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Title	Investigating the mechanism of action and potential efficacy of Apolipoprotein E mimetics as therapeutic agents for the treatment of metastatic prostate cancer
Author(s)	Johnson, Carol
Publication Date	2017-10-04
Item record	http://hdl.handle.net/10379/6867

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Investigating the mechanism of action and potential efficacy of Apolipoprotein E mimetics as therapeutic agents for the treatment of metastatic prostate cancer

A thesis submitted to the National University of Ireland in fulfilment of the requirements for the degree of:

Doctor of Philosophy

By

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March 2017

Acknowledgements

Firstly, I would like to thank my supervisors Dr. Sharon Glynn and Professor Frank Sullivan for their guidance, patience and support throughout this process and for giving me the opportunity to do this PhD.

To the Prostate Cancer Institute research group - Amy, Sarah, Ronan, Ali, Pablo and Karen. I couldn't have been more fortunate to work with such a lovely bunch! Thanks so much for all the good times and fun memories! I'll forever treasure friendships formed through tea breaks, DMCs, and CDWM nights. I'd also like to thank all the crew along the way from the NCBES and Biosciences, in particular, Dr. Alex Natoni for providing experimental ideas, discussions and expertise. Thanks Alex –You're a 'Nice Guy'!

To my amazing and loving family, thank you for your endless support and encouragement throughout the course of my academic journey. Thanks for always being there and for getting me through it!

Damien, thanks for putting up with me through the whole process and for all the love and support you have shown me. Your never-ending encouragement and pep talks kept me going.

And last but not least, Benjie and Clio, my two study buddies, foot warmers and comedians. Thanks for being a girl's best friend.

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Declaration

I, Carol Johnson, hereby declare that this is entirely my own work and that it has not been submitted as an exercise for the award of a degree at this or any other university.

Signed:_____ Date: _____

Abstract

Prostate cancer is the most prevalent non cutaneous cancer diagnosed in Irish men and the third leading cause of death in males annually in Ireland. Therapeutic strategies currently consist of prostatectomy or radiation followed by androgen ablative therapy. Typically, these treatment measures eventually fail. Prostate cancer re-occurs in the form of androgen independent prostate cancer, a lethal form of progressive and metastatic prostate cancer for which there is no cure. There is therefore an urgency for the development of more effective therapies. Apolipoprotein E (ApoE) has been reported in the literature to possess the ability to impede invasion and inhibit metastatic cell recruitment in cancer cells. ApoE has also been reported to have anti-inflammatory properties that could potentially impede pathogen recognition receptor (PRR) signalling. ONCOTIDE.inc generated a series of ApoE derived peptides modelled on the receptor binding domain of ApoE. The potential efficacy of the ApoE peptide mimetics COG112 and OP449 in prostate cancer were explored in this study. The aim of this study was to determine the toxicity of ApoE peptide mimetics to prostate cancer cells and the effect on cell cycle progression. The study also explored the ability of COG112 and OP449 to interact with the SET oncogene and modulate the SET-PP2A complex and PP2A activity. As chronic exposure to inflammatory stimuli is thought to play a role in prostate cancer progression, PRR signalling was explored to investigate potential additional benefits to PP2a stimulation. In terms of PRR signalling, the effect of the ApoE peptides on TLR and RIG-I signalling was assessed. The results demonstrated that the peptides decrease cell viability in a dose dependent manner in AR positive and AR negative cells. OP449 was shown to disrupt the progression of the cells through the cell cycle and successfully induce apoptosis in PC3 cells. The expression of SET oncoprotein was found to be up regulated in prostate cancer. COG112 and OP449 were shown to bind to SET and disrupt the protumourgenic SET-PP2A complex. Disruption of the SET-PP2A complex resulted in the upregulation of PP2A activity and negative regulation of Akt. COG112 and OP449 modulated the expression of TLRs 3 and 4 in AR negative cell lines. The peptides demonstrated an

obstruction of downstream TLR signalling and were capable of hindering the response to TLR4 stimulation. Modulation of IκBα phosphorylation and p65 translocation was also observed. The results of this study support the need for further investigation into the use of ApoE mimetic peptides, namely OP449, in the treatment of advanced prostate cancer. The results exhibit OP449 to have multipurpose benefits and demonstrate that the ApoE peptide mimetics effectively target several pro tumorigenic signalling cascades in prostate cancer cells.

Abbreviations

ADT	Androgen deprivation therapy
AIPC	Androgen Independent Prostate cancer
Akt	Protein kinase B
An V	Annexin V
ApoE	Apolipoprotein E
BPH	benign prostatic hyperplasia
CARD	caspase recruitment domain
CARD	caspase recruitment domain
CDK	cyclin-dependent kinase
CRPC	Castrate resistant prostate cancer
CSA	catalyzed signal amplification
CTD	C- terminal domain
DAMPs	Damage-associated molecular patterns
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ERK	Extracellular Signal-regulated Kinase
GSK3 α/β	Glycogen synthase kinase 3 α/β
I2PP2A	Inhibitor 2 of Protein phosphatase 2A
IAP	Inhibitors of apoptosis
IKK	inhibitor of nuclear factor kappa-B kinase
IRAK	IL-1 recepor associated kinase
IRF3	Interferon regulatory factor 3
IκB	IkappaB
JNK	c-Jun N-terminal kinases
LDLR	Low-Density Lipoprotein receptor
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related proteins
LUTS	lower urinary tract symptoms
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation-associated gene 5
mTOR	Mammalian target of rapamycin
NLR	nucleotide –binding oligomerization domain like receptors
NLR	nucleotide-binding oligomerization domain (NOD)-like receptors
NO	Nitric Oxide
NOD	nucleotide –binding oligomerization domain

OA	Okadaic Acid
PAMPs	Pathogen-associated molecular patterns
PAP	prostatic acidic phosphatase
PAP	prostatic acid phosphatase
Pca	Prostate Cancer
PGE2	prostaglandin E2
PH	plekstrin homology
PI	Propidium Iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Poly(I:C)	Polyinosinic-polycytidylic acid
PP2A	Protein Phosphatase 2A
PRR	pattern recognition receptors
PS	phosphatidyl serine
PSA	prostate specific antigen
RAGE	receptor for advanced glycation endproducts
Rig-I	retinoic acid-inducible gene I
RIP1	receptor interacting protein 1
RLR	Rig-I like receptors
RPPA	Reverse phase protein array
RTK	Receptor Tyrosine kinase
SET	Inhibitor 2 of Protein phosphatase 2A
TAK1	Transforming growth factor beta-activated kinase 1
TANK	TRAF family member-associated NF-kappa-B activator
TLR	Toll Like receptor
TRAF6	tumour necrosis factor receptor associated factor 6
TRIF	Toll/IL-1R domain containing adaptor protein inducing IFNβ

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Chapter 1: General Introduction

1. General Introduction

1.1 The prostate

The prostate gland sits in the pelvis, surrounded by the rectum posteriorly and the bladder superiorly. It is understood to produce some seminal fluid and may facilitate sperm motility. The prostate gland is composed of stromal, ductal and luminal epithelial cells and is organised by branching ducts and individual glands lined with secretory epithelial cells and basal cells [1] . The secretory epithelial cell is the chief cell type within the gland. These androgen regulated cells produce prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) [2]. Androgens play a central role in the normal growth and development of the prostate [3]. The vast majority of prostate cancers have cells that share properties with the secretory epithelial cells. Unlike the epithelial cells, the basal layer is not directly controlled by androgen signalling [4]. Neuroendocrine cells which present within both the prostate gland and the stroma, are likely to contribute to normal prostate function in a paracrine fashion [5]. The surrounding stroma is made up of fibroblasts, smooth muscle, nerves, and lymphatics. Stromal-epithelial interactions remain poorly understood, but recent insights suggest that the stroma produces multiple growth factors important for growth and development of normal prostate as well as prostate cancer (PCa) [6, 7].

The prostate gland has been defined as comprising of four major anatomic zones within the normal prostate [8, 9]. These are comprised of the peripheral, central, transitional zones (constituting 70, 20 and 5% of the glandular tissue respectively) and the anterior fibromuscular stromal zone. The peripheral zone, which extends posteriorly around the gland from the apex to the base, is the most common site for the development of prostate carcinomas. The central zone surrounds the ejaculatory duct apparatus and makes up the majority of the prostatic base. The transition zone constitutes two small lobules that abut the prostatic urethra and is

the region where benign prostatic hyperplasia (BPH) primarily originates [10, 11] (Figure 1.1).

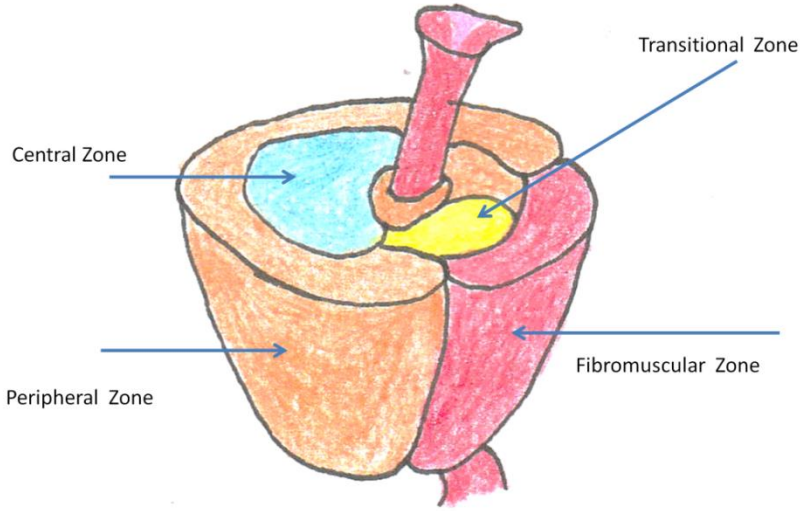


Figure.1.1 Prostate zone regions. Most cancer lesions occur in the peripheral zone of the gland, fewer occur in the transition zone and almost none arise in the central zone. Most benign prostate hyperplasia (BPH) lesions develop in the transition zone, which might enlarge considerably beyond what is shown. Both small and large carcinomas in the peripheral zone are often found in association with high-grade PIN, whereas carcinoma in the transition zone tends to be of lower grade and is more often associated with atypical adenomatous hyperplasia or adenosis, and less often associated with high-grade PIN

1.2.1 Inflammation and pathologies of the prostate

Existing literature has suggested that prostatic inflammation may be a leading contributor to prostate growth in terms of both hypergenesis or hyperplasia and neoplastic transformations. It has been proposed that inflammation plays a role in cancer whereby inflammatory conditions such as prostatitis or BPH increase the risk of cancer development among patients. For years the idea that inflammation and inflammatory illness play a close role in the development and progression of

cancer has been hypothesised and the evidence has long been accumulating to indicate so. Hanahan and Weinberg identified the hallmarks of cancer as the acquisition of functional capabilities that allow cancer cells to survive, proliferate and propagate exponentially. These changes are acquired in various tumour types at various intervals over the course of multistep tumourigenesis [12]. Emerging evidence has suggested the ability of cancer cells to evade immunological destruction with Hanahan and Weinberg adding it to the list of cancer hallmarks [12]. Inflammation can contribute to multiple hallmark attributes by providing growth, survival pro-angiogenic factors and extracellular matrix-modifying enzymes to the tumour microenvironment. These in turn promote proliferation, survival and facilitate invasion and metastasis respectively [13-16]. Inflammation is often apparent at the earliest stages of neoplastic development and evidently promotes the development of neoplasias into full-blown cancers [15, 17]. The literature has estimated that inflammatory illness and responses are somewhat related to up to 20% of cancer related deaths worldwide with several existing causes of chronic inflammation likely to act as a trigger for the onset of tumour formation, for instance Inflammatory bowel disease is associated with colon cancer while prostatitis has been linked to PCa [18-20].

1.2.2 Prostatitis

Prostatitis is an inflammatory condition of the prostate gland. Four different categories of prostatitis have been described, depending on the symptoms and manifestation of the condition [21, 22]. Three of these prototypes are classed as symptomatic and the fourth is a form that is clinically silent [21]. Acute bacterial prostatitis (category I) occurs as the result of a bacterial infection, typically of a gram negative nature such as Escherichia coli, and patients tend to present with symptoms that include significant pelvic pain, difficulty in urinating as a result of prostatic swelling and evidence of systemic infection or fever. There are rarely long term side effects of this form of prostatitis after antibiotic therapy [21-23]. The

second category, referred to as ‘Chronic Bacterial Prostatitis’, occurs as a result of a relentless bacterial infection with patients reporting both sporadic and constant pelvic pain, however these patients do not present with the symptoms of systemic infection that are reported in category I prostatitis. Prostatic fluid secretions from category II patients have been reported to contain a significant level of leukocytes and bacteria although with persistent antibiotic therapy alongside attentive awareness of possible urinary obstruction the infection can be eliminated [21-23]. The third type of prostatitis is often referred to as ‘chronic non-bacterial prostatitis’ or ‘pelvic pain syndrome’ and is the most common form of prostatitis and accounts for more than 90% of the cases with patients suffering from intermittent to regular episodes of pain in the pelvis, perineum or external genitalia [23]. Fluid secretions from patients with this type of prostatitis do not typically comprise of bacteria, however these group of patients are subcategorised into an inflammatory cell presenting group (IIIa) or a group void of leukocytes (IIIb), with both groups experiencing symptoms for weeks to possibly years [21, 22]. Therapies for this form of illness, which include antibiotics, NSAID medication and 5α-reductase inhibitors, have so far been of limited value in improving symptoms [24, 25]. The final category of prostatitis (IV) is referred to as asymptomatic prostatitis and is diagnosed by means of histological conclusion upon pathological investigation on the prostate tissue of a man who is not symptomatic of prostatitis [21, 26, 27]. It has been speculated with new interest in the past decade that a correlation exists between prostatitis and PCa; however there is a significant lacking in the evidence available in the literature. Although studies have been performed, the relationship between the two has not been very well established. This may be in part due to the possibility for potential bias resulting from the greater PCa surveillance such as PSA testing, biopsies or digital rectal examinations among patients which were previously diagnosed with prostatitis. Sutcliffe et al., performed a study investigating clinical prostatitis and the risk of PCa and reported findings which correlated with the reports of eight alternative case controlled studies, however these studies were subject to flaws due to deficiencies in urologic investigations information [28] Sutcliffe et al., reported a significant association between

prostatitis and prostate cancer among unscreened men, however in their initial investigation they did not report a correlation among screened patients [28]. There is still significant merit for the further investigation into the relationship between prostatitis and PCa with a greater emphasis to be placed on the possibility of detection bias with future studies also focusing more specifically on the 4 categories of prostatitis and their individual correlation with PCa risk.

1.2.3 Benign Prostatic Hyperplasia

BPH is an inflammatory condition characterised by enlargement of both the stromal and glandular constituents of the prostate [29]. BPH is the most common benign tumour in men with the development risk increasing with age, for instance, it has been reported to be found in approximately 70% of men at the age of 70 with prevalence increasing to almost 100% by the age of 80 and of greater occurrence in the western world [29]. In progressing BPH, many patients present with lower urinary tract symptoms (LUTS) which include frequent urination, nocturia, decrease or broken stream during urination or incomplete emptying of the bladder [30, 31]. According to Rosario and Bryant, circulating androgens along with ageing are two important prerequisites for the development of BPH. While the surrounding testosterone can be converted to dihydrotestosterone (DHT), which is strongly capable of promoting prostate growth, ageing is also thought to promote sensitivity of the prostate to androgen levels, with androgens playing a role in the development of BPH [29]. There are similarities which have led to a hypothesised link between BPH and PCa, for instance they both exhibit increasing prevalence with age, they both require androgen for growth and development and respond to antiandrogen treatment [32]. The similarities between these conditions along with their frequent coexistence has led to the postulation of an existing relationship between the two [33]. Although both conditions are hormone dependant and the existing evidence strongly suggests an important role of inflammation in both cases, there is still no proven relationship between BPH and PCa although research

is ongoing in attempting to find the link [33]. According to Bostwick et al., approximately 83% of PCas originate in men who also suffer from BPH and that the correlation is consistent across all ages [32]. A positive correlation has also been associated between fast growing BPH, PSA level and grade of PCa [34]. BPH generally originates in the transition zone and may extend to the peripheral zone and can sometimes even originate in the peripheral zone. Research has demonstrated that approximately one third of transition zone PCas originate within the BPH nodules and most other cases of PCa originate from the peripheral zone, which supports the existence of a possible link between BPH and PCa [32, 35]. There are conflicting studies which have suggested that although enlarged prostate volumes of up to 45cm^3 are associated with high grade PCa risk, when prostate volumes are above 45cm^3 the correlation is inverted [36]. Androgens typically play a critical role in the growth and development of the prostate, however they have also been established as key components in the development of BPH and PCa [33, 35, 37]. Testosterone, the most predominant circulating androgen, is converted to the more potent androgenic steroid hormone, DHT, by either of two existing isoforms (type 1 or type 2) of 5-α reductase which binds to the androgen receptor to initiate cellular differentiation and proliferation in the prostate. It is plausible that disruption of the balance between DHT regulated cellular proliferation and death, is likely to result with excessive cellular proliferation and insufficient cell death. This may subsequently result in the instigation of prostate growth and tumour development, presenting as BPH or PCa [37]. As DHT is the most potent androgen involved in prostatic enlargement and bladder obstruction which contribute to BPH, research has led to the use of 5-α reductase inhibitors to be used in the treatment of BPH [38]. Further basic and translational research is required to clarify the pathology of both diseases and the debate of an existing relationship between BPH and PCa is ongoing [39].

1.2.4 Toll like receptors in chronic inflammation

TLRs are transmembrane receptors and essential part of host's innate immune system [40]. They are generally expressed on immune cells, however they are also expressed on epithelial cells which come in to direct contact with and respond to pathogens [41, 42]. TLRs respond to two types of stimuli: exogenous and endogenous. Pathogen associated molecular patterns (PAMPs), exogenous stimuli of TLRs, are conserved molecular products derived from pathogens that include bacteria, fungi and viruses [43]. Danger associated molecular patterns (DAMPs) are endogenous molecules released from injured or dying cells such as CpG nucleotide and dsDNA [44]. Evidence provided in the literature suggests that endogenous DAMPs bind to the TLRs in the manner similar to PAMPs and activate the downstream signalling pathways leading to activation of inflammatory pathways [45]. Under normal circumstances inflammation is tightly controlled to restrict cell proliferation until infections are resolved and tissue repair is completed. In contrast, chronic inflammation establishes an environment rich in inflammatory cells, which produce growth and survival factors. This micro-environment provides a platform for surrounding cells with already sustained DNA damage or mutations, to proliferate and potentially form a tumour [46]**.** The function of TLRs is not restricted to the initiation of innate and adaptive immune reactions. Aberrant activation of TLR pathways has been implicated in various chronic and autoimmune diseases affecting the gastrointestinal tract, the central nervous system, kidneys, skin, lungs and joints. Their involvement has also been reported in non-infectious pathologies such as ischemic or traumatic injury and autoimmunity [47-51]. Evidence that intracellular proteins or products of protein cleavage can act as endogenous ligands for TLRs supported the hypothesis that TLRs are not only of importance in mediating a response to infections but to stress, damage and death of cells in general [52-54]. HSP60 was the first protein shown to act as ligand of TLR4 [55-57]; later, high mobility group protein (HMGB1) was observed to activate TLR2 and TLR4 [58]. The endogenous mRNA, ssRNA, and IgG/chromatin complexes are known to activate TLR3, TLR7, TLR8 and TLR9 respectively [45].

Targeting individual TLRs might provide a more specific target for the treatment of inflammatory diseases. Since the variety of TLR ligands released in one particular disease may be limited, specified targeting the TLRs involved would allow the function of other TLRs to remain intact. Therefore, the immune system would not be entirely affected and the side effects of the treatment reduced. Such a therapy may also disrupt the cycle of inflammation and release of danger signals. Further characterisation of the distinct TLR mechanism in specific tissues is required and consideration should be given to the dual function of TLRs in tissue protection as well as inflammation [59].

1.3.1 Prostate Cancer

PCa is the most prevalent non-cutaneous malignant tumours found in males and the third leading cause of cancer related deaths annually in Ireland following lung and large bowel cancer (Figure 1.3) [60]. The incidence of PCa is swiftly rising in parallel to the growing population of males over 50 worldwide [60, 61]. The incidence of PCa in Ireland is currently one of the highest in Europe and in 2012 the estimated incidence was approximately 1.5% higher than the U.K and Europe (Figure 1.2) [62].

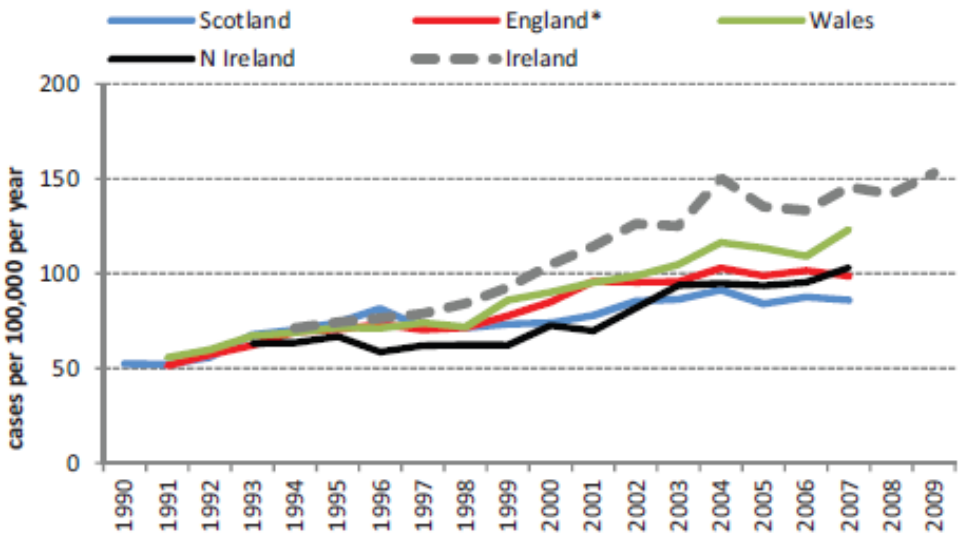


Figure 1.2 Prostate cancer incidence in Ireland The prevalence of PCa in Ireland is one of the highest in Europe. The incidence rate increased dramatically, by nearly 8% annually between 1994 and 2004 and then by 1.6% annually from 2004 to 2012. Prevalence is 1.5 times higher in Ireland than in the UK (National Cancer Registry Ireland 2014)

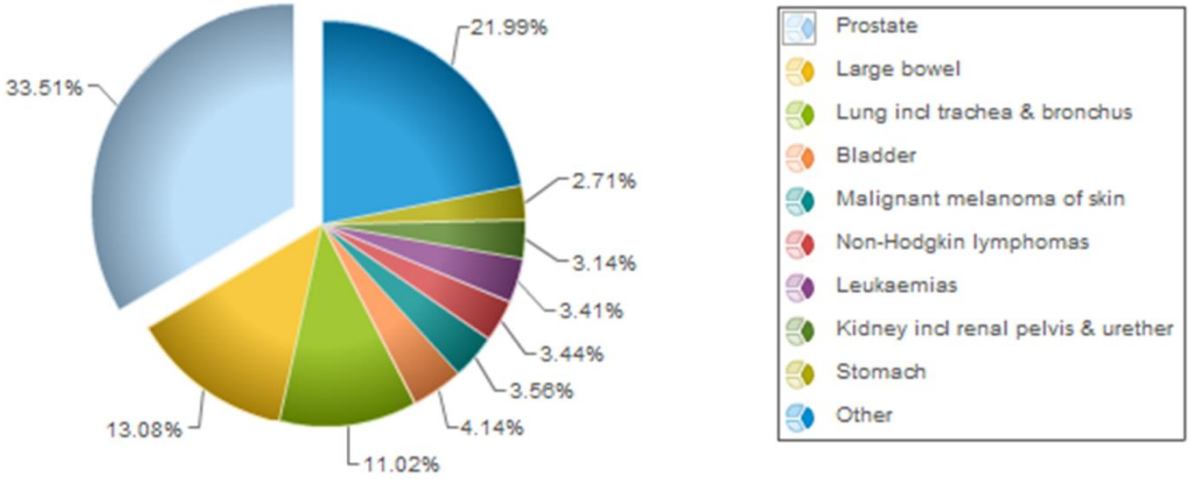


Figure 1.3 The frequency of most common invasive cancers diagnosed in men in Ireland 2012 (National Cancer Registry Ireland 2014)

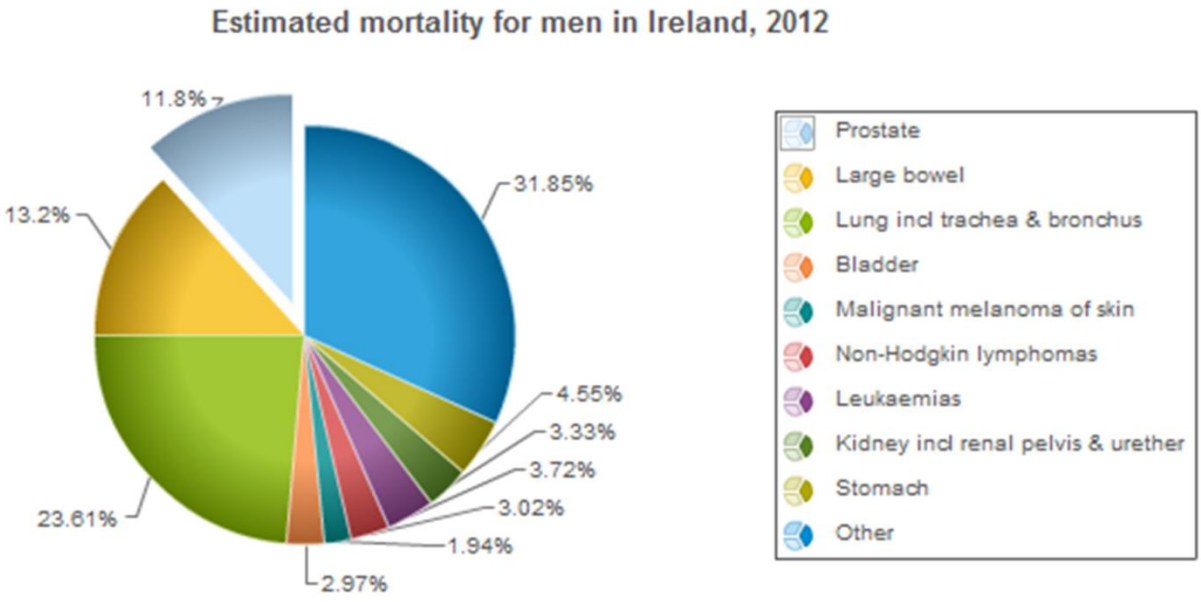


Figure 1.4 The frequency of cancer related mortality in men in Ireland 2012 Graph representation from the National Cancer Registry Ireland 2014

Both environmental and hereditary factors are contributing components to the development of PCa. Although age is considered one of the major risk factors in developing PCa, it is not the sole factor for the development of the disease as the incidence of PCa is low in men of South East Asian countries and surges following immigration to the West [63]. Chronic inflammation accounts for approximately 20% of all human cancers in adults with such inflammatory conditions stimulated by exposure to infection, environmental factors or possibly a combination of both [64, 65].

Similar to other cancer types, PCa arises through the accumulation of genetic and epigenetic changes, subsequently ensuing the inactivation of tumour suppressor genes and the activation of oncogenes, with emerging evidence indicating inflammation as a crucial component of PCa occurrence [66, 67]. Advancing age, family history, race and diet are among the key risk factors identified for the development of PCa. Another statistical finding is the significantly lower incidence and mortality rates resulting from PCa in southeast and east Asia in comparison to the United States and Western Europe. However, this discrepancy is eliminated upon emigration to the west, with Chinese and Japanese men acquiring a higher PCa risk within one generation. This reinforces the role of ones environment on the development of PCa [63, 68, 69]. Additionally, zonal preference of tumour formation in the prostate has been observed. The majority of cancer lesions are found in the peripheral zone of the gland, less occur in the transition zone almost none arise in the central zone of the gland (Table 1.1) [8]. PCa is diagnosed and graded following a typical sequence of PSA testing, digital rectal examination (DRE), transrectal ultrasound, biopsy and MRI. Detected tumours are graded by Gleeson score.

Statistical data suggests that PCa is generally a slowly progressing disease [70, 71]. Despite an increasing understanding of the contributors to PCa onset, unveiling the cellular and molecular mechanisms which enable localised PCa to invade and metastasise are a work in progress. Furthermore, there is no known specific time frame for an organ-confined primary tumour to develop into a highly invasive PCa [72]. It is not yet possible to diagnostically distinguish indolent localised prostate

tumours, which possess little metastatic potential, from aggressive localized prostate tumours with high metastatic potential. Nonetheless, in terms of relapse, biochemical recurrence (BCR) which is defined by elevated serum PSA levels following prostatectomy or radiation therapy for localised PCa, has been shown to predict metastatic progression (MP) and prostate cancer-specific mortality (PCSM) by a median of 8 years and 13 years, respectively [73, 74]. This suggests that it may take 8 to 13 years for a primary PCa to progress towards lethal metastatic disease. To date, there is no curative therapy available for metastatic PCa. It is now established that bone is the most common preferential site of PCa metastasis. An Analysis performed by Bubendorf et al., examined the reports from autopsies performed on men over forty with metastatic prostate cancer. The analyses reported that 90% of metastatic sites involved the bone, 46% the lung, 25% the liver, pleura 21% and adrenals 13% [75]. A study performed by Coleman *et al*. reported that in post-mortem examinations, approximately 70% of patients who have died from PCa complications show evidence of metastatic bone disease with the axial skeleton (skull, vertebra, ribs and collar bone, scapula, and proximal femur) being common locations for meatstasis [76, 77]. Since bone is the most common site for PCa metastasis it is crucial to understand the underlying mechanisms that facilitate this preferential migration of circulating PCa cells to the bone. There is now compelling evidence which suggests that disseminated tumour cells (DTCs) migrate to the bone marrow using mechanisms similar to those that are commonly exploited by homing hematopoietic stem cells (HSCs) during bone marrow transplantation [78] .

Table 1.1 Prevalence of prostate associated conditions in respective zonal regions

	Peripheral	Transition	Central
Acute Inflammation	*	*	
Chronic inflammation	**	**	*
Benign prostatic hyperplasia		****	*
High grade PIN	***	**	*
Carcinoma	***	**	

*Low **Low-Med *** Med-High **** High

1.3.2 Treatment strategies

Course of treatment is typically determined by cancer grade. Often risk assessment is implemented to aid in treatment strategy. A risk classification approach developed by D'Amico and colleagues is one of the most widely used assessment tools. This system uses PSA levels, Gleason score grade which is based on the microscopic appearance of the cancer cells, and tumour T stage which incorporates tumour size on rectal exam and ultrasound, as a mechanism of grouping men as low intermediate and high risk. Typically low risk patients have a PSA of less than or equal to 10, a Gleason score less than 6 and clinical stage T1-T2a. The intermediate risk category accounts for a PSA between 10 and 20, Gleason score of 7, or clinical stage of T2b. The high risk category includes patients with a PSA greater than 20, a Gleason score equal or larger than 8, or a clinical grade T2c-3a (Table 1.2) [79, 80]. Another calculated risk assessment referred to as a nomagram validation technique, pioneered by Kattan and colleagues, has also been implemented where by multiple risk variables that predict the likelihood of disease recurrence or progression are evaluated by a mathematical based calculation based assessment [81] (Figure 1.5).

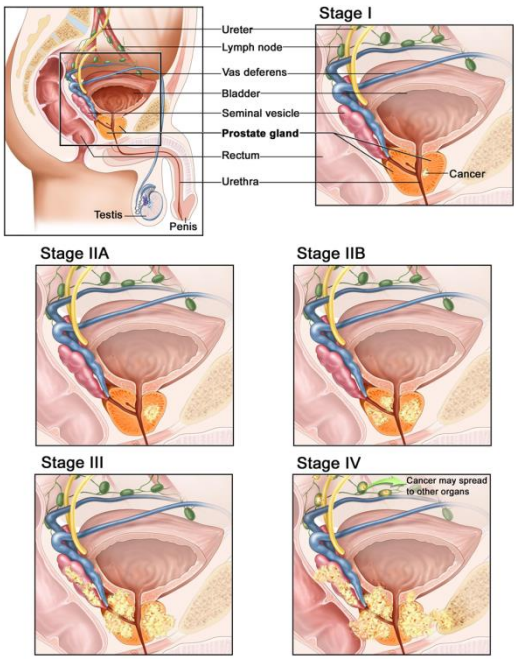


Figure 1.5 prostate cancer progresses from Stage I to Stage IV Cancer cells grow within the prostate, through the outer layer of the prostate into nearby tissue, and then to lymph nodes or other parts of the body. (https://www.cancer.gov/types/prostate/patient/prostate-treatment-pdq#section/_120)

Active surveillance (AS) is an important management strategy for men diagnosed with low-risk localised PCa. This involves regular checks to monitor progression or changes of the cancer so that early treatment intervention can be initiated if the nature of PCa changes in patients undergoing active surveillance [82].

Otherwise initial treatment consists of prostatectomy or radiation therapy in an effort to eliminate or eradicate the cancerous cells that are still confined within the prostate gland. Unfortunately, many patients are not cured by this therapy as their cancer either reoccurs or they are diagnosed after the cancer has spread [83]. To begin with, tumour growth in PCa is androgen dependent. Androgen ablation has been the main therapeutic intervention for the treatment of hormone sensitive PCas [84, 85]. Pharmacological compounds utilised include Gonadotropin releasing hormone (GnRN) superagonists (also referred to as lutenizing hormone (LH)), such as leuprolide or goserelin, which suppress the release of LH and inhibit the release of testosterone from the testis [86]. AR antagonists inhibit androgens produced by the adrenal glands from binding androgen receptors in the prostate. Maximal androgen blockade is achieved by combining and androgen receptor (AR) antagonist (anti-androgen) with a GnRH inhibitor or superagonist [87]. Unfortunately, this therapy eventually fails and PCa reoccurs in the form of androgen independent prostate cancer (AIPC).

In the event of AIPC development, subsequent second and third line treatments therapies are administered alongside continued androgen suppression (LHRH) [88]. Secondary hormonal manipulation strategies include the addition of or switch to an alternative anti-androgen such as flutamide, bicalutamide, nilutamide, enzalutamide. Another possible option is the addition of the adrenal/paracrine androgen synthesis inhibitors ketoconazole or abiraterone with predisone. Recently the results of an ongoing study presented at ASCO 2017 reported the benefits of abiraterone use in PCa treatment. An abiraterone comparison extension of the ongoing multistage STAMPEDE trial included patients with either high-risk locally advanced, metastatic prostate cancer that was newly diagnosed or relapsed

after radical prostatectomy or radiation therapy and who were starting ADT. Among the patients enrolled, metastatic disease was present in 52%, with 88% of those patients having bone metastases. In the abiraterone group, overall survival was improved by 37% compared with the standard-of-care group. Early introduction of abiraterone and prednisolone improved overall survival by 37% and failure-free survival by 71%, and reduced symptomatic skeletal events by 55%. The results show a clinically & statistically significant effect on overall survival & failure-free survival from adding abiraterone at start of ADT with a manageable increase in toxicity. The inclusion of abiratorone as a new standard of care is recommended by this group as a result of the outcome of the study. [89]. The use of an oestrogen, such as diethylstilbestrol (DES), could also be considered as a treatment option [90-95]. Enzalutamide has been documented to significantly decrease the risk of radiographic progression and delay the initiation of chemotherapy, while prolonging survival in men with metastatic PCa [92, 95]. Logithetis et al., reported significant benefits of abiraterone use in combination with prednisone in terms of pain relief in AIPC patients previously treated with docetaxel [93]. Ultimately AIPC is a lethal form of progressive and metastatic PCa, for which there are currently no available curative therapies.

Table 1.2 Tumour grading in prostate cancer

Risk	Grade	Stage	PSA (µg/L)	Gleeson Score
	T	Primary tumour		
	T0	No evidence of primary tumour		
Low Risk	T1	Clinically unapparent tumour not palpable or visible by imaging	< 10	≤ 6
Low Risk	T1a	Tumour incidental histologic finding in 5% or less of tissue resected	< 10	≤ 6
Low Risk	T1b	Tumour incidental histologic finding in greater than 5% of tissue resected	< 10	≤ 6
Low Risk	T1c	Tumour identified by needle biopsy (due to elevated PSA level); tumours found in 1 or both lobes by needle biopsy but not palpable or reliably visible by imaging	10	≤ 6
Low Risk	T2	Tumour confined within prostate	10	6
Low Risk	T2a	Tumour involving less than half a lobe	10	
Intermediate risk	T2b	Tumour involving 1 lobe or less	10-20	7
Intermediate risk	T2c	Tumour involving both lobes	10-20	7
High Risk	T3	Tumour extending through the prostatic capsule; no invasion into the prostatic apex or into, but not beyond, the prostatic capsule	10-20	8
High Risk	T3a	Extracapsular extension (unilateral or bilateral)	>20	8 - 10
Very High Risk	T3b	Tumour invading seminal vesicle(s)	>20	8 - 10
Very High Risk	T4	Tumour fixed or invading adjacent structures other than seminal vesicles (eg, bladder neck, external sphincter, rectum, levator muscles, pelvic wall)	>20	8 - 10

1.4.1 The molecular biology and signalling pathways involved in prostate cancer

Currently, histopathological analysis (Gleason scoring) and serum prostate specific antigen levels are key determinants of therapeutic decision making. However, the prospering field of molecular pathology has the potential to radically improve the way that PCa is diagnosed and managed. As the available data accumulates, it has become apparent that common epithelial malignancies comprise molecularly distinct components and the number of compounds claiming to target specific molecules or molecular pathways is expanding [96-98]. Research on the molecular biology of prostate cancer provides the potential to reveal aspects of contributing factors and, distinguish aggressive from slow growing cancers, thereby providing targets for treatment, particularly for locally advanced and metastatic disease. Advances in our understanding of PCa biology and tumour microenvironment are facilitating the development of new therapeutic strategies for advanced disease, and highlight the potential impact of molecular pathological assessment of PCa [99]. While traditionally PCa research focused on androgens, current approaches include the search for mutated genes, identification of recurrent chromosomal alterations and their associated potential tumour suppressor genes, determination of gene expression profiles, characterising tumour stages and subclasses and elucidation of the importance of epigenetic alterations. More recently, tumour suppressors and proto-oncogenes which are important in other cancers have been investigated. Molecular reshuffling of the genome, rather than changes in the sequence of base pairs appears to be the predominant genomic alteration in most prostate cancers [100]. Genomic gains, losses and structural rearrangements appear to occur in chain, with break points mapped to adjacent positions in the reference genome [101]. It is hoped that as the field of molecular pathology evolves further, the stratification of prostate tumours into distinct molecular entities, each with its own set of vulnerabilities, will be a feasible goal. Some

promising candidates for molecular classification include pathways involved in DNA repair, PI3K signalling and NF-κB.

1.4.2 Androgen receptor expression and signalling

As previously mentioned, a functional AR is essential for normal prostate development, secretory function and survival of luminal cells in the adult prostate [102-104]. Furthermore, the AR has been established as a critical driver of prostate carcinogenesis [105-107]. AR signalling has been implicated in the generation of genomic structural rearrangements that characterise many PCas [99]. Deregulated AR signalling is common during PCa development and CRPC progression due to overexpression of AR resulting from amplification, mutations, co-activator and co-repressor modifications, aberrant activation or post-translational modification, altered steroidogenesis, and generation of AR splice variants [108]. Mutations in the AR, although rare during initial stages of PCa, are common during CRPC. These mutations permit continued androgen-axis activation even in the presence of low levels of androgen in the prostate microenvironment [109, 110]. In addition, point mutations in AR also broaden the ligand pool to which AR responds. In advanced PCa, growth factors such as transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), insulin-like growth factor-1 (IGF-1), EGF, VEGF, fibroblast growth factor (FGF), interleukins (ILs), and other cytokines also act to promote synergistic activities of the androgen receptor [111, 112].

The collocation of an androgen responsive promoter such as *TMPRSS2*, next to a member of the ETS transcription factor family, typically *ERG* or *ETV1*, is the most common molecular change identified in PCa, and is typically observed in approximately 50% of cases [113]. Experimental evidence suggests that androgen signalling is central to the instigation of such rearrangements. Androgen-deprivation therapy (ADT) aims to reduce the circulating levels of androgens. Although ADT is initially effective for most patients, relapses are inevitable and are associated with re-activation of AR signalling and rising PSA levels. PCa relapse

ultimately progresses to a lethal form, known as castration-resistant prostate cancer (CRPC). A range of mechanisms have been implicated in the AR signalling of CRPC, including amplification of the *AR* locus, over-expression of its protein product, gain-of-function mutations and splice variants of the *AR* gene, altered expression of AR co regulators, ligand-independent activation of AR and *de novo* synthesis of androgens in prostate cancer cells [114]. Understanding the significance of the AR has driven the development of novel compounds capable of targeting multiple aspects of the AR signalling pathway. In the past decade, compounds have been developed to block the AR signalling axis more effectively than early anti-androgens, prolonging the life of patients with advanced-stage CRPC. Such second generation anti-androgens include Enzalutamide, an AR antagonist that blocks nuclear translocation and DNA binding of the AR with no agonistic effect on AR over expressing PCa models and Abiraterone, which is used to block the *de novo* synthesis of androgens in PCa cells that overcome androgen deprivation [115, 116].

1.4.3 p53 mutations in prostate cancer

The *TP53* gene is the most commonly mutated gene in human tumours and encodes a transcription factor that activates gene programmes required for cell-cycle arrest, DNA repair and apoptosis. It is found to be mutated in up to 40% of CRPCs [117] .Recent studies have explored the potential of targeting P53 with APR-246 (PRIMA-1MET), a novel agent that restores signalling to cells with an abnormal *p53* gene, which was recently reported as well tolerated in PCa patients in phase I and II trials [118]. Other approaches have included the development of agents, such as the Nutlins designed to disrupt the interaction between p53 and its endogenous inhibitor, the human murine double minute 2 (MDM2) protein [119]. While still at relatively early stages clinically, the pharmacological activation of functional p53 signalling in prostate cancer cells will likely require molecular profiling of p53 status in PCa patients.

1.4.4 DNA repair mechanisms

It is increasingly recognised that mutations in genes controlling DNA repair pathways, particularly homologous recombination repair and mismatch repair, may be relevant in many cancer types including prostate cancer [120]. In recent genomic sequencing efforts, the prevalence of somatic DNA repair gene mutations, primarily involving the *BRCA1/2* and *ATM* genes, is approximately 15–25% in CRPC biopsies [121]. PCa developing in patients with *BRCA1/2* mutations tend to have an associated aggressive clinical progression with increased nodal involvement, distant metastasis and poor overall PCa-specific survival [122]. Approximately half of these patients with somatic DNA repair irregularities tent to have germline defects in these same DNA repair genes (8–12% of the total) [123]. The presence of a germline or somatic mutation in a DNA repair gene may have prognostic and therapeutic implications. For example, inadequate response to ADT has been reported in these patients [124, 125]. Conversely, such patients may have a favourable response to alternative therapies including PARP inhibitors such as olaparib [126]. Intriguingly, patients with tumours that harbour DNA repair defects may exhibit higher sensitivity to platinum-containing chemotherapy, immune checkpoint inhibitors, radiopharmaceutical products or a novel approach involving high-dose testosterone treatment [127-130]. In the next few years, several on-going studies will aim to determine the predictive impact of DNA repair mutations in the context of these and other therapies [131].

1.4.5 BCl2 expression in prostate cancer

The anti-apoptotic protein BCL2 is overexpressed in approximately half of all prostate cancers, particularly in androgen-independent PCa [132]. BCL2 is not typically expressed in normal secretory epithelial cells of the prostate, however in transformed cells, from PIN to PCa, BCL2 is frequently expressed throughout the epithelium [132]. In normal cells, the cell growth signal deliberated by MYC expression is limited by the pro-apoptotic influence of protein mediated through

p14ARF1, p53, and BAX signalling. A combination of deregulated MYC and altered BCL2/BAX ratio may account for the unrestrained growth of prostate carcinoma cells [133, 134]. Growth factors are another contributing factor which not only increase cell proliferation, but also decrease apoptosis [134]. For instance, FGF-7, which is produced by prostatic mesenchymal cells, decreases apoptosis and prolongs cell survival in prostate carcinoma cells, most likely by increasing BCL2 expression [135]. Similarly, IGF-1 possesses both antiapoptotic and mitogenic properties in the prostate [136, 137]. IGF-1 has also been reported to instigate activation of the antiapoptotic PI3K/AKT pathway, stimulate the expression of BCL-like proteins and suppress BAX. Moreover, IGF-binding proteins, which also modulate apoptosis, have demonstrated modulated expression in PCa [136, 138].

1.4.6 PTEN/PI3K/Akt in prostate cancer

Alterations of the PI3K pathway can be found in as many as 70% of PCas, incidence being higher in metastatic tumours [139]. Phosphatase and tensin homologue (PTEN) protein, encoded by the *PTEN* gene is a key negative regulator of this pathway and tumour suppressor. PTEN inactivation is present in ±40% of PCas [140]. Function loss can occur due to genomic deletion, mutation, methylation, expression of pseudo genes or miRNA action, and appears to be essential for tumourigenesis in ETS-rearranged transgenic models [141]. Furthermore, genomic loss of PTEN is a strong predictor of disease aggressiveness and correlates with poor patient outcomes, regardless of Gleason grade [142].

Defects in PI3K pathway are also likely to affect the Ras/MEK/ERK pathway via amplified AKT activation. Similarly, interrelationship between AKT and IGF signalling have been reported in PCa cells. Upregulation of IGF, an upstream effector on AKT promotes PCa development in vivo [143, 144]. Myc, a downstream PI3K/AKT target also interacts with AKT to endorse PCa development and progression. PI3K/AKT pathway can function alongside other proteins such as MST1, acetate Kinase (Ack1) and Bmi1 to increase their oncogenic potential [145, 146]. PI3K/AKT can also increase the expression of metalloproteinase receptor,

MT1-MMP, which thereby favours PCa invasion and metastasis [147]. Taken together, this supports the importance of PI3K–AKT activity in PCa and suggests that pathway assessment and inhibition might be clinically useful. As changes leading to hyperactivity of the PI3K–AKT pathway can be identified in nearly all advanced-stage PCas, it is an enticing target for pharmacological intervention. Compounds that inhibit extensions of the PI3K-Akt pathway have entered clinical investigation, including PI3K and AKT inhibitors [148, 149]. Data from clinical trials using such compounds is accumulating and it is expected that, as in preclinical models, patients with identifiable PI3K–AKT hyperactivity will derive the greatest benefits. One anticipated problem is that there are numerous reciprocal feedback connections between the PI3K–AKT pathway and other pathways known to be important in PCa. Androgen signalling, for instance, is modulated by PI3K–AKT activity and the inhibition of this pathway could lead to deregulation of androgen signalling, with potentially deleterious consequences. Inhibition of multiple pathways using drug combinations may be necessary to avoid this issue [99]. It is hoped that as the field of molecular pathology evolves further, the stratification of individual prostate tumours into distinct molecular entities, will be an achievable goal with some promising candidates for molecular classification including the pathways involved in DNA repair and PI3K signalling.

1.4.7 NF-κB in prostate cancer

NF-κB is a protein complex that regulates expression of key genes required for innate and adaptive immunity, cell proliferation and survival, and lymphoid organ development. In prostate tumour cells, NF-κB is often found to be stimulated as a result of amplified levels of receptors such as tumour necrosis factor (TNF) which in turn dramatically increase IkB degradation [150]. NF-kB expression is increased in androgen independent prostate tumours at both mRNA and protein level due to elevated expression of IL-6, which occurs as a result of constitutive NF-κB activation, promoted by signal transduction via NF-κB inducing kinase (NIK) and IKK [151]. NF-κB also targets a transcription regulatory element of the prostate specific

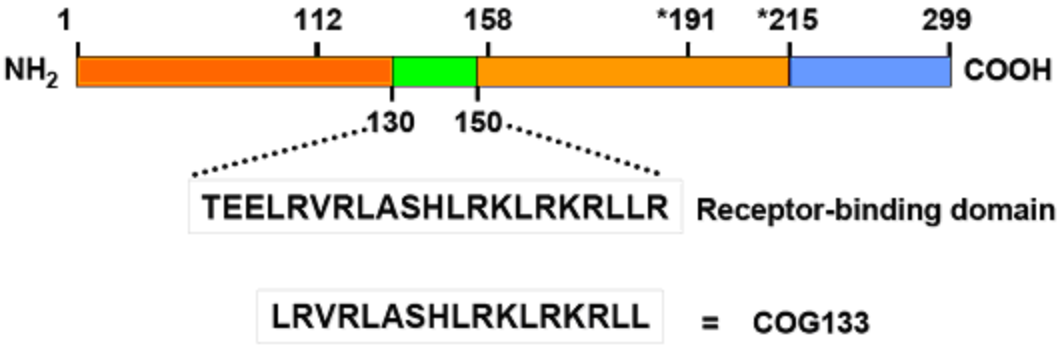
antigen PSA, a vital marker for development and progression of PCa [152, 153]. NF-kB signalling in PCa cells also correlates with cancer progression, chemoresistance, and PSA recurrence [154]. Reports also indicate that NF-κB activation contributes to soft-tissue or bone metastasis in PCa [155, 156]. TNF-a, a proinflammatory cytokine and prototypical NF-κB inducer along with its receptors TNFR1 and TNFR2 are found to be highly expressed in PCa. In Pca cells, amplified TNF-a expression has been correlated with increased proliferation and survival, angiogenesis, metastasis, and resistance to chemotherapeutic agents [157, 158]. Recent studies have also demonstrated the existence of cross-talk between NF-κB and AR signalling. NF-κB expression has been shown to augment with increased AR expression and activity in androgen-independent xenografts.

1.5.1 Apolipoprotein E peptide mimetics COG112 and OP449

Apolipoprotein (Apo) E is a multifunctional holoprotein whose role in cholesterol transport has been well documented. It is best recognised for its role in cholesterol and lipid metabolism. [159, 160]. In addition to cholesterol transport and anti-metastatic potential, ApoE has considerable immunomodulatory attributes and has been shown to modify both innate and adaptive immune responses [161]. Such qualities include the ability to suppress lymphocytic proliferation, neutrophil activation and Ig synthesis [162-164]. The impact ApoE has on immune response is of biological importance that has yet to be fully elucidated. In their endeavour to characterise the ApoE holoprotein and discover the region responsible for the immunomodulatory effects, Mike Vitek and his collaborators identified amino acid residues 133-149 of the ApoE protein to be crucial components of the anti-inflammatory effects [165](Figure 1.6).

Based on the findings that ApoE has anti-inflammatory properties, ONCOTIDE.inc., created a peptide from the amino acid residues 133–149 from the receptor binding domain of ApoE in an effort to identify the region of the ApoE holoprotein responsible for its immunomodulatory activity [165, 166]. They reported that incubation with the ApoE [133-149] peptide inhibited microglial activation in cell lines in a way that mirrored the anti-inflammatory properties of the ApoE holoprotein [166, 167]. Following on from the peptide development of COG133, the peptide was fused to a protein transduction domain derived from the Drosophila antennapedia protein in order to increase transmembrane permeability. This resulted in the formation of the ApoE peptide mimetic COG112 [168]. This molecular fusion was found to improve the bioactivity of COG133. There was significant clinical improvement and protection from both inflammation and demyelination injury in the spinal cord reported in the murine experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis [168]. Following the discovery that dimerization of ApoE peptides increased their biological activity when compared to their monomeric form, OP449 was manufactured [169]. This

compound was formed by creating a disulfide-linked dimer comprised of two covalently linked COG112 monomers. The two monomers were bridged using bismaleimido-ethane (BMOE) as a connecting component [169] (Figure 1.6) .



COG133	LRVLASHLRKLRKRLL	Monomer
COG112 (ANTP-COG133)	RQIKIWFQNRRMKWKK-C-LRVRLASHLRKLRKRLL (SEQ ID NO: 1)	Monomer
COG449 (BMOE-linked COG112)	COG112-C-BMOE-C-COG112 [BMOE is a bismalei-midoethane linker]	Dimer

Figure 1.6 Receptor binding domain sequence of ApoE ApoE-protein is 299 amino acids in length. Oncotide developed series of ApoE derived peptides starting with COG133, which spans residues from 133 to 149 adapted from http://www.cognosci.com/technology.html.

1.5.2 Mechanism of action of ApoE and ApoE mimetics

The ability of ApoE and its peptide mimetics to suppress the immune response, has been noted despite the mechanism not being completely validated [165]. The ApoE holoprotein inhibits IL-1β signalling in vascular smooth muscle cells resulting in a decline of inflammatory mediators nitric oxide (NO) and prostaglandin E2 (PGE2) being produced. This downregulation was facilitated through decreased phosphorylation and reduction of nuclear localized Nuclear factor-κappa B (NF-κB) leading to reduced production of inducible NO synthase [170, 171]. Sing et al., reported a decrease in the phosphorylation of IKB kinase (IKK) and reduced activation of NF-κB pathway in response to both ApoE and an ApoE peptide mimetic [172].

In an effort to further comprehend the role of ApoE and explore the mechanism by which these ApoE and ApoE-mimetic peptides impede inflammatory response and decrease phosphorylation of NF-κB, the ONCOTIDE.inc. manufacturers investigated if the suppression of TNF-a production following LPS stimulation was mediated by the ApoE receptors, the lipoprotein receptor-related proteins (LRP) and low density lipoprotein receptors (LDLR), and sought to identify other cellular proteins that bound to the apoE[133–149] peptide. They observed a significant dose-dependent suppression of TNF-α production by two ApoE-mimetic peptides, ApoE[133–149] and COG1410, following lipopolysaccharide (LPS) stimulation of either primary mouse peritoneal macrophages or BV2 microglial cells. This is of notable interest as both LRP and LDLR are expressed in peritoneal macrophages, but not in the BV2 cell line. This is indicative that the anti-inflammatory activity occurs via a receptor-independent mechanism [165].

Beyond the mechanism of uptake, Christensen et al., reported that ApoE and ApoE-mimetic peptides bind to the SET oncoprotein and that the ApoE holoprotein interacts with SET through its C-terminal region from amino acids 177–277 [165]. The protein SET is also known as inhibitor- 2 of protein phosphatase 2A (I2PP2A) and exists at elevated levels in the brains of those with Alzheimer's disease relative

to normal age-matched controls [173]. It has also been documented to be upregulated in leukemic cancer cells [174-176].

The activation of protein phosphatase 2A (PP2A) occurs as a consequence of the ApoE-mimetic peptide binding to SET. Following PP2A activation, subsequent dephosphorylation of PP2A targets occurs [165]. Several kinases that interact with PP2A have been identified such as the mitogen-activated protein kinase (MAPK) proteins p38, c-Jun N-terminal kinases (JNK) and extracellular Signal-regulated Kinase (ERK) [177-180]. PP2A is also negative regulator of protein kinase B (Akt) and its downstream targets which typically participate in in the activation of NF-KB transcription factor [181, 182]. (Figure 1.7)

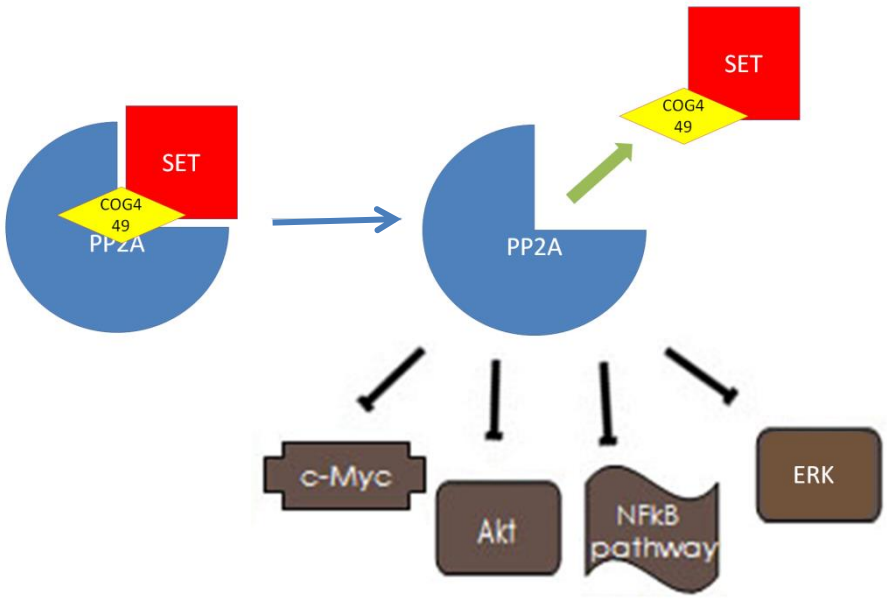


Figure 1.7 Inhibition of SET by ApoE peptide mimetic Schematic representation of the suggested mechanism by which ApoE peptides, manufactured by Oncotide, inhibit the SET protein. Inhibition of SET consequently negatively regulates cancer associated signalling. Figure adapted from http://www.cognosci.com/technology.html.

1.6.1 SET oncogene

The protein encoded by the oncogene SET (also known as I_2^{PP2A}, IGAAD and TAF-Iβ) is a 37kDa phosphoprotein and was first identified as a translocation breakpoint-encoded protein, fused to the CAN gene in acute undifferentiated leukaemia [183]. SET is widely expressed in human cells and is localised in the nucleus and cytoplasm with reported association to the endoplasmic reticulum. [184-186]. SET is a multifunctional protein which has been reported to play a role in control of cell cycle, gene transcription, apoptosis, cell migration and epigenetic regulation [187-192]. It has been shown to bind preferentially to Histone H3 and 4, exhibit histone chaperone activity, interact with various factors such as DNA binding proteins and proteases and regulate transcription, replication and apoptosis [193-201]. In addition to these, SET inhibits the activity of histone acetyltransferases, which suggests that it regulates the nuclear activity that arises from the chemical modification of core histones [192]. It has also been described as a potent inhibitor of PP2A, a protein phosphatase which plays a role in several cellular processes such as metabolism, proliferation, differentiation and DNA transcription and replication [202, 203]. In most cancer cells, including PCa, the tumour suppressor activity of PP2A is relatively low, which has been well correlated with the overexpression of SET [184, 202, 204]. It has been reported that that SET is required for the typical inflammatory responses to LPS. Christensen et al., demonstrated that transient knockdown of the SET protein significantly impedes the inflammatory response to LPS stimulation [165]. Such a role in inflammation opens the possibility of targeting SET for the downregulation of inflammatory response. Mukhopadhyay et al., assessed the expression of SET in PCa cells and normal prostate epithelial cells. They also investigated the effect of ceramide treatment on the function of SET. They implicated an association of SET expression with PCa development and reported a correlation between SET expression and the up regulation of c-Myc [205]. Their research denoted the role of SET inhibition of PP2A activity toward c-Myc and implicated SET as a therapeutic target for cancer therapy [206] (Figure 1.8).

1.6.2 PP2A

PP2A is a serine threonine phosphatase that accounts for the majority of phosphatase activity in mammalian cells. PP2A denotes a heterotrimeric complex comprised of a core dimer made up of a 36kDa catalytic C subunit and 65kDa scaffolding A subunit. This core dimer interacts with a variety of regulatory B subunits generating variations of the hetreotrimeric PP2A holoenzyme with several functions and substrate specificity [207, 208]. The literature has well documented that PP2A negatively regulates multiple signalling pathways, in particular growth and survival signalling pathways such as the Akt, β-catenin and c-myc pathways which are highly associated with cancer progression. [209-212]. PP2A has been widely described as a tumour suppressor, as inhibition of its activity or loss of function of some of its subunits are characteristic of neoplastic development [213]. Mutations of PP2A subunits have been detected in a variety of human malignancies [213, 214]. An accumulation of evidence supports the role of PP2A inhibition in the progression of PCa. Haung et al., demonstrated that the endogenous PP2A inhibitor CIP2A was frequently overexpressed in PCa and influences the sensitivity to cabazitaxel [215]. Previous studies have shown that that FTY720- dependent PP2A activation showed potent antitumor effects in PCa cells. As CIP2A was identified as a molecular target of FTY720, these results support previous findings in colorectal cancer where CIP2A down regulation by FTH720 plays a key role in the antitumor activity of this drug [216]. These findings could suggest a subset of PCa patients could benefit from treatment with PP2A activators. Numerous other mechanisms promoting PP2A inhibition have been described in PCa such as alterations affecting PP2A subunits or deregulation of the endogenous PP2A inhibitor SET [205, 216-219]. Evaluating the potential role of these alterations in modulating cabazitaxel response in PCa via PP2A inactivation remains crucial. Taken together, the data supports a role for PP2A in advanced PCa progression [220] (Figure 1.7).

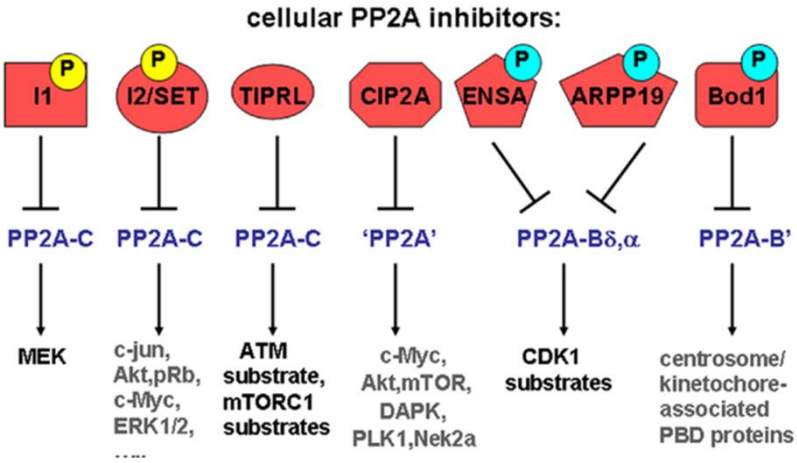


Figure 1.8 The endogenous inhibition of PP2A Schematic representation of known cellular PP2A inhibitors, highlighting their potential regulation by phosphorylation (yellow) or dependence on phosphorylation (blue), their holoenzyme specificity and the PP2A substrates they affect. Adapted from Haesen et al.,(2014) ***Front. Oncol.*** 4:**347**

1.6.3 Akt signalling

AKT/protein kinase B (PKB) is a central participant in an array of signalling cascades required for normal cellular physiology and has also been widely implicated in numerous disease states [221]. Disruption of these processes result in cellular distresses which are largely deemed hallmarks of cancer [222]. A significant mass of literature affirms the prevalent hyperactivation of Akt signalling observed in cancer. The Akt kinases are key players in signalling cascades that regulate cell proliferation and survival, cell size and response to nutrient availability, glucose metabolism, cell invasiveness, genome stability and angiogenesis [223]. Initiation of Akt signalling follows the activation of receptor protein tyrosine kinase (RPTK) [224, 225]. Stimulation of RPTK results in the production of PI(3,4,5)P3 and PI(3,4)P2 by Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) at the inner border of the cellular plasma membrane. Akt interacts with these phospholipids resulting in its translocation to the membrane where it

interacts with PI(3,4,5)P3 via its plekstrin homology (PH) domain [226]. The interaction between the Ph domain of Akt with the phosphoinositides is thought to provoke a conformational change in Akt resulting in exposure of its two main phosphorylation sites Thr308 and Ser473 [227]. Once activated, Akt controls fundamental cellular processes such as cell growth, cell cycle and cell survival by modulating a number of different downstream targets [228]. Some downstream targets affected by the activation status of Akt, in relation to cell cycle progression, include glycogen synthase kinase-3 (GSK-3), membrane translocation of the glucose transporter GLUT4, Cyclin-dependent kinase inhibitors, P21/Waf1/Cip1 and P27/Kip2 and mammalian target of rapamycin (mTOR), [229-235].

Cross et al., demonstrated that Akt inhibited GSK3 activity by direct phosphorylation of an N-terminal regulatory serine residue downstream of insulin activated PI3k [229]. Akt inhibits the anti-proliferative effects of P21/Waf1/Cip1 and P27/Kip2, by phosphorylating and retaining them in the cytoplasm [232-234, 236]. Protein synthesis and translational regulation can also be promoted by Akt signalling, for instance, Akt activates mTOR by phosphorylation [237]. Overexpression of Akt has an anti-apoptotic effect in many cell types, resulting in a resistance to or delay of cell death [238]. Akt regulates cellular survival through phosphorylation of downstream substrates that directly or indirectly control the apoptotic machinery. Akt activation also promotes prosurvival signalling with the kinase responsible for inhibiting pro-apoptotic factors such as BAD and procaspase 9 as well as Forkhead family of transcription factors that induce the expression of proapoptotic factors such as Fas ligand. [238, 239]. Akt is also capable of phosphorylating and activating IkappaB kinase α (IKKα), which, in turn, phosphorylates IkappaB (IκB), targeting it for degradation [240, 241]. This leads to the nuclear translocation and activation of NF-κB, followed by the transcription of NF-κB dependent prosurvival genes, including Bcl-XL, caspase inhibitors and c-Myc [242, 243].

1.7.1 Innate immunity

Tumours are not made of isolated masses of uncontrollably proliferating epithelial cells. Their composition relies on intricate signal exchanges between cancer epithelial cells and the surrounding stroma within the tumour microenvironment [244]. Several lines of evidence support the concept that tumour stromal cells influence growth, survival, and invasiveness of cancer cells and broadly contribute to the tumour microenvironment together with immune cells [245-248]. The stromal constitution in PCa is comprised of multiple non-malignant cells. These include fibroblasts, myofibroblasts, endothelial cells, macrophages, neutrophils, mast cells and natural killer cells, growth factors, chemokines, cytokines, extracellular matrices (ECMs), and matrix-degrading enzymes [249, 250]. They also include cells associated with the adaptive immune response such as T and B lymphocytes [251, 252]. Crosstalk between the epithelial and stromal compartments promotes tumour progression to enhance invasion and the release soluble growth factors necessary for castrate-resistant growth, and stimulating angiogenesis [253, 254].

The first line of immune defence is based on the detection of pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPS) that conjure a toxic and inflammatory response [255, 256]. PAMPs are derived from microorganisms and recognised by pattern recognition receptor (PRR)-bearing cells of the innate immune system as well as many epithelial cells. Recognition of PAMPS is a necessary/critical function of the innate immune system. PAMPS are molecular structures which include glycoproteins, lipopolysaccharides (LPS), proteoglycans and nucleic acid structures that are common to many different microrganisms and essential to the survival or infectivity of the microbe [257]. Alternatively, DAMPs are cell-derived and initiate and propagate immune response to trauma, ischemia, and tissue damage, regardless of the presence of pathogenic infection. Most PAMPs and DAMPs bind specific receptors, namely PRRs. These include toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs), AIM2-like receptors, and

the receptor for advanced glycation endproducts (RAGE) to promote immune response [43].

The innate immune system constitutes the first line of host defence during infection and therefore plays a crucial role in the early recognition and subsequent triggering of a pro-inflammatory response to invading pathogens [255]. Upon ligand recognition, TLRs and RLRs prompt proinflammatory and antimicrobial responses by activating their signalling cascade pathways which in turn leads to the activation of MAP kinases and IRFs and ultimately of NF-κB thus regulating the transcription of genes encoding inflammatory cytokines and transcription factors [258, 259]. PRR-induced signal transduction pathways result in the activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors [260]. Taken together, these orchestrate the early host response to infection and at the same time represent an important link to the adaptive immune response. The adaptive immune system is responsible for the elimination of pathogens in the late phase of infection and in the generation of immunological memory [261] (Figure 1.9).

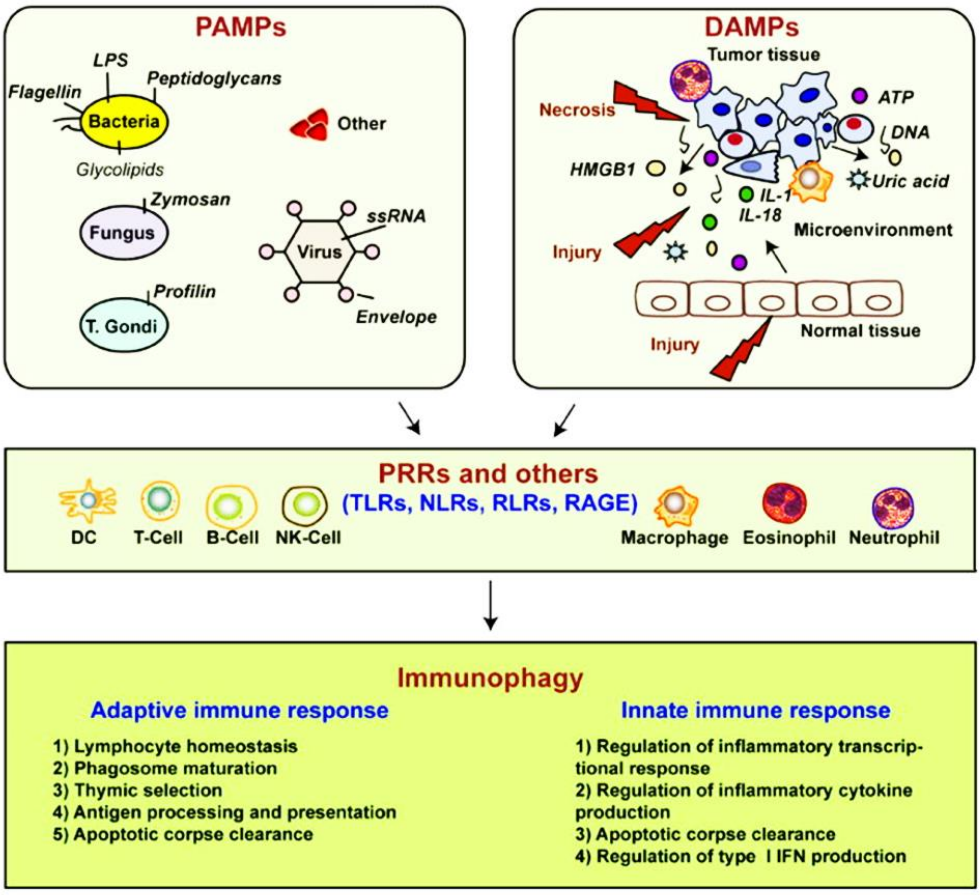


Figure 1.9 Activation of pathogen recognition receptors by PAMPS and DAMPS The binding of PAMPs and DAMPs with PRRs instigate the signal transduction of TLRs, RLRs, NLRs and RAGE. This culminates in the initiation of innate immune response. Immunological memory allows for activation of the adaptive immune response. Adapted from Tang D. et al.,(2012) Immunol Rev 249(1): 158-175 [43]

1.7.2 TLRs

Previous studies and recent advances in cancer immunobiology have emphasised TLRs as key components involved in tumour growth and progression. The normal regulation of TLRs is disrupted in cancerous epithelial cells in comparison to tissue derived from healthy individuals indicating that mutations of TLR genes or modulation of TLR signalling could be appropriate markers for diagnosis or targets for treatment strategies [262, 263]. To date, 10 TLRs have been identified in

humans. TLRs 1, 2, 4, 5and 6 are expressed on the cell surface while TLR3, 7, 8 and 9 are found exclusively within endosomes [264]. Each recognize distinct PAMPs derived from various microbial pathogens, including viruses, bacteria, fungi, and protozoa [260] TLR 10 is an orphan ligand that is highly expressed in the spleen and B cells [265, 266] (Table 1.3). The TLRs are the most comprehensively studied family of PRRs and are highly significant in the instigation of antiviral response to infection. The TLRs account for a notable proportion and best characterised of the PRRs implicated in detecting parasites, fungi, bacteria and viruses [260].

The family of TLRs is the major and most extensively studied class of PRRs. Structurally, TLRs are integral glycoproteins typically differentiated by a leucine-rich repeat (LRR) motif within their ligand binding domain and a cytoplasmic signalling Toll/Interleukin-1 (IL-1) receptor homology (TIR) domain [267]. The binding of the appropriate ligand to each respective TLR by a PAMP-TLR interaction subsequently triggers an intracellular signal transduction process. [260]. Within the TLR family, TLR1, TLR2, TLR4, and TLR6 are identified by lipids, whereas TLR3, TLR7, TLR8, and TLR9 are stimulated by nucleic acids [260]. Furthermore, it seems that TLRs can differentiate between PAMPs either via direct interaction or by means of an intermediate PAMP-binding molecule. Accordingly, TLR1/2, TLR3, and TLR9 directly bind to triacetylated lipopeptides, double-stranded RNA (dsRNA), and CpG DNA, respectively [268-270], while TLR4 binds to lipopolysaccharide (LPS) through the accessory molecule MD2 [271]. Intriguingly, some TLRs are also capable of recognising various structurally and biochemically distinct ligands, for instance, TLR4 can be stimulated by binding to LPS, the fusion protein of respiratory syncytial virus (RSV), and cellular heat shock proteins (HSPs) [260]. This may be due to the ability of different regions of the extracellular portion of TLRs to bind their related ligands or possibly due to the involvement of different PAMP-binding molecules, such as MD2 [268, 271]. Further discrimination between various PAMPs is achieved through the formation of heterodimers between TLR2 and either TLR1 or TLR6 [272].

The most significant of cell types expressing TLRs are antigen presenting cells (APCs), including macrophages, dendritic cells (DCs), and B lymphocytes [261]. TLRs have been identified in many cell types, either expressed constitutively or in an inducible manner in the course of infection [261, 273, 274]. Despite confirmation of various TLR expressions in different cell types and anatomical tissue locations, and their role in inflammatory response, much of these functional aspects remain poorly characterized. A fundamental property of the PAMP-TLR signalling complex is that a given pathogen can activate a number of different TLRs by assorted PAMPs, and similarly, numerous structurally unrelated pathogens can trigger any given TLR. The outcome of TLR engagement with the corresponding PAMP is the instigating of downstream signalling pathways. This signalling cascade ultimately results in the generation of an antimicrobial pro-inflammatory response.

Table 1.3 Human TLR receptors and their ligands

TLR Receptor	Location	Ligand PAMP	Ligand DAMP	Ligand origin
TLR 1	Cell Surface/ plasma membrane	Triacyl lipoproteins		Bacteria, mycoplasma
TLR 2	Cell Surface/ plasma membrane	Peptidoglycan Lipoprotein Lipoteichoic acid Zymosan	Heat shock proteins, HMGB1 (high mobility group box 1	Bacteria, virus, parasites
TLR 3	Endolysosome	Viral dsRNA	Self dsRNA	Virus
TLR 4	Cell Surface/ plasma membrane	Heat shock proteins, Lipopolysaccharides, RSV fusion protein, MMTV (mouse mammary tumour virus) envelope proteins, paclitaxel	Heat shock proteins, Fibrinogen, Heperan sulphate, Fibronectin, Hyaluronic acid, HMGB1	Bacteria, virus
TLR 5	Cell Surface/ plasma membrane	Flagellin		Bacteria
TLR 6	Cell Surface/ plasma membrane	Lipoteichoic acid Triacyl lipoproteins. Zymosan		Bacteria, virus
TLR 7	Endolysosome	Viral ssRNA	Self ssRNA	Virus, bacteria
TLR 8	Endolysosome	Viral ssRNA	Self ssRNA	Virus, bacteria
TLR 9	Endolysosome	Bacterial and viral DNA	Self DNA	Virus, bacteria, protazoa
TLR 10	Endolysosome	Unknown	unknown	unknown

1.7.3 RLRs

Retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) were identified as cytosolic sensors of viral RNA which could be triggered independently of TLRs. They were quickly demonstrated to play an integral role in early anti-viral response to viruses [275]. The RLR family are composed of Rig-I, Melanoma differentiation-associated antigen 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2)[276-278]. RLRs are reportedly expressed at low concentrations in the

resting cell and these concentrations are greatly increased upon stimulation [275, 278, 279].

RIG-I and MDA5 are IFN inducible RNA helicases that play a crucial role in the detection of cytoplasmic RNA [275, 278]. These RNA helicases are comprised of an N-terminal caspase recruitment domain (CARD) and a central helicase domain with ATPase activity which is obligatory for RNA-activated signalling [275]. The engagement of dsRNA or 5'-triphosphate RNA with the C-terminal domains of RLRs initiates a signalling cascade [280, 281]. This occurs via a CARD-CARD interaction between the helicase and the adaptor protein IFN-β promoter stimulator 1 (IPS-1) [282-285]. The outcome of this cascade ultimately results in an antiviral response mediated by type I IFN production [275, 286]. LGP2, a third member of the RLR family, lacks the CARD domain and therefore does not induce signalling. Instead, LGP2 inhibits RIG-I signalling [287].

Despite RIG-I and MDA5 functioning by similar mechanisms, the literature suggests variation in their roles. RIG-I is thought to be required for the response to paramyxo-viruses and influenza virus, while MDA5 is considered critical for the response to picornavirus and norovirus [288, 289]. As RIG-I and MDA5 recognize short and long dsRNAs, respectively it is possible this variation may be due to length-dependent binding of dsRNA by these two RLRs [290]. RIG-I is also capable of detecting 5'-triphosphate RNA [288, 291]. The IFN-inducible dsRNA-activated protein kinase (PKR), an enzyme that can moderate the antiviral and antiproliferative activities of IFN, can also recognise viral RNA [292-294].

The current understanding is that the major contribution to dsRNA-activated responses is mediated by RLRs, with recent data suggesting that PKR may be able to amplify RLR signalling [295, 296]. This elucidates to cross talk between these different cellular dsRNA-sensing systems encompassed in antiviral defence. [297, 298].

1.7.4 TLRs in cancer

Under normal circumstances inflammation is tightly regulated to limit cell proliferation until infections are resolved and tissue repair is complete. In contrast, chronic inflammation establishes an environment rich in inflammatory cells, which produce growth and survival factors. This micro-environment provides a platform for surrounding cells which have already sustained DNA damage or mutations, to proliferate and form a tumour [46]. Many tumours do contain increased amounts of immune cells and show all the characteristics of ongoing inflammation [299]. In previous years, specific inflammatory conditions have been associated with particular cancers. For instance, chronic ulcerative colitis and Crohns disease show a strong association with colon cancer, gastric cancer is linked to Helicobacter pylori infections, schistosomiasis and bladder cancer as well as chronic cholecystitis and gall bladder cancer [18, 65, 300-302]. On the other hand, constant intake of anti-inflammatory drugs can reduce the risk of developing certain types of cancer [300, 303]. Consequential to their capacity to induce a potent inflammatory response, TLRs have become a chief target in driving tumourigenesis. Several reports have described a correlation between certain TLR sequence variants and the risk of developing specific cancers, in particular TLR4 and PCa [299, 304-312]. Similarly, in vivo, it has been reported that Listeria monocytogenes infection promoted tumour growth in a TLR2 dependent manner [313]. The major mechanisms driving TLR-induced tumourigenesis, are suggested to be the induction of immunosuppressive cytokines, which subsequently lead to evasion of the immune response and resistance to apoptosis. In addition, the miRNAs miR-155 and miR-146 have been implicated in B-cell lymphoma, breast and lung cancers, pancreatic adenocarcinomas, prostate, stomach and papillary thyroid carcinomas [314-320]. Recent reports, have shown that miR-155 as well as miR-146 are up-regulated in response to TLR2, TLR3, TLR4, TLR5 and TLR9 stimulation indicating an alternative mechanism of how TLRs can influence tumourigenesis [321, 322].

1.7.5 TLR 4 in Prostate cancer

Recruitment of one or several adaptor molecules to a given TLR is followed by activation of downstream signal transduction pathways via phosphorylation, ubiquitination or protein-protein interactions ultimately culminating in the activation of transcription factors that regulate the expression of genes involved in inflammation and antimicrobial host defence [260]. Three major signalling pathways responsible for mediating TLR induced responses include NF-κB , Mitogen activated protein kinases (MAPKs) and IFN regulatory factors (IRFs) [259, 260, 323]. Whilst IRFs are essential for stimulation of IFN production, NF-κB and MAPKs play central roles in the induction of a pro-inflammatory response [211, 323]. In the event of TLR4 activation, four different adaptor molecules are recruited for signal transduction, namely MyD88, Mal, TRIF and TRAM; with numerous associated adaptors reflecting the intricacy of signalling downstream of this receptor [324-328]. TLR induced signalling can be largely divided into MyD88 dependent and MyD88 independent TRIF dependent pathways, both of which are capable of activating NF-κB , although each activates additional signalling components MAPKs and IRFs [42].

In response to TLR4 stimulation by an appropriate PAMP, MyD88 associates with the cytoplasmic part of the receptor and subsequently recruits members of the IL-1 receptor associated kinase (IRAK) family [329, 330]. Further downstream the associated IRAK kinase recruits TRAF6, contributing to the catalysing the formation of polyubiquitin chains of TRAF6 and substrates including transforming growth activated protein kinase 1 (TAK1) and the IKB kinase (IKK) subunit NF-κB essential modifier (NEMO) [331]. A central step in the downstream signalling events is the recruitment of TAK1 binding protein2 (TAB2) and TAB3 to ubiquitinated TRAF6 which brings TAK1 into proximity to the signalling complex, leading to its activation [332]. TAK1 stimulates two distinct pathways involving the IKK complex and the MAPK pathway respectively [333]. In the first pathway, TAK1 mediated activation of the IK complex results in site specific phosphorylation of the inhibitory IkB protein IKB. Being the point of convergence for multiple NF-κB inducible stimuli, IKK

represents an essential component in many inflammatory signalling pathways [334].

TRIF dependent activation of NF-κB occurs through binding of TRAF6 to TRIF and subsequent ubiquitination dependent recruitment and activation of TAK1 [335]. In order to obtain robust NF-κB activation, a second molecule receptor interacting protein 1 (RIP1), involved in TNF-receptor mediated NF-κB activation, is also recruited to TRIF [336]. RIP1 is poly-ubiquitinated to form a complex with TRAF6 and these two molecules appear to co-operate in facilitating TAK1 activation, resulting in IKK mediated activation of NF-KB as well as activation of the MAPK pathway [337]. Molecular signalling mechanisms within the TRIF –dependent pathway illustrate how selective binding of different molecules ie., TRAF3 or TRAF6 result in the recruitment and downstream activation of TBK1-IRF versus TAK1-IKK-NF-κB respectively [42] (Figure 1.10).

In PCa, the first evidence of a possible involvement of TLRs from epidemiological studies. A previous immunohistochemistry and qRT-PCR-based screen on 133 patients with prostate adenocarcinoma demonstrated an association between high expression of TLR3, 4 and 9 and the recurrence of PCa [338]. In vitro studies showed that rat prostate adenocarcinoma derived MAT-LU cells constitutively express TLR4 and respond to the TLR4 ligand LPS through the activation of ERK1/2 and NF-kB, thereby up-regulating numerous chemokines such as MCP1, MIP1a, IP10, RANTES and IL-8 [339]. Subsequently, Andreani and co-workers demonstrated that LPS stimulation of MAT-LU cells *in vitro* before immunization inhibited tumour growth in syngeneic rats but not in athymic nude mice. From this they concluded that TLR4 stimulation can elicit the T lymphocyte-mediated immune response against the tumour rather than directly acting on PCa cells [340]. Down regulation of the TLR4 receptor in PC3 was reported to induce a dramatic reduction of tumour cell viability and invasion [341]. Intriguingly, in accordance with a pro-tumour role of TLR4 in PCa, it was also reported that peroxiredoxin-1 is over-expressed in human PCa and that it regulates prostate tumour growth in a murine cancer experimental model through TLR4-dependent induction of prostate tumour

vasculature [342]. Considering the reported association between cancer and TLR4, targeting the receptor may be as suitable tactic for PCa treatment.

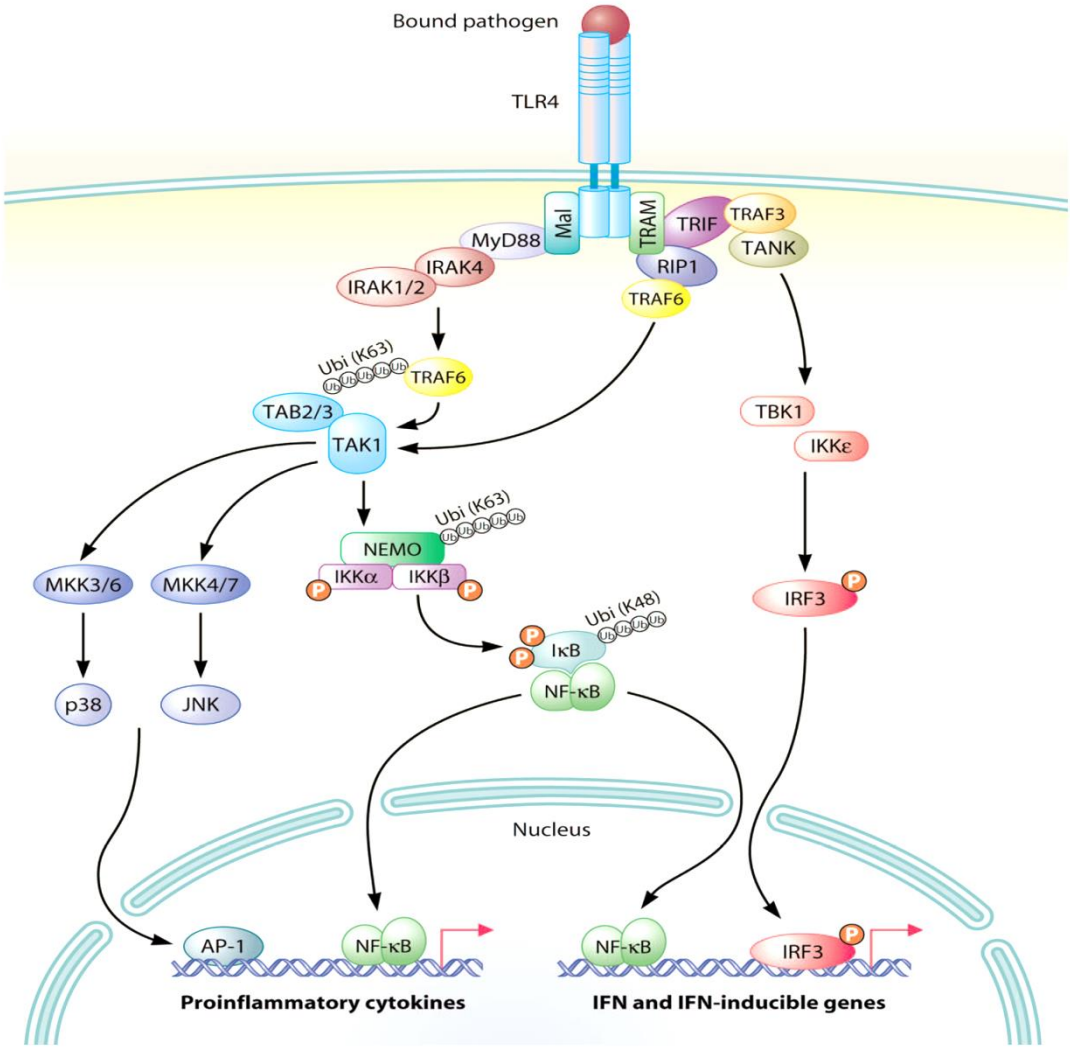


Figure 1.10 Principles in TLR signalling. Stimulation of TLR4 can activate both the MyD88 dependent and MyD88 independent, TRIF dependent pathways. The MyD88 dependant pathway is responsible for early phase NF-κB and MAPK activation which control the induction of pro-inflammatory cytokines. The MyD88 independent TRIF dependant pathways activate IRF3, which is required for the induction of IFNβ and IFN inducible genes. This pathway also mediates late phase NF-κB as well as MAPK activation thereby contributing to inflammatory responses. *Mogensen T. (2009) Clin. Microbiol. Rev 22(2):240-73*

1.7.6 TLR 3 and RIG-I in Prostate cancer

TLRs expressed at the cell surface or luminal aspect of endo-lysomal membranes, they do not seem capable of recognising intracellular cytosolic pathogens and their derivatives such as viral ssRNA, dsRNA and DNA as well as components of internalised or intracellular bacteria, the intracellular components of the innate immune response are of interest [343]. Cytosolic PRRs play a vital role here in response to pathogen recognition and inflammatory response. These include the intracellular TLRs and the TLR independent recognition receptors, namely members of the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide – binding oligomerization domain (NOD)-like receptors (NLRs). For this reason, the response of intra cellular PRRs TLR3 and the RIG-I receptor were selected as representatives of cytosolic PRRs in this study. These receptors play a critical role in recognising cytoplasmic RNA in innate immune response. Stimulation of TLR3 and RIG-I induces multiple inflammatory pathways including IRF and NF-κB.

The signalling cascade of TLR3 and RIG-I largely overlap. Viral activation of the TLR3 and RIG-I-mediated pathways can be simulated by the addition or transfection of synthetic dsRNA (poly(I:C)) into cells. The signalling pathways activated by TLR3 and RIG-I differ in their initial steps, but they converge at the end and activate the proteins kinases IKKαβ, TBK1 and IKKε. IKKαβ activates NF-κB by phosphorylating its inhibitor IκB and causing its degradation. TBK1 and IKKε, however, activate IRF-3 by directly phosphorylating it and causing its dimerization and nuclear translocation [344]. In RIG-I, IPS-1 is a point of divergence of two different signalling pathways involving either IRFs or NF-κB [345]. The IPS-1-TRADD complex recruit TRAF3 and the adapter protein TANK which subsequently activate TBK1 and IKKε, leading to IRF phosphorylation [275, 286, 288, 345-347]. Alternatively, The IPS-1-TRADD complex propagates the signal to NF-κB [348] .Viral activation of the TLR3- and RIG-I-mediated pathways can be simulated by the addition or transfection of synthetic dsRNA (poly(I:C)) into cells. Following the stimulation of TLR3 by dsRNA, the adapter protein TRIF is recruited. TRIF is responsible for initiating signalling through the TRAF3-TANK-TBK-IRF3 pathway [349]. RIG-I utilises

this route also. In these signalling cascades, NF-κB activation occurs through the binding of TRAF6 in the case of TLR3 and following TAK1 recruitment for RIG-I [335](Figure 1.11).

The literature has reported anti-tumour potential of TLR3 and RIG-I in cancer [263, 350]. Gambara et al., demonstrated that by stimulating TLR3 with Poly (I:C), proliferation was inhibited and induced apoptosis in LNCaP cells to a greater degree than PC3 PCa cells [263, 351, 352]. Subsequently, Chin et al., reported that the suppression of tumour growth of TRAMP-C2 murine PCa cells transplanted in syngeneic mice induced by poly(I: C) was dependent on T-lymphocytes and NK cell recruitment within the microenvironment [353]. Alternatively, high expression of TLR3 was shown to be associated with relapse in PCa [338]. Research exploring the effect of Sendai virus have suggested an induction of apoptosis in PC3 and DU145 PCa cells but not of non-transformed prostate epithelial PNT1 and PNT2 cells [350]. Fragments of viral RNA transported by Sendai virus are recognized by RIG-I and mitochondrial antiviral signalling (MAVS) [350, 354]. Additionally, Besh and colleagues reported that synthetic RNA induces type I IFN-independent apoptosis in human melanoma cells via the activation of RIG-I and melanoma-differentiation-associated gene 5 (MDA5) [355]. Taken together the literature supports the prospect that targeting TLR3, RIG-I and MDA5 in PCa and that further investigation is required into their role in PCa progression.

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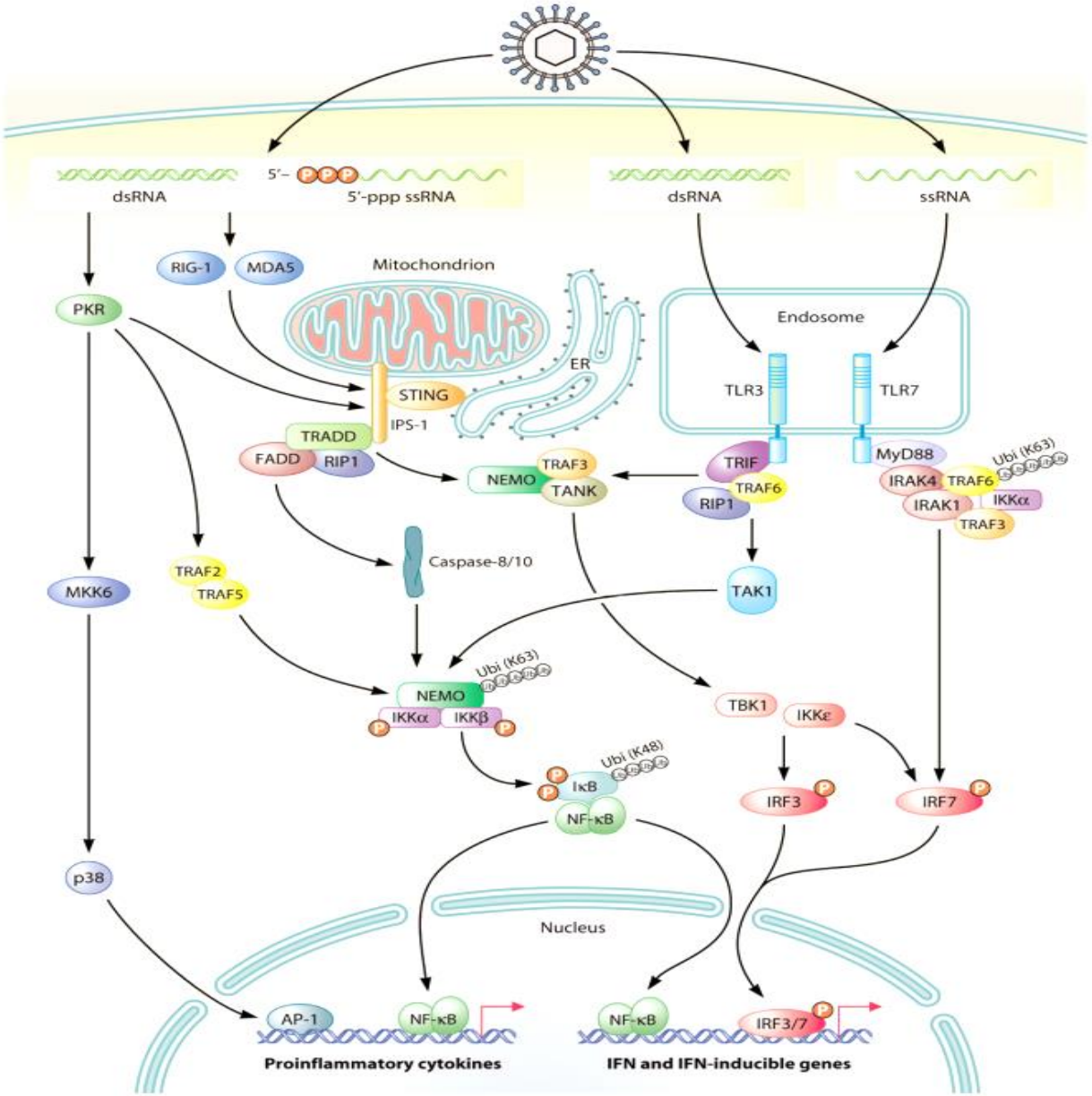


Figure 1.11 Intracellular RNA recognition and Signalling. Cytoplasmic RNA helicases RIG-I and MDA5 are stimulated by Cystolic dsRNA or 5'-triphosphate ssRNA, thereby mediating interaction with the adaptor IPS-1, localised to mitochondria. This triggers the initiation of NF-κB and IRF3 signalling via IKK and TBK/IKKε respectively. Endosomal TLR3 recognises dsRNA. Activated TLR signals through the NF-κB and IRF3 pathways. *Mogensen T. (2009) Clin. Microbiol. Rev 22(2):240-73*

1.8.1 NF-κB

The Rel/NF-κB family is a group of transcription factors that are activated by an extensive array of stimuli, such as DNA damage, cytokines and free radicals [356, 357]. NF-κB is an inducible transcription factor widely involved in immune response, carcinogenesis, and chemoresistance [358-361]. It is now established that NF-κB functions as a link between chronic inflammation and cancer [360, 362, 363]. Five proteins have been classified as members of the transcription factor family. The have been identified as p65 (RelA), RelB, c-Rel, p105/50 and p100/52. p105/50 and p100/52 are distinguished from the others as they are first synthesized as pro-forms (p105 and p100) before being proteolytically processed to p50 and p52 respectively [364]. All five members form homo- or heterodimers. [365]. RelA/p65 is responsible for most of NF-kB's transcriptional activity due to its abundancy and robust transactivation domain. NF-κB activity is tightly regulated in the cell.

In its inactive form, NF-κB is retained in the cytoplasm by dephosphorylated members of the IkB inhibitory protein family, including IkB-a and IkB-b [357] The interaction between NF-κB and IkBa prevents the nuclear localisation of NF-κB [356, 357]. Potent activators, such as TNF-a, IL-1, or LPS, induce rapid degradation of the IkBs (specifically IkBa) within minutes [366-368]. The entire process of IkBa degradation involves a series of well-characterized steps. One of the earliest events in the common activation pathway is the activation of IkB kinase (IKK) [369, 370]. IKB-α is phosphorylated at serine residues 32 and 36 by IKBa-kinase (IKKα), and thereby flagged for ubiquitination [370-372]. Following ubiquitination, IkBα is degraded by the 26S proteasome complex [370]. This results in the translocation of NF-κB to the nucleus where it triggers the transcription of a range of genes which include cytokines, cell cycle regulatory proteins, members of the IkB and Rel protein family as well as anti-apoptotic proteins [356, 370, 373, 374]. The activation of NF-κB prompts the transcriptional activation of genes that impede apoptosis. Subsequently, obstructing NF-κB activation instigates increased cell death [370, 375].

Upon classical or canonical activation of NF-KB, excitatory signalling can be instigated through Toll-like receptors (TLRs), Interleukin-1 receptor (IL-1R), tumour necrosis factor receptor (TNFR) and antigen receptors. Typical stimulating signalling molecules are tumour necrosis factor α (TNFα), lipopolysaccharides (LPS), and interleukin-1 β (IL-1β) [376, 377]. Signal transduction instigated through these receptors leads to stimulation of the IκB kinase (IKK) complex, which in turn phosphorylates IκBα primarily by IKK2. A second NF-κB signalling pathway is the alternative pathway, also referred to as the non-canonical pathway [378]. In this mechanism of signalling, a subset of receptors, including B-cell activation factor (BAFFR), lymphotoxin β-receptor (LTβR), CD40, receptor activator for nuclear factor kappa B (RANK), TNFR2 and Fn14 triggers the cascade by inducing kinase NIK. Consequently, the activation of NIK culminates in the phosphorylation and subsequent activation of IKK1 [379]. IKK1 in turn induces phosphorylation of p100 thereby calling for it to be ubiquitinated, ultimately prompting its partial degradation to p52 [380]. The mechanisms leading to activation of the non-canonical pathway are thus independent of the activity of IKK2 and the scaffolding protein NEMO [381] (Figure 1.12).

NF-κB activation typically induces the up-regulation of pro-survival genes with its activation providing the cells with the necessary survival mechanisms. Furthermore, NF-κB induces cytokines that regulate the immune and inflammatory response (such as TNFα, IL-1, IL-6 and IL-8), as well as adhesion molecules, thereby leading to the recruitment of leukocytes to sites of inflammation [382]. In relation to PCa, the literature has reported that NF-κB levels are constitutively activated in the hormone independent prostate cell lines PC-3 and DU-145, but not in the hormone responsive LNCaP cells. Furthermore, PC-3 cells demonstrate constitutive activation of IKKa, the kinase responsible for phosphorylation of IkB-a and activation of NF-κB [383] .

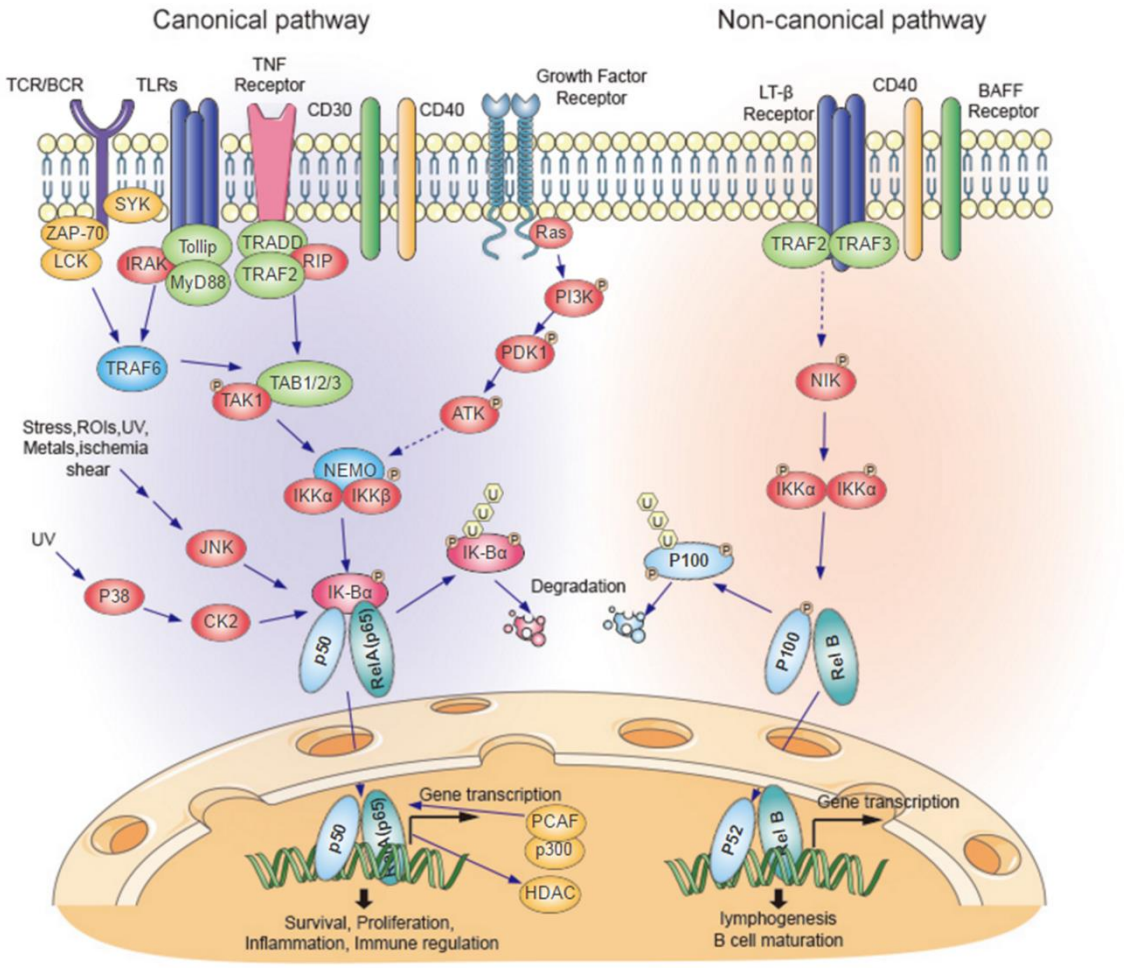


Figure 1.12 The canonical and non-canonical NF-κB signalling pathway poster image from *http://www.creative-diagnostics.com/The-NF-kB-Signaling-Pathway.htm* NF-κB can be activated by a number of stimuli, including exposure of cells to Lipopolysaccharides, inflammatory cytokines such as Tumour Necrosis Factor or Interleukin-1, growth factors, lymphokines, oxidant-free radicals, viral infection or expression of certain viral or bacterial gene products.

1.9 Experimental aims

The experimental work carried out for this thesis was designed to be carried out on cell lines representing the disease model of metastatic CRPC, comprising of the AR negative bone and brain metastasis derived PC3 and DU145 CRPC cell lines, respectively. Initial toxicity screening was also performed using non metastatic cell lines as a representative of organ confined AR dependent PCa. The following were the aims of the experiments:

1. Evaluate the toxicity of ApoE peptide mimetic compounds COG112 and OP449 in PCa cell lines and verify their effect on cell cycle progression

2. Investigate the expression of SET oncogene in PCa cell model. Validate the ability of ApoE peptide mimetic to interrupt the formation of SET-PP2A complex and modulate downstream signalling

3. Explore the anti-inflammatory properties of the ApoE peptide mimetics and elucidate the effect of COG112 and OP449 on TLR stimulation and signalling

Chapter 2: Materials & Methods

2.1 Cell culture

2.1.1 Cell lines

Human normal primary prostate epithelial cells (HPrEC), non-malignant, immortalised human prostate epithelial RWPE-1 cells, PCa cell lines 22Rv1, CWR22, PC3 and DU145 were all obtained from American tissue culture collection (ATCC; VA, USA). PC3 and DU145 cells were originally derived from the bone and brain metastatic site, respectively. The non-metastatic epithelial cell line, 22Rv1, was originally derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, human androgen-dependent CWR22 xenograft. (Table 2.1).

2.1.2 Patient RNA samples

RNA tumour-normal matched samples isolated form patient biopsies were gifted from Dr. Stefan Ambs, Laboratory of Human Carcinagenesis, National Cancer Institute, National institutes of health, Bethesda, Maryland, USA. The samples included 6 matched pairs. N&T1 and N&T2 are from African American Patients while N&T 3-6 were from European-American patients. The Ambs Prostate cancer case control study was approved by the NCI (protocol #05-C-N021) and the University of Maryland (Protocol #0298229) institute review boards.

2.1.3 Culture conditions

RWPE-1 cells were maintained in complete keratinocyte serum-free medium (KSFM), supplemented with 50 µg/mL bovine pituitary extract and 5 ng/mL epidermal growth factor (Gibco). 22Rv1 and CWR22 PCa cell lines were maintained in RPMI medium (Sigma) supplemented with 10% foetal bovine serum (FBS)

(Sigma). DU145 PCa cell line was maintained in MEMalpha medium (Gibco) and PC3 PCa cell line was maintained in Ham's F-12 nutrient mix (Gibco), both media were supplemented with 10% FBS. A pellet of Primary prostate epithelial (HPrEC) cells obtained from a lab colleague had been maintained in prostate epithelial cell basal medium, supplemented with 6 mM L-Glutamine, 0.4% Extract P, 1 µM Epinephrine, 0.5 ng/mL rh TGF-α, 100 ng/mL Hydrocortisone, 5 µg/mL rh Insulin and 5 µg/mL Apotransferrin (ATCC). All the media listed above contained 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone® Antimycotic (Gibco). Culture medium was changed every 2-3 days, and cells were passaged using 0.05% trypsin-Ethylenediaminetetraacetic acid (EDTA) (Sigma), upon reaching 70–80% confluency. Passaging of cells entailed washing the cells with Dulbecco's phosphate-buffered saline (D-PBS) (Sigma), followed by trypsinisation at 37°C for approximately 5 minutes. An equal volume of complete media was added to the detached cells, the resultant suspension was centrifuged at 300 x g for 5 minutes at room temperature, and the pellet was resuspended in the appropriate complete media. Cultures were grown in a humidified 5% CO2 environment at 37 °C.

2.1.4 Freezing and thawing of cells

All cell lines were cryopreserved in a freezing media composed of 90% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma). Cells were harvested and resuspended in 1 mL of freezing media at a concentration of 1-3 x 10^6 cells/mL and pipetted into cryovials (Nunc). The cryovials were then stored at -20°C for 2 hours, and then transferred to a -80°C freezer. Liquid nitrogen was used for the long-term storage of cells. Cell lines were restored by rapidly thawing a cryovial in a 37°C water-bath and transferring the contents to 10 mL complete media in a drop-wise manner within a 15 mL centrifuge tube (Sarstedt, Germany). The cell suspension was then centrifuged and the cell pellet resuspended in 5 mL of complete media, transferred to an appropriate tissue culture flask (Sarstedt), where a sufficient volume of media

was added to cover the entire bottom surface of the flask and the cells were allowed to attach and grow at 37ºC and 5% CO2.

Table2.1 Prostate cells used in this study

Cell Line	Site of Origin	Characteristics
Prostate epithelial cells	Prostate epithelial cells	Primary cells
RWPE-1	Prostate epithelial cells	Immortalised cell line
CWR22	PCa epithelial cells transplanted to Xenograft model	AR positive, Androgen dependent,
22RV1	PCa epithelial cells derived from xenograft propagated in mice	AR expressed, AR responsive. Androgen independent proliferation Castration induced regression.
PC3	PCa bone metastasis	No AR, androgen independent
DU145	PCa brain metastasis	No AR, androgen independent

2.1.5 COG 112 and OP449

Compounds (COG112 and OP449) were manufactured and provided by collaborator Dr. Mike Vitek, Oncotide, Inc, North Carolina, USA. Stocks were made by dissolving compound in PBS to a master stock concentration of 10mg/ML. The compound Docetaxel was purchased from Sigma-Aldrich (Sigma, Dublin, Ireland. #

01885-5MG-F). Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, Ireland.

2.2 Viability Assays

2.2.1 Trypan blue counting chamber

Proliferation and cell number was assessed by making a 1 in 2 dilution of cells suspended in media with Trypan blue (Thermo Scientific). A volume of 100µl cell suspension was transferred to a fresh eppendorf before 100µl Trypan was added. Cell suspension and trypan blue were gently mixed by pipetting up and down. Using a pipette, 20 µl of Trypan blue treated cell suspension mix was applied to a haemocytometer. Live, unstained cells which, which lay within the grid parameters of each of the four corner squares, were counted under the microscope. An average of the four squares was calculated and multiplied by 2 (dilution factor). The final value is the number of viable cells $X10^4$/mL in the cell suspension.

2.2.2 Alamar blue assay

Cells were harvested as described in section 2.1.2 and re-suspended in fresh medium at 4 x 10^4 cells/mL. One hundred microlitres of cell suspension was seeded into each well of a cell+ 96-well plate and cultured overnight in 5% CO_2 at 37°C. Media was replaced with 200 µL of the indicated concentrations of COG112 and OP449 resuspended in PBS and diluted with media followed by an incubation period at 37°C in 5% CO_2 for a further 72 hours. Following this, 40 µL of 560 µM resazurin and cells were incubated for 6 to 8 hours at 37°C. Plates were read using the Wallac plate-reader (Perkin Elmer) set at 530nm excitation and 620nm emission. A percentage (%) survival curve was calculated based on these values and

the IC50 was determined using the untreated control cultures as reference comparison for uninhibited (100%) growth. Viability assays were performed 3 times and error was presented as ± SD.

2.3 Flow cytometry

2 .3 .1 Annexin V staining

Cells were seeded in triplicate in a 6-well plate at a concentration of 2 x 10^5 cells per well and left to adhere overnight at 37°C in 5% CO_2. Cells were then and treated with 2 µM OP449, the IC_{90} value calculated from alamar blue assay (2.2.2), and 1 µM docetaxel over a 72 hours timecourse. The supernatants were transferred into tubes and the cells were harvested as described in section 2.1.2 using 0.05% trypsin-EDTA. The cell suspensions (which may contain dead floating cells) were added to the supernatants and centrifuged at 400 x g for 5 minutes. Samples were resuspended in 0.5 mL annexin staining buffer (0.5 µL annexin V stain plus 0.5 mL of 10 mM Hepes/NaOH (pH 7,4) 140 mM NaCl, 2,5 mM CaCl2) and incubated for 15 minutes at 4°C. Propidium iodide (PI) (Molecular Probes, Oregon) was added to the samples at 1 µL/sample. Samples were kept on ice until analysis using the BD Accuri flow cytometer. Cells were initially gated on morphology (FSC vs SSC), and then on annexin V (FL1) versus PI (FL3). Total dead cells were calculated by adding the percentage sum of the early-apoptotic (annexin V +/ PI -), necrotic/late-apoptotic (annexin V +/PI +) and fragments (annexin V -/ PI +). Data was processed by Flowjo v10.0.6 software.

2.3.2 Cell cycle analysis

PC3 cells were seeded into a petri dish at a density of 1.5 X 10^6 cells per plate and left to attach overnight at 37°C in 5% CO_2. This was followed by treating cells with

OP449 at the concentrations and time points outlined in the results section. The cells were harvested and filtered at the indicated time points post treatment, and 1 x 10^6 cells per sample were fixed in ice-cold 70% ethanol (Sigma) for at least 2 hours. Next, the ethanol was decanted and the cells were washed in PBS, followed by staining with 0.5 ml propidium iodide (PI)/RNase staining buffer (BD Biosciences) for 15 minutes. Samples were protected from light and kept on ice until analysis on the BD FACS Canto flow cytometer. At least 10,000 events were recorded per sample. The gating strategy was as follows: 1) cell morphology based on forward scatter (FSC) versus side scatter (SSC), 2) large event discrimination based on FSC versus SSC and 3) large event discrimination based on fluorescence. The percentage of cells in G1, S, and G2/M of the cell cycle was analysed at the time points indicated in the results section, using Flowjo v10.0.6 software using the Dean Jett Fox model.

2.4 Protein detection

2.4.1 Sample preparation

Cells pellets were obtained as described in section 2.1.2, washed with ice cold PBS, followed by lysis on ice for 15 minutes with an appropriate volume of RIPA buffer (Sigma), 1X Protease Inhibitor Cocktail III (Fisher) and 1X PhosSTOP™ protease inhibitor (Roche Life Science). The Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. Supernatants were transferred to fresh tubes and stored at –20°C. Protein concentration was determined using the BCA assay (ThermoFisher, MA).

Note: For analysis of apoptosis-related protein PARP, culture supernatants were collected, centrifuged at 100 x g for 5 minutes and the resulting pellet combined with the cell pellet prior to cell lysis.

2.4.2 Immunoblotting

NuPAGE® LDS Sample Buffer with NuPAGE® Sample Reducing Agent (Life Technologies) was added to the protein sample at a ratio of 1:4 and the samples were boiled at 95°C for 5 minutes. Equal concentrations of protein from each sample (20 - 50µg) were resolved using either hand cast Tris-Glycine gels or 8- 12% SDS-polyacrylamide pre-cast NuPAGE® Bis-Tris gels (Life Technologies). Samples loaded were run using NuPAGE® MES SDS Running Buffer (Life Technologies) or running buffer prepared in-house (10X tris-glycine, 10% SDS, dH2O). The percentage gel selected was dependent on the molecular weight of the protein of interest. Proteins were transferred to nitrocellulose membranes using the iBlot dry blotting system (Life Technologies). Membranes were then stained with Ponceau S (Sigma) stain to assess equal protein loading. Ponceau S was washed off using 1X tris buffered saline tween (0.1%), (TBS-T), (25 mM Tris; 3 mM potassium chloride (KCl); 68.5 mM sodium chloride (NaCl) (pH8) (all from Sigma), and membranes were blocked in 5% milk (Sigma) in TBS-T for 1 hour at room temperature. Membranes were then incubated in primary antibody made up with 5% milk (or 5% bovine serum albumin (BSA) for phospho-protein detection) in TBS-T, overnight at 4°C. See Table 2.3 below for antibody details. Membranes were then washed 3 times for 5 minutes each with TBS-T, followed by incubation with either IRDye 800CW goat anti-rabbit or IRDye 680LT goat anti-mouse secondary antibodies (LI-COR Biosciences) in 5% milk in TBS-T for 45 minutes at room temperature, protected from light. See Table 2.4 below for secondary antibody details. Three 5 minute washes with TBS-T and one 5 minute wash with TBS were then performed, followed by imaging of the blot using the ODYSSEY CLx Imager (LI-COR Biotechnology, NE, USA). For chemiluminescence (ECL) imaging, the washed membranes were incubated with either anti-Rabbit or anti-mouse HRP-linked IGg secondary antibody (GE Healthcare) diluted in in 5% BSA in TBS-T for 45 minutes at room temperature. Following three 5 minute washes in TBS-T, the enhanced HRP substrate was used to detect the target protein using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific).

2.4.3 Tris-Glycine gels

Tris-Glycine gels were hand cast using the Mini-PROTEAN® Tetra Cell Casting Stand with Clamp Kit (Bio-Rad Laboratories Inc., USA). Briefly, a short plate and spacer plate (1.5mm) were inserted and fastened into each casting frame, which was placed and secured into the casting stand. Depending on the % resolving gel required, the gel was made up according to the recipe below (Table 2.2) and the mixture was immediately poured between the glass plates, with enough room left over for the resolving gel. Immediately after pouring, 2-propanol was added to ensure that the gel set evenly. Once the resolving gel had set, the 2-propanol was poured off and the stacking gel solution (Table 2.2) was prepared and immediately poured on top of resolving gel. The appropriate comb (10 or 15- well) was inserted and the gel was allowed to set.

Table 2.2 Recipes for immunoblotting gels

Components (obtained from Sigma)	10% Resolving Gel (per gel)	12.5% Resolving Gel (per gel)	Stacking Gel (per gel)
Deionised H20 (ml)	2.76	1.95	3.6
30% Bis-acrylamide (ml)	3.34	4.15	0.668
1M Tris-HCl pH 8.8 (ml)	3.75	3.75	0
1M Tris-HCl pH 6.8 (ml)	0	0	0.625
10% SDS (µl)	100	100	50
10% APS (µl)	50	50	25
TEMED (µl)	5	5	5
Total (ml)	**10**	**10**	**5**

Table 2.3 Primary antibodies used for westrn blots

Antibody	Application	Dilution	Company	Product #
B-Actin	WB	1:25000	Sigma-Aldrich	A5441
SET	WB	1:500	Calbiochem	AP1134
SET	WB	1:1000	Abnova	H00006418
PP2A	WB	1:2000	millipore	05-421
p-PP2A	WB	1:1000	Santa Cruz	SC12615
AkT	WB	1:1000	CST	9272
p-Akt	WB	1:1000	CST	4058
MAPK	WB	1:1000	CST	4695

p-MAPK	WB	1:1000	CST	4370
PARP	WB	1:1000	CST	9542
GS3K α/β	WB	1:1000	CST	5676
p-GS3K α/β	WB	1:1000	CST	9331
TLR 1	WB	1:250	Millipore	06-006
TLR 3	WB	1:1000	Chemicon Int	AB4237
TLR 4	WB	1:1000	Abcam	ab30667
TLR 5	WB	1:1000	Calbiochem	AP1180
TLR 6	WB	1:500	Calbiochem	OP183
TLR 7	WB	1:000	Calbiochem	AP1181
TLR 8	WB	1:1000	Millipore	MAB10141
TLR 9	WB	1:300	Calbiochem	OP185
RIG-I	WB	1:1000	Millipore	06-1040
RIG-I	WB	1:1000	CST	3743
MDA5	WB	1:1000	CST	5321
IKKε	WB	1:1000	CST	2905
p-IKKε	WB	1:1000	CST	8766
TBK1	WB	1:1000	CST	3504
p- TBK1	WB	1:1000	CST	5483
IRF3	WB	1:1000	CST	11904
p-IRF3	WB	1:1000	CST	4947
IKBα	WB	1:1000	CST	9242
p- IKBα	WB	1:1000	CST	2859
P65	WB	1:1000	CST	8242
MAVS	WB	1:1000	CST	3993
Lammin A	WB	1:1000	Abcam	ab8980

Table 2.4 Secondary antibodies used for western blots

Antibody	Application	Dilution	Company	Product #
IRDye 680LT Anti-Mouse IgG	WB	1:20000	LI-COR	926-68020
IRDye 800CW Anti-Rabbit IgG	WB	1:15000	LI-COR	926-32211
IRDye 800CW Anti-Goat IgG	WB	1:15000	LI-COR	925-32214
ECL Anti-Rabbit IgG, HRPlinked Ab from donkey	WB	1:2500	GE Healthcare	NA934
ECL Anti-Mouse IgG, HRPlinked Ab from sheep	WB	1:2500	GE Healthcare	NA931

2.5 Protein purification

2.5.1 Immunoprecipitation

Protein was isolated and quantified as described in *2.4.1*. Immunoprecipitation (IP) was performed using an IP kit as per manuals instructions (Thermo Scientific #26148). In brief, 500µg protein lysate was pre-cleared with agarose resin to minimise nonspecific binding. PP2A antibody was prepared by making a 4µg/200µl solution using coupling buffer (4µl antibody into 196µl coupling buffer) and coupled with 20µl amino linked resin per sample in each microcentrofuge. In a

fume hood, 3µL of the Sodium Cyanoborohydride Solution (5M) was added for every 200µL of reaction volume. Spin columns were plugged and incubated on a rotator at room temperature for 2 hours before being washed twice with coupling buffer (0.01M sodium phosphate, 0.15M sodium chloride). Flow through was collected to verify antibody coupling. Antibody-resin mix was washed twice with 200µl coupling buffer and 200µL of 1M Tris-HCl. The flow through was discarded. Tubes were plugged and 200µl of 1M Tris-HCl and 3µL of Sodium Cyanoborohydride solution and was added to the resin. Tubes were incubated for 15 minutes on a rotator. Supernatant was discarded. Each resin sample was washed twice with 200µl coupling buffer and 6 times with 150µl of a 1M NaCl wash solution before proceeding to immunoprecipitation. Pre-cleared protein lysate was diluted to a final volume of 500µl with the IP Lysis/Wash Buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4). Protein samples were added to the antibody-coupled resin in the spin column, plugged, capped and incubated overnight on a rotator at 4°C. Samples were centrifuged and protein flow through was collected and stored for further analysis. Resin was washed with 200µl IP Lysis/Wash Buffer. Resin was rinsed and centrifuged an additional three times with Tris-Buffered Saline (TBS). Resin in the spin columns was washed twice with 200µL of ice-cold IP Lysis/Wash Buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4). Flow through was discarded and columns were plugged. Spin columns were placed in fresh collection tubes for elution. 25µL of Elution Buffer (0.1M Glycine pH 2.6) was added to each column before centrifugation. Another 75µL of elution buffer was added to complete the process. Samples were incubated for 10 minutes at room temperature before being centrifuged into the collection tube. Eluted samples were analysed by western blot

2.5.2 Biotin pulldown assay:

Protein concentrations of the lysate were adjusted to 1 mg of total protein per ml of solution. 90µg of biotin labelled COG112 was incubated with immobilised

streptavidin (Thermo scientific) at 4˚C for 2 hours. Biotin alone (70µg) was incubated with the streptavidin beads as a negative control. Following centrifugation at 1250 x g for 60 seconds, samples were washed twice with 250µl biotin blocking solution (0.001% BSA in PBS) in order to block free streptavidin. Samples were gently mixed by inverting 3-5 times and incubated at room temperature for 5 minutes before centrifugation at 1250 x g for 60 seconds, Each sample was then washed three times with 250µl TBS and spun at 1200 x g for 60 seconds. 200µl of cell lysate was added to the appropriate tubes with one remaining lysate free, where 200uL TBS was added instead. Samples were incubated at 4ºC for 2 hours. Following the incubation period, samples were centrifuged at 1250 x g for 60 seconds and the lysate was collected for later analysis of the flow through by western blot. Samples were washed three times with TBS with 0.05% Tween-20 and gently mixed by inverting 5 times and incubated at room temperature for 5 minutes before centrifugation at 1250 x g for 30 seconds. Prior to elution, 10µl neutralising buffer (1M Tris in ultrapure water) was added to each sample. Samples were eluted form streptavidin beads by adding 250µl elution buffer (0.1 M glycine-HCl (pH2.8)) and mixed by gentle inversion before incubating at room temperature for 5 minutes. Samples were spun at 1250 x g for 1 minute and supernatant was obtained for assessment by western blot to determine protein bound to biotin labelled COG112.

2.6 Cellular localisation

2.6.1 Subcellular fractionation

Cells were treated as required before being harvested as previously described to obtain a cell pellet. A subcellular protein fractionation kit was used, as per manufactures instructions, to isolate the nucleus and cytoplasmic fractions (Thermo Scientific 78840). In brief, lysate was extracted from $2X10^6$ cells by

incubating 200µl of hypotonic buffer (20mM HEPES pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$ (Thermo Scientific), 1X PhosSTOP™ phosphatase inhibitor (Roche Life Science) and 1X protease inhibitor cocktail III (100X stock) (Fisher Bioreagents) at 4°C for 15 minutes before 10µl 10% NP-40 (Sigma) was added per sample. Samples were vortexed to for 5- 10 seconds. Cytoplasmic extract was isolated by centrifugation at 500 x *g* for 5 minutes and the supernatant (cytoplasmic fraction) was transferred to fresh ice cold eppendorf. The remaining pellet was resuspended in a 200µl volume of the hypotonic buffer and centrifuged at 500 x *g* before discarding the supernatant. This was repeated twice before re-suspending the pellet in 100µl RIPA buffer (Sigma) supplemented with 1X PhosSTOP™ phosphatase inhibitor and 1X protease inhibitor cocktail III (Fisher Bioreagents). The samples were spun for 5 minutes at 5000 x *g* at 4°C and the supernatant (nuclear fraction) was retained and stored at -80°C until required. Cytoplasmic and nuclear fractions were analysed for protein localisation by western blot.

2.7 Kinase screening

2.7.1 Reverse phase protein array

A reverse phase protein array screen was performed, using the library resources of collaborators in Beaumont in collaboration with Prof. Byran Hennessy [384]. In brief, PCa cell lines were grown to 80% confluency in their respective media, supplemented with 10% foetal bovine serum and 1% 100X antibiotic-antimycotic, before treatment with OP449 for 48 hours. Upon harvesting, cells were washed twice in ice-cold PBS and lysed in RIPA lysis buffers as previously described. Protein concentration was determined by routine BCA assay. For quantification and accuracy purposes, protein lysates were two-fold serial diluted for four dilutions (from undiluted to 1/8 dilution) and printed onto nitrocellulose-coated glass slides. The samples were blocked and probed with antibodies by CSA amplification. The microarray slides were blocked prior to the addition of the primary antibody. The

DAKO signal amplification system was used to detect and amplify antibody-binding intensity. A biotinylated secondary antibody (anti-mouse or anti-rabbit) was used as a starting point for signal amplification. The slides were scanned on a flatbed scanner to produce 16-bit tiff images where spots were identified. Signal intensity was quantified using the MicroVigene automated RPPA module which allowed for the relative quantification of each sample. The catalogue of antibodies used in the screen were to identify modulated signalling of proteins involved in Pi3K/AKT/mTOR signalling, MAPK related signalling cascades, cell cycle and apoptosis in response to OP449

2.8 siRNA Transfection

2.8.1 Reverse transfection optimisation

siRNA reverse transfection was carried out using the specially formulated siRNA transfection reagent Dharmafect #3 (GE Dharmacon, CO, USA) supplied by Fisher Scientific (Ireland), which according to the manufacturer has been used to successfully transfect PC3 and DU145 cells with siRNA. The transfection was optimised on the AllStars Cell Death Control siRNA (Qiagen, Germany), a cocktail of siRNAs that target ubiquitously expressed human genes that are essential for cell survival. The cells were harvested as described in section 2.1.2 and a 50,000 cell/ml cell suspension was made up for each cell line in complete media (minus antibiotic). The experiment was carried out in triplicate in a 96-well plate (Cell+) (Sarstedt, Germany) for each cell line. A range of Dharmafect volumes (0.1 µl-0.5µl) and siRNA final concentration 5, 25, 50nM were assessed for toxicity alone and in combination for successful transfection were assessed to configure optimum transfection concentrations. For each well 0.1 µl-0.5µl Dharmafect was added per 100µl complete media (minus antibiotic) in separate tubes and incubated for 15 minutes at room temperature. Per well of the non-targeting siRNA control (scramble) or Dharmafect only, 1.0µl Dharmafect was added to 100µl of

incomplete RPMI-1640 media. 0.05, 0.25 and 0.5µl of 20µM siRNA (cell death siRNA or scramble) was added to each 100µl of the Dharmafect solution (100nM siRNA) and allowed to incubate for another 5 minutes. The Dharmafect only tube (to evaluate Dharmafect cytotoxicity) contained no siRNA, while siRNA only tubes contained no Dharmafect (to evaluate SiRNA cytotoxicity). Meanwhile, 100µl of the cell suspensions (5,000 cells) were each added the wells of three 96-well plates, on top of which 100µl of the appropriate Dharmafect only, siRNA only or Dharmafect - siRNA solution was added in each plate (final siRNA concentration 5, 25 and 50nM and 0.1µ- 5µl Dharmafect per ml media). The 96-well plates were transferred to the incubator at 37°C and 5% CO2. The alamar blue viability assay was carried out as outlined in section 2.2.2 on one plate each at 24, 48, and 72 hours. The viability was expressed as a percentage of the untreated (100% viability) per cell line per plate.

2.8.2 Transient gene knockdown

Cells were harvested as outlined in section 2.1.2 and a $5x10^5$ cells/ml cell suspension was made up in the appropriate complete media containing no antibiotic. Per ml of media (final volume), 1µl (PC3) or 2µl (DU145) Dharmafect was combined with 2.5µl of each siRNA (TLR3, RIG-I, scramble) (20µM stock) and the volume were brought to a volume of 500µl using complete media containing no antibiotic. Following incubation of the Dharmafect-siRNA solution at room temperature for 15 minutes, 500µl of the cell suspension (250,000 cells) and 500µl of the appropriate Dharmafect siRNA solution was combined per well of 6-well plate (Cell+). The 6-well plate was transferred to the incubator set at 37°C and 5% CO2 for 6 hours. The media was replaced with 1ml fresh complete media and the cells were incubated for a further 48 hours. The experiment was terminated and the cells were harvested as previously described. RNA and protein were isolated as

required. The cells were assessed for changes in gene and protein expression using RT-PCR or immunoblotting.

For forward transfection, cells were seeded into 6 well plates of and left to adhere overnight at 37°C and 5% CO2 prior to transfection with SiSET. Transfection reagents were prepared as described above, Per ml of media, 1µl or 2µl Dharmafect (for PC3 and DU145 cells respectively) was combined with 2.5µl of each siRNA (SET, scramble) (20µM stock) and the volume were brought up to 500µl using complete media containing no antibiotic. Following incubation of the Dharmafect-siRNA solution at room temperature for 15 minutes, the old media was removed from each well and replaced by 500µl complete media containing no antibiotic and 500µl of the appropriate Dharmafect siRNA solution per well of 6-well plate (Cell+). The experiment was terminated and the cells were harvested as previously described. RNA and protein were isolated as required. The cells were assessed for changes in gene and protein expression using qRT-PCR or immunoblotting.

2.9 Gene expression

2.9.1 RNA extraction

Cells were harvested as described in section 2.1.2 and resuspended in 1 mL of ice-cold Tri-reagent (Sigma) per sample. The cells were homogenised multiple times by pipetting and then transferred to a sterile eppendorf on ice for 15 minutes. Samples were then kept at -80ºC until further processing. Samples were thawed at room temperature for 5 minutes, followed by the addition of 200µL chloroform (Sigma) and vigorous shaking for 15 seconds. Samples were then centrifuged at 12,000 g for 15 minutes at 2-8ºC and the upper aqueous phase was transferred to a fresh eppendorf and mixed with 500µL isopropanol (Sigma). The samples were then kept at room temperature for 10 minutes and placed at -20ºC overnight. Centrifugation of the samples at 12,000 g for 10 minutes at 2-8ºC was then

performed, followed by washing of the RNA pellet with 70% ethanol (Sigma). The samples were then vortexed briefly and centrifuged at 7,500 g for 5 minutes at 2-8°C. The pellets were left to air dry for 15 minutes, resuspended in an appropriate volume of DEPC-treated water (Sigma) and quantified using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Delware).

2.9.2 cDNA synthesis

cDNA was synthesised using the Tetro cDNA Synthesis Kit (Bioline). Briefly, 1 µg total RNA was added to 1µL oligo (dT)18 primers, 1 µL 10 mM dNTP mix, 4µL 5X RT buffer, 1 µL Tetro reverse transcriptase (200 u/µL), and the volume adjusted to 20 µL with DEPC-treated water in sterile 0.2 mL tubes (Eppendorf). Samples were then incubated at 45°C for 30 minutes, followed by 85°C for 5 minutes using the Veriti Gradient Thermal Cycler (Applied Biosystems). Samples were stored at -20°C.

2.9.3 Real-time PCR

Predesigned KicqStart® SYBR Green Primers (Sigma, MO, USA) were reconstituted in molecular grade dH2O (Sigma, MO, USA) to a stock concentration of 100 µM and stored at -20 ˚C. Working stocks were made at a concentration of 10 µM and stored at -20 ˚C. Primer sequences are detailed in table 2.5. A master mix was prepared using 5 µL 2X SensiFAST SYBR Hi-ROX Mix, 0.4 µL of 10 µM forward primer, 0.4 µl of 10 µM reverse primer and 2 µL template cDNA and the final volume was adjusted to 10 µl with nuclease-free water. Samples were added to a 96-well PCR micro-plate (Thermo Scientific ABgene) and each reaction was carried out in triplicate. The cycle conditions for real-time PCR were as follows: 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. The StepOne Plus Real Time PCR System (Applied Biosystems) was used for each run. The house-keeping gene β-Actin was used to analyse the relative expression of the genes of interest. Relative gene expression data was analysed using the -ΔΔCT method.

2.9.4 Primers

Primers (Sigma) were reconstituted in molecular grade dH2O (Sigma) to a stock concentration of 100 µM and stored at -20 ˚C. Ten micro molar working stocks were made and stored at -20 ˚C. The sequences for each of the primers used can be found in Table 2.5. Where possible, primers were designed to span exon-intron boundaries so as to reduce genomic DNA amplification.

Table 2.5 RT-PCR primer sequences

Gene Name	Forward Sequence	Reverse Sequence
SET	AATCTGGAAAGGATTTGACG	ATCATCTTTGATGACCTCTCC
PP2Ac	ATGGAGGGATATAACTGGTG	CTTGGTTACCACAACGATAAC
IL-6	GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
IL-8	GTTTTTGAAGAGGGCTGAG	TTTGCTTGAAGTTTCACTGG
B-Actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

2.10 Statistical Analysis

2.10.1 Statistical Analysis

All statistical comparisons were performed with GraphPad® Prism V 5.0 (GraphPad Software, San Diego, CA, USA). Depending on the number of factors, multiple-group comparisons were made using either a single or two-way analysis of variance (ANOVA) followed by the Tukey or Bonferroni post hoc test respectively to confirm statistical differences between groups. Paired sample analysis was performed using a two-sided paired Student's t-test. Statistical significance (p-value) was assigned for values $p<0.05$.

Chapter 3: Exploring the viability and cell cycle progression of PCa cells in response to Apolipoprotein E mimetics COG112 and OP449

3.1 Introduction

Accumulating evidence supporting the potential use of ApoE as a cancer treatment strategy is emerging. For instance, Pencheva et al, identified ApoE as anti-angiogenic and metastatic suppressive factor. In their study, miR-1908 and miR-199a-3p were identified as endogenous promoters of metastatic invasion, angiogenesis and colonization in melanoma which targeted ApoE [385] Bhattacharjee and associates demonstrated that the ApoE peptide mimetic, apoEdp, had anti-angiogenic attributes in vivo through reduction of tumour growth in a mouse model and ocular angiogenesis in a rabbit eye model [386]. Singh et al., demonstrated that ApoE mimetic COG133 inhibits NF-κB, and suggest the possible therapeutic potential of ApoE peptide mimetics by inhibiting NF-κB driven proinflammatory epithelial responses [172]. Switzer et al., demonstrated the inhibition of Akt signalling and cellular proliferation of MDA-MB-231 breast adenocarcinoma cancer cells in response to COG112 [387].

Considering the supporting literature, the possibility of using ApoE peptide mimetics for the treatment of PCa was explored in the PCa cell lines CWR22, 22RV1, PC3 and DU145. In an effort to explore the effect ApoE peptides (COG112 and OP449) may have on PCa cell line proliferation, the alamar blue assay was employed to determine any reduction of cell viability. Flow cytometry analysis was employed in an effort to deduce the impact on cell death and cell cycle progression. The Annexin V assay was utilised to establish the occurrence of apoptosis and PI cell cycle analysis explored the impact that ApoE may implement on the cell cycle progression of PCa cells. As the ultimate goal in emerging therapies is the successful inhibition and elimination of cancerous cells, the execution of these experiments would determine if further investigation using these peptides is warranted. While these investigations would unveil their potential as therapeutic candidates, much more in depth investigation would be required to determine their efficacy in dampening metastasis and PCa progression

3.2 Results

3.2.1 Determination of the effect of ApoE mimetics on prostate cancer cell viability

The alamar blue assay was optimised and employed to monitor cell proliferation of PCa cell lines in response to COG112 and OP449. This is a simple fluorimetric/colourimetric assay used for the detection of metabolic activity in cells. Innate metabolic activity results in a chemical reduction of the alamar blue compound resazurin. The continued metabolic activity of growing cells promotes a reduced environment resulting in the REDOX indicator compound resazurin, to change from its oxidised non-fluorescent blue to a reduced florescent red, while inhibition of growth maintains an oxidised environment with rezasurin remaining blue and non-fluorescent [388, 389].

During the optimisation process, it was identified that seeding cells at a concentration of 4000 cells per well, performing a 72 hour treatment time course and incubating cells for 6-8 hours in 560µM alamar blue was optimal and yielded the best results and by controlling these variables, deviations were lower and background noise levels were minimal (Figure 3.1). The alamar blue assay was deemed sufficiently robust when the coefficient of variation was below ten percent.

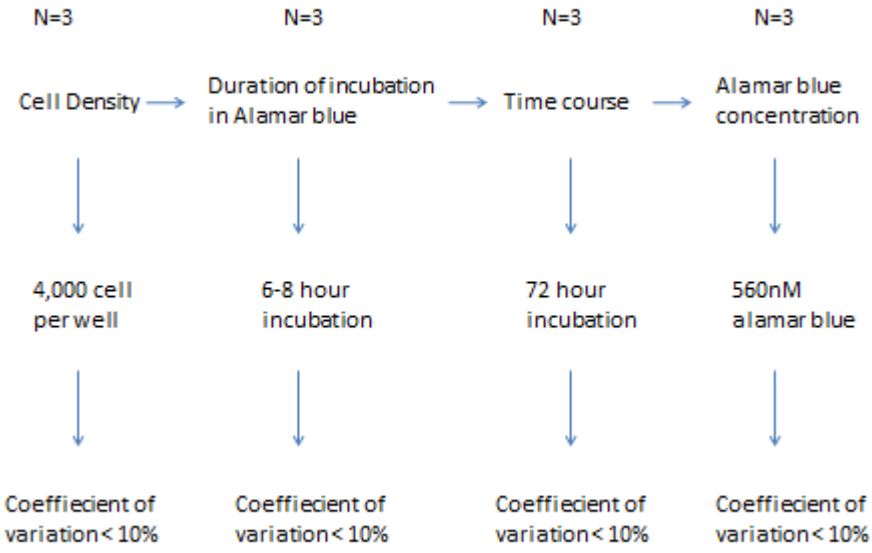


Figure 3.1 Work flow of Alamar Blue optimisation - The above parameters were identified as important variables and were optimised in order to increase the robustness of the assay. Robustness was determined wen coefficient of variation was consistently lower than 10%. The middle row is the optimal conditions that were controlled and applied for all further alamar blue assays

Following assay optimisation, 96 well plates were seeded with RWPE1 immortalised cells, CWR22, 22RV1 (AR positive), PC3 and DU145 prostate (AR negative) cancer cell lines to investigate if the ApoE mimetic peptides are capable of depleting viability in both androgen positive and androgen negative cell types. The cell lines were left to adhere overnight and then incubated with varying concentrations of either COG112 or OP449 ranging from 0nM to 2000nM over a 72 hour timecourse. Following treatment, cells were then incubated with 560µM Alamar blue for 6-8 hours. The absorbance was read using a plate reader at 530nm wavelength. Both COG112 and OP449 were effective at decreasing cellular proliferation in AR positive PCa cell lines. COG112 was shown to be considerably less effective in its ability to decrease cell viability in AR negative cell lines whereas OP449 demonstrated that while a higher concentration was required it was capable of diminishing cell viability in AR negative cell lines. The toxicity data of the individual compounds was analysed by one way ANOVA to determine statistically significant modulation in

cell viability. A relevant decrease found by the analysis is conclusive of decreased viability in response to COG112 and OP449 compared to their untreated counterparts. The effective concentrations of the peptides were also compared. Two way Anova was used to verify significant potency differences at their respective concentrations (Figure 3.2).

In the CWR22 cells treated with COG112, the lowest dose inducing a statistically significant decrease in viability was observed at 1300nM with approximately 40% reduction calculated. A 56% reduction in cell survival was observed at the highest dose of 2000nM. The OP449 peptide induced a statistically significant decrease at 600nM. At this dose, a 30% decrease in cell viability was observed. OP449 matched the maximum observed toxicity of COG112 at half the concentration of 1000nM. At 2000nM, the highest concentration used in the assay, OP449 achieved 97% reduction of cell viability when compared to the untreated control. In the comparative analysis where the difference in toxicity of the drugs at the same concentration was evaluated, OP449 was found to significantly exceed the potency of COG112 from 600nM onwards (Figure 3.2 A).

In the 22V1 cell line assessed using this assay, a significant decline in metabolic activity was identified at 1300nM in response to COG112. At this concentration, a decrease of almost 30% was detected. At 2000nM a 54% decrease in cellular metabolism was gauged. In response to OP449, a 35% decrease in viability was noted in response to 400nM. This diminishing effect on metabolic activity increased in a dose dependant manner. At 2000nM cells were reduced to 5% viability. When the effectiveness of the peptides were compared at the same concentration, OP449 was found to be pointedly more toxic than COG112 from 400nM onwards (Figure 3.2 B).

In the difficult to treat, metastatic AR negative PC3 cells, COG112 failed to decrease metabolic activity at all concentrations except for the highest dose. At

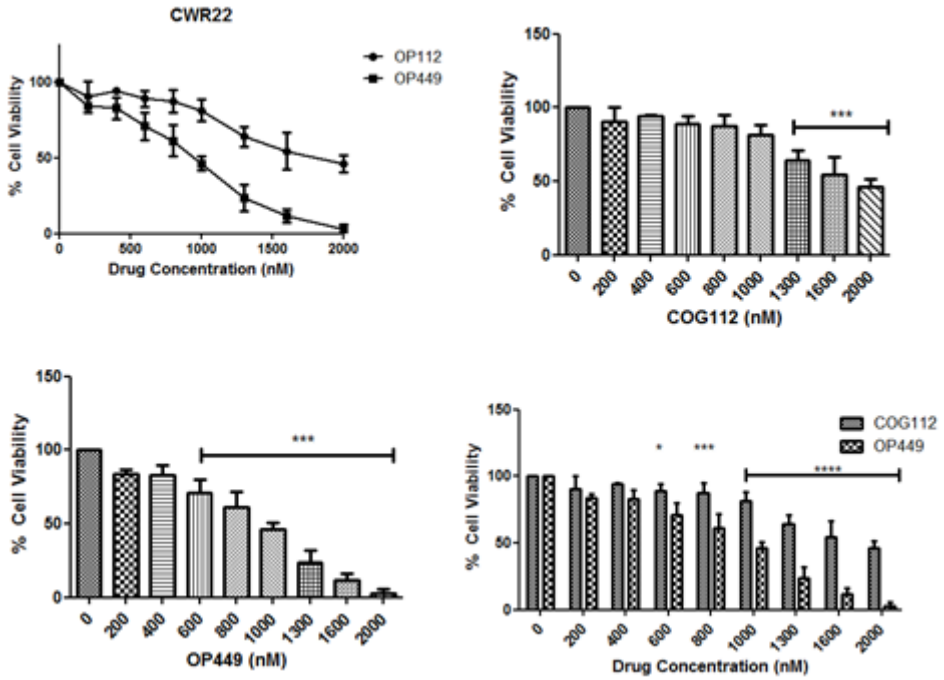
2000nm an approximate decrease in viability of 40% was detected. OP449 on the other hand, exhibited a decline, of 20%, in cell activity at a concentration of 1000nM. This response was amplified in a concentration dependant manner. At 2000nM, approximately 80% of the cells metabolic activity was diminished. When the efficiency of the two drugs was compared at the same concentration, OP449 was found to surpass COG112 from 1000nM (Figure 3.2 C).

Metastatic DU145 cells proved more difficult to inhibit with no significant decrease in viability detected in response to COG112 when compared to the untreated control. OP449 on the other hand, produced a 40% decline in viability at 1400nM which steadily increased in correlation with the dose and peaked at an approximate 70% decline in cell survival in response to 2000nM. When the efficacy of the peptides were assessed by concentration comparison using two way ANOVA, OP449 began to exceed the ability of COG112 to decrease cell viability at 1000nM (Figure 3.2 D).

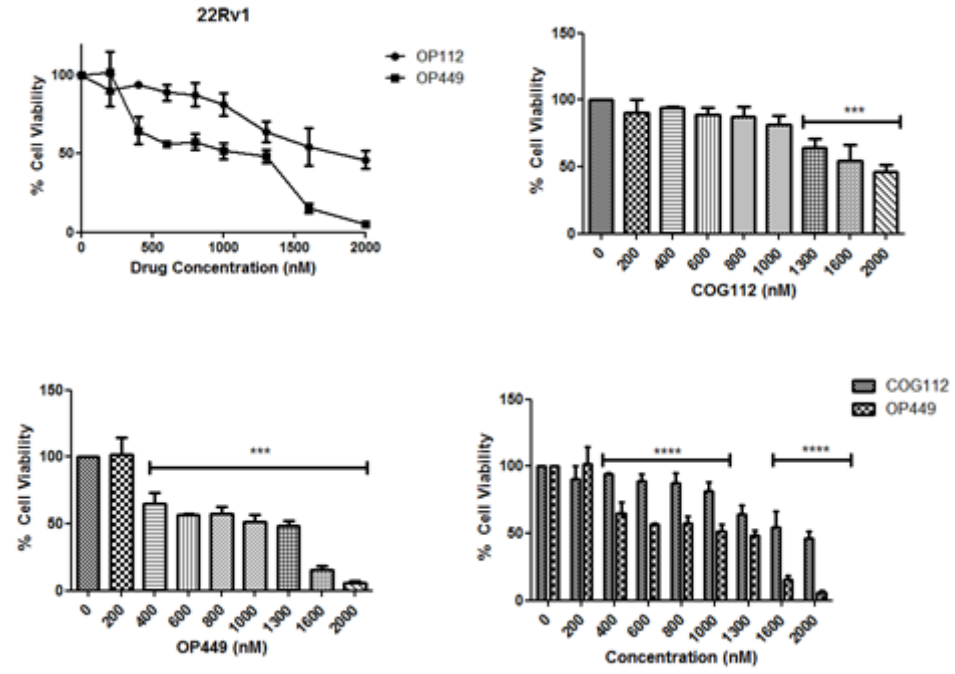
The effect of the COG112 and OP449 on RWPE1 transformed cells were also assessed using the alamar blue assay. These cells were sensitive to both drugs with IC50 valued considerably lower than that of the PCa cells (Figure 3.2 E). Complete inhibition of cell proliferation was observed following exposure to 1000nM of both COG112 and Op449.

As OP449 was unveiled to be the more potent of the two peptides and demonstrated the capability of inhibiting cell growth in the cell lines which represent advanced PCa, it was selected for further detailed analysis. Targeting cell proliferation in the advanced stages of PCa is an essential requirement for the future therapies of PCa in order to overcome the inevitable associated mortality. The sensitivity of RWPE1 cells to the peptides must be taken into consideration and possibilities for its vulnerability to the peptides explored.

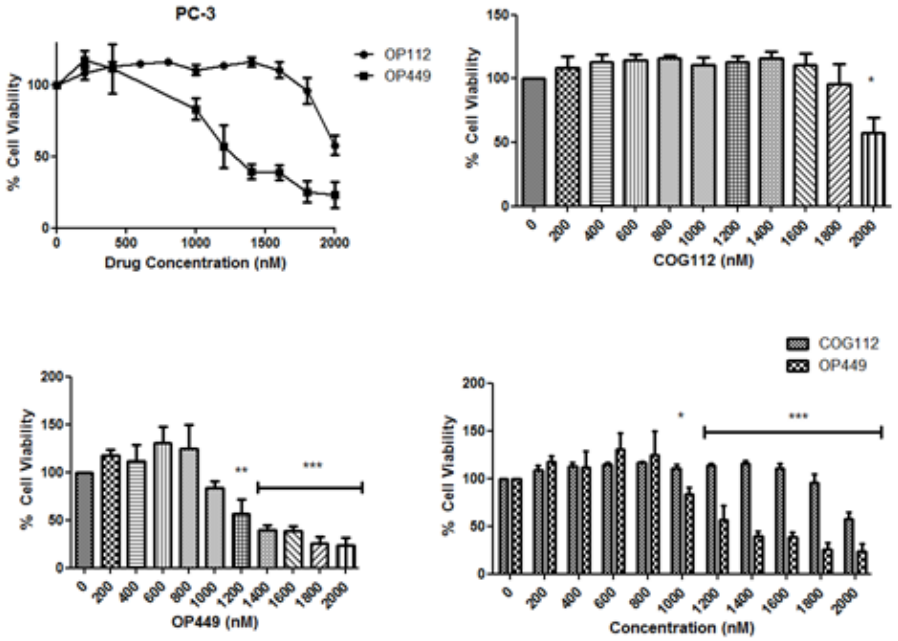
A


CWR22
OP112
OP449
% Cell Viability
Drug Concentration (nM)
COG112 (nM)
OP449 (nM)
COG112
OP449
Drug Concentration (nM)

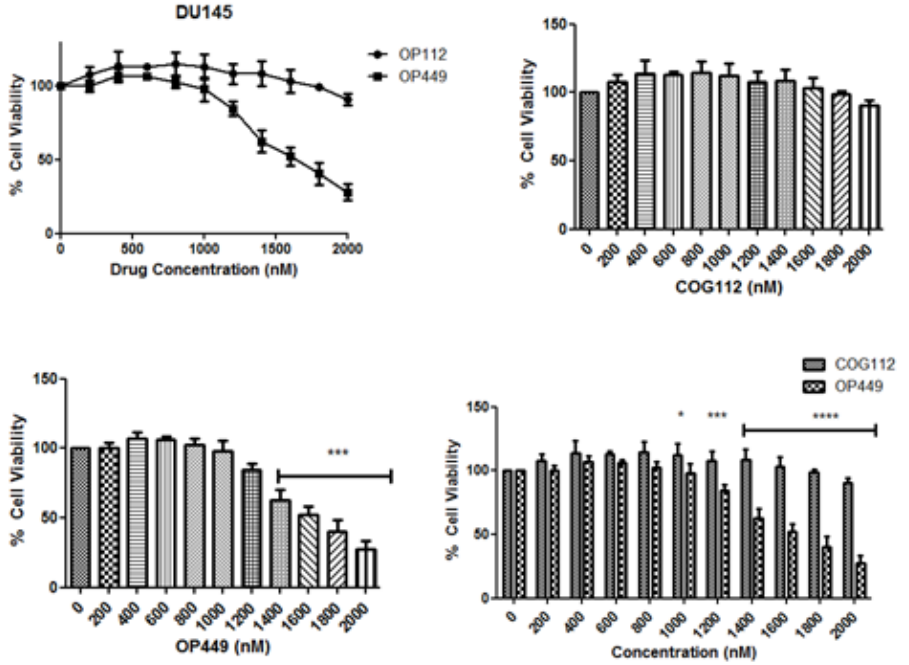
B


22Rv1
OP112
OP449
% Cell Viability
Drug Concentration (nM)
COG112 (nM)
OP449 (nM)
COG112
OP449
Concentration (nM)

C


PC-3
OP112
OP449
% Cell Viability
Drug Concentration (nM)
COG112 (nM)
OP449 (nM)
COG112
OP449
Concentration (nM)

D


DU145
OP112
OP449
% Cell Viability
Drug Concentration (nM)
COG112 (nM)
OP449 (nM)
COG112
OP449
Concentration (nM)

E

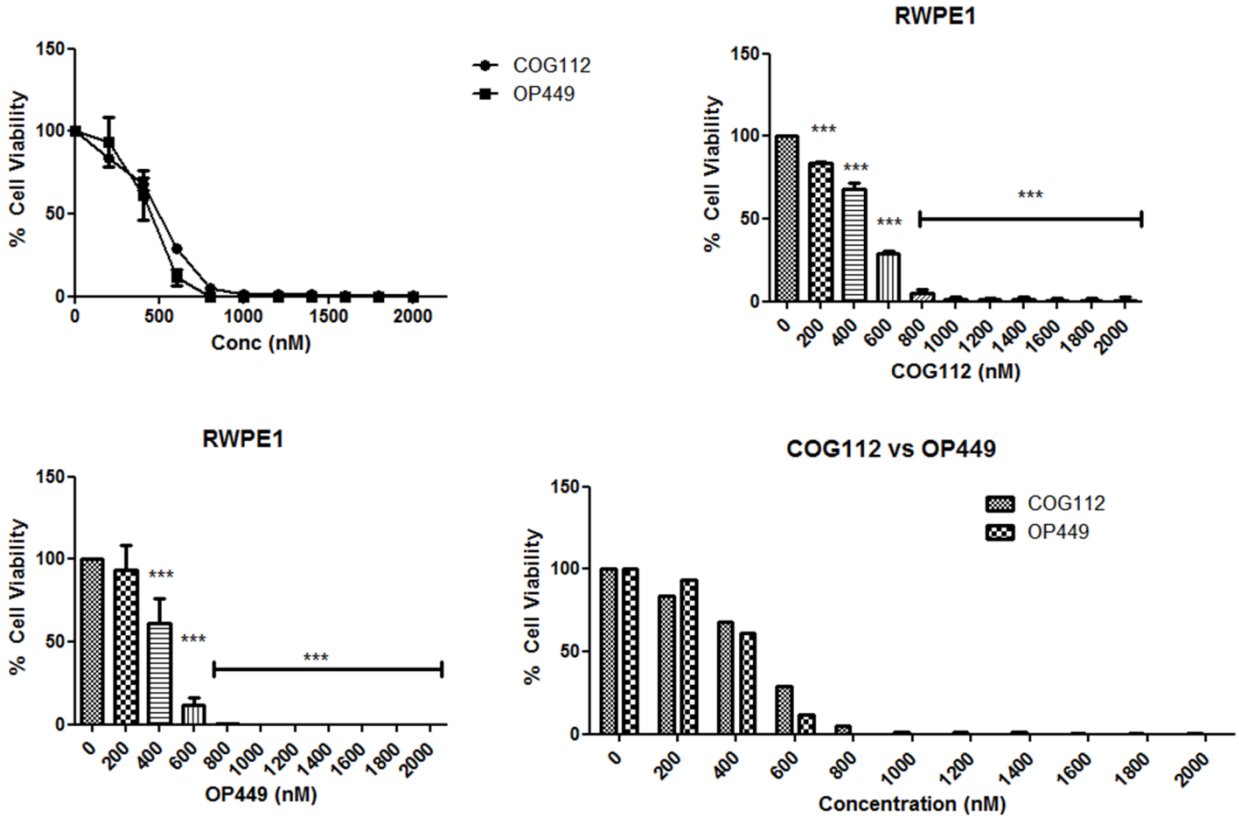


Figure 3.2 The Alamar blue Assay – performed on CWR22, 22RV1 PC3 and DU145 cell lines. CWR22, 22RV1, PC3, DU145 and RWPE1 cell lines were seeded in 96 well plates at a density of 4000 cells per well and then treated with increasing concentrations of COG112 and OP449 over a period of 72 hours this was followed by a 6-8 hour incubation with resazurin. The assay confirmed a decrease in cell viability by means of a readout measuring reductase activity in response to COG112 and OP449 in a dose dependant manner. OP449 presented as the more potent of the compounds with lower concentrations required for inhibition of cell growth by 50%. CWR22 (A) and 22RV1 (B) demonstrated a significant decrease in response to COG112 and OP449 at higher concentrations in comparison to the untreated control. In the AR negative cell lines PC3 (C) and DU145 (D), OP449 was consistently effective in decreasing viability in the cell lines at higher concentrations where COG112 was not. RWPE1 (E) were sensitive to both COG112 and OP449 at lower concentrations than the PCa cell lines with a complete loss of viable cells observed at1000nM in reponse to COG112 and 800nM in reponse to OP449. Statistical significance in cell viability was determined by one way ANOVA followed by tukey post hoc analysis, ***p<0.0001, *p<0.05. The efficiency of COG112 compared to OP449 was assessed by two way ANOVA followed by bonferroni post hoc test. OP449 presented as being significantly more potent than COG112. *p<0.05, ***p<0.001, ****p<0.0001

Table 3.1 IC50 values calculated from Alamar blue cell viability Assays

	IC50 (nM) ± SD				
	RWPE1	**CWR22**	**22RV1**	**PC3**	**DU145**
COG112	550± 6.9	1750 ± 8.9	1800 ± 8.3	2000 ± 6.7	N/A
OP449	490± 7.8	900 ±7.5	1000 ± 5.03	1300 ± 10.04	1600 ± 6.12

3.2.2 Induction of apoptosis in response to ApoE mimetic peptide

The outputs from the alamar blue assay are typically reported as cell viability; however, the assay is unable to differentiate between apoptotic or dead cells. In order to clarify if the ApoE peptide mimetics were cytostatic or cytotoxic, we investigated whether OP449 induces apoptosis/cell cycle arrest using flow cytometry. For the Apoptosis Assay, detection of apoptotic cells was determined by the binding of Annexin V to phosphatidyl serine (PS) at the cell surface of apoptotic cells. A number of morphological and biochemical changes occur at various stages of apoptosis and one of these is the exposure of PS on the outer leaflet of the plasma membrane. Annexin V binds preferably to negatively charged phospholipids such as PS in the presence of Ca_2^+ and was used in this study for the detection of early apoptosis in cells [390, 391]. Propidium Iodide (PI) was the flurochrome used to determine cells which are dying or already dead as this stain intercalates with nucleic acids. In live cells, PI is unable to penetrate the cell membrane, however where integrity of the membrane has been compromised, exposure to the internal cellular components is instigated, thus giving the capacity to identify the early and late apoptotic cells. PC3 cells were seeded into a 6 well plate with $1.5x10^6$ cells per well and left to adhere overnight. Treatment conditions included an untreated well, treatment with 2000nm OP449 and 1uM docetaxel which was used for a positive control for apoptosis, as this agent is currently available for the treatment of PCa in a clinical setting and has been shown to induce apoptotic cell death [392, 393].

Cell lines were treated over a time course of 24, 48 and 72 hours. At each time point, cells were trypsinised, washed in PBS, passed through a 40µm strainer for single cell suspension, and counted. $1x10^6$ cells were transferred to a FACS tube and resuspended in a Calcium buffer. The experiment was run using the Accuri C6 cytometer. FSC-A versus SSC-A plots were used for gating cells and to identify changes in the scatter properties of the cells.

The analysis for the 24hour time point revealed that 93% of untreated cells remained viable and negative for both Annexin V and PI. Early apoptotic cells

accounted for 1% of the population which was positive for Annexin V only and the remaining 6% of cells were deemed late apoptotic or dead, staining positive for both Annexin V and PI. Cells exposed to OP449 displayed a scatter showing 83% viable cells negative for both Annexin V and PI. Early apoptotic cells accounted for 10% of the population, positive for Annexin V only while the remaining 7% of cells were late apoptotic/dead and positive for both Annexin V and PI. The cells treated with docetaxel maintained their viability with 87% of cells negative for both Annexin V and PI at this time point. Early apoptotic, Annexin V positive cells, made up 6% of the population while the remaining 7% were late apoptotic or dead cells positive for both Annexin V and PI (Figure 3.3 A)

At 48 hours, 93% of the untreated group remained negative for Annexin V and PI and thus viable. Early apoptotic cells accounted for 2% while 5% were late apoptotic or dead. The OP449 treated cells showed an increase in apoptotic cells with a scatter exhibiting 52% remaining viable cells. Early apoptotic cells positive for Annexin V accounted for an average of 36% of the treated. The remaining 12% of the sample population accounted for the late apoptotic and dead cells which were positive for both Annexin V and PI. These values differed to the docetaxel treated cells at this time point which retained on average 75% viability. Early apoptosis was calculated to represent 10% of the cell sample. The remaining 15% of the cells made up the late apoptotic fraction of the sample (Figure 3.3 B)

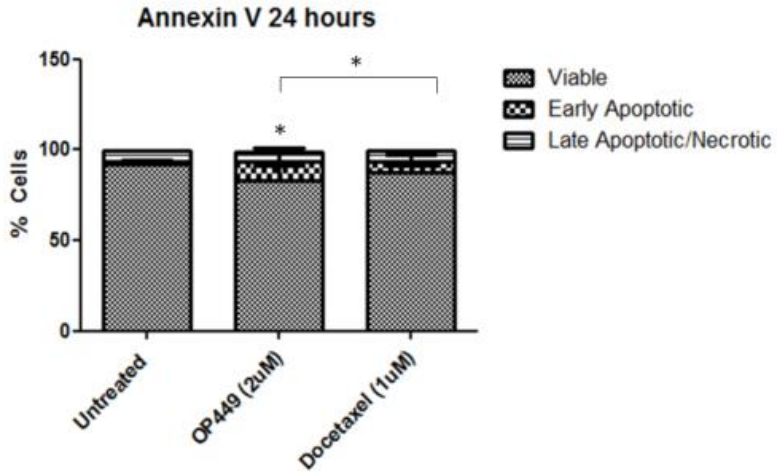
At 72 hours, the final time point of the assay, 92% of the untreated cells were gauged as viable. The remaining cells were distributed between the early and late apoptotic sub groups with 3% staining positive for Annexin V only and 5% positive for both Annexin V and PI. Treatment with OP449 returned an average of 38% viability within the sample. Annexin V positive cells accounted for 40% of the cells exposed to OP449. The remaining 22% of cells within the population made up the late apoptotic/necrotic subsection of the cells and were identified as positive for both Annexin V and PI. At the final time point of the assay, approximately 60% of cells treated with docetaxel remained intact and viable. The remaining 40% were

split evenly between the early apoptotic and late apoptotic/necrotic subgroups with 20% identified as Annexin V positive only and 20% distinguished as positive for both Annexin V and PI (Figure 3.3 C).

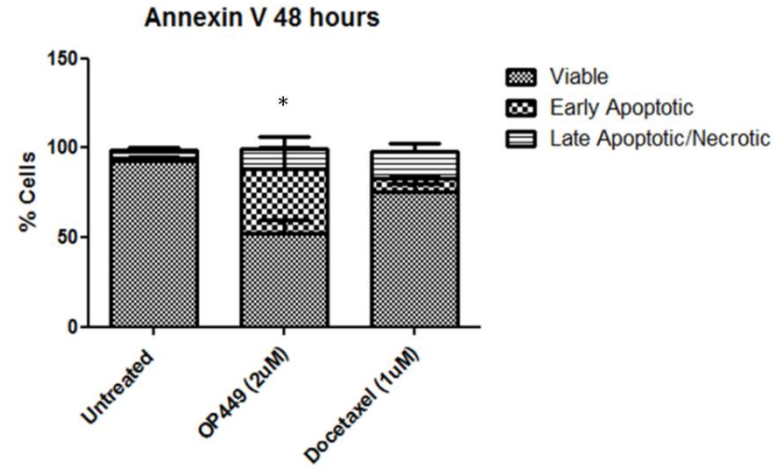
Results showed that cells shifted from an unstained viable cell to an early apoptotic phenotype at 24 hours staining positive for Annexin V only before progressing to an increasingly late apoptotic/necrotic phenotype by 72 hours, staining positive for both Annexin V and PI as exposure time to OP449 increased. This confirms that the ApoE mimetic compounds are having a cytotoxic apoptosis inducing effect on PCa cells, implicating apoptosis as one mechanism for the observed reduction in cell viability.

To validate the results obtained from flow cytometry analysis, PARP cleavage was assessed in response to 72hr incubation with OP449 (2μM) and docetaxel (1μM) in PC3 cells by western blot. The results demonstrated that cleavage of PARP was induced in response to both compounds. PARP cleavage was greater in response to OP449.

A



B



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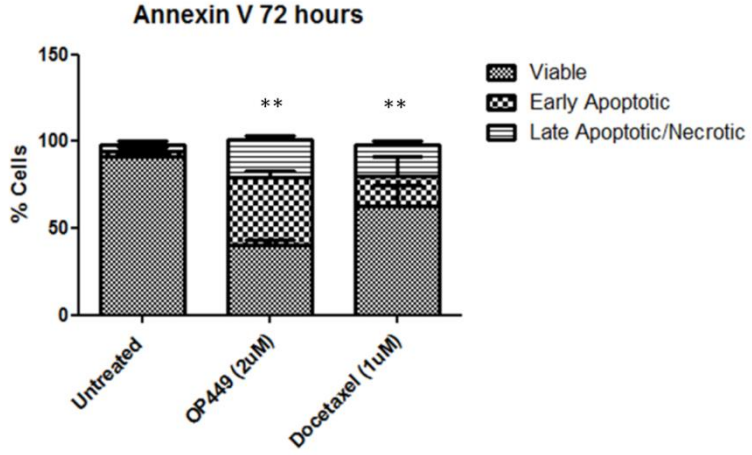
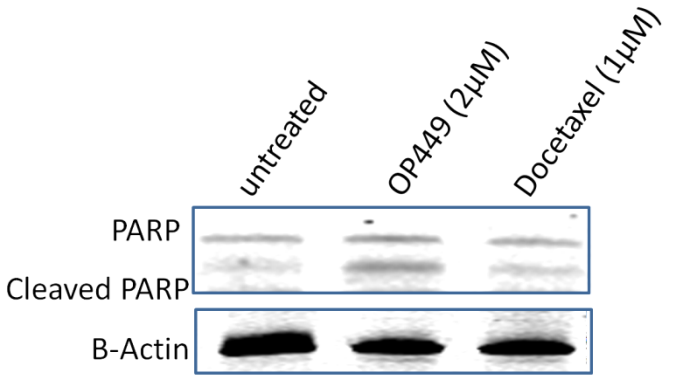


Figure 3.3 Annexin V Assay. Cells were treated with a with 2µM OP449 or 1µM docetaxel for 24 **(A)**, 48 **(B)** and 72 **(C)** hours in order to determine the induction of apoptosis in response to OP449. At each time point cells were counted and re-suspended in calcium buffer. Annexin V labelled with FITC flourochrome was added to the CA++ suspension for the detection of apoptotic cells. PI was added to the cell suspension with An V in order to detect double positive late apoptotic/necrotic. Docetaxel was used as a positive control due to its use in the clinic for the treatment of prostate cancer. Over a 72 hour period, cells transitioned from a viable state **(A)** to an apoptotic state staining positive for annexin V only. With increased time of exposure to OP449, the number of Annexin V positive cells increased. **(B & C)** As duration of exposure to the compounds increased, the cells proceeded to a late apoptotic/necrotic state. These cells were detected by staining positive for both Annexin V and Propidium Iodide. PARP cleavage in response to OP449 and docetaxel further confirmed the induction of apoptosis in PC3 cells **(D)**. Statistical significance in early apoptotic cells was determined by one way ANOVA followed by tukey post hoc analysis, **p<0.001, *p<0.05.

D



3.2.3 Morphological changes in response to ApoE mimetic peptide

Along with biochemical changes, cells also undergo morphological changes during apoptosis. Such changes include cell shrinkage, fragmentation into membrane bound bodies followed by phagocytosis by neighbouring cells [394, 395]. Previous studies in relation to programmed cell death have shown that apoptosis can be divided into biochemically and morphologically distinct phases [396, 397]. Apoptosis is initiated several hours before the morphological features become apparent [398, 399]. These morphological changes are characterised by cellular and nuclear shrinkage in addition to the compression of nuclear chromatin into sharply defined masses that become marginalised against the nuclear membranes. The nucleus progressively compacts and disintegrates. The apoptotic cells detach from their surrounding tissue and its outer membranes form intricate branch like or blebbed extensions. These membrane branches can separate from the main cell body with solid cell materials and organelles encased. These apoptotic bodies are packed with cellular organelles, nucleic splinters, membranes and mitochondria. The structures are well preserved inside the apoptotic bodies, which are rapidly engulfed by neighbouring cells. If the organelle containing bodies are not phagocytised, they undergo a degradation which resembles necrosis [394, 400].

Morphological changes of PC3 cells were assessed in untreated cells and cells exposed to OP449 and docetaxel over a 72 hour time course. Photographs were

taken of the cells under a microscope at 10X magnification. In the untreated cells, little or no changes were observed in the cell morphology although they increase in number over the time course reaching confluency and become more tightly packed at 72 hours. At 24 hours, some cell shrinkage was observed in the OP449 treated cells. As time lapsed, at 48 hours, the cells decreased in size with the occurrence of blebbing becoming apparent in some of the cells. At 72 hours, the cells have reduced further in size with some exhibiting protrusions. Many cells became detached from the petri dish. Cell numbers were considerably diminished and far from confluent. In docetaxel treated cells, only slight modification of cell shape was observed at the 24 hour timepoint, with a few cells appearing to have developed membrane projections. At the 48 hour time point, cell shape became irregular with vacuole structures become visible within the cell membranes and the cells took on an enlarged appearance. This has been initiated by the 24 hour time point, with some irregularities becoming apparent. At the final 72 hour timepoint, the docetaxel treated cells have become more rounded in appearance and have reduced in size (Figure 3.4).

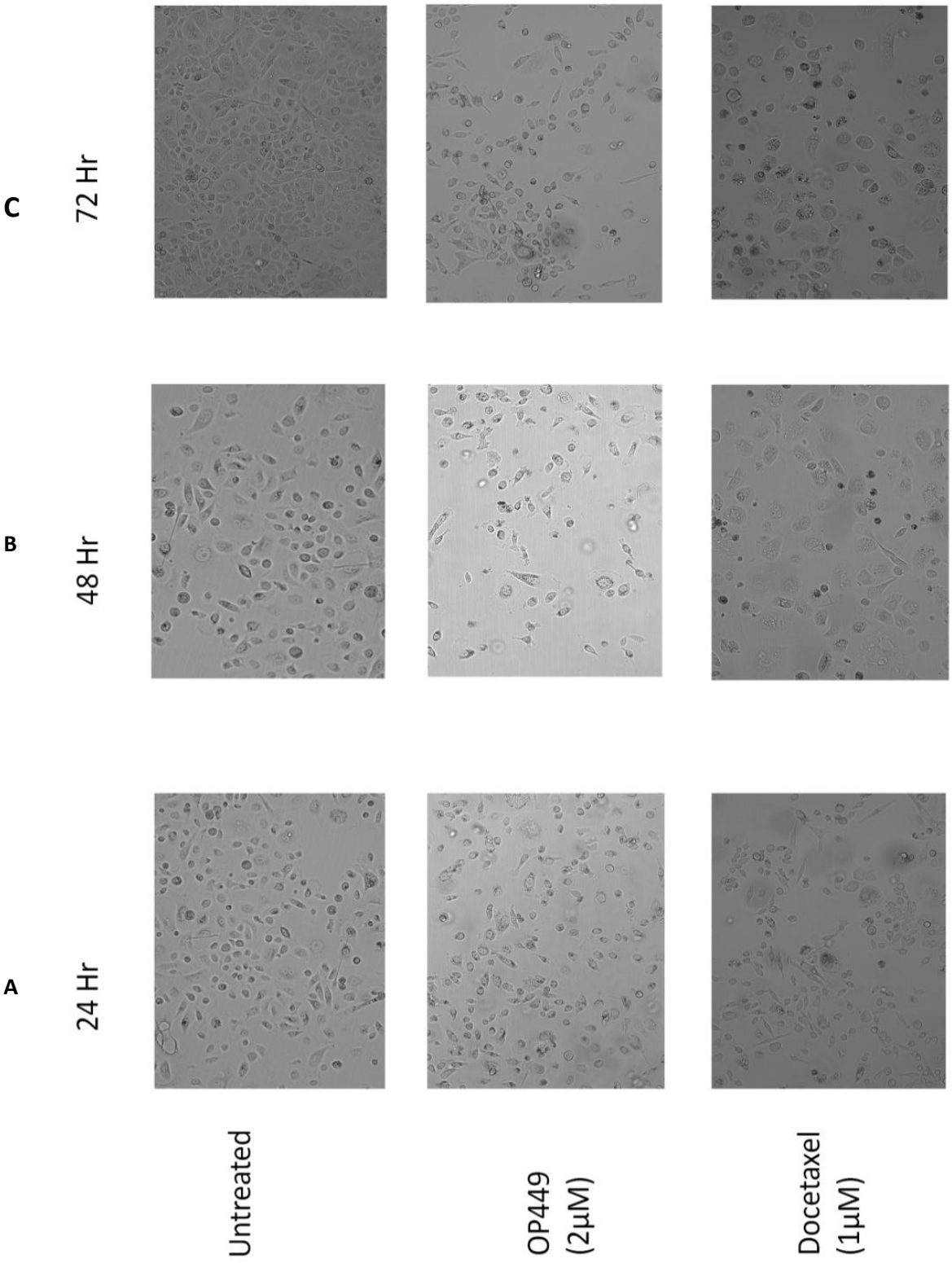


Figure 3.4 Morphological changes in response to OP449 and docetaxel. PC3 cells were either untreated **(A)** treated with 2µM OP449 **(B)** or 1µM docetaxel **(C)** for 24**,** 48 and 72 hours in order to observe morphological changes in response to OP449 and docetaxel over a 72 hour time course. The stress induced by these compounds was visible in the morphological changes observed under microscopic magnification. In contrast to the untreated controls, treated cells fail to reach confluency by 72 hours. Treated cells have taken on a rounded vacuole morphology.

3.2.3 Effect of OP449 on the cell cycle

Cell cycle analysis was performed using flow cytometry in order to investigate any influence OP449 may have on cell cycle using the Canto A cytometer. In this study, cellular DNA content was measured with the intention of verifying the distribution of cells within the major phases of the cell cycle. The cell cycle is a complex process involved in the growth, proliferation of cells regulation of DNA repair, tissue hyperplasia in response to injury and diseases such as cancer [401]. Cell cycle involves numerous regulatory proteins that direct cells through a specific sequence of events culminating in mitosis and the production of two daughter cells. Cyclin dependant kinases (cdks) and cyclin proteins are responsible for regulating the cells progression through the phases of the cell cycle. The phases have been labelled G1, S, G2 and M [401] The G1 and G2 phases of the cell cycle represent gap phases in the cycle that occur between DNA synthesis (S) and Mitosis (M) [402]. G1 phase is where cells prepare for DNA synthesis. Cells in the S phase are undergoing DNA synthesis and therefor contain aneuploidy DNA content, typically between 2N and 4N, while cells in the G2 phase are preparing for mitosis. Sub G1 or G0 account for cells not actively cycling [401]. In this study we were also able to interpret the phase profile of the cell population by classifying DNA content associated to each phase in cell cycle using PI.

The most frequently used method for measuring DNA content of cells involves the fixation and permeabilization of the cells with ethanol. The ethanol fixing permits the access and intercalation of the PI dye into the DNA grooves. PC3 cells were seeded into petri dishes at a density of $1.5X10^6$ cells per plate and left to adhere overnight. Cells were treated with OP449 at concentrations of 0, 100, 1000, 1500 and 2000nM for 24, 48 and 72 hours (2.3.2).

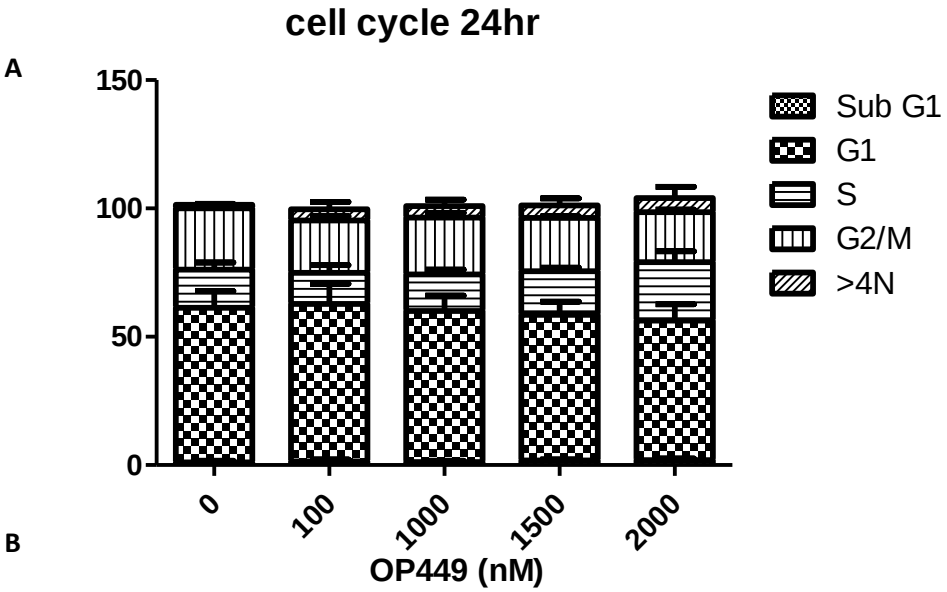
After 24 hours of exposure to OP449, approximately 60% of cells lie in the G1 phase of the cell cycle in cells exposed to 0, 100, 1000 and 1500nM OP449. At 2000nM there is a slight reduction of cells in G1 phase with 54% of cells located there. Approximately 15 % of cells lie in the S phase of the cell cycle of samples treated

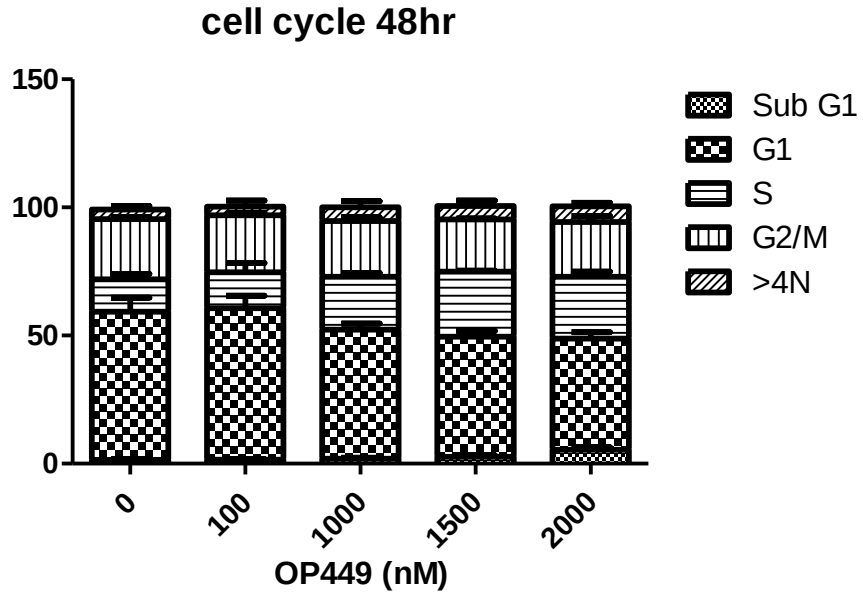
with 0, 100, 1000 and 1500nM OP449. At the highest concentration used, 2000nM, there is an increase observed in the number of cells in the S phase, with 23% of the cells resting here. The G2/M phase of the cycle accounts for approximately 20% of the cells in each sample across the entire range of treatment conditions at 24hours. As the dose increases from 0 to 2000nM there is a gradual increase in the number of cells in the sub G1 phase with the quantity increasing from 0.4% in the untreated group to 2% at 2000nM. Cells with >4N DNA content make up 2% of the untreated samples, 4% at 100nM and 2% in cells incubated with 1000, 1500 and 1% of cells incubated with 2000nM OP449 for 24 hours (Figure 3.5 A).

At the 48 hour time point of this experiment, 60% of the cells remain in the G1 phase in the samples exposed to 0 and 100nM OP449. A slight change is observed as the concentration increases and a decrease in the number of cells in the G1 phase is detected. At 1000, 1500 and 2000nM, approximately 50% of cells lie in G1. In contrast the % of cells in S phases increases from 15 % (0 and 100nM), to 20% (1000nM) to 25% (2000nM) indicating a dose dependent accumulation in S phase Within the G2/M phase, approximately 20% of cells are accounted for in each of the treatment concentrations. As the concentration of OP449 increase, there is a slight elevation in the number of cells within the sub-G1 category with 5% of cells being accounted for at 2000nM. Cells with >4N DNA content make up 2% of the untreated samples, 4% at 100nM and 5% in cells incubated with 1000, 1500 and 2000nM OP449 after 48 hours (Figure 3.5B)

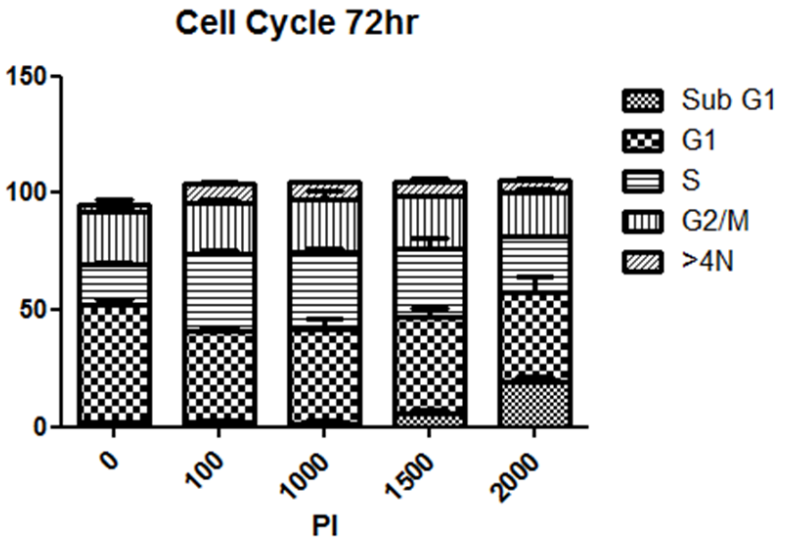
After 72 hours 50% of cells are located in the G1 phase of the cell cycle in the untreated cells. The number of cells in this phase decreased to approximately 40% at all other concentration of OP449 at this time point, ranging from 100 to 2000nM. In the untreated group, 25% lie in the S phase where as 33% of cells accumulated in this phase after exposure to 100 and 1000nM OP449. At 1500nM 30% of the cells accumulate in S phase with a decrease observed at 2000nM where 23% of cells remain. The G2/M phase accounted for 22% of cells at 0 to 1500nM with the quantity reduced to 18% at 2000nM, the highest dose OP449. Cell with a DNA content >4N make up approximately 3% of the populations across all the

concentrations at this timepoint. Cells no longer progressing through the cell cycle, having undergone arrest, make up 2% of the cells at 0, 100 and 1000nM OP449 exposure. At higher concentrations of OP449, the number of non-cycling cells increased slightly to 5% at 1500nM. A further increase in arrested cells was observed at 2000nM with 20% of cells accumulating in the Sub G1 phase. Cells with >4N DNA content make up 2% of the untreated samples, 5% at 100nM and 3% in cells incubated with 1000, 1500 and 2000nM OP449 over 72 hours (Figure 3.5 C).





C


Cell Cycle 72hr
150
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Sub G1
G1
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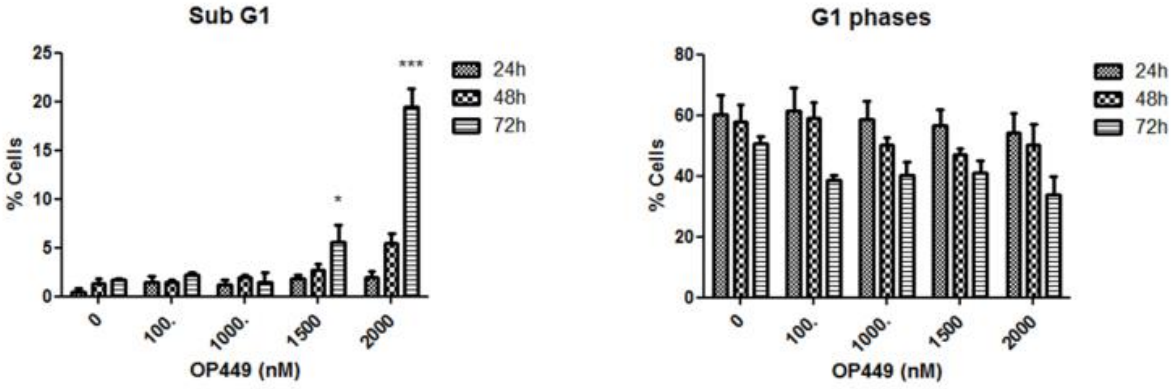
G2/M
>4N

D


Sub G1
% Cells
25
20
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*
24h
48h
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OP449 (nM)
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OP449 (nM)

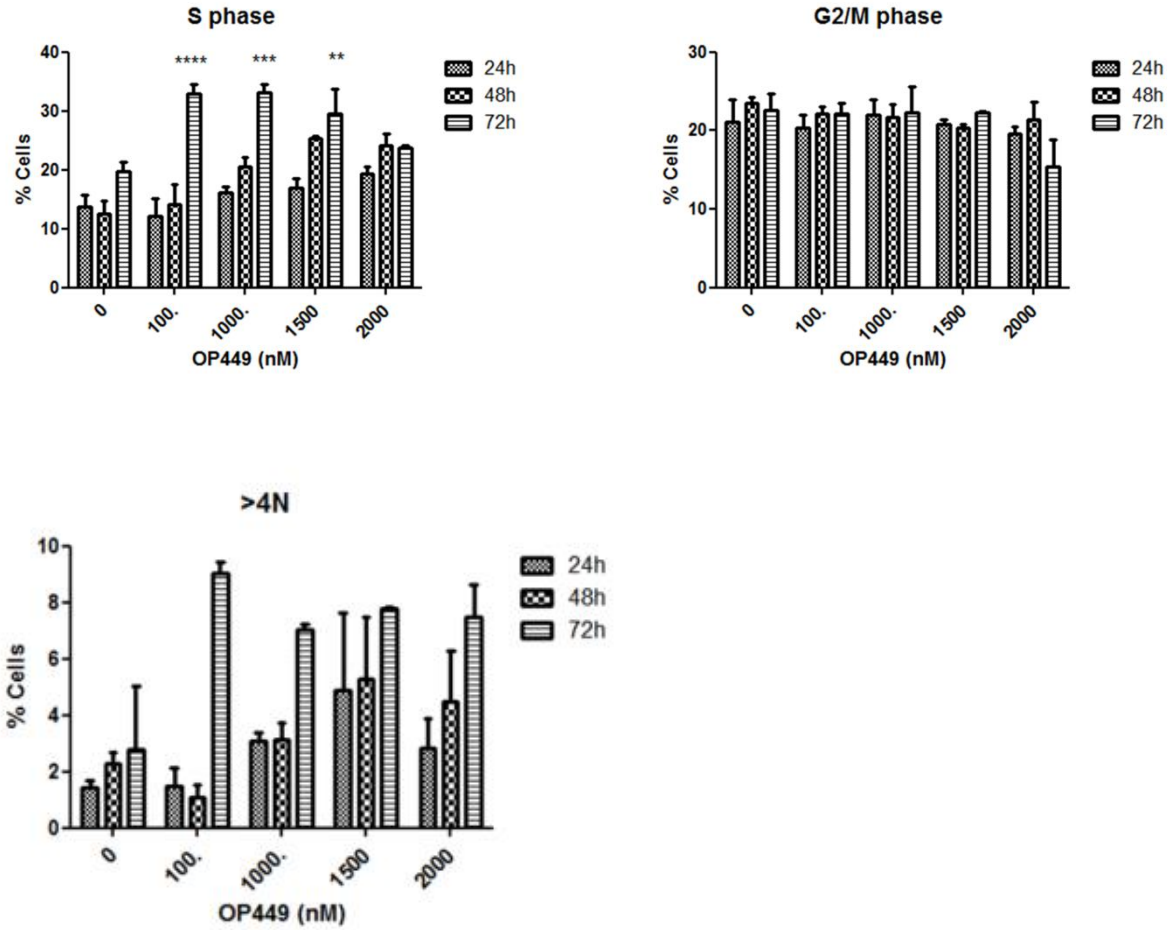


Figure 3.5 Cell cycle analysis in response to OP449. PC3 cells were treated over a 72 hour time course, counted and fixed in ethanol. Once cells were fixed they were resuspended in PI/RNASE and analysed on the CANTO A. **(A.)** After 24 hours the cell profile shows the majority of cells in the G1 phase with a slight increase in S-Phase at 2000nM treatment. **(B.)** At 48hrs, the profile follows the same trend with an increase in S-phase at 1000, 1500 and 2000nM concentrations and the occurrence of cells at >4N and Sub G1. **(C.)** At 72 hours an accumulation in S-phase is observed in response to all concentrations of OP449 along with an increase in cell number entering subG1. D Statistical significance was assessed by two way ANOVA followed by bonferroni post hoc analysis. Cell percentage in each phase of the cell cycle in response to OP449 (0, 100, 1000, 1500 and 2000nM) at 48h and 72 hours were compared to their untreated counterparts across each dose and timepoint **** P < 0.0001 *** p<0.001 p<0.01 **p<0.01, *p<0.05. P < 0.0001

3.3 Discussion

3.3.1 Authentication of ApoE mimetic peptides as cytotoxic to prostate cancer cells

Prior to investigating the interaction between the ApoE peptide mimetics provided by Oncotide, the first step was to determine the toxicity of the peptides in PCa cell lines. The alamar blue assay was optimised to ensure the most robust use of its purpose was employed. Coefficient of variation levels of 10% or lower were achieved, demonstrating regulation of the variables within the assay.

The Alamar blue assay revealed a decrease in cell number in response to both COG112 and OP449 in each of the cell lines used in the study. Although both reduced cell viability, there was a stark difference in the potency. Analysis by Anova showed OP449 induced a statistically significant decrease in cell viability at much lower concentrations than COG112 in each of the PCa cell lines (Figure 3.2). OP449 outperformed COG112 with lower IC50 values in both the AR negative CWR22, 22RV1 cells and also the more difficult to treat AR negative cell lines PC3 and DU145. There was no IC50 value calculated for the DU145 cells treated with COG112 (Table 3.1). The RWPE1, an immortalised prostate epithelial cell line, were used in this experiment as a representative of normal prostate cancer cells (Figure 3.2 E). These cells were immortalised using human papilloma virus and contain HPV sequences. In a study performed by Blanton et al., where the characteristics of epithelial cells immortalised by human papilloma viruses were explored, all transformed cell lines were reported to display morphological features which resembled premalignant lesions. Their results support the idea that immortalisation of squamous epithelial cells in culture by HPV-transforming genes generates a morphologically premalignant cell [403]. ApoE mimetic peptides have previously been reported to show limited toxicity in both whole animals and primary cells in cell culture while targeting rapidly diving cells [165, 168]. Taking these factors into consideration along with the observation that RWPE1 cells appeared to be a rapidly dividing cell line with a doubling time similar to that of the

metastatic prostate cancer cells used in this study, it is possible that the RWPE1 cells are not be a suitable representative of normal prostate epithelia.

Initial treatment for PCa includes prostatectomy or radiation to eliminate the cancerous cells that are restricted within the prostate gland. Unfortunately this approach is not a curative one for many patients as the cancer often re-occurs or metastasis has already begun prior to diagnosis [83]. As PCas are typically treated based on their discriminative dependence on androgen for growth and survival, first line therapies include androgen ablation strategies [404-407]. This treatment strategy initiates cell death or cell cycle arrest in PCa cells [408-413]. Androgen ablation instigates a regression of androgen dependent tumours, however this therapy eventually fails and patients succumb to recurrent castrate resistant prostate cancer (CRPC). CRPC is the lethal development of PC that progresses and metastasises. There is currently no effective therapy for CRPC [83, 84]. Currently the cytotoxic systemic agents used for the treatment of metastatic PCa include cytotoxic drugs docetaxel and cabazitaxel [414]. As currently used first and second line therapies are not curative for advanced and aggressive metastatic cancers, there is an urgency for new therapies capable of targeting the more aggressive cancer cell types. The ability of ApoE mimetics to significantly inhibit the growth potential of PC3 cells and DU145 cells, which represent the difficult to treat metastatic cancers, is a promising result. As COG112 and OP449 are both ApoE mimetics with the same mechanism of action, taking into account the variation in their potency, OP449 was selected for the next step of analysis to govern if cells were undergoing cell death.

The annexin V assay confirmed that OP449 was cytotoxic to PCa cells and induces cellular apoptosis. This experiment revealed not only did the ApoE peptide mimetics induce death by apoptosis in a time dependent manner but also that this compound was more effective than the traditionally used docetaxel at inducing apoptosis in the PC3 cell lines. Considering the volume of annexin v positive cells in response to OP449, there is a potential for PCa cell elimination to be amplified *in vivo,* as the exposed PS residues of early apoptotic cells could be recognised and

engulfed by macrophages. This would be worth exploring in future studies. (Figure 3.3). Given that current therapies are insufficiently effective, the use of OP449 may be feasible as an adjunctive therapeutic agent for advanced PCas although further pre-clinical testing is necessary.

The mechanism of cell death is worth exploring further, particularly in terms of apoptosis. While the results of the alamar blue assay, together with the annexin V analysis demonstrate an inhibition of cell proliferation and indicate a cytotoxic effect of ApoE peptide mimetics on PCa cells. It would also be interesting to explore the effect of the peptides on caspase activation and expression of BH3 only and Bcl2 family proteins which play a governing role in the induction of apoptosis. The BH3-only proteins of Bcl-2 family are essential initiators of apoptosis that propagate extrinsic and intrinsic cell death signals. BH3-only proteins promote apoptosis by both directly activating Bax and Bak and by suppressing the anti-apoptotic proteins at the mitochondria and the endoplasmic reticulum. Members of this diverse subset include Bad, Bim, Bid, Noxa, Puma, Bik/Blk, Bmf, Hrk/DP5, Beclin-1 and Mule [415].

Previous research using C. elegans, knock-out mice and mammalian cell-lines have shown that BH3-only proteins are critical for the induction of intrinsic apoptosis. They monitor many cellular processes and transmit death signals to the multi-region Bcl-2 family proteins at the mitochondrial outer membrane (MOM). BH3-only proteins inhibit the anti-apoptotic proteins and activate pro-apoptotic proteins thereby promoting mitochondrial outer membrane permeabilisation (MOMP)[416]. As a consequence of MOMP, proteins of the mitochondrial inter membrane space such as cytochrome c and SMAC are released into the cytoplasm where they provoke the proteolytic caspase cascade that dismantles the cell [417]. Therefore, studying the mechanisms of activation or inhibition of the membrane permeabilising proteins Bax and Bak and the activator BH3-only proteins is an important aspiration in research and would aid with the development and production of small molecules that target these processes. The effect of COG112 and OP449 have demonstrated promising results which are indicative of apoptosis

induction. The effects of the peptides on caspase activity and BH3 only and Bcl2 proteins in prostate cancer cells should be explored and is worth considering in future studies. Such experiments could include caspase activity assays and BH3 profiling .

3.3.2 Cells accumulate in the S-phases of the cell cycle in response to OP449

As the cell cycle mechanisms control cell proliferation and cancer is a disease of inappropriate cell proliferation, the cell cycle profile of PCa cells was assessed over a 72 hour time course in response to OP449 (Figure 3.5). The coordinated events that make up the process of DNA replication and cell division that is the cell cycle, are made up of distinct control mechanisms. At least two mechanisms have been identified, one of which constitutes a torrent of protein phosphorylation events that relay a cell from one phase of the cell cycle to the next. The second is a set of checkpoints which monitor the completion of critical events and delay progression to the next phase if necessary. Cell cycle regulation by phosphorylation involves a highly regulated family of kinases. The activation of each specific kinase requires association with specific cyclin subunits that are appropriately expressed at various points of the cell cycle. The cyclin and cyclin dependent kinases (CDK) form an active complex with unique substrate specificity. Regulatory phosphorylation and dephosphorylation of these CDK-cyclin complexes adjusts their activity to certify appropriate transitioning through the cell cycle phases [418]. The second form of characterised cell cycle regulation, checkpoint control, senses flaws in critical events such as DNA replication, and chromosome segregation [419]. Checkpoints can be activated due to under replicated or damaged DNA and in turn, relay signals to the cell cycle progression machinery. These signals cause a delay cell cycle progression until the danger of mutation has been averted [420]. The cell cycle checkpoints arrest or delay cell cycle progression either in the G1 phase before DNA replication, in S phase during DNA replication or in G2 phase before mitosis [420, 421]. The G1 checkpoint has been reported as compromised as a result of a

malfunctioning p53 pathway in many human cancers [422]. Consequently, cancer cells typically depend on S and G2 checkpoints for survival. Therapies that disrupt S or G2 checkpoints may selectively escalate the elimination of the cancerous cells by DNA damaging anti-cancer drugs or γ-radiation. Therefore, clarifying the molecular mechanisms of cell cycle checkpoint control is essential for more precise manipulation of the pathways for cancer therapy [423].

After surgery, radiotherapy is arguably the most common treatment of cancer, particularly for localised disease that has metastasised [423]. Although radiation is used to treat most types of solid malignancies, the degree of success varies considerably. Some tumours, such as lymphomas, are highly responsive to low doses of radiation while others such as melanoma or glioblastoma, are typically radio-resistant and tend to progress even after high radiation dosages [424]. Radiation treatment often fails in patients undergoing treatment with curable intent due to distant metastasises and local treatment site failure. There are a number of reasons radiotherapy might fail such as tumour size, location and vascular supply/hypoxia. Other factors which may be of greater importance include cellular and genetic factors such as differential tissue specific gene expression that may result in radiation resistant cellular phenotypes [425, 426]. The phases of cell cycle play a critical role in the determination of a cells relative radiosensitivity. Cells are typically most radiosensitive in the G2/M phase, Less sensitive in the G1 phase and least sensitive during the latter part of the S phase [423, 427] . This has led to the realisation that radiotherapy may work better by targeting radio-resistant cells using chemotherapeutic agents.

The cell cycle analysis from this study demonstrated that increased exposure to OP449 correlated with an increased proportion if the analysed cells detected in the S phase of the cell cycle over a 72 hour time course. At 24 hours, as concentration of OP449 increased there was a marked shift in the percentage of cells detected in the S phase with rising concentrations of OP449. Cells undergoing exposure to the ApoE peptide conformed to this profile as the proportion of S-phase cells was more pronounced at 48hrs with 25 % of analysed cells detected here at 2000nM in

comparison to 13% of the untreated population. At the final time point, 72 hours, an increase in the Sub G1 non cycling cells was observed.

While the results demonstrated that, with increasing concentration and duration of COG449 exposure the percentage of analysed cells in s-phase increased. Further investigation is required to determine if the effect of the ApoE peptide mimetics on this cell cycle pattern is a result of cells becoming blocked and unable to exit the S – phase or simply a consequence of dying cells and an overall decrease in the number of cells identified in the G2M. This could be explored by measuring DNA synthesis using a bromodeoxyuridine (BrdU) incorporation assay. This assay is used to detect DNA synthesis in vivo and in vitro. The key principle of this method is that BrdU is incorporated as a thymidine analogue, into nuclear DNA. BrdU can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle when DNA is replicated. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thereby indicating cells that are actively replicating their DNA. The colorimetric reaction produced by this assay can be detected by immunohistochemistry or monitored by flow cytometry, and using appropriate controls, it can be used for determination of proliferating properties of restricted progenitor cells. This assay could be used to identify S-phase arrest by assessing DNA replication capacity of PCa cells in response to COG112 and OP449 [428].

In terms of cell cycle progression, it would also be worth exploring the expression and status of p53 and p21 to investigate if the ApoE mimetic peptides induce a DNA damage response in PCa cells. The TP53 gene is the most frequently mutated gene in human cancer, indicating that it plays a crucial role in preventing cancer formation [429]. The *TP53* gene encodes proteins that bind to DNA and regulate gene expression to prevent mutations of the genome [430]. Although p53 mutations have also been detected in early stage prostate cancers, recent studies have found that p53 mutations are more common in advanced metastatic prostate cancer [431-435]. Interestingly, both DU145 and PC3 cells have mutant *TP53* genes, and they are also AR-negative [436]. P21 is a cyclin-dependent kinase inhibitor (CKI)

and represents a major target of p53 activity and thus is associated with linking DNA damage to cell cycle arrest [437-439]. It functions as a regulator of cell cycle progression at G_1 and S phase [440, 441]. The expression of Gamma-H2AX protein levels in response to COG112 and OP449 could also be explored in parallel with p53 and p21 as a determinant of DNA damage.

Chapter 4: Investigating the inhibition of SET and up-regulation of PP2A in response to COG112 and OP449

4.1 Introduction

The literature has well documented that PP2A as a negative regulator of multiple signalling pathways, in particular growth and survival signalling pathways such as the Akt, β-catenin and c-myc and MAPK pathways. Regulation of these pathways is often lost in cancer and their deregulation is highly associated with cancer progression. PP2A has been widely described as a tumour suppressor as, inhibition of its activity or loss of function among its subunits are implicated in neoplastic development [213].

I2PP2A/SET is a potent endogenous inhibitor of PP2A and has been reported in the literature to be overexpressed in several types of cancer including brain, lung, ovarian, Wilm tumours, leukaemia and PCa [442-450]. The SET oncoprotein plays a role in numerous cellular functions such as cell cycle regulation, gene transcription, cell migration and epigenetic regulation [187-190, 192, 212, 451]. The expression of SET has also been reported to be higher in cells with faster proliferation rates and lower in slower dividing cells. This is suggestive of a specific association for SET expression levels and cell proliferative capacity [452]. By and large, the evidence in the literature shows SET as a significant player in promoting cell growth and survival and collaborates with pathways which advocate tumour growth, progression and metastasis [212]. SET partially contributes to tumourigenesis by establishing an inhibitory complex with protein phosphatase 2A (PP2A) [453, 454]. With PP2A playing a crucial role in repressing cell signalling pathways related to cancer progression, stimulation of PP2A activity would be an appealing target for chemoprevention and chemotherapy [204, 455].

The manufacturers of the ApoE peptide mimetics have previously reported that ApoE and ApoE peptide mimetic peptides bind to and interact with the SET oncoprotein through the c-terminal region. They specifically identified amino acids 177-277 as the location of interaction [165]. The manufacturers have reported that the binding of the ApoE mimetic peptides to the SET oncoprotein prompts the

stimulation of PP2A and the conformational dephosphorylation of downstream targets of PP2A, thereby acting as a SET inhibitor [165]. With PP2A being an endogenous regulator of such pathways correlated with cancer development and whose function is typically suppressed in cancer cells, pharmacological manipulation of PP2A activity is an appealing chemotherapeutic strategy [204, 455-460]. The aim of this study was to determine expression of these proteins in PCa cells and establish if the compound efficiently targeted their activation and downstream effects. This information would support the theory of targeting SET in order to enhance the activity of PP2A in PCa, as a valid approach for the development of future therapies in PCa treatment. The COG112 and OP449 compounds are based on a short fragment of ApoE protein which has previously been reported to bind to SET and increase the activity of PP2A [454, 461]. This study focuses on targeting SET with apolipoprotein E (ApoE) mimetics in order to restore PP2A tumour suppressor function and the identification of downstream targets with the aid of the reverse phase protein array platform. The protein and RNA expression of SET in prostate epithelia was assessed and compared to that of PCa cell samples.

The rational of our focus is supported by recent contributions to the literature. In particular, our observation of increased SET expression in prostate cancer is supported by the SET profile data available from the NCBI GEO profile database. The expression profiles available within the data sets also indicated a trend of increased SET expression in PCa corresponding with recurrent disease and higher expression of SET recorded in metastatic PCa when compared to primary tumours and benign samples. The complimentary findings of a simultaneously performed study by Hu et al., 2015, reported that enhanced SET expression is sufficient to induce pathological alterations in wild type (WT) murine prostate epithelium and suggest that increased SET expression correlates with progression to androgen independence and biochemical recurrence. [462]. Their exploration of OP449 in a PTEN knockdown model of PCa also corroborated with our finding of Akt inhibition in AR positivie and AR negative cell lines [438].

4.2 Results

4.2.1 PP2A and SET are expressed in prostate cancer cell lines

Expression of SET and PP2A at a basal level in RWPE1 and PCa cell lines was investigated by isolating protein from PCa cells and probing for the proteins of interest by western blot (2.4.3). Cell lines were grown in petri dishes to 80% confluency at 37°C, 5% CO2. They were then washed in PBS and lysed using RIPA Buffer supplemented with protease cocktail inhibitor. Protein lysate was quantified by BCA Assay. The expression of SET in PCa cells was confirmed by western blot (Figure 4.1 A). As the expression of SET has previously been confirmed in breast cancer cell lines, MDA-MB-231 and MDA-MB-468 were used as positive controls [212]. Expression of the oncogene was confirmed by western blot in the transformed RWPE1 cells, both the AR negative PCa cell lines CWR22 and 22RV1 and the AR positive cell lines PC3 and DU145. Expression of PP2Ac was also verified in each of the cell lines.

Primary prostate epithelia (ATCC) were also assessed for the expression of SET by western blot in order to verify if the expression differs from PCa cell lines in comparison to normal prostate epithelial cells. Protein lysate was obtained from cultured primary epithelia and quantified by BCA. Primary prostate epithelia lysate was compared to expression levels of SET in PC3 cell lysate. The results showed no expression of SET at the protein level in the normal prostate cell lysate. (Figure 4.1 B).

RNA isolated from PCa tumour and 'normal' matched patient sample biopsies were gifted from Dr. Stefan Ambs, Laboratory of Human Carcinogenesis, National Cancer Institute, National institutes of health, Bethesda, Maryland, USA. The samples comprised of 6 matched pairs. Of the 6 patients from whom samples were taken, two were of African American decent while the rest are from European-Americans. Samples were pooled to obtain an average of SET expression in tumour and tumour adjacent tissue. The Ambs Prostate cancer case control Study was approved by the

NCI (protocol #05-C-N021) and the University of Maryland (Protocol #0298229) institute review boards. The Isolated RNA from patient matched tumour and tumour adjacent biopsies were analysed by real–time PCR to evaluate any difference in SET expression at an mRNA level. RNA was quantified using the nanodrop followed by cDNA synthesis using Bioline Sensifast cDNA synthesis technique. SYBR Green based Real-Time PCR was then performed using the Sensifast Hi-ROX mix from Bioline and KiCq start Sigma Primers on the Step one PCR machine. B-actin primers were used for housekeeping. The results exhibited a two-fold greater expression of SET mRNA levels in the prostate tumour samples when compared to the non-cancerous adjacent tissue. These results establish that SET RNA levels were elevated in patient tumour samples when compared to non-cancerous adjacent tissue. (Figure 4.1C). This signifies that the SET oncogene may be a valid target for the treatment of PCa.

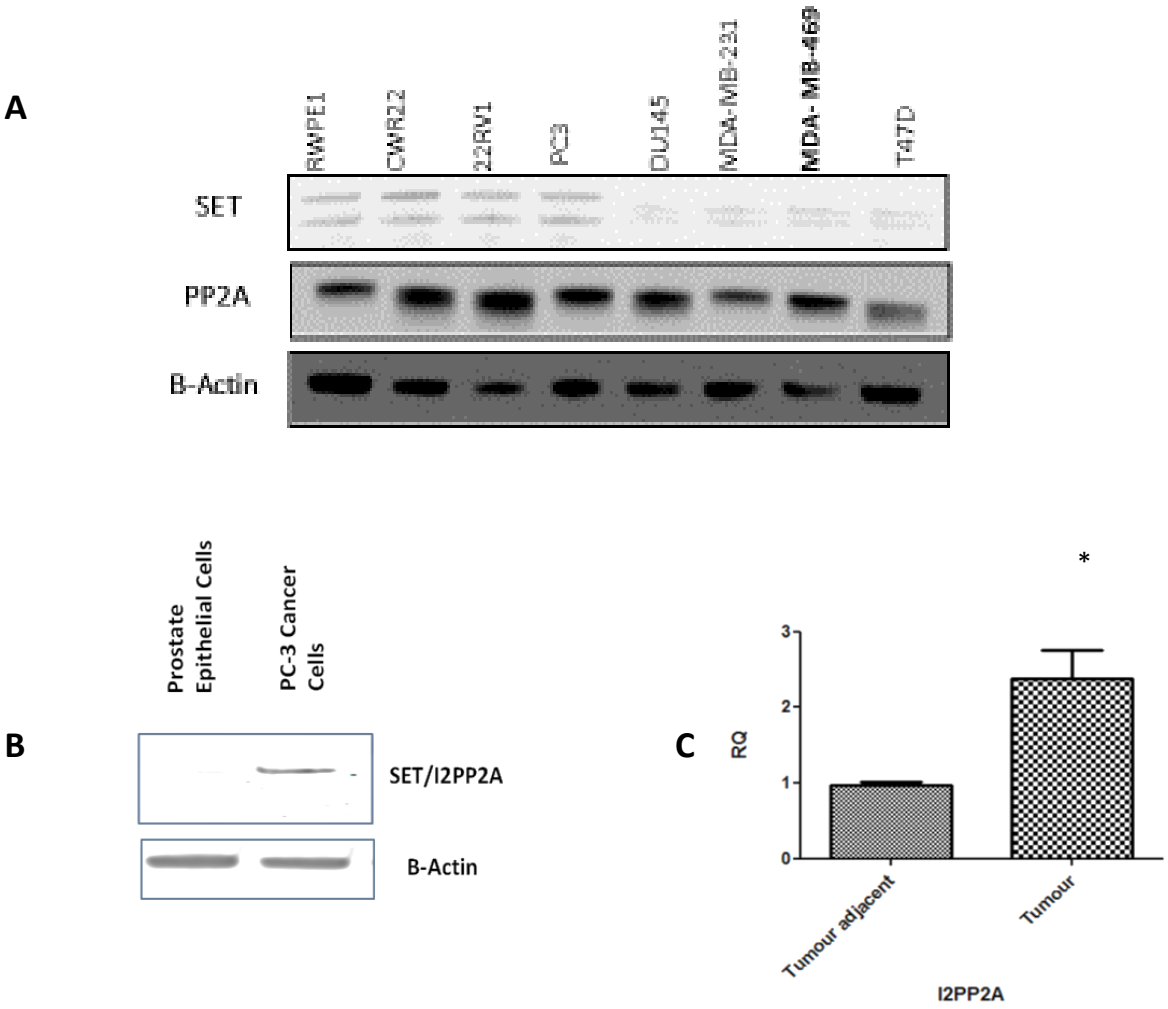


Figure 4.1 Identification of SET and PP2A expression in prostate cancer cancer Expression of SET and PP2A was identified In normal prostate epithelia and PCa at the RNA and protein level by qRT-PCR and western blot respectively. **A)** RWPE1 cells together with both androgen positive (CWR22 and 22RV1) and androgen negative cell lines (PC3 and DU145) were screened for basal expression of SET and PP2A at a protein level. Protein from breast cancer cell lines MDA-MB231 and MDA-MB469 and T47D were used as positive controls. MDA-MB-231 has been identified as a positive control for SET and PP2A expression. **B)** Protein isolated from normal prostate epithelia were probed for the expression of the SET oncoprotein by western blot. SET was undetectable at a protein level in the normal prostate epithelia. **C)** RNA isolated from patient biopsies was analysed by qRT-PCR in order to establish any difference in levels of SET between noncancerous and cancerous tissue of the prostate. SET expression was found to be two-fold higher in RNA isolated from the tumour biopsy core in comparison to RNA isolated from noncancerous tissue adjacent to the tumour site. Data analysis was performed using unpaired T-Test in order to confirm statistical significance *$p<0.05$

4.2.2 ApoE peptide mimetic binds to SET in prostate cancer cell lines

The manufacturers of the ApoE peptide mimetics suggest that the peptides interact with the SET oncogene in immune cells [165]. In order to explore the merit of this theory in PCa cells and confirm that the ApoE peptide mimetics actually do interact with the SET protein in PCa cell lines, a biotin pull down assay was performed. Either biotin labelled COG112 or biotin alone was incubated with PC3 cell lysate. Streptavadin beads were used to immunoprecipitate biotin or the biotin labelled drug out of the respective samples, bringing with it, any bound proteins, thus allowing for their identification. The flow through and elution samples were assessed by western blot to determine the binding of SET to the biotin labelled compound. Analysis revealed that lysate incubated with biotin alone did not result in protein pull down while SET was identified in the sample lysate which was incubated with the biotin labelled COG112 (Figure 4.2 A). This result corroborates with our hypothesis that the ApoE peptide mimetics do hold the capacity to bind to the SET oncoprotein in PCa cells. Detection of the protein indicates that the ApoE mimetic compound does interact with the SET oncogene.

After confirming that the ApoE mimetic is capable of binding to SET in PCa cells, the next step was to determine if the compound was capable of disrupting the interaction that occurs between SET and PP2A. As previously described, PC3 cells were seeded into petri dishes at a density of $1X10^6$ prior to 24 hour incubation with increasing concentrations of OP449. Cells were washed and lysed with RIPA buffer supplemented with complete protease cocktail inhibitor followed by protein quantification using the BCA assay. PP2A was isolated by immunoprecipitation and the integrity of the SET-PP2A complex was evaluated by western blot. As the concentration of the ApoE peptide increases, a decrease in the expression of SET bound to PP2A was established. There was also an increase in the levels of SET observed in the flow through samples as the drug concentration increased (Figure 4.2 B). This finding indicates that the ApoE mimetics interfere with the SET-PP2A protein complex and may likely disrupt the inhibitory effects of SET on PP2A.

After verifying that the ApoE peptide mimetics are capable of binding to the SET oncogene and displace the SET-PP2A complex that occurs with tumour formation and progression, it is plausible to suggest that that the inhibitory influence of SET on PP2A is also interrupted and the separation of SET from PP2A may allow for increased phosphatase activity of PP2A. The congregation of the PP2A subunits is strictly coordinated for the most part by specific post translational modifications such as methylation and dephosphorylation of the C-terminal tail of the catalytic c-subunit in order to attain the intricate functioning of PP2A [213]. The anatomical structure of PP2A can be modified either by phosphorylation of the C subunit at Tyr307 thereby preventing methylation, and successful formation of PP2A complexes while phosphorylation at Th304 constrains the enrolment of B subunits, both of which provoke the disarming of PP2A [213, 463]. With the purpose of exploring adjustment in the activity status of PP2A in response to OP449, PC3 cells were treated with increasing concentrations of OP449 for 24 hours. Cells were then washed and lysed as previously described and protein was quantified by BCA. Western blotting was used to characterise the phosphorylation status of PP2A (Figure 4.2 C). A decrease in phosphorylation was confirmed in response to OP449, which correlated with drug concentration in a dose dependant manner. This further alludes to the postulation that treatment with ApoE mimetic peptides results in pharmacological restoration of PP2A activity by binding to SET and obstructing the SET-PP2A pro tumourigenic complex resulting in a surge of PP2A signalling.

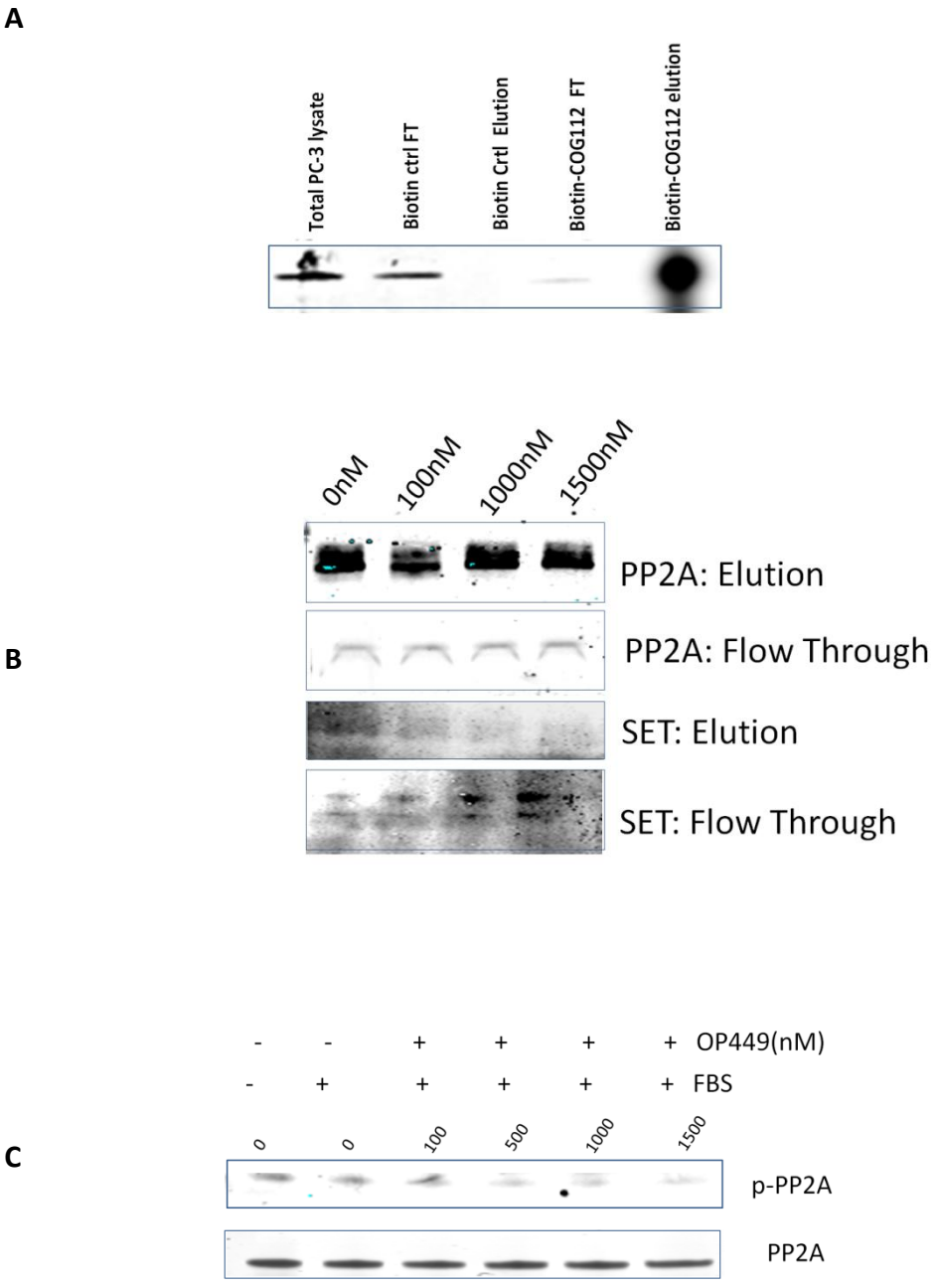


Figure 4.2 COG112 and OP449 interact with with SET and PP2A complex. A Biotin pull down assay was employed to determine if ApoE mimetic peptides were capable of binding to SET in PCa cells. Biotin only or biotin labelled COG112 was incubated with PC3 lysate. Biotin was isolated from the supernatant using streptavidin beads . Flow through (FT) and elution samples were analysed for presence of SET. SET protein bound to the biotin labelled drug was identified by western blot (far right lane) **(A)**. PC3 cells were incubated with 0, 100, 1000 and 1500nM OP449 over 24 hours. Cells were lysed and PP2A was isolated from each sample by immunoprecipitation and analysed by western blot. Results show that with increasing concentration of OP449 there is a decrease in the expression of SET protein bound to PP2A. There is a correlating increase in levels of SET in the flow through with increased concentration of OP449 indicating more non-PP2A bound SET results due to OP449 **(B)**. Phosphorylation of PP2A was shown by western blot to decrease in a dose dependant manner in response to OP449. This is indicative of increased activity of PP2A **(C)**.

4.2.3 The effect of OP449 on Akt associated signalling

In normal cellular function, phospho-regulation is maintained by a balance of phosphatase, and kinase activity [464]. Disruption of this system is a key mechanism by which cells escape normal cell regulation signals and malignant transformations occur. Many cancers are distinguished by an abnormal level of kinase activation whose activity is sometimes sufficient and frequently essential to provoke the onset of cancer [213]. Cancer cells are typically classified by modifications which result in a loss of tumour suppressive gene function and unrestrained proto-oncogene activity, which perversely amplify cell proliferation and survival capacity [465]. As many cancer provoking signals embroil serine, threonine or tyrosine phosphorylation, which act as an on/off switch for target proteins, it is reasonable to suggest that counteracting these kinases with a serine threonine phosphatase is a good anti-cancer treatment strategy [464]. As PP2A is the most abundant serine/threonine phosphatase in mammalian cells and the ApoE mimetics are capable of replenishing PP2A activity, it is rational to speculate that modulation of known PP2A targets and downstream signalling occurs in response to ApoE peptide mimetic OP449 exposure. In order to identify proteins and kinases modulated by ApoE peptide mimetics, namely OP449, a screen was performed using Reverse Phase Protein Array (RPPA) as a technique to identify target pathways (2.7.1). Proteins explored in the screen incorporated PI3K/Akt/mTOR and MAPK related signalling, cell cycle and apoptosis associated proteins.

The PI3K-Akt pathway regulates many normal cellular processes including proliferation, survival, growth and motility, all of which are critical for tumour genesis (Figure 4.3). The role of this pathway in oncogenesis has been extensively studied with altered and mutated components of the pathway being widely implicated in cancer formation and progression [466]. Akt is partially activated through phosphorylation at Threonine 308 by PDK1, while full activation is achieved by the additional phosphorylation at serine 473 in the C terminus of Akt

[467, 468]. Akt in turn regulates a wide range of target proteins that control cell proliferation, survival, growth to name a few. The Mammalian target of (mTOR) pathway is activated at least in part by the PI3K-Akt pathway which in turn controls cell growth by activating 70s6k [469].

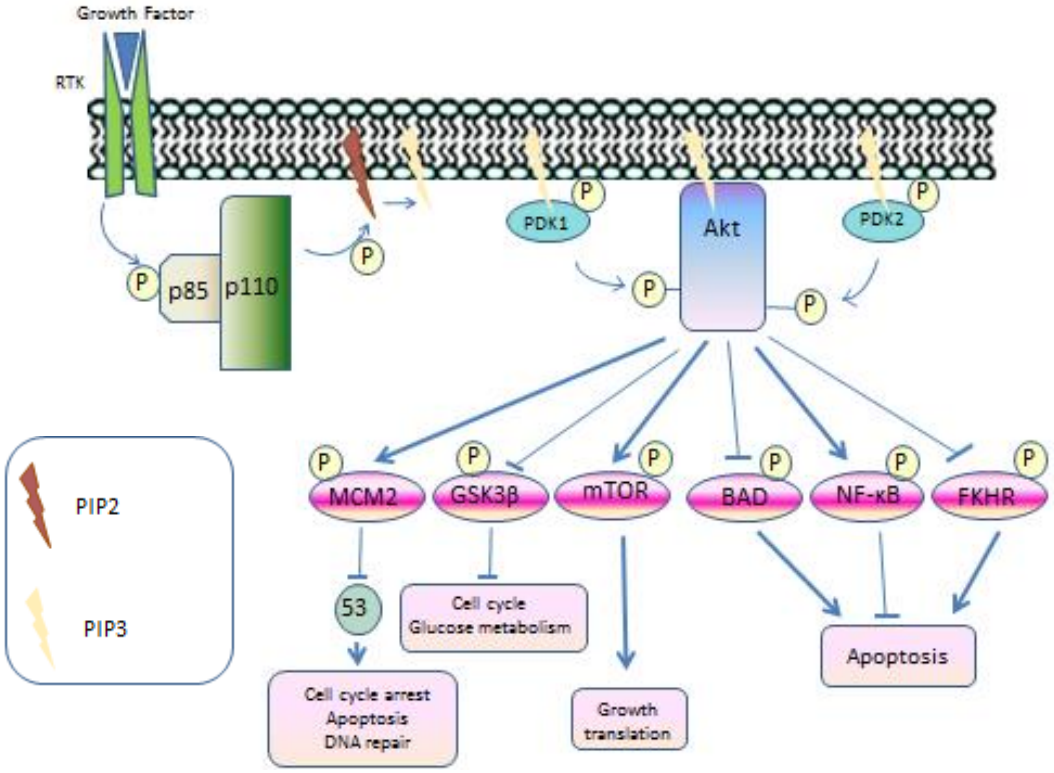


Figure 4.3 The phosphatidylinositol 3-kinase-Akt signalling pathway. Activation of PI3Ks by receptor tyrosine kinases (RTKs) activation and the associated congregating of receptor – PI3K complexes. These composites localise at the membrane where the p110 subunit of PI3K catalyses the conversion of PtdIns(4,5)P_2 (PIP_2) to PtdIns(3,4,5)P_3 (PIP_3). PIP_3 serves as a second messenger that helps to activate Akt. Through phosphorylation, activated Akt mediates the activation and inhibition of several targets, resulting in cellular growth, survival and proliferation through various mechanisms. (Adapted from vivanco and Sawyers., 2002)

Among the Akt related targets, the RPPA screen unveiled modulation of Akt, mTOR and P70sk6 activation (Table 4.1). The phosphorylation of Akt at Ser472 and T308 was assessed in response to OP449. Phosphorylation at ser473 was downregulated in response to OP449 in both AR positive cell lines CWR22 and 22RV1, and AR negative PC3 and DU145 cell lines. In CWR22 and 22RV1 there was no decrease

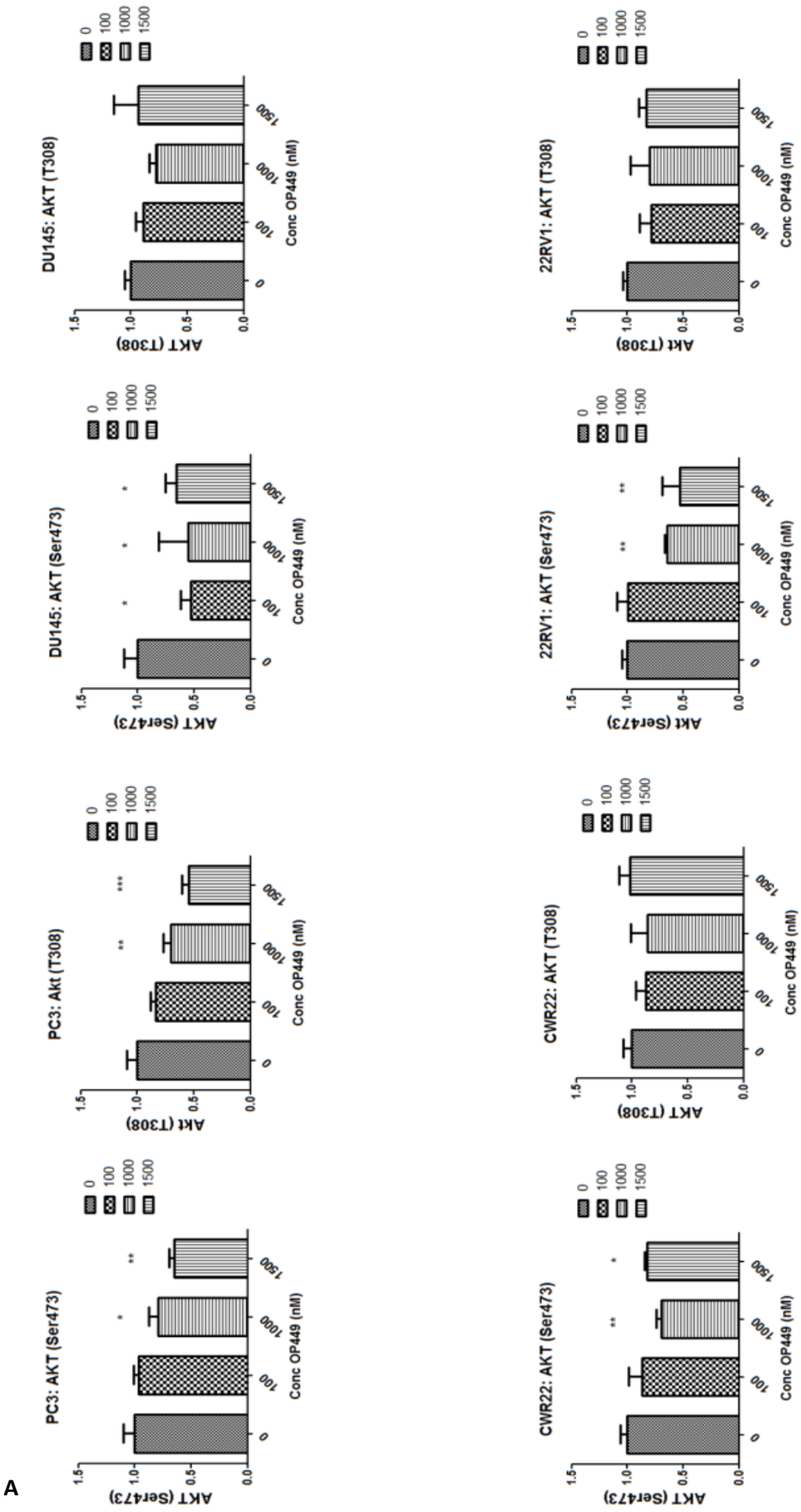
detected at 100nM when compared to control however a statistically significant reduction was observed at 1000 and 1500nM. PC3 cells saw a decrease at 1000 and 1500nM while a significant decline was detected in DU145 at 100, 1000 and 1500nM when compared to untreated samples. At T308, there was no significant decrease in phosphorylation detected in the CWR22, 22RV1 or DU145 cell lines although PC3 cells saw a reduction of phosphorylation of statistical relevance at 1000 and 1500nM (Figure 4.4 A).

Modulation of mTOR phosphorylation at S2481 and S2488 residues was investigated. There was a noted decrease in phosphorylation of mTOR at S2481 in only the DU145 cell lines at 1500nM. Downregulation at the S2488 site was detected at 1000 and 1500nM in the 22RV1 cells and from 100nm, 1000 and 1500nM in the PC3 cells when compared to their untreated control counterparts (Figure 4.4 B). Downstream of PI3K and mTOR, the screen demonstrated that P70s6k is dephosphorylated in response to OP449. Phosphorylation at T389 was decreased in CWR22 at 1000 and 1500nM. A decline in phosphorylation was also measured in the PC3 cells at 1500nM (Figure 4.4 C). Other Akt related proteins assed in this screen which did not show sufficient modulation in response to OP449 include PDK1 (S241), PI3-Kp110alpha, PTEN, VEGFR2(55) FAK, MET (T1234) and Ribosomal protein (s240/244)/(235/236) (Table 4.1).

The Akt-mTOR signalling cascade has emerged as a critical contributor to the oncogenic properties of the PI3K-Akt pathway and subsequent inhibition corresponds with an increase activity level of PP2A. PP2A has been well documented as a direct inhibitor of Akt and so increased PP2A activity in response to OP449 is a likely mechanism by which this cascade has been skewed [468].

Table 4.1 Reverse phase protein array: Statistical analysis of Akt related proteins

One Way ANOVA																				
* $p<0.05$		** $p<0.01$		*** $p<0.0001$																
	CWR22				22RV1				PC3				DU145							
	0	100	1000	1500	0	100	1000	1500	0	100	1000	1500	0	100	1000	1500				
Akt(S473)			**	*			**	**			*	**		*	*	*				
Akt(T308)							*	*			**	***								
mTOR(S2488)										*	*	*								
mTOR (S2481)																*				
P70S6K(T389)			**	**								*								
PDK1 (S241)				*																
PI3-Kp110alpha																				
PTEN																				
VEGFR2(55)																				
FAK																				
MET(T1234)																*				
RIBOSOMAL PROTEIN (S240/244)																				
S6RibP(S235/236)(2F9)																				


A
PC3: AKT (Ser473)
PC3: Akt (T308)
DU145: AKT (Ser473)
DU145: AKT (T308)
CWR22: AKT (Ser473)
CWR22: AKT (T308)
22RV1: AKT (Ser473)
22RV1: AKT (T308)
AKT (Ser473)
AKT (T308)
Conc OP449 (nM)
0
100
1000
1500

B

DU145: mTOR (s2481)
DU145: mTOR (s2488)
22RV1: mTOR (s2481)
22RV1: mTOR (s2488)
PC3: mTOR (s2481)
PC3: mTOR (s2488)
CWR22: mTOR (s2481)
CWR22: mTOR (s2488)
mTOR (s2481)
mTOR (s2488)
OP449 (nM)
0
100
1000
1500
0.0
0.5
1.0
1.5

C
D
E
PC3: P70S6K (T389)
DU145: P70S6K (T389)
CWR22: P70S6K (T389)
22RV1: P70S6K (T389)
PC3: PDK1 (S241)
DU145: PDK1 (S241)
CWR22: PDK1 (S241)
22RV1: PDK1 (S241)
PC3: PI3-Kp110alpha
DU145: PI3-Kp110alpha
CWR22: PI3-Kp110alpha
22RV1: PI3-Kp110alpha
P70S6K (T389)
PDK1 (S241)
PI3-Kp110alpha
OP449 (nM)
0
100
1000
1500

F
PC3: PTEN
DU145: PTEN
CWR22: PTEN
22RV1: PTEN
PTEN
G
PC3: VEGFR2(55)
DU145: VEGFR2(55)
CWR22: VEGFR2(55)
22RV1: VEGFR2(55)
VEGFR2(55)
H
PC3: FAK
DU145: FAK
CWR22: FAK
22RV1: FAK
FAK
0
100
1000
1500
OP449 (nM)

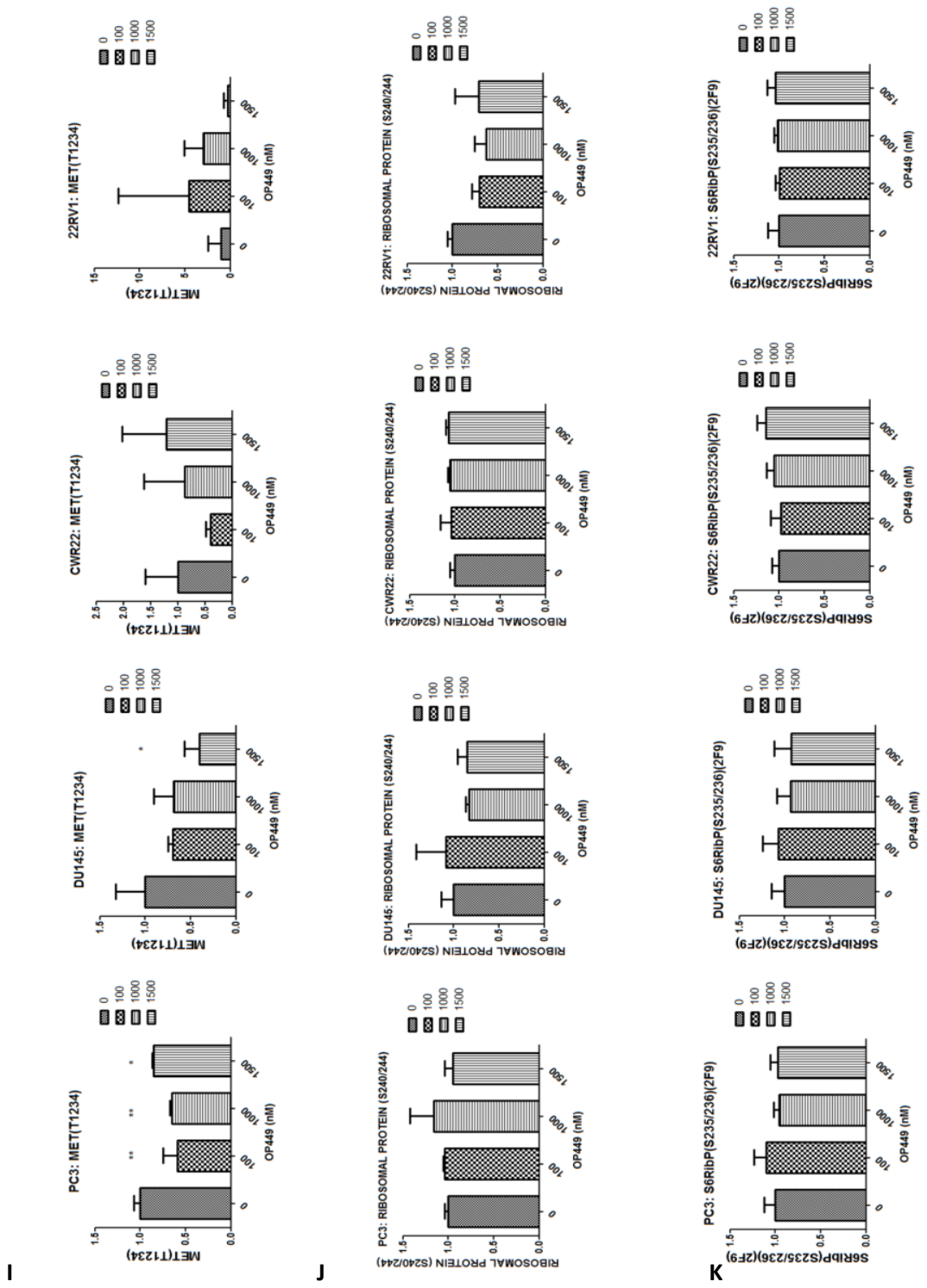


Figure 4.4 PI3K/AKTmTOR related signalling targets identified using the reverse phase protein array platform. The RPPA screen was implicated to identify alteration of the PI3K-AKT signalling cascade in response to OP449. A decrease in the phosphorylation of Akt at Ser473 and Thr308 in PC3, DU145 (AR negativie) and CWR22 and 22RV1 (AR positive) in response to increasing OP449 concentrations. mTOR and 70s6K were also identified as targets downregulated by OP449. MET (T1234) was down regulated in PC3 and DU134 cell lines. Components further upstream to Akt in the signalling cascade, PI3K, PTEN, PDK1, P53 were not demonstrated to be down regulated. Data analysis was performed using one-way Anova and Tukey post hoc test in order confirm modulation as significant.*denotes significance *p<0.05 **p<0.01 ***P<0.001

4.2.4 OP449 modulates MAPK related signalling

The MAPK pathway, in parallel with the Akt pathway, contributes to the regulation of cell cycle progression. The frequency in which this pathway is aberrantly activated has been elucidated in the literature. This has been shown in particular association with upstream epidermal growth factor receptor (EGFR) and the Ras small guanosine triphosphatases (GTPases). The up-regulation of ERK-MAPK promotes cell proliferation, cell survival and metastasis. This supports current endeavours to identify and develop approaches to pharmacologically block the EGFR-Ras-Raf-MEK-ERK MAPK signalling for the treatment of cancer [470] (Figure 4.5).

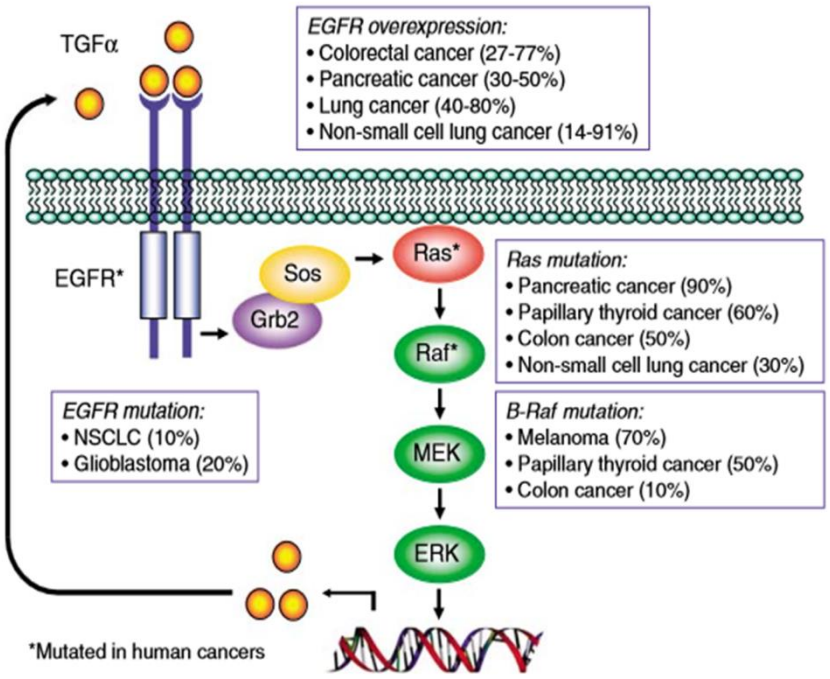


Figure 4.5 Activation of ERK MAPK cascade in cancer. Oncogene activation of the ERK MAPK cascade, mutually activated B-raf, Ras and mutually activated (by missense mutations in the cytoplasmic domain in NSCLC or by extracellular domain transductions (e.g. VIII) in gliobastomas) and/or overexpressed EGFR causes persistent activation of the ERK MAPK cascade in human cancers. Activated ERKS translocate to the nucleus, where they phosphorylate and regulate various transcription factors leading to changes in gene expression. In particular, ERK mediated transcription can result in the upregulation of EGFR ligands such as TGFα, thus creating an autocrine feedback loop that is critical for ras mediated transformation and Raf mediated gene expression changes. Adapted from *Oncogene* (*2007*) *26*, *3291–3310*

The extracellular signal-regulated kinase (ERK) MAPK pathway has been subject to intense research scrutiny leading to the development of pharmacological inhibitors for the treatment of cancer. To briefly describe this pathway, ERK is a downstream component of a conserved signalling module that is activated by the Raf serine/threonine kinases. Raf activates the MAPK/ERK kinase (MEK)1/2 dual specificity protein kinases which then activate ERK1/2. The activation of Raf in human cancers supports the important role of this pathway in human oncogenesis. The Raf-MEK-ERK pathway is a key downstream effector of the Ras, which in turn is a key downstream target of the epidermal growth factor receptor (EGFR) which is also frequently mutated and overexpressed in cancer [470]. ERK activation promotes upregulated expression of EGFR ligands, promoting an autocrine growth loop critical for tumour growth and progression. For these reasons, the EGFR-Ras-Raf-MEK-ERK signalling network has been the subject of intense research and pharmaceutical scrutiny to identify novel target-based approaches for cancer therapy. In light of this, some components of this signalling cascade were included in the RPPA screen (Table 4.2).

Among the findings, phosphorylation of P38MAPK (T180/182) was found to be decreased in both AR positive cell lines and both AR negative cell lines used in the screen. Dephosphorylation was observed at 1500nM in CWR22 cells while a decrease was measured at 1000nM and 1500nM in 2RV1 cells. A reduction in phosphorylation was detected at 100, 1000 and 1500nM in the PC3 cells exposed to OP449. P38MAPK was also down regulated in the DU145 cells at 1500nM (Figure 4.6 A).

Significant levels of MAPK-ERK (T202/Y204) dephosphorylation were observed in the 22RV1 (AR positive) cell line at 1500nM. Dephosphorylation of statistical relevance was also detected in the PC3 cells (AR negative) in response to OP449 at 100, 1000 and 1500nM (Figure 4.6 B).

Noteworthy dephosphorylation of MEK1/2 at serine21 of statistical significance was detected only in the AR negative cell lines, PC3 and DU145. In the PC3 cells, the earliest reduction in phosphorylation was detected at 100nM and was maintained at 1000 and 1500nM. In the DU145 cell line a decrease was also observed at 100, 1000 and 1500nM OP449 (Figure 4.6 C).

The phosphorylation status of EGFR was explored within the RPPA screen at sites Y992, Y1086 and Y173. EGFR showed a notable decrease in phosphorylation at Y992 in CWR22, 22RV1 and PC3 cell lines. A gradual trend in down regulation was observed in the CWR22 and a significant depletion was calculated at 1500nM. A statistically significant decrease in phosphorylation was calculated within the 22RV1 samples at 1000 and 1500nM OP449. Significant dephosphorylation at the Y1173 residue was identified in the CWR22, 22RV1 and DU145 cells lines. The decrease was calculated to occur in the CWR22 cell line at 1000nM whilst it was detected at 100, 1000 and 1500nM in the 22RV1 cell line. The AR negative cell line DU145 saw a decline in phosphorylation at 1000nM (Figure 4.6 D).

Dephosphorylation of EGFR discourages the binding of SRC domains at these sites Phosphorylation of SRC Y416 and Y572 within the screen. Down regulation was detected at the Y416 residue in the AR positive CWR22 and 22RV1 cells at 100, 1000 and 1500nM in both cell lines. There was no marked decrease detected in the AR negative cell lines. There was no significantly relevant reduction detected at the Y572 site in the CWR22, 22RV1 or PC3 cell lines. DU145 returned a decline at 1500nM (Figure 4.6 F).

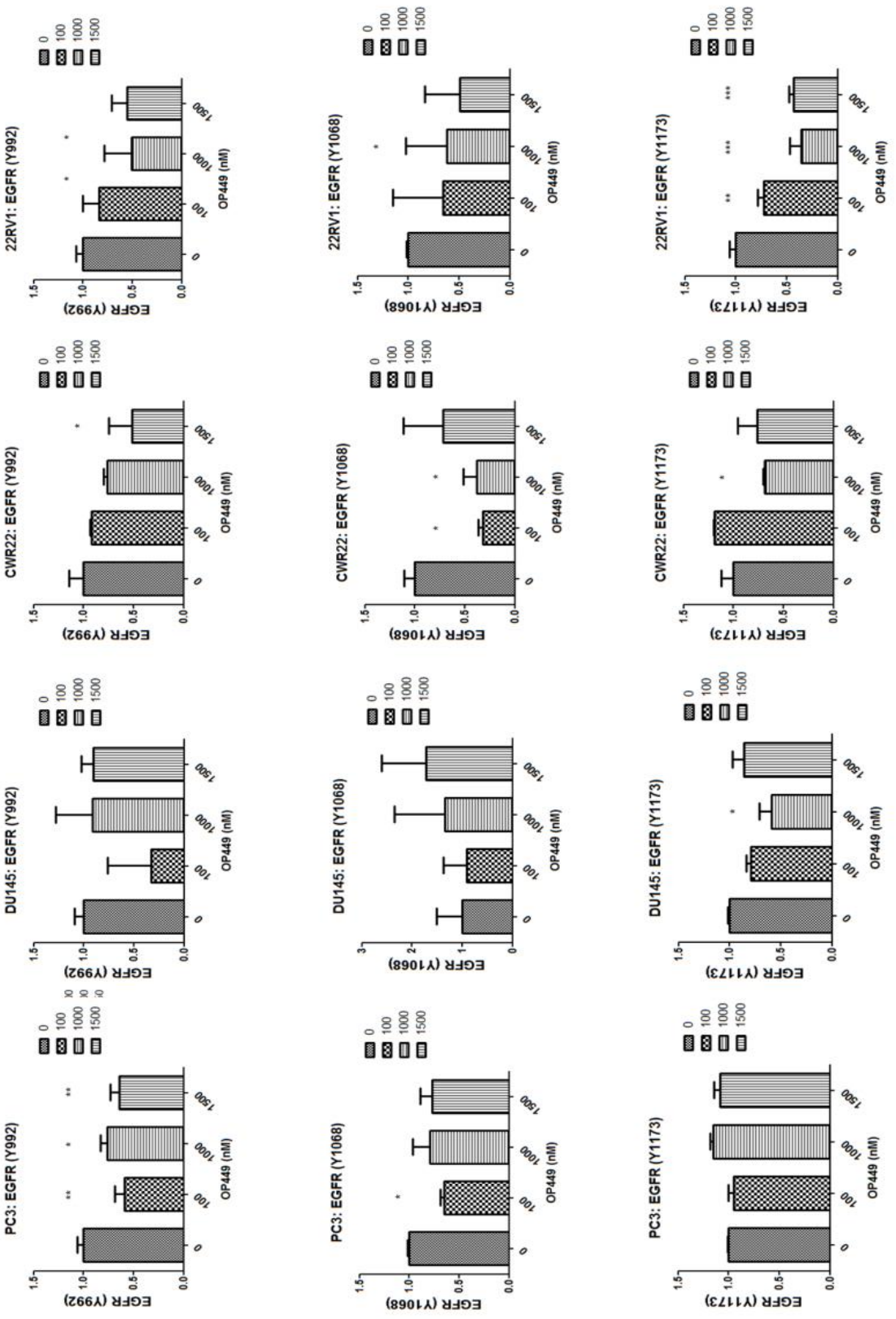
A decrease in C-Raf phosphorylation was detected only in the AR negative cell lines, PC3 and DU145. The reduction was observed in PC3 cells at the lowest concentration 100nM and was maintained at 1000 and 1500nM OP449. A decrease in phosphorylation was seen in the DU145 cell line at 1000 and 1500nM, however although there is an apparent trend, only the 1500nM concentration was gauged as statistically significant (Figure 4.6 E).

Other targets assessed in the screen include AMPK (T172), SHC (Y317), SRC (Y57) and EGFR (Y1068). There was no sufficient modulation detected within these targets in response to OP449 using the reverse phase protein array platform (Figure 4.6 G). As modulation of key MAPK signalling components were detected in this screen, it is reasonable to deduct from the findings that the OP449 peptide inhibits the MAPK- ERK pathway at various sites of the cascade.

Table 4.2 Reverse phase protein array: Statistical analysis of MAPK related proteins

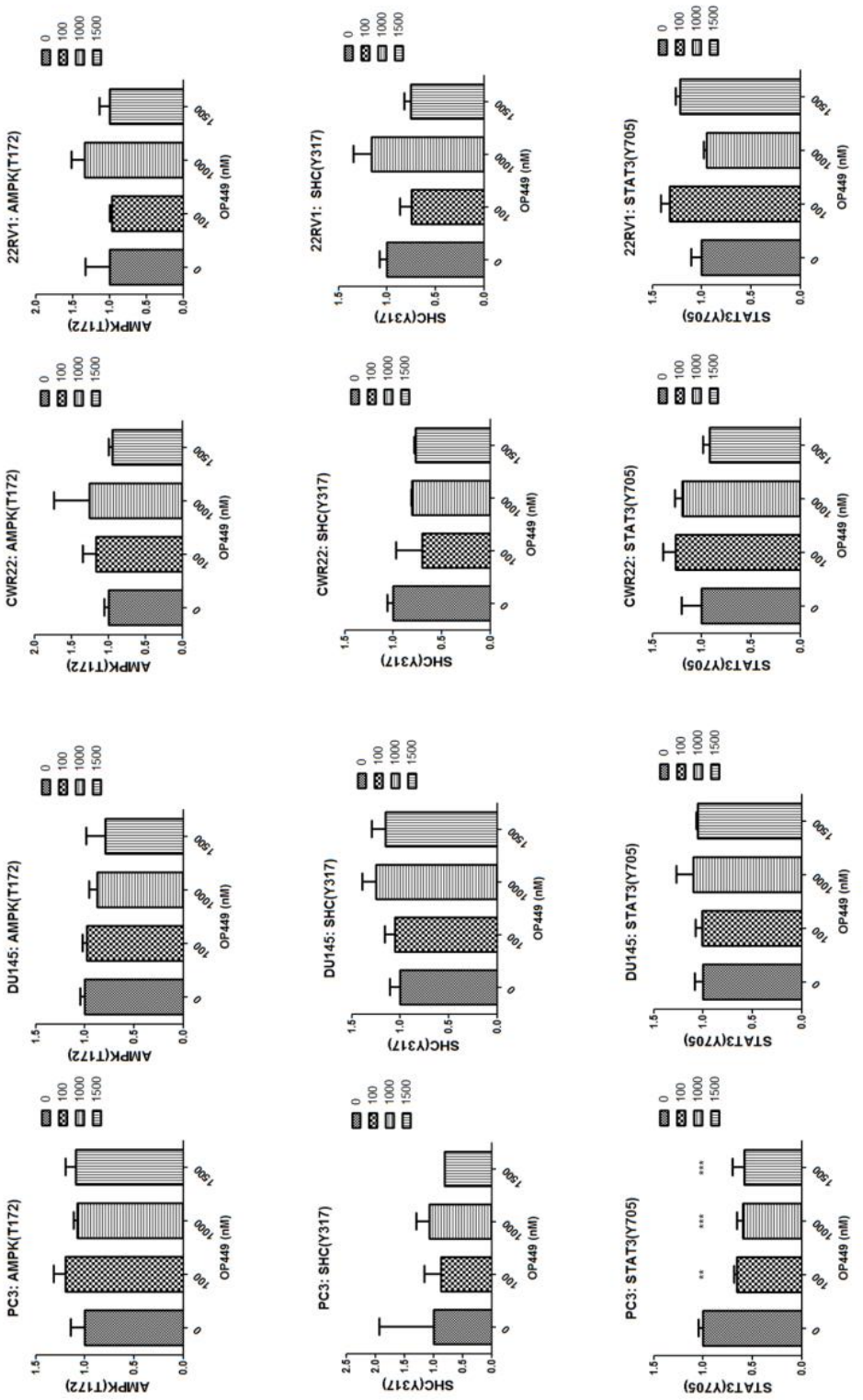
One Way ANOVA																							
	*p<0.05		**p<0.01			***p<0.0001																	
	CWR22					22RV1					PC3					DU145							
	0	100	1000	1500		0	100	1000	1500		0	100	1000	1500		0	100	1000	1500				
P38MAPK(T180/Y182)				**				**	**			**	**	***					**				
MAPK(T202/Y204)-ERK		*		**								*	*	*									
MEK1/2(s221)												*	**	**			***	***	***				
EGFR (Y992)				*				*	*			**	*	**									
EGFR (Y1068)		*	*					*				*											
EGFR (Y1173)			*				**	***	***								*						
c-RAF(S338)(S6A6)												***	**	***					*				
SRC Family (Y416)		**	**	*			*	**	*														
SRC(Y527)																			*				
AMPK(T172)																							
SHC(Y317)																							
STAT3(Y705)												**	***	***									

A
PC3: P38MAPK (T180/182)
DU145: P38MAPK (T180/Y182)
CWR22: P38MAPK (T180/Y182)
22RV1: P38MAPK (T180/Y182)
P38MAPK (T180/182)
P38MAPK (T180/Y182)
Conc OP449 (nM)
0
100
1000
1500
B
PC3: MAPK(T202/Y204)-ERK
DU145: MAPK(T202/Y204)-ERK
22RV1: MAPK(T202/Y204)-ERK
CWR22: MAPK(T202/Y204)-ERK
MAPK(T202/Y204)-ERK
OP449 (nM)
0
100
150
1000
1500
C
PC3: MEK1/2(s21)
DU145: MEK1/2(s21)
CWR22: MEK1/2(s21)
22RV1: MEK1/2(s21)
MEK1/2(s21)
OP449 (nM)


22RV1: EGFR (Y992)
22RV1: EGFR (Y1068)
22RV1: EGFR (Y1173)
CWR22: EGFR (Y992)
CWR22: EGFR (Y1068)
CWR22: EGFR (Y1173)
DU145: EGFR (Y992)
DU145: EGFR (Y1068)
DU145: EGFR (Y1173)
PC3: EGFR (Y992)
PC3: EGFR (Y1068)
PC3: EGFR (Y1173)
EGFR (Y992)
EGFR (Y1068)
EGFR (Y1173)
OP449 (nM)
0
100
1000
1500

D

F
PC3: Raf-1 c-RAF(S338)(56A6)
DU145: Raf-1 c-RAF(S338)(56A6)
CWR22: Raf-1 c-RAF(S338)(56A6)
22RV1: Raf-1 c-RAF(S338)(56A6)
c-RAF(S338)(56A6)
Raf-1 c-RAF(S338)(56A6)
OP449 (nM)
0
100
1000
1500
E
PC3: SRC Family (Y416)
DU145: SRC Family (Y416)
CWR22: SRC Family (Y416)
22RV1: SRC Family (Y416)
SRC Family (Y416)
PC3: Src(Y527)
DU145: Src(Y527)
CWR22: Src(Y527)
22RV1: Src(Y527)
Src(Y527)



G

Figure 4.6 MAPK related signalling targets identified using the reverse phase protein array platform. The RPPA screen was implicated to identify alteration of MAPK signalling cascade in response to OP449. Data analysis was performed using one-way Anova and Tukey post hoc test in order confirm modulation as significant.*denotes significance *p<0.05 **p<0.01 ***P<0.001

4.2.5 The effect of OP449 on cell cycle and cell survival cascades

Some cell cycle and cell survival related proteins were also included in the RPPA screen, namely Bcl2, Caspases 7, 8 and 9, Chk1, P27, P53, SMAC/DIABLO, XIAP and HIAP-2(cIAP1) (Table 4.3)(Figure 4.7). Among the proteins analysed, Cell cycle regulators Chk1 and P27 emerged as targets modulated in response to OP449 in multiple cell lines. The DNA damage checkpoint CDK1 was found to be dephosphorylated at Ser345 in three of the four cell lines analysed. In the CWR22, statistically significant down regulation occurred at 100, 1000 and 1500nM OP449. There were no notable changes in 22RV1. PC3 samples saw a decline in phosphorylation at 1000 and 1500nM. A small but relevant down-regulation was detected in the DU145 cell line at 100, 1000 and 1500nM (Figure 4.7 A). Previous research has previously implicated Chk1 as a drug target for cancer therapy as its inhibition has been shown to lead to cell death especially after exposure to certain antineoplastic therapies [471]. The cyclin-dependent kinase (cdk) inhibitor and tumour suppressor p27 was found to undergo dephosphorylation at T157 in the 22RV1 and DU145 cell lines. Dephosphorylation at this site has been associated with restoration of its growth inhibitory function [472]. In the 22RV1 cell line, a decrease was detected in response to 100 and 1500nM OP449. In the DU145 samples, downregulation was noted at 100 and 1000nM OP449. While there is a decrease at 1500nM, it was not found to be statistically significant. While the PC3 cell line did not return any significant changes in P27 phosphorylation, there was a trend noted as phosphorylation declined in response to OP449 (Figure 4.7 B)

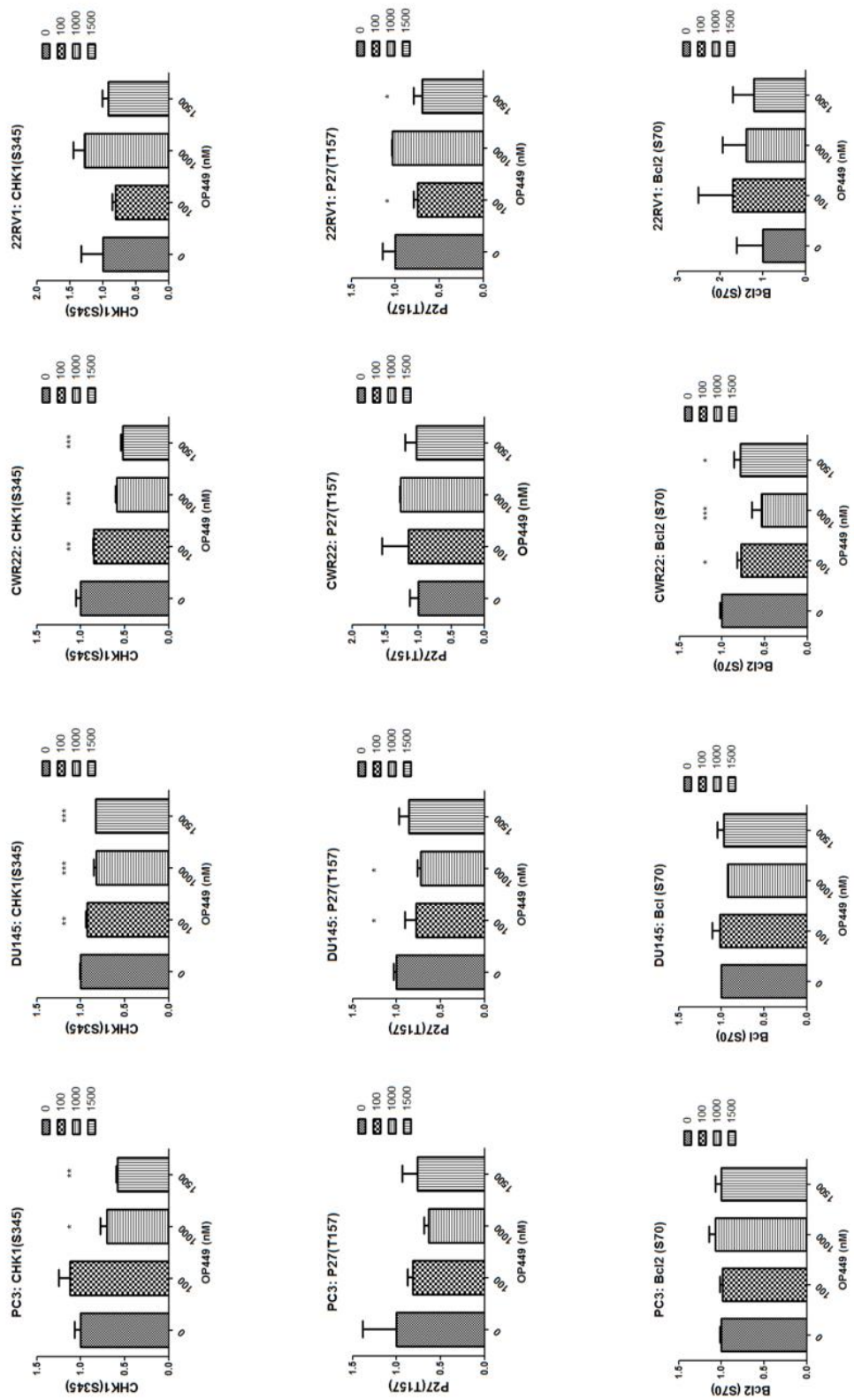
Activation of Bcl2 by phosphorylation has been implicated as a marker for mitotic events and enhanced anti-apoptotic functions [473, 474]. Phosphorylation of pro-survival Bcl2 at Ser70 and T56 were observed in the AR positive cell line CWR22 but no significant change was detected in any of the other cell lines. A decline in phosphorylation was observed at 100, 1000 and 1500nM at the S70 site, while downregulation was noted at 1000 and 1500nM at the T56 site (Figure 4.7 C).

Caspases 7, 8 and 9 were also explored in the screen, with no significant modulation being observed. Caspase 7 was down-regulated in the 22RV1 cell line only with significant modulation occurring at 100 and 1000nm before. There was also a decrease at 1500nM however this was not found to be of statistical relevance. There were no statistically significant changes in caspase 8 signalling in any of the 4 cell lines analysed. While not of statistical relevance, a trend was observed where an increase in caspase 8 was noticed. Modulation in Caspase 9 at D315 and D330 was detected in the 22RV1 cell line only, A decrease was detected at D315 in the 22RV1 cells a t 100 and 1500nM while the decrease at the D330 site occurred at 100, 1000 and 1500nM (Figure 4.7 D)

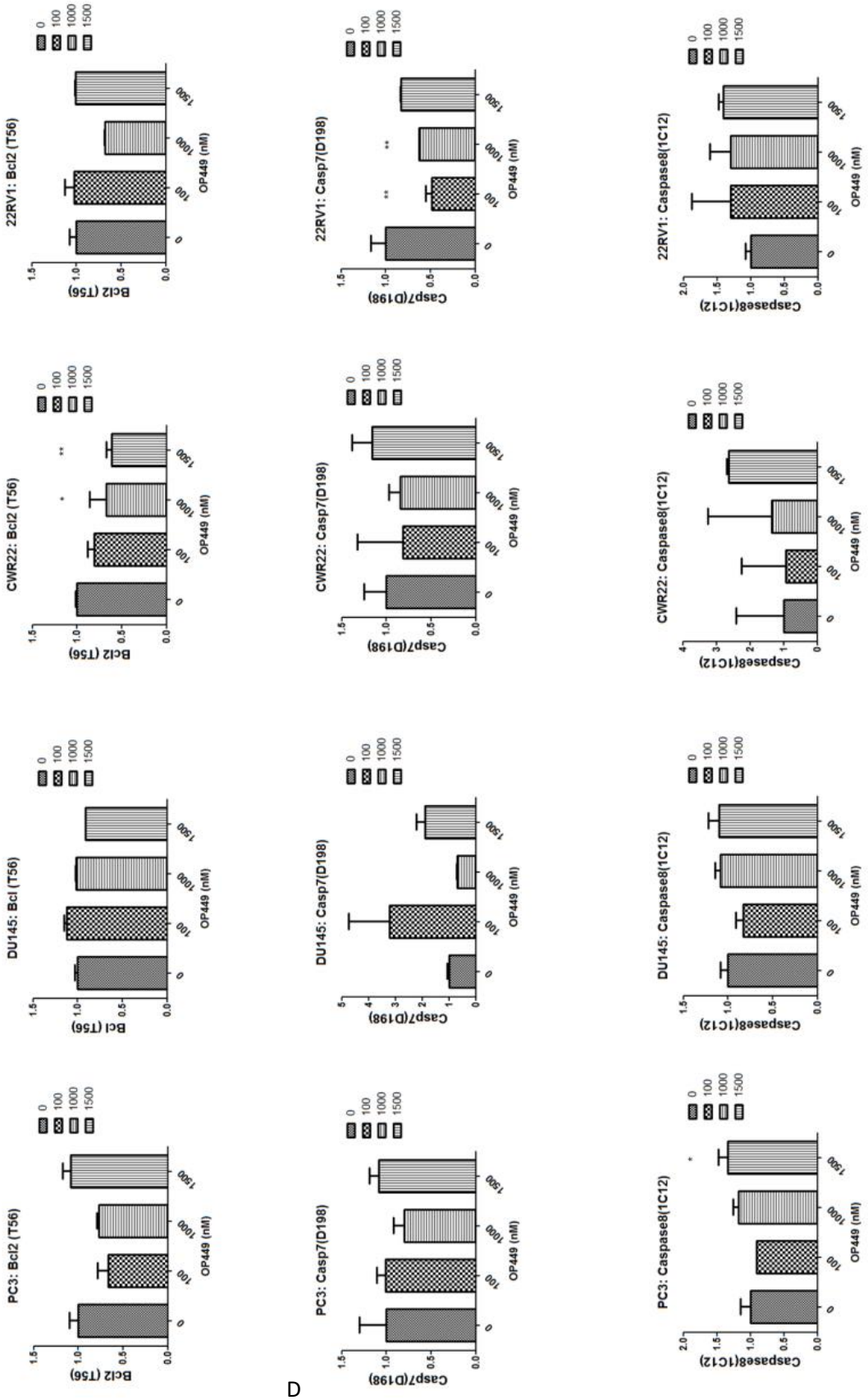
Inhibitors of apoptosis (IAP), HIAP-2(cIAP1) XIAP were included in the array however no significant modulation was detected (Figure 4.7 E) . SMAC/DIABLO, a negative regulator of IAPs was also evaluated in the included in the array; an increase was identified only in the PC3 cell line at 100 and 1000nM. No significant modulation of this protein was distinguishable elsewhere (Figure 4.7 F).

Table 4.3 Reverse phase protein array: Statistical analysis of cell cycle and apoptotic related proteins

One Way ANOVA																							
	* $p<0.05$		** $p<0.01$		*** $p<0.0001$																		
	CWR22				22RV1				PC3				DU145										
	0	100	1000	1500	0	100	1000	1500	0	100	1000	1500	0	100	1000	1500						
CHK1(S345)		**	***	***							*	*		**	***	***							
p27(T157)						*		*						*	*								
blc2(S70)		*	***	*																			
bcl2(T56)			*	**																			
Casp7(D198)						**	**																
Caspase8 (1C12)												*											
Casp9(D315)						*		*															
Casp9(D330)						**	***	**															
p53																							
SMAC/DIABLO										***	***	***											
XIAP																							
HIAP-2(cIAP1)						*	*	*															


22RV1: CHK1(S345)
CWR22: CHK1(S345)
DU145: CHK1(S345)
PC3: CHK1(S345)
22RV1: P27(T157)
CWR22: P27(T157)
DU145: P27(T157)
PC3: P27(T157)
22RV1: Bcl2 (S70)
CWR22: Bcl2 (S70)
DU145: Bcl (S70)
PC3: Bcl2 (S70)
CHK1(S345)
P27(T157)
Bcl2 (S70)
Bcl (S70)
OP449 (nM)
0
100
1000
1500

A **B** **C**


22RV1: Bcl2 (T56)
CWR22: Bcl2 (T56)
DU145: Bcl (T56)
PC3: Bcl2 (T56)
22RV1: Casp7(D198)
CWR22: Casp7(D198)
DU145: Casp7(D198)
PC3: Casp7(D198)
22RV1: Caspase8(1C12)
CWR22: Caspase8(1C12)
DU145: Caspase8(1C12)
PC3: Caspase8(1C12)
Bcl2 (T56)
Bcl (T56)
Casp7(D198)
Caspase8(1C12)
OP449 (nM)
0
100
1000
1500

D

22RV1: Casp9(D315)
22RV1: Casp9(D330)
22RV1: P53
CWR22: Casp9(D315)
CWR22: Casp9(D330)
CWR22: P53
DU145: Casp9(D315)
DU145: Casp9(D330)
DU145: P53
PC3: Casp9(D315)
PC3: Casp9(D330)
PC3: P53
Casp9(D315)
Casp9(D330)
P53
OP449 (nM)
0
100
1000
1500

E

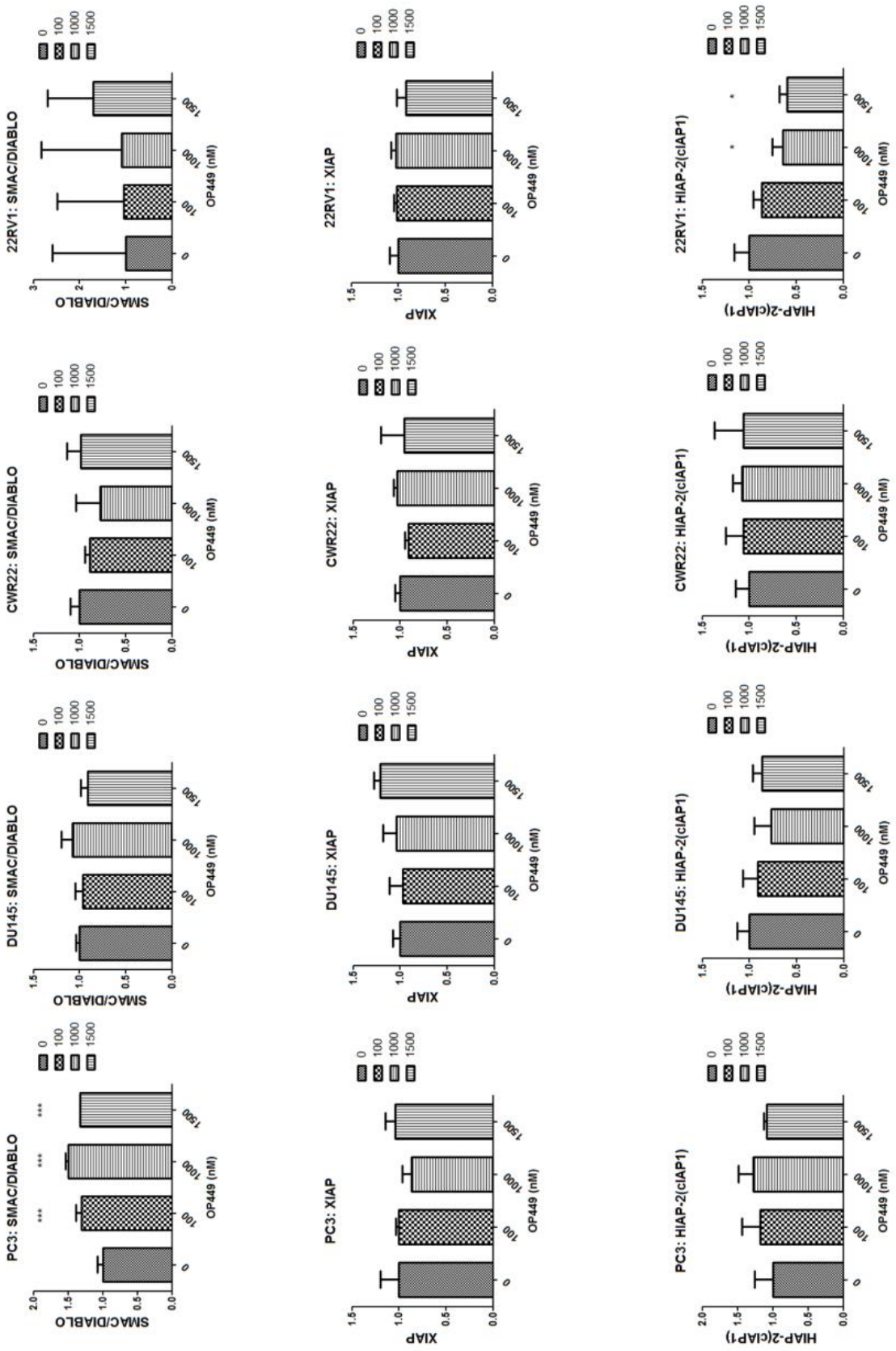


Figure 4.7 Cell cycle and apoptotic related signalling targets identified using the reverse phase protein array platform. The RPPA screen was implicated to identify modulations cell cycle and survival related proteins. Data analysis was performed using one-way Anova and Tukey post hoc test in order confirm modulation as significant.*denotes significance *p<0.05 **p<0.01 ***P<0.001

4.2.6 Inhibition of Akt stimulation by OP449

The RPPA screen indicated that OP449 is capable of interfering with the Akt signal cascade, a pathway that is widely accepted to be upregulated in cancer. Akt modulation was then assessed by western blot to confirm its inhibition by the ApoE mimetic peptide OP449. The results of this assay demonstrated that a decrease in Akt phosphorylation occurs with increasing concentrations of OP449. This was observed in all the cell lines, CWR22, 22RV1 (AR positive), PC3 and DU145 (AR negative) (Figure 4.8).

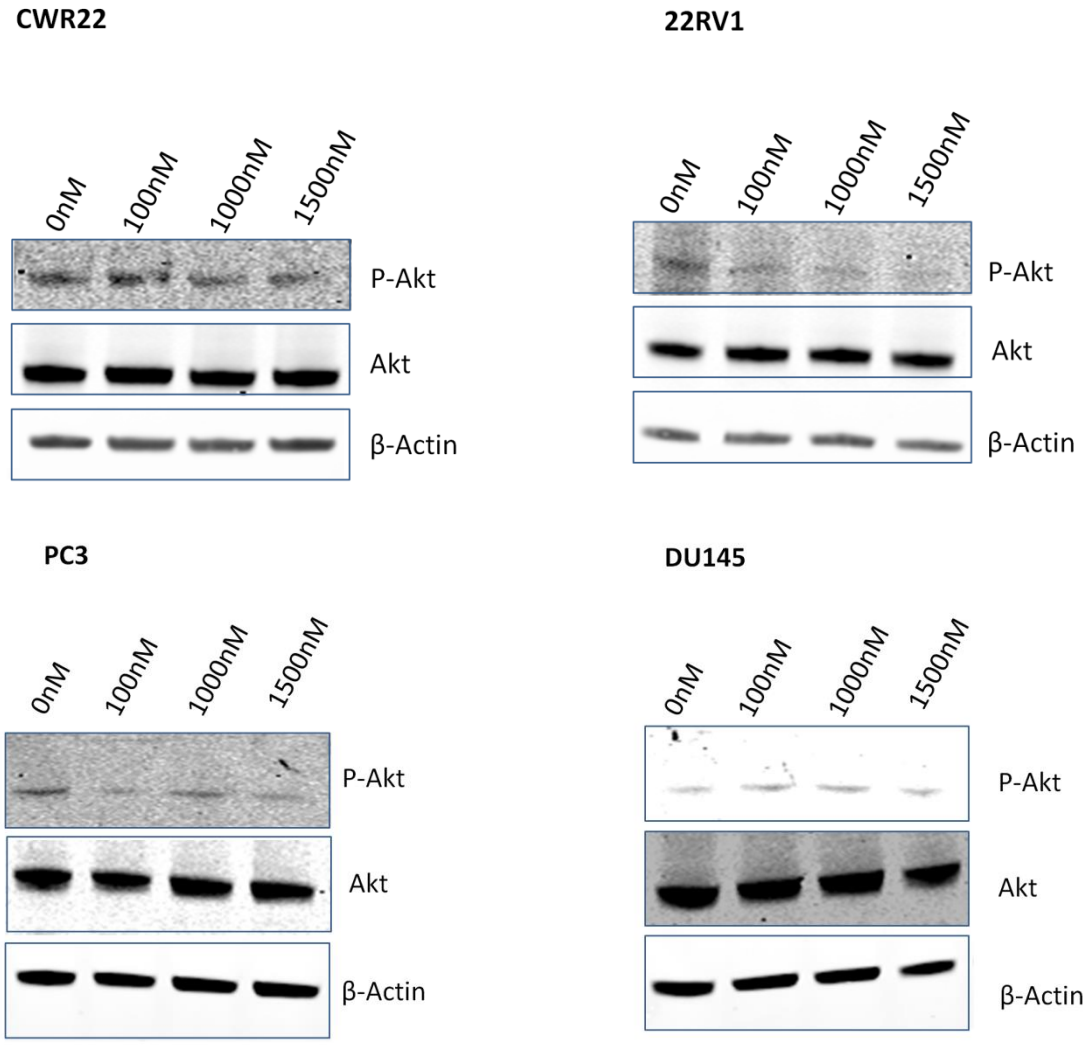


Figure 4.8 OP449 downregulates Akt signalling. The effect of OP449 was assessed by western blot with the findings being compatible with the RPPA screen. CWR22, 22RV1, PC3 and DU145 were treated with 0, 100, 1000 and 1500nM of OP449 and analysed by western blot for Akt phosphorylation (Serine 474).

In order to determine if the peptide was able to overcome the effects of Akt pathway stimulation, lipopolysaccaride (LPS) was used as a positive control to activate the tumour associated signalling cascade. Exposure to LPS has been coupled to the activation of several signalling cascades which eventually result in the production of numerous inflammatory mediators [475-477]. LPS is a known activator of PI3K and also MAPK pathways which is a necessity for the inflammatory response frequently observed in cancer and so was selected as a positive control for Akt activation in this study [478-480]. PC3 cells were seeded into petri dishes at a density of $1X10^6$ cells per dish. Prior to being exposed to treatment conditions, cells were serum starved overnight. In order to determine effect of OP449 on pathway activation, cells were either, untreated, treated with 1000nM OP449, treated with 100ng/mL LPS alone or pre-treated with LPS prior to OP449 incubation. Prior exposure to LPS was initiated to induce Akt stimulation. This was followed by OP449 treatment to investigate the ability of ApoE mimetic of reversing Akt stimulation. The findings showed that OP449 was capable of decreasing the phosphorylation of Akt on its own but also following stimulation with LPS (Figure 4.9 A). With the purpose of investigating if the mechanism of Akt inhibition was directly related to PP2A activity, the PP2A inhibitor okadaic acid (O.A) was used. PC3 cells were treated with O.A either alone, or prior to OP449 exposure. Hyper-phosphorylation of Akt was observed in the presence of O.A. The increased phosphorylation was not counteracted by OP449 following O.A exposure (Figure 4.9 B).

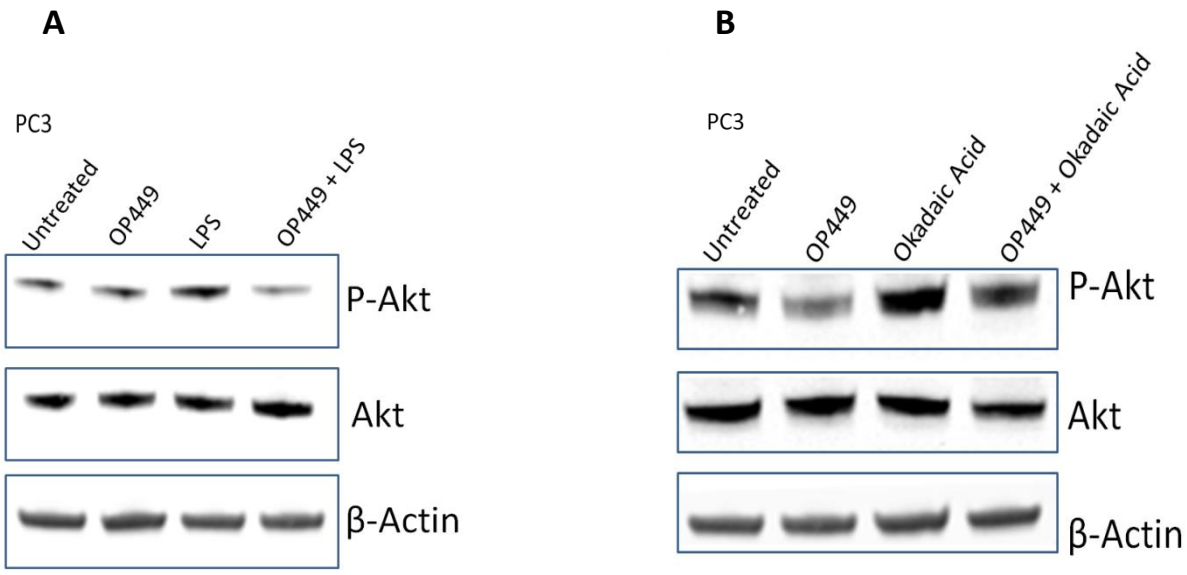


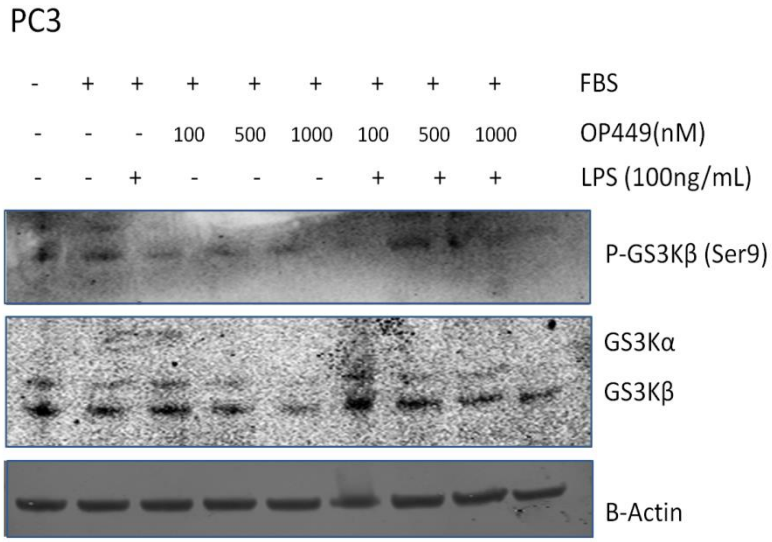
Figure 4.9 OP449 downregulates Akt signalling in response to TLR4 stimulation. The effect of OP449 was assessed by western blot with the findings being compatible with the RPPA screen. PC3 cells were treated with 0, 1000nM OP449, 100ng/mL LPS or pretreated with LPS before being treated with OP449. Protein was isolated and analysed by western blot for Akt phosphorylation (Serine 474). **(A)** Phosphorylation of Akt was stimulated by LPS. Phosphorylation induced by LPS was inhibited by OP449. **(B)** PC3 cells were treated with 0, 1000nM OP449, O.A or pretreated with O.A before being treated with OP449. Okadaic acid successfully prevented PP2A inhibition of Akt signalling and stunted Akt inhibition by OP449.

In an attempt to establish that the effects of OP449 extend beyond Akt phosphorylation and reached downstream components of this pathway, phosphorylation of GSK3α/β was also explored (Figure 4.10 A and B). GSK3β is known to play a significant role in a range of signalling pathways that control protein synthesis, cell proliferation, differentiation motility and apoptosis [481-484]. Over expression of GSK3β provokes apoptosis [454, 485, 486]. GSK3 has previously been illustrated to be negatively regulated by PI3k-Akt survival pathway primarily as a result of phosphorylation at the serine 9 residue [229, 454, 483]. Previous studies have demonstrated that inhibition of the PI3K-Akt cascade using selective PI3K inhibitors prompted GSK3 stimulation and the induction of apoptosis [454, 485, 487]. Amongst the PI3K-Akt pathway, ERK, PKA, PKC, MAP kinase activated protein kinase 1, p70S6K and Wnt signalling are also correlated with the inhibition of GSK3, some of which were flagged on the RPPA screen [188, 481, 483].

Moreover, PP2A activation may concurrently dephosphorylate and thus activate GSK3 either directly or indirectly by dephosphorylating Akt [488, 489]. In this study, Western blot analysis was used to determine if inhibition of GSK by LPS stimulation was overcome by OP449. PC3 cells were seeded into petri dishes as previously described. Cells were serum starved overnight followed by incubation with LPS alone, increasing concentrations of OP449 or pretreated with LPS followed by OP449 incubation. The results verified that inhibition of GSK by LPS stimulation was overcome by OP449 with dephosphorylation being observed in response to the compound alone. The results also demonstrate the ability of OP449 to dephosphorylate GSK3β at Ser9 following prior exposure to LPS in a dose dependant manner (Figure 4.10 A).

With the intention of confirming the role of PP2A in the dephosphorylation of GSK3β, cells were exposed to okadaic acid either alone or preceding OP449 treatment. The findings disclosed the inability of OP449 to dephosphorylate GSK3β in the presence of O.A which denotes a compelling correlation between PP2A activity and the activation of GSK3β (Figure 4.10 B).

A



B

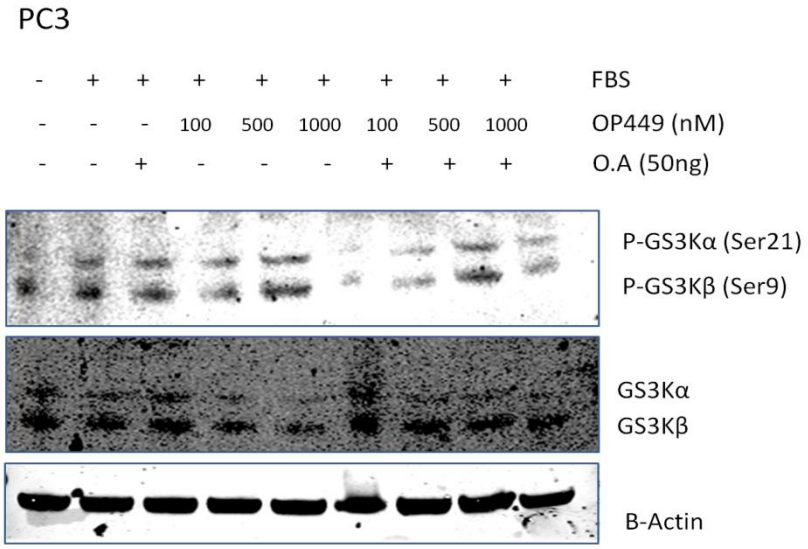
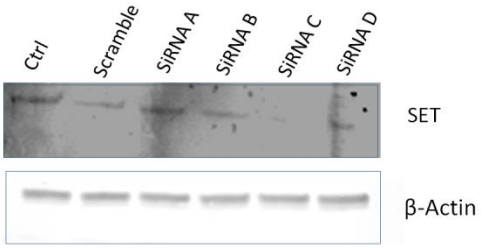


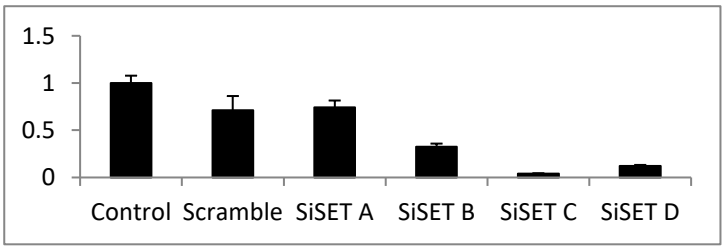
Figure 4.10 OP449 dephosphorylates GSK3β. The effect of OP449 was assessed by western blot with the findings being compatible with the RPPA screen. CWR22, 22RV1, PC3 and DU145 were treated with 0, 100, 1000 and 1500nM of OP449 and analysed by western blot for Akt phosphorylation (Serine 474). Western blot analysis of GSK-3β, a substrate of Akt, show that the Akt inhibitory effects of OP449 extend further downstream of the signalling cascade with OP449 prompting GSK3 stimulation. GSK-3β was activated by dephosphorylation in response to OP449 despite Akt stimulation with LPS **(A)**. In the presence of OA, OP449 does not stimulate GSK-3β **(B)**.

4.2.7 Down regulation of SET by SiRNA

In order to verify a direct relationship between the ability of the peptides to modulate PP2A activity and the inhibition of SET, the oncogene was depleted from PC3 cells with SiRNA (2.8.1). Following optimisation, PC3 cells were seeded into 6 well plates at a density of 1.2 $X10^5$ in antibiotic free complete media and left to adhere overnight at 37°C, 5% CO_2. Following overnight incubation the cells were transfected using a final transfection concentration of 100nM ON-TARGET plus SiRNA (Fisher) and DharmaFECT reagent (Thermos scientific formulation3). A decrease in the expression of SET was achieved by transient transfection with four different SiRNAs coding for the SET oncogene. A non-coding scramble siRNA was used as a control. RNA and protein were isolated as previously described at 24 hours and 48 hours respectively. Knockdown of SET was confirmed by western blot and real time qPCR (Figure 4.15). Western blot demonstrated effective knockdown of SET at a protein level among three of the four SET targeting SiRNAS, and protein expression was quantified by dense cytometry using image j. (Figure 4.11 A). RT-qPCR analysis confirmed knockdown of the SET oncogene in all four of the SiRNA samples when compared the control and noncoding scramble transfections (Figure 4.11 B).

A





B

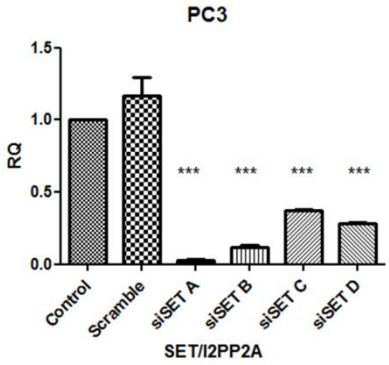


Figure 4.11 Knockdown of SET by SiRNA Transient knockdown of SET was achieved by ON-TARGET plus SiRNA transfection. Pc3 cells were transfected with 100nM SET SiRNA with dharmafdect transfection reagent. Protein was isolated following 48hr transfection and successful knock down of SET was confirmed by western blot **(A).** RNA was isolated following 24hr transfection and expression was validated by RT-qPCR **(B).** Data analysis was performed using one-way ANOVA and Tukey post hoc test in order confirm modulation as significant.*denotes significance *p<0.05 **p<0.01 ***P<0.001

4.2.8 Down-regulation of SET increases PP2A activity

Once successful knockdown of SET was confirmed, the expression of PP2A was examined with the intention of identifying an associated change in PP2A expression in transfected PC3 cells. Western blot revealed that protein levels of PP2Ac remained constant in the scramble SiRNA transfection samples and in all four of the SET coding SiRNA transfections. Variation in the activity level of PP2A was also investigated in response to SET knockdown. Western blot showcased a decrease in phosphorylation of PP2A in three of the four SET coding SiRNAs. A decrease in PP2A phosphorylation corresponds with an increase in PP2A activity (Figure 4.12 A).

PCR analysis clarified that PP2Ac expression remained stable at the RNA level with no decrease detected among the scramble and SET coding SiRNAs used in the transfection (Figure 4.12 B)

As modulation of PP2A activity was confirmed as a consequence of SET knockdown, the phosphorylation levels of Akt were explored by western blot. The result

showed that total Akt levels remained even in the control, non-coding and SET knockdown samples. Phosphorylation of Akt was impeded in the SET knockdown samples which corroborates with an increase in PP2A activity (Figure 4.12 C)

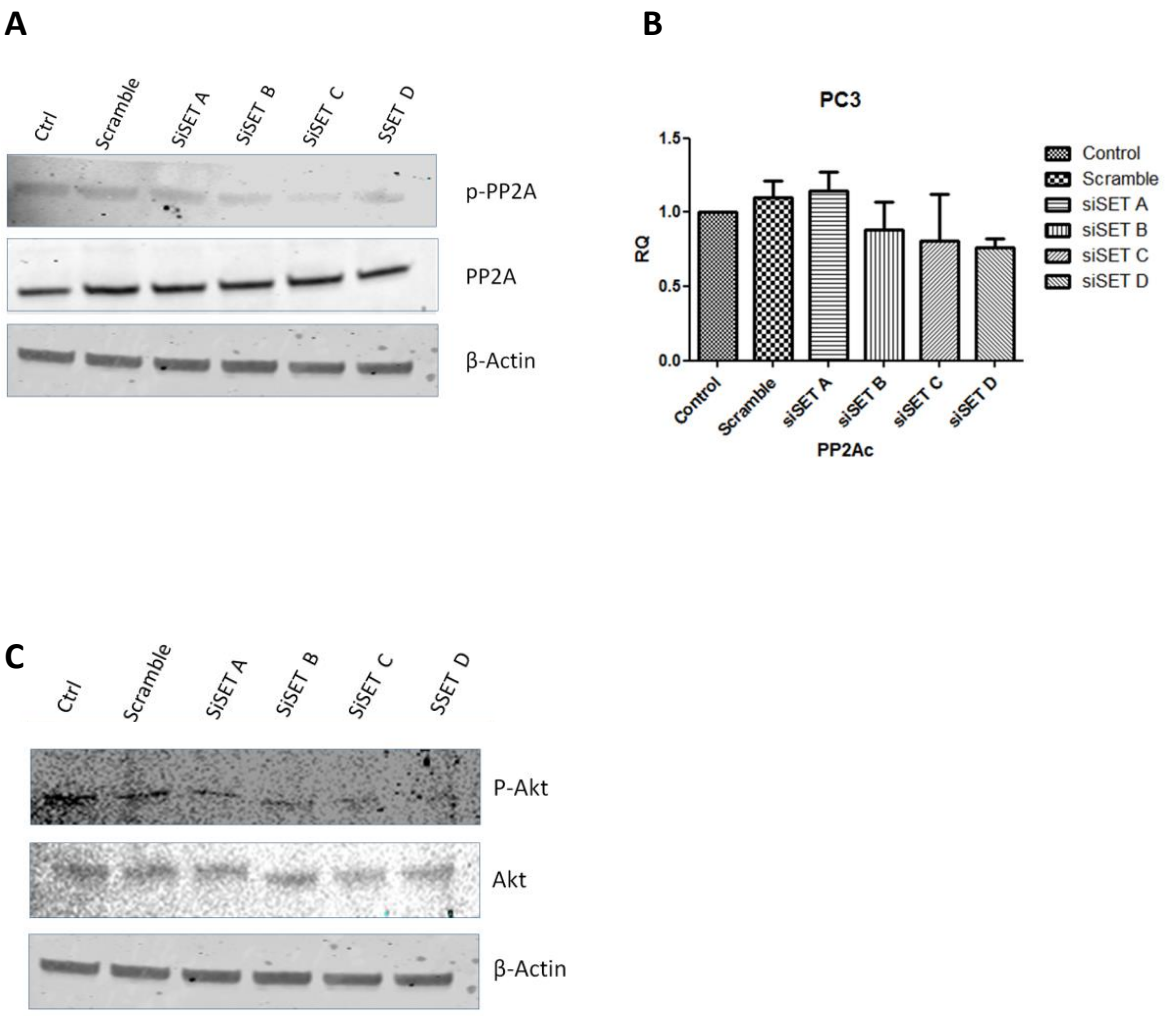
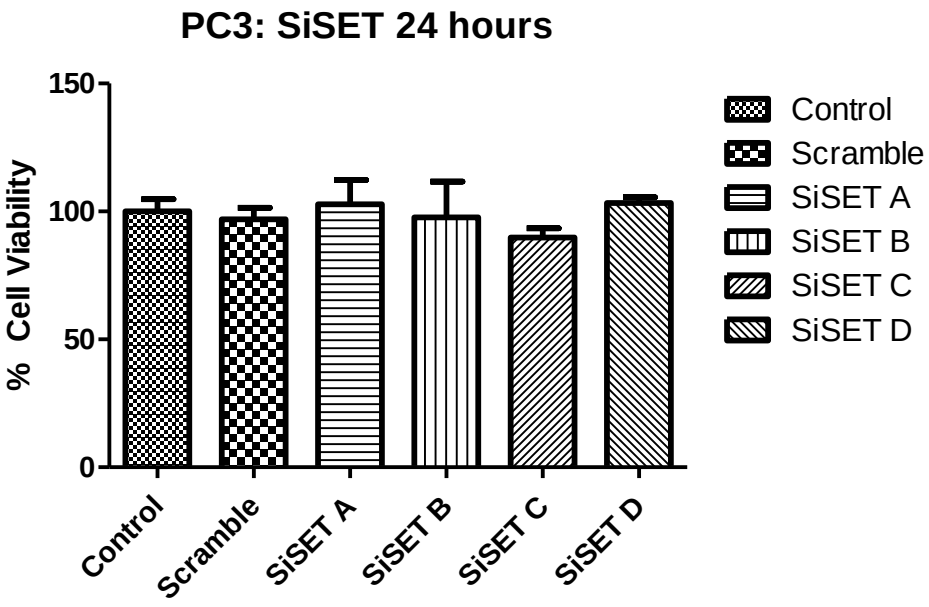


Figure 4.12 Transient knockdown of SET modulates PP2A activity. The expression and phosphorylation of PP2A was explored in SiRNA SET knock down PC3 cells. Protein expression of PP2Ac remained stable among the scramble and knockdown samples when compared to the control. Phosphorylation of PP2Ac was decreased in the SET knockdown samples **(A).** RNA levels of of PP2Ac were assessed by RT-qPCR with no significant variation detected **(B).** To confirm modulation of PP2A activity in response to SET knockdown, the phosphorylation of Akt (Ser473) was assessed by western blot. The results showed that total Akt expression remained stable while phosphorylation of Akt was decreased in the SET knockdown samples when compared to the non-coding scramble and control **(C)**. Data analysis was performed using one-way Anova and Tukey post hoc test in order confirm modulation as significant.*denotes significance *p<0.05 **p<0.01 ***P<0.001

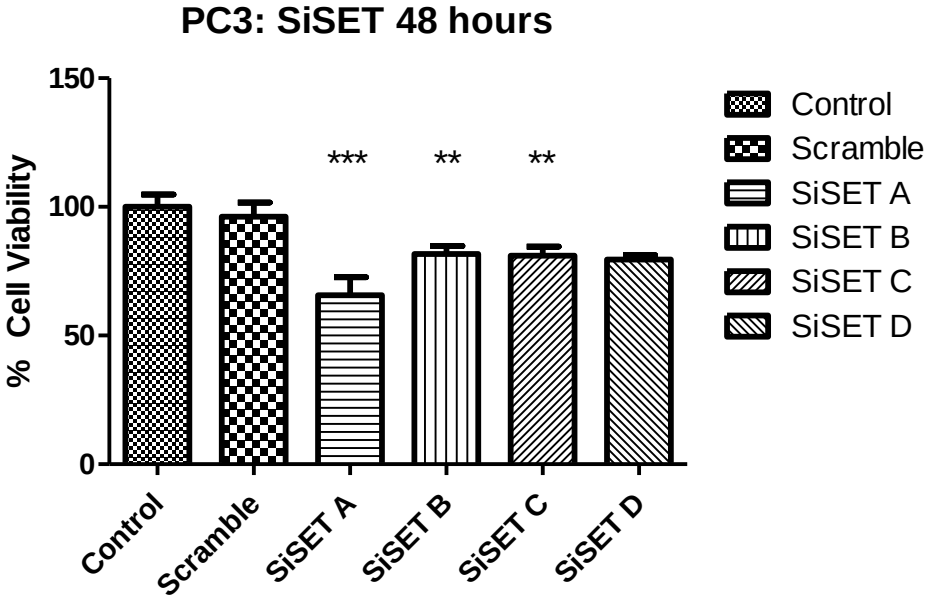
4.2.9 Knockdown of SET decreases cell viability

After demonstrating that SET knockdown triggers adjustment in the phosphorylation of PP2A and Akt at comparable levels to OP449, it seemed permissible to investigate the consequences SET knockdown may have on cell survival. The alamar blue assay was employed to detect viability levels in the transfected cells. Cells were seeded into 96 well plates and transfected with SET coding SiRNA over a 72 hour time course. Treatment conditions included incubation with the transfection reagent alone, SiRNA alone or combination of transfection reagent and SiRNA in order to distinguish toxicity caused by the reagents used and from that resulting in response the knockdown of the SET oncogene. Resazurin was added to each, as previously described at 24, 48 and 72 hour time points and incubated for a further 6 hours at 37°C, 5% CO_2 before being read on the plate reader. The outcome of this experiment at 24 hours demonstrated no decline in viability in response to transfection with SiRNA A, B or D and only a 10% decline in viability in SiRNA D transfection (Figure 4.13 A). At 48 hours, a 30% decline was detected in the cells transfected with SiRNA A and a 20% was decline was calculated in the SiRNA B, C and D transfections (Figure 4.13 B). At the 72 hour time point, the reduction in viability in response to SiRNA A remained at 30%, and also depleted to 30% reduction in viability in SiRNA C transfection. There was no further decrease in viability calculated in response to SiRNA B and D transfections (Figure 4.13 C). Although a fall in cell viability ranging from 20-30% was measured in response to SET knockdown which may reflect a SET related survival mechanism acquired by cancerous cells, it does not correlate with the loss in viability quantified in response to the OP449 and COG112.

A


PC3: SiSET 24 hours
% Cell Viability
150
100
50
0
Control
Scramble
SiSET A
SiSET B
SiSET C
SiSET D

B


PC3: SiSET 48 hours
% Cell Viability
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Control
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SiSET A
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SiSET C
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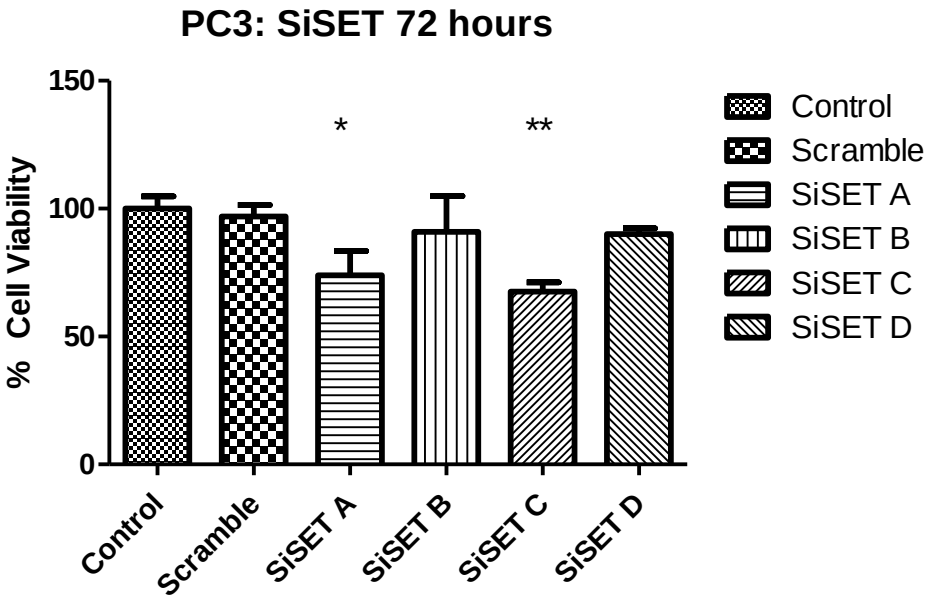


Figure 4.13 The effect of SET knockdown on cell viability. Cells seeded into a 96 well plate were treated with either transfection reagent alone, SET coding SiRNA or combination of appropriate SiRNA and transfection reagent for the knockdown of SET to assess the toxicity of the compounds on cell viability and the determine the effect of SET knockdown in cell survival over a 96 hour period. At 48 hours post transfection a 10% decline in viability was calculated **(A)**. At 72 hours a maximum loss of 30% in viability was gauged **(B)**. The reduction in cell viability remained at 30% after 96hours **(C)**. Data analysis was performed using one-way ANOVA and Tukey post hoc test in order confirm modulation as significant.*denotes significance *p<0.05 **p<0.01 ***P<0.001

4.3 Discussion

4.3.1 Upregulation of SET in prostate cancer

As SET has been reported in the literature to be upregulated in some cancer types, and the ApoE peptide mimetics have been reported to bind to SET by the manufacturers, it was important to confirm a base line expression of SET in PCa cell lines and compare to normal prostate epithelial cells. Western blot analysis confirmed the expression of SET in PCa cells, however SET was also found to be expressed abundantly in the RWPE1 immortalised normal cell line (Figure 4.1 A). With SET being a known inhibitor of PP2A, the expression of the phosphatase was also confirmed. This finding warranted further investigations in to the potential targeting of SET in PCa cells. Considering our earlier observation of rapid proliferation off RWPE1 cells, their sensitivity to COG112 and OP449, together with their abundant expression of SET, protein extracted from primary prostate epithelia lysate was also probed for the expression if SET. Western blot revealed no expression of SET at the protein level in normal prostate epithelia (Figure 4.1 B). RNA analysis of Patient matched tumour adjacent and prostate tumour samples demonstrated a two fold increase in the RNA expression of SET in tumours samples when compared to adjacent non-cancerous prostate epithelia. Given that no SET protein was identified in the normal prostate epithelia and data analysis confirmed a significantly lower expression of RNA in tumour adjoining tissue when compared to cancerous tissue from the same patient, SET may be an optimum target when treating PCa as potentially only the SET expressing cancer cells would be targeted by the SET binding ApoE mimetic peptide therapy (Figure 4.1). The results of this study are supported by the findings of Hu et al;2015 who's research strengthens the case for the correlation between SET expression and aggressive forms of prostate cancer.

Following our demonstration of SET expression in the transformed RWPE1 cells and lack thereof in normal prostate epithelial cells (ATCC), together with reporting of

SET up regulation inducing pathological alterations and a precancerous PIN like phonotype by Hu et al, RWPE1 were deemed redundant as a representation for normal prostate epithelial cells for this study.

4.3.2 ApoE peptide mimetics disrupt SET PP2A complex

After successfully identifying the expression of SET in PCa cells and clarifying its absence in normal epithelia, a biotinylation assay confirmed that the ApoE mimetic peptide successfully bound to the SET oncoprotein in metastatic PCa cells. This is supported in the literature as Switzer et al., 2011 and Christensen et al, 2011 who reported the ability of COG112 to interact with SET in breast cancer MDA-MB-231 cells and glial cells respectively [212, 461]. Collectively this indicates the ability of the peptides to interact with the SET oncogene regardless of cell type (Figure 4.2 A). Furthermore previous whole cell studies demonstrated that the interaction between COG112 and SET disrupted the formation of the SET/PP2Ac complex [212]. This further supported our finding that the dimerised peptide OP449 prompted displacement of the SET/PP2A interaction (Figure 4.2 B). As the increasing concentration of OP449 correlated with a decrease in the levels of SET bound to the purified PP2A, there was also a corresponding increase in the quantity of unbound SET oncoprotein detected in the PP2A flowthrough. In addition to the disruption of the SET/PP2A complex, western blot also exposed an increase in PP2A activity in response to OP449. This manifested as a decrease in phosphorylation of PP2A (Figure 4.2 C). This outcome corroborates with the idea that OP449 binds to SET in a similar fashion to COG112 and interferes with the SET/PP2A fusion resulting in a loss of oncogenic function of SET. This interaction would endorse pro-apoptotic signalling similar to reports in leukemic progenitor cells but would also have a desirable nontoxic profile in ex vivo primary cells and long term animal studies [165, 484, 490].

4.3.3 RPPA screen identified signalling pathways targeted by OP449

In order to gain a better understanding of the mechanism of action of OP449 at a molecular level, a range of protein targets were investigated using the RPPA platform in collaboration with the Hennessy research group in Beaumont hospital. Among the proteins assessed, a number emerged as key components modulated in response to OP449 treatment. The Akt and MAPK pathways transpired as key players targeted by the peptide. Among the Akt related proteins modulated by OP449 were Akt, mTOR and P70s6k (Figure 4.4). Within the MAPK signalling cascade, the RPPA screen unearthed P38MAPK, MAPK-ERK, MEK1/2 and C-Raf as targets modulated in response to OP449 (Figure 4.6). The screen identified some promising hit targets as both the Akt and MAPK signalling pathway pay significant contribution to the pro-survival in cancer cells and the progression of cancer. The significance of these pathways in the contribution of cancer development and progression has been well documented in the literature and the ability to target both pathways simultaneously could be a game changer for PCa treatment.

Akt itself has a range of downstream targets that regulate tumour associated development and progression such as cell growth, proliferation and survival, cell cycle progression, migration, epithelial mesenchymal transition and angiogenesis [228, 491-493]. Obstruction of the Akt signalling cascade results in apoptosis and growth inhibition of tumour cells with elevated Akt [494]. The observed dependence of many cancers on Akt signalling for survival and growth has wide implications for cancer therapy offering the potential for preferential tumour killing [494]. In Androgen resistant PCa, the PI3K-Akt-mTOR signalling pathway has emerged as a key contributor to the resistance of Androgen deprivation therapy (ADT). There is an increased appreciation for the role signal transduction pathways play in the progression of cancer. This pathway has been reported to be altered at a genomic and transcriptional level in most cases of advanced PCa [110]. It is likely that cancer cells exploit this pathway in order to adapt to the cellular stress

induced by ADT. This is likely due to the ability of the pathway to integrate several intra and extra cellular growth signals with critical cellular processes [495-497]. Recent studies have demonstrated a link between PI3K-Akt-mTOR and AR signalling, disclosing a dynamic relationship between the signalling cascade and androgen sensitivity [498, 499].

The MAPK kinase pathway, also closely associated with cancer, plays a role in cell differentiation, growth, proliferation, survival, transformation and apoptosis [343, 500]. Cells identify and interpret extracellular stimuli by engaging specific intracellular programmes, such as signalling cascades that lead to activation of the MAPKs. All eukaryotic cells possess multiple MAPK pathways which regulate a diverse range of cellular functions ranging from gene expression, mitosis and metabolism to motility, survival, apoptosis and differentiation. Of the 5 known characterised MAPK groups, the most extensively studied are the ERK1/2, JNK and p38 kinases. MAPKs can be stimulated by a wide variety of stimuli but in general, ERK1/2 are preferentially activated in response to growth factors and phorbal esters, while the JNK and p38 kinases are more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulation [501, 502].

The p38 kinase emerged as a hit in the screen. These kinases play roles in cell differentiation, growth, proliferation survival and apoptosis [503, 504]. P38 is activated in cells in response to stress signals, pro-inflammatory (TNFα, IL-6 or IL-1) or anti-inflammatory (EGF, TGFβ) cytokines, UV radiation and heat or osmotic shock [503, 505]. Activated p38 phosphorylates and regulates many transcription factors including NF-KB and P53, among other cell cycle and apoptotic mediators such Cdc25 and Bcl2 [504]. P38 has been shown to enhance cell survival in response to stress stimulus such as DNA damage [504, 506, 507]. Several studies suggest that p38 plays an important role in leukaemia, lymphomas, and a number of solid malignancies such as breast, prostate, gastric or lung cancers [505, 508-512]. Both P38 and its active form p-p38 as well as some upstream kinases are overexpressed in human cancerous prostatic epithelium [513-515]. P38 may contribute to PCa progression by promoting tumour growth androgen

independence acquisition and metastasis. It has been proposed that IL-6 may support androgen independent tumour growth by enhancing androgen receptor expression and activity. Lin et al. demonstrated that IL-6 induced androgen response depends on p38 activity [516]. P38 seems to play a critical role in hypoxia-re-oxygenation induced increases in AR activity as well as increased survival, clonogenicity and invasiveness in PCa cells, thus providing additional support for a role for p38 in androgen dependence acquisition [517]. P38 may constitute a target for PCa treatment given its demonstrated contribution to some PCa hallmarks as androgen dependence and metastatic phenotype acquisition [502].

Also upregulated in the MAPK pathway were the ERK 1 / 2 kinases. The ERK signalling pathway plays a role in several steps of tumour development [518]. Biological consequences of phosphorylation of ERK substrates include increased proliferation, differentiation, angiogenesis, motility and invasiveness [519-522]. The ERK pathway is triggered mainly by cytokines acting through receptor tyrosine kinases, G-protein coupled receptors and non-nuclear activated steroid hormone receptors [501, 505]. Some components of the Raf-MEK-ERK pathway are activated in solid tumours such as breast and PCa and haematological malignancies [523-525]. Increased expression of the Raf pathway has been associated with advanced PCa, hormonal independence metastasis and poor prognosis [526]. The literature has previously provided a link between the presence of Raf-1 And MEK1 in conjunction with elevated ERK1 and 2 and their phosphorylated forms suggesting that stimulation of cell proliferation could be triggered by IL-6 via the ERK pathway [523]. IL-6 expression is typically increased in PCa in comparison to normal tissue [523, 527].

As both MAPK and AKt play vital roles in the progression of cancer, they provide ideal targets for newer therapeutic strategies. Kinase Inhibitors typically favour one pathway over another. OP449 maybe a promising broad spectrum inhibitor as it demonstrates the capacity to target multiple aspects of the protumour signalling in both Akt and MAPK cascades. This may allow for more successful elimination of cancer cells. It would likely minimise the potential for cancer cells to overcome

stress induced by radiation or additional therapies. With much needed further investigation, Using OP449 in conjunction with current therapies used in the clinic could provide a significant advantage for the treatment of PCa.

While the RPPA provides the potential of providing high throughput screening for the quantitative measurement of signalling proteins in biological and clinical samples for the identification of therapeutic targets, it is worth mentioning that this platform is not without its limitations. This protocol is reliant on highly sensitive antibodies to be optimised for each assay. The targets explored were limited to those optimised and available within the collaborators library. For this reason, it was not possible to explore the effect of the drug in all cell cycle and cell survival related proteins and why some were included and some were not. It should also be noted that accurate identification of signal activation or down regulation is reliant on meticulous loading of samples onto the antibody coated slides. The offset of equipment calibration may also account for unexpected findings in this study. For instance, IAPs are targets of NF-κB and their lack of modulation was observed in this assay despite later findings of upstream protein inhibition. While useful leads were identified using this technique, the modulation and activity status of suspected targets should be confirmed by other means such as western blot.

4.3.4 Akt is down regulated by OP449

After identifying Akt as a target down regulated in the RPPA screen, the effect of OP449 on the expression and phosphorylation of Akt was assessed by western blot. A decrease in the phosphorylation of Akt was observed in response to increasing levels of OP449 in CWR22, 22RV1, PC3 and DU145 cells in a dose dependant manner (Figure 4.9). Once the RPPA result was confirmed by western blot, It was concluded that OP449 was capable of inhibiting the phosphorylation of Akt despite prior stimulation with LPS in PCa cells. This strengthens the argument of OP449 being a potent inhibitor of Akt signalling and activation (Figure 4.9 B). In an effort

to determine if PP2A activation is the mechanism responsible for Akt inhibition, pretreatment with Okadaic acid, a known inhibitor of PP2A was performed to inhibit any potential activation of PP2A in response to OP449. Western blot confirmed that O.A successfully prevented any downregulation of Akt phosphorylation alone but also prevented OP449 from down regulating Akt (Figure 4.9 C). This is highly suggestive that PP2A is largely responsible for the down regulation of Akt phosphorylation observed in response to OP449. This supports our previous finding and speculation that OP449 induces an increase in the activation levels of the phosphatase PP2A. Treatment with OP449 also resulted in a decrease of GSK3β downstream of Akt and mTOR (Figure 4.9 D). Similar to Akt, phosphorylation of GSK3β was increased in response to LPS and decreased in response to OP449 in a dose dependant manner. However, in the presence of O.A, OP449 did not affect the phosphorylation of GSK3β (Figure 4.9 E). As Akt and its downstream substrates are regulated by PP2A and the PP2A inhibitor, O.A, interfered with the mechanism of OP449. These results strongly support a mechanism whereby OP449 increases PP2A activity and that this increase in activity decreases pro-tumourigenic Akt signalling.

As the results of this study demonstrated the ability of OP449 to impede the binding of SET to PP2A and consequently increase PP2A activity, it could be highly beneficial to explore the effects of the peptides on additional cascades potentially regulated by SET inhibition and PP2A up regulation in prostate cancer. Janghorban et al., 2014 explored the modulation of MYC as a potential therapeutic strategy in breast cancer. In corroboration with the findings of our study, they reported a reduction of growth and tumorigenic potential of these cells in vitro and in vivo. They also reported that inhibition of SET resulted in a decrease in pS62-MYC levels and MYC transcriptional activity in breast cancer in response to OP449 [528]. The transcript encoding the proto-oncogene MYC is commonly overexpressed in prostate cancer and protein is typically exacerbated in the majority of advanced and metastatic castrate-resistant PC (mCRPC) cases [529]. The stability of MYC protein expression is regulated in part by sequential and interdependent

phosphorylation at residues, threonine 58 (T58) and serine 62 (S62)[530]. MYC is phosphorylated at S62 (pS62) through the mitogen-activated protein kinase (MAPK) pathway or cyclin-dependent kinase (CDK) activation in response to growth signals. This in turn increases its stability and oncogenic activity [530-533]. Following prior phosphorylation at S62 and termination of growth signals, GSK3, phosphorylates T58 (pT58) [530, 531]. T58 phosphorylation facilitates PP2A mediated dephosphorylation of pS62, ubiquitination and instigation of proteasomal destruction of MYC [209, 534]. Considering our finding of PP2A upregulation and the observed dephosphorylation of GSK3β in response to SET inhibition, exploring the effect of SET inhibition and PP2A / GSK3β upregulation on MYC in prostate cancer would be a logical progression for future investigations.

4.3.5 Transient knockdown of SET upregulates PP2A activity

The results of this study have so far demonstrated that OP449 is capable of interrupting the SET-PP2A complex and upregulating PP2A activity. Results have also suggested that this peptide modulates cancer promoting signalling cascades. As the Akt and MAPK modulation were thought to be a consequence of increased PP2A signalling and SET inhibition, SET protein was transiently knocked down using SiRNA targeting the SET oncogene in PC3 cells to determine if depletion of the SET oncogene in PCa had a similar effect to treating cells with OP449. Due to licensing constraints, transient knockdown was employed instead of stable knockdown. Knockdown of SET was successfully achieved and verified by western blot, protein decrease was verified by dense cytometry and RNA level determined by RT-qPCR (Figure 4.11). Knockdown of SET did not provoke any consequential depletion of PP2Ac observed by western blot or real-time qPCR (Figure 4.12). Although PP2Ac expression did not change, there was however a decrease in phosphorylation of PP2Ac detected by western Blot (Figure 4.12). This is indicative of an increase in PP2A activity in response to SET knockdown and supports the suspected mechanism of action of OP449. Knockdown of SET induces a decrease in the

phosphorylation of PP2Ac similar to that observed in PC3 cells treated with OP449. The decrease in PP2Ac phosphorylation corresponded to a decrease in Akt signalling in response to OP449. Western blot confirmed a similar decrease in Akt phosphorylation in cells where SET was transiently knocked down (Figure 4.12). This further strengthens the argument that OP449 is an effective inhibitor of SET and consequently increases activity of PP2A. Increased PP2A activity negatively regulates Akt signalling and causes a decrease in Akt phosphorylation and inhibits downstream signalling.

4.3.6 Cell viability in response to SET knockdown

Alamar blue assay demonstrated that knockdown of SET by SiRNA was not sufficient to induce the level of cell death observed in response to OP449 and COG112. Although a fall in cell viability ranging from 20-30% was measured in response to SET knockdown which may reflect a SET related survival mechanism acquired by cancerous cells, it does not correlate with the loss in viability quantified in response to the OP449 and COG112 (Figure 4.13). This is comparable to the findings of Hu et al., who reported a reduction of cell survival by 50% in response to stable knockdown of SET. Perhaps the variation in degree of survival between our studies is related to the permanency of the protein knock down. The literature has described ApoE to have immunomodulatory properties in addition to its cholesterol transport function [163, 164]. Previous studies demonstrated a suppression of microglial activation and secretion of inflammatory mediators such as TNF-α and IL-6 following treatment with both ApoE holoprotein and ApoE synthetic peptide in both primary microglial cultures and microglial cell lines [166, 167]. Although there is a lack of knowledge regarding the mechanism by which ApoE is capable of suppressing immune response, some studies have suggested that a decrease in production of inflammatory mediators in response to ApoE may occur as a result of obstructing the NF-KB signalling pathway, with a decrease in IKB kinase (IKK) phosphorylation being reported [171, 172]. As inhibition of SET is

unlikely to account for the entire mechanism by which OP449 depletes PCa cell lines, exploring the effect of the ApoE mimetic peptide on mediators of inflammation and NF-KB signalling is a viable approach in determining the mechanism of OP449 and COG112.

Chapter 5: Impact of ApoE peptide mimetics on pathogen recognition receptor signalling and NF-κB activation in AR negative PCa cells

5.1 Introduction

Inflammation has been implicated as a contributing factor of several forms of cancer with approximately 20% of adult cancers having been attributed to chronic inflammatory conditions caused by infectious agents, chronic non-infectious inflammatory disease and environmental factors. As previously mentioned, risk factors associated with the development and progression of PCa include advanced age, family history, African-American ancestry and an increased risk coinciding with the adoption of a westernised lifestyle. The pathogenesis of PCa involves environmental factors as well as hereditary factors. Chronic inflammation has gained increased recognition as a risk factor in PCa. Some causes are resulting from infections, dietary factors, physical trauma, hormonal changes, urinary reflux and other environmental exposures [61, 535]. Previous studies and recent advances in cancer immunobiology have emphasised Toll-like receptors (TLRs) as key components involved in tumour growth and progression. The normal regulation of TLRs is disrupted in cancerous epithelial cells in comparison to tissue derived from healthy individuals indicating that mutations of TLR genes or modulation of TLR signalling could be appropriate markers for diagnosis or targets for treatment strategies [262, 263].

To determine the expression of TLRs in our PCa cell lines, cells were grown to confluency as previously described. Protein was isolated and quantified before samples were assessed by western blot. To date, 10 TLRs have been identified in humans. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface while TLR 3, 7, 8 and 9 are found exclusively within endosomes [264]. TLR 10 is an orphan ligand that is highly expressed in the spleen and B cells [265, 266] (Figure 5.1). TLRs and retinoic acid-inducible gene I (Rig-I) like receptors (RLRs) represent defined families of pattern recognition receptors (PRRs) capable of detecting nucleic acids derived from viruses and initiate antiviral innate immune responses [258]. Upon ligand recognition, TLRs and RLRs prompt the activation of their signalling cascade pathways which ultimately lead to the activation of NF-κB, MAP kinases and

Interferon regulatory transcription factors (IRFs) thus regulating the transcription of genes encoding inflammatory cytokines and transcription factors [258]. Recognition of pathogen associated molecular patterns (PAMPS) and damage associated molecular patterns (DAMPS) is a necessary/critical function of the innate immune system. PAMPS are molecular structures which include glycoproteins, lipopolysaccharides (LPS), proteoglycans and nucleic acid structures that are common to many different microorganisms and essential to the survival or infectivity of the microbe [257].Recognising multiple groups of PRRs allows for the host to utilise multiple mechanisms for immediate response to a various array of pathogens [260]. Activation of the innate immune system directs the specific part of the adaptive immune system to be activated upon different threats, thereby launching the most appropriate response to any microbial invasion [536]. The detection of PAMPS by PRRs notably increases the transcription of pro-inflammatory cytokines/chemokines, type 1 interferons (IFNs), and antimicrobial proteins associated with inflammatory response. The expression patterns of the stimulated factors varies between activated PRRs [537] . To date the TLRs are the most comprehensively studied family of PRRs and are highly significant in the instigation of antiviral response to infection. The TLRs account for a notable proportion of PAMPS recognition signalling, implicated in detecting parasites, fungi, bacteria and viruses [260]. RLRs were identified as cytosolic sensors of viral RNA which could be triggered independently of TLRs. They were quickly demonstrated to play an integral role in early anti-viral response to viruses [275]. The RLR family are composed of Rig-I, Melanoma differentiation-associated antigen 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2) [276-278]. RLRs are reportedly expressed at low concentrations in the resting cell and these concentrations are greatly increased upon stimulation [275, 278, 279]

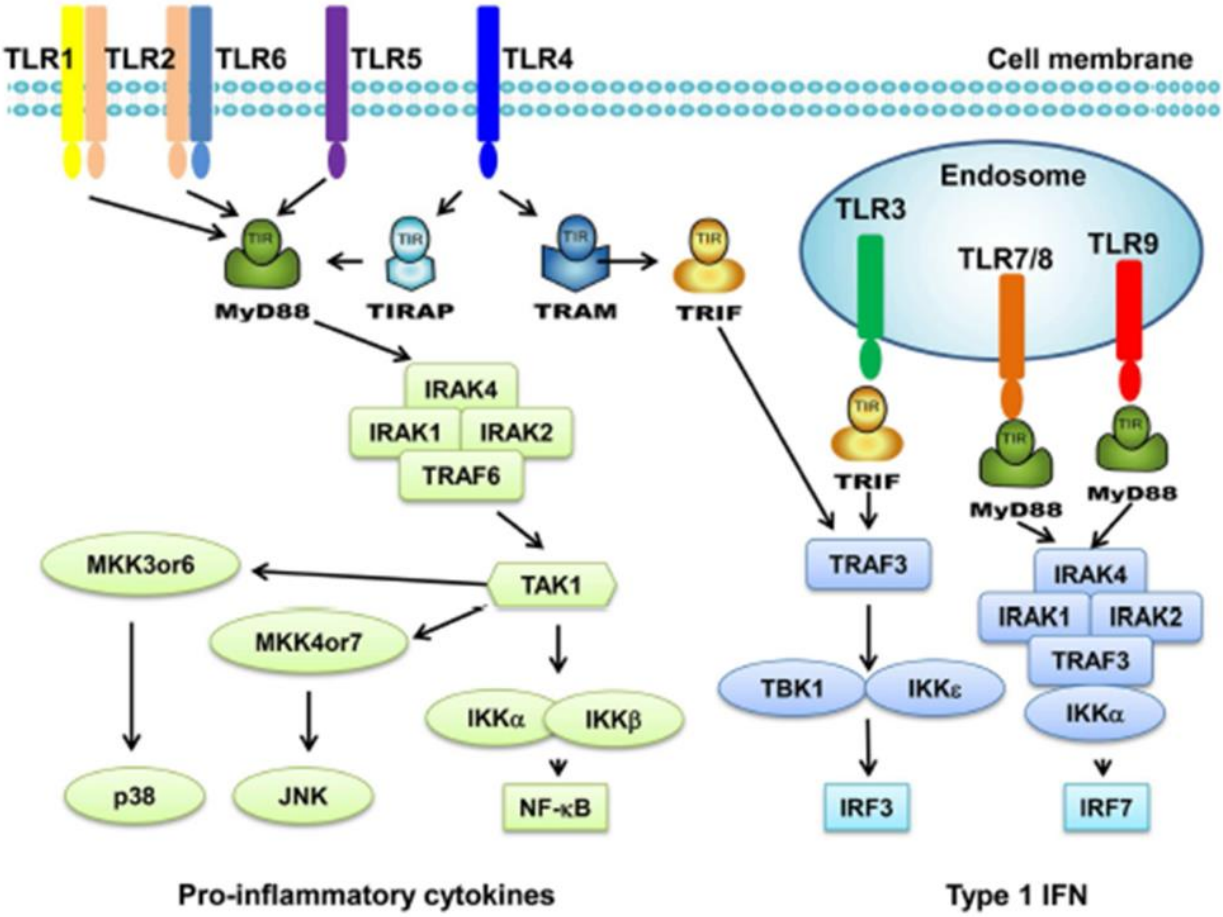
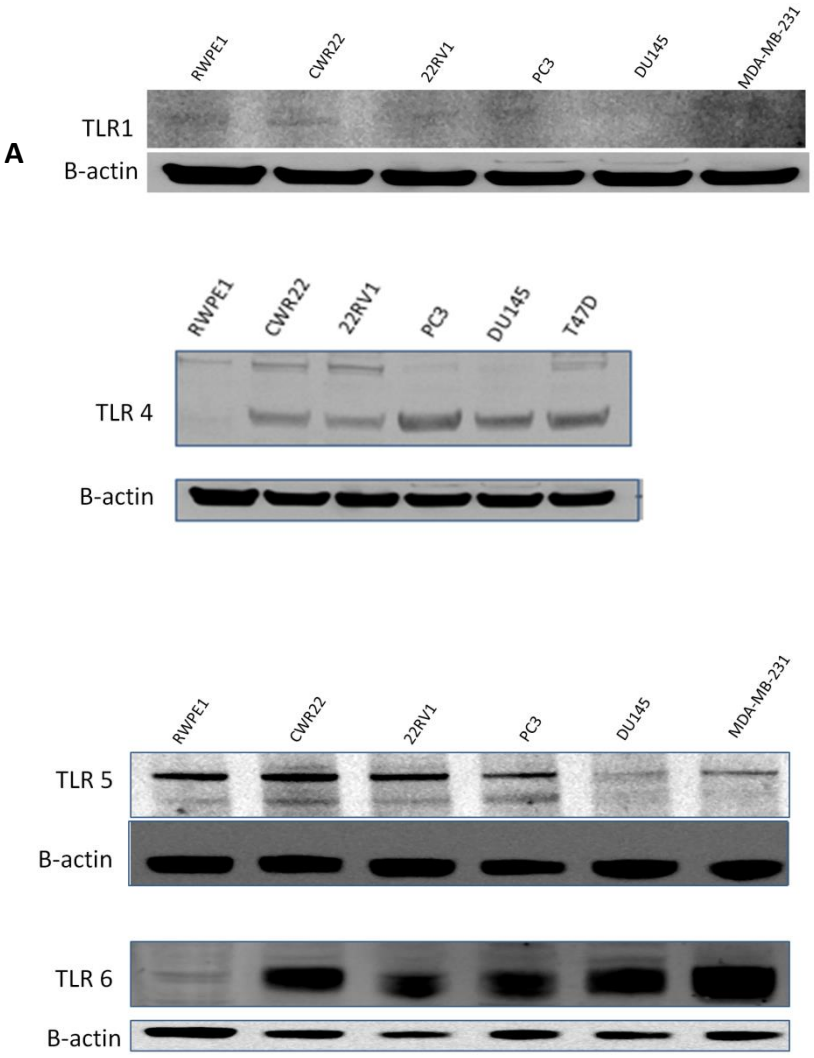


Figure 5.1 TLR signalling. TLRs 1, 3, 4, 5 and 6 are expressed on the cell surface while TLRs 3, 7, 8 and 9 and located intracellularly. TLRs 1 and 6 recognise their ligands as heterodimers with TLR2. The remaining TLRs are thought to form homodimers and initiate their signalling after interaction with their respective ligands. Most TLRs, except TLR3 signal through MyD88 to activate NF-KB . TLR3 and TLR4 can signal through the MyD88 independent TRIF pathway to activate IRF (Adapted from Toll-like Receptors in prostate cancer; Zhao et al., 2014)

5.2 Results

5.2.1 Basal expression of TLRs in Prostate cancer cells

The expression of TLRs was explored in RWPE1, CWR22, 22RV1, PC3 and DU145 prostate cell lines. Western blot was employed to determine if cell surface TLRs 1, 4, 5 and 6 are expressed in PCa cells. The expression of TLR1 was low. There was marked expression of TLR 4, 5 and 6 among the PCa cell lines (Figure 5.2 A). Expression of Intrinsically expressed Toll like receptors 3, 7, 8 and 9 was also confirmed in PCa cells. TLRs 3, 7 and 9 were found to be abundantly expressed in PCa cell lines and while TLR 8 was expressed a lower expression signal was obtained. TLRs 7, 8 and 9 demonstrated greater expression in AR positive cell lines than AR negative cell lines (Figure 5.2 B). The expression of TLRs in the RWPE1 transformed epithelial cells was also considered. While the observations of our earlier experiments (Chapters 3 and 4) indicated that the cell line is not the most ideal representative of normal prostate epithelia, it is worth nothing that the TLRs of interest and selected for further exploration in PCa, TLRs 3, 4 and 9, are not expressed in the RWPE1 epithelial cell line. Due to the shared use of primary prostate epithelial cells and the limitation of their finite passage turnover, their use in this section of the study was unfeasible. It is possible that the TLR expression in the PCa cell lines reflects their contribution and significance to the development and progression of the disease.


A
RWPE1
CWR22
22RV1
PC3
DU145
MDA-MB-231
TLR1
B-actin
RWPE1
CWR22
22RV1
PC3
DU145
T47D
TLR 4
B-actin
RWPE1
CWR22
22RV1
PC3
DU145
MDA-MB-231
TLR 5
B-actin
TLR 6
B-actin

B

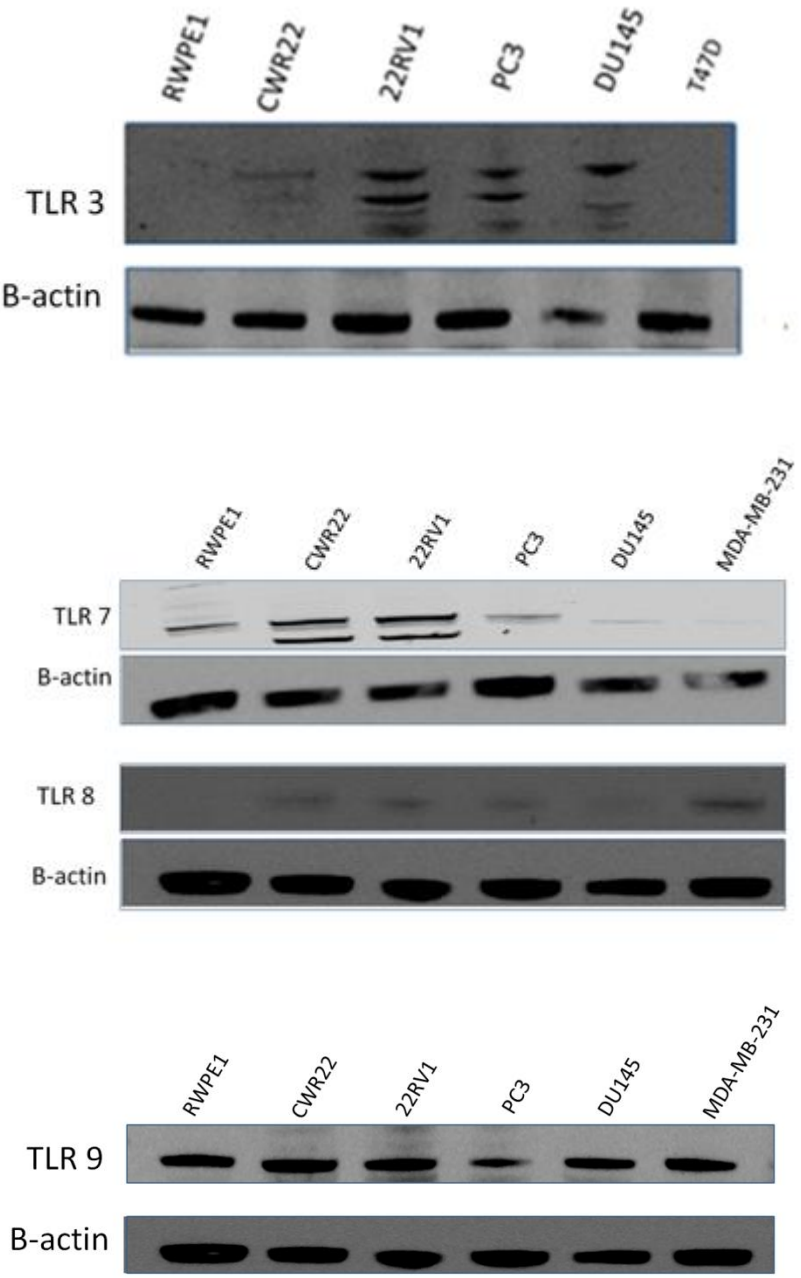


Figure 5.2 Expression of Toll like receptors in Prostate cancer cell lines. Transformed RWPE1 and PCa cells were explored for TLR expression. Cell lysates were separated by SDS PAGE and examined for the expression of Toll Like receptors. Toll like receptors are abundantly expressed in both AR positive and AR negative cell lines. Only TLRs 5, 7 and 9 were identified in RWPE1 cell.TLRs of the cell surface were confirmed by probing lysate with Anti TLR 1, 4, 5 and 6 **(A)**. Expression of intracellular toll like receptors was confirmed by probing lysates with Anti TLR 3, 7, 8 and 9 **(B)**. β actin was used as a loading control

5.2.2 Basal expression of RLRs in prostate cancer cell lines

Basal expression of the RLR Receptor family was explored in RWPE1, CWR22, 22RV1, PC3 and DU145 prostate cell lines. As shown in Figure 4.3 RIG-I is expressed at a basal level in PCa cells. MDA5 was expression was detected only in DU145 cells lines at a basal level.

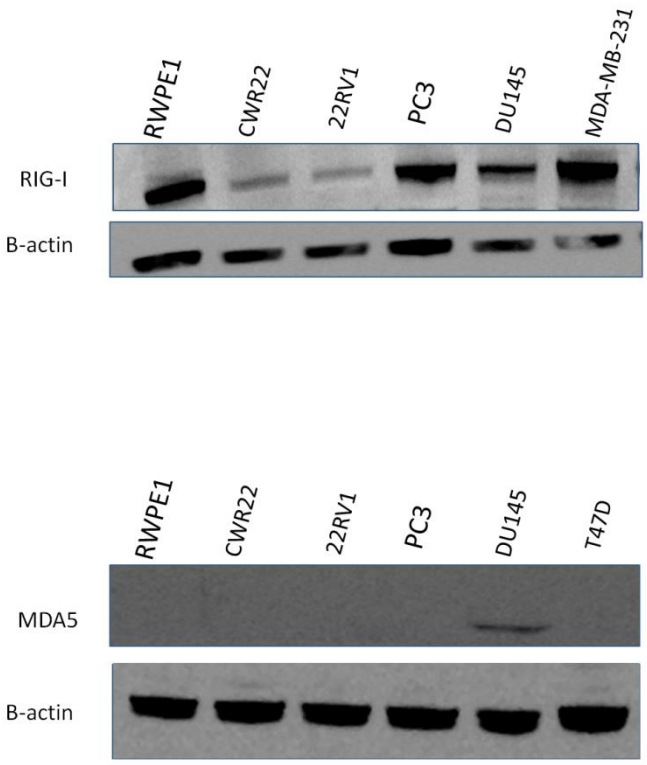


Figure 5.3 Expression of RLRs in prostate cancer cell lines. PCa cells were explored for the expression of RLRs RIG-I and MDA5. Cell lines were grown in petri dishes to confluency before harvesting. Cell lysates were separated by SDS PAGE and examined for the expression of RLRs by probing lysate with Anti RIG-I and Anti MDA5. B actin was used a s a loading control.

5.2.3 OP449 modulates basal TLR expression in prostate cancer cells

Following the confirmation of TLR and RLR expression in PCa cell lines, the ability to modulate the expression of cell surface TLRs, intracellular TLRs and RLRs in response to ApoE mimetic was explored. The consequence of incubating PCa cell lines with OP449 ranging from 0 to 1500nM was explored by western blot (Figure 5.4).

Exposure to OP449 was examined in AR positive and AR negative cell lines. The expression of cell surface receptor TLR4 was assessed in response to increasing concentrations of OP449. There was no observed change in TLR4 expression in response to OP449 among the AR positive cell lines CWR22 and 22RV1. However there was a decrease in TLR4 in response to OP449 with increasing concentrations of the peptide in AR negative cell lines PC3 and DU145 (Figure 5.4 a)

The expression of intracellular TLR3 and TLR9 were investigated in response to OP449. Within the AR positive cell lines CWR22 and 22RV1, an increase in the expression of TLR3 was observed in response to OP449 in a dose dependant manner. In contrast, AR negative cell lines PC3 and DU145 responded differently, exhibiting a decline in TLR3 expression in response to OP449. Similarly, TLR9 expression was increased in CWR22 and 22RV1 cell lines in response to OP449. TLR9 was down regulated in PC3 and DU145 with increasing concentrations of OP449 (Figure 5.4 b).

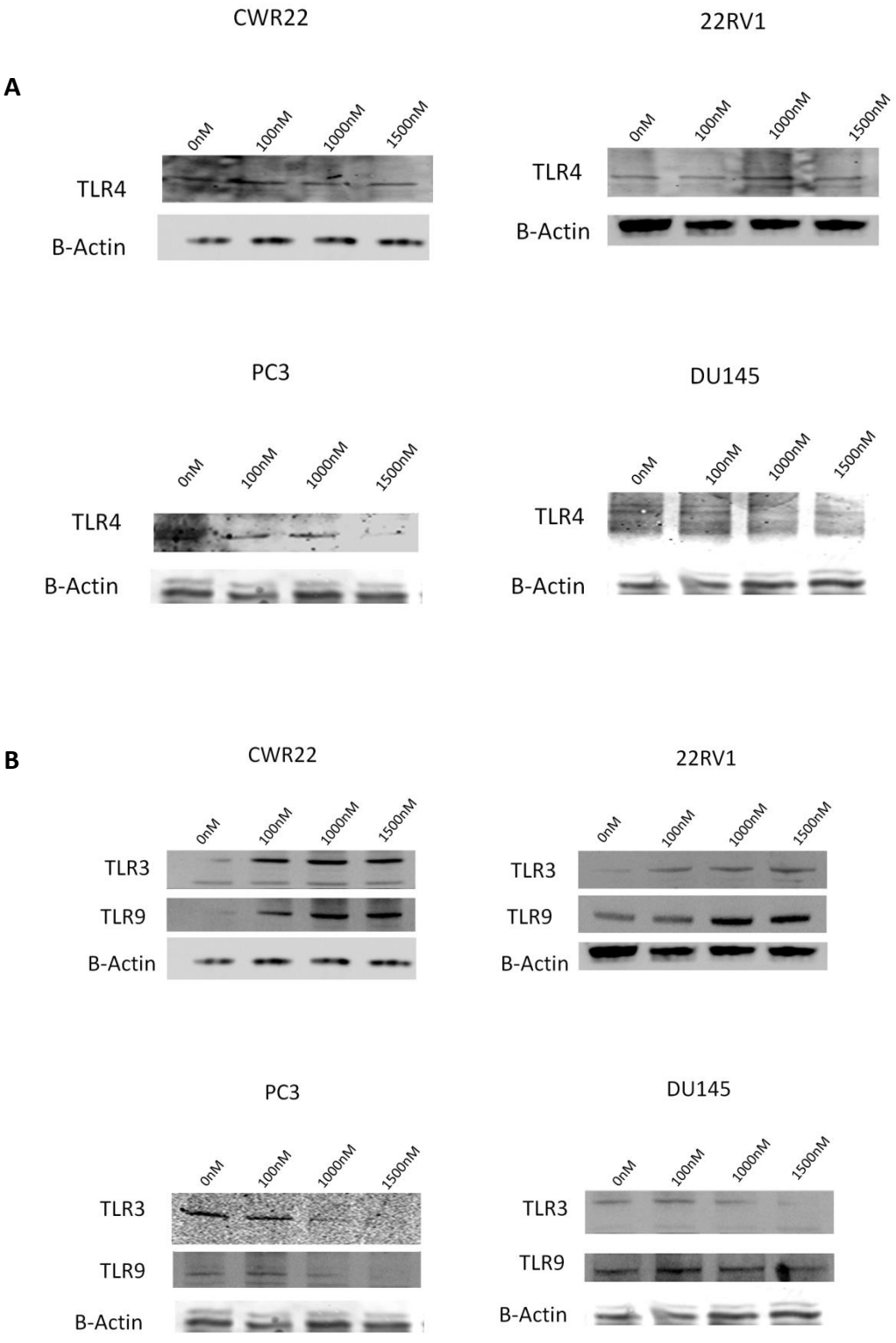


Figure 5.4 Modulation of TLR in response to OP449. AR positive (CWR22 and 22RV1) and AR negative (PC3 and DU145) cells were seeded into petri dishes at $1X10^6$ cells per dish. A dose response screen was performed using OP449 to investigate TLR modulation in response to the drug. Cell lines were exposed to OP449 for 24 hours with concentrations ranging from 0 to 1500nM. Protein was extracted and expression of TLRs -3, -4 and -9 were exploredExpression of TLR4 remained stable while an increase in TLRs -3 and -9 were observed in the AR positive cell lines. There was a correlating decrease in TLR -3, -4 and -9 expression in response to increasing concentrations of OP449 within the AR negative cell lines.

5.2.4 OP449 modulates basal RIG-I expression in prostate cancer cells

The expression of RLRs RIG-I and MDA5 was explored in response to OP449. Following the trend of the TLRs, RIG-I expression increased with increasing concentrations of OP449 in the AR positive CWR22 cell line. In the PC3 cell line, exposure to OP449 resulted in a decline of RIG-I protein expression. While there was no basal expression of MDA5, the protein was stimulated by OP449 in CWR22. There was no MDA5 detected in the AR negative PC3 cell line. The effect of OP449 on protein expression of IkappaB kinase ε (IKKε) and IRF3, targets downstream of RIG I, were also explored. Expression of IKKε increased with increasing concentrations of OP449 in the CWR22 cells while it decreased with increasing concentrations of OP449 in PC3 cells. Protein expression of IRF3 remained stable in response to OP449 in both cell lines (Figure 5.5).

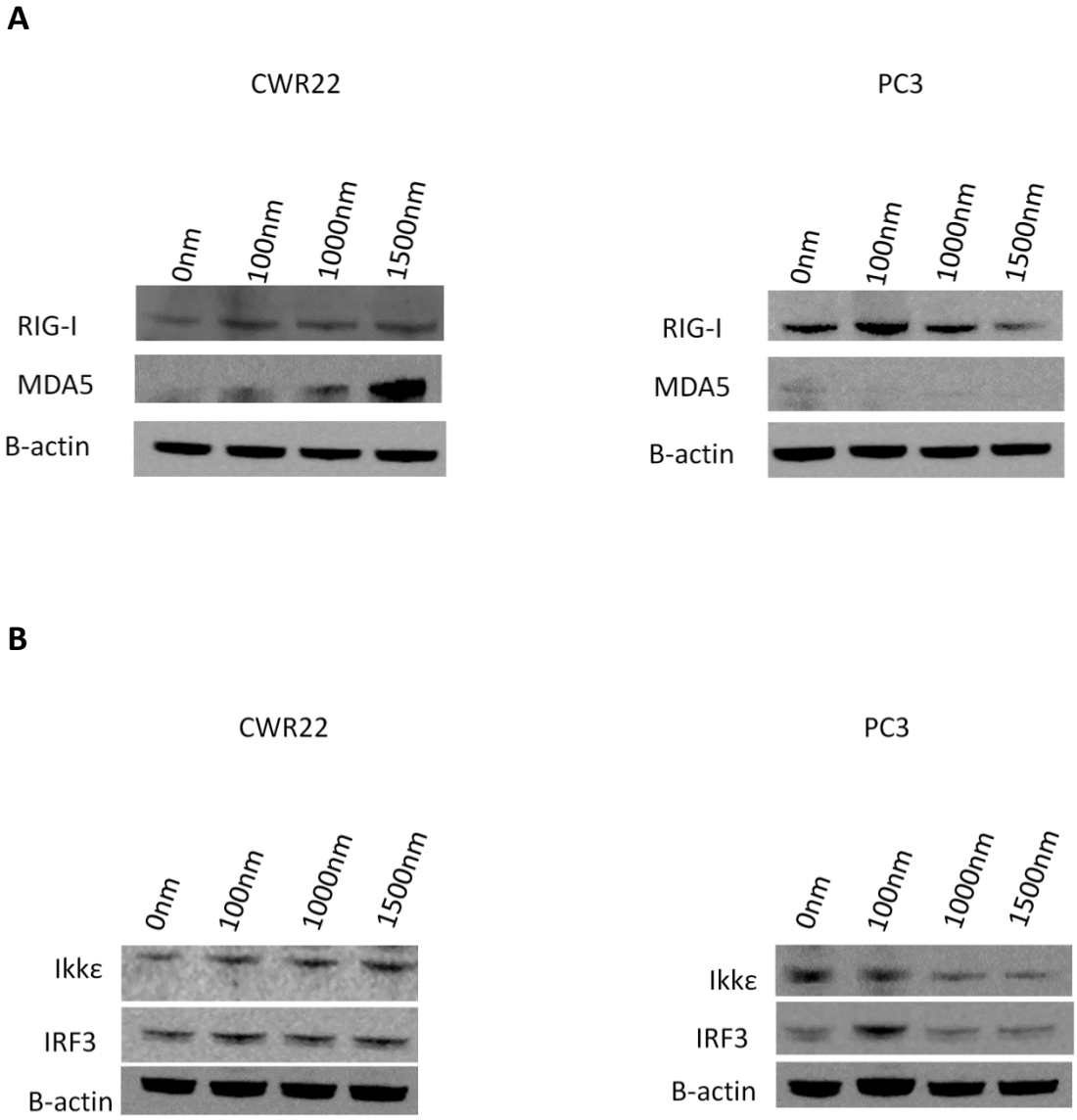


Figure 5.5 Modulation of RLRs in response to OP449 . Are positive (CWR22) and AR negative (PC3) cells lines were exposed to OP449 for 24 hours with concentrations used ranging from 0 to 1500nM in order to determine the effect of OP449 on RLRs. Cells were seeded into petri dishes at $1X10^6$ cells per dish. Protein was extracted and expression of RIG-I and MDA5 were explored by western blot. Expression of RIG-I and MDA5 increased in response to increasing concentrations of OP449 in the CWR22 cells. RIG-I was down regulated in the PC3 cells in response to OP449 and expression of MDA5 was not detected **(A)**. Protein expression of downstream targets Ikkε and IRF3 were explored in response to OP449. Western blot demonstrated Ikkε was upregulated in response to OP449 while expression of IRF3 did not change within the CWR22 cells. Protein expression of Ikkε was down regulated in the PC3 cells in response to increasing concentrations of OP449 **(B)**.

5.2.5 ApoE mimetic peptides reduce the induction of TLR4 by LPS in AR negative prostate cancer cell lines

The preliminary screening suggested modulation of innate immune signalling in AR negative cell lines to a greater degree than AR positive cells. Cell surface receptor TLR4, intracellular TLR3 and the RLR RIG-I were selected for further exploration in PCa. Subsequent studies explored the effect of ApoE peptide mimetics COG112 and OP449 on TLR and RLR activation in the AR negative PCa cell lines PC3 and DU145.

Focusing on the TLR4 receptor, the ligand LPS was used due to its specificity for binding and activating the TLR4 receptor. PC3 and DU145 cells were seeded into 6 well plates at a density of $1.6X10^5$ and left to adhere overnight. Cells were then serum starved overnight Treatment conditions included control untreated cells, cells stimulated with LPS, cells treated with COG112 or OP449, and cells pretreated with LPS followed by four hour incubation with COG112 or OP449. Protein was isolated and quantified before samples were assessed by western blot.

PC3 and DU145 cells were incubated with the ApoE mimetic COG112 for 4 hours either alone or following a 1 hour pre-treatment with 100ng/mL LPS. Concentrations of COG112 used were 500, 1000 and 1500nM. Western Blot results indicated that COG112 was capable of partially decreasing TLR4 induced by LPS in a dose dependant manner in both PC3 and DU145 cells (Figure 5.6 A).

In a similar fashion, the outcome of OP449 in response to TLR4 stimulation was assessed in AR negative PC3 and DU145 cells. Concentrations of OP449 administered were 100, 500 and 1000nM and incubation was either alone or following a 1 hour pre-treatment with 100ng/mL LPS. OP449 depletes expression of TLR4 when administered alone but also following stimulation of TLR 4 by LPS (Figure 5.6 B).

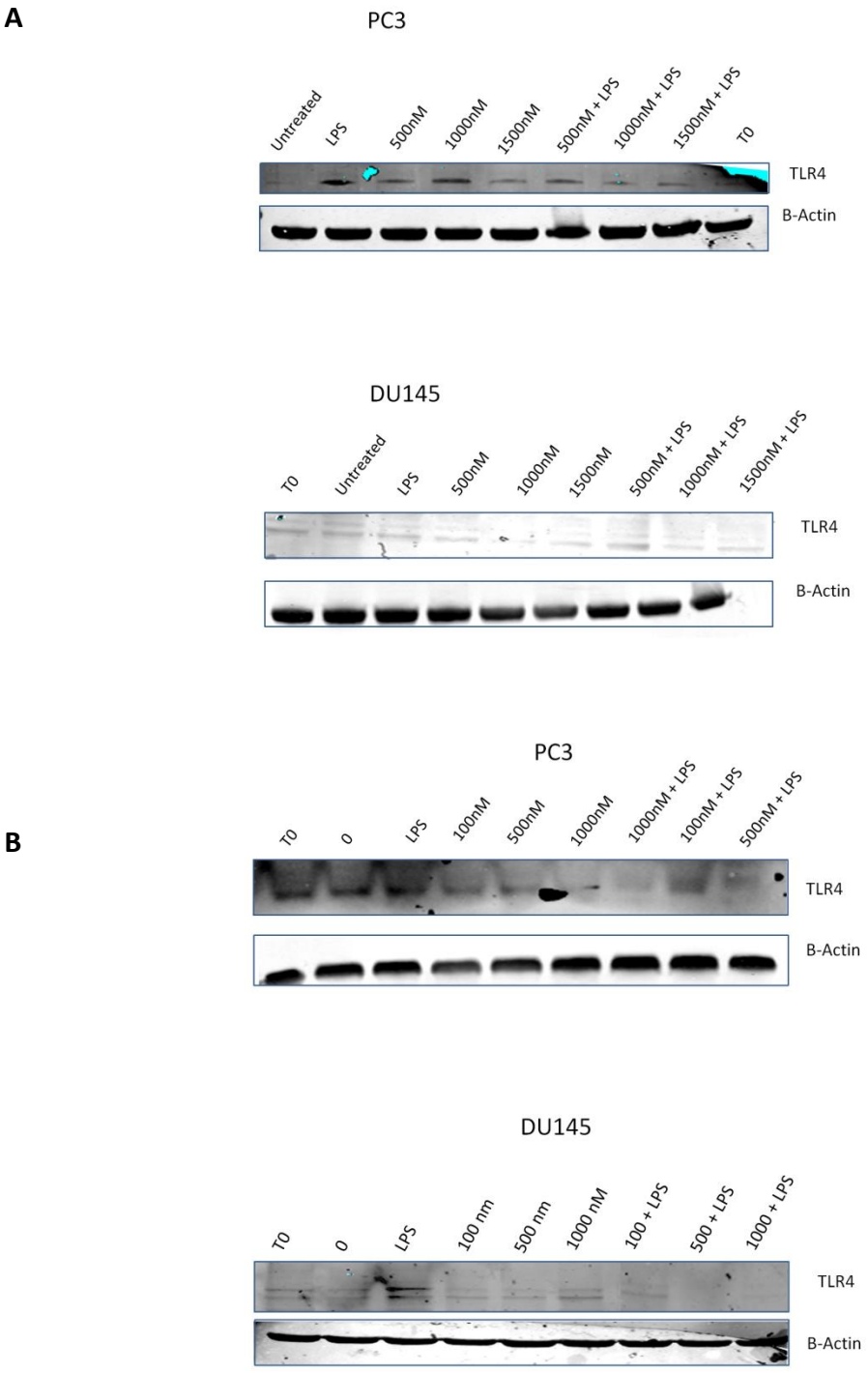


Figure 5.6 Inhibition of TLR4 activation by LPS PC3 and DU145 cells were seeded into 6 well plates and serum starved overnight. LPS was administered to stimulate TLR4. A range of concentrations of COG112 was added to the appropriate wells either as a single treatment or following stimulation with LPS. The COG112 Concentrations range was 0, 500nM, 1000nM and 1500nM. A decrease in TLR expression was observed in response to COG112 following LPS pretreatment in both PC3 and DU145 cell lines **(A)**. OP449 was administered at does 0nM, 100, 500 and 1000nM either as single individual treatments or with each dose following a 1 hour incubation with 100ng LPS. TLR4 was downregulated both in response to OP449 alone and following stimulation by LPS in a dose dependant manner in PC3 and DU145 cell lines **(B)**.

5.2.6 The effect of ApoE mimetics on TRIF-TRAF3 dependent TLR4 signalling

Upon PAMPs and DAMPs recognition, TLRs recruit TIR domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF-κB , IRFs, or MAP kinases to regulate the expression of cytokines, chemokines and type I interferons [259, 260].

TLR signalling is predominantly divided into two signalling pathways; namely the MyD88 dependent pathway and the TRIF dependent pathway. MyD88 is involved in signalling triggered by all the TLRs with the exception of TLR3 and plays a major role in TLR induced signal transduction [267, 323]. TLR3 is the only TLR that does not use MyD88 but instead is dependent on TRIF signalling [326].

Like most of the TLRs, TLR 4 signals through the MyD88 –dependent signalling pathway. Upon stimulation, this pathway in turn activates IL-1 receptor associated kinases (IRAK) [329, 538]. These in turn interact with tumour necrosis factor receptor associated factor 6 (TRAF6). Following the IRAK-TRAF6 complex association, TRAF6 undergoes polyubiquitination along with the Transforming growth factor beta-activated kinase 1 (TAK1) protein kinase complex. After associating with regulatory TAB1,2 and 3 and associating polyubiquitin chains, the activation of TAK1 occurs [332, 539, 540]. TAK1 activation in turn activates two different pathways involving the IKK complex and MAPK pathways respectively, which ultimately results in the activation of MAPK and NFκB signalling [333].

As well as the MyD88 pathway, TLR4 utilises the TRIF dependent pathway. During TLR 3 and TLR4 mediated signalling, TRIF is responsible for initiating a signalling pathway in which TRAF3 and TRAF family member-associated NF-kappa-B activator (TANK) serve as a link to bridge the IKK related kinases TBK1 and IKKε [335, 346, 541, 542]. These kinases prompt the direct phosphorylation of IRF3 and IRF7 [543, 544]. It has been noted in the literature that although TBK1 and IKKε are critical for TRIF dependent IRF3 and IRF7 phosphorylation, these kinases are not involved in TLR facilitated IκBα stimulation [545]. With this in mind, the influence of ApoE

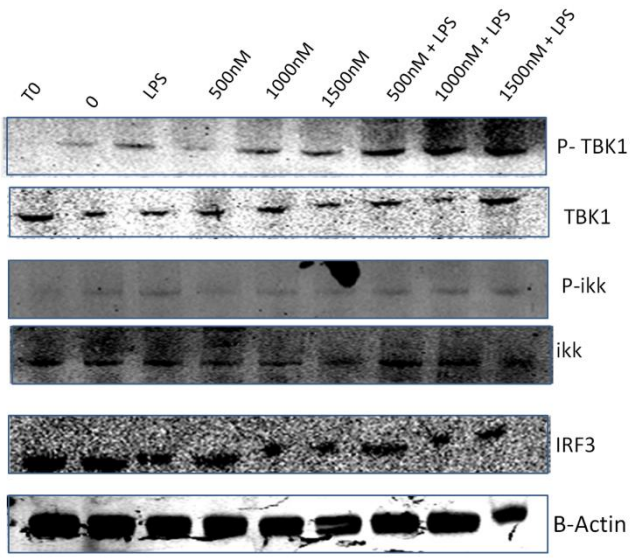
mimeic peptides on TLR4 signalling was explored by first investigating the TRIF dependant component of TLR4 signalling.

PC3 and DU145 cells were incubated with the ApoE mimetic COG112 either alone or following a 1 hour pre-treatment with 100ng/mL LPS. Concentrations of COG112 used were 500, 1000 and 1500nM. Following treatment, protein was isolated, quantified. The protein expression of TBK1, IKKε and IRF3 in response to TLR4 stimulation and ApoE peptide exposure were examined by Western Blot. Results indicated that COG112 was ineffective at decreasing TRIF dependent TLR4 signalling at the concentrations used both alone and following TLR stimulation with LPS in a in both PC3 and DU145 cells (Figure 5.7 A).

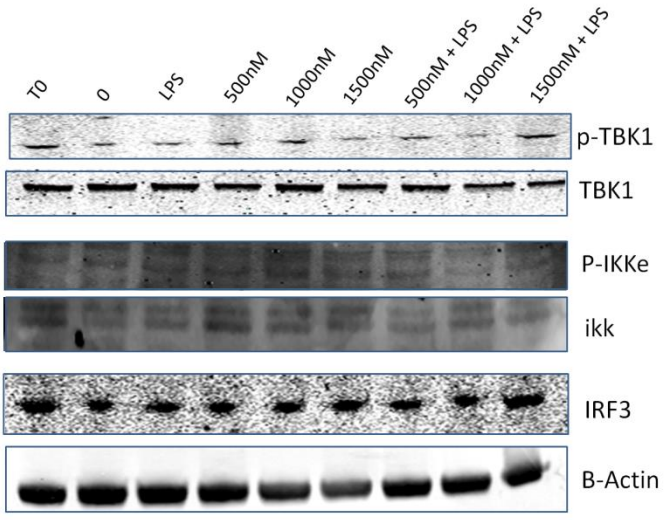
In a similar fashion, the outcome of OP449 in response to TLR4 stimulation was assessed in AR negative PC3 and DU145 cells. Concentrations of OP449 administered were 100, 500 and 1000nM and incubation was either alone or following a 1 hour pre-treatment with 100ng/mL LPS. Unlike COG112, OP449 treatment resulted in a downregulation of TBK1 and IKKε phosphorylation when administered alone and also following stimulation of TLR4 by LPS (Figure 5.7 B).

A

PC3


T0
0
LPS
500nM
1000nM
1500nM
500nM + LPS
1000nM + LPS
1500nM + LPS
P- TBK1
TBK1
P-ikk
ikk
IRF3
B-Actin

DU145


T0
0
LPS
500nM
1000nM
1500nM
500nM + LPS
1000nM + LPS
1500nM + LPS
p-TBK1
TBK1
P-IKKe
ikk
IRF3
B-Actin

B

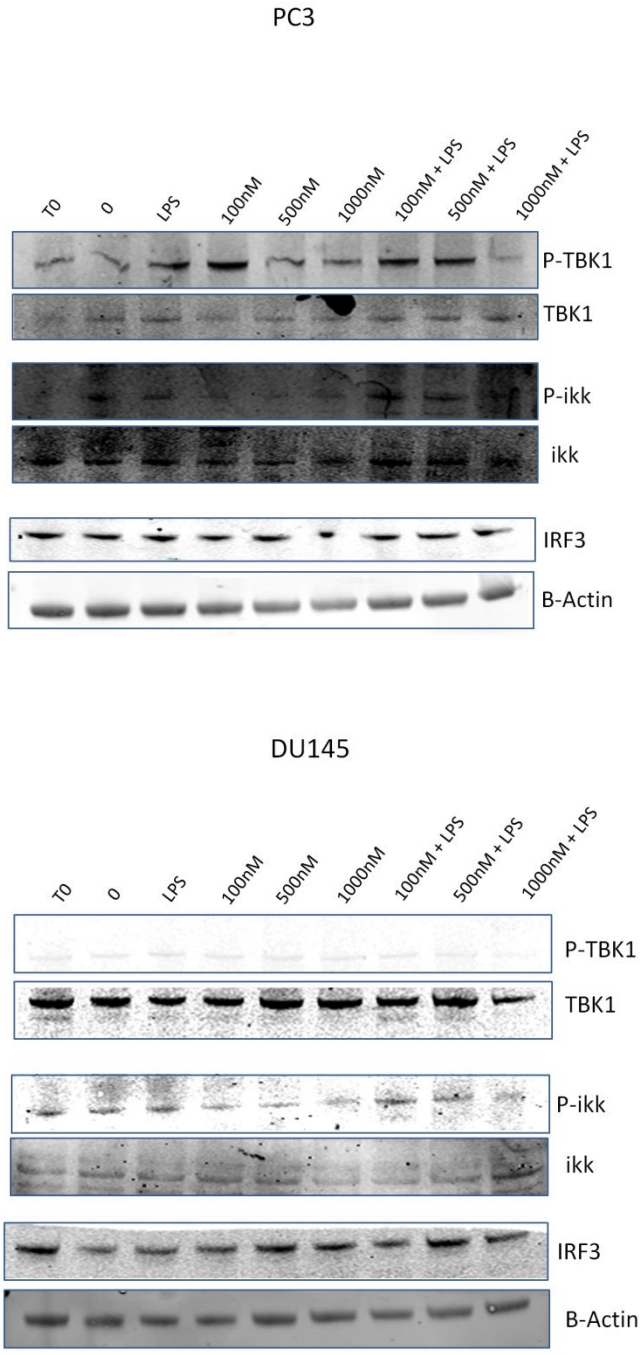


Figure 5.7 Comparison of Inhibition of LPS stimulation of TLR4 by COG112 and OP449 PC3 and DU145 cells were seeded into 6 well plates and serum starved overnight. LPS was administered to stimulate TLR4. A range of concentrations of COG112 was added to the appropriate wells either as a single treatment or following stimulation with LPS. The COG112 Concentrations range was 0, 500nM, 1000nM and 1500nM. There was no modulation of downstream signalling or activation observed in response to COG112 following LPS pretreatment in both PC3 and DU145 cell lines **(A)**. OP449 was administered at 0nM, 100, 500 and 1000nM either as single individual treatments or with each dose following a 1 hour incubation with 100ng LPS. A decrease in phosphorylation was detected in response to OP449 both alone and following stimulation by LPS in a dose dependant manner in PC3 and DU145 cell lines **(B)**.

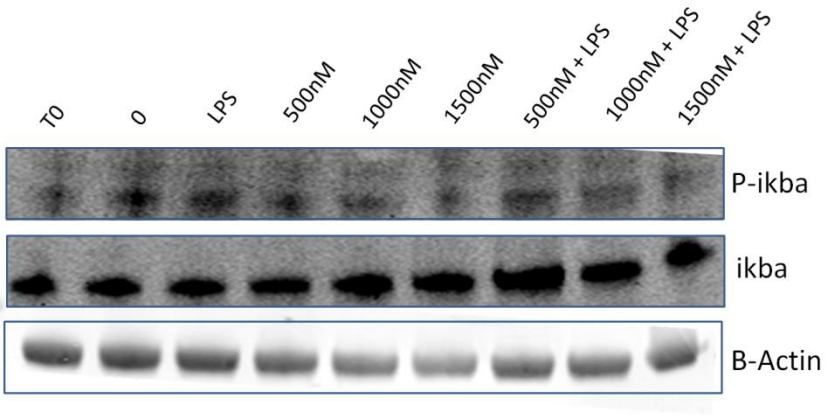
5.2.7 The effect of ApoE mimetics on TLR4 mediated NF-κB activity

The effect of ApoE mimetics on the NF-κB pathway in response to TLR4 activation was explored. Investigation involved examining the phosphorylation of IKBα in PC3 and DU145 cells exposed to the ApoE peptide mimetics COG112 and OP449 following TLR4 stimulation with the receptor agonist LPS. As previously described, $1.6X10^5$ cells per well were seeded into a 6 well plate and left to adhere before being serum starved overnight. PC3 and DU145 cells were incubated with the ApoE mimetic COG112 either alone or following a 1 hour pre-treatment with 100ng/mL LPS. Concentrations of COG112 used were 500, 1000 and 1500nM. Following treatment, protein was isolated, quantified. The protein expression IKBα and p-IKBα in response to TLR4 stimulation and ApoE peptide exposure were examined by Western Blot. Results indicated that COG112 was less effective at decreasing Trif dependent TLR4 signalling at the concentrations used both alone and following TLR stimulation with LPS in a in both PC3 and DU145 cells although a slight decrease was observed at 1500nM (Figure 5.8 A).

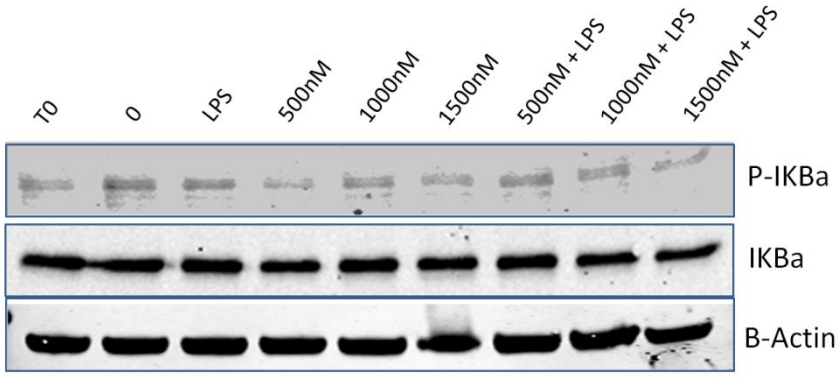
In a similar fashion, the outcome of OP449 in response to TLR4 stimulation was assessed in AR negative PC3 and DU145 cells. Concentrations of OP449 administered were 100, 500 and 1000nM and incubation was either alone or following a 1 hour pre-treatment with 100ng/mL LPS. OP449 treatment resulted in a downregulation of IκBa phosphorylation when administered alone and also following stimulation of TLR4 by LPS (Figure 5.8 B).

A

PC3


T0
0
LPS
500nM
1000nM
1500nM
500nM + LPS
1000nM + LPS
1500nM + LPS
P-ikba
ikba
B-Actin

DU145


T0
0
LPS
500nM
1000nM
1500nM
500nM + LPS
1000nM + LPS
1500nM + LPS
P-IKBa
IKBa
B-Actin

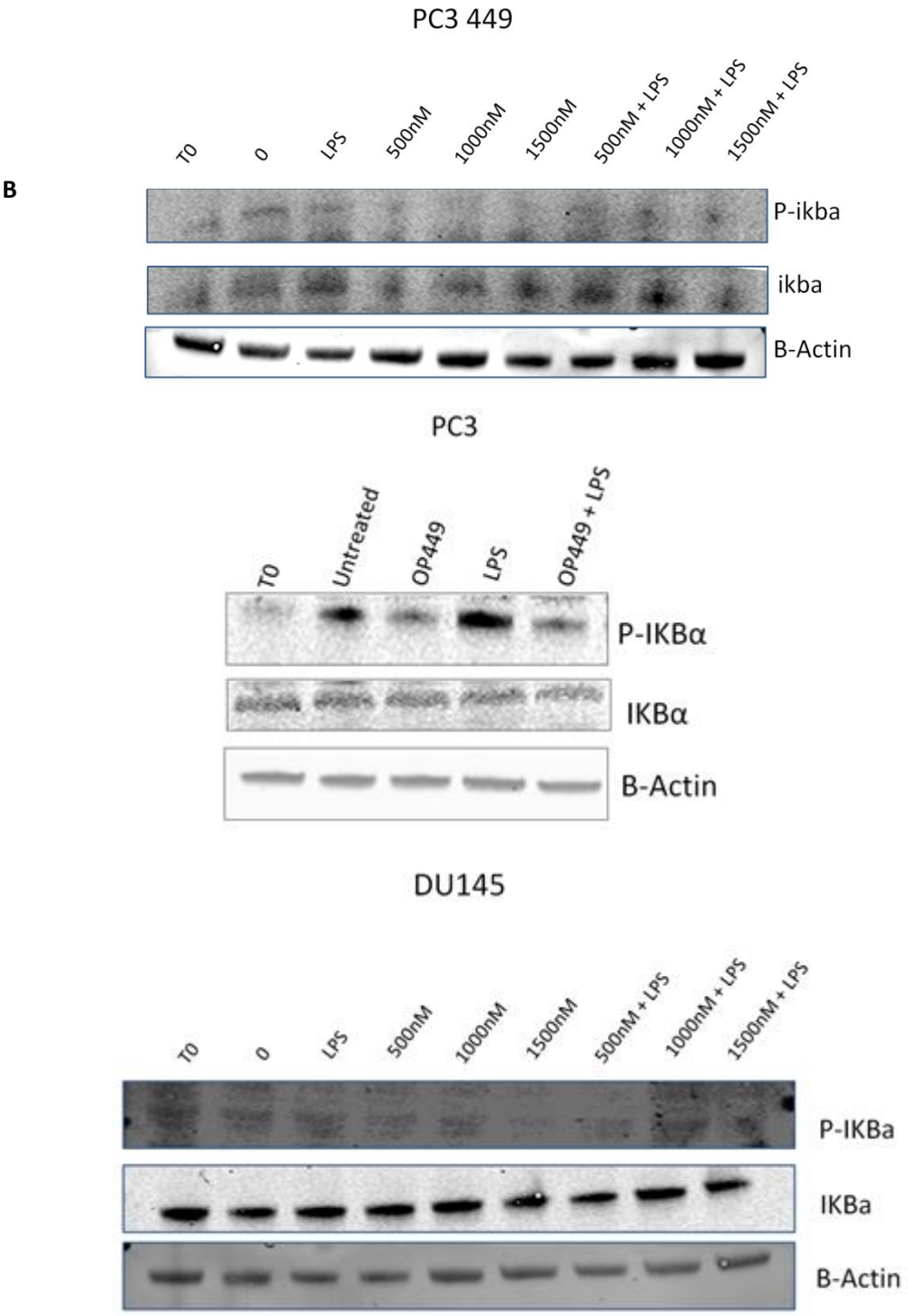


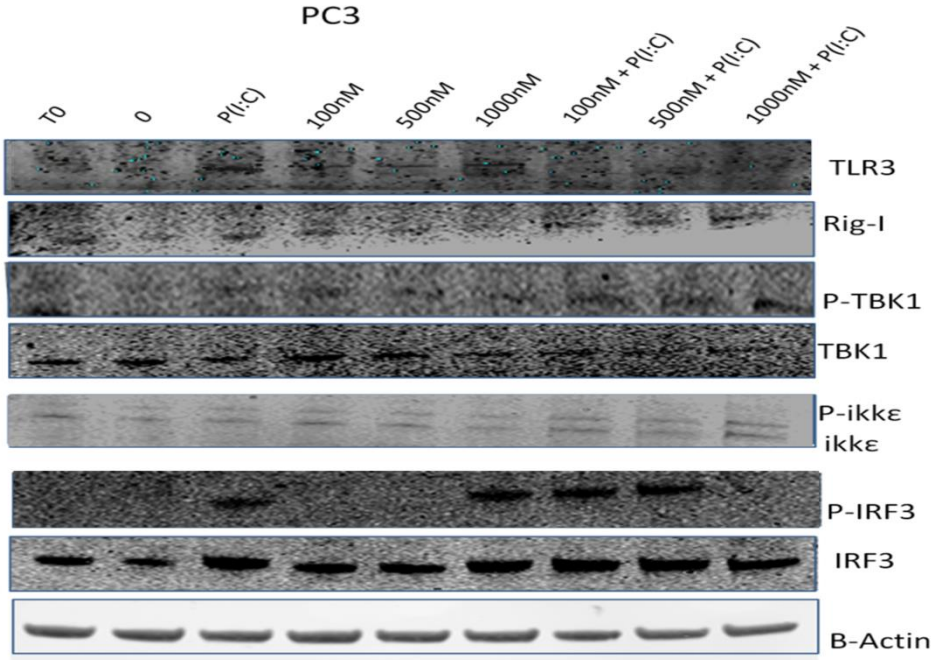
Figure 5.8 Inhibition of TLR4 induce IκBα signalling PC3 and DU145 cells were seeded into 6 well plates and serum starved overnight. LPS was administered to stimulate TLR4. A range of concentrations of COG112 was added to the appropriate wells either as a single treatment or following stimulation with LPS. The COG112 concentrations range from 0, 500nM, 1000nM and 1500nM. There was no modulation of IκBα expression in response to COG112. Phosphorylation was marginally decreased at the highest concentration 1500nM both alone and following LPS pre-treatment in PC3 and DU145 cell lines **(A)**. OP449 was administered at doses 0nM, 100, 500 and 1000nM either as a single treatment or following a 1 hour incubation with 100ng LPS. A decrease in IκBα phosphorylation was detected in response to OP449 both alone and following stimulation by LPS in a dose dependant manner in PC3 and DU145 cell lines **(B)**.

5.2.8 The effect of ApoE mimetics on anti-viral response to dsRNA

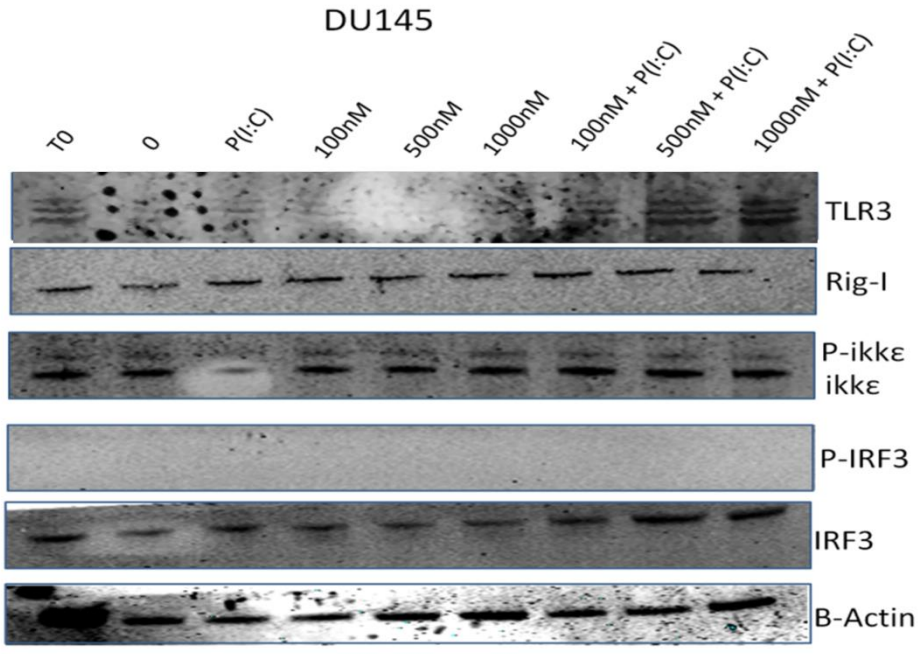
In an effort to determine if ApoE mimetic OP449 can interrupt the signalling cascade initiated by dsRNA, some shared components of the cascade were explored. Polyinosinic:polycytidylic acid (Poly (I:C)), a synthetic dsRNA immunostimulant, which simulates response to viral infection was used to induce a viral response. The compound is a ligand for the TLR3 and RIG-I receptors and so was used in this study to induce receptor stimulation. As previously described, PC3 and DU145 cells were seeded at a density of 1.6X105 cells per well in a 6 well plate and left to adhere before being subjected to an overnight serum starvation period. Cells were treated with OP449 for 4 hours either alone or following transfection with poly(I:C). OP449 with drug concentrations ranged from 0nM to 1000nM and following incubation, protein was isolated and quantified for further analysis. The expression and phosphorylation of TBK1, IKK and IRF in response to OP449 and poly(I:C) was determined by western blot by western blot (Figure 5.9).

As previously demonstrated, the OP449 compound was shown to be capable of downregulating TBK phosphorylation in PC3 cells. There was no baseline phosphorylation of IRF3 or downregulation of ikkε detected. Following Poly(I:C) transfection, OP449 failed to overcome phosphorylation of TBK1, IKKε and IRF3 (Figure 5.9A). In DU145 cells, transfection of Poly(I:C) appeared unsuccessful with no phosphorylation of IRF3 occurring. There was no downregulation of Ikkε observed although down regulation of TBK1 was detected in response to 1000nM OP449 alone (Figure 5.9 B)

A


PC3
T0
0
P(I:C)
100nM
500nM
1000nM
100nM + P(I:C)
500nM + P(I:C)
1000nM + P(I:C)
TLR3
Rig-I
P-TBK1
TBK1
P-ikkε
ikkε
P-IRF3
IRF3
B-Actin

B


DU145
T0
0
P(I:C)
100nM
500nM
1000nM
100nM + P(I:C)
500nM + P(I:C)
1000nM + P(I:C)
TLR3
Rig-I
P-ikkε
ikkε
P-IRF3
IRF3
B-Actin

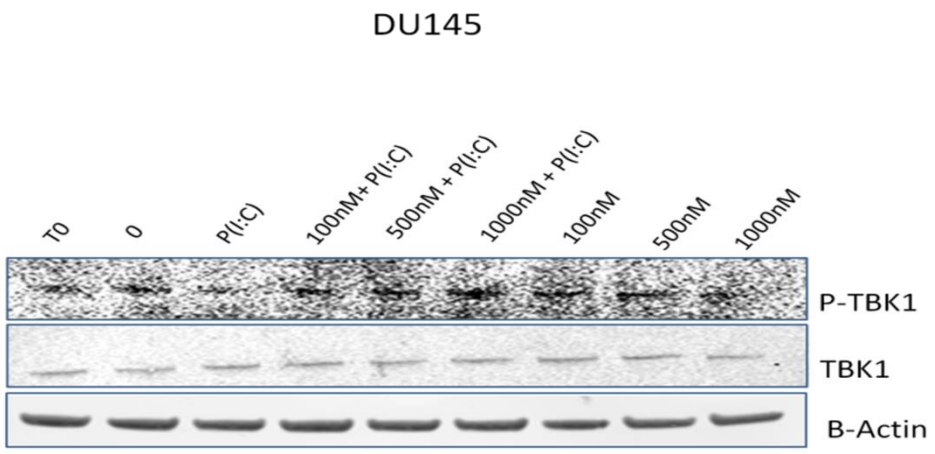


Figure 5.9 Inhibition of dsRNA stimulation of TLR3 and RIG-I PC3 and DU145 cells were seeded into 6 well plates and serum starved overnight. Poly(I:C) was transfected into cells to stimulate TLR3 and RIG-I. OP449 was administered at does 0nM, 100, 500 and 1000nM as single individual treatments or with each dose following transfection of poly(I:C). A decrease in phosphorylation of IKKε and TBK1 was detected in response to OP449 both alone and following stimulation of TLR3/RIG-I with poly(I:C) in a dose dependant manner in PC3 cells. Poly(I:C) induced phosphorylation of IRF3 in PC3 cells. OP449 failed to downregulated IRF3 phosphorylation induced by poly(I:C) **(A)**. A decrease in phosphorylation of IKKε and TBK1 was detected in response to OP449 both alone and following stimulation of TLR3/RIG-I with poly(I:C) in a dose dependant manner in DU145 cells. There was no phosphorylation of IRF3 detected in DU145 cells in response to Poly(I:C) transfection or OP449 **(B)**.

5.2.9 Transient knockdown of TLR3 and RIG-I in Androgen negative prostate cancer cells

As OP449 has been shown to impede downstream signalling of TLR3 and RIG-I in PCa cells, these receptors were silenced separately in PC3 and DU145 cells by SiRNA transfection in an effort to determine if their levels related to ApoE response. Upon seeding cells into 6 well plates at a density of of 1.2 $X10^5$, reverse transfection was employed whereby cells were transfected using a final SiRNA concentration of 25nM ON-TARGET plus SiRNA (Fisher) in PC3 cells and 50nM ON-TARGET plus SiRNA (Fisher) in DU145 cells and DharmaFECT reagent (Thermos scientific formulation3). A decrease in the expression of the receptors was achieved by transient transfection with four different SiRNAs coding for the each for TLR3 or RIG-I. A non-coding scramble siRNA was used as a control. Protein was

isolated as previously described at 48 hours. Knockdown was confirmed by western blot (Figure 5.10). Following confirmation of receptor knockdown, the expression of downstream targets were clarified.

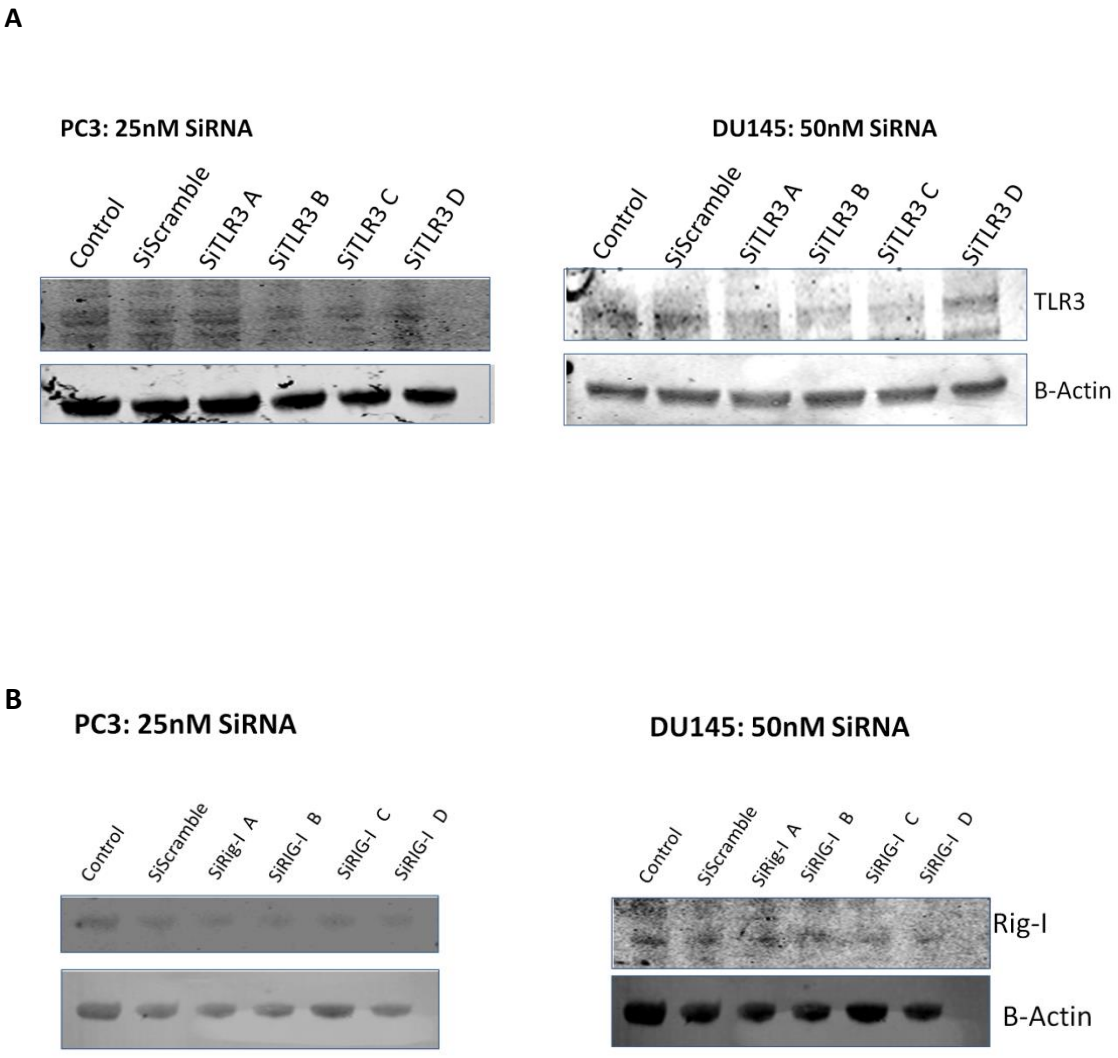
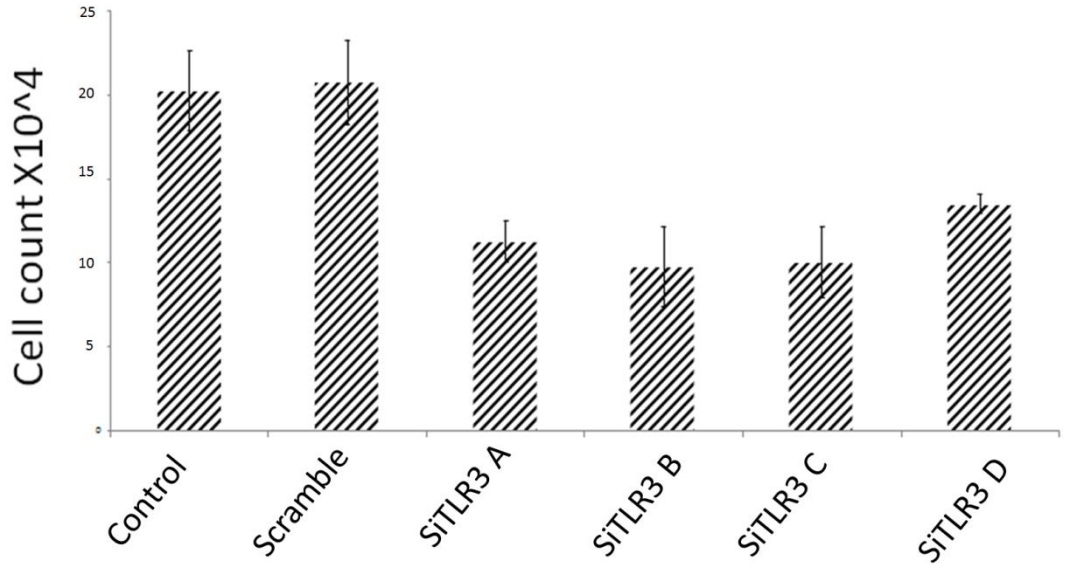


Figure 5.10 Transient knockdown of TLR3 and RIG-I The expression of TLR3 was down regulated in PC3 and DU145 cells by reverse transfection using four different SiRNAs coding for TLR3 and DharmaFECT reagent 3. A non-coding scramble siRNA was used as a control. A final SiRNA concentration of 25nM was used in the PC3 cells and 50nM for DU145 cells. Protein was isolated and successful knockdown of TLR3 was confirmed by western blot **(A)**. The expression of RIG-I was down regulated in PC3 and DU145 cells by reverse transfection using four different SiRNAs coding for RIG-I and DharmaFECT reagent 3. A non-coding scramble siRNA was used as a control. A final SiRNA concentration of 25nM was used in the PC3 cells and 50nM for DU145 cells. Protein was isolated and successful knockdown of RIG-I was confirmed by western blot **(B)**.

5.2.10 Receptor requirement for prostate cancer cell survival

Following the transient knockdown of TLR3 and RIG- by SiRNA, a decrease in adherent cells was observed at the point of protein harvesting the PC3 cells transfected with TLR3 coding SiRNA. Therefore a quantitative analysis of viable cells was performed using trypan blue staining technique Trypan blue selectively identifies dead cells. Viable cells were identified and counted using a haemocytometer for cell quantification. This assessment verified a decrease in the number of viable transfected cells. A parallel was noted between the decline in cell number and the degree of target protein expression in response to SiRNA (Figure 5.11 A). Following this observation a cell viability assessment was performed in PC3 and DU145 cells in response to both TLR3 and Rig-I knockdown using a range of SiRNA concentrations. After confirming the individual reagents were not toxic to the PCa cells, transfection was performed as previously described using 5nM, 10nM and 25nM SiRNA was used in PC3 cells and 10nM, 25nM and 50nM in DU145 cells. Quantification disclosed an increasing decline in cell number correlating with increasing SiRNA coding for TLR3. There was no correlating decline in cell viability resulting from Rig-I knock-down (Figure 5.11 B)

A



B

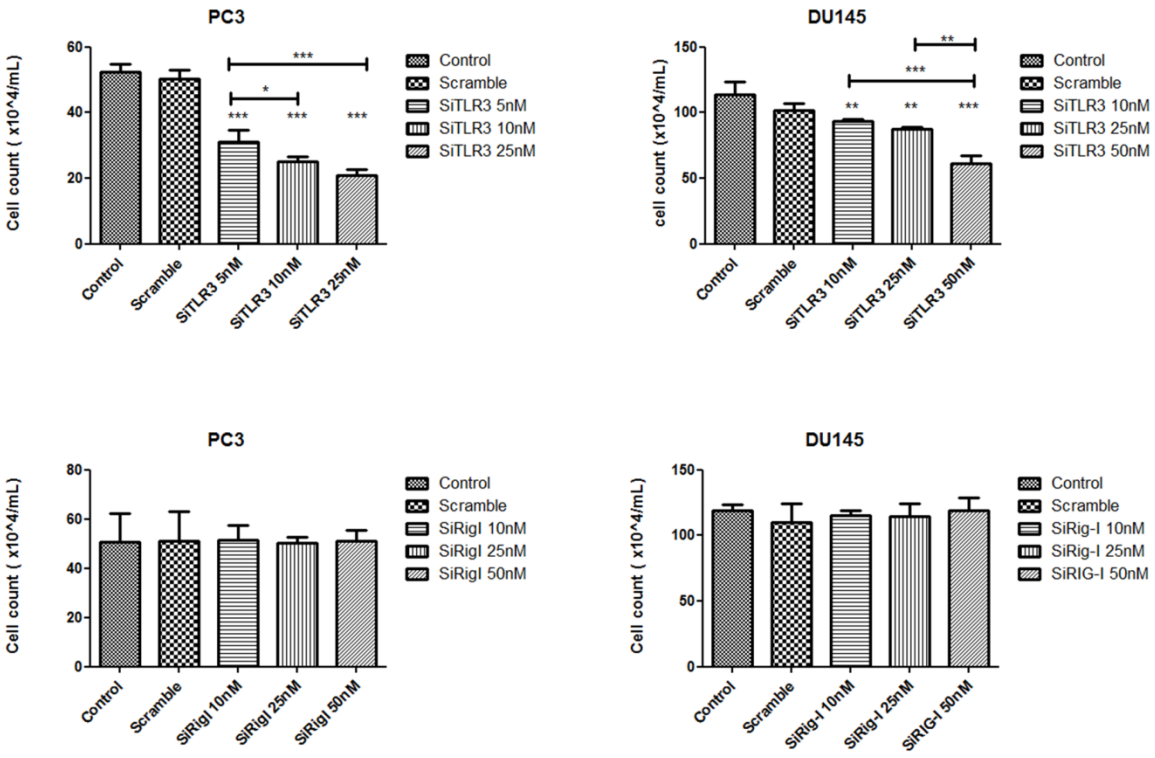


Figure 5.11 Transient knockdown of TLR3 but not RIG-I decreases cell viability Following SiRNA transfection, viable PC3 cells were identified and quantified using trypan blue staining and a haemocytometer. A decrease in the number of viable transfected with TLR3 coding SiRNA was calculated. There was no decrease in cell viability in response to scramble SiRNA transfection in comparison to non-transfected cells **(A)**. A cell viability assessment was performed in PC3 and DU145 cells in response to both TLR3 and Rig-I knockdown using a range of SiRNA concentrations. SiRNA concentration for PC3 transfections were 5nM, 10nM and 25nM SiRNA. Concentration for DU145 transfections were 10nM, 25nM and 50nM in DU145 cells. Quantification showed a decline in cell number correlating with increasing SiRNA coding for TLR3. There was no correlating decline in cell viability resulting from Rig-I knock-down **(B)** Statistical significance in cell viability was determined by one way ANOVA followed by tukey post hoc analysis, ***p<0.0001, **p<0.001, *p<0.05

5.2.11 The effect of receptor knockdown on downstream signalling

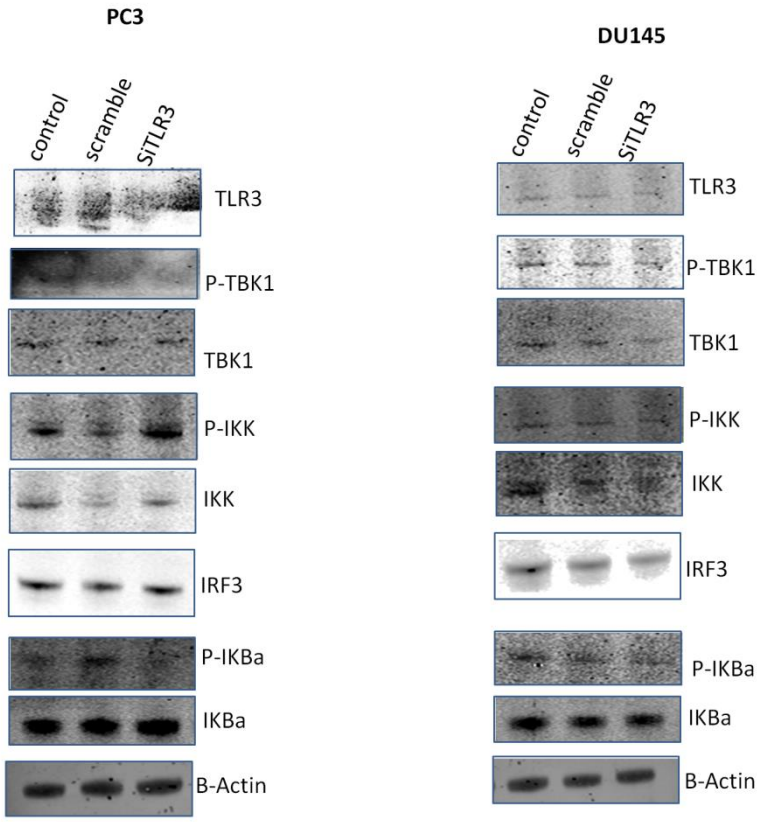
Protein isolated from TLR3 and RIG-I knockdown were assessed for subsequent changes in downstream signalling cascade in response to their individual knockdowns. This assessment was performed in an effort to connect changes in their expression to the mechanism of action of ApoE peptide mimetics. TLR3 and

RIGI receptor proteins were separately depleted in PC3 and DU145 cell lines and the expression and phosphorylation of downstream signalling was determined (Figure 5.12).

In the PC3 and DU14 cells transfected with SiRNA coding for TLR3 there was a decrease in TBK1 phosphorylation observed in PC3 and DU145 cells with no apparent modulations detected in IKKε expression or phosphorylation in either cell line. Consistent with previous findings there was a decrease in phosphorylation of IκBα in both PC3 and DU145 cells in response to TLR3 silencing (Figure 5.12 A)

Following successful transection of RIG-I targeting siRNA, there was no change in expression or phosphorylation in the IRF related proteins as western blot showed expression remained stable in TBK1, IKKε and IRF3 proteins. There was no consequential decline in phosphorylation of TBK1 or IKKε in either PC3 or DU145 cells. Similar to the TLRs knockdown samples there was a decrease in phosphorylation of the NF-κB inhibitor IKBα in PC3 and DU145 PCa cells (Figure 5.12 B)

A



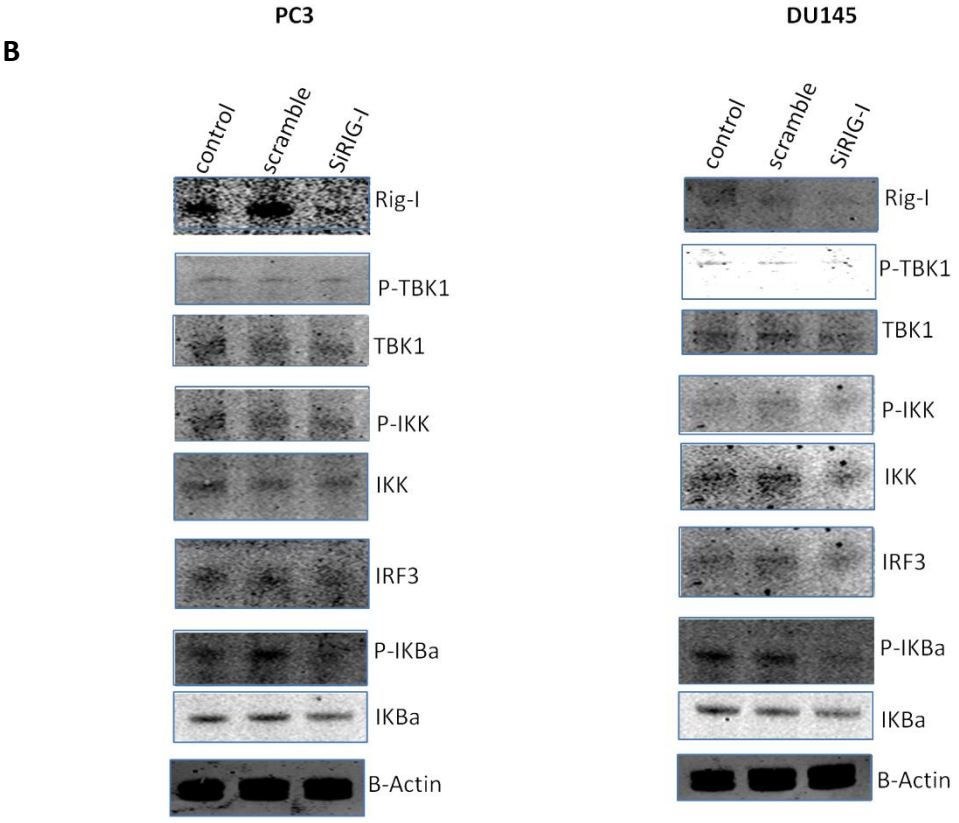


Figure 5.12 Transient knockdown of TLR3 and RIG-I modulates the expression of downstream targets. The effect of transient knockdown of TLR3 and RIG-I was explored on their downstream signalling cascade. Protein was isolated from PC3 and DU14 cells 48 hours post transfection with SiRNA coding for TLR3 and assessed by gel electrophoresis. Western blot confirmed a decrease of TBK1 phosphorylation in PC3 and DU145 cells. There were no apparent inflections detected in IKKε expression or phosphorylation in either cell line. A decrease in phosphorylation of IκBα was verified in both PC3 and DU145 cells in response to TLR3 silencing. Basal expression of IκBα remained stable following TLR3 knockdown **(A)**. Knock down of RIG-I was confirmed by western blot and downstream signalling was assessed. There was no consequential decline in phosphorylation of TBK1 or IKKε in either PC3 or DU145 cells. There was a decrease in phosphorylation of the NF-κB inhibitor IκBα in PC3 and DU145 PCa cells **(B)**.

5.2.12 ApoE mimetic impedes nuclear translocation of p65

The accumulating data of this study has implicated a role for the ApoE mimetic peptides in targeting IκBα in PCa cell lines. In its dephosphorylated state IκBα binds p65 in the cytoplasm and thereby prevents the translocation of p65 to the nucleus.

NF-κB plays a crucial role in oncogenesis and is aberrantly activated in a wide range of cancers in which it promotes cell survival and malignancy contributing to the up-regulation of pro-inflammatory cytokines, chemokines and growth factors [546].

Cellular fractionation was performed in order to determine the compartmental localisation of p65 in response to agonistic stimulation and OP449. PC3 cells were seeded into petri dishes at density of $1X10^6$ cells per well. Following overnight serum starvation, cells were either stimulated with LPS, incubated with OP449 or treated with OP449 following a 1hr retreatment with LPS. Following incubation periods, cells were washed and the cytoplasm and nuclear cellular compartments were separated by fractionation. The expression of P65 and IRF3 in response to OP449 was determined by SDS-PAGE. Western blot demonstrated an increase of p65 translocation to the nucleus in response to LPS stimulation and a decrease in response to OP449. Cells treated with OP449 following LPS exposure showed nuclear expression of P65 similar to basal levels observed in the untreated sample. Western blot did not confirm variation in IRF3 nuclear translocation in response to OP449 (Figure 5.13 A)

In an effort to uncover the most likely mechanism of NF-κB inhibition by OP449, protein was isolated form PC3 cells that were seeded into petri dishes, serum starved overnight and treated with either OP449, LPS, pp2A inhibitor okadaic acid alone or OP449 following prior exposure to LPS or okadaic acid. Western blot showed that the inhibitor of PP2A, okadaic acid, blatantly increased the phosphorylation of IκBα and while OP449 did not appear to modulate basal phosphorylation, the peptide failed to fully overcome the phosphorylation induced by okadaic acid and thus the inhibition of PP2A (Figure 5.13 B). As NF-κB appears to be inhibited as a result of PP2A upregulation, it would be worth exploring in a future analysis the status of TLR expression in response to LPS in the SET knockdown model.

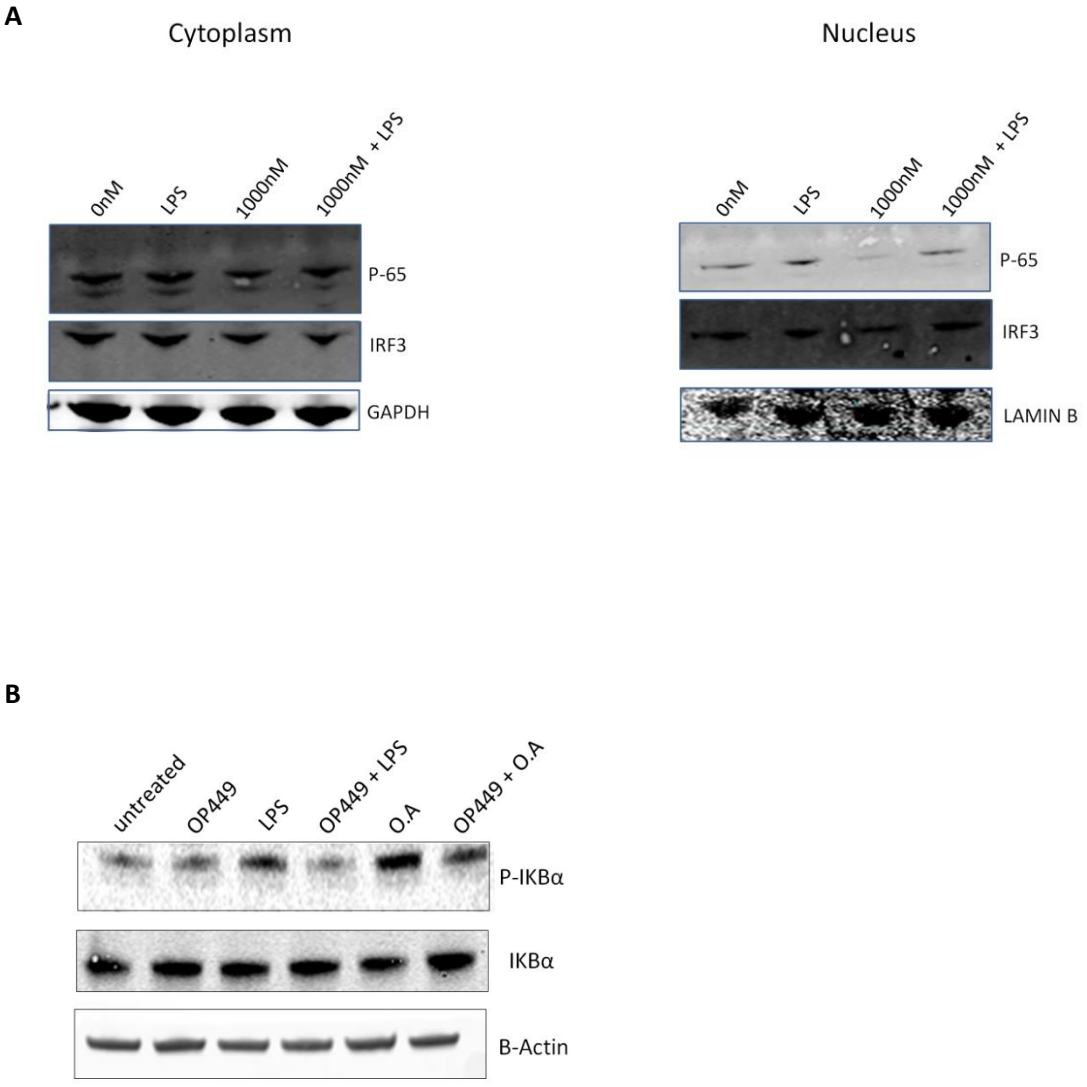


Figure 5.13 OP449 inhibits the translocation of P65 to the nucleus PC3 cells were seeded into petri dishes at density of $1X10^6$ cells per plate and serum starved overnight. Cells were either stimulated with LPS, incubated with OP449 or treated with OP449 following a 1hr pretreatment with LPS over a 4hr incubation period. Cytoplasm and nuclear cellular compartments were separated by fractionation. The expression of P65 and IRF3 in response to OP449 was determined by SDS-PAGE. Western blot demonstrated an increase of p65 translocation to the nucleus in response to LPS stimulation and a decrease in response to OP449. Cells treated with OP449 following LPS exposure showed nuclear expression of P65 similar to basal levels observed in the untreated sample. There was no variation in IRF3 nuclear translocation in response to OP449 observed **(A)**. Whole cell lysate from cells treated with OP449, LPS, OP449 after LPS pretreament, okadaic acid, and OP449 following a 1hr pretreatment with okadaic acid were analysed by gel electrophoresis. Results demonstrated a rise in p-IκBα in response to pp2a inhibitor okadaic acid. OP449 did not counteract the hyper-phosphorylation induced by okadaic acid **(B)**.

5.2.13 The effect of ApoE mimetic on proinflammatory cytokines

Pro-inflammatory cytokines are long associated with the development and progression of cancer and considered to have a pro-tumorigenic effect. It is largely accepted that chronic inflammation is an important contributing factor in generating malignancy through the exposure of pro-inflammatory cytokines and perpetual activation of pro-inflammatory pathways such as NF-κB. Cytokines contribute to tumour growth by stimulating proliferation of cancerous cells and eluding immunosurveillance [547]. IL-6, a cytokine induced by NF-κB activation, plays a key role in promoting proliferation and inhibition of apoptosis with researchers having proposed IL-6 as a therapeutic target for the treatment of cancer [548]. IL-8, a proinflammatory CXC cytokine is primarily regulated by NF-κB mediated transcriptional activity [549]. Activation of IL-8 has been established as a promoter of proliferation and survival and aids cancer cells to evade stress induced apoptosis and its expression has been correlated with angiogenesis and metastatic potential [550, 551].

As cell fractionation demonstrated that OP449 has the potential to prevent p65 nuclear translocation, the effect of the ApoE mimetic on NFκB stimulated cytokines was explored by RT PCR. In a similar fashion, cells were seeded into petri dishes at a volume of $1X10^6$ cells per plate. Following overnight serum starvation, PC3 and DU145 cells were either stimulated with LPS, incubated with OP449 or treated with OP449 following a 1hr treatment with LPS. Following incubation periods, RNA was extracted using Trizol and quantified using the nanodrop. RNA expression of IL-6 and IL-8 was assessed by RT-PCR Results revealed that OP449 had no significant effect on the basal mRNA expression of IL-6 or IL-8 although the compound hindered the surge in IL-6 and IL-8 expression in response to LPS stimulation (Figure 5.14)

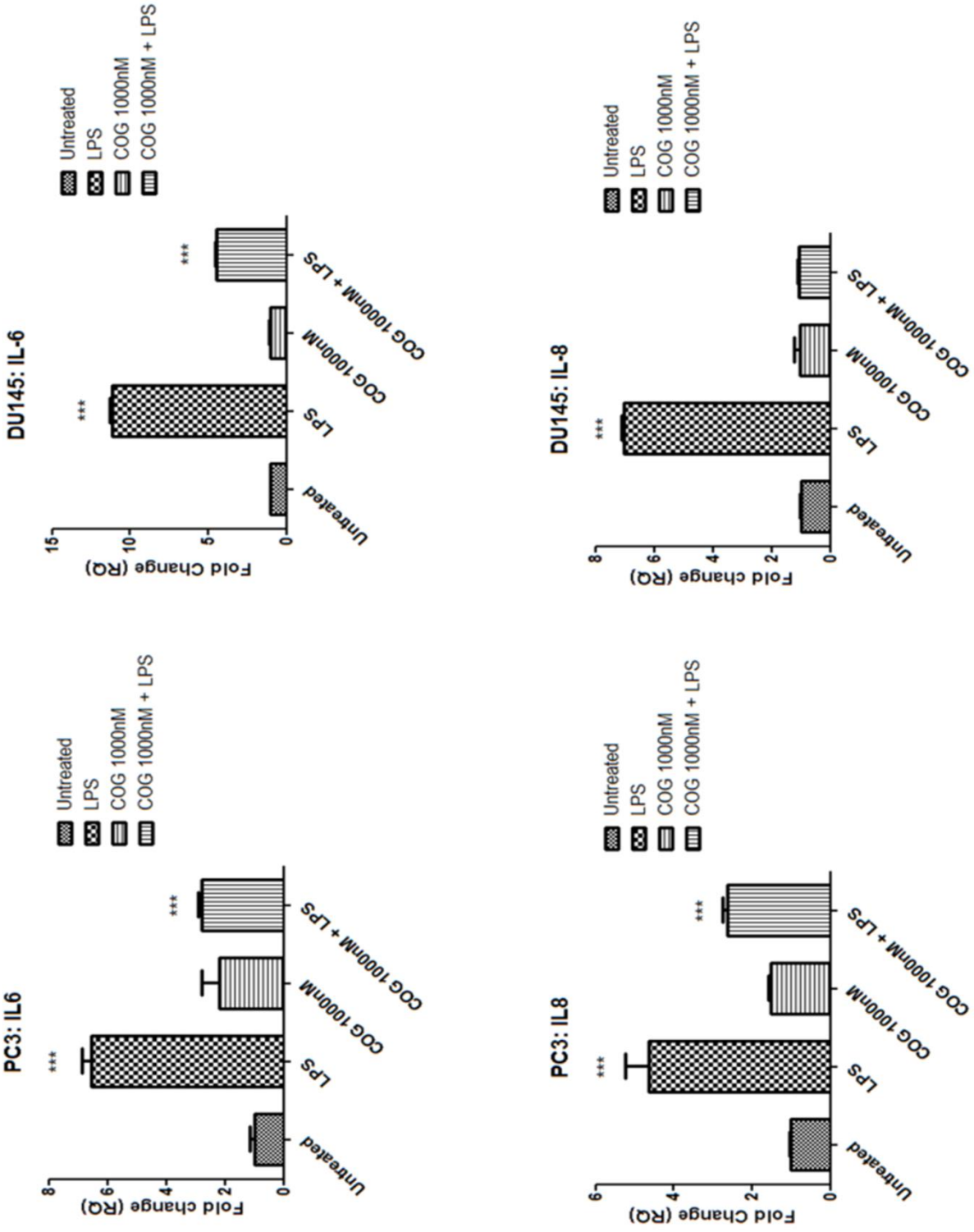


Figure 5.14 OP449 downregulated mRNA levels of IL-6 and IL-8 following LPS stimulation PC3 and DU145 cells were seeded into petri dishes at $1X10^6$ cells per dish. Treatment conditions included, 100ng/mL LPS, 1000nM OP449 and 1000nMop449 following a 1hr incubation with LPS. RNA was extracted and cDNA synthesised. Samples were analysed by RT-qPCR. OP449 had no significant effect on the basal mRNA expression of IL-6 or IL-8 although the compound hindered the surge in IL-6 and IL-8 expression in response to LPS stimulation. Statistical significance in cell viability was determined by one way ANOVA followed by tukey post hoc analysis, ***p<0.0001, **p<0.001, *p<0.05.

5.3 Discussion

5.3.1 Transmembrane TLRs are constitutively expressed in PCa cell lines

Previous studies and recent advances in cancer immunobiology have emphasised TLRs as key components involved in tumour growth and progression with normal regulation being disrupted in cancerous epithelial cells. Mutations of TLR genes or modulation of TLR signalling could potentially be suitable markers for diagnosis or targets for treatment strategies [262, 263, 338]. Exploration of TLR expression in PCa cells confirmed that TLRs are expressed in both AR positive and AR negative PCa cell lines. After the expression of cell surface receptors TLR-1, -4, -5, and -6 were identified, the trans-membrane TLRs expressed in PCa cell lines unveiled an interesting pattern of expression. While relatively low protein expression of TLR1 was detected, TLRs -4 and -6 followed a similar expression form in that they are abundantly expressed in both AR negative and AR positive cell lines. TLR5 is also expressed in each of the cell lines although detected protein expression is lower in DU145 cells in comparison to the other PCa cell lines. While expression was lower, the TLRs were also expressed in the non-cancerous RWPE1 cell line (Figure 5.1 A).

The TLRs expressed at the cell surface contain leucine rich extra cellular domains are involved in protein-protein interactions and ligand recognition [552]. TLRs expressed on the cell surface differ from the intracellular TLRs in that they recognise PAMPS on extracellular microbes and bind ligands present in the extracellular space, thereby initiating cell signalling from the cell surface. Such recognised PAMPS include gram positive and gram negative bacteria, yeast and fungi. Recognising ligands include Gram positive bacterial lipoproteins (TLR -1, -2 and -6), gram negative LPS (TLR4) and flagellin (TLR-5) [552]. The significance of cell surface TLR expression is emphasised by impaired innate immune function in response to obstruction of TLR cell surface trafficking, rendering ligands unresponsive [553].

5.3.2 Intracellular TLRs are constitutively expressed in prostate cancer cell lines

The remaining TLRs reside in intracellular compartments. Following the confirmation of the cell surface TLR expression, the expression of intracellular TLRs -3, -7, -8 and -9 were confirmed. Although detection was identified, the expression of TLR-8 was low in the cell lines explored. The TLRs -3, -7 and -9 were confirmed in PCa cell lines. Expression of TLR -7 was greater in the AR positive cell lines CWR22 and 22RV1 in comparison to the AR negative PC3 and DU145. These TLRs recognise PAMPS in nucleic acids derived from bacterial and viral pathogens [554]. TLR3 recognises double stranded RNA which is typically an artefact of viral replication in host cells. TLR7 and TLR8 recognise single stranded RNA derived from RNA viruses and small interfering RNA (siRNA). And lastly, TLR9 is stimulated by unmethylated CpG, comprising of bacterial and viral DNA. The mechanism by which intracellular TLRs interact with their ligands is not completely understood. The intracellular TLRs can be activated by the addition of their ligands to the extracellular medium, indicating that cells can transport nucleic acids from outside the cell into the TLR-containing compartments. There is a general consensus that the primary function of intracellular TLRs is to detect viruses, although they have been shown to respond to other microbes [45]. Many viruses are only identified by endosomal TLRs. The uptake of microbes into the endocytic pathway may occur by receptor-mediated endocytosis, phagocytosis, or nonspecific fluid phase endocytosis [45]. Alternatively, viruses may fuse with the plasma membrane and prior to endosome uptake either before or during the process of replication as a result of autophagy [555]. Fully replicated viruses may amalgamate into the endosomal system in the process of being released from the cell [556]. At present, little is known about the mechanisms by which TLR agonists derived from viruses or bacteria that replicate within the cell reach the intracellular TLRs [552]. Intracellular TLRs traffic from the endoplasmic reticulum (ER), through the Golgi and take up residence in the endolysosomes prior to stimulation [557-559]. The significance off the expression of these TLRs in PCa cell lines lies in the inference that these cells are equipped for immediate response to intracellular bacterial and viral pathogens.

5.3.3 Basal levels of RLRs identified in prostate cancer cell lines

The RIG-I-like receptors (RLRs) RIG-I, MDA5, and LGP2 play a major role in pathogen recognition of RNA virus infection to initiate and modulate antiviral immunity. The RLRs detect viral RNA ligands or processed self RNA in the cytoplasm to trigger signal transduction and innate immune response. Importantly, RLRs co-operate in signalling crosstalk networks with Toll-like receptors and other factors to impart innate immunity and to modulate the adaptive immune response. In an effort to further characterise the parameters of innate immune components of PCa cell lines, the expression of RLRs RIG-I and MDA5 were explored and confirmed by western blot. While RIG-I was expressed in each of the cell lines assessed, their expression was apparent greater in PC3 and DU145 cells in comparison to CWR22 and 22RV1. MDA5 was undetected at a protein level in each of the cell lines except for DU145. Previous research has suggested that MDA5 expression is inducible rather than the receptor being constitutively expressed at a basal level [560]. The RLRs expressed signal play a prominent role in triggering innate defences within myeloid cells, epithelial cells, and cells of the central nervous system [275, 278, 279, 561]. Thus, finding that extracellular TLRs, intracellular TLRs and RIG-receptors are expressed in PCa cell lines is highly suggestive that the prostate is a gland equipped with the fundamental components for instigating immediate innate immune response to pathogens and viruses. This further alludes to the role the immune and inflammatory response may play in cancer development and progression. As immune evasion is a hallmark of cancer pathogenesis, targeting PRRs is a plausible approach to modulate immune system for future cancer treatment development.

5.3.4 ApoE mimetic peptide can modulate basal PRR expression in PCa cell lines

Assessing the effect of ApoE peptide mimetic OP449 on base line PPR expression in PCa cell lines evoked thought provoking changes in receptor expression among the cell lines explored. While TLR4 expression remained stable in the AR positive cell

lines in response to OP449, a distinct decline in expression was observed in response to increasing concentrations of the peptide within the AR negative cell lines PC3 and DU145 (Figure 5.4 A). Similarly, this manifestation was observed in the intracellular TLRs -3 and -9 expressed in AR negative cell lines in response to the ApoE mimetic peptide. In contrast, the expression of TLRs -3 and -9 was upregulated in response to increasing concentrations of OP449. This finding is suggestive of variation in receptor function and performance between AR negative and AR positive cell lines. While it has not specifically been reported in the literature to the best of our knowledge, there are studies reporting a link between steroid hormones and immune response [562]. Numerous studies have postulated TLR receptors as potential targets for cancer therapy. Kanda and Yokosuka recently suggested a correlation between AR expression and apoptosis in response to TLR4 stimulation [563]. Gambana et al., demonstrated that stimulation of TLR3 with poly(I:C) sufficiently induced apoptosis in androgen-sensitive LNCaP PCa cells. They also reported that cell death was not efficiently induced upon TLR3 stimulation of AR negative PC3 cells [263]. It has also been reported TLR3 induced apoptosis in LnCaP cells is mediated through IRF3 signalling [564]. This supports our speculation of varied signalling response upon TLR stimulation in AR positive and AR negative cells. The intracellular RLR RIG-I responds in a similar manner upon exposure to increasing concentrations of OP449. CWR22 cells demonstrated a slight increase in receptor expression while the PC3 cell line exhibited a decline in RIG-I expression. This decline was most apparent at the highest concentration 1500nM. The effect of OP449 on MDA5 expression was also assessed. In the CWR22 cell line, expression of the receptor was induced by OP449. While faint expression was eliminated by OP449 in the PC3 cells (Figure 5.5). Response of the TLRs and RLRs to the peptide appears to correlate with the respective basal expression of each receptor. Lower basal expression corresponds to an increase in receptor expression while higher basal levels correspond to a decrease in receptor expression in response to OP449. , These changes drove the query of utilising ApoE peptides as an immunotherapeutic mechanism for targeting these receptors in advanced metastatic PCa.

5.3.5 ApoE interrupts TLR4 ligand stimulation

Following apparent modulation of innate receptor parameters, ApoE peptide mimetics were investigated and demonstrated to impede TLR4 expression in response to LPS. Lipopolysaccharide (LPS), a well-characterised pathogen associated molecular pattern is found in the outer leaflet of the outer membrane of most Gram-negative bacteria. It initiates a strong immune response and serves as an early warning signal of bacterial infection. LPS is detected by the TLR4–MD-2 complex [565, 566]. Aggregation of the TLR4–MD-2 complex after binding LPS leads to activation of multiple signalling components, including NF-κB and IRF3, and the subsequent production of pro-inflammatory cytokines [259, 567, 568]. TLR4 has therefore been recognized as an important pharmacological target. Previous research has demonstrated that exogenous ApoE suppressed the inflammatory response of macrophage to LPS induced TLR4 activation. [569-571]. The ability of the synthetic ApoE peptide mimetics COG112 and OP449 to respond to TLR4 stimulation in AR negative PCa cells was assessed in this study. The upregulation of TLR4 in response to LPS was apparent in the PC3 and DU145 cells with an increase in protein expression observed in both cell lines. The incubation of cells with COG112 downregulated the protein expression of TLR4 in both PC3 and DU145 cell lines, albeit at higher concentration of 1500nM in the PC3 cells and 1000 and 1500nM in the DU145s when compared to OP449. COG112 overcomes the TLR stimulation induced by LPS treatment as a decline in expression is observed in parallel to increasing concentrations of the compound in both PC3 and DU145 cell lines. The PC3 cells pre-treated with LPS showed a steady decline in TLR4 expression in response to 1000nM and 1500nM COG112 despite prior stimulation with LPS. Similarly, levels of TLR4 protein expression in DU145 cells also deteriorated with higher concentrations of the compound regardless of LPS treatment (Figure 5.6 A).

As previously mentioned, lower concentrations of OP449 were required to down regulate expression of TLR 4. Following pre-treatment with LPS, expression of TLR 4

was gradually reduced at 500nM and 1000nM in spite of prior ligand stimulation in PC3 cells. Protein expression was also diminished by OP449 in the DU145 cells pre-treated with TLR4 ligand LPS at 500 and 1000nM. This coincides with previous research which demonstrated that both exogenous and endogenous ApoE suppresses macrophage response to TLR4 agonists and inhibits agonist induced pro-inflammatory cytokine production [569, 571, 572]. These results demonstrate that exeogenous ApoE in the form of COG112 and OP449 peptide suppress PC3 and DU145 response to the TLR4 agonist LPS. In the wake of these findings the effect of ApoE on downstream signalling was explored (Figure 5.6 B).

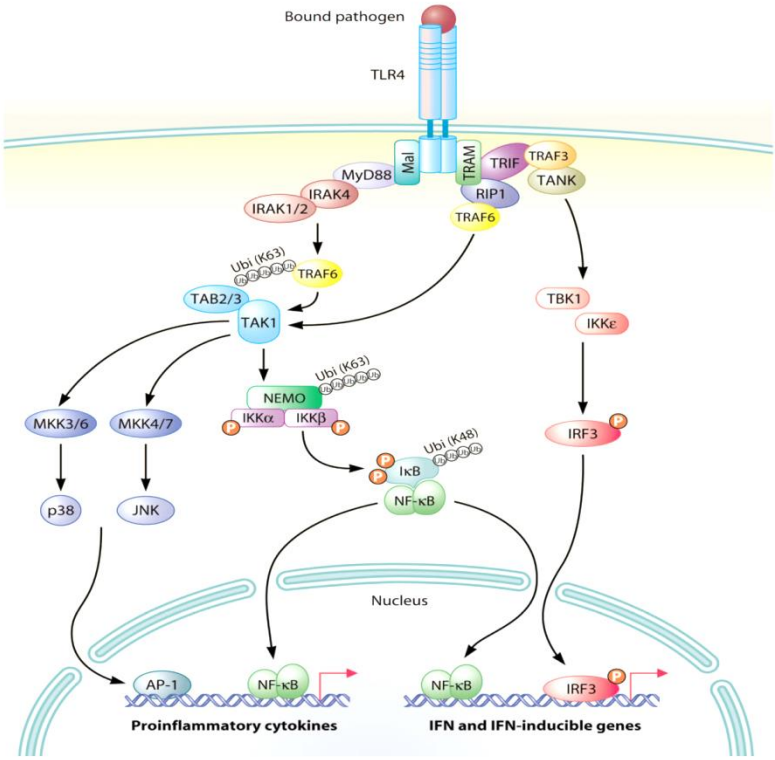


Figure 5.15 Transmembrane TLR signalling (As described in Figure 1.10).

5.3.6 *OP449 interrupts TRIF TRAF dependent TLR 4 signalling*

As previously mentioned, TLR4 signalling diverges between MyD88 and TRIF dependent pathways. In pursuit of the discovery that the ApoE peptide mimetics suppress TLR4 expression both alone and following ligand stimulation, the

influence of theses peptides on downstream signalling was explored. As the TBK1 and IKKε are necessary for TRIF dependant IRF phosphorylation and are not involved in the IκBα phosphorylation in NF-KB stimulation, these factors were selected to distinguish preference of this signalling cascade in PCa cell lines PC3 and DU145. The activation of the IKK-related protein kinases requires their phosphorylation at Ser172 [573]. Activation of this signalling cascade was assessed by phosphorylation of these proteins in response to TLR4 stimulation. While LPS successfully increased phosphorylation of TBK1 and IKKε, COG112 failed to down regulate activation of these kinases in response to TLR4 stimulation in both PC3 and DU145 cells (Figure 5.7 A). Activated Ikk-related kinases TBK1 and IKKε phosphorylate and activate IRF3 and so the phosphorylation status of IRF3 was assessed in response to the ApoE peptides and TLR4 stimulation. While expression of IRF3 was confirmed in both cell lines there was no detection of phosphorylated IRF3 either in response to COG112 or TLR4 stimulation with LPS in both AR negative PCa cell lines. However, the basal expression of IRF3 appeared to decrease in response to COG112 (Figure 5.7 A). The apparently more potent OP449 did demonstrate a decline in TBK1 and IKKε phosphorylation both alone and following TLR4 stimulation however there was again no detection of IRF3 phosphorylation (Figure 5.7 B). These results demonstrate that TLR stimulation did in fact upregulate phosphorylation of these IKK related kinases in the AR negative cell lines and that no phosphorylation of IRF3 was detected at the Ser396 residue despite activation of the Ikk related kinases being required for IRF3 activation. While one of our ApoE mimetics, COG112, was incapable of decreasing phosphorylation of the IKK related kinases following TLR4 stimulation and the other, OP449, clearly impeded their phosphorylation, and LPS stimulation failed to induce IRF3 activation following IKK related phosphorylation, it is plausible to suggest that another component is required for IRF3 activation with in the signalling cascade. It is also possible that TLR4 stimulation of innate immune response may not be the mechanism required or responsible for IRF3 signalling in PCa cells.

5.3.7 ApoE interrupts TLR 4 mediated NF-KB activity

In a bid to clarify the impact of the ApoE peptide mimetics on TLR induced NF-κB activation, the effect of the peptides on IκBα expression and phosphorylation in response to LPS was explored. Basal levels of IκBα remained stable in the AR negative cell lines in response to COG112 and OP449. The phosphorylation of IκBα was decreased in response to COG112 only at the highest concentration of 1500nM in the PC3 and DU145 cells (Figure 5.8 A). Reduction of IκBα phosphorylation was much more apparent in response to OP449 in the AR negative cells lines in comparison to COG112. OP449 effectively obstructed the phosphorylation of IκBα in response to LPS stimulation (Figure 5.8 B). Down regulation of IκBα phosphorylation indicates negative regulation of NF-κB activity in response to ApoE peptide mimetics COG112 and OP449. OP449 was evidently more potent at decreasing the phosphorylation of IκBα than COG112, and thus, likely to be retaining NF-κB bound in the cytoplasm. A study exploring the effect of COG112 on colonic epithelial cells supports the results of this study as Singh et al reported the ability of the peptide to reduce NF-κB activation through inhibition of IκBα phosphorylation in murine models of colitis [172]. While COG112 was less efficient in PCa cells, OP449 competently impeded IκB signally mediated by TLR4 stimulation.

5.3.8 ApoE interrupts anti-viral response to dsRNA independently of IRF3 signalling

As stimulation of the extracellular TLR4 with LPS increased phosphorylation of the IKK related kinases but failed to instigate an activation of IRF3, the activity status of this signalling cascade was explored in response to dsRNA Poly(I:C), a ligand for both TLR3 and RIG-I. Transfection of Poly(I:C) culminated in the upregulation of TLR3 and RIG-I receptor. As expression of these receptors are upregulated in

response to stimuli and down regulated in response to OP449, their signalling cascade may be a therapeutic target of OP449.

As demonstrated when exploring TLR4 signalling, OP449 down regulated the phosphorylation of IKK and TBK1 in both PC3 and DU145 cells induced by LPS stimulation. Despite the activation of the IKK related kinases, there was no evidence of IRF3 activation by phosphorylation. In contrast to TLR4 stimulation, transfection with Poly(I:C) resulted in an upregulation of IKKε and TBK1 that was not counteracted with subsequent OP449 exposure. Cellular transfection of The Poly(I:C) ligand induced phosphorylation of IRF3 at ser396 in PC3 cells. The treatment of PC3 cells with OP449 post transfection had no impact on IRF3 activation by Poly(I:C). Likewise, phosphorylation of the TBK1 and IKKε kinases by poly(I:C) was not reversed by OP449. Despite phosphorylation and presumed activation of the ikk related kinases there was no phosphorylation of IRF3 detected in the DU145 cell (Figure 4.9). The reason for this could be due to incomplete transfection of PRR ligand poly(I:C), although exogenous Poly(I:C) may still have induced conformational change within the cells signalling cascades. Another possible rationale could be due to an alternative kinase required in addition to TBK1 and IKK activation for IRF3 activation. As OP449 was capable of down regulating the ikk related kinases on its own and incapable following poly(I:C) transfection, the latter is a likely explanation. Although the IKK related kinases are widely believed to be solely activated by ligands that lead to the activation of IRF3 and the production if IFNβ, it has been reported that IL-1 and TNFα can activate IKKε and TBK1 in mouse embryonic fibroblasts without inducing the phosphorylation of IRF3 or IRF3 dependent gene transcription [574]. This raises the question as to how agonists such as IL-1, TNFα, LPS and Poly (I:C) activate the IKK related kinases and the roles they play that are independent of IRF3 [575].

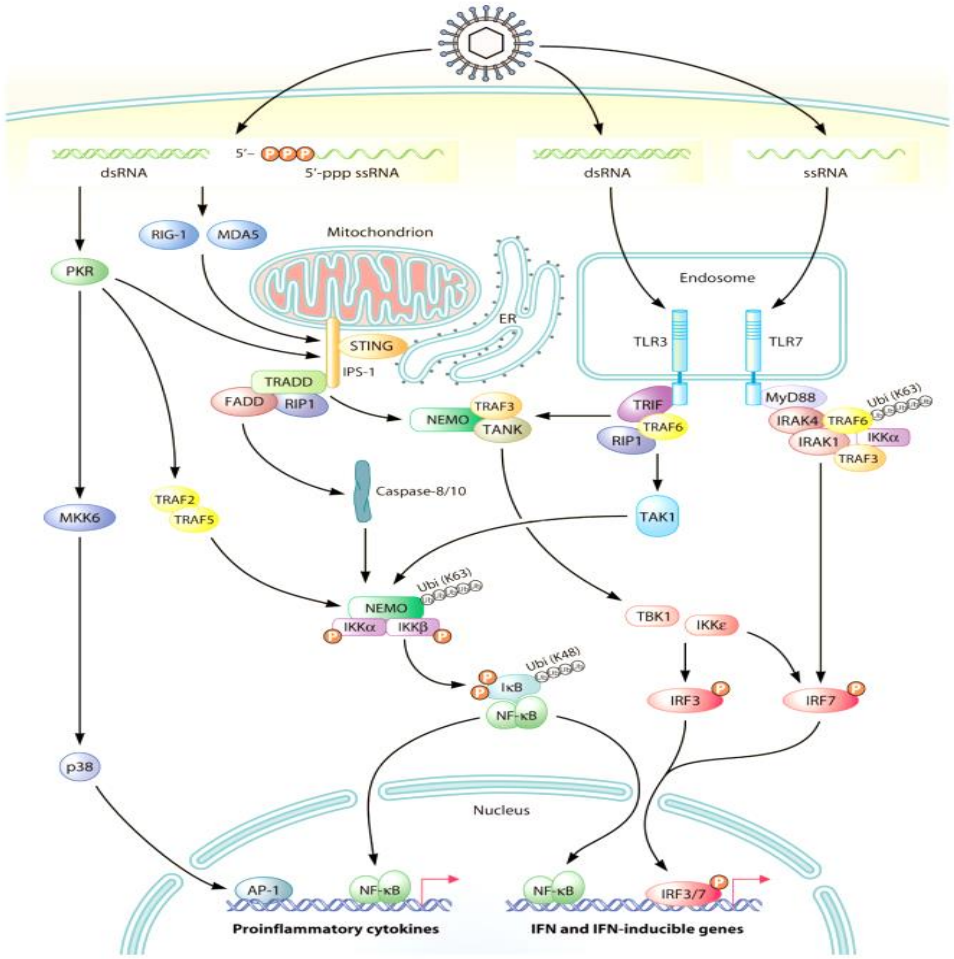


Figure 5.16 Intracellular PRR signalling cascades (as described in Figure 1.11)

5.3.9 Intracellular PPR expression is essential for cell survival in PCa cells

As expression of the intracellular TLRS and RLRs (TLR3 and RIG-I) were confirmed in the PCa cell lines and our findings demonstrate that their expression is easily up-regulated in response to stimulation and down-regulated in response to the ApoE mimetic peptides, the significance of their expression was explored in our PCa cell lines. Individual transient knockdown of TLR3 and RIG-I by reverse transfection using SiRNA was executed. In addition to confirming successful receptor knockdown by western blot, cell viability following transfection was observed. A

marked decline in cell viability was noted in response to TLR3 knockdown. In an effort to gain further understanding of the relationship of TLR expression and PCa cell survival transient knockdown was repeated using a range of SiRNA concentration Repeat transfection demonstrated that increasing concentration of SiRNA coding for TLR-3 corresponded with the decline in cell viability. Both PC3 and DU145 cells saw reduced viability in cells following transfection of SiTLR3. The lowest concentrations of SiTLR3 caused a significant reduction in the number of viable cells in comparison to the control and non-coding siRNA controls. The decline in cell viability increased in conjunction with the increase in SiTLR3 concentration in both PC3 and DU145 cell lines. The knockdown of RIG-I using a range of concentrations of RIG-I coding SiRNA was also conducted. Transient knockdown of RIG-I had no implication on cell proliferation or survival at any of the concentrations used. The decline observed in response to SiTLR3 is likely due to the degree of protein knockdown as opposed to toxicity of the SiRNA as optimisation studies performed verified that individually the transfection reagents were non-toxic to PCa cell lines and 25nM was an optimal concentration for transfection of PC3 cells and 50nM was an optimal SiRNA concentration for transfection of DU145 cells. Higher concentrations of SiTLR3 resulted in greater level of cell death although the lower concentrations of SiTLR3 also saw significant decline in cell survival in both cell lines. This finding suggests that not all dsRNA recognising PRRs are equal giving that knock down of RIG-I had no effect on cell survival when compared to the control cells and cells transfected with non-coding scramble SiRNA.

The results of this study may provide a clue to the significance TLR3 may have specifically on the survival mechanism of AR negative, metastatic prostate cancer cells. It was demonstrated earlier in the study that increasing concentrations of OP449 had opposing effects on AR negative and AR positive cell lines. The expression of TLR3 was found to increase in both CWR22 and 22RV1 cell lines in response to rising levels of OP449, while a decline in receptor expression was observed in both AR negative PC3 and DU145 metastatic cell lines. (Figure 5.4).

It is also suggestive the TLR3 may be a critical component of AR negative PCa cell lines, a finding that has not been reported in the literature to the best of our knowledge. As OP449 is capable of down-regulating the expression of these receptors and TLR3 expression is required for survival, this observation is worth exploring. The consequence of receptor knockdown was explored in downstream TLR3/RIG-I signalling. The literature has reported that treatment with poly(I:C) can induce apoptosis in some cells such as the LnCap cells although evidence is conflicting and TLR stimulation has also been suggested to induce a protumorigenic response [351, 352]. This may be explained by the lack of AR presence in PC3 and DU145 cells in comparison to the AR positive LnCaPs. Conflicting reports have been described in the literature with some findings reporting an inhibition of tumour growth in response to TLR3 stimulation and others describing a correlation between high TLR3 expression and PCa recurrence and poor prognosis. It is possible the benefits of agonising or antagonising the receptor are dependent on the degree of disease progression. To the best of our knowledge, it has not been reported that knockdown of TLR3 induces cell death in AR negative PCa cell lines and its role in PCa progression and cell survival should be explored further.

5.3.10 The effect of transient TLR3 and RIG-I knockdown models on downstream signalling

Given that the knockdown of TLR3 played a crucial role in the viability of PC3 and DU145 cells, the expression and activity status of downstream targets was explored. As the ApoE mimetic peptides used in this study do appear to signal through these cascades, receptor knockdown may provide added insight to the mechanism of action of these peptides. Knock down of TLR3 in PC3 and DU145s showed some similarity in the signalling cascade observed in response to OP449 in that TBK1 phosphorylation was down regulated, however phosphorylation of IKKε was not. There was no modulation in the expression of IRF3 (Figure 5.12 A). As TLR3s signalling can also diverge and induce NF-KB activity, the consequence of

TLR3 on IκBα was also investigated. The results demonstrated that phosphorylation if IKBα was downregulated in the knockdown samples while IKBα expression itself remained stable in the knockdown sample and the control and scramble SiRNA. (Figure 5.12 A).

As transient knockdown of RIG-I revealed no consequential decrease in phosphorylation of the IKK related kinases or modulation to IRF3, and given that a decrease in IκBα phosphorylation was detected, the results suggests that signalling through Nfκb may be the preferred route in PCa innate and inflammatory response. It is also likely the preferred pathway through why the ApoE peptide mimetics exert the effect. The results indicate that of the intracellular PRRs, TLR3 may be the preferred target of the ApoE mimetic peptides over Rig-I. Bruni et al., demonstrate in their research that TBK1 may be involved in the positive regulation of NF-κB downstream of TLR3. They also reported that that IKKε is not involved in the positive regulation of this same pathway [576]. Firstly, this emphasises a difference between the two kinases at the functional level in the TLR3 pathway. It also supports the findings of this study whereby TBK1 is downregulated in response to both the peptide and SiRNA knockdowns while IKKε is not. As we also see down regulation of the IκBα phosphorylation in parallel with TBK1 and without IKKε downregulation, the results of this study indicate that IκB activation is a preferred signalling in the PCa cells given that the IRF3 is not phosphorylated. The results also suggest that the ApoE mimetic peptide successfully targets IκBα and its activators in preference to IRF3.

5.3.11 Nuclear translocation of p65

As the data from this study has demonstrated that that the phosphorylation of IκBα is down regulated by the ApoE mimetic peptides COG112 and OP449 in PCa cells and no evidence of IκBα degredation detected, the results were highly indicative of NFκB inhibition in PCa cells in response to the peptide compounds. In an effort to ultimately confirm NFκB as a target of ApoE peptide mimetics, the cellular

localisation of NFκB was assessed in response to OP449 by assessing the sub-cellular location of p-65 in cytoplasm and nuclear compartment utilizing cellular fractionation. The NFκB transcription factor complex is held in an inactive state in the cytoplasm by binding to its inhibitor IκB. Upon activation, IκB is targeted for ubiquitination, allowing phosphorylation of the NFκB complex, facilitating its shuttling into the nucleus where it can bind to promoter sites to regulate transcription of target genes [577]. As activation of the TLR signalling cascade is known to activate NFκB, the LPS ligand was used to induce activation [578]. By isolating the cytoplasm and the nucleus from whole cell lysate it was possible to confirm the elevation of p-65 in the nucleus in response to LPS stimulation. The treatment of PC3 cells with OP449 resulted in a distinct depletion of p65 protein translocation from the cytoplasm to the nucleus. OP449 was also demonstrate to overcome p65 nuclear translocation despite prior exposure to LPS with nuclear levels of p65 being reduced (Figure 5.13). The ability of OP449 to impede mRNA levels of NFκB controlled cytokines IL-6 and IL-8 stimulated by LPS further strengthens the rationale that OP449 effectively targets NFκB activity. The peptide successfully inhibits the translocation of p65 to the nucleus and the consequential transcriptional activation of pro-inflammatory cytokines (Figure 5.14).

Abnormal NF-κB regulation has been observed in a number of cancers, including both solid and hematopoietic malignancies. NFκB can disrupt the key processes embodied in normal cell growth control and tissue homeostasis , namely the hallmarks of cancer Hanahan and Weinberg [12]. It exerts its protumour effects through the transcriptional activation of genes associated with cell proliferation angiogenesis, metastasis tumour promotion, inflammation and suppression of apoptosis [579-582]. As tumour cells often use NFκB to achieve resistance to anticancer drugs and radiations, inhibition of NFκB activation appears as a promising target option to improve the efficacy of conventional anticancer therapies [583]. The results are of particular relevance as

The progression of tumour growth to the bone is the most adverse complication of advanced PCa and is associated with substantial morbidity and significant mortality

[584]. A correlation has been documented in the literature between the overexpression of NFκB in the nucleus of PCa cells and chemoresistance, advanced stage and metastatic PCa [585-591]. A number of studies have also published data demonstrating that the NFκB pathway is a contributor to visceral or soft tissue metastasis in PCa [155, 156, 592, 593]. Jin et al previously reported that activation of NFκB signalling promotes the androgen independent growth of PCa [594]. In a more recent study it was reported that the activation of NFκB signalling increases the expression of osteoclstogenic genes in PCa cells which is sufficient for cancer cells to attach and grow in the bone environment and enhance lesion formation [595]. The literature indicates that targeting down-regulation of the NF-κB could have a major impact on reducing painful bone metastasis in advanced PCa patients. Considering the literate together with the results of this study targeting NFκB activation with an ApoE peptide mimetic for the treatment of currently untreatable advanced PCa is an auspicious prospect that should be explored further.

5.4. Conclusion

The results of this study illustrate PCa cells are well equipped for immediate TLR response which can be targeted with ApoE mimetic peptides COG112 and OP449. The findings also give justifiable reason to believe IκBα is a preferred target for the ApoE peptide mimetics to exert their effect in PCa cells. The depletion of TLR3 by SiRNA expression was demonstrated to be detrimental the survival of AR negative PCa cells, a finding that requires further exploration

Chapter 6: General Discussion

6.1 Discussion

6.1.1 ApoE mimetic induces cell death by apoptosis in prostate cancer cells

After evaluating the data acquired from this study, a number of deductions were interpreted from the findings. The preliminary results unveiled promising prospects for the role of ApoE mimetics in the future treatment of PCa. As current available therapies for PCa are limited, and with the current pharmacological interventions for castrate resistant PCa (docetaxel, abiraterone, cabazitaxel, enzalutaminde and Radium-223) being insufficient for the curative treatment of advanced metastatic PCa, there is a necessity for more effective treatment which selectively targets the cancerous cells. The first indicator of potential came after demonstrating inhibition of cellular proliferation of both AR positive and AR negative cell lines in response to COG112 and OP449 via alamar blue cell viability assay. Flow cytometry analysis provided further indication that OP449 may induce apoptosis with greater efficiency than the first line chemotherapeutic agent and positive control docetaxel with OP449 in the androgen independent PC3 cell line. The induction of apoptosis was further reinforced by the detection of PARP cleavage by western blot in response to OP449 and docetaxel. (Figure 3.3). Docetaxel was the first cytotoxic therapy to demonstrate a survival benefit in castrate resistant PCa and will most likely remain a preferred treatment choice against metastatic PCa despite the fact that most patients develop resistance, its toxicity and the limitations of its use [596, 597]. Regrettably, a substantial number of patients with androgen resistant PCa respond poorly to docetaxel and all patients eventually develop resistance to the drug. For this reason, ideally a more potent therapy solution capable of overcoming the resistance to the currently available therapies [598]. While the ApoE peptide mimetics, namely OP449, demonstrated great potential in the ability to inhibit cell viability and induce apoptosis in PCa cells, a more in depth dose response analysis is required for a more definitive comparison to docetaxel.

6.1.2 OP449 induces an S- phase accumulation in the cell cycle progression

The cell cycle analysis performed in this study demonstrated an accumulation of cells in the DNA synthesis – S phase of the cell cycle over a 72 hour time course. The results showed a steady incline in the accumulation of cell number detected within the S phase of the cell cycle as the time course progressed but also in response to increasing concentrations of the ApoE peptide mimetic. It would be extremely beneficial to explore the expression of cell cycle checkpoints and DNA replication capacity of the cells in response to OP449 in order to confirm whether our observations are a result of S-phase block or simply a consequence of dying cells and therefore fewer cells undergoing mitosis. To the best of our knowledge, this is the first reporting of this compound impacting the progression through the cell cycle phases, particularly in PCa cells. This thought provoking finding prompted careful consideration of the cell cycle effects of current therapeutic strategies for the treatment of PCa. For instance, as radiotherapy is used in the clinic for the treatment of PCa, a complimentary therapy that could maximise the potential for the successful treatment of patients is important. In particular, patients with more advanced cancer and higher Gleason score comprise a patient cohort that requires improvement in current treatment strategies as their cancer typically recurs despite having received optimal hormonal and radiotherapy treatment strategies [599]. Radiation therapy is considered to deliver lethal, potentially lethal and sublethal damage to tumour cells. The balance of the therapeutic index is to ablate the tumour compartment with as limited impact as possible on normal tissue and therefore, complimentary therapies that could potentially make the tumour cells more sensitive to radiation or protect normal cells from radiation therapy can be of significant value to the treatment of cancer. While some cellular structures such as bone marrow, have limited capacity to repair the damage induced by radiation, other cellular compartments akin those of solid tumours, have significant capability of sub lethal repair. PCa is thought to have considerable proficiency in repairing the damage provoked in response to radiotherapy and the literature has reported the

intrinsic ability of cellular repair to be restored in a short period of time following radiation therapy (minutes to hours). Taking this into consideration it would be worthwhile exploring for evidence of of DNA damage, such as γH2AX, and DNA replication, using the BrdU incorpopration assay in PCa cells in response to COG112 and OP449. Therapies designed to inhibit the repair processes during this post treatment phase may be successful in making the tumour more sensitive to radiation therapy [600, 601]. It would be worth exploring in prostate cancer and investigating the potential these peptides may hold as co-treatment. The results of this study open a platform for further investigation, where a therapeutic peptide may provide the opportunity to enhance the efficacy of currently available treatment strategies and improve the overall outcome in PCa patients.

6.1.3 OP449 targets SET and up-regulates the activity of PP2A

The inhibition of the SET oncoprotein and consequential upregulation of PP2A activity was achieved in the PCa cells in response to the ApoE peptide mimetics COG112 and OP449. As PP2A has tumour suppressor functions and negatively regulates many pathways associated with cancer progression and its activity in cancer is frequently decreased, pharmacologically increasing the phosphatase activity makes for an appealing approach to cancer therapy [204]. While this study did indeed suggest an increase in PP2A activity in PC3 cells in response to OP449, the specific function, substrate affinity and cellular localisation of the phosphatase are governed by the holoenzyme's subunit configuration. As this study demonstrated a displacement of the PP2Ac subunit from the SET/PP2A complex the results exemplify a mechanism whereby a general increase in PP2A activity is achieved in response to OP449 in PCa cells. However the displacement of the c-subunit does not determine the composition of the activated PP2A subunits and therefore the results do not define a specific subunit arrangement promoted by OP449 but instead increased levels of PP2A heterotrimers are released from conjugated SET. The findings of this study also demonstrated a correlating decrease

in Akt, mTOR, MAPK and GSK-3β phosphorylation in parallel to increased PP2A activity. As PP2A is a known negative regulator of Akt, the findings of this study are consistent with previous studies on the pharmacological activation of PP2A in breast cancer and leukaemia's [387, 458, 459, 602]. As OP449 failed to negatively regulate Akt in the presence of okadaic acid, these results strongly support a mechanism where OP449 increases PP2A and that these increases PP2A activity is responsible for the down regulation of Akt and downstream signalling in PCa cells. As many cancer promoting signals involve serine threonine or tyrosine phosphorylation that typically act as the on/off switches of their target proteins, and phospho-regulation is under the control of protein kinases and phosphatases in normal cells, disruption of this balance is a central mechanism by which cells escape regulation thereby contributing to malignant transformation [464]. The significance of PP2A upregulation demonstrated in this study regarding a therapeutic connotation is supported by the growing body of peer reviewed literature. Neviani et al., conducted a study exploring PP2A activation in CML and demonstrated that SET mediated PP2A inactivation was essential for the survival of dormant CML HSCs (hematopoietic stem cell). In their research they also demonstrated that that activation of PP2A may be achieved without causing harm to normal Hematopoietic stem cells while markedly impairing the survival of quiescent CML HSCs [603]. As the outcomes of this study are supported by the existing literature, the biological and clinical importance of our results are not limited to PCa, but may be extended to other malignancies [387, 603, 604].

6.1.4 ApoE peptide mimetics modulate innate immune response

Upon exploration of TLR and RLR protein expression in AR negative cell lines, the results concluded that the ApoE peptide mimetics successfully downregulate the expression of the extracellular TLR4 and intracellular PRRs TLR-3 and RIG-I. OP449 successfully impeded downstream TLR4 signalling following LPS stimulation with OP449 exerting a greater inhibitory effect than COG112. The difference in inhibition of the innate signalling response is likely to be a reflection of their

difference in potency and ability to inhibit cell proliferation in the PCa cell lines. Although OP449 was still capable of down regulating the activation of TBK1 in response to poly(I:C), it was less successful at targeting IKKε in response to TLR3 and RIG-I stimulation. Transient knockdown of the intracellular receptors revealed similar modulations to downstream signalling to that observed in response to OP449 but also revealed the expression TLR3 to be of critical importance for the survival of AR negative PCa cell lines. It is a possibility that down regulation of TLR3 may account for at least part of the decline in cell survival calculated in the toxicity assays performed earlier in the study. To the best of our knowledge this is the first reporting of transient knockdown of TLR3 being detrimental to the survival of AR negative PCa cells. The results are supported in the literature. While these results do not definitively conclude that AR negative cancer cell lines require TLR3 expression for survival, cell death may occur as a consequence of dismantling of a scaffolding support system composed of the adaptor proteins. As OP449 had no influence over IRF3 phosphorylation or cellular location, which is dependent on TLR3 activation, it is plausible that that the cell death observed is due to disruption of an important protein complex interaction or assembly construction as opposed to elimination of TLR3 alone. This speculation is supported by previous findings where inhibition of innate immune response is mediated by additional mechanisms. For instance, Zhu et al., have postulated that inhibition of LPS and poly(I:C) activation of macrophages by ApoE, may be due to its modulation of cell membrane microenvironment, thereby affecting TLR-mediated cell signalling events, or alternatively may be a direct result of ApoE binding to cell surface proteins in mediating cell signalling [569]. While showing that exogenous ApoE is capable of suppressing TLR4 and TLR3 mediated macrophage inflammatory response, they reported that ApoE failed to suppress poly (I:C) induced inflammatory cytokine production in macrophage depleted of cell surface heparin sulphate proteoglycans (HSPG) with heparinase treatment, while ApoE remained effective in suppressing LPS induced inflammatory response in heparinise treated RAW 264.7 cells. These findings suggested that the inhibition of poly(I:C) induced macrophage activation by ApoE, is mediated by a process via its interaction with

HSPG and is distinct from the mechanism by which it suppresses LPS induced macrophage response [569].

6.1.5 ApoE peptide mimetics target NF-κB activation

Confirmation of the ApoE peptide ability to target IκBa and P65 in androgen independent cancer cells was an intriguing innovation and further endorsed by the NFκB targeted IL-6 and IL-8 RNA upregulation. As disruption of NFκB regulation is understood to be a key manifestation contributing to malignant and progression in PCa, it is of particular interest in this study [152, 154, 592, 605, 606]. OP449 targeted IκBα and P65 in PCa cells and was demonstrated to inhibit activation of the NF-κB signalling in parallel to Akt inhibition and PP2Aactivation and in response to TLR 3 and TLR4 activation. The ability of the ApoE peptide mimetics to inhibit the various mechanisms by which NF-κB can exert its protumorigenic effects could assist the avoidance or help overcome the development of drug resistance to current therapies. This is an ideal mechanism for the successful treatment of cancer cells and could aid in overcoming resistance to current medications. Previous studies have shown that shown that NF-κB plays an important role in PCa growth, survival, angiogenesis, tumorigenesis and metastatic progression [154]. Immunohistochemistry (IHC) studies have shown that increased nuclear NF-κBstaining is a strong independent predictor of biochemical recurrence following radical prostatectomy [587, 598]. NF-κB has been proposed as a potential target for therapeutic intervention for AIPCs [592, 605]. Results of this study demonstrated that inhibition of PP2A with okadaic acid increased levels of IκBα phosphorylation, an effect OP449 failed to overcome. It is possible that the preferred mechanism of IκBα phosphorylation down regulation and inhibition of p65 activation is in response to PP2A activation but the ability of the ApoE peptide mimetic to inhibit TLR 3 and 4 induced IκB phosphorylation cannot be ignored. The constitutive activation of NF-κB has been demonstrated in primary PCas in the literature, with a correlation between loss of androgen receptor expression and castration-resistant phenotypes noted. The inhibition of NF-κB pathway may reduce the oncogenic

consequences of chronic inflammatory response, thereby representing a hopeful target for the treatment of PCa [607]. The ability to target this pathway from multiple aspects has potential to play an instrumental role in the much needed advancement of PCa therapy. Given that OP449 successfully kills metastatic PCa cell lines (potently inhibits cell proliferation, induces apoptosis and induces a block in cell cycle, targets multiple signalling cascades and blocks NFκB activation from multiple angles) it could potentially provide the possibility of a currently non-existent curative treatment for patients with advanced metastatic PCa. An avenue it would be irresponsible to not explore further. The significance of this study is supported by the previous literature. A study performed by Jin et al. postulates that activation of NFκB is sufficient to maintain androgen-independent growth of PCa by regulation AR action and that the NFκB pathway may be a potential target for therapy against androgen independent PCa [594]. In a study exploring the inflammatory response in colonic epithelial cells, Singh et al., reported that COG112 inhibited the Citrobacter rodentium stimulated induction of iNOS and chemokines KC and MIP2 to the same degree as NFκB inhibitors MG132 and BAY 11-7082 with no additive effect observed when these inhibitors were combined with COG112 [172]. Palayoor et al., placed an emphasis on the importance of targeting NFκB activation in androgen independent PCa upon reporting constitutive activation of NFκB binding activity and decreased IκBα levels in androgen-independent PC3 and DU145 cell lines. In contrast, the androgen sensitive PCa LnCaP cells displayed low levels of NFκB which were upregulated following exposure to cytokines or DNA damage [383].

6.2 Conclusion

The results of this study support the need for further investigation into the use of ApoE mimetic peptides, namely OP449, in the treatment of advanced PCa. The results exhibit OP449 to have multipurpose benefits and demonstrate that the ApoE peptide mimetics effectively target several pro tumorigenic signalling

cascades. The results demonstrate e potent inhibition of the SET protein, an oncogene reported to be up-regulated in caner and demonstrated in this study to be up-regulated in PCa also. The peptide promotes in increase of PP2A tumour suppressor activity while simultaneously down regulating key hallmark components of cancer development and progression of PCa such as Akt and NF-κB signalling and activation. Given that OP449 successfully kills metastatic PCa cell lines, potently inhibits cell proliferation, induces apoptosis and induces a block in cell cycle, targets multiple signalling cascades and blocks NFκB activation from multiple angles, and exerts stark anti-inflammatory consequences to its exposure in the cell lines used, further investigation in to potential use of this drug is highly warranted. While results are promising, the study is not without limitations. While the testing of drug response in mono layer cancer cell lines can be an informative method of identifying biochemical targets in drug development, this model type lacks the complexity of a cell growing in vivo. A cancer model that reflects the stromal surrounding and typical crosstalk would provide a more physiologically accurate representation regarding the efficacy of these drugs as therapeutic candidates.

In this study I have demonstrated the ability of OP449 to induce apoptosis and impede cell cycle progression in PCa cells. The ApoE mimetic peptides manufactured by Oncotide.inc are reported to inhibit SET, an oncoprotein with a diverse range of cellular functions. While SET is known to be an endogenous inhibitor if PP2A, it is also a regulator of DNA replication, chromatin remodelling and gene transcription [193, 608-610]. In terms of cell cycle progression, SET may influence cell cycle by regulating the activity of several cyclin-dependent kinases (CDKs). It has previously been described that SET binds to the CDK inhibitor $p21^{Cip1}$ *in vivo* and *in vitro* and that SET may reverse the inhibitory effect that $P21^{cip1}$ has on cyclinE-cdk2 activity [611]. The oncoprotein has also been reported to inhibit cyclin B-cdk1 activity and enhance the activity of $p35^{nck5a}$-cdk5 [187, 612, 613]. SET interacts specifically with B-type cyclins, although the functional significance of this interaction has not been elucidated [612]. A block in cell cycle progression at G2/M is associated with the inhibition of cyclin B-CDK1 activity, indicating that SET might

be involved in the control of mitosis entry by regulating the activity of cyclin B CDK1 complexes [187]. Results from *in vivo* analysis demonstrate association and co-localisations of SET and $p21^{Cip1}$ that these proteins may interact with each other a specific moments of the cell cycle but not others and suggests the proteins may interact during mid-late S phase [187, 610]. Findings from in vitro studies revealed that SET inhibits cyclin B-CDK1 activity in concentrations similar to those of $p21^{Cip1}$. This strongly supports the idea that SET is involved in the control of mitosis entry by regulating the activity of cyclin B CDK1 and $p21^{Cip1}$ complexes [187, 614-616]. SET has also been reported in the literature to bind to the C-terminal domain (CTD) of p53, an interaction which is dependent on the acetylation status of the CTD. SET profoundly inhibits p53 transcriptional activity in unstressed cells. SET mediated repression is eliminated by stress induced acetylation of P53 CTD. Furthermore, loss of the SET-p53 interaction activates p53 resulting in tumour regression in mouse xenograft models [617]. Considering $p21^{Cip1}$ represents a target of p53 signalling and the documented association between SET, cyclin B CDK1 and $p21^{Cip1,}$ and p53, and thus with DNA damage and cell cycle arrest, the effects of SET inhibition warrants further exploration in PCa research [437-439]. Considering that the known functions of SET clearly relate it with the control of cell cycle progression, investing the role of SET and its inhibition in response to ApoE mimetic peptides, namely COG112 and OP449, could potentially unlock further clues in the mechanism of PCa progression.

In this study, the upregulation of PP2A in response to the ApoE peptide mimetics was assessed by exploring the consequential dephosphorylation of downstream kinases. While promising targets found to be negatively regulated in a dose dependent manner, this study, like all others, is not without limitations. For instance, the upregulation of PP2A activity was demonstrated, the composition of the PP2A subunits that are upregulated in response to the peptides are not defined. As numerous combinations of Scaffolding A subunits, regulatory B subunits and catalytic C subunits exist, PP2a is a multitasking phosphatase and identifying which combinations were modulated was beyond the scope of this

study. Additionally, while the use of AR-negative cell lines allowed us to explore the effects of ApoE peptide mimetics in AR negative cells, the interactions observed in may not fully represent the pathobiology of AR negative metastatic PCa. This logic is reinforced with the awareness that while cell lines serve as useful tools for defining signalling pathways in cancer, not all of these pathways have been deemed critical for the development of tumours in a clinical setting. Taking this into consideration, a future xenograft study would provide a more representative model and help determine if the findings from our study are therapeutically relevant.

Within this study, COG112 and OP449 were shown to regulate IκBα in both a pp2A and TLR dependent manner. Having only scratched the surface exploring the immune suppressive role of ApoE peptide mimetics in AR negative PCa, further investigations are required to explore the potential cross talk between PP2A and innate immune response. It would also be worth investigating the interaction of the compounds with NF-κB subunits other than p65. Such studies proposed would enhance the findings of this research study and potentially support a role for ApoE peptide mimetics for PCa treatment in a clinical setting.

6.3 Future Direction

The aim of this research study was to investigate the potential efficacy of ApoE peptide mimetics in PCa cells. While results are promising many questions remain unanswered. Some of which are listed below with directions for future work. Further exploration into the effects of ApoE peptide mimetics on cell cycle and DNA replication are highly warranted. For instance, evidence of DNA damage could be investigated by staining for γ-H2AX and exploring the status of cell cycle checkpoints. It would also be of interest to extent the study further in terms of cell deat by exploring the mechanisms of apoptosis induction and the possibility of the peptides interfering with BH3 only and BCl2 family members.

In order to strengthen the case for the potential use of ApoE peptides in PCa, their efficacy should be explored in a 3D model, incorporating co-cultures, MSCs or the generation of spheroids in an effort to represent the tumour microenvironment. Co-cultures or 3D models response to the peptides should also be assessed under hypoxic conditions. Further studies could also incorporate exploring the effect of OP449 on components of immune response. It was shown in this study that OP449 reduced IL-6 levels in vitro. As IL-6 is produced by multiple cell types, including macrophages, endothelial cells and T lymphocytes, exploring the potential of ApoE peptide mimetics to modulate macrophage polarization and potentially enhance T cell and NK mediated response, expanding on innate immune response in PCa.

Chapter 7: Bibliography

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