conversion of non-fluorescent DCFH-DA to the highly fluorescent DCF by-product (Gomes et al., 2005). Figure reprinted with permission from copyright holder.

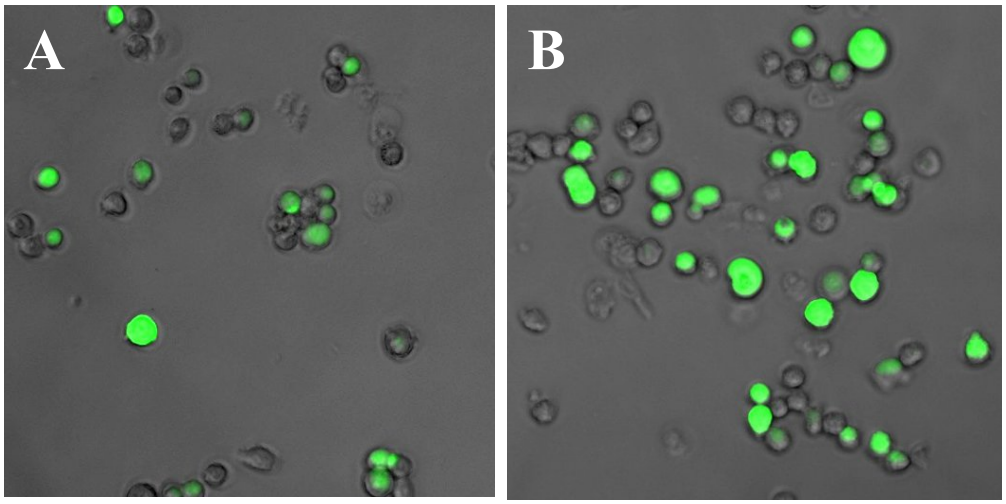


Figure 5.11. Representative images (n=2) of intracellular DCF fluorescence in PC-3 cells in basal conditions (A) or 2 h-post irradiation treatment (B).

DETECTION OF HIF-1A EXPRESSION

Immediately following drug treatment (± environmental oxygen and/or irradiation), total HIF-1α protein content was determined using a Human Total HIF-1α DuoSet IC sandwich-ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Cells were lysed using lysis buffer supplemented with 1 mM PMSF, 1X protease inhibitor cocktail, and 1X phosphatase inhibitor cocktail (Cayman Chemical). Sample protein concentration was determined using Bradford Reagent as previously described (Chapter 4). Wells of a high-bind 96-well plate were incubated over night with 4 μg/mL of kit provided capture antibody. Following incubation, wells were blocked with PBS containing 5% BSA and 0.05% tween-20 for 1 h. Sample lysates (200 μg of protein) were added to each well and incubated for 2 h at room temperature. Wells were washed before and after incubation with detection antibody (1:2000, 2 h) and HRP-linked antibody (1:500, 20 minutes) at room temperature. Substrate solution was added to wells for 20 min, and the reaction was stopped using sulfuric acid. Absorbance was read at 450 nm and HIF-1α concentrations were interpolated from a standard curve **(Figure 5.12).** A standard curve was generated with each independent experiment.

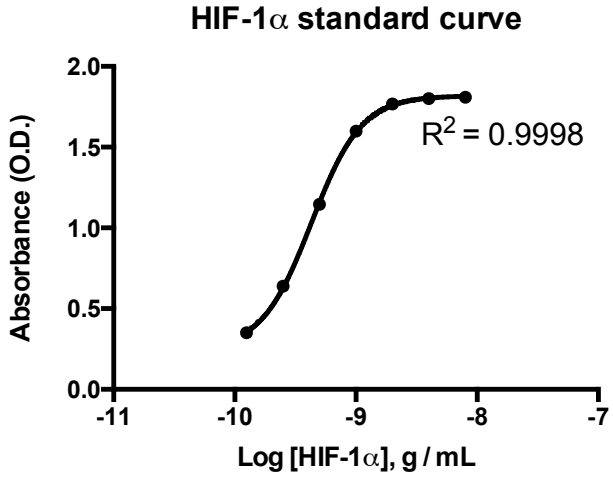


Figure 5.12. A representative non-linear (sigmoidal) regression of known concentrations of HIF-1α plotted against absorbance (at 490 nm) from which the HIF-1α concentration in collected samples was determined.

CASPASE-3 ACTIVITY ASSAY

Caspase-3 activation was used as an index of apoptotic cell death. Twenty-four hours following acute prazosin/tamsulosin treatment (± irradiation and atmospheric oxygen), caspase-3 activity was determined using the caspase-3 activity fluorescence kit (Cayman Chemicals) as previously described in Chapter 3.

CYTOID® AUTOPHAGY DETECTION ASSAY

Twenty-four hours following acute prazosin/tamsulosin treatment (± irradiation and atmospheric oxygen), change in autophagic activity was measured using a CytoID® autophagy detection kit (Enzo Sciences) as previously described in Chapter 3. Frequency of cells undergoing autophagy was determined using ImageJ analysis by representing number of autophagic cells as a percent of total number of counted cells. For each condition, a minimum sample of 100 cells was counted in all three independent experiments.

STATISTICAL ANALYSIS

Data were analysed using a one- or two-way ANOVA as appropriate. Statistical significance was determined using either Dunnett's or Tukey's post hoc test. Details of the specific tests used are indicated throughout the Results section.

5.4 RESULTS

IRRADIATION TIME-DEPENDENT CYTOTOXICITY

Irradiation of PC-3 and LNCaP prostate cancer cells was found to induce time dependent cytotoxicity, with 5 days resulting in approximately 75% and 70% reduction in cell survival compared to untreated control (**Figure 5.13).** As shown in **Figure 5.13**, irradiation had the greatest effect 5 days-post irradiation and as such, further investigations of cell proliferation assays were carried out at the 5-day post irradiation time point.

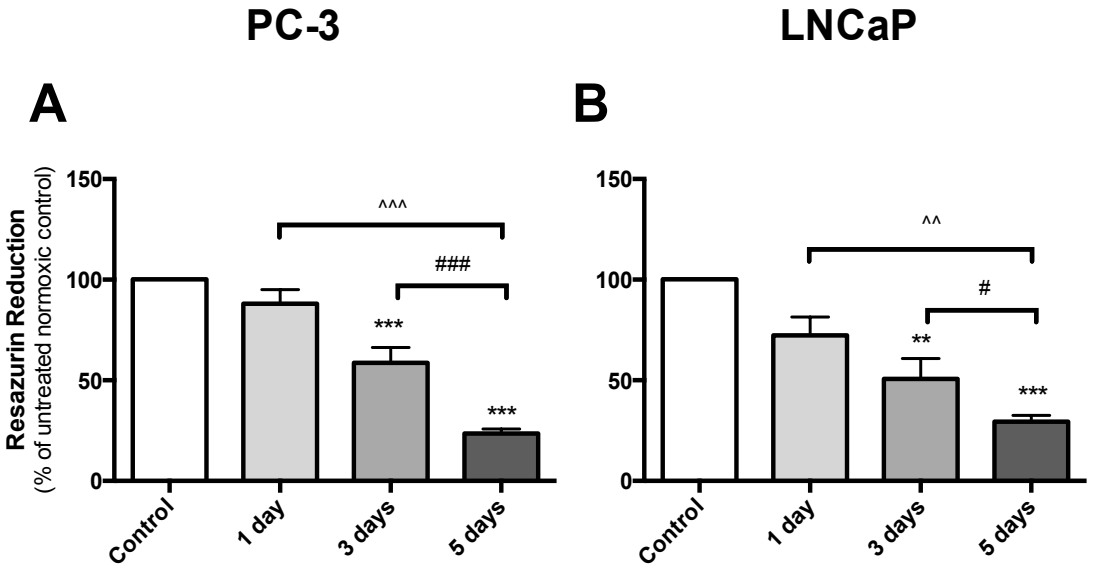


Figure 5.13. Cell proliferation of PC-3 (A) or LNCaP (B) 1, 3 or 5 days-post irradiation. Resazurin reduction was used as an index of cell proliferation and data are expressed as the mean percentage of unirradiated normoxic control (± SEM, n≥3). Statistical significance was determined using a one-way ANOVA with Tukey's post hoc test for comparisons between control and treated groups. ** P<0.01 and ***P<0.001 vs unirradiated control. For comparisons between 3- and 5-days post irradiation, # P<0.05 and ### P<0.001 and 1-day vs. 5-day post, ^^ P<0.01 and ^^^ P<0.001.

HYPOXIA-MEDIATED RADIORESISTANCE

To investigate the effect of hypoxia on the radiosensitivities of PC-3 and LNCaP cells, prostate cancer cells were irradiated in normoxic or acute hypoxic conditions (2.5 h). Cells were incubated for 5 days following irradiation and cell survival was determined by resazurin reduction. As shown in **Figure 5.14**, acute hypoxia (2.5 h) was able to significantly protect both PC-3 and LNCaP cells from irradiation-induced cell death ($P<0.001$ and $P<0.05$, respectively). Furthermore, hypoxia had a significantly greater effect in PC-3 cells than LNCaP cells **(Figure 5.15)**, increasing cell survival by 3.5- and 2.2-fold, respectively ($P<0.05$ for comparison between PC-3 and LNCaP).

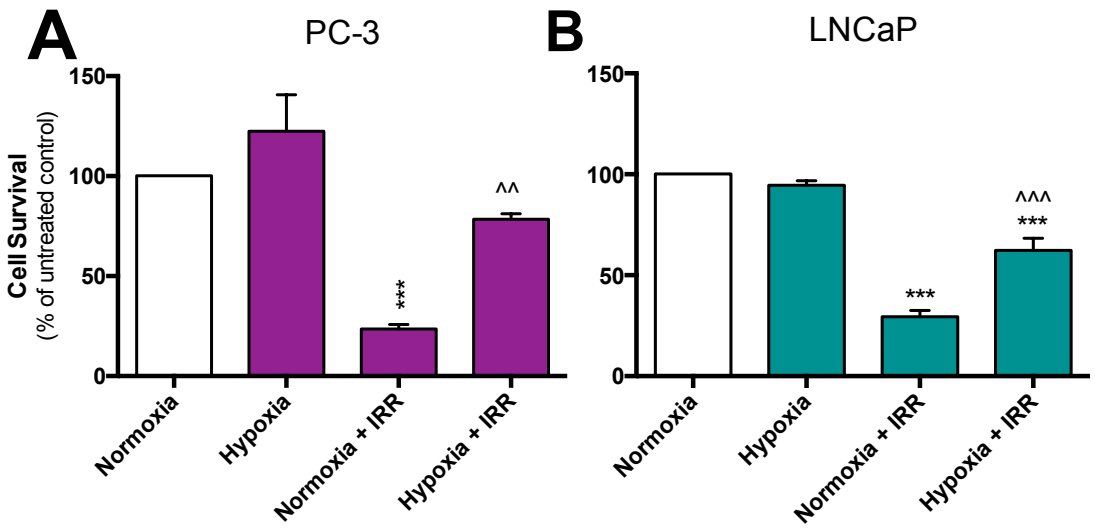


Figure 5.14. Survival of castrate-resistant PC-3 and castrate-sensitive LNCaP cells 5-days post irradiation (6.2 Gy, 6MV) in the presence or absence of acute hypoxia (2.5 h). Resazurin reduction was used as a measure of cell survival. Data are expressed as percent of untreated control and are represented as mean control ± SEM (n=5). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc. * P<0.05 and *** P<0.001 vs. respective untreated control.

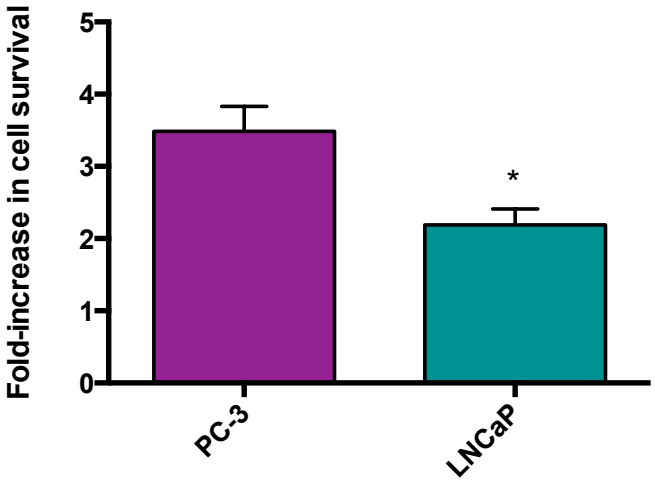


Figure 5.15. Hypoxia-mediated increase in PC-3 and LNCaP survival five days following irradiation. Cell survival was determined by reseazurin reduction. Data are represented as fold-increase in irradiated cell survival in the absence of oxygen (mean ± SEM [n=5]). Statistical significance was determined using a student's t-test. For comparison of PC-3 vs. LNCaP cells, P=0.0146.

RADIOSENSITISATION BY PRAZOSIN

To investigate whether prazosin possesses radiosensitising potential, PC-3 and LNCaP cells were treated acutely (2.5 h) in a hypoxic or normoxic environment in the presence or absence of irradiation. Five days after irradiation, cell survival was determined by resazurin reduction. In a normoxic environment, acute prazosin treatment (2.5 h, 10 – 100 μM) had no appreciable effect on PC-3 cell survival after five-day drug-free incubation (13% reduction at 100 μM), P>0.05) (**Figure 5.16 A**). In contrast, LNCaP cells appeared to be more sensitive to the short treatment with prazosin, with 30 and 100 μM significantly reducing cell survival by approximately 35% and 46%, respectively, compared to untreated normoxic control (**Figure 5.16 B**). In hypoxia treated cells, the highest concentration of prazosin (100 μM) significantly reduced survival in both cell lines compared to control (48.2%, P<0.001; and 42.2% P<0.001, respectively) (**Figure 5.16 A & B**).

In irradiated conditions, prazosin (30-100 μM) was found to significantly enhance hypoxic PC-3 and LNCaP cytotoxicity, reducing the survival fraction to that of

normoxic irradiated cells (**Figure 5.17**). In contrast, prazosin showed a statistically significant effect on normoxic irradiated prostate cancer cell survival compared to the untreated control (**Figure 5.17** [white bar]; P>0.05 for all). As shown in **Figure 5.17** (black bar), prazosin concentration-dependently reduced irradiated cell survival in hypoxic conditions. Importantly, the highest concentrations of prazosin (30 – 100 μM, PC-3 [P<0.001] and 100 μM LNCaP [P<0.01]) abolished hypoxia-mediated radioresistance. The sum of these findings indicate that prazosin selectively radiosensitises hypoxic prostate cancer cells to irradiation.

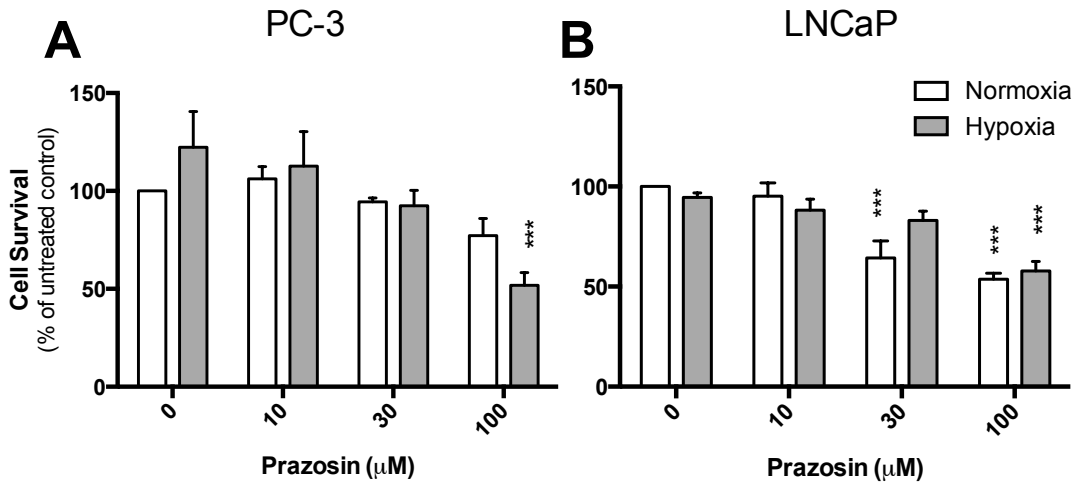


Figure 5.16. Effect of acute prazosin treatment (0-100 μM, 2.5h) on unirradiated normoxic or hypoxic PC-3 (A) and LNCaP (B) cells following five-day drug-free recovery. Resazurin reduction was used as an index of cell survival. Results are expressed as a percent of untreated normoxic control and are expressed as mean ± SEM (n=5). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. *** P<0.001 vs. respective normoxic or hypoxic control.

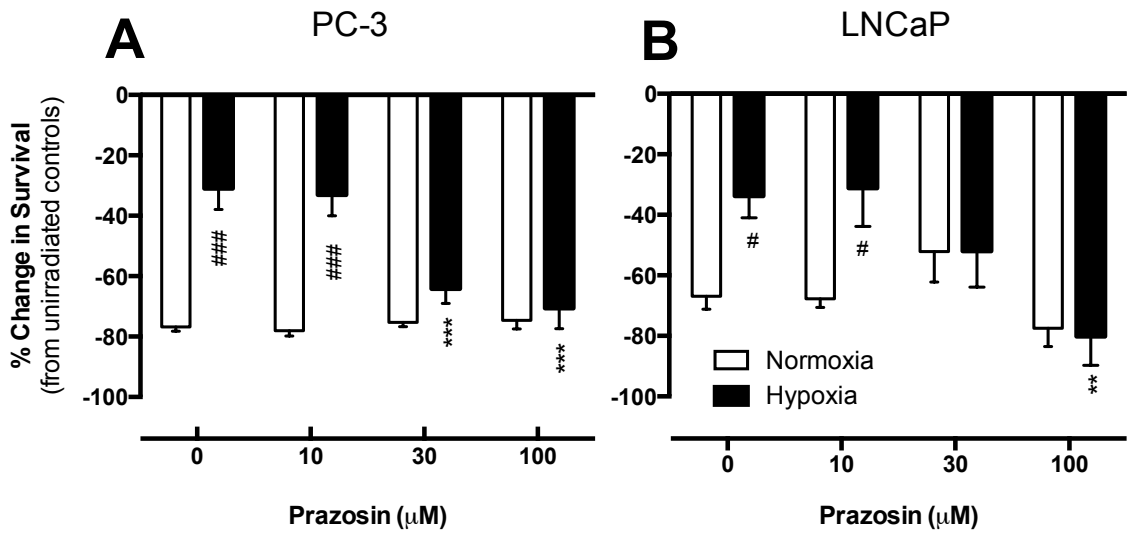


Figure 5.17. Effect of acute prazosin treatment (0-100 μM) on irradiated (6.2 Gy, 6MV) normoxic or hypoxic PC-3 (A) or LNCaP (B) cells following five-day drug free recovery. Resazurin reduction was used as an index of cell survival. Results are expressed as percent change from respective unirradiated controls (mean ± SEM, n≥5). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. ** P<0.01 and *** P<0.001 vs. respective untreated control. #P<0.05 and ### P<0.001 vs. normoxia.

INVOLVEMENT OF ALPHA1-ADR ANTAGONISM IN PRAZOSIN RADIOSENSITISATION

To elucidate whether this prazosin-mediated radiosensitisation occurred via α1-adrenoceptor-dependent mechanisms, PC-3 and LNCaP cells were treated acutely with tamsulosin (100 μM) in a normoxic or hypoxic environment in the presence or absence of irradiation (6.2 Gy, 6 MV), and cell survival was determined after five days. In contrast to prazosin, tamsulosin was unable to enhance the irradiation-mediated cytotoxicity in the normoxic or hypoxic treated PC-3 or LNCaP cells (**Figure 5.18 A & B**, respectively)

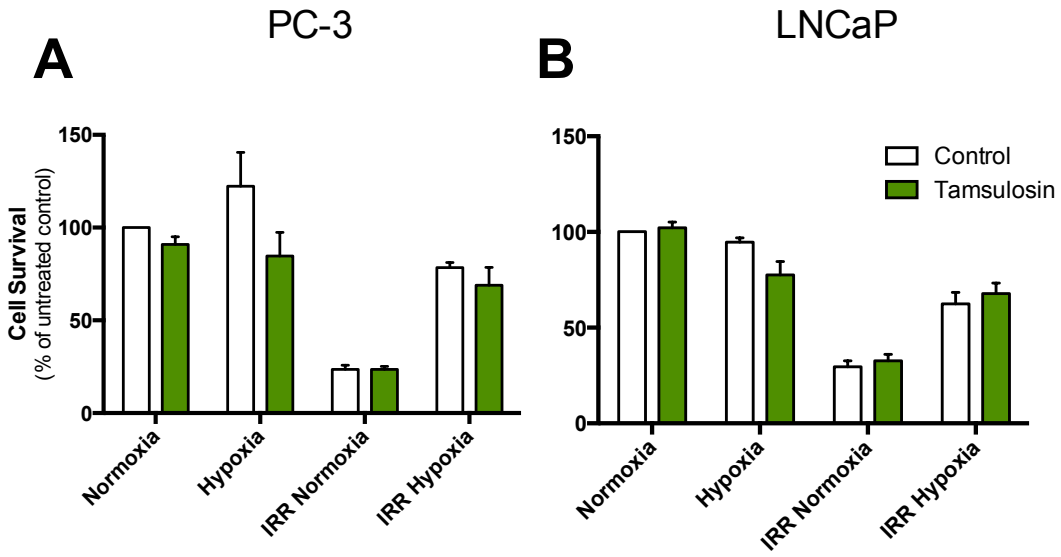


Figure 5.18. PC-3 and LNCaP survival 5 days following a combination of acute tamsulosin (100 μM, 2.5 h) and irradiation (IRR) in normoxic or hypoxic environment. Cell survival was determined by resazurin reduction assay. Data are represented as a percentage of untreated normoxic control (± SEM, n=3). Statistical significance was determined by two-way ANOVA, but no statistical differences were found between untreated controls and tamsulosin treated cells.

OXIDATIVE STRESS

Tumour hypoxia reduces the oxygen-dependent cytotoxic effect of radiotherapy, thereby promoting radioresistance. Since prazosin was able to restore radiosensitivity to hypoxic prostate cancer cells, it was postulated that prazosin may enhance free-radical production as a mechanism underlying this novel effect. To investigate this, the formation of intracellular ROS was measured using the DCF assay immediately following prazosin treatment (30-100 μM, 2.5 h) in the presence or absence of irradiation and environmental oxygen. In cell-free control experiments, prazosin did not affect DCF fluorescence (data not shown). As shown in **Figure 5.19 A & C**, prazosin was found to have a modest pro-oxidant effect that occurred in a concentration-dependent manner. At the highest concentration investigated (100 μM), prazosin alone increased ROS formation by 86% (± 43.5) and 38% (± 3.5) in PC-3 and LNCaP cells, respectively. Furthermore, this pro-oxidant effect was not altered by a hypoxic environment. As expected, irradiation increased intracellular ROS formation in both cell

lines, which was attenuated by hypoxic conditions (comparison between **Figure 5.19 A & B, C** vs. **D**). The combination of prazosin (100 μM) and irradiation further enhanced free-radical production in both normoxic and hypoxic conditions compared to unirradiated controls. However, after adjusting for the effects of prazosin alone on ROS formation in irradiated cells, it appears that the increase in ROS is merely an additive effect of irradiation and prazosin as opposed to a synergistic one (**Figure 5.20** on the following page).

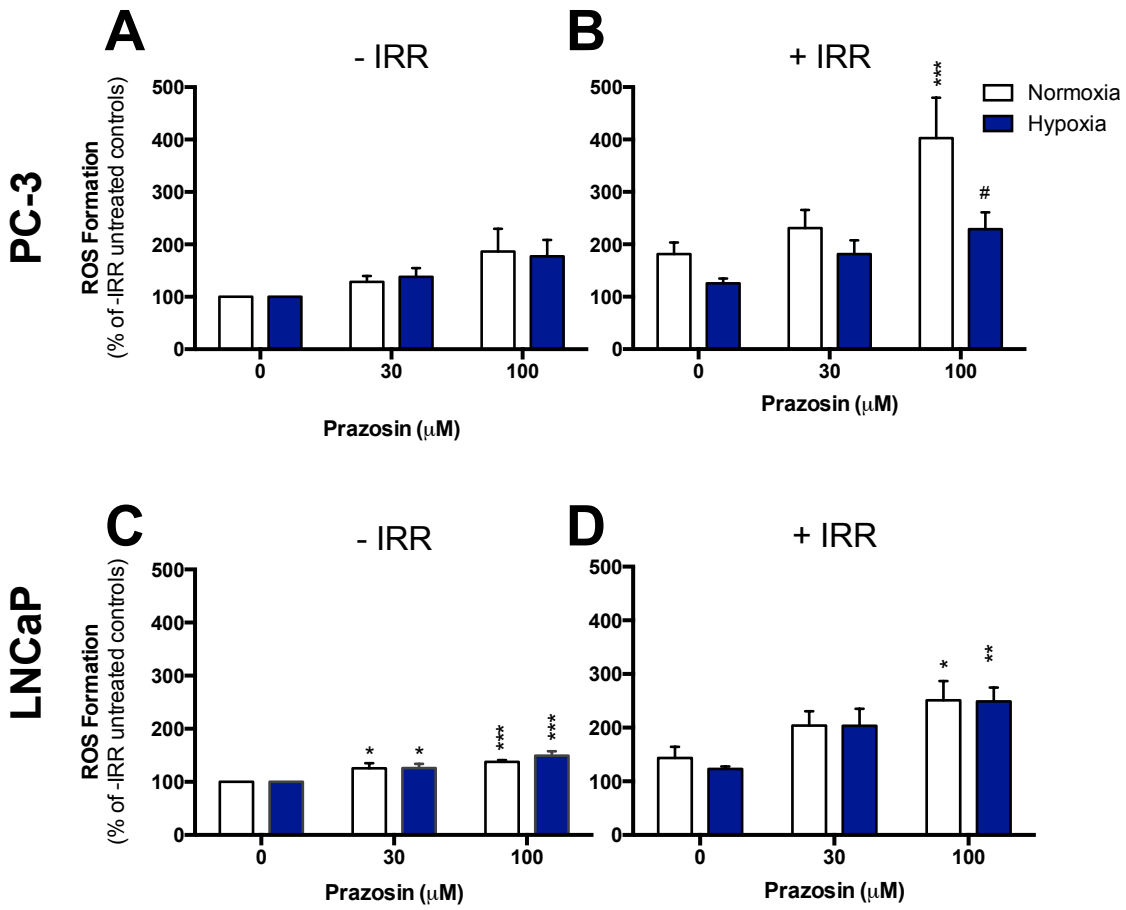


Figure 5.19. Relative intracellular ROS formation in normoxic or hypoxic PC-3 (A,B) and LNCaP (C,D) cells following treatment with prazosin (0-100uM, 2.5 h) in the absence (-IRR; A,C) or presence (+IRR, B,D) of irradiation (6.2Gy, 6MV). Relative DCF fluorescence was used as an index of ROS production. Data are expressed as percentage of unirradiated (-IRR) untreated normoxia or hypoxia controls (mean ±SEM, n=3). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01 and ***P<0.001 vs. respective untreated control. # P<0.05 vs. normoxia.

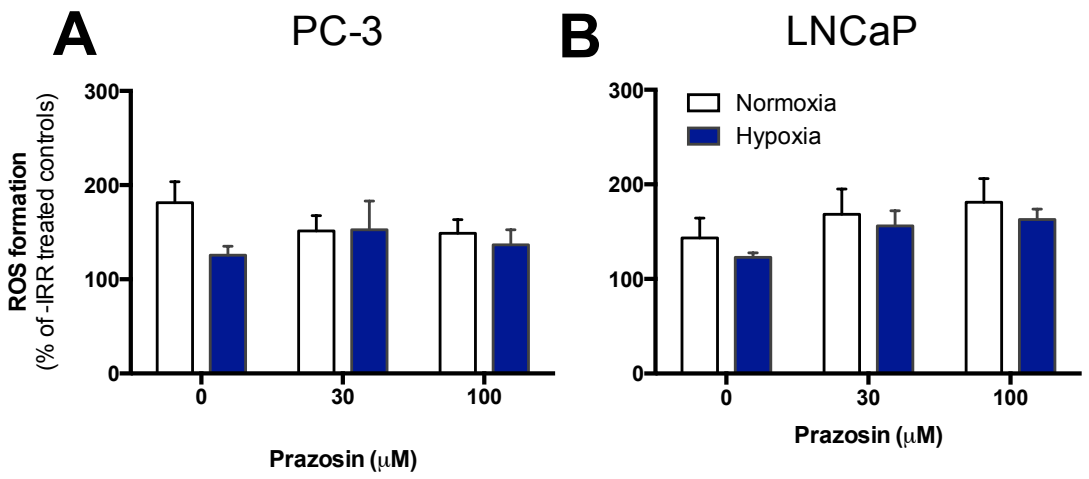


Figure 5.20. Increase in ROS formation in PC-3 (A) and LNCaP (B) cells represented as a percentage of respective unirradiated hypoxia or normoxia treated controls (mean ± SEM). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. No statistical differences were found between controls and treated cells.

EFFECT OF PRAZOSIN ON HIF-1ALPHA

To further uncover potential mechanisms of hypoxia-selective radiosensitisation, the effect of prazosin on HIF-1α expression or stabilisation was investigated. Total HIF-1α expression was quantified using an ELISA (R&D Systems) immediately following acute prazosin treatment in the presence or absence of irradiation and atmospheric oxygen. Basal expression of HIF-1α was found to be greatest in PC-3 cells, with more than 3-fold greater HIF-1α protein content than LNCaP cells (326 pg/mL vs.105 pg/mL, respectively). Acute hypoxia increased HIF-1α expression resulting in a 3.4- and 4.5-fold increase in PC-3 and LNCaP cells, respectively (**Figure 5.21**). Interestingly, treatment with prazosin (100 μM, 2.5 h) suppressed total HIF-1α expression across all treatment groups in both prostate cancer cell lines. In some instances, such as the normoxic irradiated and prazosin treated LNCaP cells, the expression of HIF-1α in samples were below the threshold of the ELISA kit sensitivity (< 125 pg/mL) and therefore were unquantifiable (**Figure 5.21 B**). In PC-3 cells, prazosin (100 μM) was able to reduce HIF-1α expression by approximately 2.3-fold in hypoxic unirradiated and

irradiated cells (**Figure 5.21 A**). Although unquantifiable, a similar trend was observed amongst LNCaP hypoxic un-irradiated and irradiated cells (**Figure 5.21 B**).

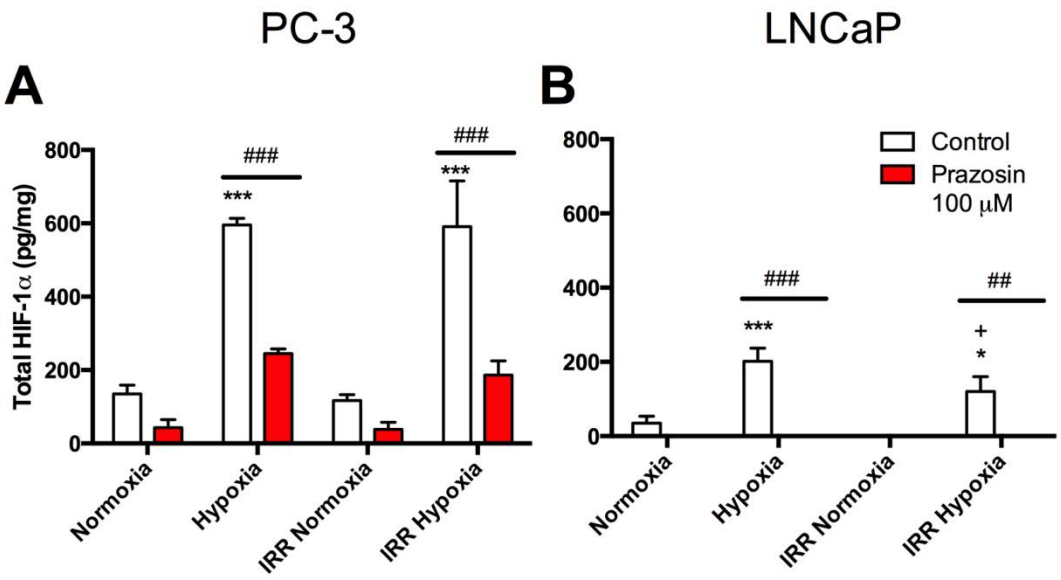


Figure 5.21. Total HIF-1α expression immediately following acute prazosin (100 μM) treatment of hypoxic or normoxic PC-3 (A) and LNCaP (B) cells in the presence or absence of irradiation. Changes in HIF-1α expression were determined using a commercially available ELISA kit. Data are expressed as picogram of HIF-1α per milligram of total protein (mean± SEM, n=3). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. * P<0.05 and ***P<0.001 vs. untreated normoxia. For comparisons of control vs. prazosin, ## P<0.01 and ### P<0.001. + P<0.05 vs. irradiated normoxia.

CELL DEATH MECHANISMS OF PRAZOSIN-INDUCED RADIOSENSITISATION

APOPTOSIS

Changes in the universal apoptotic marker caspase-3 were determined 24 h post-irradiation and acute prazosin/tamsulosin treatment of PC-3 and LNCaP normoxic or hypoxic cells and subsequently normalised to resazurin reduction. As shown in **Figure 5.22** on the following page, a similar trend in cell survival was present in 24 h recovery as 5-day recovery; however, hypoxia significantly reduced PC-3 cell survival at the 5-day time point (**Figure 5.22 A**). Consistent with previous findings, prazosin showed hypoxia selective effects by significantly reducing PC-3 cell survival in the presence or

absence of irradiation (**Figure 5.22 A & B**). In contrast, LNCaP cells were less sensitive to the hypoxia-selective effect, with an appreciable response to prazosin (100 μM) in irradiated hypoxic cells (P=0.09) (**Figure 5.22 C & D**).

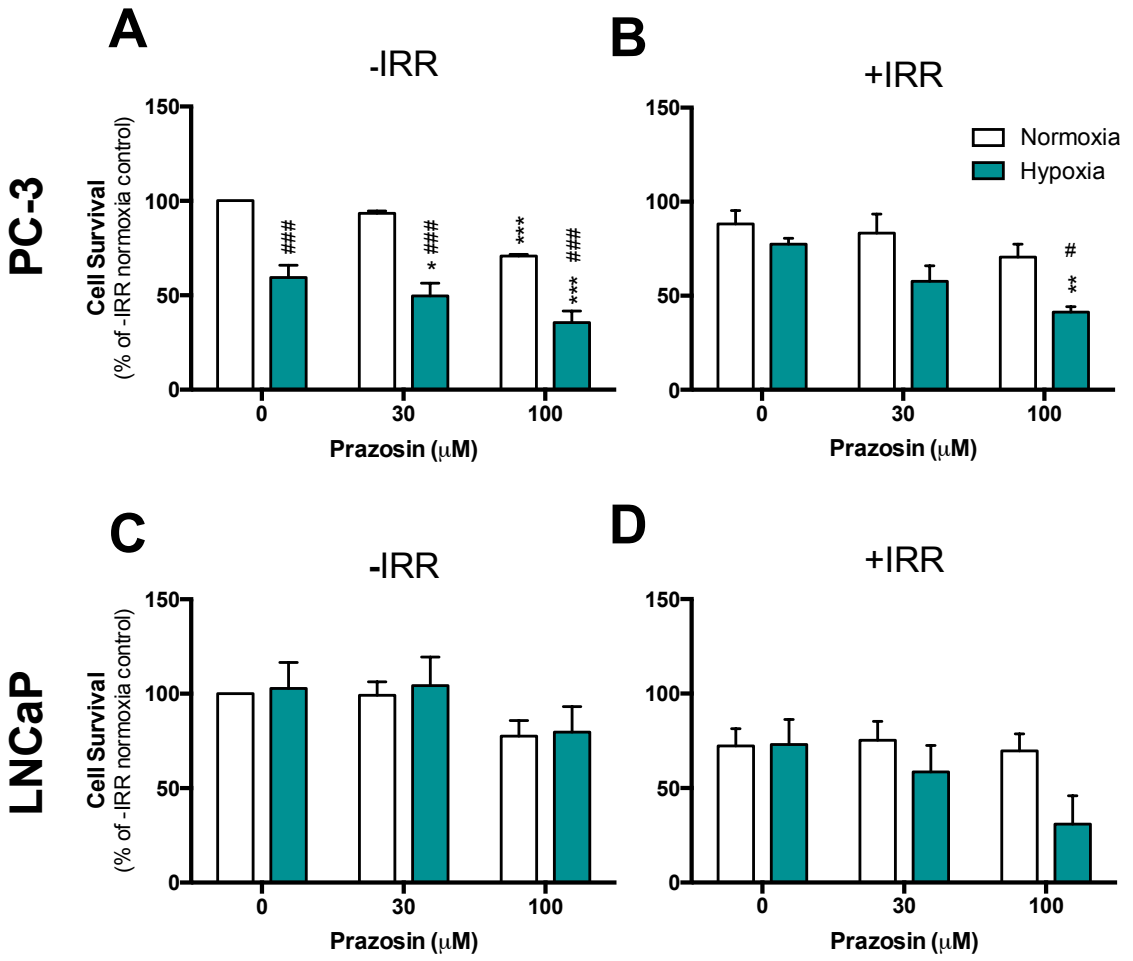


Figure 5.22. Cell survival of normoxic or hypoxic PC-3 (A,B) and LNCaP (C,D) 24 h following acute prazosin treatment (0 –100 μM, 2.5 h) in the absence (A,C) or presence of irradiation (B,D). Resazurin reduction assay was using as an index of cell survival and data are represented as percent of untreated normoxic control (mean ± SEM, n=3). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. *P<0.5, **P<0.01 and ***P<0.001 vs. respective control. #P<0.05 and ### P<0.001 vs. normoxia.

In both un-irradiated and irradiated prostate cancer cells, enhanced caspase-3 activity was only appreciable in prazosin-treated hypoxic cells (**Figure 5.23 A, B & C**) except for unirradiated LNCaP cells, where prazosin (30-100 μM) had no effect on caspase-3

activity in normoxic or hypoxic conditions (**Figure 5.23 C**). The irradiation of hypoxic prazosin treated cells modestly, yet significantly, increased caspase-3 activity in both PC-3 ($P<0.05$ for prazosin 30 and 100 μM) and LNCaP cells ($P<0.001$ for prazosin 100 μM). LNCaP cells demonstrated the greatest increase in apoptosis, with 100 μM prazosin treatment resulting in an approximately 4-fold increase in caspase-3 activation compared to unirradiated normoxic control (**Figure 5.23 D**). In contrast to hypoxia conditions, irradiation or the combination of irradiation and prazosin (30-100 μM) did not increase normoxia cell apoptosis in either cell line at the investigated time point (24 h). These findings further confirm the hypoxia-selective radiosensitisation effect of prazosin in prostate cancer cells.

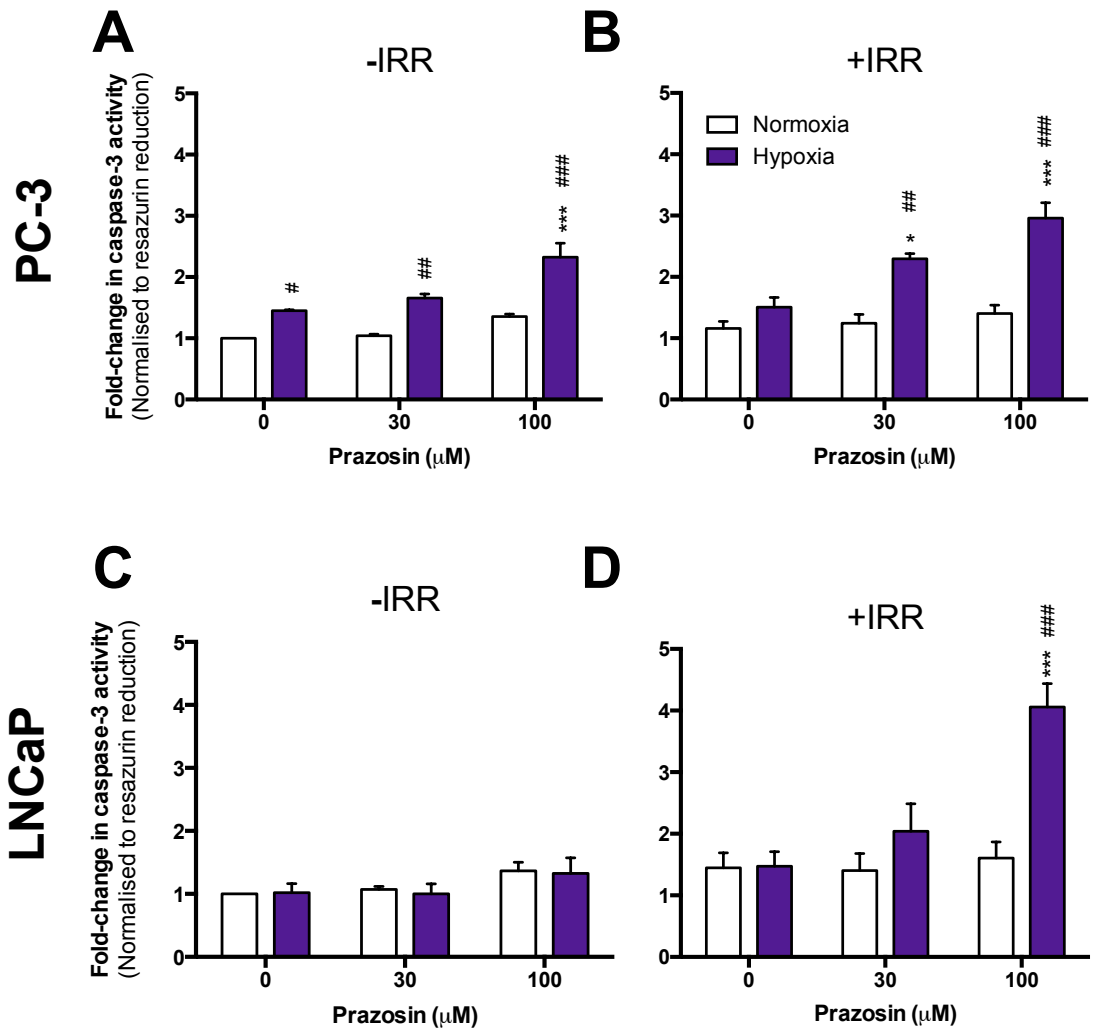


Figure 5.23. Caspase-3 activity in normoxic or hypoxic PC-3 (A,B) and LNCaP (C,D) cells was determined 24 h following acute prazosin treatment (2.5 h, 0 – 100 µM) in the absence (A,C) or presence (B,D) of irradiation. Data were normalised to resazurin reduction and are expressed as the fold-change in caspase-3 activation from unirradiated normoxic control (mean ± SEM, n = 3). Statistical significance was determined using two-way ANOVA with Tukey's post hoc test. ***P<0.001 vs. respective control. ### P<0.001 vs. normoxia.

AUTOPHAGY

It was previously shown in Chapter 3 that 24 h prazosin exposure is capable of inducing autophagy in LNCaP and PC-3 cell lines; with opposing roles by contributing to cell survival or death, respectively. However, whether acute prazosin treatment is capable of inducing sustained autophagy, and if autophagy may contribute to hypoxia-mediated radiosensitisation actions of prazosin was previously unknown. To investigate this, autophagic activity was determined using the CytoID® autophagy detection kit 24 h post-irradiation and acute prazosin treatment (0-100 μM, 2.5 h) in hypoxic or normoxic prostate cancer cells. Increase in number of autophagy cells was quantified (**Figure 5.24**) using image analysis software from fluorescence microscopy images (**Figures 5.25 & 5.26**). Measureable changes in levels of autophagy were only observed in PC-3 cells (**Figures 5.24 A & 5.25**), whereas LNCaP cells had high levels of autophagy in all conditions tested (**Figures 5.24 B & 5.26**). In PC-3 cells, hypoxia alone did not significantly affect autophagic activity in PC-3 cells, whereas irradiation treatment had the opposite effect, enhancing autophagy by approximately 4.5-fold (P=0.0579) **(Figure 5.24 A & 5.25**). The combination of irradiation and hypoxia had no effect on autophagic activity compared to normoxic conditions. Strikingly, prazosin treatment (100 μM) increased autophagy by 4- and nearly 10-fold increase in normoxic (P=0.48) and hypoxic PC-3 cells (P<0.04), respectively. A similar trend in prazosin-mediated increase in autophagy was seen amongst the irradiated normoxia and hypoxia groups, although to a lesser extent. Only 30 μM prazosin treatment significantly increased autophagy in normoxia irradiated PC-3 cells; however, prazosin (100 μM) was found to nearly double autophagy in irradiated hypoxic conditions (P>0.05). By contrast, autophagy in PC-3 irradiated in presence of 30μM prazosin was significantly reduced by hypoxia.

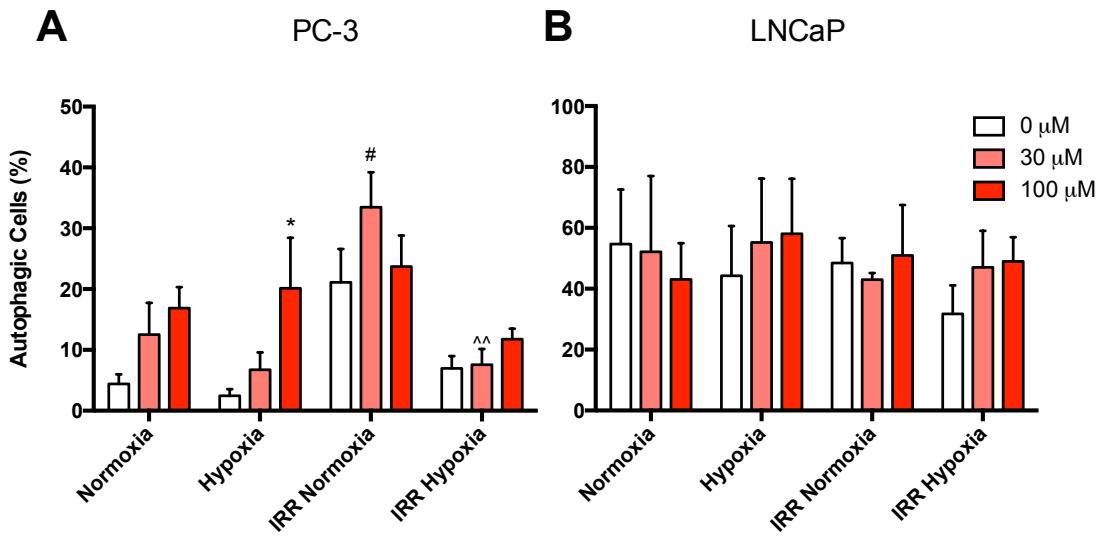


Figure 5.24. Autophagic activity following acute prazosin treatment (2.5 h, 0-100 μM) in the presence or absence of irradiation (IRR) amongst hypoxic or normoxic PC-3 (A) and LNCaP (B) cells. Autophagy was determined using a CytoID® Autophagy Detection Kit (Enzo Life Sciences). Data are expressed as percentage of autophagic cells (mean ± SEM, n=3). Data were analysed using a two-way ANOVA with Tukey's post hoc test. *P<0.05 vs. untreated hypoxia; # P<0.05 vs. prazosin-treated (30 μM) normoxia; and ^^ P<0.01 vs. prazosin-treated (30μM) irradiated normoxia cells.

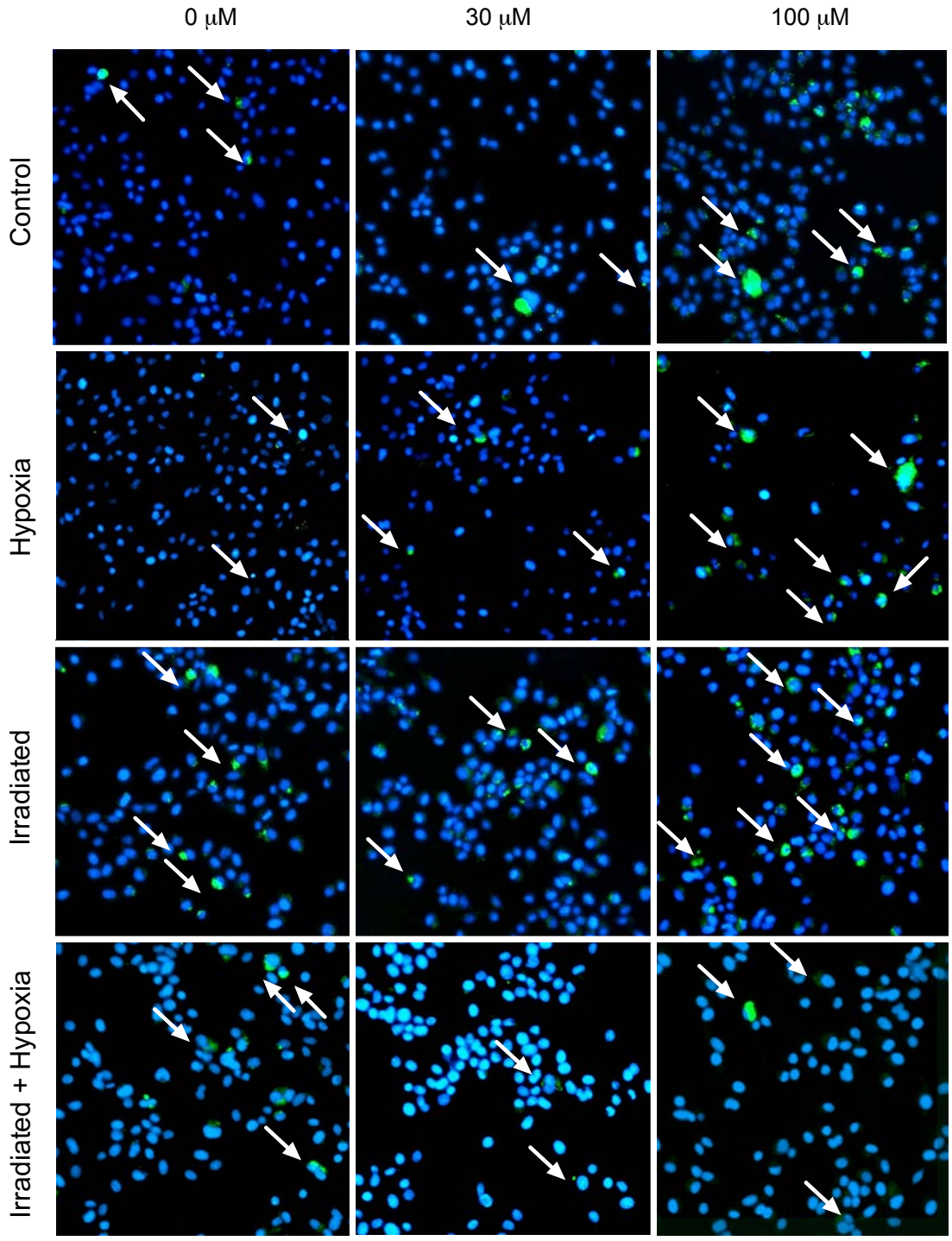


Figure 5.25. Representative fluorescent images of PC-3 cells using CytoID® Autophagy Detection Kit 24 h following acute prazosin (0-100 μM) and irradiation treatment of normoxic or hypoxic cells. Images were captured at 100x magnification using an Evos® Cell Imaging System. Arrows indicate areas with autophagic activity (Blue [DAPI] represents nuclei and green [FITC] represents autophagy-related vesicles).

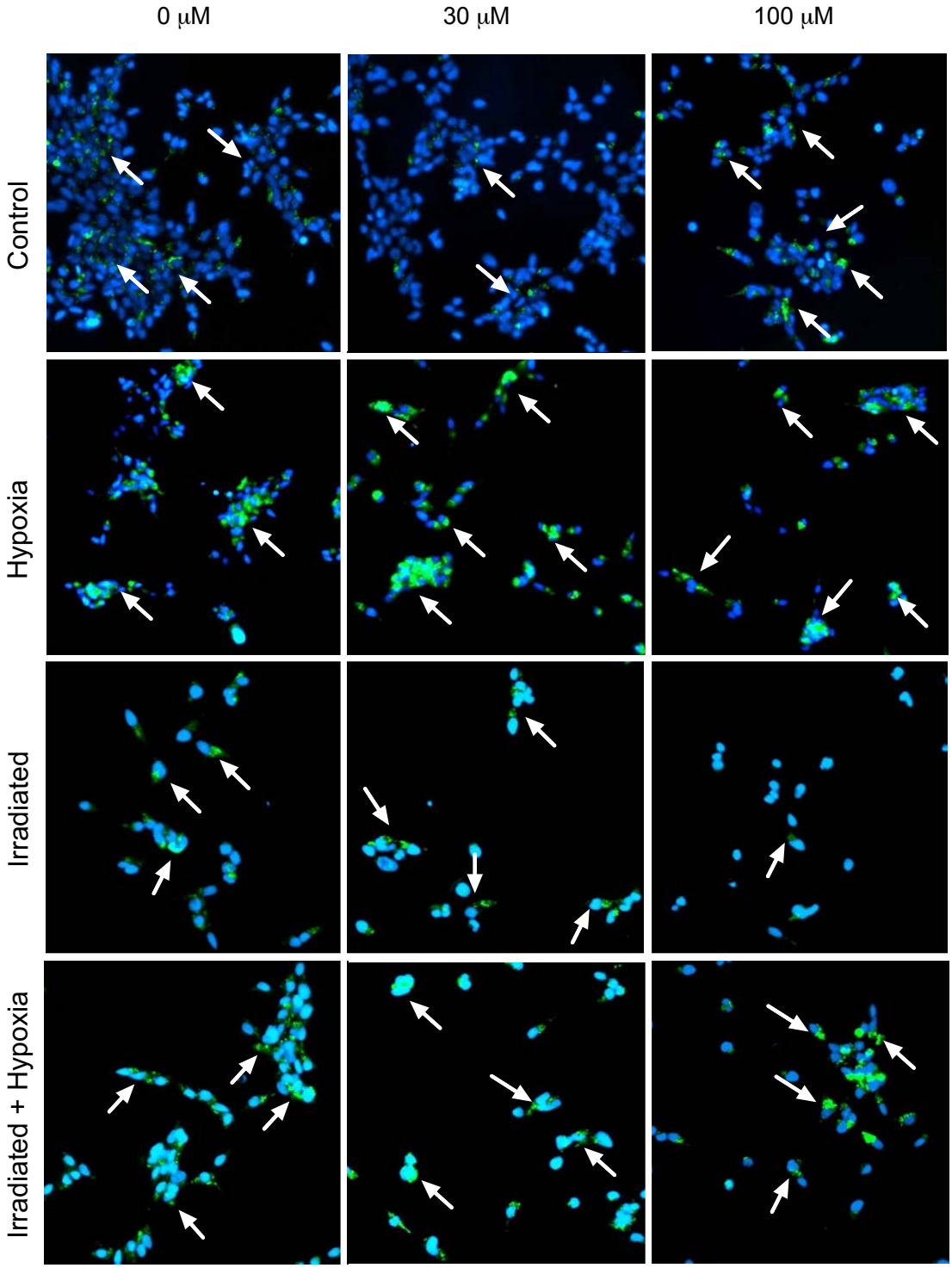


Figure 5.26. Representative fluorescent images of LNCaP cells using CytoID® Autophagy Detection Kit 24 h following acute prazosin (0-100 μM) and irradiation treatment of normoxic or hypoxic cells. Images were captured at 100x magnification using an Evos® Cell Imaging System. Arrows indicate areas with autophagic activity (Blue [DAPI] represents nuclei and green [FITC] represents autophagy-related vesicles).

5.5 DISCUSSION

The overall aim of this chapter was to determine whether prazosin treatment would enhance the cytotoxic effects of irradiation in normoxic or hypoxic conditions on AR-negative PC-3 or AR-positive LNCaP cells. While hypoxia was found to confer radioresistance, acute prazosin treatment caused hypoxia-selective sensitisation of both PC-3 and LNCaP cells to irradiation as measured after five days of drug free recovery. This effect was largely attributed to mechanisms other than antagonism of α1-ADRs and is likely to be mediated by prazosin-induced ROS formation and/or HIF-1α suppression; contributing to either autophagy and/or apoptosis in a cell type-dependent manner. Consistent with findings presented in Chapter 3, apoptosis appeared to primarily regulate prazosin hypoxic-radiosensitisation of LNCaP cells, whereas interplay between autophagy and apoptosis was likely to mediate PC-3 cytotoxicity. Lastly, the radiosensitising actions of prazosin appear to be consistent with previous reports of quinazoline-dependent cytotoxicity (discussed in Chapter 3). While it was found that the non-quinazoline tamsulosin was not cytotoxic in the presence or absence of irradiation and oxygen, further investigation is required to ascertain the importance of the quinazoline structure in the radiosensitisation of prostate cancer cells.

The formation of ROS is an important indirect mechanism underlying irradiation induced-DNA damage and subsequently cancer cell death. Low levels of molecular oxygen, as in the inner portions of solid tumours, act to circumvent the killing capacity of irradiation, and contribute to radioresistance of cancer cells (Harada, 2011). The oxygen-dependent effects of irradiation were confirmed in present study, with hypoxic conditions significantly hindering the cytotoxic effect of irradiation and the formation of intracellular ROS. Surprisingly, acute prazosin treatment was found to increase ROS in an oxygen-independent fashion in the presence or absence of irradiation. This is the first report of prazosin-mediated intracellular ROS production, and is proposed to contribute to some degree to the hypoxia-selective radiosensitising effect of prazosin through induction of oxidative stress and subsequent cell death by autophagy-mediated or apoptotic mechanisms. Briefly, oxidative stress is a state in which the accumulation of intracellular ROS overwhelms the cell's innate anti-oxidant mechanisms, which leads to a plethora of cell type- and stimuli-dependent responses including adaptation, growth-

arrest, apoptosis or necrosis (Milkovic et al., 2014, Paschos et al., 2013). Physiologically sustainable levels of intracellular ROS participate in redox-signalling cascades and are known to contribute to the pathogenesis of many diseases, including prostate cancer (Kumar et al., 2008). On the other hand, high levels of intracellular ROS are typically toxic, resulting in DNA damage and modification of the structure and functional activity of proteins and lipids (Han and Chen, 2013, Milkovic et al., 2014, Bak and Weerapana, 2015). However, the mechanism by which prazosin generates ROS production is unclear. As previously mentioned in Chapter 4, one proposed mechanism of doxazosin/prazosin toxicity may be via direct interactions with DNA causing genotoxic-stress (Arencibia et al., 2005, Lin et al., 2007). DNA damage itself is known to promote the accumulation of intracellular ROS in consequence, and acts to regulate cellular senescence or death (Nair et al., 2015). Taken together, prazosin is likely to induce DNA damage by direct-interactions or indirectly through endogenous ROS accumulation. In combination with the direct-DNA damaging actions of irradiation, this may potentiate sensitivity of prostate cancer cells to irradiation in hypoxic environments.

In support of the present findings, sources of endogenous or exogenous ROS are known to drive autophagy and/or apoptosis in prostate cancer cells by various stimuli-dependent mechanisms (Chung et al., 2013, Kim et al., 2013, Gundala et al., 2014, Shin et al., 2013, Zhou et al., 2015). Of particular interest, the literature reveals that these ROS-responsive mechanisms appear to cross-talk, converging on regulation of HIF-1α. Aside from inhibiting irradiation-induced free radical DNA damage, tumour hypoxia enhances HIF-1α levels to promote survival and adaptation, leading to further radioresistance. Consistent with previous findings (Dai et al., 2011) in these prostate cancer cell lines, acute hypoxia was found to promote accumulation of HIF-1α, cell proliferation and radioresistance; all of which were reduced in the presence of prazosin presumably via ROS-generating actions. In androgen-responsive prostate cancer cells, ROS-mediated apoptosis was recently reported to occur via the AKT/pVHL signalling pathway (Chetram et al., 2013). The authors concluded that ROS increased AKT activity through catalytic inhibition of PTEN (negative regulator of AKT), resulting in direct activation of pVHL and concomitant decrease in HIF-1α and apoptosis. Furthermore, this effect was demonstrated to be dependent on pVHL activity since genetic knockdown rescued HIF-1α and protected prostate cancer cells from apoptosis.

However, whether this pVHL-mediated apoptotic effect was dependant on changes in HIF-1α levels or other target substrates is unknown. Interestingly, these prior findings were mirrored throughout this thesis, where the ROS-inducing-prazosin increased AKT activation in androgen-responsive LNCaP cells, but not AR-negative PC-3 cells, and apoptotic cell death. While AKT activity is associated with down-regulation of autophagy, ROS also act to promote autophagy through activation of AMPK and subsequent inhibition of mTOR by depletion of energy stores or via the DNA-damage response molecule, ataxia telangiectasia mutated (ATM) (Choi et al., 2001, Alexander et al., 2010a, Alexander et al., 2010b, Kongara and Karantza, 2012). In its active state, mTOR promotes hypoxia-independent increase of HIF-1α protein translation in a separate signalling cascade to autophagy inhibition. Therefore, inhibition of mTOR by ROS/AMPK activity would effectively suppress HIF-1α accumulation and survival signalling. It was previously demonstrated in the literature and in Chapter 4 of this thesis, that prazosin and doxazosin inhibit mTOR signalling and subsequent HIF-1α expression in normoxic conditions (Park et al., 2014a, Yang et al., 2011a). In support of the proposed mTOR-dependent hypoxia radiosensitisation by prazosin, a novel inhibitor of PI3K/mTOR/p70S6K signalling was revealed to potentiate irradiation induced cytotoxicity of normoxic or hypoxic prostate cancer PC-3 cells *in vitro* and xenograft tumours *in vivo* (Potiron et al., 2013). Furthermore, the hypoxia-selective radiosensitising actions of prazosin in PC-3 cells were found to have markers of both autophagy and apoptosis, which may be a characteristic of functionally mutant p53 status. A similar effect was reported in the AR-independent and p53 mutant or null prostate cancer cell lines DU145 and PC-3, respectively, where docosahexaenoic acid (omega-3 polyunsaturated fatty acid) stimulated mitochondrial ROS production, mTOR-inhibition and subsequent autophagy and apoptosis induction (Shin et al., 2013). While further investigation is necessary to ascertain the precise molecular mechanisms underlying the hypoxia-selective radiosensitisation effect of prazosin, the following pathway is proposed. Prazosin-mediated ROS formation may act to inhibit translation of HIF-1α by ROS-dependent activation of AMPK and downregulation of the mTOR/p70S6K/S6 mRNA translation signalling axis. In a parallel and possibly cell type-dependent mechanism, prazosin-induced ROS may also promote the activation of AKT, resulting in pVHL-mediated degradation of existing HIF-1α, further contributing to the reduction of intracellular HIF-1α.

While the present study does not provide a complete evaluation of the radiosensitising effect and underlying mechanisms of prazosin, it provides a platform for further investigation. Of particular interest is whether other commonly used α1-adrenoceptor antagonists, such as alfuzosin, terazosin and doxazosin possess a similar hypoxia-selective radiosensitising effect. It was previously reported that simultaneous irradiation and doxazosin or terazosin exposure did not radiosensitise prostate cancer cells 24 h after irradiation (Cuellar et al., 2002). However, the authors report a schedule-dependent effect suggesting that doxazosin or terazosin prior to or after irradiation may enhance irradiation-induced cytotoxicity. These conflicting findings may be attributed to differences in irradiation dosing (3 Gy vs. 6.2). However, the lack of appreciable radiosensitisation is expected, since radiosensisation by prazosin was found to be both hypoxia-and time-dependent. Preliminary experimentation suggests doxazosin may have similar hypoxia-selective effects **(Figure 5.27**), which may translate into radiosensitising actions upon further testing.

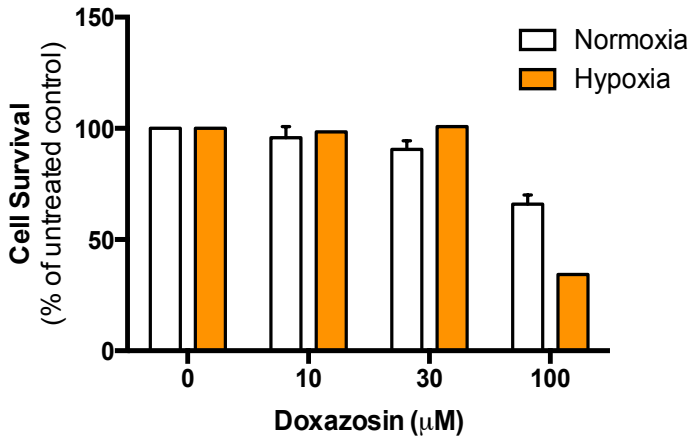


Figure 5.27. Potential hypoxia-selective effect of doxazosin (0-100 μM, 2.5 h) in PC-3 cells. Resazurin reduction was used as an indicator of cell survival following 24 h drug-free recovery. Data are expressed as percent of untreated normoxic or hypoxic control (mean ± SEM for normoxia [n=4], mean for hypoxia [n=1])

SUMMARY

The present study demonstrates that prazosin, but not tamsulosin, radiosensitises hypoxic prostate cancer cells. This effect is likely to occur via ROS-mediated inhibition of mTOR signalling and subsequently downstream suppression of HIF-1α expression and activity. Acute prazosin treatment stimulated autophagy and apoptosis in hypoxic and irradiated cells. While there was no correlation between autophagy and LNCaP cytotoxicity, autophagy may act synergistically with prazosin-induced down-regulation of HIF-1α to restore PC-3 sensitivity to irradiation.

CHAPTER 6:
FUTURE DIRECTIONS AND PRELIMINARY INVESTIGATIONS

6.1 BACKGROUND

This exploratory chapter attempts to further uncover novel uses for doxazosin and prazosin as urogenital anticancer therapies. The scope of the present experimental chapter assesses (1) the effects of these drugs on prostate stromal cells and whether these drugs alter stromal-cancer paracrine signalling; (2) investigate the potential synergism between prazosin and chemotherapeutic drugs in prostate cancer; and (3) the potential use of these drugs as intravesical treatment for non-invasive bladder cancer.

HUMAN PROSTATE STROMA AND ALPHA1-ADR ANTAGONISTS

Paracrine signalling between cancer-associated fibroblasts and prostate cancer cells is known to be reciprocal where the surrounding fibroblasts secrete growth factors (fibroblast growth factors) aiding in tumour establishment. This consequently drives a positive feedback loop where the tumour itself secretes factors, such as interleukin-6 and TGF-β, to trigger induce further growth factor release from the reactive stroma (Gandellini et al., 2015, Giannoni et al., 2010).

Prior to the revelation of the anticancer actions of doxazosin, prazosin and terazosin, these drugs were found to have a novel apoptotic effect on stromal cells in animal and human studies (Kyprianou, 2000, Turkeri et al., 2001), which was suggested to account for their sustained therapeutic effect for BPH clinically (Kyprianou et al., 2000). However, these findings preceded the current understanding of the significance of stromal-neoplastic interactions; and until recently, the relationship between antagonist-mediated stromal cytotoxicity and the consequences for prostate cancer development was unknown.

There have been several reports of alteration of stromal behaviour in response to α1-ADR antagonist treatment. Doxazosin and terazosin (25 mg/ day) were reported to alter the expression of the fibrosis-related gene and reduce basic fibroblast growth factor in the prostates of Wistar rats (Delella et al., 2012, Mitropoulos et al., 2009). Interestingly, one study demonstrated that the cytotoxic non-quinazoline naftopidil, but not silodosin

or tamsulosin, was effective in disrupting the stromal-cancer interactions of murine xenograft tumours (Hori et al., 2011). More specifically, the authors found that treatment with naftopidil reduced both stromal cell proliferation and secretion of the tumourigenic factor IL-6. It is known that these soluble factors (fibroblast growth factors and IL-6) promote the proliferation, metastasis and apoptosis-resistance of prostate tumours (Kwabi-Addo et al., 2004, Giannoni et al., 2010). While the relevance of these findings to the clinical anticancer effect of α1-ADR antagonists are less-direct, it can be hypothosised that α1-ADR antagonist-mediated suppression of secreted of fibroblast growth factors, either by indirect gene regulation or reduced number of stromal cells, may contribute to clinical anticancer findings.

Despite the clinical significance, the relative cytotoxic potencies of α1-ADR antagonists on prostate stromal cells have largely been neglected. Furthermore, whether these drugs are able to alter paracrine signalling between stromal and prostate cancer cells was previously unknown.

CHEMOTHERAPY AND A1-ADR ANTAGONISTS

The radiosensitising actions of prazosin on hypoxic prostate cancer cells shown in Chapter 5 raised the question whether prazosin may also enhance the cytotoxic potential of chemotherapies. For many cancers, chemotherapeutic agents are frequently combined with other drugs to maximize response and improve survival outcomes. Particularly in prostate cancer, docetaxel-based combinations are of significant interest to combat the resilient nature of castration-resistant prostate cancer (CRPC). Clinically investigated docetaxel-combinations such as anti-angiogenesis, anti-apoptotic inhibitors, and other cytotoxic agents were met with mixed results (Galsky and Vogelzang, 2010). Of particular interest, quinazoline-based doxazosin and prazosin are reported to have similar anti-cancer mechanisms as the aforementioned cytotoxic drugs used in combination with docetaxel including inhibition of EGFR (Hui et al., 2008) and Src (Isgor and Isgor, 2012), suppressing tumour angiogenesis (Keledjian et al., 2001), downregulating pro-survival Bcl-2 family proteins, and increasing pro-apoptotic protein expression (Chiang et al., 2005, Lin et al., 2007, Garrison and Kyprianou, 2006). Moreover, these drugs make attractive anti-cancer agents as they are safely used in clinical settings and have already been shown to have clinical anti-cancer activity (Harris et al., 2007, Keledjian et al., 2001, Tahmatzopoulos et al., 2005). While tolerable doses of doxazosin or prazosin would have little efficacy against advance stage prostate cancer, in combination with chemotherapeutics like docetaxel, these drugs may enhance prostate cancer chemosensitivity.

To date, little is known about the anticancer activity of quinazoline-based doxazosin or prazosin in combination with chemotherapeutics against cancer. One study conducted by Cal and colleagues (2000) evaluated doxazosin in combination with the cytotoxic drugs doxorubicin, etoposide, or paclitaxel for synergistic activity in prostate cancer cells. Doxazosin plus doxorubicin or etoposide resulted in synergistic effect. In contrast, doxazosin-paclitaxel treatment produced an antagonistic effect, reducing the cytotoxic potential of either drug alone (Cal et al., 2000). However, a growing body of evidence indicates that synergistic activity may occur via schedule-dependent mechanisms. Studies in other cell lines have demonstrated schedule-dependent anti-proliferative effects of the EGFR inhibitors, erlotinib and gefitinib, in combination with chemotherapeutics, including docetaxel (Morelli et al., 2005, Xu et al., 2003a) (Kassouf

et al., 2006, Mahaffey et al., 2007). Of particular interest, the quinazoline structure is common to geftinib and erlotonib, as well as, doxazosin, prazosin and terazosin (as reviewed in Chapter 4 and **Figure 4.1**). Likewise, it was previously reported that doxazosin suppressed EGFR/ERK signaling activity in breast cancer cells to a similar extent as geftinib and erlotinib(Hui et al., 2008). These findings pointed to novel EGFR inhibiting properties of quinazoline-based doxazosin. Studies evaluating the cytotoxic profile of chemotherapeutics in combination with EGFR inhibitors demonstrated greater synergism when the chemotherapeutic agent was administered first, followed by the EGFR inhibitor (Xu et al., 2003a, Morelli et al., 2005, Kassouf et al., 2006).

The effects of doxazosin, prazosin or terazosin in combination with chemotherapeutic agents are only speculative and require investigation. Therefore, the present study aimed to identify whether prazosin may have novel synergistic effects in combination with chemotherapeutic agents, such a docetaxel, in prostate cancer cells lines.

BLADDER CANCER AND A1-ADR ANTAGONISTS

Non-muscle invasive (organ-confined) bladder cancer accounts for nearly 70-80% of diagnosed urothelial tumours (Hendricksen and Witjes, 2007, Shelley et al., 2011), which is most commonly treated by a combination of transuretheral tumour resection (TURBT) and adjunct immune- or chemo-intravesical therapy to delay or prevent recurrence in moderate- to high-risk patients. In contrast to traditional systemic delivery, intravesical therapy involves administration of high concentrations of anticancer agents directly into the bladder, coming in direct contact with the bladder urothelium to target residual tumour cells (**Figure 6.1**) (Babjuk et al., 2013).

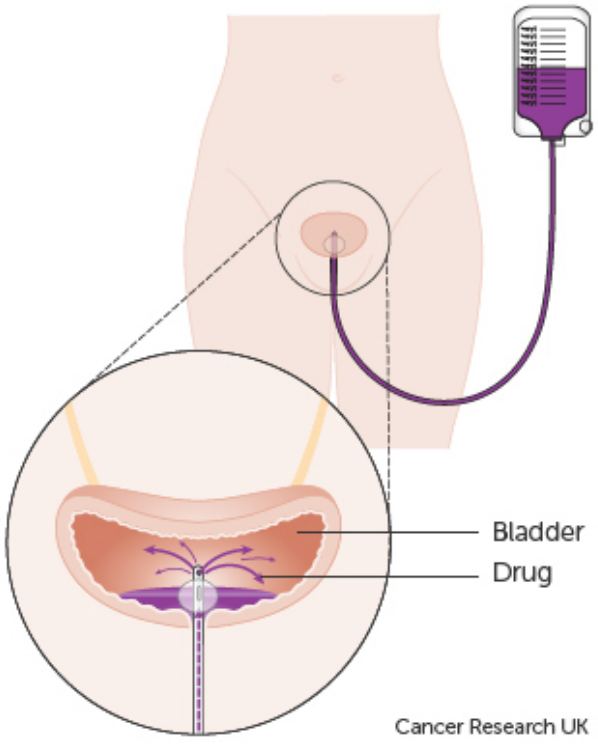


Figure 6.1. Illustration of intravesical therapy. Image provided courtesy of Cancer Research UK (2015).

In current practice intravesical therapy is carried out using Bacillus Calmette-Guerin strains or the chemotherapeutic alkylating agents, gemcitabine and mitomycin C. However, due to frequent disease recurrence and maintenance therapies (Avritscher et al., 2006), bladder cancer has the highest lifetime cost per patient compared to all cancers in the Westernised world (Sievert et al., 2009). Specifically, it is estimated that 45% of patients will develop disease recurrence following initial treatment within a 12-month window. It can be deduced that this high economic burden can be addressed in part by the reduction of intravesical drug costs or by improving treatment efficacy, thereby minimising treatment failure and subsequent salvage therapies.

Similar to prostate cancer, therapeutic concentrations of α1-ADR antagonists reportedly reduce the relative risk of developing bladder, increase apoptosis and decrease tumour vascularity of bladder tumours compared to unexposed men (Martin et al., 2008, Tahmatzopoulos et al., 2005). While only a handful, *in vitro* studies mirror findings in prostate cancer models showing comparable toxicity of supratherapeutic concentrations of these drugs in bladder cancer cell lines (Siddiqui et al., 2005, Gotoh et al., 2012) While supratherapeutic doses are unlikely to be tolerable systemically, high concentrations of these drugs might be safely used intravesically.

To date, few studies have investigated the cytotoxic effect of α1-ADR antagonists on bladder cancer cells. Furthermore, there are no published studies comparing the cytotoxic potency of these drugs and chemotherapeutic agents. The findings of this preliminary study will provide important insights for the potential use of doxazosin/prazosin as a novel intravesical therapy for the treatment of superficial bladder cancer.

6.2 AIMS

The overall aim of this study to was to investigate additional novel uses of doxazosin and/or prazosin for in the treatment of urogenital cancers.

The specific aims were to:

1. Investigate the relative cytotoxic potencies of various α1-ADR antagonists in human prostate WPMY-1 stromal cells

2. Examine the effects of indirect 2-D co-culture consisting of prostate stromal WPMY-1 and prostate cancer LNCaP and PC-3 cells, and to investigate whether the cytotoxic effects of prazosin on prostate cancer cells were altered by the presence of stromal cells.

3. Determine the relative sensitivity of prostate cancer cells to docetaxel and cabazitaxel, and investigate whether prazosin has synergistic actions when combined with docetaxel in a cellular model of castrate-resistant disease.

4. Examine the cytotoxicity of doxazosin and mitomycin C and compare the relative cytotoxic potencies of these drugs in bladder cancer T24 cells.

6.3 MATERIALS AND METHODS

AIM 1: CYTOTOXIC POTENCY OF A1-ADR ANTAGONISTS IN PROSTATE STROMAL CELLS

Human prostate stromal WPMY-1 cells were grown and maintained as previously described in Chapter 2 (General Methods). Stromal cells were seeded at $3.0x10^3$ trypan blue-excluding cells/ well in a 96-well plate and allowed to attach for 24 h. Culture medium above the cells was replaced with fresh medium containing either alfuzosin, doxazosin, prazosin, silodosin or tamsulosin (0-100 μM), and incubated for 24 – 72 h. Following appropriate treatment time, cell survival was determined using resazurin reduction as described in Chapter 2 (General Methods).

AIM 2: PRAZOSIN TREATMENT OF PROSTATE STROMAL-CANCER CO-CULTURE

Use of Indirect 2D co-culture model using Corning® Transwell ® inserts has previously been used to investigate the effects of paracrine signalling between stromal and prostate cancer cells (Li et al., 2011). In this preliminary experiment, Corning® Transwell® 0.4 µm pore polyester membrane (6.5 mm) inserts were obtained from Sigma-Aldrich (Cat. No. CLS3470) and used as described below and in **Figure 6.2**. All solutions and drugs used were described previously in Chapter 2.

PLATE PREPARATION AND SEEDING OF CELLS

Prior to use, Corning® Transwell® inserts were incubated with 200 µL of sterile complete culture medium overnight at 37˚C to equilibrate the membrane. WPMY-1 cells were seeded on the insert membrane at a density of $1.7x10^4$ trypan blue-excluding cells per insert. WPMY-1 cells were grown to confluence by incubating at 37˚C for 72 h. Two-days before the experiment, prostate cancer LNCaP cells were seeded in a 24-well plate (separate from WPMY-1) at a density of $3.0x10^5$ trypan blue-excluding cells per well and allowed to grow undisturbed for 48 h.

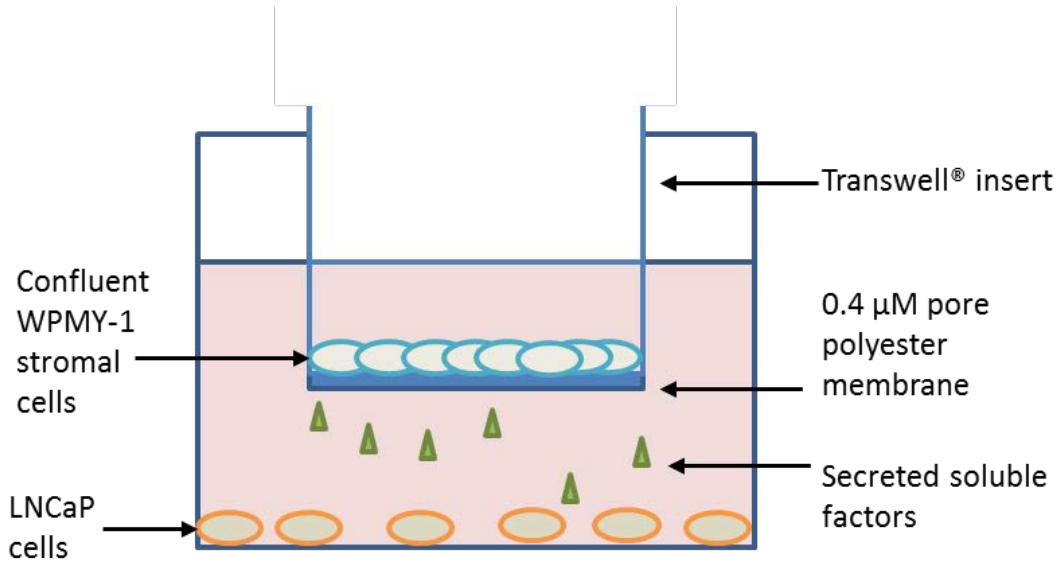


Figure 6.2. Illustration of Transwell® 2D co-culture system used.

TREATMENT OF CO-CULTURE

On the day of the experiment, Inserts containing confluent WPMY-1 cells were placed in wells containing LNCaP cells. Empty cell free inserts above LNCaP cells were included as negative controls. Spent medium was removed and replaced with fresh medium containing either vehicle or prazosin (1-30 μM) in both the upper (100 μL) and lower chambers (500 μL) of the Transwell® system. Cells were treated for 72 h and changes in cell survival or proliferation were determined by resazurin reduction assay.

AIM 3: PRAZOSIN AND CHEMOTHERAPY COMBINATION TREATMENT

RELATIVE CYTOTOXIC POTENCY

Prostate cancer cells were grown and maintained as previously described in Chapter 2 (General Methods). PC-3 and LNCaP cells were seeded at $3.0x10^3$ and $5.0x10^3$ trypan blue-excluding cells/ well in a 96-well plate and allowed to adhere for 24 or 48 hours, respectively. Following appropriate incubation time, medium above the cells was replaced with fresh complete culture medium containing either docetaxel or cabazitaxel (0-1 μM) for 24 – 72 h.

COMBINATION TREATMENT

For combination treatment of prazosin with docetaxel, cells were seeded as described above. Following appropriate incubation for cell attachment, culture medium above the cells was replaced with medium containing either single agent prazosin or docetaxel according to the schedules (A-C) shown in **Figure 6.3**. Concentrations used for combination treatments were based on pre-determined time- and cell type-dependent IC_{50} values. Each drug was used at their respective IC_{50} (nM) x 1, 0.5 and 0.25 for all treatment durations and will be indicated where appropriate throughout the Results section. Following appropriate treatment time, cell survival was determined using resazurin reduction as previously outlined in Chapter 2 (General Methods).

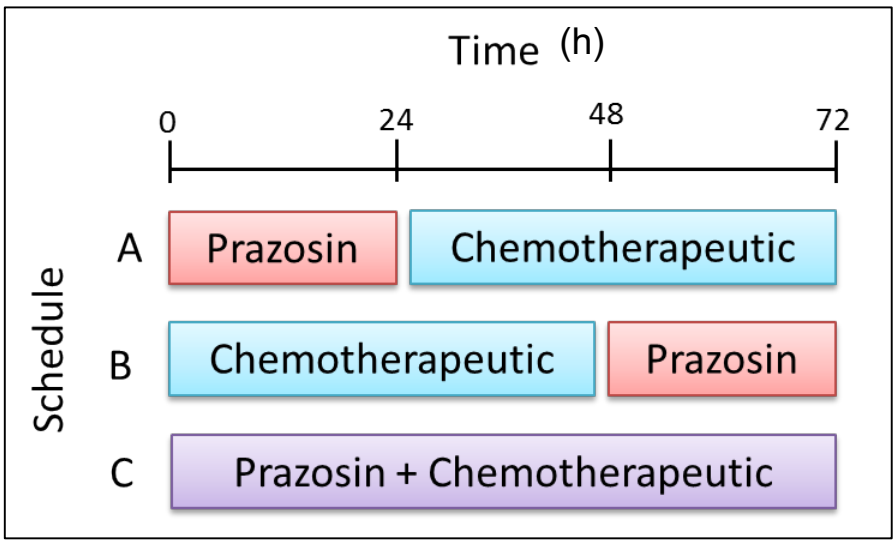


Figure 6.3. Prazosin and chemotherapeutic agent (docetaxel) combination treatment schedules.

AIM 4: COMPARISON OF DOXAZOSIN AND MMC TOXICITY IN BLADDER CANCER

Mitomycin C (MMC) is a DNA cross-linking (alkylating) agent isolated from *Streptomyces caespitosus* bacterial strain (**Figure 6.4**). In clinical settings, MMC is instilled intravesically at a concentration of 40mg per 20 ml of sterile water (5.98 mM) and is retained in the bladder for 2 hours (Au et al., 2001). For this experiment, MMC was obtained from Sigma-Aldrich. Stock solutions were made in DMSO and stored at -20˚C protected from light.

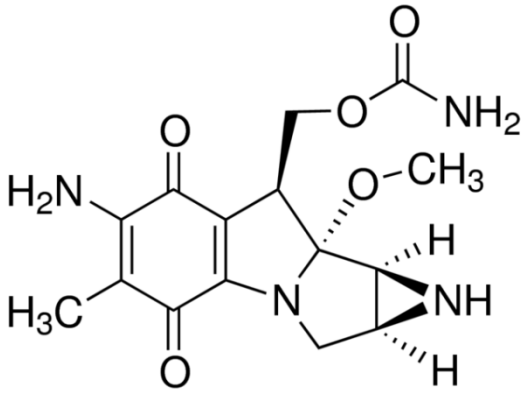


Figure 6.4. Chemical structure of mitomycin C.

CELLS AND DRUG TREATMENT

Human bladder cancer T24 cells were used to investigate the effects of doxazosin on an *in vitro* model of bladder cancer, as well as compare the relative cytotoxicity of doxazosin to the intravesical chemotherapeutic MMC. The T24 cell line is a stable model of a grade 3 transitional cell (urothelial) carcinoma (Bubenik et al., 1973, Kato et al., 1978) and has been used previously to examine the cell-based effects of intravesical treatment (Kang et al., 2013, Kang et al., 2015).

To compare the cytotoxic potency of MMC to that of doxazosin, bladder T24 cancer cells were seeded at $1.0X10^4$ trypan blue-excluding cells/well and incubated overnight (>24 h) for attachment. Culture medium above the cells was replaced with fresh complete medium containing either doxazosin (0-100 μM) or mitomycin C (MMC; 0-10 mM) for either 2 h (clinical duration) or 24 – 72 h continuous treatment. To mimic

intravesical treatment, cells that received 2 h treatment were washed with PBS and incubated for a further 24 – 72 h in drug-free complete growth medium at 37ºC. Following appropriate incubation, cell survival was determined using resazurin reduction (Chapter 2, General Methods).

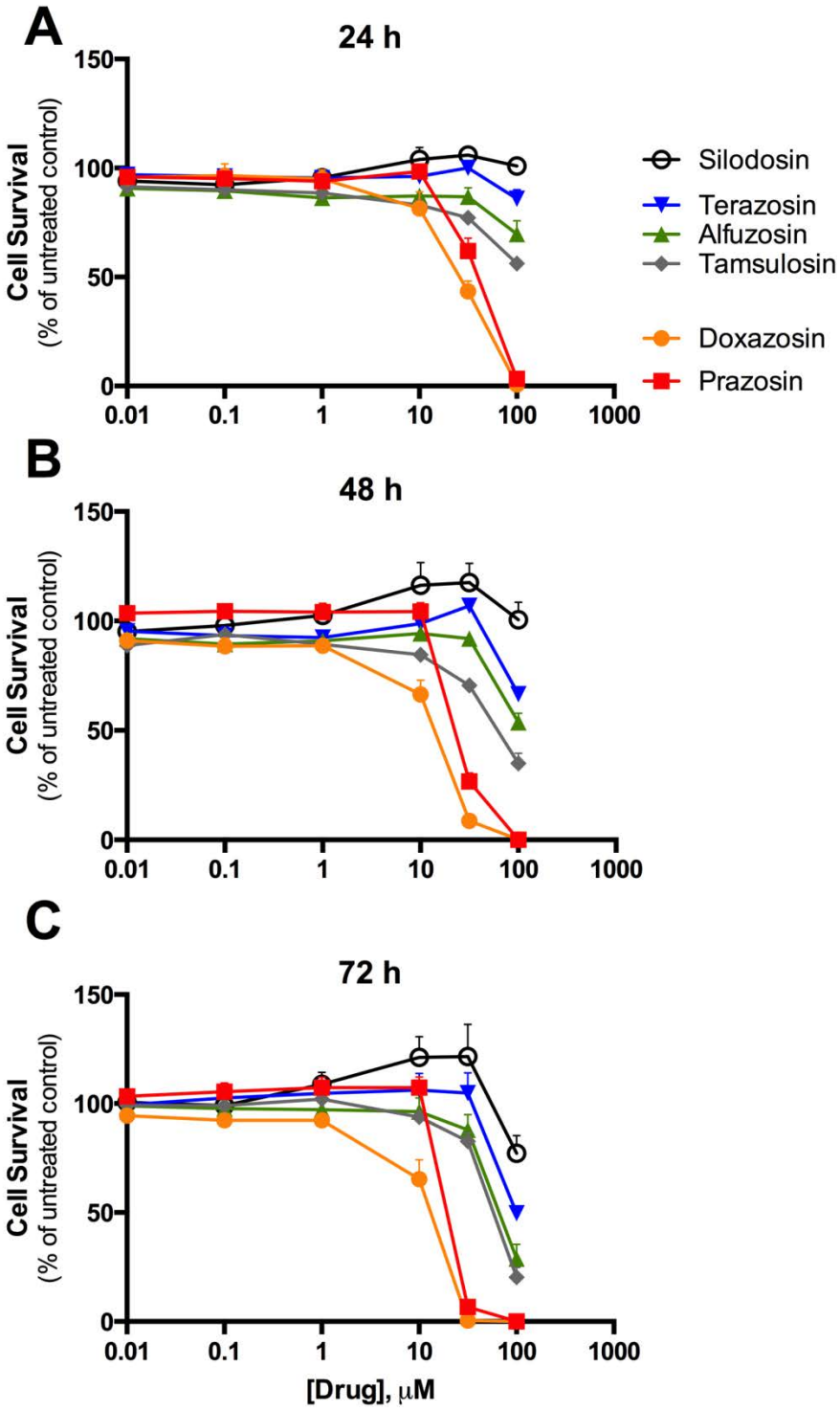
STATISTICAL ANALYSIS

Data were analysed and graphs were drawn using GraphPad Prism (ver. 6 for Mac OSX). Where appropriate, the relative IC_{50} value for each drug was determined for the concentration resulting in 50% of maximal reduction in cell survival (resazurin reduction) and IC_{50} values were obtained determined using non-linear regression. Statistical significance was determined using either one-way or two-way ANOVA as appropriate. Specific post hoc tests are indicated throughout the relevant portions of the Results section.

6.4 RESULTS

AIM 1: RELATIVE CYTOTOXIC POTENCY OF A1-ADR ANTAGONISTS ON WPMY-1 CELLS

To investigate whether α1-ADR antagonists also have cytotoxic effects on human prostate stromal cells, WPMY-1 cells were treated continuously for 24-72 hours with alfuzosin, doxazosin, prazosin, silodosin, tamsuolsin or terazosin and cell survival was determined by resazurin reduction. As shown in **Figure 6.5**, all of the investigated antagonists were cytotoxic over the concentration range tested on WPMY-1 cells. Overall, these cytotoxic effects were more pronounced in WPMY-1 cells compared to prostate cancer LNCaP or PC-3 cells (Chapter 3). Similar to findings in prostate cancer cells, 100 μM doxazosin and prazosin significantly reduced cell survival at all time points, with 100 μM completely abolishing cell survival at 72 h ($P<0.001$). In contrast to previous findings in prostate cancer cell lines (Chapter 3), alfuzosin and tamsulosin, were able to significantly reduce prostate stromal cell survival by 71.2% (± 6.62) and 79.7% (± 4.52) at 72 h, respectively ($P<0.001$ for both vs. untreated control) (**Figure 6.5 C**). Unlike findings in prostate cancer cells, terazosin was significantly less cytotoxic than alfuzosin and tamsulsoin at all time points, except for alfuzosin at 48 h, where these drugs demonstrated a similar reduction in cell survival ($P=0.064$). Interestingly, silodosin (10-30 μM) had a time-dependent hormetic effect on WPMY-1 cells, with 30 μM enhancing cell survival by approximately 6 % (± 3.5), 17.5% (± 8.9) and 21.5 % (±14.8) at 24, 48 and 72 h respectively ($P=0.015$) for 48 and 72 h). However, 100 μM silodosin also modestly, yet significantly, decreased cell survival after 72 h treatment ($P=0.009$).



.

Figure 6.5. Human prostate stromal WPMY-1 cells were treated with alfuzosin, doxazosin, prazosin, silodosin, tamsulosin or terazosin for 24 (A), 48 (B) or 72 h (C). Cell survival was determined by resazurin reduction assay. Data are expressed as percentage of untreated control (mean ± SEM, n≥4).

The relative IC_{50} values were only obtained for doxazosin, prazosin, tamsulosin 24-72 h) and alfuzosin (72 h) as determination of silodosin and terazosin IC_{50} values resulted in ambiguous results due to incomplete dose response curve. As shown in **Table 6.1 & Figure 6.6**, all three drugs were statistically equipotent at 24 h, however doxazosin and prazosin were more potent than tamsulosin at 48 and 72 h, respectively. At 24 hours, the relative cytotoxic potency was found to be: doxazosin = prazosin = tamsulosin > alfuzosin > terazosin > silodosin. The potency order was the same at the other time points, except doxazosin was more potent than both tamsulosin and prazosin at 48 h, and doxazosin > prazosin > tamsulosin at 72 h.

Table 6.1	**Relative IC_{50} values *(95% CI)* (µM)**		
	24 h	**48 h**	**72 h**
Alfuzosin	*Ambiguous*	*Ambiguous*	**69.5** *(59.0-81.8)*
Doxazosin	**70.0** *(38.0 – 129)*	**10.2** *(7.39 – 14.2)*	**11.3** *(7.82 – 16.3)*
Prazosin	**36.6** *(33.1 – 40.6)*	**24.4** *(16.1 – 36.9)*	**19.7** *(11.7-33.3)*
Silodosin	*Ambiguous*	*Ambiguous*	*Ambiguous*
Tamsulosin	**100.8** *(33.6 – 303)*	**61.3** *(50.1 – 74.9)*	**58.1** *(51.7 – 65.2)*
Terazosin	*Ambiguous*	*Ambiguous*	*Ambiguous*

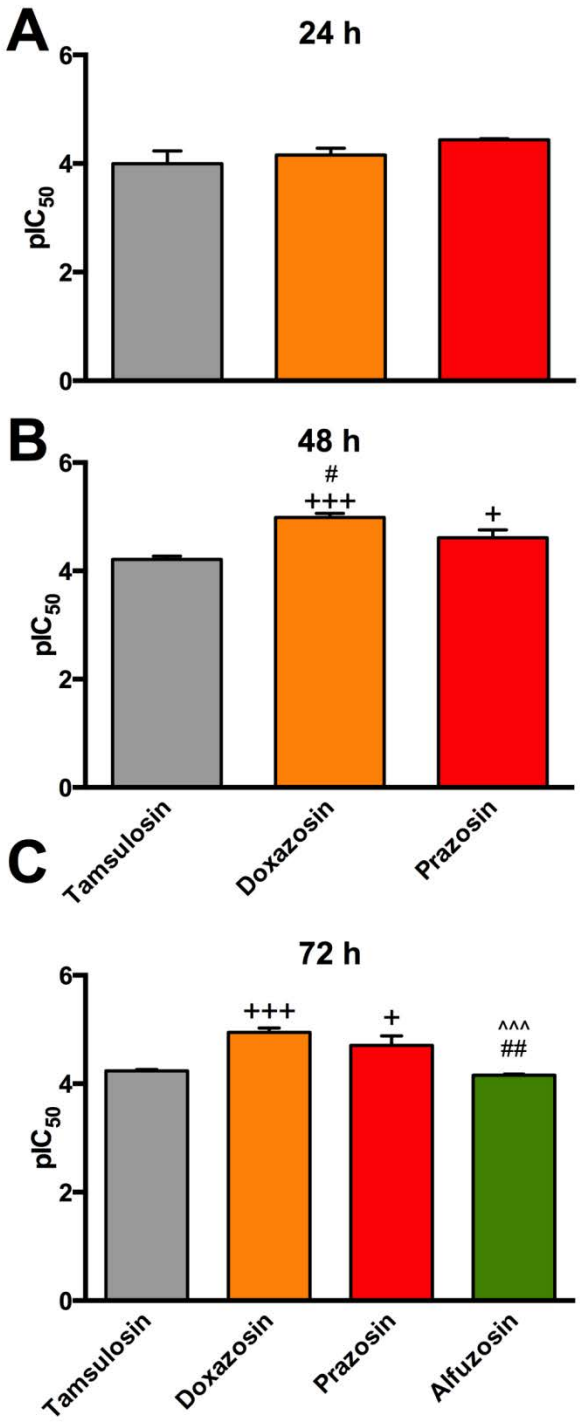


Figure 6.6. Comparison of WPMY-1 pIC_{50} values of tamsulosin, doxazosin and prazosin at 24 (A), 48 (B) and 72 h (C). pIC_{50} values were determined by non-linear regression and data are represented at the mean ± SEM (n≥4). Statistical analysis was determined using one-way ANOVA with Tukey's post hoc test. +P<0.05, ++P<0.01 and +++P<0.001 vs. tamsulosin. ## P<0.01 vs. prazosin and ^^^P<0.001 vs. doxazosin.

AIM 2: EFFECT OF PRAZOSIN ON WPMY-1 AND LNCAP CO-CULTURES

Next, it was investigated whether indirect co-culture of WPMY-1 with AR-positive LNCaP prostate cancer cells would alter the proliferation of prostate cancer cells and/or cell survival in response to prazosin treatment. To investigate this, cells were indirectly co-cultured using Transwell® tissue culture inserts, and were treated for 72 h with prazosin (0 – 30 μM). Resazurin reduction was used as an index of cell survival. As shown in **Figure 6.7**, co-culture with WPMY-1 modestly enhanced untreated LNCaP cell survival by approximately 12% at 72 h. In this experimental model, there was no significant change in LNCaP sensitivity to prazosin in the presence of stromal co-culture (**Figure 6.7**). While further investigation may be necessary, these preliminary findings suggest that prostate-stromal paracrine signalling does not alter prazosin-induced cytotoxicity of AR-positive LNCaP cells.

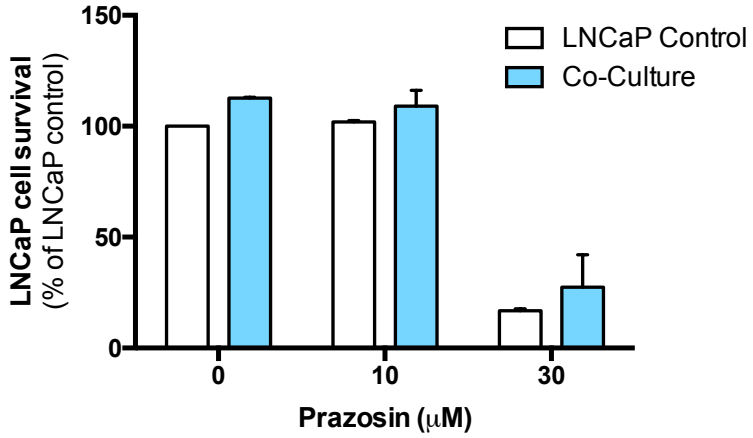


Figure 6.7. Comparison of cell survival following 72 h prazosin treatment between LNCaP mono-culture and co-culture with human stromal WPMY-1 cells. Cell survival was determined using the resazurin reduction assay. Data are expressed as percentage of LNCaP untreated control (mean ± SEM, n=3).

AIM 3: EFFECT OF PRAZOSIN IN COMBINATION WITH DOCETAXEL

CYTOTOXIC POTENCY OF DOCETAXEL AND CABAZITAXEL TREATMENT

Docetaxel is the current standard of care therapy for castrate-resistant prostate cancers. The new chemically similar taxane-based agent, cabazitaxel is currently used as a salvage therapy following docetaxel treatment failure. To determine the relative cytotoxic potency of docetaxel and cabazitaxel, PC-3 and LNCaP prostate cancer cells were treated continuously for 24 – 72 h. Cell survival was then determined using resazurin reduction. Overall, these drugs reduced PC-3 and LNCaP cell survival in a concentration- and time-dependent fashion (**Figure 6.8**). However, in both cell lines, cytotoxicity to docetaxel and cabazitaxel plateaued at approximately 10 nM at all time points, except for LNCaP where concentrations greater than 0.1 nM had no further effect at 72 h. The relative IC_{50} values for docetaxel and cabazitaxel are shown in **Tables 6.2** and **6.3** for PC-3 and LNCaP cells, respectively. Under the conditions tested, the cytotoxic potencies of these drugs were similar. Therefore, the first-line chemotherapeutic docetaxel was used for preliminary investigations of synergistic activity in combination with prazosin.

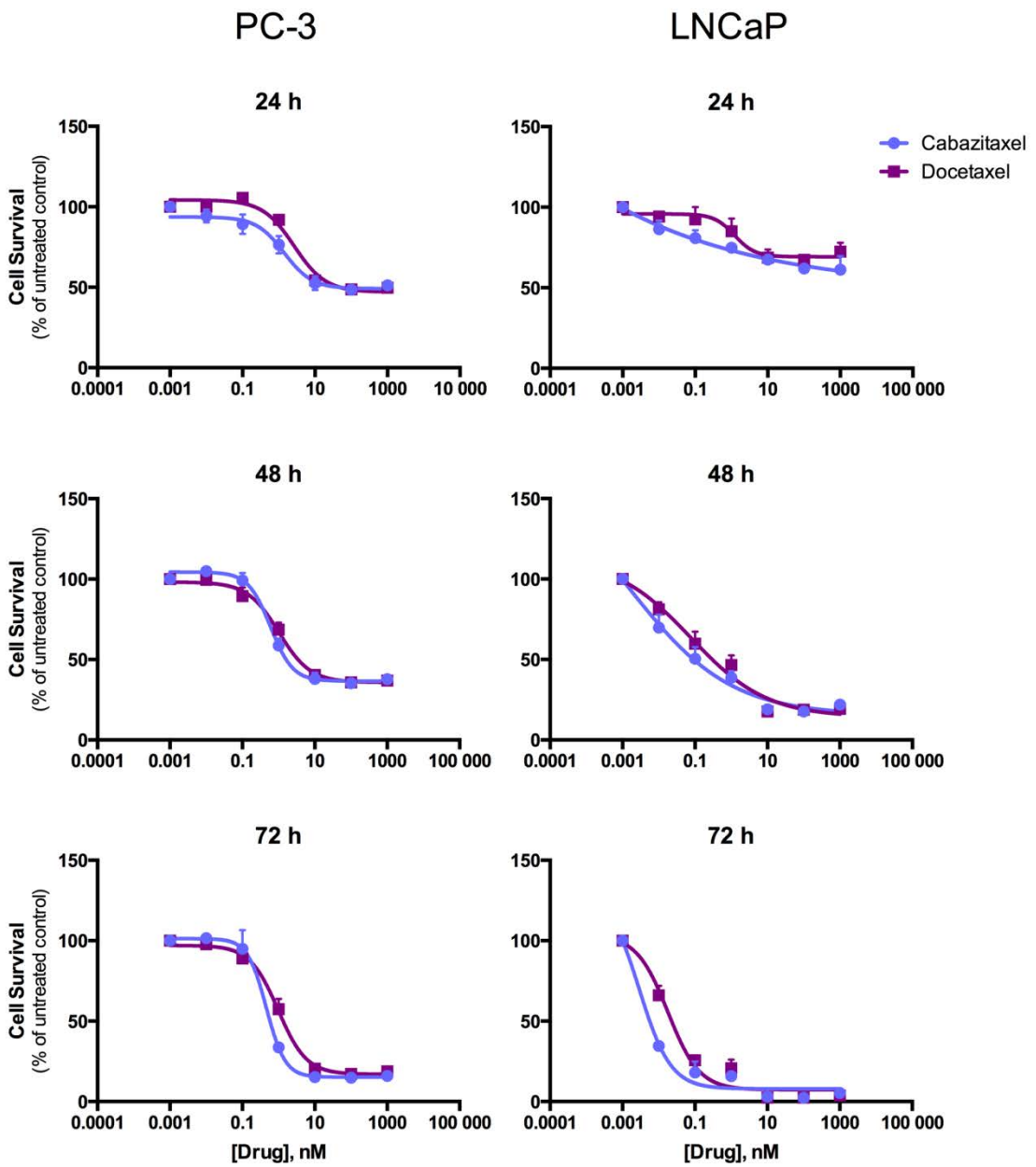


Figure 6.8. PC-3 (left) and LNCaP (right) cell survival following 24, 48 or 72 h continuous treatment with cabazitaxel or docetaxel (0-1µM). The resazurin reduction assay was used as an index of cell survival. Data are represented as percentage of untreated control (mean ± SEM, n=6).

Table 6.2	PC-3 relative IC_{50} values *(95% CI)*		
	24 h	**48 h**	**72 h**
Docetaxel (nM)	2.40 *(1.7 – 3.4)*	0.97 *(13.0 – 16.6)*	0.97 *(9.8 – 21.3)*
Cabazitaxel (nM)	1.4 *(0.6 – 3.3)*	0.60 *(0.37 – 0.97)*	0.46 *(0.27 – 0.79)*

Table 6.3	LNCaP relative IC_{50} values *(95% CI)*		
	24 h	**48 h**	**72 h**
Docetaxel (nM)	1.03 *(0.2 – 5.1)*	0.07 *(0.01 – 0.53)*	0.02 *(0.01 – 0.03)*
Cabazitaxel (nM)	0.11 *(0.6 – 3.3)*	0.023 *(0.37 – 0.97)*	0.003 *(0.002 – 0.005)*

Prazosin and Docetaxel combination treatment

Next, it was investigated whether prazosin may have a synergistic effect when combined with docetaxel. Since chemotherapy regimens are classically reserved for castrate-resistant disease, AR-negative PC-3 cells were used for clinical relevance. Prostate cancer PC-3 cells were treated according to the treatment schedule shown previously in **Figure 6.3** (Methods section). Briefly, cells were exposed to prazosin before, after or concurrently with docetaxel at the concentrations shown in **Table 6.4**. Following appropriate treatment time, cell survival was determined using resazurin reduction assay.

As shown in **Figure 6.9**, none of the investigated schedules (A, B and C) were found to enhance cytotoxicity in PC-3 cells to levels that were significantly greater than single-agent treatment with either prazosin or docetaxel.

Table 6.4. Concentrations used according to treatment schedule

Schedule	Prazosin (µM)*	Docetaxel (nM)
A	14, 28, 55	0.2, 0.48, 0.97
B		
C	5, 11, 21	0.2, 0.48, 0.97

*Schedules: [A] Prazosin 24 h, docetaxel 48 h; [B] chemotherapy 48 h, prazosin 24 h; [C] prazosin + chemotherapy 72 h. *Values were rounded to nearest whole number.*

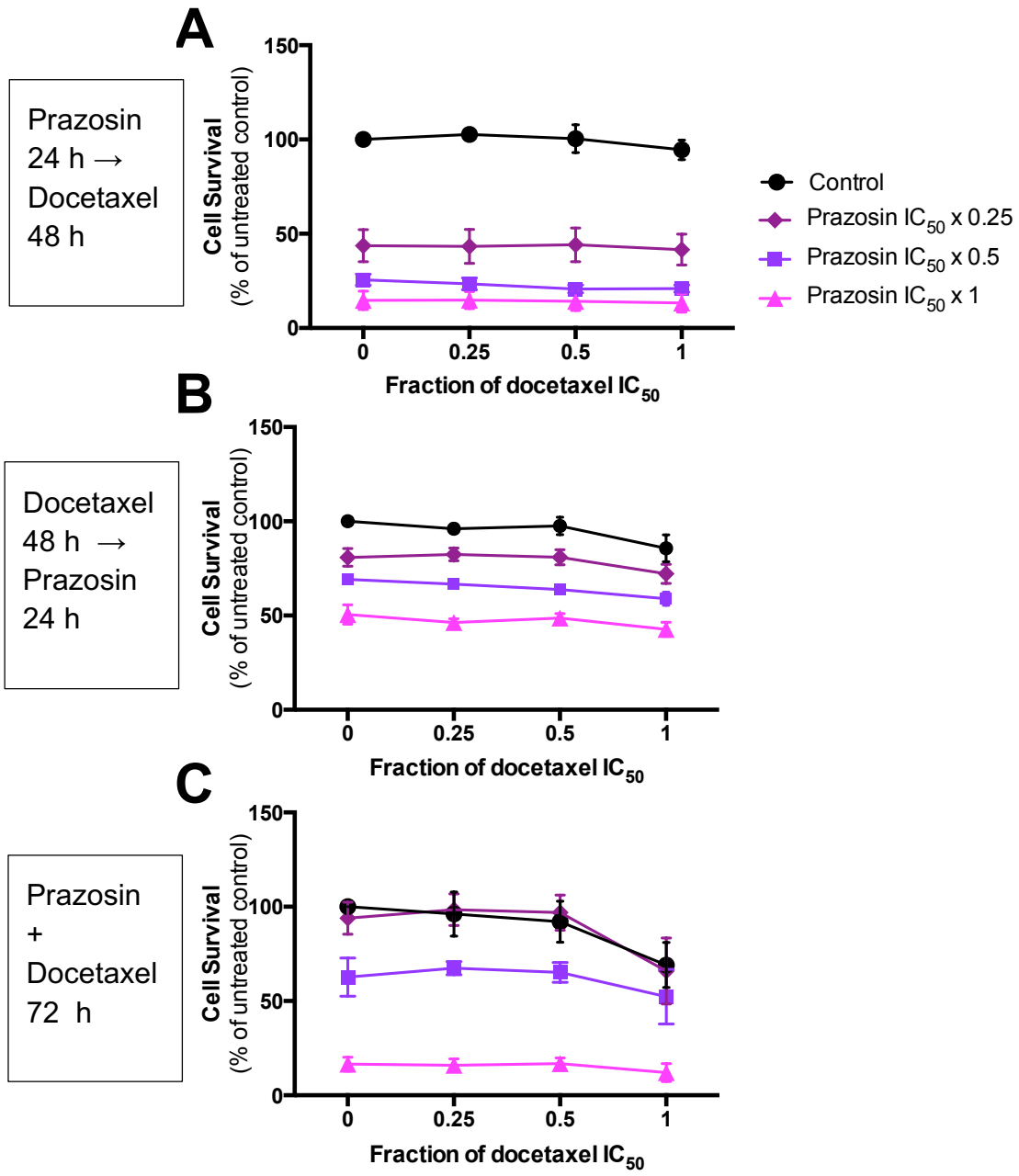


Figure 6.9. Human AR-negative PC-3 cell survival following treatment with either single-agent prazosin or docetaxel (0, 0.25, 0.5, 1 X IC_{50}) or in combination in a schedule dependent manner. Schedules were (A) 24 h prazosin followed by 48 h docetaxel; (B) 48 h docetaxel followed by 24 h prazosin; and (C) 72 h simultaneous prazosin and docetaxel combination. Cell survival was determined by resazurin reduction. Data are expressed as percentage of untreated control (mean ± SEM, n≥3).

AIM 4. COMPARE CYTOTOXIC POTENCIES OF MMC AND DOXAZOSIN IN T24 BLADDER CANCER CELLS

CONTINUOUS TREATMENT

To compare the relative cytotoxic potencies of doxazosin and the clinically used bladder cancer chemotherapeutic MMC, T24 cells were treated with doxazosin (0-100 μM) or MMC (0-10 mM) for 24, 48 or 72 h. Cell survival was determined at each time point using the resazurin reduction assay. As demonstrated in **Figure 6.10**, both MMC and doxazosin induced time-dependent reduction in T24 cell survival. However, maximal effect was observed only after 48 h continuous exposure in doxazosin treated cells. At 72 h, concentration greater than 0.1 mM and 1 μM of MMC and doxazosin, respectively, were capable of significantly reducing T24 cell survival compared to untreated control ($P<0.001$). Furthermore, doxazosin was found to be more potent than MMC at all time points investigated (**Figure 6.11**). Relative IC_{50} values for each drug and time point are detailed in **Table 6.5.** For both MMC and doxazosin, comparisons of the time point cytotoxic potencies was significantly different between 24 h vs. 48 h or 72 h ($P<0.01$ for all). However, there was no significant difference in potency between 48 vs. 72 h for either drug. In comparing the IC_{50} values, doxazosin was found to be between 7- and 36-times more potent than MMC at all time points ($P<0.001$).

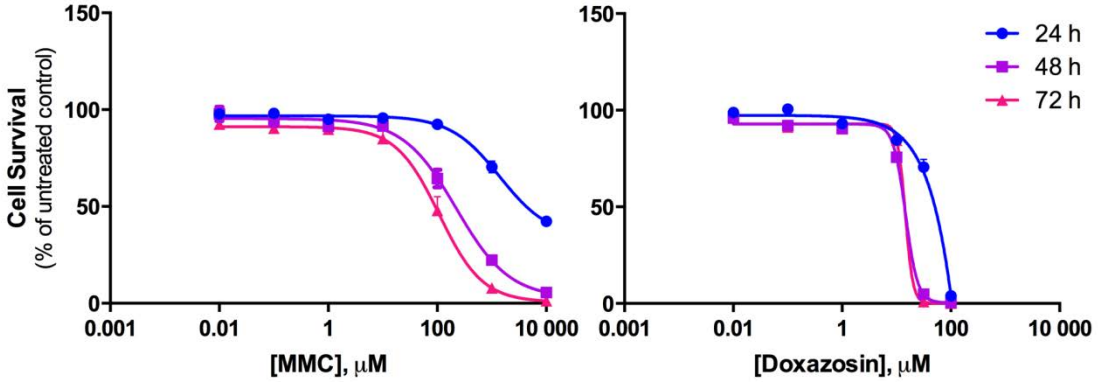


Figure 6.10. Time-dependent reduction of T24 cell survival following MMC or doxazosin treatment. Resazurin reduction assay was used as an index of cell survival. Data are expressed as percentage of untreated vehicle control (mean ± SEM, n=6).

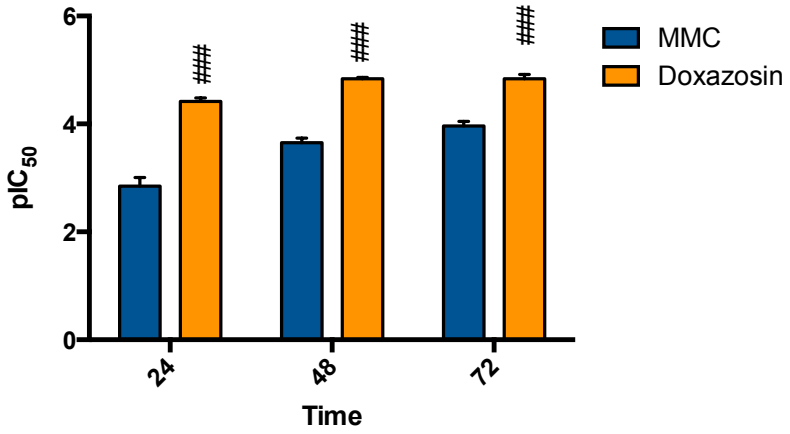


Figure 6.11. Comparison of pIC_{50} values between MMC and doxazosin following 24, 48 and 72 h treatment of T24 bladder cancer cells. pIC_{50} values were determined by non-linear regression and were represented as the mean ± SEM (n=6). Statistical significance was determined using a two-way ANOVA with Tukey's post hoc test. ### P<0.001 vs. MMC.

Table 6.5. IC_{50} values for continuous MMC or doxazosin treatment in T24 bladder cancer cells

Drug	Relative IC_{50} values (95% CI) (µM)		
	24 h	48 h	72 h
MMC	**1402** (672.0 – 2925)	**223** *(148 – 334)*	**109** *(74.2 – 159)*
Doxazosin	38.1 *(28.5-50.9)*	14.6 *(13.0 – 16.6)*	14.5 *(9.8 – 21.3)*

CLINICAL DURATION TREATMENT AND DRUG-FREE RECOVERY

Intravesical therapy is unique to bladder cancer, where high concentrations of cytotoxic agents are instilled into the bladder. This therapy is advantageous in the sense that it provides means for direct targeting of the tumour with doses that would be intolerable systemically. To mimic intravesical treatment, cells were treated acutely (2 h) with doxazosin or MMC followed by 24 – 72 h drug free recovery. Cell survival was then determined by the resazurin reduction assay. As shown in **Figure 6.12**, both doxazosin (100 μM) and MMC (1-10 mM) were effective in reducing T24 survival following 2 h treatment and drug-free recovery ($P<0.05$-0.001 for all time points). Interpolation of the clinically relevant MMC dose (6 mM) following 72 h drug-free recovery revealed an approximate 85% (95% CI = 78.4-90.3) reduction in T24 survival, which was slightly greater than doxazosin at one- one-sixtieth of the concentration (100 μM, 58% ±[7.3]). **Table 6.6** details the relative IC_{50} values for doxazosin and MMC at all time points. However, the IC_{50} values between doxazosin and MMC could not be compared statistically as the dose-response for doxazosin curve was incomplete, resulting in ambiguous values. Therefore, T24 cell survival was compared between MMC and doxazosin treatment at 100 μM. This concentration was chosen, because T24 cells displayed cytotoxicity at 100 μM for both drugs (**Figure 6.13**). At 100 μM, doxazosin was significantly superior to MMC in reducing cell survival at all time points ($P<0.001$). Specifically, doxazosin (100 μM, 2 h) was at least twice as toxic to T24 survival compared to MMC (100 μM, 2 h) treatment following 24 – 72 h drug-free recovery. These novel findings indicated that doxazosin may be more effective than the current intravesical chemotherapy agent mitomycin C.

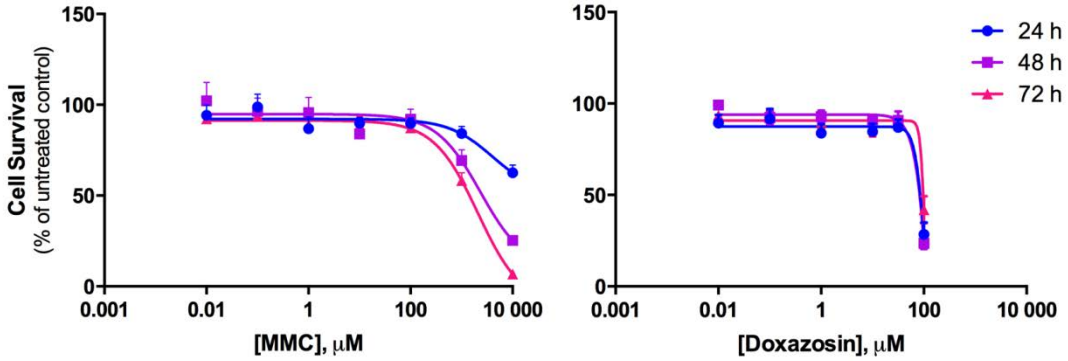


Figure 6.12. T24 cell survival following 2 h treatment with MMC (0-10 mM) or doxazosin (0-100 μM) followed by 24 – 72 h drug-free recovery. Resazurin reduction assay was used as an index of cell survival. Data are expressed as percent of untreated control (mean ± SEM, n=6).

Table 6.6. IC_{50} values for 2 h treatment with MMC or doxazosin and 24 – 72 h recovery in T24 bladder cancer cells

	Relative IC_{50} values (95% CI) (μM)		
Drug	**24 h**	**48 h**	**72 h**
MMC	**4054** (293.5 – 56000)	**2341** *(725 – 7557)*	**2130** *(1310 – 33760)*
Doxazosin	*> 100 Ambiguous*	*> 100 Ambiguous*	*> 100 Ambiguous*

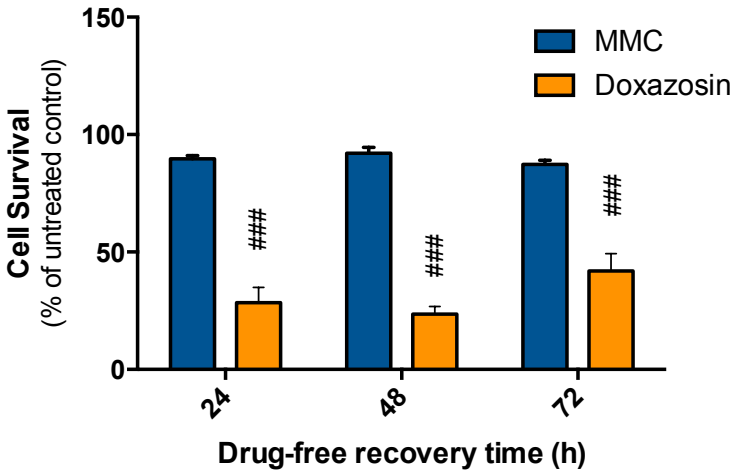


Figure 6.13. Comparison of T24 cell survival after 2 h treatment with 100 µM MMC or doxazosin followed by 24, 48 or 72 h drug-free recovery. Resazurin reduction was used as an index of cell survival. Data are expressed as percentage of untreated control (mean ± SEM, n=6). Statistical significance was determined using a one-way ANOVA with Tukey's post hoc test. ### P<0.001 vs. MMC.

6.5 DISCUSSION

The aim of this study was to investigate additional uses for doxazosin or prazosin as potential therapies for urogenital cancers. Specifically, it was investigated whether α1-prazosin may possess novel effects on prostate stromal-cancer paracrine signalling or synergistic actions in combination with chemotherapy (docetaxel). Furthermore, doxazosin was compared to MMC chemotherapy to uncover a potential novel use of α1-ADR antagonists for bladder cancer intravesical therapy.

ALPHA1-ADR ANTAGONISTS AND PROSTATE STROMAL CELLS (AIMS 1 & 2)

Consistent with previous literature, the findings in this study show cytotoxic effects of α1-ADR antagonists on prostate stromal cells. While the cytotoxic actions of doxazosin on prostate stroma are known (Kyprianou et al., 2000), the effects of other drugs such as prazosin, tamsulosin and silodosin at therapeutically relevant and supratherapeutic concentrations were previously unknown. Unlike findings in prostate cancer cells, the classically lesser-toxic agents such as the quinazoline alfuzosin and non-quinazolines silodosin and tamsulosin were found to induce significant toxicity to WPMY-1 cells in a time- and concentration-dependent manner. Furthermore, the cytotoxic effect of alfuzosin and tamsulosin (quinazoline and non-quinazoline, respectively) (100 μM) were similar to doxazosin and prazosin at all time points (24-72 h). Although the expression profile of α1-ADR subtypes in WPMY-1 cells is currently unknown, the enhanced cytotoxic potency of these drugs (including non-quinazolines silodosin and tamsulosin) suggests antagonism of α1-ADR might contribute to WPMY-1 cytotoxicity. These findings challenge previous reports of α1-ADR-independence, as well as, quinazoline-dependence. The potential involvement of α1-ADR antagonism in cell cytotoxicity is discussed in the General Discussion (Chapter 7). Therefore, it can be inferred that mechanisms driving the cytotoxic activities of these drugs may differ from prostate cancer models (as shown in Chapter 4).

Since stromal-tumour interactions play an important role in prostate tumourigenesis, it was investigated whether prazosin may alter these interactions, specifically paracrine signalling, in attempts to provide insight into the differences between *in vitro* potency

and clinical anti-tumour efficacy of these drugs. The present study did not identify any significant changes to prazosin-induced cytotoxicity on prostate cancer cell survival during co-culture with stromal cells. However, further optimisation of the co-culture methodology used here may be required. More specifically, the lack of effect of stromal paracrine signaling on prostate cancer cells cannot be ruled out until findings are confirmed with the incorporation of a positive control. These findings may conflict with a previous report (Hori et al., 2011) that cytotoxic naftopidil reduced stroma-tumour interactions *in vivo*. However, it is proposed these effects are likely mediated by disruption of direct physical interactions as opposed to altered cell-to-cell paracrine signalling. Further investigation and optimisation of 2-D, as well as 3-D co-culture models (Fong et al., 2016) may provide further insight to the changes in the prostate cancer-stroma tumour microenvironment triggered by prazosin or other quinazolines such as doxazosin and terazosin.

PRAZOSIN AND CHEMOTHERAPY TREATMENT OF PROSTATE CANCER (AIM 3)

It was found that, unlike radiotherapy, prazosin does not appear to have synergistic effects in prostate cancer cells when combined with docetaxel. These preliminary findings are the first report of attempts to enhance chemotherapeutic sensitivity through the addition of prazosin in prostate cancer cells. No evidence was found of synergy between prazosin and docetaxel cytotoxicity. At this time the reasons behind the lack of synergy between these drugs is only speculative, but may be due to incompatible cell death mechanisms or p-glycoprotein pump-mediate efflux of docetaxel and prazosin (described in more detail below). Further studies investigating other quinazoline antagonists such as doxazosin for cytotoxic synergy with docetaxel or cabazitaxel in prostate cancer cells would be particular interest.

The pharmacodynamics between doxazosin and docetaxel remain unclear, but the lack of synergistic effect may be due to incompatible cell death mechanisms between these agents. In one study evaluating doxazosin and the taxane paclitaxel found a similar lack of synergistic cytotoxicity (Cal et al., 2000). It was reasoned that paclitaxel-mediated stabilisation of microtubules may have prevent the cytotoxic actions of doxazosin. Therefore, it can be reasoned that administration of doxazosin, or other quinazoline α1-

blockers, and taxane chemotherapy such as docetaxel or cabazitaxel may exhibit reduced potency compared to either agent alone. Synergism may be achieved through schedule-dependent treatment protocols as investigated in the present preliminary study. However, further investigations are required to fully elucidate the effect of the combination of these drugs *in vitro*.

It is known that docetaxel is a substrate of p-glycoprotein pumps (Shirakawa et al., 1999), which act to detoxify the cell and are particularly important in driving innate and acquired chemoresistance (Zhang et al., 2015b). A review of the literature reveals that prazosin is also a substrate of these p-glycoprotein pumps and is commonly used to monitor p-glycoprotein pump activity *in vitro* (Shapiro et al., 1999, Rautio et al., 2006). As mentioned previously, it is unknown whether the prazosin-p-glycoprotein pump interaction contributed to the lack of appreciable synergistic effect. However, one study has reported that PC-3 cells do not express p-glycoprotein (O'Neill et al., 2011), further complicating the rationalisation for the observed lack of synergistic activity between prazosin and docetaxel in the present study.

Doxazosin was reported to inhibit p-glycoprotein and ATP-binding cassette (ABC) G2 transporter, which has been shown to reduce efflux of radiolabelled drugs and significantly enhanced the sensitivity of cells overexpressing these transport proteins to paclitaxel and mitoxantrone by 18- and 38-fold (Takara et al., 2009a, Takara et al., 2012). Interestingly, reversal of mitoxantrone resistance in cells over-expressing ABCG2 transporter was also accomplished by other α1-ADR antagonists such as naftopidil and prazosin, but not terazosin (Takara et al., 2012). Overall, these effects have been largely attributed to direct inhibitory interactions of these drugs with the transport pump as opposed to altered protein expression (Takara et al., 2009b, Takara et al., 2012). While the present preliminary findings demonstrated no change in cytotoxic potential of docetaxel in prostate cells, the literature suggests that these drugs might be useful in reversing acquired chemotherapy resistance, particularly in the event of docetaxel-treatment failure mediated by aberrant efflux pump activity. Further *in vitro* investigations using cellular models of castrate-resistance and acquired docetaxel-resistance (O'Neill et al., 2011), coupled with *in vivo* models, would be of significant interest to continue to elucidate the synergistic effects of doxazosin/prazosin in clinically relevant settings.

DOXAZOSIN AND BLADDER CANCER (AIM 4)

This exploratory chapter identified potential use for doxazosin as a novel intravesical therapy for bladder cancer. It was found that in both continual treatment 24-72 h and 2 h (clinical duration) followed by 24-72 h recovery, doxazosin demonstrated superior reductions in cell survival compared to the commonly used intravesical therapy, mitomycin C (MMC).

While previous literature has described the cytotoxic potential of doxazosin and prazosin on a plethora of urogenital cancers (Tahmatzopoulos et al., 2005, Sakamoto et al., 2011, Gotoh et al., 2012), this is the first attempt to assess the feasibility of using these drugs for anticancer clinical applications, such as intravesical therapy. These findings are particularly important as supratherapeutic concentrations of these drugs are unlikely to be both safe and tolerable for cancers which require systemic treatment. On the contrary, intravesical treatment carries little risk of systemic exposure, allowing high concentrations of drugs to be administered safely and effectively directly to the bladder tumour. Of particular interest, bladder cancer is also documented to express functional EphA2 receptors and expressional levels are strongly-associated with advanced disease (Abraham et al., 2006). In contrast to the non-specific cytotoxicity of mitomycin C and gemcitabine, the novel EphA2 agonist doxazosin may selectively target bladder cancer cells by restoration of anoikis mechanisms (Petty et al., 2012). Furthermore, this novel action may prevent tumour cells from embedding in the urothelium following TURBT. However, this doxazosin-mediated effect in bladder cancer is currently unknown and requires further investigation. Taken together, these findings suggest that the supratherapeutic anticancer potential of doxazosin may be advantageous as intravesical therapy for non-muscle invasive bladder cancer.

In future investigations, evaluating the cytotoxic effect of clinically relevant concentration of MMC (6 mM) in comparison to supratherapeutic concentrations of doxazosin (≥ 30 μM) may provide further insight to clinical benefit of these drugs intravesically. Since doxazosin is not currently available to Australian patients, whether

prazosin is able to elicit a similar anti-bladder cancer effect as doxazosin would be of significant interest to the Australian community. Therefore, further *in vitro* studies comparing prazosin to common intravesical chemotherapeutics, as well as elucidation of the underlying cytotoxic mechanisms in bladder cancer cells is needed. Likewise, use of *in vivo* bladder cancer models to assess the efficacy of doxazosin/prazosin and MMC would be beneficial to explore the clinical relevance of the present findings.

CHAPTER 7:
GENERAL DISCUSSION

The overall aim of this study was to expand upon the current understanding of the anticancer effects of various α1-ADR antagonists, including the underlying signalling molecular mechanisms, *in vitro*. This study also investigated whether these drugs may enhance efficacy of currently used prostate cancer treatments, such as radiotherapy and chemotherapy.

RELATIVE CYTOTOXIC POTENCIES AND UNDERLYING MECHANISMS OF A1-ADR ANTAGONISTS *IN VITRO*

In Chapter 3, this thesis compared the relative cytotoxic potencies of alfuzosin, doxazosin, prazosin, silodosin, terazosin and tamsulosin, as well as the cell death mechanisms underlying the actions drugs in prostate cancer cell lines. Overall it was found that prazosin and doxazosin were generally equipotent in their cytotoxic actions on AR-positive LNCaP and AR-negative PC-3 cells. Moreover, LNCaP cells were found to be more sensitive to the cytotoxic effects of these drugs. These findings were consistent with current literature, which reported that quinazoline derivatives (such as prazosin and doxazosin) have greater cytotoxic effects than non-quinazolines (tamsulosin and silodosin). Additionally, it was found that distinctly separate cell death mechanisms regulate the cytotoxic response in AR-positive LNCaP and AR-negative PC-3 cells. In LNCaP cells, it appears that these α1-ADR antagonists primarily trigger apoptotic cell death; however, prazosin possessed greater apoptotic potential than doxazosin. While autophagy was found to be elevated in both cell lines following treatment with doxazosin and prazosin; it appears that autophagy only contributed to the cytotoxicity of these drugs in PC-3 cells. In contrast, PC-3 toxicity was found to involve autophagic mechanisms which was similarly induced by both doxazosin and prazosin treatment. As previously mentioned in Chapter 4, these findings are supported by prior literature that demonstrated a paradoxical role for autophagy in PC-3 and LNCaP cells; acting as a cytoprotective or cytotoxic mechanism, respectively (Pickard et al., 2015).

Elucidation of the molecular mechanisms underlying cytotoxic activity of doxazosin, prazosin and terazosin remains incomplete. Separate studies have reported a plethora of α1-ADR independent molecular effects ranging from modulation of receptor tyrosine kinases (EGFR, VEGFR and EphA2) (Hui et al., 2008, Petty et al., 2012, Park et al.,

2014a), to activation of TGF-β/Smad-signalling (Partin et al., 2003), direct DNA interactions (Arencibia et al., 2005) and inhibition of the cell cycle (Lin et al., 2007). Unlike reports of α1-ADR-independence in cancer cell lines, quinazoline-dependent cytotoxic effects of the investigated antagonists remain to be fully elucidated. For example, the non-quinazoline naftopidil was found to have a similar potency to doxazosin and prazosin (Gotoh et al., 2012). Likewise, silodosin and tamsulosin were found to be nearly as potent as doxazosin and prazosin in WPMY-1 prostate stromal cells (Chapter 6). As such, the observed quinazoline-dependent prostate cancer cell toxicity may in fact be an coincidental finding, secondary to α1-ADR independence. Either way, it is unlikely that a singular mechanism regulates the cytotoxic actions of doxazosin/prazosin. As such, the collective findings of this study presented in Chapters 4 and 5 in combination with the current literature has led to the following hypothesised *in vitro* cytotoxic mechanism (**Figure 7.1**). Furthermore, the results of this thesis suggest that these cytotoxic mechanisms occur independent of the α1-ADRs *in vitro*.

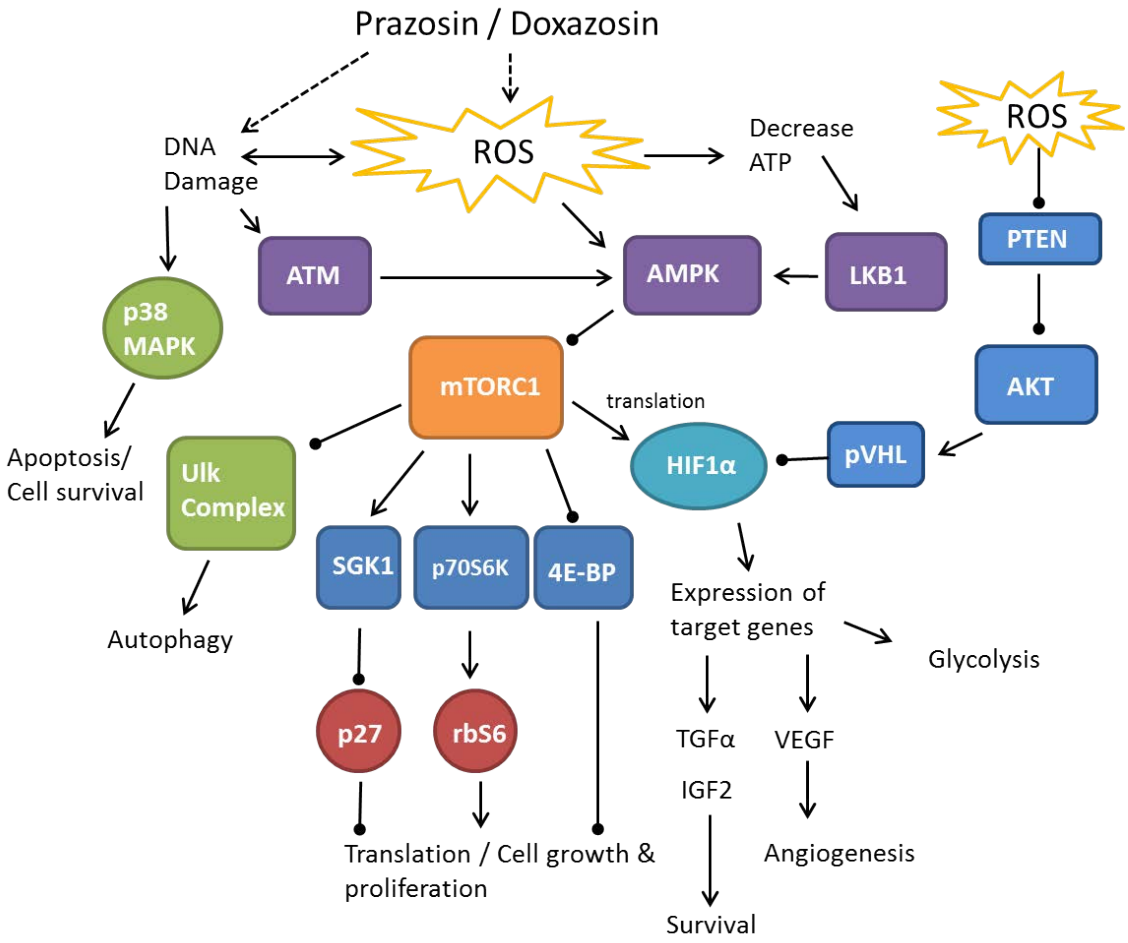


Figure 7.1. Proposed α1-ADR-independent cytotoxic mechanism of prazosin and doxazosin in prostate cancer cell lines. Activation is represented by the lines with arrows. Inhibition is represented by blunt-ended lines.

In Chapter 5, prazosin was found to trigger the accumulation of intracellular ROS. High-levels of ROS is cytotoxic (in most cases) and eventually leads to oxidative stress by overwhelming the innate anti-oxidant capabilities of the cell. Consequently, oxidative stress can result in catastrophic outcomes for the cell, causing either apoptosis or cell cycle arrest in a cell type- and stimulus-dependent manner. On the other hand, low to moderate levels of ROS are known to participate directly or indirectly in redox signalling cascades driving a plethora of cellular responses. By directly modulating cysteine residues of signalling kinases, ROS promote phosphorylation-independent activity of receptor tyrosine kinases and signalling nodes including EGFR and ERK1/2. Specific to the proposed mechanism, ROS are known to indirectly promote the

activation of the energy-sensing kinase AMPK by two mechanisms. Firstly, intracellular ROS deplete ATP stores and increase AMP:ATP radio leading to the activation of the LKB1/AMPK axis. Secondly, ATM is responsible for orchestrating cellular response to DNA damage, including ROS-mediated genotoxic stress, resulting in cytosolic activation of LKB1 and AMPK (Alexander et al., 2010a). As mentioned previously, quinazoline-based doxazosin or prazosin has been reported to activate AMPK in rodent cardiac cells (Yang et al., 2011a). Together with the present findings, it can be hypothesised that prazosin (and possibly doxazosin) activates AMPK through the accumulation of intracellular ROS, and subsequent sequestering of mTOR/p70S6K signaling cascade.

As reviewed previously in Chapters 3 and 4, in its active state mTORC1 blocks autophagy through inhibition of the Ulk complex, while also stimulating cell growth signalling through inhibition of 4E-BP and activation of p70S6 kinase (p70S6K). Importantly, S6 ribosomal protein is a substrate of p70S6K with growth promoting actions (Iwenofu et al., 2008). In the absence of mTORC1 activity, S6 ribosomal kinase activity would be suppressed, which was precisely what occurred in Chapter 4. Both mTOC1 and p70S6K were found to be suppressed in independent studies evaluating doxazosin and prazosin *in vitro* (Yang et al., 2011a, Park et al., 2014a, Park et al., 2014b). While these studies used non-prostate cancer cellular models (human ovarian cancer and rat embryonic ventricular myoblast), it can be assumed that with the presence of appropriate molecules or proteins, signalling mechanisms will be conserved between cell types. However, differences may exist in cellular responses to these signalling pathways, which will inevitably be regulated by the presence or absence of downstream effectors. This proposed signalling pathway is further supported by the results presented in Chapter 5, where prazosin was found to inhibit another downstream target of mTORC1, HIF-1α. Activation of mTORC1 triggers hypoxia-independent accumulation of HIF-1α, which has been proposed to occur via the transcription activity of p70S6K and 4E-BP1 (Dodd et al., 2015). In the present study, prazosin exposure suppressed HIF-1α accumulation in PC-3 cells, but less so in LNCaP cells. These findings are supported by previous literature, which have demonstrated doxazosin-mediated inhibition of HIF-1α levels in ovarian cancer cells (Park et al., 2014a). The importance of HIF-1α inactivation in normoxic conditions is unclear, but it may be one such mechanism by which prazosin enhances the radiosensitivity of hypoxic prostate

cancer cells. This effect will be further explored at a later point in this Chapter. In summary, it is proposed that doxazosin and prazosin elicit their cytotoxic effect to some degree by inhibition of the mTOC1/p70S6K pathway. In addition, autophagy is likely to be triggered as a consequence of mTORC1 inhibition, which either contributes to or protects against cell death in a cell-dependent manner.

As mentioned previously, the proposed cytotoxic mechanisms are likely to occur independent of the alpha1-ADR since the highly potent α1-antagonist, tamsulosin was unable to induce cell death or cytotoxic mechanisms in prostate cancer PC-3 and LNCaP cell lines. While these findings are consistent with previous literature (Benning and Kyprianou, 2002), it remains unclear if α1-ADRs play and important role in the anticancer effect of these drugs *in vivo* and in clinical settings.

PROPOSED ANTI-TUMOUR MECHANISMS OF A1-ADR ANTAGONISTS IN CLINICAL SETTINGS

The anticancer potency of α1-ADR antagonists is well documented to differ between observations *in vitro*, animal models and clinical settings. However, the "elephant in the room" has yet to be addressed in the current literature. The supratherapeutic concentrations required to induce anti-tumour effects *in vitro* (> 10 μM) are well above therapeutic concentrations identified in human plasma (33 – 391 nM) (Ahtoy et al., 2002, Zhao et al., 2009, Korstanje et al., 2011). Therefore, an unknown mechanism must exist that acts to enhance the cytotoxic potency of these drugs on hyper-proliferative cells *in vivo* and in clinical settings. Four such proposed and potentially intertwined mechanisms include: blockade of endogenous α1-ADR stimulation, ion channel interactions, effects of tumour hypoxia on receptor density, and stromal-epithelial interactions.

BLOCKADE OF A1-ADR STIMULATION

Many studies have attempted to describe the expression profile of α1-ADR subtypes in prostate cancers. Unfortunately, the majority of prior investigations have used antibody detection methods (Thebault et al., 2003, Katsogiannou et al., 2009), which have relatively recently been shown to often be non-specific (Jensen et al., 2009, White et al., 2013), making these methodologies questionable in characterising specific α1-subtype expression. Despite this, the literature continues to provide strong evidence for the role of α1-ADRs in the modulation of prostate tumourigenesis. Two separate studies reported antibody-detected α1A-expression in prostate cancer AR-positive LNCaP and androgen-independent PC-3 and DU145 cells (Thebault et al., 2003, Katsogiannou et al., 2009). In one of these studies, it was also demonstrated that chronic stimulation of LNCaP cells with the α1-ADR agonist phenylephrine enhanced cell proliferation *in vitro*, which could be abrogated in the presence of prazosin (Thebault et al., 2003). A separate study reported the presence of α1D-subtype adrenergic receptors in AR-negative PC-3 cells by antibody detection, and was loosely confirmed by the presence of α1D-subtype mRNA expression as detected by RT-PCR analysis of human benign and malignant prostatectomy tissue specimens (Morelli et al., 2014). Likewise, it was found that cancerous tissues possessed a greater expression of α1D-ADR compared to

non-cancerous prostate samples. These two studies also report the co-involvement of TRP receptors with α1-ADR in regulating prostate cancer proliferation (Morelli et al., 2014, Thebault et al., 2003). Supporting previous findings (Thebault et al., 2003), parallels between α1D-ADR and transient receptor potential (TRP) vanilloid-1 (V1) expression were observed in *in vitro* and in human prostate cancer specimens (Morelli et al., 2014). Briefly, the TRP family are plasma membrane calcium non-voltage gated channels that have been associated with increased tumourigenic potential (Liberati et al., 2014). Furthermore, stimulation with noradrenaline was found to enhance AR-negative PC-3 proliferation, in a α1D and TRPV1 dependent manner, however functionality of both α1D and TRPV1 was required for maximal proliferative effects in PC-3 cells. These reports are supported by earlier work by Thebault et al. (2003), which showed that in addition to prazosin-mediated blockage of α1-ADR, TRP channel inhibition also suppressed the proliferative effect of chronic phenylephrine stimulation in LNCaP cells. The cellular mechanism regulating the proliferative effects in prostate cancer described above is likely to involve TRP-mediated influx of calcium ions (as review in Chapter 1). As shown in **Figure 7.2**, α1-ADRs associate with TRP channels to promote calcium influx and subsequent signalling pathways to promote cell proliferation, thereby contributing to prostate cancer tumourigenesis (Deliot and Constantin, 2015).

Unlike *in vitro* studies, *in vivo* α1-ADRs are under constant stimulation by circulating catecholamines. Dysregulation of these mechanisms in prostate cancers may exhibit a tumourigenic effect. Previously, it was demonstrated that metastatic castrate-resistant prostate cancer tissues have a greater expression and activation of TRPV2 compared to primary tumours (Monet et al., 2010). This effect was postulated to be a result of androgen-deprivation, which indirectly increased TRPV2 expression, and subsequently, metastatic potential of prostate cancer cell lines. Taken together, blockade of endogenous α1-ADR stimulation and downstream TRP channel-mediated calcium influx, may reduce prostate cancer proliferation, and even delay progression to castrate-resistant state. While this mechanism is likely to negatively affect cell proliferation, it is unknown if inhibition of α1-ADR/TRP/calcium-signalling will result in apoptosis. Therefore, this mechanism alone does not fully encompass the apoptotic effects seen in animal and human studies.

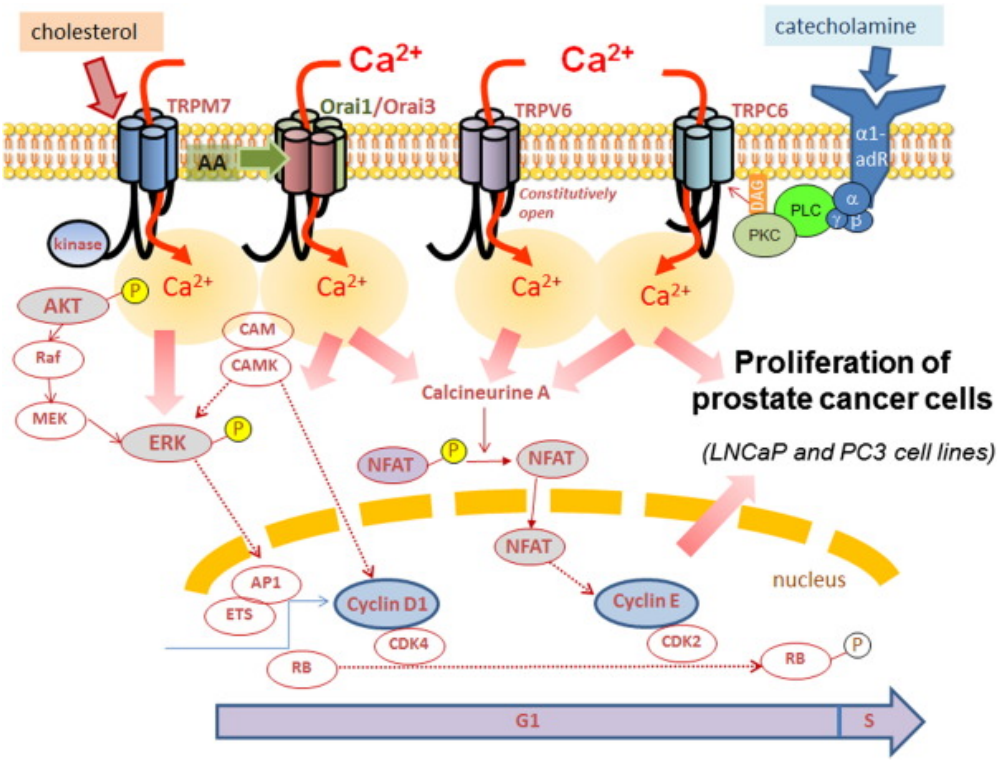


Figure 7.2. Proposed signalling mechanisms of plasma membrane calcium channels in regulating prostate cancer cell cycle and proliferation (Deliot and Constantin, 2015). In the case of α1-ADRs (right), stimulation by catecholamines promotes TRPC-mediated calcium influx, resulting in activation of NFAT. NFAT translocates to the nucleus and in-turn promotes cell cycle progression by interactions with cyclin E. Figure reprinted with permission from copyright holder.

ION CHANNEL INTERACTIONS

Ion channels, particularly voltage gated sodium and potassium channels are known to play a role in cell proliferation, tumourigenesis, invasion and metastasis of several cancers, including prostate cancer (Fraser et al., 2014). In addition to the presumed indirect effects of α1-ADR antagonists on the calcium channel TRP via interaction with α1-ADRs, doxazosin and prazosin have previously been reported to inhibit potassium and sodium channels at therapeutic concentrations (Obata and Yamanaka, 1999, Thomas et al., 2004).

A cluster of studies by the Thomas research group demonstrated that doxazosin, prazosin and terazosin bound to and inhibited the human ether-a-go-go-related gene potassium (hERG K^+) channels resulting in apoptotic cell death in various cell lines (Thomas et al., 2004, Thomas et al., 2008, Staudacher et al., 2014). At this time, hERG K^+ channels are not well documented in prostate cancer cells. However, over expression of these channels has been previously identified in androgen-independent DU145 cancer cells, and indirect down-regulation of hERG expression was associated with increased prostate cancer apoptosis *in vitro* (Ji et al., 2015). Whether these potassium channels exist in other cellular models of prostate cancer, such as PC-3 and LNCaP, remains to be elucidated.

A search of the literature also reveals an incidental report of sodium channel blocking properties of prazosin (Obata and Yamanaka, 1999). In contrast to benign prostate cells, it has been demonstrated that over-expression of functional sodium channels was characteristic of prostate cancer *in vitro* (Diss et al., 2001, Shan et al., 2014), which may function as a tumourigenic mechanism by promoting cancer cell motility and thus metastasis (Fraser et al., 2003). In theory, inhibition of sodium channels in prostate cancer might delay development of advanced metastatic disease. Despite a lack of substantial evidence for inhibitory interactions of prazosin on sodium channels, revisiting this area would provide significant insight to the mechanisms contributing to the anticancer effects clinically.

TUMOUR HYPOXIA-MEDIATED MODULATION OF RECEPTOR ACTIVATION AND EXPRESSION

As described in Chapter 5, hypoxia is well documented to promote tumourigenesis through HIF-1α stabilisation, which in turn, promotes angiogenesis. Importantly, hypoxia promotes radioresistance of tumours, particularly in prostate cancers, by suppressing irradiation-induced ROS formation and HIF-1α-mediated survival and adaptation. In addition, hypoxia is reported to alter the activation and expression of various tyrosine kinase receptors associated with tumourigenesis, metastasis and invasion, including EGFR and EphA2 (Vihanto et al., 2005, Gluck et al., 2015). Particularly, overexpression of EGFR has been strongly associated with tumour hypoxia (HIF-1α) *in vitro* and histological studies of resected tumour specimens (Hoogsteen et al., 2012, Weber et al., 2012). Less is known about the impact of hypoxia on the expression of Eph receptors, particularly EphA2. One study suggests that these receptors are up-regulated in hypoxic conditions (Cercone et al., 2009). If in fact doxazosin/prazosin act by actions on EGFR or EphA2 (Hui et al., 2008, Petty et al., 2012), it can be hypothesised that the cytotoxic potency of these drugs would be enhanced in hypoxic solid tumours. In other words, the cytotoxic outcome effect of α1-ADR antagonists in theory would be proportional to the number of receptor inhibited, producing a greater effect in hypoxic tumours compared to well-vascularised tissues. However, clinically, it is likely to be more complex than proposed, as hypoxia triggers a plethora of survival and adaption mechanisms. For example, hypoxia induced resistance to the EGFR antagonist gefitinib (similar quinazoline structure to doxazosin) by increasing activity of insulin-like growth factor 1 receptor in lung cancer cells (Murakami et al., 2014). Nevertheless, the effect of hypoxia is likely to be an important aspect to the clinical efficacy of α1-ADR antagonists as anticancer agents and warrants further investigation.

THERAPEUTIC VALUE OF DOXAZOSIN AND PRAZOSIN FOR PROSTATE CANCER

The findings presented in Chapter 5 demonstrated that doxazosin/prazosin enhanced the sensitivity of hypoxic PC-3 and LNCaP cells to radiotherapy. This effect was likely to occur independent of α1-ADR antagonism since the highly selective α1-ADR antagonist, tamsulosin, had no effect on the sensitivity of prostate cancer cells irradiated under either normoxic or hypoxic conditions. However, clinically, α1-antagonism may also be beneficial to radiotherapy treatment outcomes. It was previously reported in separate studies that rats pre-treated with α1-ADR agonist or a combination of both muscarinic and α1-ADR agonists prior to irradiation of non-cancerous salivary glands, demonstrated significantly reduced irradiation-induced tissue damage (Xiang et al., 2013, Coppes et al., 2001). In the case where α1-ADR activity acts to promotes tumourigenesis, it is unknown whether the antagonism of physiologically relevant stimulation of α1-ADR will enhance radiosensitivity of prostate cancer tumours. However, it poses an attractive mechanism where α1-ADR antagonists such as prazosin might improve radiotherapy outcomes by mitigating treatment-associated LUTS, inhibiting α1-ADR-mediated tumourigenesis and selectively targeting hypoxic prostate cancer cells.

In contrast to radiotherapy, the combination of α1-ADR antagonist prazosin and chemotherapeutic agents did not appear to have a synergistic effect. On the contrary, the combination may actually possess an antagonistic effect that is cytoprotective. While further investigations are required, this suggests that the clinical benefit of combination therapy may be clinically beneficial for men treated with radiotherapy, whereas these drugs should be used with caution in men treated with docetaxel or cabazitaxel for prostate cancer.

Clinical trials highlight the importance of autophagy in relation to prostate cancer, with modulators of autophagic activity, such as silencing of autophagy-related genes or pharmacological modulators of autophagy, possessing clinical efficacy although in a patient/disease-specific manner (Armstrong et al., 2013). The present study demonstrated that doxazosin and prazosin induced autophagy in both prostate cancer cell lines and contributed to the cytotoxic response in PC-3 cells. Nonetheless, there is no current evidence to suggest that clinically relevant doses of doxazosin or prazosin are

effective in inhibiting mTOR, thereby inducing autophagy *in vivo*. The results do suggest that these drugs may be useful in modulating autophagy and subsequent sensitivity to anticancer therapies as described by previous studies (Lin et al., 2010, Morikawa et al., 2012, Ling et al., 2014, Pickard et al., 2015). However, it is acknowledged that for some prostate cancers autophagy may act as a pro-survival mechanism and theoretical induction of autophagy by doxazosin or prazosin may be disadvantageous (Bennett et al., 2013), although this may be overcome through the addition of autophagy inhibitors (Pickard et al., 2015).

EXPERIMENTAL LIMITATIONS AND FUTURE INVESTIGATIONS

While the present study further advances the current understanding of the cytotoxic actions as well as the cell death mechanisms of doxazosin and prazosin, it is acknowledged there are experimental limitations that should be addressed in future studies. Likewise, the present findings have also raised a number of questions with potential for further investigation.

The evaluated concentration range chosen was based on a logarithmic scale to encompass a wide range of clinically-relevant and supratherapeutic doses. Prostate cancer cells were then treated with these concentrations for 24 – 72 h. To further the understanding of the cytotoxic effects of these drugs, a supratherapeutic dose-response curve containing several concentrations between 10 and 100 μM may be beneficial to more accurately determine IC_{50} values. If within solubility limits of the drugs, concentrations greater than 100 μM for the lesser toxic alfuzosin, terazosin, silodosin and tamsulosin would further clarify relative cytotoxic potency between these drugs.

Treatment times investigated (24 – 72 h) were based on typical cell culture treatment protocols used within the present laboratory. However, the clinical relevance of these treatment durations are limited since the clinical dosing of these drugs is 1-2 times daily for months to years. Therefore, extended treatment (> 72 h) *in vitro* may continue to close the gap between *in vitro* and clinical observations. Cell cultures treated beyond 72 h may require additional optimisation to prevent the negative effects of long term cell culture such as acidifying of the medium, culture medium evaporation and the loss of medium nutrients.

Two-dimensional cell culture is the current standard for pre-clinical investigations of drug cytotoxicity and molecular mechanisms. However, in recent years it has been acknowledged that the clinical relevance of 2-D culture is limited since it poorly mimics true physiological conditions. To close the gap between the cytotoxicity of doxazosin and prazosin observed *in vitro* and clinical findings, additional investigations utilising prostate cancer and stromal cell 3-D co-cultures would be of significant importance. Although the present study did not find an appreciable effect using 2-D indirect co-cultures, direct 2-D or 3-D co-cultures (scaffolding or MatriGel®) may be useful in

further exploring the anticancer effects of these drugs. The use of direct (2-D or 3-D) co-cultures more accurately mimics the tumour microenvironment, which allows the cultured prostate tumours to interact directly with stromal cells and/or the extracellular matrix as they would physiologically. Furthermore, 3-D cultures also, to some degree, can replicate characteristic tumour hypoxia and acid imbalances which are crucial to tumourigenesis (Choi et al., 2014). To the best of my knowledge, no current published literature has employed direct 2-D or 3-D co-culture technique for investigating the anticancer effects of doxazosin, prazosin or tamsulosin in prostate cancer or stromal cells. However, some limitations exist in the use of direct (2D and 3D) co-cultures. To accurately examine the potential proliferative effects of direct prostate stromal-cancer, as well as, the cytotoxic effects of these drugs, the stromal and cancer cells must be labeled and changes measureable. One such way is to use viral vectors to induce stable expression of green or red florescent proteins to distinguish and quantify changes in stromal or epithelial cell number. However, these techniques can be time consuming and costly. Furthermore, genetic modification may have unsolicited effects of cell proliferation or cytotoxic response, further removing the *in vitro* experiment from clinical relevance. Despite these limitations, the effect of these drugs on 2-D co-culture or 3-D tumour development and their cytotoxic potencies in established tumours would be of great interest.

In Chapter 4, changes in protein expression and phosphorylation of several cell stress and RTK signaling targets in response to doxazosin and prazosin were investigated. This was the first reported instance of using an array to assess the underlying cytotoxic mechanisms of these drugs following 24 h exposure *in vitro*. While these experiments provided a plethora of data, further studies investigating the identified targets of interest are required. One limitation of these array kits is the lack of a total protein expression control making changes in phosphorylation in protein difficult to interpret. In the current study, changes in protein phosphorylation status were merely correlated with changes in protein activation. While, the manufactures' optimisation data provided with the array kits suggest that phosphorylation indeed equates to activation; determination of total target protein in conjunction with phosphorylated protein is required to ascertain activation. For example, apparent changes in phosphorylation may in fact be due to changes in expression or elimination of target proteins. The use of Western blotting or

ELISA methods would be useful to measure both total and phosphorylated protein expression of the identified doxazosin/prazosin protein targets.

Another limitation of the array experiments conducted in Chapter 4 was the single investigated time-point (24 h). While it was the intention of the present study to investigate the sustained cytotoxic molecular mechanisms at 24 h to coincide with the apoptosis and autophagic data (24 h) presented in Chapter 3, future studies investigating time-dependent phosphorylation (activation) of the identified targets and related signaling pathways. This might clarify apparent discrepancies between findings in the literature and those presented in Chapter 4. For example, Hui et al. (2008), reported inhibition of EGFR following acute treatment with doxazosin, whereas the present study found no significant change to EGFR phosphorylation following 24 h doxazosin / prazosin exposure in prostate cancer cells. As discussed in the Chapter 4, EGFR activation by doxazosin / prazosin is likely to be an early signaling event which may not be sustained at 24 h. Future time course studies (5 minutes to > 24 h) using Western blotting or ELISA methods would be useful to further characterise the time-dependent effect of these drugs on the molecular targets of doxazosin / prazosin. As a last measure, the use of pharmacological inhibitors or small interfering RNA-mediated gene knockdown in combination with Western blotting and the resazurin reduction assay would be useful to confirm the proposed cytotoxic mechanisms of doxazosin and prazosin.

In Chapter 5 the fluorescent probe DCF was used to investigate the changes in intracellular reactive oxygen species following irradiation in the presence or absence of prazosin and environmental oxygen. While the present DCF data suggests prazosin enhances ROS in the presence or absence of irradiation, further investigation is required to confirm findings. As described in Chapter 5 Methods section, it is known that the DCF assay can be adversely affected by light, which was controlled for throughout the study. However, DCF-DA has been reported to not be a direct indicator of intracellular ROS, but in fact DCF-DA oxidation is dependent on ROS-mediated mitochondrial release of redox-active iron and/or cytochrome c (Karlsson et al., 2010). Additional studies using a more specific ROS probe, such as the mitochondrial superoxide indicator MitoSOX Red or Amplex Red Hydrogen Peroxide assays. Optimisation of ROS experiments will be required to overcome the challenge of treating and irradiating

in glass vessels (petri dishes or glass 96-well plates) and subsequent fluorescence reading of intracellular or extracellular ROS production or release, respectively.

In the experiments presented in Chapter 5 it was found that prazosin had radiosensitising actions on hypoxic prostate cancer cells. Whether this effect is specific to prazosin, or if it may extend to doxazosin, or the modestly cytotoxic terazosin, is unknown. Additionally, it is unknown if the observed prazosin-mediated radiosensitisation of hypoxic prostate cancer cells can be replicated in other experimental models such as 3D cultures (single cell line or co-culture). To more closely mimic clinical conditions, treatment with therapeutic concentrations with combination of a typical therapeutic irradiation regimen would be useful for the understanding of whether these drugs may possess clinical benefit. Pending studies with 3-D co-cultures, *in vivo* murine models also may help to identify to potential clinical relevance. For example, mice xenografted with AR-negative PC-3 prostate cancer cells could be treated with therapeutically-relevant doses of prazosin, doxazosin or tamsulosin and evaluated for frequency of metastasis and irradiation disease response in the presence or absence of these drugs. Furthermore, murine PC-3 tumour immunohistochemistry (or similar studies) for molecular targets previously identified (e.g. mTOR, HIF-1α) following drug treatment in the presence or absence of irradiation may help to further elucidate the anticancer mechanisms *in vivo*, and potentially clinically. As an adjunct to present study, a retrospective review of patient records conducted by a colleague at Griffith University (B. Spencer, Gold Coast, Australia) is currently underway investigating whether treatment with α1-ADR antagonists may prevent or delay radiation therapy biochemical failure. The findings of this novel study will guide further experimental investigations as well as potential prospective clinical trials.

Based on the preliminary findings presented in Chapter 6, the significant cytotoxic effect of acute doxazosin treatment on bladder cancer cells warrants further investigation. Likewise, unpublished data from our lab suggests that prazosin has a similar cytotoxic profile to doxazosin in these cell lines (P. Singh, Bond University, Australia), indicating these drugs may be useful as an alternative intravesical treatment where extremely high concentrations can be used with minimal organ-confined toxicity.

Of particular interest is *in vivo* modelling of bladder cancer and intravesical treatment with doxazosin/prazosin. Rodent models of bladder cancer can be induced using carcinogens such as N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) or transplanted via transurethral instillation of human bladder cancer cell lines including T24s (Zhang et al., 2015a). Although BBN-induced rodent bladder cancer is histologically and genetically similar to human bladder cancers, these tumours are more similar to human invasive bladder genotype making this experimental model undesirable for evaluation of non-invasive bladder cancer (Williams et al., 2008). In addition to evaluating the anticancer effects if intravesical doxazosin/prazosin, patterns of urination and assessment of released chemical mediators (e.g. ATP) might provide insight to any treatment-related bladder toxicity.

CONCLUDING REMARKS

The findings presented in this thesis demonstrate that doxazosin and prazosin exhibited the greatest cytotoxic potency of the investigated antagonist, possessing apoptotic and autophagic effects in prostate cancer cells. However, cell death mechanisms were found to be predominantly cell-type dependent, with these drugs inducing apoptotic cell death of LNCaP cells, and autophagic cell death in PC-3 cells. Furthermore, prazosin was found to sensitise hypoxic prostate cancer cells to irradiation, which likely involved ROS-mediated induction of autophagy in PC-3, and enhanced apoptosis in LNCaP cells. Mechanistic investigations implicated the inhibition of mTORC1/p70S6K-signaling as a novel mechanism underlying the cytotoxic effect of these drugs, as well as the radiosensitising effect of prazosin in hypoxic prostate cancer cells. Preliminary findings in bladder cancer T24 cells also demonstrated sustained cytotoxic effects following acute doxazosin exposure, which were significantly greater than the currently used chemotherapeutic mitomycin C.

In conclusion, these findings expand the current understanding of the mechanisms contributing doxazosin/prazosin-mediated prostate cancer toxicity *in vitro*. While further investigations are required, this thesis proposes a novel use for these drugs to improve the clinical treatment efficacy of radiotherapy for prostate cancer, or as intravesical agents for bladder cancer.

REFERENCES

ABATE-SHEN, C. & SHEN, M. M. 2000. Molecular genetics of prostate cancer. *Genes Dev,* 14**,** 2410-34.

ABRAHAM, S., KNAPP, D. W., CHENG, L., SNYDER, P. W., MITTAL, S. K., BANGARI, D. S., KINCH, M., WU, L., DHARIWAL, J. & MOHAMMED, S. I. 2006. Expression of EphA2 and Ephrin A-1 in carcinoma of the urinary bladder. *Clin Cancer Res,* 12**,** 353-60.

ABRAHAMSSON, P. A. 2010. Potential benefits of intermittent androgen suppression therapy in the treatment of prostate cancer: a systematic review of the literature. *Eur Urol,* 57**,** 49-59.

ADISSU, H. A., MCKERLIE, C., DI GRAPPA, M., WATERHOUSE, P., XU, Q., FANG, H., KHOKHA, R. & WOOD, G. A. 2015. Timp3 loss accelerates tumour invasion and increases prostate inflammation in a mouse model of prostate cancer. *Prostate,* 75**,** 1831-43.

AHTOY, P., CHRETIEN, P., DUPAIN, T., RAUCH, C., ROUCHOUSE, A. & DELFOLIE, A. 2002. Alfuzosin, an alpha1-adrenoceptor antagonist for the treatment of benign prostatic hyperplasia: once daily versus 3 times daily dosing in healthy subjects. *Int J Clin Pharmacol Ther,* 40**,** 289-94.

AL-AZAYZIH, A., GAO, F., GOC, A. & SOMANATH, P. R. 2012. TGFbeta1 induces apoptosis in invasive prostate cancer and bladder cancer cells via Akt-independent, p38 MAPK and JNK/SAPK-mediated activation of caspases. *Biochem Biophys Res Commun,* 427**,** 165-70.

ALESSI, D. R., DEAK, M., CASAMAYOR, A., CAUDWELL, F. B., MORRICE, N., NORMAN, D. G., GAFFNEY, P., REESE, C. B., MACDOUGALL, C. N., HARBISON, D., ASHWORTH, A. & BOWNES, M. 1997. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol,* 7**,** 776-89.

ALEXANDER, A., CAI, S. L., KIM, J., NANEZ, A., SAHIN, M., MACLEAN, K. H., INOKI, K., GUAN, K. L., SHEN, J., PERSON, M. D., KUSEWITT, D., MILLS, G. B., KASTAN, M. B. & WALKER, C. L. 2010a. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proc Natl Acad Sci U S A,* 107**,** 4153-8.

ALEXANDER, A., KIM, J. & WALKER, C. L. 2010b. ATM engages the TSC2/mTORC1 signaling node to regulate autophagy. *Autophagy,* 6**,** 672-3.

ALEXANDER, S. P., MATHIE, A. & PETERS, J. A. 2011. Guide to Receptors and Channels (GRAC), 5th edition. *British journal of pharmacology,* 164 Suppl 1**,** S1-324.

AMBROSINI, G. L., FRITSCHI, L., DE KLERK, N. H., MACKERRAS, D. & LEAVY, J. 2008. Dietary patterns identified using factor analysis and prostate cancer risk: a case control study in Western Australia. *Ann Epidemiol,* 18**,** 364-70.

AMERICAN SOCIETY FOR THERAPEUTIC RADIOLOGY AND ONCOLOGY CONSENSUS PANEL 1997. Consensus statement: guidelines for PSA following radiation therapy. American Society for Therapeutic Radiology and Oncology Consensus Panel. *Int J Radiat Oncol Biol Phys,* 37**,** 1035-41.

ANDERSSON, K. E. 2002. Alpha-adrenoceptors and benign prostatic hyperplasia: basic principles for treatment with alpha-adrenoceptor antagonists. *World J Urol,* 19**,** 390-6.

ANOOPKUMAR-DUKIE, S., CAREY, J. B., CONERE, T., O'SULLIVAN, E., VAN PELT, F. N. & ALLSHIRE, A. 2005a. Resazurin assay of radiation response in cultured cells. *Br J Radiol,* 78**,** 945-7.

ANOOPKUMAR-DUKIE, S., CONERE, T., CAREY, J. B. & ALLSHIRE, A. 2005b. Radical mediators and mitogen-activated protein kinase signaling in oxygen-dependent radiosensitivity of human tumor cell lines. *Free Radic Biol Med,* 39**,** 188-94.

ARENCIBIA, J. M., DEL RIO, M., BONNIN, A., LOPES, R., LEMOINE, N. R. & LOPEZ-BARAHONA, M. 2005. Doxazosin induces apoptosis in LNCaP prostate cancer cell line through DNA binding and DNA-dependent protein kinase down-regulation. *Int J Oncol,* 27**,** 1617-23.

ARMSTRONG, A. J., SHEN, T., HALABI, S., KEMENY, G., BITTING, R. L., KARTCHESKE, P., EMBREE, E., MORRIS, K., WINTERS, C., JAFFE, T., FLEMING, M. & GEORGE, D. J. 2013. A phase II trial of temsirolimus in men with castration-resistant metastatic prostate cancer. *Clin Genitourin Cancer,* 11**,** 397-406.

ASMAR, R., BEEBE-DIMMER, J. L., KORGAVKAR, K., KEELE, G. R. & COONEY, K. A. 2013. Hypertension, obesity and prostate cancer biochemical recurrence after radical prostatectomy. *Prostate Cancer Prostatic Dis,* 16**,** 62-6.

ATTARD, G., REID, A. H., A'HERN, R., PARKER, C., OOMMEN, N. B., FOLKERD, E., MESSIOU, C., MOLIFE, L. R., MAIER, G., THOMPSON, E., OLMOS, D., SINHA, R., LEE, G., DOWSETT, M., KAYE, S. B., DEARNALEY, D., KHEOH, T., MOLINA, A. & DE BONO, J. S. 2009. Selective inhibition of CYP17 with abiraterone acetate is highly active in the treatment of castration-resistant prostate cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 27**,** 3742-8.

AU, J. L., BADALAMENT, R. A., WIENTJES, M. G., YOUNG, D. C., WARNER, J. A., VENEMA, P. L., POLLIFRONE, D. L., HARBRECHT, J. D., CHIN, J. L., LERNER, S. P. & MILES, B. J. 2001. Methods to improve efficacy of intravesical mitomycin C: results of a randomized phase III trial. *J Natl Cancer Inst,* 93**,** 597-604.

AUSTIN, P. F., COOK, B. L., NIEDERHOFF, R. A., MANSON, S. R., COPLEN, D. E. & WEINTRAUB, S. J. 2004. Inhibition of mitogenic signaling and induction of apoptosis in human bladder smooth muscle cells treated with doxazosin. *The Journal of urology,* 172**,** 1662-5; discussion 1666.

AUSTRALIAN CANCER NETWORK MANAGEMENT OF METASTATIC PROSTATE CANCER WORKING PARTY 2010. Clinical practice guidelines for the management of locally advanced and metastatic prostate cancer. Sydney: Cancer Council Australia, Australian Cancer Network,.

AUSTRALIAN INSTITUTE OF HEALTH AND WELFARE 2012. Cancer Incidence Projections, Australia 2011 to 2020. Canberra.

AUSTRALIAN INSTITUTE OF HEALTH AND WELFARE 2013. Prostate cancer in Australia. *Cancer series no. 79.* Canberra: AIHW: Cat. no. CAN 76.

AUSTRALIAN INSTITUTE OF HEALTH AND WELFARE 2014. Cancer in Australia: and overview 2014. *Cancer series No 90.* Canberra: AIHW.

AUSTRALIAN INSTITUTE OF HEALTH AND WELFARE 2015. Australian Cancer Incidence and Mortality book. *In:* CANCER, P. (ed.).

AUSTRALIAN THERAPEUTIC GOODS ADMINISTRATION 2013. Australian Public Assessment Report for everolimus. Department of Health and Aging.

AUSTRALIAN THERAPEUTIC GOODS ADMINISTRATION 2014. Australian Public Assessment Report for Abiraterone Acetate. Canberra: Department of Health.

AUSTRALIAN THERAPEUTIC GOODS ADMINISTRATION 2015. Public Summary for ARTG Entry 10756.

AVRITSCHER, E. B., COOKSLEY, C. D., GROSSMAN, H. B., SABICHI, A. L., HAMBLIN, L., DINNEY, C. P. & ELTING, L. S. 2006. Clinical model of lifetime cost of treating bladder cancer and associated complications. *Urology,* 68**,** 549-53.

BAADE, P. D., GARDINER, R. A., FERGUSON, M., YOULDEN, D. R., AITKEN, J. F., YAXLEY, J. & CHAMBERS, S. K. 2012a. Factors associated with diagnostic and treatment intervals for prostate cancer in Queensland, Australia: a large cohort study. *Cancer causes & control : CCC,* 23**,** 625-34.

BAADE, P. D., YOULDEN, D. R., COORY, M. D., GARDINER, R. A. & CHAMBERS, S. K. 2011. Urban-rural differences in prostate cancer outcomes in Australia: what has changed? *Med J Aust,* 194**,** 293-6.

BAADE, P. D., YOULDEN, D. R., GARDINER, R. A., FERGUSON, M., AITKEN, J. F., YAXLEY, J. & CHAMBERS, S. K. 2012b. Factors associated with treatment received by men diagnosed with prostate cancer in Queensland, Australia. *BJU international,* 110**,** E712-9.

BABJUK, M., BURGER, M., ZIGEUNER, R., SHARIAT, S. F., VAN RHIJN, B. W., COMPERAT, E., SYLVESTER, R. J., KAASINEN, E., BOHLE, A., PALOU REDORTA, J. & ROUPRET, M. 2013. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. *Eur Urol,* 64**,** 639-53.

BAEHS, S., HERBST, A., THIEME, S. E., PERSCHL, C., BEHRENS, A., SCHEEL, S., JUNG, A., BRABLETZ, T., GOKE, B., BLUM, H. & KOLLIGS, F. T. 2009. Dickkopf-4 is frequently down-regulated and inhibits growth of colorectal cancer cells. *Cancer Lett,* 276**,** 152-9.

BAK, D. W. & WEERAPANA, E. 2015. Cysteine-mediated redox signalling in the mitochondria. *Mol Biosyst,* 11**,** 678-97.

BARRY, M. J., GALLAGHER, P. M., SKINNER, J. S. & FOWLER, F. J., JR. 2012. Adverse effects of robotic-assisted laparoscopic versus open retropubic radical prostatectomy among a nationwide random sample of medicare-age men. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 30**,** 513-8.

BARTON, W. A., DALTON, A. C., SEEGAR, T. C., HIMANEN, J. P. & NIKOLOV, D. B. 2014. Tie2 and Eph receptor tyrosine kinase activation and signaling. *Cold Spring Harb Perspect Biol,* 6.

BEER, T. M., ARMSTRONG, A. J., RATHKOPF, D. E., LORIOT, Y., STERNBERG, C. N., HIGANO, C. S., IVERSEN, P., BHATTACHARYA, S., CARLES, J., CHOWDHURY, S., DAVIS, I. D., DE BONO, J. S., EVANS, C. P., FIZAZI, K., JOSHUA, A. M., KIM, C. S., KIMURA, G., MAINWARING, P., MANSBACH, H., MILLER, K., NOONBERG, S. B., PERABO, F., PHUNG, D., SAAD, F., SCHER, H. I., TAPLIN, M. E., VENNER, P. M. & TOMBAL, B. 2014. Enzalutamide in metastatic prostate cancer before chemotherapy. *N Engl J Med,* 371**,** 424-33.

BELL, K. J., DEL MAR, C., WRIGHT, G., DICKINSON, J. & GLASZIOU, P. 2015. Prevalence of incidental prostate cancer: A systematic review of autopsy studies. *Int J Cancer,* 137**,** 1749-57.

BELLO, D., WEBBER, M. M., KLEINMAN, H. K., WARTINGER, D. D. & RHIM, J. S. 1997. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. *Carcinogenesis,* 18**,** 1215-23.

BEN SAHRA, I., LAURENT, K., LOUBAT, A., GIORGETTI-PERALDI, S., COLOSETTI, P., AUBERGER, P., TANTI, J. F., LE MARCHAND-BRUSTEL, Y. & BOST, F. 2008. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene,* 27**,** 3576-86.

BENEJ, M., PASTOREKOVA, S. & PASTOREK, J. 2014. Carbonic anhydrase IX: regulation and role in cancer. *Subcell Biochem,* 75**,** 199-219.

BENNETT, H. L., STOCKLEY, J., FLEMING, J. T., MANDAL, R., O'PREY, J., RYAN, K. M., ROBSON, C. N. & LEUNG, H. Y. 2013. Does androgen-ablation therapy (AAT) associated autophagy have a pro-survival effect in LNCaP human prostate cancer cells? *BJU international,* 111**,** 672-82.

BENNING, C. M. & KYPRIANOU, N. 2002. Quinazoline-derived alpha1-adrenoceptor antagonists induce prostate cancer cell apoptosis via an alpha1-adrenoceptor-independent action. *Cancer research,* 62**,** 597-602.

BHINDI, B., LOCKE, J., ALIBHAI, S. M., KULKARNI, G. S., MARGEL, D. S., HAMILTON, R. J., FINELLI, A., TRACHTENBERG, J., ZLOTTA, A. R., TOI, A., HERSEY, K. M., EVANS, A., VAN DER KWAST, T. H. & FLESHNER, N. E. 2015. Dissecting the association between metabolic syndrome and prostate cancer risk: analysis of a large clinical cohort. *Eur Urol,* 67**,** 64-70.

BILL-AXELSON, A., HOLMBERG, L., RUUTU, M., GARMO, H., STARK, J. R., BUSCH, C., NORDLING, S., HAGGMAN, M., ANDERSSON, S. O., BRATELL, S., SPANGBERG, A., PALMGREN, J., STEINECK, G., ADAMI, H. O. & JOHANSSON, J. E. 2011. Radical prostatectomy versus watchful waiting in early prostate cancer. *The New England journal of medicine,* 364**,** 1708-17.

BILTON, R. L. & BOOKER, G. W. 2003. The subtle side to hypoxia inducible factor (HIFalpha) regulation. *Eur J Biochem,* 270**,** 791-8.

BODDY, J. L., FOX, S. B., HAN, C., CAMPO, L., TURLEY, H., KANGA, S., MALONE, P. R. & HARRIS, A. L. 2005. The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1a, HIF-2a, and the prolyl hydroxylases in human prostate cancer. *Clinical Cancer Research,* 11**,** 7658-7663.

BOUTIN, B., TAJEDDINE, N., VANDERSMISSEN, P., ZANOU, N., VAN SCHOOR, M., MONDIN, L., COURTOY, P. J., TOMBAL, B. & GAILLY, P. 2013. Androgen deprivation and androgen receptor competition by bicalutamide induce autophagy of hormone-resistant prostate cancer cells and confer resistance to apoptosis. *Prostate,* 73**,** 1090-102.

BRAY, F., REN, J. S., MASUYER, E. & FERLAY, J. 2013. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int J Cancer,* 132**,** 1133-45.

BUBENDORF, L., SCHOPFER, A., WAGNER, U., SAUTER, G., MOCH, H., WILLI, N., GASSER, T. C. & MIHATSCH, M. J. 2000. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Human pathology,* 31**,** 578-83.

BUBENIK, J., BARESOVA, M., VIKLICKY, V., JAKOUBKOVA, J., SAINEROVA, H. & DONNER, J. 1973. Established cell line of urinary bladder carcinoma (T24) containing tumour-specific antigen. *Int J Cancer,* 11**,** 765-73.

BUIJSEN, J., VAN DEN BOGAARD, J., JUTTEN, B., BELGERS, E., SOSEF, M., LEIJTENS, J. W., BEETS, G. L., JANSEN, R. L., RIEDL, R. G., CLARIJS, R., LAMMERING, G. & LAMBIN, P. 2015. A phase I-II study on the combination

of rapamycin and short course radiotherapy in rectal cancer. *Radiother Oncol,* 116**,** 214-20.
CAI, B., CHANG, S. H., BECKER, E. B., BONNI, A. & XIA, Z. 2006. p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. *J Biol Chem,* 281**,** 25215-22.
CAL, C., USLU, R., GUNAYDIN, G., OZYURT, C. & OMAY, S. B. 2000. Doxazosin: a new cytotoxic agent for prostate cancer? *BJU international,* 85**,** 672-5.
CAREY, A. M., PRAMANIK, R., NICHOLSON, L. J., DEW, T. K., MARTIN, F. L., MUIR, G. H. & MORRIS, J. D. 2007. Ras-MEK-ERK signaling cascade regulates androgen receptor element-inducible gene transcription and DNA synthesis in prostate cancer cells. *International journal of cancer. Journal international du cancer,* 121**,** 520-7.
CARMELIET, P. & JAIN, R. K. 2000. Angiogenesis in cancer and other diseases. *Nature,* 407**,** 249-57.
CARROLL, C. E., LIANG, Y., BENAKANAKERE, I., BESCH-WILLIFORD, C. & HYDER, S. M. 2013. The anticancer agent YC-1 suppresses progestin-stimulated VEGF in breast cancer cells and arrests breast tumor development. *Int J Oncol,* 42**,** 179-87.
CERCONE, M. A., SCHROEDER, W., SCHOMBERG, S. & CARPENTER, T. C. 2009. EphA2 receptor mediates increased vascular permeability in lung injury due to viral infection and hypoxia. *Am J Physiol Lung Cell Mol Physiol,* 297**,** L856-63.
CHADE, D. C., SHARIAT, S. F., CRONIN, A. M., SAVAGE, C. J., KARNES, R. J., BLUTE, M. L., BRIGANTI, A., MONTORSI, F., VAN DER POEL, H. G., VAN POPPEL, H., JONIAU, S., GODOY, G., HURTADO-COLL, A., GLEAVE, M. E., DALL'OGLIO, M., SROUGI, M., SCARDINO, P. T. & EASTHAM, J. A. 2011. Salvage radical prostatectomy for radiation-recurrent prostate cancer: a multi-institutional collaboration. *Eur Urol,* 60**,** 205-10.
CHANG, K. H., LI, R., KURI, B., LOTAN, Y., ROEHRBORN, C. G., LIU, J., VESSELLA, R., NELSON, P. S., KAPUR, P., GUO, X., MIRZAEI, H., AUCHUS, R. J. & SHARIFI, N. 2013. A gain-of-function mutation in DHT synthesis in castration-resistant prostate cancer. *Cell,* 154**,** 1074-84.
CHANG, L., GRAHAM, P. H., HAO, J., BUCCI, J., COZZI, P. J., KEARSLEY, J. H. & LI, Y. 2014. Emerging roles of radioresistance in prostate cancer metastasis and radiation therapy. *Cancer Metastasis Rev,* 33**,** 469-96.
CHANG, L., GRAHAM, P. H., NI, J., HAO, J., BUCCI, J., COZZI, P. J. & LI, Y. 2015. Targeting PI3K/Akt/mTOR signaling pathway in the treatment of prostate cancer radioresistance. *Crit Rev Oncol Hematol,* 96**,** 507-17.
CHAO, G. F., KRISHNA, N., AIZER, A. A., DALELA, D., HANSKE, J., LI, H., MEYER, C. P., KIM, S. P., MAHAL, B. A., REZNOR, G., SCHMID, M., CHOUEIRI, T. K., NGUYEN, P. L., M, O. L. & TRINH, Q. D. 2016. Asian Americans and prostate cancer: A nationwide population-based analysis. *Urol Oncol,* 34**,** 233.e7-233.e15.
CHEN, P., HUANG, Y., ZHANG, B., WANG, Q. & BAI, P. 2014. EphA2 enhances the proliferation and invasion ability of LNCaP prostate cancer cells. *Oncol Lett,* 8**,** 41-46.
CHEN, T., REN, Z., YE, L. C., ZHOU, P. H., XU, J. M., SHI, Q., YAO, L. Q. & ZHONG, Y. S. 2015. Factor inhibiting HIF1alpha (FIH-1) functions as a tumor suppressor in human colorectal cancer by repressing HIF1alpha pathway. *Cancer Biol Ther,* 16**,** 244-52.

CHEN, Y., CLEGG, N. J. & SCHER, H. I. 2009. Anti-androgens and androgen-depleting therapies in prostate cancer: new agents for an established target. *The lancet oncology,* 10**,** 981-91.

CHENG, H. H., GULATI, R., AZAD, A., NADAL, R., TWARDOWSKI, P., VAISHAMPAYAN, U. N., AGARWAL, N., HEATH, E. I., PAL, S. K., REHMAN, H. T., LEITER, A., BATTEN, J. A., MONTGOMERY, R. B., GALSKY, M. D., ANTONARAKIS, E. S., CHI, K. N. & YU, E. Y. 2015. Activity of enzalutamide in men with metastatic castration-resistant prostate cancer is affected by prior treatment with abiraterone and/or docetaxel. *Prostate Cancer Prostatic Dis,* 18**,** 122-7.

CHETRAM, M. A., BETHEA, D. A., ODERO-MARAH, V. A., DON-SALU-HEWAGE, A. S., JONES, K. J. & HINTON, C. V. 2013. ROS-mediated activation of AKT induces apoptosis via pVHL in prostate cancer cells. *Mol Cell Biochem,* 376**,** 63-71.

CHIANG, C. F., SON, E. L. & WU, G. J. 2005. Oral treatment of the TRAMP mice with doxazosin suppresses prostate tumor growth and metastasis. *The Prostate,* 64**,** 408-18.

CHIGNELL, C. F. & SIK, R. H. 2003. A photochemical study of cells loaded with 2',7'-dichlorofluorescin: implications for the detection of reactive oxygen species generated during UVA irradiation. *Free Radic Biol Med,* 34**,** 1029-34.

CHOCKALINGAM, S. & GHOSH, S. S. 2014. Macrophage colony-stimulating factor and cancer: a review. *Tumour Biol,* 35**,** 10635-44.

CHOI, S. L., KIM, S. J., LEE, K. T., KIM, J., MU, J., BIRNBAUM, M. J., SOO KIM, S. & HA, J. 2001. The regulation of AMP-activated protein kinase by H(2)O(2). *Biochem Biophys Res Commun,* 287**,** 92-7.

CHOI, S. Y., LIN, D., GOUT, P. W., COLLINS, C. C., XU, Y. & WANG, Y. 2014. Lessons from patient-derived xenografts for better in vitro modeling of human cancer. *Adv Drug Deliv Rev,* 79-80**,** 222-37.

CHOI, Y. J., RHO, J. K., LEE, S. J., JANG, W. S., LEE, S. S., KIM, C. H. & LEE, J. C. 2009. HIF-1alpha modulation by topoisomerase inhibitors in non-small cell lung cancer cell lines. *J Cancer Res Clin Oncol,* 135**,** 1047-53.

CHON, J. K., BORKOWSKI, A., PARTIN, A. W., ISAACS, J. T., JACOBS, S. C. & KYPRIANOU, N. 1999. Alpha 1-adrenoceptor antagonists terazosin and doxazosin induce prostate apoptosis without affecting cell proliferation in patients with benign prostatic hyperplasia. *The Journal of urology,* 161**,** 2002-8.

CHOU, Y. T., HSIEH, C. H., CHIOU, S. H., HSU, C. F., KAO, Y. R., LEE, C. C., CHUNG, C. H., WANG, Y. H., HSU, H. S., PANG, S. T., SHIEH, Y. S. & WU, C. W. 2012. CITED2 functions as a molecular switch of cytokine-induced proliferation and quiescence. *Cell Death and Differentiation,* 19**,** 2015-2028.

CHOU, Y. T., WANG, H., CHEN, Y., DANIELPOUR, D. & YANG, Y. C. 2006. Cited2 modulates TGF-beta-mediated upregulation of MMP9. *Oncogene,* 25**,** 5547-60.

CHOU, Y. T. & YANG, Y. C. 2006. Post-transcriptional control of Cited2 by transforming growth factor beta. Regulation via Smads and Cited2 coding region. *J Biol Chem,* 281**,** 18451-62.

CHOUEIRI, T. K., XIE, W., D'AMICO, A. V., ROSS, R. W., HU, J. C., POMERANTZ, M., REGAN, M. M., TAPLIN, M. E., KANTOFF, P. W., SARTOR, O. & OH, W. K. 2009. Time to prostate-specific antigen nadir independently predicts overall survival in patients who have metastatic

hormone-sensitive prostate cancer treated with androgen-deprivation therapy. *Cancer,* 115**,** 981-7.
CHUNG, K. S., HAN, G., KIM, B. K., KIM, H. M., YANG, J. S., AHN, J., LEE, K., SONG, K. B. & WON, M. 2013. A novel antitumor piperazine alkyl compound causes apoptosis by inducing RhoB expression via ROSmediated cAbl/p38 MAPK signaling. *Cancer Chemother Pharmacol,* 72**,** 1315-24.
CLOUSTON, D. & BOLTON, D. 2012a. In situ and intraductal epithelial proliferations of prostate: definitions and treatment implications. Part 1: Prostatic intraepithelial neoplasia. *BJU Int,* 109 Suppl 3**,** 22-6.
CLOUSTON, D. & BOLTON, D. 2012b. In situ and intraductal epithelial proliferations of prostate: definitions and treatment implications. Part 2: intraductal carcinoma and ductal adenocarcinoma of prostate. *BJU Int,* 110 Suppl 4**,** 22-4.
CONNOLLY, R. M., CARDUCCI, M. A. & ANTONARAKIS, E. S. 2012. Use of androgen deprivation therapy in prostate cancer: indications and prevalence. *Asian J Androl,* 14**,** 177-86.
COORY, M. D. & BAADE, P. D. 2005. Urban-rural differences in prostate cancer mortality, radical prostatectomy and prostate-specific antigen testing in Australia. *Med J Aust,* 182**,** 112-5.
COPPES, R. P., ZEILSTRA, L. J., KAMPINGA, H. H. & KONINGS, A. W. 2001. Early to late sparing of radiation damage to the parotid gland by adrenergic and muscarinic receptor agonists. *Br J Cancer,* 85**,** 1055-63.
COTECCHIA, S. 2010. The alpha1-adrenergic receptors: diversity of signaling networks and regulation. *Journal of receptor and signal transduction research,* 30**,** 410-9.
CROSS, N. A., CHANDRASEKHARAN, S., JOKONYA, N., FOWLES, A., HAMDY, F. C., BUTTLE, D. J. & EATON, C. L. 2005. The expression and regulation of ADAMTS-1, -4, -5, -9, and -15, and TIMP-3 by TGFbeta1 in prostate cells: relevance to the accumulation of versican. *Prostate,* 63**,** 269-75.
CUELLAR, D. C., RHEE, J. & KYPRIANOU, N. 2002. Alpha1-adrenoceptor antagonists radiosensitize prostate cancer cells via apoptosis induction. *Anticancer Res,* 22**,** 1673-9.
CULIG, Z., HOFFMANN, J., ERDEL, M., EDER, I. E., HOBISCH, A., HITTMAIR, A., BARTSCH, G., UTERMANN, G., SCHNEIDER, M. R., PARCZYK, K. & KLOCKER, H. 1999. Switch from antagonist to agonist of the androgen receptor bicalutamide is associated with prostate tumour progression in a new model system. *British journal of cancer,* 81**,** 242-51.
CZEKANSKA, E. M. 2011. Assessment of cell proliferation with resazurin-based fluorescent dye. *Methods Mol Biol,* 740**,** 27-32.
D'AMICO, A. V. 2014. US Food and Drug Administration approval of drugs for the treatment of prostate cancer: a new era has begun. *J Clin Oncol,* 32**,** 362-4.
DAI, Y., BAE, K. & SIEMANN, D. W. 2011. Impact of hypoxia on the metastatic potential of human prostate cancer cells. *Int J Radiat Oncol Biol Phys,* 81**,** 521-8.
DAI, Z. J., GAO, J., KANG, H. F., MA, Y. G., MA, X. B., LU, W. F., LIN, S., MA, H. B., WANG, X. J. & WU, W. Y. 2013. Targeted inhibition of mammalian target of rapamycin (mTOR) enhances radiosensitivity in pancreatic carcinoma cells. *Drug Design Development and Therapy,* 7**,** 149-159.
DALBY, K. N., TEKEDERELI, I., LOPEZ-BERESTEIN, G. & OZPOLAT, B. 2010. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. *Autophagy,* 6**,** 322-9.

DARSHAN, M. S., LOFTUS, M. S., THADANI-MULERO, M., LEVY, B. P., ESCUIN, D., ZHOU, X. K., GJYREZI, A., CHANEL-VOS, C., SHEN, R., TAGAWA, S. T., BANDER, N. H., NANUS, D. M. & GIANNAKAKOU, P. 2011. Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer research,* 71**,** 6019-29.

DAVIES, R. W. & BAKER, D. J. 1970. Anoxia in radiobiology: oxygen in polystyrene petri dishes - a further experiment. *Br J Radiol,* 43**,** 496-7.

DAVIS, B. J., WIENER, M., CHAPPLE, C. R., SELLERS, D. J. & CHESS-WILLIAMS, R. 2015. Functional and radioligand binding characterization of the alpha1L-adrenoceptor subtype of the human vas deferens. *Auton Autacoid Pharmacol,* 34**,** 41-9.

DE BONO, J. S., LOGOTHETIS, C. J., MOLINA, A., FIZAZI, K., NORTH, S., CHU, L., CHI, K. N., JONES, R. J., GOODMAN, O. B., JR., SAAD, F., STAFFURTH, J. N., MAINWARING, P., HARLAND, S., FLAIG, T. W., HUTSON, T. E., CHENG, T., PATTERSON, H., HAINSWORTH, J. D., RYAN, C. J., STERNBERG, C. N., ELLARD, S. L., FLECHON, A., SALEH, M., SCHOLZ, M., EFSTATHIOU, E., ZIVI, A., BIANCHINI, D., LORIOT, Y., CHIEFFO, N., KHEOH, T., HAQQ, C. M. & SCHER, H. I. 2011. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med,* 364**,** 1995-2005.

DE BONO, J. S., OUDARD, S., OZGUROGLU, M., HANSEN, S., MACHIELS, J. P., KOCAK, I., GRAVIS, G., BODROGI, I., MACKENZIE, M. J., SHEN, L., ROESSNER, M., GUPTA, S. & SARTOR, A. O. 2010. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet,* 376**,** 1147-54.

DEBES, J. D. & TINDALL, D. J. 2002. The role of androgens and the androgen receptor in prostate cancer. *Cancer letters,* 187**,** 1-7.

DELELLA, F. K., LACORTE, L. M., ALMEIDA, F. L., PAI, M. D. & FELISBINO, S. L. 2012. Fibrosis-related gene expression in the prostate is modulated by doxazosin treatment. *Life Sci,* 91**,** 1281-7.

DELIOT, N. & CONSTANTIN, B. 2015. Plasma membrane calcium channels in cancer: Alterations and consequences for cell proliferation and migration. *Biochim Biophys Acta,* 1848**,** 2512-22.

DENMEADE, S. R. & ISAACS, J. T. 2002. A history of prostate cancer treatment. *Nature reviews. Cancer,* 2**,** 389-96.

DEVARAJAN, A., SHIH, D. & REDDY, S. T. 2014. Inflammation, infection, cancer and all that...the role of paraoxonases. *Adv Exp Med Biol,* 824**,** 33-41.

DEWHIRST, M. W., CAO, Y. & MOELLER, B. 2008. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer,* 8**,** 425-37.

DHANI, N., FYLES, A., HEDLEY, D. & MILOSEVIC, M. 2015. The clinical significance of hypoxia in human cancers. *Semin Nucl Med,* 45**,** 110-21.

DI LORENZO, G., FIGG, W. D., FOSSA, S. D., MIRONE, V., AUTORINO, R., LONGO, N., IMBIMBO, C., PERDONA, S., GIORDANO, A., GIULIANO, M., LABIANCA, R. & DE PLACIDO, S. 2008. Combination of bevacizumab and docetaxel in docetaxel-pretreated hormone-refractory prostate cancer: a phase 2 study. *European urology,* 54**,** 1089-94.

DINGS, R. P., LOREN, M., HEUN, H., MCNIEL, E., GRIFFIOEN, A. W., MAYO, K. H. & GRIFFIN, R. J. 2007. Scheduling of radiation with angiogenesis inhibitors anginex and Avastin improves therapeutic outcome via vessel normalization. *Clin Cancer Res,* 13**,** 3395-402.

DIPAOLA, R. S. & AISNER, J. 1999. Overcoming bcl-2- and p53-mediated resistance in prostate cancer. *Semin Oncol,* 26**,** 112-6.

DISILVESTRO, P. A., ALI, S., CRAIGHEAD, P. S., LUCCI, J. A., LEE, Y. C., COHN, D. E., SPIRTOS, N. M., TEWARI, K. S., MULLER, C., GAJEWSKI, W. H., STEINHOFF, M. M. & MONK, B. J. 2014. Phase III randomized trial of weekly cisplatin and irradiation versus cisplatin and tirapazamine and irradiation in stages IB2, IIA, IIB, IIIB, and IVA cervical carcinoma limited to the pelvis: a Gynecologic Oncology Group study. *J Clin Oncol,* 32**,** 458-64.

DISS, J. K., ARCHER, S. N., HIRANO, J., FRASER, S. P. & DJAMGOZ, M. B. 2001. Expression profiles of voltage-gated Na(+) channel alpha-subunit genes in rat and human prostate cancer cell lines. *Prostate,* 48**,** 165-78.

DODD, K. M., YANG, J., SHEN, M. H., SAMPSON, J. R. & TEE, A. R. 2015. mTORC1 drives HIF-1alpha and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3. *Oncogene,* 34**,** 2239-50.

DOSCAS, M. E., WILLIAMSON, A. J., USHA, L., BOGACHKOV, Y., RAO, G. S., XIAO, F., WANG, Y., RUBY, C., KAUFMAN, H., ZHOU, J., WILLIAMS, J. W., LI, Y. & XU, X. 2014. Inhibition of p70 S6 kinase (S6K1) activity by A77 1726 and its effect on cell proliferation and cell cycle progress. *Neoplasia,* 16**,** 824-34.

DUTTO, I., TILLHON, M., CAZZALINI, O., STIVALA, L. A. & PROSPERI, E. 2015. Biology of the cell cycle inhibitor p21(CDKN1A): molecular mechanisms and relevance in chemical toxicology. *Arch Toxicol,* 89**,** 155-78.

EHNMAN, M. & OSTMAN, A. 2014. Therapeutic targeting of platelet-derived growth factor receptors in solid tumors. *Expert Opin Investig Drugs,* 23**,** 211-26.

EIKENBERRY, S. E., NAGY, J. D. & KUANG, Y. 2010. The evolutionary impact of androgen levels on prostate cancer in a multi-scale mathematical model. *Biol Direct,* 5**,** 24.

FELDMAN, B. J. & FELDMAN, D. 2001. The development of androgen-independent prostate cancer. *Nature reviews. Cancer,* 1**,** 34-45.

FELDSER, D., AGANI, F., IYER, N. V., PAK, B., FERREIRA, G. & SEMENZA, G. L. 1999. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res,* 59**,** 3915-8.

FENG, S., SHAO, L., YU, W., GAVINE, P. & ITTMANN, M. 2012. Targeting fibroblast growth factor receptor signaling inhibits prostate cancer progression. *Clin Cancer Res,* 18**,** 3880-8.

FENTON, T. R. & GOUT, I. T. 2011. Functions and regulation of the 70kDa ribosomal S6 kinases. *Int J Biochem Cell Biol,* 43**,** 47-59.

FIGG, W. D., ARLEN, P., GULLEY, J., FERNANDEZ, P., NOONE, M., FEDENKO, K., HAMILTON, M., PARKER, C., KRUGER, E. A., PLUDA, J. & DAHUT, W. L. 2001a. A randomized phase II trial of docetaxel (taxotere) plus thalidomide in androgen-independent prostate cancer. *Seminars in oncology,* 28**,** 62-6.

FIGG, W. D., DAHUT, W., DURAY, P., HAMILTON, M., TOMPKINS, A., STEINBERG, S. M., JONES, E., PREMKUMAR, A., LINEHAN, W. M., FLOETER, M. K., CHEN, C. C., DIXON, S., KOHLER, D. R., KRUGER, E. A., GUBISH, E., PLUDA, J. M. & REED, E. 2001b. A randomized phase II

trial of thalidomide, an angiogenesis inhibitor, in patients with androgen-independent prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 7**,** 1888-93.

FINE, S. W. & REUTER, V. E. 2012. Anatomy of the prostate revisited: implications for prostate biopsy and zonal origins of prostate cancer. *Histopathology,* 60**,** 142-52.

FLETCHER, G. H., LINDBERG, R. D., CADERAO, J. B. & WHARTON, J. T. 1977. Hyperbaric-Oxygen as a Radiotherapeutic Adjuvant in Advanced Cancer of Uterine Cervix - Preliminary-Results of a Randomized Trial. *Cancer,* 39**,** 617-623.

FONG, E. L., WAN, X., YANG, J., MORGADO, M., MIKOS, A. G., HARRINGTON, D. A., NAVONE, N. M. & FARACH-CARSON, M. C. 2016. A 3D in vitro model of patient-derived prostate cancer xenograft for controlled interrogation of in vivo tumor-stromal interactions. *Biomaterials,* 77**,** 164-72.

FORD, A. P., DANIELS, D. V., CHANG, D. J., GEVER, J. R., JASPER, J. R., LESNICK, J. D. & CLARKE, D. E. 1997. Pharmacological pleiotropism of the human recombinant alpha1A-adrenoceptor: implications for alpha1-adrenoceptor classification. *Br J Pharmacol,* 121**,** 1127-35.

FRAGA, A., RIBEIRO, R., PRINCIPE, P., LOPES, C. & MEDEIROS, R. 2015. Hypoxia and Prostate Cancer Aggressiveness: A Tale With Many Endings. *Clin Genitourin Cancer,* 13**,** 295-301.

FRASER, S. P., OZERLAT-GUNDUZ, I., BRACKENBURY, W. J., FITZGERALD, E. M., CAMPBELL, T. M., COOMBES, R. C. & DJAMGOZ, M. B. 2014. Regulation of voltage-gated sodium channel expression in cancer: hormones, growth factors and auto-regulation. *Philos Trans R Soc Lond B Biol Sci,* 369**,** 20130105.

FRASER, S. P., SALVADOR, V., MANNING, E. A., MIZAL, J., ALTUN, S., RAZA, M., BERRIDGE, R. J. & DJAMGOZ, M. B. 2003. Contribution of functional voltage-gated Na+ channel expression to cell behaviors involved in the metastatic cascade in rat prostate cancer: I. Lateral motility. *J Cell Physiol,* 195**,** 479-87.

FREEDLAND, S. J., HUMPHREYS, E. B., MANGOLD, L. A., EISENBERGER, M., DOREY, F. J., WALSH, P. C. & PARTIN, A. W. 2005. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. *Jama,* 294**,** 433-9.

FRISCH, S. M. & FRANCIS, H. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *The Journal of cell biology,* 124**,** 619-26.

GALSKY, M. D. & VOGELZANG, N. J. 2010. Docetaxel-based combination therapy for castration-resistant prostate cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO,* 21**,** 2135-44.

GAN, L., ZHU, D. X., YANG, L. P., LIU, R. S., YAN, F. & ZHANG, J. 2008. Involvement of transcription factor activator protein-2alpha in doxazosin-induced HeLa cell apoptosis. *Acta pharmacologica Sinica,* 29**,** 465-72.

GANDELLINI, P., ANDRIANI, F., MERLINO, G., D'AIUTO, F., ROZ, L. & CALLARI, M. 2015. Complexity in the tumour microenvironment: Cancer associated fibroblast gene expression patterns identify both common and unique features of tumour-stroma crosstalk across cancer types. *Semin Cancer Biol,* 35**,** 96-106.

GAO, H., GONG, X. C., CHEN, Z. D., XU, X. S., ZHANG, Q. & XU, X. M. 2014. Induction of apoptosis in hormone-resistant human prostate cancer PC3 cells by inactivated Sendai virus. *Biomed Environ Sci,* 27**,** 506-14.

GARRISON, J. B. & KYPRIANOU, N. 2006. Doxazosin induces apoptosis of benign and malignant prostate cells via a death receptor-mediated pathway. *Cancer research,* 66**,** 464-72.

GE, X. H., LIN, Q., REN, X. C., LIU, Y. E., CHEN, X. J., WANG, D. Y., WANG, Y. Q., CAO, B., LI, Z. G. & LIU, M. L. 2013. Phase II clinical trial of whole-brain irradiation plus three-dimensional conformal boost with concurrent topotecan for brain metastases from lung cancer. *Radiat Oncol,* 8**,** 238.

GELLER, J., LIU, J., ALBERT, J., FAY, W., BERRY, C. C. & WEIS, P. 1987. Relationship between human prostatic epithelial cell protein synthesis and tissue dihydrotestosterone level. *Clinical endocrinology,* 26**,** 155-61.

GELSOMINO, F., FACCHINETTI, F., HASPINGER, E. R., GARASSINO, M. C., TRUSOLINO, L., DE BRAUD, F. & TISEO, M. 2014. Targeting the MET gene for the treatment of non-small-cell lung cancer. *Crit Rev Oncol Hematol,* 89**,** 284-99.

GIANNONI, E., BIANCHINI, F., MASIERI, L., SERNI, S., TORRE, E., CALORINI, L. & CHIARUGI, P. 2010. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. *Cancer Res,* 70**,** 6945-56.

GILLENWATER, J. Y., CONN, R. L., CHRYSANT, S. G., ROY, J., GAFFNEY, M., ICE, K. & DIAS, N. 1995. Doxazosin for the treatment of benign prostatic hyperplasia in patients with mild to moderate essential hypertension: a double-blind, placebo-controlled, dose-response multicenter study. *The Journal of urology,* 154**,** 110-15.

GIORGIO, C., HASSAN MOHAMED, I., FLAMMINI, L., BAROCELLI, E., INCERTI, M., LODOLA, A. & TOGNOLINI, M. 2011. Lithocholic acid is an Eph-ephrin ligand interfering with Eph-kinase activation. *PLoS One,* 6**,** e18128.

GLUCK, A. A., AEBERSOLD, D. M., ZIMMER, Y. & MEDOVA, M. 2015. Interplay between receptor tyrosine kinases and hypoxia signaling in cancer. *Int J Biochem Cell Biol,* 62**,** 101-14.

GOMES, A., FERNANDES, E. & LIMA, J. L. 2005. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods,* 65**,** 45-80.

GORDON, P. B., HOLEN, I. & SEGLEN, P. O. 1991. Effects of adrenergic agonists and antagonists on autophagic activity in isolated rat liver cells. *Biomed Biochim Acta,* 50**,** 383-7.

GORMAND, A., HENRIKSSON, E., STROM, K., JENSEN, T. E., SAKAMOTO, K. & GORANSSON, O. 2011. Regulation of AMP-activated protein kinase by LKB1 and CaMKK in adipocytes. *J Cell Biochem,* 112**,** 1364-75.

GOTOH, A., NAGAYA, H., KANNO, T. & NISHIZAKI, T. 2012. Antitumor action of alpha(1)-adrenoceptor blockers on human bladder, prostate and renal cancer cells. *Pharmacology,* 90**,** 242-6.

GRAHAM, R. M., PEREZ, D. M., HWA, J. & PIASCIK, M. T. 1996. alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling. *Circulation research,* 78**,** 737-49.

GREENFIELD, L. K. & JONES, N. L. 2013. Modulation of autophagy by Helicobacter pylori and its role in gastric carcinogenesis. *Trends Microbiol,* 21**,** 602-12.

GRIMM, M. O., KAMPHAUSEN, S., HUGENSCHMIDT, H., STEPHAN-ODENTHAL, M., ACKERMANN, R. & VOGELI, T. A. 2002. Clinical

outcome of patients with lymph node positive prostate cancer after radical prostatectomy versus androgen deprivation. *European urology,* 41**,** 628-34; discussion 634.

GUISE, C. P., MOWDAY, A. M., ASHOORZADEH, A., YUAN, R., LIN, W. H., WU, D. H., SMAILL, J. B., PATTERSON, A. V. & DING, K. 2014. Bioreductive prodrugs as cancer therapeutics: targeting tumor hypoxia. *Chin J Cancer,* 33**,** 80-6.

GUNDALA, S. R., YANG, C., MUKKAVILLI, R., PARANJPE, R., BRAHMBHATT, M., PANNU, V., CHENG, A., REID, M. D. & ANEJA, R. 2014. Hydroxychavicol, a betel leaf component, inhibits prostate cancer through ROS-driven DNA damage and apoptosis. *Toxicol Appl Pharmacol,* 280**,** 86-96.

GUO, S., LIANG, Y., MURPHY, S. F., HUANG, A., SHEN, H., KELLY, D. F., SOBRADO, P. & SHENG, Z. 2015. A rapid and high content assay that measures cyto-ID-stained autophagic compartments and estimates autophagy flux with potential clinical applications. *Autophagy,* 11**,** 560-72.

GUSTAVSSON, H., TESAN, T., JENNBACKEN, K., KUNO, K., DAMBER, J. E. & WELEN, K. 2010. ADAMTS1 alters blood vessel morphology and TSP1 levels in LNCaP and LNCaP-19 prostate tumors. *BMC Cancer,* 10**,** 288.

GUSTAVSSON, H., WANG, W., JENNBACKEN, K., WELEN, K. & DAMBER, J. E. 2009. ADAMTS1, a putative anti-angiogenic factor, is decreased in human prostate cancer. *BJU Int,* 104**,** 1786-90.

GWINN, D. M., SHACKELFORD, D. B., EGAN, D. F., MIHAYLOVA, M. M., MERY, A., VASQUEZ, D. S., TURK, B. E. & SHAW, R. J. 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell,* 30**,** 214-26.

HAFFTY, B. G., HURLEY, R. & PETERS, L. J. 1999. Radiation therapy with hyperbaric oxygen at 4 atmospheres pressure in the management of squamous cell carcinoma of the head and neck: results of a randomized clinical trial. *Cancer J Sci Am,* 5**,** 341-7.

HALL, E. J. G., E. 2006. Radiobiology for the Radiologist, 6th edition. Philadelphia, USA: J.B. Lippincott Company.

HAN, Y. & CHEN, J. Z. 2013. Oxidative stress induces mitochondrial DNA damage and cytotoxicity through independent mechanisms in human cancer cells. *Biomed Res Int,* 2013**,** 825065.

HARADA, H. 2011. How Can We Overcome Tumor Hypoxia in Radiation Therapy? *Journal of Radiation Research,* 52**,** 545-556.

HARADA, H., ITASAKA, S., ZHU, Y., ZENG, L., XIE, X., MORINIBU, A., SHINOMIYA, K. & HIRAOKA, M. 2009. Treatment regimen determines whether an HIF-1 inhibitor enhances or inhibits the effect of radiation therapy. *Br J Cancer,* 100**,** 747-57.

HARRIS, A. M., WARNER, B. W., WILSON, J. M., BECKER, A., ROWLAND, R. G., CONNER, W., LANE, M., KIMBLER, K., DURBIN, E. B., BARON, A. T. & KYPRIANOU, N. 2007. Effect of alpha1-adrenoceptor antagonist exposure on prostate cancer incidence: an observational cohort study. *The Journal of urology,* 178**,** 2176-80.

HATZI, V. I., LASKARATOU, D. A., MAVRAGANI, I. V., NIKITAKI, Z., MANGELIS, A., PANAYIOTIDIS, M. I., PANTELIAS, G. E., TERZOUDI, G. I. & GEORGAKILAS, A. G. 2015. Non-targeted radiation effects in vivo: a critical glance of the future in radiobiology. *Cancer Lett,* 356**,** 34-42.

HEIDENREICH, A., AUS, G., BOLLA, M., JONIAU, S., MATVEEV, V. B., SCHMID, H. P. & ZATTONI, F. 2008. EAU guidelines on prostate cancer. *European urology,* 53**,** 68-80.

HEIDENREICH, A., BELLMUNT, J., BOLLA, M., JONIAU, S., MASON, M., MATVEEV, V., MOTTET, N., SCHMID, H. P., VAN DER KWAST, T., WIEGEL, T. & ZATTONI, F. 2011. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *European urology,* 59**,** 61-71.

HEMPEL, N., CARRICO, P. M. & MELENDEZ, J. A. 2011. Manganese superoxide dismutase (Sod2) and redox-control of signaling events that drive metastasis. *Anticancer Agents Med Chem,* 11**,** 191-201.

HENDRICKSEN, K. & WITJES, J. A. 2007. Current strategies for first and second line intravesical therapy for nonmuscle invasive bladder cancer. *Curr Opin Urol,* 17**,** 352-7.

HENNESSEY, D., MARTIN, L. M., ATZBERGER, A., LYNCH, T. H., HOLLYWOOD, D. & MARIGNOL, L. 2013. Exposure to hypoxia following irradiation increases radioresistance in prostate cancer cells. *Urol Oncol,* 31**,** 1106-16.

HIGGINS, I. T. 1975. The epidemiology of cancer of the prostate. *J Chronic Dis,* 28**,** 343.

HO, T., HOWARD, L. E., VIDAL, A. C., GERBER, L., MOREIRA, D., MCKEEVER, M., ANDRIOLE, G., CASTRO-SANTAMARIA, R. & FREEDLAND, S. J. 2014. Smoking and risk of low- and high-grade prostate cancer: results from the REDUCE study. *Clin Cancer Res,* 20**,** 5331-8.

HOANG, T., HUANG, S., ARMSTRONG, E., EICKHOFF, J. C. & HARARI, P. M. 2012. Enhancement of radiation response with bevacizumab. *J Exp Clin Cancer Res,* 31**,** 37.

HONG, F., LARREA, M. D., DOUGHTY, C., KWIATKOWSKI, D. J., SQUILLACE, R. & SLINGERLAND, J. M. 2008. mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation. *Mol Cell,* 30**,** 701-11.

HONISCH, S., ALKAHTANI, S., KOUNENIDAKIS, M., LIU, G., ALARIFI, S., AL-YAHYA, H., DIMAS, K., ALKAHTANE, A. A., PROUSIS, K. C., AL-DAHMASH, B., CALOGEROPOULOU, T., ALEVIZOPOULOS, K., LANG, F. & STOURNARAS, C. 2014. A steroidal Na+/K+ ATPase inhibitor triggers pro-apoptotic signaling and induces apoptosis in prostate and lung tumor cells. *Anticancer Agents Med Chem,* 14**,** 1161-8.

HOOGSTEEN, I. J., MARRES, H. A., VAN DEN HOOGEN, F. J., RIJKEN, P. F., LOK, J., BUSSINK, J. & KAANDERS, J. H. 2012. Expression of EGFR under tumor hypoxia: identification of a subpopulation of tumor cells responsible for aggressiveness and treatment resistance. *Int J Radiat Oncol Biol Phys,* 84**,** 807-14.

HORBELT, D., DENKIS, A. & KNAUS, P. 2012. A portrait of Transforming Growth Factor beta superfamily signalling: Background matters. *Int J Biochem Cell Biol,* 44**,** 469-74.

HORI, Y., ISHII, K., KANDA, H., IWAMOTO, Y., NISHIKAWA, K., SOGA, N., KISE, H., ARIMA, K. & SUGIMURA, Y. 2011. Naftopidil, a selective {alpha}1-adrenoceptor antagonist, suppresses human prostate tumor growth by altering interactions between tumor cells and stroma. *Cancer Prev Res (Phila),* 4**,** 87-96.

HU, Z. W., SHI, X. Y. & HOFFMAN, B. B. 1998. Doxazosin inhibits proliferation and migration of human vascular smooth-muscle cells independent of alpha1-adrenergic receptor antagonism. *Journal of cardiovascular pharmacology,* 31**,** 833-9.

HUANG, L. E., GU, J., SCHAU, M. & BUNN, H. F. 1998. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A,* 95**,** 7987-92.

HUI, H., FERNANDO, M. A. & HEANEY, A. P. 2008. The alpha1-adrenergic receptor antagonist doxazosin inhibits EGFR and NF-kappaB signalling to induce breast cancer cell apoptosis. *European journal of cancer,* 44**,** 160-6.

IGEA, A. & NEBREDA, A. R. 2015. The Stress Kinase p38alpha as a Target for Cancer Therapy. *Cancer Res,* 75**,** 3997-4002.

ILIO, K. Y., PARK, II, PINS, M. R., KOZLOWSKI, J. M. & LEE, C. 2001. Apoptotic activity of doxazosin on prostate stroma in vitro is mediated through an autocrine expression of TGF-beta1. *The Prostate,* 48**,** 131-5.

INOUE, M., TAKAHASHI, Y., FUJII, T., KITAGAWA, M. & FUKUSATO, T. 2014. Significance of downregulation of liver fatty acid-binding protein in hepatocellular carcinoma. *World J Gastroenterol,* 20**,** 17541-51.

INOUE, T., HIRATSUKA, M., OSAKI, M. & OSHIMURA, M. 2007. The molecular biology of mammalian SIRT proteins: SIRT2 in cell cycle regulation. *Cell Cycle,* 6**,** 1011-8.

IP, C. K., CHEUNG, A. N., NGAN, H. Y. & WONG, A. S. 2011. p70 S6 kinase in the control of actin cytoskeleton dynamics and directed migration of ovarian cancer cells. *Oncogene,* 30**,** 2420-32.

ISGOR, B. S. & ISGOR, Y. G. 2012. Effect of Alpha-1-Adrenoceptor Blocker on Cytosolic Enzyme Targets for Ptential use in Cancer Chemotherapy. *International journal of pharmacology*.

IWENOFU, O. H., LACKMAN, R. D., STADDON, A. P., GOODWIN, D. G., HAUPT, H. M. & BROOKS, J. S. 2008. Phospho-S6 ribosomal protein: a potential new predictive sarcoma marker for targeted mTOR therapy. *Mod Pathol,* 21**,** 231-7.

JAILLON, P. 1980. Clinical pharmacokinetics of prazosin. *Clin Pharmacokinet,* 5**,** 365-76.

JANS, J., VAN DIJK, J. H., VAN SCHELVEN, S., VAN DER GROEP, P., WILLEMS, S. H., JONGES, T. N., VAN DIEST, P. J. & BOSCH, J. L. H. R. 2010. Expression and Localization of Hypoxia Proteins in Prostate Cancer: Prognostic Implications After Radical Prostatectomy. *Urology,* 75**,** 786-792.

JANSSENS, G. O., RADEMAKERS, S. E., TERHAARD, C. H., DOORNAERT, P. A., BIJL, H. P., VAN DEN ENDE, P., CHIN, A., MARRES, H. A., DE BREE, R., VAN DER KOGEL, A. J., HOOGSTEEN, I. J., BUSSINK, J., SPAN, P. N. & KAANDERS, J. H. 2012. Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. *J Clin Oncol,* 30**,** 1777-83.

JEHLE, J., STAUDACHER, I., WIEDMANN, F., SCHWEIZER, P., BECKER, R., KATUS, H. & THOMAS, D. 2012. Regulation of apoptosis in HL-1 cardiomyocytes by phosphorylation of the receptor tyrosine kinase EphA2 and protection by lithocholic acid. *Br J Pharmacol,* 167**,** 1563-72.

JENSEN, B. C., SWIGART, P. M. & SIMPSON, P. C. 2009. Ten commercial antibodies for alpha-1-adrenergic receptor subtypes are nonspecific. *Naunyn Schmiedebergs Arch Pharmacol,* 379**,** 409-12.

JEPSEN, J. V. & BRUSKEWITZ, R. C. 1998. Comprehensive patient evaluation for benign prostatic hyperplasia. *Urology,* 51**,** 13-8.
JI, N., LI, J., WEI, Z., KONG, F., JIN, H., CHEN, X., LI, Y. & DENG, Y. 2015. Effect of celastrol on growth inhibition of prostate cancer cells through the regulation of hERG channel in vitro. *Biomed Res Int,* 2015**,** 308475.
JONES, C. U., HUNT, D., MCGOWAN, D. G., AMIN, M. B., CHETNER, M. P., BRUNER, D. W., LEIBENHAUT, M. H., HUSAIN, S. M., ROTMAN, M., SOUHAMI, L., SANDLER, H. M. & SHIPLEY, W. U. 2011. Radiotherapy and short-term androgen deprivation for localized prostate cancer. *The New England journal of medicine,* 365**,** 107-18.
KAIGHN, M. E., NARAYAN, K. S., OHNUKI, Y., LECHNER, J. F. & JONES, L. W. 1979. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol,* 17**,** 16-23.
KALLBERG, E., WIKSTROM, P., BERGH, A., IVARS, F. & LEANDERSON, T. 2010. Indoleamine 2,3-dioxygenase (IDO) activity influence tumor growth in the TRAMP prostate cancer model. *Prostate,* 70**,** 1461-70.
KANG, S. H., CHESS-WILLIAMS, R., ANOOPKUMAR-DUKIE, S. & MCDERMOTT, C. 2013. Induction of inflammatory cytokines and alteration of urothelial ATP, acetylcholine and prostaglandin E2 release by doxorubicin. *Eur J Pharmacol,* 700**,** 102-9.
KANG, S. H., CHESS-WILLIAMS, R., ANOOPKUMAR-DUKIE, S. & MCDERMOTT, C. 2015. Recovery of urothelial mediator release but prolonged elevations in interleukin-8 and nitric oxide secretion following mitomycin C treatment. *Naunyn Schmiedebergs Arch Pharmacol,* 388**,** 781-91.
KAPISZEWSKA, M. 2006. A vegetable to meat consumption ratio as a relevant factor determining cancer preventive diet. The Mediterranean versus other European countries. *Forum Nutr,* 59**,** 130-53.
KARAMYSHEVA, A. F. 2008. Mechanisms of angiogenesis. *Biochemistry. Biokhimiia,* 73**,** 751-62.
KARLSSON, M., KURZ, T., BRUNK, U. T., NILSSON, S. E. & FRENNESSON, C. I. 2010. What does the commonly used DCF test for oxidative stress really show? *Biochem J,* 428**,** 183-90.
KASSOUF, W., LUONGO, T., BROWN, G., ADAM, L. & DINNEY, C. P. 2006. Schedule dependent efficacy of gefitinib and docetaxel for bladder cancer. *The Journal of urology,* 176**,** 787-92.
KATO, T., ISHIKAWA, K., NEMOTO, R., SENOO, A. & AMANO, Y. 1978. Morphological characterization of two established cell lines, T24 and MGH-U1, derived from human urinary bladder carcinoma. *Tohoku J Exp Med,* 124**,** 339-49.
KATSOGIANNOU, M., EL BOUSTANY, C., GACKIERE, F., DELCOURT, P., ATHIAS, A., MARIOT, P., DEWAILLY, E., JOUY, N., LAMAZE, C., BIDAUX, G., MAUROY, B., PREVARSKAYA, N. & SLOMIANNY, C. 2009. Caveolae contribute to the apoptosis resistance induced by the alpha(1A)-adrenoceptor in androgen-independent prostate cancer cells. *PLoS One,* 4**,** e7068.
KAYSER, S. & LEVIS, M. J. 2014. FLT3 tyrosine kinase inhibitors in acute myeloid leukemia: clinical implications and limitations. *Leuk Lymphoma,* 55**,** 243-55.
KELEDJIAN, K., BORKOWSKI, A., KIM, G., ISAACS, J. T., JACOBS, S. C. & KYPRIANOU, N. 2001. Reduction of human prostate tumor vascularity by the alpha1-adrenoceptor antagonist terazosin. *The Prostate,* 48**,** 71-8.

KELEDJIAN, K., GARRISON, J. B. & KYPRIANOU, N. 2005. Doxazosin inhibits human vascular endothelial cell adhesion, migration, and invasion. *Journal of cellular biochemistry,* 94**,** 374-88.

KELEDJIAN, K. & KYPRIANOU, N. 2003. Anoikis induction by quinazoline based alpha 1-adrenoceptor antagonists in prostate cancer cells: antagonistic effect of bcl-2. *The Journal of urology,* 169**,** 1150-6.

KELLY, W. K., HALABI, S., CARDUCCI, M., GEORGE, D., MAHONEY, J. F., STADLER, W. M., MORRIS, M., KANTOFF, P., MONK, J. P., KAPLAN, E., VOGELZANG, N. J. & SMALL, E. J. 2012. Randomized, double-blind, placebo-controlled phase III trial comparing docetaxel and prednisone with or without bevacizumab in men with metastatic castration-resistant prostate cancer: CALGB 90401. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 30**,** 1534-40.

KERR, J. F., WINTERFORD, C. M. & HARMON, B. V. 1994. Apoptosis. Its significance in cancer and cancer therapy. *Cancer,* 73**,** 2013-26.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer,* 26**,** 239-57.

KHOR, L. Y., MOUGHAN, J., AL-SALEEM, T., HAMMOND, E. H., VENKATESAN, V., ROSENTHAL, S. A., RITTER, M. A., SANDLER, H. M., HANKS, G. E., SHIPLEY, W. U. & POLLACK, A. 2007. Bcl-2 and Bax expression predict prostate cancer outcome in men treated with androgen deprivation and radiotherapy on radiation therapy oncology group protocol 92-02. *Clin Cancer Res,* 13**,** 3585-90.

KIM, K. Y., CHO, H. J., YU, S. N., KIM, S. H., YU, H. S., PARK, Y. M., MIRKHESHTI, N., KIM, S. Y., SONG, C. S., CHATTERJEE, B. & AHN, S. C. 2013. Interplay of reactive oxygen species, intracellular Ca2+ and mitochondrial homeostasis in the apoptosis of prostate cancer cells by deoxypodophyllotoxin. *J Cell Biochem,* 114**,** 1124-34.

KIMURA, K., MARKOWSKI, M., BOWEN, C. & GELMANN, E. P. 2001. Androgen blocks apoptosis of hormone-dependent prostate cancer cells. *Cancer research,* 61**,** 5611-8.

KINTSCHER, U., WAKINO, S., KIM, S., JACKSON, S. M., FLECK, E., HSUEH, W. A. & LAW, R. E. 2000a. Doxazosin inhibits retinoblastoma protein phosphorylation and G(1)-->S transition in human coronary smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology,* 20**,** 1216-24.

KINTSCHER, U., WAKINO, S., KIM, S., JACKSON, S. M., FLECK, E., HSUEH, W. A. & LAW, R. E. 2000b. Doxazosin inhibits retinoblastoma protein phosphorylation and G(1)-->S transition in human coronary smooth muscle cells. *Arterioscler Thromb Vasc Biol,* 20**,** 1216-24.

KIRSCHENBAUM, A., LIU, X., YAO, S. & LEVINE, A. C. 2001. The role of cyclooxygenase-2 in prostate cancer. *Urology,* 58**,** 127-31.

KISHI, K., YUASA, T., MINAMI, A., YAMADA, M., HAGI, A., HAYASHI, H., KEMP, B. E., WITTERS, L. A. & EBINA, Y. 2000. AMP-Activated protein kinase is activated by the stimulations of G(q)-coupled receptors. *Biochem Biophys Res Commun,* 276**,** 16-22.

KLIONSKY, D. J., ABDALLA, F. C., ABELIOVICH, H., ABRAHAM, R. T., ACEVEDO-AROZENA, A., ADELI, K., AGHOLME, L., AGNELLO, M., AGOSTINIS, P., AGUIRRE-GHISO, J. A., AHN, H. J., AIT-MOHAMED, O., AIT-SI-ALI, S., AKEMATSU, T., AKIRA, S., AL-YOUNES, H. M., AL-

ZEER, M. A., ALBERT, M. L., ALBIN, R. L., ALEGRE-ABARRATEGUI, J., ALEO, M. F., ALIREZAEI, M., ALMASAN, A., ALMONTE-BECERRIL, M., AMANO, A., AMARAVADI, R., AMARNATH, S., AMER, A. O., ANDRIEU-ABADIE, N., ANANTHARAM, V., ANN, D. K., ANOOPKUMAR-DUKIE, S., AOKI, H., APOSTOLOVA, N., ARANCIA, G., ARIS, J. P., ASANUMA, K., ASARE, N. Y., ASHIDA, H., ASKANAS, V., ASKEW, D. S., AUBERGER, P., BABA, M., BACKUES, S. K., BAEHRECKE, E. H., BAHR, B. A., BAI, X. Y., BAILLY, Y., BAIOCCHI, R., BALDINI, G., BALDUINI, W., BALLABIO, A., BAMBER, B. A., BAMPTON, E. T., BANHEGYI, G., BARTHOLOMEW, C. R., BASSHAM, D. C., BAST, R. C., JR., BATOKO, H., BAY, B. H., BEAU, I., BECHET, D. M., BEGLEY, T. J., BEHL, C., BEHRENDS, C., BEKRI, S., BELLAIRE, B., BENDALL, L. J., BENETTI, L., BERLIOCCHI, L., BERNARDI, H., BERNASSOLA, F., BESTEIRO, S., BHATIA-KISSOVA, I., BI, X., BIARD-PIECHACZYK, M., BLUM, J. S., BOISE, L. H., BONALDO, P., BOONE, D. L., BORNHAUSER, B. C., BORTOLUCI, K. R., BOSSIS, I., BOST, F., BOURQUIN, J. P., BOYA, P., BOYER-GUITTAUT, M., BOZHKOV, P. V., BRADY, N. R., BRANCOLINI, C., BRECH, A., BRENMAN, J. E., BRENNAND, A., BRESNICK, E. H., BREST, P., BRIDGES, D., BRISTOL, M. L., BROOKES, P. S., BROWN, E. J., BRUMELL, J. H., et al. 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy,* 8**,** 445-544.

KOIE, T., OHYAMA, C., YAMAMOTO, H., IMAI, A., HATAKEYAMA, S., YONEYAMA, T., HASHIMOTO, Y., YONEYAMA, T., TOBISAWA, Y., AOKI, M. & TAKAI, Y. 2014. Both radical prostatectomy following treatment with neoadjuvant LHRH agonist and estramustine and radiotherapy following treatment with neoadjuvant hormonal therapy achieved favorable oncological outcome in high-risk prostate cancer: a propensity-score matching analysis. *World J Surg Oncol,* 12**,** 134.

KONDO, Y., KANZAWA, T., SAWAYA, R. & KONDO, S. 2005. The role of autophagy in cancer development and response to therapy. *Nature reviews. Cancer,* 5**,** 726-34.

KONGARA, S. & KARANTZA, V. 2012. The interplay between autophagy and ROS in tumorigenesis. *Front Oncol,* 2**,** 171.

KORITZINSKY, M., SEIGNEURIC, R., MAGAGNIN, M. G., VAN DEN BEUCKEN, T., LAMBIN, P. & WOUTERS, B. G. 2005. The hypoxic proteome is influenced by gene-specific changes in mRNA translation. *Radiother Oncol,* 76**,** 177-86.

KORSTANJE, C., KRAUWINKEL, W. & VAN DOESUM-WOLTERS, F. L. 2011. Tamsulosin shows a higher unbound drug fraction in human prostate than in plasma: a basis for uroselectivity? *Br J Clin Pharmacol,* 72**,** 218-25.

KRISHNA, M. & NARANG, H. 2008. The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cellular and molecular life sciences : CMLS,* 65**,** 3525-44.

KUMAR, B., KOUL, S., KHANDRIKA, L., MEACHAM, R. B. & KOUL, H. K. 2008. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Res,* 68**,** 1777-85.

KUMMAR, S., RAFFELD, M., JUWARA, L., HORNEFFER, Y., STRASSBERGER, A., ALLEN, D., STEINBERG, S. M., RAPISARDA, A., SPENCER, S. D., FIGG, W. D., CHEN, X., TURKBEY, I. B., CHOYKE, P., MURGO, A. J., DOROSHOW, J. H. & MELILLO, G. 2011. Multihistology, target-driven pilot

trial of oral topotecan as an inhibitor of hypoxia-inducible factor-1alpha in advanced solid tumors. *Clin Cancer Res,* 17**,** 5123-31.
KUPELIAN, P. A., BUCHSBAUM, J. C., PATEL, C., ELSHAIKH, M., REDDY, C. A., ZIPPE, C. & KLEIN, E. A. 2002. Impact of biochemical failure on overall survival after radiation therapy for localized prostate cancer in the PSA era. *Int J Radiat Oncol Biol Phys,* 52**,** 704-11.
KWABI-ADDO, B., OZEN, M. & ITTMANN, M. 2004. The role of fibroblast growth factors and their receptors in prostate cancer. *Endocrine-Related Cancer,* 11**,** 709-724.
KYOU KWON, J., KIM, S. J., HOON KIM, J., MEE LEE, K. & HO CHANG, I. 2014. Dual inhibition by S6K1 and Elf4E is essential for controlling cellular growth and invasion in bladder cancer. *Urol Oncol,* 32**,** 51.e27-35.
KYPRIANOU, N. 2000. Induction of apoptosis by alpha1-adrenoceptor antagonists in benign prostatic hyperplasia and prostate cancer. *Prostate cancer and prostatic diseases,* 3**,** S24-S25.
KYPRIANOU, N. & BENNING, C. M. 2000. Suppression of human prostate cancer cell growth by alpha1-adrenoceptor antagonists doxazosin and terazosin via induction of apoptosis. *Cancer research,* 60**,** 4550-5.
KYPRIANOU, N., CHON, J. & BENNING, C. M. 2000. Effects of alpha(1)-adrenoceptor (alpha(1)-AR) antagonists on cell proliferation and apoptosis in the prostate: therapeutic implications in prostatic disease. *The Prostate. Supplement,* 9**,** 42-6.
KYPRIANOU, N., LITVAK, J. P., BORKOWSKI, A., ALEXANDER, R. & JACOBS, S. C. 1998. Induction of prostate apoptosis by doxazosin in benign prostatic hyperplasia. *The Journal of urology,* 159**,** 1810-5.
LAM, R. K., FUNG, Y. K., HAN, W. & YU, K. N. 2015. Rescue effects: irradiated cells helped by unirradiated bystander cells. *Int J Mol Sci,* 16**,** 2591-609.
LAMB, A. D. & NEAL, D. E. 2013. Role of the androgen receptor in prostate cancer. *Trends in Urology and Men's Health*.
LAPLANTE, M. & SABATINI, D. M. 2009. mTOR signaling at a glance. *J Cell Sci,* 122**,** 3589-94.
LAVECCHIA, A., DI GIOVANNI, C. & NOVELLINO, E. 2011. STAT-3 inhibitors: state of the art and new horizons for cancer treatment. *Curr Med Chem,* 18**,** 2359-75.
LEBEDEVA, I., RANDO, R., OJWANG, J., COSSUM, P. & STEIN, C. A. 2000. Bcl-xL in prostate cancer cells: effects of overexpression and down-regulation on chemosensitivity. *Cancer Res,* 60**,** 6052-60.
LEE, J. & KIM, S. S. 2009. The function of p27 KIP1 during tumor development. *Exp Mol Med,* 41**,** 765-71.
LEE, J. W., PARK, S., TAKAHASHI, Y. & WANG, H. G. 2010a. The association of AMPK with ULK1 regulates autophagy. *PLoS One,* 5**,** e15394.
LEE, M. C., MOUSSA, A. S., YU, C., KATTAN, M. W., MAGI-GALLUZZI, C. & JONES, J. S. 2010b. Multifocal high grade prostatic intraepithelial neoplasia is a risk factor for subsequent prostate cancer. *J Urol,* 184**,** 1958-62.
LEE, S. H., JOHNSON, D., LUONG, R. & SUN, Z. 2015. Crosstalking between androgen and PI3K/AKT signaling pathways in prostate cancer cells. *J Biol Chem,* 290**,** 2759-68.
LEHEN'KYI, V., FLOURAKIS, M., SKRYMA, R. & PREVARSKAYA, N. 2007. TRPV6 channel controls prostate cancer cell proliferation via Ca(2+)/NFAT-dependent pathways. *Oncogene,* 26**,** 7380-5.

LEPOR, H., KAZZAZI, A. & DJAVAN, B. 2012. alpha-Blockers for benign prostatic hyperplasia: the new era. *Current opinion in urology,* 22**,** 7-15.
LI, M., JIANG, X., LIU, D., NA, Y., GAO, G. F. & XI, Z. 2008a. Autophagy protects LNCaP cells under androgen deprivation conditions. *Autophagy,* 4**,** 54-60.
LI, S. H., SHIN, D. H., CHUN, Y. S., LEE, M. K., KIM, M. S. & PARK, J. W. 2008b. A novel mode of action of YC-1 in HIF inhibition: stimulation of FIH-dependent p300 dissociation from HIF-1{alpha}. *Mol Cancer Ther,* 7**,** 3729-38.
LI, Y., SIKES, R. A., MALAEB, B. S., YEUNG, F., LAW, A., GRAHAM, S. E., PEI, M., KAO, C., NELSON, J., KOENEMAN, K. S. & CHUNG, L. W. 2011. Osteoblasts can stimulate prostate cancer growth and transcriptionally down-regulate PSA expression in cell line models. *Urol Oncol,* 29**,** 802-8.
LIANG, J., SHAO, S. H., XU, Z. X., HENNESSY, B., DING, Z., LARREA, M., KONDO, S., DUMONT, D. J., GUTTERMAN, J. U., WALKER, C. L., SLINGERLAND, J. M. & MILLS, G. B. 2007. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol,* 9**,** 218-24.
LIANG, J., WU, Y. L., CHEN, B. J., ZHANG, W., TANAKA, Y. & SUGIYAMA, H. 2013. The C-kit receptor-mediated signal transduction and tumor-related diseases. *Int J Biol Sci,* 9**,** 435-43.
LIANOS, G. D., ALEXIOU, G. A., MANGANO, A., MANGANO, A., RAUSEI, S., BONI, L., DIONIGI, G. & ROUKOS, D. H. 2015. The role of heat shock proteins in cancer. *Cancer Lett,* 360**,** 114-8.
LIAO, C. H., GUH, J. H., CHUEH, S. C. & YU, H. J. 2011. Anti-angiogenic effects and mechanism of prazosin. *The Prostate,* 71**,** 976-84.
LIBERATI, S., MORELLI, M. B., AMANTINI, C., SANTONI, M., NABISSI, M., CARDINALI, C. & SANTONI, G. 2014. Advances in transient receptor potential vanilloid-2 channel expression and function in tumor growth and progression. *Curr Protein Pept Sci,* 15**,** 732-7.
LIN, C. I., WHANG, E. E., DONNER, D. B., DU, J., LORCH, J., HE, F., JIANG, X., PRICE, B. D., MOORE, F. D., JR. & RUAN, D. T. 2010. Autophagy induction with RAD001 enhances chemosensitivity and radiosensitivity through Met inhibition in papillary thyroid cancer. *Mol Cancer Res,* 8**,** 1217-26.
LIN, P. H., ARONSON, W. & FREEDLAND, S. J. 2015. Nutrition, dietary interventions and prostate cancer: the latest evidence. *BMC Med,* 13**,** 3.
LIN, S. C., CHUEH, S. C., HSIAO, C. J., LI, T. K., CHEN, T. H., LIAO, C. H., LYU, P. C. & GUH, J. H. 2007. Prazosin displays anticancer activity against human prostate cancers: targeting DNA and cell cycle. *Neoplasia,* 9**,** 830-9.
LING, S., FENG, T., KE, Q., FAN, N., LI, L., LI, Z., DONG, C., WANG, C., XU, F., LI, Y. & WANG, L. 2014. Metformin inhibits proliferation and enhances chemosensitivity of intrahepatic cholangiocarcinoma cell lines. *Oncol Rep,* 31**,** 2611-8.
LIOU, S. F., LIN, H. H., LIANG, J. C., CHEN, I. J. & YEH, J. L. 2009. Inhibition of human prostate cancer cells proliferation by a selective alpha1-adrenoceptor antagonist labedipinedilol-A involves cell cycle arrest and apoptosis. *Toxicology,* 256**,** 13-24.
LISLE, J. E., MERTENS-WALKER, I., RUTKOWSKI, R., HERINGTON, A. C. & STEPHENSON, S. A. 2013. Eph receptors and their ligands: promising molecular biomarkers and therapeutic targets in prostate cancer. *Biochim Biophys Acta,* 1835**,** 243-57.

LOBLAW, D. A., VIRGO, K. S., NAM, R., SOMERFIELD, M. R., BEN-JOSEF, E., MENDELSON, D. S., MIDDLETON, R., SHARP, S. A., SMITH, T. J., TALCOTT, J., TAPLIN, M., VOGELZANG, N. J., WADE, J. L., 3RD, BENNETT, C. L., SCHER, H. I. & AMERICAN SOCIETY OF CLINICAL, O. 2007. Initial hormonal management of androgen-sensitive metastatic, recurrent, or progressive prostate cancer: 2006 update of an American Society of Clinical Oncology practice guideline. *J Clin Oncol,* 25**,** 1596-605.

LOKE, T. W., SEYFI, D. & KHADRA, M. 2011. Prostate cancer incidence in Australia correlates inversely with solar radiation. *BJU Int,* 108 Suppl 2**,** 66-70.

LU, Y., ZHAI, L., ZENG, J., PENG, Q., WANG, J., DENG, Y., XIE, L., MO, C., YANG, S., LI, S. & QIN, X. 2014. Coffee consumption and prostate cancer risk: an updated meta-analysis. *Cancer Causes Control,* 25**,** 591-604.

LUKACS, B., GRANGE, J. C., COMET, D. & MC CARTHY, C. 1999. [Prospective follow-up of 3,228 patients suffering from clinical benign prostatic hyperplasia (BPH) treated for 3 years wi alfuzosin in general practice. BPH Group in General Practice]. *Progres en urologie : journal de l'Association francaise d'urologie et de la Societe francaise d'urologie,* 9**,** 271-80.

MACKLER, N. J. & PIENTA, K. J. 2005. Drug insight: Use of docetaxel in prostate and urothelial cancers. *Nature clinical practice. Urology,* 2**,** 92-100; quiz 1 p following 112.

MAES, C., CARMELIET, G. & SCHIPANI, E. 2012. Hypoxia-driven pathways in bone development, regeneration and disease. *Nat Rev Rheumatol,* 8**,** 358-66.

MAGNAN, S., ZARYCHANSKI, R., PILOTE, L., BERNIER, L., SHEMILT, M., VIGNEAULT, E., FRADET, V. & TURGEON, A. F. 2015. Intermittent vs Continuous Androgen Deprivation Therapy for Prostate Cancer: A Systematic Review and Meta-analysis. *JAMA Oncol***,** 1-10.

MAHAFFEY, C. M., DAVIES, A. M., LARA, P. N., JR., PRYDE, B., HOLLAND, W., MACK, P. C., GUMERLOCK, P. H. & GANDARA, D. R. 2007. Schedule-dependent apoptosis in K-ras mutant non-small-cell lung cancer cell lines treated with docetaxel and erlotinib: rationale for pharmacodynamic separation. *Clinical lung cancer,* 8**,** 548-53.

MAKAREWICZ, R., ZYROMSKA, A. & ANDRUSEWICZ, H. 2011. Comparative analysis of biological profiles of benign prostate hyperplasia and prostate cancer as potential diagnostic, prognostic and predictive indicators. *Folia Histochem Cytobiol,* 49**,** 452-7.

MARKS, C., PANG, G., COYNE, T. & PICTON, P. 2010. Cancer costs in Australia - the potential impact of dietary change. *In:* UNIT, A. F. A. N. M. (ed.). Canberra: Commonweath Department of Heath and Aged Care.

MARTIN, F. M., HARRIS, A. M., ROWLAND, R. G., CONNER, W., LANE, M., DURBIN, E., BARON, A. T. & KYPRIANOU, N. 2008. Decreased risk of bladder cancer in men treated with quinazoline-based alpha1-adrenoceptor antagonists. *Gene therapy & molecular biology,* 12**,** 253-258.

MASOUD, G. N. & LI, W. 2015. HIF-1alpha pathway: role, regulation and intervention for cancer therapy. *Acta Pharm Sin B,* 5**,** 378-89.

MCCONNELL, J. D., BRUSKEWITZ, R., WALSH, P., ANDRIOLE, G., LIEBER, M., HOLTGREWE, H. L., ALBERTSEN, P., ROEHRBORN, C. G., NICKEL, J. C., WANG, D. Z., TAYLOR, A. M. & WALDSTREICHER, J. 1998. The effect of finasteride on the risk of acute urinary retention and the need for surgical treatment among men with benign prostatic hyperplasia. Finasteride Long-Term

Efficacy and Safety Study Group. *The New England journal of medicine,* 338**,** 557-63.

MCCONNELL, J. D., ROEHRBORN, C. G., BAUTISTA, O. M., ANDRIOLE, G. L., JR., DIXON, C. M., KUSEK, J. W., LEPOR, H., MCVARY, K. T., NYBERG, L. M., JR., CLARKE, H. S., CRAWFORD, E. D., DIOKNO, A., FOLEY, J. P., FOSTER, H. E., JACOBS, S. C., KAPLAN, S. A., KREDER, K. J., LIEBER, M. M., LUCIA, M. S., MILLER, G. J., MENON, M., MILAM, D. F., RAMSDELL, J. W., SCHENKMAN, N. S., SLAWIN, K. M., SMITH, J. A. & MEDICAL THERAPY OF PROSTATIC SYMPTOMS RESEARCH, G. 2003. The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia. *N Engl J Med,* 349**,** 2387-98.

MCFADZEAN, I. & GIBSON, A. 2002. The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. *Br J Pharmacol,* 135**,** 1-13.

MCNEIL, J. J., DRUMMER, O. H., RAYMOND, K., CONWAY, E. L. & LOUIS, W. J. 1991. The influence of food on the oral bioavailability of terazosin. *Br J Clin Pharmacol,* 32**,** 775-6.

MELNYK, O., ZIMMERMAN, M., KIM, K. J. & SHUMAN, M. 1999. Neutralizing anti-vascular endothelial growth factor antibody inhibits further growth of established prostate cancer and metastases in a pre-clinical model. *The Journal of urology,* 161**,** 960-3.

MIAO, H., BURNETT, E., KINCH, M., SIMON, E. & WANG, B. 2000. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol,* 2**,** 62-9.

MIAO, H., LI, D. Q., MUKHERJEE, A., GUO, H., PETTY, A., CUTTER, J., BASILION, J. P., SEDOR, J., WU, J., DANIELPOUR, D., SLOAN, A. E., COHEN, M. L. & WANG, B. 2009. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer Cell,* 16**,** 9-20.

MICHEL, M. C., SCHAFERS, R. F. & GOEPEL, M. 2000. Alpha-blockers and lower urinary tract function: more than smooth muscle relaxation? *BJU international,* 86 Suppl 2**,** 23-8; discussion 28-30.

MIHAYLOVA, M. M. & SHAW, R. J. 2011. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol,* 13**,** 1016-23.

MILKOVIC, L., SIEMS, W., SIEMS, R. & ZARKOVIC, N. 2014. Oxidative stress and antioxidants in carcinogenesis and integrative therapy of cancer. *Curr Pharm Des,* 20**,** 6529-42.

MILOSEVIC, M., WARDE, P., MENARD, C., CHUNG, P., TOI, A., ISHKANIAN, A., MCLEAN, M., PINTILIE, M., SYKES, J., GOSPODAROWICZ, M., CATTON, C., HILL, R. P. & BRISTOW, R. 2012. Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. *Clin Cancer Res,* 18**,** 2108-14.

MINET, E., ARNOULD, T., MICHEL, G., ROLAND, I., MOTTET, D., RAES, M., REMACLE, J. & MICHIELS, C. 2000. ERK activation upon hypoxia: involvement in HIF-1 activation. *Febs Letters,* 468**,** 53-58.

MISHRA, A., BRAT, D. J. & VERMA, M. 2015. P53 tumor suppression network in cancer epigenetics. *Methods Mol Biol,* 1238**,** 597-605.

MITROPOULOS, D., KYROUDI-VOULGARI, A., CHRISTELLI, E., ZERVAS, A. & KARAYANNACOS, P. 2009. Terazosin treatment suppresses basic fibroblast growth factor expression in the rat ventral prostate. *Clin Invest Med,* 32**,** E1-7.

MOHAMMAD, R. M., MUQBIL, I., LOWE, L., YEDJOU, C., HSU, H. Y., LIN, L. T., SIEGELIN, M. D., FIMOGNARI, C., KUMAR, N. B., DOU, Q. P., YANG, H., SAMADI, A. K., RUSSO, G. L., SPAGNUOLO, C., RAY, S. K., CHAKRABARTI, M., MORRE, J. D., COLEY, H. M., HONOKI, K., FUJII, H., GEORGAKILAS, A. G., AMEDEI, A., NICCOLAI, E., AMIN, A., ASHRAF, S. S., HELFERICH, W. G., YANG, X., BOOSANI, C. S., GUHA, G., BHAKTA, D., CIRIOLO, M. R., AQUILANO, K., CHEN, S., MOHAMMED, S. I., KEITH, W. N., BILSLAND, A., HALICKA, D., NOWSHEEN, S. & AZMI, A. S. 2015. Broad targeting of resistance to apoptosis in cancer. *Semin Cancer Biol,* 35 Suppl**,** S78-S103.

MOHLER, J. L. 2014. Concept and viability of androgen annihilation for advanced prostate cancer. *Cancer,* 120**,** 2628-37.

MOLLER, H., ROSWALL, N., VAN HEMELRIJCK, M., LARSEN, S. B., CUZICK, J., HOLMBERG, L., OVERVAD, K. & TJONNELAND, A. 2015. Prostate cancer incidence, clinical stage and survival in relation to obesity: a prospective cohort study in Denmark. *Int J Cancer,* 136**,** 1940-7.

MONET, M., LEHEN'KYI, V., GACKIERE, F., FIRLEJ, V., VANDENBERGHE, M., ROUDBARAKI, M., GKIKA, D., POURTIER, A., BIDAUX, G., SLOMIANNY, C., DELCOURT, P., RASSENDREN, F., BERGERAT, J. P., CERALINE, J., CABON, F., HUMEZ, S. & PREVARSKAYA, N. 2010. Role of cationic channel TRPV2 in promoting prostate cancer migration and progression to androgen resistance. *Cancer Res,* 70**,** 1225-35.

MOON, S. Y., CHANG, H. W., ROH, J. L., KIM, G. C., CHOI, S. H., LEE, S. W., CHO, K. J., NAM, S. Y. & KIM, S. Y. 2009. Using YC-1 to overcome the radioresistance of hypoxic cancer cells. *Oral Oncol,* 45**,** 915-9.

MOREIRA, D. M., ARONSON, W. J., TERRIS, M. K., KANE, C. J., AMLING, C. L., COOPERBERG, M. R., BOFFETTA, P. & FREEDLAND, S. J. 2014. Cigarette smoking is associated with an increased risk of biochemical disease recurrence, metastasis, castration-resistant prostate cancer, and mortality after radical prostatectomy: results from the SEARCH database. *Cancer,* 120**,** 197-204.

MORELLI, M. B., AMANTINI, C., NABISSI, M., LIBERATI, S., CARDINALI, C., FARFARIELLO, V., TOMASSONI, D., QUAGLIA, W., PIERGENTILI, A., BONIFAZI, A., DEL BELLO, F., SANTONI, M., MAMMANA, G., SERVI, L., FILOSA, A., GISMONDI, A. & SANTONI, G. 2014. Cross-talk between alpha1D-adrenoceptors and transient receptor potential vanilloid type 1 triggers prostate cancer cell proliferation. *BMC Cancer,* 14**,** 921.

MORELLI, M. P., CASCONE, T., TROIANI, T., DE VITA, F., ORDITURA, M., LAUS, G., ECKHARDT, S. G., PEPE, S., TORTORA, G. & CIARDIELLO, F. 2005. Sequence-dependent antiproliferative effects of cytotoxic drugs and epidermal growth factor receptor inhibitors. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO,* 16 Suppl 4**,** iv61-68.

MORGAN, D. O. 1995. Principles of CDK regulation. *Nature,* 374**,** 131-4.

MORGAN, T. M., MENG, M. V., COOPERBERG, M. R., COWAN, J. E., WEINBERG, V., CARROLL, P. R. & LIN, D. W. 2014. A risk-adjusted definition of biochemical recurrence after radical prostatectomy. *Prostate Cancer Prostatic Dis,* 17**,** 174-9.

MORI, S., NADA, S., KIMURA, H., TAJIMA, S., TAKAHASHI, Y., KITAMURA, A., ONEYAMA, C. & OKADA, M. 2014. The mTOR pathway controls cell proliferation by regulating the FoxO3a transcription factor via SGK1 kinase. *PLoS One,* 9**,** e88891.

MORIKAWA, Y., KOIKE, H., SEKINE, Y., MATSUI, H., SHIBATA, Y., ITO, K. & SUZUKI, K. 2012. Rapamycin enhances docetaxel-induced cytotoxicity in a androgen-independent prostate cancer xenograft model by survivin downregulation. *Biochem Biophys Res Commun,* 419**,** 584-9.

MORISHIMA, S., SUZUKI, F., NISHIMUNE, A., YOSHIKI, H., AKINO, H., YOKOYAMA, O. & MURAMATSU, I. 2010. Visualization and tissue distribution of alpha1L-adrenoceptor in human prostate by the fluorescently labeled ligand Alexa-488-silodosin. *J Urol,* 183**,** 812-9.

MOTTET, N., BELLMUNT, J., BOLLA, M., JONIAU, S., MASON, M., MATVEEV, V., SCHMID, H. P., VAN DER KWAST, T., WIEGEL, T., ZATTONI, F. & HEIDENREICH, A. 2011. EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. *European urology,* 59**,** 572-83.

MUNARON, L., ANTONIOTTI, S. & LOVISOLO, D. 2004. Intracellular calcium signals and control of cell proliferation: how many mechanisms? *Journal of cellular and molecular medicine,* 8**,** 161-8.

MURAKAMI, A., TAKAHASHI, F., NURWIDYA, F., KOBAYASHI, I., MINAKATA, K., HASHIMOTO, M., NARA, T., KATO, M., TAJIMA, K., SHIMADA, N., IWAKAMI, S., MORIYAMA, M., MORIYAMA, H., KOIZUMI, F. & TAKAHASHI, K. 2014. Hypoxia increases gefitinib-resistant lung cancer stem cells through the activation of insulin-like growth factor 1 receptor. *PLoS One,* 9**,** e86459.

MYERS, A. L., ORR, W. S., DENBO, J. W., NG, C. Y., ZHOU, J., SPENCE, Y., WU, J. & DAVIDOFF, A. M. 2012. Rapamycin-induced tumor vasculature remodeling in rhabdomyosarcoma xenografts increases the effectiveness of adjuvant ionizing radiation. *J Pediatr Surg,* 47**,** 183-9.

NAIR, R. R., BAGHERI, M. & SAINI, D. K. 2015. Temporally distinct roles of ATM and ROS in genotoxic-stress-dependent induction and maintenance of cellular senescence. *J Cell Sci,* 128**,** 342-53.

NASU, K., MORIYAMA, N., KAWABE, K., TSUJIMOTO, G., MURAI, M., TANAKA, T. & YANO, J. 1996. Quantification and distribution of alpha 1-adrenoceptor subtype mRNAs in human prostate: comparison of benign hypertrophied tissue and non-hypertrophied tissue. *Br J Pharmacol,* 119**,** 797-803.

NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL 2013. Prostate-specific antigen (PSA) testing in asymptomatic men: Technical Report. *In:* NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL (ed.). Canberra.

NEWMAN, J. 1996. Epidemiology, diagnosis and treatment of prostate cancer. *Radiol Technol,* 68**,** 39-64; quiz 65-8.

NING, Y. M., BRAVE, M., MAHER, V. E., ZHANG, L., TANG, S., SRIDHARA, R., KIM, G., IBRAHIM, A. & PAZDUR, R. 2015. U.S. Food and Drug Administration Approval Summary: Enzalutamide for the Treatment of Patients With Chemotherapy-Naive Metastatic Castration-Resistant Prostate Cancer. *Oncologist,* 20**,** 960-6.

NING, Y. M., GULLEY, J. L., ARLEN, P. M., WOO, S., STEINBERG, S. M., WRIGHT, J. J., PARNES, H. L., TREPEL, J. B., LEE, M. J., KIM, Y. S., SUN, H., MADAN, R. A., LATHAM, L., JONES, E., CHEN, C. C., FIGG, W. D. & DAHUT, W. L. 2010. Phase II trial of bevacizumab, thalidomide, docetaxel, and prednisone in patients with metastatic castration-resistant prostate cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 28**,** 2070-6.

O'BRIEN, M. F., CRONIN, A. M., FEARN, P. A., SMITH, B., STASI, J., GUILLONNEAU, B., SCARDINO, P. T., EASTHAM, J. A., VICKERS, A. J. & LILJA, H. 2009. Pretreatment prostate-specific antigen (PSA) velocity and doubling time are associated with outcome but neither improves prediction of outcome beyond pretreatment PSA alone in patients treated with radical prostatectomy. *J Clin Oncol,* 27**,** 3591-7.

O'NEILL, A. J., PRENCIPE, M., DOWLING, C., FAN, Y., MULRANE, L., GALLAGHER, W. M., O'CONNOR, D., O'CONNOR, R., DEVERY, A., CORCORAN, C., RANI, S., O'DRISCOLL, L., FITZPATRICK, J. M. & WATSON, R. W. 2011. Characterisation and manipulation of docetaxel resistant prostate cancer cell lines. *Mol Cancer,* 10**,** 126.

OBATA, T. & YAMANAKA, Y. 1999. Prazosin attenuates hydroxyl radical generation in the rat myocardium. *Eur J Pharmacol,* 379**,** 161-6.

OHWAKI, K., ENDO, F. & HATTORI, K. 2015. Abdominal obesity, hypertension, antihypertensive medication use and biochemical recurrence of prostate cancer after radical prostatectomy. *Eur J Cancer,* 51**,** 604-9.

OJEMUYIWA, M. A., MADAN, R. A. & DAHUT, W. L. 2014. Tyrosine kinase inhibitors in the treatment of prostate cancer: taking the next step in clinical development. *Expert Opin Emerg Drugs,* 19**,** 459-70.

OU, G. F., ITASAKA, S., ZENG, L. H., SHIBUYA, K., YI, J. L., HARADA, H. & HIRAOKA, M. 2009. Usefulness of Hif-1 Imaging for Determining Optimal Timing of Combining Bevacizumab and Radiotherapy. *International Journal of Radiation Oncology Biology Physics,* 75**,** 463-467.

OU, S. H. & SHIRAI, K. 2016. Anaplastic Lymphoma Kinase (ALK) Signaling in Lung Cancer. *Adv Exp Med Biol,* 893**,** 179-87.

PACCEZ, J. D., VOGELSANG, M., PARKER, M. I. & ZERBINI, L. F. 2014. The receptor tyrosine kinase Axl in cancer: biological functions and therapeutic implications. *Int J Cancer,* 134**,** 1024-33.

PALLER, C. J. & ANTONARAKIS, E. S. 2011. Cabazitaxel: a novel second-line treatment for metastatic castration-resistant prostate cancer. *Drug design, development and therapy,* 5**,** 117-24.

PAN, S. L., GUH, J. H., HUANG, Y. W., CHERN, J. W., CHOU, J. Y. & TENG, C. M. 2003. Identification of apoptotic and antiangiogenic activities of terazosin in human prostate cancer and endothelial cells. *The Journal of urology,* 169**,** 724-9.

PAPADOPOULOS, G., VLACHODIMITROPOULOS, D., KYROUDI, A., KOULOUKOUSSA, M., PERREA, D. & MITROPOULOS, D. 2013. Terazosin treatment induces caspase-3 expression in the rat ventral prostate. *Journal of clinical medicine research,* 5**,** 127-31.

PARK, M. S., KIM, B. R., DONG, S. M., LEE, S. H., KIM, D. Y. & RHO, S. B. 2014a. The antihypertension drug doxazosin inhibits tumor growth and angiogenesis by decreasing VEGFR-2/Akt/mTOR signaling and VEGF and HIF-1alpha expression. *Oncotarget,* 5**,** 4935-44.

PARK, M. S., KIM, B. R., KANG, S., KIM, D. Y. & RHO, S. B. 2014b. The antihypertension drug doxazosin suppresses JAK/STATs phosphorylation and enhances the effects of IFN-alpha/gamma-induced apoptosis. *Genes Cancer,* 5**,** 470-9.

PARTIN, J. V., ANGLIN, I. E. & KYPRIANOU, N. 2003. Quinazoline-based alpha 1-adrenoceptor antagonists induce prostate cancer cell apoptosis via TGF-beta signalling and I kappa B alpha induction. *British journal of cancer,* 88**,** 1615-21.

PASCHOS, A., PANDYA, R., DUIVENVOORDEN, W. C. & PINTHUS, J. H. 2013. Oxidative stress in prostate cancer: changing research concepts towards a novel paradigm for prevention and therapeutics. *Prostate Cancer Prostatic Dis,* 16**,** 217-25.

PASQUALE, E. B. 2005. Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol,* 6**,** 462-75.

PAVITHRAN, N. M. & THOMPSON, C. S. Doxazosin causes cell death by autophagy (mitophagy) in hormone resistant prostate cancer cells. The Physiological Society, 2012 Edinburgh.

PEREZ, E. A. 2009. Microtubule inhibitors: Differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. *Molecular cancer therapeutics,* 8**,** 2086-95.

PETTY, A., MYSHKIN, E., QIN, H. N., GUO, H., MIAO, H., TOCHTROP, G. P., HSIEH, J. T., PAGE, P., LIU, L. L., LINDNER, D. J., ACHARYA, C., MACKERELL, A. D., FICKER, E., SONG, J. X. & WANG, B. C. 2012. A Small Molecule Agonist of EphA2 Receptor Tyrosine Kinase Inhibits Tumor Cell Migration In Vitro and Prostate Cancer Metastasis In Vivo. *Plos One,* 7.

PICKARD, R. D., SPENCER, B. H., MCFARLAND, A. J., BERNAITIS, N., DAVEY, A. K., PERKINS, A. V., CHESS-WILLIAMS, R., MCDERMOTT, C. M., FORBES, A., CHRISTIE, D. & ANOOPKUMAR-DUKIE, S. 2015. Paradoxical effects of the autophagy inhibitor 3-methyladenine on docetaxel-induced toxicity in PC-3 and LNCaP prostate cancer cells. *Naunyn Schmiedebergs Arch Pharmacol,* 388**,** 793-9.

PICUS, J. & SCHULTZ, M. 1999. Docetaxel (Taxotere) as monotherapy in the treatment of hormone-refractory prostate cancer: preliminary results. *Seminars in oncology,* 26**,** 14-8.

PIGNOT, G., SALOMON, L., LEBACLE, C., NEUZILLET, Y., LUNARDI, P., RISCHMANN, P., ZERBIB, M., CHAMPY, C., ROUPRET, M., PEYRONNET, B., VERHOEST, G., MUREZ, T., QUINTENS, H., LARRE, S., HOUEDE, N., COMPERAT, E., SOULIE, M. & PFISTER, C. 2015. Prostate cancer incidence on cystoprostatectomy specimens is directly linked to age: results from a multicentre study. *BJU Int,* 115**,** 87-93.

PLAZA-MENACHO, I., MOLOGNI, L. & MCDONALD, N. Q. 2014. Mechanisms of RET signaling in cancer: current and future implications for targeted therapy. *Cell Signal,* 26**,** 1743-52.

POTIRON, V. A., ABDERRAHMANI, R., GIANG, E., CHIAVASSA, S., DI TOMASO, E., MAIRA, S. M., PARIS, F. & SUPIOT, S. 2013. Radiosensitization of prostate cancer cells by the dual PI3K/mTOR inhibitor BEZ235 under normoxic and hypoxic conditions. *Radiother Oncol,* 106**,** 138-46.

POUND, C. R., PARTIN, A. W., EISENBERGER, M. A., CHAN, D. W., PEARSON, J. D. & WALSH, P. C. 1999. Natural history of progression after PSA elevation following radical prostatectomy. *JAMA,* 281**,** 1591-7.

PRICE, D. T., DELLA ROCCA, G., GUO, C., BALLO, M. S., SCHWINN, D. A. & LUTTRELL, L. M. 1999. Activation of extracellular signal-regulated kinase in human prostate cancer. *The Journal of urology,* 162**,** 1537-42.

PRICE, D. T., SCHWINN, D. A., LOMASNEY, J. W., ALLEN, L. F., CARON, M. G. & LEFKOWITZ, R. J. 1993. Identification, quantification, and localization of mRNA for three distinct alpha 1 adrenergic receptor subtypes in human prostate. *The Journal of urology,* 150**,** 546-51.

RADEMAKERS, S. E., SPAN, P. N., KAANDERS, J. H., SWEEP, F. C., VAN DER KOGEL, A. J. & BUSSINK, J. 2008. Molecular aspects of tumour hypoxia. *Mol Oncol,* 2**,** 41-53.

RAFFO, A. J., PERLMAN, H., CHEN, M. W., DAY, M. L., STREITMAN, J. S. & BUTTYAN, R. 1995. Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Res,* 55**,** 4438-45.

RANASINGHE, W. K., XIAO, L., KOVAC, S., CHANG, M., MICHIELS, C., BOLTON, D., SHULKES, A., BALDWIN, G. S. & PATEL, O. 2013. The role of hypoxia-inducible factor 1alpha in determining the properties of castrate-resistant prostate cancers. *PLoS One,* 8**,** e54251.

RAUTIO, J., HUMPHREYS, J. E., WEBSTER, L. O., BALAKRISHNAN, A., KEOGH, J. P., KUNTA, J. R., SERABJIT-SINGH, C. J. & POLLI, J. W. 2006. In vitro p-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. *Drug Metab Dispos,* 34**,** 786-92.

REESE, D. M., FRATESI, P., CORRY, M., NOVOTNY, W., HOLMGREN, E. & SMALL, E. J. 2001. A Phase II Trial of Humanized Anti-Vascular Endothelial Growth Factor Antibody for the Treatment of Androgen-Independent Prostate Cancer. *The Prostate Journal,* 3**,** 65-70.

RICHARD, D. E., BERRA, E. & POUYSSEGUR, J. 2000. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *J Biol Chem,* 275**,** 26765-71.

RODGER, J. C., SUPRAMANIAM, R., GIBBERD, A. J., SMITH, D. P., ARMSTRONG, B. K., DILLON, A. & O'CONNELL, D. L. 2015. Prostate cancer mortality outcomes and patterns of primary treatment for Aboriginal men in New South Wales, Australia. *BJU Int,* 115 Suppl 5**,** 16-23.

ROEHRBORN, C. G. & BLACK, L. K. 2011. The economic burden of prostate cancer. *BJU Int,* 108**,** 806-13.

ROEHRBORN, C. G., SIAMI, P., BARKIN, J., DAMIAO, R., MAJOR-WALKER, K., NANDY, I., MORRILL, B. B., GAGNIER, R. P., MONTORSI, F. & COMB, A. T. S. G. 2010. The effects of combination therapy with dutasteride and tamsulosin on clinical outcomes in men with symptomatic benign prostatic hyperplasia: 4-year results from the CombAT study. *Eur Urol,* 57**,** 123-31.

ROFSTAD, E. K., GALAPPATHI, K., MATHIESEN, B. & RUUD, E. B. 2007. Fluctuating and diffusion-limited hypoxia in hypoxia-induced metastasis. *Clin Cancer Res,* 13**,** 1971-8.

ROMANSKA, H., SALAGIERSKI, M., BRUTON, R., ABEL, P., SOSNOWSKI, M. & LALANI EL, N. 2010. Doxazosin induces apoptosis in PTEN-positive androgen-independent PC cells via inhibition of Akt activation. *Central European Journal of Urology,* 63.

RUSTHOVEN, C. G., CARLSON, J. A., WAXWEILER, T. V., YEH, N., RABEN, D., FLAIG, T. W. & KAVANAGH, B. D. 2014. The prognostic significance of Gleason scores in metastatic prostate cancer. *Urol Oncol,* 32**,** 707-13.

RYAN, C. J., SMITH, M. R., DE BONO, J. S., MOLINA, A., LOGOTHETIS, C. J., DE SOUZA, P., FIZAZI, K., MAINWARING, P., PIULATS, J. M., NG, S., CARLES, J., MULDERS, P. F., BASCH, E., SMALL, E. J., SAAD, F., SCHRIJVERS, D., VAN POPPEL, H., MUKHERJEE, S. D., SUTTMANN, H., GERRITSEN, W. R., FLAIG, T. W., GEORGE, D. J., YU, E. Y., EFSTATHIOU, E., PANTUCK, A., WINQUIST, E., HIGANO, C. S., TAPLIN, M. E., PARK, Y., KHEOH, T., GRIFFIN, T., SCHER, H. I. & RATHKOPF, D. E. 2013. Abiraterone in metastatic prostate cancer without previous chemotherapy. *The New England journal of medicine,* 368**,** 138-48.

SAAD, F. & HOTTE, S. J. 2010. Guidelines for the management of castrate-resistant prostate cancer. *Can Urol Assoc J,* 4**,** 380-4.

SAKAMOTO, S. & KYPRIANOU, N. 2010. Targeting anoikis resistance in prostate cancer metastasis. *Molecular aspects of medicine,* 31**,** 205-14.

SAKAMOTO, S., SCHWARZE, S. & KYPRIANOU, N. 2011. Anoikis disruption of focal adhesion-Akt signaling impairs renal cell carcinoma. *European urology,* 59**,** 734-44.

SAKR, W. A., GRIGNON, D. J., CRISSMAN, J. D., HEILBRUN, L. K., CASSIN, B. J., PONTES, J. J. & HAAS, G. P. 1994. High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. *In Vivo,* 8**,** 439-43.

SAKR, W. A., GRIGNON, D. J., HAAS, G. P., HEILBRUN, L. K., PONTES, J. E. & CRISSMAN, J. D. 1996. Age and racial distribution of prostatic intraepithelial neoplasia. *Eur Urol,* 30**,** 138-44.

SALERNO, D. M., TRONT, J. S., HOFFMAN, B. & LIEBERMANN, D. A. 2012. Gadd45a and Gadd45b modulate innate immune functions of granulocytes and macrophages by differential regulation of p38 and JNK signaling. *J Cell Physiol,* 227**,** 3613-20.

SANOFI. 2011. *Cabazitaxel Versus Docetaxel Both With Prednisone in Patients With Metastatic Castration Resistant Prostate Cancer (FIRSTANA)* [Online]. Available: http://clinicaltrials.gov/ct2/show/NCT01308567 [Accessed August 15 2013].

SANTELLA, L., ERCOLANO, E. & NUSCO, G. A. 2005. The cell cycle: a new entry in the field of Ca2+ signaling. *Cellular and molecular life sciences : CMLS,* 62**,** 2405-13.

SARBASSOV, D. D., ALI, S. M., SENGUPTA, S., SHEEN, J. H., HSU, P. P., BAGLEY, A. F., MARKHARD, A. L. & SABATINI, D. M. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell,* 22**,** 159-68.

SCHENK, J. M., TILL, C. A., TANGEN, C. M., GOODMAN, P. J., SONG, X., TORKKO, K. C., KRISTAL, A. R., PETERS, U. & NEUHOUSER, M. L. 2014. Serum 25-hydroxyvitamin D concentrations and risk of prostate cancer: results from the Prostate Cancer Prevention Trial. *Cancer Epidemiol Biomarkers Prev,* 23**,** 1484-93.

SCHER, H. I., BEER, T. M., HIGANO, C. S., ANAND, A., TAPLIN, M. E., EFSTATHIOU, E., RATHKOPF, D., SHELKEY, J., YU, E. Y., ALUMKAL, J., HUNG, D., HIRMAND, M., SEELY, L., MORRIS, M. J., DANILA, D. C., HUMM, J., LARSON, S., FLEISHER, M. & SAWYERS, C. L. 2010.

Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study. *Lancet,* 375**,** 1437-46.

SCHER, H. I., FIZAZI, K., SAAD, F., TAPLIN, M. E., STERNBERG, C. N., MILLER, K., DE WIT, R., MULDERS, P., CHI, K. N., SHORE, N. D., ARMSTRONG, A. J., FLAIG, T. W., FLECHON, A., MAINWARING, P., FLEMING, M., HAINSWORTH, J. D., HIRMAND, M., SELBY, B., SEELY, L. & DE BONO, J. S. 2012. Increased survival with enzalutamide in prostate cancer after chemotherapy. *The New England journal of medicine,* 367**,** 1187-97.

SCHER, H. I., HALABI, S., TANNOCK, I., MORRIS, M., STERNBERG, C. N., CARDUCCI, M. A., EISENBERGER, M. A., HIGANO, C., BUBLEY, G. J., DREICER, R., PETRYLAK, D., KANTOFF, P., BASCH, E., KELLY, W. K., FIGG, W. D., SMALL, E. J., BEER, T. M., WILDING, G., MARTIN, A. & HUSSAIN, M. 2008. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol,* 26**,** 1148-59.

SCHRADER, A. J., BOEGEMANN, M., OHLMANN, C. H., SCHNOELLER, T. J., KRABBE, L. M., HAJILI, T., JENTZMIK, F., STOECKLE, M., SCHRADER, M., HERRMANN, E. & CRONAUER, M. V. 2013. Enzalutamide in Castration-resistant Prostate Cancer Patients Progressing After Docetaxel and Abiraterone. *European urology*.

SCHRODER, F. H., HUGOSSON, J., ROOBOL, M. J., TAMMELA, T. L., CIATTO, S., NELEN, V., KWIATKOWSKI, M., LUJAN, M., LILJA, H., ZAPPA, M., DENIS, L. J., RECKER, F., BERENGUER, A., MAATTANEN, L., BANGMA, C. H., AUS, G., VILLERS, A., REBILLARD, X., VAN DER KWAST, T., BLIJENBERG, B. G., MOSS, S. M., DE KONING, H. J. & AUVINEN, A. 2009. Screening and prostate-cancer mortality in a randomized European study. *The New England journal of medicine,* 360**,** 1320-8.

SCHRODER, F. H., HUGOSSON, J., ROOBOL, M. J., TAMMELA, T. L., CIATTO, S., NELEN, V., KWIATKOWSKI, M., LUJAN, M., LILJA, H., ZAPPA, M., DENIS, L. J., RECKER, F., PAEZ, A., MAATTANEN, L., BANGMA, C. H., AUS, G., CARLSSON, S., VILLERS, A., REBILLARD, X., VAN DER KWAST, T., KUJALA, P. M., BLIJENBERG, B. G., STENMAN, U. H., HUBER, A., TAARI, K., HAKAMA, M., MOSS, S. M., DE KONING, H. J. & AUVINEN, A. 2012. Prostate-cancer mortality at 11 years of follow-up. *The New England journal of medicine,* 366**,** 981-90.

SCHWEIZER, M. T., ZHOU, X. C., WANG, H., BASSI, S., CARDUCCI, M. A., EISENBERGER, M. A. & ANTONARAKIS, E. S. 2014. The influence of prior abiraterone treatment on the clinical activity of docetaxel in men with metastatic castration-resistant prostate cancer. *Eur Urol,* 66**,** 646-52.

SEMENZA, G. L. 2003. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer,* 3**,** 721-32.

SERONT, E., BOIDOT, R., BOUZIN, C., KARROUM, O., JORDAN, B. F., GALLEZ, B., MACHIELS, J. P. & FERON, O. 2013. Tumour hypoxia determines the potential of combining mTOR and autophagy inhibitors to treat mammary tumours. *Br J Cancer,* 109**,** 2597-606.

SHAN, B., DONG, M., TANG, H., WANG, N., ZHANG, J., YAN, C., JIAO, X., ZHANG, H. & WANG, C. 2014. Voltage-gated sodium channels were

differentially expressed in human normal prostate, benign prostatic hyperplasia and prostate cancer cells. *Oncol Lett,* 8**,** 345-350.

SHAO, Y. H., KIM, S., MOORE, D. F., SHIH, W., LIN, Y., STEIN, M., KIM, I. Y. & LU-YAO, G. L. 2013. Cancer-specific Survival After Metastasis Following Primary Radical Prostatectomy Compared with Radiation Therapy in Prostate Cancer Patients: Results of a Population-based, Propensity Score-Matched Analysis. *European urology*.

SHAPIRO, A. B., FOX, K., LAM, P. & LING, V. 1999. Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. *Eur J Biochem,* 259**,** 841-50.

SHAW, L. M. 2011. The insulin receptor substrate (IRS) proteins: at the intersection of metabolism and cancer. *Cell Cycle,* 10**,** 1750-6.

SHAW, Y. J., YANG, Y. T., GARRISON, J. B., KYPRIANOU, N. & CHEN, C. S. 2004. Pharmacological exploitation of the alpha1-adrenoreceptor antagonist doxazosin to develop a novel class of antitumor agents that block intracellular protein kinase B/Akt activation. *J Med Chem,* 47**,** 4453-62.

SHELLEY, M. D., CLEVES, A., WILT, T. J. & MASON, M. D. 2011. Gemcitabine chemotherapy for the treatment of metastatic bladder carcinoma. *BJU Int,* 108**,** 168-79.

SHEN, H. M. & CODOGNO, P. 2011. Autophagic cell death: Loch Ness monster or endangered species? *Autophagy,* 7**,** 457-65.

SHEN, S., KEPP, O. & KROEMER, G. 2012. The end of autophagic cell death? *Autophagy,* 8**,** 1-3.

SHI, Y., HAN, J. J., TENNAKOON, J. B., MEHTA, F. F., MERCHANT, F. A., BURNS, A. R., HOWE, M. K., MCDONNELL, D. P. & FRIGO, D. E. 2013. Androgens promote prostate cancer cell growth through induction of autophagy. *Molecular endocrinology,* 27**,** 280-95.

SHIMIZU, S., YOSHIDA, T., TSUJIOKA, M. & ARAKAWA, S. 2014. Autophagic cell death and cancer. *Int J Mol Sci,* 15**,** 3145-53.

SHIN, S., JING, K., JEONG, S., KIM, N., SONG, K. S., HEO, J. Y., PARK, J. H., SEO, K. S., HAN, J., PARK, J. I., KWEON, G. R., PARK, S. K., WU, T., HWANG, B. D. & LIM, K. 2013. The omega-3 polyunsaturated fatty acid DHA induces simultaneous apoptosis and autophagy via mitochondrial ROS-mediated Akt-mTOR signaling in prostate cancer cells expressing mutant p53. *Biomed Res Int,* 2013**,** 568671.

SHIRAKAWA, K., TAKARA, K., TANIGAWARA, Y., AOYAMA, N., KASUGA, M., KOMADA, F., SAKAEDA, T. & OKUMURA, K. 1999. Interaction of docetaxel ("Taxotere") with human P-glycoprotein. *Jpn J Cancer Res,* 90**,** 1380-6.

SHOJAEI, F. 2012. Anti-angiogenesis therapy in cancer: current challenges and future perspectives. *Cancer letters,* 320**,** 130-7.

SHORT, J. D., HOUSTON, K. D., DERE, R., CAI, S. L., KIM, J., JOHNSON, C. L., BROADDUS, R. R., SHEN, J., MIYAMOTO, S., TAMANOI, F., KWIATKOWSKI, D., MILLS, G. B. & WALKER, C. L. 2008. AMP-activated protein kinase signaling results in cytoplasmic sequestration of p27. *Cancer Res,* 68**,** 6496-506.

SIDDIQUI, E. J., SHABBIR, M., THOMPSON, C. S., MUMTAZ, F. H. & MIKHAILIDIS, D. P. 2005. Growth inhibitory effect of doxazosin on prostate and bladder cancer cells. Is the serotonin receptor pathway involved? *Anticancer Res,* 25**,** 4281-6.

SIEVERT, K. D., AMEND, B., NAGELE, U., SCHILLING, D., BEDKE, J., HORSTMANN, M., HENNENLOTTER, J., KRUCK, S. & STENZL, A. 2009. Economic aspects of bladder cancer: what are the benefits and costs? *World J Urol,* 27**,** 295-300.

SMALL, E. J. & SRINIVAS, S. 1995. The antiandrogen withdrawal syndrome. Experience in a large cohort of unselected patients with advanced prostate cancer. *Cancer,* 76**,** 1428-34.

SONDERS, R. C. 1986. Pharmacokinetics of terazosin. *The American journal of medicine,* 80**,** 20-4.

STAUDACHER, I., JEHLE, J., STAUDACHER, K., PLEDL, H. W., LEMKE, D., SCHWEIZER, P. A., BECKER, R., KATUS, H. A. & THOMAS, D. 2014. HERG K+ channel-dependent apoptosis and cell cycle arrest in human glioblastoma cells. *PLoS One,* 9**,** e88164.

STEINKAMP, M. P., O'MAHONY, O. A., BROGLEY, M., REHMAN, H., LAPENSEE, E. W., DHANASEKARAN, S., HOFER, M. D., KUEFER, R., CHINNAIYAN, A., RUBIN, M. A., PIENTA, K. J. & ROBINS, D. M. 2009. Treatment-dependent androgen receptor mutations in prostate cancer exploit multiple mechanisms to evade therapy. *Cancer research,* 69**,** 4434-42.

STEPHENS, L., ANDERSON, K., STOKOE, D., ERDJUMENT-BROMAGE, H., PAINTER, G. F., HOLMES, A. B., GAFFNEY, P. R., REESE, C. B., MCCORMICK, F., TEMPST, P., COADWELL, J. & HAWKINS, P. T. 1998. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science,* 279**,** 710-4.

STEPHENSON, A. J., KATTAN, M. W., EASTHAM, J. A., DOTAN, Z. A., BIANCO, F. J., JR., LILJA, H. & SCARDINO, P. T. 2006. Defining biochemical recurrence of prostate cancer after radical prostatectomy: a proposal for a standardized definition. *J Clin Oncol,* 24**,** 3973-8.

SUN, S., SPRENGER, C. C., VESSELLA, R. L., HAUGK, K., SORIANO, K., MOSTAGHEL, E. A., PAGE, S. T., COLEMAN, I. M., NGUYEN, H. M., SUN, H., NELSON, P. S. & PLYMATE, S. R. 2010. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *The Journal of clinical investigation,* 120**,** 2715-30.

SUN, W., WANG, T., SHI, F., WANG, J., WANG, J., HUI, B., ZHANG, Y., LU, J., CHEN, H. & LIU, Z. 2015a. Randomized phase III trial of radiotherapy or chemoradiotherapy with topotecan and cisplatin in intermediate-risk cervical cancer patients after radical hysterectomy. *BMC Cancer,* 15**,** 353.

SUN, Y., HUANG, J. & YANG, Z. 2015b. The roles of ADAMTS in angiogenesis and cancer. *Tumour Biol,* 36**,** 4039-51.

SUZMAN, D. L., LUBER, B., SCHWEIZER, M. T., NADAL, R. & ANTONARAKIS, E. S. 2014. Clinical activity of enzalutamide versus docetaxel in men with castration-resistant prostate cancer progressing after abiraterone. *Prostate,* 74**,** 1278-85.

SWANSON, G. P., GOLDMAN, B., TANGEN, C. M., CHIN, J., MESSING, E., CANBY-HAGINO, E., FORMAN, J. D., THOMPSON, I. M. & CRAWFORD, E. D. 2008. The prognostic impact of seminal vesicle involvement found at prostatectomy and the effects of adjuvant radiation: data from Southwest Oncology Group 8794. *J Urol,* 180**,** 2453-7; discussion 2458.

TAHMATZOPOULOS, A., LAGRANGE, C. A., ZENG, L., MITCHELL, B. L., CONNER, W. T. & KYPRIANOU, N. 2005. Effect of terazosin on tissue

vascularity and apoptosis in transitional cell carcinoma of bladder. *Urology,* 65**,** 1019-23.

TAKARA, K., SAKAEDA, T., KAKUMOTO, M., TANIGAWARA, Y., KOBAYASHI, H., OKUMURA, K., OHNISHI, N. & YOKOYAMA, T. 2009a. Effects of alpha-Adrenoceptor Antagonist Doxazosin on MDR1-Mediated Multidrug Resistance and Transcellular Transport. *Oncology Research,* 17**,** 527-533.

TAKARA, K., SAKAEDA, T., KAKUMOTO, M., TANIGAWARA, Y., KOBAYASHI, H., OKUMURA, K., OHNISHI, N. & YOKOYAMA, T. 2009b. Effects of alpha-adrenoceptor antagonist doxazosin on MDR1-mediated multidrug resistance and transcellular transport. *Oncol Res,* 17**,** 527-33.

TAKARA, K., YAMAMOTO, K., MATSUBARA, M., MINEGAKI, T., TAKAHASHI, M., YOKOYAMA, T. & OKUMURA, K. 2012. Effects of alpha-adrenoceptor antagonists on ABCG2/BCRP-mediated resistance and transport. *PLoS One,* 7**,** e30697.

TANNOCK, I. F., DE WIT, R., BERRY, W. R., HORTI, J., PLUZANSKA, A., CHI, K. N., OUDARD, S., THEODORE, C., JAMES, N. D., TURESSON, I., ROSENTHAL, M. A. & EISENBERGER, M. A. 2004. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *The New England journal of medicine,* 351**,** 1502-12.

TAPLIN, M. E. 2003. Biochemical (Prostate-Specific Antigen) Relapse: An Oncologist's Perspective. *Rev Urol,* 5 Suppl 2**,** S3-s13.

TAYLOR, R. C., CULLEN, S. P. & MARTIN, S. J. 2008. Apoptosis: controlled demolition at the cellular level. *Nature reviews. Molecular cell biology,* 9**,** 231-41.

TEBAR, F., LLADO, A. & ENRICH, C. 2002. Role of calmodulin in the modulation of the MAPK signalling pathway and the transactivation of epidermal growth factor receptor mediated by PKC. *FEBS letters,* 517**,** 206-10.

TELLIER, C., DESMET, D., PETIT, L., FINET, L., GRAUX, C., RAES, M., FERON, O. & MICHIELS, C. 2015. Cycling hypoxia induces a specific amplified inflammatory phenotype in endothelial cells and enhances tumor-promoting inflammation in vivo. *Neoplasia,* 17**,** 66-78.

THEBAULT, S., FLOURAKIS, M., VANOVERBERGHE, K., VANDERMOERE, F., ROUDBARAKI, M., LEHEN'KYI, V., SLOMIANNY, C., BECK, B., MARIOT, P., BONNAL, J. L., MAUROY, B., SHUBA, Y., CAPIOD, T., SKRYMA, R. & PREVARSKAYA, N. 2006. Differential role of transient receptor potential channels in Ca2+ entry and proliferation of prostate cancer epithelial cells. *Cancer research,* 66**,** 2038-47.

THEBAULT, S., ROUDBARAKI, M., SYDORENKO, V., SHUBA, Y., LEMONNIER, L., SLOMIANNY, C., DEWAILLY, E., BONNAL, J. L., MAUROY, B., SKRYMA, R. & PREVARSKAYA, N. 2003. Alpha1-adrenergic receptors activate Ca(2+)-permeable cationic channels in prostate cancer epithelial cells. *The Journal of clinical investigation,* 111**,** 1691-701.

THIELE, C. J., LI, Z. & MCKEE, A. E. 2009. On Trk--the TrkB signal transduction pathway is an increasingly important target in cancer biology. *Clin Cancer Res,* 15**,** 5962-7.

THOMAS, D., BLOEHS, R., KOSCHNY, R., FICKER, E., SYKORA, J., KIEHN, J., SCHLOMER, K., GIERTEN, J., KATHOFER, S., ZITRON, E., SCHOLZ, E. P., KIESECKER, C., KATUS, H. A. & KARLE, C. A. 2008. Doxazosin induces

apoptosis of cells expressing hERG K+ channels. *Eur J Pharmacol,* 579**,** 98-103.
THOMAS, D., WIMMER, A. B., WU, K., HAMMERLING, B. C., FICKER, E. K., KURYSHEV, Y. A., KIEHN, J., KATUS, H. A., SCHOELS, W. & KARLE, C. A. 2004. Inhibition of human ether-a-go-go-related gene potassium channels by alpha 1-adrenoceptor antagonists prazosin, doxazosin, and terazosin. *Naunyn Schmiedebergs Arch Pharmacol,* 369**,** 462-72.
THOMPSON, I. M. 2001. Flare Associated with LHRH-Agonist Therapy. *Rev Urol,* 3 Suppl 3**,** S10-4.
THOMPSON, I. M., VALICENTI, R. K., ALBERTSEN, P., DAVIS, B. J., GOLDENBERG, S. L., HAHN, C., KLEIN, E., MICHALSKI, J., ROACH, M., SARTOR, O., WOLF, J. S., JR. & FARADAY, M. M. 2013. Adjuvant and salvage radiotherapy after prostatectomy: AUA/ASTRO Guideline. *J Urol,* 190**,** 441-9.
THOTA, B., ARIMAPPAMAGAN, A., KANDAVEL, T., SHASTRY, A. H., PANDEY, P., CHANDRAMOULI, B. A., HEGDE, A. S., KONDAIAH, P. & SANTOSH, V. 2014. STAT-1 expression is regulated by IGFBP-3 in malignant glioma cells and is a strong predictor of poor survival in patients with glioblastoma. *J Neurosurg,* 121**,** 374-83.
TILLEY, W. D., BUCHANAN, G., HICKEY, T. E. & BENTEL, J. M. 1996. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 2**,** 277-85.
TOMBAL, B., BORRE, M., RATHENBORG, P., WERBROUCK, P., VAN POPPEL, H., HEIDENREICH, A., IVERSEN, P., BRAECKMAN, J., HERACEK, J., BASKIN-BEY, E., OUATAS, T., PERABO, F., PHUNG, D., HIRMAND, M. & SMITH, M. R. 2014. Enzalutamide monotherapy in hormone-naive prostate cancer: primary analysis of an open-label, single-arm, phase 2 study. *Lancet Oncol,* 15**,** 592-600.
TOUAT, M., ILEANA, E., POSTEL-VINAY, S., ANDRE, F. & SORIA, J. C. 2015. Targeting FGFR Signaling in Cancer. *Clin Cancer Res,* 21**,** 2684-94.
TOURNIER, C. 2013. The 2 Faces of JNK Signaling in Cancer. *Genes Cancer,* 4**,** 397-400.
TRAN, C., OUK, S., CLEGG, N. J., CHEN, Y., WATSON, P. A., ARORA, V., WONGVIPAT, J., SMITH-JONES, P. M., YOO, D., KWON, A., WASIELEWSKA, T., WELSBIE, D., CHEN, C. D., HIGANO, C. S., BEER, T. M., HUNG, D. T., SCHER, H. I., JUNG, M. E. & SAWYERS, C. L. 2009. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science,* 324**,** 787-90.
TREINS, C., GIORGETTI-PERALDI, S., MURDACA, J., SEMENZA, G. L. & VAN OBBERGHEN, E. 2002. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *Journal of Biological Chemistry,* 277**,** 27975-27981.
TROCK, B. J., HAN, M., FREEDLAND, S. J., HUMPHREYS, E. B., DEWEESE, T. L., PARTIN, A. W. & WALSH, P. C. 2008. Prostate cancer-specific survival following salvage radiotherapy vs observation in men with biochemical recurrence after radical prostatectomy. *JAMA,* 299**,** 2760-9.
TSENG-CRANK, J., KOST, T., GOETZ, A., HAZUM, S., ROBERSON, K. M., HAIZLIP, J., GODINOT, N., ROBERTSON, C. N. & SAUSSY, D. 1995. The

alpha 1C-adrenoceptor in human prostate: cloning, functional expression, and localization to specific prostatic cell types. *Br J Pharmacol,* 115**,** 1475-85.
TURKERI, L. N., OZYUREK, M., ERSEV, D. & AKDAS, A. 2001. Apoptotic regression of prostatic tissue induced by short-term doxazosin treatment in benign prostatic hyperplasia. *Arch Esp Urol,* 54**,** 191-6.
U.S. NATIONAL LIBRARY OF MEDICINE. 2012. *How does the prostate work?* [Online]. PubMed Health,. Available: http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0072475/ 2016].
VAN BOKHOVEN, A., VARELLA-GARCIA, M., KORCH, C., JOHANNES, W. U., SMITH, E. E., MILLER, H. L., NORDEEN, S. K., MILLER, G. J. & LUCIA, M. S. 2003. Molecular characterization of human prostate carcinoma cell lines. *Prostate,* 57**,** 205-25.
VARKARIS, A., KATSIAMPOURA, A. D., ARAUJO, J. C., GALLICK, G. E. & CORN, P. G. 2014. Src signaling pathways in prostate cancer. *Cancer Metastasis Rev,* 33**,** 595-606.
VIHANTO, M. M., PLOCK, J., ERNI, D., FREY, B. M., FREY, F. J. & HUYNH-DO, U. 2005. Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin. *FASEB J,* 19**,** 1689-91.
VINCENT, J., ELLIOTT, H. L., MEREDITH, P. A. & REID, J. L. 1983. Doxazosin, an alpha 1-adrenoceptor antagonist: pharmacokinetics and concentration-effect relationships in man. *British journal of clinical pharmacology,* 15**,** 719-25.
VRIGNAUD, P., SEMIOND, D., LEJEUNE, P., BOUCHARD, H., CALVET, L., COMBEAU, C., RIOU, J. F., COMMERCON, A., LAVELLE, F. & BISSERY, M. C. 2013. Preclinical antitumor activity of cabazitaxel, a semisynthetic taxane active in taxane-resistant tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 19**,** 2973-83.
WALDEN, P. D., GERARDI, C. & LEPOR, H. 1999. Localization and expression of the alpha1A-1, alpha1B and alpha1D-adrenoceptors in hyperplastic and non-hyperplastic human prostate. *J Urol,* 161**,** 635-40.
WALDEN, P. D., GLOBINA, Y. & NIEDER, A. 2004. Induction of anoikis by doxazosin in prostate cancer cells is associated with activation of caspase-3 and a reduction of focal adhesion kinase. *Urological research,* 32**,** 261-5.
WALKER-DANIELS, J., COFFMAN, K., AZIMI, M., RHIM, J. S., BOSTWICK, D. G., SNYDER, P., KERNS, B. J., WATERS, D. J. & KINCH, M. S. 1999. Overexpression of the EphA2 tyrosine kinase in prostate cancer. *Prostate,* 41**,** 275-80.
WANG, Q., LI, W., ZHANG, Y., YUAN, X., XU, K., YU, J., CHEN, Z., BEROUKHIM, R., WANG, H., LUPIEN, M., WU, T., REGAN, M. M., MEYER, C. A., CARROLL, J. S., MANRAI, A. K., JANNE, O. A., BALK, S. P., MEHRA, R., HAN, B., CHINNAIYAN, A. M., RUBIN, M. A., TRUE, L., FIORENTINO, M., FIORE, C., LODA, M., KANTOFF, P. W., LIU, X. S. & BROWN, M. 2009. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell,* 138**,** 245-56.
WARD, J. F., SLEZAK, J. M., BLUTE, M. L., BERGSTRALH, E. J. & ZINCKE, H. 2005. Radical prostatectomy for clinically advanced (cT3) prostate cancer since the advent of prostate-specific antigen testing: 15-year outcome. *BJU Int,* 95**,** 751-6.
WATANABE, R., NAKAMURA, H., MASUTANI, H. & YODOI, J. 2010. Anti-oxidative, anti-cancer and anti-inflammatory actions by thioredoxin 1 and thioredoxin-binding protein-2. *Pharmacol Ther,* 127**,** 261-70.

WEBBER, M. M., TRAKUL, N., THRAVES, P. S., BELLO-DEOCAMPO, D., CHU, W. W., STORTO, P. D., HUARD, T. K., RHIM, J. S. & WILLIAMS, D. E. 1999. A human prostatic stromal myofibroblast cell line WPMY-1: a model for stromal-epithelial interactions in prostatic neoplasia. *Carcinogenesis,* 20**,** 1185-92.

WEBER, D. C., TILLE, J. C., COMBESCURE, C., EGGER, J. F., LAOUITI, M., HAMMAD, K., GRANGER, P., RUBBIA-BRANDT, L. & MIRALBELL, R. 2012. The prognostic value of expression of HIF1alpha, EGFR and VEGF-A, in localized prostate cancer for intermediate- and high-risk patients treated with radiation therapy with or without androgen deprivation therapy. *Radiat Oncol,* 7**,** 66.

WEI, P., ZHANG, Z. H., LI, L., DU, X. L., SHAN, C. P. & SHENG, X. G. 2015. Clinical observational study of conformal radiotherapy combined with topotecan chemotherapy in patients with platinum-resistant recurrent ovarian cancer. *Genet Mol Res,* 14**,** 3833-42.

WESTIN, P., STATTIN, P., DAMBER, J. E. & BERGH, A. 1995. Castration therapy rapidly induces apoptosis in a minority and decreases cell proliferation in a majority of human prostatic tumors. *The American journal of pathology,* 146**,** 1368-75.

WHITE, C. W., XIE, J. H. & VENTURA, S. 2013. Age-related changes in the innervation of the prostate gland: implications for prostate cancer initiation and progression. *Organogenesis,* 9**,** 206-15.

WIDMARK, A., KLEPP, O., SOLBERG, A., DAMBER, J. E., ANGELSEN, A., FRANSSON, P., LUND, J. A., TASDEMIR, I., HOYER, M., WIKLUND, F., FOSSA, S. D., SCANDINAVIAN PROSTATE CANCER GROUP, S. & SWEDISH ASSOCIATION FOR UROLOGICAL, O. 2009. Endocrine treatment, with or without radiotherapy, in locally advanced prostate cancer (SPCG-7/SFUO-3): an open randomised phase III trial. *Lancet,* 373**,** 301-8.

WIEGEL, T., BOTTKE, D., STEINER, U., SIEGMANN, A., GOLZ, R., STORKEL, S., WILLICH, N., SEMJONOW, A., SOUCHON, R., STOCKLE, M., RUBE, C., WEISSBACH, L., ALTHAUS, P., REBMANN, U., KALBLE, T., FELDMANN, H. J., WIRTH, M., HINKE, A., HINKELBEIN, W. & MILLER, K. 2009. Phase III postoperative adjuvant radiotherapy after radical prostatectomy compared with radical prostatectomy alone in pT3 prostate cancer with postoperative undetectable prostate-specific antigen: ARO 96-02/AUO AP 09/95. *J Clin Oncol,* 27**,** 2924-30.

WILLIAMS, P. D., LEE, J. K. & THEODORESCU, D. 2008. Molecular credentialing of rodent bladder carcinogenesis models. *Neoplasia,* 10**,** 838-46.

WITTEKIND, C., COMPTON, C. C., GREENE, F. L. & SOBIN, L. H. 2002. TNM residual tumor classification revisited. *Cancer,* 94**,** 2511-6.

WONG, P. F. & ABUBAKAR, S. 2008. High intracellular Zn2+ ions modulate the VHR, ZAP-70 and ERK activities of LNCaP prostate cancer cells. *Cell Mol Biol Lett,* 13**,** 375-90.

WOO, H. H., GILLMAN, M. P., GARDINER, R., MARSHALL, V. & LYNCH, W. J. 2011. A practical approach to the management of lower urinary tract symptoms among men. *Med J Aust,* 195**,** 34-9.

WU, J. & YU, E. 2014. Insulin-like growth factor receptor-1 (IGF-IR) as a target for prostate cancer therapy. *Cancer Metastasis Rev,* 33**,** 607-17.

WU, Y. T., TAN, H. L., SHUI, G., BAUVY, C., HUANG, Q., WENK, M. R., ONG, C. N., CODOGNO, P. & SHEN, H. M. 2010a. Dual role of 3-methyladenine in

modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *J Biol Chem,* 285**,** 10850-61.
WU, Z., CHANG, P. C., YANG, J. C., CHU, C. Y., WANG, L. Y., CHEN, N. T., MA, A. H., DESAI, S. J., LO, S. H., EVANS, C. P., LAM, K. S. & KUNG, H. J. 2010b. Autophagy Blockade Sensitizes Prostate Cancer Cells towards Src Family Kinase Inhibitors. *Genes & cancer,* 1**,** 40-9.
XIANG, B., LI, X. X. & ZHANG, F. Y. 2013. Underlying protective mechanism of alpha1-adrenoceptor activation against irradiation-induced damage in rat submandibular gland. *Arch Oral Biol,* 58**,** 1238-45.
XU, J. M., AZZARITI, A., COLUCCI, G. & PARADISO, A. 2003a. The effect of gefitinib (Iressa, ZD1839) in combination with oxaliplatin is schedule-dependent in colon cancer cell lines. *Cancer chemotherapy and pharmacology,* 52**,** 442-8.
XU, K., WANG, X., LING, P. M., TSAO, S. W. & WONG, Y. C. 2003b. The alpha1-adrenoceptor antagonist terazosin induces prostate cancer cell death through a p53 and Rb independent pathway. *Oncol Rep,* 10**,** 1555-60.
XU, W. & ZHOU, M. 2014. A concise update on prostate pathology. *Cesk Patol,* 50**,** 120-8.
YAMADA, D., NISHIMATSU, H., KUMANO, S., HIRANO, Y., SUZUKI, M., FUJIMURA, T., FUKUHARA, H., ENOMOTO, Y., KUME, H. & HOMMA, Y. 2013. Reduction of prostate cancer incidence by naftopidil, an alpha1 - adrenoceptor antagonist and transforming growth factor-beta signaling inhibitor. *Int J Urol,* 20**,** 1220-7.
YANG, C. C., LIN, H. P., CHEN, C. S., YANG, Y. T., TSENG, P. H., RANGNEKAR, V. M. & CHEN, C. S. 2003. Bcl-xL mediates a survival mechanism independent of the phosphoinositide 3-kinase/Akt pathway in prostate cancer cells. *J Biol Chem,* 278**,** 25872-8.
YANG, G., TIMME, T. L., PARK, S. H., WU, X., WYLLIE, M. G. & THOMPSON, T. C. 1997. Transforming growth factor beta 1 transduced mouse prostate reconstitutions: II. Induction of apoptosis by doxazosin. *The Prostate,* 33**,** 157-63.
YANG, Y. F., WU, C. C., CHEN, W. P., CHEN, Y. L. & SU, M. J. 2011a. Prazosin induces p53-mediated autophagic cell death in H9C2 cells. *Naunyn Schmiedebergs Arch Pharmacol,* 384**,** 209-16.
YANG, Y. F., WU, C. C., CHEN, W. P., CHEN, Y. L. & SU, M. J. 2011b. Prazosin induces p53-mediated autophagic cell death in H9C2 cells. *Naunyn-Schmiedeberg's archives of pharmacology,* 384**,** 209-16.
YANG, Y. F., WU, C. C., CHEN, W. P. & SU, M. J. 2009. Transforming growth factor-beta type I receptor/ALK5 contributes to doxazosin-induced apoptosis in H9C2 cells. *Naunyn Schmiedebergs Arch Pharmacol,* 380**,** 561-7.
YAO, H. P., ZHOU, Y. Q., ZHANG, R. & WANG, M. H. 2013. MSP-RON signalling in cancer: pathogenesis and therapeutic potential. *Nat Rev Cancer,* 13**,** 466-81.
YARDEN, Y. 2001. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *European journal of cancer,* 37 Suppl 4**,** S3-8.
YOKOYAMA, T., WATANABE, T., SAIKA, T., NASU, Y., KUMON, H., MIYAJI, Y. & NAGAI, A. 2007. Natural course of lower urinary tract symptoms following discontinuation of alpha-1-adrenergic blockers in patients with benign prostatic hyperplasia. *International journal of urology : official journal of the Japanese Urological Association,* 14**,** 598-601.

YOSHIMURA, M., ITASAKA, S., HARADA, H. & HIRAOKA, M. 2013. Microenvironment and Radiation Therapy. *Biomed Research International*.
YUAN, X. & BALK, S. P. 2009. Mechanisms mediating androgen receptor reactivation after castration. *Urologic oncology,* 27**,** 36-41.
ZELEFSKY, M. J., GINOR, R. X., FUKS, Z. & LEIBEL, S. A. 1999. Efficacy of selective alpha-1 blocker therapy in the treatment of acute urinary symptoms during radiotherapy for localized prostate cancer. *Int J Radiat Oncol Biol Phys,* 45**,** 567-70.
ZELIVIANSKI, S., SPELLMAN, M., KELLERMAN, M., KAKITELASHVILLI, V., ZHOU, X. W., LUGO, E., LEE, M. S., TAYLOR, R., DAVIS, T. L., HAUKE, R. & LIN, M. F. 2003. ERK inhibitor PD98059 enhances docetaxel-induced apoptosis of androgen-independent human prostate cancer cells. *International journal of cancer. Journal international du cancer,* 107**,** 478-85.
ZHANG, N., LI, D., SHAO, J. & WANG, X. 2015a. Animal models for bladder cancer: The model establishment and evaluation (Review). *Oncol Lett,* 9**,** 1515-1519.
ZHANG, W., MENG, Y., LIU, N., WEN, X. F. & YANG, T. 2015b. Insights into Chemoresistance of Prostate Cancer. *Int J Biol Sci,* 11**,** 1160-70.
ZHANG, X., MORRISSEY, C., SUN, S., KETCHANDJI, M., NELSON, P. S., TRUE, L. D., VAKAR-LOPEZ, F., VESSELLA, R. L. & PLYMATE, S. R. 2011. Androgen receptor variants occur frequently in castration resistant prostate cancer metastases. *PloS one,* 6**,** e27970.
ZHANG, X. H., CHEN, S. Y., TANG, L., SHEN, Y. Z., LUO, L., XU, C. W., LIU, Q. & LI, D. 2013. Myricetin induces apoptosis in HepG2 cells through Akt/p70S6K/bad signaling and mitochondrial apoptotic pathway. *Anticancer Agents Med Chem,* 13**,** 1575-81.
ZHAO, H., CHEN, M. S., LO, Y. H., WALTZ, S. E., WANG, J., HO, P. C., VASILIAUSKAS, J., PLATTNER, R., WANG, Y. L. & WANG, S. C. 2014. The Ron receptor tyrosine kinase activates c-Abl to promote cell proliferation through tyrosine phosphorylation of PCNA in breast cancer. *Oncogene,* 33**,** 1429-37.
ZHAO, X., LIU, Y., XU, J., ZHANG, D., ZHOU, Y., GU, J. & CUI, Y. 2009. Determination of silodosin in human plasma by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci,* 877**,** 3724-8.
ZHONG, H., CHILES, K., FELDSER, D., LAUGHNER, E., HANRAHAN, C., GEORGESCU, M. M., SIMONS, J. W. & SEMENZA, G. L. 2000. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res,* 60**,** 1541-5.
ZHOU, Z. W., LI, X. X., HE, Z. X., PAN, S. T., YANG, Y., ZHANG, X., CHOW, K., YANG, T., QIU, J. X., ZHOU, Q., TAN, J., WANG, D. & ZHOU, S. F. 2015. Induction of apoptosis and autophagy via sirtuin1- and PI3K/Akt/mTOR-mediated pathways by plumbagin in human prostate cancer cells. *Drug Des Devel Ther,* 9**,** 1511-54.
ZHU, M. L., HORBINSKI, C. M., GARZOTTO, M., QIAN, D. Z., BEER, T. M. & KYPRIANOU, N. 2010. Tubulin-targeting chemotherapy impairs androgen receptor activity in prostate cancer. *Cancer research,* 70**,** 7992-8002.
ZUMSTEG, Z. S., SPRATT, D. E., ROMESSER, P. B., PEI, X., ZHANG, Z., POLKINGHORN, W., MCBRIDE, S., KOLLMEIER, M., YAMADA, Y. &

ZELEFSKY, M. J. 2015. The natural history and predictors of outcome following biochemical relapse in the dose escalation era for prostate cancer patients undergoing definitive external beam radiotherapy. *Eur Urol,* 67**,** 1009-16.

ZUNDEL, W., SCHINDLER, C., HAAS-KOGAN, D., KOONG, A., KAPER, F., CHEN, E., GOTTSCHALK, A. R., RYAN, H. E., JOHNSON, R. S., JEFFERSON, A. B., STOKOE, D. & GIACCIA, A. J. 2000. Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev,* 14**,** 391-6.