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SIGNIFICANCE OF CYTOSOLIC PHOSPHOLIPASE A2-ALPHA IN
PTEN-NULL/MUTATED PROSTATE CANCER CELLS

SHENG HUA

A thesis submitted in fulfillment of the requirements for the degree
of Doctor of Philosophy

Discipline of Medicine, Sydney Medical School,
The University of Sydney

August 2013
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INTRODUCTORY STATEMENT

The studies presented in this thesis are the results of original research carried out while the author was enrolled as a candidate for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Sydney. These studies were conducted in the Department of Endocrinology, Discipline of Medicine, University of Sydney.

All experimental work carried out for this thesis is entirely my own original work except where stated otherwise in the text. The work presented in this thesis has not been submitted for a degree or a diploma in any other university.

Sheng Hua

August 2013
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ABSTRACT

Constitutive phosphorylation of protein kinase B (AKT) is a common feature of prostate cancer caused by genetic alteration in phosphoinositide-3-kinase (PI3K) or the phosphatase and tensin homolog (PTEN) gene and is associated with poor prognosis. Suppression of the AKT signalling pathway in cancer cells carrying genetic alterations in PI3K or PTEN has proven to be a challenge. This is due to several factors such as existence of a negative feedback loop on AKT, compensation from other oncogenic pathways including extracellular signal-regulated kinase (ERK) and potential involvement by the androgen receptor (AR) to evade androgen dependence.

Understanding and identifying novel therapeutic targets in prostate cancer may assist in the development of strategies to treat prostate cancers with aberrant AKT activation. It has been shown that cytosolic phospholipase A2α (cPLA2α) is important for the proliferation of PTEN-null prostate cancer cells. cPLA2α is responsible for production of arachidonic acid (AA), the precursor of eicosanoids, which can stimulate cell proliferation. The mechanisms of how cPLA2α action affects proliferation of prostate cancer cells is unclear. To investigate this mechanism, this thesis aims to determine the role of cPLA2α in AKT, ERK and AR signaling in PTEN-null/mutated prostate cancer cells.

Chapter 3 describes the effect of overexpression of cPLA2α in a prostate cancer cell line LNCaP, with PTEN mutation, on AKT, ERK and AR activation and/or expression. Under a tetracycline-inducible expression system, cPLA2α overexpression, coupled with
increased activity, led to an increase in phosphorylation of AKT. Phosphorylation of GSK3β, a target of AKT, and cyclin D1 levels were also increased. However, no significant increase was observed for ERK and AR. cPLA2α overexpression and activation did not cause an increase in cell proliferation.

In Chapter 4, the importance of endogenous cPLA2α expression and activation in PTEN-null prostate cancer cells were examined using siRNA. cPLA2α silencing decreased pAKT, pGSK3β and cyclin D1 levels in both PC-3 (PTEN deletion) and LNCaP cells. Additionally, silencing of cPLA2α decreased pERK and AR protein levels while total AKT and ERK were unchanged. The inhibitory effect of cPLA2α silencing on pAKT, pERK and AR were enhanced under epidermal growth factor (EGF)-stimulated conditions. In terms of cell proliferation, cPLA2α silencing significantly reduced proliferation of PC-3 and LNCaP cells compared with control. The population of G2M cells in siRNA-treated cells was significantly decreased while G0/G1-populations increased. The effect of cPLA2α on these proliferative pathways may be through an EGF receptor, which may activate PI3K/AKT and ERK.

In Chapter 5, I examined the role of AA and its metabolites in mediating cPLA2α action on AKT, ERK and AR. The inhibitory effect of cPLA2α siRNA on pAKT and AR protein levels was reduced by the addition of AA, which can increase PC-3 and LNCaP cell proliferation. AA alone can increase pAKT, pERK and AR level in both cell lines. Analysis of hydroxyeicosatetraenoic (HETE) levels following AA addition showed a prominent increase in 5-HETE in PC-3 and LNCaP cells. Inhibition of 5-HETE synthesis
abrogated the stimulatory effect of AA on pAKT, pERK and AR levels. This suggests that cPLA$_2\alpha$ action on AKT, ERK and AR is likely through AA and 5-HETE.

In summary, the studies presented in this thesis demonstrate an important role for cPLA$_2\alpha$ in sustaining AKT, ERK and AR signaling in PTEN-null/mutated prostate cancer cells and provide a potential molecular target for treating prostate cancer with constitutive phosphorylation of AKT.
PUBLICATIONS ARISING FROM WORK IN THIS THESIS


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Expression</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cPLA₂α</td>
<td>Cytosolic Phospholipase A2 alpha</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>d</td>
<td>Day</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescence Protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FLAP</td>
<td>5-lipoxygenase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>HETE</td>
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</tr>
<tr>
<td>HPETE</td>
<td>Hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>LB broth</td>
<td>Luria Bertoni broth</td>
</tr>
<tr>
<td>LC–MS</td>
<td>Liquid Chromatography – Mass Spectrometry</td>
</tr>
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<td>LOX</td>
<td>Lipoxygenase</td>
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<td>Lys</td>
<td>Lysine</td>
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<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphoinositide-dependent kinases</td>
</tr>
<tr>
<td>PHLPP</td>
<td>Pleckstrin homology domain leucine-rich repeat protein phosphatase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS and 0.05% v/v Tween-20</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>REST 2009</td>
<td>Relative Expression Software Tool 2009</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPMI Medium</td>
<td>Rosewell Park Memorial Institute Medium</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
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<td>Si-cPLA$_2$$\alpha$</td>
<td>siRNA against cPLA$_2$$\alpha$</td>
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<td>Si-Control</td>
<td>siRNA against a randomly scrambled sequence</td>
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<tr>
<td>SUPAMAC</td>
<td>Sydney University Prince Alfred Macromolecular Analysis Centre</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate, EDTA</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TBST</td>
<td>TBS and 0.05% v/v Tween-20</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline Response Element</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factors</td>
</tr>
</tbody>
</table>
CHAPTER 1

LITERATURE REVIEW
1.1 Introduction to prostate cancer (PC)

PC is one of the most common cancers. The incidence of PC varies 50-fold world-wide (1) and is most common in Western countries and least common in South-east Asia (2). In Western countries it is the second most commonly diagnosed cancer – after skin cancer – and in terms of cancer-related deaths, exceeded only by lung cancer (3; 4). In recent years, PC mortality rates have declined in many developed countries, including Australia (5), possibly due to the prevalence of screening assays such as prostate-specific antigen (PSA) testing (5; 6). Presently in Australia, there are about 20,000 new incidences and 3,300 mortalities from PC per year.

1.1.1 Prostate anatomy and pathology

The prostate gland is the size and shape of a walnut and consists of both glandular and muscular tissues (7). Located under the bladder and in front of the rectum, the prostate gland surrounds the urethra and the two ejaculatory ducts. It consists of a capsule and numerous partitions. The main function of the prostate is to store and secrete a fluid which nourishes and protects sperms, and carries them during the male orgasm. Prostate fluid constitutes 10–30% of the volume of seminal fluid (8).

The prostate gland has four distinct glandular zones: peripheral zone; central zone; transition zone; and anterior fibromuscular zone (9). The peripheral zone surrounds the anterior urethra and is the origin of 64% of prostate cancers; the central zone surrounds the ejaculatory ducts and is responsible for about 3% of prostate cancers; the transition zone surrounds the proximal urethra and is where approximately 34% of prostate cancers originate (10). The
anterior fibromuscular zone does not contain epithelial structures, thus it is not affected by adenocarcinoma (10).

1.2 Phosphoinositide-3-kinase (PI3K) / protein kinase B (AKT) / phosphatase and tensin homolog (PTEN) pathway

1.2.1 Overview

The phosphoinositide-3-kinase / protein kinase B (PI3K/AKT) pathway is one of the major signalling pathways, regulating diverse cellular processes (11). It is highly conserved in eukaryotes and its activation is tightly controlled via a multistep process (12). PI3K/AKT kinases themselves act as major switches conveying signals from transmembrane receptor tyrosine kinases (RTKs), which are activated by binding of extracellular ligands such as growth factors, hormones and insulin (11).

PI3K is a lipid-modifying enzyme that catalyses conversion of phosphatidylinositol-4,5-biphosphate (PIP$_2$) into -3,4,5-triphosphate (PIP$_3$) at the cell membrane (11). As the extra phosphate is added at the ‘3’ position in the ring, it is named PI3-kinase (13). PIP$_3$, once produced, acts as a docking site for activation of AKT by other phosphoinositide-dependent kinases (PDKs) (14).

The AKT kinase phosphorylates a wide range of downstream targets, leading to increased proliferation, survival, and cell size. AKT also influences the cellular response to nutrient availability, intermediary metabolism, angiogenesis and metastasis (14).
1.2.2 Mitogenic signals for PI3K activation

Activation of PI3K is initiated by extracellular stimuli binding to RTK. These extracellular stimuli include growth-related factors (such as epidermal growth factor (EGF); platelet-derived growth factor (PDGF); vascular endothelial growth factor (VEGF) or metabolism-related factors such as insulin and insulin-like growth factor (IGF) (13). Ligand binding to the RTK monomer leads to receptor dimerisation. The dimerisation allows for the cytoplasmic portion of the receptor to trans-phosphorylate a tyrosine residue on its partner (15). This activated form of RTK is then able to activate PI3K directly or through recruitment of adaptor molecules, such as insulin receptor substrate protein (IRS) or Grb-2 associated binder proteins (16).

1.2.3 Activation of PI3K, PIP3 and AKT and dephosphorylation

PI3K is a heterodimeric protein with two subunits: p85 (regulatory subunit) and p110 (catalytic subunit). Several isoforms of both subunits exit, although more for p110 than p85 (11). The p85 subunit is a regulatory element that inhibits and stabilises the p110 subunit. It contains a Src-Homology 2 (SH2) domain, which has affinity to RTK to bring PI3K into contact (17). Phosphorylation at tyrosine (Tyr) and threonine (Thr) residues of the p85 subunit (18; 19) leads to a conformational change and thereby releases the inhibitory effect on the p110 subunit. The catalytic p110 subunit then turns PIP₂ into PIP₃ (20). The PIP₃ serves as a docking site for AKT kinase to stay at the membrane through binding to its pleckstrin homology (PH) domain (12). This ‘docking’ method allows AKT to be brought in close proximity to other regulatory molecules.
Activation of AKT is a multistep process (21), and is important for its wide range of downstream targets. The complexity of its activation and deactivation is shown in the number of kinases and phosphatases involved in conveying its signal by phosphorylation and dephosphorylation. RTK and PI3K activation create a build-up of PIP3. Attraction of AKT to the increased PIP3 allows for a subsequent two-step phosphorylation process at the membrane region. The first phosphorylation (at Thr308) by PDK1 (also with PH domain) (22) induces a conformational change to its structure (23) and increases activity (24). This change allows a second phosphorylation of AKT (Ser473) by mTOR Complex 2 (mTORC2) (23). Thus, AKT is fully activated and can depart from the membrane to phosphorylate its target proteins.

Phosphorylation on both Thr308 and Ser473 brings a synergistic boost to AKT function and under this state AKT activity is the highest (14; 25). Mutational studies using 308D/473D AKT demonstrated an activity increase up to 40-fold compared with unstimulated wildtype (24). Both phosphorylations are required for maximum output, although single phosphorylation at either site still offers partial improvement to AKT activity and can exist independently (24). In addition, the two phosphorylation sites have their distinct purposes – Thr308 is essential for functioning of the catalytic domain and formation of the active site (26); and Ser473 broadens substrate scope to include FOXO transcription factors and proline-rich AKT substrate (PRAS40), which are not phosphorylated in the absence of Ser473 phosphorylation (27). Interestingly, one study demonstrated that only phosphorylation at Thr308 (by PDK1) is necessary for full activation and that subsequent phosphorylation at Ser473 can be achieved by AKT itself (28).
Although it is unclear which site is more important for AKT function, Ser$^{473}$ has been studied pathologically. Ser$^{473}$ phosphorylation is involved in a negative feedback loop due to mTOR inhibition in cancer cells (29). This loop renders inhibition by rapamycin somewhat ineffective (explained in more detail below). Further, about 45% of PC cases have increased levels of pAKT at Ser$^{473}$, which correlates positively with disease severity (14; 30-32).

1.2.4 Suppression of signalling by PTEN, PP2A and PHLPP

AKT activation is tightly controlled by counteraction of PTEN (33), protein phosphatase 2A (PP2A) (34-36), and pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) (37). Being a lipid phosphatase, PTEN is expressed ubiquitously to catalyse the hydrolysis of PIP$_3$ back to PIP$_2$ (16). This process counters PI3K action by reducing the source of AKT membrane binding and activation. PTEN is the cell’s most effective method of turning off AKT while also playing some part in the role of membrane remodelling (38; 39).

Apart from PTEN, AKT activation may also be controlled by phosphatases. PP2A is found to dephosphorylate AKT at Thr$^{308}$ (40). The B55$\alpha$ regulatory subunit of PP2A preferentially targets the PP2A holoenzyme to dephosphorylate AKT at Thr$^{308}$ rather than Ser$^{473}$ both in cells and in the cell-free system (41). This selective targeting can be inhibited by IEX-1, an early gene product with proliferative and survival activities, in an ERK-dependent manner (42). Two other phosphatases, PHLPP1 and PHLPP2, selectively dephosphorylate AKT at Ser$^{473}$ (37; 43). PHLPP also contains a PH domain, allowing it to migrate to a membrane
region to find substrates. Both PP2A and PHLPP1/2 are effective at reducing AKT signalling and cell proliferation (37; 41; 43).

The PI3K-AKT Pathway

![PI3K/AKT signalling pathway overview](image)

**Figure 1.1 PI3K/AKT signalling pathway overview.** Simplified diagram showing methods of AKT phosphorylation and dephosphorylation. Illustration prepared by Soma Vignarajan, a fellow PhD student.

1.2.5 Downstream signalling by AKT

The targets of AKT signalling include a number of cellular molecules that cover a large spectrum of functions, ranging from control of cell proliferation and survival to modulation of intermediary metabolism and angiogenesis. The complete list of AKT targets is unknown but most substrates share the consensus sequence for AKT phosphorylation, RXRXXS/T (14). The direct targets of AKT that will affect a single or multiple pathways are (11; 14; 40):
- GSK3β (for cyclin D1 phosphorylation and degradation; AKT inactively phosphorylates GSK3β to increase the cell cycle)
- AS160 (for increased GLUT4 translocation and glucose uptake)
- PRAS40 (required for mTORC1 formation, increases protein translation and enhance cell growth)
- BAD (for reduced Apoptosis by preventing dimerisation with Bcl-XL/Bcl-2)
- MDM2 (for inhibition of p53 in DNA repair)

The major effects of AKT signalling pass down to increase cell proliferation, cell cycle, protein synthesis and survival and reduce apoptosis.

1.2.5.1 Pro-proliferation signals

AKT is able to phosphorylate multiple substrates to increase proliferation. Phosphorylation of GSK3β by AKT prevents the degradation of cyclin D1, which is critical for complexing with CDK4 and CDK6 during the cell cycle G1/S transition (44). Phosphorylation by AKT of p21 and p27 on a site close to their nuclear localisation signal prevents these cell cycle inhibitors from entering the nucleus (45). Phosphorylated p27 binds to 14.3.3 scaffold protein and is retained in the cytoplasm (46).

1.2.5.2 Increased protein synthesis

AKT increases protein synthesis by phosphorylation of two substrates: tuberous sclerosis complex 1 and 2 (TSC1/2) and proline-rich AKT substrate 1 (PRAS40). Phosphorylation of TSC2 disrupts its interaction with TSC1 and destabilises the inhibition of the protein Ras
homolog enriched in brain (Rheb) (47; 48). Accumulation of free Rheb leads to activation of the mammalian target of rapamycin mTOR complex 1 (mTORC1). Further, AKT phosphorylates PRAS40, a component of mTORC1, to relieve the PRAS40 inhibitory effect on mTORC1 (16). Activated mTORC1 is able to phosphorylate: (i) p70 S6 kinase, which targets S6 ribosomal protein (49); and (ii) eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP), the inhibitory binding partner of eIF4E (50). Phosphorylation of both these molecules result in increased protein synthesis (51).

1.2.5.3 GSK3β and metabolism

AKT affects cell metabolism by increasing glycogen synthesis and cellular glucose uptake. GSK3β, the direct target of AKT, is involved in cell metabolism apart from the cell cycle. Inhibition of GSK3β by AKT leads to increased glycogen synthesis (52). Secondly, AKT has been found to mediate insulin-stimulated glucose transport: AKT phosphorylation of GLUT1 and GLUT4 leads to increased translocation of glucose through the membrane (53). Additionally, AKT phosphorylates the AKT substrate of 160 kDa (AS160) in adipocytes to aid the GLUT translocation process (54). Thus, the rate of glucose uptake is increased.

1.2.5.4 Survival signals

AKT signalling prevents apoptosis and increases survival signal. AKT phosphorylates BAD, the pro-apoptotic protein from the Bcl-2 family. Phosphorylated BAD binds to protein 14-3-3 and is prevented from heterodimerising with Bcl-XL/Bcl-2 to initiate apoptosis (55; 56). AKT also prevents apoptosis by inactivation phosphorylation on pro-caspase 9 (57) and activation phosphorylation on PED/PEA15, a cytosolic inhibitor of caspase-3 (58). Further,
AKT phosphorylation of p21 prevents its nuclear entry, thus abrogating its apoptosis signalling through apoptosis signal-regulating kinase 1 (59). In parallel, AKT could also activate NF-κB via phosphorylation of IκB kinase, thus resulting in transcription of pro-survival genes (14; 60). Finally, AKT can also prevent p53-mediated apoptosis by mouse double minute 2 homolog (MDM2) phosphorylation, which then complexes with p53 for ubiquination and degradation (61).

1.2.6 The PI3K/AKT pathway in PC

The PI3K/AKT pathway is one of the most prevalent pathways deregulated in cancer, including PC (11; 20). Due to the pro-proliferative nature of PI3K/AKT signaling, mutations in this pathway are more likely to result in cancer. Studies have shown over 43% of prostate cancers have either a gain of function mutation in PI3K (the oncogene) or a loss of function mutation in PTEN (tumour suppressor) (11). Thus, effective inhibition of the PI3K/AKT pathway is important in therapeutic treatment of PC.

Many epidemiological studies have linked cancer and metabolic disorders such as diabetes (62-64). Cancer cells are often glucose-dependent due to increased need for cellular building blocks. Hence, increases in insulin and IGF favours tumour growth by stimulating proliferation and survival (e.g., via insulin receptors and insulin receptor substrates) (65). However, constitutive activation of PI3K/AKT no longer requires insulin regulation and thus renders cancer cells resistant to dietary restrictions (66) and cells proceed to proliferate under high or low nutrient conditions. Therefore, PI3K plays an important role in balancing metabolism and cell proliferation and the underpins both metabolic disorders and cancer (65).
1.2.7 Increased activation of PI3K

Deregulation of the PI3K/AKT pathway can result from mutations in *PI3K*, making PI3K hyperactive. Mutations in *PIK3CA*, the gene for the p110α subunit is most common relative to other PI3K subunits (67). Somatic mutations on Glu$^{542}$, Glu$^{545}$ and His$^{1047}$ have been found to be the most common in cancer (11; 67). These mutations, which account for up to 80% of *PIK3CA* mutations (68), increase PI3K activity and lead to growth factor-independent activation of AKT (67; 69-71). The Glu$^{545} \rightarrow \text{Lys}$ mutation, for example, disrupts the inhibitory interaction with the p85-SH2 domain, and increases kinase activity of the p110 subunit (72). Constitutive activation of PI3K creates an endless supply of PIP$_3$, the docking molecule for AKT before its activation. Thus, there are more PIP$_3$ molecules present and AKT signalling is increased.

1.2.8 Loss of PTEN

Mutations in *PTEN* are far more common than in *PI3K* for PC. Inherited *PTEN* mutations cause cancer predisposition and are associated with a wide and diverse clinical spectrum of disorders, collectively known as *PTEN* hamartoma tumour syndromes, which are characterised by the development of benign tumours (73). PC cells with genetic mutations resulting in a frameshift (e.g., LNCaP) (74) or gene deletion (PC-3) (75) render the PTEN protein inactive or absent. Consequently, any build-up of PIP$_3$ cannot be converted back to PIP$_{2}$, leading to constitutive AKT activation. Further, the location of *PTEN* is at chromosome 10q23.3, a region that frequently shows loss of heterozygosity (LOH). The rate of LOH for *PTEN* is significantly high for PC with some studies showing as high as 49% (76-78). As *PTEN* is haplo-insufficient (79), losing a single copy of *PTEN* is often enough to result in cancer (79) as it provides a high selective advantage.
Other mechanisms of PTEN inactivation exist. For example, PTEN may be inactivated by promoter mutations or methylation and silencing of the PTEN promoter (80-82). Post-translational PTEN phosphorylation (by GSK3β) on its C-terminal tail, may stabilise PTEN but reduce its activity towards PIP₃ (80). In addition, reactive oxygen species (ROS) from arachidonic acid (AA) metabolism is also known to inactivate PTEN (83). Reactive oxygen species (ROS) is found to oxidise C124 of the PTEN catalytic domain and inactivate it by creating an intramolecular disulfide bond (84). Further, ROS is a by-product of AA catalysis by the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which are overexpressed in inflammation and during multistage tumour progression (85). This process is greatly enhanced in PC as the rate of eicosanoid synthesis in tumours is much higher than benign tissues (86). Taken together, PTEN inactivation in PC is common due to a variety of mechanisms.

1.2.9 Methods of PI3K/AKT inhibition

It has been suggested that tumours with increased AKT activation (e.g., from PIK3CA mutations) will indeed be addicted to PI3K as the primary source of growth, proliferation and survival signalling. Accordingly, new methods for PI3K/AKT inhibition are constantly being developed and tested (87). Key signalling molecules such as RTK, P110α or mammalian target of rapamycin (mTOR) are frequently targeted. RTK inhibitors such as Tarceva (epidermal growth factor receptor (EGFR) inhibitor) and Imatinib (PDGF inhibitor), achieve their antitumor effects at least in part by shutting off upstream signalling to the PI3K/AKT pathway (14). However, the efficacy of upstream inhibitors could be hampered by activating
mutations or gene amplifications in downstream signalling components or by the loss of PTEN.

On the level of PI3K, inhibitors such as LY294002 and wortmannin have been used extensively in research and have shown antitumour activities in PTEN-null or PI3K-overexpressing cells (88). Since PI3K is highly pleiotropic, administration of PI3K inhibitors would be associated with high toxicities. In addition, these drugs also exhibit off-target effects such as inhibition of PI3K-related kinases such as ATM and ATR (essential for DNA repair) (89).

Drugs for downstream signalling targets are also available. For example, rapamycin, the inhibitor of mTOR, has been considered for cancer treatment (90; 91). Rapamycin is effective in mTORC1 inhibition and increases the sensitivity of PTEN-deficient glioblastoma cell lines to erlotinib, a RTK inhibitor, to induce growth arrest (92). However, the therapeutic effect of rapamycin is diminished over time due to the abolishment of a negative feedback loop as described in the next section.

1.2.10 Resistance to insulin regulation and negative feedback loop

Although the PI3K/AKT pathway presents obvious targets for therapeutic intervention, the complexities in signalling regulation pose substantial challenges in therapeutic design (16). While inhibition of downstream effectors of AKT (e.g., rapamycin or RAD001) may seem effective, the process is complicated by a negative feedback loop on upstream AKT (93). While mTORC1 is inactive, its target S6 kinase is inactivated. S6 kinase serves as a negative
regulator of PI3K through phosphorylation and inhibition of IRS1 (94). IRS1 is recruited by RTK to activate PI3K (16). In this instance, mTOR inhibition results in increased PI3K signalling and AKT activation. AKT controls a host of substrates independent of mTOR signalling that may contribute strongly to oncogenesis. Thus, growth inhibition is compromised.

1.2.11 Compensation mechanism in other signalling pathways

Existing pathways inside the cell sometimes overlap or interact with each other. This horizontal effect between pathways is apparent when the inhibition of one pathway leads to upregulation of other pro-proliferative pathways. There is accumulating evidence that the Ras/Raf/ mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and PI3K/AKT pathways may cooperate to promote the survival of transformed cells and resistance to therapy (95-97). One study reported that tumour cell biopsies obtained from patients treated with the mTOR inhibitor RAD001 displayed activation of ERK (98). Similar events were observed in a breast cancer cell model (97; 98). Others have demonstrated that, in a PTEN-deficient transgenic PC model, co-inhibition of ERK1/2 (by PD0325190) and mTOR (by rapamycin) had synergistic effects in vitro and in vivo (99). This phenomenon was proposed to be due to a S6 kinase /PI3K/AKT/mTOR-dependent pathway (97; 100). Exposure of cancer cells to mTORC1 inhibition results in release of a S6 kinase-related ‘brake’ on Ras (and also on PI3K, from a negative feedback loop) (98). Inhibition of this ERK compensation in addition to mTOR shows an increase in apoptosis in animal studies, although this was not clearly evident in PC cells (98).
Interactions between PI3K/AKT and other pathways exist in PC. Androgen receptor (AR) is known to be regulated by PTEN in prostate cells (101). The interaction between PTEN and AR inhibits the AR nuclear translocation and promotes AR protein degradation that results in the suppression of AR transactivation and induction of apoptosis (101). As PTEN is often absent in PC and that AR is often overexpressed, this mechanism of control is lost. This is an example where deregulation in PI3K/AKT upregulates growth signalling in another pathway in PC.

1.3 Ras/Raf/MEK/ERK pathway

1.3.1 Overview

The Ras/Raf/MEK/ERK pathway is a part of the of MAPK pathway and is common amongst all mammals (102; 103). ERK, one of four major groups of MAPKs (ERKs, p38MAPKs, c-Jun NH2-terminal kinases and ERK5 or BMK) (103; 104), has been established as a major participant in the regulation of cell cycle progression, apoptosis and differentiation (105), but when improperly activated, it contributes to malignant transformation. The Ras/Raf/MEK/ERK pathway is regulated by multiple members of the kinase, transcription factor, apoptotic regulator and caspase activator families, which can be activated or inactivated by protein phosphorylation (95). Abnormal activation of this pathway occurs in human cancer due to mutations at upstream membrane receptors, Ras and Raf, as well as genes in other pathways (e.g., PI3K/AKT), which can influence Raf activity. Nearly 50% of human cancers have alterations in the Ras/Raf/MEK/ERK pathway or in components of Ras effector pathways that may lead to increased ERK activity (106). The pathway also influences chemotherapeutic drug resistance as ectopic activation of Raf induces drug
resistance in breast cancer cells (95). Due to the above reasons, the Ras/Raf/MEK/ERK pathway is an important pathway for therapeutic targeting in cancer treatment.

1.3.2 Mechanism of EGFR, Ras, Raf, MEK and ERK activation

The Ras/Raf/MEK/ERK signalling cascade is activated by a wide variety of receptors involved in growth and differentiation including RTK, G-protein coupled receptors (GPCR), integrin and ion channels. Specific components of the cascade vary greatly among different stimuli, but the architecture of the pathway usually includes a set of adaptor proteins linking the receptor to a guanine nucleotide exchange factor (GEF) and transducing the signal to the

Figure 1.2 Flow chart of the Ras/Raf/MEK/ERK pathway (107).
small GTP-binding Ras. For instance (in Figure 1.2), EGFR, activated by EGF binding, recruits the adaptor growth factor receptor bound protein-2 (Grb2). Grb2 binds to EGFR through its SH2 domain, which binds to the phosphotyrosine residues of the activated receptor (108). Grb2 then recruits the protein SOS (a GEF) through its double SH3 domain (108). The docking of the Grb2-SOS complex on EGFR activates SOS and allows it to catalyse the removal of GDP on Ras with a GTP (109). Ras-GTP is the active form of Ras and may bind to Raf to activate Raf’s kinase activity (110). Activated RAF kinase phosphorylates and activates MEK (MEK1 and MEK2), which in turn phosphorylates and activates ERK (ERK1 and ERK2) (95).

1.3.3 Downstream signalling by ERK

ERK1 and ERK2 are 44 and 42 kDa Ser/Thr kinases (as are Raf and MEK) and are positively regulated by MEK1 and MEK2 (95). Activated ERK has over a hundred known targets (95). ERK can enter the nucleus and directly phosphorylate many transcription factors, including Ets-1, c-Jun and c-Myc (95). ERK can also activate transcription factors indirectly. For example, ERK phosphorylates and activates the 90 kDa ribosomal S6 kinase (p90Rsk), which then leads to the activation of the transcription factor cAMP response element-binding protein (105); ERK can also activate NF-κB through phosphorylation of IκB kinase (111; 112). By altering the levels and activities of transcription factors, ERK leads to altered transcription of genes that are important for the cell cycle.
1.3.4 Deregulation of Ras/Raf/MEK/ERK signalling

As mentioned previously, mutations in the upstream receptor, Ras or Raf, are frequent causes of increased Ras/Raf/MEK/ERK signalling. Gene amplification of *Ras* and activating mutations can be as high as 30% of some cancers (113; 114). Point mutations on *Ras* may occur in codons 12, 13, 59 and 61 (105). These mutations result in the constitutive activation of the protein so it no longer requires a ligand for activation. Other mutations may activate Ras by negating the GTPase activity stimulated by the regulatory partner GTPase-activating protein (GAP). This increases the half-life of Ras-GTP (115).

Next, mutations in *B-Raf*, an isoform of Raf, have been found in about 7% of cancers (116; 117). Mutations such as Val$^{600}$→Glu (the most common) (116) results in hyperactive B-Raf, as if phosphorylated, and locks it in the active configuration (118). In this setting, B-Raf targeting of MEK is increased, leading to increased signalling.

1.3.5 Ras/Raf/MEK/ERK pathway in prostate cancer

Increased expression/activation of the Ras/Raf/MEK/ERK pathway has been associated with advanced PC, hormonal independence and a poor prognosis (119-123). Additionally, Ras/Raf/MEK/ERK signalling is also implicated in PC progression by making AR hypersensitive to androgens (121).

Prostate cancers commonly have PI3K/AKT activation and there have been evidences of cross-talk or shared jurisdiction between PI3K/AKT and the Ras/Raf/MEK/ERK pathway.
1. The two pathways may control similar downstream targets. For example, activated AKT phosphorylate caspase 9 and BAD prevent apoptosis, but ERK may also achieve this through direct phosphorylation (on caspase 9) (124) or through its downstream effector p90Rsk (on BAD) (57; 95; 125). Thus, the two pathways share a common survival mechanism.

2. AKT can also enhance MDM2-mediated ubiquitination of p53, which interacts with Ras/Raf/MEK/ERK on two levels. On one hand, p53 upregulates expression of phosphatases such as MKP1, PAC1 and DUSP5 (126-129) and these negatively regulate ERK and other MAPKs (130) in response to different stressful stimuli to lead to cell cycle arrest and apoptosis. On the other hand, induced expressions of p53 lead to activation of MEK and ERK, but not p38 (131). In this setting, p53 upregulates heparin-binding epidermal growth factor-like growth factor (HB-EGF), which (through EGFR) leads to both ERK/MAPK and PI3K/AKT activation to prevent cells from oxidative damage-induced apoptosis (130). Further, this p53-MAPK signalling can even activate cyclooxygenase-2 (COX2) for increased cell survival (132).

3. AKT may downregulate Ras/Raf/MEK/ERK signalling. Activated AKT mediates the inactivation of Raf, e.g., B-Raf (by AKT-related kinase SGK) or c-Raf, another type of Raf, by phosphorylation of Ser259 (95).

The above mentioned bring some complexity to the cross-talk between the two pathways. However, the third point above is true in PTEN-null cell lines (LNCaP and PC-3), as they express low, but detectable, levels of activated ERK (95; 133; 134). Despite this, there exists a compensation mechanism between the two pathways. Indeed, down-regulation of PI3K/AKT would remove the negative regulation of Raf and p53. Additionally, inhibition of
PI3K/AKT (such as rapamycin) leads to activation of upstream AKT and Ras through S6 Kinase and IRS1 (97; 100). Thus, this horizontal compensation mechanism may be the key to ERK activation (during PI3K/AKT inhibition) to increase survival. Further, recent evidence has highlighted that mTOR can also be activated by Ras/Raf/MEK/ERK (135; 136) and this solidifies the notion of cross-regulation. Taken together, these offer rationale for co-targeting of both signalling pathways for treatment of PC.

1.4 Androgen receptor pathway

1.4.1 Overview

The AR pathway mediates a wide range of developmental and physiological responses important in male sexual differentiation, maintenance of spermatogenesis and male hormones (137). The pathway initiates when steroidal androgens, testosterone or its metabolite 5-alpha-dihydrotestosterone (DHT) binds to AR (138; 139), a nuclear receptor (140) normally sequestered into the cytoplasm (141). Upon activation, AR translocates from the cytoplasm to the nucleus and regulates gene expression of androgen-regulated genes (140).

1.4.2 Structure and function of androgen receptor

The AR protein is composed of four functional domains: (i) an N-terminus regulatory domain; (ii) a DNA-binding domain (DBD); (iii) a hinge region; and (iv) a ligand-binding domain (LBD) (142). Each domain has a distinct role in the function and regulation of AR. The N-terminus regulatory domain encodes transcriptional activation function and homopolymeric polyglutamine (CAG) and polyglycine (GCC) repeats (143). The DBD encodes two zinc fingers essential for androgen response element (ARE) recognition and the hinge region
contains a nuclear localisation signal that directs the AR into the nucleus (144). The LBD is responsible for conformational change and optimal transcriptional activation function (TAF-1) upon ligand binding (144).

Prior to activation, the unbound AR in the cytoplasm is stabilised by forming a complex with heat-shock protein (HSP) 90 (142; 145). This complex is regulated by histone deacetylase 6 (HDAC6), which deacetylates HSP90 for AR maturation (146; 147). Androgen binding induces AR dissociation from HSPs and causes AR dimerisation, phosphorylation and nuclear translocalisation. The activated AR then interacts with AREs to recruit co-regulators, forming of a preinitiation complex, and ultimately transcriptional activation of androgen-regulated genes (148).

Figure 1.3 AR activation in normal androgen-dependent prostate cancer and reactivation after androgen deprivation therapy. Modified from Figure 1 in (149).
1.4.3 Androgen receptor in prostate cancer

AR signalling is able to increase expression of genes for cell cycle and proliferation and is required for PC growth and survival (142; 150-153). While localised cases of PC may be treated with prostatectomy and irradiation, once metastasised, androgen ablation therapy becomes the mainstay of treatment options (142). Such hormone therapies remove androgen signalling by surgical or medical castration (with leutenising hormone releasing hormone agonists and oral anti-androgen) (142). Castration induces apoptosis in the majority of PC cells, which translates clinically to improvement in cancer-related symptoms and lowering of AR-regulated genes, including prostate specific antigen (PSA).

Although PC is initially androgen dependent, over time and/or during the course of treatment, it progresses into androgen-independent PC (AIPC), through a mix of molecular and cellular changes. Growth then becomes viable despite low serum testosterone levels and eventually these PC cells stop responding to hormone therapy. After this stage treatment options become limited to suboptimal chemotherapeutic measures, allowing limited lifespans (1–2 years) (142; 154).

1.4.4 Increased androgen receptor activation

Deregulation of the AR pathway leads to increased transcription, cell proliferation and prostate carcinogenesis (144; 150; 155). Increased AR signalling can result from mutations, changes to coactivators and corepressors of AR. Studies show up to 50% of prostate tumours have AR mutations, including those that are hormone naïve and AIPC (156-158). Mutations in AR coding sequence may lead to increased AR activity. Significant AR mutations found in
tumours include Thr^{877} \rightarrow \text{Ser}, \text{ Thr}^{877} \rightarrow \text{Ala}, \text{ His}^{874} \rightarrow \text{Tyr}, \text{ Val}^{715} \rightarrow \text{Met}, \text{ Leu}^{701} \rightarrow \text{His}, \text{ Thr}^{877} \rightarrow \text{Ala} \text{ and} \text{ Tyr}^{741} \rightarrow \text{Cys} (155; 156; 159-162). These mutations generally transform AR to become ‘super receptors’, which respond to a wider variety of agonists at lower concentrations. For example, the point-mutation Thr^{877} \rightarrow \text{Ala} in the AR gene of the hormone-binding domain in LNCaP cell line and other tumour samples allows activation by progesterone, estradiol, cyproterone acetate and the anti-androgens, anandron, and hydroxyflutamide (163). Protein structural analysis shows some mutations in the ligand-binding pocket codons 670–678, 701–730, and 874–910 (164; 165) loosen the ligand specificity while other mutations (e.g., in codon 874–910 (165)) change the interaction between various components of the AR transcriptional complex in favour of growth.

1.4.5 Increase in prostate-specific antigen

The gene *kallikrein-3* is a target of AR and is one of the genes elevated under AR signalling (160; 166). The gene encodes PSA, a glycoprotein enzyme, which is secreted by the epithelial cells of the prostate gland. PSA is normally present in small quantities in serum of healthy men but often elevated in PC. Disruption of the prostate epithelium by inflammation or benign prostatic hyperplasia leads to diffusion of PSA into surrounding tissue, and causes elevated blood levels of PSA (167). PSA has been suggested as a marker for PC screening in the past (168) and although this method may be riddled with false positives it is still a useful method for PC risk-stratification and combinatorial screening (169; 170).

PSA remains present in prostate cells after they become malignant and serum PSA levels may rise as PC cell numbers increase. A vast majority of men treated with androgen deprivation
therapy show reduced serum PSA, an evidence of improvement (149). However, as PC progresses to AIPC, AR signalling becomes reactivated and cells resume expression of AR-regulated genes, which may include PSA (171-174).

1.4.6 Progression to androgen independence by androgen receptor overexpression

Following hormone therapy, prostate tumours undergo molecular or cellular changes to overcome androgen deprivation. AR protein is present in all stages of PC regardless of stage, grade or whether androgen-dependent or not (175). Thus, androgen independence often results from mutations leading to hyperactive AR or an increase in AR expression.

The level of AR is an important factor in androgen independence. Studies suggest increased that AR expression allows a mechanism to utilise low levels of androgen, following castration and hormone ablation therapy (149; 176). Increased AR mRNA can be due to gene amplification and is reported in 25–30% of AIPC cases by several studies (171; 177-179). In addition, AIPC resumes the expression of multiple AR regulated genes (e.g., PSA), indicating reactivation of AR transcriptional activity (149). Importantly, studies using PC cell lines and xenografts similarly show that progression to AIPC is associated with high levels of AR and resumed expression of androgen-regulated genes (180-182). Moreover, AR down-regulation in cell lines at this AIPC stage (by siRNA or other methods) can suppress tumour growth, indicating that AR continues to provide critical functions (176; 182; 183). Thus, the level of AR is important for its signalling, particularly for AIPC, and should be considered in PC treatments.
1.5 Arachidonic acid pathway

1.5.1 Overview

AA is a member of the ω-6 poly-unsaturated fatty acids and is the precursor to downstream eicosanoids. As a long chain fatty acid, AA normally resides in the phospholipid pool of the bilipid membrane. Free AA is metabolised by COX and LOX enzymes to form eicosanoids, which primarily stimulate inflammatory response (184). In addition to inflammation, AA and its metabolites have been shown to affect a wide array of biological functions. In particular, its role in promoting cancer proliferation has been under increasing focus in recent decades (184-186).

The majority of AA remains esterified in membrane phospholipids at the sn-2 position and can be released following phospholipase A$_2$ (PLA$_2$) cleavage in response to a number of stimuli such as cytokines, growth factors and hormones (187; 188). AA-containing phospholipid, when cleaved by PLA$_2$ releases AA and lysophospholipid. Once released, free AA can be metabolised or reinserted into the membrane. In addition to release from glycerophospholipids, free AA can also be generated from degradation of arachidonoylethanolamide via enzymatic action [182].

Inflammatory eicosanoids influence several important biological processes in cancer progression, such as inflammation, cell proliferation and tumorigenesis (189; 190) including in PC (86; 191). Therefore, inhibition of the AA pathway has been an attractive target for chemotherapeutic manipulation in PC.
1.5.2 PLA₂ enzymes

Phospholipases are important for regulation of membrane phospholipids and the cellular processes initiated by their catalytic products, which include inflammation. PLA₂ are a family of enzymes that recognises the sn-2 acyl bond of phospholipids and catalyses the hydrolysis of this bond to produce a free AA and a lysophospholipid (192; 193). PLA₂ respond to increases in cytokines, growth factors and Ca²⁺ by migration to the membrane region and increase in activity. They are central to the maintenance and turnover of membrane phospholipids and have also been acknowledged for their pathological role in the inflammation pathway (194).

PLA₂ are a family of enzymes with over 20 members identified in mammals (195-197). The family can be divided into four classes on the basis of their amino acid sequence homology. Many forms of PLA₂ are differentially expressed in a tissue-, species- and/or genotype-specific manner (195; 198).

1.5.3 Cytosolic phospholipase A₂ alpha (cPLA₂α)

Of all the PLA₂s, the cPLA₂α is perhaps the most studied. Much research in the field has focused on cPLA₂α because of its central role in the initiation of AA metabolism (199-201). cPLA₂α cleaves at the sn-2 position of membrane glycerophospholipids. As a major source of AA release, cPLA₂α preferentially targets AA-containing glycerophospholipids, which contain head groups of ethanolamine, serine, inositol and choline. Stimulation of cells with agents that mobilise intracellular calcium and/or promote the phosphorylation of cPLA₂α leads to: (i) translocation of the enzyme from the cytosol to the cell membrane, Golgi
apparatus, endoplasmic reticulum and perinuclear membranes where it may associate with the glycerol phospholipid to access AA; and (ii) a change in conformation due to phosphorylation, which gains increased phospholipid binding affinity and AA release (202; 203). cPLA₂α has attracted considerable attention as a potential therapeutic target for treatment of inflammation and cancer (134; 204). Although ubiquitously expressed, cPLA₂α knockout models exhibited a relatively normal phenotype. However, when an inflammation-related pathological process is introduced to cPLA₂α knockout animals, there is a difference between wildtype and cPLA₂α knockouts (205).

1.5.4 cPLA₂α structure

The cPLA₂α coding region is located at chromosome 1q25 and is encoded by a 3.4 kb mRNA (849 amino acid peptide length). Its promoter region contains AP1, PEA3 and NF-kB coding sequence (206). cPLA₂α is highly conserved, sharing over 95% amino acid identity between human and mouse. The homologues in chickens, zebra fish and xenopus share over 80% amino acid identity with human. The cPLA₂α has an N-terminal C2 domain and a C-terminal catalytic domain linked by a short and flexible peptide (Figure 1.4).
1.5.4.1 C2 domain

Interactions between cPLA$_2$$\alpha$ and membrane lipids occurs through the C2 domain, which consists of an eight-stranded antiparallel sandwich. Calcium binding to the C2 domain through Asp and Asn residues promotes association of hydrophobic residues with membrane phosphatidylcholine. This brings the catalytic subunit close to the membrane bilayer, allowing enzymatic activity to take place.

1.5.4.2 Catalytic domain

The cPLA$_2$$\alpha$ active site is a strictly conserved DYAD composed of a serine (Ser$^{228}$) and an aspartic acid (Asp$^{549}$). A conserved Arginine (Arg$^{200}$) is also required for activity. The Ser/Asp active site of cPLA$_2$$\alpha$ lies in a deep funnel lined by hydrophobic residues, which confers the preference for AA-containing phospholipid (207). Changes in amino acid residues

Figure 1.4 cPLA2$$\alpha$ structure in Rasmol. Ca$^{2+}$ (red), C2 domain (yellow), catalytic domain (green) and a short and flexible peptide (purple).
in the funnel in other cPLA₂s may account for decreased AA specificity and differences in sensitivity to some cPLA₂α inhibitors.

In addition to the C2 domain, cPLA₂α also contains several conserved serine residues as phosphorylation sites for further regulation. Ser⁵⁰⁵ is the most studied and recognised site for phosphorylation, followed by Ser⁵¹⁵ and Ser⁷²⁷. Although phosphorylation is not entirely necessary, each of these sites has been shown to augment cPLA₂α activity to a different degree (208-210). Multiple kinases are responsible for phosphorylation of these sites, such as MAPK (Ser⁵⁰⁵) and MNK kinase (Ser⁵¹⁵), which suggest differential regulation of cPLA₂α depending on the route of the signalling process (209; 210).

1.5.5 cPLA₂α biochemical and biological function

cPLA₂α produces free AA and lysophospholipids, contributes to the production of eicosanoids and platelet activating factors, and possesses lysophospholipase and transacylase activities (although these activities are relatively low compared to its primary AA-producing activity) (211).

1.5.5.1 Production of AA, providing substrates for eicosanoid and platelet activating factor (PAF) production

cPLA₂α is the only PLA₂ that displays a high preference for AA and has been deemed the rate-limiting step in eicosanoid synthesis (192). Free AA is rapidly oxidised by COX, LOX and P450-dependent epoxygenase to produce prostaglandins, HETE and EET respectively.
These eicosanoids regulate inflammation and promote mitogenic and angiogenic signals by binding to GPCRs, such as the prostanoid receptor, and induce downstream signalling. The other product of cPLA\(_2\alpha\) action, lysophospholipid, is the precursor of PAF and has been shown to induce its own biological activities through GPCR (212). Both of these enzymatic products have their importance in pathological settings of inflammation and cancer. Thus, targeting of cPLA\(_2\alpha\) offers an attractive option in treatment of PC.

1.5.6 cPLA\(_2\alpha\) and its native inhibitor in prostate cancer

Expression and activation of cPLA\(_2\alpha\) have been associated with cancer pathogenesis in several cancer cell types. Proliferation of PC cells is also dependent on the activation and expression of cPLA\(_2\alpha\) (134; 204; 213).

Annexin 1 and 2 (ANX1/2) are natural inhibitors of PLA\(_2\) and expressed in normal prostate epithelia. Previous studies (214; 215) have demonstrated that ANX2 was absent in 31/31 randomly selected PC cases with a Gleason score range of 3–9 (214). In benign prostatic hyperplasia, ANX2 is expressed in the same location as in a normal prostate, with comparable intensity. 65% of prostatic intraepithelial neoplasia (PIN), the precursor of PC, also showed a loss of ANX2 expression, suggesting that loss of ANX2 could be an early event (214).

Southern blot using genomic DNA from paired PC tissue-derived and blood-derived genomic DNA from the same PC patients showed no gross deletion of the ANX2 gene in either cell type (214). Hypermethylation is believed to be the cause of loss of ANX2, as sequence
analysis showed a CpG island spanning the promoter, first exon and intron of the gene. Further, treatment of the ANX2 negative PC cell line LNCaP with a demethylation agent reactivated ANX2 expression, suggesting that methylation is associated with ANX2 silencing in PC (214).

Restoration of ANX2 inhibited the migration of the transfected LNCaP cells (216). The effect on cell proliferation is uncertain. No change in cell proliferation was reported after restoration of ANX2 in the same cell line (216). Since immunohistochemical staining for ANX2 is a consistent and reliable marker of prostatic neoplasia, a potential utility as a diagnostic aid in PC histopathology has been suggested (217). The silencing of ANX1 (218) and ANX7 (219) in PC have also been reported, although the loss of ANX7 appears to occur in later stages of the disease (214).

1.5.7 PLA₂ expression in prostate cancer tissues

Studies show that secreted PLA₂ (sPLA₂)-IIA is detectable in the prostate and in seminal plasma (220). One study showed an increase in mRNA level in eight PC specimens (221). Finally, an increase in sPLA₂-IIA protein levels in PC tissues and the enhanced sPLA₂-IIA expression have been shown to be inversely related to 5-year patient survival (222; 223).

The significance of cPLA₂α in PC comes into light as it is found to be downstream of sPLA₂-IIA signalling. Previous studies demonstrated that sPLA₂ signals through cPLA₂α in inflammation in PC cells (213). The increase in sPLA₂ activity led to increased cPLA₂α phosphorylation. In addition, cPLA₂α phosphorylation, thereby activation, is higher in PC
than benign prostates. Further, this activation is yet higher after patients had undergone androgen ablation therapy (204). This gives evidence that cPLA$_2\alpha$ expression and activation is strongly correlated to PC progression and possibly its proliferation. Thus, reduction to cPLA$_2\alpha$ expression or retardation to its activity remains a possible option for treatment of advanced PC.

1.5.8 Eicosanoid-producing enzyme 5-LOX

1.5.8.1 LOX structure, function and metabolites

LOX proteins are about 75-80 kDa (224) with a small N-terminal domain and a large catalytic domain. LOX are activated when a nonheme iron of the C-terminal domain is oxidised to the active ferric iron (225). Within the LOX family, 5-LOX is unique because it owns two distinct enzymatic activities. 5-LOX can catalyse oxygenation of AA to either HpETE, which later could be reduced to 5-HETE by peroxidise, or to leukotriene A$_4$ (LTA$_4$). LTA$_4$ is an intermediate product, which can undergo enzymatic hydrolysis or be conjugated to glutathione to produce LTB$_4$ and LTC$_4$ respectively (226; 227). Another unique aspect of 5-LOX is that it is dependent on Ca$^{2+}$ and ATP, and requires interaction with a novel 18 kDa membrane protein known as 5-LOX activating protein (FLAP) to initiate the lipoxygenation (228; 229). The presence of both 5-LOX and FLAP are necessary for leukotriene biosynthesis in cells (228).

1.5.8.2 Mechanism of LOX products’ action in cancer progression

The products of LOX metabolism – including leukotriene, HETE and lipoxin – act as signalling molecules. ROS is also generated during eicosanoid synthesis. Leukotriene and
lipoxin act through GPCR on the cell surface (230) in a paracrine fashion, while HETE may act at nuclear receptors, such as peroxisome proliferator activated receptors (231). Increased ROS may damage cellular protein and DNA content, leading to increased mutations. One study has demonstrated that ROS from LOX and COX alone is enough to inhibit tumour suppressor PTEN, leading to increased AKT activation and PC cell proliferation (83).

The products of LOX metabolism may be involved in carcinogenesis by signalling for tumour cell proliferation, cell survival, adhesion, migration, invasion, angiogenesis and modulation of the immune system (232; 233). Inhibition of 5-LOX activity abrogates this process. For example, one study showed that 5-LOX activity is required for EGF-stimulated PC3 cell growth and general LOX inhibitors (NDGA and ETYA), 5-LOX inhibitors (AA861 and Rev 5901) and FLAP inhibitor (MK886) attenuated phosphorylation of EGFR, ERK and AKT in these cells (234). This effect was not induced when cells were treated with 12-LOX inhibitor (baicalin) or COX inhibitor (indomethacin).

Interestingly, PSA levels in AIPC can be reduced by inhibition of the eicosanoid pathway. In two clinical trials, the rate of increase of PSA following prostatectomy or radiotherapy was slowed in patients treated with inhibitors of eicosanoid-producing enzymes (235; 236). Members of the eicosanoid family can promote tumour cell proliferation in vitro and in vivo (201) but the long-term use of COX-2 inhibitors can lead to cardiovascular side effects (237).
1.6 Hypothesis and aim of this thesis

Prostate cancer is often characterised by loss of PTEN and constitutive activation of AKT. The existence of a negative feedback loop on AKT, horizontal compensation of ERK and an increase in AR during progression to androgen independence are all factors affecting treatment options for prostate cancer.

cPLA$_2\alpha$ has been implicated to be important for the proliferation of prostate cancer cells. Its product, AA and downstream metabolites are stimulators of cancer cell proliferation. Inhibitors of LOX and COX, the enzymes that convert AA into eicosanoids, were used or considered as anti-cancer treatment but these have been associated with adverse health risks. Inhibition of cPLA$_2\alpha$, the major enzyme releasing AA, decreases substrate supply to the LOX and COX pathways. Thus, cPLA$_2\alpha$ has been proposed to be a better target in treating prostate cancer. Inhibition of cPLA$_2\alpha$ reduces PTEN-null prostate cancer cell proliferation but the underlying mechanisms have not been well examined.

This thesis tested the hypothesis that cPLA$_2\alpha$ sustains the activation and/or expression of AKT, ERK and AR in PTEN-null prostate cancer cells. Thus, genetic silencing or pharmacological blocking of cPLA$_2\alpha$ may decrease the key oncogenic signalling leading to reduction in proliferation of PTEN-null prostate cancer cells.
The aims of this PhD thesis were:

1. To develop an inducible cPLA$_2$$\alpha$ expression system in PTEN-null prostate cancer cells and study the effect of cPLA$_2$$\alpha$ action on AKT, ERK and AR activation and expression;

2. To evaluate the effect of cPLA$_2$$\alpha$ silencing or inhibition, by either genetic silencing or pharmacological agents, on AKT, ERK and AR activation and expression;

3. To determine any possible links downstream of cPLA$_2$$\alpha$ that may mediate cPLA$_2$$\alpha$ action on oncogenic pathways.
CHAPTER 2

MATERIAL AND METHODS
2.1 Materials, catalog numbers and manufacturers

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cPLA$_2$α sequence       RC220972, Clonetech, Mountain View, CA, USA
DAB                       K346711-2, Dako
DNA isolation kit         E5038, Sigma-Aldrich
Doxycycline               D9891, Sigma-Aldrich
DMEM powder               12100061, Invitrogen
CellTiter 96 non-radioactive cell proliferation assay G4000, Promega
Cell lines
   LNCaP                    CRL-1740
   PC-3                     CRL-1435
                        ATCC, Manassas, VA, USA
EGF                       E9644, Sigma-Aldrich
EGFP-C1 vector            632470, Clonetech, Mountain View, CA, USA
FACSCalibur               BD, North Ryde, NSW, Australia
FBS                       FBS500, AusGeneX, Arundel, QLD, Australia
3x FLAG peptide           F4799, Sigma-Aldrich
FlowJo 8.0                 Tree Star Inc. Ashland, OR, USA
FLUOstar Omega            BMG Labtech, Mornington, VIC, Australia
G418                      10131035, Invitrogen
GBox imaging system       SynGene, Cambridge, England
GeneTools software        SynGene
HETEs                      
   5-HETE                  34230
   8-HETE                  34360
   11-HETE                 34510
   12-HETE                 34570
   15-HETE                 34720
                        Cayman Chemicals, Ann Arbor, MI, USA
Igepal CA-630              I7771, Sigma-Aldrich
Immulite 2000 third generation PSA kit        Siemens DPC, Bayswater, VIC, Australia
Lipofectamine 2000         11668, Invitrogen
MK886                      M2692, Sigma-Aldrich
NCSS (v12.0)              Statistical Solutions, Saugus, MA, USA
NE-PER™ nuclear and cytoplasmic extraction reagents
                        78833, Thermo Scientific, Scoresby, VIC, Australia
Nitrocellulose membrane   RPN303E, Amersham Bioscience, Rydalmere, NSW
OPTI-MEM                   11058, Invitrogen
pcDNA4 Vector             V103020, Invitrogen
pcDNA6 Vector             V1025-20, Invitrogen
pCMV6 Vector              PCMv6NEO, Clonetech
Phospholipase A2 Assay Kit E10217, Invitrogen
PI                         P4170, Sigma-Aldrich
2.2 Cell culture

2.2.1 Prostate cancer cell lines

Two established cell lines used to model PTEN-null prostate cancer PC-3 (bone metastasised, androgen receptor negative (CRL-1435) and PTEN-deletion (75)) and LNCaP (lymph node metastasised, androgen receptor positive (CRL-1740) and PTEN-frame shift mutation (74)) were purchased from ATCC. Both cell lines were maintained in Roswell park memorial institute (RPMI) 1640 Medium supplemented with 3 g/L sodium bicarbonate, 20 mM L-glutamine, penicillin at 100 units/mL, streptomycin at 100 µg/mL and 10% (v/v) FBS at 37 °C in 5% CO₂/95% air with cells passaged prior to reaching confluence. The PC-3 and LNCaP cell lines used in cell culture studies described in this thesis were between passage 30 and 40.
2.2.2 Generation of cell lines with Dox-controlled cPLA₂α expression

LNCaP cells were seeded in 10 cm dishes and grown to 100% confluence. Cells were then transfected with 24 µg pcDNA6 vector expressing TET-Repressor using lipofectamine 2000 and then treated with media containing blasticidin (10 µg/mL) for 10 d. Successfully transfected colonies were picked and passaged before transfecting with pcDNA4 vector containing human cPLA₂α cDNA sequence joined with FLAG and regulated by a promoter containing TRE sequence. Successfully transfected clones were selected with media containing G418 (300 µg/mL) for 10 d. Cells co-transfected with pCDNA6 and pcDNA4 vectors without cPLA₂α coding sequence were used as a vector control.

2.2.3 Induction of cPLA₂α using Dox

For induction of cPLA₂α, LNCaP TRE-cPLA₂α or TRE-Vector (control) cells were seeded in 6 or 96-well plates and grown until 70% confluence. The cells were then treated with or without 100 ng/mL Dox in RPMI with 10% v/v FBS for 24 h before harvest.

2.2.4 Cryopreservation of cell lines

PC-3 or LNCaP cells were prepared for cryopreservation by gently trypsinising sub-confluent cells with TrypLE. After centrifugation, the cell pellet was first resuspended in 20% (v/v) FCS RPMI and then added to an equal volume of 10% (v/v) DMSO in cell culture medium. Liquid nitrogen cryotanks were used for long-term storage of cells.

2.2.5 Genetic silencing of cPLA₂α with siRNA

PC-3 and LNCaP cells were seeded in 6-well plates. The cells were then transfected with 10 nM Si-cPLA₂α or GC-content matched si-control mixed with lipofectamine 2000 in a 1:2 v/v ratio. OPTI-MEM was used as the transfection medium. The media was replenished next day
and cells were left to propagate for 48 or 72 h before harvesting for immunoblot. All experiments were performed using cells with >70% reduction in cPLA$_2$α protein level.

### 2.2.6 EGF, Efipladib and AA treatment

For incubation with AA, PC-3 or LNCaP cells seeded in 6-well plates and grown to 70% confluency were exposed to RPMI containing 1% FBS overnight. AA was first pre-conjugated with 5 µM fatty-acid free BSA for 3 h. Cells were then treated with 20 µM AA at 37 °C for 4 h, with or without 25 µM MK886. For cells treated with Si-cPLA$_2$α or si-control, AA was introduced 4 h prior to harvesting. Commercial AA was routinely assessed by HPLC and confirmed to contain <1% oxidation products as described previously (238). For incubation with EGF, cells were treated with 20 ng/mL EGF for the last 30 min prior to harvesting. For Efipladib, PC-3 or LNCaP cells, seeded in 6-well plates for at least 24 h beforehand, were treated with 20 µM Efipladib in RPMI 10% FBS for 72 h. Cells were then lysed or harvested for subsequent analysis.

### 2.3 Molecular biology

#### 2.3.1 Cloning and sequencing

Restriction enzyme digestions, ligations and other routine DNA procedures were done according to published methods (239; 240). DNA sequencing was carried out at the Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC), Department of Molecular and Clinical Genetics, Royal Prince Alfred Hospital, NSW Australia.

#### 2.3.1.1 DNA plasmid isolation

JM109 *E. coli* cells containing plasmid vectors were inoculated into 5 mL Luria Bertani broth with 100 µg/mL ampicilin or kanamycin and grown overnight in a 37 °C incubator shaker.
The next day, cells were spun down and lysed. DNA plasmids were extracted from cell lysates using a DNA isolation kit following the manufacturers’ instructions.

### 2.3.1.2 DNA electrophoresis and gel purification

DNA fragments were separated in agarose gels (1%) with 1x tris-acetate-EDTA buffer (239) in horizontal submarine tanks. Electrophoresis was routinely performed at 100 V. DNA was visualised with ethidium bromide (0.5 mg/L) staining and a UV lamp according to routine protocols (239; 240). To purify DNA fragments, samples were first run on agarose gels. Gel pieces containing DNA were cut out under a UV lamp and put through purification using UltraClean Gelspin extraction kits according to the manufacturer’s instruction.

### 2.3.1.3 Bacterial transformation

Competent JM109 cells were incubated with DNA plasmid (1 µg) for 30 min on ice. The transformation mix was heated at 37 °C for 60 s and then incubated for 2 min on ice. The transformation mix was inoculated on a 10 cm agar plate with ampicilin or kanamycin (100 µg/mL) for overnight selection. Successful colonies were picked for DNA plasmid isolation and/or sequencing.

### 2.3.1.4 Primers

Primers were synthesised by Geneworks (Hindmarsh, SA, Australia). Primers used in this study are shown in Table 2.1.
Table 2.1 Primers sets used to measure human cPLA₂α, TBP and AR gene expression by real-time PCR and sequencing of cPLA₂α in pcDNA4 vector.

<table>
<thead>
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<th>Sequences</th>
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<td>cPLA₂α Reverse</td>
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<tr>
<td>pcDNA4 Reverse</td>
<td>5’-TAGAAGGCACAGTCGAGG</td>
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</table>

2.3.2 Reverse transcription and quantitative real-time PCR

Total RNA was isolated using an UltraClean Tissue & Cells RNA Isolation Kit according to the manufacturer’s instructions. The first strand of complementary DNA was synthesised from 500 ng of total RNA with random primers and SuperscriptIII. Quantitative PCR measurements were done with SensiMix SYBR Mastermix Kit and RotorGene 6000. Conditions for PCR were: one cycle of 10 min at 95 °C; 40 cycles of 10 s at 95 °C, 30 s at 65 °C. REST 2009 was used to calculate relative changes in cPLA₂α normalised by a TBP housekeeping gene. Amplification efficiency was determined if 3–5 points of a dilution curve were within 100 ± 3% for AR, cPLA₂α and TBP.

2.3.3 Immunoblotting

PC-3 and LNCaP cells were seeded in 6-well plates. Following treatment, cell lysates were prepared with lysis buffer (50 mM Tris-pH 8, 150 mM NaCl, 1% v/v Igepal CA-630, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS) supplemented with 1:50 v/v dilution of protease inhibitor cocktail and 50 mM sodium fluoride (as phosphatase inhibitor (241; 242)). Protein concentration was quantified using Bio-Rad DC protein assay. Proteins were separated on SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were blocked with 1% w/v non-fat milk in PBST for 30 min and incubated with primary antibody
for 1–2 d at 4 °C. The blots were then washed in PBST, and incubated with a secondary antibody conjugated with peroxidase and Precision Strep Tactin-Peroxidase Conjugate for 3 h. Immunoreactive proteins were detected by SuperSignal West Pico Chemiluminescent Substrate and the signals captured by G:Box Imaging gel doc systems. The band intensity of digital images was measured using GeneTools software and the ratio of band intensity between each protein of interest and loading control was determined before expressing changes in protein levels as a fold-change relative to control.

2.3.4 Immunocytochemistry

Expression of Ki-67 and BrdU incorporation in cells was determined by immunocytochemical staining. For determining BrdU incorporation, the experimental cells in culture medium were added to 10 μM BrdU for 3 h prior to harvest. The treated cells in T75 flasks were harvested with TrypLE, fixed in 10% buffered formalin solution at 4 °C, clotted in 1% w/v agarose and further processed into paraffin blocks. Sections (5 μm thickness) were cut and baked at 60 °C for 1 h, deparaffinised in xyline, re-hydrated in graded ethanol and distilled water, and subjected to antigen retrieval in Tris-EDTA solution (243). The sections were then incubated with 2 N HCl for 20 min. After being washed in TBST (50 mM Tris HCl, 150 mM NaCl, 0.05% v/v Tween 20, pH 7.4), the sections were blocked with 10% v/v horse serum and then incubated with either anti-Ki-67 or anti-BrdU antibody for 20 h at 4 °C. After washing in TBST, the section was sequentially labelled with a biotinylated secondary antibody and Vectastain ABC kit. To detect the immunolabelling, the sections were incubated with DAB. Thereafter, the sections were counterstained with hematoxylin and coverslipped.
2.3.5 Quantification of immunocytochemically stained cells

Digital images of the immunostained sections were obtained by using ACIS III automated cellular imaging system. Thereafter, two colour thresholds were chosen to distinguish between the positive (brown) and negative (blue) cells. Ten areas on each sample were randomly selected to determine the numbers of positive and negative cells. The percentage of positive cells of each sample was calculated using the formula: positive cells/(positive cells + negative cells) x 100%.

2.3.6 Nuclear and cytoplasmic fractionation

After treatment with Efipladib as in Section 2.2.6, cells were washed twice with cold PBS, scraped off the plate and collected into 1.5 mL microtubes. PBS was removed after centrifugation at 500 g for 3 min. Nuclear and cytoplasmic fractionation were performed using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents according to manufacturer's instructions. All procedures including centrifugation were performed at 4 °C. The isolated fractions were then used for immunoblot.

2.3.7 Fatty acid extraction from conditioned cell culture media and cell pellets

Fatty acids were extracted from cell pellet and culture medium as described in detail by Norris and Dennis (244). Cells were trypsinised, spun down and transferred to glass test tubes. The cell pellets were added with 800 µL 1 N HCl/MeOH (in 1:1 ratio) and 400 µL chloroform before being vortexed for 1 min and then centrifuged at 1000 x g for 5 min. The lower phase was transferred to a new tube and the solution was evaporated using nitrogen. 250 µL MeOH with 0.005% w/v butylated hydroxytoluene and 250 µL KOH (15% w/v) were added to each sample and vortexed for reconstitution. The solution was then incubated at 37
°C for 30 min. 1 N HCl was added to each sample until pH < 3. Heptadecanoid acid (100 µg/mL, 20 µL) was added before extraction by hexane as below.

Briefly, media samples (2 x 250 µL from each sample) were diluted into a mixture of MeOH (400 µL), heptadecanoic acid (100 µg/mL, 10 µL) and HCl (1 M, 25 µL). Treated samples of pellet and media were then mixed with 2 mL hexane, vortexed for 30 s and centrifuged at 1000 x g for 5 min. The upper (hexane) layer was removed from the aliquots and combined into a 25 mL pear-shaped flask with a ground glass joint. The hexane extraction was repeated and the combined fractions were then evaporated under vacuum, reconstituted with 200 µL isopropanol and stored at –80 °C before analysis using LC-MS.

2.4 Assays

2.4.1 Bio-Rad DC protein assay

Reagent A (25 µL) was mixed with 5 µL protein sample (diluted) in 96-well plates. 200 µL of Reagent B was then added before 10 min incubation at room temperature in the dark. Standards of 0, 0.25, 0.5, 1, 2, 3, and 4 mg/mL were made from BSA. Absorbance was read at 690 nm to create a standard curve using absorbance of standard (y-axis) and concentration (x-axis). Sample protein concentration was then worked out based on the equation of trendline and their dilution factors.

2.4.2 SYBR green assay for measurement of DNA synthesis

Prostate cancer cells were seeded to 96-well plates at 2 x 10³ cells/ well for PC-3 cells and at 5 x 10³ cells/ well for LNCaP cells. After at least 24 h seeding, Efipladib was then added at 0, 5, 10, 15, 20 and 25 µM in fresh medium and incubated at 37 °C for 72 h. The same number of cells was aliquoted as at baseline and stored at –80 °C until use. 3 d after treatment, the
medium was gently removed from each well and 100 µL of lysis buffer containing SYBR Green I DNA gel stain at 1:10,000 v/v dilution was added. The lysis buffer consisted of buffer A (10 mM Tris-pH 7.5 and 2 M NaCl) and buffer B (100 mM Tris-pH 7.5, 200 mM EDTA and 10% v/v Triton X-100) in a 9:1 ratio. The cells were lysed in the dark for 30 min. Previously frozen aliquots were thawed at room temperature, lysed in the same buffer and transferred to the treatment plate containing samples from the same type of cells. The fluorescence intensity of SYBR Green was measured using a FLUOstar Omega plate reader and the gain in the cellular DNA content during the treatment was estimated by subtracting baseline fluorescence intensity from the value obtained from experimental cells.

2.4.3 MTT assay for cell proliferation based on mitochondrial function

Prostate cancer cells were seeded to 96-well plates in a similar fashion to the SYBR Green assay for at least 24 h before treatment. At end of treatment, culture media was aspirated and cells were added with RPMI (10% FBS) and dye solution from a CellTiter 96 Non-Radioactive Cell Proliferation Assay in a 9:1 ratio. The cells were then incubated at 37 °C for 30 min before being taken for absorbance reading at 414 nm.

2.4.4 Determination of HETEs

PC-3 and LNCaP cells were harvested, and the cell pellets incubated with 0.1 mM purified AA at 37 °C for 1 h. Total lipids were extracted into chloroform/ methanol/ water (5:1:1 v/v/v) and centrifuged (3600 x g, 15 min). The chloroform phase was dried and the resultant lipids reconstituted with methanol/water (9:1 v/v), were used for high-performance liquid chromatography (HPLC) separations as described previously (238). The identity of HETEs in the HPLC profile was confirmed by spiking with the authentic HETEs obtained commercially.
HPLC separation of HETE was performed by PhD student, Dr Marzieh Niknami, in the same Laboratory in Bosch Institute, The University of Sydney, NSW, Australia.

2.4.5 PSA assay
LNCAp cells in 6-well plates were treated with 20 µM Efipladib for 72 h. Following treatment, 200 µL media were collected, centrifuged (300 x g, 5 min), and measured with Immulite 2000 Third Generation PSA kit at the Royal Prince Alfred Hospital. The intra- and inter-assay coefficients of variation were 5% and 7% respectively for the dose over the range of standard curves. Cells in each well were harvested for protein assay and the PSA value was normalised by protein concentration.

2.4.6 Transfection efficiency testing by Block-IT Stealth oligo
PC-3 and LNCAp cells were grown in T75 flasks until semi-confluent. Then they were trypsinised and seeded in 6-well plates at 500 k cells/well and transfected with or without 10 nM Block-IT Stealth oligo mixed with lipofectamine 2000 in a 1:2 v/v ratio. OPTI-MEM was used as the transfection medium. The cells were incubated at 37 °C for 24 h before being trypsinised, spun down and resuspended in PBS with 5% (v/v) FBS. The cells were taken for flow cytometry analysis using FACSCalibur machine. Positively transfected cells showing intracellular fluorescence at 485 nm (245) were gated and counted by Flow-Jo 8.0.

2.4.7 Transfection efficiency testing by EGFP-C1 vector
PC-3 or LNCAp cells were seeded in 6-well plates at 100 k cells/well. Following 24 h, the cells in each well were transfected with or without 4 µg EGFP-C1 Vector mixed with lipofectamine 2000 in a 1:1, 1:2 or 1:4 ratio. OPTI-MEM was used as the transfection medium. The cells were then incubated at 37 °C for up to 72 h. After 24, 48 and 72 h, light
contrast and fluorescence images of cells expressing EGFP were taken under an UV microscope. The percentage of cells expressing EGFP was measured using flow-cytometric analysis or manual counting.

2.4.8 Flow cytometry analysis of the cell cycle using PI
PC-3 or LNCaP cells seeded in 6-well plates were treated with Si-cPLA2α or Efipladib and control according to above procedures. At the end of treatment, the cells were trypsinised, spun down and re-suspended in 300 µL PBS. The cells were then fixed by adding 800 µL ethanol drop-wise to each sample under a constant vortex and then stored at 4 °C for at least overnight. Following fixation, cells were washed in PBS and incubated in PBS containing 20 µg/mL PI and 100 µg/mL RNase for 1 h. The cells were then washed and resuspended in PBS with 5% (v/v) FBS before being measured by flow cytometry analysis. Positive nuclei stained with PI were gated and cells in each cell cycle phase measured using Flow-Jo 8.0.

2.4.9 Trypan blue exclusion assay
At end of treatment, PC-3 or LNCaP cells in 6-well plates were trypsinised, spun down and resuspended in PBS. The cell suspension was then mixed with trypan blue in a 1:1 ratio and loaded onto a Haemacytometer. For each sample, five 0.1 x 0.1 mm squares were counted. Trypan blue positive cells were subtracted from total number of cells to get cell viability.

2.4.10 cPLA2α immunoprecipitation and activity assay
LNCaP cells transfected with TRE-cPLA2α or TRE-empty vector control were induced with Dox as described in Section 2.2.3 and then lysed with lysis buffer (50 mM Tris-pH 7.4, 150 mM NaCl, 1% v/v Triton X-100, 1 mM EDTA, 0.1% v/v NP-40). The cell lysates were centrifuged at 8200 g/10 min/4 °C and the protein concentration in the supernatant was
quantified as in Section 2.4.1. Each sample (final protein concentration 250 μg/mL) was mixed with 40 μL Anti-FLAG M2 Affinity Gel in a microtube and followed by 1 mL of lysis buffer. The mixtures were agitated for 2 h/4 °C and then centrifuged for 30 s/8200 g before removing the supernatant. The bead pellets were washed three times with 500 μL TBS for 5 min/4 °C. Bound antigens were eluted with 3× FLAG peptide and assayed using the Phospholipase A2 Assay Kit according to the manufacturer's specifications.

2.4.11 Liquid chromatography–mass spectrometry (LC–MS)/MS analysis

Fatty acid from the cell pellet and culture medium were extracted as in Section 2.3.7 and resuspended in 200 μL isopropanol. Samples were stored at –80 °C for less than one week before LC-MS/MS analysis as described in Hua et al. (134). MS was performed by Leila Hejazi in the Mass Spectrometry Facility, University of Western Sydney, Sydney, Australia.

2.5 Statistical analysis

Statistical software NCSS (v12.0) was used for analysis. One-way analysis of variance (ANOVA) was used to determine differences between groups of data. Fisher’s Least Significant Difference Multiple-Comparison Test was used to determine the difference between individual groups (P value < 0.05 was considered significant). Data that failed the test for normal distribution or homogeneous variance were analysed with the Kruskal-Wallis test of ranked variable and post-hoc Kruskal-Wallis Multiple-Comparison Z-Value Test (a z-value of > 1.96 was considered significant).
CHAPTER 3

EFFECT OF OVEREXPRESSION OF cPLA$_2\alpha$ ON PTEN-NULL PROSTATE CANCER CELLS
3.1 Introduction

The mechanisms by which eicosanoids promote cancer progression include activation of the PI3K/AKT and Ras/Raf/MEK/ERK pathways (246). AA metabolites such as PGE2, PGF2α, 5-HETE and 12-HETE have all been implicated to activate pAKT and/or pERK in various cancer types (247-250). Prostanoid receptors EP1, EP4 and FP have been shown to convey the eicosanoid signals to AKT and ERK pathways via transactivation of EGFR (249; 251). Naturally, the inhibition of eicosanoid synthesis has shown effects of cancer inhibition (237). Blockade of eicosanoid production by drugs, commonly termed non-steroidal anti-inflammatory drugs (NSAIDs), decreases the levels of pAKT (234), phospho-PI3K (on p85 subunit) (252) and pERK (251).

LOX and COX, the enzymes that metabolise AA to produce eicosanoids, have been identified as potential targets for both chemoprevention and treatment of several cancers (85; 253). However, the long-term use of some inhibitors such as COX-2 inhibitors can lead to cardiovascular side effects (237) and gastrointestinal bleeding (254). Further, a single, specific inhibitor alone cannot inhibit the action of all LOX and LOX enzymes. The current hypothesis proposed by others and us is that inhibition of cPLA2α could be a more effective therapeutic approach than inhibition of COX or LOX alone against prostate cancer (201; 237).

cPLA2α is the rate-limiting enzyme that releases AA from membrane phospholipids for eicosanoid production (255). Although concentrations of free AA are maintained at low levels in resting cells, alterations in AA production, often resulting from dysregulation of cPLA2α activity, are observed in transformed cells. Studies have shown alterations in the levels of cPLA2α expression or functional activity in several types of cancers (256). However, in previous experiments conducted in our laboratory, we were first to demonstrate expression
of total and phospho-cPLA\(_2\alpha\) (pcPLA\(_2\alpha\)) in prostate cancer cell lines and an aberrant increase in pcPLA\(_2\alpha\) in hormone-refractory prostate cancer (204).

This presence of total and phospho cPLA\(_2\alpha\) coincides with an aberrant increase in AKT signalling in advanced prostate cancer cell lines LNCaP and PC-3. Given the fact that AA metabolites lead to AKT and ERK activation and promote prostate cancer progression (257-260), we examined the hypothesis that aberrantly-increased cPLA\(_2\alpha\) is required for sustaining AKT and ERK signalling in PTEN-null/mutated prostate cancer cells. The aim of the work described in this chapter was to establish a model of inducible expression of cPLA\(_2\alpha\) in PTEN-null LNCaP cells and investigate the role of cPLA\(_2\alpha\) in oncogenic signalling pathways in prostate cancer cells.

3.2 Materials and methods

3.2.1 TetOn system used in overexpression of cPLA\(_2\alpha\)

Within tetracycline inducible system, tetracycline may induce or suppress the transcription of downstream gene depending on the types of tetracycline binding protein used (261). The TetR gene from pcDNA6 vector was originally isolated from the Tn10 transposon which confers resistance to tetracycline in \(E.\ coli\) and other enteric bacteria (262). It encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa (262). Under unstimulated conditions, TetR protein binds to the 2x tetracycline operator sequence (5'-TCCCTATCAGTGATAGAGA) (261) on the CMV promoter of \(\text{pTetOn:cPLA}_2\alpha\) vector. This is able to block the transcription of downstream cPLA\(_2\alpha\) gene. Addition of tetracycline releases this inhibition as tetracycline molecules bind to TetR, induce conformational change, and allow it to dissociates from the operator sequence. The transcription of downstream cPLA\(_2\alpha\) is highly upregulated under CMV promoter.
Figure 3.1 TetOn system. The TetOn system used in this study involves two vectors working together in presence or absence of tetracycline. TetR (repressor) is transcribed from pcDNA6 vector and will suppresses transcription on pTetOn:cPLA\(_2\)\(\alpha\) vector. Presence of tetracycline binding to TetR releases this inhibition.

3.2.2 Vectors and DNA sequence

For establishment of tetracycline-inducible expression of cPLA\(_2\)\(\alpha\), three vectors were used in the cloning process. Ampicillin and kanamycin were used for selection during DNA isolation. Restriction enzyme digestion and SUPAMAC sequencing were employed to verify the cloned product. Transfection efficiency tests were done using an EGFP-C1 vector and assessed by flow cytometry and cell counting. G418 and blasticidin were used for selection of stably transfected clones. Successfully transfected clones (transient or stable) were induced with or without doxycycline (Dox) and verified by immunoblot. Effect of cPLA\(_2\)\(\alpha\) overexpression on oncogenic pathways was measured by immunoblot. Effect of cPLA\(_2\)\(\alpha\) overexpression on proliferation was measured by cell counting and MTS assay. I assessed the activity of induced cPLA\(_2\)\(\alpha\) using PLA\(_2\) activity assay and LC-MS.
Figure 3.2 pCMV6-cPLA\(_2\alpha\) vector. The vector contained human PLA\(_2\)G4A (cPLA\(_2\alpha\)) cDNA sequence followed by Myc and DYKDDDDK (FLAG) epitope. Gene expression is driven by a CMV promoter and vector is selected by a neomycin resistance gene for bacterial and mammalian culture selection.
Figure 3.3 pcDNA6 vector. The pcDNA6 vector contained Tet-repressor sequence driven by a CMV promoter for constitutive expression. The vector contained ampicillin and blasticidin resistance genes for bacterial and mammalian culture selection respectively.

Figure 3.4 pcDNA4 Vector. The pcDNA4 vector contained a CMV promoter harbouring a 2x tetracycline operator sequence. The vector also contained ampicillin and zeocin resistance genes for bacterial and mammalian culture selection respectively.
3.2.3 Restriction enzyme digestion

For DNA digestion, 2 µg of vector was added with 40 U of one or two restriction enzymes to 10% v/v BSA and 10% v/v restriction enzyme buffer. Digestion was performed at 37 °C. The result of the DNA digest was run on agarose gel (see 2.3.1.2) and visualised under UV.

3.2.4 DNA ligation

For DNA ligation, 100 ng of vector was added with 100 ng of insert to 1 U T4 DNA ligase and 10% v/v T4 DNA ligase buffer. Ligation was performed at 15 °C for 16 h. The ligation mix was then used for transformation (see 2.3.1.3) and plated on agar plates containing the corresponding antibiotic. Successful colonies were picked for DNA plasmid isolation (see 2.3.1.1) and subsequent reconfirmation by restriction digestion. The successful product was then sent for sequencing (see 2.3.1).

3.3 Results

3.3.1 Creation of a pTetON:cPLA₂α vector

To achieve a pTetON:cPLA₂α vector, pCMV6 cPLA₂α (Figure 3.2) was used as a template for the majority of the plasmid backbone, as the cPLA₂α coding sequence was on pCMV6 (Figure 3.3) from the manufacturer. Additionally antibodies against FALG and myc epitope (attached to the cPLA₂α sequence) and the antibiotics for selecting the Neo/KanR gene are all readily available.

pCMV6 cPLA₂α (Figure 3.2) and pcDNA4 (Figure 3.4) vectors both have a single NdeI and Acc65I restriction site in their sequence. Cleavage by these two enzymes gave 406 bp and 500 bp fragments respectively (Figure 3.5).
Figure 3.5 *NdeI* and *Acc65I* digestion of pCMV6 cPLA$_2$α and pcDNA4 vectors. Lane 1, double digest of pCMV6 vector yielding 406 bp fragment; Lane 2, DNA 1 kb ladder; Lane 3, double digest of pcDNA4 vector yielding 500 bp fragment; Lane 4, undigested pcDNA4 vector.

The major portion of pCMV6 (Figure 3.5 lane 1 top band) and the 500 bp product from pcDNA4 (Figure 3.5 lane 3 bottom band), which contains the 2x tetracycline operator sequence, were both isolated through purification and ligated together to create pTetOn: cPLA$_2$α (Figure 3.6). The ligation mix was subsequently transformed into JM109 for plasmid isolation. Successfully isolated vectors (from miniprep) were digested to confirm fragment size (Figure 3.7). The plasmid was then sent for sequencing to check the fidelity of plasmid amplification on the cPLA$_2$α coding sequence.
Figure 3.6 pTetOn:cPLA₂α vector. The pTetON: cPLA₂α vector contained cPLA₂α coding sequence and largely pCMV6 plasmid elements. A 500 bp fragment was taken from pcDNA4 vector to replace the coding sequence between NdeI and Acc65I sites on pCMV6 to produce the final vector.
A **pTetOn:control** vector without cPLA$_2$α cDNA was used as a vector control. This was created by restriction enzyme digest of *XhoI* and *SalI* sites, which flanked the cPLA$_2$α sequence, and rejoining the compatible loose ends. The ligation mix was transformed and plated to form colonies for DNA isolation. Successfully isolated clones were tested by *NdeI*, digested and compared with a *XhoI* and *SalI* double digest of **pTetOn:cPLA$_2$α** vector (**Figure 3.8**). Vector without cPLA$_2$α sequence (sample 1 and 3, **Figure 3.8**) was sent for sequencing.
3.3.2 Testing of transfection efficiency by EGFP

To test transfection efficiency, LNCaP cells were seeded into 6-well plates and transfected with EGFC-C1 vector for constitutive expression of enhanced green fluorescence protein. Percentage of successfully transfected cells from different lipofectamine to DNA (µL:µg) ratio and different duration of transfection were examined by flow cytometry and manual counting (Figure 3.9). There appeared to be a positive correlation between transfection efficiency and vector amount or duration. The efficiency peaked at about 45% for 48 h transfections (Figure 3.9C). However, the percentage of cell death also rose, peaking at 1:2. Thus a 1:1 ratio was chosen as the optimal ratio in 48 h.

Figure 3.8 *NdeI* digestion of isolated *pTetOn*:control vector. Lane 1–3, *NdeI* digestion of purified vector from individual colonies; Lane 4, DNA 1 kb ladder; Lane 5, double digest of isolated *pTetOn*:cPLA₂α by *XhoI* and *SalI*.
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Patient ID: LNCaP cells

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Gate: G1
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Total Events: 11550

X Parameter: FL1-H GFP (Log)
Y Parameter: SSC-H Side Scatter (Linear)

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Patient ID: LNCaP cells

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X Parameter: FL1-H GFP (Log)
Y Parameter: SSC-H Side Scatter (Linear)

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Y Parameter: SSC-H Side Scatter (Linear)

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b) Control

1:1

1:0.5

1:2

c) Percentage of positive cells

Control 1 to 0.5 1 to 1 1 to 2

24h 48h

![Percentage of positive cells graph]

63
Figure 3.9 Testing transfection efficiency on LNCaP cells. a) 24 h and b) 48 h transfection of EGFP-C1 vector in LNCaP cells and measured by flow cytometry. c) Histogram of measurements by flow cytometry. d) Phase contrast and e) fluorescent image of LNCaP cells under microscope. f) Measurement of transfection efficiency by manual cell counting. g) Measurement of cell viability after transfection by trypan blue exclusion assay.
3.3.3 Transfection of pTetOn:cPLA2α and pcDNA 6 into LNCaP cells

To produce the tetracycline inducible cell line (LNCaP TRE-cPLA2α), pcDNA6 and pTetOn:cPLA2α vectors were transfected into LNCaP cells (performed by Soma Vignarajan). For the control cell line (LNCaP TRE-Vector), pTetOn vector without the cPLA2α coding sequence was stably transfected into pcDNA6 containing LNCaP cells according to Section 2.2.2. Stably transfected LNCaP TRE-cPLA2α and TRE-Vector clones were then tested by immunoblot for cPLA2α expression under induced conditions. 39 clones of LNCaP TRE-cPLA2α were picked and screened (20 shown in Figure 3.10). Four clones (namely 101, 105, 117 and 119) were picked based on their elevated expression of cPLA2α following Dox induction. Two clones (101 and 105) showed increased pcPLA2α following Dox induction (Figure 3.11). LNCaP TRE-Vector clones, which also contained two stably transfected vectors, were cultured based on their antibiotic resistance and tested for Dox treatment. In TRE-Vector cells, the level of cPLA2α was comparable to parental LNCaP and did not increase following Dox treatment (Figure 3.12).
Figure 3.10 Expression of cPLA\(_2\alpha\) for LNCaP TRE-cPLA\(_2\alpha\) clones following Dox induction. a)--d) clones of stably transfected clones (namely 101, 105, 110, 111, 116–120, 122–124, 126–128, 132, 136–140) were induced by Dox (1 mg/ml) and screened for cPLA\(_2\alpha\) overexpression by immunoblot. Hela and PC-3 cells were used as controls.
Figure 3.11 Overexpression of cPLA$_2$$\alpha$ in LNCaP TRE-cPLA$_2$$\alpha$ clones with or without Dox. a) total and b) pcPLA$_2$$\alpha$ levels were probed by immunoblot after the four clones were induced with or without Dox (1 mg/ml) for 24 h.
Figure 3.12 Expression of cPLA$_2$α for LNCaP TRE-Vector transfected cells following Dox induction. LNCaP TRE-Vector cells were treated with or without Dox (1 mg/ml) for 24 h. The expression of cPLA$_2$α was compared to parental LNCaP cell lines under similar treatments.

3.3.4 Optimisation on time and dose of Dox induction

To find out the optimal dose and time for induction of cPLA$_2$α, LNCaP TRE-cPLA$_2$α was treated with a range of Dox concentrations. The amount of induction on cPLA$_2$α protein was measured by immunoblot. Dox was able to induce high levels of total and phospho cPLA$_2$α at concentrations as low as 0.01 µg/mL (Figure 3.13 C and D). However, the results of Dox induction appeared to be more consistent at concentrations over 0.1 µg/mL. Thus 0.1 µg/mL was chosen as the concentration to use in subsequent experiments.
### a) LNCaP - LNCaPTRE-cPLA₂α (117) - LNCaPTRE-cPLA₂α (105) - LNCaP TRE-Vector

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- **cPLA₂α**
- **α-tubulin**

### b) LNCaPTRE-cPLA₂α (105)

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- **pcPLA₂α**
- **α-tubulin**

### c) LNCaPTRE-cPLA₂α (105) - PC-3

<table>
<thead>
<tr>
<th>Dox (µg/ml)</th>
<th>Ladder</th>
<th>HeLa</th>
<th>LNCaPTRE-cPLA₂α (105)</th>
<th>PC-3</th>
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<tbody>
<tr>
<td>0</td>
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<td>si-cPLA₂α</td>
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<td>0.5</td>
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- **cPLA₂α**
- **GAPDH**
Figure 3.13 Dose response of cPLA₂α protein expression following Dox induction. a)–d) cPLA₂α overexpression clone 105 and 117 were treated with or without Dox 0.01–2 µg/mL. The level of total and phospho cPLA₂α protein induced were analysed by immunoblot. Hela cells treated with or without siRNA targeting cPLA₂α and PC-3 cells were used as controls. The amount of GAPDH in d) for LNCaP TRE-cPLA₂α (105) cells is equivalent to that of c).

To find out the optimal duration of Dox induction, LNCaP TRE-cPLA₂α cells were treated with Dox for up to 48 h (Figure 3.14). cPLA₂α induction began to show following 8 h Dox treatment and appeared to be greatest at 24 and 48 h. As the cPLA₂α level was similar between those two time points, 24 h was chosen as the best time for all subsequent experiments.
3.3.5 Biochemical changes in LNCaP cells following cPLA$_2$$\alpha$ overexpression

3.3.5.1 Measurement of ectopically expressed cPLA$_2$$\alpha$ by FLAG tag

Ectopically induced cPLA$_2$$\alpha$ was probed by targeting its recombinant FLAG or myc epitope. In so doing, comparison between total cPLA$_2$$\alpha$ levels and ectopically induced cPLA$_2$$\alpha$ was made. A clear increase of ectopically expressed cPLA$_2$$\alpha$ following Dox treatment was seen.
by antibody against FLAG (Figure 3.15). Control cells showed no detectable levels of FLAG or any increase in total or phospho cPLA\(_2\alpha\) following induction. The ectopically expressed cPLA\(_2\alpha\) was also shown to be phosphorylated.

![Figure 3.15 Induction of cPLA\(_2\alpha\) in TRE-cPLA\(_2\alpha\) compared to TRE-Vector. LNCaP TRE-cPLA\(_2\alpha\) and TRE-Vector were induced with Dox for 24 h and then analysed by immunoblot.](image)

3.3.5.2 Measurement of cPLA\(_2\alpha\) activity after overexpression and measurement of extracellular AA

To determine Dox-induced cPLA\(_2\alpha\) enzyme activity, LNCaP TRE-cPLA\(_2\alpha\) and Vector were induced with Dox and the FLAG-tagged cPLA\(_2\alpha\) was immunoprecipitated and assayed for activity. Consistent with the increased levels of pcPLA\(_2\alpha\) (Figure 3.15), Dox-induced cPLA\(_2\alpha\) showed a ~2.5–3-fold increase in enzyme activity compared with cells in the absence of Dox (Figure 3.16). TRE-Vector control cells without FLAG-tagged cPLA\(_2\alpha\) showed no detectable cPLA\(_2\alpha\) activity. The amount of free AA released into culture medium increased significantly in the cells treated with Dox compared with controls (Figure 3.16). There was no significant change in released AA after Dox treatment in control cells.
Figure 3.16 Enzyme activity and AA release. LNCaP TRE-cPLA\(_{2}\alpha\) and TRE-Vector cell lysates were tested for cPLA\(_{2}\alpha\) enzyme activity. AA released into culture medium was analysed by LC-MS. The activity assay detection limit was defined as two standard deviations above the mean value of the blank control. Histograms are represented as mean ± SD (n=3) * P < 0.05 versus no Dox treatment by One-Way ANOVA.

3.3.5.3 Effect of ectopic cPLA\(_{2}\alpha\) expression on the PI3K/AKT pathway

The effect of cPLA\(_{2}\alpha\) expression positively upregulated the AKT pathway. Overexpression of cPLA\(_{2}\alpha\) caused a significant (P < 0.05) increase in pAKT (both at Ser\(^{473}\) and Thr\(^{308}\)) and its downstream effectors, pGSK3β and cyclin D1 (Figure 3.17), whereas total AKT and GSK3β were not significantly (P > 0.05) altered. The same dose of Dox in cells transfected with the empty vector control showed no significant increase in pAKT, pGSK3β or cyclin D1 levels (Figure 3.17). Overall, overexpression of cPLA\(_{2}\alpha\) led to increased levels of pAKT and its downstream targets.
Figure 3.17 Effects of cPLA2α overexpression on AKT pathway. LNCaP TRE-clones were treated with or without Dox for 24 h and examined by a) immunoblot on pAKT (Ser\(^{473}\) and Thr\(^{308}\)), pGSK3β and cyclin D1. b) Quantified band intensity represented as the mean ± SD (n = 4 independent experiments, pAKT values are for Ser\(^{473}\). * P < 0.05 versus no Dox treatment One-Way ANOVA.
3.3.5.4 Effect of cPLA$_2$$\alpha$ overexpression on ERK and AR

The effect of cPLA$_2$$\alpha$ expression on ERK and AR was also examined. Unlike the effect on the AKT pathway, cPLA$_2$$\alpha$ induction did not induce a change in pERK, ERK (Figure 3.18) or AR (Figure 3.19) levels.

**Figure 3.18** Effect of cPLA$_2$$\alpha$ overexpression on ERK. LNCaP TRE-cPLA$_2$$\alpha$ clones were treated with or without Dox for 24 h and examined by immunoblot for pERK and ERK levels.

**Figure 3.19** Effect of cPLA$_2$$\alpha$ overexpression on AR. LNCaP TRE-cPLA$_2$$\alpha$ were treated with or without Dox for 24 h and examined by immunoblot for AR levels.
3.3.6. Biological changes in LNCaP cells following cPLA$_2$$\alpha$ overexpression

3.3.6.1 Measurement by cell counting

Although cPLA$_2$$\alpha$ overexpression leads to increased levels of pAKT and its downstream targets, this increase in signal did not ultimately translate to cell proliferation. Although there was significant increase in cell number between Dox induced and non-induced cells for some clones (e.g. 105 at 48 h, $P < 0.05$), no significant difference was detected at 72 h for all clones (Figure 3.20). LNCaP TRE-Vector cells showed no increase but rather a decrease in proliferation after 72 h treatment (Figure 3.21). Therefore, cPLA$_2$$\alpha$ overexpression did not seem to increase LNCaP cell number.
Clone 101

Clone 105

Cell number

Time (h)

Clone number

No Dox

Dox
Figure 3.20 Cell counting on cPLA$_2$α overexpression clones. a-d) LNCaP TRE-cPLA$_2$α clones were treated with or without Dox for up to 72 h. At end of each time point, cells were trypsinised, spun down and counted by haemacytometer. Cell number per well is represented as the mean ± SD (n = 3 independent experiments). * P < 0.05 versus no Dox treatment.
Figure 3.21 MTS assay on LNCaP TRE-Vector cells. LNCaP TRE-Vector cells were treated with or without Dox for 72 h. At end of treatment, cells’ proliferation were measured by MTS assay. Cell proliferation is represented as the mean ± SD relative to no Dox (n = 3 independent experiments). * P < 0.05 versus no Dox treatment.

3.3.6.2 Measurement of cell cycle by flow cytometry

To find out whether ectopic expression of cPLA_2α affected normal cell cycle, LNCaP TRE-cPLA_2α clones were analysed by flow cytometry using PI staining. Similar to the results of cell counting, cPLA_2α-overexpressing cells showed no significant change in percentage of cells in G_1/G_0 phase after either 24 or 48 h (Figure 3.22 A–D). Control cells that did not overexpress cPLA_2α also did not show a change after 48 h, as expected (Figure 3.22 E).
Clone 101 24 h treatment

Clone 101 48 h treatment
c)

Clone 117 24 h treatment

![Graph showing cell cycle distribution for Clone 117 24 h treatment with and without Dox.]

- G1/G0
- S
- G2/M

% of total cells

Dox
No Dox

d)

Clone 117 48 h treatment

![Graph showing cell cycle distribution for Clone 117 48 h treatment with and without Dox.]

- G1/G0
- S
- G2/M

% of total cells

Dox
No Dox
Figure 3.22 Cell cycle distribution in LNCaP cells with or without cPLA$_2$$\alpha$ overexpression. a-d) LNCaP TRE-cPLA$_2$$\alpha$ clones were induced with or without Dox for 24 or 48 h. e) LNCaP TRE-Vector were induced with or without Dock for 48 h. At end of treatment, cells were harvested, fixed in 80% ethanol and stained with PI. Flow cytometric analysis showed cell cycle distributions and LNCaP TRE-Vector cells were used as control. Histograms show mean ± SD (n = 2 independent experiments).

3.4 Discussion

Previous studies have shown the importance of cPLA$_2$$\alpha$ action in several cancers (204; 256; 263). cPLA$_2$$\alpha$ action leads to increased AA and eicosanoid synthesis, both of which have been frequently implicated in giving pro-proliferative signals in cancer (247-250). AA and eicosanoids regulate signalling in the AKT, ERK and AR pathways through multiple ways: (1) eicosanoids may transactivate EGFR through prostanoid receptors to activate PI3K/AKT and ERK (249; 251); (2) inactivation of PTEN through ROS produced from eicosanoid synthesis (83) leads to increased AKT signalling and removal of AR inhibition (101); or (3) AA induces de novo androgen synthesis, which is required for AR signalling, particularly to overcome hormone therapy (260). However, few studies have documented any mechanism or direct correlation between cPLA$_2$$\alpha$ action and prostate cancer.
The importance of cPLA$_2$α came to light when previous findings from our lab showed that cPLA$_2$α is expressed in prostate cancer cells and pcPLA$_2$α, which increases AA generation (264), is increased in hormone refractory cancers (204; 205; 265; 266). In addition, ANX2, the inhibitor of cPLA$_2$α is also absent in late stages of prostate cancer (214). Given the extensive link between AA and the pro-proliferative pathways driving prostate cancer, we propose that cPLA$_2$α itself plays a central role in mediating these signals.

The aim of developing the current cPLA$_2$α overexpression model was to test the importance of cPLA$_2$α in PTEN-null prostate cancer, where signalling from AKT, ERK and AR pathways drives proliferation (20; 97; 267; 268). The mechanism of how cPLA$_2$α influences cancer through these pro-proliferative pathways may also be explored. Further, this model manipulated cPLA$_2$α directly, rather than as many studies did by focusing on AA, eicosanoid-producing enzymes, or regulators of cPLA$_2$α, then implying a cPLA$_2$α effect on AKT or ERK.

If cPLA$_2$α action leads to the activation of proliferative pathways, its overexpression should show an increase of this effect. Using the LNCaP TRE-cPLA$_2$α model, we found that cPLA$_2$α can be induced by Dox at concentrations well below the concentration (> 5 µg/mL) of Dox’s other reported activities, such as inhibition of matrix metalloproteinases (269). This induced cPLA$_2$α can be phosphorylated and is enzymatically active (Figure 3.15 and 3.16). Overexpression of cPLA$_2$α led to an increase of pAKT (Ser$^{473}$ and Thr$^{308}$) and its immediate downstream effector pGSK3β. An increase in cyclin D1 expression was also observed, consistent with an inactivation of GSK3β by AKT phosphorylation (52). In addition, pharmacological blockade of cPLA$_2$α with Efipladib was able to attenuate the pAKT increase induced by cPLA$_2$α overexpression (Chapter 5). However, this increase in signalling in AKT (due to cPLA$_2$α overexpression) was not accompanied by an equal increase in pERK and AR.
(Figure 3.18 and 3.19). In accordance with findings on pERK and AR, cPLA$_2$α overexpression did not stimulate an increase in cell proliferation within 72 h and cell cycle within 48 h (Figure 3.20–3.22).

It is possible that the apparent increase in cPLA$_2$α was limited by the amount of AA present in the phospholipid membrane in vitro. This speculation is in accordance with previous literature (252; 270), which documented significant increases in pAKT and cell proliferation in PTEN-null prostate cancer cells following AA treatment at micromolar concentrations. LNCaP TRE-cPLA$_2$α cells showed a mere 2.5–3 fold increase in AA at nanogram concentrations following overexpression (Figure 3.16) and this level of AA is well below the concentrations inducing cell proliferation from previous reports (270). Further, findings from this study (AA treatment, Chapter 5) show that AA was best at increasing PTEN-null prostate cancer cells at ~2.5 µM and not effective below the micromolar range. This may also be the reason for the lack of increase in pERK and AR.

In summary, work in this chapter describes a model of a cPLA$_2$α-inducible PTEN-null prostate cancer cell line. This model reflects the conditions of prostate cancer in terms of increased AKT signalling and cPLA$_2$α activation. Our studies here suggest while increased cPLA$_2$α expression and phosphorylation do not necessarily lead to increased cell proliferation and ERK and AR activation over the study period, significant metabolic changes could already be detected in PI3K/AKT pathway. Therefore, the link between cPLA$_2$α and PI3K/AKT exists and is quite active.
CHAPTER 4

EFFECT OF GENETIC SILENCING

OF cPLA$_2$α ON PTEN-NULL

PROSTATE CANCER CELLS
4.1 Introduction

An aberrant increase in AKT signalling is a salient feature of advanced PC and can be used to predict disease severity and outcome (11; 30; 32; 271). Activated AKT can be increased by many factors as described in Chapter 1. The aberrant activation of AKT is one of the main sources of proliferative signalling in PC and can even affect other parallel pro-proliferative pathways. For example, loss of PTEN, which increases AKT activation, in PC increases AR nuclear localisation and reduces AR degradation (101).

Few options exist to inhibit activated AKT without causing a vertical compensation, such as a negative feedback loop involving S6 kinase and IRS1 or a horizontal compensation in other oncogenic pathways (97). Under AKT inhibition, ERK activation is increased due to (i) p53-mediated HB-EGF expression (131), (ii) removal of AKT suppression on B-Raf and c-Raf signalling (95) and (iii) Ras activation by the aforementioned negative feedback loop of AKT pathway (93; 94; 98). Therefore, inhibition of AKT signalling per se is not enough to prevent PC cell proliferation.

The inducible cPLA2α expression model described in the last chapter, showed a positive link between cPLA2α and AKT signalling in PTEN-null PC cells, despite no increase in cell proliferation. In this chapter, the role of cPLA2α in sustaining AKT signalling is explored further. The hypothesis is that if increased cPLA2α expression and activity is able to upregulate AKT signalling, an inhibition of cPLA2α should down-regulate AKT signalling. Indeed, much previous literature has documented how inhibition of eicosanoid-producing enzymes, which are downstream of cPLA2α, reduces cell proliferation.
The limited available literature on the role of cPLA$_{2\alpha}$ in cancer points to a link between cPLA$_{2\alpha}$ expression/activity and activation of AKT. In breast tumour lysates, cPLA$_{2\alpha}$ expression is correlated with mTORC1/2 signalling (272). Inhibition of cPLA$_{2\alpha}$ in lung tumour cells by the non-selective inhibitor AACOCF3 prior to irradiation prevented radiation-induced AKT and ERK phosphorylation, suppressed tumour growth and decreased overall tumour blood flow and vascularity (263; 273). Further, decreases in cPLA$_{2\alpha}$ activity have been shown to reduce AKT phosphorylation in PC cells (204).

cPLA$_{2\alpha}$ has also been shown to affect cancer cell proliferation. Inhibition of cPLA$_{2\alpha}$ by pyrrolidine reduced non-small cell lung cancer cell growth and BrdU incorporation (274). Inhibition of cPLA$_{2\alpha}$ also reduced colon cancer cell proliferation via inhibition of the PKA and AKT pathways (275). In in vivo settings, cPLA$_{2\alpha}$ knockout mice are viable and appear phenotypically normal. The females are infertile and both genders have impaired eicosanoid generation from inflammatory cells (200). Importantly, the mice exhibited reduced small bowel (276; 277) and lung tumourigenesis (278). Thus, cPLA$_{2\alpha}$ expression and activity is required for tumourigenesis and may very well be important for proliferation of PC. This chapter use genetic silencing for cPLA$_{2\alpha}$ inhibition to further determine the role of cPLA$_{2\alpha}$ in AKT, ERK and AR signalling.

4.2 Materials and methods

Refer to Chapter 2 for methodologies and an outline on conditions used for cell culture. All experiments in this Chapter were repeated at least three times, unless indicated otherwise.
4.2.1 siRNA transfection

LNCaP, PC-3 or A431 cells were grown in T75 flasks until semi-confluent. Then they were trypsinised and seeded in 6-well plates in different cell numbers depending on duration of treatment (150 k cells for 2 d; 125 k cells for 3 d and 75 k cells for 4 d). Immediately after seeding, they were transfected with 10 nM si-cPLA2α or si-control, which was mixed with lipofectamine 2000 in 1:2 v/v ratio (for 20 min prior to transfection). OPTI-MEM was used as the transfection medium for initial dilution of si-cPLA2α, si-control or lipofectamine 2000. Cells were incubated overnight at 37 °C before the media with 10% FBS was replenished and the cells were left to propagate for another 48–96 h. At end of treatment cells were lysed for immunoblot and RNA extraction. RT-PCR analysis was done to confirm significant reduction of cPLA2α mRNA in si-cPLA2α versus si-control before proceeding to immunoblot.

4.3 Results

4.3.1 cPLA2α silencing by siRNA

4.3.1.1 Measurement of transfection efficiency

To transfect PTEN-null PC cells with siRNA against cPLA2α, we first tested the transfection efficiency of stealth® siRNA using fluorescent oligonucleotides made using the same stealth® technology. Successfully transfected cells fluoresce and were detected by flow cytometry using laser against fluorescein isothiocyanate. Transfection efficiency in LNCaP cells was significantly greater (80%) compared to control (0.17%) (Figure 4.1).
Transfection efficiency of stealth oligonucleotide in LNCaP cells. Typical plot of forward scatter versus side scatter of cells and side scatter versus fluorescence for a) control and b) 10 nM fluorescent oligo-transfected LNCaP cells. c) Histogram of cells versus fluorescent transfected cells. Transfection efficiency represented as mean ± SD (n=3 independent experiments).
4.3.1.2 Changes in mRNA level

To silence cPLA$_2\alpha$, LNCaP cells were treated with 10–40 nM of siRNA and changes to cPLA$_2\alpha$ mRNA were measured by RT-PCR analysis (Figure 4.2 C). The cPLA$_2\alpha$ mRNA level was reduced by over 91% following 10 nM siRNA (si-cPLA$_2\alpha$) treatment compared with siRNA targeting a randomly-scrambled sequence of similar GC-content (si-control).
Figure 4.2 cPLA$_2$α mRNA level following treatment. LNCaP cells were treated with si-cPLA$_2$α or si-control for 48 h. Cells were harvested for mRNA extraction and analysed by RT-PCR. a)–b) cDNA dilutions of cPLA$_2$α or TBP amplification were made to create a standard curve for calculation of concentration. c) Histograms are mean mRNA level ± SEM (n=2 independent experiments). * P < 0.05 versus si-control (One-Way ANOVA).
4.3.1.3 Optimisation of treatment durations

To choose an optimal length of siRNA treatment, LNCaP cells were treated with si-cPLA₂α over 96 h to compare the best rate of mRNA knockdown between each time point. Results show cPLA₂α knockdown is most effective at 48 h after treatment with over 95% reduction in cPLA₂α mRNA (Figure 4.3).

![Figure 4.3 cPLA₂α mRNA level following treatment](image)

**Figure 4.3 cPLA₂α mRNA level following treatment.** LNCaP cells were treated with si-cPLA₂α or si-control for 48, 72 and 96 h. Cells were harvested for mRNA extraction and analysed by RT-PCR. Changes in mRNA level are represented as means ± SEM (n=2 independent experiments).

To confirm that changes in cPLA₂α mRNA lead to a decrease in cPLA₂α protein level, the same treatment was carried out in LNCaP cells for 2, 4 or 6 d before cell lysis for immunoblot. The cPLA₂α protein levels were reduced at all time points examined but si-cPLA₂α was most effective at 4 d after treatment (Figure 4.4). Result from the 6 d treatment shows that si-cPLA₂α set 2 was more effective than set 1. Since there was about a 3–5 fold-increase in cPLA₂α protein levels following Dox induction, a shorter exposure time for imaging was used in Chapter 3. To objectively demonstrate the silencing of cPLA₂α, a
longer exposure was used in this chapter. This explains the difference in endogenous cPLA$_2$$\alpha$ levels between figures from the two chapters.

a) 2 day treatment

![Image](cPLA2alpha_2_day.png)

b) 4 day treatment

![Image](cPLA2alpha_4_day.png)

c) 6 day treatment

![Image](cPLA2alpha_6_day.png)

**Figure 4.4** cPLA$_2$$\alpha$ protein level following treatment. LNCaP cells were treated with two si-cPLA$_2$$\alpha$ targeting different sections, si-Control or lipofectamine alone for a) 2 day, b) 4 day and c) 6 day. Amount of cPLA$_2$$\alpha$ remaining after siRNA knockdown was probed by immunoblot. Lipofectamine alone and si-control were used as controls. Overexpression clone LNCaP TRE-cPLA$_2$$\alpha$ 105 were used as positive controls for b) and c).
4.3.1.4 siRNA treatment on PC-3 and LNCaP cells

Using the time and dose calibrated from previous experiments, PC-3 and LNCaP cells were treated with si-cPLA$_2$$\alpha$ or si-control. Total cPLA$_2$$\alpha$ levels were reduced by ~70% in both cell lines (Figure 4.5). The reduction in cPLA$_2$$\alpha$ protein levels lead to a concurrent decrease in pcPLA$_2$$\alpha$ to over 70% and around 50% in PC-3 and LNCaP cells respectively.

![Figure 4.5](image)

**Figure 4.5 cPLA$_2$$\alpha$ protein level following treatment.** A decrease in total and phospho-cPLA$_2$$\alpha$ in PC-3 and LNCaP cells following a 48 h/37 °C treatment with si-cPLA$_2$$\alpha$ relative to si-control. The marked reduction of bands at 85 kDa by cPLA$_2$$\alpha$ siRNA also verified that the bands detected by a) anti-total and b) phospho cPLA$_2$$\alpha$ antibodies were cPLA$_2$$\alpha$. c) Histograms representing the densitometric analysis are mean ± SD (n=6 independent experiments). * P < 0.05 versus si-control.
4.3.2 Effect of cPLA$_2$$\alpha$ silencing on the PI3K/AKT pathway

Incubation with si-cPLA$_2$$\alpha$ led to a significant ($P < 0.05$) decrease in pAKT levels relative to the si-control in both cell lines (Figure 4.6 and 4.7). There was a trend of decrease in cyclin D1 in PC-3 and pGSK3$\beta$ in LNCaP after incubation with si-cPLA$_2$$\alpha$ relative to the si-control (Figure 4.6 and 4.7), but both did not reach statistical significance ($P = 0.1$).

To determine the role of cPLA$_2$$\alpha$ in influencing the AKT response to mitogens, PC-3 and LNCaP cells were treated with si-cPLA$_2$$\alpha$ or si-control in the presence or absence of EGF (Figure 4.7, 4.8 and 4.9). In these studies, incubation of PC-3 cells treated with si-control/EGF elicited a significant ($P < 0.05$) increase in pAKT (Ser$^{473}$) relative to the si-control alone (Figure 4.7), but this stimulation was not observed in LNCaP cells (Figure 4.8). Both cell lines showed increased pAKT (Thr$^{308}$) (Figure 4.9). However, in the presence of cPLA$_2$$\alpha$ siRNA and EGF (si-cPLA2$\alpha$/EGF), pAKT (both Ser$^{473}$ and Thr$^{308}$) levels diminished significantly ($P < 0.05$) in both cell lines relative to the si-control/EGF. In addition, relative to the si-control, levels of pGSK3$\beta$ were significantly ($P < 0.05$) increased after incubation of PC3 or LNCaP cells with si-control/EGF (Figure 4.7 and 4.8). For cyclin D1, the increase in si-control/EGF was significant ($P < 0.05$) in both cell lines. By incubation of cells with si-cPLA$_2$$\alpha$/EGF as opposed to si-control/EGF (Figure 4.7 and 4.8), the levels of pGSK3$\beta$ and cyclin D1 were significantly decreased ($P < 0.05$) in both cell lines. Total AKT and GSK3$\beta$ were not significantly altered between different treatments. It was noted that EGF treatment alone in LNCaP and PC3 cells increased endogenous pcPLA$_2$$\alpha$, but total cPLA$_2$$\alpha$ was unchanged (Figure 4.7). Overall, these experiments show that silencing of cPLA$_2$$\alpha$ leads to down-regulation of pAKT and its signalling in these cells.
Figure 4.6 Genetic silencing of cPLA$_2$α and EGF treatment in PC-3 and LNCaP cells.
a) PC-3 and b) LNCaP cells were treated with si-cPLA$_2$α for 48 h. 30 min before treatment, EGF (20 ng/mL/30 min) was used to stimulate the cells. Immunoblots are representative of 6 independent experiments.
Figure 4.7 Genetic silencing of cPLA₂α in PC-3 cells diminishes AKT signalling by immunoblot. PC-3 cells treated with si-cPLA₂α for 48 h showed reduced pAKT (Ser⁴⁷³), pGSK3β and cyclin D1 levels under basal or EGF-stimulating (20 ng/mL/30 min) conditions. a) Immunoblots are representative of 4 independent experiments; b) Histograms representing densitometric analysis are mean ± SD (n=4). * P < 0.05 versus si-control. # P < 0.05 versus si-control/EGF.
Figure 4.8 Genetic silencing of cPLA$_2$α in LNCaP cells diminishes AKT signalling by immunoblot. LNCaP cells treated with si-cPLA$_2$α for 48 h showed reduced pAKT (Ser$^{473}$), pGSK3β and cyclin D1 levels under either basal or EGF-stimulating (20 ng/mL/30 min) conditions. Immunoblots in a) are representative of 4 independent experiments, while the histograms in b) representing the densitometric analysis are mean ± SD (n=4 independent experiments). * P < 0.05 versus si-control. # P < 0.05 versus si-control/EGF.
Figure 4.9 Genetic silencing of cPLA₂α in PC-3 and LNCaP cells diminishes AKT phosphorylation at Thr³⁰⁸. PC-3 a) or LNCaP b) cells treated with si-cPLA₂α for 48 h showed reduced pAKT at Thr³⁰⁸. Immunoblots in a-b) were representative of 3 independent experiments (n=3).

4.3.3 Effect of cPLA₂α silencing on ERK and AR

It is known that blocking the PI3K/AKT pathway may induce a compensatory response in ERK (97), and thus could be ineffective at reducing cell proliferation. To investigate whether this phenomenon exist following cPLA₂α silencing (which reduces pAKT) total and phospho ERK were probed by immunoblot (Figure 4.10 and 4.11). In these studies, pERK 1/2 levels were examined under basal or EGF-stimulated conditions with or without si-cPLA₂α or si-control. The levels of pERK 1/2 were relatively low under basal culture conditions in PC-3 cells and no significant (P > 0.05) change was noted after treatment with cPLA₂α siRNA relative to the si-control (Figure 4.10). For LNCaP cells under basal culture conditions there was a marginal decrease (P = 0.07) in pERK 1/2 levels after treatment with cPLA₂α siRNA relative to the si-control (Figure 4.11). However, after incubation with the si-control/EGF, there was a marked (P < 0.05) increase in pERK 1/2 levels relative to the si-control in both cell-types (Figure 4.10 and 4.11). The level of pERK 1/2 was significantly (P < 0.05)
reduced in both PC-3 and LNCaP cells upon incubation with si-cPLA₂α/EGF relative to treatment with the si-control/EGF. Under all treatment conditions, total ERK 1/2 levels were not significantly altered in either cell lines (Figure 4.10 and 4.11).

Figure 4.10 Genetic silencing of cPLA₂α in PC-3 cells diminishes ERK signalling under EGF stimulated conditions. PC-3 cells transfected with cPLA₂α siRNA for 48 h/37 °C show reduced pERK 1/2 levels relative to the scrambled control under EGF-stimulating (20 ng/mL/30 min) conditions. a) Immunoblots are representative of 4 independent experiments, while the histograms representing the densitometric analysis are mean ± SD (n = 4). * P < 0.05 versus si-control. # P < 0.05 versus si-control/EGF.
Figure 4.11 Genetic silencing of cPLA₂α in LNCaP cells diminishes ERK signalling under EGF stimulated conditions. LNCaP cells transfected with cPLA₂α siRNA for 48 h/37 °C show reduced pERK 1/2 levels relative to the scrambled control under EGF-stimulating (20 ng/mL/30 min) conditions. The level of AR was reduced under basal and EGF-stimulating conditions after incubation of LNCaP cells with si-cPLA₂α relative to the scrambled control. a) Immunoblots are representative of 4 independent experiments, while the histograms representing the densitometric analysis are mean ± SD (n = 4 independent experiments). * P < 0.05 versus si-control. # P < 0.05 versus si-control/EGF.
An increase in AR levels is an important mechanism involved in the progression of hormone-refractory PC (149; 176). The effect of cPLA₂α silencing on AR expression was therefore determined next. Since PC-3 cells do not express AR (279), LNCaP cells were used to examine the change in AR levels (Figure 4.10). Interestingly, although AR protein levels were significantly (P < 0.05) increased in response to si-control/EGF relative to the si-control, there was a significant (P < 0.05) reduction of AR protein level after cPLA₂α siRNA treatment, regardless of the presence or absence of EGF (Figure 4.11). Treatment with si-cPLA₂α negated the EGF effect on AR increase. However, no significant change in AR mRNA level was observed under the same incubation conditions (Figure 4.12). Collectively, these experiments show that silencing cPLA₂α does not lead to a compensatory increase in the oncogenic pathways mediated by pERK 1/2 or the AR, suggesting that cPLA₂α may be a useful therapeutic target.

![Graph showing AR mRNA levels](image)

**Figure 4.12** AR mRNA levels remain unchanged after incubation with si-cPLA₂α relative to si-control. LNCaP cells were transfected with si-cPLA₂α (10 nM, 48 h) in the presence or absence of EGF (20 ng/mL/30 min). The levels of AR mRNA were determined by RT-qPCR, normalised by levels of TBP and expressed relative to si-control. Histograms represent mean AR mRNA levels ± SD (n=3 independent experiments).
4.4 Effect of cPLA\textsubscript{2}\textalpha silencing on PTEN-null prostate cancer cell proliferation

4.4.1 Measurement by cell number and DNA synthesis

Changes in biochemical pathways underpin changes in cell proliferation. To find out whether the biochemical changes following cPLA\textsubscript{2}\textalpha silencing lead to inhibition of proliferation, PC-3 and LNCaP cells were treated with si-cPLA\textsubscript{2}\textalpha or si-control and measured by cell counting (Figure 4.13) and/or SyBr green assay (Figure 4.14 and 4.15). cPLA\textsubscript{2}\textalpha silencing reduced LNCaP cell numbers by up to 24% following 4 d treatment (Figure 4.13). The decrease in proliferation was dose dependent for both cell lines, with reduction reaching as high as 49% in both cell lines (Figure 4.14 and 4.15). For LNCaP, the magnitude of this decrease increased over time, with highest at 5 d. For PC-3, the decrease in proliferation reduced from 49% (3 d) to 34% (5 d) over time. Taken together, cPLA\textsubscript{2}\textalpha silencing reduced PC-3 and LNCaP cell proliferation over time in a dose dependent manner.

![Figure 4.13](image)

Figure 4.13 siRNA against cPLA\textsubscript{2}\textalpha reduced LNCaP cell proliferation by cell counting. LNCaP cells were transfected with 10 nM or 20nM si-cPLA\textsubscript{2}\textalpha or si-control and left to propagate at 37 °C for 96 h. Cells are trypsinised, spun down and counted by haemacytometer. Histograms represent average cell number ± SD normalised to si-control (n=3 independent experiments). * P < 0.05 versus si-control.
Figure 4.14 siRNA against cPLA₂α reduces LNCaP cell proliferation by SyBr assay. LNCaP cells were transfected with 10, 20 or 40 nM si-cPLA₂α or si-control and left to propagate at 37 °C for 72, 96 or 120 h. At end of treatment, cells were added with SyBr green I DNA dye in lysis buffer. The amount of DNA present in each well of the culture plate was measured by fluorescence at 485 nm. Histograms represent mean fluorescence of each treatment ± SD and normalised to si-control (n=3 independent experiment). * P < 0.05 versus si-control.
Figure 4.15 siRNA against cPLA$\alpha$ reduced PC-3 cell proliferation by SyBr assay. PC-3 cells were transfected with 10 nM si-cPLA$\alpha$ or si-control and left to propagate at 37 °C for 72, 96 or 120 h. At end of treatment, cells were added with SyBr green I DNA dye in lysis buffer. The amount of DNA present in each well of the culture plate was measured by fluorescence at 485 nm. Histograms represent mean fluorescence of each treatment ± SD and normalised to si-control. (n=3 independent experiments). * P < 0.05 versus si-control.
4.4.2 Flow cytometry analysis on cell cycle

A reduction in cell number may result from a decrease in cells undergoing the cell cycle or an increase in apoptosis. To investigate the underlying cause of reduction in cell numbers, propidium iodide (PI) staining and flow cytometry was used. PC-3 and LNCaP cells treated with si-cPLA$_2$α or si-control were stained with PI before being analysed by flow cytometry. cPLA$_2$α silencing in PC-3 and LNCaP cells significantly increased cells in G$_1$/G$_0$ phase (Figure 4.16 and 4.17). This was due to a significant decrease in cells in G$_2$/M phase in both cell lines. There was a significant reduction in S phase for LNCaP cells treated with si-cPLA$_2$α compared to control, but not for PC-3. There was also an increase in Sub-G$_1$ (2.4%) for PC-3 cells, but this was not observed in LNCaP cells. Overall, flow analysis showed cPLA$_2$α silencing significantly reduced PC-3 and LNCaP cell proliferation through an increase in G$_1$/G$_0$ phase and a decrease in G2/M phase.
Figure 4.16 cPLA₂α silencing increased G₁/G₀ in PC-3 cells. PC-3 cells treated with si-cPLA₂α or si-control were grown for 48 h before being harvested and fixed in 80% ethanol. Cells were then added with PI (20 µg/mL) and RNase (100 µg/mL) for 1 h and then analysed by flow cytometry. Appropriate single cells were gated from multiple cell clusters and separated according to fluorescence intensity. Cells in each cell cycle phase were gated according to DNA content (2N for G₁/G₀, 4N for G₂/M, >2N and <4N for S; those <2N were sub-G₁). Histograms represent mean percentage of cell population ± SD (n=3 independent experiments). * P < 0.05 versus si-control.

Figure 4.17 cPLA₂α silencing increased G₁/G₀ in LNCaP cells. LNCaP cells treated with si-cPLA₂α or si-control were grown for 48 h before being harvested and fixed in 80% ethanol. Cells were then added with PI (20 µg/mL) and RNase (100 µg/mL) for 1 h and then analysed by flow cytometry. Appropriate single cells were gated from multiple cell clusters and separated according to fluorescence intensity. Cells in each cell cycle phase were gated according to DNA content (2N for G₁/G₀, 4N for G₂/M, >2N and <4N for S; those <2N were sub-G₁). Histograms represent mean percentage of cell population ± SD (n=3 independent experiments). * P < 0.05 versus si-control.
4.5 cPLA$_2$α silencing reduces pEGFR signalling in A431 cells

Previous experiments show that cPLA$_2$α inhibition reduces activation of AKT and ERK pathways under basal and mitogens stimulated conditions (Chapter 4). EGFR is a RTK that may pass on a signal to the PI3K/AKT and ERK pathways and is likely to play a role in mediating cPLA$_2$α action. Eicosanoids synthesised from AA have been shown to transactivate EGFR via prostanoid receptors (249; 251). LNCaP cells have very low levels of pEGFR, which is difficult to detect. To find out whether EGFR activation is involved in mediating cPLA$_2$α signal, some pilot experiments were done using A431 cancer cells, which have increased EGFR due to a B-Raf mutation (280) so pEGFR can be easily detected.

To show cPLA$_2$α is indeed be reduced by siRNA, A431 cells were incubated with si-cPLA$_2$α or si-control. qRT-PCR analysis showed a decrease in cPLA$_2$α mRNA level in si-cPLA$_2$α treated cells compared with si-control (Figure 4.18). Next, EGFR phosphorylation and its signalling were examined. Incubation with si-control/EGF increased EGFR phosphorylation significantly compared to si-control alone (Figure 4.19). This effect was also seen in pERK but not in pAKT. However, treatment of si-cPLA$_2$α/EGF showed significant decrease in pEGFR and pERK compared with si-control/EGF (P < 0.05). pAKT (Ser$^{473}$) was not significantly affected, and neither were total levels of AKT or ERK. Taken together, these results suggest that EGFR may at least partially mediate cPLA$_2$α action on ERK signalling in A431 cells. Thus, similar results may be hypothesised for PTEN-null PC cells.
Figure 4.18 cPLA₂α mRNA was reduced by si-cPLA₂α in A431 cells. A431 cells were treated with si-cPLA₂α or si-control for 48 h. Cells were harvested for RNA extraction and RT-PCR analyses. Histograms are mean values of mRNA ± SD corrected by values of TBP and normalised si-control (n=2 independent experiments). * P < 0.05 versus si-control.
Figure 4.19 cPLA$_2$α reduced EGFR activation under stimulated conditions in A431 cells. A431 cells were treated with si-cPLA$_2$α or si-control for 48 h. Cells were lysed at the end of treatment and used for immunoblot. a) Immunoblots are representative of 2 independent experiments, while the histograms b) representing the densitometric analysis are mean ± SD (n=2). * P < 0.05 versus si-control. # P < 0.05 versus si-control/EGF.
4.6 Discussion

In this chapter, the aim was to study the effect of cPLA$_2$$\alpha$ silencing on PI3K/AKT, AR and ERK pathways. Following cPLA$_2$$\alpha$ silencing, all three pathways showed levels of down-regulation. Further, this effect was more pronounced under mitogen-stimulated conditions. Importantly, while cPLA$_2$$\alpha$ overexpression had no effect on PTEN-null PC cell proliferation, the inhibition of cPLA$_2$$\alpha$ caused a significant reduction of cell proliferation (Figure 4.13–15). This demonstrates the vital role of cPLA$_2$$\alpha$ in sustaining signalling in these cells. The effects of cPLA$_2$$\alpha$ action were most likely due to its product AA and eicosanoids through a paracrine or autocrine fashion. Pilot experiments on A431 cells also suggest the possible involvement of EGFR in mediating cPLA$_2$$\alpha$ action on ERK. The role of EGFR in mediating cPLA2$\alpha$ action on pAKT and AR is uncertain at present (Figure 4.19).

Previous studies have shown an increase in pAKT together with a decrease in pERK 1/2 in poorly differentiated PC (30; 32; 66). This inverse relationship is likely due to AKT phosphorylation of B-Raf at Ser$^{259}$ that provides a binding site for 14-3-3 protein (281) and other mechanisms discussed in Section 4.1. This is likely the reason for the low levels of endogenous pERK found in PC-3 and LNCaP cells as pAKT was aberrantly increased in these two PTEN-null cell lines, as well as the possible explanation for the compensational increase in ERK following AKT inhibition (97). However, no compensatory increase in ERK was observed following cPLA$_2$$\alpha$ silencing in this chapter. We speculate that cPLA$_2$$\alpha$ is able to decrease activation of EGFR in addition to the effect on AKT. Under this setting, a decrease in AKT signalling is also met with a concomitant reduction in signalling upstream ERK. Hence, the compensatory mechanism from the ERK pathway is difficult to manifest.
Previous literature has not documented any link between cPLA$_2$α and AR. However, our study demonstrated that cPLA$_2$α is crucial for sustaining the AR level in PTEN-null PC cells. Previous publications from our lab have documented a concomitant decrease in cPLA$_2$α activation and AR level by Mediterranean food extracts (282), however it does not provide any link in between. Although cPLA$_2$α is important in providing AA, which induces androgen synthesis for AR signalling (260), cPLA$_2$α inhibition perhaps affects AR through some undefined mechanisms. It is possible that this mechanism is post-translational, as EGF treatment increases AR protein levels within 30 min (Figure 4.11) and cPLA$_2$α silencing can nullify this stimulation and keep AR levels down.

In conclusion, cPLA$_2$α inhibition by genetic silencing in PTEN-null PC cells down-regulates activation of the PI3K/AKT, ERK and AR pathways without any compensation between each pathway. This finding indicates that cPLA$_2$α is an attractive target for inhibition of PC.
CHAPTER 5

PHARMACOLOGICAL INHIBITION OF cPLA$_2$$\alpha$ AND AA ADD-BACK IN PTEN-NULL PROSTATE CANCER CELLS
5.1 Introduction

Eicosanoids are synthesised from intracellular AA, which is released from membrane glycerophospholipids by PLA$_2$ (283; 284). As cPLA$_2$$\alpha$ is the most predominant enzyme responsible for AA supply to eicosanoid-producing enzymes, a better outcome may be achieved with cPLA$_2$$\alpha$ inhibition than with a COX or LOX inhibitor alone. While inhibitors such as COX have cardiovascular side effects (237) and cause gastrointestinal bleeding (254), knocking out cPLA$_2$$\alpha$ in mice was not associated with disease (199). Highly specific inhibition of cPLA$_2$$\alpha$ may be achieved through genetic silencing using siRNA as shown in Chapter 4. However a pharmacological agent targeting cPLA$_2$$\alpha$ is also a viable option and it is able to target all cPLA$_2$$\alpha$ enzymes present.

In Chapter 4, experimental data from cPLA$_2$$\alpha$ silencing demonstrated a decrease in activation of PI3K/AKT, ERK and AR pathways. The aim of the studies in this Chapter were to demonstrate whether Efipladib, a new and potent inhibitor of cPLA$_2$$\alpha$ (285), could be used to reduce cPLA$_2$$\alpha$ activity and decrease the activation and expression of AKT, ERK, AR and cell proliferation. Moreover, an effort was made to verify the effect of AA and its metabolites in the presence or absence of Efipladib, on AKT, ERK and AR activation and expression.

5.2 Materials and methods

5.2.1 PLA$_2$ activity assay

In this chapter, PLA$_2$ activity assay was performed using complete cell lysates instead of immunoprecipitated cPLA$_2$$\alpha$ (Section 2.4.10). LNCaP cells were treated with Efipladib (15 $\mu$M) or DMSO for 24 h and then washed twice with Hank’s balanced salt solution (5.33 mM KCl, 0.441 mM KH$_2$PO$_4$, 4.17 mM NaHCO$_3$, 137.93 mM NaCl, 0.338 mM Na$_2$HPO$_4$ and 5.56 mM D-glucose). Cells attached in monolayer were scraped off the culture plate using a
plastic scraper and resuspended in 400 μL Hank’s balanced salt solution. The cells in suspension were lysed using a probe sonicator and the protein concentration was measured by Bio-Rad DC protein assay (see Section 2.4.1). A 100 μL cell homogenate was used per sample for the PLA₂ activity assay according to manufacturer’s instructions. The results obtained for each sample were normalised by protein concentration for that sample.

5.2.2 Efipladib and LY294002 treatment

Efipladib (in powder) was dissolved in DMSO to make 50 μM stock. LY294002 was pre-dissolved in DMSO. Both drugs were diluted to working concentrations in 10% FBS media. DMSO was used as the vehicle control.

5.2.3 Nuclear fractionation

Nuclear fractionation for AR studies was done as described in Section 2.3.6.

5.2.4 AA-BSA conjugation

5 μM fatty acid-free BSA was first added to 0% FBS RPMI media. The media was stirred until BSA was completely dissolved then sterile filtered. 100 μM AA or ethanol (vehicle control) was then added to the BSA media and the mixture was incubated at 37 °C on a rocking platform for 3 h. The BSA-AA conjugated media was then added with 1% FBS and diluted to working concentrations using sterile 5 μM BSA 1% FBS media. For conditions of treatment for AA studies see Section 2.2.6.

5.2.5 AA spiking and 5-HETE measurement

Cell culture, AA spiking and HPLC measurement of HETEs were done as described in Section 2.4.4.
5.2.6 Cell growth, SyBr green assay, PSA assay and immunostaining

Refer to Chapter 2 for details.

All experiments in this Chapter were repeated at least three times, unless indicated otherwise.

5.3 Results

5.3.1 Inhibition of cPLA$_2$$\alpha$ by Efipladib

Efipladib is a specific inhibitor of cPLA$_2$$\alpha$. A decrease in cPLA$_2$$\alpha$ activity in PC-3 cells following Efipladib treatment was demonstrated previously by our lab (204; 265). To repeat the experiment in LNCaP cells, Efipladib or control treatment was given and the cells were analysed by PLA$_2$ assay. After 24 h treatment, Efipladib significantly reduced total PLA$_2$ activity from LNCaP cell lysates compared with control by 32% (Figure 5.1). Taken together, Efipladib reduced cPLA$_2$$\alpha$ activity of both PC-3 and LNCaP cells.

![Figure 5.1 cPLA$_2$$\alpha$ inhibition results in decreased activity. LNCaP cells treated with Efipladib (20 µM) or control (DMSO) for 24 h/37 °C were lysed and assayed for PLA$_2$$\alpha$ activity. Mean activity normalised by protein concentrations were expressed as relative to DMSO (n=3 independent experiments). * P < 0.05 versus DMSO by One-Way ANOVA.](image-url)
5.3.2 Effect of cPLA$_2$α inhibition on AKT, ERK and AR activation and/or signalling

We have seen that genetic silencing of cPLA$_2$α reduced activation or expression of AKT, ERK 1/2 and AR in Chapter 4. To evaluate the effect of Efipladib on these pathways, we examined the treated PC-3 and LNCaP cells by immunoblot. To understand the effect duration of down-regulation of cPLA$_2$α may have on AKT, PC-3 and LNCaP cells were incubated with Efipladib for 24, 48 or 72 h. pAKT (Ser$^{473}$) was reduced at all time points compared with control for both cell lines while total AKT stayed the same (Figure 5.2, 5.3 and 5.4). Moreover, Efipladib caused a significant (P < 0.05) decrease in pERK 1/2 in PC-3 cells, but an increase in pERK 2 (42 kDa) in LNCaP cells relative to the control (Figure 5.3 and 5.4). Notably, a significant (P < 0.05) decrease in AR protein levels was also noted in LNCaP cells after incubation with Efipladib relative to the control (Figure 5.3).

![Image](image_url)

**Figure 5.2** Efipladib reduced pAKT (Ser$^{473}$) in PC-3 and LNCaP cells. PC-3 and LNCaP cells were incubated with Efipladib (20 μM) or the vehicle control (0.04% DMSO) for 24 or 48 h/37 °C. Immunoblots of phospho and total AKT are representative of 3 independent experiments. The vertical line indicates the pictures are combined but all from the same gel.
Figure 5.3 Efipladib reduced AKT and ERK activation in PC-3 cells. PC-3 cells were incubated with Efipladib (20 μM) or the vehicle control (0.04% DMSO) for 72 h/37 °C. a) Immunoblot of phospho and total AKT and ERK 1/2 levels in PC-3 cells (n=3 independent experiments); b) histograms representing densitometric analysis are mean ± SD (n = 3 independent experiments). * P < 0.05 versus vehicle-treated controls by One-Way ANOVA.
Figure 5.4 Effect of Efipladib treatment on AKT, ERK and AR in LNCaP cells. LNCaP cells were incubated with Efipladib (20 μM) or the vehicle control (0.04% DMSO) for 72 h/37 °C. a) Immunoblot of phospho and total AKT, pERK 1/2 and AR levels in LNCaP cells. Immunoblots in a) are representative of 3 independent experiments and the histograms in b) representing the densitometric analysis are mean ± SD (n = 3). * P < 0.05 versus vehicle-treated control by One-Way ANOVA.
As AKT is dephosphorylated by PHLPP at Ser\(^{473}\) (14), we investigated if PHLPP is involved in Efipladib action on pAKT (Ser\(^{473}\)) (Figure 5.2–5.4). Levels of PHLPP phosphatase were analysed by immunoblot. Incubation of Efipladib on LNCaP cells for 24, 48 or 72 h showed reduction of PHLPP 1 (140 and 190 kDa) (37) and PHLPP 2 (150 kDa) (43) at all time-points (Figure 5.5). This result suggests a decrease in AKT phosphorylation after cPLA\(_{2}\alpha\) inhibition is not due to an increase in PHLPP action.

![Figure 5.5 Efipladib reduces PHLPP 1/2 LNCaP cells. LNCaP cells were incubated with Efipladib (20 μM) or the vehicle control (0.04% DMSO) for 24, 48 or 72 h/37 °C. Immunoblots of PHLPP 1 and PHLPP 2 were representative of 3 independent experiments. The vertical line indicates the pictures are combined but all from the same gel.](image)

### 5.3.3 Combination of PI3K/AKT and cPLA\(_{2}\alpha\) inhibition

Inhibition of AKT activation in PC cells is often ineffective due to processes such as the negative feedback loop and so-called horizontal compensation from other oncogenic pathways (97). As shown in Chapter 4, cPLA\(_{2}\alpha\) inhibition by siRNA reduces AKT activation as well as proliferation of PC cells. To find out how cPLA\(_{2}\alpha\) inhibition may help further decrease PC cell proliferation in addition to AKT inhibition, a combinatorial
treatment of Efipladib and LY294002 (a PI3K inhibitor) were used in this study. LNCaP cells were treated with varying doses of LY294002 and/or two dose of Efipladib (10 or 20 µM). After 3 d treatment, LY294002 (5, 10 or 20 µM) or Efipladib (10 and 20 µM) significantly (P < 0.05) reduced LNCaP cell numbers compared with control (Figure 5.6). In addition, co-treatment with Efipladib (10 µM) significantly (P < 0.05) decreased cell numbers further than 5, 10 or 20 µM of LY294002 alone. The same synergistic effect was observed for 20 µM Efipladib and 20 µM LY294002 as their co-treatment significantly (P < 0.05) reduced cell number than either inhibitor alone. These results suggest that combination treatment of LY294002 and Efipladib significantly reduced LNCaP cell proliferation more than PI3K inhibition alone.
Figure 5.6 Concomitant treatment of Efipladib and LY294002 reduced LNCaP cell numbers greater than either inhibitor treatment alone. LNCaP cells seeded in 96 well plates were treated with Efipladib (10 or 20 µM) or LY294002 (5, 10 or 20 µM) or a combination of the two inhibitors at varying concentrations for 72 h/37 °C. 0.04% DMSO was used as the vehicle control. Cells were subjected to SyBr green assay at the end of treatment. A standard curve based on accurately dispensed cells was used to work out the cell numbers based on fluorescence. Histograms represent mean cell numbers ± SD of 2 independent experiments. # P < 0.05 versus control, * P < 0.05 versus 10 µM Efipladib or 5 µM LY294002, ** P < 0.05 versus 10 µM Efipladib or 10 µM LY294002, *** P < 0.05 versus 10 µM Efipladib or 20 µM LY294002, **** P < 0.05 versus 20 µM Efipladib or 20 µM LY294002 by One-Way ANOVA.

5.3.4 Effect of Efipladib on AR levels, nuclear localisation and PSA

From Figure 5.4, we saw a significant decrease in AR level in LNCaP cell following cPLA2α inhibition. To confirm this, we conducted immunostaining on paraffin-embedded Efipladib-treated LNCaP cells, which showed ~50% decrease in AR staining compared to control (Figure 5.7). AR functions as a nuclear transcription factor and its nuclear translocation is crucial for its activity (137; 148). To examine the effect of cPLA2α inhibition on AR level inside and outside of the nucleus, we employed nuclear fractionation techniques. Cytosolic
and nuclear fractions were separated from LNCaP cells treated with Efipladib or control for 72 h. GAPDH (cytosolic) and Histone deacetylase 1 (HDAC1) (nuclear) were used as controls for the effectiveness of fractionation technique and as loading controls in immunoblot (Figure 5.8a). The immunoblot showed that AR levels in both nuclear and cytosolic fractions were reduced significantly (P < 0.05) compared with control (Figure 5.8b). The effect of Efipladib on PSA, a downstream target of AR (166), was then assessed. Relative to the control, Efipladib caused a significant (P < 0.05) decrease in PSA levels in the medium overlying the LNCaP cell monolayer (Figure 5.9). Taken together, these results indicate inhibition of AR and its signalling from cPLA₂α blocking.
Figure 5.7 Decrease in AR staining following Efipladib treatment. LNCaP cells in T75 flasks were incubated with 0.04% DMSO control or Efipladib (20 µM) for 72 h/37°C before being harvested and processed for paraffin embedding (as described in Section 2.3.4). Sectioned slides were stained with anti-androgen receptor antibody or an isotype control. Results are typical of 3 experiments. Histograms are mean percentage of positive cells ± SD (n=3 independent experiments). * P < 0.05 versus DMSO by One-Way ANOVA. Larger image can be found online in supplementary data of Hua et al., (2013) (134).
Figure 5.8 Decrease in AR level following Efipladib treatment. LNCaP cells were incubated with Efipladib (20 µM) for 72 h/37 °C. After treatment, cells were lysed and the cytosolic fractions were separated from the nuclear fraction. Levels of AR in each fraction were determined by immunoblot. Immunoblots in a) are representative of 3 independent experiments and the histograms in b) representing the densitometric analysis are mean ± SD (n = 3). * P < 0.05 versus vehicle-treated control by One-Way ANOVA).
Figure 5.9 Efipladib treatment lead to a reduction of PSA secretion. Media from LNCaP cells incubated with Efipladib or 0.04% DMSO control in 6-well plates for 72 h were taken for PSA assay. The values obtained from each well were normalised by total protein concentration for that well. Histograms represent mean PSA value ± SD (n = 3 independent experiments). * P < 0.05 versus vehicle-treated control by One-Way ANOVA.

5.3.5 AA increases PTEN-null prostate cancer cell proliferation

From results above we saw decreased cPLA$_2$$\alpha$ action leads to decreased levels of pAKT, pERK and AR. Since cPLA$_2$$\alpha$ produces AA, a change in AA level is likely a link in affecting other signalling pathways. If so, an increase in AA level should increase PC cell proliferation. Indeed, treatment with BSA-conjugated AA in low serum conditions significantly increased (P < 0.05) the proliferation of PC-3 cells at 0.3125–2.5 µM (Figure 5.10) and LNCaP cells at 1.25–5 µM (Figure 5.11). In summary, AA is able to increase PC-3 and LNCaP cell proliferation.
Figure 5.10 AA treatment in PC-3 cells. PC-3 cells seeded in 96 well plates were put under 1% serum for 16 h then given BSA (5 µM) conjugated with AA at varying concentrations or BSA alone for 72 h/37 °C. Cell proliferation was analysed by SyBr green assay at the end of treatment. Histograms representing fluorescence intensity relative to control are mean ± SD (n = 3 independent experiments). * P < 0.05 versus control by One-way ANOVA.

Figure 5.11 AA treatment in LNCaP cells. LNCaP cells seeded in 96 well plates were put under 1% serum for 16 h then given BSA (5 µM) conjugated with AA at varying concentrations or BSA alone for 72 h/37 °C. Cell proliferation was analysed by SyBr green assay at the end of treatment. Histograms representing fluorescence intensity relative to control are mean ± SD (n = 3 independent experiments). * P < 0.05 versus control by One-way ANOVA.
5.3.6 AA add-back against genetic or pharmacological inhibition of cPLA$_2$α

To assess if AA mediates the effect of cPLA$_2$α action on pAKT, pERK 1/2 and AR levels, we next examined the effect of adding exogenous AA to PC-3 and LNCaP cells in the presence or absence of cPLA$_2$α siRNA. Incubation with si-cPLA$_2$α in the absence of AA significantly (P < 0.05) decreased pAKT and pERK 1/2 levels relative to the si-control in both cell lines (Figure 5.12 and 5.13). Addition of AA in the presence of the si-control (si-control/AA) significantly (P < 0.05) increased pAKT and pERK 1/2 levels in both cell lines relative to the si-control alone (Figure 5.12 and 5.13). The suppressive effect of si-cPLA$_2$α on pAKT in both cell-types relative to the si-control was abolished following incubation with AA (si-cPLA$_2$α/AA). However, although si-control/AA significantly (P < 0.05) increased pERK 1/2 levels relative to the si-control, si-cPLA$_2$α/AA did not completely abolish the suppressive effect of cPLA$_2$α siRNA alone on pERK 1/2 (Figure 5.12 and 5.13). Notably, pERK 2 (42 kDa) relative to pERK 1 (44 kDa) was mostly affected by incubation with AA alone or cPLA$_2$α siRNA plus AA. None of the treatments had any significant effect on total AKT or ERK 1/2. Incubation of LNCaP cells with si-control/AA also significantly (P < 0.05) increased AR levels relative to cells incubated with the si-control alone (Figure 5.13). Further, AA reduced the suppressive effect of cPLA$_2$α siRNA on AR protein levels such that the effect of cPLA$_2$α siRNA was no longer significant relative to si-control/AA (Figure 5.13).
Figure 5.12 The effect of AA on pAKT and pERK after cPLA$_2$$\alpha$ siRNA treatment in PC-3 cells.
a) PC-3 cells were treated with or without AA (20 μM) for 4 h/37 °C following a 48 h/37 °C incubation with si-cPLA$_2$$\alpha$ relative to the si-control. Immunoblots are representative of 3 independent experiments, while b) the histograms representing the densitometric analysis are mean ± SD (n = 3). * P < 0.05 versus si-control, # P < 0.05 versus si-control/AA by One-Way ANOVA.
Figure 5.13 The effect of AA on pAKT, pERK and AR after cPLA₂α siRNA treatment in LNCaP cells. a) LNCaP cells were treated with or without AA (20 μM) for 4 h/37 °C following a 48 h/37 °C incubation with si-cPLA₂α relative to the si-control. Immunoblots are representative of 3 independent experiments, while b) the histograms representing the densitometric analysis are mean ± SD (n = 3). * P < 0.05 versus si-control. # P < 0.05 versus si-control/AA by One-Way ANOVA.
5.3.7 Increase in HETE levels following AA spiking

Previously, we showed that the 5-LOX product, 5-HETE, was markedly reduced after blocking cPLA$_2$α activity (265). To examine whether 5-HETE levels were increased after the addition of AA, we incubated PC-3 and LNCaP cells with AA and determined the levels of HETE products by HPLC. Indeed, 5-HETE and 15-HETE were the most markedly increased HETEs after treatment with AA in both PC3 and LNCaP cells (Figure 5.14).

![Figure 5.14](image)

**Figure 5.14** Efipladib treatment lead to AR degradation over time. PC-3 and LNCaP cell lysates were incubated with AA (0.1 mM/1 h) and the levels of HETE were then determined by HPLC and expressed as relative to the 12-HETE. Histograms are mean HETE levels ± SD (n=3 independent experiments).

5.3.8 MK886 negates AA effect on pAKT, pERK and AR

Considering that 5-HETE is decreased after inhibiting cPLA$_2$α (265) and increased after AA addition we then determined whether 5-HETE mediates the effect of AA on AKT, ERK 1/2 and AR, by incubation of cells with AA in the presence or absence of MK886, an inhibitor of 5-HETE production (286). In these studies, AA significantly (P < 0.05) increased the levels of pAKT and pERK 1/2 in PC-3 and LNCaP cells relative to the control, and also AR in
LNCaP (Figure 5.15 and 5.16). Importantly, MK886 diminished the stimulatory effect of AA in both cell lines. Again, it is pERK 2 (42 kDa) relative to pERK 1 (44 kDa) that was typically affected by incubation with AA alone or AA plus MK886. Total AKT and ERK 1/2 were not significantly altered by any of the treatments relative to the control (Figure 5.15 and 5.16). Hence, these studies indicate that 5-HETE plays an important role in the effect of AA on pAKT, pERK 1/2 and AR expression.
Figure 5.15 The effect of AA on AKT and ERK 1/2 levels in PC-3 cells. a) PC-3 cells treated with AA (20 μM/4 h/37 °C) in the presence of the 5-LOX inhibitor, MK886. After treatment, cells were lysed and analysed by immunoblot. Immunoblots are representative of 3 independent experiments, while b) the histograms representing the densitometric analysis are mean ± SD (n = 3 independent experiments) * P < 0.05 versus vehicle-treated control, # P < 0.05 versus AA alone by One-Way ANOVA.
Figure 5.16 The effect of AA on AKT, ERK 1/2 and AR levels in LNCaP cells. a) LNCaP cells treated with AA (20 μM/4 h/37 °C) in the presence of the 5-LOX inhibitor, MK886. After treatment, cells were lysed and analysed by immunoblot. Immunoblots are representative of 3 independent experiments, while b) the histograms representing the densitometric analysis are mean ± SD (n = 3 independent experiments) * P < 0.05 versus vehicle-treated control. # P < 0.05 versus AA alone by One-Way ANOVA.
5.3.9 Measuring cell proliferation by cell counting and SyBr green assay

cPLA$_2$$\alpha$ silencing by siRNA reduces cell proliferation (Chapter 4). To test the same effect from Efipladib, PC-3 and LNCaP cells were treated with various concentrations of Efipladib for 72 h. Cell numbers after 72 h were significantly reduced: at 15 and 20 µM for PC-3 and at 20 µM for both cell lines compared with DMSO control (Figure 5.17). This reduction in proliferation was explored further in measurement of DNA content by SyBr green assay. DNA content, and thereby proliferation, was significantly reduced for PC-3 cells treated with 15 µM or higher and LNCaP cells treated with 10 µM or higher concentrations of Efipladib (Figure 5.18). These results indicated that Efipladib reduced PC cell proliferation in a dose-dependent manner.
Figure 5.17 PC-3 and LNCaP cell numbers were reduced following Efipladib treatment. a) PC-3 or b) LNCaP cells were seeded in 6-well plates for 48 h before being treated with various doses of Efipladib or 0.04% DMSO (control) and propagated for 72 h/37 °C. After treatment, cells were trypsinised and harvested for cell counting by haemacytometer. Histograms represent mean cell numbers ± SD (n=3 independent experiments). * P < 0.05 versus DMSO by One-way ANOVA.
Figure 5.18 PC-3 and LNCaP proliferations were reduced following Efipladib treatment. PC-3 or LNCaP cells were seeded in 96-well plates for 48 h before being treated with various doses of Efipladib or 0.04% DMSO (control) and propagated for 72 h/37 °C. After treatment, cells were washed and added to lysis buffer and SyBr green I DNA dye. Histograms represent mean fluorescence intensity ± SD (n= 3 independent experiments). * P < 0.05 versus DMSO by One-Way ANOVA.

5.3.10 Measurement of cell cycle by PI-based flow cytometry

Efipladib treatment in PC-3 and LNCaP cells showed a decrease in proliferation. This should be underpinned by a decrease in proliferating cells. To confirm this, PC-3 and LNCaP cells treated with Efipladib (20 µM) or DMSO for 72 h were stained with PI for cell cycle analysis by flow cytometry. Both cell lines showed a significant increase in G1/G0 cells compared with DMSO control (Figure 5.19). This was accompanied by a significant decrease in cells in S and G2/M phase. Although there was a significant increase of Sub G1 population in Efipladib-treated LNCaP cells (0.22 → 1%), the percentage of cell death was very small. These results demonstrated that cPLA2α inhibition reduced PC-3 and LNCaP cells through a reduction in the population of dividing cells.
Figure 5.19 Efipladib reduced PC-3 and LNCaP in G2M and S phase population. a) PC-3 or b) LNCaP cells were seeded in 6-well plates for 48 h before treated with 20 µM of Efipladib or 0.04% DMSO for 48 h/37 °C. After treatment, cells were trypsinised, stained with PI (20 µg/mL) and RNase (100 µg/mL) for 1 h and then analysed by flow cytometry. Histograms represent mean cell population ± SD (n=3 independent experiments). * P < 0.05 versus DMSO by One-Way ANOVA.

5.3.11 Measurement of BrdU incorporation and Ki-67 staining

Cells undergoing a specific phase during the cell cycle may be identified based on DNA content or by specific markers of a particular phase. To investigate further the reduction of proliferating cells, PC-3 and LNCaP cells treated with Efipladib or DMSO were analysed by BrdU incorporation and Ki-67 staining. In both cell lines, the percentage of cells undergoing
DNA synthesis (positive BrdU staining) was significantly reduced (P < 0.05) in Efpladib-treated populations compared with control (Figure 5.20, 5.21 and 5.22). Next, Ki-67 revealed a significant (P < 0.05) decrease of cells undergoing cell cycle after Efpladib treatment (Figure 5.20, 5.21 and 5.22). Taken together, the results suggested that Efpladib reduced cells undergoing cell cycle and forced them into quiescent status.
Figure 5.20 Decrease in BrdU and Ki-67 staining following Efipladib treatment in PC-3 cells. PC-3 cells in T75 flasks were incubated with Efipladib (20 µM) for 72 h/37°C before being harvested and processed for paraffin embedding (as described in the Section 2.3.4). Sectioned slides were stained with anti-AR antibody or an isotype control. Results are typical representative of 3 independent experiments.
Figure 5.21 Decrease in BrdU and Ki-67 staining following Efipladib treatment in LNCaP cells. LNCaP cells in T75 flasks were incubated with Efipladib (20 µM) for 72 h/37°C before being harvested and processed for paraffin embedding (as described in Section 2.3.4). Sectioned slides were stained with anti-AR antibody or an isotype control. Results are typical of 3 independent experiments.
Figure 5.22 Quantifications of BrdU and Ki-67 staining. The fraction of Ki-67 and BrdU positive cells from immunostaining experiments in Figure 5.20 and 5.21. Histograms are mean percentage of positive cells ± SD (n=3 independent experiments). * P < 0.05 versus vehicle-treated control by One-Way ANOVA.

5.4 Discussion

In this chapter, Efipladib was used to inhibit cPLA$_2$$\alpha$ in PC-3 and LNCaP cells and the subsequent biological/biochemical changes were examined. The effect of Efipladib was in accordance with the effects of cPLA$_2$$\alpha$ siRNA. Efipladib significantly inhibited (32% reduction) the total PLA$_2$$\alpha$ activity in LNCaP cells (Figure 5.1). The remaining 68% PLA$_2$ activity following Efipladib treatment was likely due to iPLA$_2$ and sPLA$_2$ present in LNCaP cells (213). As sPLA$_2$ is very low in PC-3 cells this may explain the reason why PLA$_2$ activity was reduced to a greater extent (40%) (204; 265).

The significant reduction in cPLA$_2$$\alpha$ activity from Efipladib treatment offers support for its effect in subsequent experiments. We examined the biochemical changes following Efipladib treatment that may underlie the change in cell proliferation. Efipladib is an indole derivative and is thought to be highly specific for cPLA2$\alpha$ (285). It binds to the single site of cPLA2$\alpha$ reversibly in a 1:1 stoichiometry (285; 287). Efipladib is currently under preclinical
evaluation for non-cancer-related diseases (285). Previous studies have shown that Efipladib specifically inhibits cPLA2α enzyme activity at 5–20 μM (204; 288; 289). Consistent with the results obtained with cPLA2α siRNA, incubating PC-3 and LNCaP cells with Efipladib decreased pAKT levels (Figure 5.2–5.4). This inhibitor also reduced pERK 1/2 levels in PC-3 cells, but unexpectedly increased pERK 2 in LNCaP cells (Figure 5.4). We interpret our data to indicate that Efipladib triggered a mechanism to compensate for the inhibition of AKT signalling by an increase in pERK1/2 in LNCaP cells. However, this compensatory mechanism may not operate in PC-3 cells. Hence, it is likely that in addition to cPLA2α, Efipladib has additional off-target effects, which need to be taken into consideration when contemplating further studies using Efipladib.

The mechanism underlying the decrease in pAKT is not clear. Since PHLPP dephosphorylates pAKT, one would expect an increase in PHLPP following Efipladib treatment (Figure 5.5). However, to our surprise there was a decrease in PHLPP. Hence, the down regulation of AKT following Efipladib treatment must result from other mechanisms, e.g., decreased PI3K activation and PIP3 level.

Notably, Efipladib also decreased AR protein expression (Figure 5.4 and 5.7) in the nucleus and cytosol (Figure 5.8), as well as PSA levels in culture medium (Figure 5.9). Considering the role AR plays in PC cell proliferation and survival (149; 176), it may explain why AR-positive LNCaP cells are more sensitive to Efipladib treatment compared with AR-negative PC-3 cells (Figure 5.18).

The AR is crucial for proliferation and survival of PC cells (142; 151-153) and can promote G1–S phase progression via enhanced translation of cyclin D1 (267). Since cPLA2α inhibition
had such great effect on AR, it is interesting to see its effect in AIPC cells with increased AR levels. As a change in AR levels via alteration of its synthesis and/or degradation can influence PC progression (142), it would be interesting to see if long-term blockade of cPLA$_2$$\alpha$ leads to a population with less dependence on cPLA$_2$$\alpha$.

Although we were unable to identify any studies in the literature examining the role cPLA2$\alpha$ in regulating AR expression, the available evidence implicates AA in androgen and/or AR signalling. In fact, AA induces androgen synthesis (260), which is required for AR stability (290) and inhibition of masculinisation (a manifestation of androgens) by estradiol-17$\beta$ or cyproterone acetate can be completely corrected by AA (291). Furthermore, indomethacin (a non-selective COX inhibitor) blocks the corrective effect of AA on masculinisation (291), suggesting the involvement of eicosanoids in mediating the AA effect. Given that AA can influence the AKT signalling pathway as shown herein, and that AKT can up-regulate AR protein levels (292; 293), the effect of AA on the AR could also be via AKT.

To investigate the downstream link under of cPLA$_2$$\alpha$, we tested the effect of AA in stimulating PC cells. AA treatment significantly stimulated PC-3 and LNCaP cell proliferation at low doses (Figure 5.10 and 5.11). We then examined the mechanism underlying the effect of cPLA$_2$$\alpha$ activity on pAKT, pERK 1/2 and AR levels. We reasoned that if the effect of genetic silencing of cPLA$_2$$\alpha$ on the aforementioned signalling pathways is mediated by a decrease in the pool of available AA, replenishment of AA to cPLA$_2$$\alpha$ siRNA-treated cells should rescue the suppression of pAKT, pERK 1/2 and AR levels. Consistent with this hypothesis, we demonstrated that incubation of PC-3 and LNCaP cell lines with AA alone significantly increased pAKT, pERK 1/2 and AR levels (Figure 5.12 and 5.13). Importantly, supplementation of cells with AA reversed the effect of cPLA$_2$$\alpha$ siRNA on
pAKT and to some extent AR, while the rescue effect of AA on ERK 1/2 was less apparent. Our working hypothesis is that the AA effect on AKT is more direct compared with the effect on ERK 1/2 and AR. We also noted the effect of AA on pAKT is stronger in si-cPLA2α than si-control PC3 cells, suggesting that AA treatment unleashes a stronger pAKT response under conditions where cPLA2α is blocked.

We then examined which metabolite of AA was likely to mediate its action. We have shown previously that blocking cPLA2α resulted in reduced accumulation of 5-HETE (265). To verify this finding, we measured the (S)-HETE products in PC-3 and LNCaP cells incubated with AA (Figure 5.14) and demonstrated a prominent increase in 5-HETE, a product of 5-LOX enzymatic action on AA (201). This outcome points to a role for 5-LOX products in mediating the effect of AA on pAKT, pERK 1/2 and AR levels. Indeed, the stimulatory effect of AA on pAKT, pERK 1/2 and AR is diminished in the presence of the 5-LOX inhibitor, MK886, confirming that 5-LOX products can mediate the action of AA on AKT, ERK 1/2 and AR (Figure 5.15 and 5.16). Recently, AA has been shown to up-regulate pAKT via mTORC2-dependent and PI3K-independent mechanisms in breast cancer cell lines (272). Interestingly, this effect was blocked by LOX inhibitors (272). As the increase in 15-HETE after addition of AA is comparable to 5-HETE, further study is needed to determine its role in mediating AA action on pAKT, pERK 1/2 and AR levels. In addition, it remains to be determined if AA can also have a direct effect independent from 5-HETE on pAKT, pERK 1/2 and AR levels.

Lastly, we examined the effect of cPLA2α treatment on proliferation of PTEN-null PC cells. Inhibition of cPLA2α by Efipladib hampered proliferation (Figure 5.17 and 5.18) in both cell lines. We next examined how cPLA2α inhibition reduced the proliferation of these cells. Flow
cytometry showed it that was through down regulation of cells in G2/M phase (Figure 5.19). Further analysis showed that, in addition to decreased DNA synthesis, cPLA2α inhibition draws cells into the G0 quiescent state (Figure 5.20–5.22). This is in accordance with flow cytometric results in that the majority of change in the G2M population was to shift into G1G0 phase and not to apoptotic Sub G1. Thus, cPLA2α inhibition was not cytotoxic and may be a therapeutic option in cancer. Indeed, co-targeting of cPLA2α and PI3K using two inhibitors significantly reduced more cell numbers than either inhibitor alone (Figure 5.6).

Together, these results show that cPLA2α inhibition by Efipladib decreased PTEN-null PC cells through reduction of pAKT, pERK and AR. The effect of cPLA2α action is likely mediated by its downstream AA and 5-HETEs. Further studies, such as AA and inhibitors of COX or LOX, may identify other eicosanoids or downstream molecules mediating cPLA2α action on AKT, ERK or AR.
CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS
6.1 Introduction

Cancer is a multi-step process driven by inherited and/or somatic genetic and epigenetic alterations. Thus, to understand cancer aetiology, progression and patients’ prognosis, it is necessary to identify these alterations and find their links with cancer initiation and progression.

Our laboratory has shown in advanced prostate cancer there is an increase in the protein levels of sPLA$_2$ and cPLA$_2$$\alpha$ (204; 213; 266), a key enzyme cleaving membrane-bound AA for lipid metabolism and production of inflammatory mediators. Blocking either sPLA$_2$ (213) or cPLA$_2$$\alpha$ (204) inhibits proliferation of PTEN-null prostate cancer cells \textit{in vitro} and \textit{in vivo}. A patent application for using the PLA$_2$ inhibitor to treat advanced prostate cancer was granted in the USA in 2011 (294) (US Patent I.D. 7919458). The importance of sPLA$_2$ and cPLA$_2$$\alpha$ in cancer biology is now recognised by inclusion in the 2\textsuperscript{nd} edition of the Cancer Encyclopedia (295). Moreover, our laboratory has identified natural products that are able to block cPLA$_2$$\alpha$ action (282; 296). As part of the overarching effort to identify the mechanism underlying cPLA$_2$$\alpha$ action on key oncogenic pathways, my PhD Project focused on the role of cPLA$_2$$\alpha$ in activation and/or expression of AKT, ERK and AR.

6.2 Effect of cPLA$_2$$\alpha$ on AKT activation

The first study in this thesis (Chapter 3) examined the effect of cPLA$_2$$\alpha$ overexpression and activation on AKT activation and signalling in PTEN-null prostate cancer cells. It was found that increased cPLA$_2$$\alpha$ expression and activation, with increased AA secretion, increased phosphorylation of AKT. This increased AKT activation was accompanied by increased phosphorylation of AKT targets. However, this effect could not be somehow translated into increased cell proliferation.
Both genetic silencing and pharmacological inhibition of cPLA$_2$α (Chapter 4 and 5) led to a reduction of AKT phosphorylation. The phosphorylation of AKT target, GSK3β, and levels of cyclin D1 were also reduced. This effect was not due to the increase of PTEN, as these are PTEN-null cells (74; 75), and unlikely due to actions from a negative regulator of AKT, PHLPP 1/2, as PHLPP was reduced by Efispladib treatment. The effect of cPLA$_2$α inhibition on AKT was enhanced under mitogenic stimulation. In EGF-stimulated cells, the rise in AKT activation and signalling was abrogated by cPLA$_2$α silencing. However, this effect of cPLA$_2$α silencing could be reverted by reintroduction of AA from an extracellular source. Indeed, AA, the product of cPLA$_2$α, stimulated pAKT and increased prostate cancer cell proliferation. The stimulation of AA on pAKT was likely through 5-HETE because inhibition of 5-LOX by MK886 abrogates the AA effect.

Inhibition of cPLA$_2$α by siRNA or Efispladib significantly reduced PTEN-null prostate cancer cells in a dose-dependent manner. In addition, the co-inhibition of PI3K and cPLA$_2$α significantly reduced cell proliferation more than either inhibitor alone. These effects likely resulted from decreased AKT activation and signalling as aberrant AKT activation is a common characteristic crucial for prostate cancer. cPLA$_2$α inhibition not only reduced the amount of dividing cells and DNA synthesis, but also forced cells into a G$_0$ quiescent state.

6.3 Effect of cPLA$_2$α on ERK activation

Increase in cPLA$_2$α expression and activation did not significantly change in pERK. But cPLA$_2$α inhibition reduced pERK under basal and EGF-stimulated conditions. Although it has been suggested that ERK activation may increase following AKT inhibition (95; 97), no significant increase in pERK was detected when pAKT was down-regulated by cPLA$_2$α.
inhibition. Addition of AA significantly increased pERK, and was able to abolish the down-regulation of pERK brought on by cPLA₂α inhibition. In addition, MK886 treatment reduced the AA effect on pERK, suggesting the role of 5-HETE in mediating AA effect on ERK. Finally, there is possible involvement of EGFR in mediating cPLA₂α on ERK, but more work is required to ascertain this link in prostate cancer.

6.4 Effect of cPLA₂α on AR expression

The effect of cPLA₂α or AA on AR expression has not been documented in the literature before. Results from this study found that there was a very clear and strong link between cPLA₂α action and AR levels in PTEN-null prostate cancer cells. Inhibition of cPLA₂α by genetic silencing or pharmacological agent significantly reduced AR expression. This reduction in AR level was significant following 24 h Efipladib treatment and existed under EGF-stimulated conditions. cPLA₂α inhibition reduced both nuclear and cytosolic AR as well as the secretion of PSA, whose gene is upregulated by AR. The effect of cPLA₂α inhibition on AR was also likely through AA and 5-HETE. Addition of AA significantly increased AR levels in LNCaP cells after 4 h and this was abrogated in MK886-treated cells.

Collectively, I have now found an important role for cPLA₂α in sustaining AKT signalling under basal and/or EGF-stimulating conditions in PTEN-null/mutated prostate cancer cells. Moreover, a reduction of pAKT levels by genetic silencing of cPLA₂α caused no compensational increase in pERK and AR levels. The mechanism of cPLA₂α action on AKT, ERK and AR is likely via AA and its metabolite, 5-HETE. Hence, restriction of AA supply to PTEN-null prostate cancer cells by inhibition of cPLA₂α could have a therapeutic value by inhibiting constitutively activated AKT signalling.
Figure 6.1 Schematic diagram of cPLA$_2$α affecting AKT, ERK 1/2 and AR levels. The studies herein demonstrate using genetic approaches that cPLA$_2$α positively regulates pAKT, pERK 1/2 and AR levels via AA and its metabolite 5-HETE derived from the activity of 5-LOX (as confirmed with the 5-LOX pharmacological inhibitor, MK886) in PTEN null/mutated prostate cancer cells. Pharmacological inhibition of cPLA$_2$α causes reduction in proliferation of PTEN null/mutated prostate cancer cells.

6.5 Future directions

This PhD project has laid foundation for the overarching goal to develop a novel strategy to inhibit constitutively-activated AKT signalling in PTEN-null prostate cancer.

Firstly, the potential of blocking cPLA$_2$α in preventing prostate cancer development can be examined by determination of the time of onset of prostate cancer after blocking cPLA$_2$α in prostate specific PTEN knockout mice. This can be done by administering Efipladib to PTEN knock-out mice and measure the timing and frequency of prostate cancer occurrence. Secondly, the effect of blocking cPLA$_2$α on cell cycle re-entry by (serum starved or contact inhibited) quiescent PTEN-null prostate cancer cells can be examined. These quiescent
cancer cells are thought to be central for cancer recurrence after cycling cancer cells are removed by chemo- or radiotherapy. Lastly, it is worthwhile determining if blocking cPLA$_2$α concomitantly with androgen ablation therapy can delay the occurrence of resistance to medical castration. A mouse model of androgen ablation therapy and Efipladib treatment can be used in preliminary studies.

The work described in this thesis has provided a new perspective in our understanding of cPLA$_2$α and AA in activation of classical oncogenic pathways in PTEN-null prostate cancer cells. It is my hope that this work can contribute to the development of novel therapies for advanced prostate cancer with constitutively activated AKT signalling pathway.


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Cytosolic phospholipase A$_2$α sustains pAKT, pERK and AR levels in PTEN-null/mutated prostate cancer cells

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ABSTRACT

Constitutive phosphorylation of protein kinase B (AKT) is a common feature of cancer caused by genetic alteration in the phosphatase and tensin homolog (PTEN) gene and is associated with poor prognosis. This study determined the role of cytosolic phospholipase A$_2$α (cPLA$_2$α) in AKT, extracellular signal-regulated kinase (ERK) and androgen receptor (AR) signaling in PTEN-null/mutated prostate cancer cells. Doxycycline (Dox)-induced expression of cPLA$_2$α led to an increase in pAKT, pGSK3β and cyclin D1 levels in LNCaP cells that possess a PTEN frame-shift mutation. In contrast, silencing cPLA$_2$α expression with siRNA decreased pAKT, pGSK3β and cyclin D1 levels in both PC-3 (PTEN deletion) and LNCaP cells. Silencing of cPLA$_2$α decreased pERK and AR protein levels. The inhibitory effect of cPLA$_2$α siRNA on pAKT and AR protein levels was reduced by the addition of arachidonic acid (AA), whereas the stimulatory effect of AA on pAKT, pERK and AR levels was decreased by an inhibitor of 5-hydroxyeicosatetraenoic acid production. Pharmacological blockade of cPLA$_2$α with Etipifl%d reduced pAKT and AR levels with a concomitant inhibition of PC-3 and LNCaP cell proliferation. These results demonstrate an important role for cPLA$_2$α in sustaining AKT, ERK and AR signaling in PTEN-null/mutated prostate cancer cells and provide a potential molecular target for treating prostate cancer.

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1. Introduction

Phosphoinositide-3-kinase (PI3K) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) are positive and negative regulators of phosphorylation of protein kinase B (AKT), respectively [1]. Genetic alteration of PI3K and PTEN has been found in at least sixteen types of human cancers [2]. Nearly one-third of prostate cancer cases have either a gain of function mutation in PI3K or loss of function mutation or deletion in PTEN [2]. Accordingly, about 45% of prostate cancer cases have increased levels of pAKT at Ser473, which correlates positively with disease severity [3–5]. The loss of PTEN or increase in pAKT, has been used as a predictor of advanced prostate cancer that fails to respond to treatment [6,7].

Suppression of the AKT signaling pathway in cancer cells carrying genetic alterations in PI3K or PTEN has proven to be a challenge. Constitutively-activated AKT renders cancer cells resistant to manipulation by insulin or insulin-like growth factor [8]. Inhibition of downstream effectors of AKT such as the mammalian target of rapamycin complex 1 (mTORC1), results in AKT activation due to removal of the negative feedback effect on pathways upstream of AKT [9]. In addition to this vertical compensation mechanism, a horizontal compensation mechanism is reported to involve increasing RAS/RAF/MEK/ERK activity in the presence of inhibitors of the AKT pathway [10]. Another factor crucial for survival and proliferation in prostate cancer cells is the androgen receptor (AR) [11–14]. It has been shown that an increase in AR expression alone is sufficient to enable cancer cells to progress to a hormone refractory status [15,16]. Thus, a potential impact on AR signaling should be considered in any attempt to block an oncogenic pathway in prostate cancer cells.

Pro-inflammatory eicosanoids (prostaglandins, leukotrienes or hydroxyeicosatetraenoic acid) are produced by various types of cancer cells as well as their surrounding tissues and are often associated with poor prognosis [17–19]. The mechanisms by which eicosanoids promote cancer progression include activation of the PI3K/AKT and RAS/ERK pathways [19]. Inhibition of eicosanoid production in PTEN-null prostate...
cancer cells decreases the levels of pAKT [20] and phospho-PI3K (p85 subunit) [21]. In two clinical trials, the rate of increase of prostate-specific antigen (PSA) following prostatectomy or radiotherapy was slowed in patients treated with inhibitors of eicosanoid-producing enzymes [22,23]. However, members of the eicosanoid family can promote tumor cell proliferation in vitro and in vivo [24] and the long-term use of cyclooxygenase-2 (COX-2) inhibitors can lead to cardiovascular side effects [25]. The current hypothesis proposed by others and us is that inhibition of cytosolic phospholipase A2α (cPLA2α) could be a more effective therapeutic approach than inhibition of COX or lipoxygenase (LOX) alone [26,27]. Significantly, cPLA2α catalyzes the hydrolysis of arachidonic acid (AA) selectively at the sn-2 position of glycerophospholipid [28]. After hydrolysis, the non-esterified AA is enzymatically converted to eicosanoids by the action of COX or LOX [29]. Hence, the outcome is not influenced by the effect of serum deprivation. For the Efplabidib incubation, PC-3 or LNCaP cells were seeded in 6-well plates for at least 24 h and then treated with 20 μM Efplabidib (Sanmar Chemicals, India) in RPMI/FBS (10% v/v) for 72 h before harvesting.

2.3. Doxycycline (Dox)-controlled cPLA2α expression

LNCaP cells were seeded in 10 cm dishes and grown to 100% confluence. Cells were then transfected with 24 μg of pcDNA6 (Cat.#: V1025-20; Invitrogen) vector expressing TET-Repressor using Lipofectamine 2000 (Cat.#: 11668; Invitrogen). These cells were then incubated with media containing Blasticidin (10 μg/mL) for 10 days. Successfully transfected colonies were then selected and passaged before further transfection with the pcDNA4 (Cat. #: V1030-20; Invitrogen) vector containing the human cPLA2α cDNA sequence with Flag regulated by a promoter containing the tetracycline-response element (TRE-cPLA2α). Successfully transfected clones were selected using media containing G418 (300 μg/mL; Cat.: 10131; Invitrogen) for 10 days/37 °C. Cells co-transfected with the pcDNA6 and pcDNA4 vectors without the cPLA2α coding sequence were used as a vector control (TRE-Empty). Four of the clones showing a positive increase in cPLA2α due to doxycycline induction were chosen and two clones were used in the present study. For induction of cPLA2α, cells were seeded in 6-well plates and treated with 100 ng/mL of Dox in RPMI/FBS (10% v/v) for 24 h/37 °C before harvest. There is a significant increase in cPLA2α protein level 8 h after Dox induction and it reaches a maximum as it approaches 24 h.

2.4. cPLA2α immunoprecipitation and activity assay

LNCaP cells transfected with cPLA2α or the empty vector control were induced with Dox and then lysed with buffer (50 mM Tris-pH 7.4, 150 mM NaCl, 1% v/v Triton X-100, 1 mM EDTA, 0.1% v/v NP-40). The cell lysates were centrifuged at 8200 g at 4 °C for 10 min. The cell pellets were washed three times with 500 μL TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4) for 5 min/4 °C. Bound antigens were eluted with 3 × FLAG peptide (Cat. #: F4759; Sigma) and assayed using the Phospholipase A2 Assay Kit (Cat. #: E10217; Invitrogen) according to the manufacturer’s specifications.

2.5. Fatty acid extraction from conditioned cell culture media

Fatty acids were extracted from cell culture medium as described in detail by Norris and Dennis [37]. Briefly, samples of media (2 × 250 μL from each sample) were diluted into a mixture of MeOH (400 μL), heptadecanoic acid (100 μg/mL, 10 μL) and HCl (1 M, 25 μL). Treated samples were then mixed with 2 ml hexane, vortexed for 30 s and centrifuged at 1000 × g for 5 min. The hexane layer was removed from the aliquots and combined into a 25 mL pear-shaped flask with a ground glass joint. The hexane extraction was repeated and the combined fractions were then evaporated under
vacuum, reconstituted with 200 μL isopropanol and stored at −80 °C before analysis using LC-MS.

2.6. LC-MS/MS

A Xevo-Triple quadruple mass spectrometer (Waters, Micromass, UK) coupled to a Phenomenex Kinetex 1.7 μm C18 100A (2.1 × 150 mm) was used for analysis. Fatty acids (10 μL) were separated using a binary gradient program, at a flow rate of 0.1 mL/min (mobile phase A: 0.2% formic acid in water; and mobile phase B: isopropanol.) The gradient used for separation of fatty acids analytes was as follows: 0 min, 20:80 A/B; 3 min, 0:100 A/B; 6 min, 0:100 A/B; 7 min, 20:80 A/B. Mass spectra of individual analytes was acquired in MS/MS mode under different collision energy ranging from 5 to 40 eV. The mass spectrometer was operated in negative ESI mode with capillary voltage of 2.30 kV, cone voltage of 25 V and desolvation temperature of 500 °C. The collision induced dissociation (CID) gas used was argon. Fragments with highest intensity were chosen at optimum collision energy (CE) for analysis; analytes were identified using the following mass transitions: for heptadecanoic acid, m/z 269.14 → 251.2 (CE 25 eV), for arachidonic acid m/z 303.3 → 59 (CE 20 eV), m/z 303.3 → 205 (CE 15 eV), m/z 303.3 → 259 (CE 15 eV), m/z 303.3 → 285 (CE 15 eV). Standard curves were constructed using linear normalization of the peak areas of the analytes (arachidonic acid) over internal standard (heptadecanoic acid) against the corresponding regression of the normalized peak areas of the analyte (arachidonic acid).

2.7. Genetic silencing of cPLA2α with siRNA

PC-3 and LNCaP cells were seeded in 6-well plates and then transfected with 10 nM siRNA against cPLA2α or the scrambled control (Cat. #: 45-2001; Invitrogen) mixed with Lipofectamine 2000 in a 1:2 v/v ratio. In these studies, OPTI-MEM (Cat. #: 11058; Invitrogen) was used as the transfection medium. The media was then replenished the next day and the cells were left to proliferate for 32 h/37 °C before harvesting for immunoblot. All experiments were performed using cells with a >70% decrease in cPLA2α protein level.

2.8. Immunoblotting

PC-3 and LNCaP cells were seeded in 6-well plates. Following treatment, cell lysates were prepared for immunoblots as described previously [38]. The band intensity of digital images was measured by using GeneTools software (SynGene, Cambridge, UK) and the ratio of band intensity between each protein of interest and the loading control was determined before expression changes in protein levels as a fold-change relative to the control. Two loading controls, α-tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were used to avoid overlapping signals from proteins of interest which had a similar molecular weight. Primary antibodies against: cPLA2α (Cat. #: SC-454), phospho-cPLA2α at Ser505 (Cat. #: SC-34391), AKT (Cat. #: SC-8312), phospho-AKT at Ser473 (Cat. #: SC-7985) and HDAC1 (Cat. #: SC-7872) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); ERK 1/2 (Cat. #: 9102), phospho-ERK 1/2 (Cat. #: 91065), GSK3β (Cat. #: 9315) and phospho-GSK3β (Cat. #: 9336S) were bought from Cell Signaling Technology (Danvers, MA); androgen receptor (Cat. #: MS-443-P) was purchased from Lab Vision (Kalamazoo, MI); cyclin D1 (Cat. #: C7464) and FLAG-peptide (Cat. #: A8592) were purchased from Sigma-Aldrich; and α-tubulin (Cat. #: ab7291) and GAPDH (Cat. #: ab8245) were obtained from Abcam (Boston, MA).

2.9. Reverse transcription- and quantitative real-time PCR

Total RNA was isolated using the UltraClean Tissue & Cells RNA Isolation Kit (Mo Bio Laboratories, CA) according to the manufacturer’s instructions. The first strand complementary DNA was synthesized from 500 ng of total RNA with random hexamers and SuperscriptIII (Cat. #: 48190 and P/N 56575, Invitrogen). The primers used for AR were forward 5′-AATGTCACCTCCAGATGCT and reverse 5′-CCATCCA CGGAATAATGCT. The TATA box binding protein (TBP) was used as the housekeeper gene, forward 5′-GAACCCGGCACTGATTTTC, reverse 5′-CCCCACCATGTCTGAACTC. Quantitative PCR measurements were performed using a SensiMix SYBR Mastermix Kit (Cat. #: QT605, Bioline, Sydney, NSW, Australia) and a RotorGene 6000 PCR machine (Qiagen, Santa Clarita, CA). Conditions for PCR were 1 cycle of 10 min at 95 °C; 40 cycles of 10 s at 95 °C and 30 s at 65 °C. The Relative Expression Software Tool 2009 (Qiagen) was used to calculate relative changes in cPLA2α normalized to the housekeeping gene. Amplification efficiency was determined using a 5-point dilution curve and was within 100% ± 3% for AR and TBP.

2.10. Nuclear and cytoplasmic fractionation

After treatment with Effipladib, cells were washed twice with cold PBS, scraped off the plate and collected into 1.5 mL microtubes and PBS removed after centrifugation at 500 g for 3 min. Nuclear and cytoplasmic fractionation were performed using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Cat. #: 78833 Thermo Scientific) according manufacturer’s instructions. All procedures including centrifugation were performed at 4 °C.

2.11. Immunocytochemistry

Expression of Ki-67 and BrdU incorporation were determined by immunocytochemical staining. For assessing BrdU incorporation, cells were added with 10 μM BrdU for 3 h/37 °C prior to harvest. The treated cells in T75 flasks were harvested and processed for immunocytochemistry as previously described [38]. Primary antibody against Ki-67 and BrdU were purchased from Lab Vision (Cat. #: RM-9106-S) and Sigma (Cat. #: B2531) respectively. Digital images of the immuno-stained sections were obtained by using an automated cellular imaging system (ACIS III; Dako). Two color thresholds were chosen on the images to distinguish between the positive (brown) and negative (blue) cells. Ten areas on each sample were randomly selected to determine the numbers of positive and negative cells. The percentage of positive cells of each sample was calculated using the following formula: positive cells / (positive cells + negative cells) × 100%.

2.12. Determination of hydroxyeicosatetraenoic acids (HETEs)

PC-3 and LNCaP cells were harvested and the cell pellets were incubated with 0.1 mM purified arachidonic acid for 1 h/37 °C. Total lipids were extracted into chloroform/methanol/water (5:1:1 v/v/v) and centrifuged (3600 g/15 min/20 °C). The chloroform phase was dried under vacuum and the resultant lipids, reconstituted with methanol/water (9:1 v/v) then separated by HPLC as described previously [36]. The identity of HETEs in the HPLC profile was confirmed by addition of authentic HETEs obtained commercially (Cayman Chemicals, Ann Arbor, MI). As 12-HETE level was the lowest, it was used for normalization.

2.13. Prostate specific antigen (PSA) assay

LNCaP cells in 6-well plates were treated with 20 μM Effipladib for 72 h/37 °C. Following treatment, 200 μL media were collected, centrifuged (300 g/5 min/4 °C) and measured using an Immulite 2000 Third Generation PSA kit (Siemens DPC, Sydney, NSW, Australia). The coefficient of variation of intra-assay and inter-assay variability was...
5% and 7%, respectively, for the dose over the range of standard curves. Cells in each well were harvested for protein assay and the PSA value was normalized by protein concentrations. The protein concentration was quantified using the Bio-Rad DC Protein Assay.

2.14. Measurement of DNA synthesis

Prostate cancer cells were seeded into 96-well plates at 2 × 10³ cells/well for PC-3 cells and at 2 × 10³ cells/well for LNCaP cells. Efladiplab was then added at the dose indicated in the figure legends and incubated for 72 h/37 °C. The same number of cells was aliquoted as a baseline and stored at −80 °C until use. Three days after treatment, the medium was gently removed from each well. Then, 100 µl of lysis buffer containing SYBR Green (Cat.: # S-7563, Invitrogen) was added at a 1:10,000 v/v dilution. The lysis buffer consisted of buffer A (10 mM Tris-pH 7.5 and 2 M NaCl) and buffer B (100 mM Tris-pH 7.5, 200 mM disodium EDTA and 10% v/v Triton X-100) in a 9:1 ratio. The cells were lysed in the dark for 30 min/4 °C. Previously frozen aliquots were thawed at room temperature, lysed in the same buffer and transferred to the treatment plate containing samples from the same type of cells. The fluorescence intensity of SYBR Green was measured using a plate reader (FLUOstar Omega, BMG Labtech) and the gain in the cellular DNA content during the treatment was estimated by subtracting baseline fluorescence intensity from the value obtained from experimental cells.

2.15. Statistical analysis

The statistical software NCSS (v12.0; Kaysville, UT) was used for analysis. One-Way ANOVA was implemented to determine the difference between individual groups of data. Fisher’s LSD Multiple-Comparison Test was used to determine whether the difference between individual groups (P < 0.05) was considered significant.

3. Results

3.1. Dox-induced cPLA2α expression increases AKT signaling in PTEN-mutated prostate cancer cells

To determine the effect of an increase in cPLA2α levels on AKT signaling in PTEN-mutated prostate cancer cells, we established a Dox-controlled cPLA2α expression system in the prostate cancer cell line, LNCaP (PTEN frame-shift mutation; [39]). The induction of ectopic cPLA2α by Dox was confirmed by detection of the Flag-tagged protein, which was also verified by the size of the molecule (85 kDa; Fig. 1A). Hence, the 85 kDa band detected using anti-total and phosphorylated cPLA2α antibodies correspond to cPLA2α (Fig. 1A).

The Dox-induced cPLA2α was phosphorylated at Ser505 (pCLPA2α; Fig. 1A), as shown previously with endogenous cPLA2α [29]. To determine Dox-induced cPLA2α enzyme activity, LNCaP cells transfected with cPLA2α or the empty vector control expression system were induced with Dox and the Flag-tagged cPLA2α was immuno-precipitated and assayed for activity. Consistent with the increased levels of pCLPA2α (Fig. 1A), Dox-induced cPLA2α showed an ~2.5–3-fold increase in enzyme activity compared with cells in the absence of Dox (Fig. 1B). Control cells without Flag-tagged cPLA2α showed no detectable cPLA2α activity. The amount of free AA released into culture medium increased significantly in the cells treated with Dox compared to the controls (Fig. 1B). There was no significant change in released AA after Dox treatment in control cells.

Over-expression of cPLA2α caused a significant (P < 0.05) increase in pAKT and its downstream effectors, glycogen synthase kinase 3β (pGSK3β) and cyclin D1 (Fig. 1C and D), whereas total AKT and GSK3β were not significantly (P > 0.05) altered. The same dose of Dox in cells transfected with the empty vector control showed no increase in cPLA2α expression or phosphorylation (Fig. 1A), nor was there any significant increase in pAKT, pGSK3β or cyclin D1 levels (Fig. 1C and D). In conclusion, over-expression of cPLA2α leads to increased levels of pAKT and its downstream targets.

3.2. Genetic silencing of cPLA2α decreases AKT signaling in PTEN-null mutated prostate cancer cells

To further determine the role of cPLA2α in AKT signaling, we genetically silenced cPLA2α in PC-3 (with a deletion in PTEN) and LNCaP cells (Fig. 2). Genetic silencing of cPLA2α using si-cPLA2α in PC-3 and LNCaP cells significantly (P < 0.05) decreased levels of cPLA2α and pcPLA2α by ≥70% relative to the scrambled control (si-control) in both cell lines (Fig. 2A). Since there was about 3–5 fold-increase in cPLA2α protein level following Dox induction, a short exposure for imaging was used in Fig. 1. To objectively demonstrate the silencing of cPLA2α, a long exposure was used in Fig. 2. This explains the difference in endogenous cPLA2α levels between Figs. 1 and 2. Incubation with si-cPLA2α led to a significant (P < 0.05) decrease in pAKT levels relative to the si-control in both cell lines (Fig. 2B and C). There was a trend of decrease in cyclin D1 in PC-3 and pGSK3β in LNCaP after incubation with si-cPLA2α relative to the si-control (Fig. 2B and C), but both did not reach statistical significance (P = 0.1).

To determine the role of cPLA2α in influencing the AKT response to mitogens, we incubated PC-3 and LNCaP cells with cPLA2α siRNA or the corresponding si-control in the presence or absence of EGF (Fig. 2B and C). In these studies, incubation of PC-3 cells with the si-control and EGF (si-control/EGF) elicited a significant (P < 0.05) increase in pAKT relative to the si-control alone (Fig. 2B), but this stimulation was not observed in LNCaP cells (Fig. 2C). However, in the presence of cPLA2α siRNA and EGF (si-cPLA2α/EGF), pAKT levels diminished significantly (P < 0.05) in both cell lines relative to the si-control/EGF. In addition, relative to the si-control, levels of pGSK3β were significantly (P < 0.05) increased after incubation of PC3 or LNCaP cells with si-control/EGF (Fig. 2B and C). For cyclin D1, the increase was significant (P < 0.05) in both cell lines. By incubation of cells with si-cPLA2α/EGF relative to the si-control/EGF (Fig. 2B and C), the levels of pGSK3β and cyclin D1 were significantly decreased (P < 0.05) in both cell lines. Total AKT and GSK3β were not significantly altered. It was noted that EGF treatment alone in LNCaP and PC3 cells increased endogenous phospho-cPLA2α, but total cPLA2α was unchanged (data not shown). Overall, these studies show that silencing of cPLA2α leads to down-regulation of pAKT and this likely impacts on its signaling in these cells.

3.3. Genetic silencing of cPLA2α diminishes pERK and AR in PTEN-null mutated prostate cancer cells

Considering the possibility that blockade of one oncogenic pathway may induce a compensatory response [10], and thus could be detrimental, we examined whether a decreased pAKT, after silencing of cPLA2α, caused an increase in pERK 1/2 (Fig. 3). In these studies, pERK 1/2 levels were examined under basal or EGF-stimulated conditions with or without cPLA2α siRNA or the corresponding si-control. The levels of pERK 1/2 were relatively low under basal culture conditions in PC-3 cells and no significant (P > 0.05) change was noted after treatment with cPLA2α siRNA relative to the si-control (Fig. 3A). Experiments examining LNCaP cells under basal culture conditions demonstrated there was a marginal decrease (P = 0.07) in pERK 1/2 levels after treatment with cPLA2α siRNA relative to the si-control (Fig. 3B). However, after incubation with the si-control/EGF, there was a marked (P < 0.05) increase in pERK 1/2 levels relative to the si-control in both cell types (Fig. 3A and B). The level of pERK 1/2 was significantly (P < 0.05) reduced in both PC-3 and LNCaP cells upon incubation with si-cPLA2α/EGF relative to treatment with the si-control/
EGF. Under all treatment conditions, total ERK 1/2 levels were not significantly altered in either cell lines (Fig. 3A and B).

An increase in AR levels has been shown to be an important mechanism involved in the progression of hormone-refractory prostate cancer [15,16]. Therefore, we next determined whether silencing cPLA2α had an effect on AR expression. As PC-3 cells do not express AR [40], studies examining AR levels were conducted using the LNCaP cell line only (Fig. 3B). Interestingly, although AR protein levels were significantly (P < 0.05) increased in response to si-control/EGF relative to the si-control, there was a significant (P < 0.05) reduction of AR protein level after cPLA2α siRNA treatment regardless of the presence or absence of EGF (Fig. 3B). However, no significant change in AR mRNA level was observed under the same incubation conditions (Supplementary Fig. 1). Collectively, these experiments showed that silencing cPLA2α does not lead to a compensatory increase in the oncogenic pathways mediated by pERK 1/2 or the AR, suggesting that cPLA2α may be a useful therapeutic target.

3.4. Arachidonic acid and 5-HETE can mediate cPLA2α action on AKT, ERK 1/2 and AR expression

To assess if AA mediates the effect of cPLA2α action on pAKT, pERK 1/2 and AR levels, we next examined the effect of adding exogenous AA to PC-3 and LNCaP cells in the presence or absence of cPLA2α siRNA (Fig. 4). Incubation with si-cPLA2α in the absence of AA significantly (P < 0.05) decreased pAKT and pERK 1/2 levels relative to the si-control in both cell lines (Fig. 4A and B). Addition of AA in the presence of the si-control (si-control/AA) significantly (P < 0.05) increased pAKT and pERK 1/2 levels in both cell lines relative to the si-control alone (Fig. 4A and B). The suppressive effect of si-cPLA2α on pAKT in both cell-types relative to the si-control was abolished following incubation with AA (si-cPLA2α/AA). However, although si-control/AA significantly (P < 0.05) increased pERK 1/2 levels relative to the si-control, si-cPLA2α/AA did not completely abolish the suppressive effect of cPLA2α siRNA alone on pERK 1/2 (Fig. 4A and B). Notably, it is pERK 2 (42 kDa) relative to pERK 1 (44 kDa) that is mostly affected by incubation with AA alone or cPLA2α siRNA plus AA. None of the treatments had any significant effect on either total AKT or ERK 1/2. Incubation of LNCaP cells with si-control/AA also significantly (P < 0.05) increased AR levels relative to cells incubated with the si-control alone (Fig. 4B). Further, AA reduced the suppressive effect of cPLA2α siRNA on AR protein levels such that the effect of cPLA2α siRNA was no longer significant relative to si-control/AA (Fig. 4B).

Previously, we showed that the 5-LOX product, 5-HETE, was markedly reduced after blocking cPLA2α activity [41]. To examine whether 5-HETE levels were increased after the addition of AA, we incubated PC-3 and LNCaP cells with AA and determined the levels of HETE products by HPLC. Indeed, 5-HETE and 15-HETE were the most markedly increased HETEs after treatment with AA in both PC3 and LNCaP cells (Supplementary Fig. 2). Considering that 5-HETE is decreased after inhibiting cPLA2α [41], we then determined...
whether this eicosanoid mediates the effect of AA on AKT, ERK 1/2, and the AR, by incubation of cells with AA in the presence or absence of MK886, an inhibitor of 5-HETE production [42]. In these studies, AA significantly (\( P < 0.05 \)) increased the levels of pAKT and pERK 1/2 in PC-3 and LNCaP cells relative to the control, and also the AR in LNCaP (Fig. 4C and D). Importantly, MK886 diminished the stimulatory effect of AA in both cell lines. Again, it is pERK 2 (42 kDa) relative to pERK 1 (44 kDa) that is typically affected by incubation with AA alone or AA plus MK886. Total AKT and ERK 1/2 were not significantly altered by any of the treatments relative to the control (Fig. 4C and D). Hence, these studies indicate that 5-HETE plays an important role in the effect of AA on pAKT, pERK 1/2 and AR expression.

3.5. Pharmacological inhibition of cPLA2\(^\alpha\) on AKT, ERK 1/2 and AR expression in PTEN-null/mutated prostate cancer cells

To evaluate the potential of targeting cPLA2\(^\alpha\) using a pharmacological approach, we examined AKT, ERK 1/2 and AR signaling in PC-3 and/or LNCaP cells treated with Efipladib (Fig. 5), a new and potent inhibitor of cPLA2\(^\alpha\) [43]. Incubation with Efipladib (20 \( \mu \)M, 72 h/37 °C), which we have previously shown to decrease cPLA2\(^\alpha\) activity in PTEN-null cells [30,41], significantly (\( P < 0.05 \)) reduced pAKT levels in both cell lines relative to the control samples (Fig. 5A and B). Moreover, Efipladib caused a significant (\( P < 0.05 \)) decrease in pERK 1/2 in PC-3 cells, but an increase in pERK 2 (42 kDa) in LNCaP cells relative to the control (Fig. 5A and B). Notably, a significant (\( P < 0.05 \)) decrease in AR protein levels was also noted in LNCaP cells after incubation with Efipladib relative to the control (Fig. 5B and Supplementary Fig. 3). The decrease in AR protein was apparent in both nuclear and cytoplasmic fractions (Fig. 5C). The effect of Efipladib on a downstream target of the AR, namely prostate-specific antigen (PSA) [44], was then assessed. Relative to the control, Efipladib caused a significant (\( P < 0.05 \)) decrease in PSA levels in the medium overlaying the LNCaP cell monolayer (Fig. 5D).

Efipladib also reduced PC-3 and LNCaP cell proliferation in a dose-dependent manner as judged by assessing DNA content using SYBR...
Green (Fig. 5E). Further, the percentage of Ki-67 and BrdU positive cells was significantly \( P < 0.05 \) reduced after incubation with Eflidib in PC-3 cells (Fig. 5F, Supplementary Fig. 4) and LNCaP cells (Fig. 5F and Supplementary Fig. 5). These investigations indicate that cPLA\(\alpha\) could be an important molecular target for the development of effective anti-tumor therapies in prostate cancer and that further pre-clinical studies with Eflidib or an active derivative are warranted.

4. Discussion

An aberrant increase in AKT signaling is a salient feature of advanced prostate cancer and can be used to predict disease severity and outcome [2,4,6,7]. Few options exist for inhibiting activated AKT without causing an increase in other oncogenic pathways [10]. We previously demonstrated an aberrant increase in pcPLA\(\alpha\), which increases AA generation [29], in hormone-refractory prostate cancer [30]. Considering this and that the cPLA\(\alpha\) enzymatic product, AA, is required for sustaining AKT signaling in \( P T E N \)-null/mutated prostate cancer cells (see schematic: Fig. 6).

The role for cPLA\(\alpha\) was first assessed using a Dox-induced cPLA\(\alpha\) over-expression system. Induced cPLA\(\alpha\) expression resulted in a concomitant increase in pcPLA\(\alpha\) protein levels, enzymatic activity and AA release, leading to an increase of pAKT and its immediate downstream effector, pGSK3\(\beta\). An increase in cyclin D1 expression was also observed, consistent with an inactivation of GSK3\(\beta\) by AKT phosphorylation [45]. In addition, pharmacological blockade of cPLA\(\alpha\) with Eflidib is able to attenuate the pAKT increase induced by cPLA\(\alpha\) over-expression (data not shown). In contrast to over-expression of cPLA\(\alpha\), genetic silencing of cPLA\(\alpha\) led to a decrease in pAKT and its downstream effectors in PC3 and LNCaP cells (Fig. 2). Both these cell lines have constitutively activated AKT due to a \( P T E N \) deletion or frame-shift mutation [39,46]. The role of cPLA\(\alpha\) in the regulation of AKT phosphorylation became more apparent after EGF-stimulation. These results support the hypothesis that cPLA\(\alpha\) is required for sustaining AKT signaling in \( P T E N \)-null/mutated prostate cancer cells.

An increase in an alternative oncogenic pathway has been shown in various types of cancer cells when one oncogenic pathway is blocked [10]. Hence, we examined the consequence of silencing cPLA\(\alpha\) on key oncogenic proteins in the context of prostate cancer, namely ERK 1/2 and the AR [47,48]. Previous studies have shown an increase in pAKT together with a decrease in pERK 1/2 in poorly differentiated prostate cancer [4,6,8]. One possible mechanism underlying this inverse relationship is that AKT phosphorylates RAF at Ser^259 that provides a binding site for the 14–3–3 protein [49], resulting in down regulation of RAF activity [50]. Hence, we sought to determine whether a decrease in pAKT signaling as a result of genetic silencing of cPLA\(\alpha\) leads to an increase in pERK 1/2 levels. Our experiments examining this possibility revealed no increase in pERK 1/2, and instead, a decrease in pERK 1/2 was noted under EGF-stimulating conditions in the \( P T E N \)-null/mutated PC3 and LNCaP cell lines (Fig. 3). This effect occurred despite the fact that pAKT levels decrease after genetic silencing of cPLA\(\alpha\), with parallel decreases in the immediate downstream targets, pGSK3\(\beta\) and cyclin D1 (Fig. 2). Hence, inhibition of cPLA\(\alpha\) is able to abrogate both AKT and ERK 1/2 activation. Notably, it is pERK 2 (42 kDa) relative to pERK 1 (44 kDa) that is mostly affected by incubation with AA alone, or the combination of cPLA\(\alpha\) siRNA with added AA. Considering p44 (ERK1) and p42 (ERK2) are phosphorylated by MEK1 and MEK2, respectively [51], and MEK1 has been shown to interact with A-Raf or C-Raf whereas MEK2 interact with C-Raf only [52], our finding suggests that the role of cPLA\(\alpha\) in these pathways may be mediated through C-Raf or MEK2.

The AR is crucial for proliferation and survival of prostate cancer cells [11–14] and can promote G1–S phase progression via enhanced translation of cyclin D1 [53]. Studies in animals bearing a human prostate cancer xenograft have suggested that an increase in AR alone is sufficient to enable cancer cells to progress to hormone-refractory status [14]. Thus, a change in AR levels via alterations in its synthesis and/or degradation, can influence prostate cancer progression [14]. Herein, we showed that silencing of cPLA\(\alpha\) with siRNA reduced AR protein levels with no change in AR mRNA levels, suggesting post-transcriptional regulation of AR by cPLA\(\alpha\). Although we were unable to identify any studies in the literature examining the role of cPLA\(\alpha\) in regulating AR expression, the available evidence implicates AA in androgen and/or AR signaling. In
Fig. 4. The effect of arachidonic acid (AA) on AKT, ERK 1/2 and AR levels as determined by immunoblot. (A) PC-3 and (B) LNCaP cells were treated with or without AA (20 μM) for 4 h/37 °C following a 48 h/37 °C incubation with si-cPLA₂α relative to the scrambled control. * P < 0.05 versus si-control. # P < 0.05 versus AA si-control. (C) PC-3 and (D) LNCaP cells treated with AA (20 μM/4 h/37 °C) in the presence of the 5-LOX inhibitor, MK886, show the diminished effect of AA alone on pAKT, pERK 1/2 and AR levels. P < 0.05 versus vehicle-treated control. # P < 0.05 versus AA alone. (A–D) Immunoblots are representative of 3 independent experiments, while the histograms representing the densitometric analysis are mean ± SD (n = 3 independent experiments).
fact, AA induces androgen synthesis [34], which is required for AR stability [54] and inhibition of masculinization (a manifestation of androgens) by estradiol-17β or cyproterone acetate can be completely corrected by AA [55]. Furthermore, indomethacin (a non-selective COX inhibitor) blocks the corrective effect of AA on masculinization [55], suggesting the involvement of eicosanoids in mediating the AA effect. Given that AA can influence the AKT signaling pathway as shown herein and that AKT can up-regulate AR
membrane

\[ \text{cPLA}_2 \]

\[ \text{5-LOX} \]

\[ \text{5-HETE} \]

\[ \text{AKT} \]

\[ \text{ERK} \]

\[ \text{GSK3β} \]

\[ \text{AR} \]

\[ \text{Cyclin D1} \]

\[ \text{si-cPLA}_2 \alpha / \text{Efpladib} \]

\[ \text{MK886} \]

\[ \text{AA} \]

Inhibition

Stimulation

Increase in cell proliferation

Fig. 6. Schematic diagram of cPLA2α affecting AKT, ERK 1/2 and AR levels. The studies herein demonstrate using genetic approaches that the cPLA2α positively regulates pAKT, pERK 1/2 and AR levels via AA and its metabolite 5-HETE derived from the activity of 5-LOX (as confirmed with the 5-LOX pharmacological inhibitor, MK886) in PTEN null/mutated prostate cancer cells. Pharmacological inhibition of cPLA2α causes reduction in proliferation of PTEN null/mutated prostate cancer cells.

protein levels [56,57], it can be suggested that the effect of AA on the AR can also be via AKT.

Our investigation then examined the mechanism underlying the effect of cPLA2α activity on pAKT, pERK 1/2 and AR levels. We reasoned that if the effect of genetic silencing of cPLA2α on the aforementioned signaling pathways is mediated by a decrease in the pool of available AA, replenishment of AA to cPLA2α-silenced cells should rescue the suppression of pAKT, pERK 1/2 and AR levels. Consistent with this hypothesis, we demonstrated that incubation of PC-3 and LNCaP cell lines with AA alone significantly increased pAKT, pERK 1/2 and AR levels. Importantly, supplementation of cells with AA reversed the effect of cPLA2α siRNA on pAKT and to some extent AR, while the rescue effect of AA on ERK 1/2 was less apparent. Our working hypothesis is that the AA effect on AKT is more direct compared with the effect on ERK1/2 and AR. We also noted the effect of AA on pAKT is stronger in si-cPLA2α than si-control PC3 cells, suggesting that AA treatment unleashes a stronger pAKT response under the condition when cPLA2α is blocked. The reason is unknown and further studies aimed at understanding this response are planned.

We then examined which metabolite of AA was likely to mediate its action. We have shown previously that blocking cPLA2α resulted in reduced accumulation of 5-HETE [41]. To verify this finding, we measured the (S)-HETE products in PC-3 and LNCaP cells incubated with AA (Supplementary Fig. 2) and demonstrated a prominent increase is 5-HETE, a product of 5-LOX enzymatic action on AA [24]. This outcome points to a role for 5-LOX products in mediating the effect of AA on pAKT, pERK 1/2 and AR levels. Indeed, the stimulatory effect of AA on pAKT, pERK 1/2 and AR is diminished in the presence of the 5-LOX inhibitor, MK886, confirming that 5-LOX products can mediate the action of AA on AKT, ERK 1/2 and AR. Recently, AA has been shown to up-regulate pAKT via a mTOR complex 2-dependent and PI3K-independent mechanism in breast cancer cell lines [58]. Interestingly, this effect was blocked by LOX inhibitors [58]. As the increase in 15-HETE after addition of AA is comparable to 5-HETE, further study is needed to determine its role in mediating AA action on pAKT, pERK 1/2 and AR levels. In addition, it remains to be determined if AA can also have a direct effect independent from 5-HETE on pAKT, pERK 1/2 and AR levels.

Next, we determined the effect of the cPLA2α inhibitor, Efpladib, on AKT, ERK 1/2 and AR signaling pathways. Efpladib is an indole derivative and is thought to be highly specific for cPLA2α [43]. It binds to the single site of cPLA2α reversibly in a 1:1 stoichiometry [43,59]. Efpladib is currently under preclinical evaluation for non-cancer-related diseases [43]. Previous studies have shown that Efpladib specifically inhibits cPLA2α enzyme activity at 5–20 μM [30,60,61]. Consistent with the results obtained with cPLA2α siRNA, incubating PC-3 and LNCaP cells with Efpladib decreased pAKT levels. This inhibitor also reduced pERK 1/2 levels in PC-3 cells, but unexpectedly, increased pERK 2 in LNCaP cells. We interpret our data to indicate that Efpladib has triggered a mechanism to compensate for the inhibition of AKT signaling by an increase in p-ERK1/2 in LNCaP cells. This compensatory mechanism may not be operating in PC-3 cells. Hence, it is likely that in addition to cPLA2α, Efpladib has additional off-target effects, which need to be taken into consideration when further studies using Efpladib are contemplated. Notably, Efpladib also decreased AR protein expression, as well as PSA levels in culture medium. Considering the role AR plays in prostate cancer cell proliferation and survival [15,16], it may explain why AR-positive LNCaP cells are more sensitive to Efpladib treatment compared with AR-negative PC-3 cells (Fig. 5D). Further study is needed to determine if long-term blockade of cPLA2α will lead to a population with less dependence on cPLA2α. Considering the effect of cPLA2α on AR expression, it would be interesting to compare the AR expression in, and proliferation of, cells under long-term treatment with Efpladib versus those exposed to Efpladib over a short-term.

In conclusion, in this investigation we showed that cPLA2α can sustain AKT signaling under basal culture and/or EGF-stimulating conditions in PTEN-null/mutated prostate cancer cells. A reduction of pAKT levels by genetic silencing of cPLA2α caused a concomitant decrease in pERK 1/2 and AR levels. The mechanism of cPLA2α action on AKT, ERK 1/2 and AR was via AA and its metabolite, 5-HETE. Hence, the inhibition of cPLA2α action could be considered as an option to inhibit activated AKT in advanced prostate cancer. Our study further supports the case for restricting dietary AA intake in patients with...
diagnosed prostate cancer and utilizing agents blocking cPLA2 as a novel therapy for this disease.

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Appendix A. Supplementary data

Supplementary data can be found at http://dx.doi.org/10.1016/j.bbapal.2013.02.011.

References


