Pre-clinical targeting of enzalutamide-resistant prostate cancer

by

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M.D. Queen's University, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

Doctor of Philosophy

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2016

Abstract

Prostate cancer causes morbidity and mortality for thousands of Canadian men each year. Castration remains the primary treatment for recurrent or metastatic prostate cancer. However, castration is never curative, and the cancer inevitably recurs as castration resistant prostate cancer (CRPC). Newer androgen receptor (AR) antagonists such as enzalutamide(ENZ) demonstrate significant benefit in CRPC patients, but these agents remain non-curative. Therefore, the goal of this thesis was to explore novel strategies to target ENZ-resistant prostate cancer using previously developed models of resistance. Our models suggest that similar resistance patterns to CRPC may be found in ENZ-resistance, including the upregulation of steroidogenesis and activation of survival pathways such as the PI3K/Akt pathway. Unlike inhibition of the AR pathway, we found that inhibition of different nodes of the PI3K/Akt pathway had limited efficacy as monotherapy and we therefore focused on combination strategies to target this prominent survival pathway. We found that combined Akt and MEK pathway inhibition demonstrates only moderate synergy in AR-positive models of prostate cancer, and this did not appear significantly greater in ENZ-resistant than ENZ-sensitive prostate cancer. However, we did find that blockade of Akt signaling in combination with ENZ significantly delays the development of resistance to ENZ through a very significant induction of apoptosis and cell cycle arrest. Further, co-targeting of the Akt and AR pathways appears more effective at earlier stages of prostate cancer progression, when the tumours are still castrate-sensitive. Finally, to further evaluate the combination of PI3K/Akt pathway and AR blockade, we investigated the pre-clinical rationale for the use of bromodomain inhibitors, which indirectly targets both the AR and myc transcription factors. Upregulation of myc was observed following PI3K/Akt inhibition, but overall our studies did not support the combination of bromodomain inhibitors in combination with PI3K/Akt inhibition in prostate cancer models. Taken together, our pre-clinical results highlight several treatment strategies and pitfalls in targeting ENZ-resistant prostate cancer. These studies enhance our understanding of therapeutic approaches to target resistant prostate cancer with several novel agents and combination strategies. Further evaluation in clinical trials is warranted and ongoing.

Preface

All of the work presented henceforth was completed at the Vancouver Prostate Centre, affiliated with Vancouver Coastal Health Research Institute and the University of British Columbia. All animal experimentation was carried out in accordance with Canadian Council on Animal Care guidelines and with approval of the Animal Care Committee of the University of British Columbia (protocol # A12-0210).

Figures 1.3, 1.4, 1.5 and Table 1.2 and Table 1.3 in Chapter 1 are used with permission. Portions of the introductory text are used with permission from review articles of which I am an author as follows: Toren P, Zoubeidi A. Targeting the PI3K/Akt pathway in prostate cancer: challenges and opportunities (review). Int J Oncol. 2014 Nov;45(5):1793-801.PubMed PMID: 25120209 and Toren PJ, Gleave ME. Novel non-AR therapeutic targets in castrate resistant prostate cancer. Toren PJ, Gleave ME. Novel non-AR therapeutic targets in castrate resistant prostate cancer. Transl Androl Urol . 2013;2(3):265-77.

A version of chapter 2 has been published in Molecular Cancer Therapeutics as follows: Toren P, Kim S, Pham S, Mangalji A, Adomat H, Tomlinson Guns ES, Zoubeidi A, Moore W, Gleave M. Anti-cancer activity of a novel CYP17A1 inhibitor in pre-clinical models of castrate and enzalutamide resistant prostate cancer. Mol Cancer Ther. 2015 Jan;14(1):59-69. PMID 25351916. Dr Martin Gleave was the principal investigator. I designed the studies with input from Dr Amina Zoubeidi, Dr William Moore and Dr Martin Gleave. I also performed *in vitro* and *in vivo* experiments with assistance for qRT-PCR experiments and western blots from Soojin Kim and Azzra Mangalji. Stephen Pham and Hans Adomat performed the LC-MS analyses. Dr Tomlinson Guns, Dr Amina Zoubeidi, Dr William Moore and Dr Martin Gleave reviewed the manuscript and provided insightful critiques and suggestions.

A version of Chapter 3 has been published in PLoS One as follows: Toren P, Kim S, Johnson F, Zoubeidi A. Combined Akt and MEK pathway blockade in pre-clinical

models of enzalutamide resistant prostate cancer. PLoS One. 2016 Apr 5;11(4):e0152861. PMID: 27046225. Dr Amina Zoubeidi was the principal investigator and originally designed the studies. I performed the *in vitro* and *in vivo* studies. Soojin Kim performed the qRT-PCR experiments and assisted with western blots. Fraser Johnson assisted with the caspase-3 activity assays. I performed data analysis and drafted the manuscript. Dr. Zoubeidi provided critical feedback and reviewed the manuscript.

A version of Chapter 4 has been published in European Urology as follows: Toren P, Kim S, Cordonnier T, Crafter C, Davies BR, Fazli L, Gleave ME, Zoubeidi A. Combination AZD5363 with Enzalutamide Significantly Delays Enzalutamide-resistant Prostate Cancer in Preclinical Models. Eur Urol. 2015 Jun;67(6):986-90. Epub 2014 Aug 20. PubMed PMID: 25151012. Dr Zoubeidi was the principal investigator for this study. Dr. Zoubeidi and myself designed the experiments. I performed the *in vivo* studies and most of the *in vitro* experiments. Dr. Cordonnier assisted with the first LNCaP CRPC *in vivo* study. Soojin Kim assisted with the western blots and qRT-PCR. Dr. Fazli performed the immunohistochemistry analysis. I performed data analysis and drafted the final manuscript. Drs Davies, Crafter, Gleave and Zoubeidi provided critical input and reviewed the manuscript.

A version of chapter 5 is under preparation for submission as an original research manuscript.

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List of Abbreviations

ABI	Abiraterone
ADT	Androgen Deprivation Therapy
AMPK	Adenosine Monophosphate–activated Protein Kinase
AR	Androgen Receptor
ARE	Androgen Response Element
BET	Bromodomain and Extra-Terminal
BRCA1	Breast Cancer 1 susceptibility protein
BRCA2	Breast Cancer 2 susceptibility protein
CI	Combination Index
AR-V7	Androgen Receptor Splice Variant 7
CAD	Continuous androgen deprivation
cfDNA	Circulating free DNA
CRPC	Castrate Resistant Prostate Cancer
CSS	Charcoal Stripped fetal bovine Serum
CTC	Circulating tumour cell
CTLA4	cytotoxic T-lymphocyte antigen 4
CYP	Cytochrome P450
CYP17A1	Cytochrome P450 17A1
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid

EBRT	External Beam Radiation Therapy
EGFR	Epidermal Growth Factor Receptor
ENZ	Enzalutamide
ERK	Extracellular Signal-Regulated Kinase
EtOAc	Ethyl acetate
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HDAC	Histone Deacetylases
HER2	Human Epidermal growth factor Receptor 2
Hex	Hexane
HMGCR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
IGF-1	Insulin Growth Factor-1
IL-6	Interleukin-6
IL-11Ra	Interleukin-11 receptor alpha;
KLK3	Kallikrein-related peptidase 3
LBD	Ligand Binding Domain
LC-MS	Liquid Chromatography tandem Mass Spectroscopy
LHRH	Luteinizing Hormone-Releasing Hormone
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK/ERK Kinase
MMLV	Moloney Murine Leukemia Virus
mRNA	messenger Ribonucleic Acid

mTOR	Mammalian target of rapamycin
PARP	Poly Adenosine diphosphate Ribose Polymerase
PCR	Polymerase Chain Reaction
PD1	Programmed cell death protein 1
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin homolog
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SE	Standard Error
SEM	Standard error of the Mean
SERCA	Sarcoplasmic/Endoplasmic Reticulum Calcium Adenosine triphosphatase
SREBP-1	Sterol regulatory element-binding protein 1
SRD5	Steroid 5α-reductase
StAR	Steroidogenic acute regulatory protein
TCGA	The Cancer Genome Atlas
TMA	Tissue Microarray
VEGF	Vascular Endothelial Growth Factor

Acknowledgements

This has been an unexpected but welcomed chapter in my life and I wish to convey my sincere thanks to my numerous colleagues and friends at the Vancouver Prostate Centre. There are so many who have been helpful throughout this journey and I would like to thank them for their support as I have worked to complete this thesis.

I first all would like to express my sincere gratitude to Dr. Amina Zoubeidi for her mentorship and support throughout my graduate studies. Her genuine enthusiasm, patience in teaching, and persistent pursuit of excellence was greatly appreciated. I would also thank Dr. Martin Gleave for inviting me to work with such a dynamic group and for the financial support to my training which the centre, through his direction, was able to provide. I am also grateful for his mentorship and support as I entered an entirely new field of work from prior clinical responsibilities. I do not believe I could have found better mentors and their passion and excellence in translational research is truly inspiring.

I would also express my sincere gratitude to the members of my graduate supervisory committee which included at various times Dr. Vincent Duronio, Dr. Yuzhou Wang, Dr Ralph Buttyan and Dr Christopher Ong. Their encouragement, helpful comments and input was exemplary.

Many others have contributed to my laboratory research training; I will highlight a few in particular. Thanks to my fellow students Soojin Kim, Daksh Thaper, Chris Wang, Peter Raven and Fraser Thompson for the invaluable assistance and advice with laboratory techniques and protocols. To the numerous post-docs who provided helpful discussions I am grateful, including Shusuke Akamatsu, Jenna Bishop, Thomas Cordonnier, Kirsi Ketola, Yohann Loriot, Nader Al-Nakouzi, Yoshi Yamamoto and Alex

Wyatt. Thanks to Mary Bowden, Darrell Trendall and Virginia Yago for much help with all the murine xenograft studies. Many thanks to Eliana Beraldi, Na Li, Fan Zhang and Ka Mun Nip who provided invaluable advice and support on the cell culture studies performed.

Finally, I would like to acknowledge with gratitude the continued support of my family and friends. I am blessed to have such unwavering support from my family and was privileged to make so many new wonderful friends during my time in Vancouver.

To the men diagnosed this year with prostate cancer.

1 Introduction

1.1 Epidemiology of Prostate Cancer

Prostate cancer has a very high prevalence in our society, affecting approximately 1 in 8 men over their lifetime. New cases diagnosed every year in Canada represent about ¼ of all new cancer diagnoses in men, with the annual number expected to exceed 34,000 cases annually in the years 2018-2022(1). With widespread use of PSA testing, approximately 92% of prostate cancer patients present with localized or regional disease and only 4% present with metastatic disease(2).

1.2 Natural History of Prostate Cancer

Prior to the introduction of PSA screening, almost all men presented with advanced disease which was incurable with local therapy. In this era, prostate cancer was detected due to symptoms such as bone pain, urinary obstruction, paresthesia and inability to walk due to spinal cord compression or generalized fatigue and weakness.

With the use of screening serum PSA measurements, the disease is often detected early while still localized and thus permitting treatment with curative intent. Indeed, since the onset of PSA screening in the 1990s, there has been a significant decrease in the incidence of metastatic disease and subsequent death from prostate cancer (Figure 1.1).

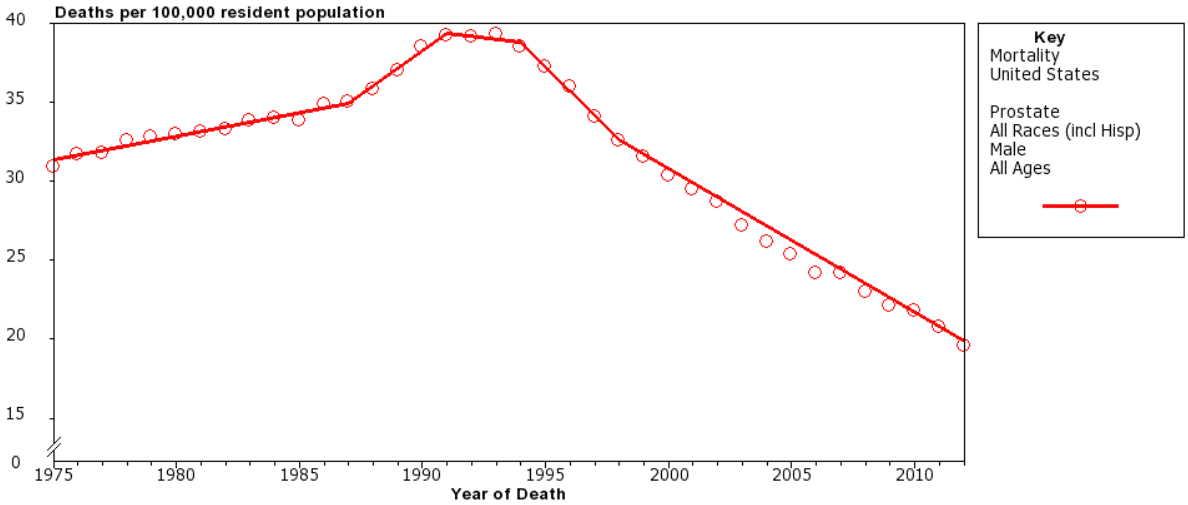


Figure 1.1 Historical trends in prostate cancer mortality in the United States. Created by statecancerprofiles.cancer.gov on April 16, 2016. Regression lines calculated using the Joinpoint Regression program version 4.2. Source: Death data provided by the National Vital Statistics System public use data file. Death rates calculated by the National Cancer Institute using SEER*Stat. Death rates (deaths per 100,000 population per year) are age-adjusted to the 2000 US standard population (19 age groups: <1, 1-4, 5-9, …80-84, 85+). Population counts for the population shifts due to hurricanes Katrina and Rita for 62 counties in Alabama, Mississippi, Louisiana, and Texas. 1969-2013 US Population Date File is used with mortality data.

The natural history of prostate cancer is varied, but often relatively slow compared to most cancers. Seminal studies by indicate that the natural history of prostate cancer progression depends on the pathological grade. Historically untreated low grade, or Gleason 6 prostate tumours are estimated to have a up to 30% mortality 20 years from the time of diagnosis of localized disease until death, whereas high grade, or Gleason 8-10 tumours are estimated to have approximately 40% mortality after only 5 years(3). Due to a stage migration in the pathological classification and long-term experience with surveillance of lower grade tumours, the vast majority of Gleason 6 tumours are not recommended for initial active treatment (4)

1.3 Staging of Prostate Cancer

1.3.1 Pathological Staging

The pathologic staging of prostate cancer is based on the Gleason grading system. T Gleason grading system is based on the macroscopic architecture of the tumour and has undergone several modifications over the years (5). The Gleason score consists of the sum of the primary and secondary grade patterns observed, which are ranked on a historical scale of1-5, though in actuality only 3,4 and 5 are considered cancer. There is considerable evidence suggesting Gleason grade 3 does not possess the biologic capacity to metastasize (6).

1.3.2 Clinical Staging

Clinical staging of prostate cancer is performed based on clinical digital rectal exam of the prostate, serum PSA values and results of biopsy. In some cases, imaging for metastases such as bone scans or computed tomography scans will also be utilised. The American Joint Committee on Cancer TNM staging system is widely used to stage cancers. In prostate cancer, the majority of tumours are T1 tumours, indicating there is no tumour palpable on exam. T2 tumours are clinical palpable, but localized within the prostate. T3 tumours have extended outside the prostate and T4 tumours have invaded adjacent organs. The N category may be N0 if no nodes harbor tumour, N1 if tumour is confirmed in the nodes or Nx in unknown cases. The M category indicates whether there are metastases (M1) or not (M0). This information can be grouped into four stage

categories to allow for relative comparisons with other cancers, though this four-stage classification system is rarely used in the clinic.

1.4 Prostate Cancer Clinical States

Treatments for prostate cancer are tailored to the extent of disease, as well as the disease state. Depending on the time of presentation, the disease may pass from various disease states and lines of treatment, outlined in Figure 1.2. This approach to describing the stage and progression of prostate cancer is most commonly employed in the clinic.

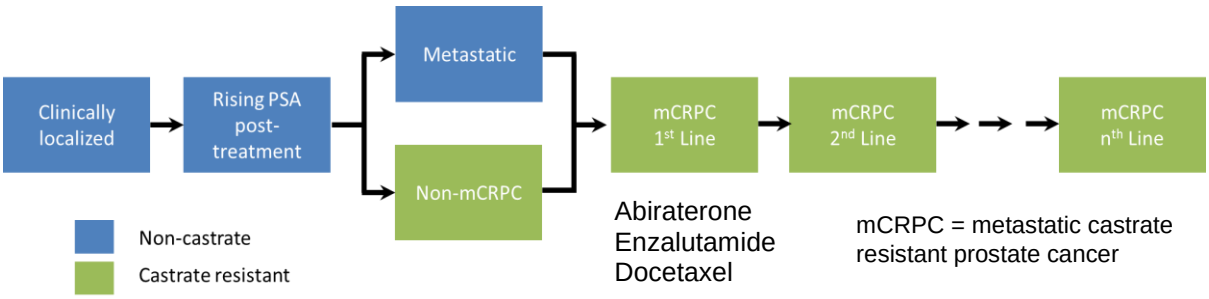


Figure 1.2 Clinical states of prostate cancer. Adapted from Scher et al(1).

1.4.1 Localized Disease

Prostate cancer which is localized to the prostate is treated according to the pathologic Gleason score. Recommended treatment of Gleason 6 tumours is active surveillance. Active surveillance is a strategy which aims to minimize overtreatment of indolent cancer while preserving the option to change to curative, definitive treatment. Repetition of prostate biopsies with or without prostate magnetic resonance imaging are performed at intervals of 1-2 years to confirm that no higher grade disease exists. Thus this strategy is pursued with the intent to treat only patients who are later proven to have

more aggressive disease. This is to be distinguished from the strategy of watchful waiting, where patients are not actively treated as it is estimated the probability of complications or death from their prostate cancer is less than the probability of death from other causes; in this case, surveillance biopsies or imaging are not therefore indicated. For prostate tumours with Gleason scores of 7 or higher (ie the presence of Gleason pattern 4 disease), active treatment is recommended in patients whose life expectancy is greater than 10 years(7). The two established modalities of treatment for localized prostate cancer are radiation therapy and surgical therapy. Radiation therapy is typically given in combination with ADT and may include various modalities, though the evidence for external beam intensity modulated radiottherapy is the best established(7). Surgical therapy for localized prostate cancer has evidence from randomized clinical trials that it decreases mortality in men with prostate cancer under the age of 65(7, 8). Surgery consists of removing the prostate and pelvic lymph nodes using an open or laparoscopic surgical approach. Accumulating evidence suggests that long term oncologic outcomes are better for men treated initially with surgery rather than radiotherapy, and as such surgery is considered the preferred option for younger men without significant co-morbidities (9, 10). This may be in part related to the possibility of salvage radiation following biochemical relapse in men who undergo radical prostatectomy.

1.4.2 Recurrent or Metastatic Disease

Despite local therapy, up to 35% of men will have a local or systemic recurrence of prostate cancer (11, 12). A recurrence of prostate cancer is readily detectable after surgery as a detectable and rising PSA, and is similarly eventually detected as a rising

PSA after radiation therapy. For prostate cancers which recur following initial therapy or present with metastatic disease, there remains no curative therapy. With the recent decline in PSA screening, the proportion of men who present with metastatic disease is expected to increase. The current standard of care for treatment of recurrent prostate cancer is androgen deprivation therapy (ADT). ADT consists of surgical castration or medical castration using luteinizing-hormone releasing hormone (LHRH) agonists or antagonists. In many cases in men with less aggressive disease, a slowly rising PSA or significant co-morbidities, initiation of treatment may be delayed to minimize the side effects of treatment. Men with metastatic prostate cancer at presentation are also treated with ADT, although recent studies suggest that men with a significant burden of metastases may benefit from concomitant docetaxel chemotherapy with ADT (13).

1.4.3 Castrate Resistant Prostate Cancer

Castrate resistant prostate cancer (CRPC) is determined by the presence of a rising PSA or progressive disease on imaging despite serum testosterone levels in the castrate range. CRPC is a heterogeneous and progressive stage of prostate cancer, including both symptomatic and asymptomatic men with or without clinical metastases (Figure 1.2). Approximately 10-20% of men castrated for prostate cancer treatment develop CRPC within 5 years of follow-up (14, 15).

CRPC is defined according to the Prostate Cancer Clinical Trials Working Group as progression of prostate cancer despite castrate levels (<1.7ng/mL) of testosterone. Progression may be biochemical (3 consecutive PSA rises >2ng/mL above nadir, minimum 1 week apart) or radiological or symptomatic (16). Radiological progression is

defined as the appearance of two or more new lesions on bone scan or enlargement of a soft tissue lesion according to RECIST criteria (17).

The median age of men with CRPC is in the seventies (14, 18). Up to 85% of patients at diagnosis of CRPC will have metastases (15). By comparison, approximately 4% of all newly diagnosed prostate cancer patients present with metastatic disease (19). The classification of metastatic versus non-metastatic CRPC is important to distinguish. Prognosis and natural history is variable, with a less aggressive course in non-metastatic CRPC. One study estimated approximately 30% of men with a rising PSA and no bone metastases developing bone metastases at 2 years (18). Notably, absolute PSA levels and PSA kinetics continue to maintain usefulness in CRPC as biomarkers predictive of prognosis (18, 20).

Historically, treatment for metastatic CRPC was largely palliative. Mitoxantrone showed a benefit for palliation (21), but it was not until the SWOG 9916 and TAX 327 studies that an improvement in overall survival was noted with chemotherapy (22, 23). Anti-androgens, particularly bicalutamide, have also been used in combination with ADT, but with only a modest survival benefit(24). Bone targeted therapy with zoledronic acid also entered clinical practice at a similar time as the early chemotherapies(25) and may improve quality of life by decreasing the incidence of skeletal related complications. Radiopharmaceuticals are known to have a palliative benefit, but until recently have been sparsely used, in part due to hematologic toxicity. External beam radiotherapy to symptomatic sites was and continues to be an effective and widely used treatment for bone metastases in CRPC patients, though its incident use is not well reported (15).

Historically, the median survival of men with metastatic CRPC was reported to be as low as one year (21) . With improvements in care as well as earlier detection of the CRPC state, the median survival now exceeds 2.5 years. Moreover, both survival and quality of life continue to improve with the introduction of new therapies. However, not all the improvements in survival are related to new therapies available. Palliative care and supportive care have also improved. It is noteworthy that the median overall survival (OS) in the placebo arm of CRPC trials appears to have increased over time. For example, the survival in the docetaxel/prednisone control arm of the CALGB 90401 trial reported in 2012 was 21.5 months, 2.6 months longer than the original TAX327 study reported in 2004 (22, 26). Finally, while not extensively studied, there are suggestions that palliative external beam radiotherapy may also provide some survival benefit (27).

1.5 Mechanisms of Prostate Cancer Resistance

Since the initial experiences of Charles Huggins treating with advanced prostate cancer, it has been clear that the prostate cancer does not uniformly and completely regress as a result of androgen ablation(28, 29). Decades of research continue to identify and characterize pathways and targets which allow prostate cancer to progress despite androgen deprivation therapy. In this process, it has become clear that the androgen receptor continues to remain a principal oncogenic driver in castrate resistant prostate cancer (CRPC). The continued usefulness of PSA as a prognostic marker in CRPC highlights how the AR axis remains a principal target (20). Molecular resistance pathways can be classified in different ways. Figure 1.3 demonstrates how current treatments under investigation can be classified as cellular, micro-environmental or

systemic targets. Alternatively, molecular pathways of resistance may be classified according to the hallmarks of cancer (Figure 1.4). Given the importance of the AR in prostate cancer and the widespread use of PSA as a marker of disease progression, the categorization AR and non-AR pathways of resistance is widely used.

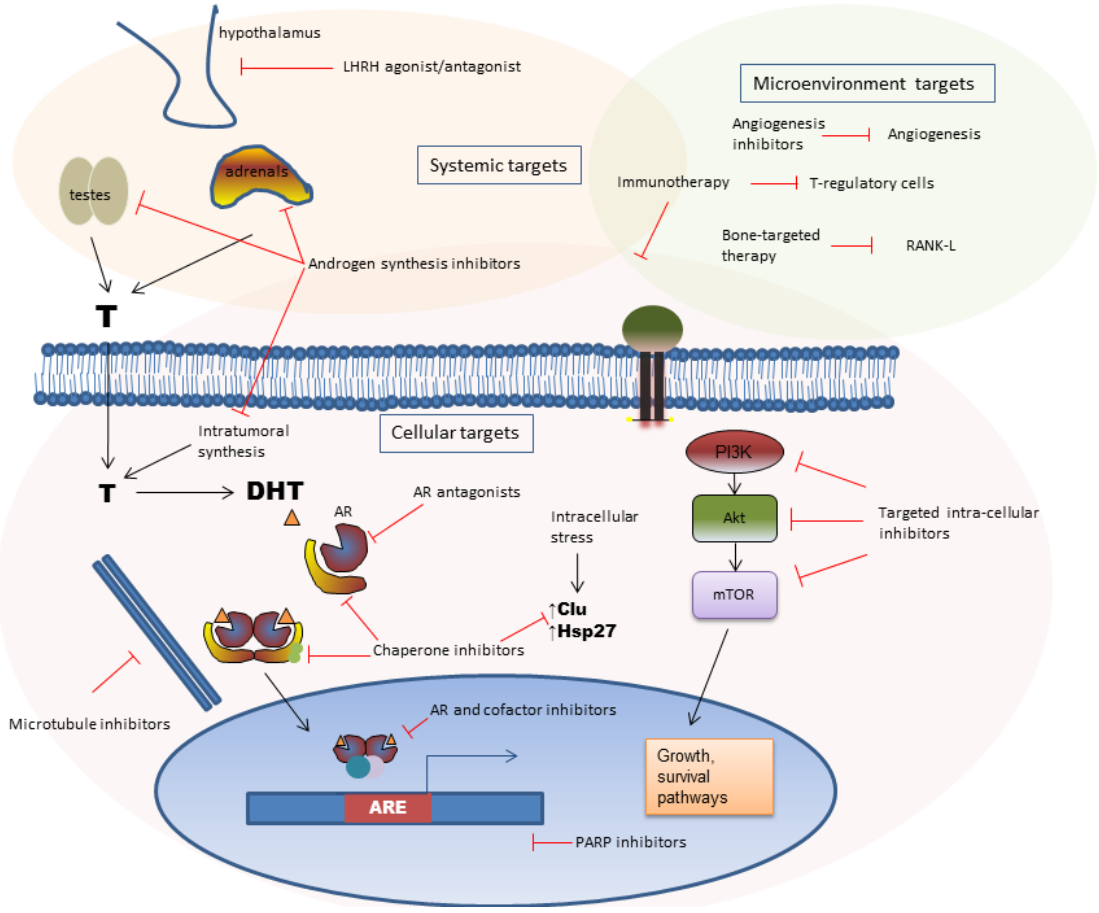


Figure 1.3 Various targets for prostate cancer treatment in use or under development.

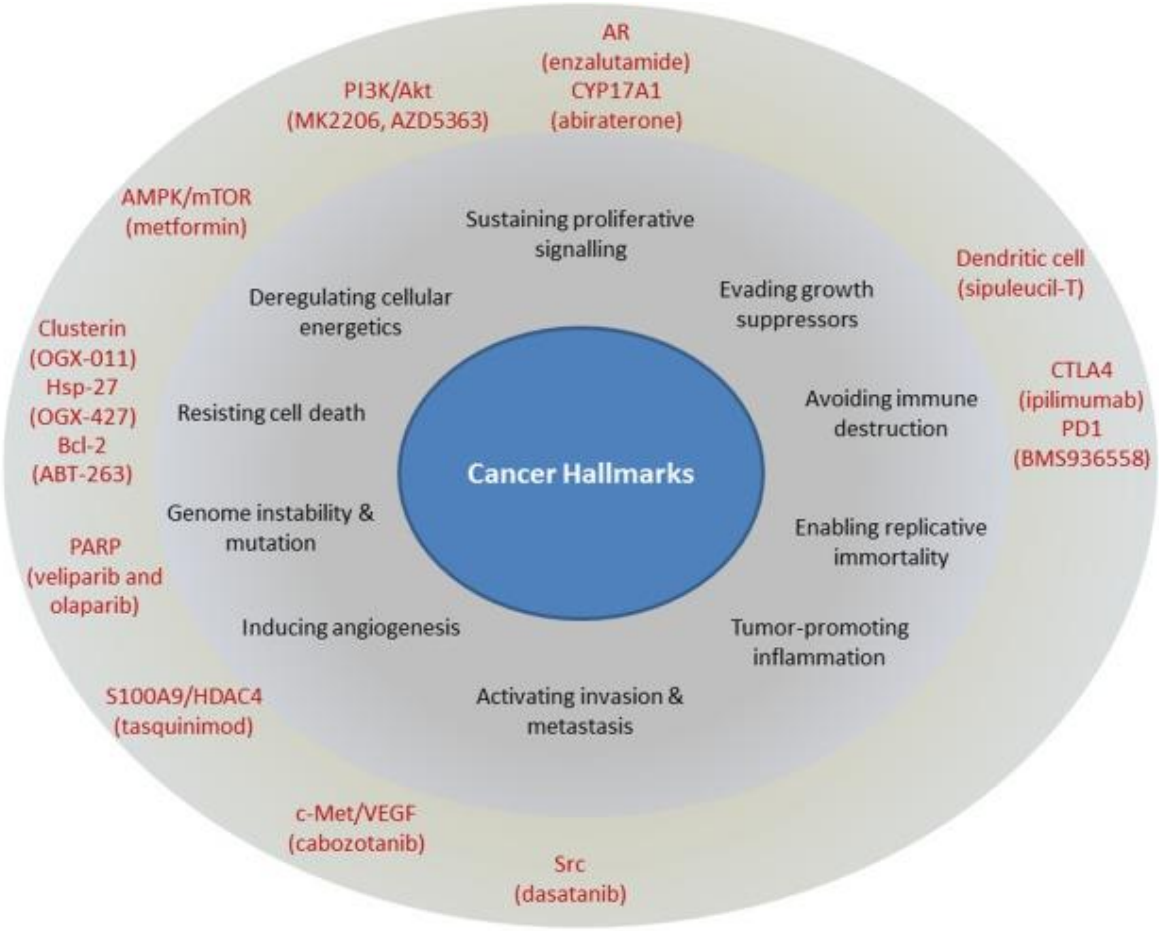


Figure 1.4 Selected therapeutics in use or development against castrate-resistant prostate cancer with corresponding cancer hallmarks. Adapted from Hanahan et Weinberg(30).

1.6 Androgen Receptor Pathway

The androgen receptor(AR) was first characterized in the 1960s and continues to be an enduring target in prostate cancer(29). The AR gene is encoded on Xq11-12 and consists of eight exons which correspond to different parts of the modular protein. The receptor is similar to other nuclear binding receptors, and has several components: the regulatory N-terminal domain, the DNA-binding domain, a hinge region, and the carboxy-terminal ligand binding domain. Upon binding to dihydrotestosterone (DHT) in the

cytoplasm, AR dimerization occurs and it subsequently is translocated into the nucleus. There, the AR initiates translation of mRNA through formation of transcriptional complexes and binding to androgen response elements in the DNA, which eventually drives cell growth and proliferation.

Testosterone and DHT are the principal ligands to the AR, with DHT possessing greater affinity. Androgen biosynthesis follows general steps from precursors to DHT, though there exists redundancy and overlap between various enzymes, with a 'backdoor' pathway to DHT identified (Figure 1.5). The CYP17 p450 enzyme has dual functions, catalyzing lyase and hydroxylase reactions in the pathway for the biosynthesis of androgens. Through blockade of the cytochrome p450 enzyme CYP17A1, there is a decrease in the production of testosterone (and DHT) ligand available to bind the AR in prostate cancer cells. Although castration effective decreases testosterone levels

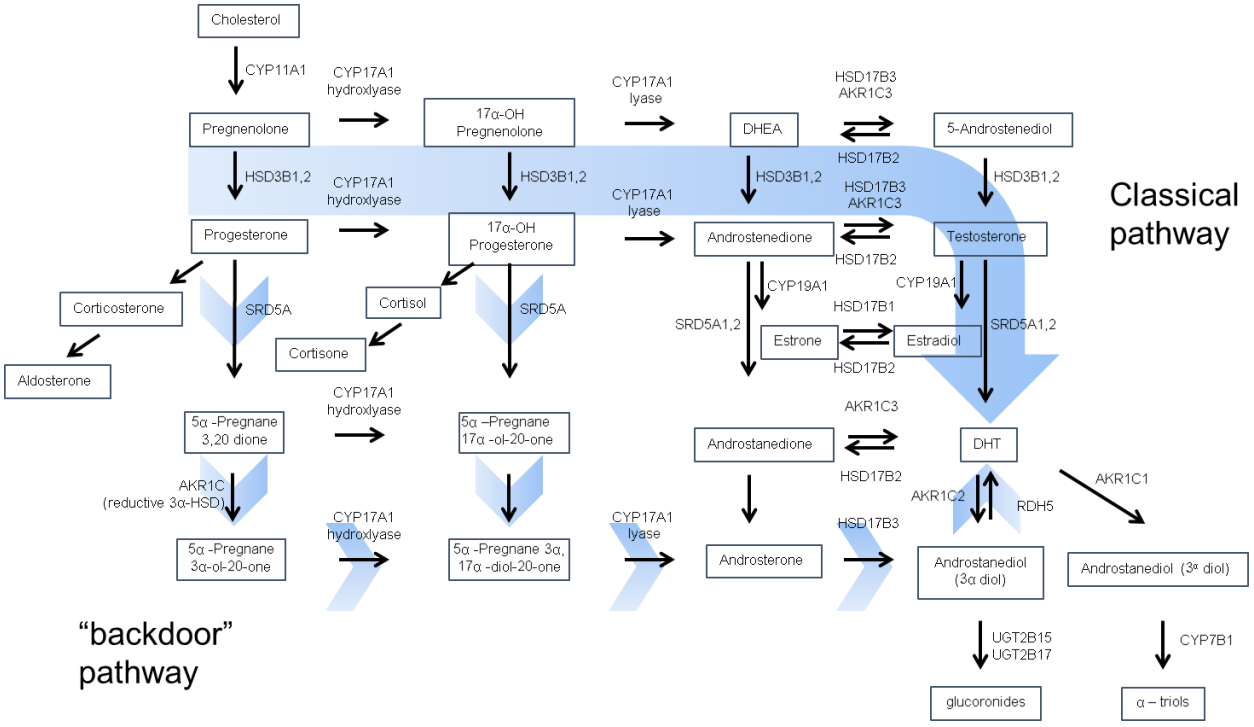


Figure 1.5 Schematic of the androgen biosynthesis pathway. Adapted from reference (34).

produced in the testis, there remains potential adrenal or Intratumoral sources of testosterone available to tumours. Some suggest that even very low levels of testosterone may be important to fuel persistent tumour growth (31, 32).

1.6.1 The Androgen Receptor in CPRC

It was previously thought that once prostate cancer progressed despite androgen deprivation that the androgen receptor was no longer driving tumour growth. Thus, there was stronger rationale to target rapidly dividing cells with non-androgen receptor targeted therapy; the success of docetaxel targeting rapidly dividing cells strengthened this viewpoint (22). This perspective on CRPC has changed through a better understanding of the biology of CRPC, as well as the recent success of androgen synthesis inhibitor abiraterone and AR antagonist enzalutamide for treating CRPC. Thus, the terms ‘hormone-refractory’, or ‘androgen-independent’ are inappropriate descriptors and are no longer in use. Recent research suggests that the AR pathway continues to remain active in resistance to the newer AR-targeted therapies enzalutamide and abiraterone (33, 34).

Several AR-mediated mechanisms of resistance have been proposed and include activation of a promiscuously mutated AR, AR gene amplification, intratumoral production of androgens, recruitment of transcriptional co-factors, and gene fusions or re-arrangements(35-39). Deep sequencing has identified genomic alterations of the AR in CRPC in approximately 50% of cases(40). More broadly, the AR pathway is estimated to be altered in ~50% of primary prostate cancers and 100% of metastases (39). The role of the AR pathway in resistance to newer AR-targeted therapies continues to be explored. Up-regulation of certain enzymes in the steroidogenesis pathway and constitutively active

AR splice variants without the ligand binding domain are two mechanisms of interest (33, 34).

1.6.2 Genomic AR Aberrations

Persistent activation of AR receptor signalling, a hallmark of CRPC, may be related to AR genomic aberrations. Changes in the AR genome including AR amplification and AR mutations are known to increase as prostate cancer progresses to castrate resistance (41, 42). Similar genomic changes occur as CRPC becomes resistant to newer AR pathway inhibitors, with a greater prevalence of AR mutations and possibly the development of genomic rearrangements resulting in AR splice variants which are ligand unresponsive(43, 44).

Several mutations of the AR ligand binding domain (LBD) have been described, including T877A and H874Y (45-47). These may be promiscuously activated by other sex steroids, such as progesterone DHEA and estrogens, driving AR-dependent gene transcription more than wild-type AR (45, 48). Promiscuous activation of AR LBD mutations by glucocorticoids has also been reported (49, 50). Recently, a novel F877L LBD mutation associated with ENZ-resistance was discovered which results in ENZ becoming an AR agonist (44, 51). In patients who received abiraterone prior to prostatectomy as part of a clinical trial, it was reported that there was a significant increase in the prevalence of promiscuously activated AR LBD mutations, which concomitant increases in tumoral progesterone levels (52).

1.6.3 Androgen Receptor Splice Variants

There are various splice variants of the androgen receptor reported to exist, but the AR-V7 variant appears to be the most relevant to prostate cancer resistance. This splice variant is a truncated AR protein which lacks the ligand binding domain. As a result of the absence of the LBD, this variant may constitutively remain active and bound to the DNA in the presence or absence of androgens. As a result, it would be expected that AR agonists which target cancer cells where AR-V7 is present instead of AR will be ineffective

Androgen receptor splice variants appear to be commonly found in very low levels in the serum in both healthy and cancerous patients, but it appears elevated in patients which harbour increased tumour levels of the splice variant present(53, 54). A prospective clinical trial has indicated that circulating levels of AR-V7 mRNA can be used to predict response to enzalutamide or abiraterone treatment(54). Indeed, it is the case that AR-V7 has been demonstrated clinically to be a biomarker of response to AR directed therapy, but not to taxane-based therapy (54, 55). Further validation studies of circulating AR-V7 levels in men with CRPC will allow it to enter mainstream clinical practice as a predictive biomarker. Validation in cohort of men with less advanced disease may also help distinguish whether the presence of these splice variants are only a marker of heterogeneity and advanced disease or a true biology driver of resistance to AR targeted agents.

1.6.4 Intratumoral Androgen Synthesis

Several pre-clinical studies have demonstrated the importance of intratumoral synthesis of androgens as a mechanism of castration resistance. Locke et al demonstrated that LNCaP tumours have the capacity to synthesis their own androgens from precursors (35). Similarly, Montgomery et al demonstrated in prostate cancer metastases that androgen levels were maintained through upregulation of steroidogenic enzymes despite systemic castration (56). These seminal pre-clinical studies are now supported by several clinical studies indicating steroidogenic enzymes and androgen levels are elevated in metastases of prostate cancer(57-59).

Despite widespread acceptance that inhibition of intratumoral androgen synthesis is an valid strategy to treat tumour CRPC, several points remain controversial. Androgen synthesis within the tumour may be produced *de novo* from cholesterol or testosterone or DHT may be formed as a result of conversion of circulating adrenal androgens such as DHEA (60). Epidemiologic studies have found low serum cholesterol protective against the detection of high grade prostate cancer (61) and high serum cholesterol a risk factor for prostate cancer mortality (62). In pre-clinical models, higher circulating levels of cholesterol have been reported to accelerate prostate cancer growth (63). This suggests *de novo* synthesis may be relevant, though there are other potential ways cholesterol may alter prostate cancer progression. A study of culturing *ex vivo* portions of prostate normal prostate or CRPC tissue with radiolabelled steroid precursors suggests that the dominant source of intratumoral androgens originates from conversion of adrenal precursors (64). Similarly, it is also not clear whether tumour cells themselves

predominantly produce the androgens or whether they are mostly synthesized in adjacent stroma cells (65).

Intratumoral steroidogenesis also appears to be a relevant mechanism of resistance to newer AR pathway inhibitors. Upregulation of steroidogenic enzymes has been observed in patient-derived xenografts following abiraterone treatment and has been proposed as a mechanism of abiraterone resistance (34, 66). However, this has not been confirmed in patients, nor was the increase in steroidogenenic enzyme transcription linked with concomitant increases in androgen levels. Interestingly, in patients who progressed on enzalutamide, increases in serum testosterone levels have been observed, suggesting that increased androgen synthesis may be related to resistance to enzalutamide (59).

1.7 The PI3K/Akt Signaling Pathway

The more prevalent use of potent AR pathway inhibitors may likely select for the survival of tumours driven by alternate survival pathways such as the PI3K/Akt pathway. Activation of the PI3K/Akt pathway is implicated in many aggressive human cancers(67, 68). Accordingly, there has been significant pharmaceutical investment toward developing targeted inhibitors of this pathway in various hematologic and solid cancers. As detailed below, the PI3K/Akt pathway is also particularly important in resistant, aggressive prostate cancer.

The PI3K/Akt signaling pathway regulates cellular metabolism, tumour development, growth, proliferation, metastases and cytoskeletal reorganization. It is part of a complex intracellular cell signalling cascade (Figure 1.6). PI3K is a plasma

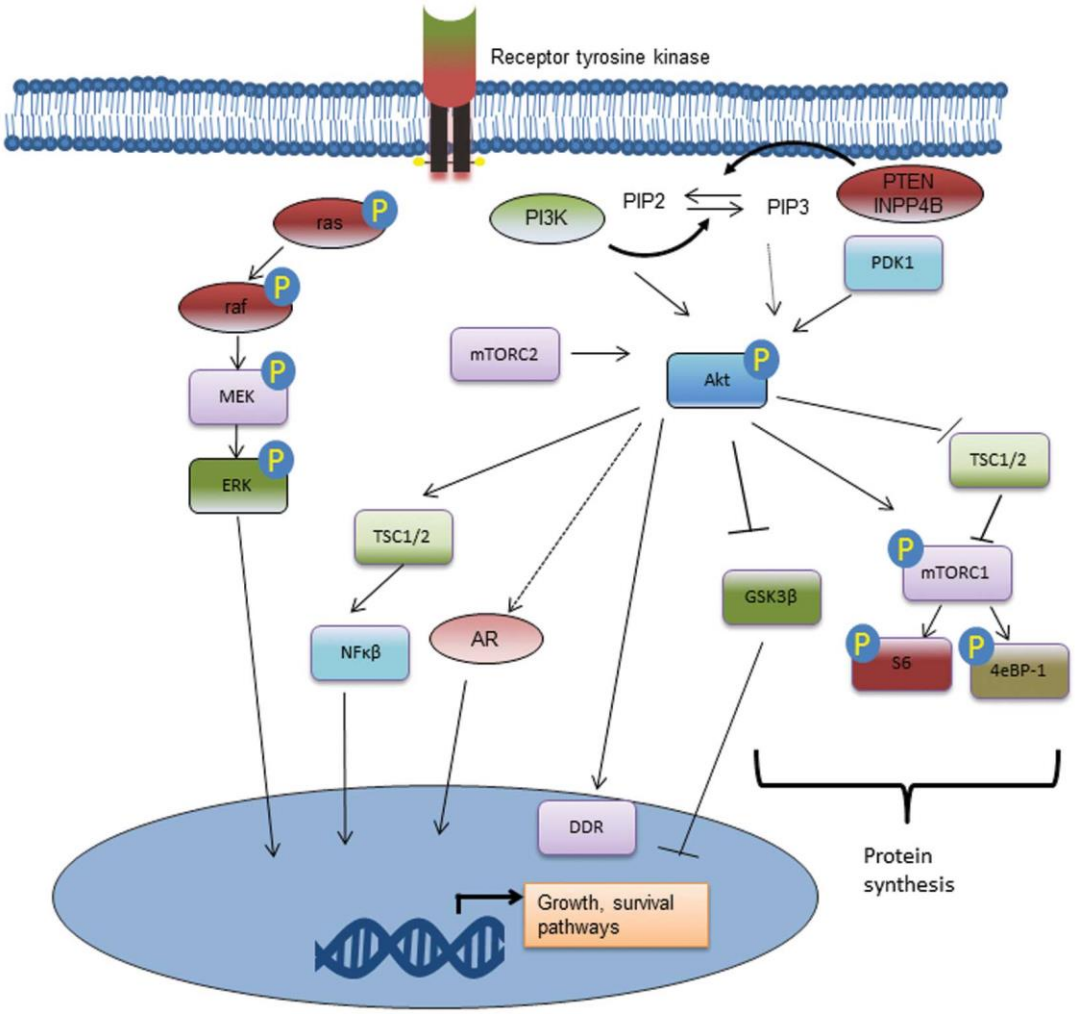


Figure 1.6 Schematic of the PI3K/Akt pathway.

membrane-associated protein kinase consisting of three subunits: the regulatory subunits p85 and p55; referred collectively by convention as p85 and a catalytic subunit, p110(69). There are three classes; it is the class IA PI3Ks which are the most clearly implicated in human cancer, including prostate cancer. Moreover, this class is usually acting downstream of receptor tyrosine kinases. Activation of receptor tyrosine kinases at the cell membrane results in conformational changes which removes auto-inhibition of the catalytic domain of PI3K. Three catalytic isoforms p110α, p110β, and p110δ are respectively the product of the genes PIK3CA, PIK3CB and PIK3CD. Once activated, PI3K catalyzes the phosphorylation of Phosphatidylinositol 4,5-bisphosphate (PIP2) to

produce Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 then activates intracellular signalling through its binding to pleckstrin homology (PH) domains of many signalling proteins, including Akt. In prostate cancer, it appears that the p110β isoform is most relevant to prostate cancer progression and resistance (70). It has been associated with basal activation of Akt in prostate cancer models (71).

The PI3K/Akt pathway functions downstream of receptor tyrosine kinases (RTKs) as well as independently of RTKs. Non-RTK activation of this pathway may be from other intracellular signalling pathways or from other membrane receptors including G-protein coupled receptors. The main upstream activators likely are context specific. In autopsy specimens of metastatic prostate lesions, various RTKs were associated with Akt activation (72). Of note, many of the RTKs which activated the PI3K/Akt pathway, including EGFR, IGF-IR, FGFR and c-MET receptors, are actively researched as targets in CRPC. Nonetheless, some *in vitro* studies in prostate cancer cells suggests that basal activation of this pathway occurs independently of RTKs (71). Notably, phospho-proteomic analysis of metastatic tumour samples collected on rapid autopsy found that Akt was the tyrosine kinase most commonly found to be active in metastatic prostate cancer(72). Activated Akt is a kinase which in turn phosphorylates and activates many oncogenic features within cancer cells. Upon recruitment to the cell membrane, it is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1), a reaction catalyzed by PIP3 binding to the PH domains of both molecules. Once phosphorylated at both Ser473 and Thr308 phosphosites occurs, activated Akt can activate many downstream functions via its intrinsic kinase activity.

Mammalian target of rapamycin (mTOR) is a major downstream signalling protein involved in protein translation via the eIF4E complex and S6K which is activated by Akt. Both mTOR and S6K are found in higher levels in prostate cancer compared to benign controls (73). The proteins differentially associated with mTOR defined the TORC1 andTORC2 complexes. These have overlapping, but different functions, with TORC2 providing negative feedback regulation on the PI3K/Akt pathway via S6K (74, 75). There are many other downstream oncogenic effects of Akt phosphorylation. Cell survival is promoted through anti-apoptotic effects, particularly inhibition of the pro-apoptotic Bcl-2 family members BAD and BAX(76). Transcription factor FOX01 acts as a tumour suppressor and its phosphorylation by Akt induces its ubiquitation and degradation by the proteasome. Further, inhibition of glycogen synthase kinase 3 (GSK-3) increases cellular translation of proteins as does phosphorylation of 4eBP-1. Regulation of cell growth and survival by Akt also occurs by the NF-κB pathway via activation of IκB kinase (IKK) (Figure 1.5). Further, the PI3k/Akt pathway in prostate cancer appears to be involved with modulation of the DNA damage repair pathway(77). More recently, the PI3K/Akt pathway has been implicated in modulating a more aggressive phenotype through modulation of cholesterol ester formation in prostate cancer cells (78). This suggests a possible relationship with metabolic pathway disturbances and the development of aggressive prostate cancer. Overall, there are a plethora of downstream cellular functions of the Akt pathway which correspond to a clinically aggressive phenotype.

The PI3K/Akt pathway is antagonized by several phosphatases, including phosphatase and tensin homolog gene (PTEN), PH and leucine-rich repeat protein phosphatase (PHLPP), cellular prostatic acid phosphatase, PP2A and INPP4B(79-81).

Genetic loss or other inactivation of these phosphatases results in greater amounts of phospho-Akt and subsequent increased or sustained oncogenic signalling. Notably, the PTEN gene on chromosome 10q23.3 is the most-commonly deleted gene in prostate cancer(82). However, genomic loss of PTEN does not always correlate with activation of the PI3K/Akt pathway(83). Pre-clinical models and patient samples also show that loss of PTEN results in a particularly aggressive phenotype when found in combination with activation of receptor tyrosine kinases (84, 85). PHLPP is regulated by the AR via FKBP5 and explains in part the up-regulation of the PI3K/Akt pathway seen following androgen deprivation (81). INPP4B is decreased following androgen deprivation and may be another mechanism through which the Akt pathway is activated resulting in earlier disease recurrence (80).

1.7.1 PI3K/Akt Signalling in Prostate Cancer

The phosphatidylinositol 3-kinase (PI3K) /Akt pathway represents the most commonly activated kinase signalling pathway in CRPC. Genomic alterations in this pathway have been identified in 42% of primary tumours and up to 100% of metastases (42). Loss of function of the PTEN repressor results in increased levels of activated Akt and downstream effectors, as does an activating mutation of the PIK3A gene. The activated downstream effectors, including GSK3β and S6 kinase, result in cell survival, proliferation, migration and invasion (Figure 1.4) (68, 86-88). Activation of the PI3K/Akt pathway is associated in prostate cancer patients with higher Gleason score, and decreased metastasis-free survival(88).

It is estimated that genomic PTEN alterations are found in 9-45% of high grade prostate intra-epithelial neoplasia(HG-PIN), increase to 20-60% in localized prostate cancer, and are altered in up to 100% of cases of metastatic prostate cancer(42, 89). Homozygous deletion of PTEN is linked with CRPC (90). Further, PTEN is the most common gene with loss of heterozygosity in circulating tumour cells CTCs(91). Similarly, mutations in the PI3K pathway occur more frequently in metastatic tissue compared to primary tumours. In the Taylor et al dataset, mutations in the PI3K regulatory genes PIK3R1, PIK3R3 and PIK3CS occur at a frequency of 22%, 2%, and 6 percent% in primary tumours, respectively. In metastatic tissues, the frequency increases to 58%, 16% and 16%, respectively (42). Mutations in PIK3CA catalytic gene of PI3K are known to be activating to the pathway and also may predict response to therapy with PI3K inhibitors, though they are not highly prevalent in prostate cancer (92). Mutations in the regulatory phosphatase PHLPP gene can also result in activation of the Akt pathway, occurring at 11% and 37% in primary and metastatic tumours, respectively (42).

On immunohistochemistry, the loss of PTEN staining in localized prostate cancer samples correlates with higher Gleason score and pathologic stage(93, 94) as well as an increased risk of positive lymph nodes(95). PTEN protein loss on immunohistochemistry of the primary tumour has also been associated with shorter time to biochemical recurrence post radical prostatectomy, but not consistently (96). Levels of phospho-Akt increase with higher Gleason grade(97, 98) and are associated with poorer survival in CRPC(99). However, it is unclear whether they hold any prognostic significance in low and intermediate grade disease (Gleason score 6-7)(98). Levels of phospho-Akt also

predict for biochemical recurrence post radical prostatectomy, with improved prediction when used in combination with PTEN protein loss (100).

1.7.2 Role of PI3K /Akt in Prostate Cancer Carcinogenesis and Progression

The PI3K pathway may be important to induce carcinogenesis and contribute to prostate cancer progression through the activation of growth and survival pathways. Activation of this pathway may further alter epigenetic regulators such as BIM1 (101). Unsurprising given its role of cell survival, the PI3K/Akt pathway appears highly important for the survival and proliferation of prostate cancer stem cells (102).

PTEN deletion in mice is commonly used to model prostate cancer progression in mice (103, 104). PTEN loss in mice has been shown to suppress androgen-responsive genes and promote cell autonomous growth (105). Activation of the P3K/Akt pathway in mice may also occur using myristolated Akt or constitutive activation of p110β. In a murine subrenal xenograft model, activation of both AR and Akt has been noted to synergize to increase prostate tumour growth (106). Nonetheless, the exact role of this pathway in carcinogenesis in humans is uncertain. On the contrary, a recent genome wide sequencing analysis suggests that PTEN loss is a late-stage feature in the progression of prostate cancer (107).

Pre-clinical studies suggest that concomitant loss of certain proteins together with PTEN loss appear to accelerate prostate cancer progression. This has been demonstrated in mice and correlated with features of aggressiveness, such as Gleason score, in patient samples for the tumour suppressors NKX3.1, EAF2/U19, Gata3 and

Sox9 (108-112). B-raf and Stat3 activation and loss of SMAD4 and p53 signalling have also been shown in murine models to cooperate with PTEN loss to enhance prostate cancer progression (104, 113-116). This complex network with other pathways highlights why monotherapy against the PI3K/Akt pathway may not be an optimal strategy. Table 1.1 lists different combination strategies which have been explored targeting the PI3K/Akt/mTOR pathway in pre-clinical models of prostate cancer.

Table 1.1 Selected pre-clinical combination studies of PI3K/Akt/mTOR inhibitors.

PI3K/Akt/mTOR pathway inhibitor	Co-target	Selected outcomes assessed
ZSTK474 (Pan- PI3K inhibitor)	PSMA	*In vitro* and *in vivo* (C4-2luc) tumour growth(117)
BEZ235 (PI3K-mTOR inhibitor)	HDAC	Attenuation of DNA damage repair protein ATM(77)
AZD5363 (Akt inhibitor)	AR	Apoptosis, proliferation, LNCaP tumour growth(118)
BEZ235 (PI3K/mTOR inhibitor)	Microtubules	*In vitro* and *in vivo* tumour growth(119)
AZD5363 (Akt inhibitor)	Autophagy	Apoptosis, tumour growth(120)
Akt inhibitors	Pim-1	Apoptosis, tumour growth (121)
Rapamycin (mTOR inhibitor)	MEK	*In vitro* and *in vivo* tumour growth(122)
Everolimus (mTOR inhibitor)	Propachlor (from drug screen panel)	Authophagic cell death(123)
Perifosine (Akt inhibitor)	EGFR	Apoptosis(124)

1.7.3 Biomarkers of PI3K/Akt Pathway Activity

With the aggressive oncogenic characteristics of the PI3K/Akt pathway, there has been significant interest to use this pathway as a biomarker to differentiate more significant, lethal prostate cancer from more indolent disease. While it does appear from the current data that this pathway does provide prognostic information, it is unclear that it provides any significant improvement over currently used clinical and pathologic

markers. However, there is ongoing interest to use activation of this pathway as a predictive biomarker for newer targeted agents against the PI3K/Akt axis.

The challenges of using this pathway as a biomarker relate in large part to the complexity of the biology in advanced prostate cancer and tumour heterogeneity. Firstly, there is a large diversity of mutations and genomic alterations which may activate this pathway, making any single marker less sensitive. Further, the context of this pathway in prostate cancer differs from other malignancies where this pathway also plays an important role. For example, while activating PI3KCA mutations are relatively common among advanced malignancies, they are not common in prostate cancer (42). Factors in the tumour microenvironment can also influence signalling of this pathway, which is downstream of various cell surface receptors. Therefore, both tumour and patient heterogeneity contribute to a complexity making biomarker evaluation and validation more challenging. For example, in circulating tumour cells PTEN allelic loss has significant heterogeneity when analyzed by fluorescent in situ hybridization(125). Further, on immunohistochemistry, analysis of downstream targets does not clearly correlate in patient samples with the phosphorylation of Akt. In one study, phosphorylation of downstream GSK3β and a forkhead transcription factor was noted in only 29 and 40% of cases, respectively, in localized prostate cancer samples with phospho-Akt(126). Finally, technical variation, antibody limitations, tissue acquisition and processing also present challenges to use of this pathway as a clinical biomarker, though progress is being made(127).

PTEN status and phospho-Akt are the most commonly investigated biomarkers. PTEN loss may be detected by genomic microarray, sequencing, fluorescent or

chromogenic *in situ* hybridization or immunohistochemistry techniques in tumor samples, circulating tumour cells or in cell free DNA, while phospho-Akt levels rely on immunohistochemical approaches. Akt can be phosphorylated at Thr308 and Ser473; it appears that both sites are usually phosphorylated in the active state. It does appear that Akt staining is specific to tumour cells, without any staining in adjacent stromal tissue (126). As detailed above, several studies indicate the prognostic importance of PTEN loss and phospho-Akt levels in localized prostate cancer. Loss of INPP4B has been noted to be a good marker of aggressive breast cancer(128), and recently has been reported to also predict for poor survival in prostate cancer patients(129). An area which remains to be more fully explored is the cellular localization of phospho-Akt. It is unclear whether increased nuclear staining improves prognostication, as suggested by one study which found that greater nuclear phospho-Akt staining was associated with higher Gleason grade (130).

In addition to the use of PTEN, phospho-Akt or other related proteins as prognostic biomarkers, there is significant interest into the use of predictive biomarkers for novel PI3K/Akt inhibitors. Only a few studies to date have investigated predictive biomarkers, but this area is expected to increase as more inhibitors of this pathway enter clinical evaluation. In unselected men with CRPC, PTEN status on immunohistochemistry did not predict response to everolimus (131). Activating mutations in mTOR were found in one patient with an excellent response to combination everolimus and pazopanib (132). Similar anecdotal responses to AZD5363, a pan-Akt inhibitor has been reported to be associated with genetic alterations, but not yet in prostate cancer patients(133). Prospected clinical investigation and validation is ongoing to identify and evaluate

appropriate predictive biomarkers in patients for response to PI3K/Akt inhibitor therapy in prostate cancer patients.

1.7.4 Interactions of the PI3K /Akt and the AR Pathway

The relationship between the PI3K /Akt and AR pathways is of significant interest as a co-targeting strategy in prostate cancer (81, 134). Reciprocal interactions between these pathways have been demonstrated in several pre-clinical studies (81, 135, 136). Blockade of the AR pathway results in PHLPP-mediated Akt inactivation via a decrease in androgen regulated FKBP5 (81, 105). Inhibition of the PI3K/Akt pathway may result in up-regulation of AR transcriptional activity via activation of membrane signalling proteins such as HER3 (81, 137). Direct AR phosphorylation by Akt appears to predominantly be relevant in the low-testosterone state (ie during androgen deprivation therapy)(106, 138, 139). Akt has been shown to phosphorylate the AR at Ser-213 and Ser-791, but the significance of these phosphosites is unclear (106). *In vitro* models suggest that Akt may regulate AR transcription (106), but that this is not by direct phosphorylation of the AR(106, 140).

The combination of bicalutamide and a pan-Akt inhibitor, AZD5363 have been noted in *in vitro* and *in vivo* LNCaP models to synergize to decrease tumour growth(118). Recent results suggest markedly decreased *in vivo* tumour volumes with the use of potent pan-Akt or PI3K isoform specific inhibitors in combination with potent AR inhibitors(141, 142). These pre-clinical findings have supported the development of clinical trials of combination blockade of both the AR and the PI3k/Akt/mTOR pathways. Further

understanding of the interactions between these pathways in pre-clinical models may aid in design of future clinical trials.

1.8 Other Mechanisms of Resistance in CRPC

Several other recognized mechanisms of resistance have been recognized to be relevant to castrate-resistant prostate; these may also be relevant in enzalutamide or abiraterone resistant prostate. This includes other kinase signalling pathways such as IRS-1, c-met, or MAPK(also known as MEK/ERK) signalling (143-145), activation of cellular stress and survival networks, changes in the tumour microenvironment. Neuroendocrine prostate cancer represents a divergent form of resistant prostate cancer which may does not resemble typical prostate cancer, but may emerge following treatment pressure.

1.8.1 MEK/ERK Signalling

Similar to the PI3K/Akt pathway, the ras/raf/MEK/ERK pathway is an intracellular kinase signalling pathway which is commonly activated by receptor tyrosine kinases implicated in cancer. This may include c-MET, HER2 and IGF-1. The MEK/ERK pathway is activated in many cancers, including prostate cancer (146). High ERK expression has been suggested to be a prognostic factor for localized prostate cancer (147). However, due to the rarity of activating ras mutants in prostate cancer, there is relatively less interest in this pathway in prostate cancer than in cancers where activated ras mutants act as drivers, such as in colon and pancreatic cancer. Nonetheless, there remains considerable cross-talk between this pathway and other intracellular signalling pathways.

There is much overlap and significant cross-talk between kinases which activate the PI3K/Akt and the MEK/ERK signalling pathway(74). Pre-clinical studies suggest that concomitant activation of both PI3K/AKT and MEK/ERK pathways may signal more aggressive, resistant prostate cancer (146, 148-150). Mechanistically, the MEK/ERK pathway regulates the nuclear transcription factor YB-1 important in prostate cancer progression (147).

There is also interest whether co-targeting the MEK/ERK pathway may be done concomitantly with AR blockade. Activation of this pathway may result in phosphorylation and subsequent stabilization of the AR to maintain AR signalling (151). For example, pre-clinical studies suggest a synergist effect of targeting of both AR and signal transduction pathways such as PI3K/Akt and MAPK pathways (81, 152). Clinical studies into the effects of combined AR and MEK inhibition with trametinib in prostate cancer have recently started (eg NCT01990196).

1.8.2 Cellular Stress and Survival Pathways

Molecular chaperones have an important role in the cellular stress response through maintaining protein homeostasis and regulating pro-survival networks. Chaperone proteins stabilize intracellular proteins against against misfolding and aggregation during stress, as well as facilitating intracellular and compartmental transport (153). Co-targeting the stress response, activated by AR inhibition and mediated through stress-activated cytoprotective chaperones like clusterin or Hsp27, may create conditional lethality and improve outcomes (153, 154). In CRPC, two stress-activated cytoprotective chaperones, clusterin and Hsp27, are targets in ongoing clinical trials.

Clusterin exists in two forms, nuclear clusterin and secretory clusterin. Secretory clusterin(sCLU) functions as a cytoprotective chaperone which is up-regulated in CRPC. sCLU has been demonstrated to play a role in inhibiting endoplasmic reticulum stress, cytosolic protein aggregation and also inhibits mitochondrial apoptosis(155-157). Custirsen (OGX-011) is a second-generation antisense oligonucleotide against the clusterin mRNA. Preclinical studies demonstrate that OGX-011 potently suppresses sCLU levels *in vitro* and *in vivo* (158, 159). Further, co-targeting of sCLU and AR delays CRPC progression in pre-clinical models through inhibiting the adaptive stress response and regulating AR stability (160). In a Phase II clinical trial, a survival advantage of 6.9 months for custirsen plus docetaxel and prednisone over docetaxel and prednisone was seen(161), though Phase III results did not demonstrate an overall survival benefit. Heat shock protein-27 (Hsp27) is another abundant stress-induced cellular chaperone protein implicated with the AR signalling and treatment resistance (162, 163). OGX-427 is a second-generation antisense oligonucleotide against Hsp27 now in phase II trials as second line treatment in metastatic CRPC in combination with abiraterone.

Deregulation of normal cellular functions of apoptosis is one of the common characteristics of cancer. Bcl-2 is a regulator of apoptosis; several other anti-apoptotic family members include Bcl-2, Bcl-XL, Mcl-1, BCL-W and BFL-1(164) . The balance of cell survival or cell death is further regulated by multidomain pro-apoptotic proteins such as BAX, BAK and BOK. As in several advanced cancers,Bcl-2 gene expression it is up-regulated in CRPC(165), presenting a targetable oncogene.

1.8.3 Changes in the Tumour Micro-environment

With the increased understanding of the importance of the tumour microenvironment on the progression of CRPC, therapeutic strategies are emerging to target molecular originating from or acting within the adjacent tumor stroma (166, 167). This includes transcription factors such as Stat3 and cytokines such as NFκB and IL-6 which are implicated in CRPC progression(168).Notably, some current and novel targets for CRPC such as AR, IGF-IR and c-MET are active in both stromal and epithelial compartments (169-171). However, with the failure to date of several angiogenesis inhibitors in CRPC, agents targeting the microenvironment are likely best evaluated in rationale combination strategies with other treatments. For example, pre-clinical research suggests that IGF-IR blockade may enhance Src inhibition (172).

Hedgehog signalling is an important paracrine factor during organogenesis and appears to be de-regulated during prostate cancer progression. Sonic hedgehog secreted by the tumour appears to alter the tumour microenvironment to ultimately increase oncogenic Gli -1/2 transcription factors through paracrine signalling. Sonic hedgehog ligands signal via Patched-1 and results in the loss of the Smoothened repression on Gli-1 and Gli-2. Hedgehog signalling appears to be up-regulated following androgen deprivation conditions (173, 174). Preclinical data on TAK-441 and GDC-0449 (vismodegib) in CRPC models and an ongoing neo-adjuvant study of GDC-0449 may lead to clinical trials of these hedgehog signalling agents in CRPC patients(174, 175).

Another novel drug which targets the tumour microenvironment is the monoclonal antibody sibrotuzumab. It targets fibroblast-activated protein (FAP). This protein

expressed in cancer-associated stroma, but not normal stroma-associated with epithelial cancers. It is considered to play a role in tumour growth and proliferation(176).

Changes in the immune composition of the prostate tumour microenvironment are of significant interest given the effectiveness of immune checkpoint inhibition in renal and bladder cancer. Increased numbers of M2 polarized macrophages and myeloid derived suppressor cells appear important in maintaining an immune-suppressive phenotype and preventing activation of innate immunity. Tasquinimod, which targets the S100A9 protein which is a key surface regulator protein on myeloid derived suppressor cells recently completed a phase III trial of over 1200 patients where it demonstrated a significant improvement in radiographic progression free survival, but not in overall survival(177).

1.8.4 Neuroendocrine Transdifferentiation

Neuroendocrine prostate cancer (NEPC) represents a subset of prostate cancers which undergo radical genomic changes and are no longer AR-driven. It is possible the increased use of more potent AR-directed therapy such as enzalutamide may result in a greater prevalence of NEPC. Whether this type of prostate cancer arises from neuroendocrine cells located in the basal layer of prostate glands or are derived from prostate carcinomas which undergo a process of transdifferentiation is controversial.

Neuroendocrine prostate cancer is a separate entity from most cases of CPRC (178). Clinically, it remains a relatively rare and thoroughly aggressive phenotype. Disease progression appears entirely unrelated to the AR axis with patients usually identified through a disproportionately low PSA. Visceral and brain metastases are more common. Serum and tissue markers of chromogranin A, NSE and synaptophysin are

commonly elevated. Recently, protocadherin-PC has also been suggested to be a marker of neuroendocrine transdifferentiation(179).

At the genomic level, neuroendocrine prostate cancer demonstrates large scale differences from CRPC, with frequent chromosomal aberrations and large rearrangements. Few drivers of NEPC have been identified; several potential targets include Aurora kinase B, PEG10, DEK and BRN2(180, 181). Sequencing studies have identified overexpression and gene amplification of aurora kinase A and N-myc in 40% of NEPC vs 5% of prostate cancers(178). Phase II trials of MLN8237 are ongoing in men with elevated NEPC markers.

1.9 Current treatments for CRPC

There have been multiple new agents approved for treatment of CRPC in the last decade (182-184). This has impacted clinical practice significantly and changed the treatment landscape or CRPC. The following paragraphs summarize the currently approved treatments for CRPC in 2016.

1.9.1 Enzalutamide

AR antagonists function by competitively inhibiting the binding of the AR ligand (ie DHT), thereby inhibiting nuclear translocation and binding of the AR to DNA (185) Enzalutamide (previously known as MDV3100) is a potent AR ligand binding domain antagonist (185). It is significantly more potent than the previous drugs in its class, with an 5-8 fold high affinity for the AR compared to bicalutamide (185). Phase III clinical trials have demonstrated improvements in overall survival of 3-5 months, with a relative

decrease in the death rate of 30%. There have also been highly significant improvements in time to disease progression measured radiologically or symptomatically (182, 186). These results have prompted regulatory approval and the current use of enzalutamide as first line therapy in men with CRPC. Side effects of enzalutamide include fatigue, diarrhea and hot flashes. Seizures rarely occurred, with the majority of seizures occurring in patients having a pre-existing disposition to seizures.

1.9.2 Abiraterone

The inhibition of steroidogenic enzymes in prostate cancer dates back to the use of ketoconazole in advanced prostate cancer(187). Abiraterone is a steroidal inhibitor of CYP17, which is given as the form abiraterone acetate(AA) to improve oral absorption. Similar to enzalutamide, AA has been demonstrated in Phase III studies to improve overall survival by several months when given to men with CRPC as first-line therapy or following docetaxel treatment(183, 184) . Mineralocorticoid-related adverse events such as fluid retention, hypertension, and hypokalemia were more common in the AA group. Like enzalutamide, AA is also now used as first line therapy for CRPC.

In the absence of clinical trials, it remains unclear whether AA or enzalutamide is best selected as initial therapy for men with CRPC. With the later entry and regulatory approval of enzalutamide, there has been greater clinical experience with abiraterone. However, the inclusion criteria for the enzalutamide trials were broader, including men with visceral metastases. Aside from exclusion due to side effects, such as risk of seizure of prior side effects from prednisone, there remains little to guide clinicians as to the optimal choice. As well as comparative clinical trials, a further understanding of the

biology of resistance to these agents could prove to be useful in establishing the optimal clinical sequencing.

In the short term, it is clear both are effect agents, and they have changed the treatment landscape, as well as the patterns of resistance which develop following initial therapy for CRPC. It is notable that docetaxel, the prior first line of therapy demonstrated effectiveness when patients were re-challenged (188, 189). However, existing data do not suggest that following clinical progression on enzalutamide or AA that patients frequently respond when re-challenged with either agent(190-192).

1.9.3 Taxanes

Docetaxel and cabazitaxel are two taxanes approved for use in CRPC based on Phase III clinical trials. Taxanes function by stabilizing the dynamic polymerization of microtubules. The ability of microtubules to assemble and disassemble is critical for mitosis and therefore inhibiting microtubules preferentially halts rapidly dividing cancer cells. It also may inhibit androgen receptor (AR) signalling through its alteration of microtubule-associated AR cellular transport and nuclear translocation (193, 194). Docetaxel was the first agent outside of hormonal therapy which a demonstrated survival benefit in CRPC (22, 195).

Cabazitaxel is a newer taxane which was selected through pre-clinical studies which found it had the greatest activity against docetaxel-resistant cell lines *in vitro* and *in vivo*(196). Cabazitaxel improved overall survival by a median of 2.4 months in part previously treated with docetaxel. Cabazitaxel has a higher rate of adverse effects than docetaxel, particularly neutropenia and diarrhea (22, 23).

Resistance to taxane therapy may be mediated through overexpression of the multi-drug resistant P-glycoprotein efflux pump(197), mutations in the microtubule binding sites, and mutations in microtubule-associated proteins giving greater stability to cellular microtubule assembly (198, 199).

1.9.4 Bone Targeting Agents

Therapeutic targeting of the bone microenvironment addresses side effects associated with androgen deprivation therapy. Several clinical trials have now established new treatment options for patients with CRPC and should be used appropriately alongside lifestyle changes and calcium supplementation. Bisphosphonates were the first bone-targeting agents approved for men with metastatic prostate cancer. Zoledronic acid was approved based on studies which demonstrated an improvement in skeletal related events, though no survival benefit was observed (21, 25). More recently, denosumab has been approved has been approved for me with CRPC. Denosumab functions as a monoclonal antibody against receptor activator of nuclear factor kappa-B ligand, which prevents bone loss through the inactivation of osteoclasts. Further, denosumab also appears to have an effect on the metastatic niche, with a delay in the appearance of bone metastasis (200). Compared to zoledronic acid, denosumab appears to have superior potency, with a greater reduction in skeletal-related events (201).

Radiopharmaceuticals also target the bone metastatic environment. Historical radiopharmaceuticals such as Rhenium-186 and Samarium-135 demonstrated improved bone pain in patients with metastatic CRPC in small randomized trials (202, 203).

Strontium-89 is as a calcium mimetic and as such has a strong propensity for the bone microenvironment (204, 205). Radium-223 chloride is the newest and most effective radiopharmaceutical agent; it also acts as a calcium mimetic. In contrast to the aforementioned agents which emit beta-radiation, radium-223 emits alpha radiation. Alpha-radiation has a shorter penetration depth with higher energy and is therefore less toxic to the bone marrow. Bone marrow toxicity is a challenging toxicity in men with CRPC and bone metastasis who often have anemia. Notably, radium-223 has also demonstrated an improvement in overall survival in recent Phase III clinical trials of men with painful bone metastasis (206, 207).

1.9.5 Sipuleucil-T

The goal of immunotherapy is to boost the tumour suppressive response of the patient's own immune system. This approach has been validated in CPRC with the approval of sipuleucil-T. Phase III randomized trials demonstrate an overall survival benefit (208) in men with minimally or asymptomatic metastatic prostate cancer, though an effect on progression-free survival or PSA-response was not seen. The treatment consists of re-infusing patient's autologous peripheral blood monocytes and antigen-presenting cells which have been exposed *ex vivo* to the fusion protein of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor. However, the cost of this treatment remains a challenge to its implementation in many jurisdictions and it is not available in Canada.

1.10 Emerging Treatments for CRPC

The increased understanding of the molecular biology of CRPC has led to a proliferation of novel targeted therapeutics in clinical evaluation. Table 1.2 lists some of the non-AR targets which are in clinical evaluation in CRPC. These novel targets emerge as CRPC develops more genetic and epigenetic alterations accumulate over time(209). In addition, tumours acquire resistance through alternative pathways as a result of the selective pressure of current treatments. Accordingly, the potent AR-targeting agents abiraterone and enzalutamide will likely lead to more cancers that survive and proliferate through activation of alternate pathways. In the following paragraphs, we review a few key classes of therapies for CRPC in clinical development.

Table 1.2 Selected approved and experimental non-AR targeting therapeutics agents for CRPC. Source: clinicaltrials.gov.

	Stress response pathways	**Proliferative signal transduction targets**	**Immune Escape**	**Critical cellular proliferative components**	**Tumour microenvironment**
Targets	Clusterin Hsp90 Bcl-2 Hsp27	PI3K Akt mTOR Mu-opoid receptor eIF4E IGF-IR Her-2	Dendritic cells CTLA-4 PD-1	Microtubules PARP1 SERCA pump	Osteoclasts IL-11Ra RANK-L FAP Endoglin alpha V integrin VEGF/FGFR Neurotransmitters Somatostatin receptor
Approved therapeutics			Sipuleucil-T	Docetaxel Cabazitaxel	Denosumab Radium-223
Experimental therapeutics	OGX-011 OGX-427	BEZ235 BKM120 AZD5363 MK2206 AZD8186 Naltrexone ISIS 183750 Everolimus Temsirolimus Linsitinib Lapatanib	Ipilimumab BPX-201 BMS-936558 Pidilizumab	Tesetaxel Patupilone Ixabepilone G-202	Sibrotuzumab TRC-105 EMD 525797 BMTP-11 Dovitinib Bevacizumab Pazopanib Phenelzine Pasireotide

1.10.1 Androgen Receptor Antagonists

Several novel AR inhibitors are currently in various stages of development(210). ARN-509 is an AR inhibitor in Phase II trials which is structurally similar to enzalutamide with reportedly greater *in vivo* activity (211). ARN-509 inhibits nuclear translocation of the AR as well as AR binding to androgen response elements (AREs). It has a potential advantage as to the risk of seizures compared to enzalutamide because of its slightly different chemical structure which changes its permeability to the blood-brain barrier. A novel strategy against the AR includes inhibitors of the N-termimus domain (NTD) of the AR(212). This strategy is appealing given the recent evidence suggesting the importance of androgen receptor splice variants in enzalutamide resistance(33), though their mechanistic importance to resistance remains to be fully elucidated. Splice variants lack the ligand-binding domain and appear to constitutively activate AR-related genes with only the NTD and the DNA-binding domain present. EPI-001 is a small-molecular inhibitor which specifically targets the N-terminus domain and does not block ligand binding (213). Other investigational methods to abrogate AR-driven growth in CRPC cells include antisense approaches, and second-site androgen receptor antagonists, such as targeting the BF-3 domain of the AR (214, 215).

1.10.2 Steroidogenesis Inhibitors

Several novel CYP17-20 inhibitors are under evaluation. These next-generation inhibitors selectively inhibit the lyase-function of the CYP17A1 enzyme. As a results of

less hydroxylase inhibition with lyase-selectivity, there is less cortisol suppression and subsequently less mineralocorticoid suppression. This specificity is hoped to lead to less mineralocorticoid-related side effects and may avoid prednisone co-administration.

Orteronel (TAK-700) is a more selective, but less potent 17,20-lyase activity inhibitor compared to abiraterone which has undergone two phase III trials(216). The first study evaluated orteronel plus prednisone versus prednisone in patients previously treated with chemotherapy and found significant improvements in radiographic progession free survival, but did not meet its primary endpoint of overall survival (217). The subsequently completed trial in patients who were chemotherapy naïve also did not demonstrate a significant improvement in overall survival (218). As a result, development of this agent has ceased, though evaluation of this agent in a trial of men with castrate sensitive prostate cancer is ongoing.

Galeterone (TOK-001) is another novel lyase-selective CYP17 inhibitor in development. In addition to the CYP17 inibition, it has been reported to increase AR degradation (219, 220). Results from its Phase I and II studies demonstrated significant PSA responses and a Phase III trial is expected to begin by the end of 2016. VT-464(named seviteronel in late 2015) is another novel Cyp450 inhibitor which has specificity for the lyase function of this dual hydroxylase/lyase enzyme. The first published pre-clinical results in prostate cancer models are discussed in Chapter 2.

1.10.3 Bromodomain Inhibitors

The bromodomain and extraterminal (BET) family of proteins are important in the epigenetic regulation of gene transcription. The BET proteins function as adaptor proteins

to specific acetylation marks on the chromatin; they form part of a class known as “readers”. BET inhibitors such as JQ1 and I-BET have been shown to have anti-proliferative activities in multiple malignancies. In particular, JQ1 has been shown to indirectly target the transcription factor c-myc, which otherwise is not a very druggable target (221). BRD4, a BET family protein, has been demonstrated to bind directly to the AR and inhibit the AR transcriptome(222). There are now ongoing trials of several BET inhibitors in prostate cancer patients (eg, clinicaltrials.gov IDs NCT01587703, NCT02711137, NCT02698176 and NCT02705469) Based on a recent pre-clinical study, one trial is evaluating the combination of BET inhibition and enzalutamide in CRPC (NCT02711956) (223).

1.10.4 PI3K/Akt/mTOR Inhibitors

Multiple new inhibitors of this pathway have been developed in the last decade (224). The novel agents now in development (Table 1.3) differ significantly from the only agent, perifosine, which has completed clinical trials in prostate cancer. Perifosine did not show any activity as monotherapy agents in CRPC in two small clinical trials (225, 226). Perifosine is an alkylphospholid with allosteric Akt inhibitor properties. Akylphospholipids are known to accumulate in cell membranes, but the exact reason for the anti-cancer activity is unclear, but this is presumed due to its capacity to inhibit the Akt pathway.

Table 1.3 Inhibitors of the PI3K/Akt/mTOR pathway in clinical evaluation in prostate cancer.

Drug		Phase	Regimen	Trial Registry Number
AZD8186 (AstraZeneca)	PI3K β and δ inhibitor	I	Monotherapy	NCT01884285
BKM120 (Novartis)	Pan-PI3K inhibitor	I	+Abiraterone acetate	NCT01634061

Drug		Phase	Regimen	Trial Registry Number
		I	+Abiraterone acetate	NCT01741753
		II	Monotherapy	NCT01385293
PX-866 (Oncothyreon)	Pan-PI3K inhibitor	II	Monotherapy	NCT01331083
BEZ235 (Novartis)	PI3K/mTOR inhibitor	I	+Abiraterone acetate	NCT01634061
GDC-0980 (Genetech)	PI3K/mTOR inhibitor	I/II	+Abiraterone acetate	NCT01485861 2011-004126-10
GSK2636771	PI3K inhibitor	I	+ enzalutamide	NCT02215096
LY3023414	PI3K/mTOR inhibitor	I	+ enzalutamide	NCT02407054
AZD5363	Akt inhibitor	I	Monotherapy	NCT01692262
(AstraZeneca)		II	+ enzalutamide	2013-004091-34
		I/II	+ docetaxel	NCT02121639
GDC-0068 (Genetech)	Akt inhibitor	I/II	+Abiraterone acetate	NCT01485861 2011-004126-10
MK2206 (Merck)	Akt inhibitor	II	+ bicaluatmide	NCT01251861
Everolimus (Novartis)	mTORC1 inhibitor			
		II	+ pasireotide (somatostatin)	NCT01313559
		I/II	+Docetaxel, bevacizumab (VEGF inhibitor)	NCT00574769
		I/II	+Carboplatin, everolimus, and prednisone	NCT01051570
		II	+Bicalutamide	NCT00814788
Temsirolimus (Wyeth)	mTORC1 inhibitor	I/II	+ bevacizumab	NCT01083368
		I	+Vorinostat	NCT01174199
		II	Monotherapy	2011-002087-24
		I/II	+Docetaxel	NCT01206036 2010-018370-21
		I/II	+Cixutumumab	NCT01026623

Newer PI3K/Akt/mTOR pathway inhibitions are small molecule reversible catalytic site inhibitors. Common adverse effects inhibitors include insulin resistance, hyperglycemia, nausea and mood alterations. PI3K inhibitors include non-specific and isoform-specific inhibitors. The three isoforms (p110α, β, and δ) of Class IA PI3K may play relatively different roles in the progression of prostate cancer(71). Isoform-specific inhibitors in prostate cancer aim to inhibit only the PI3Kβ and δ isoforms to decrease insulin resistance and hyperglycemia associated with PI3K α inhibition(227). Inhibitors of

Akt may act as competitive inhibitors of its ATP-binding pocket (eg AZD5363, ipatasertib); isoform specificity is difficult to achieve due to high homology of the binding sites between isoforms(228).

BKM120 is an oral pan-PI3K inhibitor with reported clinical data in advanced solid tumours, including prostate cancer(229). Among 31 patients in the phase I trial, one had a partial response and 12 (52%) had stable disease. Treatment-related adverse events include rash, hyperglycemia, diarrhea, anorexia, mood alteration, nausea and fatigue. Reversible neuropsychiatric adverse events may be due to BKM120 crossing the blood-brain barrier and inhibiting PI3K/Akt/mTOR signalling modulating neurotransmitter concentrations. Similarly, results with the oral pan-Akt inhibitor AZD5363 showed two partial responses out of 92 patients in two Phase I trials(133). Notably, both patients had mutations in Akt1 or PI3KCA, suggesting mutations in these genes could be predictive of response.

Results to date with inhibition of downstream TORC1 or TORC2 in prostate cancer have been disappointing. Rapalogs such as everolimus, temsirolimus, and ridaforolimus have had poor results as single agents in prostate cancer clinical trials (131, 230). This may be due in part to TORC2-mediated feedback on Akt(74). Dual TORC1/TORC2 inhibition has demonstrated improved inhibition of downstream effectors such as the eIF–4E protein translation complex not seen with mTORC1 inhibition (231). Dual mTORC1/mTORC2 inhibitors have entered clinical testing for advanced solid tumours, including prostate cancer(232).

1.10.5 DNA-Damage Repair Inhibitors

PARP inhibitors represent a class of therapeutics under development for many cancers including prostate cancer; examples include veliparib and olaparib. The enzyme poly-ADP ribose polymerase (PARP) is responsible for repairing single strand breaks in DNA. Inhibition of this enzyme leads to alterations in the ability of DNA replication to occur, causing cell death (233). It may induce synthetic lethality in tumours with BRCA1 or BRCA2 mutations, both of which are implicated in more aggressive prostate cancer (234). BRCA1 and BRCA2 proteins are responsible for repairing double-strand DNA breaks in DNA. With the inhibition of PARP, single-strand breaks may become non-repairable (and thus lethal) double-strand breaks in BRCA1/2 mutant cancers. Similarly, a synthetic lethality using DNA-damage repair inhibitors has also been proposed to apply to the common PTEN-deletion CRPC tumours, which are reported to have defects in homologous recombination (235). Therefore, this represents a possible tailored therapy for patients with these mutations. A clinical trial is also underway for patients with ETS fusions, based on pre-clinical data suggesting the involvement of TMPRSS2 gene fusions with DNA repair cellular machinery, including PARP1 and topoisomerase II. (236)

Results of the PARP inhibitor olaparib as a single agent against CRPC have been promising (237). In a phase II trial in 50 patients who had received multiple lines of CRPC therapy, overall 30% of patients had a response to therapy, with 12 patients having a response beyond 6 months. What was most notable about the trial was that among patients who had genomic alterations in DNA-repair genes, the response rate was 88%, suggesting that alterations in these genes could be used to select patients for therapy. This suggests the future of effective treatments for resistant CRPC may lie in the ability

to identify which biologic aberration the tumour possess and treat with a targeted agent accordingly.

1.10.6 Immunotherapy

Ongoing immunotherapies under investigation in prostate cancer include the prostate cancer vaccines and immune checkpoint inhibitors. The PROSTVAC-VF vaccine aims to boost natural immunity against tumour cells in CRPC. The vaccine consists of transgenes for PSA, as well as three co-stimulatory molecules (B7.1, leukocyte function-associated antigen-3 (LFA-3), and intercellular adhesion molecule-1 (ICAM-1)) to enhance immune memory against the weakly immunogenic PSA antigen (31). A priming injection is followed by monthly booster injections. Checkpoint modulators of the immune system aim to remove the negative feedback signals in the patient's own immune system, thereby decreasing the immune system's tolerance of tumour antigens. PD-1 is an immune inhibitory receptor expressed on T cells which modulates the immune response to prevent activation of auto-antigens. Blocking this receptor reduces some of the negative self-regulation of the immune system. Despite impressive results in other cancers, results in prostate cancer to date have been disappointing, perhaps related to the low levels of PD-1 expression in prostate cancer (238).

1.11 Biomarkers Platforms in CRPC

With a host of various therapies in development, it is increasingly clear that the development of predictive biomarkers will be essential for implementing novel treatment for resistant prostate cancer. While prognostic biomarkers such as PSA give information

on the natural history of prostate cancer, predictive biomarkers are those which indicate what kind of response can be expected from a certain therapy. Biomarkers may be based on clinical features, imaging features, though the majority of current investigations focus on molecular or biologic markers which are mechanistically related to the therapy.

Several biomarker platforms have emerged in recent years for use in advanced prostate cancer. Though there have been many new treatments introduced to treat CRPC over the last decade, the optimal timing and sequencing of these agents is unknown. Currently, treatments are administered sequentially based on whether the prior treatment has failed. As multiple lines of treatment are given, CRPC often becomes more heterogeneous. Biopsies of tumours are difficult to do as most metastases are often osseous. Therefore, liquid biopsy approaches are of great interest as biomarker platforms to facilitate longitudinal and accessible indices of tumour progression.

Various platforms for biomarkers are currently in development. The majority utilise blood based biomarkers, since this may assist in minimizing the effect of heterogeneity, though urine or semen-based biomarkers may take advantage of the prostate's location in the male genitourinary system. Biomarker platforms may use various levels of PCR technologies, mass-spectroscopy or enzyme-base biochemical assays. To obtain information of tumours in real time and to avoid invasive and often difficult biopsies of tumor tissue, platforms aim to assess circulating tumour cells, cell-free tumour DNA, exosomes or other constituents in the blood or urine such as cytokine markers or steroids. Blood-based biomarker platforms such as circulating cell free DNA (cfDNA) and circulating tumour cells have potential advantages of averaging intertumoral heterogeneity between lesions. However, it is not clear if the intrapatient tumoural

heterogeneity is actually large, with several studies now suggesting low intrapatient heterogeneity (72, 239) exists between metastatic lesions.

1.12 Prostate Cancer Models

1.12.1 LNCaP Cell Lines

Compared to many other cancers, there are relatively few prostate cancer cell lines widely available for study. LNCaP cells were derived from the supra-clavicular lymph node of a 50 year Caucasian man with metastatic prostate cancer(240).It is the most widely used model of human prostate cancer. It expresses the AR and PSA protein and is very sensitive to androgen levels. The androgen receptor harbours a point mutation in the ligand binding site of the ligand binding domain, T877A, which permits promiscuous activation of the AR by progesterone and related steroids(241). Various derivatives of this cell line have been created, included C4-2, LNCaP-95. C4-2 cells were generated in the early 1990s at MD Anderson Cancer Centre through passage of LNCaP cells co-inoculated with a bone stromal cell line in castrated mice. C4-2 cells are derived from the second sub-line thus generated and represented the only sub-line which was capable of forming colonies in soft-agar and also grew upon inoculation in castrate mice(242). Other androgen-independent sub-lines of LNCaP included LNCaP-AI(243) and LNCaP95(244).

1.12.2 22RV1 Cell Line

The human prostate cancer 22RV1 cell line is unique in that it has very high levels of constitutive production of AR splice variants (245). This cell line was derived from the human prostate xenograft CWR22R (246). It grows in castrate conditions and is weakly

responsive to androgens. It is a PSA producing cell line and forms rapidly growing xenografts. It harbours the H874Y mutation in the ligand binding domain of the AR (241). Recently, it has been demonstrated that this cell line has an incorporated xenovirus which may influence its' behavior (247).

1.12.3 Other Prostate Cancer Cell Lines

Other widely-used prostate cancer cell lines include VCaP, DU145 and PC-3. PC-3 cells are widely used, but do not harbour the AR, nor do they produce PSA. Similarly, DU145 cells are AR and PSA-negative. VCaP, which is derived from a vertebral metastasis, is androgen dependent, AR positive and PSA producing. It thus represents a more valid model for the PSA-producing nature of clinical prostate cancer. However, among the cell lines, it is the most difficult to maintain in culture, and it possess a relatively slow doubling time. LuCAP and LAPC cell lines were derived in Seattle and Los Angeles, respectively, but are not available from the American Tissue Culture Collection (ATCC). LuCAP xenografts were derived from a rapid autopsy program; however, these various models are limited as there are no stable cell lines produced; xenograft tumours must be passaged in mice.

1.12.4 Animal Models

Several *in vivo* models have been developed for prostate cancer. One of the earliest was the Shionogi model, which is actually a murine breast cancer cell line. Its murine compatibility and dependancy on androgens made it a convenient model to evaluate the effects of castration and AR-antagonists. Studies using the Shionogi model

were important in the early development of intermittent androgen deprivation therapy as a treatment option for men requiring systemic therapy (248).

Xenograft models in immunocompromised mice are commonly performed using common human prostate cancer cell lines. Patient derived xenografts represent are similarly developed and may recapitatulate key events in prostate cancer progression (249). The most widely used transgenic model is the transgenic adenocarcinoma mouse prostate (TRAMP) model, from which arises the C2 derived murine prostate cancer cell line. In this model, mature tumours are predominantly neuroendocrine-like and tend to metastases to lymph nodes (249). A newer transgenic model, referred to as the RapidCaP model, uses surgical injection of lentivirus to result in prostate-specific transformation of prostate cancer (250). The model has the advantage of being faster than transgenic models, which often take 6 months to develop metastases.

1.12.5 Generation of Enzalutamide-Resistant Cell Lines

As enzalutamide is more cytostatic than cytotoxic *in vitro*, it was decided to derive resistant models using xenograft models at the Vancouver Prostate Centre to avoid the possibility of very slow cell growth impeding development of resistant models. Briefly,

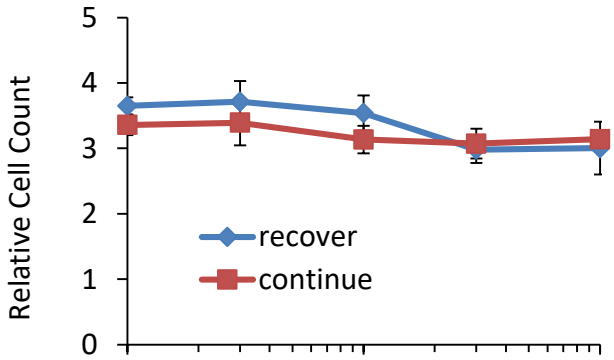


Figure 1.7 MR49C cells are resistant to ENZ. 5X104cells were seeded in 12-well plates, then treated with different concentrations of ENZ (0, 0.1, 0.3, 1, 3, 10uM). After 72 hours exposure, culture medium changed fresh media alone (Recover) or with ENZ (continue).

LNCaP cells were injected to mice and when the serum PSA reached 50ng/ml mice were castrated. When the serum PSA again reached 50ng/ml 5-6 weeks later, tumors were called castrate resistant. V16D cells were derived from an LNCaP xenograft resistant to castration. Tumors were excised and cell lines were generated in androgen-free media. For MR49C and MR49F cell lines, when LNCaP xenografts reached the castrate resistant state, they were treated with 10mg/Kg of ENZ with a subsequent PSA decline to low serum values. When the serum PSA returned to castrate resistant levels or higher, tumors were called ENZ-resistant. These tumors were then passed 3 times in castrate mice treated with ENZ and cell lines were generated. MR49C and MR49F were identified as enzalutamide-resistant cell lines which were AR positive and PSA producing.

1.13 Scope of Thesis

1.13.1 Hypotheses

- Targeting of steroidogenesis will remain a viable treatment strategy in ENZ-resistant prostate cancer
- Selective pressure by enzalutamide will increase the importance and efficacy of combination approaches against PI3K/Akt and MEK/ERK signalling pathways in ENZ-resistant prostate cancer
- Combination strategies will be critical to effectively delay development of resistant prostate cancer

1.13.2 Rationale and Specific Aims

The AR remains a critical target in prostate cancer. Recently, the approval of enzalutamide, and AR antagonist, and abiraterone, a CYP17 inhibitor, have strengthened the view that the AR is also important in CRPC. Nonetheless both these agents are never curative, with the development of resistance inevitable. With enzalutamide and abiraterone now in widespread use, there is a need to identify and develop treatment strategies which may be effective to treat or delay the development of resistant prostate cancer.

The development of ENZ-resistant models as detailed above permits the evaluation of novel strategies. Prior work in our laboratory has demonstrated that these resistant cell lines remain dependent on the AR pathway and sensitive to novel AR antagonists (251). Further, sequencing results (not shown) suggest that mechanisms may be similar to those which have been identified in CRPC, which include activation of PI3K/Akt and MEK survival pathways. In the remaining chapters, we will evaluate several specific aims to test our general hypotheses.

1. Evaluate the efficacy of a novel, selective CYP17A1 inhibitor in models of CRPC and ENZ-resistant prostate cancer

a. Evaluate effect on AR pathway activation *in vitro* and *in vivo*

b. Evaluate changes in steroidogenesis and androgen synthesis *in vitro* and *in vivo*

c. Compare efficacy and inhibiting tumour growth with abiraterone

2. Evaluate the efficacy of co-targeting MEK and Akt pathways in ENZ-resistant prostate cancer

a. Compare relative growth inhibition in models of various stages of prostate cancer progression

b. Evaluate *in vivo* efficacy of the combination compared to monotherapy approach in different xenograft models

3. Evaluate PI3K or Akt inhibition as a strategy against ENZ-resistant prostate cancer

a. Investigate co-targeting approaches involving direct or indirect inhibition of the AR pathway

b. Evaluate combination AR and PI3K/Akt pathway inhibition in various stages of the LNCaP xenograft models

In addressing the *first* aim, we demonstrate in **Chapter 2** that a novel lyase-selective CYP17 inhibitor VT-464 (now known as seviteronel) demonstrates superior suppression of the AR pathway compared to abiraterone, the only currently-approved CYP17 inhibitor for prostate cancer. Moreover, we discovered novel direct AR-antagonism of VT-464, and corroborate prior reports that abiraterone also is a direct AR antagonist. Overall, the data established in our ENZ-resistant models was critical in the launching of multiple Phase II clinical trials of this agent in men with ENZ-resistant and abiterone-resistant CRPC. Notably, our results demonstrating superior results with once-daily dosing and excellent suppression of the androgen synthesis correlate with the initial clinical results of this agent. The publication of this work represents the first pre-clinical report of this novel agent (Mol Cancer Ther Jan 2015)(252).

In **Chapter 3**, we assess the *second* aim of this thesis and evaluate whether combined inhibition of both MEK and Akt pathways is an effective strategy in prostate cancer. With both resistance pathways relevant to advanced prostate cancer, this combination was not previously evaluated in prostate cancer models. In our various *in vitro* cell lines and using different inhibitors of both pathways, we find only moderate synergy following combined blockade of both pathways. Evaluation at various stages of prostate cancer using different LNCaP-based models and in both MR49F and 22RV1 xenografts suggests that the combination is best used only in tumours which possess activation of both pathways. Analysis of xenograft tumour samples suggests that in some cases, activation of the MEK pathway may explain a poor response to AKT inhibitor monotherapy. Overall, our published results emphasize the need for biomarker characterization of both pathways to justify combination therapy (PLoS One 2016) (253).

Using novel PI3K (AZD8186) and Akt (AZD5363) inhibitors from AstraZeneca, we evaluated their efficacy in the LNCaP CRPC xenograft model. Together with *in vitro* data, we demonstrate on-target activity of both agents (**Chapters 4 and 5**). In combination with enzalutamide, we demonstrated never-seen-before efficacy when combining AZD5363 with enzalutamide in the LNCaP CRPC model, with sustained regression of tumours (**Chapter 4**). Our published results (Eur Urol 2015)(254) spurred the development of a multi-national Phase II clinical trial of AZD5363 plus enzalutamide in men with CRPC. Evaluation of this combination given at time of castration in the LNCaP model demonstrate an even more dramatic response, suggesting this combination is most effective when given earlier in the course of the disease.

With the recent development of bromodomain inhibitors which inhibit both the AR and myc, we evaluated a rationale for combining PI3K inhibition with the bromodomain inhibitor JQ1(**Chapter 5**). Evaluation of our AZD8186 treated LNCaP CRPC tumours demonstrated high levels of myc, which we identified as a potential mechanism of resistance. We confirm *in vitro* upregulation of myc mRNA and protein levels are induced by PI3K/Akt inhibition. Gene profiling and *in vitro* experiments however suggest that there is not significant synergy with this approach. This work has been presented at national and international conferences with submission for publication expected shortly.

In the last chapter (**Chapter 6**), we discuss the findings in previous chapters and provide the clinical relevance to our findings. In particular, we discuss the pertinence of model systems, the need for development of biomarkers and the important of correlative science components being integrated into clinical trials. Finally, we also discuss the relevance of our findings to further research in the field of resistant prostate cancer.

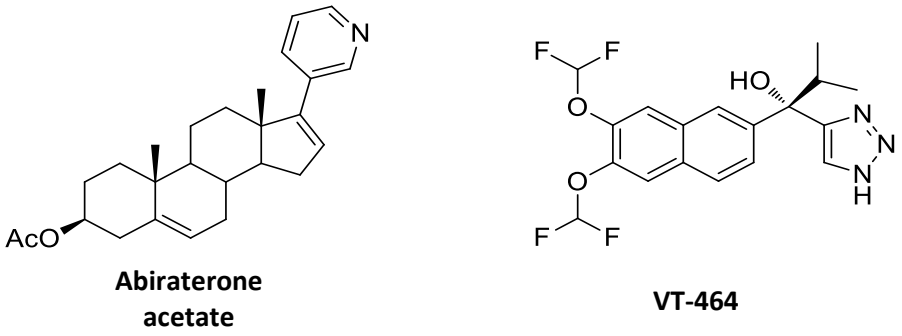
2 Anti-Cancer Activity of a Novel CYP17A1 Inhibitor in Pre-clinical Models of Castrate Resistant Prostate Cancer

1.1 Introduction

Recent clinical trials have clearly established that castrate resistant prostate cancer (CRPC) continues to remain sensitive to agents that inhibit the AR pathway (182, 184). Targeting androgen biosynthesis in CRPC dates back to the use of ketoconazole almost 25 years ago(255). More recently, abiraterone acetate (AA) in combination with prednisone has demonstrated improved overall survival (OS) in both pre- and post-chemotherapy patients with CRPC in phase III trials (183, 184). AA specifically and irreversibly inhibits both CYP17 hydroxylase and lyase (256). Inhibition of 17□-hydroxylase by AA prevents the synthesis of glucocorticoids and produces side-effects due to mineralocorticoid excess, which are partially suppressed by co-administration of the cortisol replacement, prednisone(257). The development of selective CYP17A1 lyase inhibitors has potential to obviate the need for concomitant prednisone administration in patients which is associated with its own toxicities.

CYP17A1 is the rate-limiting enzyme in the biosynthesis of androgens. CYP17A1 is a dual function enzyme with both17-hydroxylase and 17, 20 - lyase functions sharing the same active site, which makes selective therapeutic inhibition of the lyase function more challenging. Inhibition of the hydroxylase function in patients results in an ACTH feedback-mediated mineralocorticoid excess which can be suppressed through prednisone administration. In addition to avoidance of side effects of prednisone, avoiding prednisone may have potential oncologic benefits in patients (258, 259).

VT-464 is a novel small molecule CYP17A1 inhibitor with selectivity for the 17, 20-lyase activity of this dual enzyme (260-262)(Figure 2.1). The objective of this study was to evaluate the anti-cancer activity of VT-464 compared to abiraterone in pre-clinical models of CRPC. With the use of potent AR pathway inhibitors shifting earlier in the CRPC treatment paradigm, we used both a CRPC cell line model (C4-2) and two enzalutamide (ENZ)-resistant cell lines (MR49C, MR49F) to model advanced disease responses to this novel inhibitor *in vitro* and *in vivo*.



Abiraterone acetate **VT-464**

Figure 2.1 Molecular structure of abiraterone acetate (AA) and VT-464, a highly-selective 4-(1,2,3-Triazole)-Based P450c17a 17,20-Lyase Inhibitor.

1.2 Materials and Methods

2.1.1 Materials

Stock solutions of testosterone (T) (Sigma) 4-androstene-3,17-dione (Sigma), 4-pregnen-17-ol-3,20-dione (Steraloids, Inc.), 5α-androstan-17β-ol-3-one (Sigma), dihydrotestosterone (DHT) (Sigma), 5β-pregnan-3α-27-diol-20-one (Steraloids), 5β-pregnan-3,20-dione (Steraloids), androsterone (Aldrich), cortisol (Sigma), pregnenolone (Sigma), progesterone (Sigma), R1881 (DuPont), and were prepared in 100% methanol as MS standards. VT-464, abiraterone, enzalutamide, and TAK-700 were provided by

Viamet Pharmaceuticals, Inc.; stock solutions were prepared in DMSO. Abiraterone acetate (MedChem Express) was used for *in vivo* evaluations. VT-464 design and synthesis has been described elsewhere(261).

2.1.2 *In Vitro* Models

C4-2 cells were provided as a gift from Dr. Leland Chung and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2013. MR49C and MR49F cells were derived from LNCaP cells through serial xenograft passage in ENZ-treated mice as described previously(251). All cell lines were treated in RPMI 1640 in 10% charcoal-stripped serum (CSS; Hyclone), with 10μM ENZ present in the media of MR49C and MR49F cells. For all 6-day experiments, media with corresponding treatments were changed at 72 hours.

2.1.3 *In Vivo* Models

All animal experimentation was conducted in accordance with institutional standards and the Canadian Council of Animal Care (CCAC). Male athymic mice (Harlan Sprague-Dawley, Inc.) were castrated 1-2 weeks prior to tumour inoculation with enzalutamide 10mg/kg being started the day prior to inoculation and continued until the total tumour volume was >200mm^3. MR49F cells were grown in both flanks of mice for the pharmacodynamic study and in the left flank for the full study, similar to previous experiments(251). ENZ 10mg/kg was given on a 5 day on, 2 day off cycle starting the day of tumour inoculation and stopped at the time of treatment initiation. PSA levels were measured by tail vein sera samples weekly using the Cobas automated enzyme

immunoassay (Montreal, Québec). Tumor measurements were made twice weekly using calipers (volume = length x width x weight x 0.5326). Treatments began once the total tumour size exceeded 200mm^3. For the pharmacodynamic study, tumors were collected after 3-10 days (for each treatment: 2 mice after 3 days, 1 after 7 days and 2 after 10 days) and fragments were either immediately frozen in liquid nitrogen, fixed in formalin or preserved in RNAlater (Ambion). Treatments were cycled for 5 days on, 2 off. All xenograft tumors were harvested approximately 3 hours after their last treatment dose. Mice were sacrificed when tumour volume exceeded 1500mm^3 or loss of >20% body weight.

2.1.4 Steroid Analysis

Cell pellets and tissue homogenates were extracted 1:4 (v:v) with 60/40 hexane/ethyl acetate (hex/EtOAc) and media with EtOAc. Extracted steroids were dried (CentriVap) and reconstituted in 50-100 µL of 50 mM hydroxylamine, incubated 1hr at 65˚C and the resulting oximes analyzed using a Waters Aquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer. Separations were carried out with a 2.1x100mm BEH 1.7µM C18 columns, mobile phase water (A) and 0.1% formic acid in acetonitrile (B) (gradient: 0.2min, 20%B; 8min, 80%B; 9-10min, 100%B; 10.2min, 20%B; 12min run length). All data was collected in ES+ by multireaction monitoring (mrm) with instrument parameters optimized for the m/z's and corresponding fragments of the oxime-steroids. Data processing was done with Quanlynx (Waters) and exported to Excel for addition normalization to weights and volumes as required. Deuterated T and DHT were used as internal standards (IS) and a curve of 6 calibration standards (0.01-

10ng/ml) used for quantification (R2> 0.98). Recoveries were greater than 80% including extraction, conversion and matrix effects. The extraction protocol was found to also be effective for VT-464 and ABI and they withstood the derivatization procedure essentially intact, however some conversion of ABI to DHEA was observed (<0.01%) rendering samples very high in ABI unusable for that endpoint. Deuterated T was used as internal standard and a curve of 6 calibration standards (0.005-10μM, VT-464; 0.0015-3μg/ml, ABI) used for quantification (R2> 0.98). Samples were diluted as needed with IS blank to be within calibration range.

2.1.5 PCR and Western Blots

Steroidogenic enzyme RNA quantification in tumour samples was assayed by quantitative reverse transcription-PCR (qRT-PCR) with primers for STAR, HSD3B2, CYP17A1, HSD17B3, SRD5A1 and AKR1C3. Reactions were conducted with 0.4 μL of RT-PCR cDNA, 0.8 μL each of forward and reverse primers (3μM), 2 μL nuclease-free water (Ambion), and 4 μL Roche SYBR Green qPCR MasterMix. Triplicates of samples were run on the default settings of the ABIViia7 real-time PCR machine. For western blot analysis, cell pellets or homogenized tumour tissue was processed in RIPA buffer (50 mM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mM NaCl and 1× Roche complete protease inhibitor cocktail). 30-50ug of protein were loaded and run on gel electrophoresis. After washing thrice with washing buffer, membranes were incubated with Alexa Fluor 680 or 800 secondary antibodies (Invitrogen) for 1h. Detection of specific bands with their densitometric quantification was done using the ODYSSEY IR imaging system (Li-COR Biosciences). AR (sc-816 / sc-7305), PSA (sc-7638 / sc-69664), and

CYP17A1 (sc-46084) antibodies were purchased from Santa Cruz Biotechnology and Vinculin (V 9131) with an antibody from Sigma-Aldrich (St. Louis, MO).

2.1.6 AR Transactivation Assays

C4-2, MR49C or MR49F cells were plated on 6 well plates and transfected with 1.0 µg per well of ARR3-Luciferase-Plasmid using lipofectin (4.5uL per well; Invitrogen Life Technologies, Inc.) overnight in serum-free media. Treatments in 10% CSS RPMI 1640 with VT-464, ABI or DMSO control +/- 0.1nM R1881 (PerkinElmer) were performed at indicated times. Luciferase activity (relative light units) was measured using a microplate luminometer (Tecan) in duplicate and normalized to the cell lysate protein concentration. All experiments were carried out in triplicate.

The AR agonist / antagonist activity of VT-464, abiraterone, and TAK-700 was assessed at Indigo Biosciences (State College, PA) using CHO cells transfected with an expression vector that encoded a hybrid receptor comprised of full length wild-type AR and an AR response element functionally linked to firefly luciferase. Reporter cell AR response was validated using 6α-fluoro-testosterone as an agonist (EC50 = 60 pM). For antagonist assays (n = 3), cells were co-mixed with 2X the EC80 of 6α-fluoro-testosterone and the CYP17 inhibitor. Per cent antagonist inhibition was calculated as: 100*(1-[Average relative light units (RLU) test compound / Average RLU EC80 agonist). Dose-response non-linear curve-fits were generated using GraphPad Prism software and IC50s were calculated.

2.1.7 Statistical Analysis

Tumor growth velocity was calculated using linear regression of the log tumour volume over time. Student t-test was used to compare means. Means were plotted +/- SEM. Significant differences ($p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.00$) were assessed using a Student's t-test. Kaplan-Meier curves compared overall (OS) and cancer-specific survival. Cancer-specific survival was defined by the time from treatment until animal sacrifice for tumour size exceeding endpoint.

2.2 Results

2.2.1 VT-464 Suppresses the AR Signalling Pathway

As VT-464 targets CYP17A1 lyase-activity, we began by confirming the presence of CYP17A1 protein in the cell lines selected for study. Surprisingly, protein levels of CYP17A1 in LNCaP cells were not previously demonstrated in the literature. Unsurprisingly, protein levels were low and required immunoprecipitation to enrich prior to western blotting (Figure 2.2)

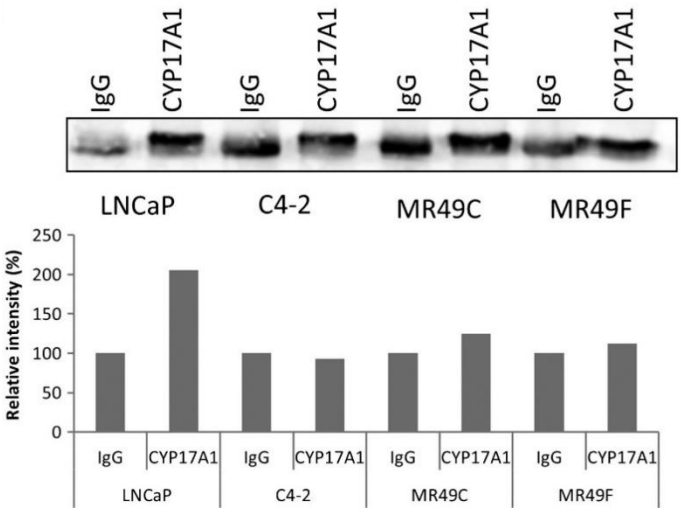


Figure 2.2 Immunoprecipitation demonstrating CYP17A1 protein in indicated LNCaP based cell lines with densitometry quantification below.

To evaluate the activity VT-464 on AR signalling pathway, we first analyzed its effect on AR transactivation using an ARR3 luciferase reporter assay following treatment with 1, 5, and 10uM of ABI, VT-464 or control for six days in androgen-depleted media. In C4-2 cells, both ABI and VT-464 significantly decreased AR-transactivation (Figure 2.3). At the 1uM and 5uM doses, this was significantly greater with VT-464 compared to ABI ($P<0.01$). Notably, in the ENZ-resistant MR49C and MR49F cell lines, only VT-464 demonstrated decreases compared to control, while ABI was similar to controls or increased transactivation. With VT-464 treatment, a dose-dependent trend in decreasing transactivation was also observed in both MR49C and MR49F cells. Similar results were observed for all cell lines and doses after 72h of treatment (data not shown).

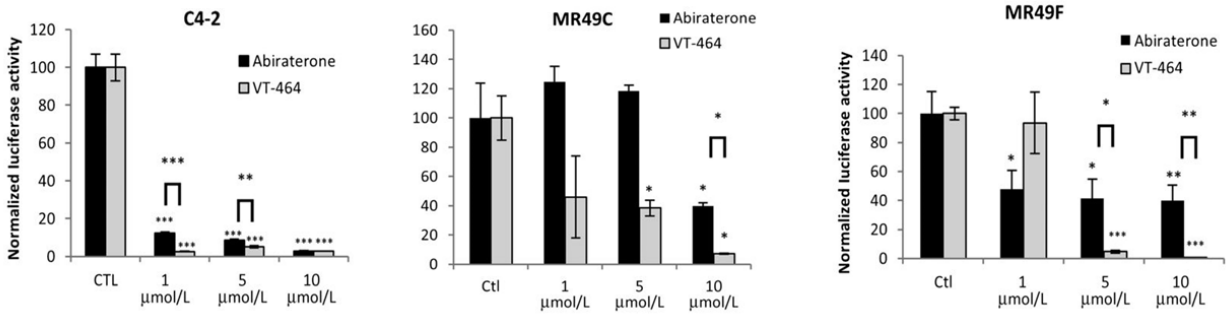


Figure 2.3 AR-transactivation following abiraterone and VT-464 treatment. C4-2, MR49C and MR49F cell lines were transfected with an ARR3 luciferase plasmid and treated in androgen-depleted media for 6 days with 1, 5 or 10µM of treatment with ABI or VT-464(VT). Results are normalized to total protein

Since VT -464 induced a decrease in AR transactivation, we next assessed changes in AR-dependent genes were evaluated following six days in androgen-deprived conditions. The treatment-containing media was changed after 72h. In C4-2 cells, we found that both ABI and VT-464 stimulated an increase in AR mRNA transcripts, but a decrease in AR-dependent mRNA transcripts (e.g. PSA, NKX3.1) with greater decreases seen with VT-464 (Figure 2.4). In the ENZ-resistant MR49C cell line, ABI did not produce any decrease in AR-dependent transcript levels relative to control; VT-464 at equivalent doses lowered transcript levels compared to ABI. Similar to C4-2 cells, there was a VT-464 dependent decrease in AR-dependent genes compared to AR transcript levels suggesting a feedback-induced increase in AR levels due to greater androgen suppression with VT-464. In MR49F cells, 10 µM VT-464 induced a sharp decrease in AR and PSA mRNA and PSA protein (Figure 2.4) possibly due to tumour cell apoptosis as evidenced by increased PARP cleavage (Figure 2.5). VT-464 and abiraterone dose-dependent increases an apoptosis also occurred in MR49C cells as evidenced by increased PARP cleavage and decreased AR-signalling (luciferase). Those cells that survived in the presence of 10 µM abiraterone had higher PSA:AR mRNA and protein levels while those treated with 10 µM VT-464 had increased AR and PSA mRNA levels

The increases in AR and PSA seen between 5 and 10μM doses of VT-464 in MR49C cells suggests a selection for MR49C cells that were highly dependent on AR-transcription may have occurred

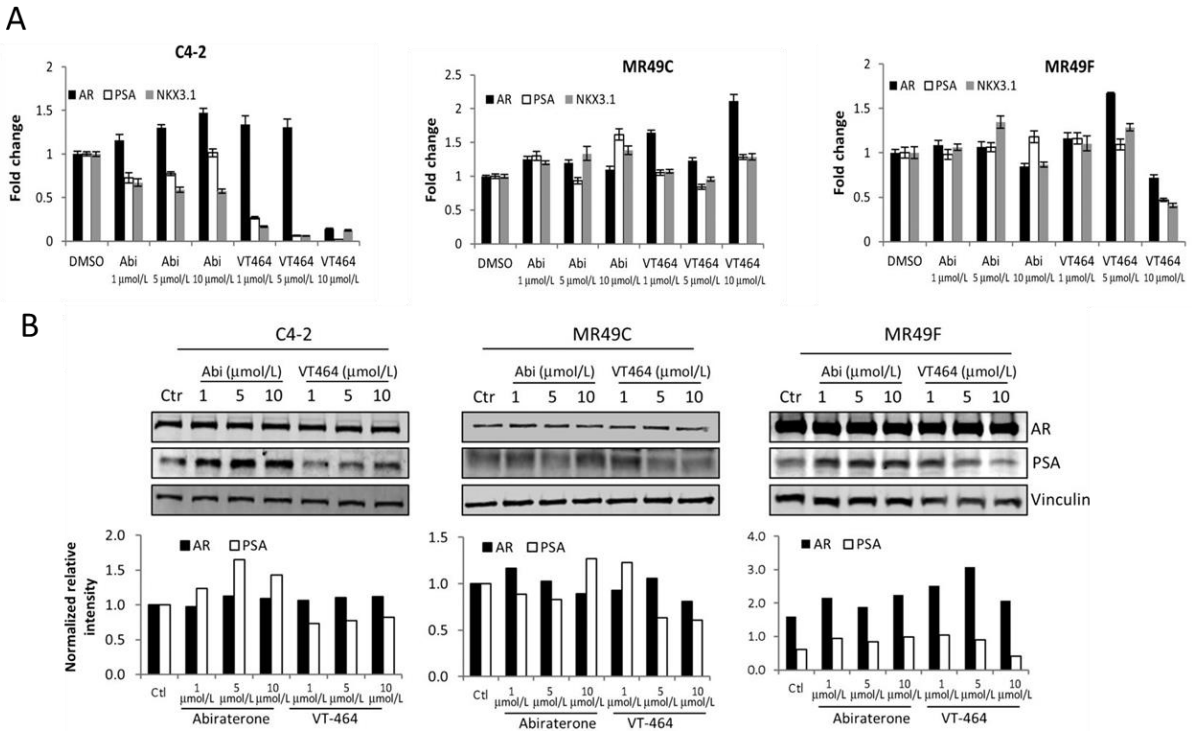


Figure 2.4 Androgen receptor and PSA mRNA transcripts and protein levels following abiraterone and VT-464 treatment. **A**.AR and AR-dependent transcript levels were assessed using quantitative real-time PCR. **B.** AR and PSA protein levels were assessed in all three cell lines. Densitometry was relative to vinculin and control treatment. The media and drug treatments were changed at 72hrs for all experiments. For PCR and protein results, representative results of at least duplicate experiments are presented.

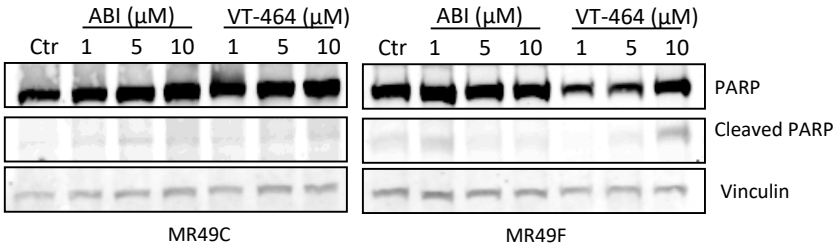


Figure 2.5 Induction of cleaved PARP by abiraterone and VT-464 in ENZ-resistant cell lines. Western blotting for PARP and cleaved PARP (~90kD fragment) was performed on MR49C and MR49F cells treated in 10%CSS media with indicated treatments for 6 days, with treatment media containing ENZ 10μM.

There were no differences in AR protein levels in C4-2 and MR49C cells following ABI or VT-464 treatment at the dose range tested, though AR levels did seem to increase more in response to VR-464 in MR49F cells as noted above (Figure 2.4). However, PSA protein levels were significantly lower after VT-464 treatment, with a dose-dependent decrease in the ENZ-resistant cell lines, compared to ABI treated cells.

2.2.2 Changes in the Androgen Synthesis Pathway Following CYP17A1 Inhibition *In Vitro*

Since VT-464 and ABI inhibit the AR signalling pathway by inhibiting CYP17, we evaluated mRNA transcript levels for selected steroidogenesis enzymes in all three cell lines following 6 days of treatment in androgen-depleted media. Both ABI and VT-464 treatments increased StAR, HSD3B2, HSD17B3, AKR1C3, and SRD5A1 enzyme transcript levels in a dose-dependent fashion (Figure 2.6); VT-464 was much more potent than ABI, presumably due to greater suppression of androgens. Similar trends were seen with upstream steroid and fatty acid pathway transcription factor SREBP-1 and HMGCR gene transcripts (Figure 2.7). With the exception of outliers, the levels of CYP17A1 expression were higher in VT-464- than ABI-treated cells with no evident dose response observed (Figure 2.6).

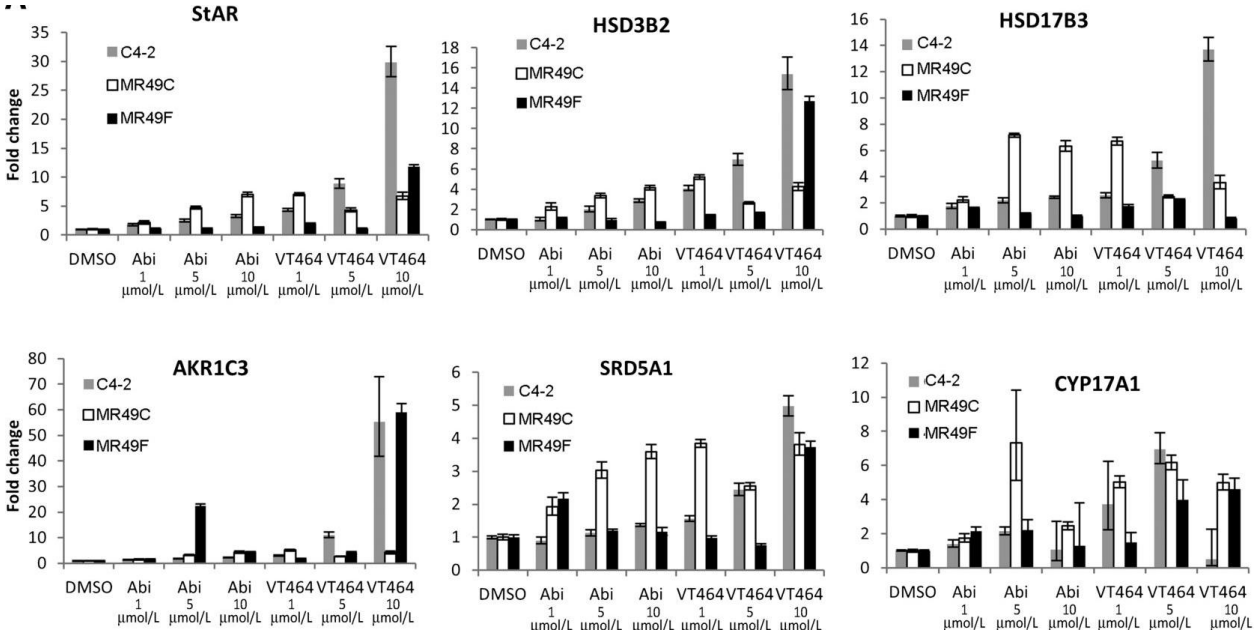


Figure 2.6 The effect of VT-464 and abiraterone on the steroid synthesis pathway enzymes *in vitro*. C4-2, MR49F and MR49C cells were cultured in 1, 5 or 10µM of abiraterone, VT-464 for 6 days. The media and drug treatments were changed at 72hrs and MR49C and MR49F cells were maintained in 10µM ENZ. Transcript levels of STAR, HSD3B2, CYP17A1, HSD17B3, AKR1C3 and SRD5A1 enzyme mRNA transcripts were measured using SYBR green primers (see methods) for all cell lines. Values relative to DMSO control are plotted +/-SEM.

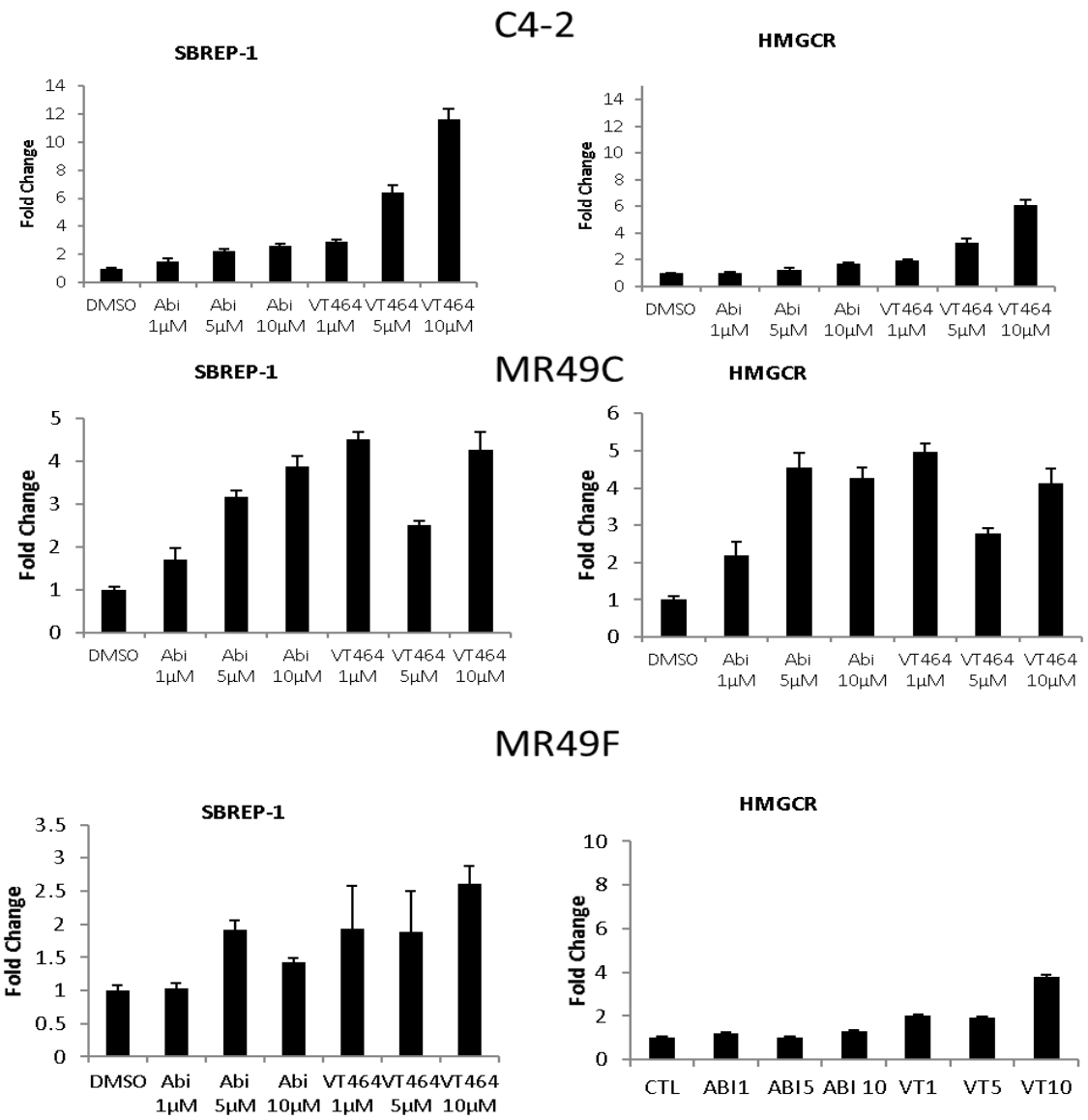


Figure 2.7 Expression of HMGCR and SBREP-1 *in vitro*. Following treatment with indicated doses of abiraterone and VT-464 in androgen depleted media for 6 days, RT-qPCR was used to assess steroid transcripts in C4-2(A), MR49C (B) and MR49F cells(C).

Steroid level measurements were attempted for all cell lines but were only feasible in C4-2 cells due to detection limitations in MR49C and MR49F cells. Surprisingly, testosterone and dihydrotestosterone (DHT) levels were increased in ABI-treated cells after 6 days of treatment. This was consistent with the increase in PSA protein levels and AR and PSA mRNA transcripts observed in ABI-treated, but not VT-464-treated cells (Figure 2.5). Decreasing concentrations of progesterone and increasing concentrations of pregnenolone occurred with higher doses of ABI, consistent with prior reports of HSD3B2 inhibition with higher doses of ABI (263) (Figure 2.8). In C4-2 cells, the decreased AR transactivation with ABI compared to DSMO control, despite increased concentrations of the androgens DHT, T, and androsterone, as well as pregnenolone, suggesting that non-steroid factors must be involved, such as drug AR antagonism. DHEA levels were very low in VT-464 treated cells, but could not be measured accurately

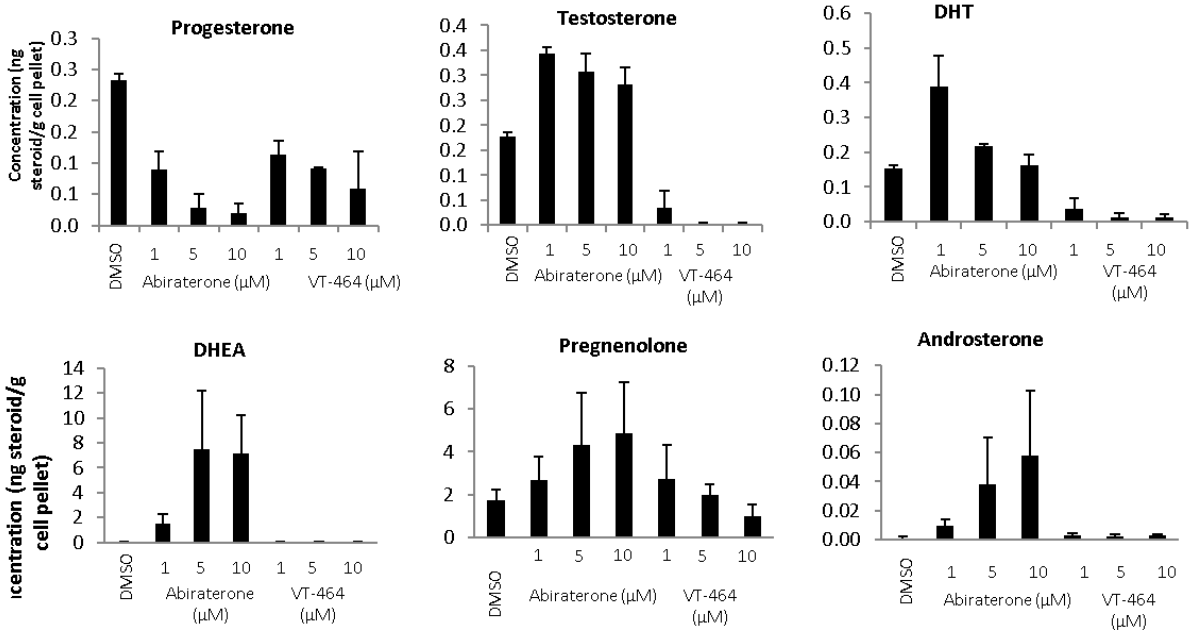


Figure 2.8 LC-MS measurement of steroid levels in C4-2 cells. Cells were grown in androgen free media treated with DMSO, 1, 5 or 10μM of abiraterone or VT-464 for 6 days.

in abiraterone-treated cells due to the low level conversion of abiraterone to DHEA noted in the methods. Overall, these results indicated that in C4-2 cells, suppression of the AR pathway by ABI was not due to androgen concentration decreases. Further, for VT-464, the increase in steroid enzyme transcripts did not appear to be related to resistance as observed increases did not result in increased androgen biosynthesis or AR transactivation. In contrast, the increased androsterone and DHT that occurred in response to ABI in C4-2 cells does suggest that increased expression of AKR1C3, and SRD5A1 may be relevant to resistance through activation of alternate androgen synthetic pathways as previously reported (34, 264).

2.2.3 Direct AR Antagonism by VT-464

To investigate a possible AR antagonistic effect, we repeated transactivation assays with ABI and VT-464 in the presence of 0.1nM R1881. ENZ as well as the CYP17 inhibitor TAK-700, were also tested as controls. Both abiraterone and VT-464 suppressed R1881-stimulation of AR transactivation (Fig. 2.9). In C4-2 cells, the decrease in R1881-

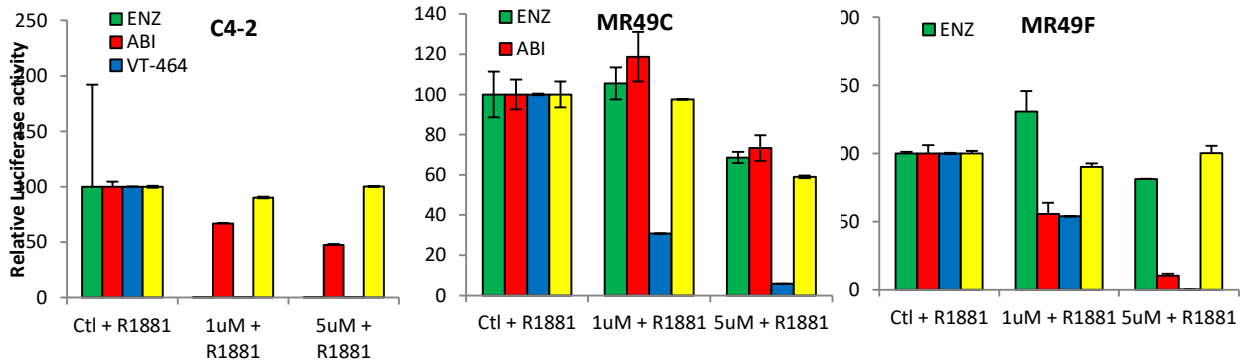


Figure 2.9 AR antagonism with VT-464 and abiraterone. AR transactivation results using transient transfection of an ARR3 luciferase reporter in androgen-depleted conditions in C4-2, MR49C and MR49F cell lines. In the presence of 0.1nM R1881, both abiraterone 5μM and VT-464 5μM decreased AR-transactivation after 24h of treatment (**A**) in all cell lines. No decrease was seen with the negative control TAK-700 in all cell lines; ENZ potency was also consistent as a positive and negative control in C4-2 and ENZ-resistant cells, respectively. Representative results of at least 3 replicates +/- SEM are displayed.

stimulated transactivation due to VT-464 was similar to that observed with ENZ, while no effect was seen with TAK-700. As expected, ENZ and TAK-700 again showed minimal changes in reporter activity in the ENZ-resistance cell lines. Both abiraterone and VT-464 decreased R1881-induced AR-transactivation in MR49F cells. Corroborating prior results, VT-464 demonstrated greater suppression of transactivation in the presence of R1881 than abiraterone in C4-2 and MR49C cells.

To further evaluate a direct antagonistic effect on the AR, VT-464 and ABI were tested in a wild-type AR luciferase assay transfected into CHO cells. AR-antagonism was observed with both ABI and VT-464, but not with TAK-700. Assessment of AR protein levels following VT-464 treatment at different time points and different doses indicated that AR degradation is not a major contributor to the AR-antagonism observed (Figure 2.10)

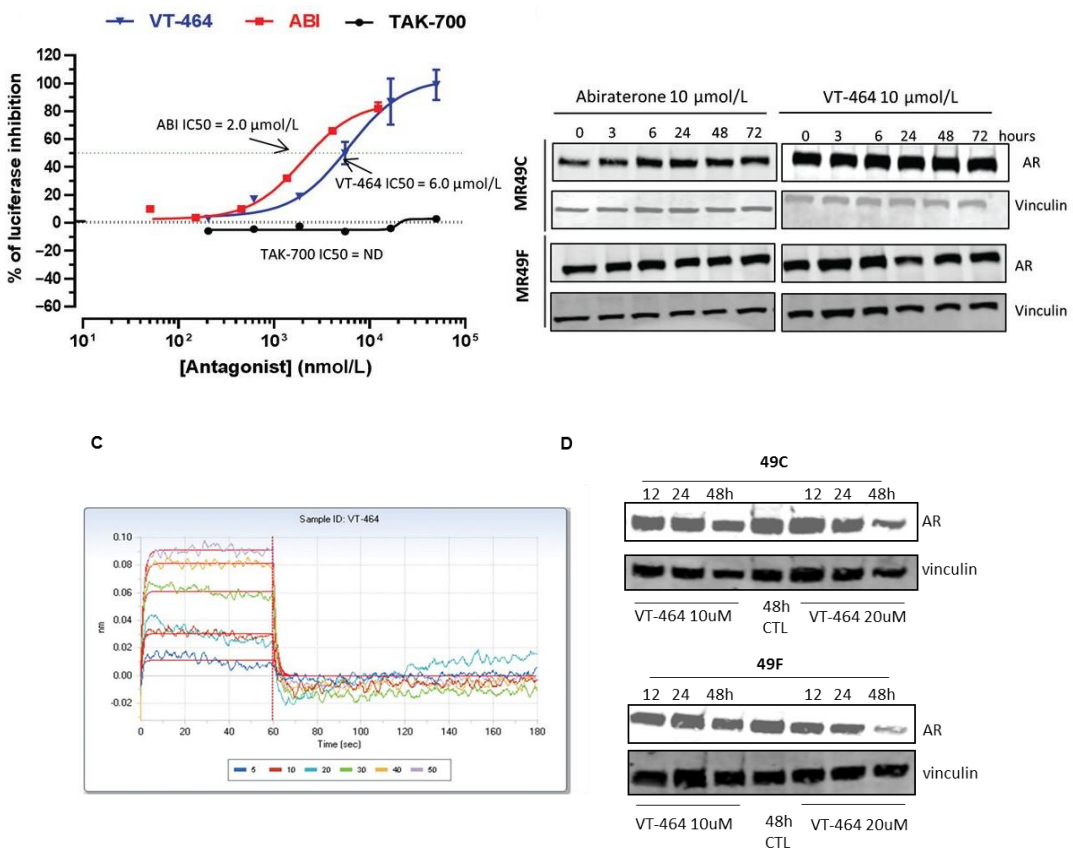


Figure 2.10 AR antagonism with abiraterone and VT-464, but not TAK-700 was further seen using a wild-type AR luciferase reporter assay in CHO cells. **A.** No AR degradation was noted in time course of AR protein levels over time under treatment with 10 µM ABI(left) or 10 µM VT-464(right) in MR49C and MR49F cell lines(**B**). Representative results of duplicate time courses are presented. **C**. Bio-Layer interferometry assay demonstrating a dose-dependent effect with VT-464 binding to a purified androgen receptor ligand binding domain. Doses listed are in µM. **D**. AR protein degradation is seen on western blotting only at 20uM dose of VT-464 after 48h of treatment in MR49F cells. No AR degradation was seen in MR49C cells. Representative results of duplicate experiments are presented.

2.2.4 VT-464 and Abiraterone Acetate Decrease Tumour Growth and Serum PSA Levels in an *In Vivo* Model of Enzalutamide-Resistance

Castrated mice inoculated with MR49F cells were randomized to oral gavage treatment with vehicle (0.5% methylcellulose) twice-daily (BID), abiraterone acetate (AA) 196mg/kg BID, VT-464 75mg/kg BID and VT-464 150mg/kg once-daily (QD). The AA oral gavage dose of 1 mmol/kg daily is higher than previously published doses of 0.5mmol/kg/d(34), but was tolerated in prior unpublished experiments; the VT-464 dose of 0.375 mmol/kg daily (75 mg/kg BID or 150 mg/kg OD) was used due to mouse weight loss at higher BID doses. Efficacy of the VT-464 150mg/kg OD arm was assessed preliminarily in 5 mice. AA and both VT-464 regimens demonstrated tumour growth inhibition (Figure 2.11). Mean tumour growth velocity was significantly lower in the VT-464 BID arm compared to vehicle (P=0.03); tumour growth velocity in AA arm did not different statistically compared to vehicle (P=0.06). Differences between AA and VT-464 were not significant (p=0.64).

Serum PSA was suppressed in all three treatment arms. After 3 weeks of treatment the median PSA was lowest in the VT-464 BID arm (Figure 2.11). The mean PSA velocity was significantly lower for AA compared to vehicle (P=0.03), but not for VT-464 BID (P=0.38). The waterfall plots after three weeks (Figure 2.11) show that there was a small subgroup of AA-treated mice with very low PSA that skewed the mean versus median values. Similarly, there were high PSA outliers in the VT-464 BID treatment arm.

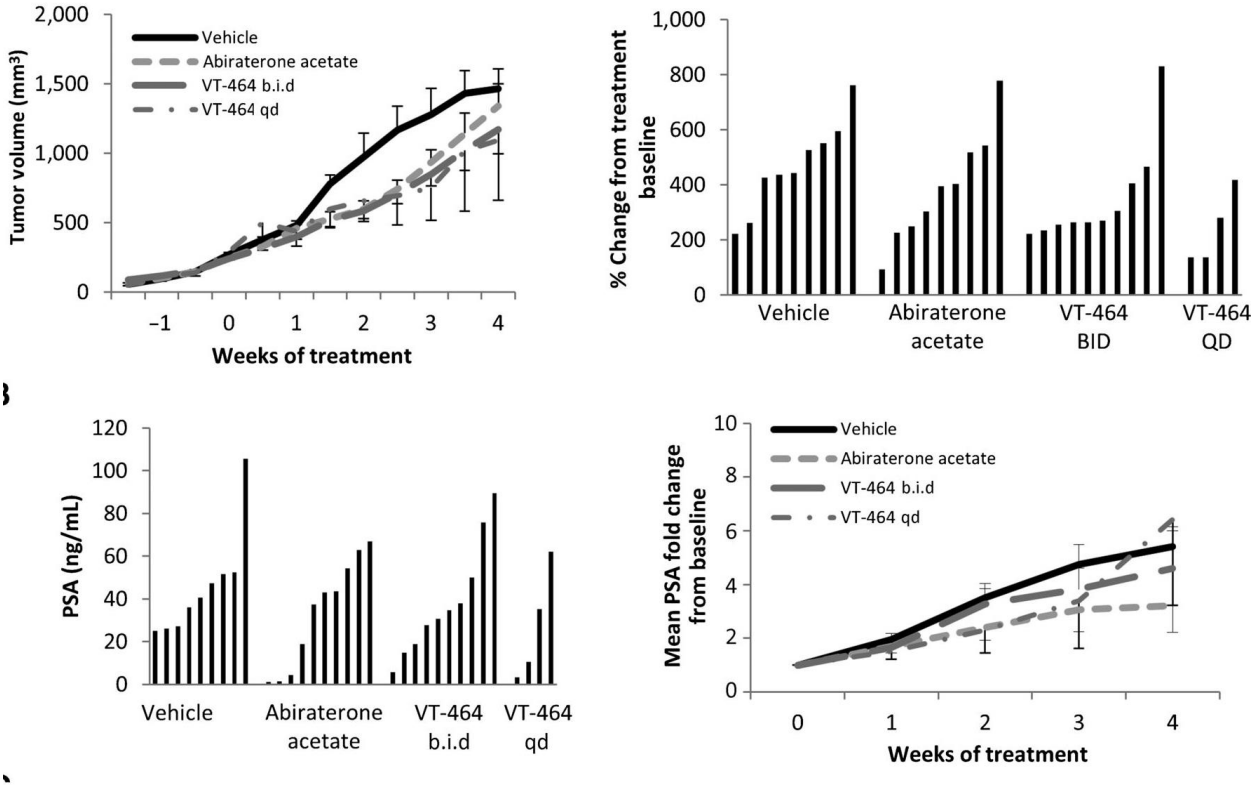


Figure 2.11 *In vivo* evaluation of VT-464 and abiraterone. Castrated MR49F xenografts were randomized to treatment once tumour size exceeded 200mm3. Treatments were vehicle (0.5% MC) BID, abiraterone 196mg/kg BID, VT-464 75mg/kg BID and VT-464 150mg/kg QD. Mean tumour size is plotted +/- SEM (A, left). The waterfall plot details % change from baseline tumor size after 3 weeks of treatment for all mice in study (A, right). Waterfall plot of serum PSA after 3 weeks of treatment for all mice in study (B, left). Mean PSA values +/-SEM are plotted (B, right).

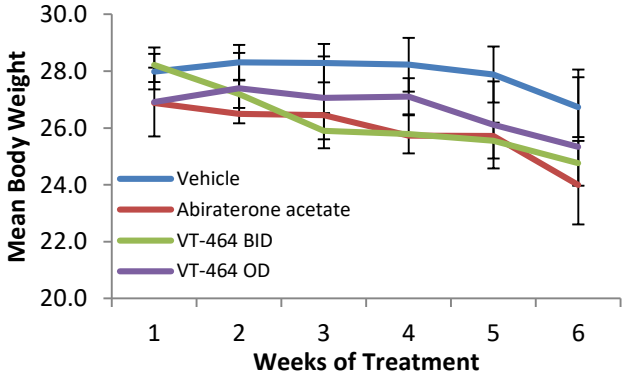


Figure 2.12 Mean weight of mice with MR49F xenografts treated with vehicle, abiraterone acetate or VT-464. Mean body weights(g) are displayed according to week of treatment.

Results in the VT-464 150mg/kg QD arm were generally similar to 75 mg/kg BID regimen. Weight loss was similar between all treatment arms (Figure 2.12). Two mice in

the AA arm and one mouse in the VT-464 75mg BID arm were euthanized for weight loss >20% after 19, 33 and 39 days of treatment, respectively. One mouse in the VT-464 150mg OD arm was found dead one day after starting treatment. Significant improvements in OS and CSS were found only in the VT-464 75mg/kg BID arm compared to vehicle (p=0.03 and p=0.009, respectively) (Figure 2.13).

End-of-study intratumoral AR-dependent mRNA transcripts in VT-464 75mg/kg BID treated-mice were lower compared to AA (Figure 2.14). End-of-study tumour samples also had steroidogenesis enzyme mRNA transcripts (Figure 2.14) which were lower in both AA and VT-464 treatment arms compared to vehicle, with the lowest levels in the VT-464 arm.

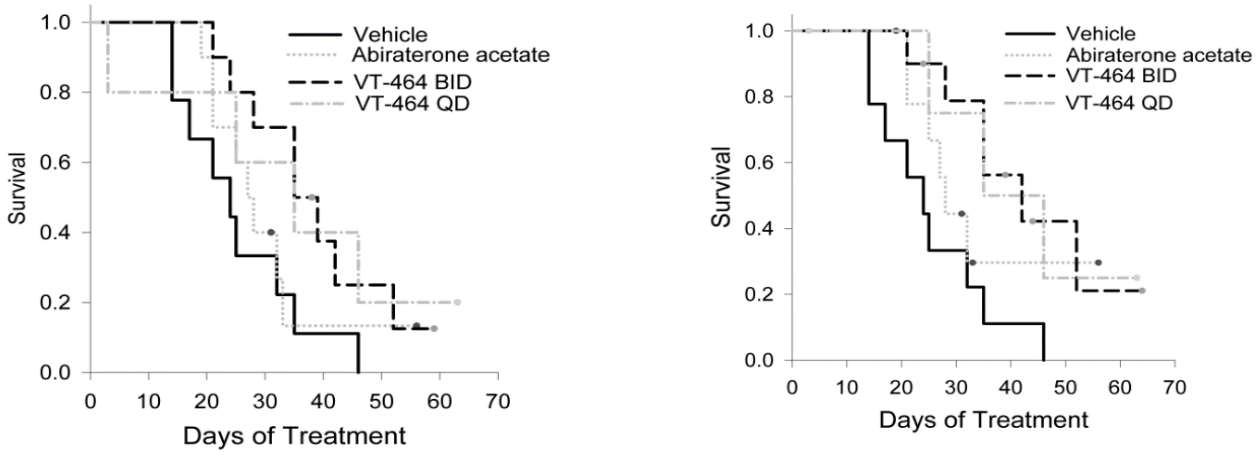


Figure 2.13 Cancer specific survival and overall survival of MR49F murine xenografts following abiraterone and VT-464 treatment. Kaplan-Meier analysis of treatments: cancer specific survival(left) and overall survival (right).

End-of-study intratumoral AR-dependent mRNA transcripts levels (Figure 2.14) were lower in VT-464 75mg/kg BID treated-mice compared to AA. Abiraterone-treated mice had AR mRNA transcript levels slightly increased compared to vehicle-treated mice.

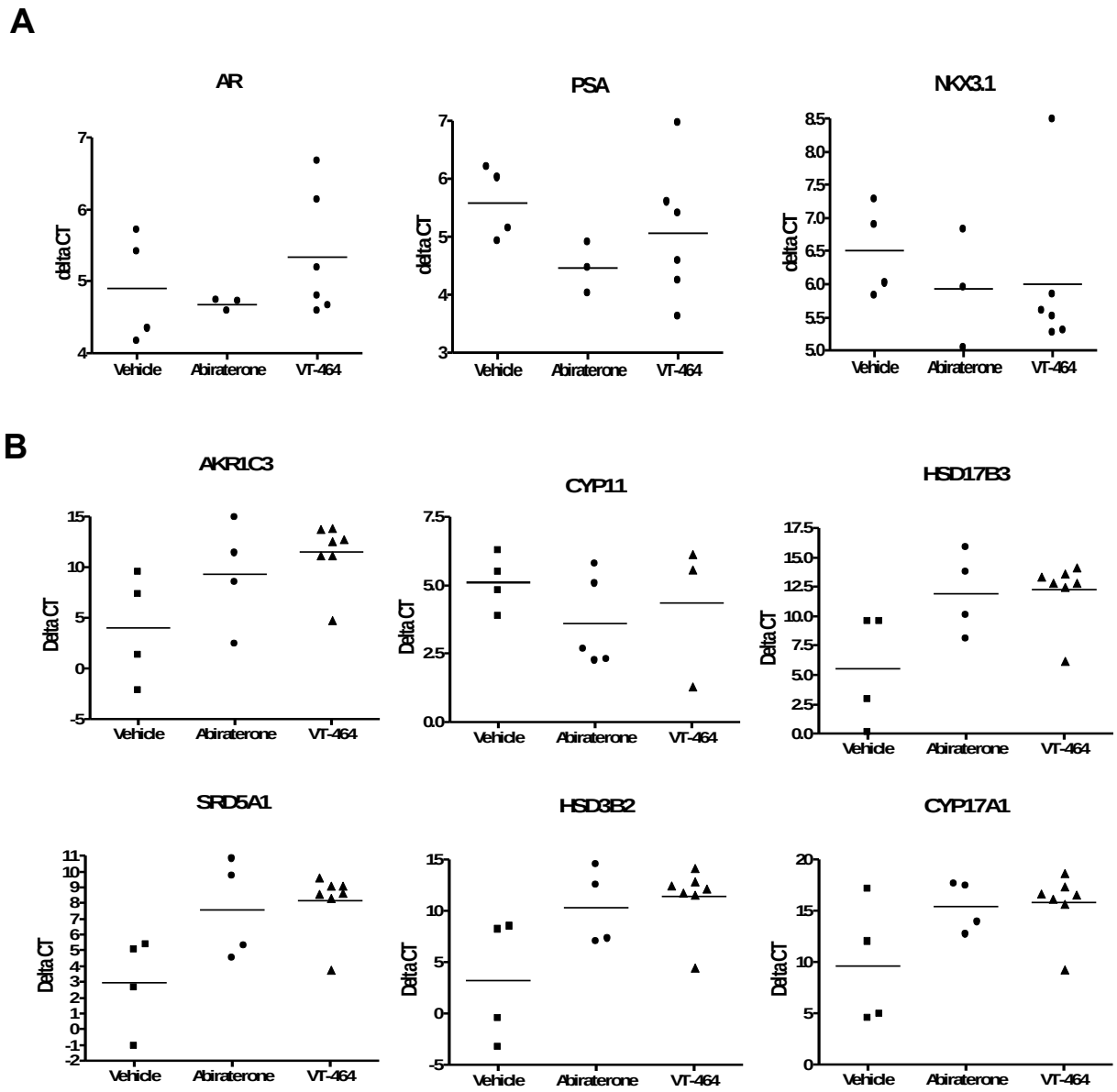


Figure 2.14 AR-dependant and steroidogenesis transcripts in MR49F xenografts. RT-qPCR was performed on 4-6 xenograft samples collected per group at the end of the study. Transcript levels were normalized to GAPDH. A relative decrease in AR and AR-dependent mRNA transcripts was seen with VT-464 compared to abiraterone. (A). Further, there was a significant decrease in six steroid enzyme transcripts (normalized to beta-actin) in abiraterone and VT-464 treated mice compared to vehicle-treated mice from the same samples (B).

Changes in AR-regulated genes PSA and NKX3.1 were also lower in the VT-464 compared to the AA arm, which were increased relative to vehicle. TMPRSS2 showed a different trend, with higher values in the VT-464-treated group compared to AA and vehicle. In contrast to the *in vitro* results above, select steroid synthetic enzyme mRNA transcripts (Figure 2.14) were significantly lower intratumorally in the treatment arms (AA and VT-464) compared to vehicle, with the lowest levels in the VT-464 arm.

2.2.5 Pharmacodynamic Study

To investigate pharmacodynamic effects, castrated mice implanted with MR49F xenografts in both flanks were treated orally with VT-464 at 100mg/kg BID or AA at 196mg/kg BID once tumors reached 200mm^3 in size as detailed in methods. AR-dependent gene transcripts and AR and PSA protein level data collected from the tumors demonstrated significant heterogeneity of response (Figure 2.15), with a similar suppression of the AR-pathway transcripts with both abiraterone and VT-464. Analysis

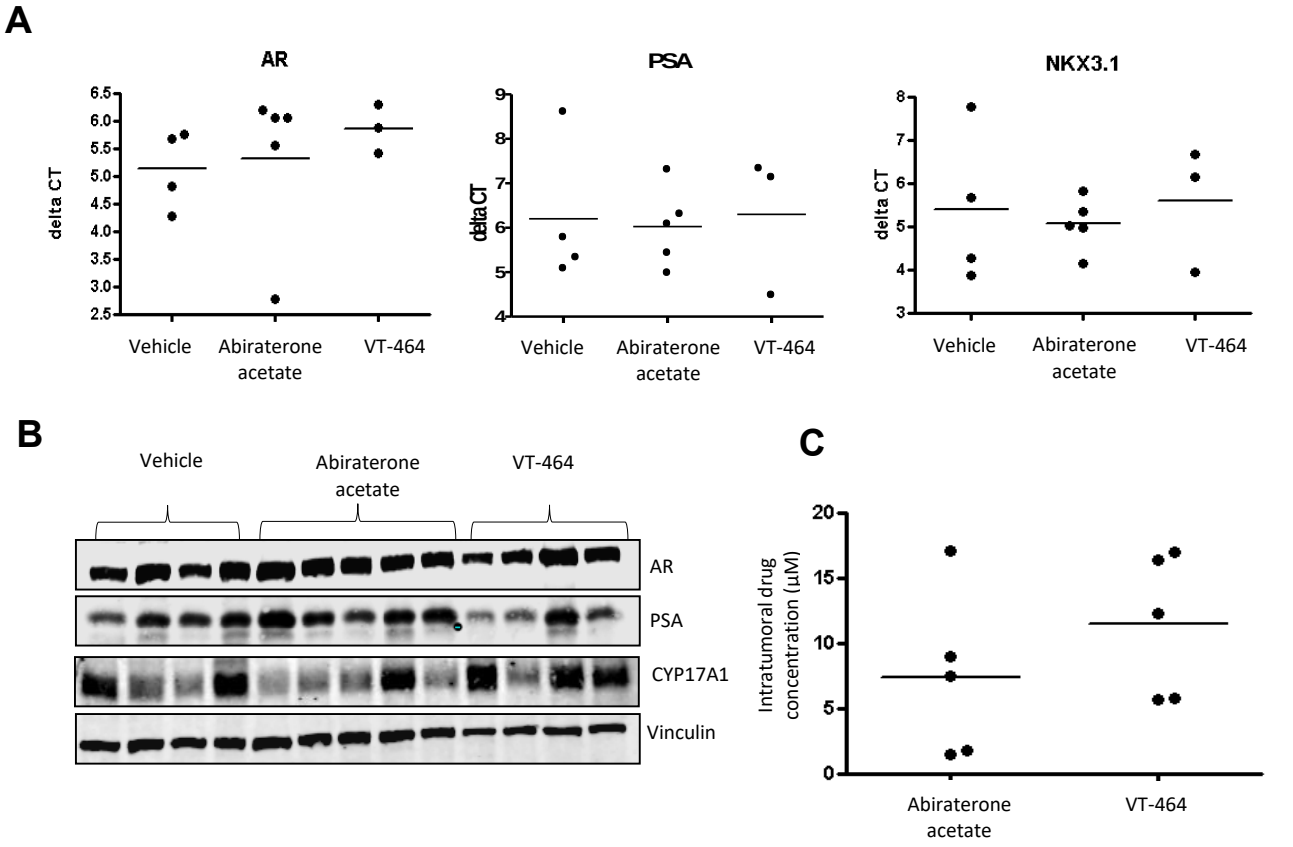


Figure 2.15 Pharmacodynamic evaluation of VT-464 and abiraterone in MR49F xenograft model. Fifteen xenograft–bearing mice were treated for 3-10days with abiraterone 196mg/kg BID, VT-464 100mg/kg BID or vehicle and tumors were harvested. All mice were sacrificed approximately 3 hours after last dose (for details, see methods). **A.** mRNA transcript levels of AR related transcripts in tumor samples were evaluated with qRT-qPCR relative to GAPDH. **B.** Western blot analysis for AR, PSA and CYP17A1 levels demonstrates significant tumor heterogeneity between tumours from individual mice. **C.** Intratumoral drug levels for abiraterone and VT-464 as measured using LC-MS chromatography in triplicate.

of intratumoral drug levels indicated similar heterogeneity in intratumoral drug levels (Figure 2.15) though the intratumoral VT-464 concentrations were greater on average and somewhat less variable.

Analysis of intratumoral steroid levels demonstrated that testosterone and DHT levels were significantly lower following VT-464 or AA treatment compared to vehicle, with the greatest decreases seen with VT-464 (Figure 2.16). Upstream pregnenolone levels were significantly higher in tumors of AA-treated animals (P=0.03). Steroidogenic enzyme mRNA transcripts were assessed in the collected tumour samples. Here, in contrast to the prior *in vivo* findings, but similar to the *in vitro* findings, we found a trend

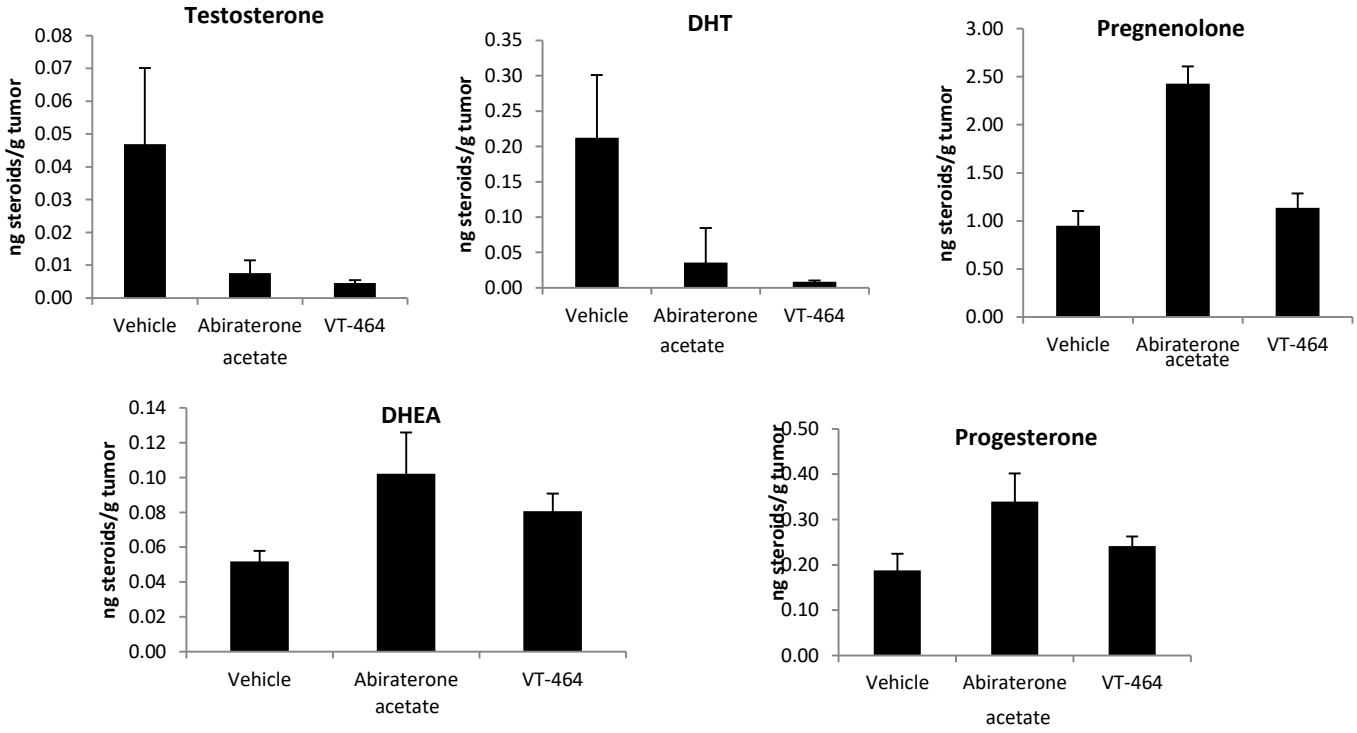


Figure 2.16 LC-MS analysis of steroid levels in MR49F tumours treated in pharmacodynamic study.

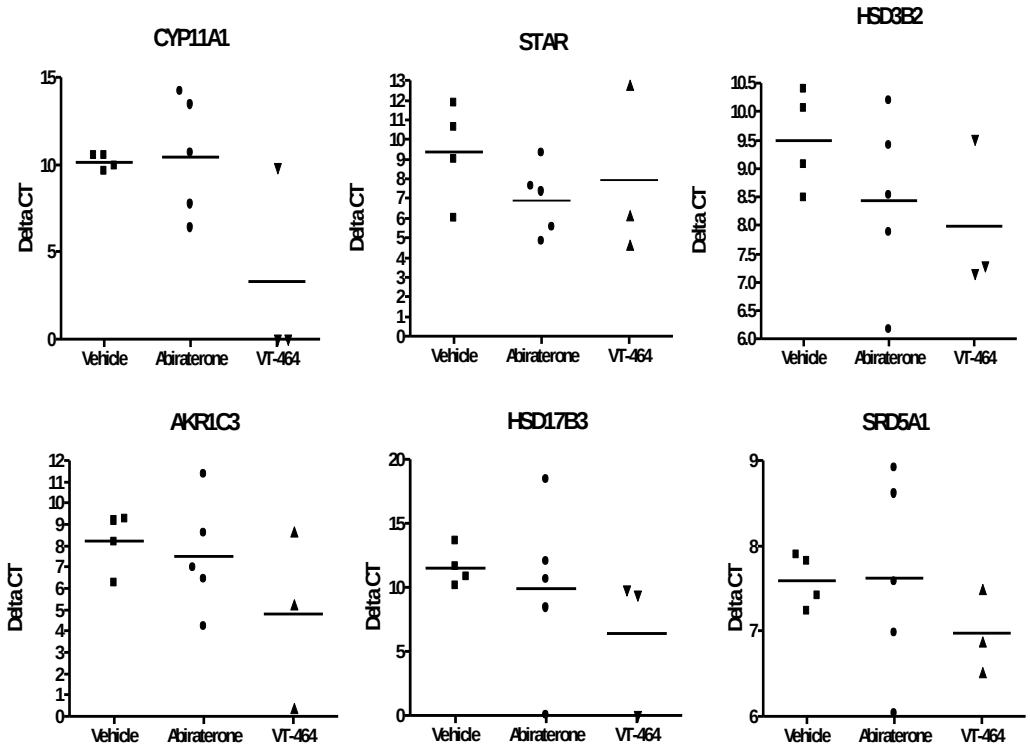


Figure 2.17 Levels of intratumoral steroidogenesis enzyme synthesis mRNA transcripts collected from the pharmacodynamic study. MR49F mice were randomized to treatment with Vehicle, Abiraterone 196mg/kg BID or VT-464 100mg/kg BID as detailed in methods. Tumors were collected after 3-10days of treatment. From tumor samples stored in RNAlater, qRT-PCR was used to amplify indicated SYBR Green primers, with results normalized to beta-actin.

toward increased steroid enzyme synthesis in AA- and VT-464 treated mice (Figure 2.17), with the greater up-regulation seen in the VT-464 treated mice, though androgen levels were lower in this group (Figure 2.16).

2.3 Discussion

This study demonstrated more potent inhibition of androgen synthesis by VT-464 in CRPC models compared to abiraterone. Further, direct AR antagonism was demonstrated as a novel mechanism of action of VT-464. The use of LNCaP-based models mirrors the clinical situation wherein the T887A AR mutation renders tumors

sensitive to activation by non-canonical androgens such as progesterone, pregnenolone, and prednisone (265, 266).

AR antagonism has been demonstrated in pre-clinical studies for the steroid-based CYP17 inhibitors AA and TOK-001(267-269) but this mechanism has not previously been reported for any non-steroidal CYP17A1 inhibitor. The clinical results with TAK-700(21) and enzalutamide (1) confirm that selective androgen synthesis inhibition or AR antagonism alone have activity in CRPC. Combination AR antagonism with

P17A1 inhibition has previously been reported to contribute to the pre-clinical efficacy of galeterone and abiraterone (267, 268). Our results suggest that AR antagonism is best combined with selective inhibition of CYP17A1 lyase, compared to more potent inhibition of both lyase and hydroxylase functions.

Prior *in vitro* studies evaluating the selectivity for the lyase versus hydroxylase activity of CYP17A1 suggest VT-464 has approximately 50X more selectivity compared to abiraterone(270). In castrate rhesus monkeys, similar to our results, decreases in testosterone with VT-464 were not accompanied by accumulation of progesterone or pregnenolone seen with abiraterone(271). This selectivity allows for dosing of VT-464 without prednisone in ongoing Phase II trials; the oncologic significance of avoiding prednisone continues to be explored.

Recent pre-clinical studies suggest glucocorticoids may activate canonical AR pathways following ENZ-resistance(258). Therefore, the combination of selective CYP17 inhibition and AR antagonism is a promising strategy for patients who progress on ENZ. Other pre-clinical studies have identified the F876L mutation to be another potential

mechanism of ENZ-resistance found in approximately 10% of patients (44, 51). This mutation alters the AR ligand binding domain, resulting in ENZ acting as an agonist. In our study, we found VT-464 had anti-AR activity against both MR49C and MR49F cells which have this mutation.

The lack of relative up-regulation of steroidogenic enzymes in VT-464-treated tumours collected at animal endpoints differs from our *in vitro* and pharmacodynamic study results. This may be related to upregulated metabolism of VT-464 observed in regulatory safety studies in rodents, where the exposure to the drug after 3 weeks is diminished by >50% (data not shown). This is consistent with the diminished PSA response observed in the VT-464 once daily arm after 3 weeks. Our *in vivo* results are also limited by the heterogeneity observed. Nonetheless, this heterogeneity mirrors early clinical results of CYP17A1 inhibition in ENZ-resistant patients (272, 273)

In summary, the novel CYP17 inhibitor VT-464 demonstrated anti-cancer activity in pre-clinical models of CRPC and ENZ-resistance, decreasing androgen levels significantly in castrate mice. The *in vitro* and *in vivo* results suggest greater suppression of the AR-axis with VT-464 compared to abiraterone due to the selective suppression of androgen synthesis through CYP17-lyase inhibition, as well as AR.

3 Combined AKT and MEK Pathway Blockade in Pre-Clinical Models of Enzalutamide-Resistant Prostate Cancer

3.1 Introduction

Medical or surgical castration remains the first line of systemic therapy for metastatic prostate cancer since its discovery over 70 years ago (28). Unfortunately, cure remains elusive following castration and patients inevitably progress to develop castrate resistant prostate cancer (CRPC). Potent androgen receptor (AR) pathway inhibitors such as enzalutamide (ENZ) and abiraterone are now commonly used in the treatment of patients with CRPC. While survival is improved, resistance nonetheless inevitably develops to these agents (274). It is anticipated that with the increased clinical use of these more potent AR pathway inhibitors that targeting approaches against non-AR driven resistance pathways will gain increasing importance (262). Therefore, understanding and targeting pathways implicated in resistance has important clinical relevance.

The PI3K/AKT/mTOR and RAF/MEK/ERK signalling pathways play an important role in cell survival, treatment resistance, and cooperate to facilitate prostate cancer progression to CRPC (146, 275-278). Both AKT (97, 279) and ERK (162, 280) signalling pathways are up-regulated with CRPC and are associated with poor outcome (122, 281). There is extensive cross-talk between these two pathways as well as with other oncogenic pathways (282, 283). We have previously showed that both AKT and ERK are activated following treatment with ENZ in prostate cancer cells (160). Targeting AKT alone is not sufficient to induce conditional lethality due the feedback signalling leading

to activation of AR and therefore targeting AKT alone is not a good strategy to combat ENZ resistance (81). Interestingly, dual inhibition of PI3K/AKT and MEK/ERK pathways has shown promise in pre-clinical models of other cancers (284-287). Results of the combination of an mTOR inhibitor with a MEK inhibitor in the transgenic *NKx3.1-PTEN* murine prostate cancer model further supports the rationale for a combined approach in prostate cancer (122)therapy.

Therefore, we set to investigate combination AKT plus MEK inhibitor therapy in human prostate cancer models, particularly ENZ-resistant prostate cancer models. We selected a panel of cell lines including ENZ-resistant LNCaP-derived cell lines as well as the 22RV1 cell line. The 22RV1 prostate cancer cell line possesses activation of the MEK/ERK pathway (288), while the ENZ-resistant MR49C and MR49F are recognized to be more dependent on the AKT pathway (254). We demonstrate that combination blockade of the AKT and MEK does improve responses compared to monotherapy in some of ourin *in vitro* and *in vivo* prostate cancer experiments. Notably, the results vary considerably between model systems with the absence of additional benefit in some cases, highlighting the need to appropriately identify which patients will benefit most from a combination approach.

3.2 Materials and Methods

3.2.1 Prostate Cancer Cell Lines

The human prostate cancer LNCaP and 22RV1 cell lines used in this study were kindly provided by Dr. Leland W.K. Chung(289)(1992, MDACC, Houston Tx). V16D (castrate resistant), MR49F and MR49C cells (enzalutamide resistant) were derived

through serial xenograft passage of LNCaP cells as previously described (251) (Figure 1.7). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO2 atmosphere, with 10μM ENZ added to all media for MR49C and MR49F cells.

3.2.2 Reagents

The AKT inhibitor AZD5363 was provided by AstraZeneca (Macclesfield, UK). ENZ was purchased from Shanghai Haoyuan Chemexpress (Shanghai, China), PD0235901 from Selleck Chem (Houston, TX) and LY294002 and UO126 from Sigma-Aldrich (St Louis, MO). LY294002 is a reversible PI3K inhibitor; AZD5363 is a competitive pan-AKT inhibitor (290). PD0325901 is a competitive MEK1/2 inhibitor while UO126 inhibits MEK1/2 in a non-competitive, selective manner (291). Stock solutions of AZD5363, PD0235091, UO126 and LY294002 were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich); ENZ was prepared in H_2O.

3.2.3 Cell Proliferation Assays

Cell viability was assessed in 96-well culture plates using the WST-1 reagent and/or crystal violet assay, as described previously(251).

3.2.4 Cell Cycle Analysis

Cell cycle analysis with propidium iodide staining was performed as previously described (163). Relative DNA content was analyzed by FACS Canto II flow cytometer using the cyflogic v1.2.1 software (www.cyflogic.com) for analysis.

3.2.5 Caspase-3 Activity Assay

Caspase-3 activity was assessed using the Caspase 3 Assay kit, with the acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) fluorometric substrate (Enzo Scientific). Thirty micrograms of whole cell lysate was incubated with caspase-3 substrate AC-DEVD-AMC at 37.5°C for 2.5h and caspase-3 activity was quantified with a fluorometer with excitation set at 365nm and emission 460nm. Fold change differences from control were calculated following subtraction of readings from blank wells without lysate.

3.2.6 Western Blot Analysis

Total proteins were extracted in RIPA buffer as previously described (163). 30-50 µg of protein lysate was separated by SDS-PAGE, and western blot was performed using primary antibodies PSA, AR (Santa Cruz Biotechnology), vinculin (Sigma-Aldrich, St. Louis, MO), PARP, p-AKT Ser473, AKT, p-ERK, total ERK, total S6 and p-S6 (Cell Signaling Technology, Danvers, MA). Detection of secondary antibodies was performed using the ODYSSEY IR imaging system (Li-COR Biosciences) or ECL (Amersham Biosciences, Piscataway, NJ, USA).

3.2.7 Quantitative RT PCR

RNA extraction and RT-PCR were performed as previously described (31). Real time monitoring of PCR amplification of cDNA was performed using the following primer pairs and

probes:*AR* (Hs00171172_m1), *PSA* (Hs00426859_g1), and *GAPDH* (Hs03929097_g1) (Applied Biosystems, Foster City, CA) on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using TaqMan Gene Expression Master Mix (Applied Biosystems). Target gene expression was normalized to *GAPDH* levels in respective samples as an internal control.

3.2.8 Animal Treatment

Six week old male castrated athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected subcutaneously with $2x10^6$ MR49F cells (suspended in 0.1ml Matrigel; BD Biosciences) on both flanks. Nine mice per arm were randomized to vehicle (0.1% methylcellulose), AZD5363 100mg/kg BID, PD0325901 5mg/kg OD or the combination. ENZ 10mg/kg was administered prior to inoculation and continued until tumors reached $200mm^3$. Tumor measurements were measured biweekly and tumour volume calculated using the formula l x w x d x 0.5236. Drugs were administered as an oral gavage 5 days on, 2 off. PSA levels were measured weekly using automated enzymatic immunoassay (Cobas, Montreal, Quebec, Canada). Mice were sacrificed if the total tumour burden was $>2000mm^3$ or had >20% loss of body weight. For the 22RV1 xenografts, $2x10^6$ cells were inoculated into the left flank of nude castrate mice. Eight mice per arm were randomized to vehicle, AZD5363 100mg/kg BID, selumitinib (AZD6244; ARRY-142886) 25mg/kg BID, and the combination. The 22RV1 xenografts were sacrificed when tumour volume was $>1500mm^3$. All mice were monitored regularly for their clinical condition under the supervision of a veterinarian at the University of British Columbia. At sacrifice, all mice were deeply anesthetized with isoflurane prior to being euthanized with CO2. Tumors

collected at sacrifice were divided into parts and snap frozen in liquid nitrogen or fixed in formalin. All animal procedures were performed according to Canadian Council on Animal Care guidelines and with approval of the Animal Care Committee of the University of British Columbia (protocol # A12-0210).

3.2.9 Immunohistochemistry

A tissue microarray of all MR49F xenograft tumors was constructed using a manual tissue microarrayer (Beecher Instruments, Inc., Sun Prairie, WI). Immunohistochemical staining was performed as previously reported (292). All comparisons of staining intensities were done at 20X magnification on triplicate samples by a pathologist blinded to treatment assignment.

3.2.10 Statistical Analysis

All results are expressed as the mean ±SEM, with one-way ANOVA used to detect significant differences between multiple treatments. When ANOVA showed significant differences ($p<0.05$), Tukey's HSD post-hoc test was used to compare means. Tumor growth velocity was calculated using linear regression. Kaplan Meier survival analysis compared cancer specific survival (CSS) and overall survival, with the log-rank test used to compare groups. CSS was defined as time from treatment start until total tumour volume exceeded $2000mm^3$ and overall survival as the time from treatment start until sacrifice. The combination index (CI) was calculated using the Calcusyn software (Biosoft, Cambridge, UK). A CI <1 indicates synergy, a CI >1 indicates antagonistic interactions.

3.3 Results

3.3.1 Effect of Targeting MEK and AKT Pathways on AR Signalling

To investigate the relevance of AKT and MEK pathways in prostate cancer, we targeted respective pathways using AZD5363 (AKT inhibitor) and PD0325901 (MEK inhibitor). We evaluated their effects alone and in combination in models of different stages of prostate cancer including androgen sensitive cells (LNCaP), castrate resistant (V16D, 22RV1) and ENZ-resistant cells (MR49C and MR49F) (Figure 3.1).

Blocking AKT with AZD5363 was evaluated by its effect on AKT downstream effector p6SK and not on AKT phosphorylation itself because of the nature of AZD5363. Basically, AZD5363 induces increased AKT phosphorylation which is inactive, a phenomenon occurs with many ATP competitive, catalytic inhibitors of AKT, and is due to the protein being held in a hyper-phosphorylated but catalytically inactive form as a consequence of compound binding as has been established previously. AZD5363 was found to induce a decrease in S6K phosphorylation in all cell lines; this effect was more pronounced in the PTEN null cells MR49C, MR49F, LNCaP and V16D cells compared to PTEN positive 22RV1 cells (Figure 3.1). Similar effects were observed using other MEK inhibitor (UO126) and AKT inhibitor (LY294006) or in combination with Ay (Figures 3.1, 3.2). In 22RV1 cells, PD325901 completely abrogates ERK phosphorylation while AZD5363 had no effect on AKT downstream effector S6K phosphorylation which was only with combination of AZD5363 and PD0325901 (Figure 3.1). However, no additional decrease in pERK levels was observed with the combination compared to MEK inhibitor monotherapy.

Moreover, we observed that AKT inhibition increased both AR and PSA at protein and RNA levels in LNCaP and its derivatives (Figure 3.1). Although AZD5363 increased AR and PSA levels in all cell lines, the effect of PD0325901 on AR and PSA levels alone or in combination with AZD5363 varied between cell lines (Figure 3.1). In contrast to LNCaP-based cell lines, PSA was further increased by the combination of AZD5363 and PD0325901 in 22RV1 cells. Notably, these results mirrored similar data with the previously tested successful combination of AZD5363 and ENZ where the changes in AR expression also differed between 22RV1 and LNCaP-based cell lines(Figure 3.2)(254). Taken together, we conclude that AKT and MEK cooperate to regulate the AR pathway.

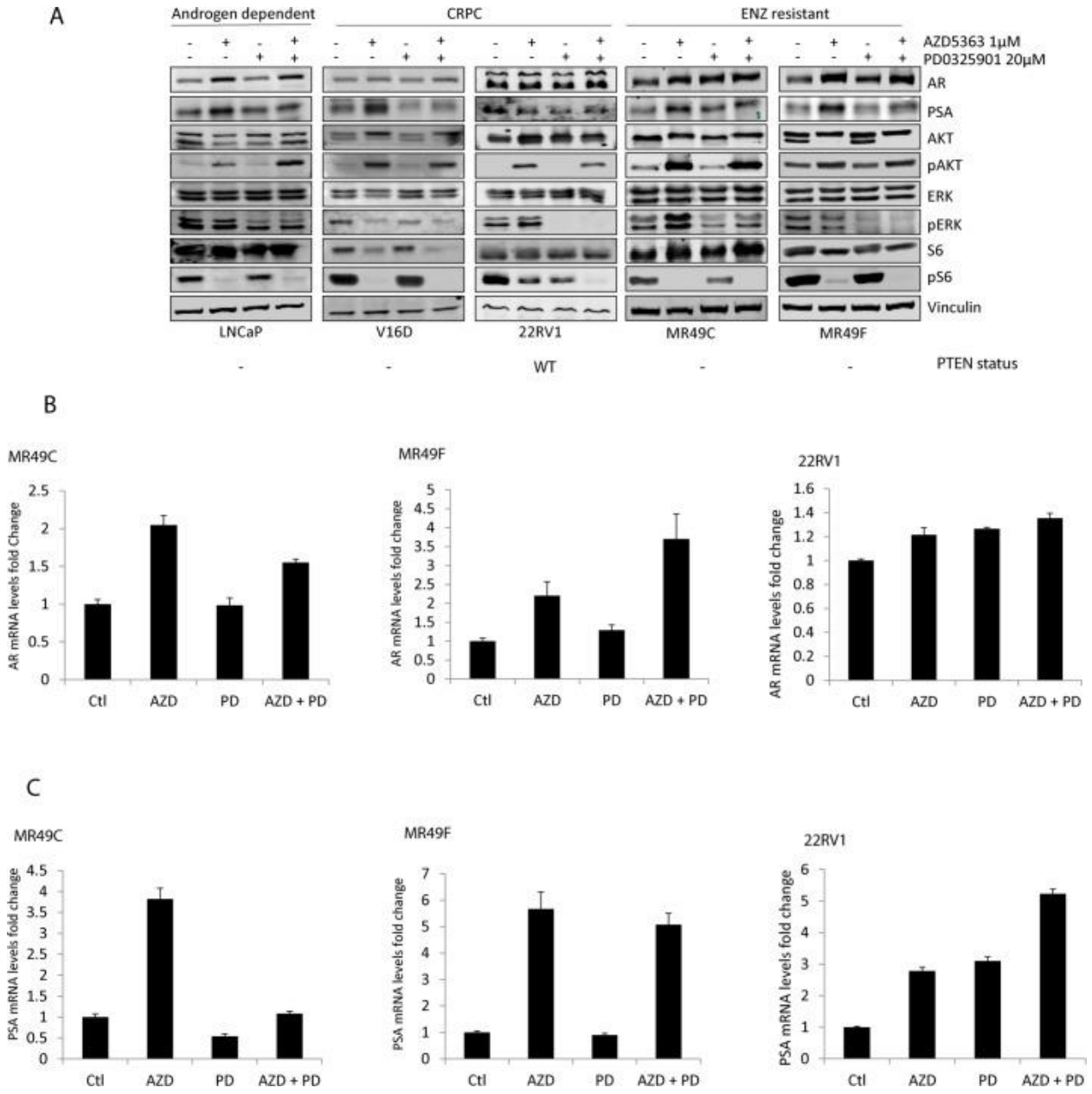


Figure 3.1 Effect of AKT and MEK inhibition on downstream signaling pathways an AR signaling pathway. **A.** Effect of AKT and MEK inhibition on downstream signaling pathways. Androgen dependent prostate cancer cell line LNCaP, CRPC (V16D and 22RV1) and ENZ-resistant cell lines MR49C, MR49F cell lines were treated with AZD5363 1μM, PD0325901 20 μM alone or in combination for 48 hours. Total proteins were extracted and western blots were performed using AR, PSA and PI3K/AKT pathway signalling proteins as indicated. Representative blots of duplicate experiments are shown. **B-C.** Effect of AKT and MEK inhibition on AR pathway. MR49C, MR49F and 22RV cell lines were treated with AZD5363 1μM, PD0325901 20 μM alone or in combination for 48 hours. RNA was extracted from different cell lines and quantitative real time were performed using Taqman probes for AR (**B)** and AR target gene PSA **(C)**. Representative results of biologic duplicates with technical triplicates are shown.

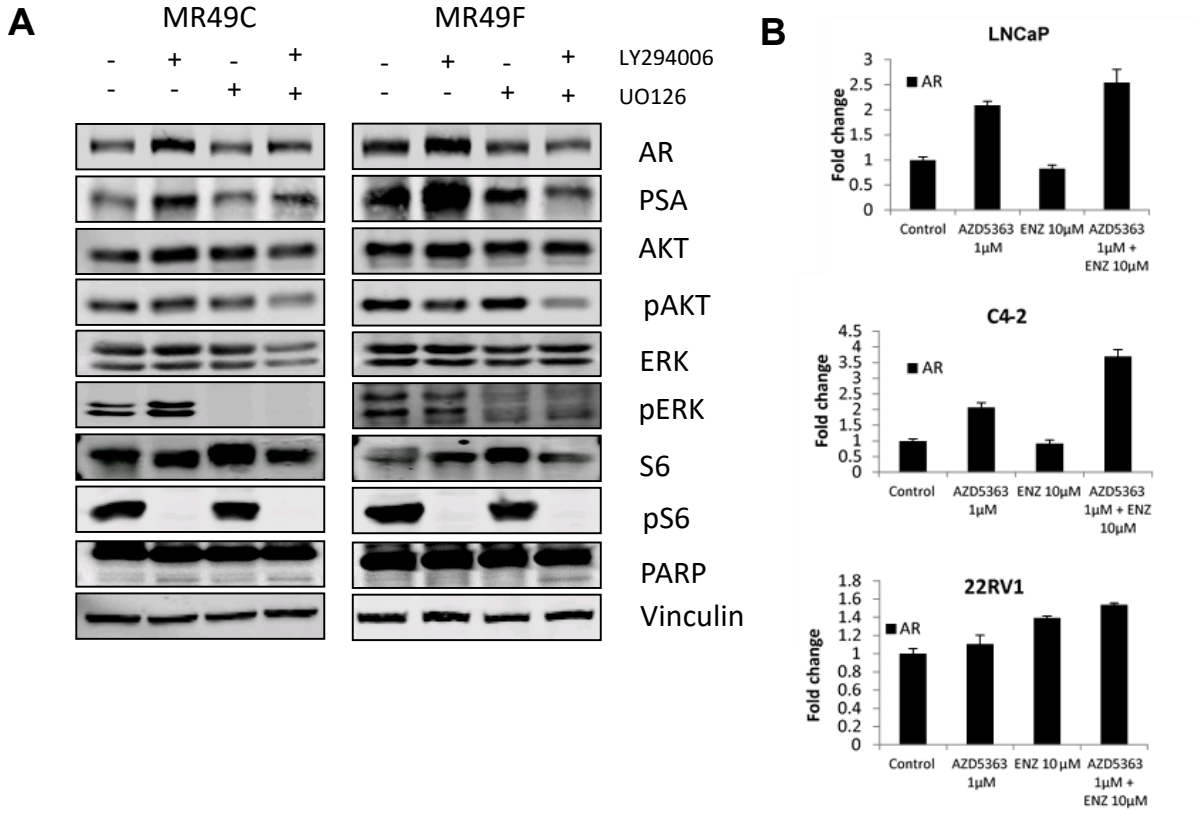


Figure 3.2 Effect of combination AKT plus MEK inhibition with alternate inhibitors on cell signalling. **A.** MR49C and MR49F cell lines were treated with LY294006 20μM, UO126 10 μM, or combination for 48 hours. Total protein was extracted and wester blots were performed using AR, PSA and PI3K/AKT pathway signalling proteins. **B.** MR49C and MR49F cell lines were treated with LY294006 20μM, UO126 10 μM, or combination for 24 hours. RNA was extracted and quantitative real time PCR results of Taqman probes for AR.

3.3.2 Effect of Combination MEK and AKT Blockade on Cell Apoptosis and Proliferation

To determine the biological activity of targeting AKT and MEK signalling pathways on cell apoptosis, we treated our panel of cell lines with AZD5363, PD0325901 or the combination. Our data showed that combination of targeting AKT and MEK induces

apoptosis as shown by increased sub G0/G1 cell cycle population. This effect was greater than AZD5363 monotherapy in CRPC cells V16D (17% vs 10%, P=0.009) and ENZ-resistant MR49C (29% vs 12%, p= 0.005) and MR49F (12% vs 3%, p=0.006) cells. None of the treatments exhibited any significant changes in the sub G1/G0 fraction in 22RV1 cells (Figure 3.3). In contrast, in androgen-sensitive LNCaP cells, combination treatment therapy did not show further induction of sub G1/G0 compared to AZD5363 monotherapy (17% vs 16%, p=0.76). S-phase and G2/M fractions appeared to decrease to a similar amount in MR49C, MR49F and V16D cells with AZD5363 or the combination, with significant differences for both treatments from control(P<0.001). Moreover, AZD5363 and PD325901 combination treatment induced cleaved PARP in LNCaP-based cell lines compared to PTEN positive cells 22RV1 cells (Figure 3.3). While not statistically different, similar trends were observed with caspase 3 activity assays (Figure 3.3).

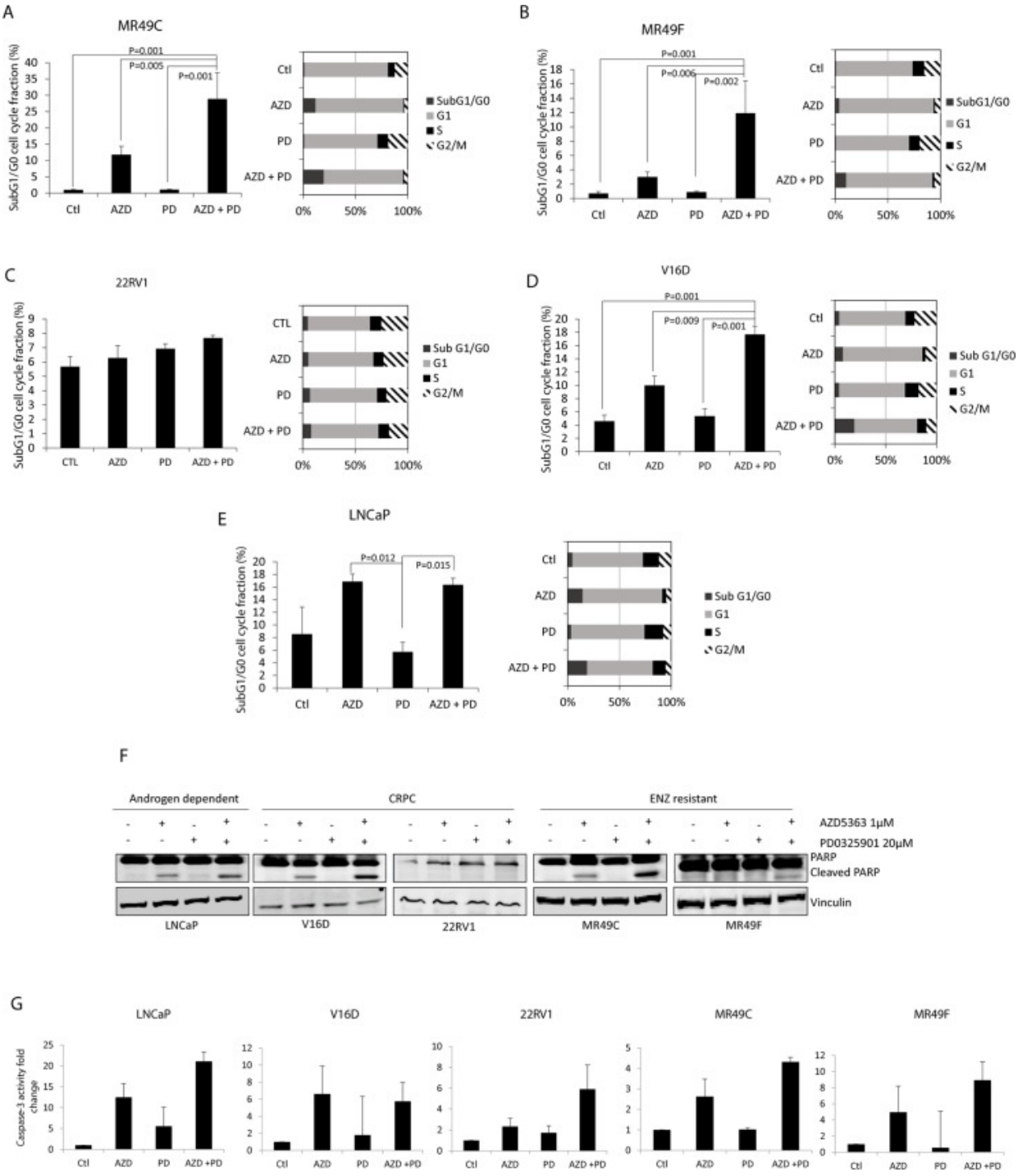


Figure 3.3 Combination AZD5363 + PD325901 increases apoptosis. **A-E.** Propidium iodide flow cytometry cell cycle analysis of indicated cell lines shows increased apoptotic cell cycle fraction (SubG1/G0) (left panels). Means of triplicate experiments are plotted +/- SEM. Representative results of all cell cycle populations are shown in right panels. **F.** Indicated cells were treated with AZD5363 1μM, PD0325901 20μM, or the combination for 48 hours. Proteins were extracted and western blot was performed using PARP antibody, vinculin was used as a loading control. Representative blots of two or more experiments are shown. **G.** Caspase-3 activity in MR49C, MR49F, 22RV1, LNCaP and V16D cell lines treated with AZD5363 and/or PD0325901 for 24 hours. Mean fold change +/-SEM of pooled values from at least two biologic duplicates are shown.

We next investigated if the effect observed in cell apoptosis can be translated to cell viability. Our data show that targeting AKT using AZD5363 affects cell viability in all cell lines tested (Figure 3.4) while targeting MEK using either PD0325901 (Figure 3.4) or UO126 (Figure 3.5) had greater activity in 22RV1 cells compared to the other cell lines further confirming our data on cell cycle population and PARP cleavage. Although we observed trends for decreased viability and synergy with the combination compared to monotherapy in LNCaP cells and V16D cells, in MR49C and MR49F cells the combination did not appear synergistic (Figure 3.5). This was further confirmed using the combination of MEK inhibitor U0126 with the AKT inhibitor LY294006 in MR49C, MR49F cells (Figure 3.5). Synergy was observed in 22RV1 cells with a combination index <1 (Figure 3.5,3.6).

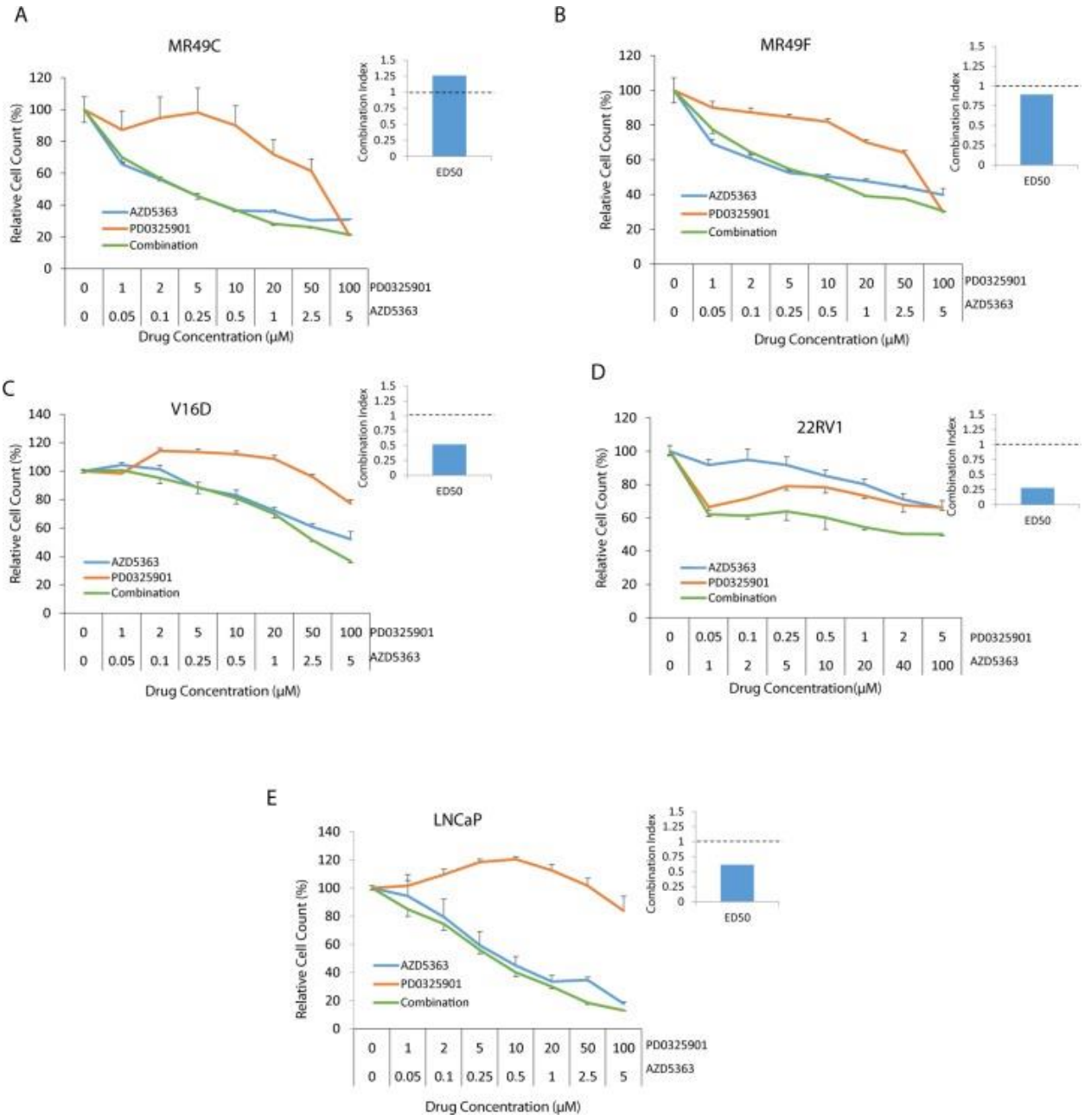
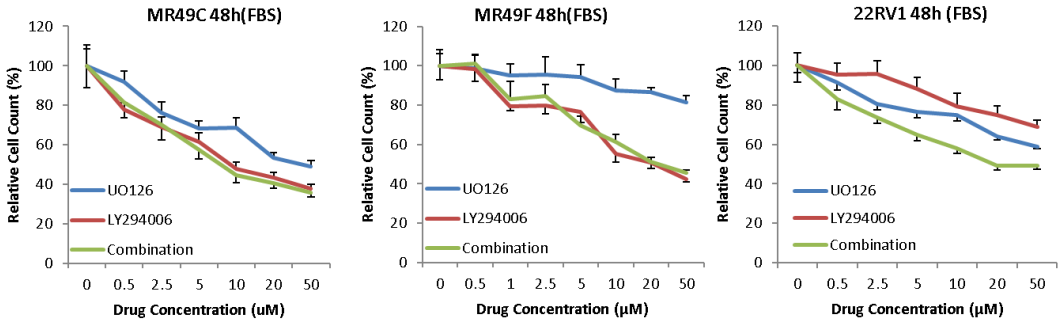


Figure 3.4 Effect of AZD5363 and PD0325901 on cell viability. **A-E.** MR49C, MR49F, 22RV1, LNCaP and V16D cell lines were treated with AZD5363 and/or PD0325901 as indicated for 48 hours and cell viability was assessed using WST-1 assay. Pooled results of biologic triplicates with technical triplicates are shown. Combination indices are shown inset, with values <1 indicating synergy.

A



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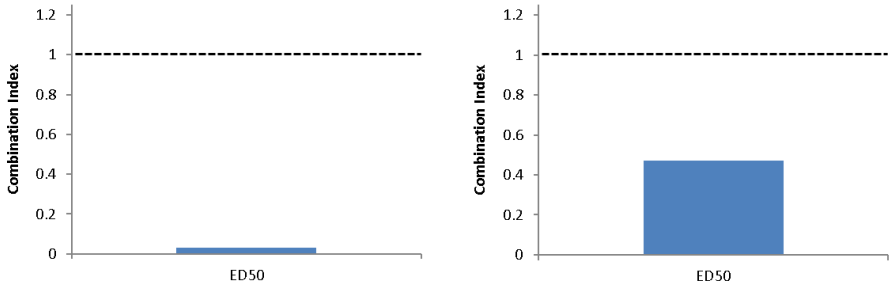

Figure 3.5 Effect of combination AKT plus MEK inhibition with alternate inhibitors on cell proliferation. **A.** Indicated cell lines were treated with LY294002 and UO126 at indicated doses and cell viability was assessed using crystal violet. Results shown are pooled values of triplicate repeats of biologic triplicate experiments +/- SEM. **B.** Combination indices calculated for AZD5363 + PD0325901 combination (left) and UO126 + LY294002(right) from pooled crystal violet proliferation results. Values <1 indicate synergy.

3.3.3 Targeting AKT and MEK Pathways in Combination Inhibits Tumour Growth and Improves Cancer Specific Survival

Since the activity of combination therapy showed differences related to PTEN expression, we investigated *in vivo* how targeting AKT and MEK will influence tumour growth in two possible clinical settings (PTEN positive and negative tumors). We used ENZ-resistant MR49F cells and CRPC 22RV1 cells to assess the *in vivo* efficacy of the

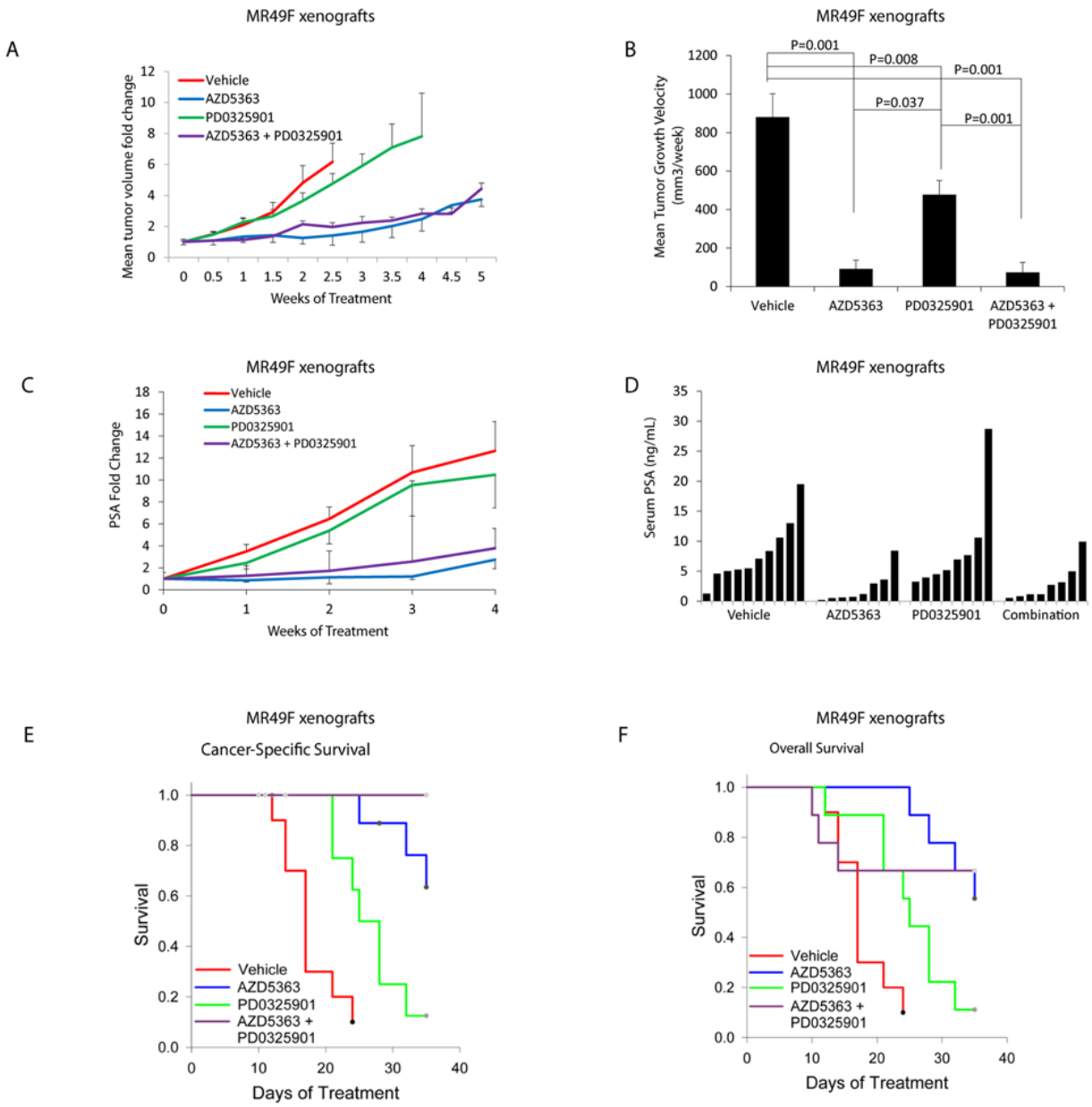

Figure 3.6 Effect of targeting MEK and AKT using PD0325901 and AZD5363 in MR49F xenografts. After establishment of tumors (200mm3) from subcutaneous injection of ENZ resistant MR49F cells in castrated mice under the pressure of 10mg/kg daily of ENZ, mice were treated with 100mg/kg, AZD5363 100mg/kg BID, PD0325901 5mg/kg QD or AZD5363 100mg/kg BID + PD0325901 5mg/kg QD. **A.** Representative data of MR49F mean tumor volume over 4 weeks is shown. **B.** Mean MR49F tumor growth velocity for each treatment group +/-SEM calculated using linear regression estimation of tumor growth velocity for each mouse. **C.** Mean MR49F weekly PSA plotted as fold change from baseline for each treatment group +/-SEM. **D.** Waterfall plot of individual PSA measurements after 3 weeks of treatment for all MR49F xenografts in the study. **E-F.** Kaplan-Meier cancer specific survival and overall survival curves for treatment arms of MR49F xenografts.

combination of targeting AKT and MEK pathways. The MR49F xenografts were sensitive to AZD5363, making assessment of additional benefit with MEK blockade challenging. Monotherapy treatment with PD0325901 demonstrated tumour growth inhibition and demonstrated a non-significant trend to lower serum PSA compared to vehicle (Figure 3.6). Combination treatment with AZD5363 and PD0325901 in the MR49F xenografts did not result in greater decreases in tumour volume compared to AZD5363 monotherapy, but the combination did have significantly improved cancer specific survival ($P<0.001$) compared to vehicle- and PD0325901- treatment arms (Figure 3.6). Median cancer specific survival was 17 days for vehicle-treated mice, 25 days for PD0325901-treated mice, and was not reached for either AZD5363 alone or in combination with PD0325901. No mice in the combination arm were euthanized due to tumour size after 35 days of treatment. Serum PSA was significantly reduced in both the AZD5363 and the combination treatment arms compared to vehicle (Figure 3.6).

We next assessed the combination of AKT and MEK pathway inhibition using 22RV1 xenografts in castrated mice. AZD5363 was again used as an AKT inhibitor while selumetinib was chosen as a MEK inhibitor which may be more clinically relevant as it is currently in clinical evaluation and was recently approved for treatment of melanoma. In contrast to the MR49F model, 22RV1 tumors were relatively insensitive to AZD5363, but did demonstrate tumour growth inhibition to both selumetinib and the combination of selumetinib plus AZD5363. With all 22RV1 tumors growing relatively robustly despite treatment, no significant differences were found between treatment arms, though the greatest tumour growth inhibition was seen with combination treatment (Figure 3.7).

Analysis of the individual mice tumour volumes in the MR49F model suggests that combined blockade may benefit a subset of mice (Figure 3.8). In the AZD5363 monotherapy arm, while most tumors responded well, in select cases, the tumour growth inhibition was minimal. However, in the combination therapy group, no outliers were noted (Figure 3.8). Notably, immunohistochemistry staining of the MR49F tumors demonstrated pERK staining in the mice which demonstrated resistance to AZD5363 (Figure 3.8). No significant pERK staining was seen in the mice who responded to the treatment (Figure 3.8) nor were differences in pS6 staining or Ki67 staining detected between combination treatment and the AZD5363 monotherapy arm (Figure 3.8).

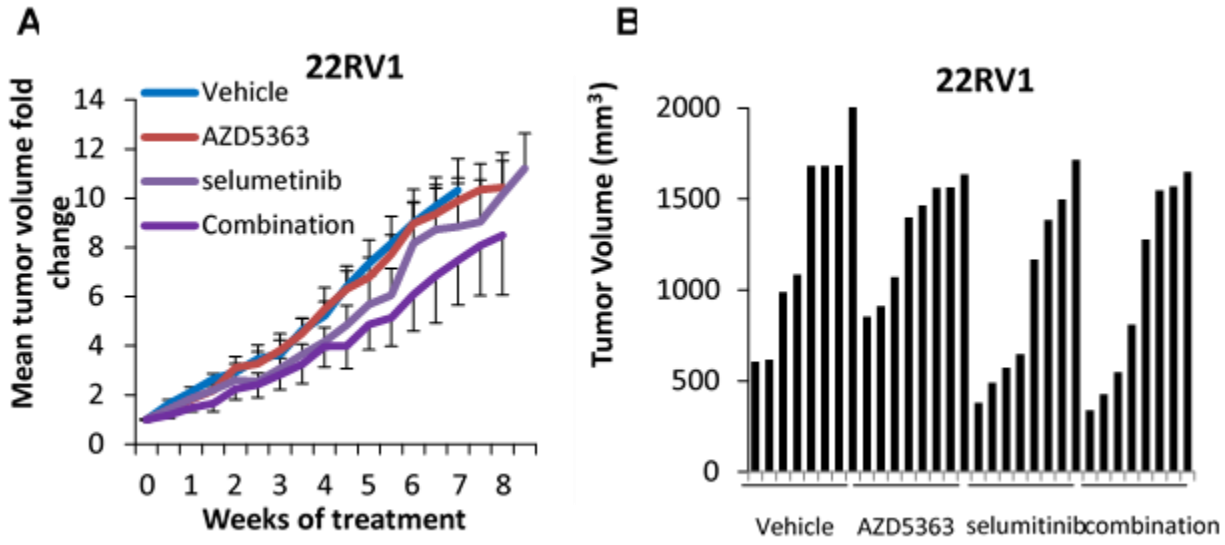

Figure 3.7 Effect of targeting MEK and AKT using selumetinib and AZD5363 in 22RV1 xenografts. **A.** Mean 22RV1 xenograft volume following treatment with vehicle, AZD5363 100mg/kg BID, selumetinib 25mg/kg QD or AZD5363 100mg/kg BID + selumetinib 25mg/kg QD. Mean fold change in tumor size is plotted +/-SEM. **B.** Waterfall plot of 22RV1 xenograft tumor volumes after 6 weeks of treatment.

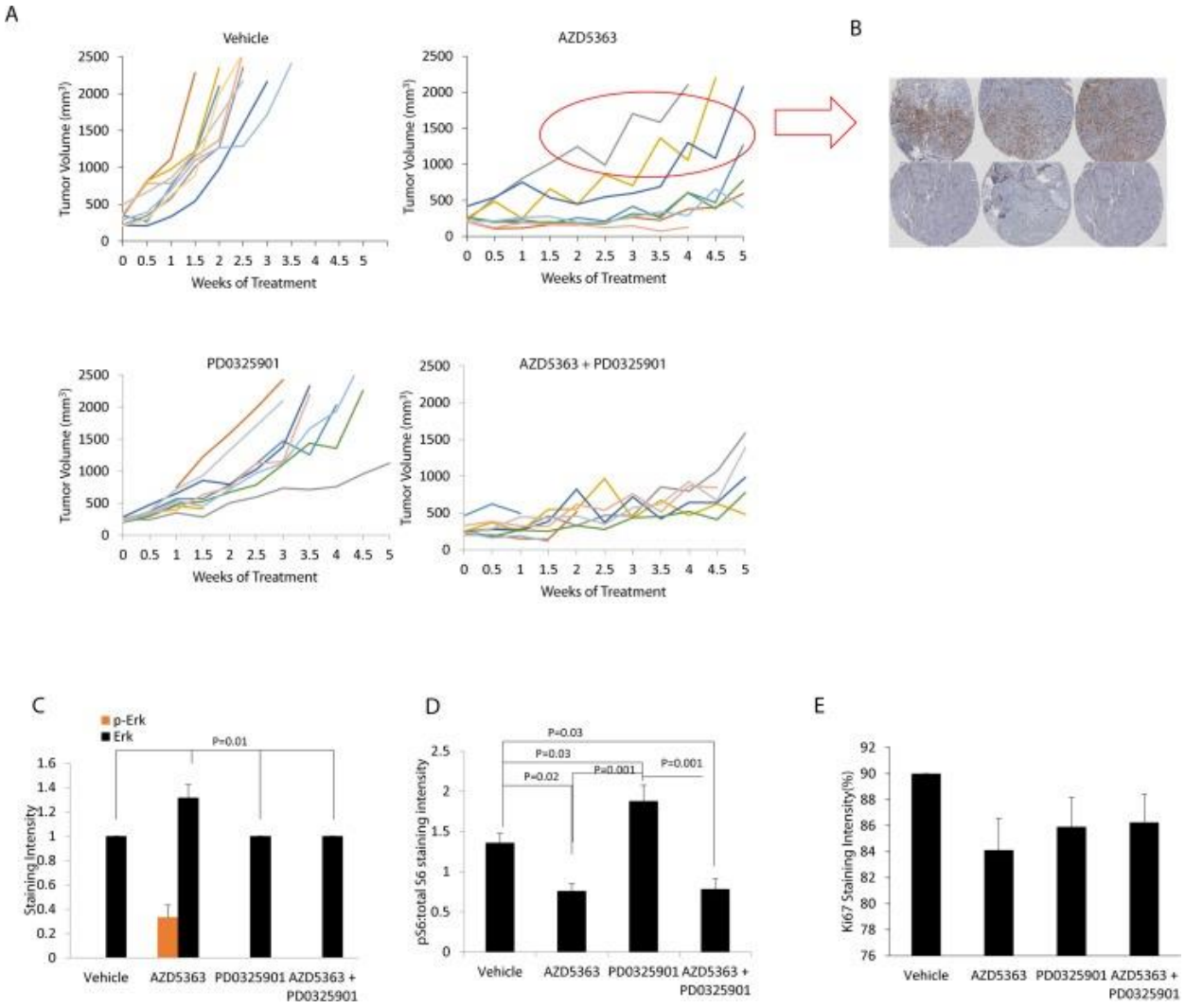

Figure 3.8 Analysis of tumors treated with combination MEK + Akt inhibition. **A.** Individual tumor growth curves for all mice in the study grouped by treatment. **B.** Immunohistochemistry of pERK demonstrates detectable levels in 3 mice with early resistance to AZD5363 treatment (top); 3 other mice treated with AZD5363 are shown for comparison (bottom). **C.** Staining of microarray specimens demonstrates that positive pERK staining was only evident in these mice. **D.** The proportion of pS6 is decreased with AZD5363 and to a greater extent with combination AZD5363 + PD0325901. **E.** Ki67 staining as a marker of proliferation. A tissue microarray was constructed using tumor samples in each group (7-9 per group). Mean scores of staining intensity graded by a blinded pathologist are shown +/- SEM.

3.4 Discussion

Despite the success of castration and newer potent AR pathway inhibitors such as enzalutamide, treatment resistance occurs in all cases. Persistent AR signalling is usually clinically evident by a rising PSA. The persistence of AR-signalling may be driven by several mechanisms of resistance including AR splice variants, intratumoral production of androgens and activation of alternate pathways which support AR signalling, including the AKT and MEK pathways (293). Combination therapy in prostate cancer holds potential for increasing patient survival by blocking these resistance pathways.

Early clinical experience in prostate cancer patients suggests that targeting AKT alone is not an effective strategy. Clinical trials of AKT inhibitor monotherapy in CRPC have demonstrated limited success with this approach (225, 226). Prior *in vitro* work suggests that PI3K/AKT inhibition can result in up-regulation of the Raf/MEK/ERK pathway (294), and our results in the MR49F xenograft model also suggests this may occur in some cases. The well-recognized crosstalk between these pathways provides a rationale for combining both of these inhibitors (284-287, 295-297). Clinical experience with dual targeting of MEK and AKT pathways is limited; early results in advanced malignancies suggests that dual targeting of both pathways improves oncologic efficacy at the cost of greater toxicity(298).

Our study highlights the differences which can occur between AR positive prostate cancer models and especially the relevance of PTEN status when targeting AKT and MEK pathways. Interestingly, targeting the AKT pathway increased PSA transcription in the surviving cells and this effect, noted previously by others (244), may be important to consider in the design of future clinical trials with these targeted agents since PSA is

commonly used as a marker of progression. In the LNCaP-derived ENZ-resistant models, MEK inhibition added a modest benefit compared to AKT inhibition; interestingly the synergy appeared greater *in vivo* than *in vitro*. In 22RV1 cells, MEK inhibition alone had significant impact on cell proliferation and signalling, but the combination of MEK and AKT inhibition demonstrated minimal additional benefit *in vitro*. Thus, while improvements in anticancer activity were noted by some metrics with the combination in each model, each model appeared relative addicted to AKT and MEK, respectively, thus limiting the benefit of combination therapy.

Combined targeting of the MEK and AKT pathway has been investigated in several other cancers in pre-clinical models, with results supporting the use of the combination (284-287, 295-297). In a pre-clinical prostate cancer *in vivo* study, PD0325901 was assessed in combination with the mTOR inhibitor rapamycin (122). While we did not observe any significant decrease in Ki67 with the combination, this may be related to collecting all of our tumors at the end of study at which point the xenograft tumors were treatment-resistant. Recently, Park et al, showed the combination of selumitinib and the pan-PI3K inhibitor GSK2126458 improved tumour growth inhibition compared to either monotherapy in AR-negative DU145 and PC3 xenografts(299). Overall, their results in AR-negative models are comparable to our results. Taken together, our data suggest that the efficacy of targeting AKT and MEK is independent of the disease state of prostate cancer and that the efficacy correlates with the activation of these pathways in the tumour.

Our study is limited by several aspects of our prostate cancer models. LNCaP cells appear to be highly dependent on the AKT pathway. Nonetheless, as AR positive, PSA-producing cell lines with an activated PI3K/AKT pathway, they do resemble a common

CPRC phenotype encountered (72). Moreover, our ENZ-resistant models demonstrate several phenotypes consistent with clinical ENZ-resistant disease, such as persistent AR nuclear localization, AR F876L mutation, and upregulation of steroiodogenic enzymes (251, 252, 300). 22RV1 cells harbor androgen splice variants, which predict a poorer response to AR-pathway inhibitors (54). The presence of the enzalutamide-agonistic F876L mutation and the presence of dominant AR splice variants limited our ability to test for the efficacy of combined AKT and MEK inhibition together with AR inhibition in advanced prostate cancer models. Further, while our *in vitro* studies were not performed in androgen deficient media, it is unclear whether this would result in significantly different results, particularly as CRPC tumors can produce their own androgens through *de novo* synthesis pathways (35). Accordingly, our results in castrate conditions *in vivo* were not substantially different.

In conclusion, our results in ENZ-resistant CRPC cell models suggest that combination treatment with AKT and MEK inhibition may be a rational combination in a subset of resistant prostate cancer cases needing further investigation. Our results also suggest the importance of targeting inhibitors to tumour pathways which are known to be activated; in well-selected patients monotherapy may be as effective as combination therapy. Overall, this emphasizes the need for rational, biomarker-driven selection of patients as targeted therapeutics are evaluated in clinical trials.

4 Combination AZD5363 with Enzalutamide Significantly Delays Enzalutamide Resistant Prostate Cancer

4.1 Introduction

Prostate cancer continues to be a common cancer in western countries, and a leading cause of cancer-related death. While new AR-targeting therapies such as abiraterone and enzalutamide highlight the success of targeting the androgen receptor (AR) axis (183, 274), these therapies are not curative and resistance inevitably develops. Therefore, there remains a need for rationale therapeutic strategies to delay the development of resistance.

The PI3K/Akt-pathway is up-regulated in CRPC and predicts a poorer prognosis (97, 100, 301). Akt signalling is involved in numerous cellular processes including cell growth, survival, and metastases (74, 302). Pre-clinical research demonstrates that the PI3K/Akt/mTOR pathway and AR pathways interact reciprocally both in settings of resistance and carcinogenesis (81, 105, 135, 136, 303, 304). Further, these experiments provide a rationale explaining disappointing results of prior trials of PI3K/Akt/mTOR pathway inhibitors as monotherapy in advanced prostate cancer (225, 226, 305).

AZD5363 functions as an orally available ATP-competitive pan-Akt inhibitor(306). Prior work has demonstrated that it synergizes with autophagy inhibitors(120) and with bicalutamide(134). Interestingly, the synergy with bicalutamide was greater in the androgen resistant C4-2 cell lines than LNCaP(134). In this study, we sought to investigate the relevance of Akt inhibition with AZD5363 in the context of the development of ENZ-resistance prostate cancer.

4.2 Materials and Methods

4.2.1 Cell Culture Reagents

The human prostate cancer LNCaP, C4-2 and 22RV1 cell lines were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal bovine serum (FBS) and cultured without antibiotics at 37°C in 5% CO_2 atmosphere. MR49C and MR49F cells were derived as previously described(251) and were maintained in RPMI 1640 supplemented with 10% FBS and 10μM ENZ. For the *in vitro* studies, AZD5363(AstraZeneca, Macclesfield, UK) was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) at 10mM stock solutions and stored at -20ºC. For *in vitro* use, ENZ (Shanghai Haoyuan Chemexpress, Shanghai, China) was dissolved in H_2O and stored at 4 ºC.

4.2.2 Cell Proliferation and Apoptosis Assays

Cell proliferation was assessed in 96 well plates using the crystal violet assay as previously described(251). Synergy was determined using the method of median effect principle first described by Chou and Talalay (39) and calculated using the Calcusyn™ software (Biosoft, Cambridge, UK). Caspase-3 activity was assessed using the Caspase-3 Assay kit (Enzo Life Sciences, Farmingdale, NY), with the acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) fluorometric substrate incubated with 30μg of cell lysate at room temperature for 2h and fluorescence quantified with a fluorometer with excitation set at 365nm and emission 460nm. Cell cycle analysis with propidium iodide

staining was performed after 24 hours of treatment as previously described using a FACS Canto II flow cytometer(120).

4.2.3 Protein Detection

Western blots were probed with antibodies against Cyclin D1, PSA, AR N-20 (Santa Cruz Biotechnology, Dallas, TX); PARP, p-Akt Ser473, Akt, p-mTOR, mTOR, p-4E-BP1, 4E-BP1, S6 and p-S6 (Cell Signaling Technology, Danvers, MA) and vinculin (Sigma-Aldrich, St. Louis, MO) as previously reported(120). Immunohistochemical staining was performed as previously reported and graded on triplicate samples by a pathologist blinded to treatment assignment at 20x magnification(120).

4.2.4 Animal Treatment

Six week old male castrated athymic nude mice (Harlan Sprague-Dawley, Inc.) were subcutaneously injected with $2x10^6$ MR49F cells (suspended in 0.1ml Matrigel; BD Biosciences) on both flanks. ENZ 10mg/kg was started prior to inoculation and continued until tumours exceeded $200mm^3$, when mice were randomized to treatment. Dosing for AZD5363 in MR49F xenografts was 100mg/kg without ENZ and later 75mg/kg and 37.5mg/kg with 10mg/kg ENZ with the control arms receiving vehicle and ENZ, respectively. Mice were sacrificed when the total tumour burden was $>2000mm^3$ for the initial MR49F xenograft study (bilateral flank injection) or $>1500\ mm^3$ for the second MR49F xenograft study (unilateral flank injection) or animals lost >20% of body weight.

For the LNCaP CRPC xenografts, the six week old male athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected subcutaneously with $2x10^6$ LNCaP cells (suspended in 0.1ml Matrigel) on both flanks. Mice were castrated when PSA values exceeded 50ng/mL. Treatment started when the PSA rose to pre-castration levels or were there was two consecutive rises above nadir with concomitant tumour regrowth. For treatment, 10 mice per arm mice were randomized to vehicle (1% Tween 80 + 10% DMSO), AZD5363 BID (37.5 mg/kg), ENZ OD (10mg/kg) or the combination in cycles of 5 days on, 2 days off. Animals were sacrificed when tumour volume reached $\geq$ 10% of body weight or animals lost >20% body weight.

Tumor volume was measured with calipers (l x w x d x 0.5236) and serum PSA measurements taken from tail vein were measured using automated immunoassay (Cobas, Montreal, Quebec, Canada). Tumors collected at sacrifice were divided into parts and snap frozen in liquid nitrogen or fixed in formalin. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care along with appropriate institutional certification.

4.2.5 Statistical Analysis

All results are expressed as the mean $\pm$ SE, a student t-test compared means. To compare the tumour growth velocity between treatment groups, we took the linear regression for the log of each mouse total tumour volume over time and compared groups using a student t-test. PSA velocity was calculated using linear regression. Kaplan-Meier

survival analysis was performed for cancer specific survival and overall survival. Cancer specific survival was defined as the time until tumour volume exceeded study limits.

4.3 Results

4.3.1 The Akt Pathway is a Key Pathway Active in ENZ-Resistance

Concordant with prior reports, we found the Akt pathway is activated following treatment with ENZ (Figure 4.1)(81). Basal Akt and p-Akt levels in LNCaP, MR49C and MR49F cells demonstrated that the Akt pathway was most active in the MR49F cells

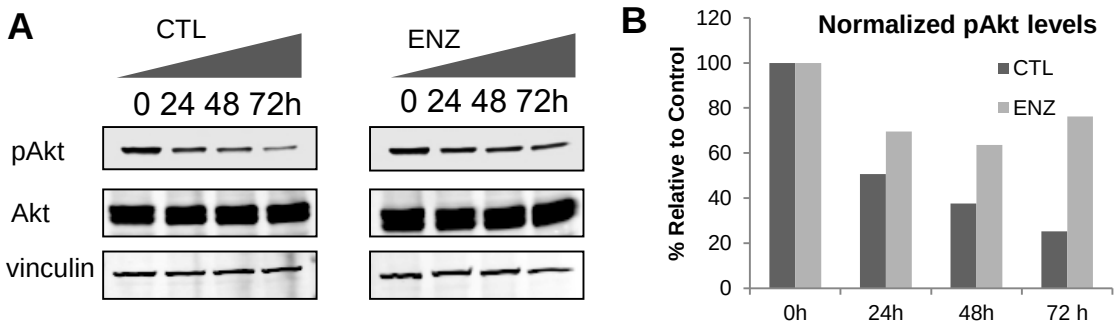

Figure 4.1 Time course demonstrating an increase of phospho-Akt protein levels with enzalutamide 10μM treatment relative to DMSO control over 72h of treatment. Bar graph (right panel) shows densitometric intensity of the bands on the left panel with pAkt levels normalized to vinculin levels.

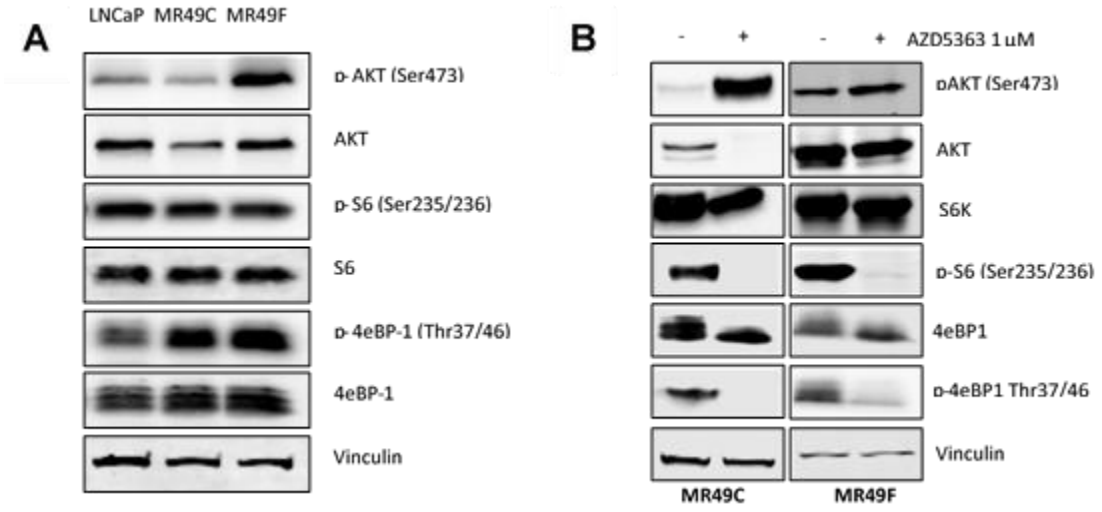

Figure 4.2 The Akt signalling pathway in LNCaP, MR49C and MR49F cells. **A.** Western blot showing basal levels of pAkt and Akt and downstream effectors S6, 4eBP-1 in ENZ-resistant cell lines MR49C, MR49C and parenteral LNCaP. Following culture in 10% CSS for 24h, total protein was extracted from MR49F and MR49 cells and western blots were performed for indicated antibodies. **B.** Effect of AZD5363 on Akt signaling pathway in ENZ-resistant cells: MR49C and MR49F were treated with 1μM AZD5363 for 24h in 10% FBS, total protein was extracted and western blots were performed on key phosphorylated proteins in the Akt signaling pathway.

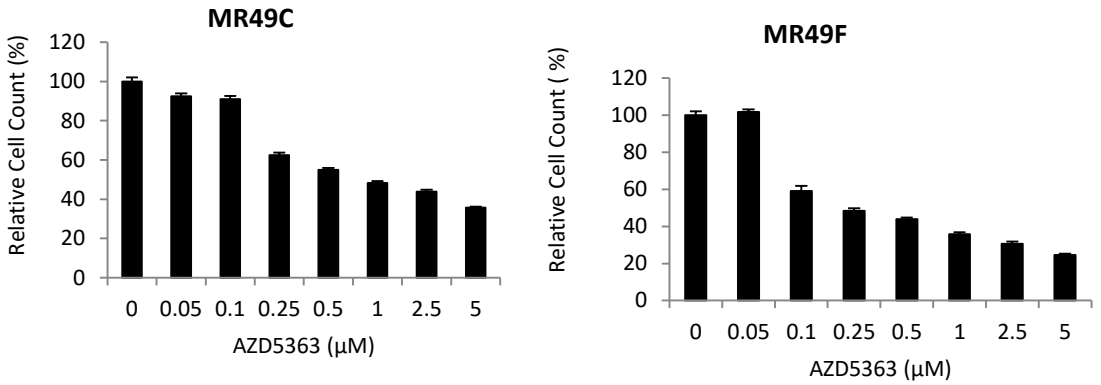

Figure 4.3 AZD5363 decreases MR49C and MR49F cell number in a dose-dependent manner. Crystal violet assay was performed after 48h of treatment with indicated doses of AZD5363 in 10%FBS. Pooled means +/- SEM from 3 experiments with triplicates are shown.

(Figure 4.2A). Consistent with other ATP competitive, catalytic inhibitors of Akt, AZD5363 resulted in an accumulation of inactive p-Akt (120, 290), evidenced by downstream decreases in p-S6 and 4e-BP-1 in both cell lines (Figure 4.2B, see also Figure 3A).

These signalling effects in MR49C and MR49F cells corresponded with dose-dependent decreases in cell viability (Figure 4.3). Cell-cycle analysis after AZD5363

treatment demonstrated an increase in subG1/G0 population, as well as a decrease in the S-phase for both cell lines (Figure 4.4). Similarly, we found a 3-4-fold increase of caspase-3 activity and an increase in cleaved PARP protein levels (Figure 4.5).

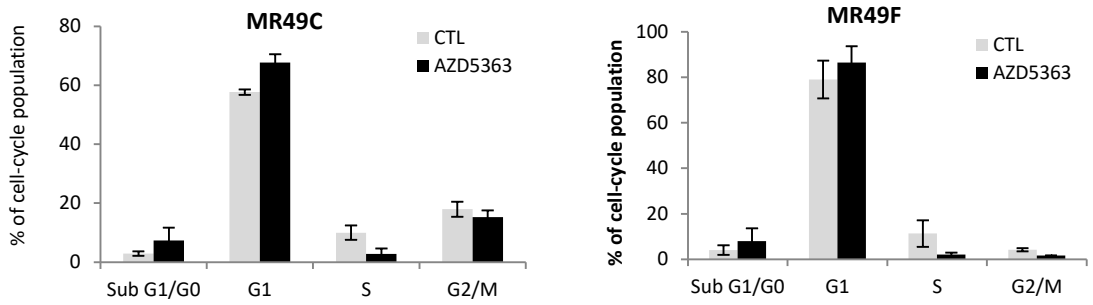

Figure 4.4 AZD5363 effect on MR49C and MR49F cell cycle. MR49C and MR49F were treated with AZD5363 for 24 hours and then cells were fixed and stained with propidium iodide and analyzed by flow cytometry.

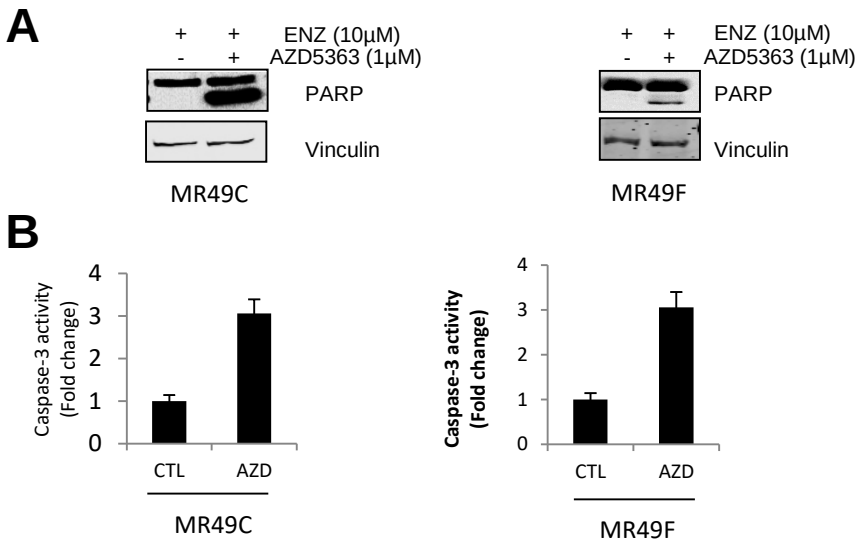

Figure 4.5 AZD5363 induces apoptosis in MR49C and MR49F. **A.** Cleaved PARP protein levels in MR49C and MR49F cells after 24h of treatment with 1μM AZD5363 in RPMI with 10% FBS. **B.** Caspase-3 activity fluorescence assay results on 30ug protein from cells treated for 24h.

4.3.2 AZD5363 has Significant Activity in an ENZ-Resistant Xenograft Model

With consistent anti-cancer activity of AZD5363 *in vitro*, we tested its activity in vivo as monotherapy in castrate mice using the MR49F xenograft model (262). We observed significant response to AZD5363 treatment in terms of tumour growth inhibition and PSA response (Figure 4.6). The tumour growth velocity on treatment was significantly different (173mm^3/week vs 599mm^3/week, P<0.001). Similarly, the PSA velocity was significantly different (P<0.01). Median cancer specific survival was 18 days

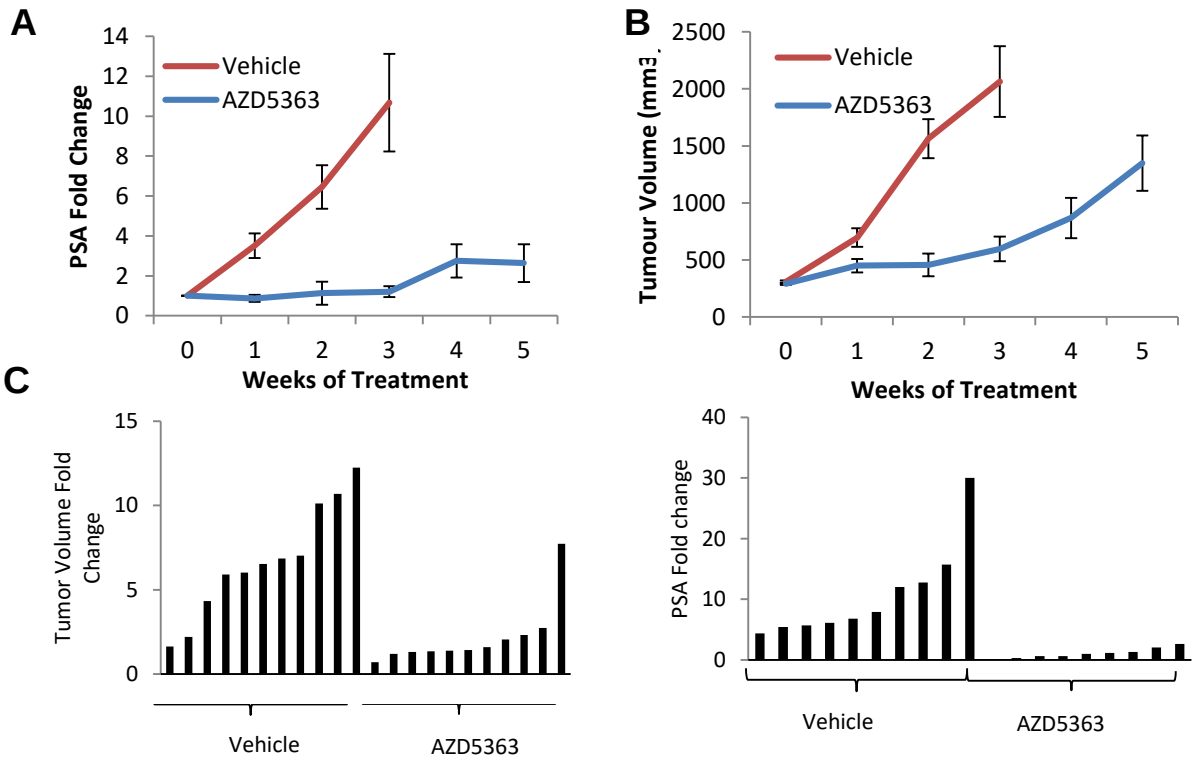

Figure 4.6 Effect of AZD5363 alone on MR49F tumour growth in castrated mice. **A.** Weekly mean tumour volume in mice treated with AZD5363 100mg/kg BID 5 days on 2 days off vs vehicle control once tumours reached 200mm3. ENZ was stopped when tumour size reached 200mm^3 **B**. Effect of AZD5363 on MR49F serum PSA: mean weekly serum PSA value in mice treated with AZD5363 100mg/kg. **C**. Waterfall plot showing individual responses in tumour volume and serum PSA change from baseline between groups at 3 weeks.

in vehicle treated mice and not reached after 5 weeks of treatment in the AZD5363 arm. (Log rank P<0.001; Figure 4.7). The only toxicity noted was a trend toward more weight loss in the AZD5363 arm, with 1/10 mice euthanized after 28 days of treatment for >20%

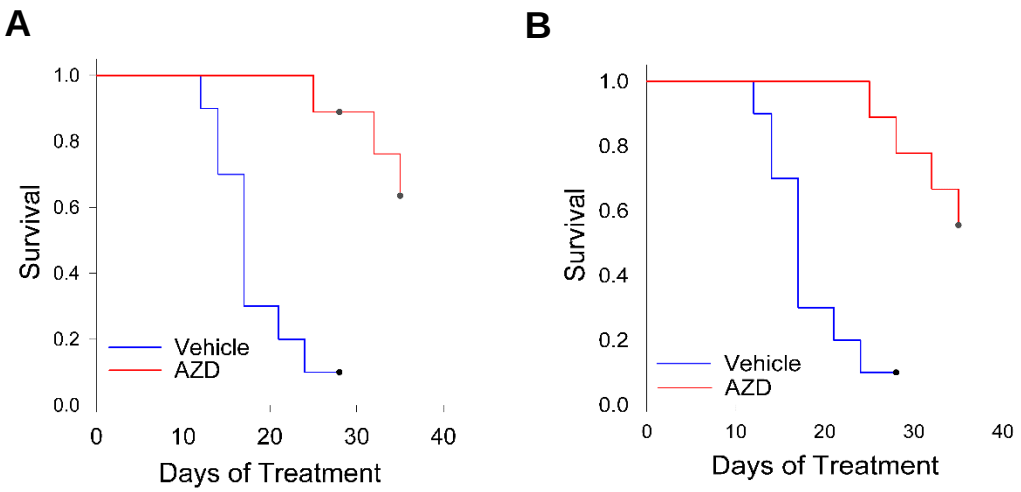

Figure 4.7 Effect of AZD5363 on survival of MR49F xenografted mice. Kaplan-Meier plot of cancer-specific survival (**A**) and overall survival (**B**) of mice with MR49F xenografts treated with 100mg/kg BID AZD5363 or vehicle.

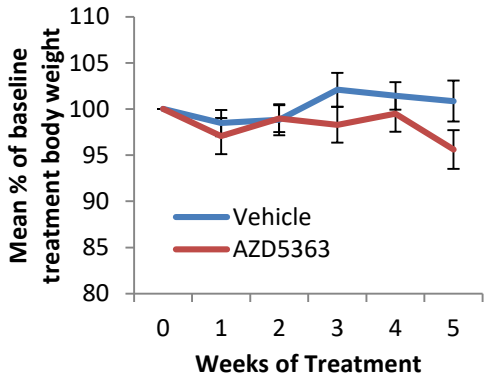

Figure 4.8 On-treatment weight in AZD5363-treated mice versus control. Weekly treatment weight as percentage of baseline treatment weight among mice receiving AZD5363 100mg/kg or vehicle.

weight loss (Figure 4.8).

Notably, the correlation between the rate of individual tumour growth and PSA velocity both calculated by linear regression was higher for AZD5363-treated tumours

(R^2=0.96) than vehicle tumours (R^2= 0.38, Figure 4.9). This high correlation as well as the continued PSA rise (Figure 4.6, right panel) suggests that resistance to AZD5363 was AR-driven.

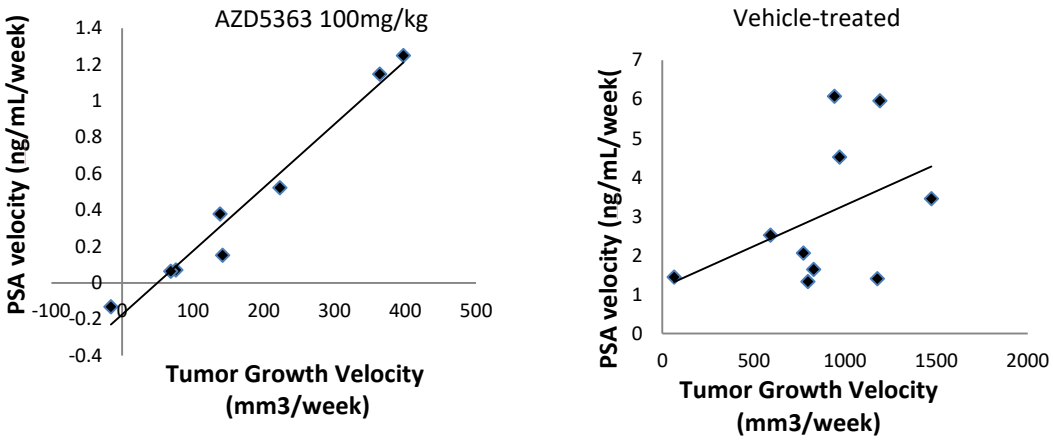

Figure 4.9 Correlation between tumor growth and serum PSA in MR49F murine xenografts. A greater correlated in AZD5363-treated MR49F xenograft mice was observed, suggesting the AR axis is important in resistance to AZD5363.

Immunohistochemistry of collected tumours demonstrated very high levels of Akt in the vehicle-treated mice (Figure 4.10). Increased pAkt levels, an expected on-target effect of AZD5363 were observed. A decrease in Ki67 staining confirms the anti-proliferative action *in vivo*.

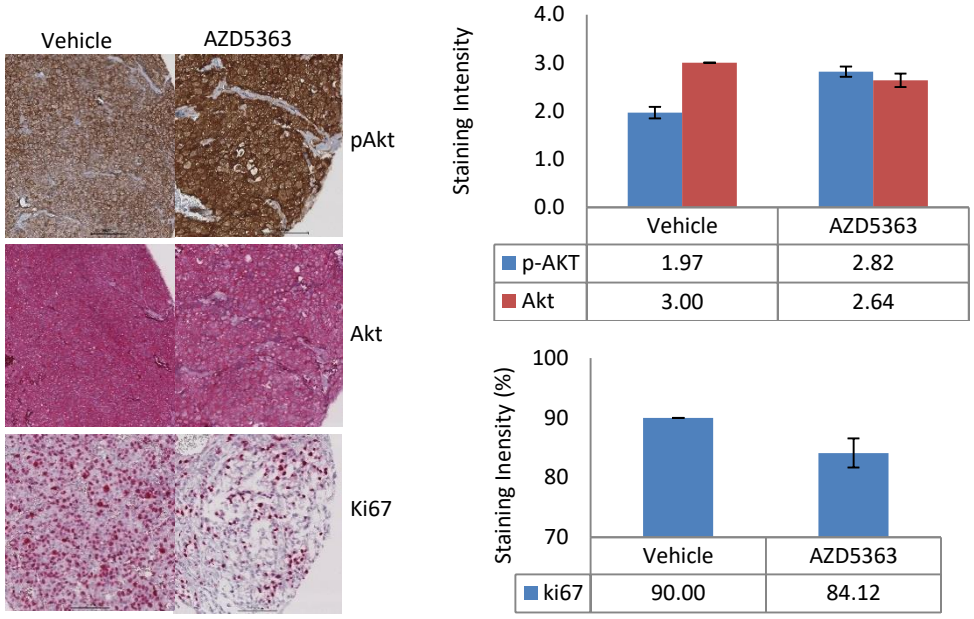

Figure 4.10 Immunohistochemical evaluation of effect of AZD5363 on collected tumor tissue. Ki67, pAkt and Akt immunohistochemistry on MR49F xenografts collected at the end of study after dosing of 100mg/kg AZD5363 alone. A microarray of these tumours (10 vehicle and 8 AZD5363 samples with triplicates) was stained with 1/500 Ki67, 1/25 pAkt (Ser473), and 1/150 Akt. Representative images (left panel) demonstrate trends seen on mean staining intensity scores (right panel) by a blinded pathologist at 20X. Means are plotted ±SEM.

We further assessed if the combination of AZD5363 with ENZ would induce synergy in MR49F xenografts at lower doses of AZD5363. The presence of the ENZ-agonistic F876L mutation was confirmed to be present in these cell lines after these experiments were completed. In this experiment, MR49F cells were injected into one flank and AZD5363 dosed at 37.5mg/kg BID and 75mg/kg BID together with daily ENZ 10mg/kg compared to ENZ alone in the control arm. Due to lower tumour take, we halted accruing mice to the 37.5mg/kg arm mid-study to ensure adequate statistical numbers in the other arms. At the 75mg/kg dose, mean PSA velocity was lower in the AZD5363 arm compared to the control arm (P=0.03) (Figure 4.11). Tumor growth velocity showed a similar, non-significant trend to be lower with AZD5363 75mg/kg (P=0.08). Weight loss appeared greater than the prior study (Figure 4.12), likely as a result of larger tumours (based on single tumour size vs combined volume of bilateral tumours), as well as

multiple gavages (3/day in AZD5363 + ENZ arm). No differences in cancer specific survival was observed (Figure 4.13). In retrospect, the ENZ-agonist F876L in the MR49F cells (discovered after this study started) mutation helps explain the less dramatic results seen in this *in vivo* study, where AZD5363 nonetheless had significant effects, even at lower doses.

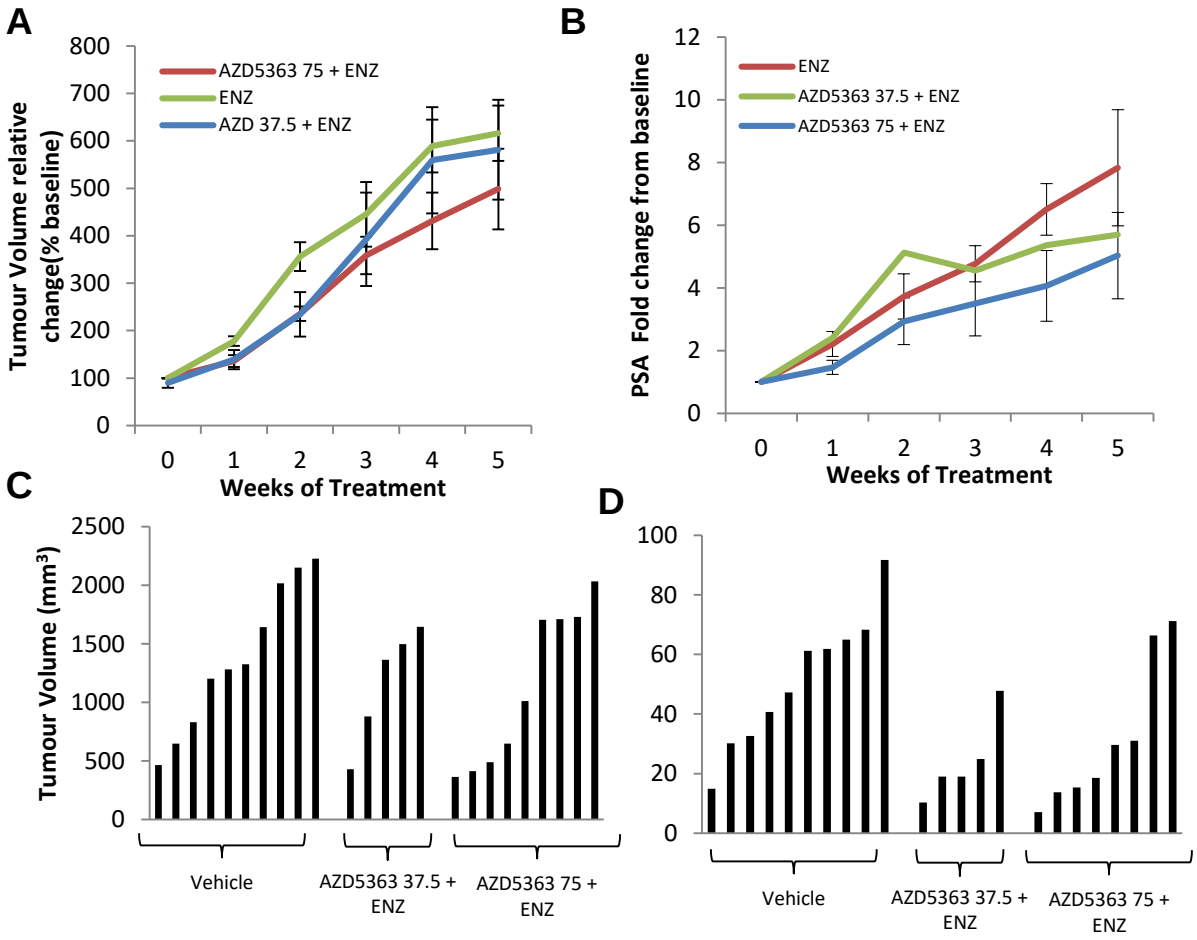

Figure 4.11 Effect of dosage of AZD5363 on MR49F tumour growth in castrated mice. **A**. Weekly mean tumour volume in mice treated with AZD5363 75mg/kg + ENZ 10mg/kg or ENZ 10mg/kg control in MR49F xenografts **B.** Effect of AZD5363 on MR49F serum PSA: mean weekly serum PSA value in mice treated with or without AZD5363 in addition to ENZ. **C.** Waterfall plot showing individual responses in tumour volume and serum PSA change from baseline between groups at 3 weeks. **D.** Waterfall plot showing individual responses in tumour volume and serum PSA change from baseline between groups at 3 weeks.

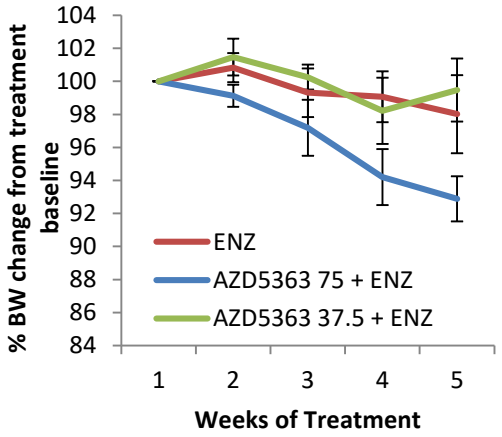

Figure 4.13 Weight of mice treated with different doses of AZD5363. Weekly treatment weight as percentage of baseline treatment weight among mice receiving AZD5363 37.5mg/kg, 75mg/kg or vehicle in addition to ENZ.

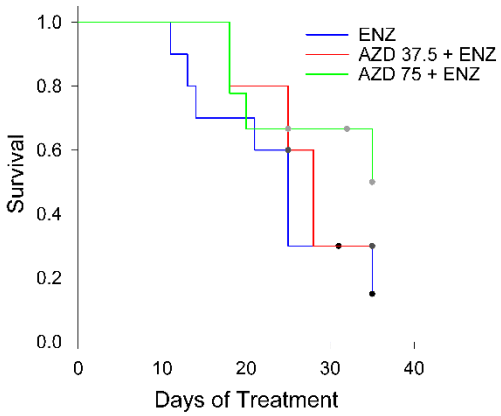

Figure 4.12 Survival of MR49F mice treated with different doses of AZD5363. Kaplan-Meier plot of overall survival of mice with MR49F xenografts treated with AZD5363 37.5mg/kg or 75mg/kg versus control, with all mice receiving ENZ throughout study.

4.3.3 Combination AZD5363 and ENZ Induces Apoptosis Through Synergistic Effects on Downstream Survival Pathways and Apoptosis

We hypothesized that earlier targeting may provide greater benefit and therefore evaluated the effect of dual Akt and AR blockade to delay the onset of ENZ-resistance. Using LNCaP, C4-2 and 22RV1 cells, protein signalling pathways were analyzed following 24h of treatment of with 1μM AZD5363 +/- 10μM ENZ (Figure 4.14). The combination of ENZ and AZD5363 resulted in a greater amount of inactive pAkt, evidenced by downstream decreases in p-S6 and 4e-BP1. Cyclin D1 levels also had greater decreases with combination treatment in all three cell lines. Cleaved PARP levels were highest in the combination group, indicating that a synergistic increase in apoptosis occurs with combination therapy. Interestingly, this was seen in the PTEN wild type 22RV1 cells, which demonstrated no cleaved PARP in response to AZD5363 monotherapy. Caspase-3 activity demonstrated 40-fold and 25-fold increases with AZD5363 + ENZ in LNCaP and C4-2 cell lines, respectively. A similar non-significant trend was seen in 22RV1 cells (Figure 4.15).

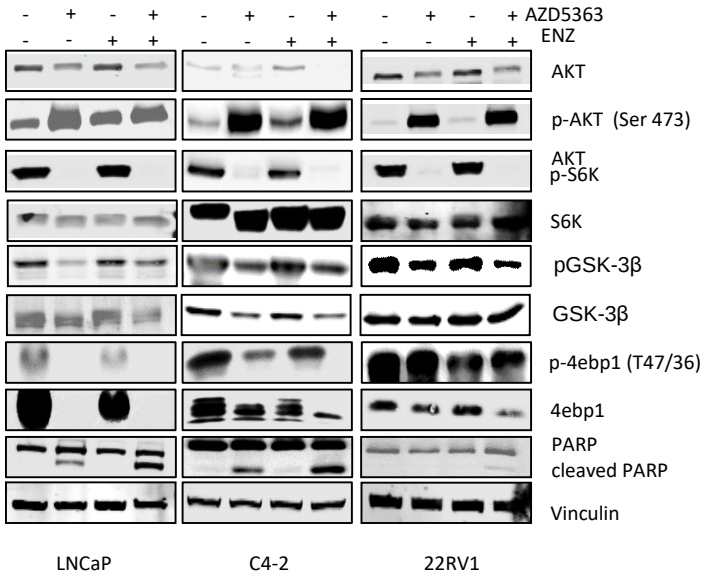

Figure 4.14 Effect of combination of AZD5363 and ENZ on Akt signaling pathway and apoptosis. Total proteins were extracted after treatment with control DMSO, 1μM AZD5363, 10μM ENZ or 1μM AZD5363 + 10μM ENZ and western blots were performed to evaluate Akt downstream effectors, apoptotic and cell cycle markers.

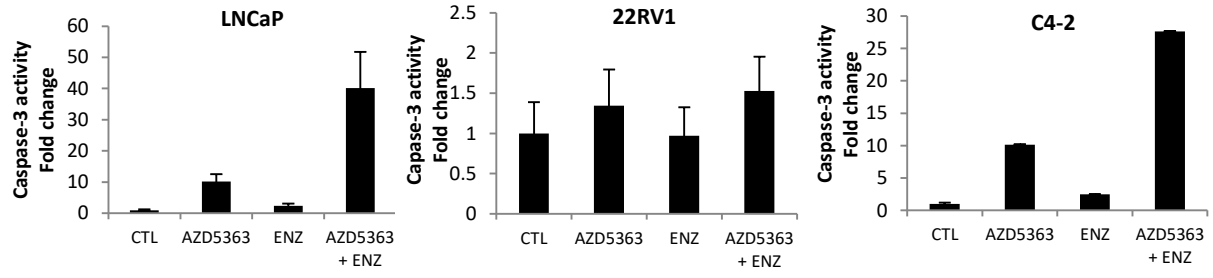

Figure 4.15 Induction of Caspase-3 with combination AZD5363 and enzalutamide. LNCaP, C4-2 and 22RV were treated DMSO, 1μM AZD5363, 10μM ENZ or 1μM AZD5363 + 10μM ENZ and proteins were extracted to evaluate caspase-3 activity by fluorescence assay. Means are plotted ±SEM.

We further sought to confirm the synergistic effects of combination AZD5363 and ENZ in these cell lines through proliferation assays and cell cycle analysis. After 24 hours of treatment an increase in subG1/G0 fraction and a decrease S-phase was seen in all three cell lines (Figure 4.16). As expected, cell viability after 48 hours of treatment was decreased in LNCaP and C4-2 cell lines with either monotherapy (Figure 4.17). However, the combination was most effective in reducing viability in all three cell lines. Calculated combination indices determined at ED_{50}, and ED_{75} (using Calcusyn software) revealed values below 1 in all cell lines (Figure 4.17), indicating a strong synergy. Notably, synergy was found even in the PTEN wild-type and ENZ- resistant 22RV1 cells (307).

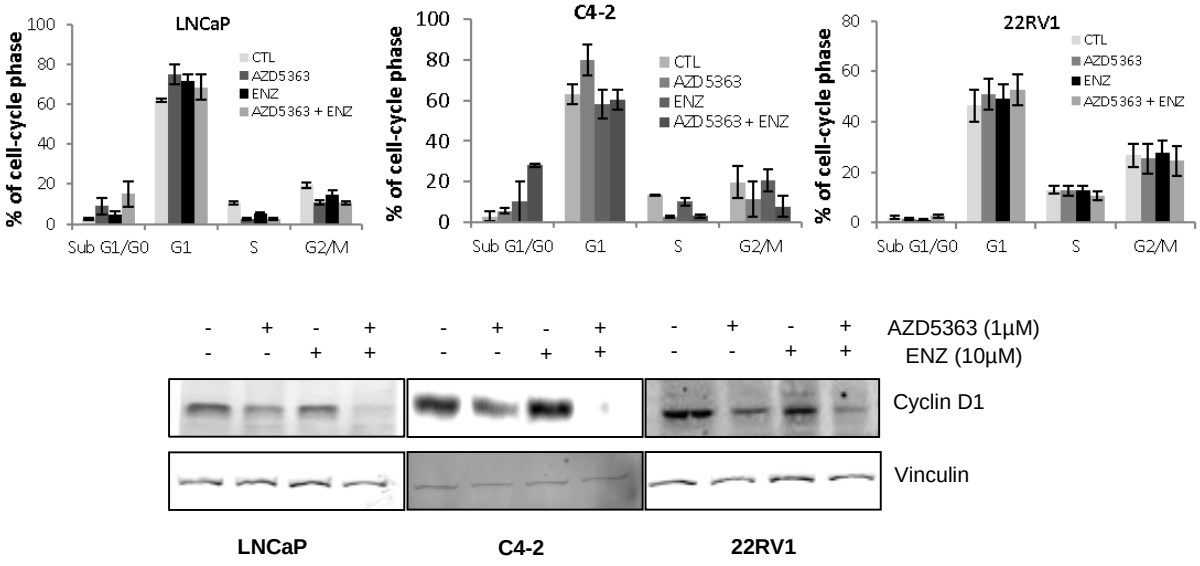

Figure 4.16 Effect of combination of AZD5363 and ENZ on cell cycle. LNCaP, C4-2 and 22RV1 cells were treated with monotherapy or combination AZD5363 + ENZ for 24 hours. **A.** Cells were fixed and stained with propidium iodide and analyzed by FACS. Pooled mean results of at least two separate experiments are shown. **B.** Western blotting of whole cell lysates and detection with cyclin D1 antibody demonstrate lower levels with combination therapy in all three cell lines. Representative results of two biologic duplicates are shown.

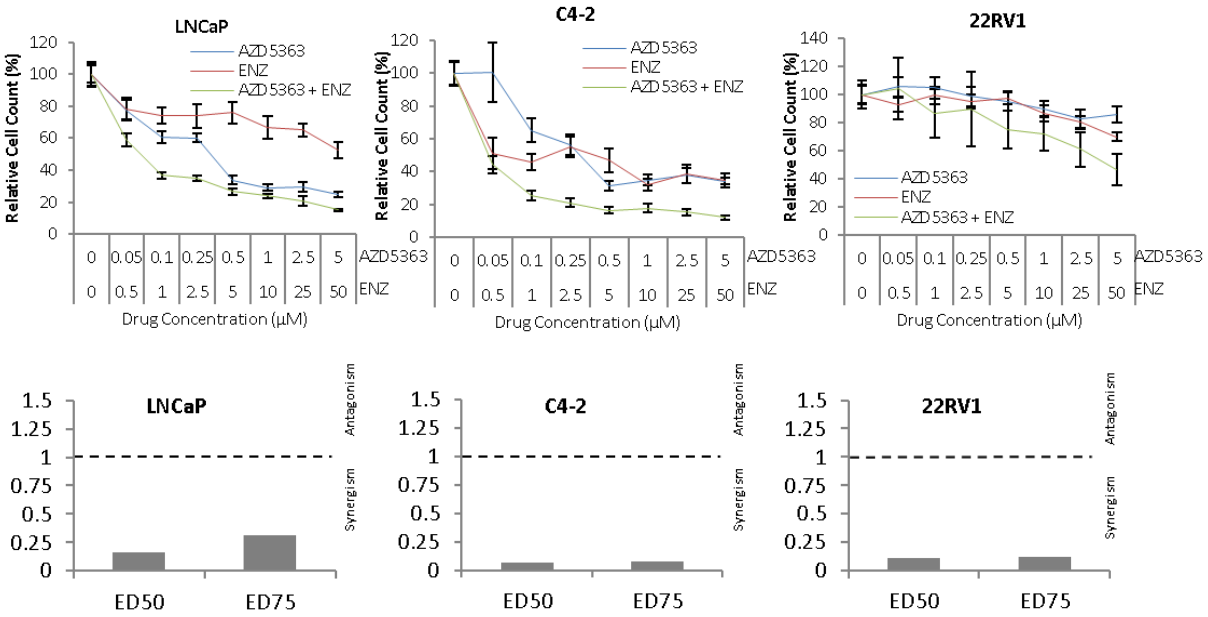

Figure 4.17 Cell proliferation with AZD5363, ENZ or combination treatment. Crystal violet assay was performed after 48h of treatment with indicated doses of AZD5363 and ENZ. Pooled results from 3 experiments with triplicates are shown. Calculated combination index demonstrates synergy for all 3 cell lines. Means are plotted ±SEM.

4.3.4 Combination AZD5363 and ENZ Significantly Delays CRPC Progression *In Vivo*

To assess if the combination of ENZ and AZD5363 can delay progression of castration-resistant prostate cancer (CRPC) to be ENZ-resistant *in vivo*, we used the CRPC LNCaP xenograft model. Mice were castrated once serum PSA reached levels of 50ng/mL and randomized to treatment once CRPC was demonstrated (Figure 4.18). Treatment arms included AZD5363 37.5mg/kg BID, ENZ 10mg/kg daily, and the combination of both drugs.

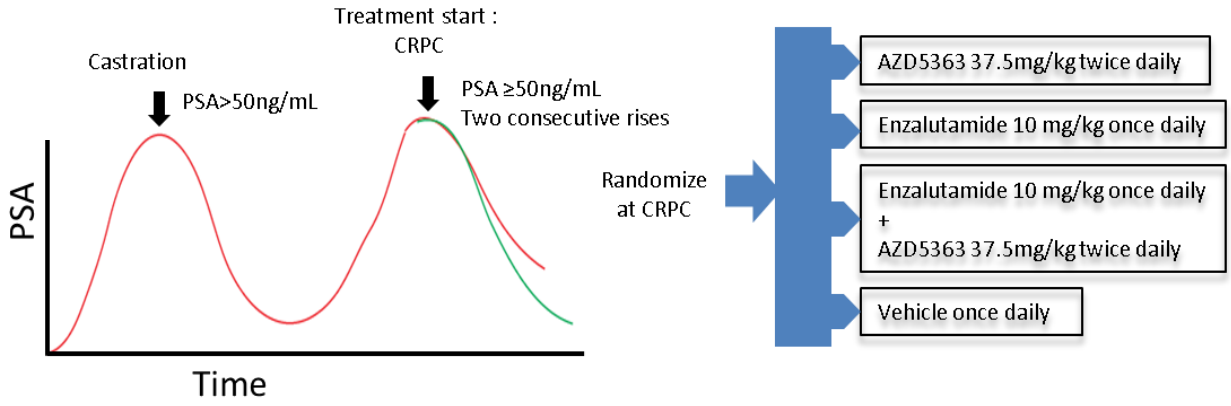

Figure 4.18 Study design for LNCaP castrate resistant prostate cancer (CRPC) model.

Combination therapy resulted in significant regression of tumours without regrowth as well as a significant and sustained PSA decline (Figure 4.19). Tumor growth velocity was significantly different between all treatment arms ($P<0.05$). The time to first tumour doubling (25 days to 44 days; $p<0.05$) and cancer-specific survival were improved with AZD5363 + ENZ compared to vehicle (Figure 4.20). A waterfall plot of maximal percent growth and regression from baseline tumour volume summarizes evident effectiveness of the combination over the course of the study. Due to this dramatic response, mice from the combination group were followed beyond 12 weeks, with no tumour growth or significant PSA rises observed up to 19 weeks. Further, the mean PSA nadir (outlier excluded) was 12.8X lower in the combination arm compared to ENZ alone ($p=0.06$, Figure 4.19D). Adverse effects included a slight initial weight loss, which did not persist (Figure 4.21). One mouse in each of the ENZ and AZD5363 arms was euthanized for weight loss >20% and one mouse in the combination arm were euthanized due to a skin abscess.

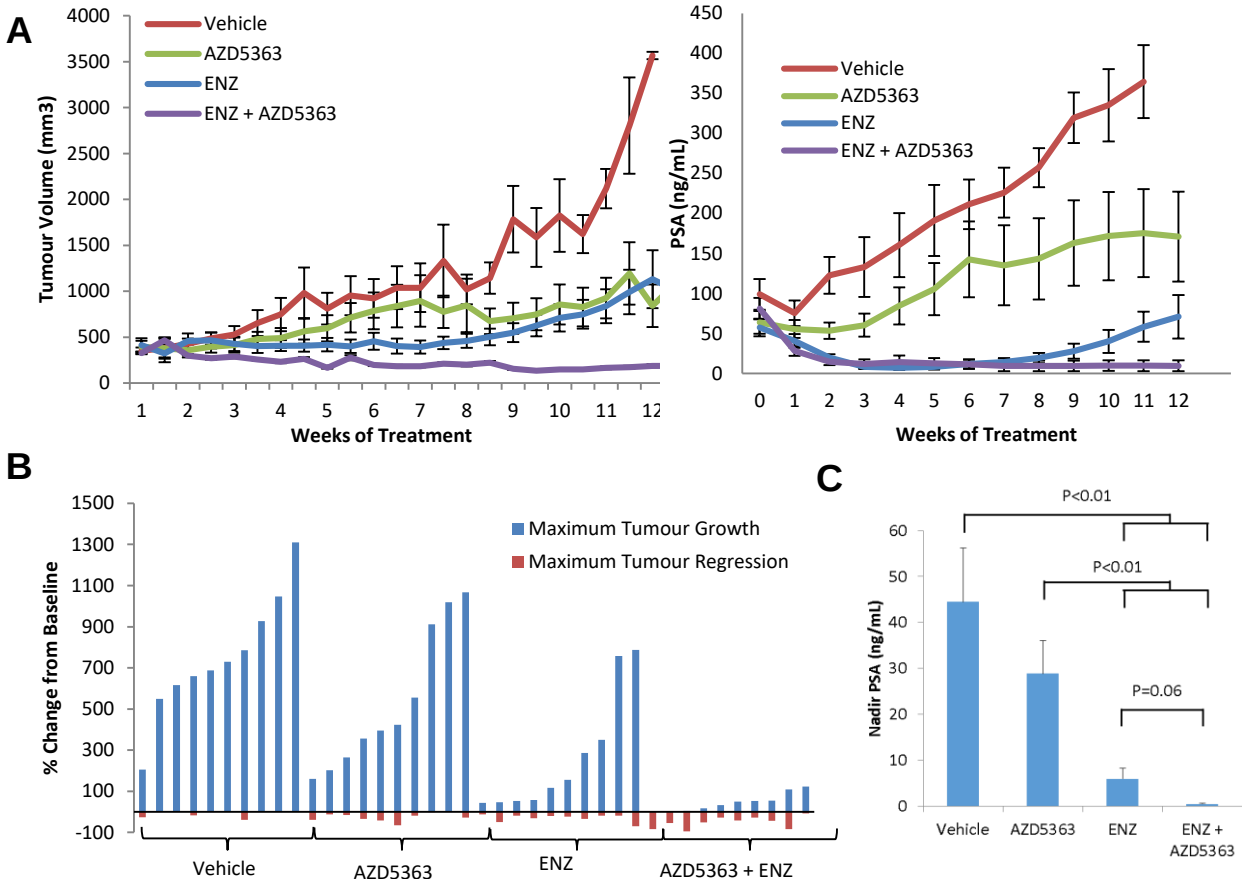

Figure 4.19 Combination AZD5363 and ENZ results in profound tumour regression and PSA decline in LNCaP CRPC xenografts. **A.** Effect of combination therapy on tumour volume and PSA. Mean tumour volume (left panel) and serum PSA (right panel) values in LNCaP castrate resistant prostate cancer (CRPC) xenografts treated with vehicle, 37.5mg/kg AZD5363 BID 5 days on, 2 days off 10mg/kg ENZ OD, or AZD5363 37.5mg/kg BID + 10mg/kg ENZ. Mice started treatment at the onset of CRPC. **B.** Plot showing individual maximal percent growth and regression from baseline tumour volume for all mice. **C.** Mean nadir serum PSA values for all treatment groups. One outlier from the ENZ + AZD5363 group was excluded.

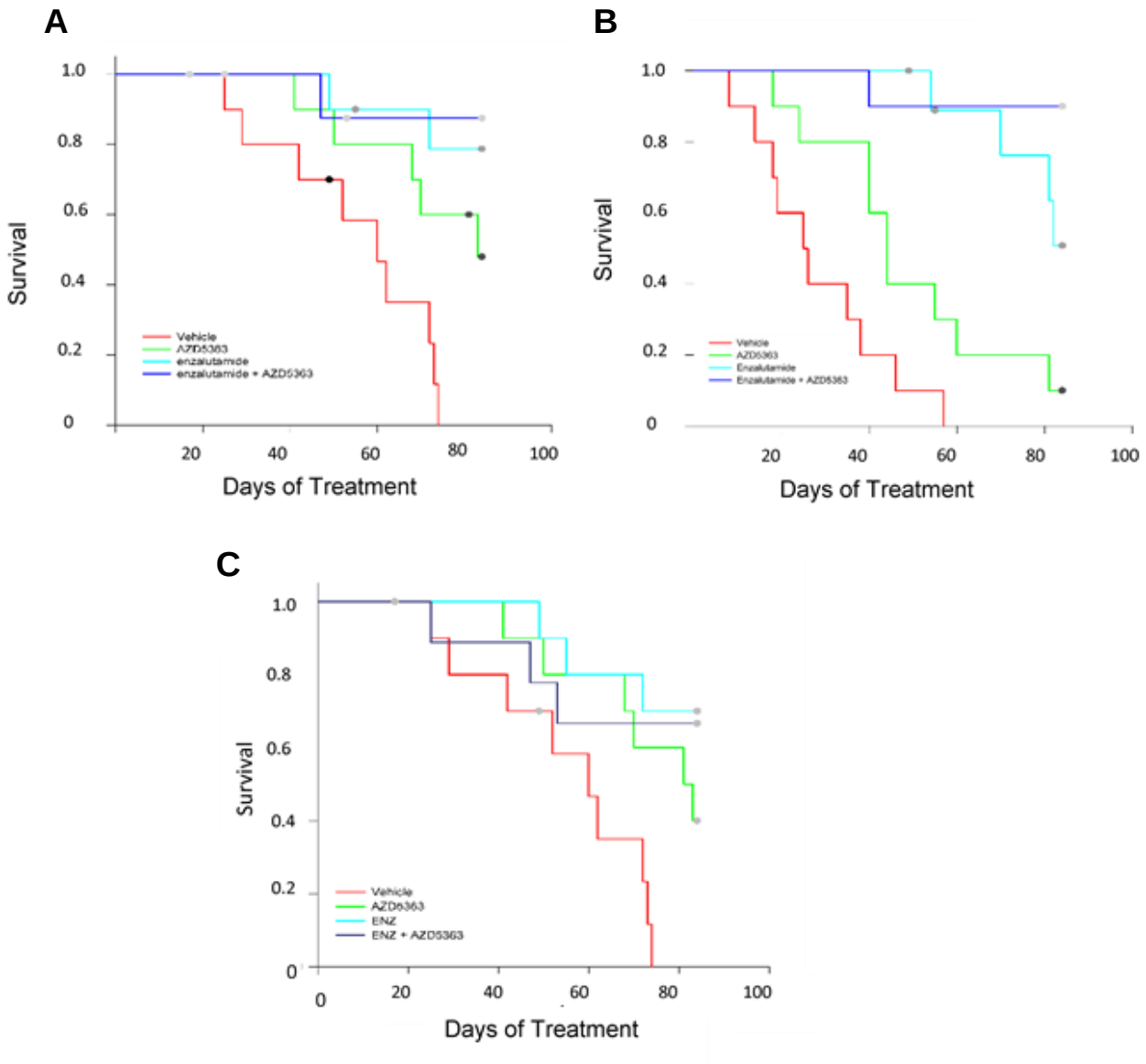

Figure 4.21 Kaplan-Meier survival in LNCaP CRPC xenografts. Survival curves for time to first tumour doubling **(A)** and cancer specific-survival (**B**) and overall survival(**C**) after 12 weeks of treatment.

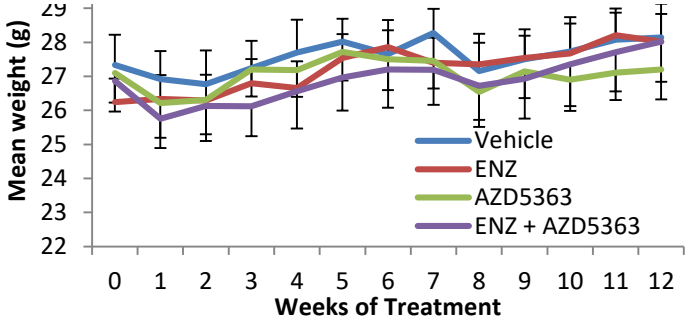

Figure 4.20 Weight of mice treated with AZD5363, ENZ or the combination in LNCaP CRPC model. Mean values ± SEM are shown.

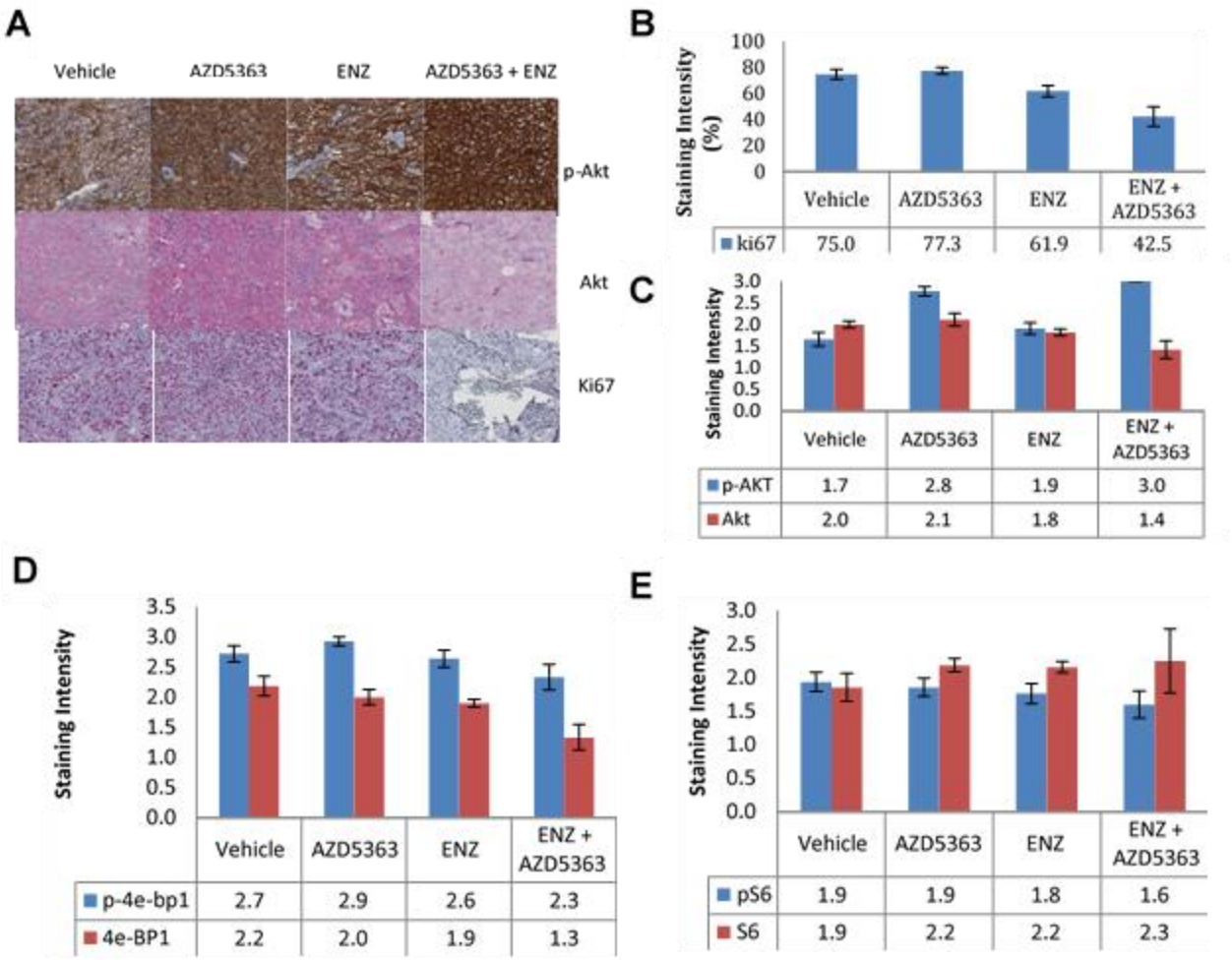

Figure 4.22 Immunohistochemistry results from treated LNCaP CRPC xenografts. A tissue microarray was constructed from 5 xenograft samples collected at the end of the study from each group. Staining intensity was graded by a pathologist blinded to group. **A.** Immunohistochemistry of representative samples of pAkt, Akt and Ki67 stained xenografts from each group collected at the end of study with mean scoring intensity of microarray. **B**. Ki67 microarray staining results show lower values for combination AZD5363 +ENZ. **C.** Total and phospho-Akt staining results for tissue microarray demonstrate on-target effects, with high levels of inactive pAkt(see Figure 4.15). **D.** Decreases in p-4e-BP1 and total 4e-BP-1 were seen with combination AZD5363 + ENZ compared to monotherapy and vehicle. **E.** Decreases in p-S6 were seen with either monotherapy, with the greatest increase seen with combination treatment.

Immunohistochemistry was performed on a microarray of 5 tumour samples per group collected at time of animal sacrifice. Ki67 staining indicated decreased proliferation in the combination arm compared to either monotherapy (Figure 4.22). Notably, pAkt increased to the greatest extent in the combination group, suggesting that blockade of the Akt pathway was pivotal in the dramatic regression seen *in vivo*. As seen in the *in vitro* data,

significant decreases in phospho- and total 4e-BP1 as well as phospho- and total S6 levels were found in the combination treatment arm.

4.3.4 Combination AZD5363 and ENZ Appears More Potent Earlier in the Course of Disease Progression

With our findings of delayed development of ENZ-resistant prostate cancer very evident in the LNCaP CRPC model, we next asked whether earlier use or later use of combination treatment in this model has similar efficacy. We elected to focus on the question of earlier treatment given we had already evaluated the combination indirectly in the MR49F model. This also avoided the very long time required for testing the combination in ENZ-resistant, CRPC LNCaP xenografts model and the challenges of treating mice over 6 months of age. Using a similar study design as above, we randomly administered AZD5363 37.5mg/kg plus ENZ 10mg/kg or vehicle to 10 mice per arm at time of castration. In these castrate-sensitive tumours, we found that tumour volume dropped similarly between the two groups and PSA levels dropped to a relatively greater extent compared to the prior study in CRPC LNCaP xenografts (Figure 4.23). The mean PSA nadir was lowered to a greater extent with combination therapy given at castration relative to vehicle (51-fold difference; $p = 0.064$) compared to AZD5363 plus ENZ given at time of CPRC relative to ENZ treatment (12-fold difference; $p = 0.065$) among mice with >4wk of treatment. Although two separate studies, it is notable that in comparison with the highly effective response in castrate resistant LNCaP xenografts, the PSA response to combination AZD5363 was even more pronounced in mice treated at time of castration.

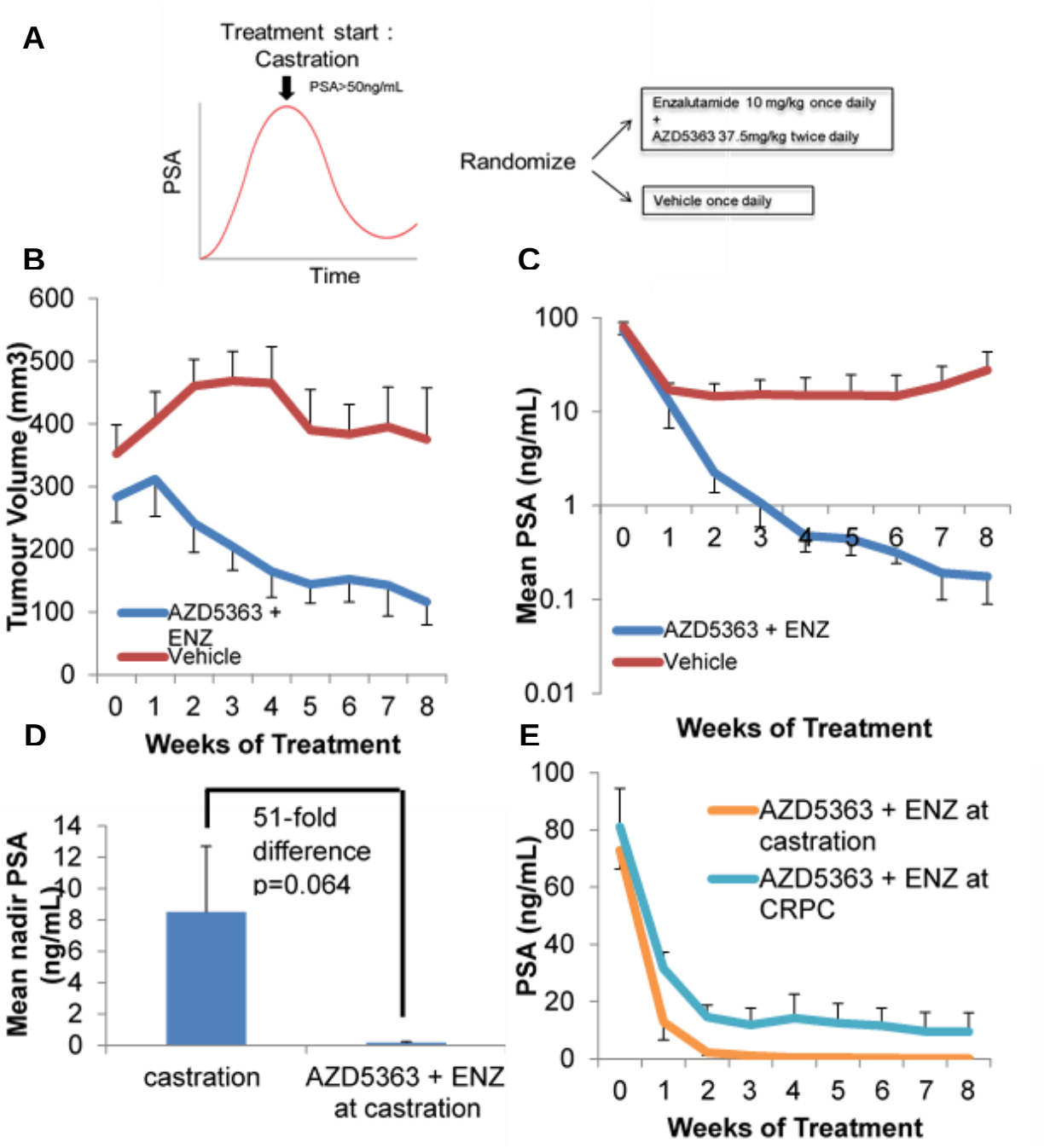

Figure 4.23 Results of combination AZD5363 and ENZ in castrate sensitive prostate cancer model. **A.** Schematic of *in vivo* study in LNCaP xenografts. **B.** Mean tumour volume following treatment at time of castration. **C.** Mean weakly serum PSA following treatments. **D.** Mean PSA nadir values between treatment arms. **E.** Comparison of weekly mean serum PSA values between this study and prior study in CRPC LNCaP xenografts in nude mice.

A second, related, question which arises with earlier administration combination therapy is whether such potent treatment may select for more aggressive clones, which could ultimately negate the improved potency with earlier treatment. To address this

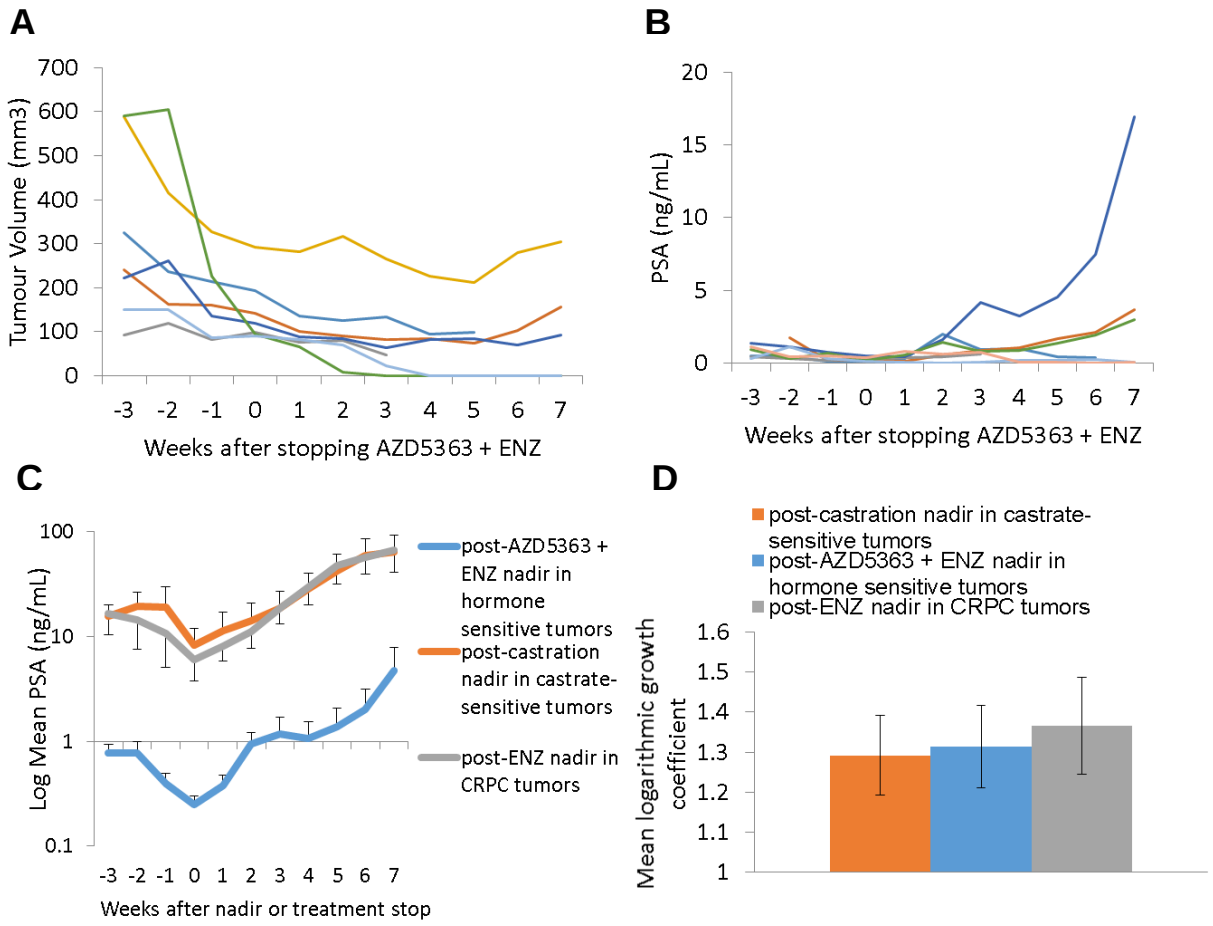

Figure 4.24 Exploratory analysis of tumor regrowth following treatment with combination AZD5363 and ENZ in castrate sensitive prostate cancer model. Individual tumor volumes(**A**) and serum PSA(**B**) of mice treated with AZD5363 37.5mg/kg po BI 5days on 2off which discontinued therapy after 6 weeks or weekly serum PSA values consecutively <0.5ng/mL. **C.** Graphical comparison of rate of PSA rise following discontinuation of AZD5363 and ENZ compared to PSA rise following castration or ENZ treatment in the LNCaP CRPC model. **D.** Mean logarithmic growth coefficients of PSA rise do not significantly differ between groups.

question, 7 mice were treated with AZD5363 plus ENZ at time of castration, but these mice stopped treatment after 6 weeks, or once the PSA nadir was <0.5ng/mL for two consecutive weeks. Drug treatment was then stopped and mice were monitored to see if tumours regrew; serum PSA continued to be monitored weekly. This could test both if the tumours were truly eradicated, and whether they regrew at a faster rate following treatment.

Analysis of these mice demonstrated continued regression or stability of each tumour size, but PSA rises were detectable after a couple weeks off treatment(Figure

4.24). As a comparator for tumour re-growth rate, we compared the rate of regrowth of these tumours to the rate of tumour regrowth in the same model following castration and following ENZ treatment of CRPC tumours(using our above data). As our results demonstrate PSA to be a more sensitive marker of tumour regrowth, we compared only serum PSA values and not tumour size. Graphically, this is illustrated in Figure 4.24) Using mean linear and mean logarithmic analyses of PSA rise following treatment, there was no significant difference between the mean rate of PSA rise in LNCaP tumours post AZD5363 plus ENZ at time of castration compared to controls post-castration($p=0.11$ and $p=0.88$, respectively).

4.4 Discussion

This study presents pre-clinical evidence that combined targeting of the Akt and AR pathways with AZD5363 and ENZ induces significant apoptosis which delays the development of ENZ-resistant disease. While ENZ improves survival and quality of life in CRPC patients(274), resistance invariably develops and thus there is a need for successful strategies to delay resistance to ENZ.

Given the early disappointing results of Akt inhibitor monotherapy in prostate cancer (225, 226), combination strategies are under investigation. Recent research provides a strong rationale for combined blockade of AR and Akt pathways (81, 303) with clinical trials in various phases of development(308). Targeting mTORC1 with rapamycin analogues along with AR antagonism is another strategy which has been pursued, though initial clinical experience has been disappointing (309). Similar to AR blockade, mTOR inhibition is known to result in feedback up-regulation of the PI3K/Akt pathway

(310, 311), leading to dual targeting approaches of the mTOR pathway. Advantages to upstream targeting of Akt include avoidance of these feedback loops (74).

While the underlying mechanisms of the synergy between AZD5363 and ENZ remain to be fully elucidated, our results suggest that suppression of downstream pathways may be important. In contrast to TORC1 inhibitors, our results with AZD5363 show an *in vitro* and *in vivo* effect on the downstream eIF4E translation complex, involved in prostate cancer progression and resistance (312, 313). 4-eBP1 acts as a translation suppressor and is phosphorylated into the inactive form by many pathways, including the PI3K/Akt/mTOR pathway. It has been implicated in mTOR resistance (313); decreased active levels appear related to aggressive tumours (314, 315). The complete abrogation of the p-4-eBP1 in our PTEN-negative cells with combination of AZD5363 and ENZ *in vitro* and *in vivo* therefore suggests both mechanistic and clinical significance.

In conclusion, in our pre-clinical models of resistant prostate cancer we show an impressive response in delaying and treating ENZ-resistant disease using the novel Akt-inhibitor AZD5363 in combination with ENZ. The best clinical response may be expected among men whose cancer harbors a PTEN-mutation or demonstrated activation of this pathway. These results provide a strong preclinical evidence to evaluate the combination of AZD5363 with ENZ in patients with CRPC in the clinic.

5 A Pre-Clinical Rationale for Combination PI3K Inhibition and BET Inhibition in Prostate Cancer.

5.1 Introduction

Prostate cancer remains one of the most common cancers among men, with a lifetime incidence of one in eight(2). Medical or surgical castration remains the mainstay of treatment for recurrent or metastatic prostate cancer. While effective, it is not curative and resistance inevitably develops. An understanding of the molecular pathways that drive resistant cancer is needed to drive the development of further targeted therapeutics and better improve outcomes for men with lethal prostate cancer.

The phosphatidylinositol-3-kinase(PI3K)/Akt/mammalian target of rapamycin(mTOR) pathway is an oncogenic pathway active in many cancers, including aggressive prostate cancer(224). The incidence of alterations in this pathway increases as prostate cancers become more resistant(42). The development of targeted inhibitors against this pathway is therefore of significant interest in prostate cancer. The use of mTOR inhibitors to date has been hampered by relatively poor responses coupled with significant side effects(131, 316). Prior research suggests the use of Akt inhibitors as monotherapy has not been successful at least in part due to reciprocal activation of feedback pathways, including the AR and receptor tyrosine kinases(RTK) pathway(81, 225, 226).

Class I PI3Ks are most implicated in human cancers and consist of the regulatory p85 and p55 subunits and the p110 catalytic subunit. Three catalytic isoforms p110α, p110β, and p110δ are recognized and play distinct roles in membrane-associated signalling involving the conversion of PIP2 to PIP3. It is recognized that p110β appears

to have the greatest relevance in prostate cancer signalling(70). Further, it appears that inhibition of either p110α or p110β results in upregulation of signalling through the other isoform(317, 318). Pharmacologically, inhibition of p110β has the advantage of less hyperglycemia, which is one of the significant patient side effects of targeting this enzyme.

Development of the PI3K β and δ selective small molecular inhibitor AZD8186 has previously been described(319). In this study, we aim to assess its efficacy in human prostate cancer models. We identify the transcriptional upregulation of the oncogene myc as a potential mechanism of resistance. Co-targeting of the PI3K/Akt pathway inhibition using the BET inhibitor JQ1 shows promise in our pre-clinical models. Importantly, inhibiting the bromodomain protein BRD4 with JQ1 co- targets both the AR pathway as well as myc-activated RTKs implicated in resistance, suggesting it may be a superior method to co-targeting the AR alone with an AR antagonist such as enzalutamide. In summary, our results present a pre-clinical rationale for combining BRD4 inhibitors with PI3K inhibitors in the treatment of PTEN-deficient prostate cancer.

5.2 Methods

5.2.1 Prostate Cancer Cell Lines

The human prostate cancer LNCaP and 22RV1 cell lines used in this study were kindly provided by Dr. Leland W.K. Chung (1992, MDACC, Houston Tx). V16D(castrate resistant), MR49C cells (enzalutamide resistant) were derived through serial xenograft passage as previously described(251). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and cultured without

antibiotics at 37°C in 5% CO2 atmosphere. MR49C cells were maintained and treated in media supplemented with 10μM ENZ.

5.2.2 Reagents

The PI3K inhibitor AZD8186 and the pan-Akt inhibitor AZD5363 were provided by AstraZeneca (Macclesfield, UK). ENZ was purchased from Shanghai Haoyuan Chemexpress (Shanghai, China) and JQ1 was generously supplied by the Bradner Laboratory. For the *in vitro* studies, AZD8186, AZD5363 and JQ1 were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) as 10mM stock solutions, respectively, and stored at -20ºC. For *in vitro* use, ENZ was dissolved in H_2O at 4 ºC. Based on prior work and the literature, doses selected for *in vitro* studies were AZD5363 1μM, AZD8186 0.5 μM and JQ1 0.5 μM(222, 292, 318).

5.2.3 Cell Proliferation Assays

Cell growth was assessed using the crystal violet assay, as described previously(251). Cells were plated in 96-well plates and treated with drugs at indicated concentrations. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware) at 562nm.

5.2.4 Western Blot Analysis

Whole cell lysates were collected in RIPA buffer (50nM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 10nM NaCl, Roche complete protease inhibitor cocktail) after 24 hours of treatment, incubated on ice for at least 60min and centrifuged at 13.000

rpm for 20 min at 4°C. 30-50 µg of whole cell lysate was subjected to SDS-PAGE, transferred to nitrocellulose filters and immunoblotted with primary antibodies and secondary antibodies. Detection of specific bands and densitometric quantification was done using the ODYSSEY IR imaging system (Li-COR Biosciences) or ECL (Amersham Biosciences, Piscataway, NJ, USA). AR (N-20) was assessed with antibodies from Santa Cruz Biotechnology and vinculin from Sigma-Aldrich (St. Louis, MO). PARP, p-Akt Ser473, Akt, c-myc were from Cell Signaling Technology (Danvers, MA).

5.2.5 RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies, Burlington, ON). Total RNA (2 µg) was reversed transcribed using MMLV reverse transcriptase and random hexamers (Invitrogen) as previously reported(292). Real-time monitoring of PCR amplification of cDNA was performed using SYBRGreen ROX Master Mix (Roche Applied Science, Indianapolis, IN, USA) and the following primer pairs and probes (Invitrogen): GAPDH, AR, PSA(KLK3), myc, EGFR and IGF-IR (for sequences see Table 5.1). Target gene expression was normalized to GAPDH levels in three technical replicates per sample with each experiment performed in at least biologic duplicates.

Table 5.1 Primer Sequences for qRT-PCR SYBRgreen target genes.

Gene	Reverse Primer	Forward Primer
EGFR	AGGCTGATTGTGATAGACAGG	CCAGTGCCTGAATACATAAACC
IGF-1R	TTCCACTTCACGATTAACTGAG	CACCAATGCTTCAGTTCCT
myc	AACATCGATTTCTTCCTCA	TGAGGAGACACCGCCCA

5.2.6 Animal Treatment

Six week old male castrated athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected subcutaneously with $2x10^6$ LNCaP cells (suspended in 0.1ml Matrigel; BD Biosciences) on both flanks. Mice were castrated when PSA values exceeded 50ng/mL. Treatment started when the PSA rose to pre-castration levels. Serum PSA measurements taken from tail vein were measured using automated immunoassay (Cobas, Montreal, Quebec, Canada). For treatment, mice were randomized to vehicle (0.5% methylcellulose), AZD8186 BID (at 10 and 25 mg/kg doses) 4 days on, 3 off. A separate study under identical conditions consisted of vehicle or AZD5363 treatment dosed at 75mg/kg 5 days on, 2 off). Body weight, tumour measurements and volume (l x w x d x 0.5236) were recorded weekly. Mice were sacrificed when the total tumour burden was >1500mm^3 or >20% loss of body weight. Tumor samples were stored in RNAlater(Invitrogen) and snap frozen in liquid nitrogen. Glucose measurements were taken on a consecutive sample of 5 mice per treatment arm 3 weeks after initiating treatment. Whole heparinized blood collected by tail vein draw of ~65uL was immediately measured using the glucose cartridge in the iStat 1 handheld analyzer (Abaxis, Union City, CA, USA). All mice were monitored regularly for clinical condition under the

supervision of a veterinarian at the University of British Columbia. At sacrifice, all mice were deeply anesthetized with isoflurane prior to being euthanized with $C0_2$. Tumors collected at sacrifice were divided into parts and snap frozen in liquid nitrogen or fixed in formalin. All animal procedures were performed according to Canadian Council on Animal Care guidelines and with approval of the Animal Care Committee of the University of British Columbia (protocol # A12-0210).

5.2.7 Statistical Analysis

All results are expressed as the mean ± SEM, and means were compared by a student t-test. Tumour growth velocity was calculated using linear regression on the tumour burden for each individual mouse. Kaplan-Meier survival analysis compared cancer specific survival and overall survival, with the log-rank test used to compare groups. Cancer specific survival was defined as time from treatment start until total tumour volume exceeded $1500mm^3$. The combination index (CI) was calculated for the effective doses (ED50 and ED75) using Calcusyn software (Biosoft, Cambridge, UK). A CI <1 indicates synergy, a CI >1 indicates antagonistic interactions.

5.3 Results

5.3.1 AZD8186 Targets the PI3K Pathway in *In Vitro* and *In Vivo* Prostate Cancer Models

In LNCaP cells, which have an inactivated phosphatase and tensin homolog(PTEN) resulting in activation of the Akt pathway, AZD8186 decreased pAkt(Ser473) and downstream pS6 in a dose-dependent fashion. After 24h, there was a

reactivation downstream of S6 as other have also described(318). As expected, inhibition of PI3K with AZD8186 resulted in increased apoptosis as measured by flow cytometry, PARP cleavage and caspase-3 activity (Figure 5.1). In addition to apoptotic increases in G0/SubG1 cell cycle fraction, there were also significant decreases in S-phase fraction of cells. However, despite these changes, relative cell survival after 24h of treatment with AZD8186 up to doses of 5μM was over 50%.

For an *in vivo* model, we utilized castrate resistant LNCaP xenografts grown in nude mice and treated with 10mg/kg and 25mg/kg doses of AZD8186. Our results demonstrate dose-dependent decreases in mean tumour size and mean tumour growth

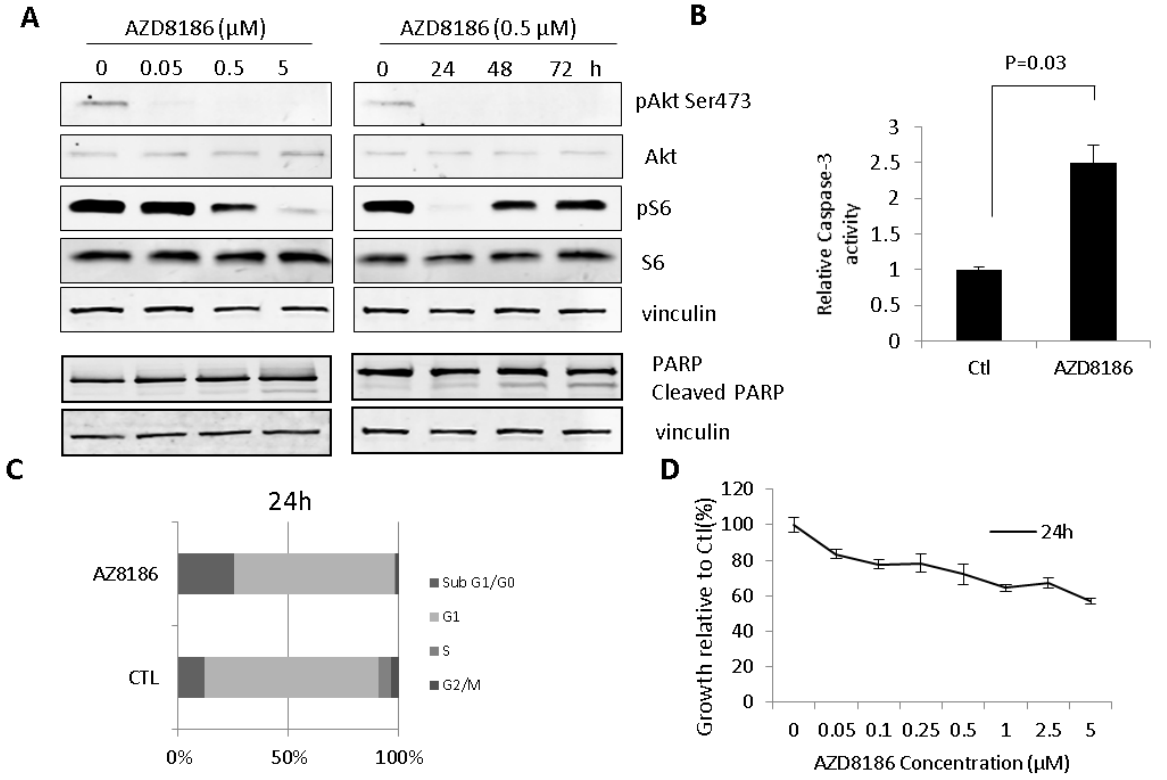

Figure 5.1 PI3K inhibitor AZD8186 in LNCaP prostate cancer cells *in vitro*. **A.** Dose- and time-course experiments with AZD8186 in LNCaP cells evaluating pAkt/Akt and pS6/S6 protein levels using western blotting. Cleaved PARP levels indicating apoptosis are also shown. **B.** Relative caspase-3 activity in LNCaP following 24h of treatment with DMSO control or AZD8186 0.5μM **C.** Propidium iodide cell cycle analysis demonstrates increased G0/Sub G1 and decrease S-phase following AZD8186 0.5μM treatment for 24h. **D.** Cell viability following 24h of treatment with AZD8186 at indicated doses relative to control.

velocity, though the difference between vehicle and AZD8186 25mg/kg dose tumour growth velocity did not reach statistical significance(p=0.09) (Figure 5.2).

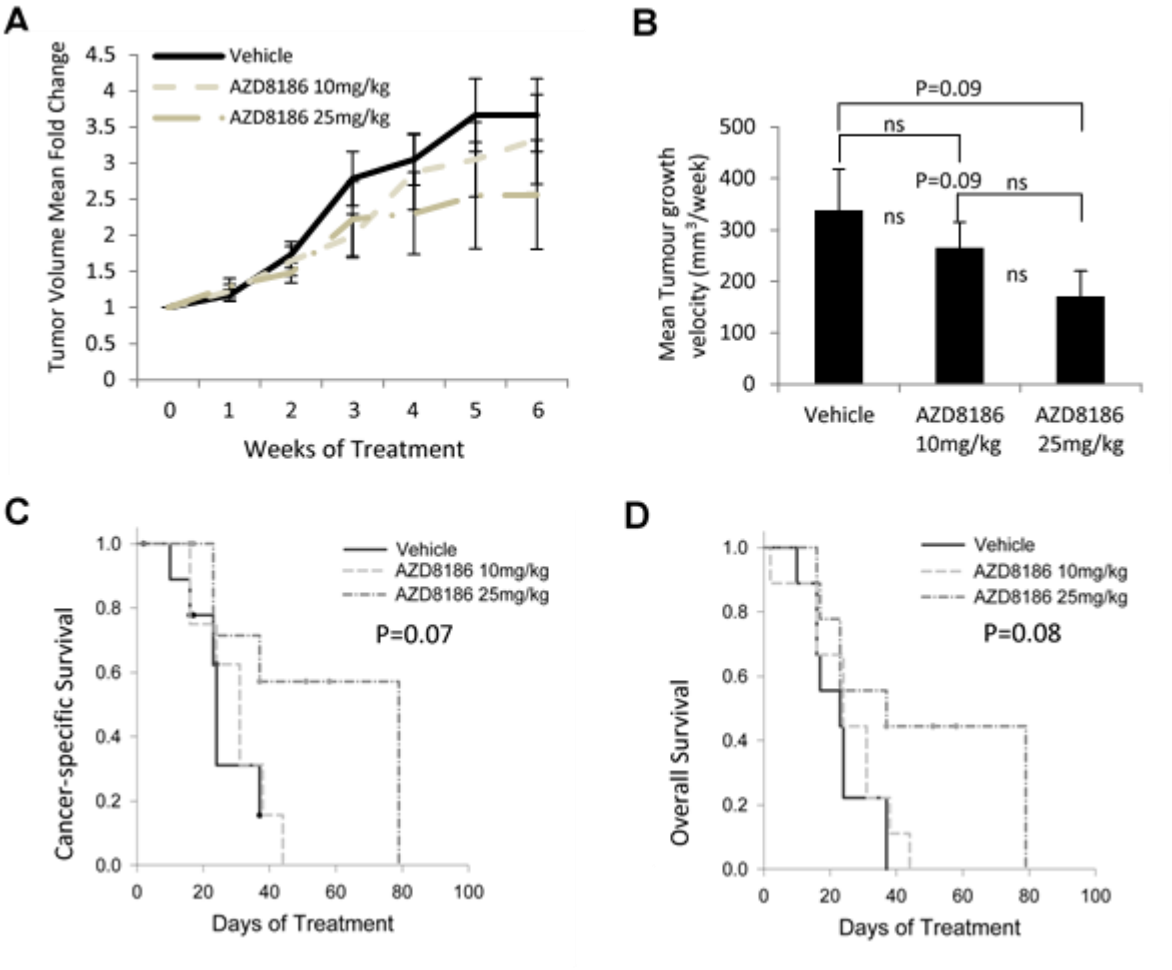

Figure 5.2 PI3K inhibitor AZD8186 treating LNCaP prostate cancer cells *in vivo*. **A**. Mean tumour volume fold change of LNCaP castrate-resistant xenografts treated with vehicle, AZD8186 10mg/kg or AZD8186 25mg/kg dosed orally 4 days on, 3 off. **B**. Mean tumour growth velocity for indicated treatment arms(right). Eight mice per arm were evaluated. Cancer-specific survival(**C**) and overall survival(**D**) for LNCaP castrate-resistant xenografts treated with AZD8186 at indicated doses compared to vehicle-treated mice.

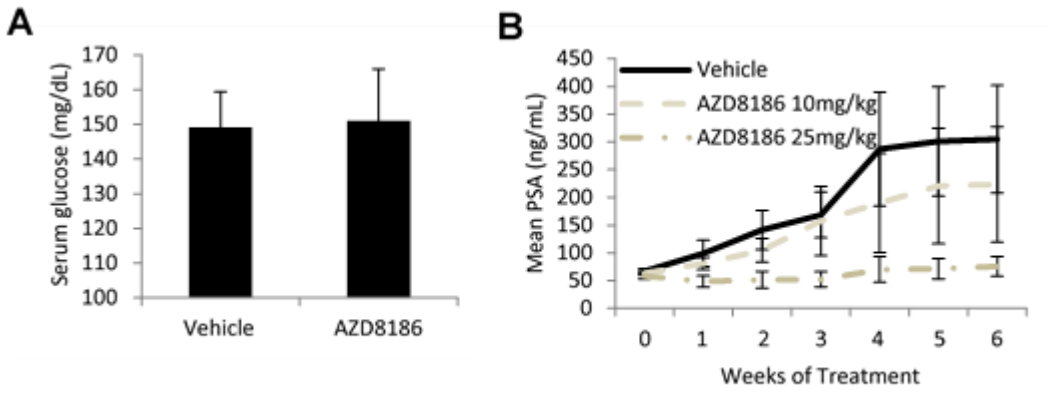

Figure 5.3 Additional details of AZD8186 results in LNCaP CRPC *in vivo* prostate cancer model. **A.** Serum glucose measurements following 3 weeks of treatment. Tail vein samples were measured using blood gas analysis in 5 consecutive mice per arm. **B.** Mean serum PSA following treatment with AZD8186.

Similarly, improvements in cancer specific survival and overall survival differences were short of reaching statistical significance (p=0.07 and p=0.08, respectively). To evaluate for differences in glucose homeostasis, serum glucose was measured by blood gas analysis and did not show significant increase after 3 week of treatment, confirming the expected benefit of PI3K inhibition (Figure 5.3). Serum PSA declines were seen with treatment, being most notable at the 25mg/kg dose.

5.3.2 C-myc Expression Increases in Response to PI3K Inhibition

Feedback signalling via receptor tyrosine kinases(RTKs) as a mechanism of resistance to PI3K inhibitors has been previously demonstrated to be important in prostate cancer(318). For several reasons we decided to further investigate myc as a transcription factor which may be implicated in resistance to PI3K inhibition. First, many RTKs possess promoter sequences for myc, including IGF-IR and EGFR, both of which are implicated in prostate cancer. IGF signalling has previously been implicated in

AZD8186 resistance(318). Further, it has previously been demonstrated that PB-myc mice are resistant to PI3K inhibition(81). Very recently, others have presented data suggesting that myc increases following PI3K inhibition(320).

Despite the large heterogeneity prevalent in castrate resistant LNCaP xenograft tumours, we did find higher levels of myc protein in the castrate resistant tumours treated with AZD8186 compared to vehicle-treated tumours (Figure 5.4). Further, in individual tumours the elevated c-myc protein levels corresponded to the decreases in pAkt levels observed with AZD8186 treatment. Eliminating one outlier per group, these differences were significant when compared using densitometric quantification ($p=0.49$). In the same set of 12 tumours, we amplified RNA and performed qRT-PCR for to evaluate myc transcript levels. Using the same set of 5 tumours per group, we found significantly higher levels of myc mRNA transcripts in the AZD8186-treated castrate-resistant LNCaP xenograft tumours. In LNCaP CRPC tumours treated with AZD5363, we found an analogous increase in myc mRNA transcript levels compared to vehicle-treated mice in the same cohort. Notably, analysis of EGFR and IGF-1R levels in the same and AZD5363-treated tumours revealed a trend for higher levels in the treated tumours AZD8186- compared to control (Figure 5.5)

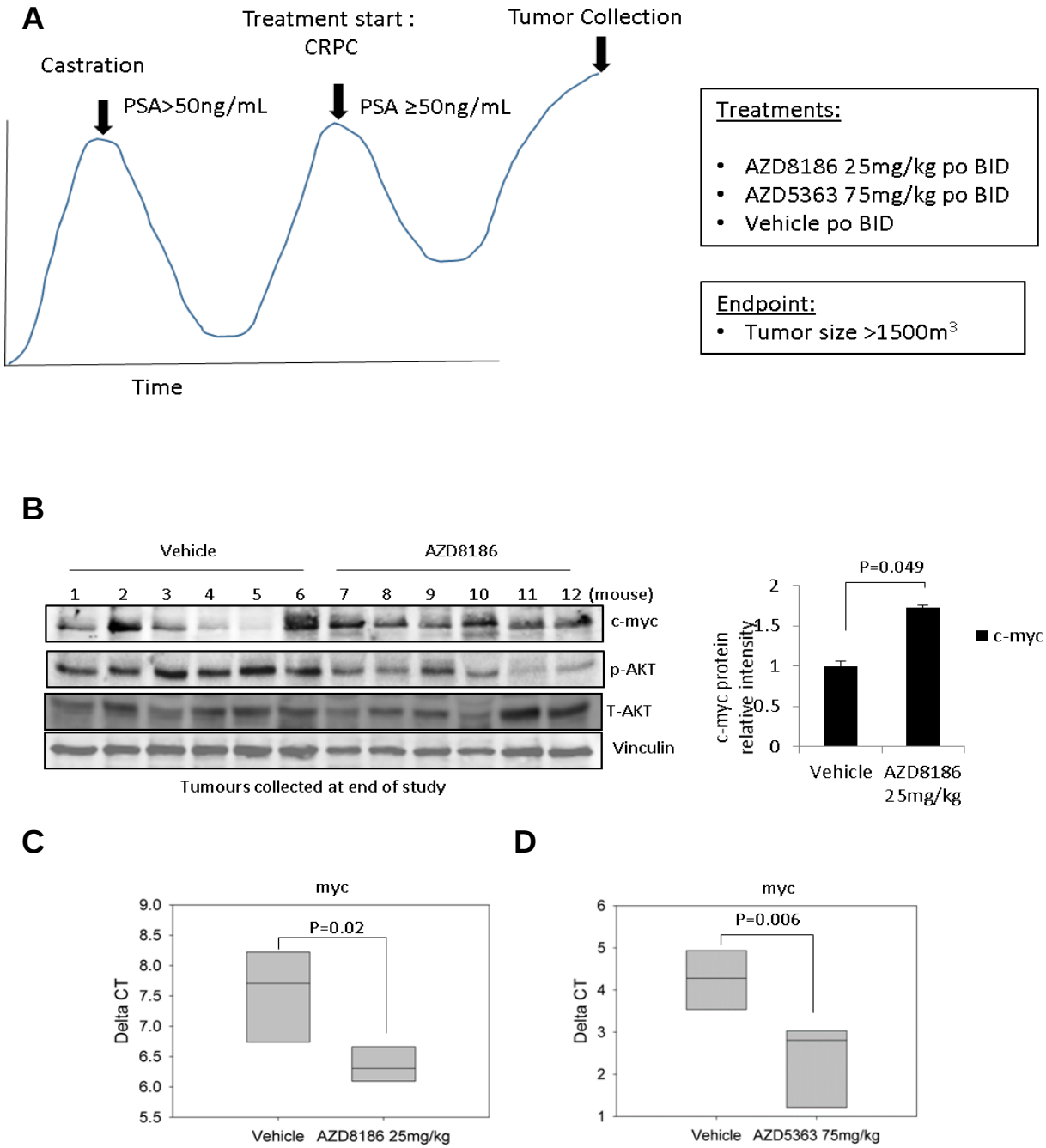

Figure 5.4 Analysis of myc mRNA and protein levels in castrate-resistant LNCaP xenografts treated with AZD8186 or AZD5363. **A.** Schematic of murine study and collection of samples after subsequent treated with castration and PI3K/AKT inhibitor. **B.** Western blotting for c-myc and p-Akt (Ser473) of LNCaP castrate-resistant xenografts treated with AZD8186 25mg/kg or vehicle. Tumours were collected and snap frozen when tumours exceeded endpoints. Right shows comparison of densitometric quantification of 5 tumours per group (one outlier per group excluded). **C.** RT-qPCR of for myc levels. Isolated RNA from individual tumours was stored in RNAlater(same 5 tumours per group as in B). Values were calculated relative to GAPDH control. **D**. myc levels in AZD5363-treated LNCaP castrate-resistant xenografts.

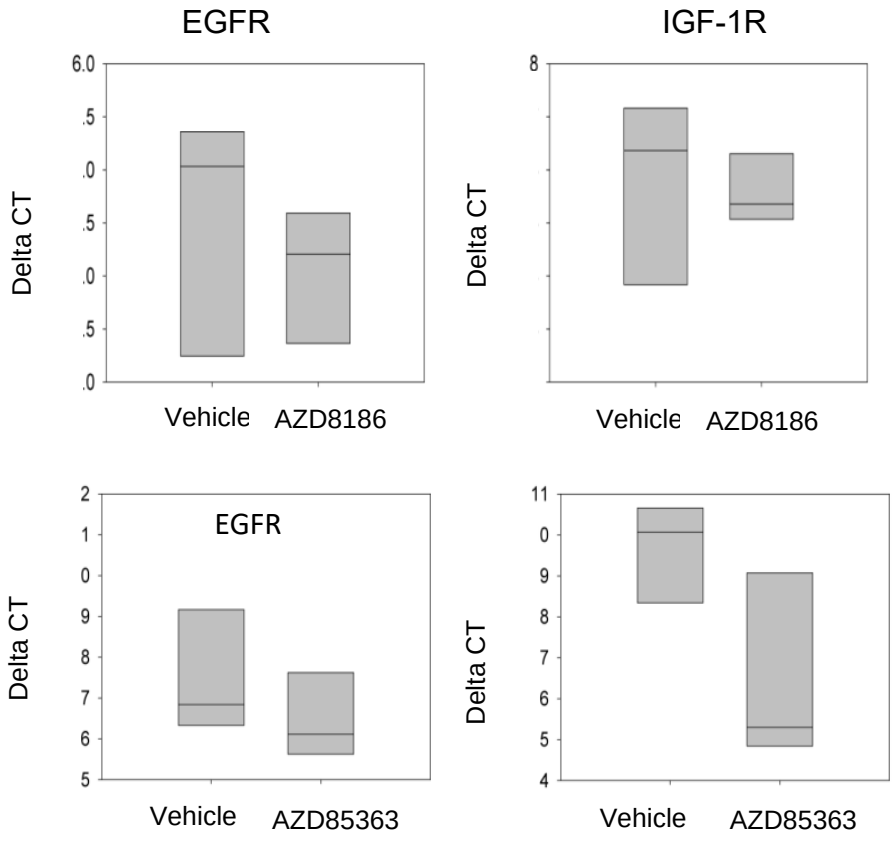

Figure 5.5 Increased EGFR and IGF-1R mRNA levels in castrate-resistant LNCaP xenografts treated with AZD5363 or AZD8186. Delta CT values were calculated as the difference between CT values from RT-PCR for indicated primers versus GAPDH as control. Means with interquartile ranges are displayed.

To understand our findings of myc upregulation following PI3K inhibition in castrate resistant xenografts, we investigated publically available genomic databases available at cbioportal.org (Figure 5.6). In the recently released Stand Up 2 Cancer cohort of metastatic prostate tumours, we found that myc was amplified in 18/118(15%) patients; moreover, this was mutually exclusive(p=0.012) from those patients with PTEN deletion (34/118, 24%)(321). PTEN loss was used as a surrogate for PI3K/Akt pathway activation. The tendency for mutually exclusivity was non-significant in the smaller Nelson cohort of men with metastatic CRPC from the University of Washington(239). However, in the Beltran cohort of patients with neuroendocrine patients, myc and PTEN alterations were not significantly co-occurent(322). The Cancer Genome Atlas(TCGA) prostate cancer

dataset is the largest cohort of localized prostate cancer samples containing extensive genomic and proteomic data. In our analysis of this information, we found that deletion of PTEN resulted in significantly lower myc protein levels($p=0.010$). Similarly, overexpression of myc mRNA was significantly associated with lower levels of pAkt(Thr308) expression($P=0.028$).

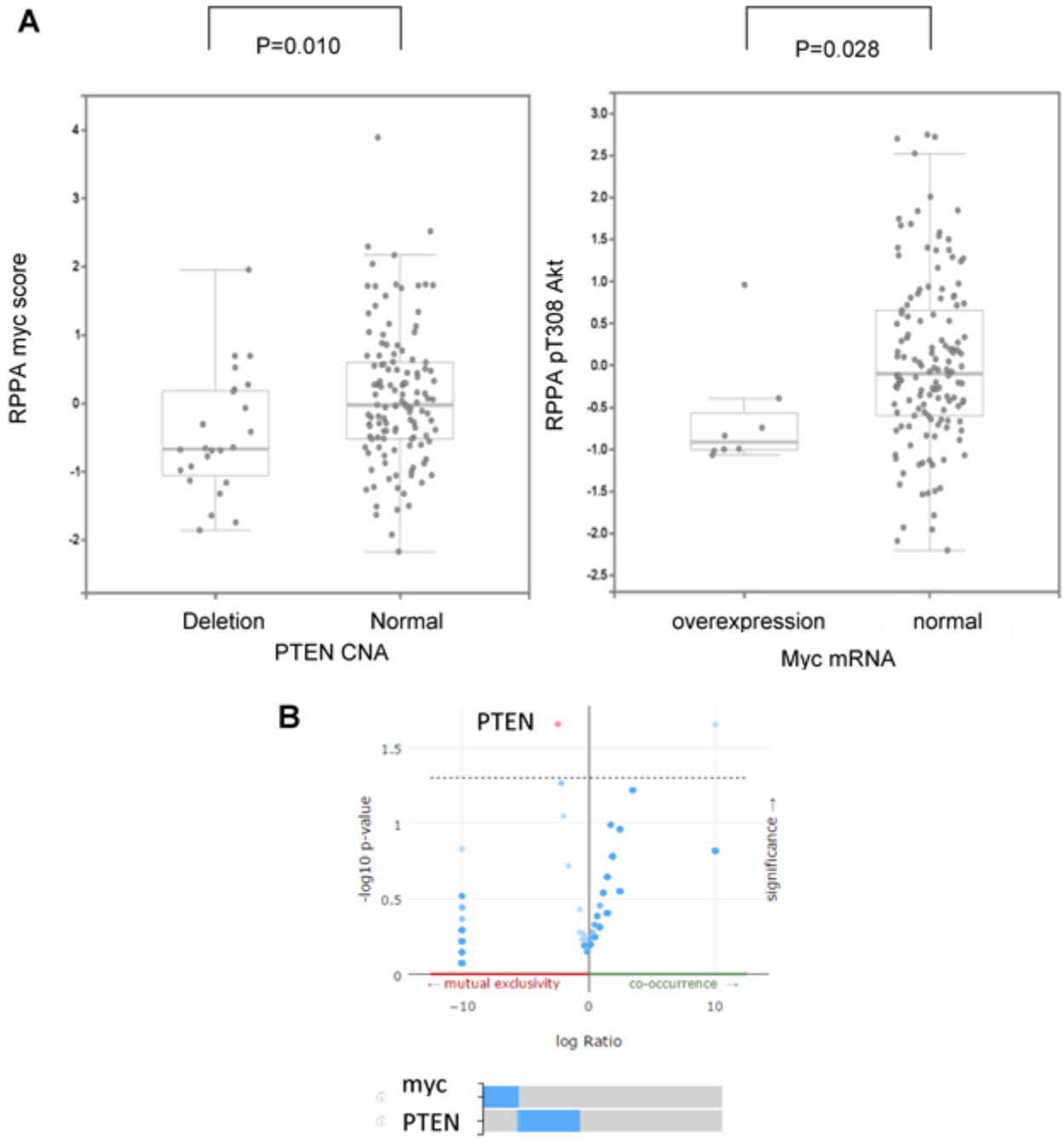

Figure 5.6 Mutually exclusive activation of myc and PI3K/Akt pathway in publically available prostate tumor patient datasets. **A**. The Cancer Genome Atlas(TCGA) data demonstrating myc protein levels are decreased in patient prostatectomy samples with PTEN deletion(left). The corollary of decreased pAkt(Thr308) levels was also seen in patient prostatectomy samples with myc mRNA over-expression(right). **B**. Stand Up 2 Cancer metastatic prostate cancer samples showing mutually exclusive amplification of myc and PTEN loss.

These data suggest that myc and PI3K/Akt pathways may be mutually exclusive oncogenic pathways in patient tumors. Their mutually exclusivity suggest that activation

of one may be more likely to occur if the other is blocked.

To investigate experimentally if upregulation of myc occurs following PI3K inhibition, we treated LNCaP cells with AZD8186 and evaluated myc levels at different time intervals. We found that AZD8186 treatment of LNCaP cells increased myc transcript levels in a time-dependent manner (Figure 5.7). Induction of c-myc protein levels over time was also observed (Figure 5.7).

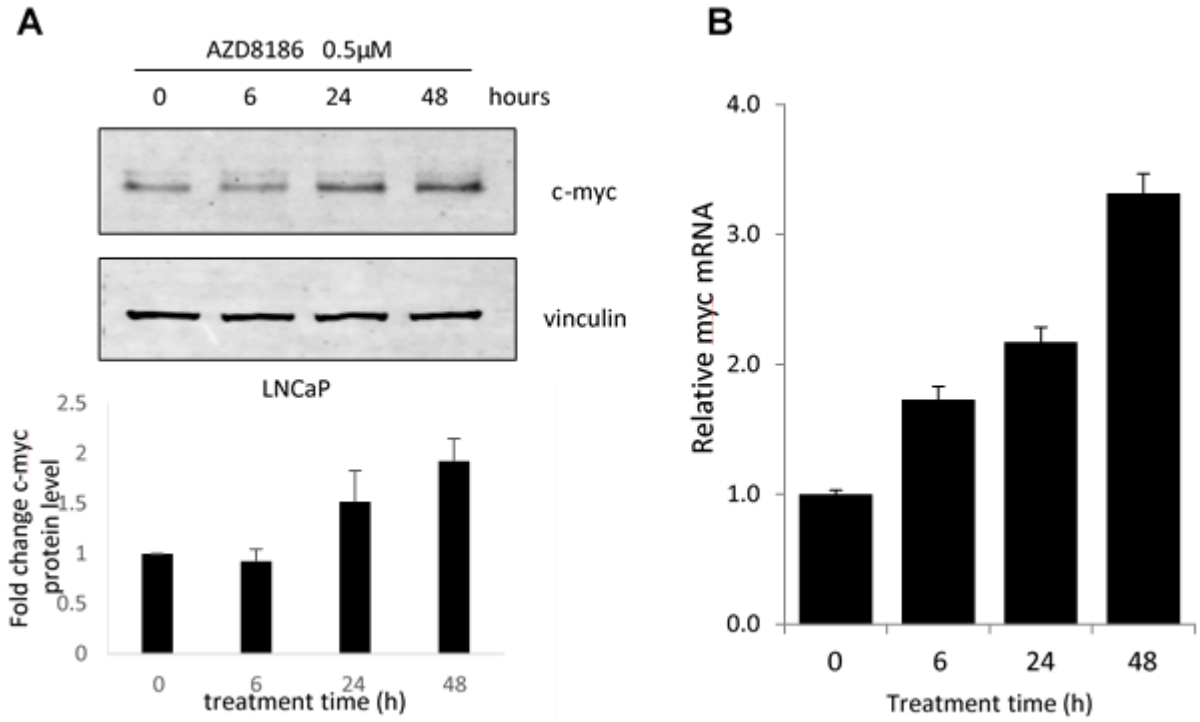

Figure 5.7 AZD8186 induces myc mRNA and protein levels in a time-dependent manner. **A.** Western blot showing increase in c-myc protein levels increase following treatment of LNCaP cells with AZD8186 0.5μM. Densitometry studies showing average of biologic duplicates. **B**. Increase in mRNA levels of myc following treatment of LNCaP cells with AZD8186 0.5μM; results are normalized to GAPDH levels.

5.3.3 AZD8186 Does Not Induce Myc-Dependent Gene Expression

To assess whether PI3K inhibition with AZD8186 induces myc-dependent gene expression and thus further establish the observed increases in myc as a mechanism of resistance, we performed gene profiling. LNCaP cells were grown to 70-80% confluence and treated as above with AZD8186 0.5μM for 24h. Triplicate samples of isolated RNA were analyzed using the Affymetrix single colour human gene expression chip. Our results demonstrated over 283 genes with significant fold changes following correction for multiple analyses. Using only those genes with a corrected P-value of <0.1, we found significant enrichment in AR-dependant genes using gene set enrichment analysis(GSEA) of all hallmark gene sets available from the Broad Institute (Table 5.2). Similar results were obtained using all genes with GSEA(Figure 5.8).

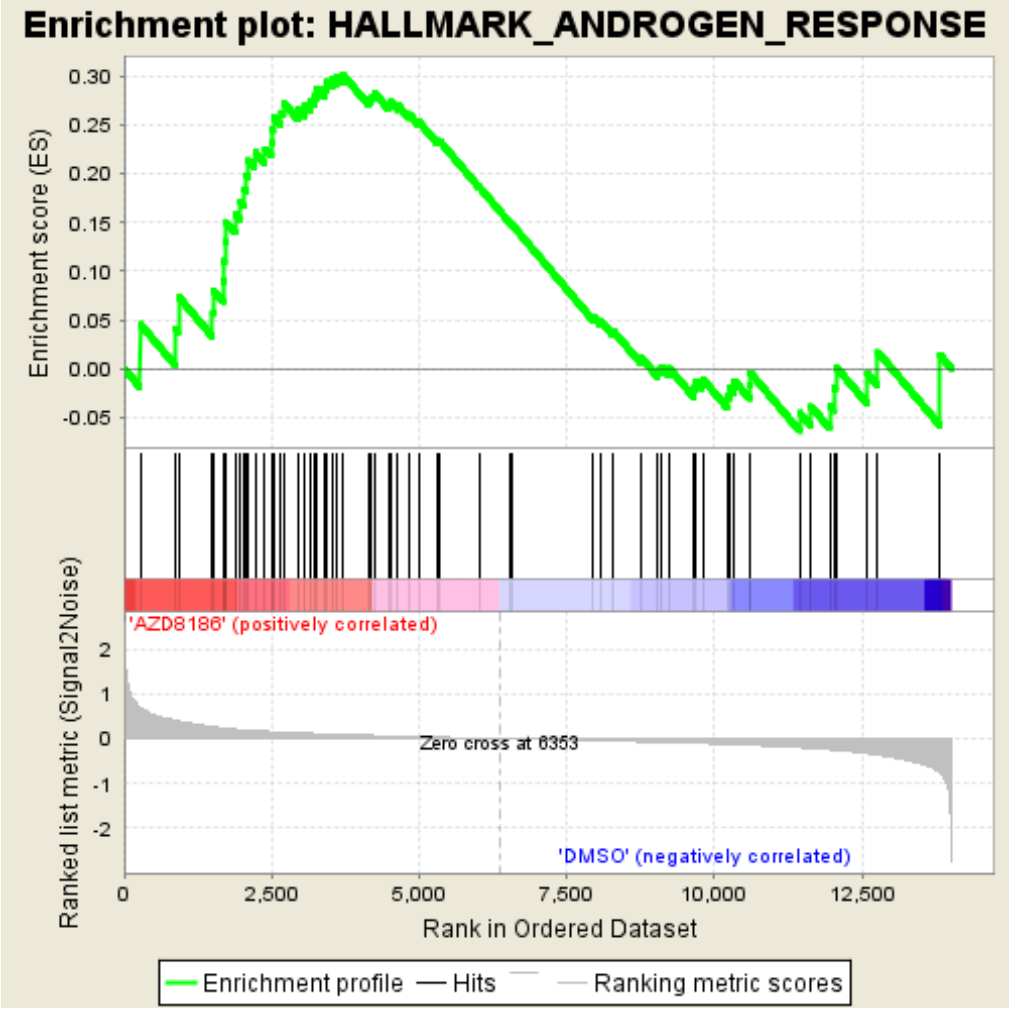

Figure 5.8 Androgen-dependent genes are enriched in LNCaP cells following treatment with AZD8186 0.5μM for 24h.

Table 5.2 Gene Set Enrichment analysis of microarray results following treatment with AZD8186 using genes with significant upregulation or downregulation (P_{corr}<0.1). FDR, false discovery rate.

Gene Set Name	# Genes in Gene Set (K)	Description	# Genes in Overlap (k)	k/K	p-value	FDR q-value
Upregulated Genes						
HALLMARK_TNFA_SIGNALING_VIA_NFKB	200	Genes regulated by NF-kB in response to TNF [GeneID=7124].	5	0.025	3.73E-07	1.86E-05
HALLMARK_ANDROGEN_RESPONSE	101	Genes defining response to androgens.	3	0.0297	5.87E-05	1.47E-03
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	200	Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis and metastasis.	3	0.015	4.40E-04	3.67E-03
Downregulated genes						
HALLMARK_E2F_TARGETS	200	Genes encoding cell cycle related targets of E2F transcription factors.	43	0.215	1.26E-68	6.31E-67
HALLMARK_G2M_CHECKPOINT	200	Genes involved in the G2/M checkpoint, as in progression through the cell division cycle.	39	0.195	2.90E-60	7.25E-59
HALLMARK_MYC_TARGETS_V1	200	A subgroup of genes regulated by MYC - version 1 (v1).	15	0.075	5.45E-17	9.08E-16

These results validate the quality of our results, as well as the finding of significantly upregulated IGF-1R (Fold change 3.3, P_{corr}=0.044). However, we did not find the same degree of myc upregulation as we found in our PCR results. In the microarray, myc was only upregulated 1.7 fold (P_{corr}=0.09). Further, gene expression analysis of hallmark myc target sets showed significant downregulated of myc target genes (Figure 5.9). Taken together, these findings suggest that while myc upregulation may occur, it may represent a feedback response which does not necessarily drive subsequent resistance. Consistent with this concept is a weak correlation found between AR and myc

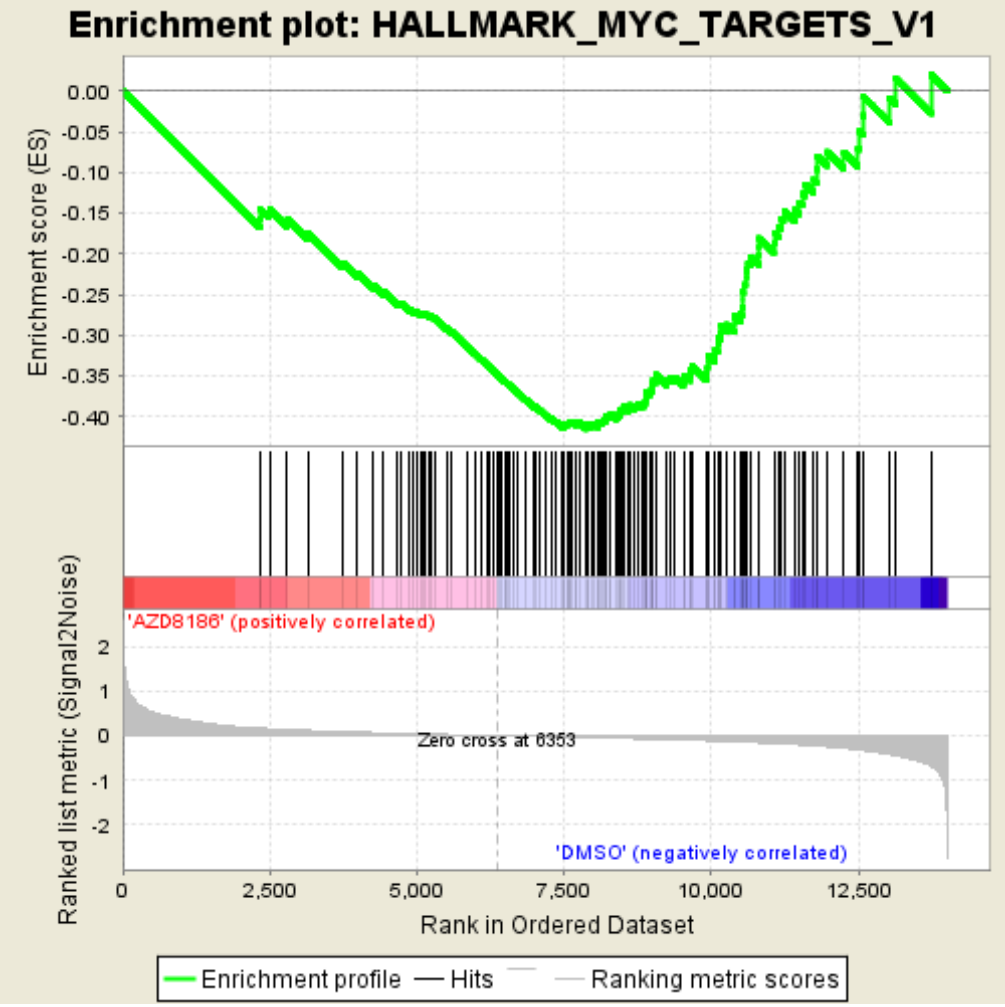

Figure 5.9 Myc target genes are downregulated in LNCaP cells following treatment with AZD8186 0.5μM for 24h. Gene set enrichment analysis was performed using hallmark gene sets available from the Broad Institute.

expression in clinical metastatic CRPC samples in the Nelson(Pearson = 0.35) and Beltran(Pearson=0.365) cohorts.

5.3.4 Combination JQ1 and AZD8186 Treatment in Prostate Cancer Cells

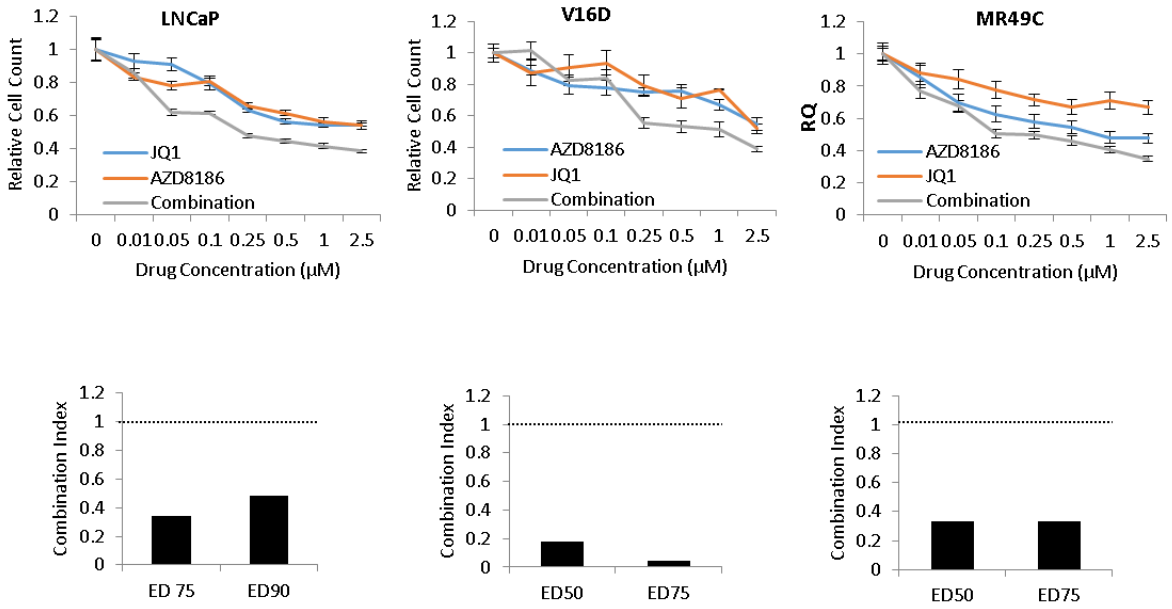

Figure 5.10 Cell proliferation following combination AZD8186 + JQ1. LNCaP, V16D or MR49C cells were treated with AZD8186 alone or in combination with JQ1 at indicated doses for 48h. Media for MR49C cells was supplemented with 10μM enzalutamide. Below graphs demonstrate combination indices calculated using Calcusyn software (Biosoft, Cambridge, UK).

Prior work has demonstrated that targeting the epigenetic bromodomain protein BRD4 inhibition using JQ1 disrupts the transcription and production of myc, as well as indirectly disruption AR transcriptional function(222). Therefore, we set to evaluate the combination of AZD8186 and JQ1 in LNCaP cells as an approach to co-target the observed increase in myc transcription which we had observed. Using JQ1 has the advantage of concomitantly targeting the AR, which we evaluated in Chapter 4 as an important feedback pathway activated after PI3K/Akt pathway inhibition (81, 254). We

further evaluated the combination of AZD8186 and JQ1 in castrate-resistant V16D cells and enzalutamide-resistant MR49C cells. In all three cell lines, the combination demonstrated a greater inhibition of cell growth with the combination compared to monotherapy with either JQ1 or AZD8186(Figure 5.10).

Similar findings were observed with the combination of the Akt inhibitor AZD5363 +/- JQ1(Figure 5.11). To confirm the effect of JQ1 on myc transcription, we evaluated myc transcript levels following monotherapy and combination therapy. As previously noted, AZD8186 increased myc levels; the addition of JQ1 abrogated the increase in LNCaP and V16D cells, and partially abrogated the increase in myc levels in MR49C cells (Figure 5.12). Combination with JQ1 partially abrogated AZD8186-induced increases in IGF-IR and EGFR transcript levels. PSA transcript levels were also increased by PI3K inhibition, as expected with known reciprocal activation of the AR axis(81, 254). However, PSA transcript levels were completely abrogated by the combination of JQ1 and AZD8186 in all three cell lines.

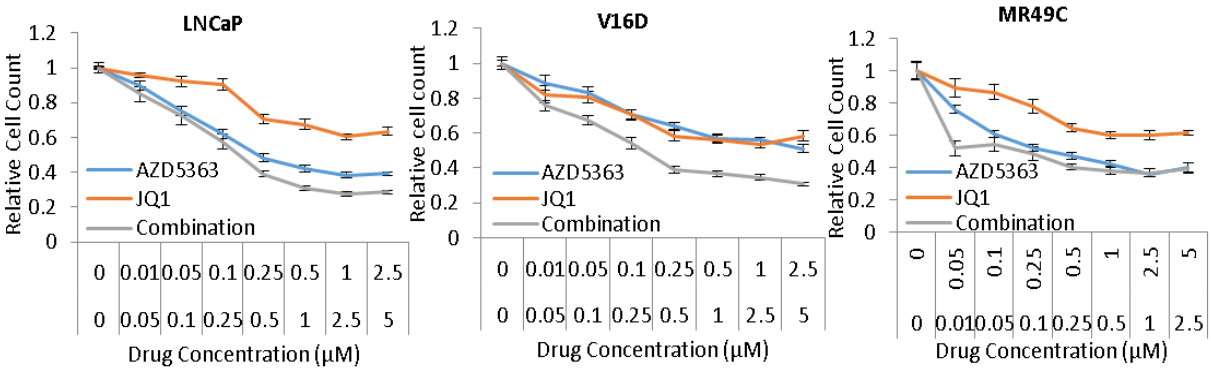

Figure 5.11 Cell proliferation following combination AZD5363 + JQ1. LNCaP, V16D or MR49C cells were treated with AZD5363 alone or in combination with JQ1 at indicated doses for 48h. Media for MR49C cells was supplemented with 10µM enzalutamide. Below graphs demonstrate combination indices calculated using Calcusyn software (Biosoft, Cambridge, UK).

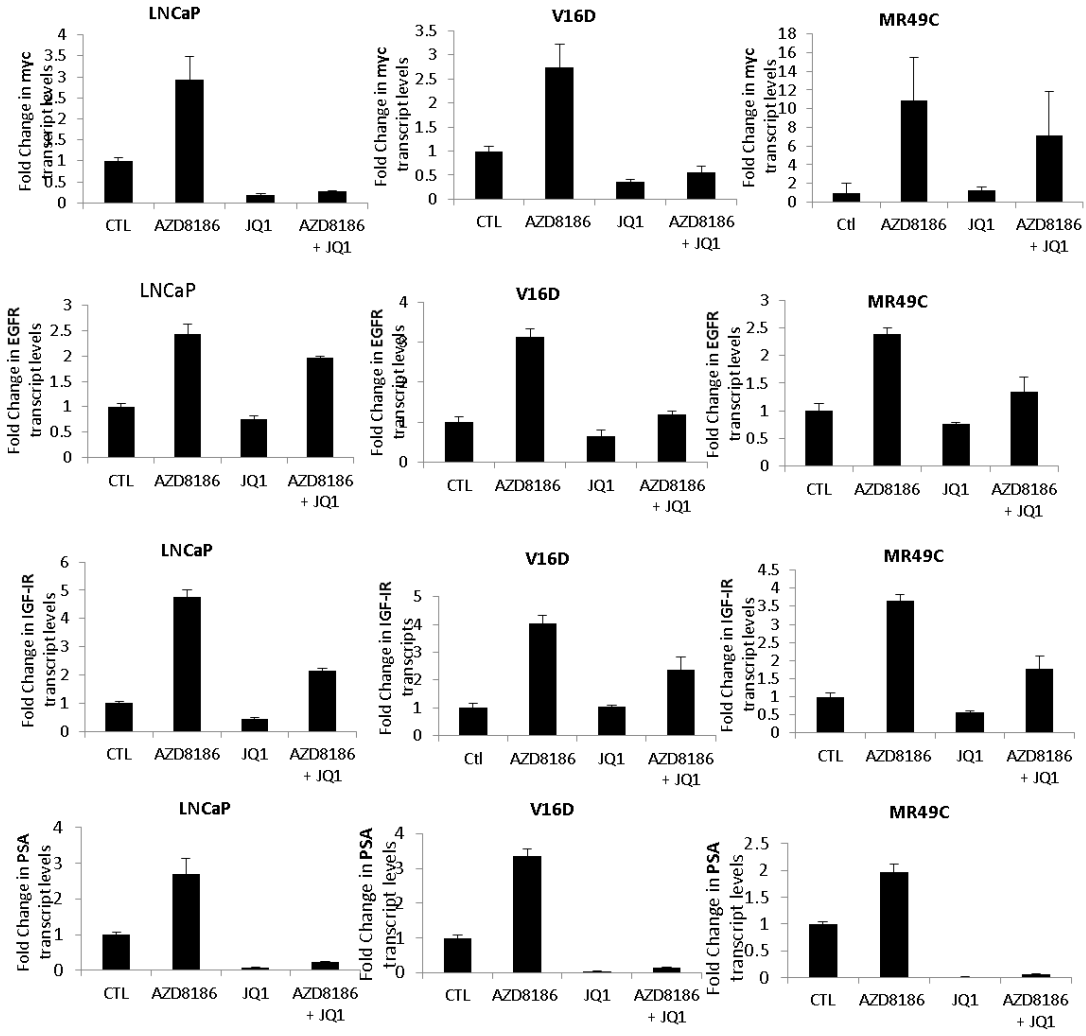

Figure 5.12 Relative changes in myc, EGFR, IGF-IR and PSA mRNA transcript levels following treatment with combination AZD8186 and JQ1. LNCaP, V16D and MR49C cell lines were treated with DMSO control, AZD8186 0.5 μM, JQ1 0.5 μM or the combination for 24h. Levels are normalized to cellular GAPDH as control. Enzalutamide 10 μM was added to media for MR49C cells throughout experiments. Representative experiments of biological triplicates are shown.

Given the differences observed between cell lines in suppression of myc feedback activation, we set to evaluated whether the increase was related to the androgen milieu or concomitant AR antagonism. We found that in the presence of ENZ in both CSS and FBS conditions, AZD8186 still induced myc levels 3 fold, with similar increases in PSA transcript levels (Figure 5.13), suggesting these differences represented cell autonomous changes which occurred with resistance.

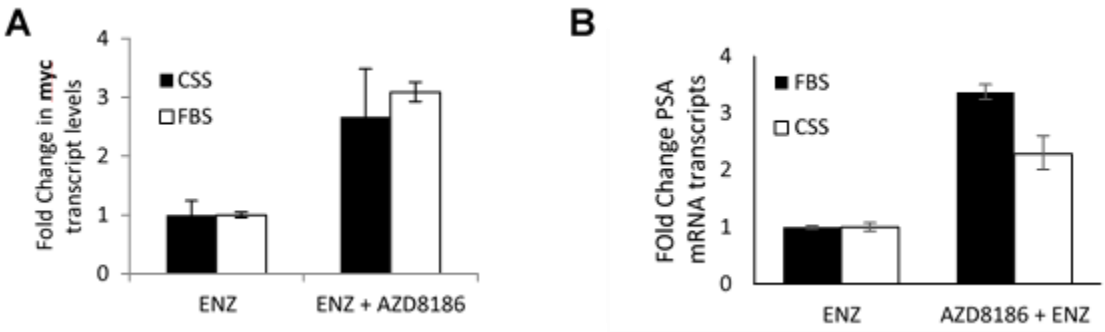

Figure 5.13 AZD8186-induced changes in myc and PSA are independent of AR suppression. **A.** Relative myc mRNA transcript levels following treatment with AZD8186 0.5 μM for 24h in androgen free media (10% CSS) and 10% fetal bovine serum(FBS) in media supplemented with 10μM enzalutamide. **B**. Relative changes in PSA mRNA transcripts under the same experimental conditions. Representative experiments of biologic duplicates with technical triplicates are shown.

We next evaluated whether co-targeting of AZD8186 and JQ1 results in greater reductions in cell growth than the combination of AZD8186 + ENZ. Examination of PSA protein levels demonstrated increases in PSA protein levels following treatment with AZD8186. Notably, the decrease in PSA protein levels was greatest with JQ1 0.5uM and this greater than ENZ 10uM (Figure 5.12). The combination of JQ1 and AZD8186 almost completely abrogated the increase of PSA seen with AZD8186 treatment. In contrast, ENZ + AZD8186 only partially abrogated the increase in PSA with AZD8186 monotherapy. Similarly, when evaluating proliferation of LNCaP cells at different time points, a relative reduction in cell number was seen with combination AZD8186 and JQ1 which was greater than ENZ in combination with AZD8186 (p=0.12). These findings

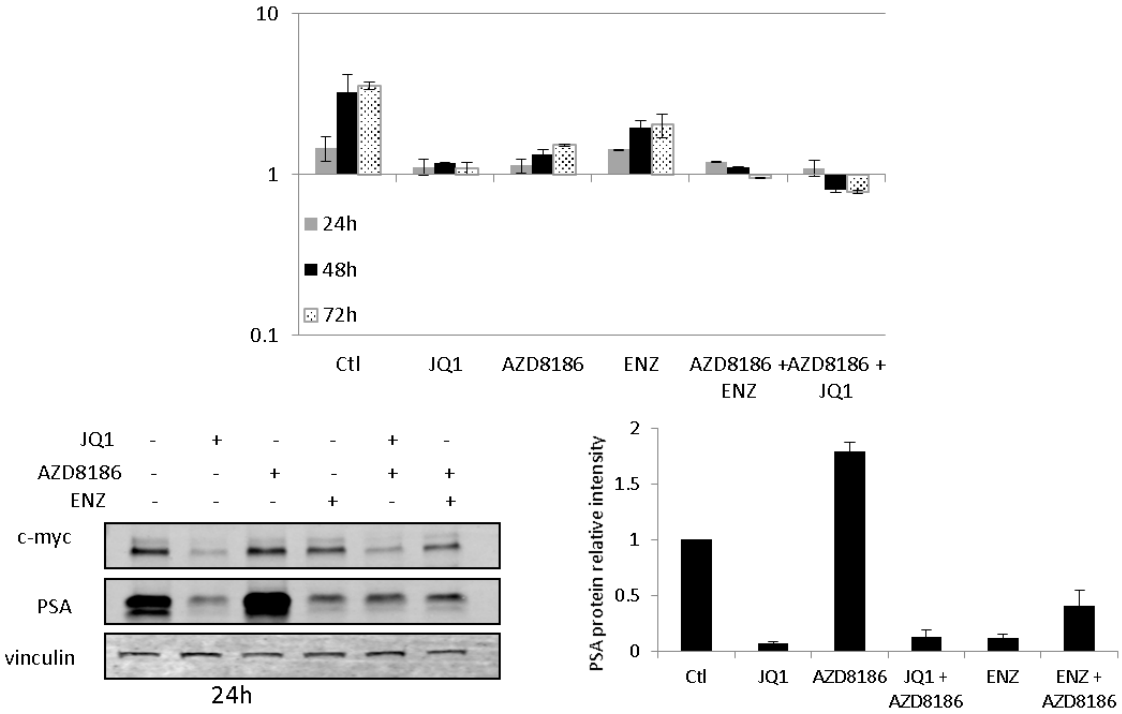

Figure 5.14 Combination AZD8186 and JQ1 appears slightly more potent than combination AZD8186 and enzalutamide. **A**. Western blot of PSA protein levels in LNCaP cells treated with monotherapy, AZD8186 + JQ1 or AZD8186 + enzalutamide(ENZ). Densitometry of triplicate western blots is shown on the right. **B**. Relative cell viability following treatment with monotherapy, AZD8186 + JQ1 or AZD8186 + enzalutamide(ENZ) for the indicated times. Means of triplicate experiments +/-SEM are shown.

suggest that the additional blockade of the myc pathway with JQ1 may improves the cellular growth inhibition and PSA production compared to combined blockade with an AR antagonist.

5.4 Discussion

There continues to be a strong clinical need to develop better strategies to combat therapeutic resistance in CRPC. Synergistic combination strategies have the potential to maximize patient tumour response while lowering the effective dose of each drug required. Our investigation of the novel PI3K inhibitor AZD8186 do not demonstrate strong synergy and raise questions as to the biologic rationale, despite some of our results couple with prior studies suggesting a theoretical benefit.

There is growing interest in epigenetic approaches to treat resistant prostate cancer. BET inhibitors have shown promise in pre-clinical models of prostate cancer and clinical trials are ongoing(222, 323, 324). Our results confirm the efficacy of these inhibitors in prostate cancers and the increased myc which we observe following PI3K inhibition suggests BET inhibitors might be of benefit in combination with PI3K/Akt inhibitors. However, the gene profiling study and the non-significant differences in cell proliferation studies do not suggest a strong synergy, despite some suggestions it may be better than combination with pure AR antagonism.

The combination of BET inhibitors with PI3K inhibitors has been reported in breast cancer(320). Similar to our results with PI3K inhibition prostate cancer, the reported feedback activation of both RTKs and myc was observed. Similar to their results, we also found a decrease in RTK gene expression following BET inhibitor therapy in LNCaP cells. In T-cell adult lymphoblastic leukemia, the combination of BET inhibition with PI3K inhibition was not as effective as the combination of BET inhibition and HDAC inhibitors, suggesting the efficacy of this combination may be cancer- or tumour-specific(325). Moreover, the much larger numbers of breast cancer models available may have aided in finding models where the synergy was greater(320). Identify what may be features of responders to this combination will be essential for further studies in prostate cancer.

The use of combined blockade of PI3K/Akt/mTOR and AR pathways is currently in multiple early stage clinical trials in men with CRPC(326). However, given the higher side effects with multiple agents and the probability that only select patients will have dramatic results as seen in pre-clinical studies, biomarkers will need to be developed to select for patients who will respond. Our results highlight that c-myc upregulation may not

necessary indicate that this is a targetable driver of cancer progression, when in fact it is a feedback response. Indeed, in LNCaP cells following a week of treatment with AZD8186, myc levels have been found to decrease on gene profiling studies pending publication (personal communication, Simon Barry. This highlights some of the dynamic challenges in assessing transcription factors as biomarkers.

The AR-positive LNCaP model is robust and well characterized to mimic many features prostate cancer as it responds to treatment and progresses, but the generalizability of our results is nonetheless limited by the pre-clinical models which we used. Indeed, there was significant heterogeneity in the castrate-resistant LNCaP model, and some of our findings did not reach significance. While our results are supported by *in vitro* and *in vivo* data, we did not include an *in vivo* evaluation of the combination of AZD8186 and JQ1. With marginal synergy found, we judged that the combination did not merit further evaluation. Indeed, even if the marginal differences observed *in vitro* were present in an *in vivo* evaluation, the size of the study would need to be very large to detect significant differences. Of note, our laboratory has also performed *in vivo* studies of different PI3K/Akt inhibitors in combination with another BET inhibitor and did not find any synergy. Another limitation of this study is the lack of validation of c-myc upregulation in clinical prostate tumour samples treated with PI3K/Akt inhibitors. This validation in clinical samples may be more feasible as more trials with targeted inhibitors in prostate cancer are performed.

In conclusion, our results do not support further evaluation of the BET inhibitor JQ1 to co-target both c-myc and AR in combination with PI3K/Akt inhibitors. Further

investigation in clinical samples may be required to identify which subset, if any, may benefit from this combination treatment strategy.

6 Discussion

6.1 Our Results in Context

The global burden of prostate cancer is significant; the morbidity and mortality related to prostate cancer stems almost entirely from men with castration-resistant disease. The recent improvements in overall survival with abiraterone and enzalutamide represents significant progress, but as resistance inevitably develops, there remains a need to develop newer therapeutic approaches. In widespread clinical use now for several years, enzalutamide has now shaped the clinical landscape and there remains a strong need to develop strategies to target enzalutamide-resistant cancer or delay its onset.

While focusing our studies on enzalutamide-resistance, there is some reason to believe our results may also, at least in part, apply to abiraterone-resistance. In addition to its CYP17-inhibiting properties abiraterone also acts as a direct AR antagonist, as we demonstrate in Chapter 2. Thus, it may be expected to share some mechanisms of resistance with the pure AR antagonist enzalutamide. While there are limited studies on abiraterone-resistance due to the challenges of working with this drug *in vitro*(327), the limited mechanisms to date suggested do not vastly differ from those of enzalutamide(66).

As is perhaps best illustrated with highly active anti-retroviral therapy(HAART) for human immunodeficiency virus, combination therapies are likely to be the most successful strategy against the evolving and inevitable resistance to AR-pathway inhibition. Notably, in patients with resistant prostate cancer, therapies currently are given sequentially and as monotherapy, based on whether there is clinical progression evidenced by a rising PSA or new metastases on imaging. Further, unlike HER2 positive

versus negative breast cancers, or ALK mutations in lung cancer, there are currently no biological markers which are used to differentiate which treatments are preferable for prostate cancer patients.

The models of ENZ-resistance developed at the Vancouver Prostate Centre were among the first in the world to be developed. The MR49C and MR49F cell lines reflect several key features of ENZ-resistant prostate cancer. They are AR-positive, prostate specific antigen (PSA)-producing with the AR predominantly located in the nucleus(251). They also have increased levels of steroidogenic enzymes. Unlike the enzalutamide resistant models reported in publications published during the period of this research, these models were developing *in vivo* and not in cell culture(44, 51). Interestingly, the F876L AR LBD mutation found in these models was not identified in the sequencing studies done on the original resistant xenograft models, though the mutation was later identified in the derived cell lines. While this may be related to technical limitations, it suggests that ENZ-resistant models developed *in vitro* may have greater tendency to develop resistance due to selectively advantageous mutations. Conversely, the *in vivo* derived resistance may possibly be more representative of the clinical situation, where the frequency of such of the F876L mutation in ENZ-resistant patients is very low(44, 328).

6.2 Limitations of Our Results

Notwithstanding the advantages of the models used for our studies, there remain limitations to the available prostate cancer models which is highlighted by some of our results. The number of prostate cancer cell lines which are widely used and characterized

is limited compared to other cancers, with under 10 principle cell lines used for virtually all studies. Moreover, only LNCaP, VCaP, 22RV1, and LAPC4 are widely used AR positive cell lines. Given the prominence of the AR in prostate cancer progression, is it questionable how representative non-AR cell lines are of CRPC. This shortage of models represents one of the global limitations of our pre-clinical targeting studies.

It is clear that the amount of heterogeneity present in resistant prostate cancer is not represented in the cell lines which are widely available. Recent studies suggest that the degree of tumour heterogeneity increases as prostate tumours become more resistant(329). While evaluation in more than one ENZ-resistant model (ie MR49C and MR49F) increases the robustness of our findings of the potency of VT-464, it is nonetheless desirable to have models that cover the spectrum of resistance. Our studies focused on AR positive prostate cancer since the vast majority of resistant lethal prostate tumours are accompanied clinically by a rising PSA, indicating the AR axis remains central in ENZ-resistant tumours.

At this juncture, it is helpful to appreciate that clinical categories of ENZ-resistance are only beginning to develop. A hallmark paper has described the importance of the presence of the AR-V7 splice variant as a strong predictor of both enzalutamide and abiraterone resistance(54), with validation studies near press. However, whether this is a true biologic driver of resistance or a passenger to an increasingly chaotic and heterogeneous tumor type remains to be determined. Whether the Akt or MAPK pathway activation represents a distinct driving oncogenic resistance pathway specific to ENZ-resistances remains to be determined. It may form a spectrum with neuroendocrine differentiation, which like that Akt pathway, is associated with higher Gleason

scores(330). Limited clinical studies to date suggests elevations in testosterone and steroidogenesis can occur following enzalutamide treatment, but more research is required to identify whether this represents a unique subclass of ENZ-resistant tumours(58, 59). Finally, patients with mutations in DNA-repair pathways may represent a distinct pattern of resistance(237).

In particular, our results in Chapter 3 highlight some of the limitations of pre-clinical models. We found only moderate synergy with the combination of MEK and Akt inhibition, with the effect appearing to be largely reflective of the relative importance of each pathway in the 22RV1 and LNCaP models, respectively. Cell culture is by its nature a simplification; it remains possible that in the more complex tumour microenvironment dual blockade of MEK and Akt pathways may be more relevant. While xenograft models permit greater complexity, including some interaction between murine fibroblasts, endothelial cells and macrophages and the tumour, the improvements are nonetheless limited. While these limitations may hamper pre-clinical research efforts, it is still important to note the clear importance of multiple oncogenic signalling pathways in lethal prostate cancers(72).

While it remains possible the combination of MEK and AKT inhibition will be more effective in the complex milieu of patient tumours than in model systems, our modest results concur with others(portrayed perhaps in an overly positive manner)(299) and do not support clinical evaluation of this combination for prostate cancer. It remains possible that other model systems may demonstrate better utility of the combination, but for the moment our results may signify the end of further evaluation of this combination in prostate cancer models.

6.3 Clinical Translation of Our Results

Our results confirm the hypothesis that targeting of the AR pathway via steroidogenesis in ENZ-resistant remains pertinent, but we add an important nuance to this conclusion. The nuance is that we found that VT-464 also concomitantly directly inhibits the AR. Despite the strong pre-clinical data which we have developed, it is impossible to complete dissect these different mechanisms of action apart and it remains possible that our results are driven by direct AR antagonism, as our lab has previously shown for another agent(251) . Nonetheless, the next steps from our research on this novel inhibitor are quite clear and have progressed rapidly since the publication of our research detailed in **Chapter 2**. Even prior to publication, our data was instrumental in securing several larger studies. A new company, Innocrin Pharma, has been spun off the parent company Viamet Pharmaceuticals Inc to facilitate the development of VT-464 as a cancer therapeutic. A one-million-dollar Prostate Cancer Foundation Challenge Award was awarded in 2014 to a team of researchers from Seattle and New York for the project titled “The Novel CYP17 Lyase Inhibitor VT-464 for Patients with Advanced Prostate Cancer Resistant to Enzalutamide: Use of Predictive Biomarkers during Drug Development Process Is Essential for Improved Patient Management and Time to Drug Approval”. Integrated as part of this award was a Phase II trial of VT-464 in patients with CRPC progressing on ENZ or ABI(NCT02445976). The once-daily dosing in this study was supported by our *in vivo* study as well as preliminary patient results. Two other Phase II trials of VT-464 in prostate cancer are ongoing: the Phase I/II open label study in CRPC patients sponsored by Innocrin (NCT02012920, NCT02361086), with initial results presented at the 2015 Genitourinary Symposium(331) and the Phase II National Cancer

Institute study in patients with mCRPC resistant to ENZ (NCT02130700). Finally, evaluation of this agent in breast cancer is now ongoing (NCT02580448). Thus, the results of our pre-clinical work has supported a rapid advance to ongoing clinical evaluation.

The initial results of VT-464 in patients to date confirm our pre-clinical findings(331). While initial numbers are small, 2/7 patients treated with VT-464 who received prior ENZ had responses: one patient(post-chemo) had a maximum PSA reduction of ≥50% and one patient (pre-chemo) had a maximum PSA reduction of ≥90%. While this response rate is not high, it exceeds previously reported data for abiraterone(190). The very low levels of testosterone which we noted in xenograft tumours was also confirmed in patients. In castrate patients taking the three higher dose echelons (450mg BID, 450mg QD or 600mg QD), the mean % decline of testosterone from baseline to nadir was over 80%. 12/16 of the same patients had testosterone levels after one month of therapy at levels below the lower limit of quantification on LC-MS. Pharmacokinetic(PK) analysis studies indicated the AUC for Cmax values were highest in these same groups. In particular, the PK analysis supported once daily dosing, which was presaged with our initial results, albeit in a small number of mice.

These clinical results are particularly notable given that the main competitor in the market for lyase-selective CYP17 inhibitors for prostate cancer, galeterone, has not reported significant responses in ENZ-resistant patients. This may be one reason why their Phase III trial, recently begun, focuses on targeting patients with the presence of the AR-V7 splice variant. While AR-V7 is present at low levels in LNCaP cells, further studies

may be warranted to assess whether VT-464 has specific activity against AR-V7. Similarly, galeterone has been reported to result in AR degradation, and though our initial results do not demonstrate significant AR degradation at the doses studied, it remains to be seen if clinically significant AR degradation may occur in patients.

We hypothesized that combination therapy would be needed to co-target oncogenic signalling that emerges as prostate tumors become more resistant. However, two of our studies did not demonstrate a strong synergy, despite an established rationale. During the course of our studies, results on co-targeting the MEK and Akt pathways in AR-negative prostate cancer models was reported(299). On close inspection, their results in AR-negative models are similar to ours; nonetheless, Park et al propose MEK plus Akt inhibition is an effective strategy for CRPC. Our modest results with this combination as well as that of BET inhibition plus PI3K inhibition may reflect a limitation of the available models. Nonetheless, our results do raise questions as to whether combinations are necessarily needed or if accurate characterization of patient or tumor features may allow for similar results with less side effects in patients. Ultimately, clinical evaluation will be required to answer these questions, but promising results with biomarker-based selection and HDAC inhibitors suggest characterization of patient or tumor features is a viable strategy to move novel treatments and combination strategies forward.(237). Further, combination strategies will also need to demonstrate clear synergy via multiple outcomes in pre-clinical models for advancement to clinical evaluation, which has not been the case for many of the prior combinations assessing co-targeting of PI3K/Akt in pre-clinical studies (Table 1.3).

Our results with combination AZD5363 and enzalutamide do demonstrate clear synergy across multiple pre-clinical outcomes. To the best of our knowledge, our results with combination AZD5363 and enzalutamide represent the best response ever reported for any treatment or combination in the aggressive LNCaP CRPC model. Accordingly, AstraZeneca decided to initiate a Phase II trial of AZD5363 in combination with ENZ in the UK. The recommended Phase II dosing of AZD5363 is 320 mg BID 4 days on/3 days off based on a phase I study in combination with docetaxel(332). This has since become a multi-national trial, named RE-AKT, with Dr. Johann De Bono as the Primary Investigator (NCT02525068). While our results were the first to suggest the combination may be more effective in castrate-sensitive disease, it is much more feasible to obtain results in the more advanced CRPC space where measurable clinical events needed for regulatory approval occur more rapidly. Studies in castrate-sensitive prostate cancer require significantly more follow-up time and therefore incur much higher costs. Over the last several years, multiple clinical studies have now emerged in CRPC to test various combinations of PI3K/Akt/mTOR inhibition in combination with AR blockade (Table 6.2).

Table 6.1 Ongoing trials featuring combination of AR pathway inhibitors and PI3K/Akt/mTOR pathway inhibitors in prostate cancer.

PI3K/Akt/mTOR inhibitor	Target class	AR pathway inhibitor	Disease State	Phase	Trial Registry Number
GSK2636771 (GlaxoSmithKline)	PI3K-beta inhibitor	+enzalutamide	mCRPC	I	NCT02215096
BKM120 (Novartis)	Pan-PI3K inhibitor	+Abiraterone acetate	Post-abiraterone CRPC	I	NCT01634061
		+Abiraterone acetate	mCRPC	I	NCT01741753
BEZ235 (Novartis)	Dual PI3K/mTOR inhibitor	+Abiraterone acetate	Post-abiraterone CRPC	I	NCT01634061
GDC-0980 (Genetech)	Dual PI3K/mTOR inhibitor	+Abiraterone acetate	Post-docetaxel	I/II	NCT01485861
AZD5363 (AstraZeneca)	Akt inhibitor	+enzalutamide	mCRPC	II	NCT02525068
GDC-0068 (Genetech)	Akt inhibitor	+Abiraterone acetate	Post-docetaxel	I/II	NCT01485861
MK2206 (Merck)	Akt inhibitor	+Bicalutamide	Biochemical recurrence	II	NCT01251861
		+Bicalutamide	Recurrent or mCRPC	II	NCT00814788

It is notable that while we did detect synergy with AZD5363 and ENZ in 22RV1 cells (PTEN wild-type), the synergy was clearly more dramatic in LNCaP-based cells (PTEN loss). The clinical use of PI3K/Akt inhibitors will allow for assessment of reciprocal activation of AR in patient specimens, as we observed following PI3K/Akt inhibition in pre-clinical model. This is particularly important given all the studies to date on the combination of AR and PI3K/Akt/mTOR blockade for prostate cancer treatment have been done in pre-clinical models. Early anecdotes suggest that PSA elevation on monotherapy with PI3K/AKT inhibitors may occur, but the published literature is sparse

on this topic. Developing clinical experience with PI3K/Akt inhibitors in prostate cancer patients will also allow assessment of relative tolerability. In our murine studies, the drugs AZD5363 and AZD8186 were both well tolerated, with less hyperglycemia observed with PI3K inhibition.

As BET inhibitors are developed for various malignancies, it remains unknown whether they will be effective in prostate cancer, and if so, whether they are best employed as monotherapy or in combination with other agents. One clinical trial has recently begun evaluating the combination of BET inhibitor therapy with enzalutamide, based on one pre-clinical study(223). Given that BET inhibitors work by blocking transcription; it remains plausible that their use will be most effective in tumours which have high transcriptional activity. In a similar way, the greatest effect of the combination which we observed with JQ1 and PI3K inhibition was at the level of mRNA transcription of key genes induced following PI3K inhibitor therapy. Analysis of gene expression in CTCs or tumour biopsies following PI3K/Akt or BET inhibitor treatment may therefore be important to understand potential reasons for resistance to these agents.

6.4 Development of Biomarkers

While the results of these trials will be pivotal in further development of these novel therapies, there clearly remains a need to integrate biomarkers for the use of targeted inhibitors. The failure of multiple studies in metastatic castrate-resistant prostate cancer over the years (Table 6.3) highlights the need to develop better approaches to advancing novel agents and combination therapies into clinical practice. The development of accurate and reliable biomarkers can be expected to depend on a thorough and

contextually knowledge of the relevant pathways in prostate tumours and will require rigorous validation.

Table 6.2 Selected list of novel agents given which failed Phase III evaluation in metastatic CRPC.

Regime evaluated	Mechanism of action
Docetaxel + Estramustine(23)	alkylating agent
Docetaxel + High-dose calcitriol (333)	vitamin D
Docetaxel + Bevacizumab(26)	angiogenesis inhibitor
Docetaxel + Atrasentan(334)	Endothelin A receptor antagonist
Docetaxel + GVAX(335)	immunotherapy
Docetaxel + Zibotentan(336)	endothelin A receptor antagonist
Docetaxel + Aflibercept(337)	VEGF-inhibitor
Docetaxel + Lenalidomide(338)	Tumor microenvironment
Docetaxel + Dasatinib(339)	tyrosine kinase inhibitor
Docetaxel + OGX-011(340)	antisense clusterin inhibitor

The of use biomarkers to enrich for populations of patients who are likely to respond to therapy can drastically reduce the number of patients required in Phase III trials from over a thousand to several hundred or less. In addition to requiring fewer patients in a context where it can be difficult to recruit patients, this also significantly decreases the costs involved to run trials. Further, when testing for a larger benefit in a subset of patients, the potentially higher side effects with combination therapy may be justifiable if significant responses can be demonstrated. Biomarker-based studies are now underway for galeterone(NCT02438007), as well as olaparib(NCT01682772).

The importance of biomarkers is supported by our results evaluating the combinations of Akt inhibitor and MEK inhibitors. Identification of which kinase pathways

are active and significant in patient tumours in real time can provide stronger justification to combination therapies, potentially with very significant responses. Thus, the use of the combination could be rapidly justified, sparing unnecessary toxicity in patients in whom the combination would not be beneficial. A phospho-proteomic analysis of multiple tyrosine kinases suggests the intra-patient heterogeneity of prostate cancer is low(72), supporting the concept of targeting relevant kinase signalling pathways. However, recent data on the intra-patient heterogeneity of genomic alterations in patients with metastatic prostate cancer is more varied(239, 322, 341). Nonetheless, it needs to be considered that genomic alterations which result in activation of the MEK/ERK pathways are rare in prostate cancer and so activation of this pathway and others may be underappreciated in genomic studies. Conversely, PTEN loss is one of the most common genomic alterations in CRPC, but is not always completely penetrant likely due to compensation and cross-talk of related pathways. Circulating tumour cells may have PTEN loss, but the identification does not appear sufficiently reliable in clinical studies to date as a marker of PI3K/Akt pathway activation (221). The use of propriety platforms such as Epic Bioscience permits the rapid analysis of selected protein staining of CTCs. While cost and labour currently remains prohibitive for regular clinical use, with sufficient validation this approach may potentially be useful to identify patients with activation of MEK and/or PI3K/Akt pathway. Finally, molecular-based imaging techniques, including PET scanning may represent a biomarker which gives both metabolic and spatial information on tumour progression. For example, preliminary studies now suggest that (18)F-

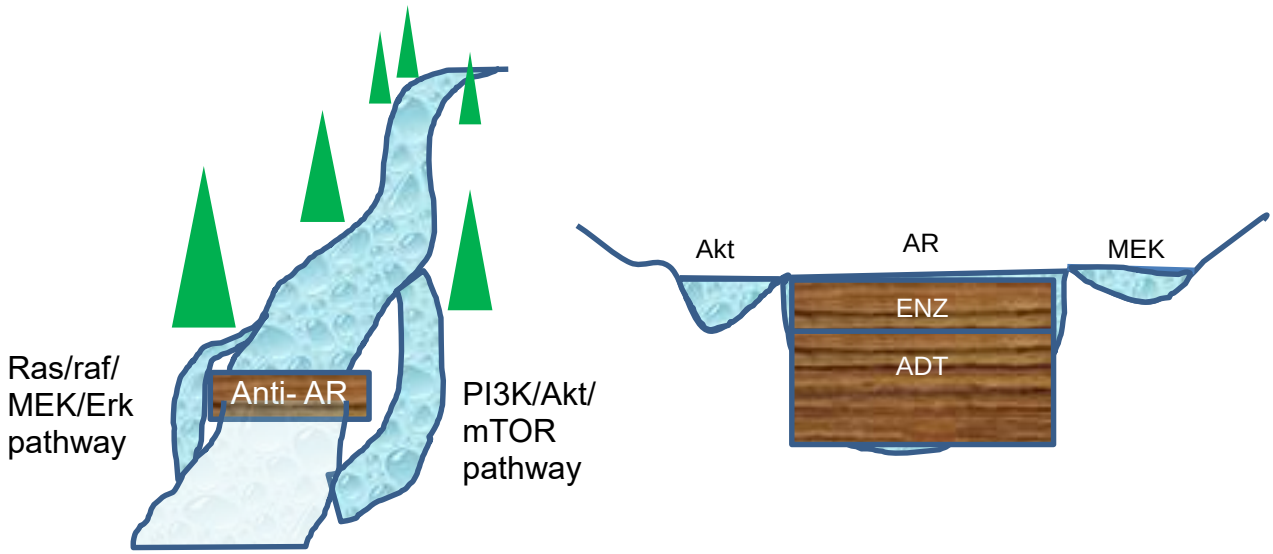

Figure 6.1 Illustration of potential rationale for dual kinase inhibition in enzalutamide-resistant cancer. As the principal driver of prostate cancer progression, blockade of the AR pathway with androgen deprivation therapy is effective; more effective blockade of the pathway with the addition of enzalutamide(ENZ) has proven to be effective. However, this is expected to result in further activation of alternative survival pathways, such as the Akt and MEK signaling pathways.

fluorodeoxyglucose positron emission tomography ((18)F-FDG PET) may be a pathway specific biomarker of sensitivity to AZD8186(342).

Prior work indicates that significant changes in prostate tumour characteristics occur as a result of treatment stress(107). With our LNCaP models demonstrating persistent dependence on Akt signalling despite being castrate-sensitive or castration-resistant, another area which remains unexplored is whether biomarkers may predict which kinase pathways are more likely to become activated following AR-directed therapy. While it is clear that the incidence of PTEN and other genomic abnormalities increase as tumors become more resistant, it remains speculative whether certain patterns of protein or gene expression in the primary tumour may herald certain resistance pathways to develop. In other words, much like how a landscape dictates which route a dammed river will flow, can certain primary tumour characteristics predict which kinase signalling pathway will be activated once the primary river (ie AR pathway)

is effectively inhibited? This is illustrated in **Figure 6.1**. Supporting for this hypothesis is the tendency of higher grade tumours to eventually develop neuroendocrine differentiation (330) as well as the ability of serum testosterone levels in patients on ADT to predict the development of CRPC years later (343).

6.5 Conclusions

We demonstrate in these studies several important findings which we believe will help move the field of prostate cancer treatment forward. Firstly, we provide strong evidence for further evaluation of two treatment strategies in the clinic, with mulit-national Phase II trials of both VT-464(now known as seviteronel) and the combination of AZD5363 and enzalutamide ongoing in patients with CRPC. Secondly, we cast doubt on two potential combination treatment strategies, which we hope will save unnecessary evaluation in patients. Thirdly, we also found contrary to our hypothesis that combination targeting of kinase pathway signalling is not necessary more effective in advanced cancer, and perhaps in select patients, combination therapy such as AZD5363 and enzalutamide is better administered earlier to delay the development of resistance. Finally, our results support the importance of patient and tumour characterization in order to optimize selection of the best treatment strategy.

To move forward care of patients with CRPC and discover, develop and adopt new therapeutic treatments and combination strategies, a close interaction between pre-clinical models and clinical trials is required. The chapters in this thesis demonstrate how work performed in pre-clinical studies can be rapidly transferred to clinical evaluation through the support of industry partners. It is equally important to evaluate at a biological

level the reasons why therapies do or do not succeed in clinical trials. It is now standard practice to include correlative science components as part of clinical trials to assist in identifying biomarkers. Closing the gap between laboratory science and clinical medicine will be important for future improvements in clinical outcomes for patients with lethal prostate cancer.

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