Investigation into the role of protein phosphatase 4 catalytic subunit on the survival of leukaemic cell lines

By

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Dedication

To

My precious parents, I hope this achievement make you proud

My beloved husband and ever supportive, Hayder

My gorgeous daughter, Laalie, I love you to the moon and back.

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Abl	Abelson leukaemia viral oncogene
Akt	Protein kinase B (PKB)
ALL	Acute lymphocytic leukaemia
AML	Acute myelogenous leukaemia
APL	Acute promyelocytic leukaemia
ASK1	Apoptosis signal regulating kinase 1
Bcr	Breakpoint cluster region protein
CLL	Chronic lymphocytic leukaemia
CML	Chronic myelogenous leukaemia
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
HDAC3	Histone deacetylase 3
HL-60	Human acute promyelocytic leukaemic cell line
HPK1	Haematopoietic progenitor kinase 1
K562	Human chronic myeloid leukaemic cell line
MAPK	Mitogen activated protein kinase
MKK4	Map-Kinase-Kinase-4
mTOR	Mammalian target of rapamycin pathway
NF-κB	Nuclear factor-kappa B
PBS	Phosphate buffered saline
PDGFR	Platelet derived growth factor receptor
PEA-15	Phosphoprotein Enriched in Astrocytes
PI3K	Phosphatidylinositol 3- kinase
PP4	Protein phosphatase 4

PP4c	Protein phosphatase 4 catalytic subunit
PPP	Phosphoprotein phosphatases
RNA	Ribonucleic acid
siRNA	Small interfering RNA
TCR	T-cell receptor
TNF	Tumour necrosis factor
UV	Ultraviolet ray
γ-H2AX	γ-histone H2A variant X

Abstract

Background: Serine/Threonine protein phosphatase 4 (PP4) plays a crucial role in cell signalling, proliferation and apoptosis, and its catalytic subunit has been reported as an important regulator in many types of cancer, including breast and lung cancers.

Aims: Evaluate the effects of PP4c endogenous modulation in leukaemic cell lines; K562 for chronic myeloid leukaemia and HL-60 for acute promyelocytic leukaemia, on cell viability, apoptosis, and proliferation.

Methods: K562 and HL-60 cells were transfected with pcDNA3.1-PP4c to over-express or with specific siRNAs to down-regulate PP4c. Following transfection, cell viability, total apoptosis percentage, proliferation rate and cell cycle were assessed. The response of these cells to four different therapeutic drugs (imatinib, cisplatin, doxorubicin and rapamycin) was assessed post PP4c silencing.

Results: This study demonstrated that PP4c over-expression in K562 cells significantly reduce cell viability and cell proliferation, accompanied by a significant increase in apoptosis at 24 and 48 hours post re-plating. It also altered the cell cycle profile by increasing the number of cells in G0/G1 phase and reducing the cell number in S phase. On the other hand, PP4c silencing significantly increased cell viability in K562 cells at 48 hours. PP4c down-regulation also enhanced and promoted the action of both imatinib and rapamycin by increasing apoptosis in K562 cells. PP4c over-expression in HL-60 decreased the viable cell number at 48 hours, while PP4c down-regulation increased the viable cell number at 72 hours and altered the cell cycle profile by reducing the cell number in S phase and increasing the cells number in G2/M phase. PP4c silencing in HL-60 cells had no effect on the action of the chemotherapeutic drugs.

Conclusion: The results suggested that PP4c regulates cell proliferation, cell death, and cell cycle in K562 cell line in addition to enhancing their response to imatinib and rapamycin. Such studies are important to identify molecular regulators of the pathways involved in the pathogenesis of leukaemia and will contribute to greater understanding of the mechanism(s) by which phosphatases regulate cell survival in cancer and may allow design of optimised targeted therapies in the future.

1. General Introduction

Protein post translational modifications are known to play crucial roles in many cellular processes. One of the most biologically important post translational modifications is protein phosphorylation (Khoury et al., 2011). Reversible protein phosphorylation controls a wide spectrum of cellular processes, including gene transcription and translation, cell-division cycle, cell proliferation, apoptosis and invasion (Bauman and Scott, 2002).

Cancer cells are characterised by modifications in specific signalling pathways leading to the loss of tumour suppressor gene function, and the gain of unrestrained proto-oncogene expression and activity (Griffiths et al., 2000). Increased expression and activity of oncoproteins cause an abnormal increase in cell proliferation and survival accompanied with inhibition of apoptosis and differentiation, promoting the expansion of tumour cell clones (Perrotti and Neviani, 2013). The gain of oncogene function associated with loss of tumour suppressors is now widely accepted as a hallmark of cancer initiation and progression (Hanahan and Weinberg, 2011).

1.1. Leukaemia

Haematological malignancies are a group of heterogeneous diseases that are initiated by leukaemic stem cells that are able to self-renew and differentiate similarly to the normal haematopoietic stem/progenitor cells (HSCs) but in an abnormal way, i.e. characterised by the excessive build up or accumulation of either immature or abnormal blood cells (Orkin and Zon, 2008). Four large groups of haematological malignancies have been described; Acute Lymphocytic Leukaemia (ALL), Chronic Lymphocytic Leukaemia (CLL), Acute Myelogenous Leukaemia (AML), and Chronic Myelogenous Leukaemia (CML). They are classified depending on the stage of the disease (acute or chronic leukaemia), and immunophenotype of the cells (myeloid or lymphoid) (Warr et al., 2011).

1.1.1. **Chronic Myeloid Leukaemia (CML)**

Chronic myeloid leukaemia (CML) was the first human malignancy shown to be associated with a specific chromosomal aberration (Nowell & Hungerford, 1960). It is a clonal disorder of the pluripotent haematopoietic stem cells characterised by an increase in myeloid, erythroid cells and platelets in peripheral blood and marked myeloid hyperplasia of the bone marrow (Rowley, 1973). Clinically, CML is characterised by two distinct stages, an extended myeloproliferative disorder named the chronic phase (CML-CP) that progresses to a rapidly fatal blast crisis (CML-BC) (Calabretta and Perrotti, 2004).

The Philadelphia chromosome is the cytogenetic hallmark of CML. It is the product of the reciprocal translocation between chromosome 9 and chromosome 22, t(9;22)(q34;q11) (Groffen et al., 1984). This translocation results in the fusion of the breakpoint cluster region (Bcr) from chromosome 22 to the Ableson leukaemia virus (c-Abl) gene from chromosome 9 (Nowell and Hungerford, 1960; Groffen et al., 1984; Rowley, 1973). The Bcr-Abl fusion gene encodes for a 210 kDa protein, p210- Bcr-Abl and is another hallmark of CML, which displays constitutive tyrosine kinase activity and confers on leukaemic cells a growth and proliferation advantage, as well as resistance to apoptosis (Melo, 1996; Ren, 2005). Depending on the location of the break point on the Bcr locus, the product of the t(9;22) can also be a p190- or a p230- Bcr-Abl, which are normally detected in Philadelphia-positive B-acute lymphoblastic leukaemia (Ph1 ALL) and chronic neutrophilic leukaemia (CNL) respectively (Ravandi et al. ,1999; Pane et al., 1996; Wilson et al., 1997).

The unrestrained tyrosine-kinase activity of Bcr-Abl leads to the stimulation of different signalling pathways that transduce oncogenic signals leading to growth factor-independent proliferation, genetic instability, increased survival, altered differentiation of haematopoietic progenitors and resistance to apoptosis (Thielen et al., 2011; Druker, 2008;

Huang et al., 2011; Mancini et al, 2012). These pathways include RAS/MAPK (Sawyers et al., 1995; Thielen et al., 2011), PI3K/Akt (Varticovski et al., 1991; Skorski et al., 1997) and nuclear factor-kappa B (NF-κB) (Reuther et al., 1998; Asimakopoulos et al., 1999). In fact, the effects of Bcr-Abl transformation is mostly dependent on phosphorylation of key signalling molecules involved in RAS/MAPK, PI3K/Akt and NF-κB pathways (Thielen et al., 2011; Druker, 2008). Phosphorylation of these proteins control cell growth, survival and differentiation of haematopoietic cells by modifying the activity or expression of downstream effectors in their signalling pathways (Druker, 2008; Huang et al., 2011; Mancini et al, 2012).

Since the constitutive Bcr-Abl kinase activity is the characteristic cause of CML, drugs such as imatinib and other tyrosine kinase inhibitors, which target the kinase catalytic domain and inhibit it, are considered the most effective for CML treatment (Druker et al., 1996; Druker et al., 2001a; 2001b; Kantarjian et al., 2006; Talpaz et al., 2006). However, some patients fail to respond, or develop resistance, to this treatment due to intrinsic heterogeneity of the disease (Druker et al., 2006; Irvine et al., 2010). Resistance to the kinase inhibitors could be the result of Bcr-Abl 1 amplification or over-expression and mutations in the ATP-binding domain and catalytic domain activation loop (Hughes et al., 2003; Druker et al., 2006). Thus, many studies are now dedicated to dissecting the signalling pathways involved in the pathogenesis of CML. This could lead to the development of novel therapeutic targets needed in order to improve the survival and long-time cure of CML patients (Irvine et al., 2010).

1.1.2. **Acute Promyelocytic Leukaemia (APL)**

Acute myeloid leukaemia (AML) is a heterogeneous group of genetically and phenotypically aggressive disorders where the differentiation of haematopoietic progenitors (HPs) is blocked, increasing their self-renewal ability and disturbing the normal regulation of proliferation (Frohling et al., 2005). AML is classified based on morphology, immunophenotyping, genetics and clinical manifestations, into four main groups: AML with recurrent genetic abnormalities, AML with myelodysplasia (MDS)-related changes, therapy-related AML and MDS or AML that does not fit into any of these groups (Estey and Döhner 2006; Mrózek et al., 2007). In this type of leukaemia, constitutive activation of the PI3K/Akt signalling pathway is frequently detected in approximately 50–70% of patients (Martelli et al., 2006). Generally, acute myeloid leukaemia is characterised by mutations and deregulation of various types of tyrosine kinases such as c-KIT (CD117; a transmembrane protein encoded by the oncogene c-KIT), platelet derived growth factor receptor (PDGFR) and receptors Fms-like tyrosine kinase (Flt-3) (Smith et al., 2011).

In 1957, Leif Hillestad was the first who described APL as a unique subtype of AML that has a distinct morphological appearance, caused by an arrest of leukocyte differentiation at the promyelocyte stage, and associated the pattern of an acute leukaemia with coagulopathy and some clinical features including fibrinolysis, hypofibrinogenemia and catastrophic haemorrhage (Hillestad, 1957). The unique morphology of APL has both classical hypergranular and variant microgranular forms and they are characterized by a balanced translocation between the long arms of chromosomes 15 and 17, [t(15;17)(q22;q21)], giving rise to a unique fusion gene PML-RARa (RARa: retinoic acid receptor-a), an abnormal chimeric transcription factor, which disrupts normal myeloid differentiation programs (Rowley et al., 1977; Testa et al., 1978). The

discovery and elucidation of the molecular pathogenesis for APL has turned it from being once considered ‘the most malignant form’ to 'the most curable form' of AML (Lo-Coco et al., 2016).

According to European registries, APL represents 3-13% of AML cases (Grimwade et al., 2010; Lehmann et al., 2011) and its therapy has improved over 50 years of research from traditional chemotherapy started in the mid-1960s, through to the empirical discovery of targeted therapies including all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) agents that directly target the molecular foundation of the disease, in which a complete remission can occur in most APL patients (Lo-Coco et al., 2016).

1.2. **Hallmarks of Cancer**

Cellular homeostasis is maintained through signalling pathways that promote survival and growth, while preventing unrestrained proliferation by inducing cell senescence and apoptosis in response to DNA damage and other factors such oxidative stress and ageing.

In 2011, Hanahan and Weinberg described the existence of eight hallmarks of cancer, which are defined as distinctive and complementary abilities that permit the growth of tumours and the development of metastasis (Hanahan and Weinberg, 2011). The eight hallmarks of cancer are detailed in the following sections.

1.2.1. **Self-sufficiency in growth signals**

In contrast to normal cells that require growth molecules to divide and grow, cancer cells do not respond to these signals that regulate cell growth and division and instead maintain prolonged proliferation even without any external growth signal (Hanahan and Weinberg, 2011). This results in overproduction of growth factors, which in turn interact with cell-surface receptors leading induction of the tyrosine kinase activity of these receptors, including transforming growth factor-β (TGF-β) (Cheng et al., 2008a), constitutively

activating signalling pathways leading to altered gene expression, such as the NF-κB signalling pathway, which is reported in several cancer types (Karin et al., 2002). Moreover, in cancer cells the alterations in cell cycle due to the activation of these signalling pathways, initiate uncontrolled cell division and growth because of the alteration of the proteins themselves that control the timing of the events in the process so that each individual process is turned on and off at an appropriate time (Cheng et al., 2008a, Hanahan and Weinberg, 2011).

1.2.2. **Insensitivity to growth-inhibitory (antigrowth) signals**

The second hallmark involves evading growth suppressors. In order to sustain cell proliferation, even when the cell has severe abnormalities, cancer cells must avoid the actions of tumour suppressors, which are responsible for the negative regulation of cell proliferation such as TP53 (also known as p53) and RB proteins (retinoblastoma-associated protein) and its two relatives, p107 and p130. In cancer, these tumour suppressors are mutant or altered so that they lose their signalling abilities as cell division controllers so the cancer cells continue to divide and consequently they accumulate even more damaged DNA (Hanahan and Weinberg, 2011). On the other hand, normal cells show contact inhibition, so that they will stop dividing when they are confluent and start touch each other, while cancer cells lose this characteristic and will grow and divide continuously despite the overgrowth that surrounds them, causing a large mass of cells to form (McClatchey and Yap, 2012).

1.2.3. **Evasion of programmed cell death (apoptosis)**

Defects in programmed cell death or apoptosis is considered as one of the essential changes in cells that cause malignant transformation, and its resistance plays a critical and a vital role in carcinogenesis (Kerr et al., 1972; Norbury and Hickson, 2001). Normal cells switch on molecular pathways of apoptosis in response to stress-inducing events such as DNA damage, while cancer cells develop resistance to cell death machinery regardless of their abnormalities, thereby resulting in continuous cell growth (Ghobrial et al., 2005). This is carried out by the alteration of apoptosis signalling pathways that detect the damage or abnormalities by disrupting the balance of pro-apoptotic and anti-apoptotic proteins, reducing caspase function or by death receptor signalling impairment (Elmore, 2007).

1.2.4. **Limitless replicative potential**

Another hallmark of cancer is enabling replicative immortality; most normal cells possess cellular mechanisms that limit cell division to a finite number, blocking further growth (Hanahan and Weinberg, 2011). This is called the Hayflick limit or Hayflick phenomenon, and represents a finite number of times that normal human cells will divide before they become senescent or die, due to the shortening of telomeres (specific DNA-protein structures found at both ends of each chromosome) slightly with each cell division (Hayflick, 1965). Normal cells can divide only about fifty times before they die; this is related to their ability to replicate DNA only a limited number of times while many lines of evidence indicate that in cancer cells, telomerase, an enzyme that maintains telomere length, is activated (Blasco, 2005; Shay and Wright, 2000). By extending telomeric DNA, telomerase is able to prevent the progressive telomere erosion that would otherwise occur in its absence, allowing cell proliferations to continue (Blasco, 2005, Shay and Wright, 2000; Hanahan and Weinberg, 2011).

1.2.5. **Sustained angiogenesis or inducing angiogenesis**

Tumour angiogenesis is a very important multistep process that involves signalling input from several pro-angiogenic growth factors (Hicklin and Ellis, 2005). The process is crucial for continuous cancer growth and metastasis by providing increased oxygen, nutrients and growth factors to benefit cancer cell growth (Hicklin and Ellis, 2005). In other words, when the tumour grows larger, the cancerous cells produce new vasculature by increasing the production of growth factors which induce formation of new capillary blood vessels to supply the cells in the middle of the tumour with nutrients and oxygen (Hanahan and Weinberg, 2011).

1.2.6. **Tissue invasion and metastasis**

Another cancer hallmark that distinguishes malignant tumours from benign tumours is metastasis. In contrast to normal cells, cancer cells have the ability to penetrate the blood vessel walls and spread from an initial or primary site to a different or secondary site due to genetic alterations that develop their ability to proliferate rapidly and indefinitely (van Zijl et al., 2011). Activating invasion and metastasis processes encompass molecular changes in the way cells attach to other cells and extracellular matrix components (Hanahan and Weinberg, 2011). This is usually initiated through local tissue invasion, intravasation, transition through blood and lymphatic system and colonisation of foreign tissue (Hanahan and Weinberg, 2011).

1.2.7. **Reprogramming energy metabolism**

In order to maintain unrestrained proliferation, cancer cells alter their energy production by reprogramming glucose metabolic rate via aerobic glycolysis, or the Warburg effect, a very important process for cancer cell growth, migration and invasion (López-Lázaro, 2008). The cancerous cells up-regulate glucose transporters and glycolytic enzymes such as hexokinases (HK1 and HK2) by the action of the transcription factor, Hypoxia-inducible factor-1α (HIF1α), to accelerate cell proliferation and survival (Hanahan and Weinberg, 2011). On the other hand, the role of some pro-apoptotic Bcl-2 family proteins, such as BAD, in the regulation of reprogrammed metabolism has been reported (Danial et al., 2003). Up-regulation of the glucose transporters activates many Bcl-2 family proteins, increasing their expression and subsequently preventing apoptosis (Danial et al., 2003).

1.2.8. **Evading immune destruction**

Immune surveillance is the process responsible for preventing tumour formation in the body by eliminating the vast majority of cancer cells before forming a tumour mass (Hanahan and Weinberg, 2011). Cancer cells can escape the immune system and consequently, become immune to its elimination processes (Teng et al., 2008). Studies have suggested that the immune system can block tumour formation and progression in non-virus induced cancer (Hanahan and Weinberg, 2011). Others supported the antitumoral immune responses in patients with colon and ovarian tumours that are infiltrated with CTLs and NK cells, having a better prognosis than those that lack such abundant killer lymphocytes (Nelson, 2008).

Figure 1.1 illustrates the eight hallmarks of cancer.

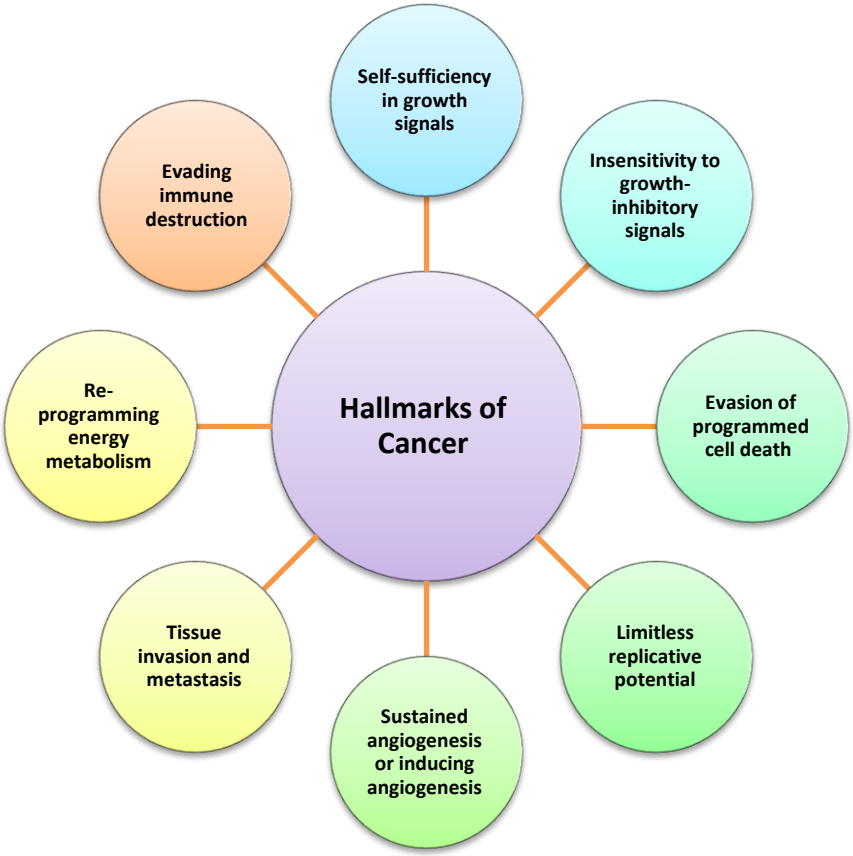

Figure 1.1 The hallmarks of cancer. The eight hallmark capabilities of cancer as illustrated by Hanahan and Weinberg, 2011.

1.3. Programmed cell death (Apoptosis)

Maintaining self-sufficiency in proliferation and/or growth signals and limitless replicative potential require cancer cells to withstand stimuli that promote the initiation of cellular apoptosis. The most obvious strategy for cancer therapy is to target the lesions that suppress apoptosis in tumour cells, this and mechanisms responsible for drug resistance and endogenous mechanisms of apoptosis evasion, continue to be an interesting area for research.

Apoptosis is a tightly regulated process, it was first defined in 1972 by Kerr and colleagues as spontaneous cell deletion which occurs either normally i.e. aging, or as a defense mechanism in some pathological conditions such as cancer (Kerr et al., 1972). It is

considered the most common type of programmed physiological cell death (PCD) and is essential during stages of embryonic development and throughout the lifespan (Green and Evans, 2002). Apoptosis plays a crucial role in the maintenance of tissue homeostasis, eliminating unfit cells from the body and balancing cell proliferation (Claveria et al., 2013; Czabotar et al., 2014), as well as playing an important role in the regulation of immune system function (Jacobson et al., 1997; Baehrecke, 2002). Therefore, any abnormality in this type of programmed cell death will result in various pathological problems, including cancer (Okada and Mak, 2004). Apoptosis is a complex and energy-dependent process characterised by distinctive morphological changes and biochemical mechanisms. The morphological changes include; rounding of the cell, cell shrinkage leading to chromatin condensation, nuclear DNA fragmentation and extensive plasma membrane blebbing, and the shedding of apoptotic bodies, vacuoles containing cytoplasm and intact organelles (Kerr et al., 1972; Hacker, 2000; Green, 2005).

Apoptosis has two main signalling pathways: the intrinsic (also called mitochondrial pathway) and the extrinsic (also called death receptor pathway; acts by binding to cell surface death receptors, such as tumor necrosis factor-α (TNF1α), Fas (CD95/APO1) and TNF related apoptosis inducing ligand (TRAIL) receptors), whilst the two pathways are also linked and can be influenced/activated by each other (Cowling and Downward, 2002; Elmore, 2007). Stimulation of both pathways results in the activation of caspases; a family of inactive proenzymes (cysteine proteases) that have proteolytic activity and the ability to cleave proteins next to aspartate residues (Degterev et al., 2003; Green, 2005). Caspase activation leads to the permeabilisation of the mitochondrial membrane, chromatin condensation and DNA fragmentation, therefore leading to cell death (Green, 2005). Once a caspase is activated, it can lead to the activation of other procaspases by cleavage at identical sequences to initiate a protease cascade, therefore, the apoptotic signalling

pathway is amplified leading to many of the morphologic changes (Degterev et al., 2003). Numerous caspases have been identified and classified according to their roles. Caspases that have known roles in apoptosis are divided into two subgroups; the initiator caspases (caspase- 8 and -9) and effector or executioner caspases (caspase-3, -6, and -7). Caspases that have known roles in inflammation are caspase-1, -4, -5, -11, and -12, whilst the roles of caspase-2, -10, 13, and -14 are not well categorised (Elmore, 2007; Mcllwain et al. 2013).

Many important proteins including Bcl-2 family proteins that control apoptosis are reversibly phosphorylated. The activity of these proteins is determined by their phosphorylation status which is the result of the balance between the activities of protein kinases and phosphatases (Ruvolo et al., 1998). The Bcl-2 family, which consists of 25 genes including Bcl-2 itself, BAD, Bid and Bim, have the ability to inhibit or induce apoptosis by governing mitochondrial outer membrane permeabilisation (MOMP) (Chipuk and Green, 2008). Another essential protein involved in apoptotic signalling pathways is cytochrome c (Cyt c) (Huttemann et al., 2011). It is a small haemoprotein located in the inner membrane of the mitochondrion and has many important roles besides its intermediate role in apoptosis; for example in oxidative phosphorylation or as a radical scavenger (Huttemann et al., 2011). By the action of some Bcl-2 family members, especially the pro-apoptotic Bax and Bak, Cyt c releases from mitochondria into the cytosol where it binds with the apoptotic protease-activating factor 1 (Apaf-1), and subsequent formation of the apoptosome, leading to activation of caspase 9 and initiation of a caspase cascade, including the activation of the effector caspases 3 and 7, which regulate the cell death process (Figure 1.2; Lopaz and Tait, 2015; Cardone et al., 1998; Wang, 2001).

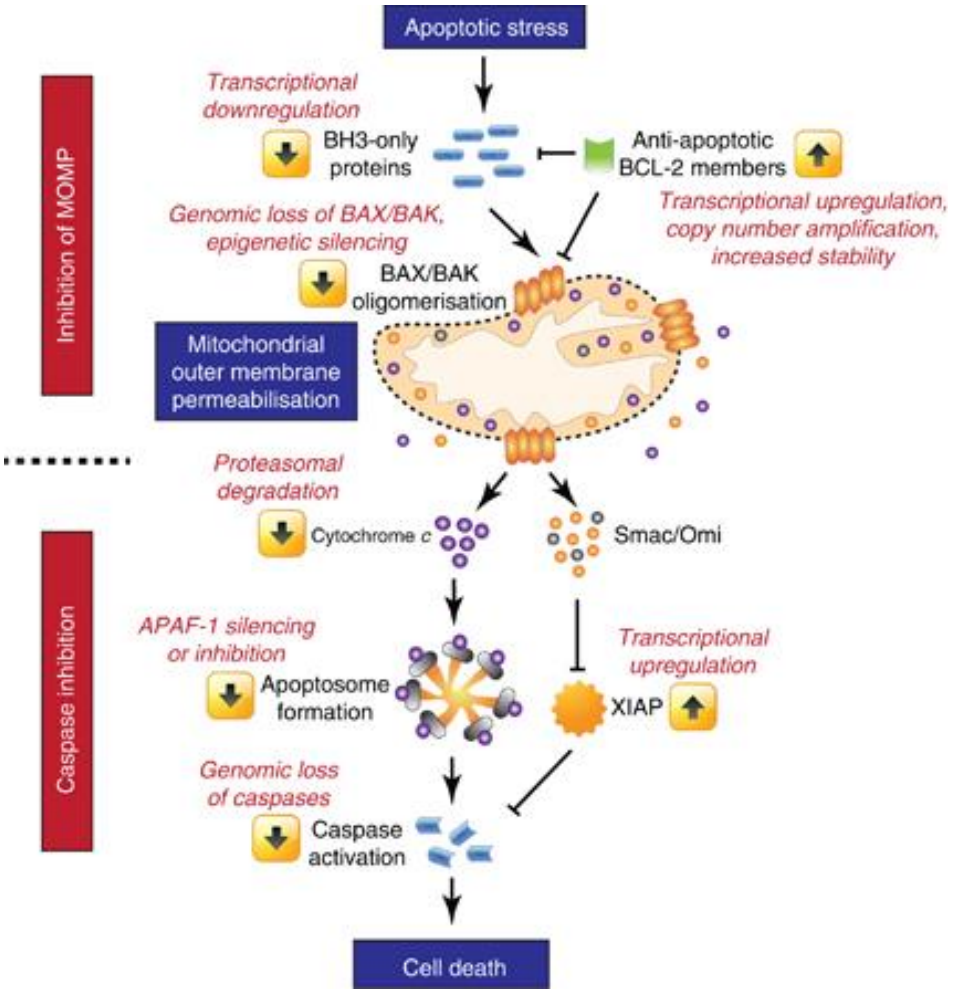

Figure 1.2 Intrinsic or mitochondrial apoptosis pathway. Death stress signals induce death receptors from extrinsic pathway that affects the intrinsic pathway by promoting the accumulation of BH3-only proteins leading to oligomerisation of BAX/BAK, mitochondrial outer membrane permeabilisation (MOMP) and release of Cytochrome c and Smac/Omi proteins. Cytochrome c leads to caspase activation and apoptosis (From Lopez and Tait, 2015).

1.4. **Reversible protein phosphorylation**

Mammalian cells must have the ability to adapt to external stimuli in order to survive when conditions change and a way in which cells manage this is the post-translational modification of their proteins. One form of which is reversible protein phosphorylation, which is able to cause distinct changes in the structure and activity of proteins and is considered as the most important type of post-translational modification used in signal transduction (Khoury et al., 2011). It is believed that approximately one third of cellular proteins are partly regulated via phosphorylation. Reversible phosphorylation controls metabolism, gene transcription and translation, cell-cycle progression, cell proliferation, cell differentiation, apoptosis and invasion (Virshup and Shenolikar, 2009).

1.4.1. **Kinases and phosphatases: types and roles**

A protein can be phosphorylated by kinases and dephosphorylated by phosphatases, therefore this is a reversible reaction and for each phosphorylation of a protein that can occur by a kinase, a phosphatase exists with the ability to dephosphorylate that same protein. This process of phosphorylation occurs mainly on three hydroxyl-containing amino acids, serine, threonine, and tyrosine, with serine being the predominant target (Olsen et al., 2006). The mechanism of action of phosphatases is the ability to nucleophilically attack phosphate groups that exist on the hydroxyl-containing amino acid residues of a phosphorylated protein in the presence of water resulting in the de-phosphorylation of that residue (Seshacharyulu et al., 2013). It has been established that there are more kinases in existence within cells than there are phosphatases, therefore one phosphatase will be responsible for reversing the activity of more than one kinase (Ubersax and Ferrell, 2007).

Five hundred and eighteen putative protein kinase genes were found in the sequence of the human genome, these kinases were classified into two families; 90 Tyrosine kinases (PTKs) and 428 serine/threonine (Ser/Thr) kinases (PSKs) (Lander et al., 2001; Venter et al., 2001; Johnson and Hunter, 2005). On the other hand, only 125 putative Protein Tyrosine Phosphatases (PTPs) and 30 Protein Serine/Threonine Phosphatases (PSTPs) were identified (Alonso et al., 2004; 2016; Alonso and Pulido, 2016), and within these two classes, further sub-classes exist. Protein Serine/Threonine phosphatases can be classified based on their amino acid sequence homology, structures and biochemical properties (e.g. metal dependence) into three distinct gene families; phosphoprotein phosphatases (PPPs), metal ion-dependent (Mg^{2+} or Mn^{2+}) protein phosphatases (PPMs), and aspartate-based phosphatases (Figure 1.3; Zhang et al., 2013; Eichhorn et al., 2009; Shi, 2009a).

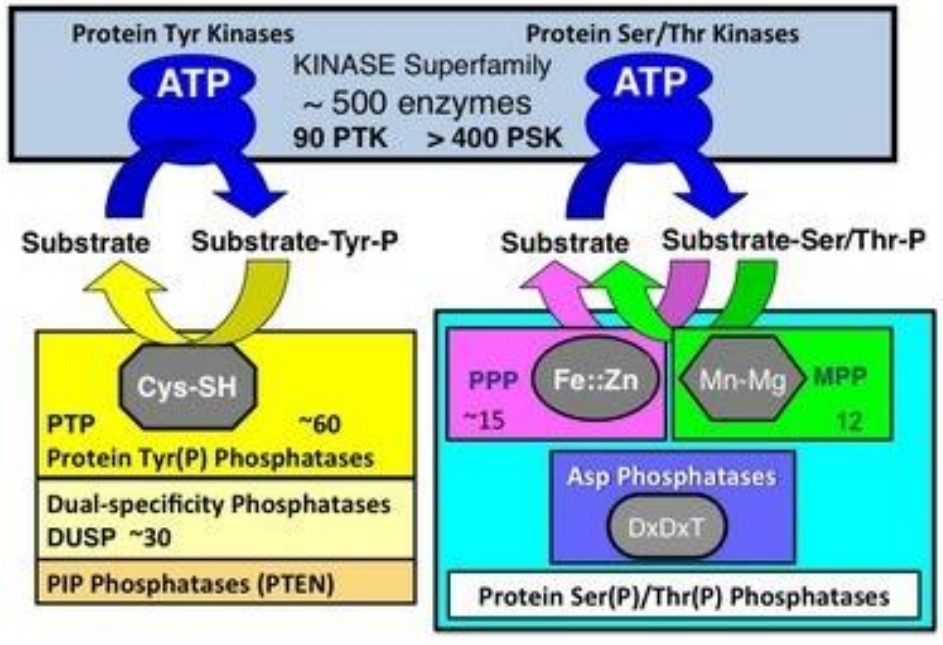

Figure 1.3 Protein kinases and protein phosphatases. Protein kinases divided into Tyr kinases (PTK 90 enzyme) and Ser/Thr kinases (PSK>400 enzyme). Protein phosphatases divided into Tyr phosphatases (PTP) and Ser/Thr phosphatases (PSTP). The PTP family subdivided into different groups (PTP, DUSP and PTEN), while Ser/Thr phosphatases are classified into three families; PPP, PPM, and aspartate-based phosphatases (From Brautigan, 2013).

1.4.2. **Kinases and cancer**

Ser/Thr protein kinases (STK) have been considered as the largest group of kinases that encode about 2% of proteins in the genomes of most eukaryotes and play a crucial role in every aspect of cellular function, such as cell cycle, proliferation and survival (Krupa et al., 2004; Cohen, 2002). They act as key mediators of many signalling pathways, including cancer pathways and consequently they are found to be one of the best anticancer-drug targets (Krupa et al., 2004; Cohen, 2002; Han and McGonigal, 2007; Hardie, 2007). Tyrosine kinase activity enhancement is a hallmark of many types of cancers (Blume-Jensen and Hunter, 2001); likewise, serine/threonine kinases have also been implicated in human cancer (Freeman and Whartenby, 2004). For example, the deregulation of the mitogen-activated protein kinases (MAPK) family has been associated with tumour growth, metastasis, and poor clinical outcome (Freeman and Whartenby, 2004). CML is characterized by the activation of the Bcr-Abl fusion gene that leads to the activation of many vital pathways including PI3K/Akt, RAS/MAPK, STAT5 and NF-κB pathways (Figure 1.4; Zaharieva et al., 2013; Druker, 2008; Thielen et al., 2011; Jacquel et al., 2003). These signal transduction pathways become constitutively activated leading to increased cell movement, uncontrolled cell proliferation and consequently cell death inhibition (Jacquel et al., 2003; Thielen et al., 2011). When Bcr-Abl oncogene interacts with the growth factor receptor-binding protein (Grb-2)/Gab2 complex, this results in the activation of Sos (Son of Sevenless), a guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor of Ras; that leads to the continuous Ras activation (Zaharieva et al., 2013; Thielen et al., 2011). In addition, the constitutive activation of the signal transducer and activator of transcription 5 (STAT5) has been reported in leukaemia (Jacquel et al., 2003). Bcr-Abl activates PI3K/Akt pathway through various proteins such as Gab2, Grb2 and Cbl,

resulting in BAD phosphorylation and inactivation of the anti-apoptotic protein Bcl-2 (Zaharieva et al., 2013; Thielen et al., 2011).

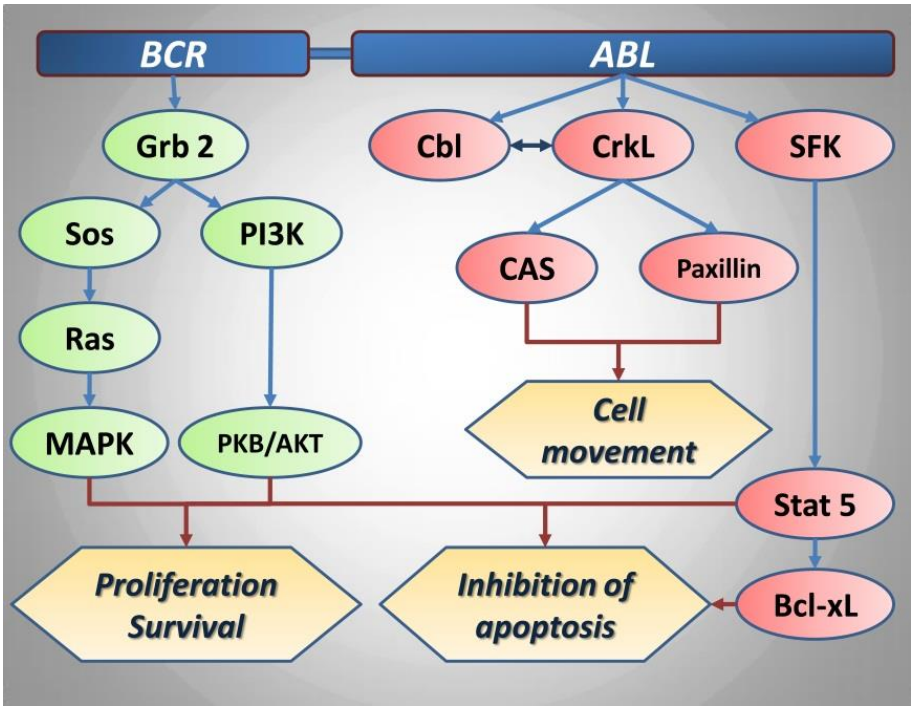

Figure 1.4 Bcr-Abl activates some downstream pathways. Bcr-Abl oncogene activates several important pathways such as PI3K/AKT, RAS, MAPK, and STAT5 leading to cell proliferation, survival and inhibit cell death (From Zaharieva et al., 2013).

Due to the expanded studies on how to inhibit their activity with small molecules, kinases are considered as extremely attractive anti-cancer therapeutic targets. Therefore, selective inhibitors that can block or modulate diseases with abnormalities in specific signalling pathways for many protein kinases such as Bcr-Abl and protein kinase C (PKC), have been developed and applied in cancer therapy (Scapin, 2002).

Imatinib mesylate (also called Gleevec®) is an example of such a kinase inhibitor, being the first of these used in clinical trials against Philadelphia chromosome positive CML (Druker, 2008). It is a highly potent cancer drug because of its activity as an inhibitor of the critical CML pathogenetic event; the Bcr-Abl tyrosine kinase; and thus it is active in

about 90% of patients with CML (Druker, 2008). Inhibition of Bcr-Abl tyrosine kinase activity results in inhibition of proliferation and induction of apoptosis in only Bcr-Abl - positive cells, while normal cells will not be affected (Peng et al, 2005). Furthermore, imatinib has also been successfully used in the treatment of gastrointestinal stromal tumours (GIST), where mutations in KIT produce ligand-independent constitutive activation of KIT (Also called CD117 or stem cell factor receptor; a tyrosine kinase receptor used as a tumour marker) (Din and Woll, 2008). It has been found that imatinib interrupts KIT-mediated signal transduction and PDGF-receptor tyrosine kinases in a manner analogous to its inhibition of Bcr-Abl (Peng et al, 2005; Savage and Antman, 2002).

1.4.3. **Phosphatases and cancer**

As they work oppositely to kinases, phosphatases are integral to various signalling pathways including kinase signalling pathways and protein-protein interaction (Chen et al., 2017). Protein phosphatases, including phosphatase and tensin homologue deleted on chromosome 10 (PTEN), Src homology 2 (SH2)-containing inositol 5′-phosphatase (SHIP) (Di Cristofano and Pandolfi, 2000), and the serine/threonine phosphoprotein phosphatase (PPP) family member PP2A (Shi, 2009b), have been reported in cancer.

An intracellular signalling pathway that exists in all types of cells, involving PI3K/Akt/mTOR contains three major junctions; i) Phosphatidylinositol 3-kinase (PI3K), ii) a serine/threonine kinase known as either Akt or PKB and iii) mammalian target of rapamycin (mTOR), a key regulatory pathway of many physiological processes such as cell growth, proliferation, and survival (Franke et al., 1997; Hennessy et al., 2005). In this pathway, the activation of PI3K results in phosphorylation and activation of Akt, and consequently leads to activation of the mTOR pathway (Franke et al., 1997; Hennessy et

al., 2005). Activation of the PI3K pathway has been identified as a critical event in tumour survival and development (Hennessy et al., 2005).

The phospholipid phosphatase PTEN, also called mutated in multiple advanced cancers (MMAC), dephosphorylates protein substrates on serine, threonine and tyrosine residues (Myers et al., 1997). It was identified as one of the most powerful and distinct tumour suppressors that is mutated in many types of cancers at high frequency (hence the name). Normally, it inhibits the PI3K/Akt signalling pathway by dephosphorylating phosphoinositide substrates such as phosphatidylinositol (3,4,5) tri-phosphate (PIP3), thus suppressing tumour formation (Stambolic et al., 1998; Sun et al., 1999). In other words, PTEN can suppresses the cancer formation process by different mechanisms including the inhibition of cell-division cycle progression, leading to arrest cell proliferation and increase programmed cell death (apoptosis) by blocking PI3K/Akt activation that activates Bad and Caspase-9 (Song et al., 2012). Therefore, many studies revealed that any PTEN mutation or loss of function leads to increased concentrations of PIP3, resulting in constitutive activation of downstream components of the PI3K signalling pathway, including the kinases Akt and mTOR leading to increased cell proliferation and reduced cell death that develops in different types of cancers such as breast, lung and prostate cancers (Song et al., 2012; Yin and Shen, 2008; Sansal and Sellers, 2004).

Another negative regulator of the PI3K/Akt signalling pathway is SHIP1. Similarly to PTEN, many studies suggested a tumour suppressor function of SHIP1 (Luo et al., 2003; Horn et al., 2004). The role of SHIP1 has been implicated in many types of leukaemias and lymphomas and a reduction in SHIP1 activity as a result of a mutation in a patient with AML has been reported (Luo et al., 2003; Gilby et al., 2007). Another study suggested that restoration of SHIP1 activity in Jurkat cells, a SHIP1 deficient leukaemic cell line, resulted in an increased transit time through the G1 phase of the cell cycle and consequently

reduced cell proliferation (Horn et al., 2004). Moreover, the low expression or absence of SHIP due to Bcr-Abl oncogene activity contributes to the pathogenesis of CML (Sattler et al., 1999).

The following sections will focus on the serine/threonine phosphoprotein phosphatases (PPPs) family, particularly serine/threonine phosphoprotein phosphatase 4, and their role in apoptosis and cancer.

1.5. **Phosphoprotein phosphatases Family (PPP)**

The phosphoprotein phosphatases family (PPP) is a group of enzymes responsible for dephosphorylating almost all the phospho-Ser/Thr residues in cells. A key defining feature of all the PPP members is that they are multimeric holoenzyme complexes and the complexity in their function lies well beyond the number of genes encoding their catalytic subunits (Zhang et al., 2013). While only 13 human genes encode PPP catalytic subunits, these are associated with several regulatory subunits (Cohen, 2009). Each catalytic subunit is able to combine with several regulatory subunits to maintain specificity and enormous diversity of PPPs functions. These phosphatase regulatory subunits do not share extensive sequence conservation and are identified by their physical association and function (Cohen, 2009). They determine the subcellular localisation, substrate specificity, and fine-tuning of catalytic subunit activity, conferring significant complexity and specificity to the PPP family (Virshup and Shenolikar, 2009).

In addition to the regulatory subunits, an increasing number of scaffolding and inhibitory proteins have been characterised and reported to control the activity of the PPP catalytic subunits (Virshup and Shenolikar, 2009). The PPP family consists of protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B), protein phosphatase 4 (PP4), protein phosphatase 5 (PP5), protein phosphatase 6 (PP6), and

protein phosphatase 7 (PP7). These phosphatases exhibit high amino acid sequence homology within their catalytic domains and are reported to share similar catalytic mechanisms (Table 1.1; Honkanen and Golden, 2002). It has been reported that PP1, PP2A, and PP2B share a common catalytic domain of 280 residues but they are contrasting and antithetical within their noncatalytic N- and C-terminal domains and consequently they are distinguished by their regulatory subunits to form a diverse variety of holoenzymes (Bradford et al., 1998). Other studies supported the above findings and revealed that PP1, PP2A, PP4 and PP6 are highly homologous phosphatases, but they differ in their N- and C-terminal domains (Figure 1.5; reviewed in Honkanen and Golden, 2002; Shi, 2009a). While the other three phosphoprotein phosphatases; PP2B, PP5 and PP7, have distinct structures. PP2B and PP7 contain an insert in the catalytic core, PP2B has a $Ca2^{+}$-calmodulin (CaM) binding site, PP5 has an extended N-terminal region with three tetratricopeptide (TPR) domains and PP7 has five EF- and EF-like hand domains in the C-terminal calcium binding domain (reviewed by Honkanen and Golden, 2002). Moreover, figure 1.5 shows the similarity in the characteristic motifs; GDxHG, GDxVDRG, GNHE, HGG, RH and H (G, glycine; D, aspartic acid; x, any amino acid; H, histidine; V, valine; R, arginine; N, asparagine; E, glutamic acid) of the all phosphoprotein phosphatases family members (Shi, 2009a).

The differences in the subunits' architecture gave each catalytic subunit of PPP family members' unique physiological roles in which influencing the catalytic activity, targeting it to a particular region of the cell and modulating substrate specificity (Honkanen and Golden, 2002). Table (1.2) summarises some of the characteristics of phosphoprotein phosphatases (PPP) family members.

Table (1.1) The catalytic domain sequence homology of PPP family members

	Amino acids sequence homology percentage	Reference
PP1	40% with PP2A	Eichhorn et al., 2009
PP2A	40% with PP1 65-66% with PP4 57% with PP6	Eichhorn et al., 2009 Chen et al., 2008 Chen et al., 1998
PP4	65-66% with PP2A	Chen et al., 2008
PP5	35-45% with other PPP members	Swingle et al., 2004
PP6	57% with PP2A	Chen et al., 1998
PP7	35% with other PPP members	Huang and Honkanen, 1998

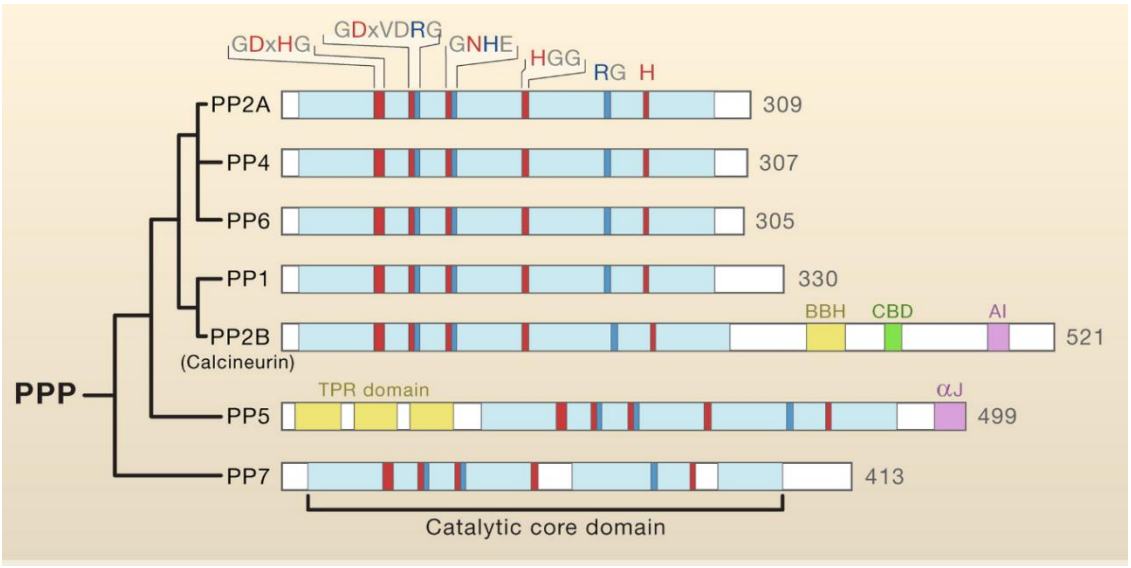

Figure 1.5 The Phosphoprotein Phosphatases (PPP) family's strucures. This figure shows that Ser/Thr Protein Phoshatases contain a common catalytic core domain, but they differ primarily in their N- and C-terminal domains. Moreover, PP2B has a Ca2+-calmodulin (CaM) binding site, PP5 contains an extended N-terminal region with three tetratricopeptide (TPR) domains while PP7 contains gaps inside the catalytic core domain. This figure is adapted from Shi, 2009a.

Table (1.2) The structural subunits of PPP family members (Adapted from Lee and Chowdhury, 2011; Moorhead et al., 2007).

	Genes encoding catalytic subunit isoforms	Catalyst subunit	Regulatory subunit	Subunit composition
PP1	3	Cα, Cβ, Cγ1, Cγ2	PP1-interacting proteins (PIPs, more than 90)	Heterodimer and higher order
PP2A	2	Cα, Cβ	Scaffolding A subunit (α, β), Regulatory B subunit (PR55, PR61, PR72)	Heterodimer and higher order
PP2B	3	PP3Cα, PP3Cβ, PP3Cγ	Calcineurin B or CNB	Heterodimer and higher order
PP4	1	C	PP4R1, PP4R2, PP4R3α, PP4R3β, PP4R4	Monomer and higher order
PP5	1	C	None	Monomer and higher order
PP6	1	C	PP6R1, PP6R2, PP6R3	Monomer and higher order
PP7	2	C	Unknown	Monomer

The role of reversible protein phosphorylation in different diseases, including cancer, has received a huge amount of interest in the last decades. While much of the attention has focused on modulating the activity of the kinases responsible for phosphorylation, the role that protein phosphatases play has received relatively little attention. Whilst the focus of this thesis is on the role of PP4, the role of the other PPP family members in apoptosis and cancer is outlined in the following section.

1.5.1. **Role of phosphoprotein phosphatases in apoptosis and cancer**

Most of protein phosphatase inhibitors such as okadaic acid, calyculin, cyclosporine A, FK506, and viral proteins (E4orf4) have been shown to induce apoptotic cell death via protein phosphatase inhibition (McCluskey et al., 2002). On the other hand, studies on the role of protein kinases and phosphatases in cellular signal transduction revealed that 16% of protein phosphatases are linked to human cancer (Forbes et al., 2015).

PP2A, one of the most abundant and best studied members of the PPP family, has a well-established role as a regulator of cell cycle progression and apoptosis (Sangodkar et al.,

2016). It has an essential role in controlling apoptosis by regulating both intrinsic and extrinsic apoptotic pathways (Sun and Wang, 2012), including by its interaction with caspase-3 (Allan et al., 2003) and by regulating the activity of other key players of apoptosis namely members of the Bcl2-family such as BAD and Bcl-2 (Marcellus et al., 2000; Shtrichman et al., 1999; 2000). Phosphorylation of BAD suppresses, and its dephosphorylation by PP2A promotes its pro-apoptotic activity (Chiang et al., 2001), while phosphorylation of Bcl-2 activates, and its dephosphorylation by PP2A suppresses its anti-apoptotic activity (Lin et al., 2006). Xu et al. (2013) have reported that the tyrosine kinase Src, which is able to prevent apoptosis by the inactivation of caspase-8, is also regulated by PP2A. PP2A inhibits the action of Src through its de-phosphorylation leading to the activation of caspase-8 resulting in increased apoptosis (Xu et al., 2013). Inhibition of its activity or loss of some of its functional subunits is a characteristic of neoplastic transformation; therefore PP2A is now widely designated as a tumour suppressor (Sun and Wang, 2012). The first evidence that PP2A was a tumour suppressor was based on studies where mice treated with okadaic acid, a specific inhibitor of PP2A, developed tumours (Fujiki et al., 1991).

PP2A has been found to regulate a number of pathways that are deregulated in cancer. It regulates the RAS-RAF-mitogen activated protein kinase (MAPK) signalling pathway by targeting multiple kinases in the pathway (Eichhorn et al., 2009). Studies using both PP2A and okadaic acid have also implicated PP2A in the inhibition of the Wnt signalling pathway (Perrotti and Neviani, 2013). Deregulation of the Wnt signalling pathway is involved in the control of cell growth and cell survival, and is believed to increase cell proliferation, often resulting in tumour formation (Perrotti and Neviani, 2013). The tumour formation is due to a member of the Wnt signalling pathway called β-catenin, which upon stabilisation, migrates and accumulates in the nucleus of cells, causing tumour growth

(Perrotti and Neviani, 2013). An increase in PP2A levels was found to correlate with an increase in β-catenin degradation and an increase in the PP2A inhibitor okadaic acid caused a decrease in β-catenin degradation (Perrotti and Neviani, 2013).

It is now well established that deregulation of Akt activity is associated with various human malignancies and that PP2A can regulate the activity of Akt by de-phosphorylating its Thr^{308} and Ser^{473} residues (Li et al., 2005; Sangodkar et al., 2016). Co-expression of both Akt and the catalytic subunit of PP2A resulted in a significant decrease in the activity of Akt (Li et al., 2005). In addition, the inhibition of PP2A by okadaic acid was found to increase Akt activity (Li et al., 2005). PP2A activity is lost in colorectal cancer as a result of its catalytic subunit PP2Ac hyperphosphorylation and the anti-tumoural effects of forskolin-mediated PP2A activation suggests that PP2A could be a promising therapeutic target in colorectal cancer (Cristóbal et al., 2014).

In addition to PP2A, other PPP family members are reported to be involved in controlling apoptosis and many other studies have linked them to cancer development (Klumpp and Krieglstein, 2002). Studies have also shown that PP1 inhibition by over-expression of PP1 inhibitor-1 resulted in the prevention of cardiomyocyte apoptosis (Nicolaou et al., 2009), whilst protein phosphatase 1 inhibitor 5 (IPP5) was characterised to be a potential growth inhibitor for cervical carcinoma cells (Zeng et al., 2012). Using systematic affinity proteomics analysis, it has been found that okadaic acid disrupts the specific interactions of PP1 and PP2A, inhibiting their activity, supporting the potential of therapeutics targeting them (Yadav et al., 2017). Moreover, Yadav et al., (2017) also describe the structural heterogeneity and the functional interaction that link phosphatases to the regulation of signalling pathways and to several diseases including distinct cancers; including brain tumours, and leukaemia. Down-regulation in PP2B activity was reported in squamous cell carcinoma of cervix with high risk HPV infection, indicating that perturbations in PP2B

signalling pathways may be involved in development of cervical neoplasia (Padma et al., 2005).

PP5 is involved in regulating the activity of the stress and DNA-damage-regulated protein kinases (Hinds and Sanchez, 2008). In response to either prolonged hypoxia or acute oxidative stress, PP5 binds to ASK1 (apoptosis signal regulating kinase 1) (Morita et al., 2001; Zhou et al., 2004); this interaction prevents the sustained activation of JNK (c-Jun-N-terminal kinase) and apoptosis by suppressing ASK1 dependent activation of MKK4 (Map-Kinase-Kinase-4) (Morita et al., 2001; Huang et al., 2004; Zhou et al., 2004). Additionally, PP5 overexpression blocks ASK1 activation and promotes tumour growth in a MCF-7 mouse xenograph model (Golden et al., 2004) and it is also reported that overexpression of PP5 prevented ASK1 activation and blocked apoptosis in p53 mutant rhabdomyosarcoma (Rh30) cell line by inhibiting the activity of the protein kinase mTOR using rapamycin, and thus inducing a stress response that includes activation of ASK1 (Huang et al., 2004). PP7 was also reported to act as a potent negative regulator of ASK1 (Kutuzov et al., 2010). Elevated levels of PP5 were detected in mantle-cell lymphoma and human breast cancer (Ghobrial et al., 2005; Atiye et al., 2005; Golden et al., 2008a; Golden et al., 2008b). Recently, it has been found that PP5 knockdown suppresses the proliferation and migration of hepatocellular carcinoma cells (Feng et al., 2015), human glioma cells (Zhi et al., 2015) and acute myeloid leukaemic cells (Li et al., 2016a).

PP6c silencing in HeLa cells, cervical carcinoma cells, showed more than a two fold increase in the level of apoptosis (MacKeigan et al., 2005). PP6c is overexpressed in high grade human ductal carcinoma in situ of the breast (Adeyinka et al., 2002), paediatric acute lymphoblastic leukaemia (Ross et al., 2003), pancreatic tumours (Sato et al., 2004), and is induced with androgen treatment of LNCaP prostate cancer cells (Nantermet et al., 2004). The regulatory subunit of PP6, PP6R1, is one of the top 20 genes overexpressed in

hepatocellular carcinoma and metastatic liver tumours (Tackels-Horne et al., 2001). In patients with glioblastoma multiforme (GBM), PP6 knockdown inhibits the glioblastoma growth and potentiates the sensitivity of GBM cells to radiation (Shen et al., 2011).

1.5.2. **PP2A role in leukaemia**

Numerous studies have highlighted the importance of protein phosphatases in leukaemia and their potential as targets for novel therapies (McConnell and Wadzinski, 2009; Neviani et al., 2005; 2007). In particular, PP2A has emerged as an important tumour suppressor in myeloid leukaemia and strategies aimed at reactivating this phosphatase, in addition to the inhibition of the tyrosine kinase activity, have shown great promise for a new generation of leukaemia therapies (McConnell and Wadzinski, 2009; Neviani et al., 2005; 2007).

Another study on B55α, a subunit of PP2A regulatory subunit which is identified as a specific regulator of the levels of Akt phosphorylation at Thr308, showed that this subunit was inhibited and loss of complete haematological remission in AML patients (Ruvolo et al., 2011). In CML, it has been reported that PP2A over-expression suppresses the activity of Bcr-Abl kinase, promotes apoptosis and enhances imatinib activity (Neviani et al., 2005; 2007). PP2A inhibition by Bcr-Abl is essential for the development of positive Philadelphia chromosome (Ph+) leukaemias including CML, and this occurs as a result of the over-expression of SET, a PP2A endogenous inhibitor (also called I2PP2A, TAF-1β or PHAP1) that was isolated from a patient with acute undifferentiated leukaemia (Neviani et al. 2005; 2007; Adachi et al., 1994). On the other hand, imatinib treatment inhibits Bcr-Abl that lead to reduced SET protein expression, which results in the reactivation of PP2A leading to dephosphorylation of several substrates such as ERK1/2, Akt, and BAD, which are shared by both Bcr-Abl and PP2A (Neviani et al. 2005; Calabretta and Perrotti 2004; Janssens and Goris 2001). Restoration of PP2A activity results in PP2Ac association with Bcr-Abl via the SHP1 tyrosine phosphatase, Bcr-Abl dephosphorylation, and proteasomal

degradation (Neviani et al. 2005). These findings suggested that SET-dependent inhibition of PP2A is required for the transduction of aberrant mitogenic, survival and anti-differentiation signals that contribute to the development of CML from the chronic phase into blast crisis (Neviani et al. 2005).

1.6. **Protein phosphatase 4 (PP4)**

In the last decades, few studies have highlighted the role of PP4 in apoptosis and diseases such as cancer, therefore, this research will focus on the structure, functions and roles of PP4, specifically in leukaemia.

1.6.1. **PP4 structure**

Protein phosphatase 4 (PP4), also known as PPP4, PPX, and PPH3, is a ubiquitous and highly conserved serine/threonine phosphatase. The PP4 holoenzyme consists of a catalytic subunit, PP4c, which interacts with different structural and regulatory subunits (Chen et al., 2008). PP4c was first cloned from a human teratocarcinoma library and named PPX by Brewis and Cohen (1992). The human PP4c is mainly localized to the centrosomes with a molecular mass of 35 kDa and consists of 307 amino acids (Hastie et al., 2000). Human PP4c is 100% identical to the mouse protein, differs from that of the rabbit by only two amino acids and shares 91% amino acid identity with the Drosophila PP4c (Hastie et al., 2000), suggesting a highly conserved protein of functional importance. The Human PP4c gene is located on chromosome 16 p11.2, which is linked to the translocational mutation associated with acute leukaemia (Bastians et al., 1997).

PP4c shares about 65-66% identity in the amino acid sequences with the PP2A α and PP2A β catalytic subunits, yet its regulatory subunits are very distinct from those of PP2A and this elucidates the difference between PP4 and PP2A and their roles in the regulation of the cellular functions (Chen et al., 2008; Mourtada-Maarabouni, and Williams, 2008; 2009).

Several PP4c-interacting proteins have been identified, including PP4 regulatory subunit 1 (PP4R1), PP4 regulatory subunit 2 (PP4R2), PP4 regulatory subunit 3 alpha (PP4R3α), PP4 regulatory subunit 3 beta (PP4R3β), PP4 regulatory subunit 4 (PP4R4), and alpha 4/ immunoglobulin (CD79A) binding protein 1 (α4/ IGBP1) (Hastie et al., 2000; Carnegie et al., 2003; Gingras et al., 2005; Chen et al., 2008). PP4R1 was one of the first regulatory subunits identified to interact with the catalytically active subunit PP4c (Kloeker and Wadzinski, 1999; Wada et al., 2001). Stable complex formation between PP4R1 and PP4c was further confirmed by independent proteomic screening approaches (Chen et al., 2008; Gingras et al., 2005). PP4R2 is predominantly located in the centrosomes in human cells suggesting that it could be involved in targeting PP4c to this location (Hastie et al., 2000). Other studies have suggested that PP4R1 and PP4R2 may form PP4c-R1 and PP4c-R2 complexes that become attached to a third variable subunit to form different PP4 complexes with different functions (Carnegie et al., 2003; Janssens and Goris, 2001). PP4R3 (also known as suppressor of Mitogen-activated Protein/extracellular signal-regulated Kinase (Smek) is also a regulatory subunit of PP4c (Gingras et al., 2005; Chowdhury et al., 2008). In Dictyostelium cells, overexpression of Smek and PP4c increased the nuclear accumulation of PP4c; leading to the suggestion that Smek regulates PP4c by controlling its localisation to the nucleus (Mendoza et al., 2005). In addition, Smek dephosphorylates the phosphorylated histone H2A variant X (γ-H2AX) in mammalian cells (Chowdhury et al., 2008) and enhances hepatic gluconeogenesis in vivo (Yoon et al., 2010). The fourth regulatory subunit characterised was PP4R4 which displays some sequence homology with PP2A scaffolding subunit A (Chen et al., 2008). Despite this homology, PP4R4 interacts only with PP4c and this interaction occurs in a direct manner and independently of other subunits (Chen et al., 2008). Finally, α4/IGBP1 is the only regulatory subunit of PP4c that is reported to bind to both PP4c and PP2Ac (Hastie et

al., 2000; Kloeker et al., 2003). McConnell et al. (2010) reported that α4/IGBP1 plays a role in the regulation of PP2Ac ubiquitination and protects it from proteasomal-mediated degradation, by acting as an adaptor protein that provides a platform for both PP2Ac and the E3 ubiquitin ligase Mid1 (Kong et al., 2009; McConnell et al., 2010). Depletion of α4/IGBP1 caused a rapid decrease in PP2Ac, PP4c and PP6c levels leading to the inhibition of the dephosphorylation of their substrates (Kong et al., 2009).

1.6.2. **PP4 functions**

The interaction between the different targeting regulatory subunits of PP4 appears to be central to PP4c function. PP4 plays an essential role in human centrosome maturation, cell migration (Martin-Granados et al., 2008), DNA damage checkpoint signalling (Nakada et al., 2008), and DNA repair (Lee et al., 2010). It is also involved in tumour necrosis factor (TNF)-alpha signalling, NF-κB regulation, activation of c-Jun N-terminal kinase (Zhou et al., 2002), nutrient sensing pathway (Bertram et al., 2000; Wu et al., 2004), apoptosis (Mourtada-Maarabouni et al., 2003; Mourtada-Maarabouni and Williams, 2008) and regulation of histone acetylation (Zhang et al., 2005). A study suggested that both excessive and insufficient activity of PP4c could be fatal to Human Embryo Kidney 293T cells (Mourtada-Maarabouni and Williams, 2008).

PP4c expression was reported to be altered in different tissues in the murine embryo, indicating a potential role in embryonic development (Hu et al., 2001). Knockout mice studies have confirmed a vital and non-redundant role for PP4c in ontogenesis and tissue development (Shui et al., 2007). In addition, PP4c gene deletion in the T-cell lineage resulted in abnormal thymocyte development and maturation, indicating that PP4c is essential for thymocyte development and pre-T cell antigen receptor (pre-TCR) signalling (Shui et al., 2007). Knockout mice studies have also identified an essential role for PP4c in

B cell development, since PP4c knockout mice showed a disruption in pro-B cell differentiation leading to a complete absence of mature B cells (Su et al., 2013).

Studies have also shown that PP4c plays an important role in the spliceosomal assembly via interaction with the survival motor neurons (SMNs) complex (Carnegie et al., 2003). PP4c is also reported to regulate the activity of Histone deacetylase 3 (HDAC3), a member of the human class I HDACs that regulates gene expression by deacetylation of histones and non-histone proteins (Zhang et al., 2005). In HeLa cells, the down-regulation and over-expression of PP4c showed that the activity of HDAC3 is inversely proportional to the cellular abundances of PP4c (Zhang et al., 2005).

PP4c is reported to act as a positive regulator of NF-κB activity in human cervical carcinoma SiHa (Yeh et al., 2004), whilst in T Lymphocytes, PP4c negatively regulates the activity of NF-κB (Brechmann et al., 2012). Such counteracting effects of PP4c functions have been related to the existence of different PP4c complexes that have different compositions as a result of interaction with its different regulatory subunits (Cohen et al., 2005); for example, the negative regulation of the activity of NF-κB in T lymphocytes has been linked to PP4c-PP4R1 complex, therefore, substrate specificities, subcellular localisations, activities and functions of PP4c depend largely on the composition of its different complexes (Brechmann et al., 2012). T cell receptor or tumour necrosis factor receptor 1 (TNFR1) stimulation leads to the phosphorylation of IκB kinase (IKK) complex followed by the proteasomal degradation of subsequent inhibitory κB proteins (IkBs) resulting in the translocation of NF-κB proteins to the nucleus, where they bind to DNA and activate the transcription of NF-κB-regulated genes (Hayden and Ghosh, 2008). Brechmann et al. (2012) have showed that PP4R1 potentiates the activity of PP4c and allows its association with IKK complex leading to its dephosphorylation and inactivation and consequently the inhibition of the aberrant NF-κB signalling activity. These findings

suggested that PP4R1 deficiency leads to inactivation of PP4c that causes PP4c disability to dephosphorylate the IKK complex, resulting in uncontrolled IKK phosphorylation and abnormal NF-κB activity (Brechmann et al., 2012).

1.6.3. **PP4 role in apoptosis, DNA damage repair and cancer**

Many studies have supported the hypothesis that PP4c may function as a tumour suppressor gene and implicated its role in the regulation of apoptosis (Mourtada-Maarabouni et al., 2003; Mourtada-Maarabouni and Williams, 2008; 2009). The first experiment that suggested that PP4 plays an important role in apoptosis was carried using mouse lymphoma W7.2c cells, where over-expression of PP4c resulted in an increase in cell death and a decrease in cell proliferation (Mourtada-Maarabouni et al., 2003). Subsequent studies on HEK 293T human embryonic cells and both leukaemic human T cells and primary human peripheral blood T-cells also showed that over-expression of PP4c enhanced apoptosis, while PP4c down-regulation suppressed apoptosis (Mourtada-Maarabouni and Williams, 2008; 2009). These studies also showed that modulation of PP4 expression affects the phosphorylation status of proteins that are involved in apoptosis and cell proliferation such as extracellular signal-regulated kinase 1 (ERK1), extracellular signal-regulated kinase 2 (ERK2) (Mourtada-Maarabouni and Williams, 2008) and Phosphoprotein Enriched in Astrocytes (PEA15), a member of the death effector domain (DED) protein family involved in regulating both ERK1 and ERK2 (Krueger et al., 2005, and Mourtada-Maarabouni and Williams, 2008). PEA-15 inhibits apoptosis through its death effector domain (DED) which interacts with the DED of other proteins such as apoptotic adaptor protein (FADD) and inhibits apoptosis via inhibiting assembly of apoptotic signalling complexes (Greig and Nixon, 2014). PEA-15 function is tightly regulated by phosphorylation; the anti-apoptotic effect of PEA-15 is stabilised by Akt/PKB, which promotes its phosphorylation (Toker and Newton, 2000). Previous studies

suggested that the level of PP4 influenced the gene mutation rate, which is crucial to oncogenesis (Mourtada-Maarabouni and Williams, 2008; 2009). PP4c over-expression reduced the mutation in the indicator gene, hypoxanthine phosphoribosyl transferase (hprt), whilst the PP4 down regulation increased the mutation frequency of this gene (Mourtada-Maarabouni and Williams, 2008; 2009). The PEA-15 gene has been found to be over-expressed in human breast cancer and other human cancers (reviewed in Krueger et al., 2005) and such over-expression was associated with a resistance to a broad range of anti-cancer drugs (Stassi et al., 2005). Akt, which phosphorylates and stabilises the anti-apoptotic action of PEA-15, is also up-regulated in breast cancer and the hyper activation of PI3K/Akt/mTOR pathway is implicated in the tumorigenesis of ER+ breast cancer and resistance to endocrine therapy, suggesting that Akt and PEA-15 might function cooperatively in breast tumorigenesis (Ciruelos Gil, 2014). A recent study suggested that modulating the levels and activities of PP4c and/or PEA-15 may have a novel role in the treatment of breast cancer (Mohammed et al, 2016). Mourtada-Maarabouni and Williams (2009) have also reported that the pro-apoptotic effects of PP4c in leukaemic cells was partly mediated by the dephosphorylation of PEA-15, providing evidence that the interaction between PEA-15 and PP4c may be critical in leukaemogenesis and/or leukaemia progression (Mourtada-Maarabouni and Williams, 2009).

On the other hand, other studies have supported the hypothesis of the role of PP4c in the progression of several cancers suggesting that PP4c might function as oncogene and this action could be a result of the deregulation in the expression or activity of PP4 regulatory subunits (Martin-Granados et al., 2008; Wang et al., 2008; Weng et al., 2012; Li et al., 2016b). It has been reported that depletion of PP4c in HEK293 cells resulted in severely decreased cell migration and suggested that PP4c complexes may coordinate centrosome maturation and cell migration via regulation of Rho GTPases (Martin-Granados et al.,

2008). The high expression of PP4c has been also reported in breast and lung tumours (Wang et al., 2008). In their study, Wang and colleagues (2008) also suggested that the inhibition of PP4c expression may increase the sensitivity of breast and lung cancer cells to cisplatin treatment (Wang et al., 2008). In addition, an immunohistochemical study on stage II pancreatic ductal adenocarcinoma (PDAC) samples revealed that PP4c over-expression is associated with poor prognosis and this over-expression was associated with a distant metastasis (Weng et al., 2012). This study also suggested that PP4c could act as a marker for the diagnosis of invasive PDAC and a new approach for the treatment of this disease via targeting its signalling pathways (Weng et al., 2012). Recently, a study suggested that PP4c also plays an oncogenic role in glioma's development and progression (Li et al., 2016b).

Studies have also implicated PP4 in DNA repair mechanisms. The loss of DNA repair systems that prevent the fixation of pre-mutagenic lesions in the genome is another major cause of oncogenesis in humans and the accumulation of these lesions is essential for carcinogenesis (Shimada and Nakanishi, 2013). The PP4c-PPP4R2 complex regulates the phosphorylation status of the replication protein A2 (RPA2) which is essential in the DNA double strand break (DSB) repair pathway (Davis and Chen, 2012). Dephosphorylation of RPA2 is essential for the continuation of post damage DNA synthesis and this in turn allows the cell to restart the cell cycle (Lee et al., 2010). In response to DNA double-strand breaks, the histone H2A variant H2AX is rapidly phosphorylated by ATR to produce γ-H2AX. γ-H2AX functions to stabilise cell-cycle checkpoint proteins and DNA repair factors at the break site (Chowdhury et al., 2008). A complex containing PP4c, PP4R2, and PP4R3β is reported to dephosphorylate γ-H2AX generated during DNA replication, a process required for DNA damage repair (Chowdhury et al., 2008). Another finding suggests PP4c is involved in controlling the proliferation and differentiation of neural

progenitor cells in the mouse neocortex by regulating the phosphorylation status of nuclear distribution protein nudE-like1 (Ndel1) (Xie et al., 2013).

The role of PP4c regulatory subunits in cancer is also studied. *In vitro*, PP4R1 knockdown inhibits cell proliferation and colony formation in breast cancer cells (Qi et al., 2015), hepatocellular carcinoma cells (Wu et al., 2015) and lung cancer cells (Zhu et al., 2016). A recent study suggested that insufficient PP4R2 may contribute to the pathogenesis of AML by increased DNA damage (Herzig et al., 2017). PP4R2 suppression results in de-phosphorylation impairment of phosphorylated DNA damage response proteins such as γH2AX and p53 and thus developed AML (Herzig et al., 2017).

1.7. **The aims of the study**

Many studies support the hypothesis that protein phosphatase 4 catalytic subunit (PP4c) may function as a tumour suppressor gene. PP4c has also been implicated in the regulation of the growth and survival of leukaemic T-cells and untransformed human peripheral blood T-cells (Mourtada-Maarabouni and Williams, 2008; 2009). While others suggest that PP4c might function as oncogene, since high expression of PP4c levels have been reported in breast and lung tumours (Wang et al., 2008) and pancreatic ductal adenocarcinoma (PDAC) (Weng et al., 2012).

Therefore, the overall aims of this study are:

- The evaluation of the role of PP4c in leukaemia, using two types of model system cell lines; K562 for chronic myeloid leukaemia and HL-60 for acute myeloid leukaemia.

The objectives are:

- To over-express and down-regulate PP4c in two types of leukaemic cell lines, K562 and HL-60 cells, and evaluate the effect on cell viability, apoptosis, and proliferation.
- To investigate the response of K562 and HL-60 cells to the first line therapeutic drugs post PP4c silencing. The functional effects of PP4c down-regulation on the cellular response to cisplatin, doxorubicin, imatinib (only with K562) and rapamycin was investigated by measuring changes in apoptosis and cell viability.

2. Materials and Methods

2.1. Materials

Roswell Park Memorial Institute (RPMI)-1640 medium (R0883), gentamicin (G1272), L-glutamine (200 mM solution), trypan blue (0.4%), dimethyl sulphoxide (DMSO), β-actin antibody (#A5441), horseradish peroxidase-conjugated (HRP-conjugated) anti-goat IgG secondary antibody (#A5420), doxorubicin hydrochloride (#25316-40-9) and Cis-Diammineplatinum (II) dichloride (cisplatin) (#15663-27-1) were purchased from Sigma-Aldrich. Imatinib mesylate (#A03-901B) was purchased from SignalChem and rapamycin (#1292) from TOCRIS bioscience. Nucleofection solution V (MIR 50115) was purchased from Mirus Bio LLC, USA, HiPerFect transfection reagent (#301704) was purchased from Qiagen. Scrambled or negative control siRNA (-siRNA) (#4605), PP4c siRNA1 (#105835) and PP4c siRNA2 (#105834), were purchased from Ambion, while PP4c siRNA8 (S102658698) and PP4c siRNA9 (S102658705) were purchased from Qiagen. All these siRNAs were annealed and ready to use. The primary anti-PP4c (PPX/PP4 (C-18), a goat polyclonal IgG, #sc-6118 & (C-6), a mouse monoclonal IgG, #sc-374106) were purchased from Santa Cruz Biotechnology. HRP-conjugated anti-mouse IgG secondary antibody (#P0447) was purchased from Dako. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega.

The Muse Count and Viability Kit (#MCH 100102), Muse Annexin V and Dead Cell Kit (#MCH 100105), Muse Cell Cycle Kit (#MCH 100106), and Muse Ki67 Proliferation Kit (#MCH 100114) were purchased from Merck Millipore, Germany. The 6, 24, and 96 well plates and tissue culture flasks were purchased from Greiner.

2.2. **Cell lines and routine cell culture**

Human chronic myelogenous leukaemia (CML) cell line K562 and acute promyelocytic (APL) cell line HL-60 were used in this study. The K562 cell line was established from the pleural effusion of a 53-year-old female with CML in terminal blast crises and is characterised by a positive Philadelphia chromosome (Lozzio and Lozzio, 1975), while HL-60 (a non-adherent human leukocyte cell line) was derived from a 36-year-old Caucasian female with APL (Collins et al., 1978).

Both cell lines were originally purchased from the ATCC (K562; CRL-343™ and HL-60; CCL-240™), cell lines were provided with a specification document containing STR profiles and expressed isoenzymes. Secondary stocks were generated and frozen down within two weeks receipt from the ATCC. Cultured cells were replaced from these secondary stocks after a maximum culture period of 2 months, with cellular morphology and growth characteristics monitored routinely throughout culture periods.

K562 and HL-60 cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mmol/L L-glutamine and 10 mg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO_2:95% air. K562 and HL-60 cells are suspension cells and they were sub-cultured three times per week at 2×10^5 cells/mL for K562 cells and 3×10^5 cells/mL for HL-60 cells.

2.3. **Freezing and thawing of the cell lines**

For long term storage of the cells, 1×10^6 cells were harvested and re-suspended in 1.0 mL of cryoprotectant medium comprising 40% FBS-supplemented RPMI-1640 medium with 10% dimethyl sulphoxide (DMSO). The cell suspension was transferred into a cryo-tube and immediately stored at -80 °C or at -140°C in liquid nitrogen for long term storage.

For thawing cryopreserved cells, cells were rapidly thawed at 37°C and immediately re-suspended in 10 mL of complete RPMI-1640 medium to dilute the DMSO, then centrifuged at 1200×g for 5 minutes and the cell pellet re-suspended in an appropriate volume of fresh culture medium (section 2.2).

2.4. **Plasmid preparation and purification**

Prior to the current study, the PP4c EST (accession #BG913014), containing the full length cDNA encoding the catalytic subunit of PP4, was directionally cloned into pcDNA3.1 using the EcoRI and XhoI restriction sites of the vector. Recombinant (pcDNA3.1-PP4c) and native (pcDNA3.1) vectors were transformed into *E.coli* by electroporation and positive (ampicillin resistant) colonies isolated; insert incorporation into the recombinant vector was confirmed by sequencing (MWG) using vector-specific primers, and glycerol bacterial stocks prepared for long-term storage at -80C (Mourtada-Maarabouni et al., 2003).

The preparation and purification of the plasmids pcDNA3.1 and pcDNA3.1-PP4c were carried out using the Qiagen® Plasmid Maxi Kit. Briefly, around 10 µL of frozen bacterial glycerol suspension was added to a starter culture of 2-5 mL Lennox broth (LB) medium containing 50 µg/mL of ampicillin and then incubated at 37°C for eight hours with vigorous shaking (at 300 rpm). The starter medium was then diluted in a ratio of 1/500-1/1000 in LB media containing 50 µg/mL ampicillin and incubated at 37°C for up to 16

hours with vigorous shaking (at 3000 rpm). The purification protocol was based on an alkaline lysis procedure; 100 mL of the LB culture bacterial cell were pelleted by centrifuging at 6000×g for 15 minutes at 4°C, then 10 mL of re-suspension buffer composed of 50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 μg/mL RNase A was added to homogenize the bacterial pellet. A further 10 mL of a lysis buffer composed of 200 mM NaOH and 1% SDS (w/v) was added and the samples mixed by vigorously inverting the tube 4-6 times, followed by incubation at room temperature (15-25°C) for 5 minutes. Plasmid DNA was then isolated by initially binding to a QIAGEN resin under low-salt and pH conditions by adding 10 mL from a chilled neutralization buffer composed of 3.0 M potassium acetate, pH 5.5, then the bacterial lysate was transferred to a barrel of the QIAfilter Cartridge and incubated at room temperature (15-25°C) for 10 minutes and then the lysate was filtered into a 50 mL tube. By a medium-salt wash (1.0M NaCl; 50 mM MOPS [free acid], pH 7.0; 15% isopropanol (v/v)), the column was washed twice (30 mL each wash) so that all impurities including RNA, proteins, and low-molecular–weight particles were removed. Plasmid DNA was then eluted with 15 mL of a high-salt buffer (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)) and then concentrated and desalted by 10.5 mL isopropanol precipitation followed by centrifugation ≥ 15000×g at 4°C for 30 minutes. The extracted plasmid DNA concentration and purity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), any samples with A260/A280 value that was less than 1.8 were discarded. The identity of the plasmid DNA was verified by a diagnostic digest and sequence analysis (MWG Biotech); sequencing results were checked against the ncbi entry for human PP4c (accession #BG913014).

2.5. **Plasmid DNA transfection**

The effects of PP4c over-expression on cell viability, proliferation and apoptosis in CML and APL were investigated by introducing the pcDNA3.1-PP4c expression construct or the empty pcDNA3.1 vector (negative control) into the K562 and HL-60 cell lines using nucleofection as a transfection method. (2×10^6 cells) were re-suspended in 0.1 mL nucleofection solution V containing 2 μg of plasmid DNA then transfected by Nucleofector ™ II 2b using programme T-016 for K562 cells and T-019 for HL-60 cells (see chapter 3 section 3.4.1). Cells were then seeded in 3.0 mL of pre-incubated growth medium in 6-well plates and incubated for 24 hours to allow recovery and PP4c expression, cells were then re-plated at 2×10^5 cells/mL for further functional assays (see section 2.7).

2.6. **RNA interference**

Small interfering RNA (siRNA), also called short interfering RNA or silencing RNA, are double-stranded RNA molecules, usually 20-25 pairs in length with phosphorylated 5' ends and hydroxylated 3' ends, which can be introduced into cells by various transfection methods to silence any gene. When the siRNA molecules enter the cell they incorporated into a siRNA-protein complex called siRNA induced silencing complex (RISC), which contains helicase activity that unwinds the two strands of the siRNA molecule (Zamore et al., 2000). The siRNA/RISC complex is responsible for the degradation of the target RNAs that contain homologous sequences to the siRNA (Figure 2.1; Tuschl, 2001).

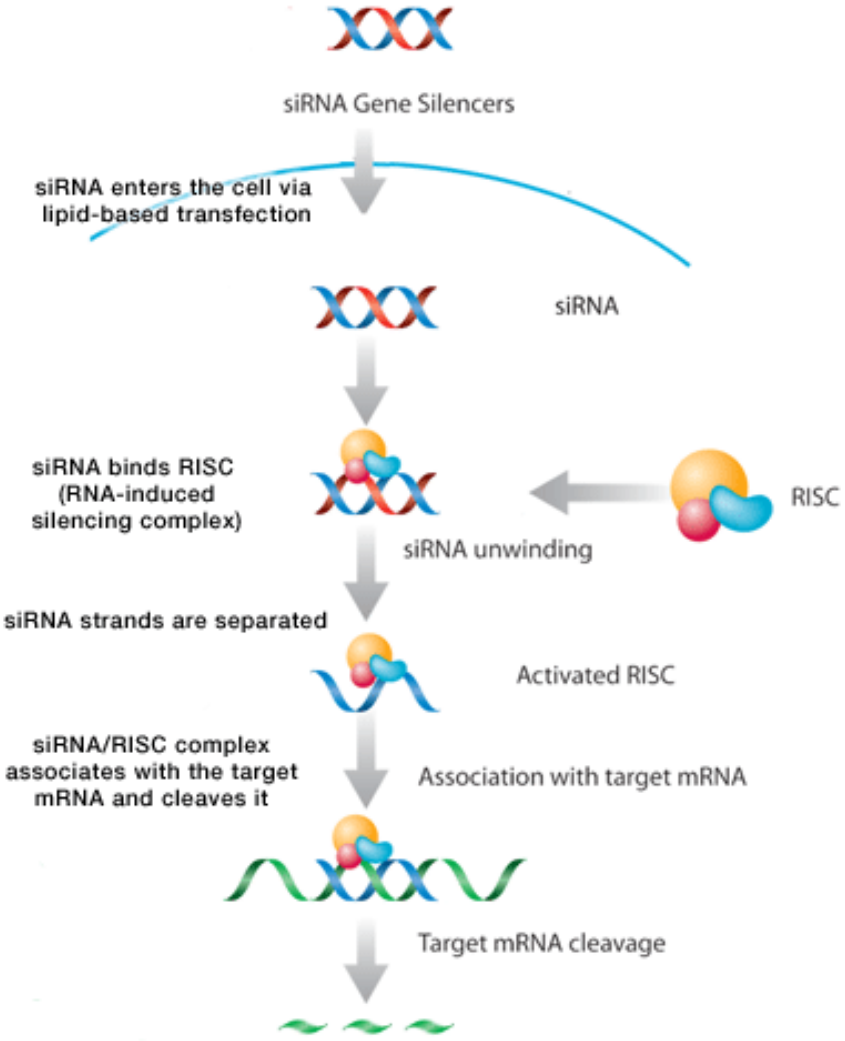

Figure 2.1 How do siRNA Gene Silencers work. From Santa Cruz Biotechnology Inc.

2.6.1. **K562 cell line**

In this study, the effects of PP4 down-regulation on the K562 cell viability, proliferation and apoptosis were also conducted using nucleofection as a transfection method. K562 cells were incubated in the log phase for 24 hours before 1-2×10^6 cells per transfection were harvested. Cells were re-suspended in 100 µL nucleofection V solution and then nucleofected using 50 nM of either the non-silencing negative control siRNA, PP4c siRNA1 (targeting exon 4 and 5), PP4c siRNA2 (targeting exon 7), PP4c siRNA8 (targeting exon 7, distinct sequence from PP4c siRNA2) or PP4c siRNA9 (targeting exon 6), using the Nucleofector ™ II 2b. The transfected cells were then transferred to 7.0 mL of pre-incubated complete growth medium in 25cm^2 flasks and incubated for 72 hours before being re-plated at 2×10^5 cells/mL for further functional assays (see section 2.7).

2.6.2. **HL-60 cell line**

HiPerFect transfection reagent (Qiagen) was used to introduce the siRNAs into HL-60 cells. Cells were seeded in 6 well plates at a density of 8×10^5 cells per well in 0.4 mL complete culture medium. A mixture of 0.4 mL of Opti-MEM™ medium, 12 µL HiPerFect transfection medium and 3.2 µL siRNAs (200 ng) were allowed to form transfection complexes for 5-10 minutes at room temperature and added dropwise to the cells. Plates were incubated for 6 hours before 1.6 mL of complete culture medium was added to each well and the plates incubated for 72 hours at 37°C in a 5% CO_2 humidified incubator (see chapter 3 section 3.4.2.2). The cells were re-plated in 6-well plates at 2×10^5 at 72 hours post transfection for further functional assays.

Previous studies investigating PP4c knockdown in HEK 293T, CEM-C7 and Jurkat cell lines, cells were analysed for PP4c expression by qRT-PCR and Western blotting 48 hours post-transfection, yielding a 80-90% decrease (Mourtada-Maarabouni and Williams, 2008; 2009). Further investigations prior to the current study also determined a comparable

reduction in expression levels at 48-72 hours post transfection and therefore cells were harvested at 72 hours post transfection in this study to ensure efficient knock-down of PP4c at the mRNA and protein level.

Whilst there is a 60-65% sequence homology between PP4 and PP2A, as well as homology with other PPP family members (see Chapter 1, Table 1), selectivity of the siRNAs towards only PP4c (hence precluding any off-target effects on down-regulation of other PPPs) is validated by the manufacturers, with no 'off-target' effects reported, even with high homology transcripts. Both Western blot and qRT-PCR techniques could be used in the current study to confirm the specificity of the PP4c siRNAs manually, however in-house validation (prior to the current study) similarly observed no effect of PP4c siRNAs on the expression levels of PP2A in Jurkat, CEM7 and MCF-7 cells, similarly there was no effect of PP2A siRNAs on the expression of PP4c (Mourtada-Maarabouni et al., 2003; Mourtada-Maarabouni and Williams 2008; 2009).

2.7. **Determination of cell viability**

2.7.1. Trypan blue dye exclusion

Viable cell number was determined by Trypan blue exclusion analysis in 6-well plates following plasmid DNA and siRNA transfection and re-plating after 24 or 72 hours, respectively, or in 24-well plates for drug treatment (see section 2.12), at 2×10^5 cells/mL. Using a haemocytometer, 10 µL of the cell suspension from each well of the plates were mixed with an equal volume of 0.4% Trypan blue dye and 10 µL loaded to the counting chamber with coverslip. The cells in the four corner 4x4 grids were counted as viable if they excluded the dye and dead if they stained blue. Trypan blue is taken up by all cells but is actively excluded by viable cells, which retain membrane integrity. The results were

represented either as the number of viable cells/mL ($\times 10^5$) or viability expressed as a percentage of the total cell number (% viability).

2.7.2. MTS assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is another cell viability method used in this research. It is a colorimetric method to determine the viability of cells in proliferation or drug dose response assays (The CellTiter 96® AQueous One Solution Cell Proliferation Assay user guide). The reagent contains MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), which acts as inner salt and PES (phenazine ethosulfate), which acts as an electron coupling reagent and enhances the chemical stability by forming a stable solution when combined with MTS. MTS tetrazolium is converted to a soluble formazen, this conversion is carried out by NADPH-dependent dehydrogenases (NADPH or NADH) and the quantity of this product is directly proportional to the number of living cells in culture. The assay was performed by adding 20 µL of the CellTiter 96® AQueous One Solution Reagent directly to cultures in 96 well plates and incubating the plates for 3 hours at 37°C in a 5% CO_2 humidified incubator. Absorbance was then measured at 490 nm on a 96-well plate reader and results expressed as cell viability as a percentage of untreated control cells after taking the blank wells values out of the values of all wells containing cell cultures (% viability).

2.8. **Determination of programmed cell death (Apoptosis)**

Programmed cell death or apoptosis was assessed by the Muse™ Annexin V and Dead Cell Assay kit. This method depends on the externalization of phosphatidylserine (PS), as an early hallmark of apoptosis, translocated to the outer surface of the cell membrane which binds Annexin V, a 35-36 kDa calcium-dependent phospholipid-binding protein, which has a high affinity for phosphatidylserine. (Tait et al., 1989; van Engeland et al.,

1998; Arur et al., 2003). The dead cell marker (7-amino actinomycin D; 7-AAD) is used as an indicator of cell membrane structural integrity and it is excluded from live and early apoptotic cells. The percentage of live (non-apoptotic), early apoptotic, late stage apoptotic, dead cells, and debris were therefore determined in this method. This technique was used to determine the percentage of cells undergoing apoptosis 24 and 48 hours after re-plating the cells. 24 hours post-transfection with plasmids and 72 hours post-transfection with siRNAs, cells were re-plated in fresh medium at 2×10^5 cells/mL in 6 well plates. 24 hours post re-plating; 100 µL of cell suspension was isolated from culture plates and mixed with 100 µL of Annexin V reagent. Samples were incubated for 20 minutes in the dark before reading the result using the Muse cell analyser. The results were expressed as total apoptotic percentage (% Total apoptotic).

2.9. **Clonogenic assay**

Clonogenic or long term survival assay was performed to determine the cell's ability to proliferate and form colonies. An equal cell number proportion of parental, transfected or treated cells was diluted in 3.0 mL Iscove's medium (Sigma) containing 20% heat-inactivated foetal bovine serum (FBS), 10% cell-specific conditioned medium and 10% noble agar solution (0.5% w/v, Difco) and plated in 6-well plates. Each well was overlaid with 1.5 mL Iscove's complete medium containing 10% cell conditioned medium. The number of colonies formed was counted following 2-3 weeks incubation at 37ºC in a 5% CO_2 humidified incubator.

2.10. **Cell cycle analysis**

The cell-division cycle represents the series of significant processes that take place in eukaryotic cells resulting in their replication. The Muse™ Cell Cycle premixed reagent, a mixture of propidium iodide (PI) (a nuclear DNA intercalating stain) and pancreatic ribonuclease (RNAse A) that cleaves single-stranded RNA, discriminates and measures the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle (Darzynkiewicz et al., 2001). In G0/G1 phase, metabolic changes prepare the cell for division and moves into the S phase where DNA synthesis replicates the genetic material so each chromosome now consists of two sister chromatids. In G2/M phase, the metabolic changes assemble the cytoplasmic materials necessary for mitosis and cytokinesis/a nuclear division (mitosis) followed by a cell division (cytokinesis) and ends with the formation of two daughter cells. After 24 hours of re-plating cells following transfection, at least 2×10^5 cells were collected and washed once with phosphate buffered saline (PBS), centrifuged at 300×g for 5 minutes, the supernatant discarded and the cells fixed in 1.0 mL ice cold 70% ethanol/30% PBS and incubated at -20°C for at least 3 hours. After the incubation, cells were centrifuged at 300×g for 5 minutes, the supernatant was discarded, and the cells were re-suspended in 200 µL of Muse™ Cell Cycle reagent. They were then incubated for 30 minutes in the dark before reading the result by Muse cell analyser. The results were expressed as percentage of cells in each phase of the cell cycle (% Cells).

2.11. **Ki67 proliferation analysis**

The nuclear antigen Ki67 is one of several markers that have been used to assess cell growth and proliferation (Iatropoulos and Williams, 1996). Ki67 is considered as a cell-division cycle related nuclear protein because it is expressed in all active phases of the cell-division cycle (G1, S, G2 and M phases) and is absent in the resting phase (G0), making Ki67 a good marker of cell proliferation (Scholzen & Gerdes, 2000). A fluorescent based analysis distinguishes between the proliferating Ki67 (+) cells and non-proliferating Ki67 (-) cells. After 24 hours post-transfection with plasmids and 72 hours post-transfection with siRNAs, cells were harvested and plated in fresh growth medium at 2×10^5 cells/mL in 6 well plates. Following 24 hours incubation, up to 1×10^5 cells for each sample were harvested and washed once with 200 µL PBS. 50 µL of a 1×Fixation Solution were added to each sample and mixed thoroughly, followed by incubation at room temperature for 15 minutes and then samples washed once in 1×Assay Buffer. A volume of 150 µL of the 1×Assay Buffer was added to each sample and centrifuged for 5 minutes at 300×g. The supernatant was discarded and 100 µL of Permeabilisation Buffer added to each sample. These were mixed thoroughly and incubated for 15 minutes at room temperature, before a further wash in 1×Assay Buffer. Another 100 µL of the 1×Assay Buffer was added to each sample and centrifuged for 5 minutes at 300×g then the supernatant was discarded and 50 µL of 1×Assay Buffer was added. Samples were mixed thoroughly and incubated at room temperature for 15 minutes, before 10 µL of either the Muse® Hu IgG1-PE or Muse® Hu Ki67-PE reagent was added to each sample, mixing thoroughly. The samples were incubated for 30 minutes at room temperature in the dark. After incubation, 150 µL of 1×Assay Buffer was added to each sample and the sample were run on the Muse cell analyser, with the results expressed as proliferation percentage (% Proliferation).

2.12. **Drug treatment**

In some experiments, the effect of PP4c down-regulation on the cytotoxic response of the cells to four different drugs was investigated. K562 and HL-60 parental cells were initially treated with five different, increasing concentrations of imatinib (0.0-1.5 μM) (K562 cells only), cisplatin (0.0-15.0 μM), doxorubicin (0.0-0.6 μM) and rapamycin (0.0-100.0 nM) using 96-well plates. K562 cells were plated at a density of 75,000 cells/mL, while HL-60 cells were plated at 100,000 cells/mL and incubated for 24 and 48 hours before assessment of cell viability, to determine the optimum concentration to use in subsequent investigations. To determine effect of PP4c siRNAs on viability response to drug treatment, after 72 hours of PP4c down-regulation, the transfected cells were re-plated in 24-well plates at 2×10^5 cells/mL and treated with 0.2 μM imatinib (K562 cells only), cisplatin (2.0 μM for HL-60 cells and 10.0 μM for K562 cells), doxorubicin (0.1 μM for HL-60 cells and 0.2 μM for K562 cells) and 20 nM rapamycin (both cell lines). The transfected cell's viability/apoptosis was measured as described in sections 2.7, 2.8 and 2.9.

2.13. **Analysis of the level of PP4c expression by Western blotting**

Samples for protein analysis were collected from all transfected cells from every experiment in order to examine the level of PP4c expression to ensure that the effects observed in the results can be attributed to the changes in PP4c expression levels. A total of 1×10^6 cells were collected by centrifuging at 1200×g and washed twice in 500 μL PBS. Cells were then suspended in 20 μL of RIPA (Radioimmunoprecipitation Assay) buffer containing protease inhibitor (at a 1:100 dilution), followed by 20 μL of Laemmli sample buffer (Sigma). Samples were then heated at 95C for 10 minutes to denature and solubilise proteins. Samples were then loaded (40 μL/lane) on SDS-PAGE gels using a 12% resolving gel and separated at 150V for 60 minutes in a 1× SDS-PAGE running buffer

(0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.5). Proteins were then electro-transferred onto a polyvinylidenedifluoride (PVDF) membrane (BIO-RAD), which had been pre-wetted in 100% methanol, using either a semi-dry transfer unit (at 0.8mA/cm^2) for 1 hour, or a wet transfer system at 30V, 90mA overnight at 4°C with stirring. The blot was incubated in 5% non-fat milk in TBST (Tris Buffered Saline Tween 20; 25 mM Tris, 0.13M NaCl, and 0.1% Tween) for 1 hour to block free binding sites. The membrane was washed three times with TBST and probed with primary anti-PP4c (PPX/PP4c) using either a goat-polyclonal IgG (C-18, 1:1000 dilution) or a mouse-monoclonal IgG (C-6, 1:200 dilution) at 4°C for 12 hours (or overnight). The membrane was then washed three times with TBST for 10 minutes each to remove residual primary antibody and then incubated with a 1:2500 dilution of HRP-conjugated anti-goat IgG secondary antibody (C-18) for 1 hour or 1:800 dilution of HRP-conjugated anti-mouse IgG secondary antibody (C-6) overnight followed by washing five times with TBST for 10 minutes each. Protein bands were visualised by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (BIO-RAD, # 170-5060) and imaged and quantified using the C-DiGit blot scanner (LI-COR Biosciences). After analysis, blots were stripped by incubating in stripping buffer (Thermo, # 46430) for 30 minutes and re-probed with a mouse-anti-β-actin antibody (1:5000 dilution), followed by a HRP-conjugated anti-mouse IgG secondary antibody (1:800 dilution) with bands visualised as before.

2.14. **Statistical analysis**

Data are presented as the mean and standard error of the mean (SEM) (mean ± SEM). Data were analysed using Graghpad prism 7.0 statistical programme. One-way ANOVA and two-way ANOVA followed by Bonferroni post-hoc test were used to compare the test and control values. *P*-value of <0.05 was considered as statistically significant.

3. **Optimisation**

3.1. **Introduction**

There is a need to choose optimal conditions that maximise the accurate output of each experiment, minimises the errors and troubleshoots the difficulties. For example, optimal siRNA interference or DNA delivery into mammalian cell lines depends on the cell specificity besides other factors. The conditions that published by the manufacturing companies or previous studies can be used as a guide, but for best results it is recommended to optimise the transfection conditions for the cell of interest by evaluate the transfection efficiency, low cytotoxicity and reproducibility in each lab.

Hence, this chapter will focus on the cell lines that have been selected in this research and why they have been considered as models to use *in vitro*, the structures of the chemotherapeutic drugs and their mode of action, the growth inhibition or dose response optimisation and lastly the optimisation of the transfection methods that have been used in this research. This is necessary to i) establish appropriate culture and transfection procedures with the selected cell lines for further investigation into functional effect of manipulating PP4c levels and ii) to establish dose-response profiles and optimisation of treatment conditions with a range of chemotherapeutic drugs for further work into the effects of PP4 silencing on drug toxicity.

3.1.1. **Cell lines**

Two leukaemic cell lines have been used in this study, K562 cells for chronic myelogenous leukaemia (CML) and HL-60 cells for acute promyelocytic leukaemia (APL).

3.1.1.1. **K562 cell line**

The human myelogenous leukaemic cell line, K562, was the first myeloid cell line that was derived from a 53-year-old female chronic myelogenous leukaemia patient at the terminal stage of blast crisis (Lozzio and Lozzio, 1975). It is an erythro-megakaryoblastic leukaemia cell line that is characterised by positive Philadelphia (Ph) chromosome t(9;22); a specific genetic abnormality that results in the Bcr-Abl fusion gene and consequently activates tyrosine kinases, and also by a second reciprocal translocation between the long arm of chromosome 15 with chromosome 17 (Lozzio and Lozzio, 1975; 1979). These cells are a non-adherent, suspension cell line with rounded morphology and can spontaneously develop characteristics similar to early-stage erythrocytes, granulocytes and monocytes (Lozzio and Lozzio, 1979; Lozzio et al., 1981). It has been reported that K562 cells can express many phosphatases, including PTEN and SHIP2, but they fail to express others such as SHIP1 (Sattler et al., 1999; Bruecher-Encke et al., 2001). Owing to the lack of Major Histocompatibility Complex (MHC) class I antigens on their membrane that are required for inhibiting natural killer (NK) cells activity, K562 cells are very sensitive to NK cell activity (Lozzio and Lozzio, 1979).

Several pathways have been reported to be deregulated by Bcr-Abl fusion gene in myeloid leukaemia (Jacquel et al., 2003). Activation of NF-κB by the IKK pathway may play an important role in the pathogenesis of myeloid leukaemia induced by Bcr-Abl, resulting in targeting NF-κB and IKKs as therapeutic agents of Philadelphia-positive leukaemias (Hsieh and Van Etten, 2014). The mammalian target of rapamycin (mTOR) pathway is one

of the multiple signalling pathways (canonical PI3K/Akt/mTOR) activated by Bcr-Abl (Ly et al., 2003). mTOR is a serine/threonine kinase that forms two different complexes; mTORC1 and mTORC2, mTORC1 positively regulates the biosynthesis of proteins necessary for cell growth, and proliferation, and is also known to inhibit the autophagy process, while mTORC2 is a critical regulator of Akt (Gentzler et al., 2012).

The K562 cell line is considered as the most appropriate CML cell line model to detect differentiation *in vitro* for different reasons. They are highly undifferentiated cells with an active proliferative capacity, which were simultaneously accompanied by apoptosis and autophagy (Lozzio and Lozzio, 1979). Moreover, K562 cells are less clumping than other suspension cell lines due to the Bcr-Abl activity that results in down-regulation of surface adhesion molecules (Lozzio and Lozzio, 1979).

3.1.1.2. **HL-60 cell line**

The human promyelocytic leukaemia cell line, HL-60, was derived from a 36-year-old woman with acute promyelocytic leukaemia (APL) (Collins et al., 1977). Unlike other acute promyelocytic leukaemia cell lines that are characterised by a specific chromosome translocation t(15;17), which results in the promyelocytic leukaemia (PML) fusion gene and retinoic acid receptor α gene (RARα), HL-60 cell line lack this chromosomal translocation (Lee et al., 2002). It has been reported that HL-60 cells have amplified c-*myc* oncogene expression from 16 to 32 fold (Dalla Favera et al., 1982), altered N-*ras* and c-*neu* gene expression (Murray et al., 1983) and lack p53 expression due to a deletion of a large proportion of the gene that encodes it (Wolf and Rotter, 1985).

Using cDNA microarray assay, granulocytic differentiation induced by all-*trans* retinoic acid (ATRA) was observed in two acute promyelocytic leukaemia cell lines; HL-60 and NB4 (Lee et al., 2002). Lee and colleagues observed that ATRA-treated HL-60 up-

regulated 35 genes and down-regulated 36 genes as compared to untreated HL-60 cells, while ATRA-treated NB4 cells up-regulated 119 genes and down-regulated only 17 genes as compared to untreated NB4 cells, however, there were no common gene expression profiles regulated by the ATRA, suggesting that the molecular mechanisms and genes involved in ATRA-induced differentiation of APL cells may be different and cell type specific (Lee et al., 2002).

HL-60 cells are rounded, highly proliferative suspension cells with doubling time in culture of approximately 36-48 hours (Collins, 1987). Moreover, HL-60 cells express both transferrin and insulin receptors on their cell surface, which are believed to be required for cell proliferation, growth and differentiation (Collins, 1987). HL-60 cells are predominantly a neutrophilic promyelocyte (precursor) and they can differentiate to eosinophils and eosinophilic precursors when cultured in mildly alkaline medium (Breitman et al., 1980; Birnie, 1988). One of the major properties that have made the HL-60 cell line an attractive model for studies of human myeloid cell differentiation is the expressing of an amplified c-myc proto-oncogene (Birnie, 1988). In contrast to the c-myc gene of the normal cells which is localized to chromosome 8q24, it has been found that the c-myc mRNA levels in HL-60 cells are 30-fold amplification of the c-myc gene located in an abnormal banded region on chromosome 8q24 (Birnie, 1988).

From the previous studies highlighting the characteristics and features of K562 and HL-60 cells, these two cell lines were selected for use in this study as well-defined models of CML and AML respectively (Lozzio and Lozzio, 1979; Birnie, 1988).

3.1.2. **Drugs used in cancer treatment and their targets**

Chemotherapy is a term that refers to one or a group of toxic medications (chemicals) that are used to treat cancer by killing the cancer cells and preventing them from dividing and growing. The best anticancer agents would attack the cancer cells only without harming normal tissues but unfortunately, such agents are not available (Moschovi et al., 2015).

In the last century, when the era of chemotherapy had begun, Mustard Gas was used as chemotherapy for the first time during World War II, with nitrogen mustard being found to be active against Lymphoma (DeVita and Chu, 2008; Galmarini et al., 2012). This compound was used as a model for alkylating agents, a group of similar but more effective agents to nitrogen mustard, which killed cancer cells by activation of DNA damage pathways (DeVita and Chu, 2008; Galmarini et al., 2012). After the discovery of nitrogen mustard, another compound related to folic acid called aminopterin, has been used in childhood acute leukaemia to produced remissions by blocking DNA replication (DeVita and Chu, 2008; Galmarini et al., 2012). Aminopterin is considered as the predecessor of methotrexate, a cancer treatment drug commonly used nowadays (DeVita and Chu, 2008; Galmarini et al., 2012). Over the years, continued research has led to the development of further drugs that block crucial functions such as cell cycle checkpoints that affect cell growth and replication. These chemotherapeutic drugs have been successfully applied in long-term remissions and even cure of many people with cancer (DeVita and Chu, 2008; Galmarini et al., 2012).

Over the past decades, many studies have reported the discovery and validation of novel cancer therapeutics called targeted therapies. These drugs can be used individually or in combination with other conventional therapies including surgery, radiation or traditional chemotherapy (Meiler and Schuler, 2006). For example, rapamycin can be used in combination with imatinib to induce apoptosis by inhibition the mTORC1 (Ly et al., 2003;

Sillaber et al., 2008) and to prevent outgrowth of resistant subclones and consequently improve the overall outcome in CML patients (Sillaber et al., 2008). These anti-cancer drugs act to prime the apoptotic machinery, as promising apoptosis-inducing agents, such as by targeting the cell cycle either by slowing it down or by blocking it at checkpoints (Makin, 2002). The DNA damage caused by the anti-cancer drugs leads to cell cycle arrest at the S phase or G2/M phase, resulting in cell death, bearing high hopes for the management of cancers resistant to conventional treatments (Makin, 2002). Traditional chemotherapy drugs are cytotoxic agents that act on all rapidly dividing cells, while targeted therapies interact with their specific molecular targets that are associated with cancer and block tumour cell proliferation (Charlton and Spicer, 2016).

There are two major types of targeted therapies; small molecule drugs such as tyrosine kinase inhibitors, and monoclonal antibodies including blinatumomab, inotuzumab and rituximab that are used in the treatment of different types of lymphomas and leukaemias (Jabbour et al., 2015; Charlton and Spicer, 2016). The first very potent and advances in cancer targeted therapy were made with haematological malignancies (Druker et al., 2001a; 2001b). Imatinib, which is used in this study, is an example of a small molecule targeted therapy, which inhibits the signalling of the Bcr-Abl tyrosine kinase, the genetic abnormality causing CML (Druker et al., 2001a; 2001b).

This section will highlight the structure and mode of action for four anti-cancer drugs used in this study.

3.1.2.1. **Cisplatin**

Cisplatin, *cis*-diamminedichloroplatinum (II), is a neutral, square-planar, coordination complex of divalent platinum that has been used for many years to treat different kinds of cancers, inducing cytotoxicity via cell cycle inhibition as a result of the formation of DNA adducts and subsequent induction of apoptosis (Dasari and Tchounwou, 2014; Johnstone et al., 2016). It is classified as an 'alkylating-like agent not alkylating agent' since it has no alkyl group and therefore cannot carry out alkylating reactions.

Cisplatin's mode of action has been linked to its ability to crosslink with purine bases in DNA in a process known as aquation, in which one of the two chloride ligands is slowly displaced by water forming *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (Basu and Krishnamurthy, 2010; Johnstone et al., 2015). The aquo complex itself is easily displaced by the N-heterocyclic bases on DNA; interfering with DNA repair mechanisms and displaying cytotoxicity towards the fastest proliferating cells leading to cell cycle arrest and subsequently cell death (Figure 3.1; Basu and Krishnamurthy, 2010; Johnstone et al., 2015).

Cisplatin affects many signalling pathways and induces apoptosis via p53 or its family of transcription factors. p53 can regulate cisplatin-induced apoptosis by several mechanisms including direct binding and counteracting the anti-apoptotic function of B-cell lymphoma-extra-large (Bcl-xL) or phosphatase and tensin homolog (PTEN) up-regulation (Basu and Krishnamurthy, 2010). Moreover, p38 MAP kinase pathway activation may mediate and regulate cell death in response to cisplatin. p38 mediates stress-induced EGFR (epidermal growth factor receptor) phosphorylation that triggers cisplatin-induced receptor incorporation (Winograd-Katz and Levitzki, 2006). Cisplatin induces cell death also by JNK pathway activation via p73. This can occur directly by p73 phosphorylation or indirectly through c-Jun, and forms a complex at several serine and threonine residues leading to cell death (Jones et al., 2007). Moreover, cisplatin activates extracellular signal-

regulated kinase (ERK) in many types of cancer cells via p53 phosphorylation, causing p21 up-regulation and Gadd45 (45kd-growth arrest and DNA damage) leading to activation of cell cycle checkpoints, resulting in either DNA repair or, if the damage cannot be repaired, the cells are permanently eliminated by inducing cell death (Dasari and Tchounwou; 2014). Cisplatin also induces DNA damage by BAD phosphorylation at Ser-136 via PI-3K-PKB/Akt or at Ser-112 via an ERK cascade (Hayakawa et al., 2000; Dasari and Tchounwou; 2014); inhibition of either of these cascades sensitizes both Caov-3 and A2780 ovarian cancer cells to cisplatin (Hayakawa et al., 2000).

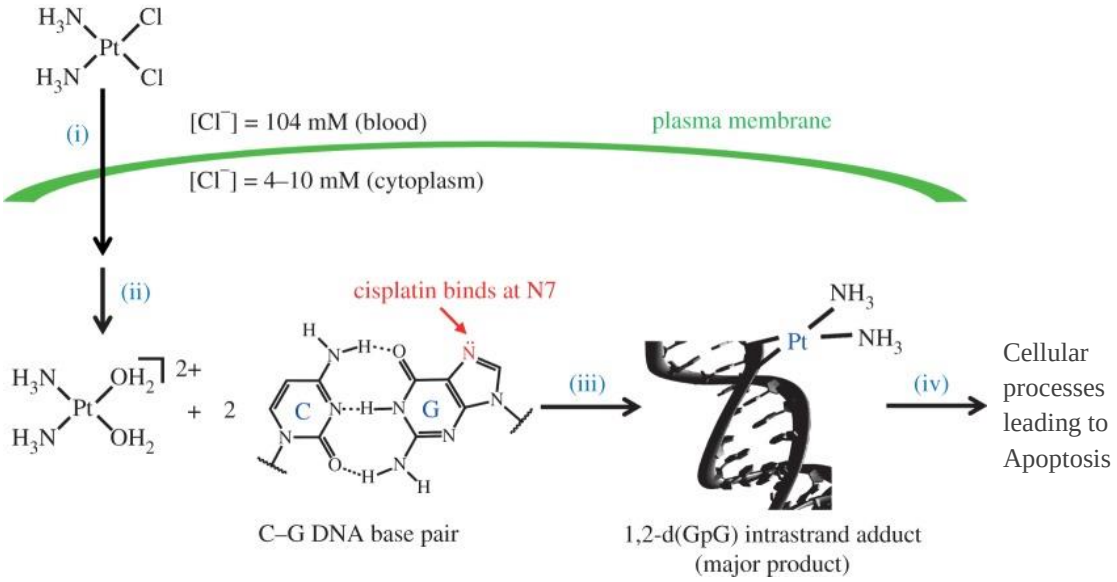

Figure 3.1 Cisplatin mechanism of action. This mechanism of action can be divided into four distinct sequential steps: (1) cellular uptake, (2) aquation/activation, (3) DNA platination, and (4) cellular processing leading to apoptosis. This figure is adapted from Johnstone et al., 2015.

3.1.2.2. **Doxorubicin**

Doxorubicin (14-hydroxydaunomycin), also known as Adriamycin, is an anthracycline anti-tumour antibiotic produced by *Streptomyces peucetius var. caesius* after introducing a genetic mutation using N-nitroso-N-methyl urethane (Arcamone et al., 1969). Since the 1970's, doxorubicin has been used as a potent and safe treatment of a wide variety of cancers including solid tumours, lymphomas, and leukaemias (Cortes-Funes and Coronado, 2007).

The action of doxorubicin in cancer cells can be summarised by two major mechanisms (Figure 3.2). The first one by a process called intercalation in which doxorubicin interacts with DNA and inhibits the progression of topoisomerase-II, an enzyme that controls supercoils in DNA by cutting and resealing both DNA strands (Nitiss, 2009). In other words, inhibition of topoisomerase-II results in unwinding of the DNA and double-strand breakage, preventing the DNA double helix from being resealed and thereby stopping the process of replication (Nitiss, 2009). Pang and colleagues (2013) found that doxorubicin intercalation could also induce histone eviction from transcriptionally active chromatin leading to a DNA damage response. The second mechanism by which doxorubicin affects cancerous cells is by increasing the production of reactive oxygen species (ROS), particularly the quinone type, and subsequently leading to lipid peroxidation, damage to cellular membranes, DNA and proteins, hence contributing to doxorubicin cytotoxicity (Gewirtz, 1999).

It has been reported that doxorubicin-induced apoptosis occurs through several signal transduction mechanisms (Wang et al., 2004a). Numerous studies have revealed that doxorubicin treatment activates the tumor suppressor protein, p53, leading to apoptosis (Nobori et al., 2002; Lorenzo et al., 2002), while others reported that the transcription factor NF-κB, is also involved in doxorubicin-induced apoptosis (Wang et al., 2004a).

Some studies have reported NF-κB as up-regulator of p53 gene expression (Pei et al., 1999), while others reported that both p53 and NF-κB work as inhibitors for each other (Wang et al., 2004b).

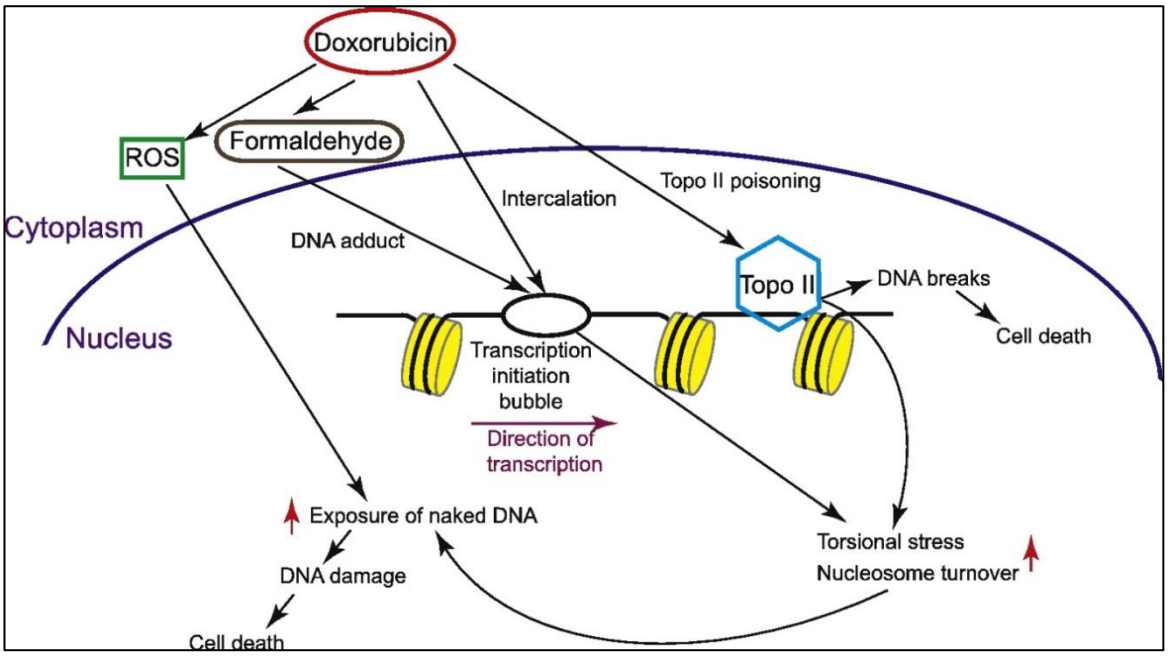

Figure 3.2 Doxorubicin mechanism of action. Two mechanisms used by doxorubicin to cause DNA damage and consequently cell death. In the first mechanism, which is called intercalation, doxorubicin interacts with DNA and inhibits the progression of topoisomerase-II and results in break the DNA double-strand and thereby stopping the process of replication and cause cell death. While in the second mechanism doxorubicin treatment increase the production of reactive oxygen species (ROS) in the cytoplasm then ROS inter the nucleus, the high exposure of the DNA to ROS causes DNA damage and consequently cell death (From Yang et al., 2014).

3.1.2.3. **Imatinib mesylate**

Imatinib mesylate (Novartis compound STI-571) is a tyrosine kinase inhibitor with activity against Abl, Bcr-Abl, PDGFR, and c-KIT. Imatinib has been used as a first-line treatment for Philadelphia chromosome-positive CML since the late 1990s (Li et al., 2015a). In Bcr-Abl-positive cells, the activities of STAT5, Akt/PKB and NF-κB are inhibited by imatinib treatment (Deininger et al., 2000). It is also a potent inhibitor of receptor tyrosine kinases encoded by c-KIT, PDGFR and stem cell factor (SCF) oncogenes, and inhibits PDGF- and SCF-mediated cellular events. *In vitro*, imatinib inhibits proliferation and induces apoptosis in those cells overexpressing c-KIT such as gastrointestinal stromal tumour (GIST) cells (Li et al., 2015a).

Generally, imatinib works by occupying the tyrosine kinase active site, leading to tyrosine de-phosphorylation and subsequently enhances cell death (Bende et al., 2010; Yada et al., 2012; Li et al., 2015a). In leukaemias, imatinib inhibits Bcr-Abl tyrosine kinase activity and restores interleukin-3 (IL-3) dependent growth resulting in decreased cell proliferation and enhanced apoptosis in CML and ALL by binding close to the ATP binding site of Bcr-Abl tyrosine kinase, locking it in a closed or self-inhibited conformation and leading to the inhibition of the protein semi-competitively and thereby inhibition of the downstream signalling pathways that promote leukaemogenesis (Figure 3.3; Lee et al., 2011; Bende et al., 2010; Yada et al., 2012; Li et al., 2015a). It has been reported that imatinib inhibits the colony-forming growth of CML myeloid cells (about 92%-98%), yet it has minimal effect on colony formation from normal cells (Deininger et al., 1997; Druker et al., 1996).

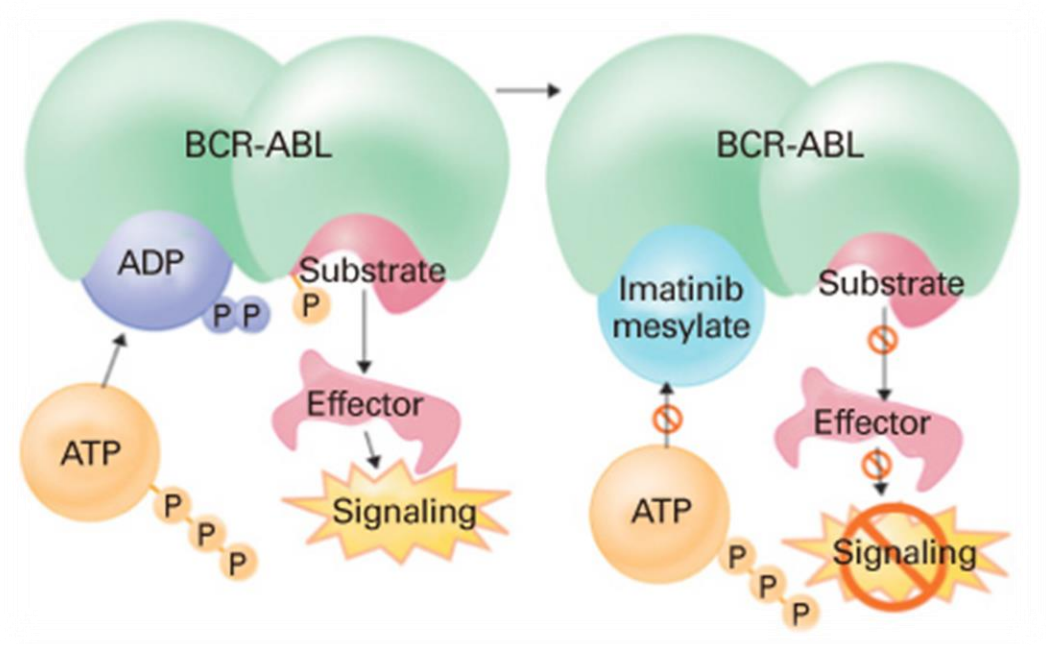

Figure 3.3 Imatinib mechanism of action. Imatinib binds to the amino acids of the Bcr-Abl ATP binding site leading to preventing tyrosine autophosphorylation and subsequently the phosphorylation of its substrates resulting in inhibiting the downstream signalling pathways that promote leukaemogenesis (From Lee et al., 2011).

3.1.2.4. **Rapamycin**

A natural macrocyclic lactone (also known as sirolimus), produced by the *bacterium Streptomyces hygroscopicus*, with both immunosuppressant (Vezina et al., 1975; Sehgal et al., 1975) and anti-tumor properties (Douros and Suffness, 1981).

Rapamycin is a potent cell growth inhibitor and its mechanism of action can be summarized as the following: sirolimus binds to the cytosolic protein FK-binding protein 12 (FKBP12) (a 12 kDa FK506-binding protein; also known as FKBP1A), leading to the formation of the active sirolimus-FKBP12 complex (Sabers et al., 1995). This then binds to and inhibits the activation of the key regulatory kinase pathway, mammalian Target Of Rapamycin (mTOR), directly by binding to mTOR Complex 1 (mTORC1) (Figure 3.4; Belinda, 2012; Huang and Houghton, 2003; Sawyers, 2003).

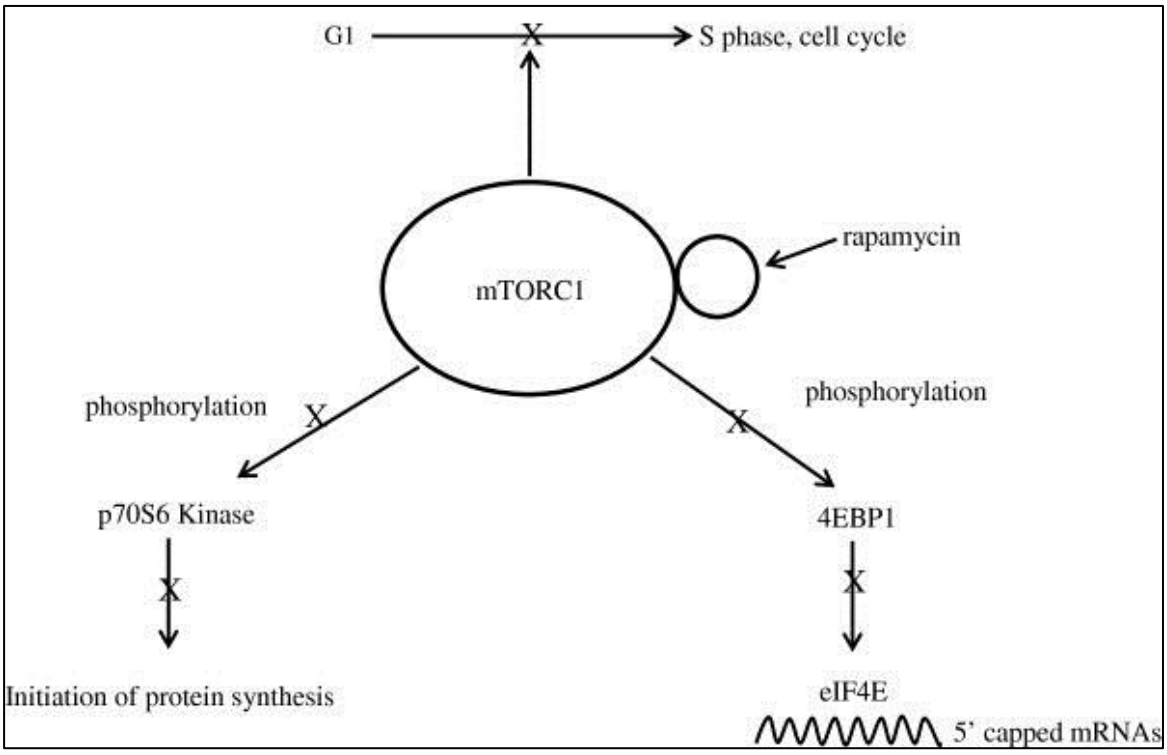

Figure 3.4 Rapamycin mechanism of action. Rapamycin binds directly to mTORC1 leading to inhibition of cell cycle progression, cell proliferation and antibody production (From Belinda, 2012).

This immunosuppressive complex has no effect on PP2B activity but it is believed to suppress cytokine-driven T-cell proliferation, inhibiting cell cycle progression from the G1 to S phase (Figure 3.4; Brown et al., 1994; Hashemolhosseini et al., 1998).

Studies showed that inhibiting mTOR with rapamycin decreased the phosphorylation of some proteins involved in mTOR signaling pathway, induced cell cycle arrest at G0/G1 phase and distinctly rescued the differentiation of normal haematopoietic stem cells (HSCs) and depleted leukaemic stem cells (Cheng et al., 2008b).

3.1.3. **Transfection methods**

Transfection techniques are analytical tools that facilitate the study of various cellular functions such as genetic functions, protein synthesis, cell growth and development. Transfection can be defined as a process of introducing genetic materials such as DNA or RNA into eukaryotic cells, carried out via physical, chemical or biological techniques to deliberately modify the genome or the protein production of the targeted cell (Prathees et al 2011). The transfection could be temporary for short period without integration of the DNA with the cellular chromosomes (transient transfection) or long period (stable transfection) to integrate plasmid DNA into the cellular genome so it will be passed on to the daughter cells. This process is routinely used in *in vitro* studies of molecular biology including gene expression and gene function.

The preferred transfection method is characterised by high transfection efficiency accompanied with minimum toxicity. Four most commonly used techniques are:

1. Electroporation; an easy, non-chemical technique in which the cell membrane is exposed to high-intensity electric pulses that cause a transient aqueous channels in the membrane for DNA to enter the cytoplasm, resulting in high transformation efficiency and no alteration in the cell morphology or functions (Chang et al.,

1992). It is a simple and rapid method, however; the transfected cells have high cytotoxicity after shocking due to the electric pulses.

2. Liposome-mediated transfection (lipofection); one of the most efficient procedures of transfection that involve the use of liposome to form cationic and neutral lipids polymers in which the DNA is complexed within lipid droplets. These droplets interact directly with the cell membrane and fuse and then the DNA is liberated into the cytoplasm, resulting in highly efficient gene knockdown even when using low siRNA concentrations (1 to 5 nM) without concomitant activation of the cells (Felgner et.al, 1987).
3. Calcium phosphate precipitation; the oldest method of transfection with main advantage is that it is cheap and easy to perform but it is less efficient than other methods and the precipitates often cause cytotoxicity.
4. Viral delivery; using either adenovirus or retrovirus vector as a carrier to introduce DNA into the cells.

In this research, two of the mentioned methods were used; the electroporation-based transfection (nucleofection V reagent) and the lipofection-based transfection (HiPerFect reagent).

The aims of this chapter are:

- To determine the optimum concentrations of the anti-cancer drugs that have been used in this study (cisplatin, doxorubicin, imatinib and rapamycin) to use the most effective dose of these anticancer drugs post PP4c silencing in K562 and HL-60 cell lines. This will initially be carried out by a dose response; screened using the MTS assay, with the confirmation of cytotoxicity of selected drug doses from the MTS based on Trypan blue vital dye exclusion assay, flow cytometry (MUSE Annexin V) and long-term survival methods.
- To evaluate the efficiency of the transfection reagents, nucleofection V and HiPerFect, based on their transfection efficiency percentage, cytotoxicity and reproducibility in order to select the most appropriate transfection method for PP4c silencing or up-regulation in K562 and HL-60 cells.

3.2. **Materials and Methods**

Before starting the drug dose response analysis, optimisation of cell density for use in the MTS assay was undertaken using a range of different densities (0.5-2.0×10^5 cells/well) of K562 and HL-60 parental cells seeded in 96-well plates and incubated for 24, 48 and 72 hours at 37ºC in a 5% CO_2 humidified incubator. Cell viability was then assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega), from which the optimum density to use in subsequent investigations was determined (see chapter 2 section 7.2).

K562 and HL-60 parental cells were treated with five different, increasing concentrations of imatinib (0.0-1.5 μM) (K562 cells only), cisplatin (0.0-15.0 μM), doxorubicin (0.0-0.6 μM) and rapamycin (0.0-100.0 nM) for 24 or 48 hours at 37°C in a humidified 5% CO_2 incubator, with cell viability measured using the MTS assay with absorbance measured at 490 nm on a 96-well plate reader and results expressed as cell viability as a percentage of untreated control cells after taking the blank wells values out of the values of all wells containing cell culture (% viability).

Trypan blue exclusion method was used for viable cell count and re-plating (see chapter 2 section 7.1). Confirmation of drug-induced cytotoxicity was assessed by the Muse™ Annexin V and Dead Cell Assay kit, with results expressed as total apoptotic percentage (% Total apoptotic) (see chapter 2 section 8). The ability of K562 and HL-60 cells to form colonies (long-term survival) after treatment with certain drugs was also determined (see chapter 2 section 9). The number of colonies formed was counted following 2-3 weeks incubation at 37ºC in a 5% CO_2 humidified incubator.

In this research, different transfection methods have been used to study the modulation of PP4c gene expression in leukaemic cell lines. Here, transfection methods for plasmid DNA transfection in K562 and HL-60 cells were optimised using nucleofection (V solution),

Lipofectamine 2000 Reagent and Lipofectamine 3000 Reagent, while nucleofection (V solution) and HiPerFect reagent were used for the optimisation of siRNA delivery in HL-60 cells.

For plasmid DNA transfection, transfection efficiency was determined using pmaxGFP in both K562 and HL-60 cells. Cells after 48 hours post pmaxGFP transfection were harvested and centrifuged at 1200×g then washed once in PBS, around 10 µL from the cells added to a microscope slide with coverslip and counted (100 cells) with fluorescence and light microscopy to calculate the transfection efficiency.

For optimisation of siRNA transfection, a scrambled negative siRNA labelled with Cyanine dye 3 (Cy3) (labelled using the Silencer siRNA labelling kit from Ambion (#1632)) was transfected in parallel transfections to determine transfection efficiency in K562 and HL-60 cells using nucleofection V reagent and HiPerFect reagent respectively. Cells 72 hours post Cy3 transfection were harvested and centrifuged at 1200×g, washed once in PBS, then 10 µL from the cells added to a microscope slide with coverslip and counted (100 cells) with fluorescence and light microscopy to calculate the transfection efficiency percentage.

3.3. **Results**

3.3.1. **Optimisation of cell density**

To optimise cell density for use in the MTS assay, K562 parental cells were seeded in 96-well plates (0.5-2.0×10^5 cell/well) and cultured for 24-72 hours before assessment of viability. The results showed that the absorbance at 24 hours increased for all cultures, in other words, the cell number in every culture has increased or approximately doubled. On the contrary, the absorbance readings for some cultures, especially higher densities, decreased at 72 hours suggesting density arrest at these higher cell numbers. Therefore, the optimum density for K562 cells in which they continued their proliferation from day 1 to day 3 was (0.75×10^5 cell/well) (Figure 3.5).

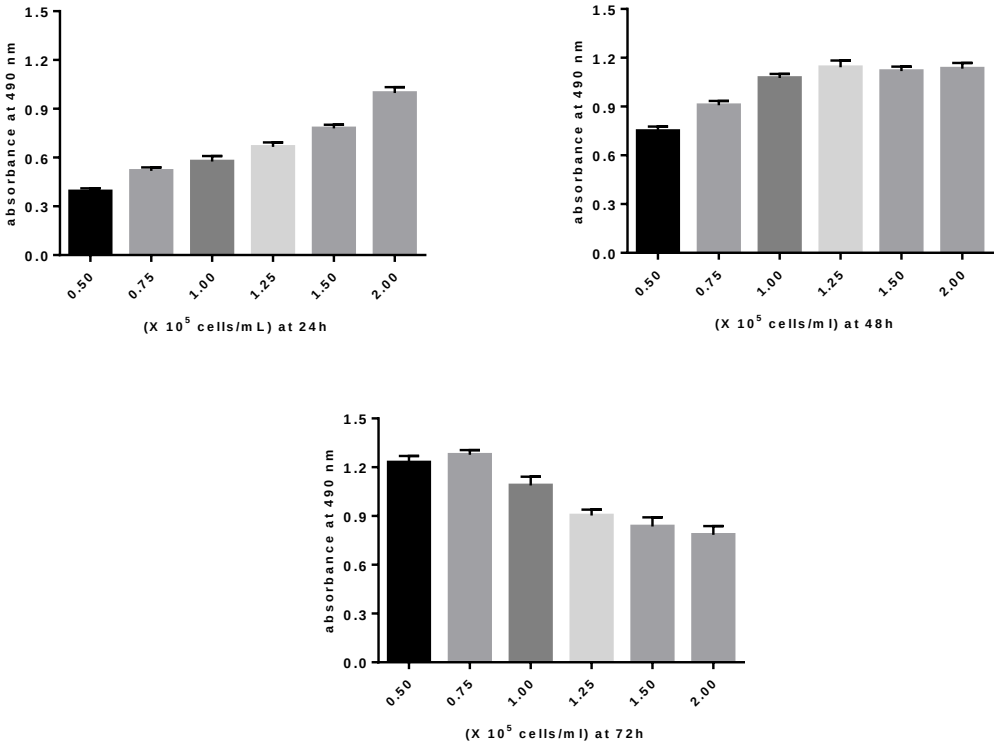

Figure 3.5 Seeding density of parental K562 cells affected over the period of time. Parental K562 cells where plated 24h, 48h and 72 hours. This figure shows the growth of K562 cells according the density of seeding (n=3).

Similarly to K562 cells, HL-60 parental cells (0.5-2.0×10^5 cell/well) were seeded in 96-well plates prior to drug dose response assay. The results showed that all absorbance readings increased for all cultures from 24-72 hours, suggesting an increase in their cell number and in some of them doubled. (1.25×10^5 cell/well) density has been selected for HL-60 cells in which they continued their proliferation without confluence from day 1 to day 3 (Figure 3.6).

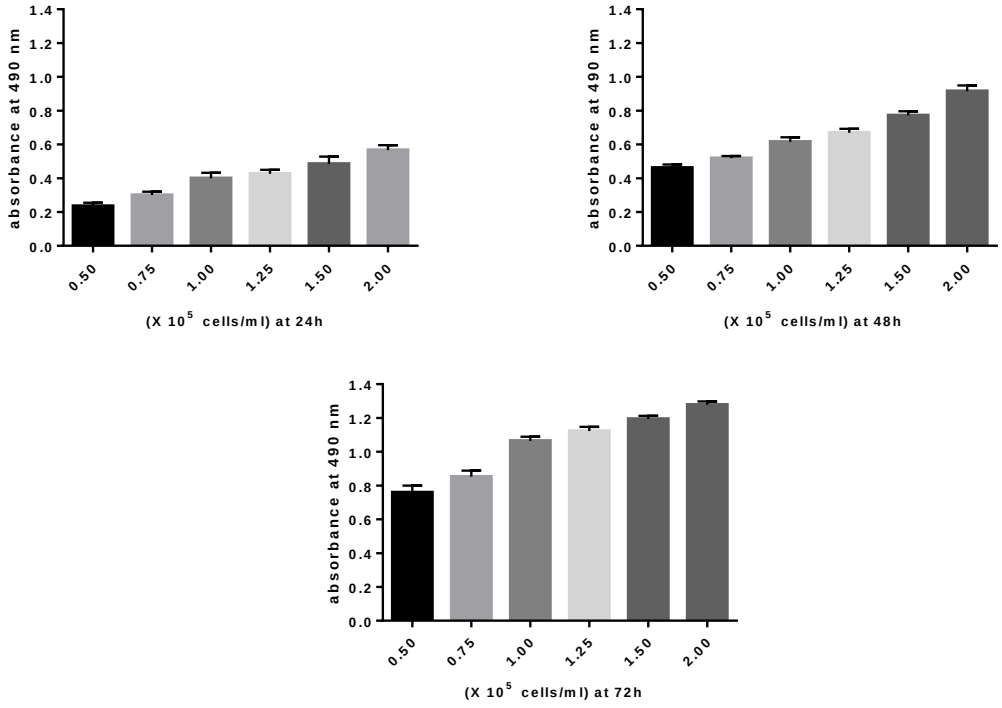

Figure 3.6 Seeding density of parental HL-60 cells affected over the period of time. Parental HL-60 cells where plated 24h, 48h and 72 hours. This figure shows the growth of HL-60 cells according the density of seeding (n=3).

3.3.2. **Drug dose response**

Following the optimisation of cell density, (0.75×10^5 cell/well) of parental K562 cells were treated with cisplatin (0.0-15.0 μM), doxorubicin (0.0-0.6 μM), imatinib (0.0-1.5 μM), and rapamycin (0.0-100.0 nM) for 24 or 48 hours. Cell survival was measured by MTS assay and the results indicated that cell viability decreased with increased drug doses and incubation periods (Figures 3.7, 3.8, 3.9, and 3.10).

In figure 3.7, the cell viability percentage significantly decreased (**$p<0.01$) when K562 cells were exposed to high-doses of cisplatin (15.0 μM) at 24hours and 48 hours, as compared to untreated cells. On the other hand, treating the cells with medium- to high-doses (0.2-0.6 μM) doxorubicin significantly decreased cell viability at 24 hours (around 40% reduction; *$p<0.05$, **$p<0.01$; figure 3.8 A), accompanied with further reduction (45%-60%) in cell viability starting from low-doses (0.1 μM) at 48 hours (Figure 3.8 B).

K562 cells treatment with imatinib showed a noticeable reduction, as expected, with all-doses starting from low-doses to high-doses at 24 hours (Figure 3.9 A). After 48 hours of treatment, about 40% reduction (*$p<0.05$) in K562 cell viability treated with 0.1-0.2 μM imatinib and about 80-90% (**$p<0.01$, ***$p<0.001$) reduction when treated with medium- to high doses (0.5-1.5 μM) (Figure 3.9 B) was observed.

Moreover, K562 cells treatment with all-doses of rapamycin resulted in 20-50% reduction in cell viability at 24 hours (Figure 3.10 A), with a sustained reduction (about 60%) observed after 48 hours (Figure 3.10 B).

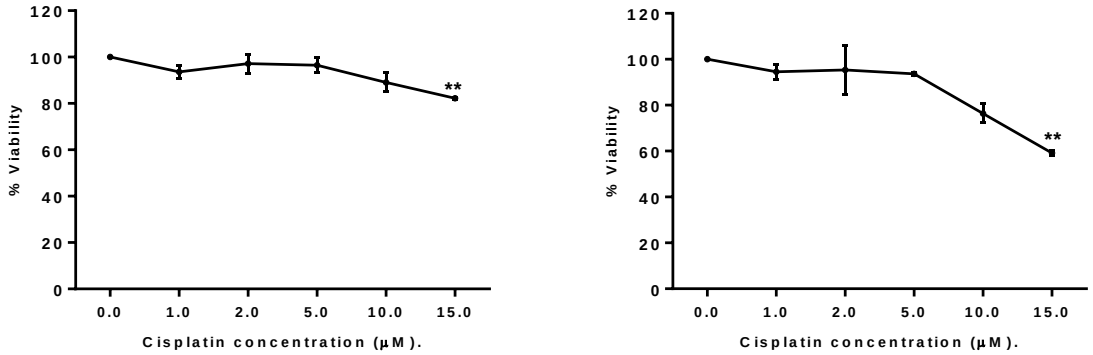

Figure 3.7 Cytotoxic effect of cisplatin on K562 cells. Cells were treated with or without 1, 2, 5, 10 and 15 µM of cisplatin for 24h (A) and 48h (B). Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (**p<0.01; One-way ANOVA test followed by Bonferroni test; n=3).

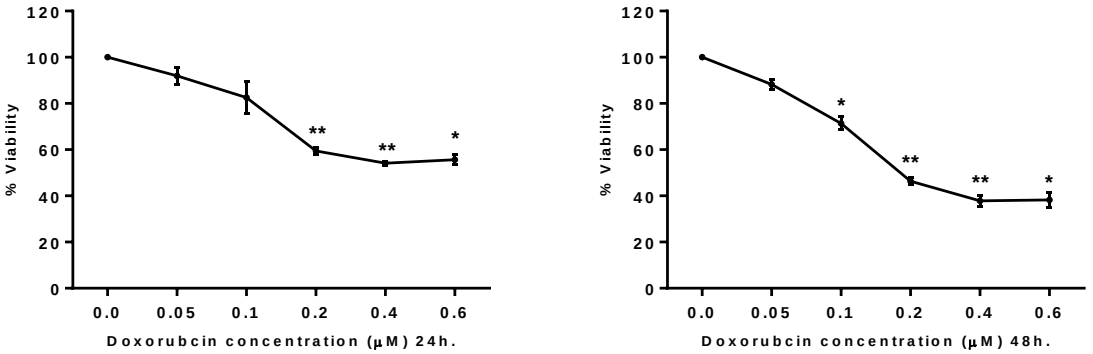

Figure 3.8 Cytotoxic effect of doxorubicin on K562 cells. Cells were treated with or without 0.05, 0.1, 0.2, 0.4, and 0.6 µM of doxorubicin for 24h (A) and 48h (B). Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (*p<0.05, **p<0.01; One-way ANOVA test followed by Bonferroni test; n=3).

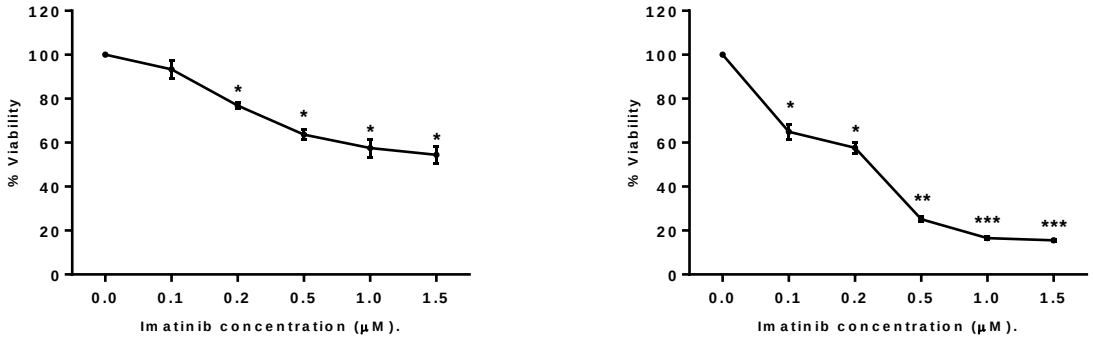

Figure 3.9 Cytotoxic effect of imatinib on K562 cells. Cells were treated with or without 0.1, 0.2, 0.5, 1.0 and 1.5 µM of imatinib for 24h and 48h. Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (*p<0.05, **p<0.01, ***p<0.001; One-way ANOVA test followed by Bonferroni test; n=3).

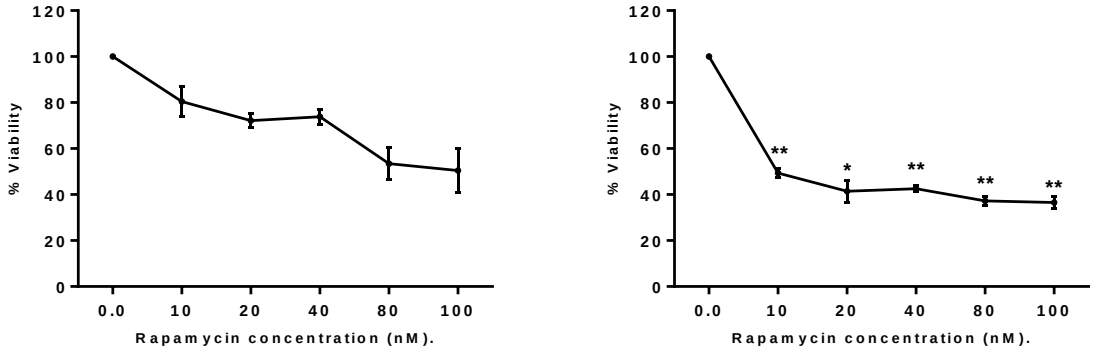

Figure 3.10 Cytotoxic effect of rapamycin on K562 cells. Cells were treated with or without 10.0, 20.0, 40.0, 80.0, and 100.0 nM of rapamycin for 24h and 48h. Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (*p<0.05, **p<0.01; One-way ANOVA test followed by Bonferroni test; n=3).

(1.25×10^5 cell/well) of parental HL-60 cells were treated with cisplatin (0.0-15.0 μM), doxorubicin (0.0-0.6 μM), and rapamycin (0.0-100.0 nM) for 24 or 48 hours. Cell survival was measured by MTS assay and the results expressed as viability percentage. Figures 3.11-3.13 showed that the cell viability was decreased with increased drug doses and incubation periods.

Around 30-40% reduction in cell viability was seen in HL-60 cells when treated with high-doses (10-15 μM) of cisplatin at 24 hours (Figure 3.11A) and this reduction continued at 48 hours post-treatment to reach 95% with high-doses as well as a further reduction seen with low- and medium doses (Figure 3.11B). Similarly to cisplatin, treating HL-60 cells with medium- to high-doses (0.2-0.6 μM) doxorubicin significantly decreased cell viability after 24 hours (20-40% reduction; figure 3.12 A), further reducing (up to 96%) after 48 hours (Figure 3.12 B). Similarly to K562 cells, treatment with 10-100 nM rapamycin resulted in up to 20% reduction in HL-60 cell viability after 24 hours (Figure 3.13 A), sustained to up to 60% after 48 hours post-treatment (Figure 3.13 B).

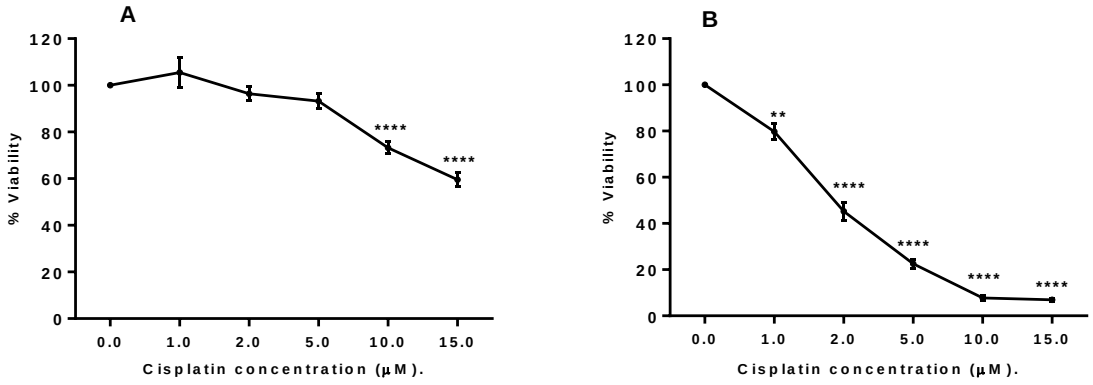

Figure 3.11 Cytotoxic effect of cisplatin on HL-60 cells. Cells were treated with or without 1, 2, 5, 10 and 15 μM of cisplatin for 24h (A) and 48h (B). Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (**p<0.01, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

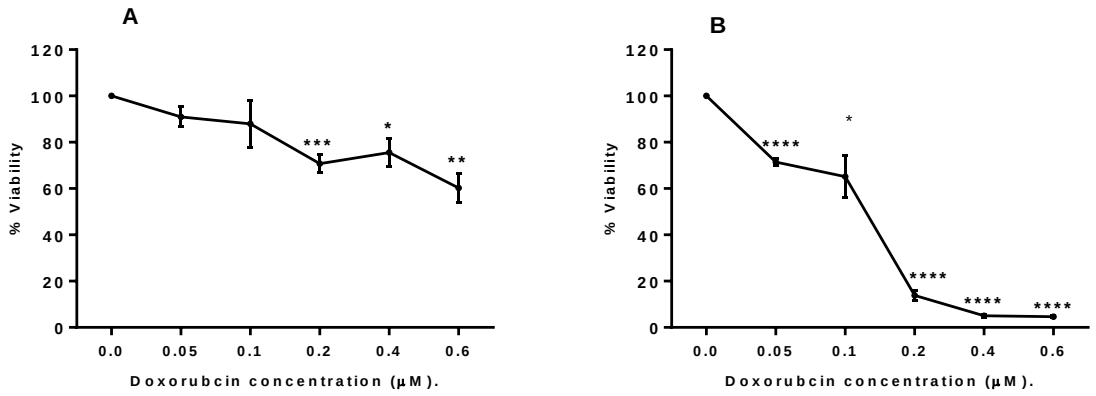

Figure 3.12 Cytotoxic effect of doxorubicin on HL-60 cells. Cells were treated with or without 0.05, 0.1, 0.2, 0.4 and 0.6 µM of doxorubicin for 24h (A) and 48h (B). Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

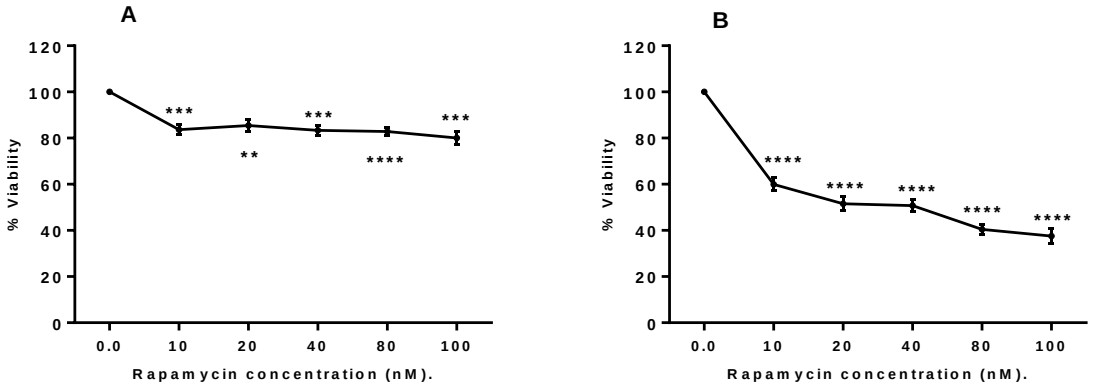

Figure 3.13 Cytotoxic effect of rapamycin on HL-60 cells. Cells were treated with or without 10, 20, 40, 80 and 100 nM of rapamycin for 24h (A) and 48h (B). Cell viability was tested by cell proliferation assay (MTS) with absorbance measured at 490 nm and results expressed as viability percentage (viability %) (**p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

3.3.3. **Confirmation with Trypan blue dye, Annexin V and long-term survival**

According to the results of MTS assay, the following drug concentrations were selected for use in further studies on K562 cells; 0.2 µM for doxorubicin and imatinib, 10 µM for cisplatin and 20 nM for rapamycin. Confirmation of drug toxicity at these selected doses was then assessed by viable cell counting using the Trypan blue exclusion method and MUSE Annexin V and dead cell assay at 24 and 48 hours post treatment in 24-well plates. The results showed the viable cell number (viability %) of K562 cells decreased from 3.54×10^5 cells/mL (100%) without treatment to 2.31×10^5 cells/mL (65.22%) with imatinib, 2.16×10^5 cells/mL (61.06%) with cisplatin, 1.69×10^5 cells/mL (47.56%) with doxorubicin and 2.88×10^5 cells/mL (81.44%) with rapamycin at 24 hours (Figure 3.14 A). The results at 48 hours showed further decrease in viable cell count (viability %) from 6.35×10^5 cells/mL (100%) without treatment to 2.53×10^5 cells/mL (39.97%) with imatinib, 1.76×10^5 cells/mL (27.39%) with cisplatin, 1.57×10^5 cells/mL (24.65%) with doxorubicin and 4.66×10^5 cells/mL (71.34%) with rapamycin (Figure 3.14 B).

Moreover, the findings of total apoptosis percentage showed similar effects on parental K562 cells after treatment; total apoptosis increased from 4.61% without treatment to 9.20% with imatinib and 7.93% with cisplatin at 24 hours, and from 5.75% without treatment to 32.55% with imatinib and 15.18% with cisplatin at 48 hours (Figure 3.15). In addition, rapamycin treatment showed no difference in total apoptosis percentage at 24 and 48 hours, suggesting that rapamycin only reduced cell growth, rather than inducing death by apoptosis (Figure 3.15). Due to a low number of cells, the apoptotic percentage for cells treated with doxorubicin could not be established.

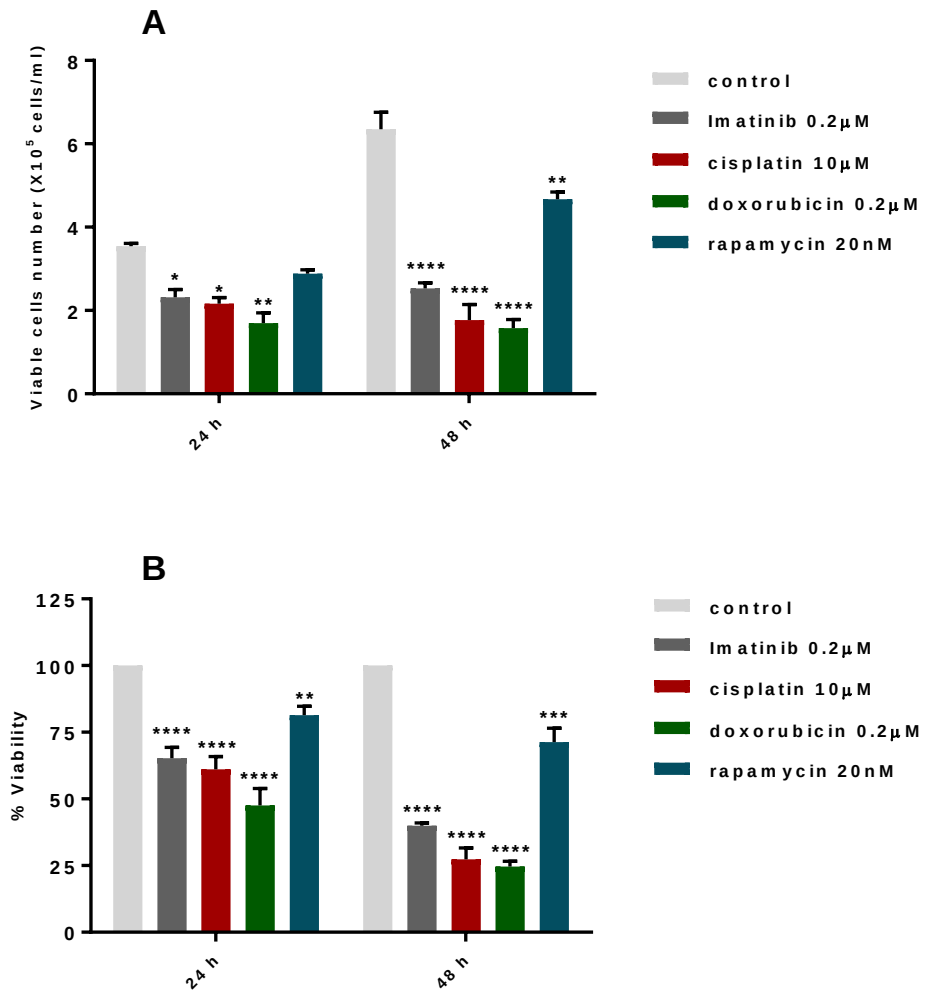

Figure 3.14 Effects of selected drug doses on the cell viability of K562 parental cells. Parental K562 cells were treated with 0.2µM imatinib, 10µM cisplatin, 0.2µM doxorubicin or 20nM rapamycin and viable cell number assessed by Trypan blue vital dye exclusion at 24 and 48 hours, expressed as both absolute cell numbers/mL (A) or as a percentage viability (B) (**p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

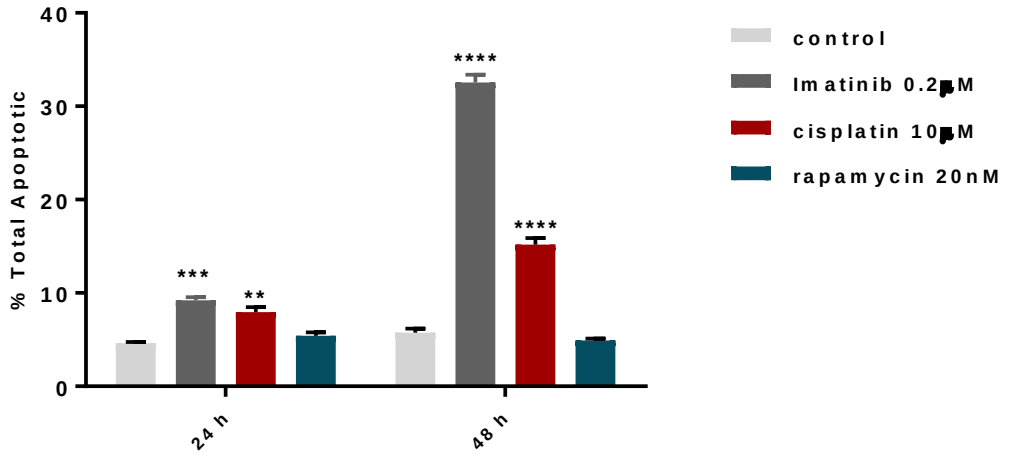

Figure 3.15 Effects of selected drug doses on the total apoptosis percentage of K562 parental cells. Parental K562 cells were treated with 0.2µM imatinib, 10µM cisplatin or 20nM rapamycin and apoptosis rate assessed by the Muse™ Annexin V and Dead Cell Assay kit at 24 and 48 hours expressed as apoptosis percentage (**p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

For long-term survival assays, investigations initially focused on those concentrations established from the drug dose-response assay using MTS method, with confirmation of data from cell counts and apoptosis assays however, the number of colonies for cells treated with cisplatin and doxorubicin was zero, suggesting that whilst cell viability in the short-term may be measureable, that long-term survival is more greatly affected under these conditions. Therefore the investigation was repeated with a lower dose range of 0.0-5.0 µM cisplatin, 0.0-0.1 µM doxorubicin, 0.0-0.1 µM imatinib and 0.0-20.0 nM for rapamycin. The results showed that treating K562 cells with 1.0, 2.0 and 5.0 µM cisplatin, 0.02 µM doxorubicin, 0.2 µM imatinib and 20.0 nM rapamycin significantly reduce their ability of colony formation (Figure 3.16).

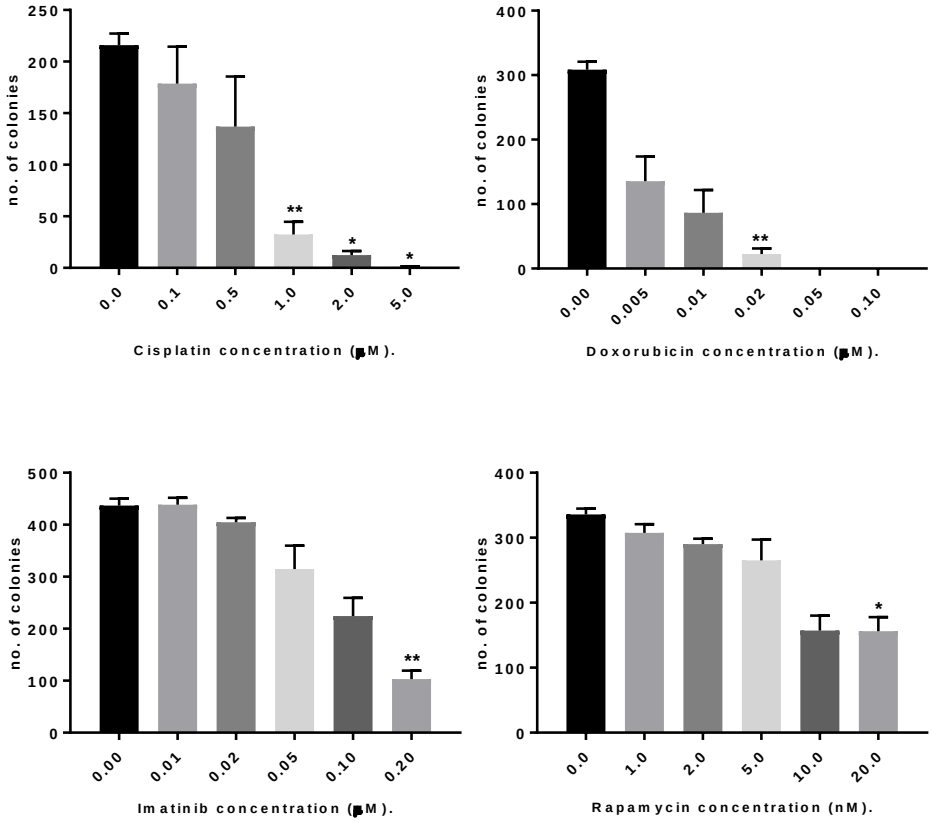

Figure 3.16 Effects of selected drug doses on the colony formation in K562 parental cells. Parental K562 cells were treated with (0.1-5.0) µM cisplatin (A), (0.005-0.1) µM doxorubicin(B), (0.01-0.2) µM imatinib (C) or (1.0-20.0) nM rapamycin (D) and colonies number assessed by long-term survival assay (**p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

Based on these optimisation results, concentrations that will be used as the optimised doses in subsequent chapters following PP4c silencing in K562 cells with Trypan blue exclusion and apoptosis assays are 10 µM cisplatin and 0.2 µM doxorubicin, while concentrations of imatinib and rapamycin will be used at 0.2 µM and 20 nM respectively for all assays.

Similarly, the following drug concentrations were selected for use in further studies on HL-60 cells; 0.1 µM for doxorubicin, 2.0 µM for cisplatin and 20 nM for rapamycin. Because of the high sensitivity of HL-60 cell as seen in MTS assay, the confirmation of drug toxicity at these selected doses as assessed by Trypan blue exclusion and MUSE Annexin V and dead cell assay was conducted only at 24 hours post treatment.

The results showed the viable cell number (viability %) of HL-60 cells decreased from 2.6×10^5 cells/mL (100%) without treatment to 1.71×10^5 cells/mL (66.24%) with cisplatin, 0.93×10^5 cells/mL (35.91%) with doxorubicin and 2.56×10^5 cells/mL (87.74%) with rapamycin (Figure 3.17). Moreover, the findings of total apoptosis percentage showed similar effects on parental HL-60 cells after treatment; total apoptosis increased from 5.56% without treatment to 10.61% with cisplatin, 75.36% with doxorubicin and 8.66% with rapamycin after 24 hours (Figure 3.18).

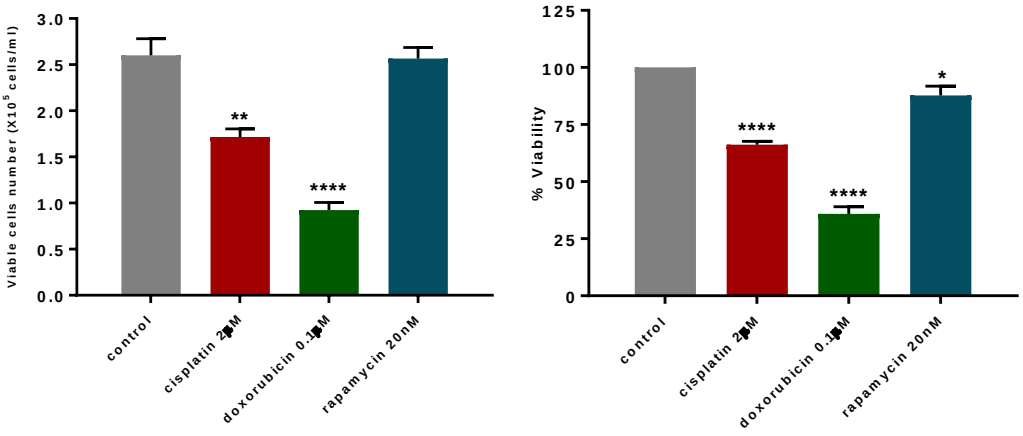

Figure 3.17 Effects of selected drug doses on the cell viability of HL-60 parental cells. Parental HL-60 cells were treated with 2µM cisplatin, 0.1µM doxorubicin or 20nM rapamycin and viable cell number assessed by Trypan blue vital dye exclusion at 24 expressed as both absolute cell numbers/mL (A) or as a percentage viability (B) (*p<0.05, **p<0.01, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

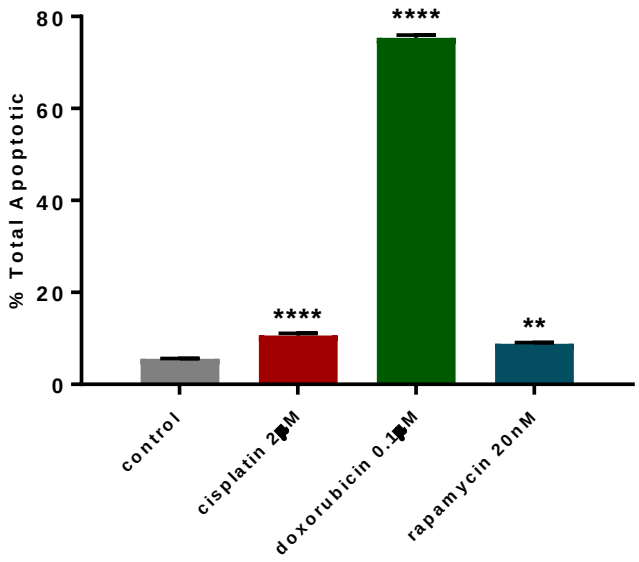

Figure 3.18 Effects of selected drug doses on the total apoptosis percentage of HL-60 parental cells. Parental HL-60 cells were treated with 2µM cisplatin, 0.1µM doxorubicin or 20nM rapamycin and their apoptosis rate assessed by the Muse™ Annexin V and Dead Cell Assay kit at 24 hours expressed as apoptosis percentage (**p<0.01, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

Similarly to K562 cells, the long-term survival assay was initially carried out using the same drug doses used in the MTS method, with colony number for cells treated with cisplatin and doxorubicin zero. This was then repeated with a lower dose range from 0.0 to 5.0 µM with cisplatin, 0.0-0.1 µM with doxorubicin and 0.0-20.0 nM with rapamycin (Figure 3.19). The long-term survival assay results showed that 0.5 µM and 1.0 µM cisplatin and 0.005 µM and 0.01 µM doxorubicin concentrations significantly inhibited the cells' ability to form colonies, while 20 nM rapamycin did not inhibit HL-60 cells' colony forming ability. Therefore, 0.5 µM cisplatin and 0.01 µM doxorubicin concentrations will be used as the optimised doses following PP4c silencing in HL-60 cells with Trypan blue exclusion and apoptosis assays instead of the previous concentrations 2.0 µM cisplatin and 0.1 µM doxorubicin.

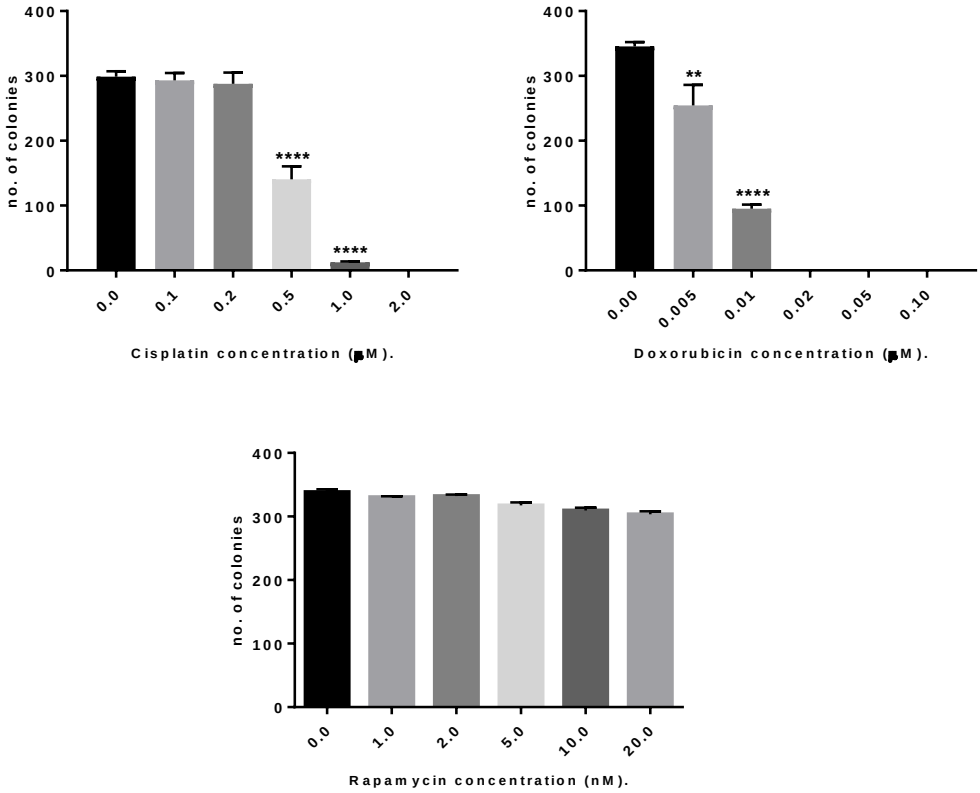

Figure 3.19 Effects of selected drug doses on the colony formation in HL-60 parental cells. Parental HL-60 cells were treated with (0.1-5.0) μM cisplatin (A), (0.005-0.1) μM doxorubicin (B) or (1.0-20.0) nM rapamycin (C) and colonies number assessed by the long-term survival assay (**p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA test followed by Bonferroni test; n=3).

3.3.4. **Transfection methods optimisation**

As mentioned previously (see section 3.1.3), a preferred transfection method needs to yield high transfection efficiency, minimum toxicity and subsequently, gives high quality and reproducible results, therefore, this section will focus on the results of inroducing DNA or RNA into K562 and HL-60 cells.

3.3.4.1 **Plasmid DNA transfection**

3.3.4.1.1. **K562 cell line**

The transfection efficiency at 48 hours post GFP transfection in K562 cells using nucleofection method was more than 80% at (Figure 3.20).

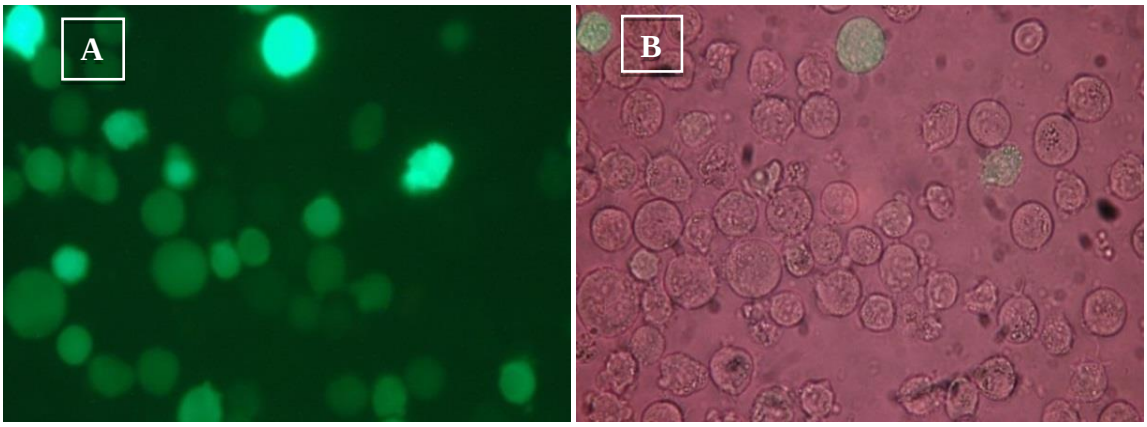

Figure 3.20 Transfection efficiency of K562 leukaemic cell line using nucleofection. Representative images showing transfected K562 cells with enhanced pmaxGFP by nucleofection using programme T-016. The transfection efficiency was more than 80% at 48 hours post transfection using **[A]** fluorescence microscopy and **[B]** light microscopy.

3.3.4.1.2. **HL-60 cell line**

In this research and after using different transfection reagents that have been used for plasmid DNA transfection, HL-60 cells were found to be difficult to transfect and very sensitive to these reagents. Similarly to K562 cells, HL-60 cells were initially transfected with a GFP-expressing plasmid using nucleofection solution V, however; this resulted in low transfection efficiency (<35%, table 3.1). This is contrast to the use of nucleofection with K562 cells where transfection efficiency was greater than 80% (see 3.3.4.2.1). Alternative methods for plasmid delivery into HL-60 cell using Lipofectamine 2000 Reagent and Lipofectamine 3000 Reagent were also explored, however; this resulted in no visual GFP expression and a negligible transfection rate.

Table 3.1 pmax-GFP transfection optimisation for HL-60 cell line. Three experiments using nucleofection V reagent showed very low level of transfection efficiency percentage.

	Transfection efficiency %
Replicate 1	26%
Replicate 2	34%
Replicate 3	33%
Total	31.0 ± 2.5 %

Following the same steps used with K562 cells, figure 3.21 showed the results of the transfection efficiency in HL-60 cells at 48 hours post GFP transfection.

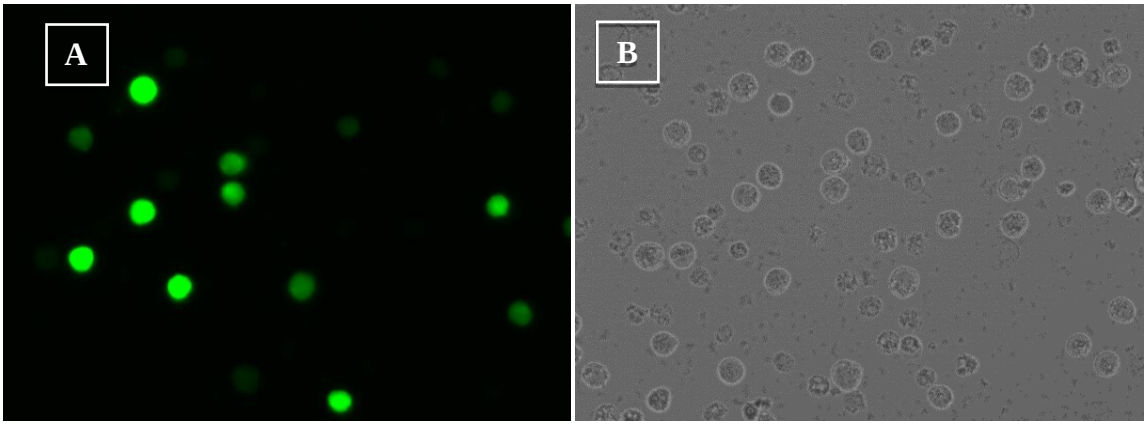

Figure 3.21 Transfection efficiency of HL-60 leukaemic cell line using nucleofection. Representative images showing transfected HL-60 cells with enhanced pmaxGFP by nucleofection V solution using programme T-019. The transfection efficiency was less than 40% at 48 hours post transfection using **[A]** fluorescence microscopy and **[B]** light microscopy.

3.3.4.2. **Small interfering RNA (siRNA)**

3.3.4.2.1. **K562 cell line**

The results of current study revealed that the transfection efficiency of Cy3 labelled siRNA in K562 cells was 95% at 72 hours post-transfection using nucleofection method (Figure 3.22).

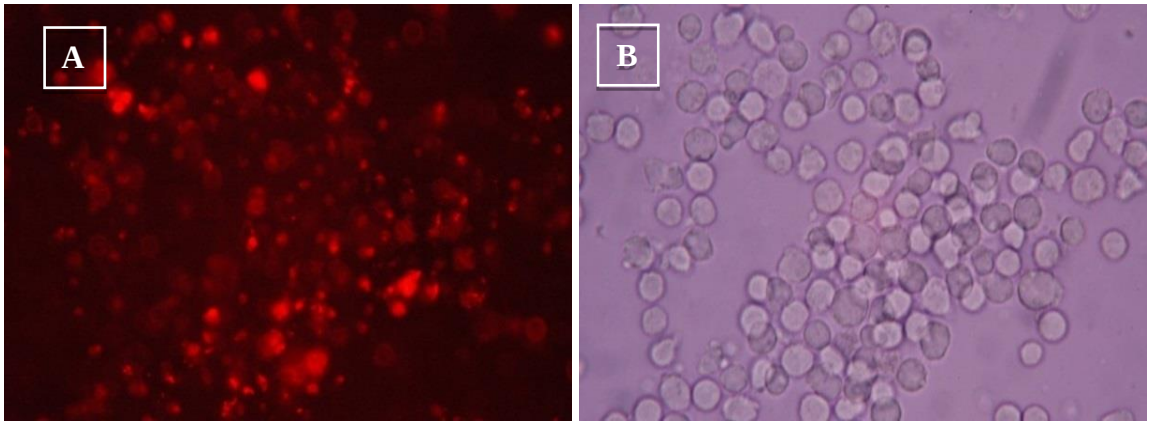

Figure 3.22 Transfection efficiency of K562 leukaemic cell line using nucleofection as a transfection method. Representative images showing transfected K562 cells with Cy3-labelled (-) siRNA. The transfection efficiency was 95% at 72 hours post transfection using **[A]** fluorescence microscopy and **[B]** light microscopy.

3.3.4.2.2. **HL-60 cell line**

Owing to low transfection efficiencies and/or poor recovery of cells post-transfection, a variety of transfection methods for the introduction of siRNAs into HL-60 cells were explored. HiPerFect transfection reagent (a lipid-based transfection method from Qiagen) was used to introduce the siRNAs into HL-60 cells. Optimal transfection efficiency was achieved by culturing HL-60 cells for 24 hours prior to transfection to ensure that the cells were in logarithmic growth phase. After which, 2×10^5 cells per well were seeded in 24 well plates in 100 µL complete culture medium. Transfection effeciency was determined using three different volumes of HiPerFect according to the manufactorer's protocol; 750 ng scrambled negative siRNA labelled with Cy3 was diluted in 100 µL Opti-MEM™ culture medium (Gibco) and either 3, 6 or 9 µL HiPerFect transfection reagent was added to the diluted siRNA. Samples were mixed by vortexing then incubated at room tempeartaure for 5-10 minutes to allow the formation of transfection complexes. The complexes were added drop-wise onto the seeded cells in 24-well plates and the plates gently swirled to ensure uniform distribution of the transfection complexes. Plates were then incubated for 6 hours to allow siRNA delivery and then 400 µL complete RPMI culture medium was added to all wells. Cell viability on HL-60 cells transfection with HiPerFect at different volumes and Cy3-siRNA amounts resulted in consistent high viable cell numbers and cell viability in the region of 95% (Table 3.2).

Transfections were later repeated using a reduced amount of scrambled negative siRNA labelled with Cy3 (200 ng) and, following the same steps above, comparable results for transfection efficiency were obtained (Figure 3.23).

Table 3.2 HL-60 HiPerFect reagent 48 hours in 24-wells plate (n=3)

Cy3	HiPerFect reagent	Total cell number×10^5 (mean±SEM)	% Death	Transfection efficiency %
200 ng	3 µL	7.90 ± 0.78	4.94 ± 0.97	100 %
375 ng	3 µL	8.61 ± 2.04	5.48 ± 0.96	100 %
750 ng	3 µL	8.51 ± 3.05	6.40 ± 3.06	100 %
750 ng	6 µL	10.87 ± 2.6	5.24 ± 0.45	100 %

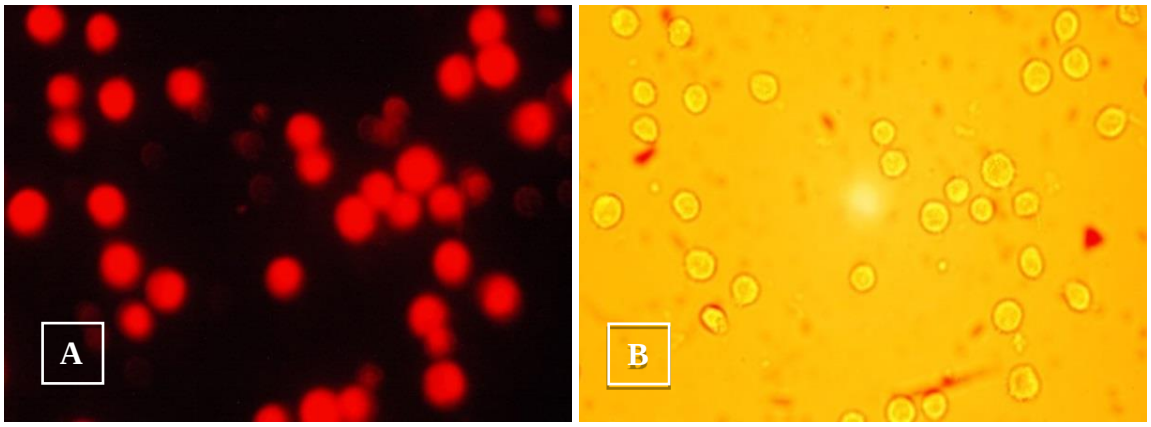

Figure 3.23 Transfection efficiency of HL-60 cell line using HiPerFect as a transfection method. Representative images showing transfected HL-60 cells with 200 ng Cy3-labelled (-) siRNA. The transfection efficiency was 100% at 72 hours post transfection using **[A]** fluorescence microscopy and **[B]** light microscopy.

3.4. **Discussion**

This study aimed to confirm the cytotoxicity of a range of chemotherapeutic drugs against K562 and HL-60 cells in order to establish preliminary dose response relationships and determine suitable concentrations for use in further studies. Both K562 and HL-60 cells were treated with increasing concentrations of cisplatin, doxorubicin, rapamycin, and only K562 cells were treated imatinib for 24 and 48 hours, with cell viability assessed using the MTS assay. The data obtained from the MTS assay indicated that the cell viability was significantly decreased with increased drugs doses and incubation period. This could be explained as the drug treatment decreased the number of viable cells either by inducing cell death directly or by decreasing their proliferation rate in a concentration- and time-dependent manner.

In consistence with the results of this study, previous studies showed that cisplatin and doxorubicin inhibit cell proliferation rate in other types of cancers such as breast cancer, ovarian cancer and sarcoma (Tsimberidou et al., 2009). The present data showed that the cytotoxicity or the inhibition of cell proliferation in HL-60 cells post cisplatin and doxorubicin treatment was much higher (up to 95% at 48 hours) than the inhibition level in K562 cells (up to 40% with cisplatin and 60% with doxorubicin at 48 hours), and consequently, the viability of HL-60 cells post cisplatin or doxorubicin treatment was much lower than the viability of K562 cells at 48 hours post-treatment. This may be related to the kinases linked with AML and CML that may be interacting with some of the downstream targets as well as the phosphatases that these two cells express or lacked. In a previous study, a comparison between the response of HL-60 and K562 cells to the same stimuli over long time period (up to 14 days) indicated that the execution phase of programmed cell death in K562 cells is delayed compared with HL-60 cells (Martins et al., 1997). Moreover, Martins and colleagues reported that the levels of activation of the

effector caspases can vary in these two cell lines undergoing apoptosis after treatment with the same apoptosis-inducing stimulus (Martins et al., 1997). Compared to HL-60 cells, the caspase activation was delayed in K562 cells, suggesting that the Bcr-Abl kinase acts upstream of events that result in cytochrome c release and caspase activation (Martins et al., 1997). In addition, by two-dimensional gel electrophoresis assay, three caspase species and three active forms of caspase-3 were detected in K562 cytoplasm and nuclei, while HL-60 cells contained one active form of caspase-3 and another caspase species (Martins et al., 1997). Another study revealed that in contrast to HL-60 cell line, K562 cells represent a cell line with an earlier arrest in myeloid differentiation, therefore when the K562 cells treated with dimethyl sulfoxide (DMSO) did not induce morphological or histochemical changes as it did in HL-60 cells, suggesting that K562 cells did not exhibit morphological or histochemical myeloid differentiation (Collins et al., 1978).

Many studies have suggested that the activation of p53 might increase the cell's sensitivity to cisplatin (Bressac et al., 1990; Qin and Ng, 2002). As K562 cells do not express the p53 gene and HL-60 cells also lack this oncoprotein (Wolf and Rotter, 1985; Prokocimer et al., 1986; Durland-Busbice and Reisman, 2002), they thus show reduced sensitivity to cisplatin compared to other cells, which express p53 protein (Bressac et al., 1990; Dasari et al., 2015). Nevertheless, cisplatin does induce apoptosis and causes changes in cell cycle progression in cells, whether they express p53 or not, through both p53 dependent and independent pathways (Qin and Ng, 2002; Dasari et al., 2015). In a study on p53-deficient renal cells, Jiang et al. (2009) revealed that apoptosis can be induced by cisplatin via the intrinsic or mitochondrial pathway. Cisplatin induces apoptosis by interdependent activation of Bcl-2 members, Bax and Bak, resulting in c cytochrome release and subsequently activation of caspases (Jiang et al., 2009).

Dasari and colleagues reported that cisplatin induced lipid peroxidation and DNA damage, irreversibly inhibiting cell proliferation in HL-60 cells by arresting them in G1 phase of cell cycle (Dasari et al., 2015). Cisplatin causes DNA cleavage in APL cells by modulating c-jun expression and the protein kinase C-dependent signalling pathway (Rubin et al., 1992). Inhibition of cell proliferation or cytotoxicity induced by cisplatin in HL-60 cells could be the result of collective mechanisms including c-jun expression, protein kinase C-dependent signalling pathways, Bcl-2 down regulation and inhibition of cell cycle progression (Saad et al., 2004; Kumar and Tchounwou, 2015).

As the mammalian Target Of Rapamycin (mTOR) deregulation has been implicated in cancer (Li et al., 2014), rapamycin, a potent inhibitor of mTOR with its ability to inhibit cell growth and trigger a nutrient deprivation-like response, was used in this study. The results showed a significant decrease in the viability of K562 cells at 24 and 48 hours and a decrease in their ability to form colonies when they treated with 20 nM rapamycin, while a significant decrease at 48 hours was seen in HL-60 cell when treated with rapamycin, associated with significant increase in apoptosis rate.

This study used a range of complementary methods for the assessment of cell viability, apoptosis, or long-term survival following drug treatment. Of the many methods that have been developed to measure cell health, these can be divided in to three categories: those that exploit loss of membrane integrity, those that directly measure metabolic markers, and those that assess metabolic activity. In this research, the Trypan blue dye exclusion assay (cell membrane integrity) and the MTS assay (a measure of metabolic status) were used as complementary methods for assessing cell viability. In the Trypan blue dye exclusion assay, viable cell numbers are counted directly by the ability of viable cells (which retain membrane integrity) to actively exclude the dye. While MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) indirectly reflects cell

viability by measuring the mitochondrial metabolic rate (Berridge and Tan, 1993). Differences in mode of action in terms of assessment of cell viability, can explain slight variation of the data obtained from these two methods and emphasises the importance of using a number of complementary methods in these approaches. The effect of a concentration of a drug on cell survival measured with long term survival, an important method for the assessment of survival by measuring the ability of a single cell to form colonies. K562 and HL-60 cells were highly sensitive to both doxorubicin and cisplatin so that long-term survival method was carried out first with the same concentration of these drugs that been used with Trypan blue exclusion and Muse™ Annexin V and Dead Cell Assay kit (10 µM cisplatin and 0.2 µM doxorubicin with K562 cells and 2 µM cisplatin and 0.1 µM doxorubicin with HL-60 cells), but the results showed that the cells were incapable of forming colonies. The experiment was then repeated with new set of lower concentrations to select another concentration in which the cells can form colonies.

Each cell line has its own characteristics that determine a special requirement for the transfection method. A comparative study on many transfection methods demonstrated that nucleofection is more effective at delivering DNA for different cell types than chemical transfection reagents such as Lipofectamine 2000 and Lipofectamine Plus, resulting in high levels of transfection efficiency associated with low toxicity (Maurisse et al., 2010). These findings are in agreement with the results of this study in term of K562 cells and support the present results in HL-60 cells despite the low transfection efficiency (less than 35%; due to the high toxicity results from electric pulses) when they transfected by nucleofection solution V, while they showed a negative response to Lipofectamine 2000 and Lipofectamine 3000 reagents. On the other hand, in this research HiPerFect reagent has been used instead of nucleofection V reagent to down-regulate PP4c in HL-60 cells when

the first experiments showed high toxicity, as HiPerFect reagent is recommended for rapidly growing cells.

Collectively, these two different models of leukaemic cell lines showed different response to the transfection methods that been used to deliver plasmid DNA or small interfering RNA as well as their response to the selective drugs applied in this research. In depth studies, different types of cell viability assays, proliferation and apoptosis are crucial to assess cell health, monitor the growth rate and to examine how the cells conducted programed cell death by gene modulation or by exposing to pharmacological compounds. The next chapter will focus on the effects of protein phosphatase 4 catalytic subunit's modulation on cell viability, proliferation and apoptosis in both K562 and HL-60 cell lines using some of the methods optimised in this chapter.

4. The effects of modulating the endogenous level of PP4c on the viability, apoptosis and proliferation of leukaemic K562 and HL-60 cell lines

4.1. Introduction

As several cancers are characterized by the unrestrained activation of oncogenic kinases, it is not surprising that many protein phosphatases can counteract the activity of these kinases and function as tumour suppressors (Lee and Muller, 2010; Perrotti and Neviani, 2013). The study of protein phosphatases and their regulation has therefore become an expanding field of research aimed at determining the importance of these proteins in cancer (Tonks, 2006; Perrotti and Neviani, 2006; 2013).

One of the best known example of oncoproteins with unrestrained tyrosine kinase activity is the product of the Philadelphia chromosome t(9;22) translocation, Bcr-Abl. The Bcr-Abl fusion protein is a constitutively active tyrosine kinase that acts as a driving force in the induction of chronic myeloid leukaemia (CML) and the survival of CML cells (Nowell and Hungerford, 1960; Rowley, 1973). Mutations and dysregulation of a number of tyrosine kinase including receptors Fms-like tyrosine kinase (Flt-3), c-KIT and platelet derived growth factor receptor (PDGFR) are also characteristics of the heterogeneous disease Acute myeloid leukaemia (AML) (Neviani et al., 2005). While much of the focus for targeted therapies in myeloid leukaemias has concentrated on the tyrosine kinases responsible for phosphorylation events, research over the past decade has now started to highlight the importance of protein phosphatases in myeloid leukaemias and their potential as targets for novel therapies (Neviani et al., 2005; 2007).

PP2A is the most studied member of the PPP family due to discoveries indicating its important role as a tumour suppressor in many cancers, including myeloid leukaemia (McConnell and Wadzinski, 2009). Many studies have implicated PP2A in a wide range of cellular signalling pathways, such as Wnt, mTOR and MAPK pathways, many of which

are involved in cellular proliferation, apoptosis, cell-cycle division and differentiation (McConnell and Wadzinski, 2009). The inactivation of PP2A by mutant c-KIT receptors that consequently altered expression of the endogenous PP2A inhibitory protein SET, was found to be a common theme in acute myeloid leukaemia, therefore, re-activation of PP2A results in dephosphorylation, and deactivation, of the kinase and subsequent inhibition of leukaemogenesis (Roberts et al., 2010). Therefore, re-activation of PP2A is considered as an attractive strategy for leukaemia therapy (Neviani et al., 2005; 2007; Kalla et al., 2007; Cristóbal et al., 2012; Smith et al., 2016). The expression of PP1 and PP2B was also evaluated in various acute myelogenous leukaemic (AML), acute lymphocytic leukaemic (ALL) and chronic lymphocytic leukaemic (CLL) cells compared to normal peripheral leukocytes (Yamamoto et. al., 1999). The results showed that the levels of these protein phosphatases were lower in leukaemic cells compared to normal peripheral leukocytes, suggesting that they play a role in regulating important signalling pathways that contribute to leukaemogenesis (Yamamtoto et.al., 1999).

4.1.1. **Protein phosphatase 4**

Due to its location on chromosome 16 p11.2, the human PP4c gene is linked to the translocational mutation associated with acute leukaemia (Bastians et al., 1997). PP4c interacts with four regulatory subunits which regulate and control its activity (Martin-Granados et al., 2008) and is believed to be involved in many signalling pathways such as tumour necrosis factor (TNF)-alpha signalling and regulation of histone acetylation (Zhang et al., 2005). It has been reported that the silencing of PP4R1, a constitutive interacting partner of PP4c, causes cell cycle arrest at G2/M phase leading to decreased cell proliferation and colony formation in hepatocellular carcinoma cell lines (Wu et al., 2015). PP4R1 silencing also inhibits cell growth and proliferation in breast cancer cells (Qi et al., 2015) and lung cancer cells (Zhu et al., 2016).

Brechmann and colleagues (2012) have reported that a specific PP4c-PP4R1 interaction occurs in human malignant T lymphocytes as a negative regulator of inhibitor of NF-κB kinase (IKK) activity (Brechmann et al., 2012). T cell receptor or tumour necrosis factor (TNF) receptor 1 (TNFR1) stimulation leads to the phosphorylation of NF-κB kinase (IKK) complex followed by the proteasomal degradation of subsequent inhibitory kB proteins (IkBs) resulting in the translocation of NF-κB proteins to the nucleus, where they bind to DNA and activate the transcription of NF-κB-regulated genes (Hayden and Ghosh, 2008). PP4R1 enhances PP4c activity and allows its association with IKK complex leading to its dephosphorylation and inactivation and consequently the inhibition of NF-κB activity. So, PP4c is unable to dephosphorylate the IKK complex in the absence of PP4R1 resulting in unrestrained IKK phosphorylation and abnormal NF-kB activity (Brechmann et al., 2012).

PP4 plays a crucial role in different cellular functions such as human centrosome maturation, cell migration (Martin-Granados et al., 2008), DNA damage checkpoint signalling (Nakada et al., 2008), and DNA repair (Lee et al., 2010). In numerous types of cancers including breast and lung tumours (Wang et al., 2008), pancreatic ductal adenocarcinoma (PDAC) (Weng et al., 2012), and in glioma development and progression (Li et al, 2016), PP4 catalytic subunit is over-expressed and these findings suggested that PP4c might function as an oncogene. It is also reported that depletion of PP4 catalytic subunit in HEK293 cells resulted in a decrease in cell migration and PP4c complexes may coordinate centrosome maturation and cell migration via regulation of Rho GTPases (Martin-Granados et al., 2008).

An experiment carried out on W7.2c mouse lymphoma cells implicated that over-expression of PP4c results in an increase in cell death and a decrease in cell proliferation suggesting that PP4 has an important role in apoptosis regulation and may function as a

tumour suppressor gene (Mourtada-Maarabouni et al., 2003). Moreover, other studies on HEK 293T human embryonic cells and on both human leukaemic T cells and primary human peripheral blood T-cells, as well as various breast cancer cells, have also shown that over-expression of PP4c enhances apoptosis, while PP4c down-regulation suppresses apoptosis (Mourtada-Maarabouni and Williams, 2008; 2009; Mohammed et al., 2016).

Changes in PP4c expression levels affect the phosphorylation status of many proteins involved in apoptosis and cell proliferation, extracellular signal-regulated kinase 1 (ERK1) and extracellular signal-regulated kinase 2 (ERK2), and their regulator; PEA-15 (Phosphoprotein Enriched in Astrocytes), which is also identified to be significantly over-phosphorylated when PP4c expression was suppressed (Krueger et al., 2005; Mourtada-Maarabouni and Williams, 2009; Mohammed et al, 2016). Increasing evidence also supports a role for PP4c in MAPK-mediated apoptosis. MAPK cascades consist of three major branches: p38, JNK (JUN-N-terminal protein kinase), and ERK pathways which are involved in the regulation of apoptosis and cell proliferation (Rose et al., 2010; Cohen et al., 2005).

Therefore, the aim of this chapter is:

- To evaluate the effects of the modulation of the PP4c endogenous level on the viability, apoptosis, proliferation rate and cell long-term survival of K562 and HL-60 cell lines using the most appropriate transfection method for each cell line to ultimately determine if this phosphatase plays a role in the cell fate of CML and/or AML cell models.

4.2. **Methodology**

K562 and HL-60 cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 10 mg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO_2 (see chapter 2 section 2).

To over-express PP4c in these cells, (1-2×10^6) K562 or HL-60 cells per transfection were harvested and re-suspended in 100 µL nucleofection V solution containing 2 µg of plasmid DNA (either pcDNA3.1-PP4c or pcDNA3.1) and the transfection was achieved by Nucleofector TM II 2b, Lonza using programme T-016 for K562 cells and T-019 for HL-60 cells. Following transfection, cells were seeded in 3.0 mL of RPMI medium in 6-well plates and incubated for 24 hours to allow PP4c expression, before being re-plated according to the type of the following functional assay (see chapter 2 sections 5.1 and 5.2).

PP4c down-regulation was carried out in these cell lines using different methods depending on their sensitivity to the transfection reagent (see chapter 3 section 3). (1-2×10^6) K562 cells per transfection were harvested and re-suspended in 100 µL nucleofection V solution containing 50 nM of either -siRNA, PP4c siRNA1, PP4c siRNA2, PP4c siRNA8 or PP4c siRNA9, using Nucleofector ^{TM} II 2b. The cells were then transferred to 7.0 mL of pre-incubated growth medium in 25cm^2 flasks and incubated for 72 hours before being re-plated at 2×10^5 cells/mL for further functional assays (see chapter 2 section 6.1). HiPerFect transfection reagent was used to introduce the above siRNAs into HL-60 cells in 6-well plates using 250 ng/well, following the method described in chapter 2 section 6.2.

Western blotting was performed to determine the level of PP4c expression and confirm that Plasmid DNA transfection or RNA interference were accomplished correctly (section 2.13).

Cell viability and apoptosis were assessed using a variety of complementary methods following re-plating of cells after plasmid or siRNA transfection as described in sections

2.7.1 and 2.8. Briefly, count and cell viability were performed by manual Trypan blue exclusion method. Programmed cell death (Apoptosis) was determined by Muse™ Annexin V and Dead Cell Assay kit using Muse™ Cell Analyser and cell cycle analysis was carried out using Muse™ Cell Cycle kit (section 2.10).

Methods for assessment of cell proliferation were also used in this study. Determination of the cell's ability to form colonies was performed by long term survival assay over a period of 2-3 weeks (see section 2.9) and the Muse® Ki67 Proliferation Kit (section 2.11) was used to determine the percentage of cells positive for the proliferation marker Ki67.

4.3. **Results**

4.3.1. **K562 cell line**

4.3.1.1. **PP4c over-expression (up-regulation)**

The effects of PP4c over-expression on the K562 cell line were investigated and compared to negative control (vector only). K562 cells were transfected with pcDNA3.1-PP4c (accession #BG913014) or pcDNA3.1 empty vector. The cells were counted and plated after 24 hours post-transfection and the effects of PP4c over-expression on the cell count, viability, apoptosis, Ki-67 proliferation and cell-division cycle were examined 24 and 48 hours post-plating.

The degree of over-expression of PP4c was determined by Western blotting, with a 1.5 fold increases in the level of PP4c protein observed in K562 cells transfected with pcDNA3.1-PP4c as compared to mock transfected cells and cells transfected with pcDNA3.1 only (Figure 4.1, $p<0.05$ vs vector only transfection).

Overexpression of PP4c in K562 cells showed a significant decrease in viable cell number from 2.25×10^5 cells/mL with pcDNA3.1 empty vector to 1.5×10^5 cells/mL ($p<0.05$), as assessed by Trypan blue vital dye exclusion assay (Figure 4.2). When the effects of PP4c overexpression on the basal apoptosis rate were studied, a significant increase in the percentage of cells scored as apoptotic was observed in PP4c overexpressing cells at 48 hour post plating ($p<0.01$) (Figure 4.3).

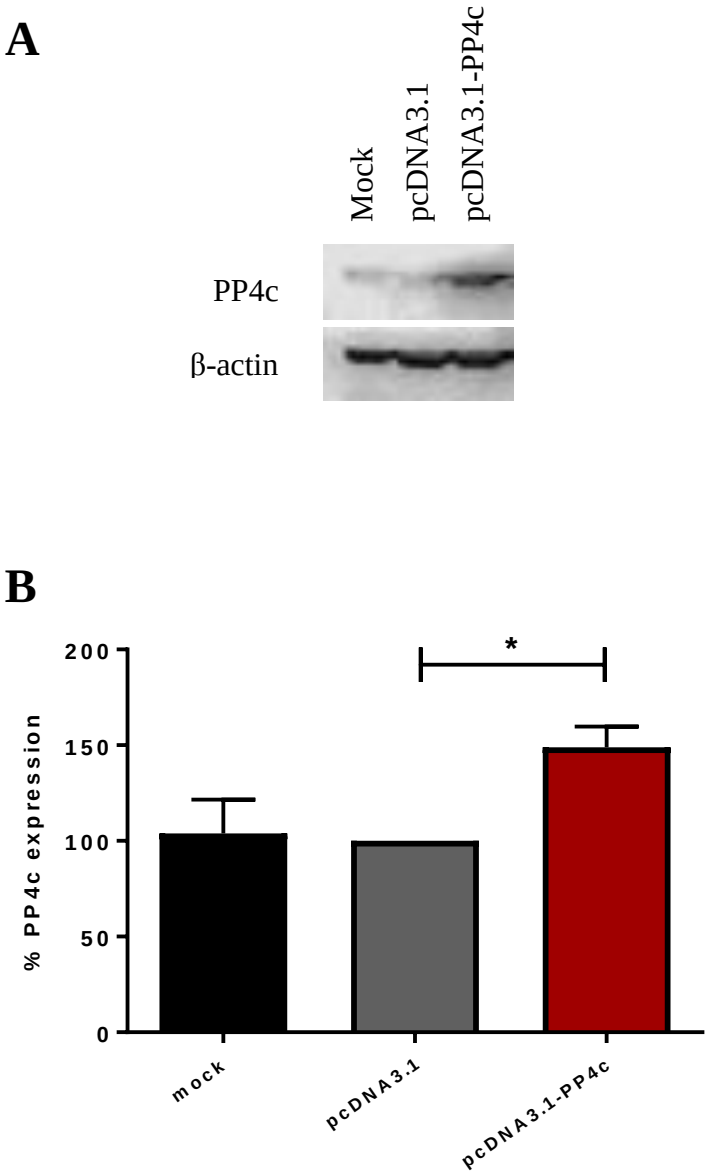

Figure 4.1 Effects of pcDNA3.1-PP4c transfection on endogenous PP4c levels in K562 cells. Western blotting and subsequent quantification shows a significant elevation in the expression of PP4c in transfected cells as compared to control (*$p<0.05$; one-way ANOVA test followed by Bonferroni test). **A**) Representative immunoblot of PP4c protein expression in K562-pcDNA3.1-PP4c-transfected cells, K562-pcDNA3.1-transfected cells and K562 parental cells. Cells lysates of $1x10^6$ cells/lane were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of PP4c. Blots were stripped and re-probed for β-actin. **B)** A bar graph represents three independent experiments expressed as mean ± SEM. Relative expression is the ratio of PP4c level versus β-actin as determined using Licor image analysis software. (n=3).

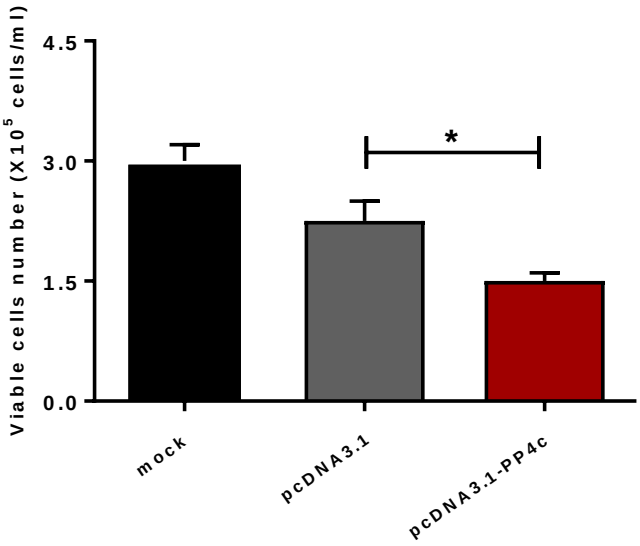

Figure 4.2 PP4c over-expression in K562 cell line decreases the viable cell count. K562 cells were transfected with empty vector pcDNA3.1 and pcDNA3.1-PP4c. The number of viable cells was determined by Trypan blue vital dye stain 24 hours post-plating. Cells transfected with pcDNA3.1-PP4c were significantly less viable than cells transfected with pcDNA3.1 only (*p<0.05; one-way ANOVA test followed by Bonferroni test; n=3).

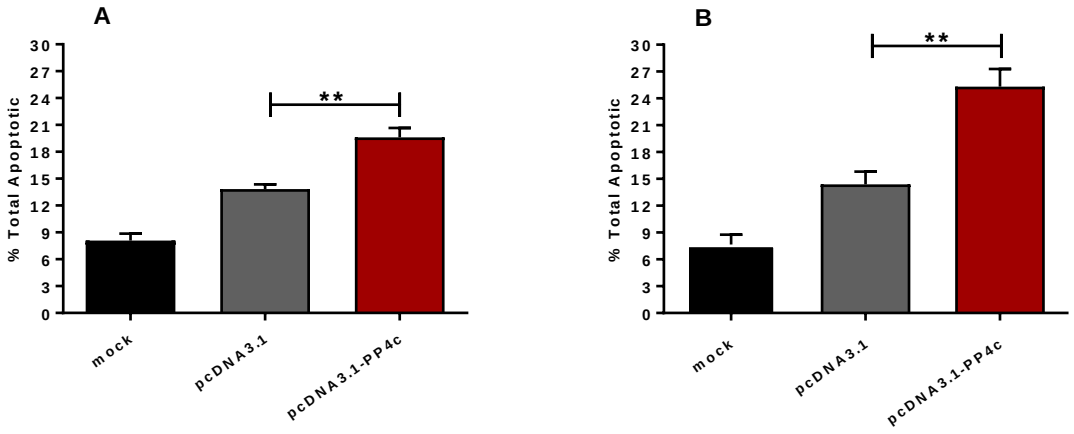

Figure 4.3 PP4c over-expression in K562 cell line increases the percentage of total apoptosis. K562 cells were transfected with empty vector pcDNA3.1 and pcDNA3.1-PP4c and apoptosis levels were measured by Muse annexin V & dead cell assay at different time points. After 24 and 48 hours of the plating, the percentage of total apoptosis is higher in the cells transfected with pcDNA3.1-PP4c than that in the pcDNA3.1 transfected cells (**p<0.01; one-way ANOVA test followed by Bonferroni test) **A**) 24 hours post plating **B**) 48 hours post plating (n=3).

The viable cell count, assessed by Trypan blue exclusion, at 24 hours was almost halved compared to only a 6% increase in the basal apoptosis rate in cells transfected with pcDNA3.1-PP4c, so effects on reduced viable cell number may suggest an effect on PP4c on the proliferation rate. Therefore, the effect of PP4c up-regulation on the proliferation of K562 cells was further assessed by Ki-67 labelling of proliferating cells (Iatropoulos and Williams, 1996). Despite a high proliferation rate observed in parental (mock transfected) and pcDNA3.1 expressing cells, PP4c over-expression in K562 cells showed a significant decrease of almost 10% (from 82.08% with pcDNA3.1 to 75.74% with pcDNA3.1-PP4c) in the number of Ki67-positive cells (Figure 4.4).

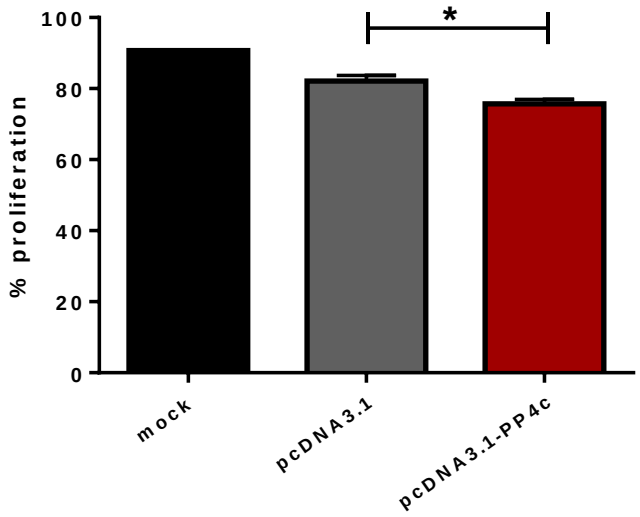

Figure 4.4 PP4c over-expression in K562 decreases the cell proliferation. The proliferation of PP4c transfected cells and control cells were determined by Ki-67 staining and Muse Cell analyser. PP4c over-expression decreases the proliferation rate of pcDNA3.1-PP4c transfected cells as compared to pcDNA3.1 (*$p<0.05$; one-way ANOVA test followed by Bonferroni test; n=3).

To determine whether this reduction in the viable cell count, induction of apoptosis and reduced cellular proliferation of K562 cells that were transfected with pcDNA3.1-PP4c were due to cell cycle arrest, cell cycle analysis was performed at 24 hours post-plating. Cell cycle analysis revealed that in G0/G1 phase the percentage of the proliferating cells with PP4c over-expression was significantly higher as compared to the control cells associated with a concomitant decrease in the percentage of cells in S phase (Figure 4.5). These findings support the previous results of Ki67 proliferation assay.

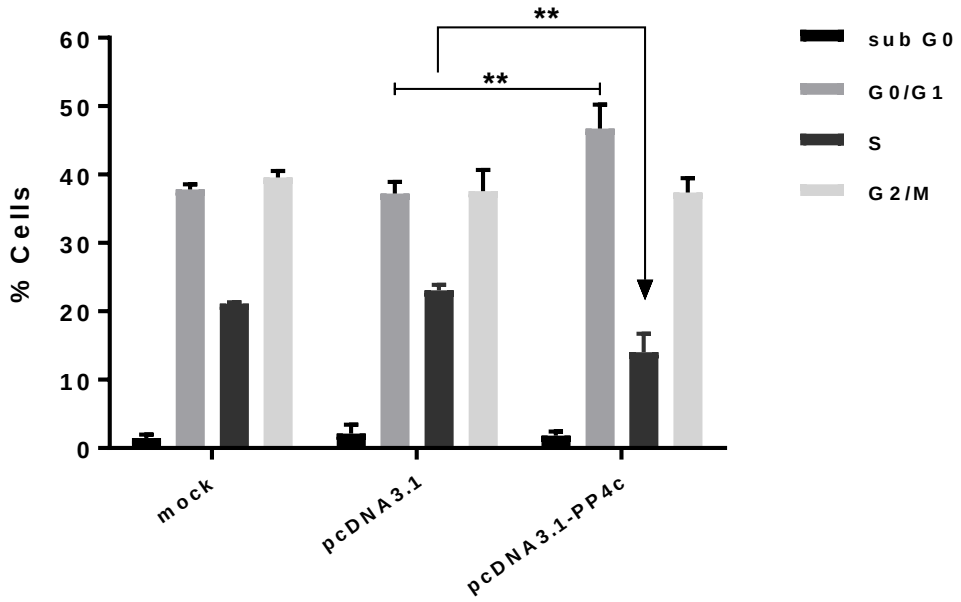

Figure 4.5 PP4c over-expression alters the cell cycle profile of K562 cells. Cell cycle analysis was performed by Muse™ Cell Cycle kit which revealed a significant elevation in the number of the K562-pcDNA3.1-PP4c transfected cells in G0/G1 phase compared to mock transfected cells and cells transfected with pcDNA3.1 (**p<0.01), this was accompanied with a significant reduction in the number of the transfected cells in S phase (**p<0.01) as compared to mock and pcDNA3.1 transfected cells (Two-way ANOVA test followed by Bonferroni test; n=3).

Collectively, these results suggest that PP4c over-expression in K562 cells decreases cell viability and proliferation rate, as well as increasing basal apoptosis. So the next section focused on the effects of PP4c down-regulation in these cells.

4.3.1.2. **PP4c down-regulation (knock-down)**

The effects of PP4c on K562 cells were further investigated by using specific siRNAs to down-regulate endogenous PP4c expression in these cells. Cells were transfected with negative or scrambled siRNA, siRNA1, siRNA2, siRNA8 and siRNA9.

Western blotting technique was used to confirm that the siRNAs effectively down-regulated PP4c expression at the protein level and the results showed that all targeted PP4c siRNAs reduced the expression of endogenous PP4c, with a 33.19%, 57.69%, 65.54% and 65.78% decrease in PP4c expression levels observed in cells transfected with PP4c siRNA1, PP4c siRNA2 & PP4c siRNA8, and PP4c siRNA9 respectively, as compared to the cells transfected with -siRNA (Figure 4.6). Following confirmation of the silencing of PP4c and because of the significant lower expression of PP4c, only cells transfected with PP4c siRNA 8 and PP4c siRNA 9 were assessed for viability, cell cycle and apoptosis.

PP4c down-regulation results showed a significant increase in the viable cell number of the cells transfected with PP4c siRNA 8 ($p<0.05$) at 48 hours post-plating as assessed by Trypan blue vital dye exclusion assay (Figure 4.7). The knockdown of PP4 by siRNA 8 and 9 had no effect on the basal apoptosis rate in K562 cells which was measured using Muse™ Annexin V and dead cell assay and Muse analyser at 48 hours post-plating the cells, as compared to control cells (Figure 4.8).

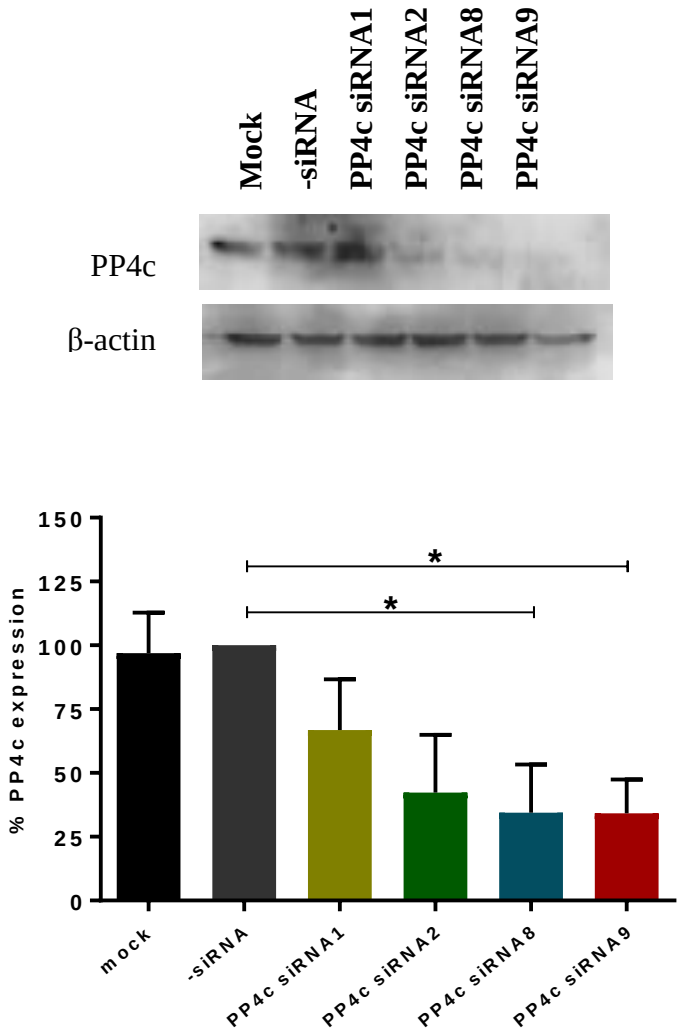

Figure 4.6 Effects of PP4c specific siRNA transfection on endogenous level of PP4c in K562 cells. Western blotting and subsequent quantification shows a significant reduction in the expression of PP4c in transfected cells as compared to control (*$p<0.05$ for PP4c siRNA8 and PP4c siRNA9; two-way ANOVA test followed by Bonferroni test). **A**) Representative immunoblot of PP4c protein expression in PP4c siRNA1, PP4c siRNA2 & PP4c siRNA8, PP4c siRNA9, -siRNA and K562 parental cells. Cells lysates of $1x10^6$ cells/lane were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of PP4c. Blots were stripped and re-probed for β-actin. **B)** A bar graph represents three independent experiments expressed as mean ± SEM. Relative expression is the ratio of PP4c level versus β-actin as determined using Licor image analysis software (n=3, see appendix A, page 191).

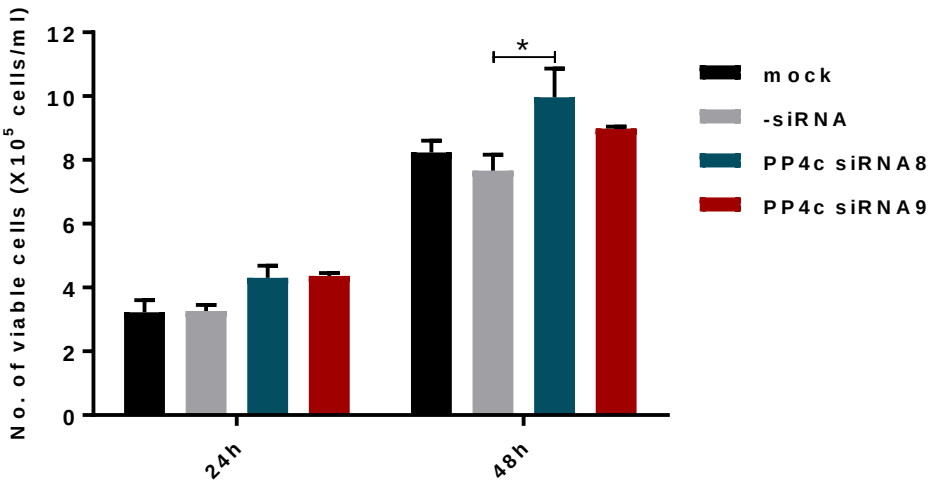

Figure 4.7 PP4c down-regulation in K562 leukaemic cell line increases viable cell count. K562 cells were transfected with scrambled siRNA and PP4c siRNA 8 and PP4c siRNA 9. Viable cell count carried out by Trypan blue vital dye exclusion shows a significant elevation in viable cell count of K562 cells with PP4c siRNA 8 at 48 hours post-plating the cells as compared to the negative control (*$p<0.05$; two-way ANOVA test followed by Bonferroni test; n=3).

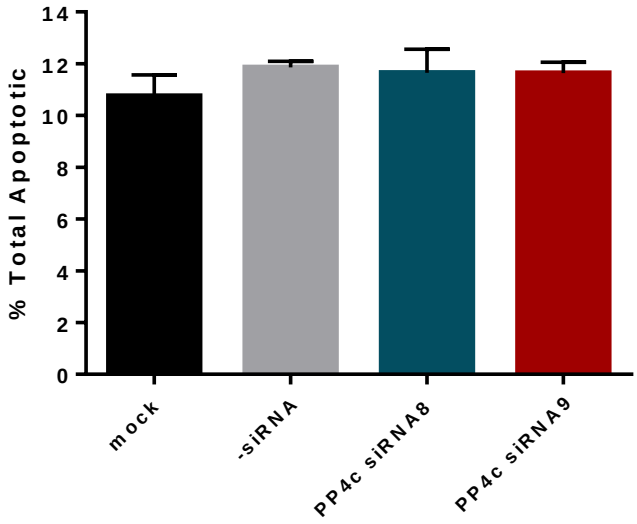

Figure 4.8 PP4c down-regulation in K562 cells has no effect on the level of basal apoptosis. At 48 hours post-plating, the apoptosis percentage was measured using the Muse annexin V & dead cell assay carried out by Muse cell analyser. Transfected cells with PP4c siRNA8 and PP4c siRNA 9 show no difference in total apoptotic percentage as compared to -siRNA (n=3).

Given that PP4c silencing increased viable cell number but with no reduction in basal apoptosis, the effects on cell proliferation was also measured. The Ki67 proliferation assessment showed no difference in the percentage of Ki-67 positive cells between the cells transfected with PP4c siRNA 8 or PP4c siRNA 9 and -siRNA transfected cells (Figure 4.9). This figure shows that the basal proliferation rate (mock and -siRNA) is already very high, making any further increases difficult to detect over these short time points and therefore the long-term ability of the cells to form colonies was investigated.

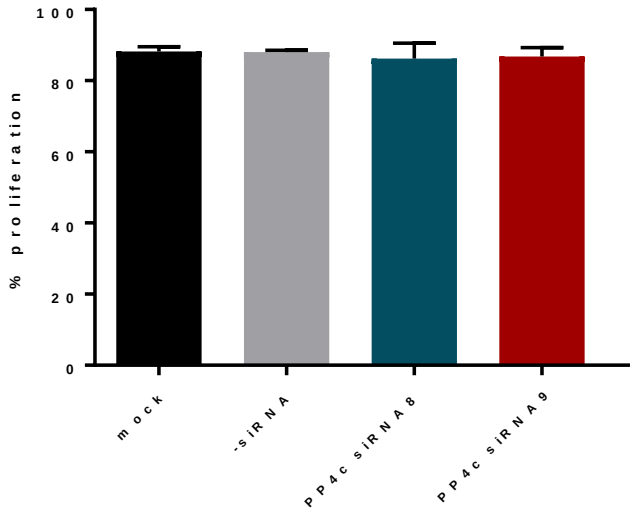

Figure 4.9 Effects of PP4c down-regulation on K562 cell proliferation. The proliferation of PP4c transfected cells and control cells were determined by Ki-67 staining and Muse Cell analyser. PP4c down regulation has no effect on the proliferation rate of K562 cells (n=3).

It was found that K562 cells transfected with the selected siRNAs (PP4c siRNA 8 and PP4c siRNA 9) have no significant difference in colony forming ability in comparison to the negative control -siRNA and mock cells (Figure 4.10).

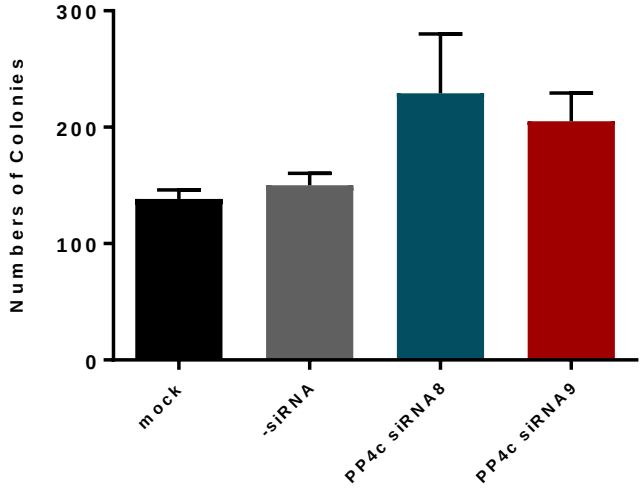

Figure 4.10 PP4c knockdown affects colony forming ability in K562 cells. Long term survival is compromised following transfection of the cells with siRNA 8 and PP4c siRNA 9 as compared to the cells transfected with scrambled siRNA. The figure shows no significant difference in colony forming ability of siRNA 8 and PP4c siRNA transfected cells in comparison to the negative control -siRNA and mock cells (n=3).

A cell cycle analysis was performed to study the effect of PP4c down-regulation on the growth and proliferation of K562 cells. Cells transfected with PP4c siRNA 8 and siRNA 9 showed no significant differences in the percentage of cells in all cell cycle phases in comparison to the cells transfected with negative control (Figure 4.11).

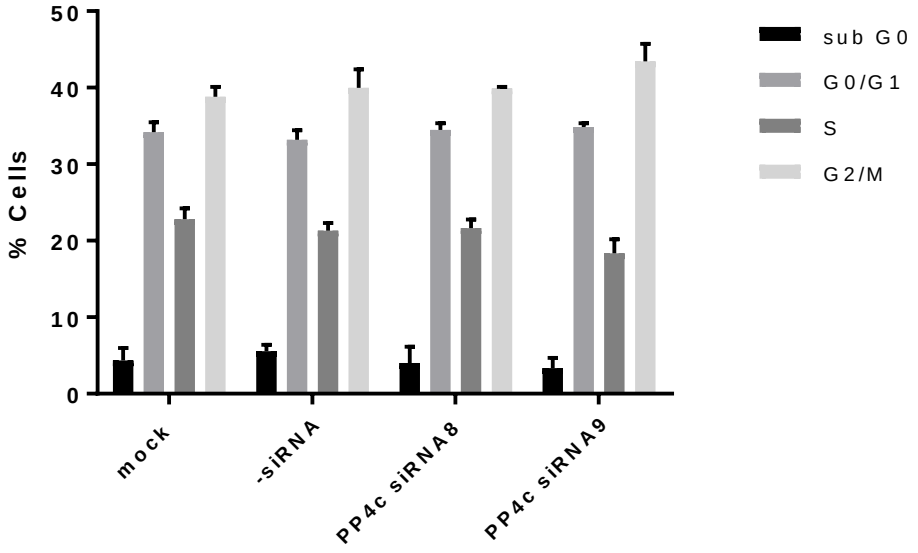

Figure 4.11 The effects of PP4c down-regulation on the cell cycle profile of K562 cells. Cell cycle analysis was performed by Muse™ Cell Cycle kit and the results showed no significant difference in the number of the PP4c siRNA 8 and PP4c siRNA 9 transfected cells in all phases compared to mock transfected cells and cells transfected with -siRNA (n=3).

In summary, PP4c down-regulation in K562 cells increased the number of viable cells, however with no effect on their proliferation rate, colony formation or apoptosis. These effects of modulating PP4c endogenous level on cell survival in the CML cell line model (K562) arises the importance of investigation of these effects in different leukaemic cell models, such as AML (HL-60).

4.3.2. **HL-60 cell line**

4.3.2.1. **PP4c over-expression**

The effects of modulating the endogenous level of PP4c were also investigated in the HL-60 cell line. HL-60 cells were also transfected with pcDNA3.1 empty vector and pcDNA3.1-PP4c for 24 hours before being re-plated and again the effects of PP4c over-expression on cell viability, Ki-67 proliferation and cell-division cycle were examined after a further 24 and 48 hours post-replating.

Due to the transfection method used, there were problems in isolating sufficient cell numbers for viability, proliferation or cell cycle analysis and for Western blotting analysis, and due to technical problems with this technique, out of four experiments, only one immunoblot observed the expression of PP4c in HL-60 cells, suggesting only modest over expression of PP4c in those cells that could be recovered for analysis post-transfection (Figure 4.12). Over-expression of PP4c in HL-60 cells showed no significant but lower viability in pcDNA3.1-PP4c transfected cells as compared to pcDNA3.1 transfected cells at 24 hour post-plating, with a significant decrease in viable cell number from 4.04×10^5 cells/mL with pcDNA3.1 empty vector to 1.63×10^5 cells/mL with pcDNA3.1-PP4c ($p<0.05$), as assessed by Trypan blue vital dye exclusion assay, observed at 48 hours post-plating (Figure 4.13).

On the other hand, PP4c up-regulation in HL-60 cells has no effect on the proliferation rate of cells transfected with pcDNA3.1-PP4c compared to cells transfected with the empty vector; pcDNA3.1 as assessed by Ki-67 proliferation assay at 24 hours (Figure 4.14). The lack of significance with the Ki67 proliferation assay supports the cell count at 24 hours, collectively this may be related to poor transfection efficiency and recovery of the cells, given that even under mock transfection conditions only 54% of cells were Ki-67 positive compared to 90% with K562 cells under the same experimental conditions (Figure 4.4).

Cell-division cycle assay was performed 24 hours post-plating and showed no difference in the cell cycle profile of the cells transfected with pcDNA3.1-PP4c as compared to mock cells or pcDNA3.1 transfected cells (Figure 4.15).

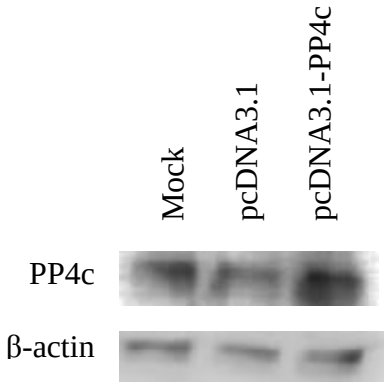

Figure 4.12 Effects of pcDNA3.1-PP4c transfection on endogenous PP4c levels in HL-60 cells. The expression of PP4c gene was hardly assessed by Western blotting technique and it shows a slightly elevation in transfected cells as compared to control. Immunoblot of PP4c protein expression in HL-60-pcDNA3.1/PP4c-transfected cells, HL-60-pcDNA3.1-transfected cells and HL-60 parental cells.

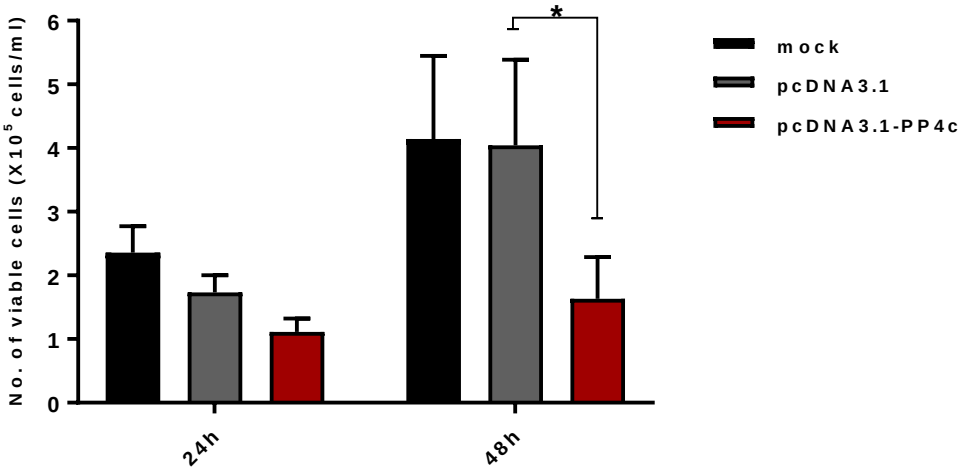

Figure 4.13 PP4c over-expression in HL-60 cell line decreases the viable cell count. HL-60 cells were transfected with empty vector pcDNA3.1 and pcDNA3.1-PP4c. The number of viable cells was determined by Trypan blue vital dye stain 24 and 48 hours post-plating. At 48 hours post-plating, cells transfected with pcDNA3.1-PP4c were significantly less viable than cells transfected with pcDNA3.1 only (*$p<0.05$; two-way ANOVA test followed by Bonferroni test; n=3).

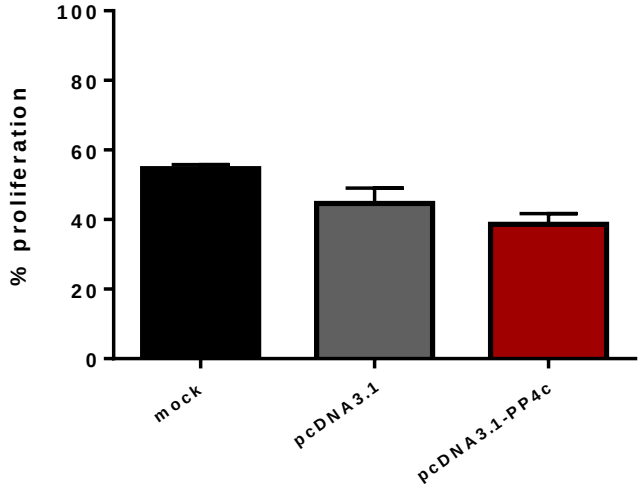

Figure 4.14 Effects of PP4c expression on HL-60 cell proliferation. The proliferation of PP4c transfected cells and control cells were determined by Ki-67 staining and Muse Cell analyser. PP4c over-expression has no effect on the proliferation rate of pcDNA3.1-PP4c transfected cells as compared to pcDNA3.1 (n=3).

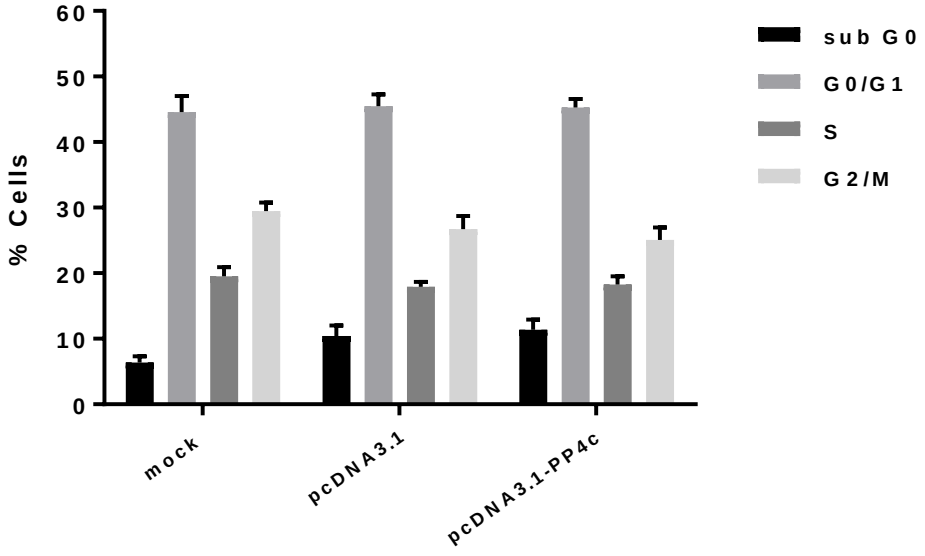

Figure 4.15 PP4c overexpression has no effect on the cell cycle profile of HL-60 cells. Cell cycle analysis was performed by Muse™ Cell Cycle kit which showed no difference in the number of the pcDNA3.1-PP4c transfected cells in all phases compared to mock transfected cells and cells transfected with pcDNA3.1 (n=3).

4.3.2.2. **PP4c down-regulation**

The effects of PP4c on HL-60 cells were also investigated by using siRNAs to down-regulate endogenous PP4c expression in these cells. Similarly to K562 cells, HL-60 cells were transfected with negative or scrambled siRNA, siRNA1, siRNA2, siRNA8 and siRNA9 but using the HiPerFect transfection method instead (Chapter 3, section 3). Western blotting technique was used to confirm that the siRNAs effectively down-regulated PP4 expression at the protein level and the results showed that all targeted PP4c siRNAs reduced the expression of endogenous PP4c by 23.11%, 14.55%, 41.30% and 34.88% in the cells transfected with PP4c siRNA1, PP4c siRNA2 & PP4c siRNA8, and PP4c siRNA9 respectively, as compared to the cells transfected with -siRNA (Figure 4.16). Following silencing of PP4c, only cells transfected with PP4c siRNA 8 and PP4c siRNA 9 were assessed for viability, cell cycle and apoptosis.

PP4c down-regulation in HL-60 cells showed no significant increase in the viable cell number at 24 and 48 hours, whereas a significant increase was observed at 72 hours post-plating in PP4c siRNA 8 transfected cells ($p<0.001$) and PP4c siRNA 9 transfected cells ($p<0.0001$), as assessed by Trypan blue vital dye exclusion assay (Figure 4.17). Similarly to K562 cells, the knockdown of PP4 by siRNA 8 and 9 had no effect on the basal apoptosis rate in HL-60 cells which was measured using Muse™ annexin V and dead cell assay and Muse analyser at 48 hours post-plating the cells, as compared to control cells (Figure 4.18). Moreover, long-term survival was also carried out at 48 hours post-plating. The results showed no difference in the ability of cells transfected with PP4c siRNA 8 or PP4c siRNA 9 to survive and to form colonies as compared to the cells transfected with negative control (-siRNA) (Figure 4.19).

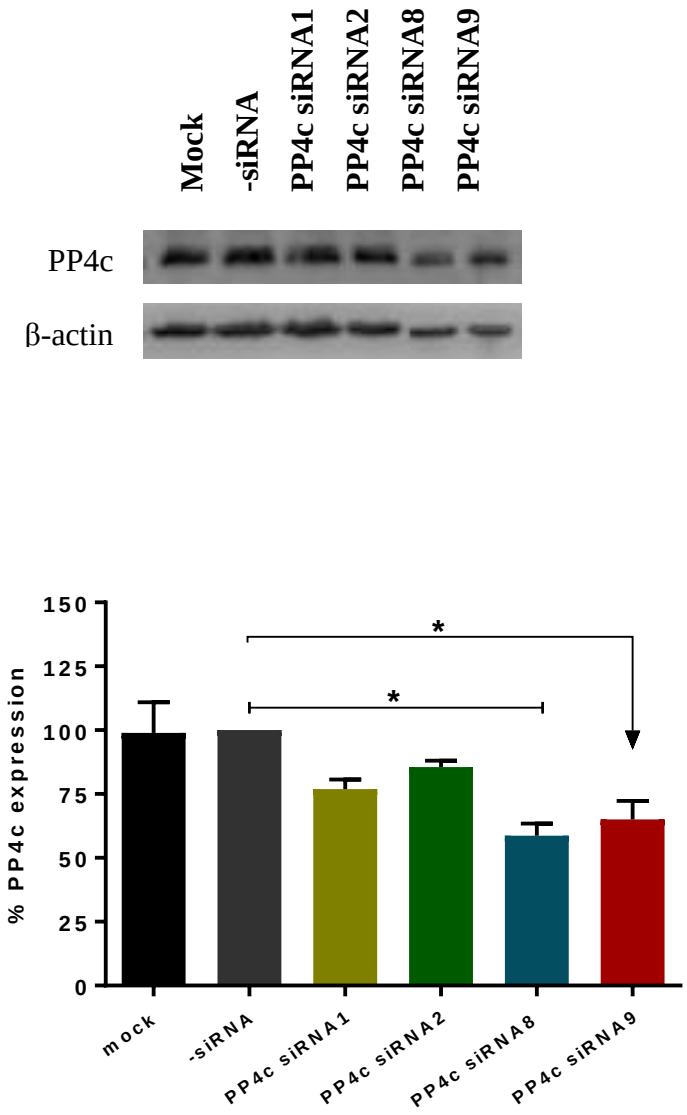

Figure 4.16 Effects of PP4c specific siRNA on endogenous level of PP4c in HL-60 cells. Western blotting and subsequent quantification shows a significant reduction in the expression of PP4c in transfected cells as compared to control (**p<0.01 for PP4c siRNA8 and *p<0.05 for PP4c siRNA9; two-way ANOVA test followed by Bonferroni test). **A)** Representative immunoblot of PP4c protein expression in PP4c siRNA1, PP4c siRNA2, PP4c siRNA8 & PP4c siRNA9, -siRNA and HL-60 parental cells. Cells lysates of $1x10^6$ cells/lane were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of PP4c. Blots were stripped and re-probed for. **B)** A bar graph represents mean ± SEM from 3 independent experiments. Relative expression is the ratio of PP4c level versus β-actin as determined using Licor image analysis software (n=3, see appendix A, page 191).

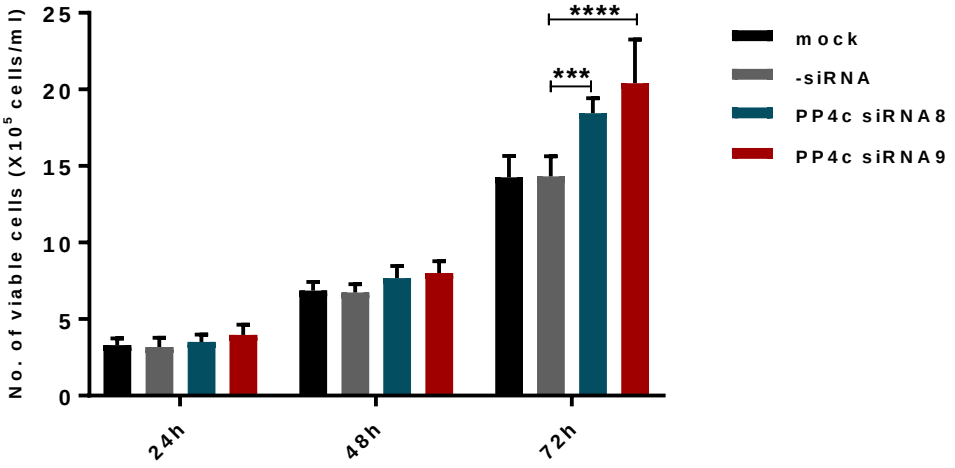

Figure 4.17 PP4c down-regulation in HL-60 cell line increases viable cell count. HL-60 cells were transfected with scrambled siRNA and PP4c siRNA 8 and 9. Viable cell count carried out by vital dye exclusion shows an elevation in viable cell count of HL-60 cells with PP4c siRNA 8, and 9 at 72 hours post-plating the cells as compared to the negative control (***p<0.001 for PP4c siRNA 8 and ****p<0.0001 for PP4c siRNA 9; Two-way ANOVA test followed by Bonferroni test; n=3).

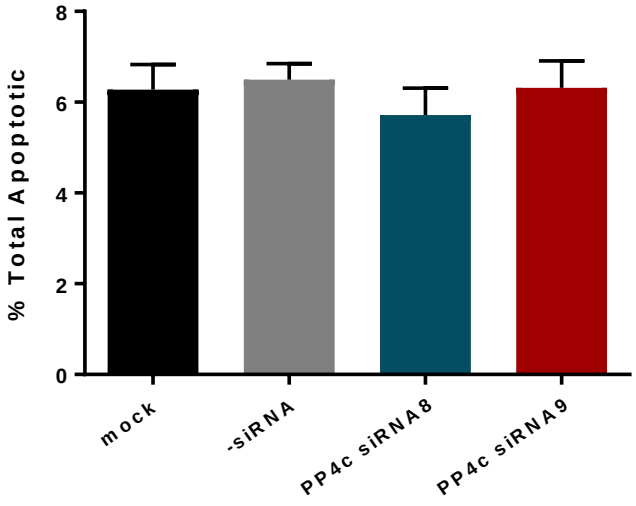

Figure 4.18 PP4c knockdown has no effect on the level of spontaneous apoptosis of HL-60 cells. Cells were transfected with -siRNA, PP4c siRNA 8 and 9. At 48 hours post-plating, the apoptosis percentage was measured using the Muse annexin V & dead cell assay carried out by Muse cell analyser. Transfected cells with PP4c siRNA8 and 9 show no difference in total apoptotic percentage as compared to -siRNA (n=3).

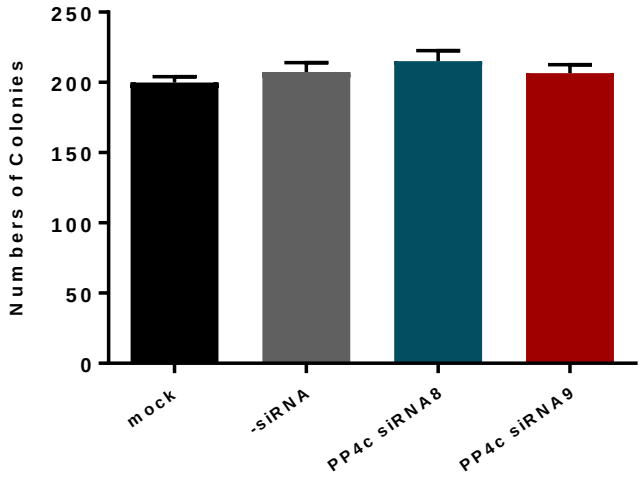

Figure 4.19 PP4c knockdown has no effect on colony forming ability in HL-60 cells. Long term survival was assessed following transfection of the cells with siRNA 8 and 9 and compared to the cells transfected with scrambled siRNA. The figure shows no differences in colonies formation in cultures transfected with the PP4c siRNA 8 and PP4c siRNA 9 as compared to control (n=3).

The previous findings that showed an increase in viable cell number only at 72 hours post plating, the lack of any change in basal apoptosis and the lack of effect on long term survival suggest any effect on cell growth may be limited, in comparison to K562 cells. Additionally, cell proliferation was also measured by Ki-67 staining and Muse cell analyser at 24 hours post-plating, to determine if an increase in the proliferation marker at earlier time points could account for higher cell number seen at 48-72 hours. The assessment showed that there was no difference in the percentage of Ki-67 positive cells between the cells transfected with PP4c siRNA 8 or PP4c siRNA 9 and negative control transfected cells (Figure 4.20).

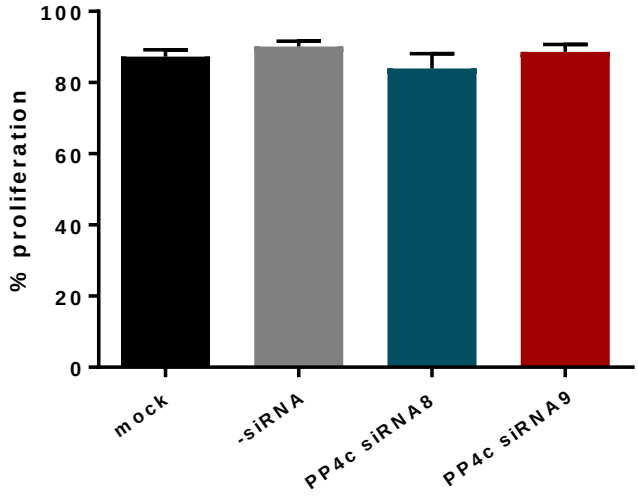

Figure 4.20 PP4c down-regulation has no effect on HL-60 cell proliferation. The proliferation of PP4c transfected cells and control cells were determined by Ki-67 staining and Muse Cell analyser. PP4c down regulation has no effect on the proliferation rate of K562 cells (n=3).

Similarly to PP4c silencing in K562 cells, the high proliferation rate of HL-60 mock transfected cells assessed by Ki67 proliferation assay makes the assessment for effect of PP4c silencing difficult, so a cell cycle assay was also performed at 24 hours post re-plating. PP4c down regulation in HL-60 cells affects the cell-division cycle and the results showed a significant decrease in cells proportion of S phase in cells transfected with PP4c siRNA 8 ($p<0.05$), and a significant increase in cells proportion of G2/M phase in cells transfected with PP4c siRNA 8 ($p<0.05$) and PP4c siRNA 9 ($p<0.01$) as compared to the corresponding cells transfected with negative control siRNA (Figure 4.21).

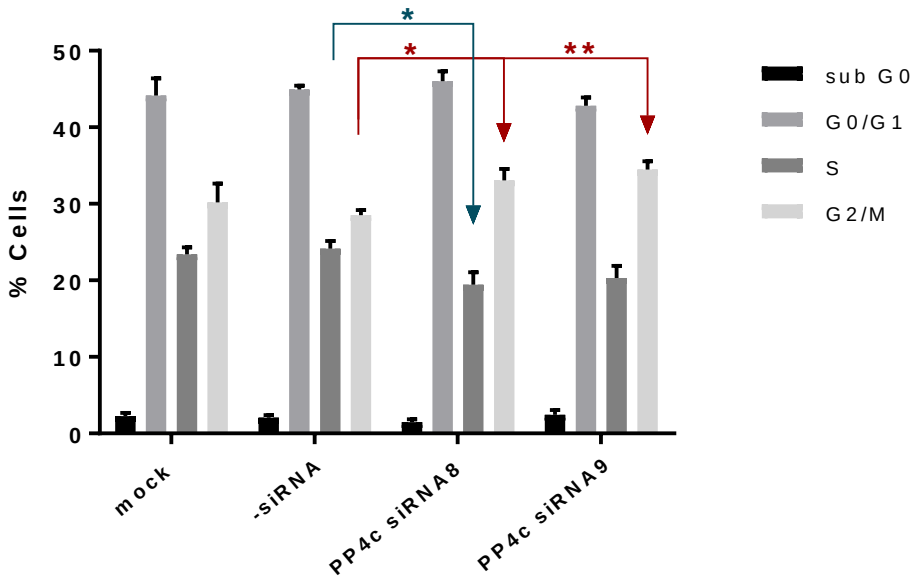

Figure 4.21 PP4c down-regulation affects the cell cycle profile of HL-60 cells. PP4c silencing decreases the proliferating cells count in S phase and increases the proliferating cells count in G2/M phase. This figure revealed a significant reduction in the number of the PP4c siRNA8 transfected cells in S phase and a significant elevation in the number of the PP4c siRNA8 and PP4c siRNA9 transfected cells in G2/M phase as compared to control (-siRNA) (*$p<0.05$; **$p<0.01$; two-way ANOVA test followed by Bonferroni test; n=3).

4.4. **Discussion**

Many studies have suggested that PP4c has a crucial role in controlling cell growth, survival and proliferation in different types of cancers such as breast cancer, lung cancer and lymphoma (Wang et al., 2008; Mourtada-Maarabouni and Williams, 2008; 2009; Brechmann et al., 2012).

The aim of this chapter was to investigate the effects of modulating the endogenous level of PP4c in two different types of model system cell lines; K562 for CML and HL-60 for AML. As mentioned previously, the kinases that are activated in K562 cells differ from those activated in HL-60 cells, and on the other hand, the difference in their response to transfection methods (see chapter 3), particularly the poor recovery of HL-60 cells, suggests that their sensitivity to changes in PP4c will be different. Collectively however, the results implicate that PP4c has an effect on the growth and survival of specific leukaemic cells. Over-expression of PP4c in K562 cells resulted in suppression of cell viability and proliferation rate, and subsequently resulted in an increase in apoptosis at different time points (24 and 48 hours post plating). The experiments have also demonstrated that over-expression of PP4c in K562 cells increases the percentage of cells in G0/G1 phase and decreases the percentage of cells in S phase suggesting that PP4c over-expression commits the cells to the G0/G1 transition leading to G1 phase arrest and accumulation instead of progressing to S phase, possibly contributing to increased cell death. On the other hand, over-expression of PP4c in HL-60 cells resulted in suppression of cell viability. The lack of significance in some assays with HL-60 cells may be related to the low transfection efficiency and the high toxicity of the nucleofection method that used to over-express PP4c that led to poor recovery of the HL-60 cells post transfection.

The findings of the effect of the modulation of the endogenous level of PP4c in K562 and HL-60 cells are in agreement with previous studies on CEM-C7 and Jurkat cells

(Mourtada-Maarabouni and Williams, 2009) and on MCF7 and MDA-MB-231 cells (Mohammed et al., 2016). PP4c over-expression increases cell death and decreases cell viability and proliferation in both leukaemic T-cells and untransformed human peripheral T-cells (Mourtada-Maarabouni and Williams, 2009) and in breast cancer cells (Mohammed et al., 2016). Previous results have also shown that PP4c is an important modulator of apoptosis and cell proliferation in the human embryonic kidney cell line HEK 293T (Mourtada-Maarabouni and Williams, 2008).

This study also showed that down-regulation of PP4c by around 65% in K562 cells, using specific PP4 siRNAs, significantly increased the number of viable cells at 48 hours post-plating, while down-regulation of PP4c by 34-42% in HL-60 cells significantly increased the viability of the cells after 72 hours post-plating. Whilst expression levels of PP4c were normalised to the levels of beta actin, as an endogenous loading control, small variations in expression levels of such 'housekeeping' proteins, sample loading and/or signal saturation may skew data normalised in this way. Further quantitation with total protein strategies such as the anionic dyes Coomassie blue or Ponceau S, or in-gel stain free labelling, can reduce uncertainty from minor experimental variation in sample loading or signal saturation of single endogenous controls. Though such a strategy would have been applicable in the current study, blots were imaged using the Licor C-digit scanner rather than conventional radiography. Such technology is reported to minimise signal saturation owing to advanced imaging optics of the scanner (Licor, undated) and the blot images for PP4c and actin in siRNA transfected cells indicate lack of saturation in these bands.

The differences in the expression and the response of these two cell lines to PP4c knock-down may be related to differences in kinases expressed in these cells and the downstream signalling pathways that they are associated with, as well as differences in levels of the phosphatase regulatory subunits expressed, which may impact the sensitivity of the cells.

These results are also in agreement with studies on HEK293 cells and both leukaemic T-cells and untransformed human peripheral blood T-cells which showed that PP4c silencing increases cell viability, decreases apoptosis and alters the cell cycle profile by increasing the number of the cells in G1 phase in HEK293, CEM-C7 and Jurkat cells (Mourtada-Maarabouni and Williams, 2008; 2009). It was also reported that silencing PP4c increase the cell proliferation rate and migration in oestrogen receptor-positive and triple negative breast cancer (TNBC) cell line types (Mohammed et al, 2016). Silencing PP4c by gene specific siRNAs in K562 cells increased their ability to form colonies more than mock cells and scrambled siRNA transfected cells. In contrast to over expression results, PP4c down-regulation has no effect on basal apoptosis and on the proliferation rate in both K562 cells and HL-60 cells; this might be related to the high proliferation rate of the basal cells, making any further decrease difficult to detect over these short time points. PP4c silencing in HL-60 cells alters the cell-division cycle by increasing the cell number in G2/M phase which confirm the results of increased cell viability in these cells post PP4c knock-down.

Whilst limited studies have investigated the role of PP4c in leukaemic cell line models, the impairment in the regulation of the activity of PP2A has been reported. Since PP4c is a PP2A-related Ser/Thr protein phosphatase and shares about 65-66% identity in the amino acid sequences with the PP2A α and PP2A β catalytic subunits, though their regulatory subunits are very distinct (Chen et al., 2008), PP2Ac and PP4c have been shown to dephosphorylate the same residue on a substrate (*in vitro*, both phosphatases dephosphorylate nucleosomal γ-H2AX in a dose-dependent manner (Chowdhury et al., 2008)), and so similar modes of action between these two phosphatases can be suggested. This overlap in substrate specificity may allow dephosphorylation to occur at a different subcellular location or under different cellular conditions (Lee et al., 2010). Moreover,

PP2Ac has been suggested to be involved in DNA repair after exogenous DNA damage, while PP4c plays a greater role in the repair during DNA replication (Lee et al., 2010). Also due to their homology, certain cellular functions of PP2A may in fact be those of PP4 (Shui et al., 2007; Zhou et al., 2002). The above studies suggested that PP4c might have a similar role in leukaemic cells to that of PP2Ac. Many studies have implicated the role of PP2Ac in leukaemia; a study showed that B55α, a subunit of PP2A regulatory subunit which is identified as a specific regulator of the levels of Akt phosphorylation at Thr308, was inhibited and loss of complete haematological remission in acute AML patients. Thus it could serve as a biomarker in AML or could be a novel target for AML chemotherapy (Ruvolo et al., 2011). Whereas in CML, it has been reported that PP2A over-expression suppresses the activity of Bcr-Abl kinase, promotes apoptosis and enhances imatinib activity (Neviani et al., 2005; 2007). The findings of PP2A over-expression in K562 cells are similar to the findings of the current study.

Many studies on PP4c have suggested that it might function as an oncogene. It has been reported that PP4c is over-expressed in breast and lung cancers and any inhibition of PP4c expression may increase the sensitivity of breast and lung cancer cells to cisplatin treatment (Wang et al., 2008). An immunohistochemical study on stage II pancreatic ductal adenocarcinoma (PDAC) samples showed that over-expression of PP4c is associated with poor prognosis and suggest that PP4c could act as a marker for the diagnosis of invasive PDAC and a new approach for the treatment of this disease via targeting its signalling pathways (Weng et al., 2012). Recently, a study revealed that PP4c also plays an oncogenic role in glioma's development and progression (Li et al., 2016b).

It is interesting to note that when scaffold and regulatory subunits of PP2A are deregulated in cancer cells, the cell tries to maintain a constant level of functional catalytic subunit activity (Seshacharyulu et al., 2013). Therefore, the over-expression of PP4c in these types

of cancer could be the results of the deregulation in the expression or activity of PP4 regulatory subunits. For example, PP4R1 depletion in cutaneous T cell lymphoma (CTCL) causes constitutive IKK/NF-κB signalling (Brechmann et al., 2012). This study showed that PP4R1 forms part of a PP4 holoenzyme and bridges the inhibitor of NF-κB kinase (IKK) complex and the phosphatase PP4c, thereby directing PP4c activity to dephosphorylate and inactivate the IKK complex. Deficiency of PP4R1 leads to an inactive PP4c and causes sustained and increased IKK activity, T cell hyperactivation, and aberrant NF-κB signalling in NF-κB-addicted T cell lymphomas (Seshacharyulu et al., 2013). Whilst another study on colorectal carcinoma suggested that PP4c up-regulated MMP-2 and MMP-9 via PI3K/Akt pathway, associated with lymphatic nodes and consequently promotes distant metastasis (Li et al., 2015b).

The findings of this study showed many distinct differences in the response of K562 and HL-60 cells to PP4c endogenous modulation. The different kinases linked with CML and AML may be altered by PP4 levels/activity by interacting with some of the downstream targets, alongside possible differences in regulatory subunit interactions. Previous studies on K562 cell line involving the cell survival assessment at 4 to 24 hours post treatment with an inducing stimulus have demonstrated that Bcr-Abl kinase expression restores the cells resistant to the induction of apoptosis (McGahon et al., 1997). Furthermore, Martins et al. (1997) suggested that this expression of Bcr-Abl kinase in K562 cells delays cell death rather than preventing it (Martins et al., 1997).

With PP4c over expression in K562 cells a decrease in viable cell number at 24 hours and decreased proliferation rate, suggested an increase in cell death, supported by changes in the cell-division cycle in G0/G1 and S phases, while PP4c over expression in HL-60 only affected viable cell numbers after 48 hours. Moreover, the results revealed that the K562 mock transfected cells show higher Ki67 staining than the HL-60 mock transfected cells

under similar experimental conditions, supporting the lower tolerance of HL-60 cells to the nucleofection method. Transfection efficiencies of PP4c over-expression in HL-60 cells was very low (less than 40%), unlike the transfection efficiency of PP4c over-expression in K562 cells which was more than 80% (see chapter 3 section 3.4.1), supporting poor cell recovering, possibly accounting for the limited effect of PP4c over-expression in HL-60. Moreover, the effects of PP4c over-expression in HL-60 were difficult to conclude because of the cell loss during transfection, affecting data from the apoptosis assay in HL-60 cells due to low cell numbers.

Differences were also observed with PP4c siRNA; in comparison to K562, the extent of down-regulation in HL-60 cell is not as great. PP4c down regulation in K562 cells increased the viable cell number at 24 and 48 hours, while PP4c down regulation in HL-60 cells only increased the viable cell number at 72 hours, this could be related to differences in the extent of PP4c down-regulation but may also suggest a differential sensitivity of the two models to changes in PP4c levels, which may also in part reflect the data from PP4c over-expression. Unlike K562 cells where there was a clear trend on long-term survival post PP4c silencing, this was not apparent in the colony forming assay with HL-60. PP4c down-regulation in these two cell lines also altered the cell-division cycle profile differently. These differences and the sensitivity of these cells towards PP4c endogenous modulation might be related to the different mutations and dysregulation of tyrosine kinase activity, the translocation chromosomes that they possess, oncoprotein expression, and the signalling pathways involved. So, further studies are needed to understand the signalling pathways impacted on by PP4c in K562 and HL-60 cells.

From the findings of this chapter, it can be concluded that the effects of PP4c over-expression and down-regulation in both K562 and HL-60 cells are very distinct and the observation that PP4c down-regulation under basal conditions has no effect on both apoptosis rate and Ki67 proliferation assay in K562 and HL-60 cells needs to be further explored. Moreover, previous study indicated that PP4c silencing may increase the sensitivity of cells in other cancer models such as breast and lung cancer cells to cisplatin (Wang et al., 2008). For these reasons, the next chapter will focus on the effects of PP4c silencing on the K562 and HL-60 cells response to some anti-cancer drugs in term of the apoptosis rate and proliferation rate of these leukaemic cells.

5. Effect of PP4c silencing in K562 and HL-60 cells on the response to anti-cancer drugs

5.1. Introduction

The importance of PP4c in cancer progression by regulating many signalling pathways such as NF-κB, JNK and m-TOR pathways has been demonstrated previously (Zhou et al., 2004; Mourtada-Maarabouni and Williams, 2008). Any excessive or insufficient activity of PP4c could be lethal to the cells and this could be associated with cisplatin sensitivity (Zhou et al., 2004; Mourtada-Maarabouni and Williams, 2008; 2009). Another study has reported that the low expression of PP4c results in increased sensitivity of breast and lung cancer cells to cisplatin treatment (Wang et al., 2008). *In vitro*, the effects of UV-C radiation treatment post PP4c endogenous modulation in HEK 239T, CEM-C7, and Jurkat cell lines have been studied (Mourtada-Maarabouni and Williams, 2008; 2009). The results showed that PP4c over-expression enhanced the cellular proliferation inhibition of the UV-exposed cells, while PP4c silencing in these cells enhanced their ability to form colonies and consequently resist the cell death that induced under these conditions (Mourtada-Maarabouni and Williams, 2008; 2009). In addition, these studies demonstrated that other cytotoxic stimuli including cisplatin showed similar effects of radiation therapy on HEK 239T, CEM-C7, and Jurkat cell lines suggesting that PP4c plays an important role in apoptosis (Mourtada-Maarabouni and Williams, 2008; 2009).

The findings of the current study in the previous chapter suggested that PP4c knock-down under basal conditions has no effect on the apoptosis and proliferation rates in both K562 and HL-60 cells, therefore, the aim of this chapter is:

- To evaluate the effects of the PP4c silencing in K562 and HL-60 cells on their response to the selected anti-cancer drugs; cisplatin, doxorubicin, imatinib and rapamycin, by measuring their cells viability percentage and total apoptosis rate. In other words, to examine if PP4c down-regulation protects against anticancer drugs-induced apoptosis and consequently affects some important pathways such as caspase-dependent pathways and MAPK pathways in which PP4c plays an important role.

5.2. **Methodology**

In this chapter, the effect of PP4c down-regulation on the cytotoxic response of the cells to four different drugs was investigated. Seventy-two (72) hours post PP4c silencing in K562 cells by nucleofection V reagent (Chapter 2 section 6.1) and HL-60 cells by HiPerFect transfection reagent (Chapter 2 section 6.2), the cells were re-plated with the anti-cancer agents; cisplatin, doxorubicin, imatinib (only K562 cells) and rapamycin.

According to the findings of the current study in chapter three, K562 cells were re-plated with 10 μM cisplatin, 0.2 μM doxorubicin, 0.2 μM imatinib, or 20 nM rapamycin at 2×10^5 cells/mL in 24-well plates, while HL-60 cells were re-plated with 2 μM cisplatin, 0.1 μM doxorubicin, or 20 nM rapamycin also at 2×10^5 cells/mL in 24-well plates and incubated for 24 and 48 hours before assessment of cell viability and apoptosis as described in chapter 2 sections 7.1 and 8.

Trypan blue exclusion method was used to count the number of viable cells 24 and 48 hours post treatment as described in chapter 2 in section 7.1 and the results represented in this chapter as the number of viable cells/mL ($\times10^5$) and viability expressed as a percentage of the total cell number (% viability).

Apoptosis was also assessed by Muse™ Annexin V and Dead Cell Assay kit using Muse™ Cell Analyser according to manufacturer's instruction as explained in chapter 2 section 8. The results were expressed as total apoptotic percentage (% Total apoptotic).

5.3. **Results**

5.3.1. **Effects of PP4c down-regulation in K562 cells on their response to some of the anti-cancer drugs**

In chapter 4, the results of PP4c down-regulation in K562 cells showed an elevation in cell viability but with no effect on basal apoptosis. Therefore, in this chapter, the effect of some of chemotherapeutic drugs on the viability of K562 cells following silencing of PP4c was investigated. At 72 hours post nucleofection, the cells were counted, treated with drugs and plated at 2×10^5 cells/mL in 24-well plates to evaluate the viable cell number, viability and total apoptosis.

Mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected K562 cells were treated with 0.2 µM doxorubicin and the results showed that doxorubicin treatment decreased the number of viable cells under all transfection conditions after 24 hours (Figure 5.1A), which was further decreased after 48 hours (Figure 5.1B). Thus, figures 5.1C and D (data expressed as percentage viability) showed no significant difference in cell viability of PP4c-transfected cells at 24 and 48 hours post-treatment with doxorubicin when compared to negative siRNA (-siRNA) suggesting no effect of PP4c silencing on the K562 cells' sensitivity to doxorubicin.

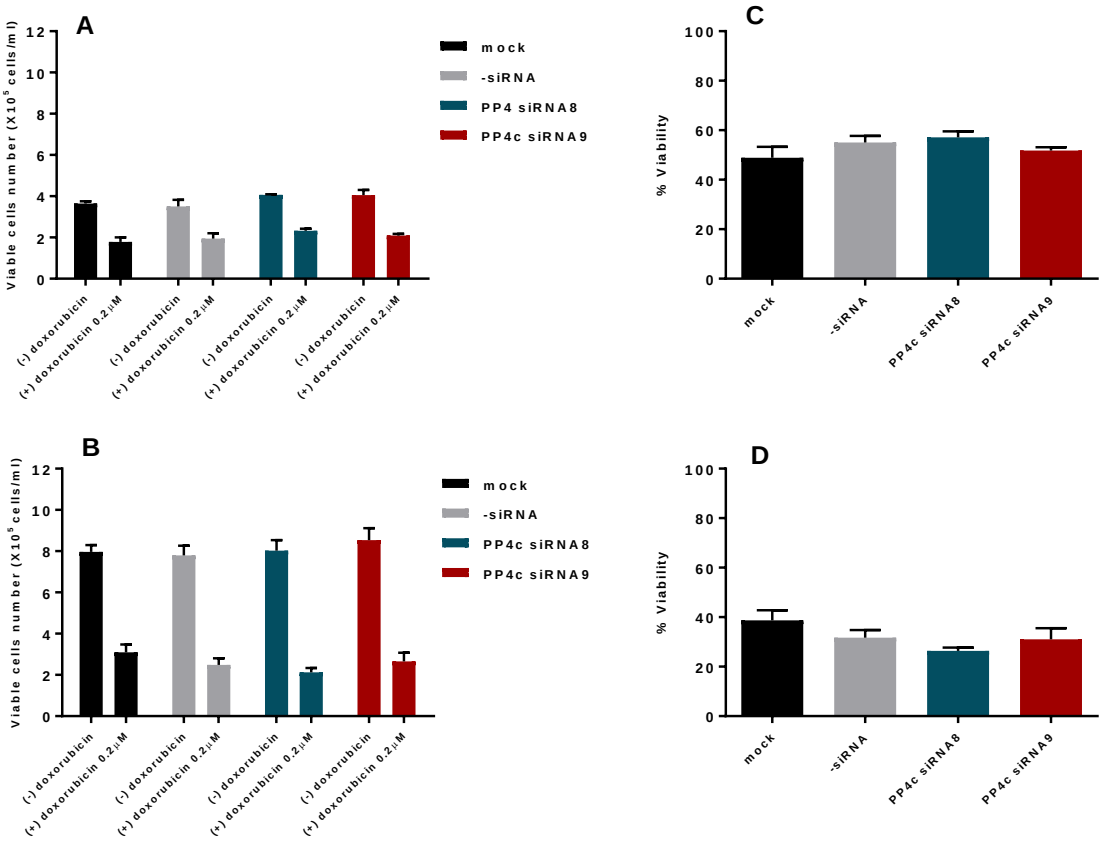

Figure 5.1 PP4c silencing has no effect on K562 cells viability in response to doxorubicin treatment. Transfected K562 cells were treated with 0.2 µM doxorubicin. Viable cell count carried out by Trypan blue dye exclusion at 24 and 48 hours showed a decrease in viable cell count (A, B) and the viability (C, D) of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9. PP4c down regulation in K562 cells showed no resistance to doxorubicin treatment (n=3).

Total apoptotic percentage was also analysed at 24 hours post doxorubicin treatment (Figure 5.2a), and the results showed around a 6-fold increase in the apoptosis rate of all transfected K562 cells. Representative scatter plots from the Muse analyser showed a characteristic shift from viable pre-treatment, to early apoptotic stage post-treatment, and no differences in the apoptosis profiles of PP4c-siRNA transfected K562 cells as compared to mock or -siRNA (Figure 5.2b). These results, similar to Figure 5.1, suggested that PP4c silencing has no effect on the sensitivity of K562 cells towards doxorubicin treatment.

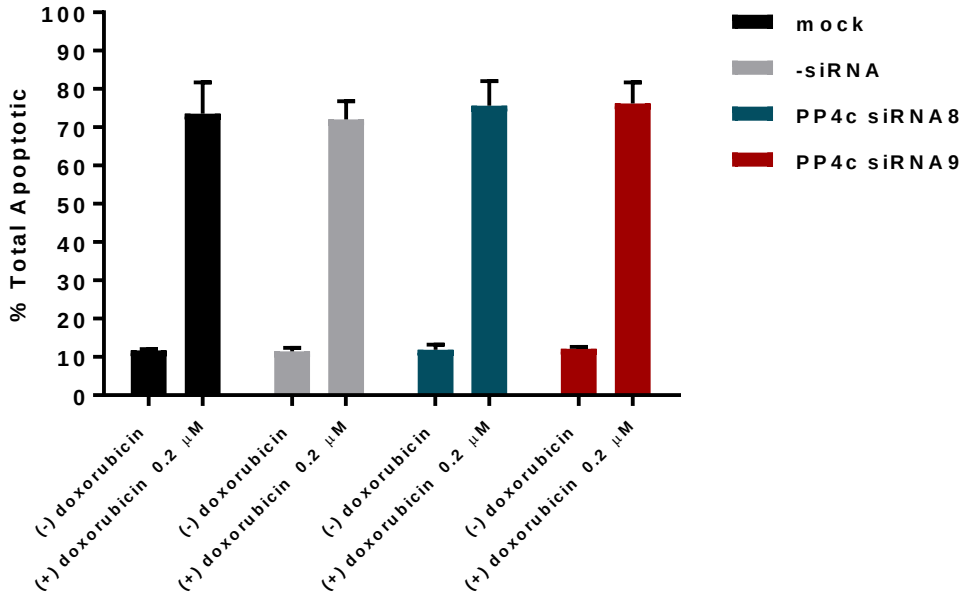

Figure 5.2a PP4c silencing has no effect on K562 cells apoptosis in response to doxorubicin treatment. At 24 hours post 0.2 µM doxorubicin treatment, the apoptosis percentage was measured using the Muse annexin V & dead cell assay carried out by Muse cell analyser. Transfected cells with PP4c siRNA8 and PP4c siRNA 9 show no difference in total apoptotic percentage as compared to -siRNA (n=3).

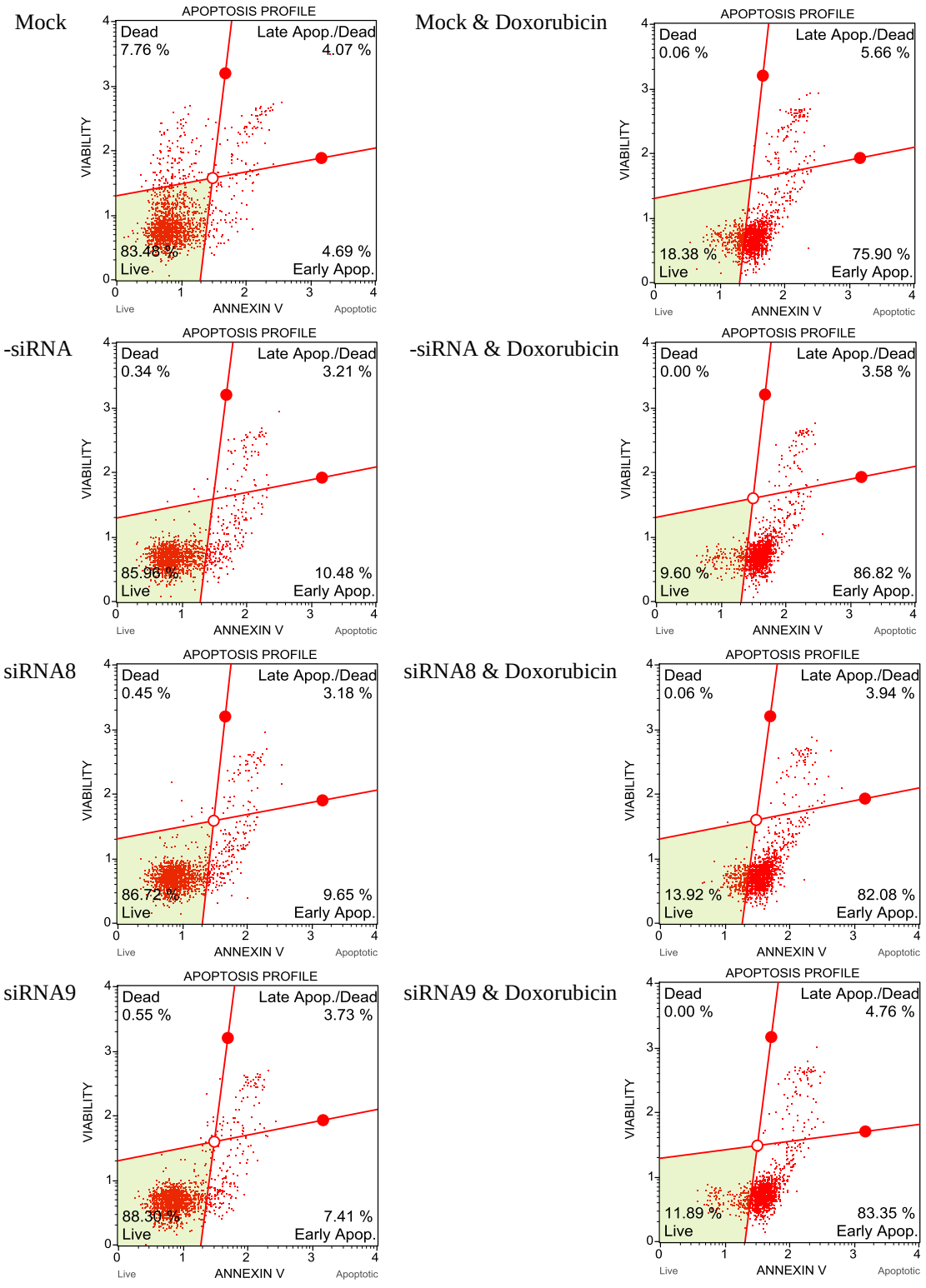

Figure 5.2b PP4c silencing has no effect on K562 cells apoptosis in response to doxorubicin treatment. Representative pictures from Muse analyser showed no significant difference in the early and late apoptotic percentages of the K562-PP4c siRNAs transfected cells as compared to mock and negative control cells at 24 hours.

Transfected K562 cells were also treated with 10 µM cisplatin and the viable cell number and cell viability percentage were assessed at 48 hours post-treatment according to the optimisation results (Chapter 3) which showed that the treatment of parental K562 cells with cisplatin becomes significantly effective at 48 hours.

The results of treating K562 cells with 10 µM cisplatin showed a comparable reduction in the number of viable cells in mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected K562 cells at 48 hours (Figure 5.3A) and consequently, there was no significant difference in cell viability (expressed as a percentage) of PP4c-transfected cells at 48 hours post-treatment as compared to mock or -siRNA transfected cells (Figure 5.3B). These findings suggested that PP4c silencing in K562 cells has no effect on the sensitivity to cisplatin treatment.

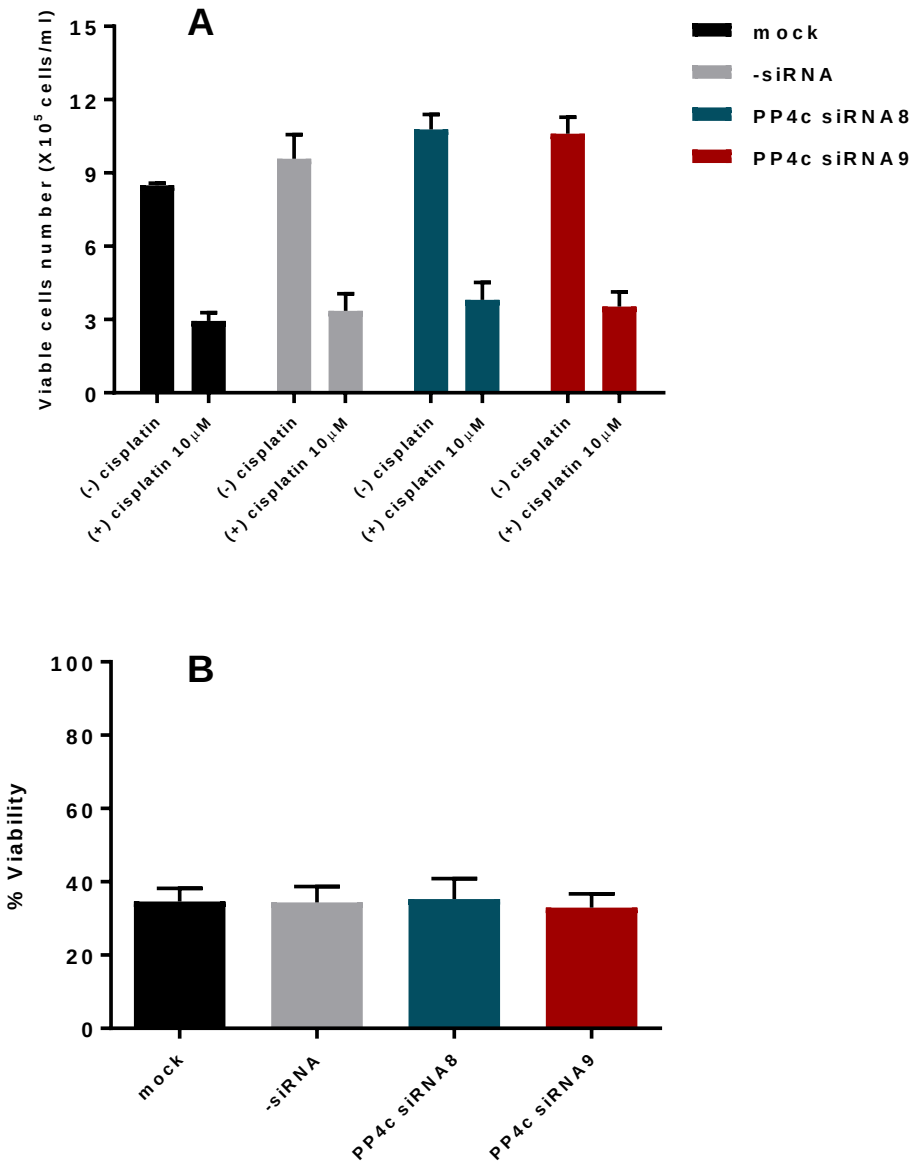

Figure 5.3 PP4c silencing has no effect on K562 cells viability in response to cisplatin treatment. Transfected K562 cells were treated with 10 µM cisplatin. Viable cell count carried out by Trypan blue dye exclusion at 48 hours showed a decrease in viable cell count (A) and the viability (B) of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9. PP4c down regulation in K562 cells showed no resistance to cisplatin (n=3).

K562 cells were treated with 0.2 μM imatinib post PP4c down-regulation and the results showed a noticeable reduction in the viable cell number of K562-PP4c siRNA8 and K562-PP4c siRNA9 at 24 and 48 hours (Figures 5.4A and B).

A significant inhibition in percentage cell viability of PP4c siRNA9 (34.6%, $p<0.05$) transfected cells can be seen as compared to the -siRNA cells at 24 hours (Figure 5.4C), whilst at 48 hours a significant decrease in the viability level of both PP4c siRNA8 (27.5%, $p<0.05$) and PP4c siRNA9 (31.8%, $p<0.05$) transfected cells as compared to the negative control -siRNA cells was observed (Figure 5.4D).

These results suggested that PP4c silencing enhances the action of imatinib in K562 cells, leading to a further reduction in cell viability.

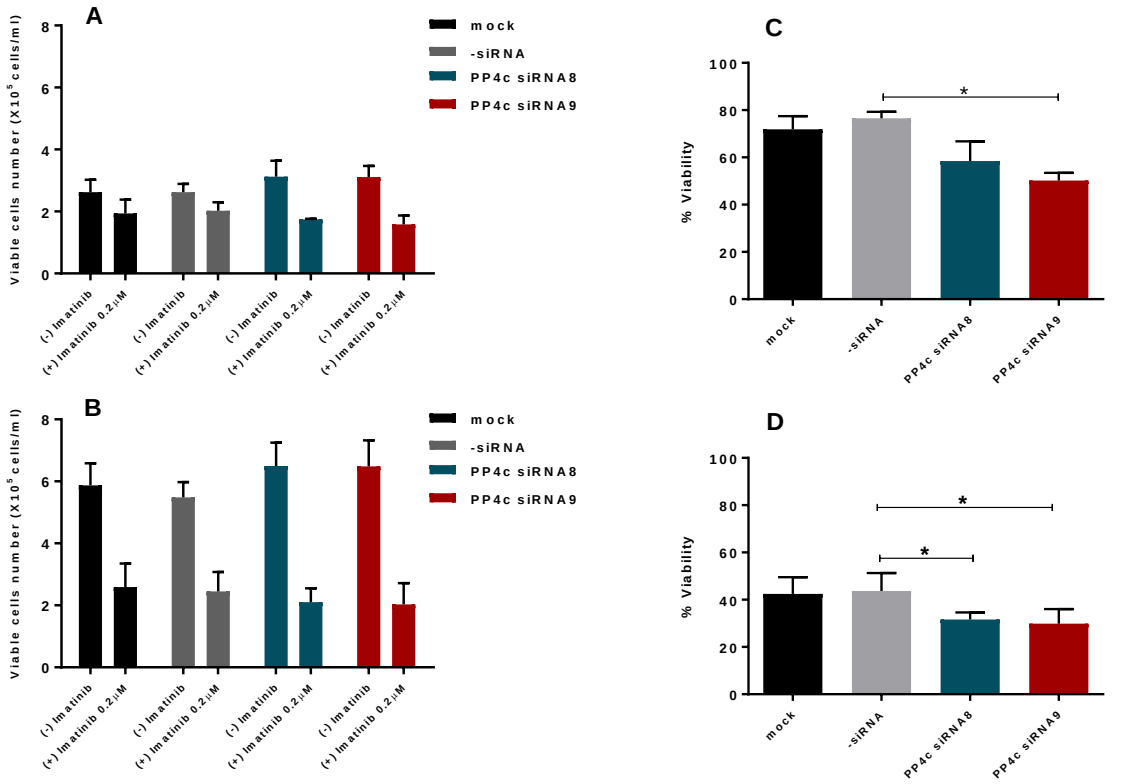

Figure 5.4 PP4c silencing decreases the viability of K562 cells in response to imatinib treatment. Transfected K562 cells were treated with 0.2 μM imatinib. Viable cell count carried out by Trypan blue dye exclusion at 24 and 48 hours showed a reduction in the number of viable cells of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9 (Figures A, B). Figures (C) and (D) show significant reduction in the viability percentage of the PP4c siRNA8 and PP4c siRNA9 cells compared to negative control cells (-siRNA) 24 and 48 hours respectively (*p<0.05; one-way ANOVA test followed by Bonferroni test; n=3)

Moreover, total apoptosis percentage was assessed 24 and 48 hours post imatinib treatment, and the results showed a significant elevation in the apoptosis rate at 48 hours of treating K562-PP4c siRNA8 cells (23.0%, $p<0.05$) and K562-PP4c siRNA9 cells (24.4 %, $p<0.05$) with 0.2 µM imatinib as compared to negative control (-siRNA) (Figure 5.5a). Representative scatter plots from the Muse analyser showed the apoptosis profiles of transfected K562 cells (mock, -siRNA, PP4c siRNA8 and PP4c siRNA9) pre and post treatment (Figure 5.5b), with an increased shift in the percentage of cells scored as apoptotic. Together, these results suggest that PP4c silencing may promote the action of imatinib and increase the apoptosis percentage significantly in K562 cells.

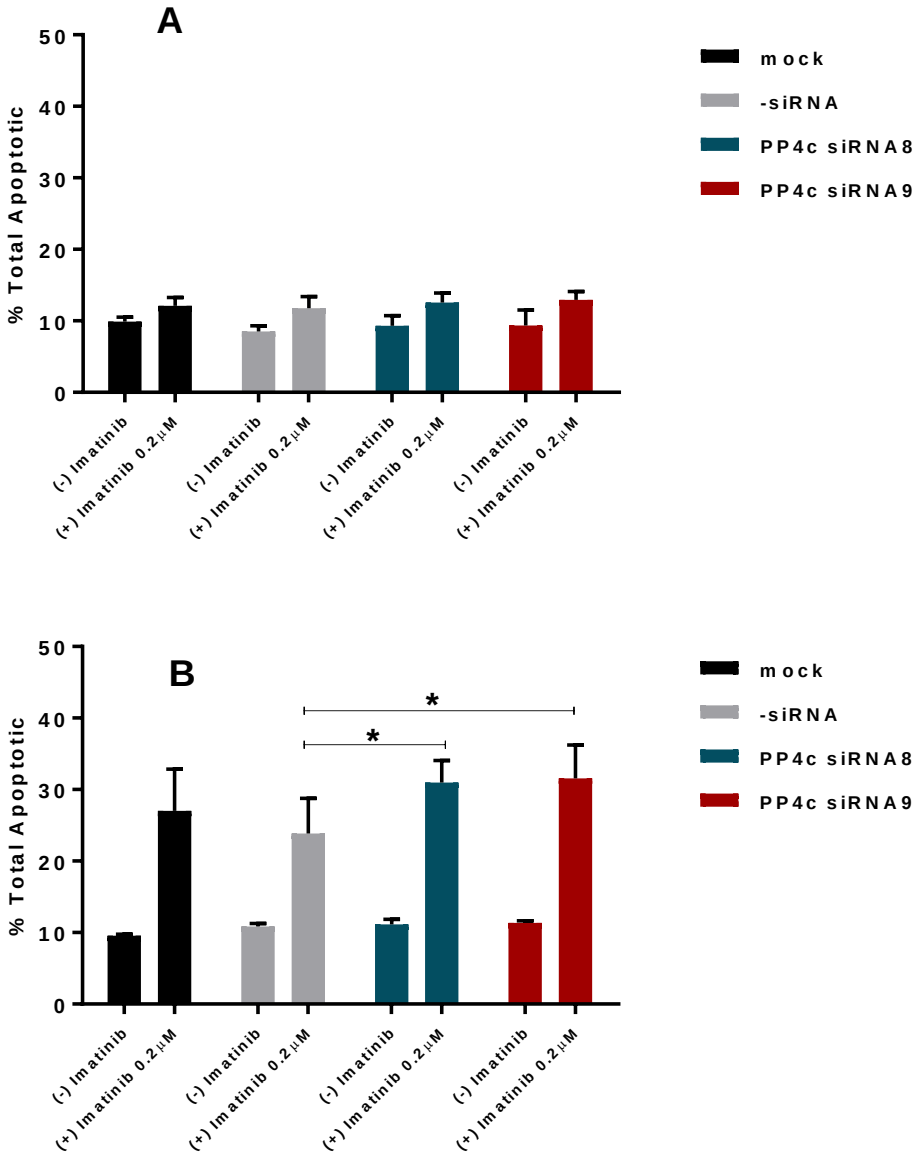

Figure 5.5a PP4c silencing increases the total apoptotic percentage of K562 cells in response to imatinib treatment. At 24 and 48 hours post 0.2 µM imatinib treatment, the apoptosis percentage was measured using the Muse annexin V & dead cell assay carried out by Muse cell analyser. PP4c silencing has no effect on total apoptotic percentage of PP4c siRNA8 and PP4c siRNA9 transfected cells when treated with 0.2 µM Imatinib at 24 hours as compared to -siRNA (A). PP4c silencing significantly increased the total apoptotic percentage of PP4c siRNA 8 and PP4c siRNA 9 at 48 hours post-treatment as compared to -siRNA (*$p<0.05$; two-way ANOVA test followed by Bonferroni test; n=3).

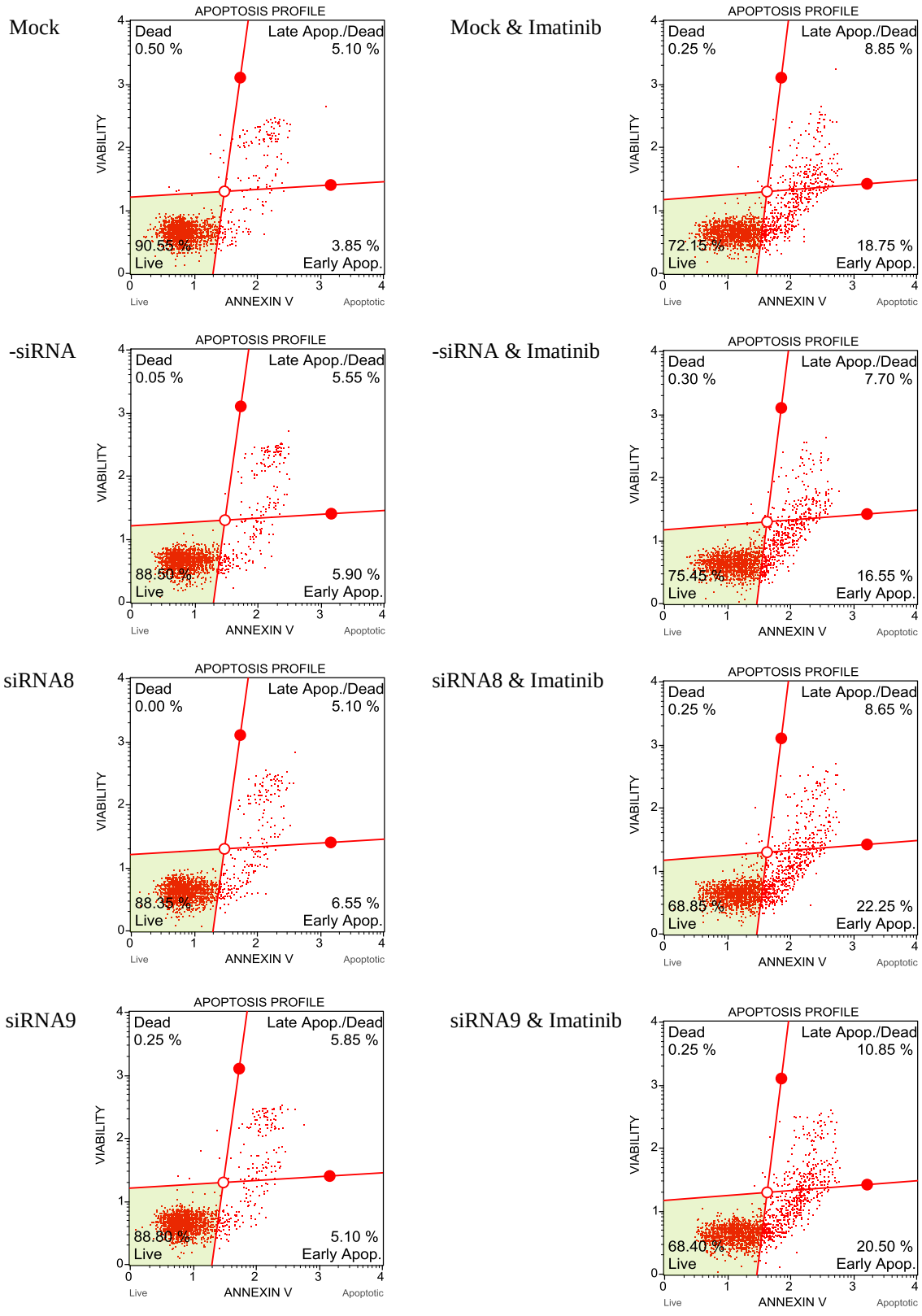

Figure 5.5b PP4c silencing increases the total apoptotic percentage of K562 cells in response to imatinib treatment. PP4c down regulation increase the total apoptotic percentage of K562 cells 48 hours post-treatment with Imatinib. Representative pictures from Muse analyser show elevation in the early and late apoptotic percentages of the PP4c siRNAs K562 transfected cells compared to mock and negative control cells (n=3).

Similar results were observed when K562 cells treated with 20 nM rapamycin post PP4c silencing. Rapamycin treatment post PP4c silencing reduced the viable cell number of mock, -siRNA, PP4c siRNA8, and PP4c siRNA9 transfected cells at 48 hours (Figure 5.6A). A significant reduction (about 25%; $p<0.05$) in the viability level of both PP4c siRNA8 and PP4c siRNA9 transfected cells as compared to the -siRNA cells at 48 hours (Figure 5.6B) was thus observed, suggesting that PP4c silencing enhanced the action of rapamycin in K562 cells leading to a reduction in viable cell numbers.

To summarise the effects of PP4c down-regulation in K562 cells on the action of the anti-cancer agents, the results showed that PP4c silencing has no effect on the sensitivity of the cells towards doxorubicin or cisplatin treatment; however, it enhanced the action of imatinib and rapamycin action.

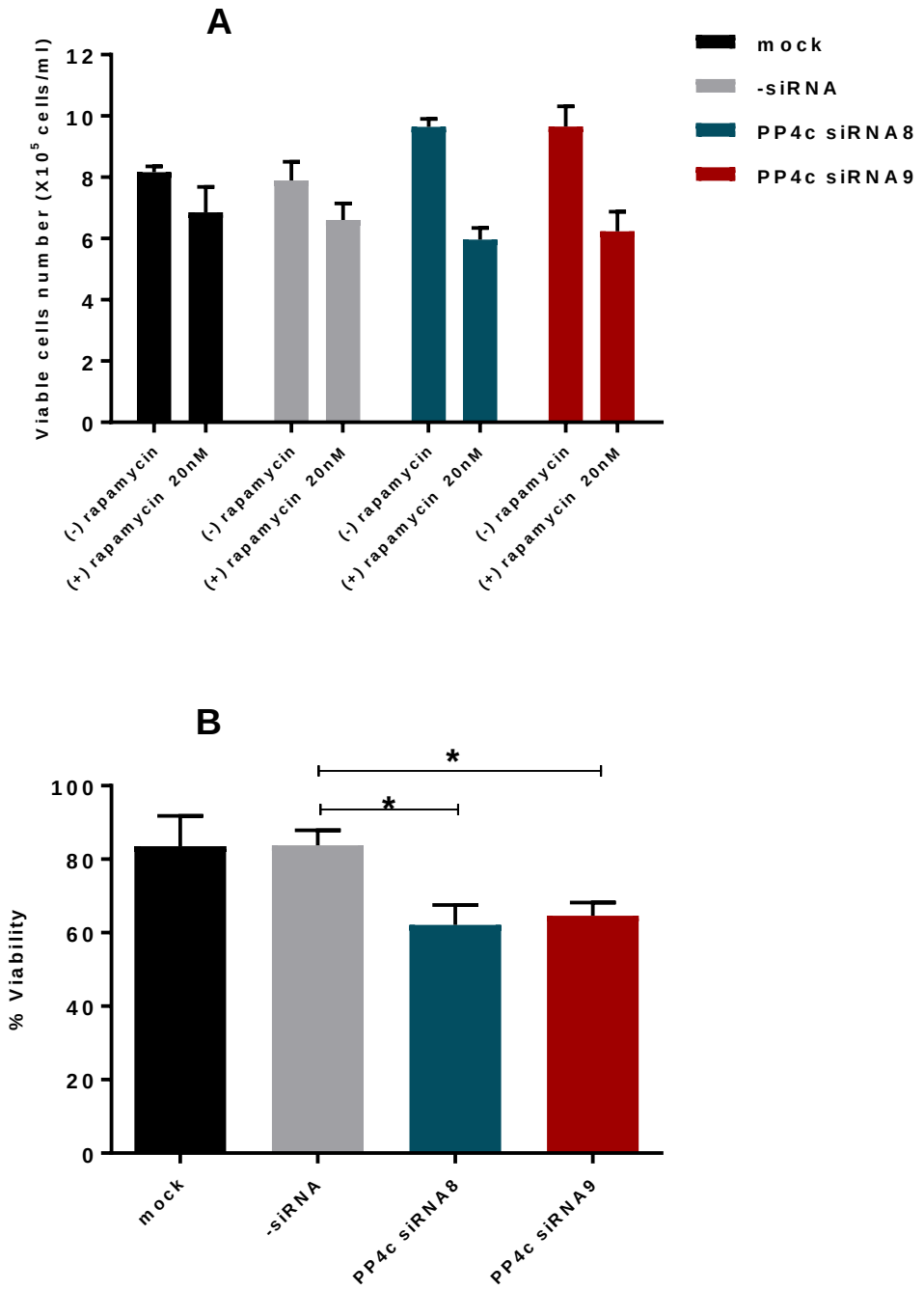

Figure 5.6 PP4c silencing decreases the viability of K562 cells in response to rapamycin treatment. Transfected K562 cells were treated with 20 nM rapamycin. Viable cell count carried out by Trypan blue dye exclusion at 48 hours showed a reduction in the number of viable cells of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9 (Figure A). Figure (B) shows a significant reduction in the viability percentage of the PP4c siRNA8 and PP4c siRNA9 cells compared to negative control cells (-siRNA) 48 hours (*$p<0.05$; one-way ANOVA test followed by Bonferroni test; n=3)

5.3.2. **Effects of PP4c down-regulation in HL-60 cells on their response to some of the anti-cancer drugs**

The effect of the same chemotherapeutic drugs (except imatinib) on cell viability of HL-60 cells following silencing of PP4c was also investigated. HL-60 cells were counted 72 hours post nucleofection, treated with drugs and plated at 2×10^5 cells/mL in 24-well plates to evaluate cell viability and total apoptosis.

PP4c down-regulation in HL-60 cells showed no effect on basal apoptosis, therefore, mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected HL-60 cells were treated with 0.1 µM doxorubicin and the results showed that doxorubicin decreased the number of viable cells of mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected cells at 24 hours (Figure 5.7A), which was further reduced at 48 hours (Figure 5.7B). Figures 5.7C and D show no significant difference in cell viability of PP4c-transfected cells at 24 and 48 hours post-treatment when compared to -siRNA suggesting no effect of PP4c silencing on the HL-60 cells' sensitivity to doxorubicin.

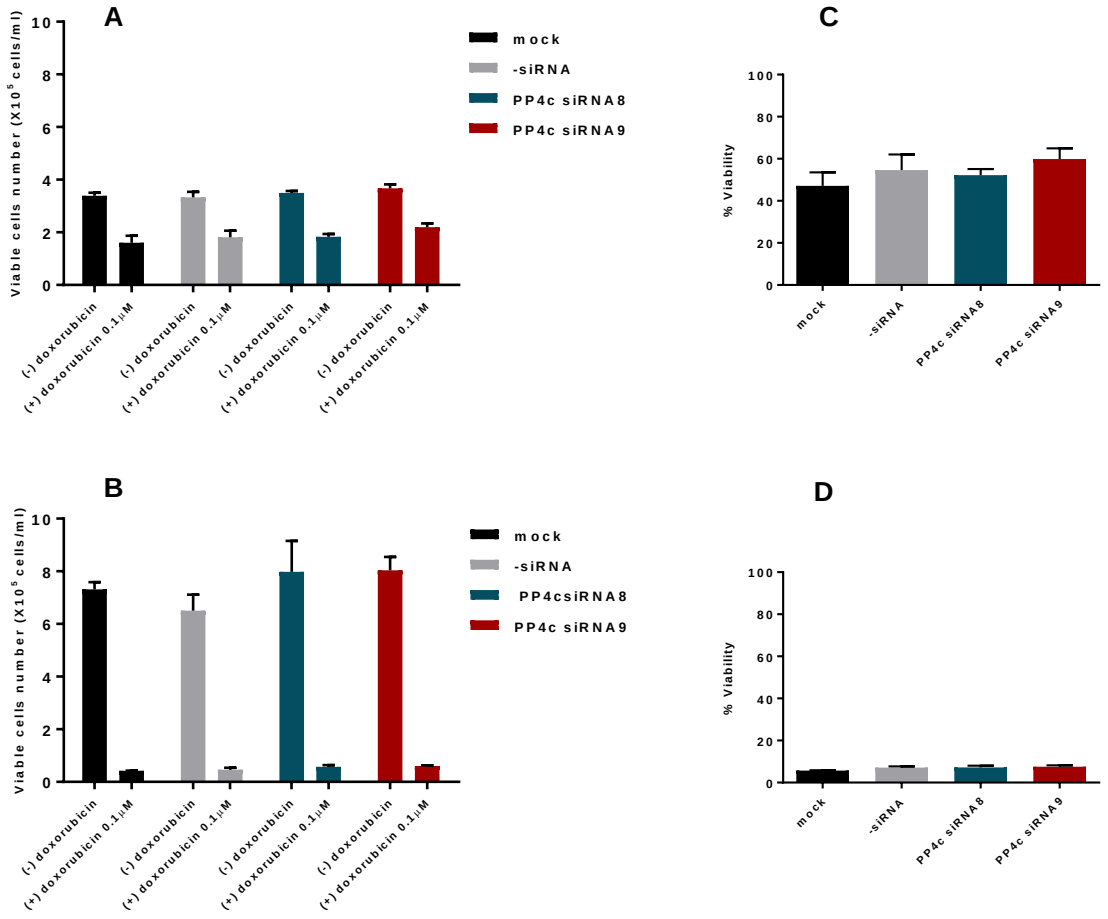

Figure 5.7 PP4c silencing has no effect on HL-60 cells viability in response to doxorubicin treatment. Transfected HL-60 cells were treated with 0.1 μM doxorubicin. Viable cell count carried out by Trypan blue dye exclusion at 24 and 48 hours showed a decrease in viable cell count (A, B) and the viability (C, D) of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9. PP4c down regulation in HL-60 cells showed no resistance to doxorubicin treatment (n=3).

Total apoptotic percentage was also analysed at 24 hours post doxorubicin treatment (Figure 5.8), and the results showed a comparable elevation in the apoptosis rate of all transfected HL-60 cells (mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected cells). These results, similar to Figure 5.7, suggest that PP4c silencing has no effect on the sensitivity of the HL-60 cells towards doxorubicin treatment.

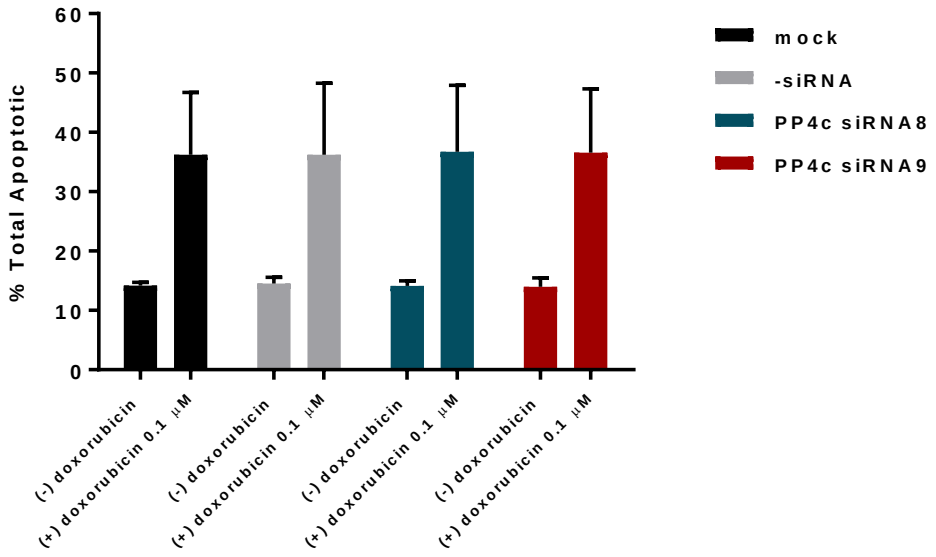

Figure 5.8 PP4c silencing has no effect on HL-60 cells apoptosis in response to doxorubicin treatment. At 24 hours post 0.1 µM doxorubicin treatment, the apoptosis percentage was measured using Muse annexin V & dead cell assay carried out by Muse cell analyser. Transfected cells with PP4c siRNA8 and PP4c siRNA 9 show no difference in total apoptotic percentage as compared to -siRNA post doxorubicin treatment (n=3).

Mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected HL-60 cells were treated with 2 μM cisplatin and the results showed that the number of viable cells of all transfected cells decreased at 24 hours post cisplatin treatment (Figure 5.9A). Similarly to 48 hours post doxorubicin treatment findings, a further reduction in the number of viable HL-60 cells of all transfected cells post cisplatin treatment (Figure 5.9B) was observed, regardless of PP4c silencing and consequently, there was no significant difference in cell viability of PP4c-transfected cells at 24 and 48 hours post-treatment as compared to mock or -siRNA transfected cells (Figures 5.9 C and D). These findings suggested that PP4c silencing in HL-60 cells has no effect on the sensitivity to cisplatin treatment.

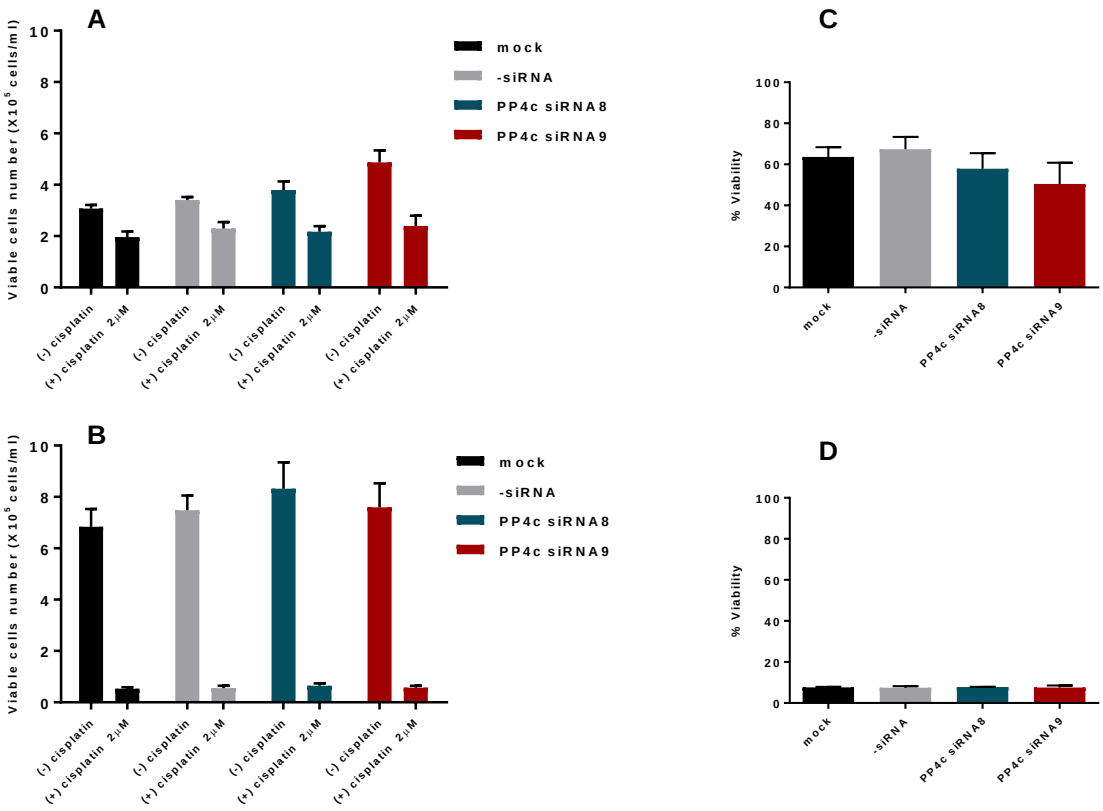

Figure 5.9 PP4c silencing has no effect on HL-60 cells viability in response to cisplatin treatment. Transfected HL-60 cells were treated with 2 µM cisplatin. Viable cell count carried out by Trypan blue dye exclusion at 24 and 48 hours showed a decrease in viable cell count (A, B) and the viability (C, D) of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9. PP4c down regulation in HL-60 cells showed no resistance to cisplatin treatment (n=3).

Likewise, HL-60 cells were treated with 20 nM rapamycin, the mTOR inhibitor, post PP4c silencing and the results showed that rapamycin has the lowest effect on these cells comparing to doxorubicin or cisplatin. The results revealed that rapamycin treatment decreased the number of viable cells of mock, -siRNA, PP4c siRNA8 and PP4c siRNA9 transfected cells at 24 and 48 hours (Figure 5.10). Similarly to doxorubicin and cisplatin results, rapamycin treatment post PP4c silencing showed no significant difference in viable cell number and cell viability of PP4c-transfected cells at 24 and 48 hours post-treatment when compared to mock or -siRNA.

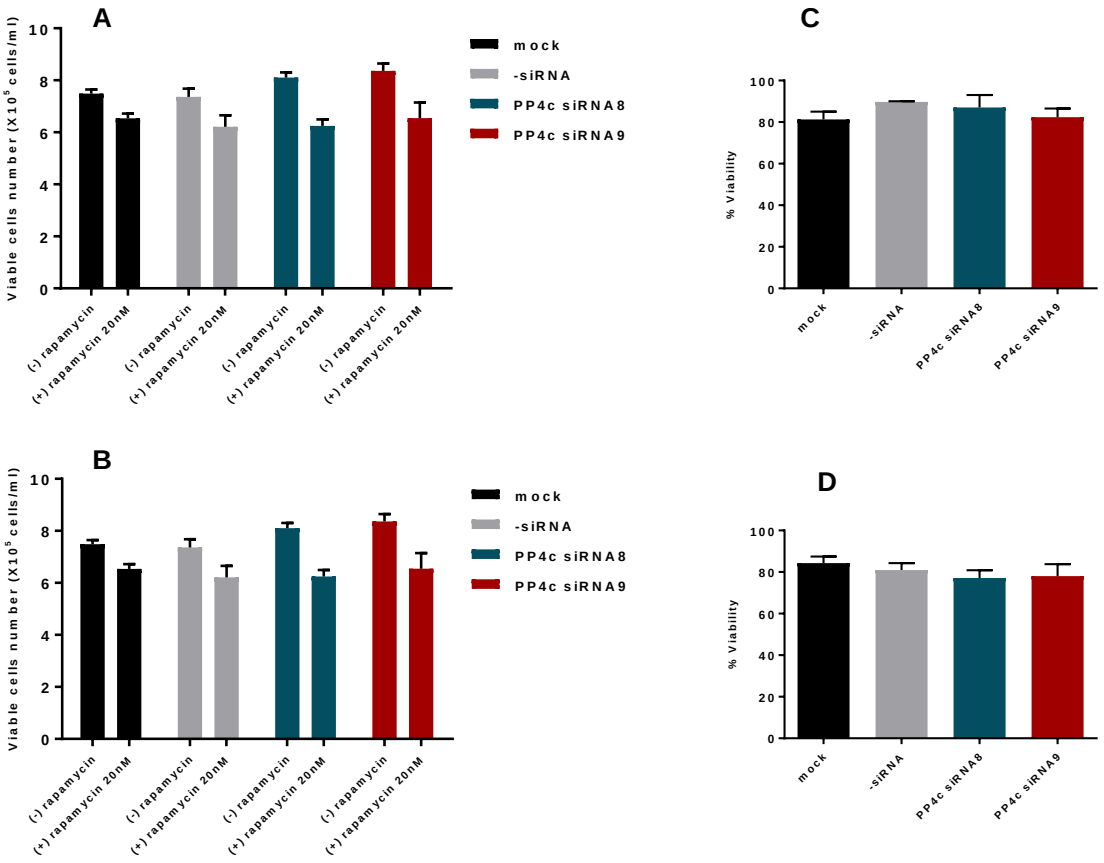

Figure 5.10 PP4c silencing has no effect on HL-60 cells viability in response to rapamycin treatment. Transfected HL-60 cells were treated with 20 nM rapamycin. Viable cell count carried out by Trypan blue dye exclusion at 24 and 48 hours showed a decrease in viable cell count (A, B) and the viability (C, D) of mock, -siRNA, PP4c siRNA 8 and PP4c siRNA 9. PP4c down regulation in HL-60 cells showed no resistance to rapamycin treatment (n=3).

Collectively, PP4c silencing in HL-60 cell has no effect on the cell viability and apoptosis when they treated with doxorubicin, cisplatin or rapamycin.

5.4. **Discussion**

The results in this chapter describe the effects of PP4c down-regulation on the response of K562 and HL-60 cells to cisplatin, doxorubicin, imatinib and rapamycin. For K562 cells, the results showed that down-regulation of PP4c with siRNA8 and PP4c siRNA9 significantly decreased cell viability and increased apoptosis at 24 and 48 hours post imatinib treatment. Moreover, rapamycin treatment significantly decreased cell viability of K562-PP4c siRNA8 and K562-PP4c siRNA9 cells as compared to -siRNA. Silencing PP4c had no effect on the sensitivity of the K562 cells towards doxorubicin and cisplatin treatment. On the other hand, the results of HL-60 cells treatment with cisplatin, doxorubicin and rapamycin post PP4c silencing showed no effect on the response of HL-60 cells to these anti-cancer agents.

The above results suggested that PP4c silencing may enhance the action of both imatinib and rapamycin in K562 cells and may have a relation the protein tyrosine kinase gene Bcr-Abl expressed by K562 cells only.

Cisplatin is a platinum-based genotoxic agent that has successfully used in treating different types of solid tumours including breast and lung cancer (Rabik and Dolan, 2007). In contrast to this study, several cancer cell lines develop cellular resistance to cisplatin and this resistance may be in part due to PP4's integral role in regulating cell cycle checkpoints or DNA damage repair by γ-H2AX de-phosphorylation generated during DNA replication (Rabik and Dolan, 2007; Wang et al., 2008). The over-expression of PP4c-PP4R2-PP4R3α complex that was reported in breast and lung tumours dephosphorylates γ-H2AX and promotes DNA replication, regardless of replication block and consequently cells escaped cisplatin-induced apoptosis (Chowdhury et al., 2008; Wang et al., 2008). Therefore, PP4c silencing sensitised breast and lung cancer cells to cisplatin treatment (Wang et al., 2008). In addition to other cytotoxic stimuli, cisplatin resistance post PP4c

modulation has been reported in leukaemic and T-lymphoid cell lines, HEK 239T, CEM-C7, and Jurkat (Mourtada-Maarabouni and Williams, 2008; 2009). PP4c silencing enhanced the viability of HEK 239T, CEM-C7 and Jurkat cells and protected them from losing their ability of colony-forming that induced by cisplatin treatment (Mourtada-Maarabouni and Williams, 2008; 2009).

On the other hand, some studies correlated imatinib-induced apoptosis to γ-H2AX phosphorylation to understand the mechanism of apoptosis induced by imatinib in CML and why some patients develop imatinib resistance (Zhang et al., 2012). In their study, Zhang and colleagues demonstrated that H2AX C-terminal phosphorylation is involved in K562 cell apoptosis and that treatment with imatinib for 24 or 48 hours induced H2AX C-terminal phosphorylation at Ser139 and Tyr142 (Zhang et al., 2012). It has been reported that PP4c regulates cell cycle checkpoints and DNA damage repair by dephosphorylation of γ-H2AX (Chowdhury et al., 2008; Wang et al., 2008). Moreover, PP4 has been shown to be a positive regulator of haematopoietic progenitor kinase 1 (HPK1) and the HPK1-JNK signaling pathway (Zhou et al., 2004), and its down-regulation activates PEA-15 (Mourtada-Maarabouni and Williams, 2009). Other studies revealed that higher concentrations of imatinib (more than 1 μM) had no further effect on Bcr-Abl kinase activity and the viability of K562 cells (Majsterek et al., 2006). Both K562 and HL-60 cells are p53-deficent; however, some studies have revealed that imatinib treatment activates p53 in Bcr-Abl-expressing cells as a result of Bcr-Abl kinase inhibition (Wendel et al., 2006). Taken together, this can explain the enhancement of imatinib action that significantly decreased cell viability and proliferation and increased apoptosis post PP4c silencing seen in the present study.

Furthermore, targeting the mTOR pathway has been established as potential strategy in the treatment of patients with imatinib-resistant chronic myeloid leukaemia (Sillaber et al.,

2008). It has been reported that PI3K/Akt/mTOR signalling pathways were activated in Bcr-Abl fusion protein positive K562 cells and that PI3K/Akt/mTOR deregulation might contribute to the development of CML (Ly et al., 2003; Cheng et al., 2008b). Rapamycin enhances the action of imatinib and increases cell death in CML cell lines when they are combined *in vitro* and may control imatinib resistance (Ly et al., 2003; Sillaber et al., 2008). As PP4c has reported to regulate many pathways including mTOR (Cohen et al, 2005), this suggests that PP4c may directly or indirectly deregulate the Bcr-Abl kinase activity or its downstream target signaling pathways.

Again, HL-60 cell showed a different response from K562 cells to the same chemotherapy drugs, suggesting that the different signalling pathways activated and the kinases that are expressed by these two cell lines have a vital link to their viability and proliferation. In the present study, HL-60 cells showed a 50% reduction in cell viability during the first 24 hours when treated with cisplatin, while K562 cells showed a reduction in cell viability at 48 hours post cisplatin treatment. Moreover, a large reduction (up to 95%) in HL-60 viable cell number and cell viability was observed following treatment with both doxorubicin and cisplatin at 48 hours, while K562 cells showed a 70% reduction in cell viability when treated with doxorubicin and cisplatin for the same period of time. Although the response of mock and -siRNA transfected HL-60 cells was similar to that of mock and -siRNA transfected K562 cells post rapamycin treatment, PP4c silencing showed a significant decrease in K562 cell compared to no difference in HL-60 cells treated with rapamycin under these conditions.

More studies are needed to explain and understand the differences of these cell line models in term of their response to the anti-cancer agents and the effect of PP4c and its regulatory subunits on the signalling pathways that associate with leukaemia and the kinases that over-expressed.

6. General discussion

In the last decades, much attention has focused on the role that protein kinases play in cancer targeted therapies including leukaemia, more than the role of protein phosphatases (Bamborough, 2012), with targeted therapies in leukaemia treatment concentrating on strategies that inhibit the activity of these kinases (McConnell and Wadzinski, 2009). One such successful targeted therapy is imatinib, a small-molecule drug designed to interfere with the continuous Bcr-Abl tyrosine kinase activation in chronic myeloid leukaemia and consequently inhibit this activation (Druker et al., 1996; 2001; Talpaz et al., 2006).

The effects of serine/threonine protein phosphatases on cell signalling and other cellular processes are complex. These enzymes act on a number of target proteins, many of which have yet to be identified. Numerous studies have highlighted the importance of protein phosphatases in leukaemia and their potential as targets for novel therapies (McConnell and Wadzinski, 2009). In particular, the serine/threonine phosphatase PP2A has emerged as an important tumour suppressor in myeloid leukaemia and strategies aimed at reactivating this phosphatase in addition to the inhibition of the tyrosine kinase activity have shown great promise for a new generation of leukaemia therapies (McConnell and Wadzinski, 2009; Neviani et al., 2005; 2007).

Another of these protein phosphatases is PP4, which is comparatively less studied than other PPP family members such as PP1 and PP2A, therefore, the effects of PP4c modulation in leukaemia was studied in the current research. Studies have shown that PP4 regulates an increasing number of cellular functions in different cellular locations and have resulted in the identification of several PP4 regulatory subunits and binding proteins (Shui et al., 2007; Xie et al., 2013; Brechmann et al., 2012). Similar to other serine/threonine phosphatases, PP4 is likely to be targeted to its specific sites of action by these regulatory subunits. It is also likely that the interchange between the different regulatory subunits and

binding proteins plays an important role in regulating the activity of PP4 complexes (Brechmann et al., 2012; Xie et al., 2013).

PP4 is implicated in NF-κB (Gingras et al., 2005), it also recognised to regulate other cellular pathways including the DNA damage response, the activation of c-Jun N-terminal kinase MAPK 8 (Mihindukulasuriya et al., 2004; Mourtada-Maarabouni et al., 2003), interacts with the target of rapamycin (TOR) pathway (Bertram et al., 2000) and with insulin receptor following TNFα stimulation (Mihindukulasuriya et al., 2004). The role of PP4 in T-cell signalling has been reported by Zhou and colleagues as they suggested that PP4 positively regulates the activity of haematopoietic progenitor kinase 1 (HPK1) in a T-cell receptor (TCR)-dependent manner (Zhou et al., 2004). In addition, PP4 has been reported to associate with HDAC3, an important gene expression regulator, and to inhibit its activity (Zhang et al., 2005), with depletion of PP4c resulting in a prolonged checkpoint arrest in human cells, suggesting that PP4c plays a critical role in dephosphorylating H2AX after DNA damage (Nakada et al., 2008). Many studies suggested that PP4c has a crucial role in controlling cell growth, survival and proliferation in different types of cancers such as breast cancer, lung cancer, lymphoma and leukaemic T cells (Wang et al., 2008; Mourtada-Maarabouni and Williams, 2008; 2009; Brechmann et al., 2012). Moreover, the role of PP4c regulatory subunits is also studied, especially PP4R1 and PP4R2. It has been reported that PP4R1 knockdown inhibits cell proliferation and colony formation in breast cancer cells (Qi et al., 2015), hepatocellular carcinoma cells (Wu et al., 2015) and lung cancer cells (Zhu et al., 2016). While PP4R2 suppression results in de-phosphorylation impairment of phosphorylated DNA damage response proteins such as γH2AX and p53 and thus contribute to the pathogenesis of AML (Herzig et al., 2017).

On the other hand, many studies have implicated PP2A in a wide range of cellular signalling pathways, many of which are involved in cellular proliferation, apoptosis and

differentiation (McConnell and Wadzinski, 2009). The inactivation of PP2A by leukaemia associated tyrosine kinases was found to be a common theme in myeloid leukaemia (Roberts et al., 2010). Importantly, re-activation of PP2A results in dephosphorylation, and deactivation, of the kinase and subsequent inhibition of leukaemogenesis. Therefore, re-activation of PP2A is considered as an attractive strategy for leukaemia therapy (Neviani et al., 2005; 2007; Kalla et al., 2007). Based on the hypothesis that PP2A act as a tumour suppressor besides the previous studies on PP4c modulation in other cell models including lymphoma and breast cancer that support this hypothesis (Mourtada-Maarabouni and Williams, 2008; 2009; Mohammed et al., 2016) and the similarity between PP2Ac and PP4c structures, besides the importance of PP4c in many pathways, therefore, this study aimed to investigate the role of endogenous PP4c modulation on the cell fate of CML and AML cell models.

The current study found that PP4c over-expression in K562 resulted in suppression of cell viability and induction of apoptosis. The experiments have also demonstrated that over-expression of PP4c in K562 cells can suppress their proliferation and promote cell cycle arrest in G0/G1. On the other hand, an increase in PP4c protein level in HL-60 cells was associated with a decrease in cell viability only. These findings are in agreement with studies on CEM-C7, Jurkat cells (Mourtada-Maarabouni and Williams, 2009) and on MCF7 and MDA-MB-231 cells (Mohammed et al., 2016). These studies revealed that any increase in PP4c protein levels negatively regulates the survival of leukaemic T-cells, untransformed human peripheral blood T cells, oestrogen receptor-positive and TNBC cells and is consistently associated with a decrease in cell viability and long term survival as well as apoptosis induction in the absence of extracellular cytotoxic stimuli (Mourtada-Maarabouni and Williams, 2009; Mohammed et al., 2016). Previous results have also shown that PP4c is an important modulator of apoptosis and cell proliferation in HEK

293T cell line (Mourtada-Maarabouni and Williams, 2008). PP4c significantly decreased the mutation rate of human embryonic kidney 293T cells and induced cell cycle arrest in phase G1 (Mourtada-Maarabouni and Williams, 2008). The current study revealed differences in the response of K562 and HL-60 cells to PP4c endogenous modulation. When PP4c was over-expressed in K562 cells the results showed a reduction in viable cell number and in their proliferation rate, accompanied with an increase in apoptosis and changes in the cell-division cycle profile, while PP4c over-expression in HL-60 cells only decrease the viable cell numbers. However, these differences could be related to the sensitivity of HL-60 cells towards the nucleofection solution V. The lower tolerance of HL-60 cells to the nucleofection reagent was supported by observations with the Ki67 proliferation assay showing that K562 mock transfected cells had a much higher proliferation rate than the HL-60 mock transfected cells under similar experimental conditions.

In contrast to PP4c up-regulation, the present study also reveals that PP4c down-regulation increased the viable cell number with K562 cells but had no effect on basal apoptosis and on the proliferation rate. PP4c silencing in HL-60 cells increased cell viability and affected their cell cycle profile by decreasing the cell population in S phase and increasing the cell population in G2/M phase. Furthermore, PP4c down-regulation also had no effect on basal apoptosis and on the proliferation rate percentage in HL-60 cells. These results are partially in agreement with studies on MCF7 and MDA-MB-231 cells (Mohammed et al., 2016) and HEK293 cells and both leukaemic T-cells and untransformed human peripheral blood T-cells (Mourtada-Maarabouni and Williams, 2008; 2009). PP4c silencing in breast cancer cells caused an increase in cell viability and migration, decreased the proportion of cells in subG0 and increased the proportions of cells in S and G2/M phases, effects associated with a decrease in the apoptosis rate (Mohammed et al., 2016), while PP4c silencing in

HEK293, CEM-C7 and Jurkat cells increased their viability and decreases apoptosis (Mourtada-Maarabouni and Williams, 2008; 2009). Moreover, some of the pathways that are regulated by PP4 are also reported to be deregulated in myeloid leukaemia. It has been reported that NF-κB activation through the IKK pathway may play an important role in the pathogenesis of myeloid leukaemia induced by Bcr-Abl (Hsieh and Van Etten, 2014). PP4R1 deficiency leads to an inactive PP4c and causes sustained and increased IKK activity, T cell hyperactivation, and aberrant NF-κB signalling in T cell lymphomas (Seshacharyulu et al., 2013), while PP4c or PP4R2 depletion in human osteosarcoma cells delayed γH2AX dephosphorylation during recovery from irradiation (Nakada et al., 2008). Recently, the role of PP4R2 has been also reported in AML (Herzig et al., 2017). PP4R2 suppression leads to the impairment of DNA damage response proteins such as γH2AX and p53 and consequently leads to AML development (Herzig et al., 2017).

The effects of PP4c down-regulation in HL-60 cells were different from those seen in K562 cells. Firstly, transfection of HL-60 cells with PP4c siRNAs did not yield a high transfection efficiency using nucleofection method, unlike with K562 cells, so that, HiPerFect, a different method was used. PP4c down-regulation in K562 cells showed an elevation in viable cell number at 48 hours post-plating associated with a clear trend on long-term survival, while PP4c down-regulation in HL-60 cells increased the viable cell number at 72 hours post-plating and affected the cell cycle profile by decrease the cell population in S phase and increase the cell population in G2/M phase.

In the current study, the PP4c endogenous modulation in acute myeloid leukaemia models, HL-60 and NB4 (initial experiments, results are not shown), revealed a very low expression using nucleofection solution V and no expression using both Lipofectamine 2000 and Lipofectamine 3000 reagents. On the other hand and despite the high percent of transfection efficiency, the Western blot results of PP4c silencing in HL-60 cells

comparing to the results of K562 cells demonstrated a relatively weak gene knock-down and this could be due to the poor release of the siRNA from the complex, despite high efficiency of delivery to the cell, as assessed by Cy3-labelled siRNA delivery. It has been reported that transfection method has many limitations including toxicity, lack of cell-type specificity and targeting nonspecificty, therefore, a new powerful method called CRISPR/Cas9 could be more useful to manipulating the PP4c gene in these types of cells. CRISPR/Cas9 is a genetic engineering technique in which can either splice out part of a gene to disrupt its function, or insert a new sequence into the genome to code for a new function (Wang et al., 2016). CRISPR/Cas9 has two key components: CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), a short strand of RNA which is a chemical messenger; and Cas9, a special CRISPR-associated nuclease that can cut a double DNA strand at a very precise point, nick it, or block its gene expression. The CRISPR helps guide the Cas9 enzyme to bind with a specific sequence on the genome where it will make a specific cut (Wang et al., 2016). The CRISPR/Cas9 method could be considered as another technique to investigate the role of PP4c in other cell line models for other types of leukaemia such as acute lymphocytic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL).

As PP4c down-regulation in both K562 and HL-60 cells had no effect on apoptosis rate, four different anti-cancer drugs have been used post PP4c silencing to examine if these drugs cause apoptosis or the cells develop resistance. Treating K562 cells with cisplatin and doxorubicin post PP4c silencing showed no effect on the cell viability as well as the apoptosis rate of the mock or negative control transfected cells. Treating HL-60 cells with doxorubicin and cisplatin post PP4c silencing also showed no different effect on the cell viability and the apoptosis rate of control cells. This study revealed that K562 and HL-60 cells could not escape cisplatin- or doxorubicin-induced apoptosis and develop resistance,

these findings are in disagreement with previous studies which suggested that PP4c down-regulation increases the resistance of breast and lung cells (Wang et al., 2008) and the T-leukaemic cell lines CEM-C7 and Jurkat (Mourtada-Maarabouni and Williams, 2009) to cisplatin treatment. Additionally, PP4c silencing partially protects T-leukaemic cells against Fas-induced apoptosis leading to cisplatin-induced apoptosis and consequently developed resistance (Mourtada-Maarabouni and Williams, 2009). This difference in cell response might be related to the oncoproteins that expressed by each type of cells. For example, it has been reported that cisplatin activates extracellular signal-regulated kinase (ERK) in cancer cells via p53 phosphorylation (Dasari and Tchounwou; 2014) while K562 and HL-60 are p53-deficient, so that these cells could not escape cisplatin-induced apoptosis and consequently develop resistance.

The results of the current study showed that K562 cells treatment with imatinib post PP4c silencing significantly decreased their viability and increased the apoptosis rate. In addition, rapamycin treatment post PP4c silencing decreased K562 cell viability. In opposition to the findings of K562 cells, treating HL-60 cells with rapamycin showed no different effect on the cell viability of control cells. The crucial role of PP4c in regulating cell cycle checkpoints and DNA damage repair by γ-H2AX de-phosphorylation has been reported in breast and lung cancer cells suggesting that PP4c down-regulation in these cells promotes DNA replication regardless of replication block and consequently sensitized them to cisplatin treatment (Rabik and Dolan, 2007; Chowdhury et al., 2008; Wang et al., 2008). In addition to that, imatinib-induced apoptosis has been correlated to γ-H2AX phosphorylation (Zhang et al., 2012). Although K562 cells are p53-mutant, p53 might be activated by imatinib treatment as a result of Bcr-Abl kinase inhibition (Wendel et al., 2006). In CML, the mammalian target of rapamycin (mTOR) pathway is one of the multiple signalling pathways activated by Bcr-Abl (Ly et al., 2003). mTOR is a

serine/threonine kinase that forms two different complexes; mTORC1 and mTORC2. mTORC1 positively regulates the biosynthesis of proteins necessary for cell growth, and proliferation, and also known to inhibit the autophagy process, while mTORC2 is a critical regulator of Akt (Gentzler et al., 2012). mTOR is usually described as a member of the canonical PI3K/Akt/mTOR pathway. It was reported that together with imatinib, inhibition of mTORC1 has been shown to induce apoptosis to treat patients with imatinib-resistant chronic myeloid leukaemia (Ly et al., 2003; Sillaber et al., 2008), while mTORC1 and mTORC2 inhibitions could induce apoptosis in cells expressing the T315I-mutated Bcr-Abl gene (Carayol et al., 2010). It was also reported that the oncogenic kinase Bcr-Abl activates signalling pathways, such as PI3K/Akt, STAT5 and Ras/MEK/ERK1/2 (Jacquel et al., 2003). Given PP4 has been described to be a positive regulator for haematopoietic progenitor kinase 1 (HPK1) and the HPK1-JNK signaling pathway (Zhou et al., 2004), and silencing PP4 amplified PEA-15 activation (Mourtada-Maarabouni and Williams, 2009), therefore, the K562 cells are more sensitive to the chemotherapeutic drugs imatinib and rapamycin post PP4c silencing, suggesting a relation between PP4c and the tyrosine kinase gene Bcr-Abl and/or its pathway and collectively, the current study suggested that PP4c may be involved in the regulation of signalling pathways of growth factor-independent proliferation and survival of myeloid leukaemic cells.

Using proteomic analysis, it has been shown that PP4 expression changes have important effects on the phosphorylation status of various proteins that are involved in regulating apoptosis and cell proliferation such as the critical apoptosis regulators Bad, PEA-15, cofilin, STAT3, and ERK2 (Mourtada-Maarabouni and Williams, 2008). This study reported that the phosphorylation of Bad and PEA-15 proteins increased when PP4c levels are suppressed, and it is reduced when PP4c levels are increased (Mourtada-Maarabouni and Williams, 2008). The dephosphorylation of PEA-15 by PP4c may counteract the

effects of AMPK by switching the activity of PEA15 from a tumour promoter to a tumour suppressor (Mourtada-Maarabouni and Williams, 2008). In the same concept, this balance in the activities of AMPK and PP4c are likely to be crucial in the development and progression of breast cancer (Mohammed et al., 2016). Thus, further studies are required to investigate whether the effects of PP4c on K562 and HL-60 cells are mediated through the alteration of the phosphorylation status of these proteins. In addition, more investigations are needed to give more explanations to the results of this research and to understand why the K562 cells response to PP4c endogenous modulation and to the anti-cancer agent, rapamycin, was different from the response of the HL-60 cells.

6.1. **Limitations and Recommendations**

Chronic myeloid leukaemia (CML) has unusual protein tyrosine-kinase activity (Ren, 2005), and this study provided strong evidence that protein phosphatases, represented by PP4, play a role in the survival of K562 and HL-60 cells; an area where further study is now required to continue to explore the signalling pathways targeted by PP4c and their significance to cell survival. These include the mTOR (mammalian target of rapamycin), protein kinase B (Akt), the extracellular signal-regulated kinase 1 (ERK1) and ERK2 pathways, as well as the critical apoptosis regulators BAD and PEA-15 (Phosphoprotein Enriched in Astrocytes). Similar to previous multiplexed phosphorylation screens in HEK 293T cells (Mourtada-Maarabouni and Williams, 2008), the characteristics of the signalling pathways targeted by PP4c in myeloid leukaemic cell lines can be studied by Kinetworks phosphoprotein analysis (an antibody based protein microarray incorporating over 600 phospho-specific antibodies), or other phosphoproteomic approaches, to examine the phosphorylation status of key cellular proteins/signalling pathways in lysates of cells where PP4c expression levels is modulated.

Whilst this study reports effects based on alteration in expression of the PP4 catalytic subunit, it is known that PP4c interacts with many proteins; regulatory subunits, including one shared with PP2A (α4/IGBP1), as well as other scaffolding and inhibitory proteins. Therefore, further studies should not only focus on phosphoproteomic analysis to detect changes in the phosphorylation status in signalling pathways downstream of PP4c, but should also address the complexity of the associations between PP4c and its interacting proteins. This will better define the critical importance of PP4c in determining cell fate in CML and AML models through a better understanding of not only it's downstream signaling effects, but also factors controlling endogenous PP4c activity. Such interacting proteins are also important in controlling subcellular targeting of the PP4 holoenzyme as well as its catalytic activity (Chowdhury et al., 2008; Lee et al., 2010; Davis and Chen, 2012) and in particular, the roles of the PP4 regulatory subunits, PP4R1 and PP4R2, have been studied in various cancer models (Brechmann et al., 2012; Seshacharyulu et al., 2013; Qi et al., 2015; Wu et al., 2015; Zhu et al., 2016; Herzig et al., 2017). For example, it has been reported that PP4R1 deficiency leads to an inactive PP4c and causes sustained and increased IKK activity, T cell hyperactivation, and aberrant NF-κB signalling in NF-κB-addicted T cell lymphomas (Seshacharyulu et al., 2013), while PP4R2 depletion in U2OS human osteosarcoma cells delayed γH2AX dephosphorylation during recovery from irradiation (Nakada et al., 2008), with PP4R2 having previously been shown to be of importance for PP4c targeting to centrosomes. Similarly, the overexpression of both PP4R3/Smek and PP4c increased nuclear accumulation of the complex (Mendoza et al., 2005; Hastie et al., 2000).

Therefore, experiments on PP4 regulatory subunits, PP4R1, PP4R2, PP4R3, PP4R4, and α4/IGBP1 should be expanded to determine their role in mediating the PP4c effects on cell proliferation and cell death in myeloid leukaemic cell lines, as reported in the current

study. Initial studies should determine the expression levels of PP4c (as well as other PPP family members) and its regulatory subunits at the mRNA level using TissueScan™ Lymphoma and Normal Tissue cDNA Arrays or real-time PCR approaches; with endogenous expression levels of select proteins of interest (based on the findings from initial array/RT-PCR screens) confirmed by Western blotting. Additionally, this strategy can also be employed using samples in which the endogenous levels of PP4c have been modulated (as in the current study) to determine if changes in PP4c expression lead to alterations in the basal expression of its interacting proteins, as well as other PPP members. This, alongside phosphoproteomic analysis in PP4c modulated cells will further contribute to defining a critical role of PP4c in CML and AML models. A better understanding of the functional roles of relevant PP4c interacting proteins in K562 and HL-60 cells (as well as other relevant cell line models) could be further explored in studies analysing effects on cell viability, apoptosis and drug response following their endogenous modulation by the regular transfection methods that have been used in this study, or using CRISPR/Cas9 approaches (particularly given the limitations of the transfections methods used with HL-60 cells in the current study). Further analysis of how changes in the expression level of PP4c interacting proteins control PP4c activity should also be undertaken. In view of the fact that PP4c-containing complexes are found throughout the cell, ranging from cytosolic to nuclear or centrosomal localisations with different regulatory protein compositions, substrate specificities, and activities (Chen et al., 2008; Cohen et al., 2005), immunocytochemistry could be used to study PP4c subcellular localisation following changes in the expression of interacting proteins. This should also be considered alongside phosphoproteomic analysis to identify changes in PP4c activity in these models.

Furthermore, since CRISPR/Cas9 methodology could be applied to studies furthering mechanistic insight into the role of PP4c and its interacting proteins, following the

demonstration of significant functional effects in the current study, this technology could also be applied to better define the importance of specific PP4c interactions. Since, whilst PPP family members display high sequence homology in the core catalytic domain, differences in the N- and C- terminal regions are thought to control, in part, the interactions with regulatory proteins (reviewed by Honkanen and Golden, 2002). Therefore CRISPR/Cas9 (or other site-directed mutagenesis techniques) could be employed to generate truncation mutants of PP4c, alongside immuno-precipitation approaches to identify specific regulatory protein interactions (or the lack of), together with immunocytochemistry (to investigate subcellular localisation) and functional assays of cell viability, apoptosis and drug sensitivity, to further the understanding of how PP4c complexes control cell fate in the current models.

Additional studies to expand the drug sensitivity data post changes in PP4c levels, as demonstrated in the current study with imatinib and rapamycin in K562 cells, could also be explored. Whilst the current study utilised a range of standard and targeted (e.g. imatinib) drugs individually, as a rationale to investigate potential downstream signaling pathways impacted on by alterations in PP4c, such as possible effects of PP4c on the mTOR pathway relating to enhanced sensitivity to rapamycin, combination drug treatments could also be explored. A basis for combination chemotherapy is to use drugs that work by different mechanisms, thereby reduces drug resistance, reducing tumour growth and metastatic potential, arresting mitotically active cells, reducing cancer stem cell populations, and inducing apoptosis. Further studies could therefore use combined doses of the drugs employed, possibly requiring lower individual doses selected on the basis of the preliminary MTS assay screens in the current study. Investigations into the relationship between PP4c expression levels and drug sensitivity could also be expanded to explore mechanisms underpinning the functional effects (changes in cell viability and apoptosis

rate) already demonstrated. Imatinib-induced apoptosis has been correlated to γ-H2AX phosphorylation (Zhang et al., 2012) and p53 may be activated by imatinib treatment as a result of Bcr-Abl kinase inhibition (Wendel et al., 2006). In addition to that, PP4c has a crucial role in regulating cell cycle checkpoints and DNA damage repair by γ-H2AX de-phosphorylation (Chowdhury et al., 2008); an observation that can also be related to PP4c subcellular localisation controlled by interactions with regulatory partners and so drug sensitivity can also be explored in further studies investigating functional roles of PP4c interacting proteins. Additionally, the mTOR pathway has been reported in CML models as one of the multiple signalling pathways activated by Bcr-Abl (Ly et al., 2003). Thus, combined effects of imatinib and rapamycin, which showed positive effects individually with enhanced reduction in cell viability in K562 cells post-PP4c silencing, should also be explored after lowering their doses. This approach would also be supported by previous studies using a combination of imatinib and rapamycin to treat patients with imatinib-resistant CML, with results showing that rapamycin enhanced imatinib's action and induced apoptosis (Ly et al., 2003; Sillaber et al., 2008), further highlighting an important relationship between Bcr-Abl and the mTOR pathway, together with the observation in the current study that PP4c silencing enhanced the action of both drugs individually.

Finally, to further investigate the role of PP4c in leukaemia, PP4c endogenous modulation (and its interacting proteins) in cell line models of other types of leukaemia such as acute lymphocytic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL) using appropriate transfection methods for each cell line would support the results of this research and continue to better define the importance of PP4c in this context.

6.2. **Conclusion**

Protein phosphatase 4 catalytic subunit plays an important and critical role in the regulation of different cellular processes. Modulating the level of PP4c expression in two leukaemic cell line models has demonstrated to have an effect on their proliferation, survival, as well as cell cycle progression. PP4c over-expression in K562 cells decreased their viable cell number and survival sharply. An obvious reduction in cell proliferation of those transfected with PP4c and an elevation in the number of cells in G0/G1 in the cell cycle associated with high amount of cell death are strong evidences that PP4c may play a crucial role in chronic myeloid leukaemia cell fate. On the other hand, silencing PP4c gene in K562 cell line increased cell viability and cell proliferation and, additionally, enhanced their response to imatinib and rapamycin.

In addition, PP4c over-expression in HL-60 cells showed a decrease in viable cell number while PP4c silencing in these cells elevated the viable cell number and the number of the cells in G2/M in cell cycle. Unlike K562 cells, HL-60 cells displayed no enhanced response post PP4c down-regulation towards the same anti-cancer drugs.

The differences and the sensitivity of K562 and HL-60 cells towards the PP4c endogenous modulation might be related to the different mutations and dysregulation of tyrosine kinase activity, the expression levels and roles of PP4c interacting proteins and the downstream signalling pathways. These areas warrant further study, since the current research highlights the importance of studying the modulation of the endogenous level of PP4c in leukaemic cell lines as a starting point to widening the knowledge and understanding of the pathways are that involved in leukaemia pathogenesis; the mechanisms by which phosphatases regulate cell fate may allow the development of novel anti-cancer therapeutic strategies in the future.

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Appendix A

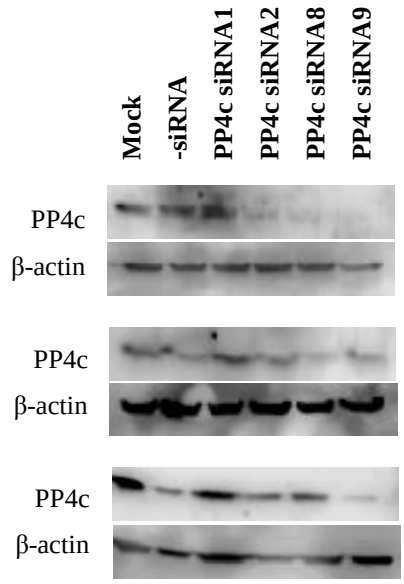

Figure A. Replicate Western blots for PP4c down-regulation in K562 cells. Three immunoblots of PP4c protein expression in PP4c siRNA1, PP4c siRNA2 & PP4c siRNA8, PP4c siRNA9, -siRNA and K562 parental cells. Cells lysates of $1x10^6$ cells/lane were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of PP4c. Blots were stripped and re-probed for β-actin.

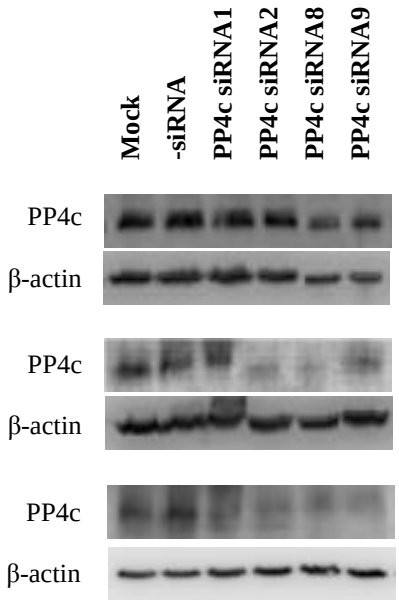

Figure B. Replicate Western blots for PP4c down-regulation in HL-60 cells. Three immunoblots of PP4c protein expression in PP4c siRNA1, PP4c siRNA2 & PP4c siRNA8, PP4c siRNA9, -siRNA and HL-60 parental cells. Cells lysates of $1x10^6$ cells/lane were separated by SDS-PAGE on 12% resolving gels followed by transfer to PVDF and detection of PP4c. Blots were stripped and re-probed for β-actin.