TARGETING THE SPLICE FACTOR KINASES SRPK1 AND CLK1 IN LEUKAEMIA

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A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy

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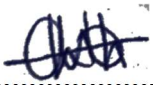
AUTHOR'S DECLARATION

"This PhD thesis is submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy in Biomedical Science and except where duly acknowledged or referenced it is entirely my own work. It has not been submitted, either in whole or in part, for any other award at UWE or elsewhere."

Signed  **Date** 21/04/2021

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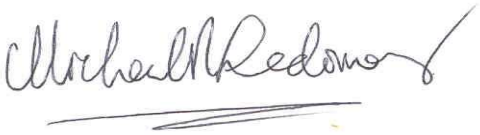

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DEDICATION

Dedicated to the loving memory of Late Mrs Glady I.A. Wodi (1945-2015) and Ms Stella E. Ozomaro (1971-2013). The women whom I called mother. May your early departed souls continue to rest in peace.

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LIST OF ABBREVIATIONS

aa	Amino acid
AD	Accessory domain
AKT/PKB	Protein kinase B
ALL	Acute Lymphoblastic Leukaemia
AML	Acute myelogenous Leukaemia
ANOVA	Analysis of Variance
AP	Accelerated phase
AP	Ammonium Per sulphate
APAF	Apoptotic protease activating factor 1
AS	Age Standardised
AS	Alternative splicing
ASF/SF2	Alternative splicing factor 1 (ASF1) / pre-mRNA-splicing factor SF2
ATP	Adenosine triphosphate
Aza	Azacytidine
Az	Azacytidine
BAP-1	BCR associated protein 1
BC	Blast crisis
BCL	B-cell lymphoma-2
BCL/ABL	B-cell lymphoma-2 / Abelson murine leukaemia virus
BCR	Breakpoint cluster region
µ- BCR	Micro Breakpoint cluster region
BM	Bone marrow
BPS	Branch point sequence
BRCA-1	Breast cancer gene-1
BSA	Bovine Serum Albumin
CBL	Casitas b-lineage lymphoma protein
cDNA	Complimentary Deoxyribonucleic acid
CEBPA	CCAAT/enhancer binding protein alpha gene
CLL	Chronic lymphoblastic leukaemia
CLK1	CDC-like kinase-1
CML	Chronic Myeloid Leukaemia

CO^2 Carbon (IV) Oxide

COOH Carboxylic group

CP Chronic phase

CRIBS Centre for Research in Biosciences

CRKL CRK oncogene like protein

CRUK Cancer Research UK

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleic acid

DYRK Dual-specificity tyrosine phosphorylation-regulated kinases

ssDNA Single Stranded Deoxyribonucleic acid

dsDNA Double Stranded Deoxyribonucleic acid

dNTP Deoxynucleotide triphosphates

EBV Epstein-Barr virus

EGF Epidermal growth factor

ESEs Exonic splicing enhancers

ESSs Exonic splicing silencers

FAB French-American-British system

FAK Focal adhesion kinase

FBS Foetal Bovine Serum

FISH Fluorescence in situ hybridization

FITC Fluorescein isothiocyanate

GEF GDP-GTP exchange factor

G_1/S Growth phase 1/Synthesis phase

GRB2 Growth factor receptor bound protein 2

GvHD Graft versus host disease

HBV Hepatitis-B Virus

hnRNPs Heterogenous nuclear ribonucleoproteins

HPV-1 Human Papilloma Virus-1

HSC Haematopoietic stem cell

HSCT Haematopoietic stem cell transplant

HSP Heat shock proteins

HTLV-1 Human T-cell lymphoma virus-1

IC Intensive chemotherapy

IFN-α Alpha interferon

IM Imatinib mesylate

ISEs Intronic splicing enhancers

ISEs Intronic splicing silencers

JAK Janus kinase

LDAC Low-dose cytosine arabinoside

MDS Myelodysplastic syndromes

MLL Myeloid-lymphoid or Mixed lineage leukaemia

mRNA Messenger ribonucleic acid

mTOR Mammalian target of rapamycin

NOS Not otherwise specified

NLS Nuclear localization signal

OD Optical density

OH^- Hydroxyl ion

ORF Open reading frame

OS Overall survival

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PFS Progression free survival

PDGFR+ Platelet-derived growth factor receptor (+) positive

PHLPP PH domain and leucine rich repeat protein phosphatase

Ph Philadelphia chromosome

PI3 Phosphatidylinositol 3-kinase

Plk Polo-like kinase

PML Promyelocytic Leukaemia

p-NA p-nitrianiline

PRP4K Pre-mRNA processing factor 4 kinase

PSI Percentage of splice inclusion

PTEN Phosphatase and tensin homolog

PVDF Polyvinylidene Fluoride

qPCR Quantitative PCR

RCC Renal cell carcinoma

RRM RNA recognition motif

RNA Ribonucleic acid

RT-PCR Real-Time Polymerase Chain Reaction

SDS Sodium dodecyl sulphate

SHC SRC homology 2-containing protein

siRNA Small interference ribonucleic acid

snRNA Small nuclear ribonucleic acid

snRNPs Small nuclear ribonucleoproteins

SOS Son of sevenless

SPHINX 5-methyl-N-[2-(morpholin-4-yl)-5-(tri-fluoromethyl) phenyl] furan-2-Carboxamide

SRPIN340 N-[2-(1-piperidinyl)-5-(trifluoromethyl) phenyl] isonicotinamide

SRPK1 Serine-rich protein kinase-1

SRPK2 Serine-rich protein kinase-2

SRPK3 Serine-rich protein kinase-3

SRSF-1 Serine-rich splice factor-1

STAT Signal transducer and activators of transcription

TBST Tris-buffered saline and Tween-20

TBI Total body irradiation

TEMED Tetramethylethylenediamine

TF Tissue factor

TGF-β Transforming growth factor-β

TG003 1-(3-ethyl-5-methoxy-2, 3-dihydrobenzothiazol-2-ylidene) propan-2-one

TIMP-1 Tissue inhibitor of metalloproteinase-1

TKI Tyrosine kinase inhibitor

UTR Untranslated region

VEGF Vascular endothelial growth factor

VEGFxxxb Vascular endothelial growth factor 165b isoform

WB White blood cell

WT-1 Wilms' tumour-1

ABSTRACT

This study was aimed at investigating the effect of inhibiting SRPK1 in leukaemic cells. It was also aimed at exploring the potential utility of combining conventional leukaemia chemotherapy (such as imatinib) with compounds that inhibit SRPK1.

SRPK1 is best known for its role in the phosphorylation of serine/argenine rich proteins (SR-proteins) which are responsible for constitutive and alternative mRNA splicing. Studies have associated elevated levels of SRPK1 with tumour growth, proliferation and invasiveness with inhibition resulting in decreased tumour growth and altering the choice of alternative splice site.

Imatinib mesylate and azacytidine remain the drugs of choice for the management of chronic myeloid leukaemia (CML) and acute myelogenous leukaemia (AML) respectively. Studies have shown that both imatinib and azacytidine are able to reduce the growth of proliferating Bcr/Abl^{+} and AML cells principally through the induction of apoptotic cell death.

SRPK1 was inhibited using the small molecule inhibitor SPHINX. SPHINX was combined with either imatinib in a CML cell line (K562) or azacytidine in an AML cell line (Kasumi-1) for up to 72hrs. Results suggest that the SPHINX compound affects the ability of SRPK1 to phosphorylate its substrates in all three cell lines (TK6, K562 and Kasumi-1). Inhibition of SRPK1 was found to reduce cell viability in Kasumi-1 cells and at higher concentration, affect K562 cell viability consistent with the work of Sanidas *et al*.,(2010). There was also an indication that SRPK1 could be regulating its own expression through a feedback loop in a cell line-dependent manner.

Studies with imatinib mesylate and azacytidine showed that both imatinib mesylate and azacytidine are able to reduce cell growth and viability in a dose and time-dependent manner. On combining them with SPHINX, a combination of azacytidine and SPHINX had an additive effective on Kasumi-1 cells but not with imatinib mesylate in K562 cells. Results also showed that imatinib affected the alternative splicing of caspase 9 favouring a pro-apoptotic isoform, caspase 9a. Imatinib mesylate alone also caused an apparent reduction in the expression of SRPK1, CLK1 and SRSF1, suggesting that pathways imatinib affects cell signalling pathways that regulate the expression of these oncogenic splice factor kinases and splice factors. In summary, this thesis presents

evidence that targeting SRPK1 could potentially provide therapeutic benefit in the treatment of a range of leukaemias; further research is now needed to explore this novel approach.

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CHAPTER 1

Introduction

1.1 Haematopoiesis

The blood system is established and maintained by self-renewing haematopoietic stem cells found in the bones marrows of adult humans. This process known as haematopoiesis is the process by which circulating blood stem cells called haemocytoblasts go on to become committed progenitor cells giving rise to erythroid, megakaryocytic, granulocytic, monocytic, basophilic, eosinophilic, or lymphoid lineages over the course of a lifetime (Kondo *et al*., 1997; Akashi *et al*., 2000) (Figure 1.1). An earlier study suggests that not all haematopoietic stem cells become committed to a lineage as some cell which express high levels of interleukin-7 (IL-7) neither adopt erythroid or megakaryocyte lineage fates (Adolfsson *et al*., 2005). The process of cell differentiation in haematopoiesis is mediated by transcription factors (TFs) (AML-1, GATA-2), cytokines (such as granulocyte–macrophage CSF(GM-CSF) receptors, interleukins (ILs), interferons) and other factors (Ikros, Hox, Notch) with variable level of expression of these molecules observed at different stages of cell differentiation (Klug *et al*., 1998; Pan and Simpson, 2001; Enciso *et al*., 2016).

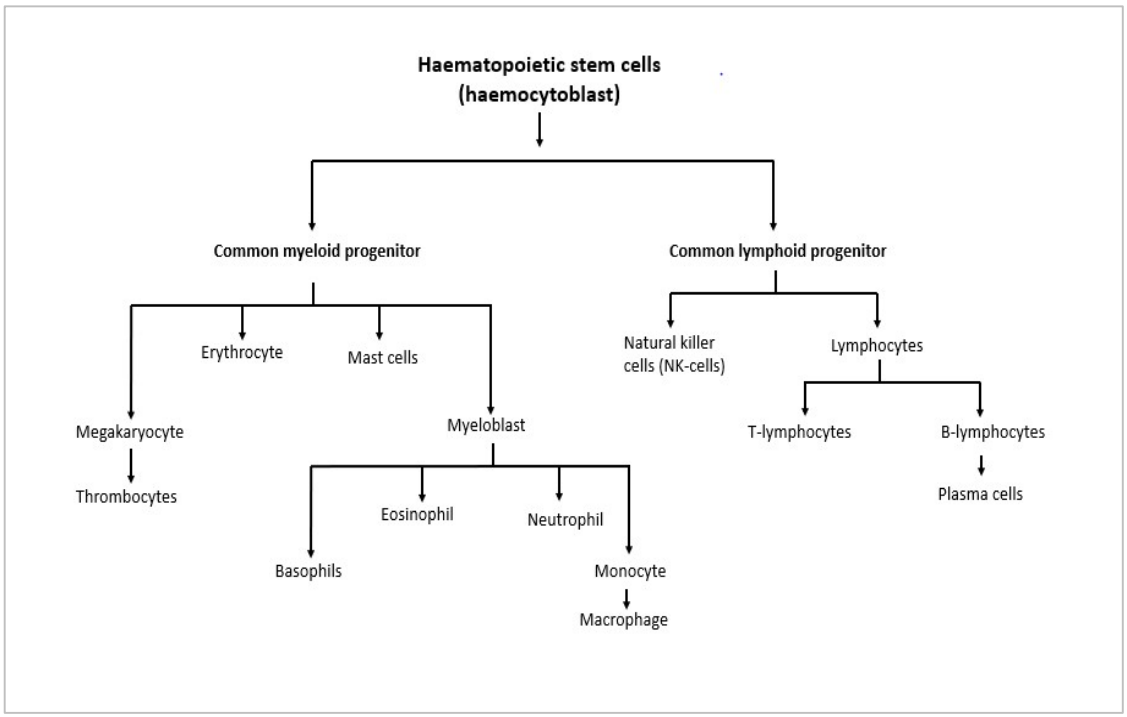


Figure 1.1: Stages of cell differentiation in haematopoiesis. Pluripotent haematopoietic stem cell differentiates into myeloid and lymphoid precursor cells.

While this process is tightly controlled to achieve stability and homeostasis, genetic alteration which can be in the form of variation, mutation and deletion in this process could result in disease conditions (Owen *et al*., 2008). For example, germline mutation of the *RUNX1* gene leading to its deficiency cause familial platelet disorder and predisposes an individual with such mutation to myeloid leukaemia (Owen *et al*., 2008). Deficiency of the *RUNX1* gene also correlates with deficiency in megakaryocyte colony formation thus, implicating *RUNX1* as a regulator of megakaryopoiesis. This links *RUNX1* haploinsufficiency and predisposition to malignant haematological conditions (Owen *et al*., 2008) possibly due to increased circulating haematopoietic progenitor cells and defective T- and B-lymphocyte development observed in mice deficient of AML-1/RUNX-1 (Ichikawa *et al*., 2004).

Further studies suggests that bone marrow tumour microenvironment is initiated by pro-inflammatory cues within the bone marrow and is responsible for the maintenance of ALL precursor cells at the expense of normal hematopoietic cells, through aberrant expression of NF-κB induced by intrinsic and extrinsic factors (Enciso *et al*., 2016). It is also known that an interplay between genetic and epigenetic changes is responsible for the development of B-cell acute lymphoblastic leukaemia. These changes lead to excessive production of malignant B-lymphoid precursor cells within the bone marrow (BM) (Pelayo *et al*., 2012; Purizaca *et al*., 2012).

Failure in the differentiation of immature cell into myeloid or lymphoid cell is a characteristics of blast phase in chronic myeloid leukaemia (CML) (Hehlmann, 2012). A study has shown that although the blast phase CML is driven by genetic instability and additional mutation through the *BCR-ABL* fusion gene, cross-talk between the signalling network involving Sonic hedgehog (Shh), Wnt, Notch and Hox are responsible for blast transformation of CD34+ CML cells (Sengupta *et al*., 2007). Interestingly, haematopoietic stem cell transcription factors; most of which are DNA binding proteins such as RUNX1, TEL/ ETV6, SCL/Tal1, and LMO2 have been linked to leukaemia-associated somatic mutations and translocations in patients. The result is deregulation of the locus or generation of chimeric fusion proteins (Golub *et al*., 1995; Krivtsov *et al*., 2006; Regha *et al*., 2015). These findings suggest the role dysregulation in both biochemical and molecular pathway during haematopoiesis plays in the initiation of leukaemia and related myelodysplastic syndromes.

1.2 Leukaemia

1.2.1 *Definition, types and classification*

Leukaemia results from the proliferation of an abnormal clone of haematopoietic stem cells that has become insensitive to regulatory, differentiation and apoptotic signal (Chu *et al*., 2012). These cells are known to multiply at the expense of normal haematopoietic cells (Lee *et al*., 2007). Signs of leukaemia are commonly related to the leucocytes infiltrating the lymphatic nodes and organs. However, most patients will present with symptoms related to bone marrow failure such as spontaneous bruising or abnormal bleeding, recurrent infection due to neutropenia or symptoms relating to severe anaemia (Grigoropoulos *et al*., 2013). Leukaemia is known to have a poor prognosis (Huang *et al*., 2014). It could result in osteolytic bone destruction, impaired haematopoiesis, and progressive renal failure (Allegra *et al*., 2010). Despite the advancement in understanding the molecular pathogenesis of leukaemia, improvement in therapy and introduction of novel drugs, most patients will relapse (Allegra *et al*., 2010). Relapse in leukaemic cancers is a common event and has been associated to the heterogeneous nature of the tumour cells due to genomic instability and accumulation of multiple mutations (Schlenk *et al*., 2008; Casado *et al*., 2013). Such heterogeneity disrupts the homeostasis of the signalling network which is maintained by complex crosstalk and feedback (Sugawara *et al*., 1998; Kahlert *et al*., 2014). Therefore, regulation of protein signalling networks in leukaemic cells is difficult to predict, as it is also difficult to predict the influence of oncoproteins on the cells, as well as determine the most effective method to reverse the adverse effects of these oncoproteins on the cell (Quail and Joyce, 2013; Bailey *et al*., 2018). For example, the RUNX1/ETO oncoprotein responsible for leukaemic transformation in AML. In a complex network, RUNX1/ETO downregulates several DNA repair proteins such as BRCA2 and ATM and increase the phosphorylation of TP53 gamma H2AX (γH2AX) (Forster *et al*., 2016). The EVI1 (ecotropic viral integration site 1) oncoprotein is a transcriptional regulator with an essential role in haematopoiesis. Overexpression of EVI1 in acute myeloid leukaemia (AML) confers extremely poor prognosis through transcriptional regulation, signalling, and epigenetic modifications by interacting with DNA, proteins and protein complexes (White *et al*., 2013). Other oncoproteins which are well characterized and associates with complex protein-protein network driving leukemogenesis include the

MYC (Yun *et al*., 2018), BCR-ABL oncoproteins (p210 and p230) (Hantschel *et al*., 2014), the RAS family proteins (Liang *et al*., 2006) and the tyrosine kinases which are activated in this network (Scheijen and Griffin, 2002).

Leukaemia's are either of pre-B cell or pre-T cell phenotype, meaning that they exhibit cell surface markers of normal pre-B and pre-T cells and appear to be clonal outgrowths of normal precursor cells whose differentiation has not progressed but stopped at a particular stage (Aziz *et al*., 2015). Leukaemic cells are capable of extravasation since they are blood cells. There are mounting evidence that common genetic mutation in leukaemia such as translocations involving t(12;21)*TEL-AML1,* t(8;21)*AML1-ETO,* inv(16)*CBFB-MYH11* occur prenatally evidenced by its presence in neonatal blood spots at birth in children who contract leukaemia later in life (McHale *et al*., 2003; Zuna *et al*., 2011). Further studies have shown that mutations associated with leukaemia increase susceptibility to the condition but do not result in the acquisition of the disease itself (Mori *et al*., 2002). This is true for *TEL-AML1 and AML1-ETO,* the most common translocations for ALL and AML, respectively suggesting that a percentage of normal individuals carry preleukaemic clones (Mori *et al*., 2002; Zuna *et al*., 2011).

Classification of leukaemia is based on cell type and stage of leukocyte differentiation. Leukaemia classification can either be acute or chronic. Leukaemia is said to be acute when the proliferating blood cells are immature white blood cells or cells in blast phase. Whereas the proliferation of mature cells is considered chronic. Further classification is based on the origin of the leukaemic cells. Leukocytes are usually of lymphoid origin and, hence, leukaemic cells can be classed as T-cell or B-cell leukaemia whereas, neutrophils, basophils, eosinophils and monocytes which are of myeloid origin are classed as myeloid leukaemia (Grigoropoulos *et al*., 2013).

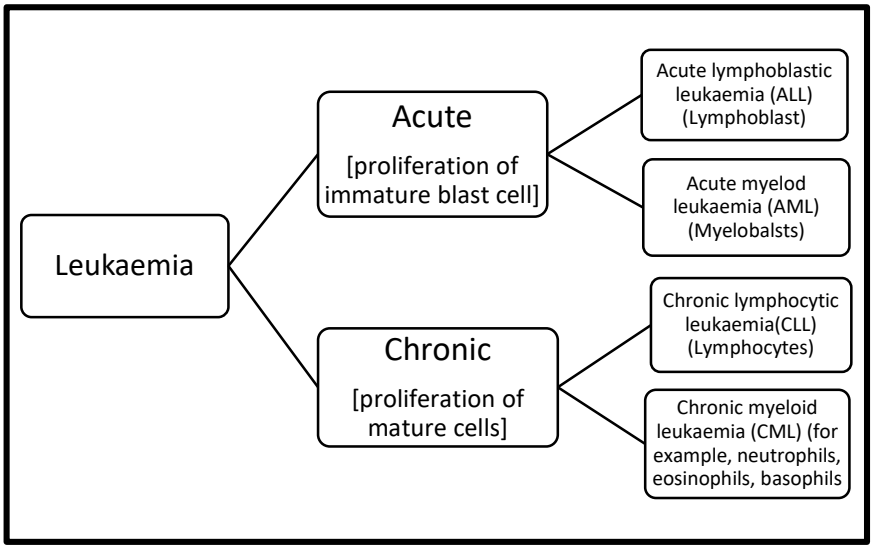


Figure 1.2. **Summary of the broad classification of leukaemia**

The World Health Organization (WHO) in 2016 revised haematopoietic and lymphoid tissue related neoplasms. The revision (Table 1.1) reflects the opinion of haematopathologists, haematologists, geneticists and oncologists and incorporates updated data to include new clinical, prognostic, morphologic, immunophenotypic and genetic characteristics (Arber *et al*., 2016).

Table 1.1. ***Summary of WHO classification of myeloid neoplasms and acute Leukaemia. Reported in Arber et al., 2016***

Myeloproliferative Neoplasms (MPN)
○ Chronic Myeloid Leukaemia (CML), BCR-ABL11^{+} ○ Chronic Neutrophilic Leukaemia (CNL) ○ Polycythaemia Vera (PV) ○ Primary Myelofibrosis (PMF) • PMF, prefibrotic/early stage • PMF, overt fibrotic stage ○ Essential Thrombocythemia (ET) ○ Chronic Eosinophilic Leukaemia, not otherwise specified (NOS) ○ MPN, unclassifiable ○ Mastocytosis

Myeloid/Lymphoid Neoplasms with eosinophilia and rearrangement of PDGFRA, PDGFRB, or FGFR1, or with PCM1-JAK2

- Myeloid/Lymphoid Neoplasms with PDGFRA rearrangement
- Myeloid/Lymphoid Neoplasms with PDGFRB rearrangement
- Myeloid/lymphoid Neoplasms with FGFR1 rearrangement
- Provisional entity: Myeloid/Lymphoid Neoplasms with PCM1-JAK2

Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN)

- Chronic Myelomonocytic Leukaemia (CMML)
- Atypical Chronic Myeloid Leukaemia (aCML), BCR-ABL1^{-}
- Juvenile Myelomonocytic Leukaemia (JMML)
- MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)
- MDS/MPN, unclassifiable

Myelodysplastic syndromes (MDS)

- MDS with single lineage dysplasia
- MDS with ring sideroblasts (MDS-RS)
    - MDS-RS and single lineage dysplasia
    - MDS-RS and multi-lineage dysplasia
- MDS with multi-lineage dysplasia
- MDS with excess blasts
- MDS with isolated del(5q)
- MDS, unclassifiable
- Provisional entity: Refractory cytopenia of childhood
- Myeloid neoplasms with germ line predisposition

Acute Myeloid Leukaemia (AML) and related neoplasms

- AML with recurrent genetic abnormalities
    - AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
    - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
    - APL with PML-RARA

- AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
- AML with t(6;9)(p23;q34.1);DEK-NUP214
- AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
- AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
- Provisional entity: AML with BCR-ABL1
- AML with mutated NPM1
- AML with biallelic mutations of CEBPA
- Provisional entity: AML with mutated RUNX1

o AML with myelodysplasia-related changes

o Therapy-related myeloid neoplasms

o AML, NOS

- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute Myelomonocytic Leukaemia
- Acute Monoblastic/Monocytic Leukaemia
- Pure erythroid leukaemia
- Acute Megakaryoblastic Leukaemia
- Acute Basophilic Leukaemia
- Acute panmyelosis with myelofibrosis

o Myeloid sarcoma

o Myeloid proliferations related to Down syndrome

- Transient Abnormal Myelopoiesis (TAM)
- Myeloid leukaemia associated with Down syndrome

Blastic plasmacytoid dendritic cell neoplasm

Acute leukaemia's of ambiguous lineage

- Acute undifferentiated leukaemia
- Mixed phenotype acute leukaemia (MPAL) with t(9;22)(q34.1;q11.2); BCR-ABL1
- MPAL with t(v;11q23.3); KMT2A rearranged
- MPAL, B/myeloid, NOS
- MPAL, T/myeloid, NOS

B-Lymphoblastic Leukaemia/Lymphoma   ○ B-lymphoblastic leukaemia/lymphoma, NOS   ○ B-lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities
○ B-lymphoblastic leukaemia/lymphoma with t(9;22)(q34.1;q11.2);BCR-ABL1   ○ B-lymphoblastic leukaemia/lymphoma with t(v;11q23.3);KMT2A rearranged   ○ B-lymphoblastic leukaemia/lymphoma with t(12;21)(p13.2;q22.1); ETV6-RUNX1 B-lymphoblastic leukaemia/lymphoma with hyper-diploidy   ○ B-lymphoblastic leukaemia/lymphoma with hyp-odiploidy   ○ B-lymphoblastic leukaemia/lymphoma with t(5;14)(q31.1;q32.3) IL3-IGH   ○ B-lymphoblastic leukaemia/lymphoma with t(1;19)(q23;p13.3);TCF3-PBX1   ○ Provisional entity: B-lymphoblastic leukaemia/lymphoma, BCR-ABL1–like   ○ Provisional entity: B-lymphoblastic leukaemia/lymphoma with iAMP21
T-Lymphoblastic Leukaemia/Lymphoma
○ Provisional entity: Early T-cell precursor Lymphoblastic Leukaemia   ○ Provisional entity: Natural killer (NK) cell Lymphoblastic Leukaemia/Lymphoma

1.2.2 *Prevalence and statistics*

According to Cancer Research UK (CRUK), leukaemia is the 12th most common cancer in the UK with about 9,900 new cases of leukaemia reported each year. The incident rate is higher in male than in females. It is projected that the incidence rate of leukaemia will rise by 5% between 2014 and 2035 to 19 cases per 100,000 people while the mortality rate will rise by 18%. The Caucasian population are known to be more affected than Black or Asian populations. Current statistic on leukaemia incidence and survival according to CRUK are shown in Figure 1.3 and 1.4.

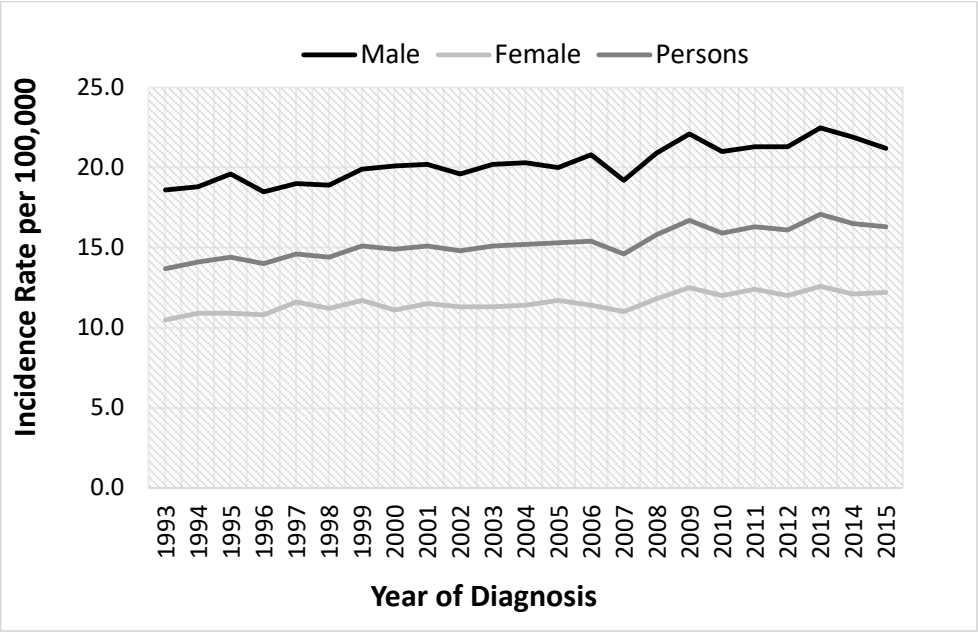


Figure 1.3. UK leukaemia incidence rate per 100,000 population by sex from 1993 to 2015. Increase rate in leukaemia in all age group. Age standardized (AS) incidence rate in males and females between 1993-1995 and 2013-2015 was 15% and 14% respectively (adapted from CRUK).

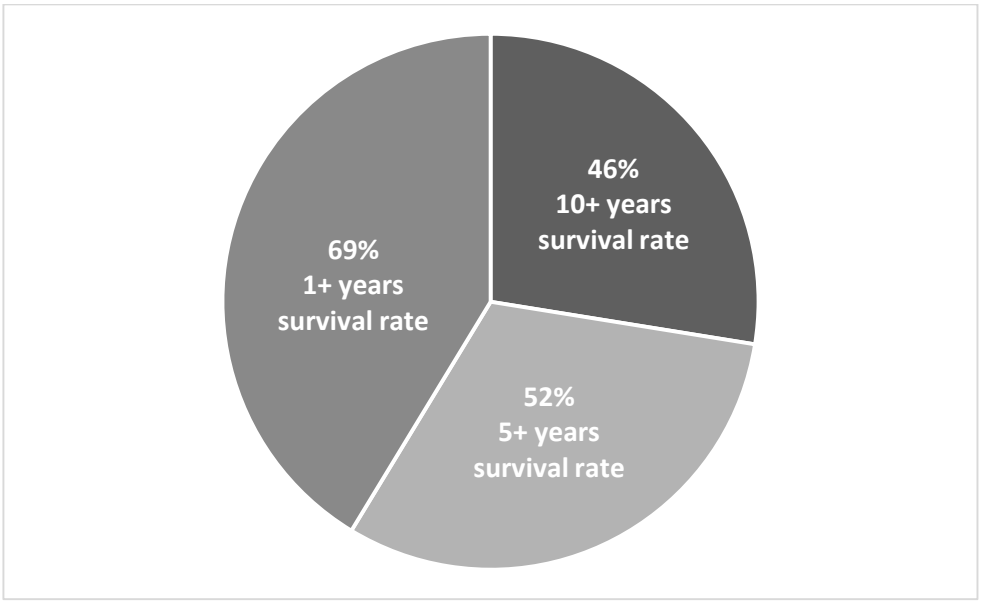


Figure 1.4. Survival rate of leukaemia in England and Wales between 2010-2011. Chart showing overall survival at 69%, 52% and 46% for one, five and ten years from the time of diagnosis (adapted from CRUK).

More comprehensive data compiled from several databases was recently published by Adalberto *et al*., (2018) (Table 1.2). The collected data was consistent with reports that leukaemia is more common in males than female. Australia and New Zealand were identified as having the highest incidence of leukaemia. Among children, acute

lymphoblastic leukaemia (ALL) subtype was more prevalent. In adults, subtypes were found to be diverse. European and North American adults had a relatively higher incidence of chronic lymphoblastic leukaemia (CLL) whereas, adults in South American, Caribbean, Asian and African had higher rates (Adalberto *et al*., 2018).

Table 1.2. Summary of finding on the global incidence of leukaemia

Continent/ region	Sex	Incidence rate
Australia and New Zealand	Males	11.3
	Females	7.2
North America	Males	10.5
	Females	7.2
Western Europe	Males	9.6
	Females	6.0
Western Africa	Males	1.4
	Females	1.2

Adalberto *et al*., 2018

It has been observed that adults have a higher incidence of CML, AML, myelodysplastic and myeloproliferative syndrome when compared to children (Adalberto *et al*., 2018). Whereas childhood leukaemia is more prevalent in B and T-cell acute lymphoblastic leukaemia (ALL). The former subtype which has shown a higher incidence in adults are of myeloid origin and are derivative of precursor cells critical in innate immunity rather than adaptive immunity (Abbas *et al*., 2005).

1.2.3 *Aetiology and Pathogenesis*

The table below summarises some factors which are thought to increase the risk of developing leukaemia.

Table 1.3. Genetic and environmental risk factor implicated in leukaemia

Environmental factors
• Radiation and electromagnetic exposure • Chemical exposure e.g. benzene • Viral infection e.g. human T-cell lymphoma virus (HTLV-1), Epstein-Barr virus (EBV) • Therapy-related predisposition e.g. radiotherapy and chemotherapy
Congenital and inherited syndromes
Inherited predisposition factors • Fanconi anaemia • myelodysplasia • Neurofibromatosis DNA repair defects • Bloom syndrome • Li-Fraumeni syndrome • Ataxia-telangiectasia Chromosomal associated syndrome • Down syndrome • Klinefelter syndrome
Other factors
• Age • Gender • Race • Family history • Preventable cases e.g. lifestyle Extensive review by Buffler *et al*., 2005.

As mentioned earlier, in adults there was a higher incidence of leukaemia of myeloid origin, myeloid cells are derivatives of precursor cells critical in innate immunity rather than adaptive immunity (Abbas *et al*., 2005). Cells involved in innate immunity are known to produce large number of enzymes (e.g., myeloperoxidase) that can produce cytotoxic mediators as part of their normal function. The cytotoxic mediator produced can activate environmental chemicals (e.g., benzene), reactive oxygen and nitrogen species through chronic inflammation that can reach the bone marrow and produce genotoxic intermediates (van der Vliet *et al*., 1997; Eiserich *et al*., 1998). Benzene is a known culprit in leukaemia and lymphoma development as well as causes haemopoietic

defects (Hayes *et al*., 2000; Lan *et al*., 2004). Myeloperoxidase in the bone marrow activates benzene metabolites to nucleophilic compounds causing DNA damage (Wiemels *et al*., 1999; Eastmond *et al*., 2005). Bioactivated benzene metabolite, hydroquinone (BAHQ) has also been shown to inhibits topoisomerase-II (Topo II) *in vitro* and *in vivo* during the DNA binding stage and at the closed clamp stage in the catalytic cycle thereby interfering with either binding of Topo II to the DNA or the release of synthesized DNA. This could result in DNA breakage, chromosomal aberration and leukaemic-associated chromosomal translocation in bone marrow (Eastmond *et al*., 2001; Mondrala and Eastmond, 2010).

A recent study using a genome wide association study and meta-analysis found a 20-fold increased risk of acute lymphoblastic leukaemia (ALL) in children with Down syndrome (DS).It also demonstrated distinct somatic features, including *CRLF2* rearrangement in approximately 50% of cases with susceptibility loci for single nucleotide polymorphisms in *IKZF1*, *CDKN2A*, *ARID5B* and *GATA3* which was independent of DS-ALL subtype (Brown *et al*., 2019). Knockdown of *IKZF1* resulted in increased cell proliferation in lymphoblastoid cell lines (Brown *et al*., 2019). Lifestyle factors such as smoking and increased BMI associated with obesity have been identified as risk factors for other cancers and leukaemia (Poynter *et al*., 2016). Owing to the conflicting results published, a meta-analysis of a pool of existing data found an association between being overweight and obesity with increased incidence of AML (Li *et al*., 2017). However, they only found an association between acute promyelocytic leukaemia (APL) with short overall survival (OS) and high risk of differentiation syndrome but not with AML (Li *et al*., 2017). Among the several mechanisms which have been suggested through which obesity can increase susceptibility and promote cancer is the activation of free radicals through mitochondrial and tissue stress which are capable of inducing DNA damage by release of reactive intermediates (Inoue and Kawanishi, 1995; Collado *et al*., 2012). Through the production of leptin and adiponectin which are adipokines, obesity promotes cancer cell proliferation and survival via activation of PI3K, MAPK, and STAT3 (Jaffe and Schwartz, 2008; Gao *et al*., 2009).

1.2.4 *Chronic Myeloid Leukaemia (CML)*

Chronic Myeloid Leukaemia (CML) is a myeloproliferative disorder of transformed haematopoietic stem cells and progenitors such as common myeloid progenitor and granulocyte-macrophage progenitor cells (Jamieson *et al*., 2004). CML is characterized by a balanced and reciprocal translocation of chromosome t(9;22)(q34;q11) with resultant fusion of the breakpoint cluster region protein (BCR) and Abelson Murine Leukaemia viral oncogene homolog 1 (ABL1) (Figure 1.5) (Li *et al*., 1999). The resultant *BCR-ABL* fusion gene is commonly referred to as the Philadelphia (Ph) chromosome (Tough *et al*., 1961; Nowell, 2007) and is found in over 90% of CML patients. The initial phase of CML is characterised by expansion of premature myeloid precursors and mature cells which have the capacity to differentiate normally.

Studies (Daley *et al*., 1990; Li *et al*., 1999) suggests that three main types of BCR-ABL protein are formed depending on the breakpoint (Figure 1.5). A breakpoint that occurs in introns 1 or 2 of ABL and in cluster region (M-*bcr*) of BCL between exon 13 and 14 (b2) or exon 14 and 15 (b3) produce BCR-ABL fusion gene that is transcribed into b2a2 or b3a2 mRNA (where b = *BCL* gene and a = *ABL* gene). The resultant fusion protein, $p210^{BCR-ABL}$ is 210kDa, and sufficient for the malignant transformation of CML and responsible for the phenotypic characteristics such as elevated WBC, splenomegaly and anaemia observed in the chronic phase of CML. Atypical causes are those resulting from BCR-ABL transcripts involving ABL exon a3 instead of a2, e1a2 encoding a protein about 190kDa, $p190^{BCR-ABL}$ and *BCR* exon 19/ABL exon a2 (e19/a2) encoding a larger protein of about 230kDa, $p230^{BCR-ABL}$. A study (Sawyers *et al*., 2002) suggested that although these fusion proteins differ in their BCR component, they express the same level of c-ABL tyrosine kinase activity.

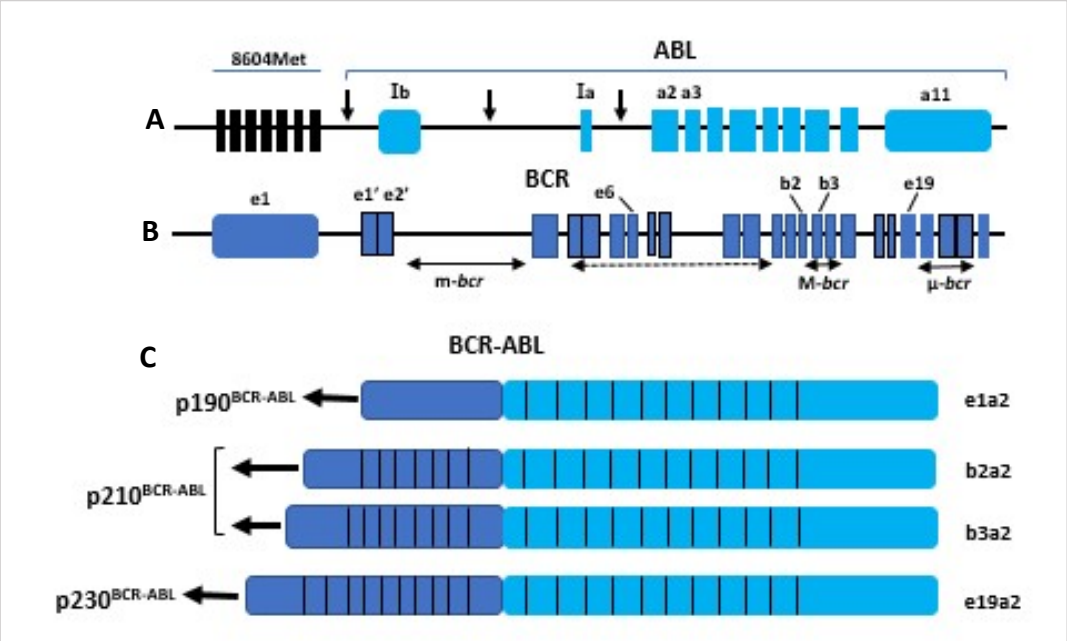


Figure 1.5. Schematic representation of BCR and ABL gene, translocation and resultant protein. Boxes represent exons while connecting horizontal lines represents introns. **A**. Breakpoints in the *ABL* gene are indicated by the arrows and occur at intron 1 or 2 of the *ABL* gene. **B**. *BCR* alternative first (e1') and second (e2') exon. Breakpoints in BCR occur within the cluster region (m-*bcr*, M-*bcr* and µ-*bcr*) shown by the double-headed horizontal arrows. **C.** Represents the structure of the various BCR-ABL mRNA transcript which are formed and in accordance with the position of the BCR breakpoint. Breaks in m-*bcr* gives rise to mRNA molecule with e1a2 junction. Breaks in M-*bcr* generate fusion transcripts with a b2a2 or a b3a2 junction, respectively. Breakpoints in µ-bcr, result in BCR-ABL transcripts with an e19a2 junction. (where: I = intron; e=exon; b=*BCL* gene and a=*ABL* gene) (Adapted from Li *et al*., 1999).

Functional domains (Figure 1.6) in the BCR-ABL protein that could be responsible for the cellular transformation in leukaemia have been identified (Pendergast *et al*., 1991). In the ABL portion, the Src-homology domain 1 and 2 (SH1 and SH2) and the actin-binding domain have been named. While in the BCR portion, the coiled-coil oligomerization domain (*aa*-1-63), the Grb-2 binding site (Tyr-177) and the phosphoserine/threonine-rich SH2 binding domain have been identified to aid cellular transformation (Pendergast *et al*., 1991; McWhirter *et al*., 1993; Fredericks and Ren, (2013).

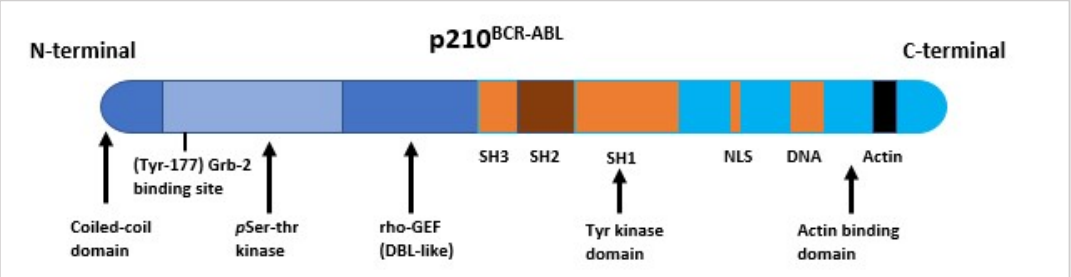


Figure 1.6. Schematic illustration showing the functional domains of BCR-ABL protein using the p210$^{BCR-ABL}$ example. Shown on the BCR portion is the coiled-coil domain, Grb-2 binding site, the phosphoserine/threonine-rich SH2-binding domain, the rho-GEF domain. The ABL portion consist of the regulatory src-homology regions SH3 and SH2, the SH1 tyrosine kinase domain, the nuclear localization signal (NLS) and the DNA and actin binding domain (Adapted: Salesse and Verfaillie, 2002).

The resultant BCR-ABL fusion protein exhibits a constitutively active tyrosine kinase activity because of autophosphorylation of the activation loop via SH2 binding (Hantschel *et al*., 2014). The tyrosine kinase activity of the chimeric protein is found exclusively in the cytoplasm of the cell complexed with several cytoskeletal proteins (Figure 1.7) (Puil *et al*., 1994; Raitano *et al*., 1995). The constitutively active tyrosine kinase activity and the interaction with cytoskeletal proteins underlie the mechanism of induction and pathophysiology of the leukaemic phenotype. The cytoskeletal proteins and their role in cell signalling have been listed in Table 1.4.

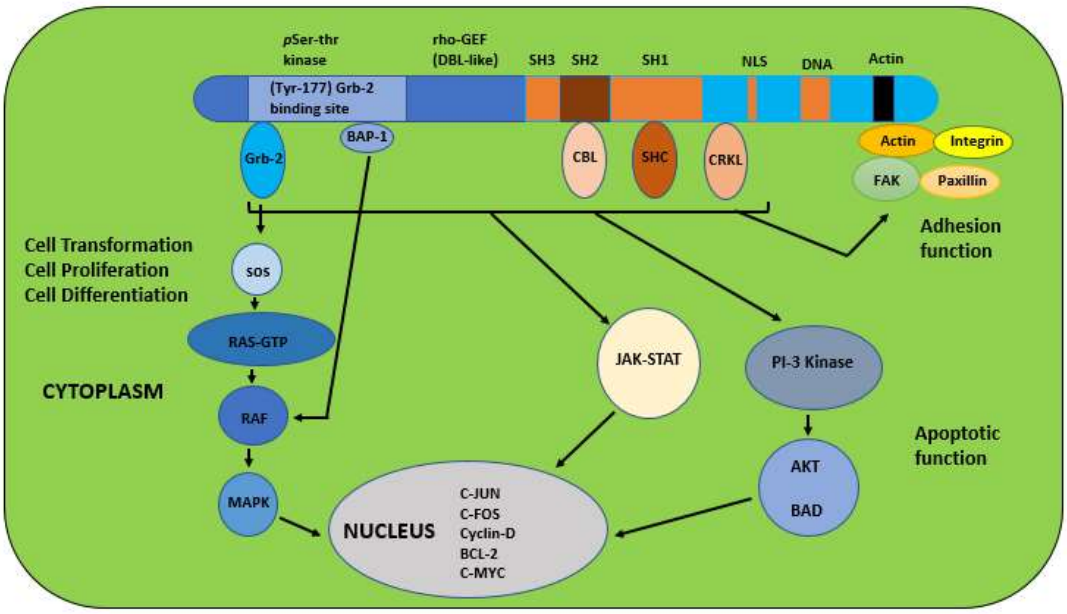


Figure 1.7. Basic representation of the signalling pathway of the BCR-ABL proteins. Activation of the RAS-JAK/STAT-PI-3 pathways and the FAK complex (actin, paxillin and integrin) which are multi-protein structures that link the extracellular matrix (ECM) to the cytoplasmic cytoskeleton result in increased cell proliferation, differentiation and decreased apoptosis in CML progenitor cells. Binding of these proteins to adaptor protein such as GRB2, CBL, SHC, and CRKL result in their activation. BAP-1 denotes BCR-associated protein 1; GRB2: growth factor receptor-bound protein 2; CBL: casitas B-lineage lymphoma protein; SHC: SRC homology 2-containing protein; CRKL: CRK-oncogene-like protein; JAK-STAT: Janus kinase-signal transducers and activators of transcription; FAK: focal adhesion kinase, SOS: son-of-sevenless and GEF: GDP-GTP exchange factor (Adapted: Salesse and Verfaillie, 2002).

In CML, the abnormal cells function adequately, allowing a mild initiation of the disease; a benign chronic phase (CP). This then progresses to an accelerated phase (AP), which is more difficult to control and characterised by an enlarged spleen and increased number of blast cells. The final stage of CML is the acute or blast crisis (BC) phase characterised by blast cells domination of the bone marrow and blood. At the blast phase, CML presents as ALL in 25% of patients, while the remaining 75% present as AML (Hamerschlak, 2008).

Table 1.4. The Role of signalling pathways and transcriptional factors dysregulated in response to BCR-ABL activation

Pathway	Role	Effectors
Wnt/β-catenin	-HSCs self-renewal -Interaction with BM niche -CML progression	Wnt β-catenin GS3K
Notch	-Interaction between leukemic, HSCs, and BM niche -CML advanced stages	ϒsecretase
PI3K/AKT/mTOR	Normal haemopoiesis	PI3K AKT mTOR1/2
JAK/STAT	-Normal haemopoiesis -A key player in a variety of myeloproliferative disorders	JAK 1/2
FoxO/TGF-β	-Expression of genes involved in cell growth, proliferation and differentiation -Involvement in BCR-ABL activated PI3K/AKT pathway	TGF-β
PML	-Critical role in haemopoiesis -Dysregulated in CML -LSC maintenance	PML

Abbreviations: CML, chronic myeloid leukaemia; HSC, hematopoietic stem cells; BM, bone marrow. PI3K, phosphoinositide 3-kinase; PML, promyelocytic leukaemia; PP2A, protein phosphatase 2A; TGF-β, transforming growth factor-β. (Jilani *et al*., 2008; Naka *et al*., 2010)

Diagnosis of CML is through a basic blood test which looks for elevated peripheral WBC count that is dominated by granulocytes. A confirmation is achieved by examination of the bone marrow, which shows a large proportion of matured white cells when compared to the blast cells. Bone marrow aspiration is also useful for staging between phases. Chromosomal abnormalities can be identified in bone marrow samples using fluorescence *in-situ* hybridization (FISH) and reverse transcriptase polymerase chain reaction (RT-PCR) technique (Schoch *et al*., 2002; Jabbour and Kantarjian, 2018). The FISH analysis relies on co-localization of large genomic probes specific for BCR and ABL genes. The error margin for a false positive test is between 1-5% depending on the probe used (Jabbour and Kantarjian, 2018). PCR can be either qualitative or quantitative. A qualitative assessment provides information about the presence of BCR-ABL transcript whereas, quantitative PCR assesses the amount of transcript present. Qualitative PCR is preferred in the diagnosis of CML whereas quantitative PCR is best used to monitor the amount of residual disease (Jabbour and Kantarjian, 2016; 2018). Both FISH and PCR

techniques can also be used to assess response to treatment and differentiate CML from other myeloproliferative diseases with similar presentation (Schoch *et al*., 2002).

A cost-effective chemiluminescence clinical diagnosis method has been recommended by Xu and colleagues (Xu *et al*., 2016). WHO has outlined criteria for CML diagnosis listed in Table 1.5 (Arber *et al*., 2016).

Table 1.5. WHO haematological/cytogenetic criteria for the diagnosis of CML Report: Arber *et al*., 2016.

Chronic phase (CP) CML • Cytogenetic evidence of Ph^+ chromosome in peripheral blood or bone marrow • Examination of haematological cells from bone marrow (BM) aspiration with less than 10% blast cells
Acute phase (AP) CML (diagnosed if any one or more of the following exist) • Persistent or increasing WBC (>10 x 10^9/L), unresponsive to chemo or radiation therapy • Persistent or increasing splenomegaly, unresponsive to chemo or radiation therapy • Persistent thrombocytosis (>1000 x 10^9/L), unresponsive to chemo or radiation therapy • Persistent thrombocytopenia (<100 x 10^9/L) unrelated to chemo or radiation therapy • 20% or more basophils in the peripheral blood • 10%-19% blasts in the peripheral blood and/or bone marrow • Any new clonal chromosomal abnormality in Ph^+ cells that occurs during therapy   ***Tyrosine kinase inhibitor (TKI) response criteria*** • Hematologic resistance to the first TKI (or failure to achieve a complete hematologic response to the first TKI) • Any haematological, cytogenetic, or molecular indications of resistance to two sequential TKIs • The occurrence of two or more mutations in *BCR-ABL1* during TKI therapy
BC CML (diagnosed if any one or more of the following exist) • ≥20% of peripheral blood or bone marrow cells are blasts • Onset lymphoblast in the peripheral blood or bone marrow cells

Earlier treatment and or management of CML was initially based on cytoreductive therapy such as hydroxyurea. This method of therapy only provides temporary disease control but does not limit disease progression with a median survival of about 45months from diagnosis. Later immunology-based therapy was introduced which, includes the use of interferon alpha (IFN-α) (Hehlmann *et al*.,1994). This proved to be superior to cytoreductive therapy with an increase in the median survival rate of 60months from diagnosis and patients going into complete cytogenic remission (Allan *et al*., 1995; Bonifazi *et al*., 2001).

The use of allogeneic haematopoietic stem cell transplant (HSCT); a process in which stem cells from a matching donor is infused into a recipient to re-establish haematopoietic function, offered effective and lasting curative potential for patients with CML. However, this method was less applicable than alternative therapies due to the challenge in finding a suitable donor, the morbidity of the procedure, tissue rejection known as graft versus host disease (GvHD) and the age of the recipient (McGlave *et al*.,2000; Curtler *et al*., 2001). Further challenges in the use of HSCT as a lasting solution is the theory of "bystander effect". A theory in which the naïve haematopoietic stem cells transplanted are reprogrammed within the new host microenvironment and becomes cancerous (Shen *et al*., 2012). This has been blamed on the total body irradiation (TBI) required for conditioning regimen (Shen *et al*., 2012).

The use of tyrosine kinase inhibitors (TKI), which function by blocking adenosine triphosphate (ATP) binding sites in the BCR-ABL kinase proved efficacious in patients who were nonresponsive to IFN-α (Kantarjian *et al*., 2002a). The first TKI, imatinib became the front-line drug for the management of CML (O'Brian *et al*., 2003; Baccarani *et al*.,2009). Imatinib, when administered at a standard daily dose of 400mg/day in a patient, is relatively tolerated but not without side effects which include nausea, oedema, and diarrhoea (Druker *et al*., 2001). Following its success with an estimated progression-free survival of 90% in 5years (Kantarjian *et al*., 2002b), the newer generation of imatinib analogue; nilotinib, dasatinib, and bosutinib has been developed and approved by the FDA as front-line treatment of patients with newly diagnosed CML in chronic phase (CP) (Talpaz *et al*., 2006; Kantarjian *et al*., 2007; Cortes *et al*., 2011). This newer generation of drugs was developed to solve the problem of resistance with imatinib due to mutations in the BCR-ABL ATP binding site (Talpaz *et al*., 2006; Kantarjian

et al., 2007; Cortes *et al*., 2011). Patients who develop the T315I "gatekeeper" mutation display resistance to all currently available TKIs except ponatinib (O'Hare *et al*., 2009). Individuals with the advanced disease do not respond to imatinib and hence, are offered induction chemotherapies such as etoposide, cytarabine and carboplatin with the hope of restoring patients to the chronic phase. Kuroda *et al*., (2013) argues that molecular remission (which is a complete remission with no evidence of the disease in the blood and or bone marrow using sensitive monitoring test such as PCR) is rarely achieved even when haematological and cytogenetic remission is achieved. This is due to the protective effect conferred on these cells by the extensive network of *BCR-ABL1* gene with a normal haematopoietic and signalling pathway (Kuroda *et al*., 2013). Allogeneic stem cell HSCT remains an important therapeutic option for patients with CML-CP who have failed at least two TKIs, and for all patients in CML advanced phases (Jabbour *et al*., 2011).

Studies have shown that combining kinase inhibitor with cytoreductive drugs could improve the efficacy of TKI in overcoming the challenge of mutation in the BCR-ABL1 oncogene (Fava *et al*., 2015). Combination of imatinib and interferon has been suggested to improve the cytogenic response (Palandri *et al*., 2008; Fava *et al*., 2015). A phase III randomized study combining imatinib and peginterferon alfa-2a obtained a higher rate of major molecular response (MMR) (i.e., a BCR-ABL RNA level ≤0.1%) (Preudhomme *et al.*, 2010). Another study found that co-treatment with imatinib and amiloride re-sensitized BCR-ABL1 T3151 mutant cells to imatinib treatment by modulating alternative splicing (Cheng *et al*., 2011). These studies suggest that combined kinase therapy in CML treatment may offer a better cytogenic response.

1.2.5 *Acute myeloid leukaemia (AML)*

Acute myeloid leukaemia (AML) is a heterogeneous malignancy that is characterized by exaggerated growth or clonal expansion of blast cells (myeloid progenitors) in the bone marrow and peripheral blood (Narmala *et al*., 2010). This results in haematopoietic insufficiency with or without leucocytosis (Narmala *et al*., 2010). It was earlier reported that 40-50% of patients with AML do not have clonal chromosomal aberration (Schlenk *et al*., 2008). In recent years, with the advances in technology, several acquired genetic mutation and deregulated gene expression profiles have been identified. The cytogenetic risk for AML has been classed as favourable prognosis, intermediate prognosis and poor prognosis and have been summarized in Table 1.6. Two classes of

somatic mutations have been observed in AML. Class I includes mutation that activates the signal transduction pathway; mainly the FMS-related tyrosine kinase 3 gene (*FLT3*) and RAS family of viral oncogenes (Schlenk *et al*., 2008). While class II involves mutations that affects transcriptional factors and components of the transcriptional co-activation complex e.g. mutations in CCAAT/enhancer binding protein α gene (*CEBPA*), myeloid–lymphoid or mixed-lineage leukaemia gene (*MLL*) and nucleophosmin gene (*NPM1*) (Schlenk *et al*., 2008). There has also been a report on an early acquisition of somatic mutation in genes encoding epigenetics modifiers such as *DNMT3A, ASXL1, TET2, IDH1* and *IDH2* (Ley *et al*., 2010; Rocquain *et al*., 2010).

Table 1.6 Prognostic cytogenetics risk for acute myeloid leukaemia

Prognosis	Cytogenetics
Favourable	Balanced structural rearrangements; t(15;17) (q22;q 12-21) t(8; 21) (q22; q22) inversion (16)(p13q22)/t (16;16)(p13 ;q22)
Intermediate	Normal karyotype Balanced structural rearrangements t(9 ;11)(p22 ;q23) Unbalanced structural rearrangements del (7q), del (9q) , del (11q) , del (20q) Numerical aberrations -Y, +8, +11, +13, +21
Poor	Balanced structural rearrangements; Inversion (3)(q21q26)/t (3 ;3)(q21 ;26) t(6 ;9)(p23 ;q34) t(6 ;11)(q23 ;p13,1) Unbalanced structural rearrangements del (5q) Numerical aberrations -5, -7

(Adapted: Hamerschlak, 2008)

Another sub-classification of AML using M0-M7 has been described. M0 and M1, immature myeloblastic; M2, mature myeloblastic; M3, promyelocytic; M4, myelomonocytic; M5, monocytic; M6, erythroleukaemia; and M7, megakaryocytic based on the different cell types observed in the blood and bone marrow (Bennett *et al*., 1985; Basharat *et al*., 2019).

Following the WHO update, the European leukaemic network has published a more comprehensive cytogenetic/mutational prognostic stratification and is described by Döhner *et al*., (2017) in Table 1.7.

Table 1.7. Update summary of AML genetics/mutational prognostic stratification

Prognosis	**Genetic abnormality**
Favourable	t(8;21)(q22;q22.1);RUNX1-RUNX1T1 Inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated NMP1 without *FLT3*-ITD or with *FLT3*-ITD^{low} (allelic ratio <0.5); Biallelic mutated *CEBPA*
Intermediate	Mutated NPM1 and FLT3-ITD^{high} (allelic ratio ≥0.5) Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* Cytogenetic abnormalities not classified as favourable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM* (*EVI1*) -5 or del(5q); -7; -17/abn(17p) Complex karyotype (three or more unrelated chromosome abnormalities), Monosomal karyotype (defined by the presence of 1 single monosomy) Wild-type *NPM1* and *FLT3*-ITD^{high} Mutated *RUNX1* Mutated *ASXL1* Mutated *TP53*

(Adapted: Döhner *et al*., 2017)

Development of AML has been associated with exposure to benzene, ionizing radiation, chemotherapy and inherited syndrome such as Fanconi anaemia and Downs syndrome (Tsai *et al*., 2014). The statistical record for AML incidence in the UK is approximately 3.9 and 5.2/100,000 in females and males accounting for 43% and 57% respectively (CRUK) with a diagnostic median age of 75years (Cartwright *et al*., 2002; Bhayat *et al.,* 2009). AML is more common in older people than children and adolescent (Bhayat *et al.,* 2009). The five years relative survival rate in England is 16% and 14% respectively for women and men compared to 18% and 15% reported for available data for Europe (CRUK). Techniques such FISH, PCR, flow cytometry and array technology, which can detect gain and loss of genetic materials can be used for the diagnosis of AML (Döhner *et al*., 2010).

1.2.6 *AML therapy and management*

Due to the wide range of cytogenetic and mutational events that occur in AML, treatment is based on presentation. Intensive chemotherapy (IC) is often employed in 30-60% of the elderly with adverse prognosis but results in poor performance and treatment-related mortality of about 10-25% (Burnett *et al*., 2007). Overall survival (OS) when IC is feasible is 13months. Low-dose cytosine arabinoside (LDAC), the farnesyltransferase inhibitor tipifarnib, and gemtuzumab ozogamicin have shown improved outcome with patients going into remission but without improvement in OS (Burnett *et al*., 2007; Burnett *et al*., 2013).

Older patients with AML and high-risk myelodysplastic syndrome (MDS) who have never received treatment showed increased therapy response with clofarabine. However, OS, relapse and treatment co-morbidity were unchanged when compared to patients who had LDAC treatment (Burnett *et al*., 2013). Sapacitabine alone, a nucleoside analogue, has also been used in a randomized study in older patients with no improvement in OS when compared to LDAC alone. Both Sapacitabine and LDAC were found to result in a similar outcome except for sapacitabine whose side effect was well tolerated (Burnett *et al*., 2015). Barasertib showed an OS of 8.2 months against 4.5 months with LDAC but with more toxic effect. The population for this study was notably small at 74 (Kantarjian *et al*., 2013).

Volasertib, a selective inhibitor of polo-like kinase (Plks) which is responsible for mitotic checkpoint regulation and cell division in a phase 2 trial, was found unsuitable for intensive induction therapy when compared to LDAC (Dohner *et al*., 2014). Although a significant increase in event-free and OS rate were achieved when combined with LDAC, volasertib led to an intolerable increase in the frequency of adverse effect (Dohner *et al*., 2014).

Azacytidine, a DNA methylation inhibitor administered at 75mg/m^2/d in a phase-III trial significantly prolonged OS in patients with intermediate and high-risk myelodysplastic syndrome. Patients for this study were classified using the French American British (FAB) classification with one-third meeting the WHO definition of AML at ≥20% BM blasts (Fenaux *et al*., 2010; Dombret *et al*., 2015). In phase I/II trial, azacytidine was combined with midostaurin; a broad-spectrum tyrosine kinase inhibitor of both wild type and mutated FLT-3 type. The result showed better response and longer median remission duration in patients who have not been previously exposed to FLT3 inhibitor and previously transplanted (Strati *et al*., 2014). On Table 1.8 is listed the current regiment used in AML management and the drug performance according to (Almeida and Romas, 2016).

Table 1.8. Summary of therapeutic efficacy of drugs used for AML in older patients

Regimen	Complete remission (%)	Median overall survival (OS) (months)
Intensive chemotherapy	50-60	6–12
LDAC	10-25	6
Azacytidine in low-blast count AML	25	24.5
Azacytidine in AML >30%	25	12.1
Decitabine in AML >30% bone marrow blast	18	7.7
Clofarabine	38	11.4
Sapacitabine	37	7.9
Barasertib	35	8.2
Volasertib (+ LDAC)	31	8
Tipifarnib	8	3.6
Midostaurin (+Aza)	2	NA
Quizartinib	54	<12
Vorinostat (+LDAC)	46	NA
Gemtuzumab Ozogamicin	30	11

(Almeida and Romas, 2016)

1.3 Constitutive and alternative Splicing

RNA splicing is a post-transcriptional modification required for the maturation of RNA. There are two types of splicing events; constitutive splicing (Figure 1.7A) in which all introns are removed and exons are joined together (Ding and Elowitz, 2019) while alternative splicing (Figure 1.8B-F) is a regulatory mechanism of post-transcriptional mRNA processing that selectively removes or retains introns, which allows for multiple expression of several mRNAs from a single gene. The result is the generation of two or more proteins from a single gene (Liu *et al*., 2017; Bush *et al*., 2017). Other processes through which multiple mRNA transcript can be generated apart from alternative splicing are alternative polyadenylation and alternative promoter usage (Pecci *et al*., 2001; Hilgers, 2015). The close coupling of constitutive splicing with transcription (Fong and Zhou, 2001) is thought to serve as a signal-processing filter that regulates the amount of mature mRNA depending on the rate of transcription (Ding and Elowitz, 2019). There are indications that constitutive splicing provides several possible functions including intron-enhanced transcriptional efficacy (Brinster *et al*., 1988), increased retention of intronic miRNAs and other non-coding RNAs in constitutively spliced introns (Rodriguez *et al*., 2004; Rearick *et al*., 2011) and could reflect or promote the evolutionary selection of new phenotypes (Lev-Maor *et al*., 2007). Thus, constitutive splicing could be playing roles beyond isoform diversification.

An earlier study suggests that the size of an intron determines whether an exon is constitutively or alternatively spliced. The study found that when intron size is between 200 and 250 nucleotides, the splice sites across the introns are not recognized resulting in exonic splice site recognition with inclusion of weak exonic splice site (Fox-Walsh *et al*., 2005). Other factors such as the exon and intron architecture have been shown to influence the choice of splice site such that large exons result in exon skipping but could be included when flanked by small introns (Fox-Walsh *et al*., 2005; Roy *et al*., 2008). This suggests that constitutive splice-site recognition is more efficient in small exons and introns.

It is estimated that 90-95% of human genes are alternatively spliced (Wang *et al*., 2008). It is also apparent that alternative splicing plays a role in physiological functions and developmental processes and in recent times has been found to aid the progression of

several diseases such as cancer, neurological conditions like Alzheimers, vascular defects of the eyes and metabolic disorders (Climente-González *et al.,* 2017; Han *et al*., 2018; Batson *et al*., 2017; Wong *et al*., 2018). A proteomic mapping performed on human tissue identified several new protein-coding regions in genes due to alternative splicing and selection of an alternative splice site thus indicating that alternative splicing also contributes to proteomic complexity (Kim *et al*., 2014). Alternative splicing can modify the properties of the encoded protein which affects the stability, binding pattern, cellular localization and activity (Bush *et al*., 2017). With a better understanding of the regulatory mechanism of alternative splicing, studies are now geared toward understanding the functional consequence of splicing events.

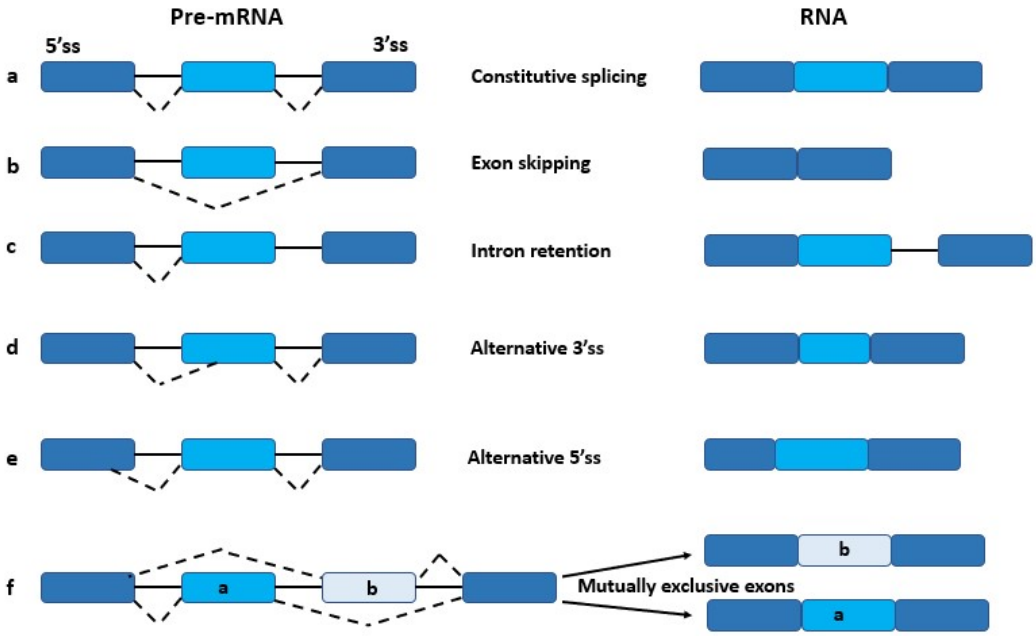


Figure 1.8. **Representation of constitutive and alternative splicing**. Alternative splicing of pre-mRNAs occurs when an exon is skipped, intron retained, an alternative 3'- (acceptor) or 5'- (donor) sites are used and from selection of mutually exclusive exons. Exons (rectangles) are connected by introns (black lines). Broken lines depict regions which are spliced (adapted: Li *et al*., 2007).

1.3.1 Precursor messenger RNA (Pre-mRNA) splicing by spliceosome

Pre-mRNA splicing is a process whereby the intron sequence is identified and excised from pre-mRNA with the ligation of adjoining exons (Figure 1.7). Binding of the splicing machinery to the splice site results in the assembly of the spliceosome. Splicing is catalysed by the spliceosome, which is a large ribonucleoprotein complex formed from an assembly of five small nuclear ribonucleoproteins (snRNP) (Makarov *et al.*, 2002). Each snRNP is composed of a single uridine-rich small nuclear RNA (snRNA U1, U2, U4, U5, and U6) (shown in Figure 1.9) and multiple proteins such as the U1 specific proteins, SR-proteins and U2AF protein required for RNA-protein interaction during the splicing process (Makarov *et al.*, 2002; Saulière *et al.*, 2006).

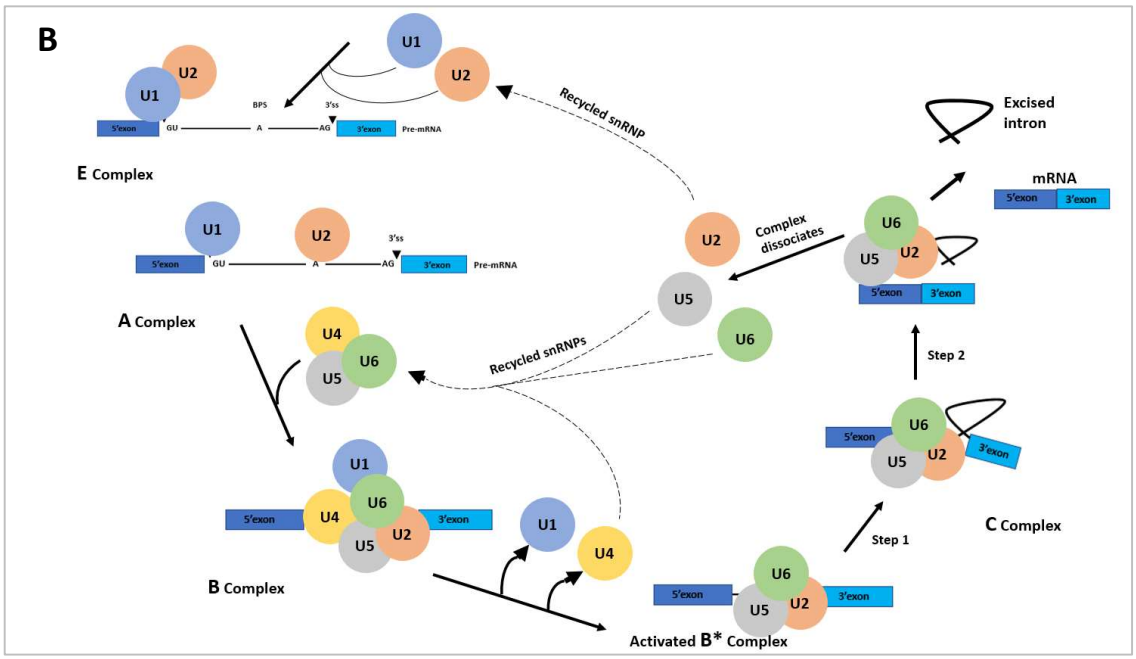


Figure 1.9. Illustration of the mechanism of spliceosome assembly. Representative pre-mRNA showing binding site. GU sequence is binding site for U1snRNP, AG sequence is binding site for U2AF splice factors and the branchpoint sequence (A) which is binding site for U2 snRNP. Inactive Complex B formed from combination of the U1, U2 snRNP with the pre-assembled tri-snRNP. Activated Complex B is formed after dissociation of U1 and U4. Formation of a lariat intron and excision of the intron result in complex C and matured mRNA. Dissociated snRNP are recycled into the system (Adapted: Wahl *et al.*, 2009).

During the initiation of splicing, there are characteristics of the RNA which determines spliceosome assembly and includes;

- Recognition of the 5’ and 3’ splice site (5’ss and 3’ss) that determine the exon-intron and intron-exon boundaries.
- The polypyrimidine tract binding protein (PTB/hnRNP I) which recognizes short motif such as UCUU and UCUCU associated with the polypyrimidine tract upstream of the 3’ss of alternative exons
- The branchpoint sequence (BPS) which provides activity site for the 2’hydroxyl group during the first transesterification reaction.

The mechanism of spliceosome assembly (Figure 1.9) begins with the U1 snRNP recognising the 5’ splice site. The U1 snRNP is recruited together with SR proteins by RNA polymerase II and ensure the coupling of splicing to transcription and they are co-transcriptionally deposited on the 5’ splice sites of nascent transcripts (Staknis, and Reed, 1994; Das *et al*., 2007). The co-transcriptional deposition of SR proteins on nascent pre-mRNA transcripts contributes to genome stability and prevents the formation of R-loops due to hybridization of the neosynthesized RNA to the complementary strand of the DNA template (Li and Manley, 2005). The U2 snRNP recognises functional 3’ splice site by base pairing with the branch-point sequence (Shao *et al*., 2014). This pairing between U2 and BPS requires the assistance of auxiliary splice factors like U2AF due to the degenerate nature of the branch point sequence (Zhang *et al*., 1992). The U2AF heterodimer consist of two subunit U2AF65 and U2AF35 which binds the polypyrimidine tract downstream of the BPS and the AG dinucleotide, respectively (Singh *et al*.,1995; Valcárcel *et al*., 1996). This result in the stability of the branch point. SR proteins such as SF1 interact with U1 snRNP and the 35kDa subunit of the heterodimeric factor, U2AF, bridging the gap between U1 and U2AF snRNP (Wu and Maniatis, 1993). This pre-spliceosome assembly is the earliest splicing-specific complex to form and is known as complex E. Conversion of complex E to complex A involves an ATP-dependent association of U2 snRNP at the branchpoint sequence of the intron (Shao *et al*., 2014). The tri-snRNPs U4/U6/U5 which forms the A complex associates with U1 and U2 snRNPs and pre-mRNA forming the pre-catalytic B complexes. Following this, U5 snRNP interacts with the 5’ and 3’ splice site, leading to the destabilization and dissociation of the U1 and U4 snRNPs association and activating the B complex. The U5

snRNP binds to the 5' splice site and U6 associates with U2 snRNPs resulting in the U2/U5/U6 complex. With the activation of the B complex and looping of the spliced intron, complex C is formed, and splicing is processed. Intron is spliced out and a matured mRNA is formed. U2/U5/U6 complex then dissociates and is recycled.

These signals responsible for recruitment of the spliceosome are thought to be recognized multiple times during the spliceosome assembly to coincide with the internal rearrangements resulting in the assembly of spliceosome complexes (Lim and Hartel, 2004; Schneider *et al*., 2010). A critical step in the mRNA splicing is the recruitment of pre-assembled tri-snSNP U4/U6/U5 to Complex A which interacts with 5' and 3' SS to form the pre-catalytic Complex B. Formation of activated Complex B result from structural and conformational changes of the pre-catalytic Complex B This step is associated with the release of U1 and U4 and the hydrolysis of ATP and GTP catalysed by Brr2 and Snu114 proteins, respectively (Häcker *et al.,* 2008). The splicing reaction is completed by Complex C whose formation is catalysed by activated Complex B (Wickramasinghe *et al*., 2015). The kinetics of splicing involves two esterification reaction. The first which results in the formation of a lariat structure after the 2'OH group of the branch adenosine of the intron carries out a nucleophilic attack on the 5'ss. This results in cleavage at this site and ligation of the 5' end of the intron to the branch adenosine. Next, the 3'OH group of the 5' exon attacks the 3'ss, leading to the ligation of the 5' and 3' exons and formation of the mRNA with a corresponding release of the intron (Will and Lührmann, 2007).

1.3.2 *Regulation of constitutive and alternative splicing by regulatory elements (SRE) and splice factors*

Spliceosome assembly is highly dynamic, and tightly controlled in its rearrangement, (Makarov *et al*., 2002) as splicing errors involving a single nucleotide either by addition or removal will affect the open reading frame (ORF) of an mRNA resulting in the use of alternative splice site (Krawczak *et al*., 1992; 2007). To overcome such error, snRNAs target specific phosphate bonds for cleavage once the spliceosome is assembled. The spliceosome, however, needs to be able to recognize the correct splice site. This is aided by regulatory *cis* elements known as exonic and intronic splicing enhancers (ESEs and ISEs) and exonic and intronic splicing silencers (ESSs and ISSs) (Will and Lührmann, 2007).

Exons contain short and classical splice site sequences, whereas, introns contain numerous pseudo splice sites which have sequence similar to a true splice site (Sun and Chasin, 2000; Lim and Burge, 2001). Since splicing needs to be precise and definite, the *cis*-regulatory elements (ESEs and ISEs; ESSs and ISSs), which either promote or inhibit the inclusion of an exon or intron or the use of adjacent splice site, regulate this process by recruiting trans-acting splice factors that activate or suppress recognition of a splice site or spliceosome assembly (Chasin, 2007).

It has been shown that ESEs function by recruiting serine/arginine (SR) protein family members such as SRSF1 and SRSF2 (previously known as SC35) which bind to the RNA recognition motif (RRM) domains on the N-terminal of the ESEs and mediates protein-protein interaction that facilitates spliceosome assembly (Graveley *et al*., 1998). The heterogenous nuclear RNA proteins (hnRNP) are class of protein associated with heterogenous nuclear RNA (hnRNA or pre-mRNA). These were found to be associated with the splicing machinery where they act as splice repressors. The splice repressors of the hnRNP class often bind the ESSs. hnRNP such as PTB (hnRNP I) can act by blocking interaction between U1 and U2 snRNPs causing skipping of the exon (Sharma *et al*. 2005) while hnRNP A1 either displace U1 snRNP or binds on either side of the exon forming a loop (Nasim *et al*. 2002;). Both ESEs and ESSs have been identified based on their enrichment and depletion from the authentic exon sites so that exons which are constitutively spliced have abundant ESEs bound to it. Similarly, there are fewer ESSs bound to authentic exons (Fairbrother *et al*., 2002). The abundance of ESEs in constitutively spliced exons suggested that while enhancers play a dominant role in constitutive splicing, silencers play more prominent role in alternative splicing (Fairbrother *et al*., 2002; Wang *et al*., 2004).

ISEs such as the G-triplet (GGG) have been well characterized and are found in clusters. They enhance the recognition of adjacent 5’ and 3’ splice site (McCullough and Berget 2000). Intronic C-A repeats are known to enhance splicing of upstream exons through binding of hnRNP L (Hung *et al*. 2007). Some neuron-specific ISEs such as YCAY (Y=C or U) and UGCAUG have been identified which are responsible for brain-specific splicing event and recognized by the brain and muscle-specific factor Fox-1 and Fox-2 (Wang *et al*., 2012).

It is known that the SR-proteins, a family of splice factors with a characteristic serine-arginine (SR)-rich domain, play a critical role in the assembly of the spliceosome and interact with regulatory elements and cofactors during splicing. SRSF1 is known to regulate the use of 5'ss which does not involve the binding of SRSF1 to ESE via hyper-phosphorylation by CDC-like kinase 1 (CLK1) and serine-rich protein kinase-1 (SRPK1) (Bourgeois *et al*., 2004). The study suggests that SRSF1 acts by increasing the recruitment of U1 snRNP to the 5'ss by interaction with the U1-70k subunit with the alternate use of proximal 5'ss (Bourgeois *et al*., 2004). An earlier study has also reported a novel tri-snRNP-specific 27K SR-protein, which is thought to be phosphorylated by the snRNP associated kinase, is responsible for the recruitment of the tri-snRNP (U4/U6/U5) during spliceosome assembly (Fetzer *et al*., 1997). SRPK2 has been associated with the tri-snRNP via stabilization of PRP28 (DDX28), which is required for the formation of the B-complex whereas, SRPK1 is associated with the U1 snRNP in spliceosome assembly (Mathew *et al*., 2005). Another protein kinase, the pre-mRNA processing factor 4 kinase (PRP4K or PRPF4B), through phosphorylation of PRP6 and PRP31, has been shown to interact with pre-mRNA splicing factor PRP6, copurify with U5 snRNP and regulate the U4/U6/U5 tri-snRNP assembly during B complex formation (Dellaire *et al*., 2002; Schneider *et al*., 2010).

1.3.3 *Aberrant alternative splicing in cancer*

Considering the increased likelihood of the choice of alternative splicing event and the resemblance between a true and pseudo splice site, it is not unlikely that the splicing machinery can be hijacked and manipulated resulting in diseases such as cancers. The consequence of which, results in aberrant splicing through the choice of an alternative pseudo-splice site. Alternative splicing can occur in untranslated 3' and 5' regions (UTR) of mRNA resulting in altered mRNA stability and translation efficiency (Hughes 2006). Aberrant splicing can also result in the introduction of premature stop codon which can render mRNA transcript inactive, alter protein function or result in an encoded protein with an antagonistic function (Solier *et al*., 2005).

Changes in alternative splicing have been observed to affect several aspects of tumorigenesis to include cell cycle control of tumour growth, migration and proliferation, cell apoptosis, tumour metabolism and angiogenesis (Inoue and Fry, 2015;

Mavrou *et al*., 2014; Christofk *et al*., 2008). In recent times, studies have continued to show that alternatively spliced gene can be used for diagnostic and prognosis purposes, as well as therapeutic targets in malignancies (Amin *et al*., 2011; Mavrou *et al*., 2014). For example, inhibition of SRPK1 using a small molecule inhibitor, SPHINX, result in alternative splicing of VEGF to its anti-angiogenic isoform, thus, inhibiting growth of blood vessel in models of choroidal angiogenesis *in vivo* (Batson *et al*., 2017)

Breast cancer has been associated with aberrant splicing of the *TP53*, BRCA1 and *PTEN* genes (Okumura, *et al*., 2011), and *CDC25* encoding phosphatase (Albert, *et al*., 2011). In ovarian cancer, the growth factor receptor-bound protein-7 (GRB7) has been identified to be alternatively spliced and its spliced variant GRB7v function as an adaptor for extracellular signalling ligand responsible for cell proliferation (Wang, *et al*., 2010). In colon cancer, tissue inhibitor of metalloproteinase-1 (TIMP-1) and cell adhesion molecule CD44 are alternatively spliced and increase the metastatic potential of colon cancer cells (Usher *et al*., 2007; Gotley *et al*., 1996). A wide variety of genes (*BCL2L1, CD44, VEGFA, CCND1*) with spliced variants were found in lung cancer that confer increased proliferation and resistant to apoptosis to tumour cells (Cheung *et al*., 1998; Nguyen *et al*., 2000; Shabnam *et al*., 2004; Li *et al*., 2008). Other splicing events, which have also been observed, include CLK1 (Simon, *et al*., 2018), VEGFA (Mavrou and Oltean, 2016), BCL2L2 (Mercatante *et al*., 2001 and ERG (Hagen *et al*., 2014) splicing in prostate cancer, Caplin-3 in melanoma and Kruppel-like factor-6 (KLF6) in liver cancer (Hanoun *et al*., 2010).

It is evidenced that isoform switches resulting from translation of alternative spliced gene or use of alternative reading frame affect protein-protein interactions in cancers due to loss or gain of the functional domain. Loss of function has been observed in *NFE2L2* (Goldstein *et al*., 2016) when alternatively spliced and consequently, activates an alternative mechanism for oncogenesis when interacting with a negative regulator such as *KEAP1* (Goldstein *et al*., 2016; Climente-González *et al*., 2017).

1.3.4 *Aberrant alternative splicing in leukaemia*

Aberrant splicing has also been reported in leukaemic cancers and has been shown to drive leukaemogenesis. For example, DNA methyltransferase 3A (DNMT3A) and its transcripts (DNMT3A2; DNMT3A4) are known to be key players in haematopoietic cell

differentiation and proliferation (Božić, *et al*., 2018). An alternative spliced variant of transcript DNMT3A2; DNMT3A2V when overexpressed has been shown to delay cell proliferation whereas a mutated isoform DNMT3A2V R882H promotes cell proliferation in haematopoietic cell. A switch from DNMT3A1 to DNMT3A2V is implicated in the pathophysiology of AML (Lin, *et al*., 2017). Further RNA-seq analysis of AML has shown variations in isoform levels of key genes (such as *MYB*, *BRD4* and *MED24*) known to be involved in leukaemogenesis following inhibition of SRPK1 (Tzelepis *et al*., 2018).

In Philadelphia positive (Ph^+) pre-B-lymphoblastic leukaemia and ALL cells, aberrant splicing induced by BCR-ABL1 gene has been found in IKAROS; a transcription factor belonging to the zinc-finger family, with the resultant isoform (IK6) exerting a negative dominant effect on early lymphoid commitment of B-cells (Klein *et al*., 2006). Increased expression of IK6 isoform correlates with the high percentage of blast cells in ALL and TKI resistance (Klein *et al*., 2006; Iacobucci *et al*., 2008).
An earlier study (Liu *et al*., 2012) has found differential expression of the gene (*CENPE, SLC4A1*, *WT1* and *E2F7*) responsible for the cell cycle in K562, a CML cell line. However, a reversed gene expression level was observed with genes which were previous upregulated (*E2F7*, *WT1*, *CCNE2* and *CHEK2*) becoming downregulated due to imatinib-induced alternative splicing of the T-box transcription factor (TBX3) (Liu *et al*., 2015). A similar experiment has found a change in splicing in Bclx protein with the anti-apoptotic variant, Bcl-xL showing increased expression after treatment with imatinib in K562 cell line (Liu *et al*., 2012).

A key gene *c-Myb* involved in the regulation of proliferation and differentiation in several cells including haematopoietic cells (Emambokus *et al*., 2003) has been found to be alternatively spliced with its spliced variant highly expressed in leukaemia and implicated in leukaemic cell transformation (Zhou *et al*., 2011). Expression of *c-Myb* variant correlated with poor survival in a cohort of B-ALL samples (Zhou *et al*., 2011). The alternative spliced *Myb* variants are formed using alternate exon 8A,9A.9B.10A,13A and 14A. The levels of these spliced variant transcripts were shown to be regulated independent of one another during haematopoietic cell differentiation (O'Rourke and Ness, 2008).

1.4 Serine/arginine-rich (SR) proteins

An essential class of splicing regulators is the serine/arginine (SR) family of proteins. SR proteins are a highly conserved family of splicing factors and regulators which contain an arginine/serine (RS)-rich domain. The SR protein have a characteristically organized structure which contain one or two N-terminal RNA-recognition motifs (RRM), C-terminal rich in arginine and serine (RS) dipeptides repeats (Krainer *et al*., 1990; Fu and Maniatis, 1992). Using the mAb104 monoclonal antibody, specific for phosphoepitopes, members of the SR protein family were identified and classed as a family (Roth *et al*., 1991). Members of the 'classical' SR protein family has been designated splice factor, serine/arginine (SRSF) 1-9 and 11. These differs either in the presence or absence of the RRMH, the zinc knuckle and the number of arginine/serine repeats (Figure 1.10) (Boucher *et al*., 2001).

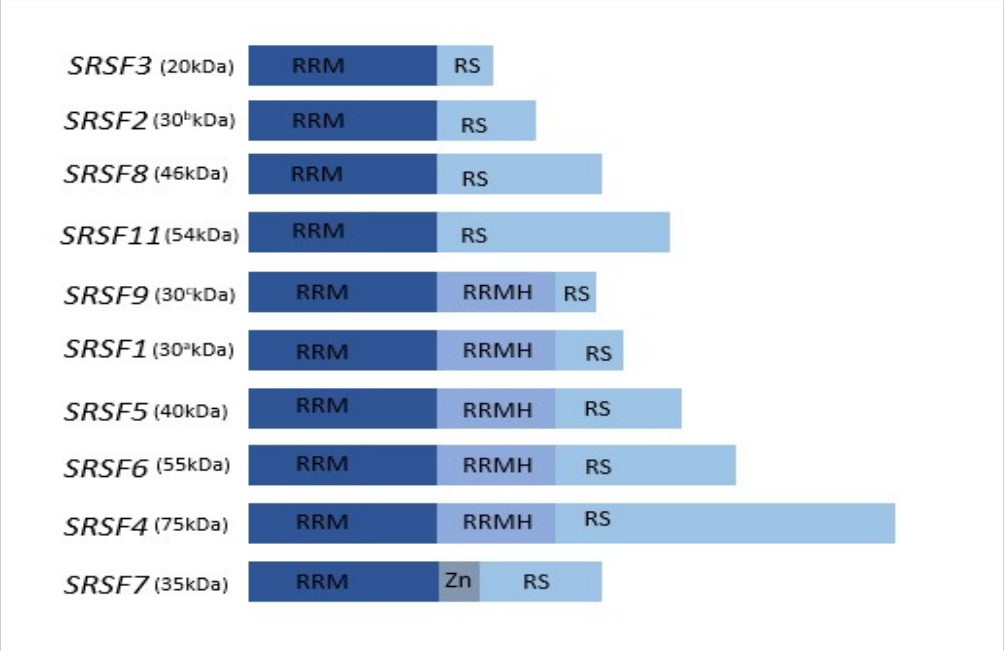


Figure 1.10. Schematic showing structural organization of the common human SR proteins. RRM, RNA recognition motif; RRMH, RRM homology; RS, arginine/serine-rich domain; Zn, Zinc knuckle (Shepard and Hertel, 2009).

Localization of SR proteins are in 'speckles' which are subnuclear structures found in the interchromatin region of the nucleoplasm of mammalian cells from where the SR proteins are mobilized during active transcription (Cazalla *et al*., 2002).

SR proteins have been shown to determine and regulate splice-site selection during constitutive and alternative splicing (Chandler *et al*., 1997), a function performed by the RRM while the RS domain mediate protein-protein interaction in several steps during spliceosome assembly (Kohtz *et al*., 1994; Tronchère *et al*., 1997). They have also been implicated in crucial aspects of mRNA metabolism including export, localization, translation, and nonsense-mediated decay (NMD) (Krainer *et al*. 1990; Cáceres *et al*., 1998; Sanford *et al*.,2004). Another study reports that SR proteins binds noncoding RNAs and exhibit positional RNA binding during regulated alternative splicing events (Bradley *et al*., 2014). The choice of alternative promoter and polyadenylation site selection are also affected by levels of SR proteins (Bradley *et al*., 2014). SR proteins act as activators during alternative splicing by binding to exonic splicing enhancers (ESEs) in pre-mRNA and recruiting the splicing machinery to the splice site through RS-domain-protein interaction (Bradley *et al*., 2014).

Other essential function of the SR protein in cell viability and embryogenesis has been demonstrated in cultured cell and xenograft study using mice with germline deletion of *Srsf1, Srsf2* and *Srsf3* resulting in cell death and early embryonic death in mice respectively (Jumaa *et al.,* 1999; Wang *et al*., 1996, 2001; Xu *et al*., 2005). In contrast *Srsf10* null mice were observed to have severe cardiac defect to include septa defect and myocardial thinning (Feng *et al*., 2009). These findings underline the key role played by SR proteins in early development and active gene transcription.

The role of SR proteins in splice site selection and spliceosome assembly is regulated by their phosphorylation. In vitro phosphorylation of RS domain affects both protein-protein and protein-RNA interactions and prevents nonspecific binding during early spliceosome assembly (Xiao and Manley, 1997; Tacke *et al.,* 1997). Dephosphorylation on the other hand, results in the resolution of the splice machinery and release of processed RNA (Cao *et al*., 1997). Thus, suggesting that phosphorylation-dephosphorylation cycle of the RS domain of SR proteins play a role during the splicing cycle. Further supporting the effect of phosphorylation on the function of SR protein is evidenced by the intracellular and intranuclear trafficking mediated by RS domain

phosphorylation (Yeakley *et al*., 1999) and suggests the regulation of SR proteins by specific kinases.

Protein kinases which have been well characterized and specific for SR protein are the SRPK and CLK families of kinases. Kinases belonging to these two families are structurally distinct, are differentially expressed and exhibit different substrate specificities (Gui *et al*., 1994; Colwill *et al*., 1996).

1.5 CDC-like kinase-1 (CLK1)

1.5.1 Structure and function of CLK1

CDC-like kinase-1 (CLK1) is a member of an evolutionarily conserved group of kinases. There are four CLKs in humans, termed CLK1-4. It is widely expressed and can phosphorylate protein substrates at serine, threonine and tyrosine residue (Moeslein *et al*., 1999). CLK1 was first identified by Ben-David et *al*., (1991) where it was closely associated with protein kinases involved in the regulation of the cell cycle. The human *CKL1* gene consists of 13 exons. Its full-length protein consists of 484 amino acids (aa) with the first 130aa constituting a regulatory region required for CLK1 interaction with SR proteins. The remaining amino acids forms its catalytic domain (Uzor *et al*., 2018).

Within the motif HTDLKPEN, CLK has a threonine where most other kinases have arginine (Ben-David et *al.,* 1991). The CLK consist of 19 amino acid (19-aa) inserts in between the HTDLKPEN and highly conserved *DFG* motif relative to other protein kinases (Ben-David et *al*.,1991; Hanks *et al*., 1998). The CLKs can phosphorylate both Arg-Ser and Ser-Pro dipeptides common in all serine-rich proteins ((Aubol *et al*., 2013).

Localization of CLK1 is mainly in the nucleus where its function is to phosphorylate SR proteins and facilitate the release of SR proteins from nuclear speckles during splicing and regulation (Aubol *et al*., 2013). High level of CLK1 is thought to inhibit the recognition of splice sites by SR proteins and regulate SRPK1 nuclear presence (Aubol *et al*., 2016). Phosphorylation and activation of the protein-tyrosine phosphatase 1B (PTP-1B) is achieved with the action of CLK1 resulting in fold increase of phosphatase activities in HEK-29 cells *in vivo* (Moeslein *et al*., 1999)

It is suggested that CLK1 is required for cell cycle progression as inhibition or depletion of CLK1 results in cell death or G1/S phase arrest due to a defect in mitosis (Dominguez *et al*., 2016).

1.5.2 *Regulation of CLK1 expression and activity*

Studies on the regulation of CLK1 expression appear to be sparse. An earlier study has suggested that CLK1 is regulated through its pre-mRNA splicing yielding a catalytically active CLK1 and a truncated inactive polypeptide ($CLK1^{T}$) (Duncan *et al*., 1997). This was further demonstrated in a recent study, which showed that CLK1 auto-regulates itself through exon-4 skipping and intron-4 retention in the presence of environmental or biological stress by altering the balance between its full length and truncated form Uzor *et al*., 2018).

Aubol *et al*., (2016; 2018) has shown that mobilization of SRSF1 from the nuclear speckle by SRPK1 is enhanced by CLK1 and this, in turn, result in fold increase in the level of CLK1 in the nuclei as it forms a complex with SRPK1. In a similar study, it was observed that human CLK1 activity is dependent on CLK1 concentration (a concentration fold change which was associated with its quaternary structure). It was also observed that the N-terminus of CLK1 is necessary for speckle location of CLK1 and induces oligomerization, which determines CLK1 substrate specificity (Keshwani *et al*., 2015)

1.5.3 *Molecular interactions of CLK1*

The association of CLK1 with SRPK1 in the mobilization of SR proteins, during pre-mRNA splicing is well described (Figure 1.11). The release of SR protein from nuclear speckles, following phosphorylation, requires the CLK1-SRPK1 hetero-kinase complex (Aubol *et al*., 2016). It is also reported that this process results in the nuclear regulation of SRPK1 by CLK1 (Aubol *et al*., 2014; 2016).

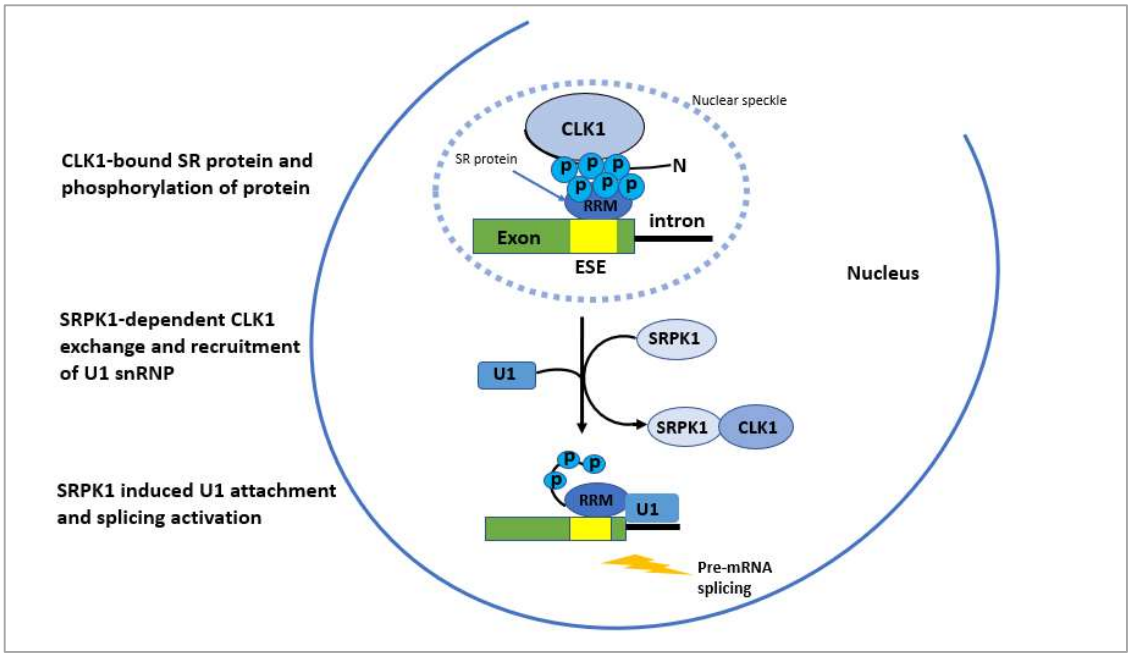


Figure 1.11: Schematic diagram showing CLK1-SRPK1 interaction during SR protein phosphorylation for the initiation of pre-mRNA splicing. CLK1 bounds to the RNA recognition motif (RRM) of SR protein and phosphorylates SR protein. With the nuclear import of SRPK1, SRPK1-CLK1 complex is formed which recruits the U1 snRNP to the splice site. With the attachment of U1 snRNP to the splice site, complete phosphorylation and release of SR protein from nuclear speckle, splicing is initiated (Adapted: Aubol *et al.,* 2016).

Like the association between CLK1 and SRPK1, another protein kinase Prp4 has been shown to interact with CLK1 carboxylic terminus resulting in phosphorylation of Prp4 arginine/serine-rich domain by CLK1 (Kojima *et al*., 2001). The protein kinase PTP-1B which is required for regulation of intracellular protein phosphorylation is itself enzymatically activated by CLK1 phosphorylation (Moeslein *et al*., 1999). Myelin basic proteins and Histone H1 have also been identified as CLK1 substrates.

There are also indications that CLK1 may interact with some apoptotic genes such as *Caspase, MCL-1, BCL-X and Survivin* as inhibition of CLK1, using the benzothiazole TG003, one of the first established inhibitors of CLKs (Muraki *et al*., 2004) was found to alter levels of pro and anti-apoptotic isoforms of these genes (Uzor *et al*., 2018).

1.5.4 *The role of CLK1 in cancer*

With the critical function of CLK1 synergizing with SRPK1 in the release of SR protein and consequent regulation of SRPK1 (Aubol *et al*., 2016) during alternative mRNA splicing, and its role in cell cycle regulation and progression (Dominguez *et al*., 2016), it is not

surprising that CLK1 could play a role in carcinogenesis. Varying levels of CLK1 have been found in different cancers with the overexpression indicative of poor disease prognosis and survival (Dominguez *et al*., 2016). Silencing of CLKs in MCF7 breast cancer cells and MCF10 female xenograft mice using shRNA plasmid or lentivirus has been shown to inhibit breast tumour growth indicated by expression of smooth muscle epithelium (Yoshida *et al*., 2015) as well as a change in the splicing pattern of epithelial-mesenchymal transition (EMT) genes such as *ENAH* gene (Yoshida *et al*., 2015). CLK1/4 has also been shown to regulate alternative splicing of tumour suppressor gene *TP53* in breast cancer where it influences the choice of alternatively spliced p53β and p53Y isoforms (Marcel *et al*., 2014; Czubaty and Piekiełko-Witkowska, 2017).

In lung cancer, CLK1/4 prevents the stimulation of hypoxia-induced angiogenesis through reduction of an alternatively spliced isoform of tissue factor (asTF), which is known to stimulate the expression of proangiogenic genes (Eisenreich *et al*., 2013). A recent study also showed that CLK1 is upregulated in PC3 prostate cancer cell lines and inhibition of CLK1 in prostate cancer significantly resulted in decreased expression of anti-apoptotic caspase 9b isoforms (Bowler *et al*., 2018).

CLK1 has also been implicated in therapy-related resistance due to its direct regulation of the SPF45 human splice factor (Liu *et al*., 2013). Knockdown of CLK1 using siRNA was found to degrade SPF45 with consequent reduction in SPF45-induced exon 6 exclusion from Fas mRNA (Liu *et al*., 2013) implicating CLK1 as a druggable target.

There are insufficient data on the role of CLK1 in leukaemia and blood-related malignancy. One study found that upregulation of CLK1 and other members of the CLK family have been observed in hexamethylene-bis-acetamide (HMBA)–induced erythroleukaemia cell differentiation and, thus it is implicated in the erythroid cell transformation in leukemogenesis (García-Sacristán *et al*., 2005).

Owing to the fact that the effect of CLK1 in carcinogenesis is related to its role in alternative splicing of TNF-stimulating genes (TSGs) and oncogenes and with the established role alternative splicing play in tumorigenesis and cancer progression, it is therefore, arguable that the control of alternative splicing in cancers through manipulation of splice factors and their kinases such as SRPK1 and CLK1, could be key in

the treatment and management of most cancer types where aberrant splicing is key to progression and metastasis.

1.5.5 CLK1 as a therapeutic target

As the search for targeted therapeutics with high efficacy and minimal side effect continues, the use of small molecule inhibitors against protein molecules and their substrates has continued to increase. This has shown prospect as some of these molecules have moved on to clinical trials (extensive review by Gatzka, 2018). Targeting protein kinases involved in alternative splicing is gaining grounds and an emerging therapy due to the key role they play in gene regulation. As such, several molecules are currently being developed targeting the CLK1 and the CLK family of kinases such as CLK2 and CLK4 (Araki *et al*., 2015; Iwai *et al*., 2018).

Mounting evidence continues to show that targeting the CLKs significantly affect tumour behaviour and significantly change splicing. Araki *et al*., (2015) showed that CLK inhibition leads to modulation in ribosomal protein kinase, *S6K* splicing, splicing alterations in several genes and protein depletion for multiple genes including those involved in tumour growth and survival pathways such as endothelial growth factor receptor (EGFR), eukaryotic translation initiation factor 3 (EIF3D) and poly (ADP-ribose) polymerase (PARP). A similar finding was also published by ELHady *et al*., (2017) in which CLK1 and CLK4 led to the depletion of EGFR, histone deacetylases (HDAC1) and S6K1 kinase in cancer cells.

A study investigated 169 cell lines, of which, 19 were haematological cancers and 150 were solid tumours. The results showed that a reduction in CLK-dependent phosphorylation, led to alternate skipping of exons in CLK-regulated genes, increased apoptosis and growth suppression both *in vitro* and *in vivo,* of which, *MYC*-driven cancers were found to be the most sensitive (Iwai *et al*., 2018).

Compound screening has identified CC-671 as a potential and selective inhibitor of CLK1/2 in triple-negative breast cancer. Mechanism of action of this compound involves the inhibition of SRSF4 through reduced phosphorylation which resulted in the selection of an alternate splice site leading to increased cell apoptosis (Zhu *et al*., 2018).

1.6 Splice factor kinase-1 (SRPK1)

1.6.1 *Structure and function of SRPK1 kinase*

The serine/arginine-rich protein-specific kinase-1 (SRPK1) belongs to a family of protein kinases which phosphorylates RS-domain of proteins rich in serine/arginine repeats. Members of this kinase family (SRPK1 and SRPK2) are known to be conserved in eukaryotes (Yun and Fu, 2000). The human *SRPK1* gene is located on chromosome 6p21.2-p21.3 (Figure 1.12A) (Wu *et al*., 2013). Like most protein kinases, SRPK1 is characterized by two conserved kinase domains separated by a spacer sequence; an accessory domain (Figure1.12B) (Ding *et al*. 2006; Zhong *et al*., 2009).

The accessory domain is known to regulate SRPK1 cytoplasmic-nuclear shuttling as deletion of the accessory domain results in exclusive nuclear localization (Wang *et al*., 1998; Zhong *et al*., 2009). This observation suggests the role of *SRPK1* in spliceosome assembly and in the mediation of trafficking of splice factors and subsequent phosphorylation of SR proteins during constitutive and alternative splicing in mammalian cells (Zhou *et al*., 2012). A further study has also shown that the accessory domain in addition to stabilizing the catalytic loop is essential for protein substrate phosphorylation by increasing the exchange rate in the glycine-rich and activation loop which drives phosphoryl transfer from ATP (Plocinik *et al*., 2011).

The N-terminus and C-terminus of this kinase are not known to be conserved and are thought to play an auxiliary role (Ngo *et al*., 2005). Deletion of the N- and C-terminal does not inactivate the kinase catalytic activity (Ngo *et al*., 2007). However, another study demonstrated that the N-terminus stabilizes the docking groove of the kinase domain which enhances high-affinity binding and efficient phosphorylation of its substrate, the SR proteins (Ngo *et al*., 2005). Consequently, deletion of the N-terminus reduces SR-protein recognition and binding affinity (Plocinik *et al*., 2011).

X-ray structures of SRPK1 reveal an insert for mitogen-activated protein kinase (MAPK) which connects helices αG and αH, a loop connecting helices αF and αG and a docking groove generated by the MAPK insert which contains eight amino-acid residues (191-198) (Ngo *et al*., 2007). Contrary to other proteins, which require regulation by diverse mechanism, *SRPK1* and its family members are constitutively active and require no post-translational modification or additional subunits for optimal activity (Ngo *et al*., 2007).

This is because SRPK1 has a relatively short activation loop that lacks a reversible phosphorylation site, allowing for a stable conformation and access of substrates to the constitutively active site (Ngo *et al*., 2007).

The presence of a short activation loop in addition to the malleability of the activation loop, and the ability of the kinase catalytic loop to extract hydroxyl hydrogen (OH^-) from the substrate serine; a critical step for phosphorylation, has been shown to be responsible for the maintenance of a constitutively active conformation for SRPK1 (Ngo *et al*., 2007).

Other family members of the SRPK family have been identified that are expressed in the nervous system (*SRPK2*) and muscle cells (*SRPK3*) respectively. In addition, a spliced isoform of SRPK1 known as SRPK1a, which result from the inclusion of 513bp segment is exclusively expressed in the testis (Sanidas *et al*., 2010; Nikolakaki *et al*., 2001). This implies that the members of this kinase family have their distinct cellular localization and functions in different cell types (Nakagawa *et al*., 2005; Zhou and Fu, 2013).

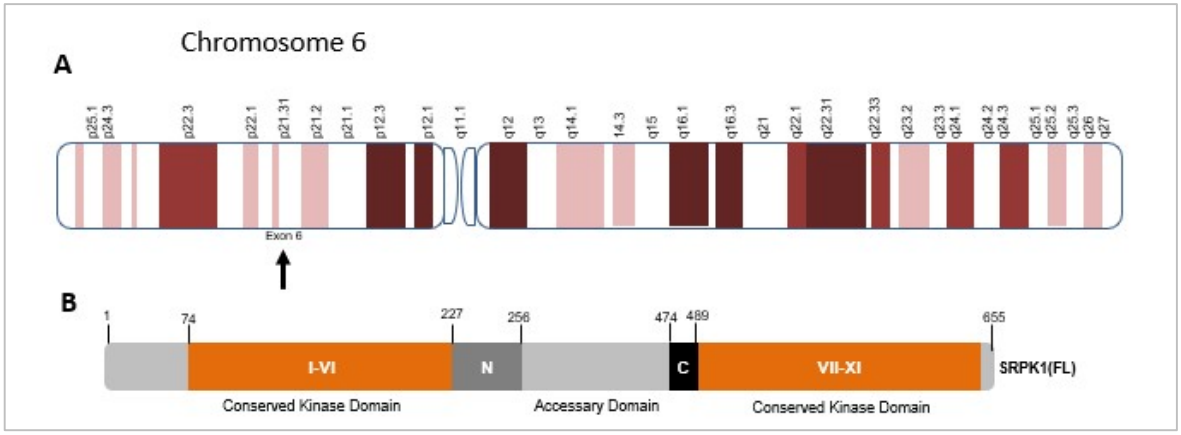


Figure 1.12 Gene location and protein structure of SRPK1. Structure of chromosome 6 showing the location of the *SRPK1* gene on p21.2-p21.3 (**A**). Structure of full length SRPK1 protein (**B**). The accessary domain (AD) located between aa 256-474 is flanked at both end by N-terminal (aa 227-255) and C-terminal (aa 474-489) and divides the kinase domain into two (Ngo *et al*., 2007).

1.6.2 *Regulation of SRPK1 activity*

The accessory domain (AD) of SRPK1 has been shown to affect the localization of SRPK1 Zhong *et al*., 2009. An earlier study has shown that deletion of the accessory domain results in SRPK1 being sequestered in the nucleus (Wang *et al*., 1998; Zhong *et al*., 2009). It is possible that the AD function as a cytoplasmic anchor for SRPK1. The accessory domain of SRPK1, in part, regulates its function by providing a binding surface for chaperone complex assembly. Plocinik *et al*., (2011), in an X-ray diffraction study, discovered that although the AD is large with about 200 amino acid, it lacks a stable structure and proposed that the bulk of the AD which has been previously described constitute a large intrinsically disordered region in SRPK1. It suggests that this region could provide a large unstructured surface for chaperones which are known to bind to unfolded proteins (Plocinik *et al*., 2011).

Chaperones, which have been identified are the heat shock family and co-chaperones Hsp40/Hsp70 and Hsp90/Aha1(Zhong *et al*., 2009) (Figure 1.13). While Hsp40 targets region outside the accessory domain, Hsp90 targets the accessory domain (Zhong *et al*., 2009). Chaperone complex assembly is initiated by the binding of Hsp40/Hsp70 to SRPK1 followed by the binding of Hsp90 and Aha1, a co-chaperone (Figure 1.13). Formation of SRPK1/chaperone/co-chaperone complex also ensures the folding of SRPK1 into its active conformation and protects it from proteasome degradation. This model has been validated in an experiment which showed that inhibition of the chaperone Hsp90 ATPase activity resulted in dissociation of the chaperone complex and SRPK1 nuclear localization (Pratt and Toft, 2003; Zhong *et al*., 2009).

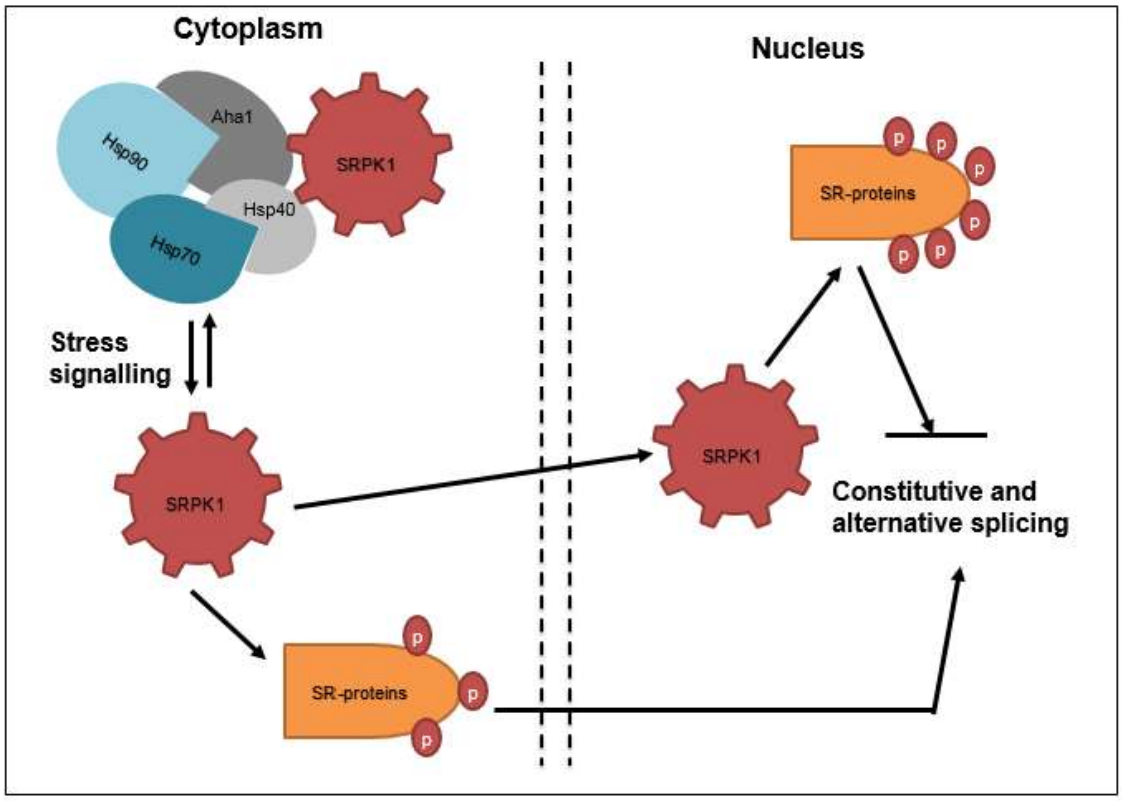


Figure 1.13. Model for SRPK1 regulation showing the binding of chaperone and co-chaperone proteins to SRPK1. Signalling such as those required for transcription result in dissociation of SRPK1 from the chaperone molecules and it's binding to SR-protein with nuclear translocation which results in transcription and constitutive and alternative splicing (Zhong *et al*., 2009).

1.6.3 *Molecular interactions of SRPK1*

Like other protein kinases, SRPK1 interacts with several molecules, which either serve as a substrate or control its association with substrates. Such interactions result in the regulation of their activity either directly or indirectly. Phosphorylation of SR proteins by SRPK1 results in nuclear import of SR-proteins and their localization into nuclear speckles.

In relation to SRPK1 phosphorylation of SR proteins, SRPK1 also interacts with the splice factor protein kinase CLK1. SRPK1 forms a complex by interacting with the RS-domain in the N- terminus of CLK1 in a mechanism to release the phosphorylated SR protein (Aubol *et al*., 2016 and 2018).

SRSF1 is a member of the SR protein family which has been extensively studied and strongly phosphorylated by SRPK1. SRSF1 has two RRM and an RS domain of about 50 amino acids. It is known that SRPK1 interacts with about 10-12 amino acids of the RS

domain (Plocinik *et al*., 2011); an interaction which produces high-affinity binding which is considered critical for the regulation of the extent to which SRSF1 is phosphorylated (Ngo *et al*., 2007; Plocinik *et al*., 2011). Phosphorylation of SRSF1 by SRPK1 has been shown to be in C- to N-terminal direction irrespective of the presence (SRPK1-FL) or absence of N-terminal (SRPK1ΔN) and spacer domain (SRPK1ΔS) (Plocinik *et al*., 2011).

Cellular interactions of SRPK1 with SRSF1 affects several pathways including those involved in apoptosis and cell proliferation (Anczuków *et al*., 2012) as well upstream signalling pathways of MYC and PI3K such as S6K1 and BIN1 (Karni *et al*.,2007). Studies (Amin *et al*., 2011; Oltean *et al*., 2012) have also shown that SRPK1 and its key substrate, SRSF1 is part of an essential pathway responsible for the regulation of the alternative splicing of vascular endothelial growth factor (VEGF) into pro-/anti-angiogenic isoform in renal epithelial cells (podocytes) and in colon carcinoma cells. These studies further demonstrate that a knockdown of SRPK1 increased levels of VEGF anti-angiogenic isoform and prevents tumour growth in xenograft by reducing micro-vessel density. A similar finding has been observed in a study using prostate cancer cell line PC3 (Mavrou *et al*., 2014). Furthermore, interaction of SRPK1 with the Wilms' tumour-1 (WT1) has resulted to hyper-phosphorylation of SRSF1 and hence splice shift in VEGF to proangiogenic isoform (Amin *et al*., 2011).

Studies (Wang *et al*., 2014; Chang *et al*., 2015) suggests that SRPK1 is a downstream target of activated protein kinase-B (Akt/PKB). The studies showed that SRPK1 interacts and modulates the Akt pathway through recruitment of pH domain leucine-rich repeat protein phosphatase (PHLPP), an Akt phosphatase. SRPK1 was shown to induce Akt activation by interfering with PHLPP mediated dephosphorylating of Akt, which suggest that SRPK1 could be a regulator of Akt. A positive feedback mechanism between SRPK1 and Akt was also suggested where elevated SRPK1 induced activation of Akt and the activated Akt then binds and stimulates SRPK1 auto-phosphorylation (Chang *et al*., 2015).

Also, SRPK1 has been shown to regulate epidermal growth factor (EGF) activation; a proto-oncogene which is upregulated in tumour cells and capable of activating several oncogenic pathways in cancers (Zhou *et al*., 2012). This study suggests that EGF can be activated by SRPK1 via the phosphatidylinositol-3-kinase/protein kinase-B (PI3/Akt) pathway other than the well-known downstream mammalian target of rapamycin

(mTOR) pathway to induce large splicing response in the nucleus (Zhou *et al*., 2012). Supporting this finding is a mass spectrometry analysis of SRPK1 phosphopeptides showing multiple phosphorylation sites, which could be induced by EGF or Akt (Zhou *et al*., 2012). There are also indications that down-regulation of SRPK1 or CLK1 blocks EGF stimulation of nuclear SRSF1 mobilization (Aubol *et al*., 2018).

Since SRPK1 has been shown to display both cytoplasmic and nuclear localization (Aubol *et al*., 2016), it is speculated that SRPK1 may have other substrates which drive its cellular function even though it is known to be highly specialised to target SR proteins and related splicing factors (Aubol *et al*., 2013).

Other named possible substrate of SRPK1 are viral proteins. Nuclear import of herpes simplex virus-1 protein, ICP27 due to interaction with SRPK1, was found to reduce splicing activity with subsequent intron-less viral mRNA formed (Sciabica *et al*., 2003).

In hepatitis B virus (HBV) infected cells, SRPK1 phosphorylates the viral protein which results in the encapsulation of the viral genetic material (Zheng *et al*., 2005). This was further evidenced by the failure in encapsulation of Sindbis virus following inhibition of SRPK1 by a small molecule, SRPIN340 (Fukuhara *et al*., 2006). The E1^E4 protein of human papillomavirus 1(HPV-1) has also been named as a substrate for SRPK1, where it inhibits SRPK1 phosphorylation of viral SR protein thus, regulating posttranscriptional processing of viral transcript by SR protein (Prescott *et al*.,2014).

In addition, phosphoprotein such as human P1 protamine has been identified as substrates for SRPK1. SRPK1 phosphorylation of human P1 protamine results in its exchange with histone H1 and H3 during the process of spermiogenesis (Papoutsopoulou *et al*., 1999).

1.6.4 *The role of SRPK1 in cancer*

Interestingly, upregulation of SRPK1 has been observed in prostate cancer (Mavrou *et al*., 2014), glioma (Wu *et al*., 2013), colon cancer (Amin *et al*., 2011), breast cancer (van Roosmalen *et al*., 2015) and hepatocellular carcinoma (Zhou *et al*., 2013). It is also apparent that the role and expression of SRPK1 in different cancers are heterogeneous and not yet entirely clear (Zhou *et al*., 2013). While SRPK1 upregulation has been observed to promote tumour growth, progression, metastatic dissemination in prostate,

breast and hepatocellular cancers, and an indication for chemo-resistance in cancers with the proangiogenic phenotype (Oltean *et al*., 2012; Mavrou *et al*., 2014; van Roosmalen *et al*., 2015). Tumours of germinal origin have shown progressive and metastatic dissemination characteristics following downregulation of SRPK1 with associated cisplatin resistance (Krishnakumar *et al*., 2008; Wang *et al*., 2014). van-Roosmalen and colleagues have associated high levels of SRPK1 with poor prognosis, tumour aggressiveness, dissemination and distant metastases in breast cancer and have suggested SRPK1 as a potential target for drug therapy (van Roosmalen *et al*., 2015). That SRPK1 is a druggable target has already been demonstrated as its inhibition blocks angiogenesis and tumour growth *in vivo* (Amin *et al*., 2011; Mavrou *et al*., 2014).

1.6.5 *SRPK1 in leukaemia*

The complexity of protein network involved in molecular signalling and its interaction with kinases and other molecules has become the target in leukaemia therapy due to their role in genomic instability and dysregulation of these signalling pathways in leukaemia (Paulsen *et al*., 2009). Research into molecular and cellular mechanisms can provide clues on the behaviour of leukaemia cells and possible therapeutic interventions (García-Sacristán *et al*., 2009; Jang *et al*., 2008; Paulsen *et al*., 2009).

The need to maintain cell homogeneity and homeostasis in leukaemia has implicated SRPK1 as candidate for drug targeting due to its involvement in wide cellular interaction responsible for pre-mRNA splicing (Sanidas *et al*., 2010; Zhou *et al*., 2012), angiogenesis (Amin *et al*., 2011; Oltean *et al*., 2012), cell survival and proliferation (Sanidas *et al*., 2010) even in leukemic cells. A study using K562 CML demonstrated that SRPK1 is highly expressed in erythroid and lymphoid cells and associated increased expression of SRPK1 to cell proliferation and tumour grade (Sanidas *et al*., 2010). Another review suggests that repression of SRPK1 in acute myelogenous leukaemia (AML) could modify the choice of a splice site in VEGF (Mohamed *et al*., 2014). The mechanism is such that repression of SRPK1 results in hypo-phosphorylation of SRSF1 and hence, the selection of 3' proximal (pro-angiogenic) splice site during VEGF splicing (Oltean *et al*., 2012; Mohamed *et al*., 2014). Several studies (Daub *et al*., 2002; Hishizawa *et al*., 2005; Zheng *et al*., 2005; Fukuhara *et al*., 2006;) have shown that SRPK1 is able to interact with viral protein associated with several viral diseases which include the human T-cell leukaemia

virus 1. Thus, the pharmaceutical targeting of SRPK1 could benefit not just leukaemia and other cancer treatments, but also antiviral treatments,

1.6.6 SRPK1 and leukaemia therapy

There has been increasing interest towards targeting SRPKs in the management of several cancers as SRPKs have shown increased expression in tumours of the pancreas, colon, breast as well as viral-induced T-cell leukemic cells (Fukuhara *et al*., 2006). More so, experiments using several cell line models (U87, CaCo2 MiaPaCa2, HL60) have also shown diminished activities in pathways relating to cell proliferation, invasion, migration, and increased apoptotic potential and sensitivity to common chemotherapeutics following inhibition of SRPK1 (Hayes *et al*., 2007; *al*., 2011; Wu *et al*., 2013; Siqueira *et al*., 2015). Earlier studies have demonstrated increased expression of SRPK1 as an indication for resistance to chemotherapeutic agents such as oxaliplatin, gemcitabine and cisplatin using HT29 colon cancer cell and K562 leukaemic cell lines (Plasencia *et al*., 2006; Sanidas *et al*., 2010). In myeloma, combined therapy of bortezomib, a proteasome inhibitor and thalidomide, a cell cycle inhibitor has been investigated which only showed improved progression-free survival (PFS) on long-term administration but not overall survival (OS) with increased toxicity even in a high-risk patient with cytogenic profile t(4;14).

Over time, second-generation proteasome inhibitors and immunomodulatory agent such as pomalidomide, alkylphospholipid (an Akt inhibitor) perifosine and heat shock protein (HSP) inhibitors have undergone varying level of clinical evaluation in leukaemia (Anderson *et al*., 2007; Mitsaides *et al*., 2009; Allegra *et al*., 2011). In phase III clinical trial by Palumbo *et al*., (2010) combined bortezomib-melphalan-prednisone-thalidomide (VMPT), which combines a proteasome inhibitor, alkylating agent, immune cells and cell cycle respectively was used followed by maintenance with bortezomib-thalidomide (VMPT-VT). This was compared with bortezomib-melphalan-prednisone (VMP) treatment alone in untreated multiple myeloma patients who are ineligible for autologous stem-cell transplantation. Patients showed only three years of overall survival (OS). This was not free of toxicity relating to cardiologic and thromboembolic event which result in treatment-related death of 4% of the 38% who completed the therapy. It was shown that the inclusion of thalidomide greatly improved the efficacy of

the treatment (Palumbo *et al*., 2010). The current regimen for the management of leukaemia is not without toxic side effects, which have affected therapy as most of these drugs were found to have reduced potency at lower doses (Palumbo *et al*., 2010).

Drug-like imatinib mesylate, a tyrosine kinase inhibitor which was developed since the 1990s to specifically target the breakpoint cluster region (BCL/ABL^{+}) fusion protein in CML has remained the first line treatment for this condition. Although not curative, imatinib is only known to manage the condition through inhibition of cell proliferation and induction of apoptosis (Liu *et al*., 2015) with up to 37% discontinuing the therapy due to suboptimal response or tolerance (Jangamreddy *et al*., 2013).

Following increased treatment failure and relapse in patients with leukaemia in novel therapeutic regimens, investigations are further targeted towards specific signalling pathways that are known to be deregulated (for example, constitutively activated) in leukemic cancers (Allegra *et al*., 2011). The evidence strongly suggests that targeting splice factor kinases in leukaemic patients could prove to be beneficial. There is however very little work on targeting SRPK1 as a novel method in improving existing drugs in leukaemia treatment. The aim of this thesis is to explore the potential benefits of combining SRPK1 inhibition with the effect of existing leukaemic drugs.

1.7 Hypothesis and aims

1.5.1. *Hypothesis*

Targeting SRPK1 in leukaemia will enhance the efficacy of conventional chemotherapy.

1.5.2. *Aims and objectives*

The aims of this research are;

Aim A

To investigate the consequences of targeting SRPK1 in K562 and Kasumi-1 leukaemic cells.

This will be achieved through the following specific objectives;

i. Investigate the effect of SRPK1 chemical inhibition using small molecule inhibitors such as SPHINX and its knockdown using small interfering RNA (siRNA) on leukaemic cell growth, proliferation and apoptosis (chapters 3 and 4).

ii. Investigate the effect of SRPK1 chemical inhibition and knockdown on the alternative splicing of a panel of apoptotic genes; *BCL2L1, APAF1 and CASPASE 9* (chapters 3 and 4).

Aim B

To investigate the potential of combining conventional chemotherapeutic drugs (imatinib mesylate and azacytidine) with SRPK1 (and for comparison CLK1 inhibition) in leukaemic cells;

This will be achieved using the following specific objectives;

i. Treat leukaemic cell lines (K562 and Kasumi-1) with established chemotherapeutic drugs; Imatinib mesylate and azacytidine and observe the effect on cell activities such as cell growth, proliferation and apoptosis (chapter 4).

ii. Combine SPHINX and TG003 treatment with conventional chemotherapeutic drugs to see if it augments their effects (chapter 4).

iii. Investigate the effect of this combination on the alternative splicing of a panel of apoptotic genes; *BCL2L1, APAF1 and CASPASE 9* (chapter 4).

CHAPTER 2

2 Materials and methods

2.1 Cell and tissue culture

Human lymphoblastic cell line, TK6, chronic myeloid leukaemia cell ($Phil^+$), K562 and acute myeloblastic leukaemic cell line, Kasumi-1 were used in this experiment which targets the effect of SRPK1 on these cell lines. Cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC). All three cell lines are suspension cells and were cultured using RPMI-1640 culture medium with L-glutamine from (Sigma Aldrich, UK). Culture media was further supplemented with 10% foetal bovine serum (Sigma Aldrich, UK) for TK6 and K562 cell lines while Kasumi-1 cells were cultured in a 20% foetal bovine serum enhanced media. Cells used were between passage six to nineteen. Sub-culturing of all three cell lines involves the collection of the cell suspension into a centrifuge tube and centrifuged at 106 x g for 5mins. Following centrifugation, the supernatant was aspirated and discarded, and cell pellets were re-suspended in an appropriate volume of fresh media. Cells were seeded at densities of $5x10^5 – 1.0x10^6$ in a T_{25} flask and were sub-cultured every 48hours. All cell lines used for this experiment were incubated at 5% CO_2 at 37^oC.

2.2 Cell viability and growth count

Cell count and viability were determined using basic trypan-blue stain. Ten microlitre of cell suspension was diluted with an equal amount of 0.4% trypan-blue dye in a ratio of 1:1. Using a haemocytometer counting chamber, an aliquot of the stained cell is viewed and counted under a microscope at x10 magnification. Cells which absorbed the dye and stain blue were considered non-viable/dead. Alternatively, the Luna FL automated cell counter (Logos Biosystems, France) was used and result compared to manually counted cells. Following cell count, cell viability and proliferation were estimated using standard formula;

$$cell\ viabilty = \frac{live\ cell}{live + dead\ cells}$$

$$Cell\ count\ (N) = \left({}^{x}/_{y}\right) \times a \times b \times c$$

where;
x=Total live cell count; y= Number of grids counted (4); a= Dilution factor; b=Fixed factor ($1x10^4$) and c=Total volume of cell suspension

2.3 Chemical inhibition of splice factor kinases

Either TK6, K562 or Kasumi-1 cell line was treated with SRPK1 specific small molecule inhibitors 5-methyl-N-[2-(morpholin-4-yl)-5-(tri-fluoromethyl)phenyl] furan-2-carboxamide commonly known as SR Protein Inhibitor X (SPHINX) which was purchased from Enamine (Kiev, Ukraine). SRPK1 chemical inhibitors were dissolved in 100% sterile dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) soluble at 25mM. Using culture media, a serial dilution of 10nM, 100nM, 1µM, 10µM was prepared. The equivalent of the amount of DMSO (1%) in each dilution was used as positive control while negative control was represented by an untreated cell culture. The experimental setup involved seeding $1.0x10^6$/ml of cells in a T_{25} culture flask for each treatment. The experimental setup was done in duplicate. Cells were incubated for the period of treatment up to 72hrs.

Similarly, K562 and Kasumi-1 cell were also treated with 1-(3-ethyl-5-methoxy-2, 3-dihydrobenzothiazol-2-ylidene) propan-2-one, known as TG003, a competitive inhibitor for CLK1. The experimental setup was like those of SPHINX treatment although the concentration of TG003 up to 50µM was used to determine the inhibitory concentration. The subsequent experiment was performed using the highest concentration of 50µM.

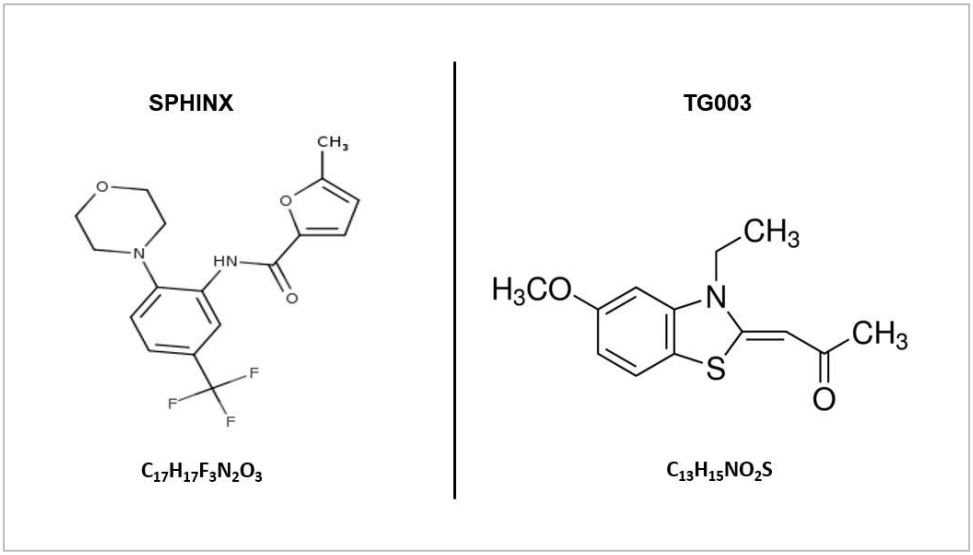


Figure 2.1: Structure and chemical formulae for SRPK1 and CLK1 small molecule inhibitors.

2.4 Treatment with chemotherapeutic drugs

Imatinib mesylate (Gleevec, formally; STI571) is an orally bioavailable mesylate salt of Imatinib which a multi-target inhibitor of Abl-Bcr^{+}. The tyrosine kinase inhibitor, [N-(4-methyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)phenyl)-4-(4-methylpiperazin-1-yl)methyl)benzamide methanesulfonic acid] was the drug of choice for K562 treatment. Whereas, 4-amino-1--D-ribofuranosyl-1,3,5-triazin-2(1H)-one; azacytidine, a DNA methyltransferase inhibitor was used to treat Kasumi-1 cells. Both drugs were sourced from Selleckchem. The initial dose of imatinib and azacytidine treatment was up to 20µg/ml and 1.5µg/ml respectively. These were later maintained at 3-5µg/ml and 750ng/ml respectively corresponding to the peak serum concentration when a standard clinical dose of 400mg and 75mg/ml/m^2 is administered to a patient.

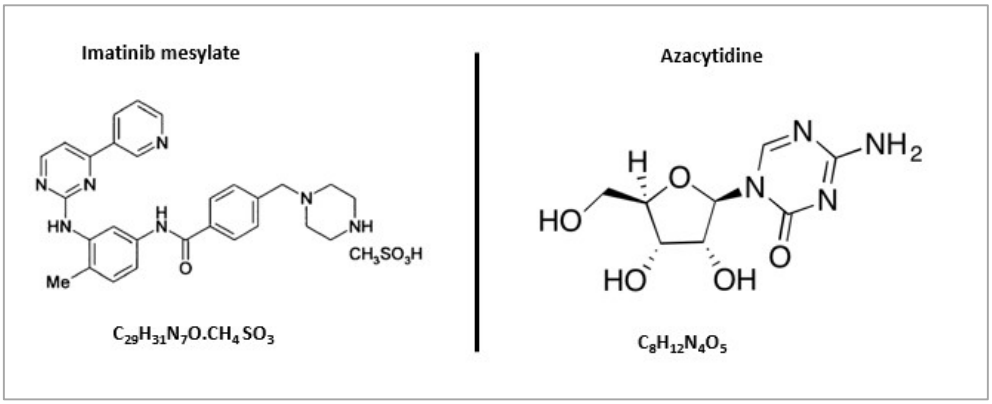


Figure 2.2: Structure and chemical formula for imatinib mesylate and azacytidine.

2.5 Caspase-3 colorimetric assay

To determine Caspase 3 activity in all leukaemic cell lines, caspase-3 colorimetric assay kit sourced from Abcam (ab39401) was used. The protocol used were those provided by the manufacturer. Cells were treated with kinase inhibitor or drug of choice for the treatment duration. At the end of the treatment period, cells were collected in a microcentrifuge tube, pelleted and lysed using the lysis buffer provided. Extracted proteins were quantified using the Bradford assay method. Aliquot of 1.5mg/ml of extracted protein was transferred into a 96-well plate in triplicates (x3). Appropriate volumes of a cocktail mixture containing 2x reaction buffer, dithiothreitol (DTT) and a labelled substrate, DEVD-p-nitroaniline (DEVD-p-NA) was added to each well. Plates were incubated at $37^{O}C$ for one hour, protected from light. Spectrophotometric detection of the chromophore p-nitroaniline after cleavage was read at 405nm corresponding to caspase-3 activity in the cells. Caspase-3 activity was determined by subtracting reading of background wells from those of the actual sample.

2.6 Microscopy

2.6.1 *Examination of cell morphology using acridine orange fluorescence stain*

The compound 3, 6-Bis(dimethylamino) acridine hydrochloride zinc chloride double salt also known as acridine orange hemi (zinc chloride) salt; is a cell-permeable, nucleic acid selective dye that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA (Sigma Aldrich). Since it is a cationic dye, it also enters

acidic compartments such as lysosomes which in low pH conditions, will emit orange light. Twenty thousand of K562 cells were collected post-treatment into a microcentrifuge tube and spun at 106 x g for 10minutes. The supernatant was aspirated and discarded. The cell pellet was re-suspended in 150µL of sterile phosphate buffered saline (PBS) and transferred into a cytofunnel. The cytofunnel which has a microscope slide attached was spun at 20,000 x g for 8minutes in a Cytospin 4 (Thermo Scientific). Cells were then fixed using 90% methanol for 10mins and air dried.

For staining, slides were dipped in fresh phosphate buffer (0.66% (w/v) potassium phosphate mono-basic + 0.32% (w/v) sodium phosphate dibasic, at pH 6.4-6.5) to rehydrate and stained in a solution of acridine orange (0.12 mg/ml in phosphate buffer; Sigma Aldrich, UK) for 45 seconds. Removal of excess stain was achieved by dipping slides in two changes of fresh buffer for 10 minutes and 15 minutes, respectively. Post-staining, slides were air dried and stored away from light. Microscopic examination of slides using phosphate buffer as a mountant. Slides were analysed using the Nikon Eclipse 80i (upright) fluorescence microscope. Nuclei morphology was analysed using a BG-12 excitation filter and 0-530nm barrier filter under x40 magnification. Scoring was achieved by manually counting 2,000 cells per slide.

2.6.2 *Examination of cell morphology using caspase 3/7 fluorescence stain*

Cells pre-treated with a combination of kinase inhibitor and chemotherapeutic agent were stained with the ThermoFisher scientific CellEvent^{TM} Caspase 3/7 green detection reagent. The detection reagent was diluted (1:100) in PBS. Twenty thousand pelleted cells were suspended in 150µl of the reconstituted reagent and allowed to stand for 45minutes at room temperature away from light. Counterstaining was done using the Hoechst at a 1:5000 in PBS for one minute after which cells were transferred into a cytofunnel (Fisher Scientific, UK) and spun onto a microscope slide using the cytospin 4 (Thermo Scientific, UK) at 20,000 x g for 8minutes. Air drying of slides was in the dark. Slides were mounted using Mowiol aqueous mounting media (24g Glycerol, 9.6g Mowiol, 24ml Ultra dH_20 and 48ml 0.2M Tris-HCl, pH 8.5). The Nikon Eclipse 80i fluorescent microscope was used for imaging. Nuclei morphology was analysed using a BG-12 and FITC excitation filter and 0-530nm barrier filter under X40 magnification.

The principle of action is such that the caspase 3/7 green detection reagent which is a four-amino acid peptide (DEVD) is activated in apoptotic cells following cleavage of the peptide (Thermofisher). This enables the dye to bind to DNA producing a bright green fluorescence emission of about 530nm.

2.7 RT-PCR and Agarose gel electrophoresis

2.7.1 RNA isolation and DNase treatment

The Agilent absolute RNA miniprep kit (cat: 400800) and its protocol for total RNA isolation were adapted. Post treatment, cells were transferred into a microcentrifuge tube and centrifuged (Beckman Allegra x-22R, Rotor: F2402H) at 9,650g for 5minutes. Cell pellets were lysed using a reconstituted lysis buffer containing a 1:100 dilutions of β-mercaptoethanol and the lysis buffer. Homogenization of cells was achieved either by vortexing for a minute or passing solution through a 21guage needle. Up to 700µl of the cell homogenate was transferred to a pre-filter spin column placed in a receptacle and spun at 9,650g for 5min. The spin column was discarded while the filtrate retained. An equal amount of 70% ethanol was added to the filtrate and vortexed until mixed thoroughly. Up to 700µl of the filtrate was then transferred to an RNA binding spin column seated in a sterile receptacle and spun at maximum speed for a minute. The filtrate was discarded, and the spin cup which contains the bound RNA was retained.

For DNase treatment, 600µl of 1x low-salt wash buffer was added into the RNA binding column and spun at maximum speed for 3mins. A constituted aliquot of DNase digestion buffer and reconstituted RNase buffer was added to the matrix of the spin column and incubated at 37^{o}C for 15mins. This was followed by several washes with 1x high and low salt wash buffer. Purified RNA was eluted in 30µl of elution buffer into a micro-centrifuge tube following a 2minute incubation at room temperature. The final product was stored in -20 or -80^{o}C. The total RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). 1.5µl of each RNA sample was used to determine RNA purity, reading absorbance on the spectrophotometer at 260/280nM.

2.7.2 *Complimentary DNA (cDNA) synthesis*

Post RNA extraction, cDNA synthesis was performed using the ProtoScript first strand cDNA synthesis kit protocol described by New England Biolabs (NEB). Two micrograms (2µg) of RNA for each treatment category was collected in a 0.5ml sterile microcentrifuge tube. In each tube, a cocktail (2µl of a mixture of random primer and oligodT at a 1:1, 4µl of 2.5mM dNTP) was added and brought to a final volume of 16µl with nuclease-free water (Qiagen). This was followed by incubation at 70^{o}C on a heat block for 5mins. The reaction product was spun down and held immediately on ice. While on ice, another cocktail (2µl of 10x M-MLV reverse transcriptase buffer, 1:1 mix of 2x reverse transcriptase and RNA inhibitor; NEB, UK) was added to the tube followed by incubation at 42^{o}C for one hour. The transcriptase enzyme was deactivated at 90^{o}C for 10 mins and the final product stored at -20^{o}C.

2.7.3 *Standard PCR and Agarose gel electrophoresis*

Following cell treatment with chemical inhibitors, RT-PCR was carried out to measure the levels of SRPK1, and the alternative splicing of the gene of interest, which included *Caspase 9, Bcl-x, APAF-1, CLK1 and VEGF*. Beta-actin (β-actin) was also amplified and used to determine the quality of the cDNA as well as a loading control. Table 2.1 describes all primer sequence used for standard PCR and the annealing temperatures (Tm) for individual primers.

2.7.4 Primer sequences and design

Gene name	Primer sequence/ length	Alignment	Tm(^{0}C)	Amplicon length (bp)
SRSF protein kinase 1 (*SRPK1*) NM_003137	**F**: TTCCTCAACTGTAGGTCAGTCATTC (25) **R**: TGTTCTTGCTCTTGTTCATCTTCAC (25)	**F** :1329 – 1353 **R** :1430 – 1403	49	102
Beta – actin (*β-actin*) NM_001101	**F**: TTAAGGAGAAGCTGTGCTACG (21) **R**: GTTGAAGGTAGTTTCGTGGAT (21)	**F**: 719 – 739 **R**: 924 – 904	52	206
Vascular endothelial growth factor A (*VEGF-A*) NM_001025366	**F**: GTAAGCTTGTACAAGATCCGCAGACG (26) **R**: ATGGATCCGTATCAGTCTTTCCTGG (25)	**F**: 1616 – 1641 **R**: 1814 – 1790	54	199
Vascular endothelial growth factor A (*VEGF-A165b*) NM_001171629	**F**: GGCAGCTTGAGTTAAACGAACG (22) **R**: ATGGATCCGTATCAGTCTTTCCTGG (25)	**F**: 1562 – 1583 **R**: 1625 – 1601	52	64
Vascular endothelial growth factor A (*VEGF-A165b*) NM_001033756	**F**: GGCAGCTTGAGTTAAACGAAC (21) **R**: ATGGATCCGTATCAGTCTTTCCTGG (25)	**F**: 1560 – 1580 **R**: 1623 – 1599	52	64
BCL2 like 1 (*BCL2L1*) xl	**F**: CATGCCAGCAGTGAAGCAAG (20)	**F**: 627 – 646	53	351

NM_138578	**R**: GCATTGTTCCCGTAGAGATCC (21)	**R**: 977 – 957		
BCL2 like 1 (*BCL2L1*) xs NM_001191	**F**: CATGCCAGCAGTGAAGCAAG (20) **R**: GCATTGTTCCCGTAGAGATCC (21)	**F**: 857 – 876 **R**: 1018 - 998	53	162
Apoptotic peptidase activating factor 1 (*APAF1*) NM_013229	**F**: CAGCTGATGGAACCTTAAAGC (21) **R**: GTCTGGTCATCAGAAGATGTC (21)	**F**: 2829 – 2849 **R**: 3258 – 3238	48	430
Apoptotic peptidase activating factor 1 (*APAF1*) NM_001160	**F**: CAGCTGATGGAACCTTAAAGC (21) **R**: GTCTGGTCATCAGAAGATGTC (21)	**F**: 2829 – 2849 **R**: 3129 – 3109	48	301
Caspase 9 Variant alpha (*CASP9α*) NM_001229	**F**: GCTCTTCCTTTGTTCATCTCC (21) **R**: CATCTGGCTCGGGGTTACTGC (21)	**F**: 220 – 240 **R**: 961 – 941	50	742
Caspase 9 Variant beta (*CASP9β*) NM_001278054	**F**: GCTCTTCCTTTGTTCATCTCC (21) **R**: CATCTGGCTCGGGGTTACTGC (21)	**F**: 450 – 470 **R**: 741 – 721	50	292
CDC like kinase 1 (*CLK1*), Exon 4 retention NM_004071	**F**: (1FB) CAAGGATGTGAACCTGGACATCGC (24) **R**: (3RB) CTCCTTCACCTAAAGTATCAAC (22)	**F**: 339 – 362 **R**: 606 – 585	48	268
CDC like kinase 1 (Δ*CLK1*), Exon 4 skipping	**F**: (1FB) CAAGGATGTGAACCTGGACATCGC (24) **R**: (3RB) CTCCTTCACCTAAAGTATCAAC (22)	**F**: 339 – 362 **R**: 606 – 585	48	187

NM_004071				
CDC like kinase 1 (Δ*CLK1x*), intron 4 retention NM_004071	**F**: (2FB) GGAGGGTCACCTGATCTGTCAG (22) **R**: (3RA) CTGCTACATGTCTACCTCCCGC (22)	**F**: 530 – 551 **R**: 663 – 642	57	516
CDC like kinase 1 (*CLK1x*), intron 4 skipping NM_004071	**F**: (2FB) GGAGGGTCACCTGATCTGTCAG (22) **R**: (3RA) CTGCTACATGTCTACCTCCCGC (22)	**F**: 530 – 551 **R**: 663 – 642	57	134

Table2.1: Forward (F) and reverse (R) primer sequences for all human genes amplified using standard PCR.
The target sites of the primers, and amplicon size are shown. All primers were obtained from Eurofins Ltd, (Germany).

First, a gradient PCR was performed to determine the optimum annealing temperature (Tm/^{0}C) for each primer, and these are shown on Table 2.1. General cycling conditions for all primers are as follows; denaturation at 95°C for 30 seconds, elongation at 68°C for 1minute and final extension was at 68°C for 5minute. A total of 35 cycles at 95°C for 15 seconds was performed. PCR amplicons were electrophoresed on a 2% agarose gel using 1x tris-acetate-EDTA (TAE) buffer at 95V for 45minutes. Gels were stained with 0.01% of 10µg/ml ethidium bromide (Sigma Ltd, UK). A 100bp DNA ladder (NEB, UK) was loaded alongside the PCR products to determine amplicon size.

Image acquisition from gels was performed using the LI-COR Odyssey FC imaging system (USA) at 600nm.

2.7.5 *Normalization of RT-PCR*

To ensure that the level of gene expression observed was due to experimental treatments, gels were analysed using LI-COR odyssey FC software (Image studio Lite 5.2) that measured RT-PCR band densities. Obtained values were normalized to β-actin for the same treatment. Final values were exported to GraphPad statistical software (version 7). Corresponding graphs were generated for each RNA and their splice variants. Splice variants were plotted as the percentage of splice inclusion (PSI-Ψ). Data were presented as mean ± 95% confidence interval (CI).

2.8 SDS-PAGE and immunoblotting

2.8.1 *Protein extraction and quantification*

Proteins were extracted from cells using the radioimmunoprecipitation assay (RIPA) lysis buffer (10 mM Tris-Cl (pH 8.0), 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl). Post-treatment harvested cells were suspended in ice-cold lysis buffer (0.1mL per 1 x 10^6 cells) supplemented with protease inhibitor (1:1000) (Roche Diagnostics, UK) for 30minutes. This was followed by centrifugation at 3000 x g at 4^{o}C for 10minutes. Supernatant which contained proteins were aspirated into a clean sterile microcentrifuge tube and held on ice for quantification.

Protein samples were diluted in a 1:1 ratio with nuclease free water for quantification. The Pierce^{TM} BCA assay kit (Thermo Scientific) was used. Five protein standards using

bovine serum albumin (BSA) was prepared in the following concentrations: 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml. The working solution was prepared using the Pierce BCA suggested dilution of 50:1 (BCA reagent A: BCA reagent B). An aliquot of 200µl of the working solution was added to each well of a 96well plate containing the sample to be quantified. The plate was incubated in the dark for 30minutes. All samples including controls were prepared in duplicates and read at 560nm on a plate reader (Fluostar Optima, BMG lab tech, Germany). Absorbance values were exported into an excel sheet (Microsoft) where a standard curve was generated, and protein concentration determined using the graph equation generated. Final protein concentration was brought to 20µg/µl using sterile water and were mixed with 2x Laemmli buffer (Sigma, UK) in 1:1 dilution. The mixture was then boiled at 100^{o}C for 5minutes before being held on ice for loading into gels.

2.8.2 *Acrylamide gel*

Proteins were separated on a 10% acrylamide gel. Each gel contains 30% (v/v) acrylamide mix (29:1; acrylamide: bis-acrylamide) (Sigma Aldrich), 1.5M resolving buffer (18.17g tris and 100ml diH_2O; pH 8.8), distilled water, 10% (w/v) ammonium persulphate (AP) (Sigma Aldrich) and tetramethylethylenediamine (TEMED) (Sigma Aldrich). This cocktail was carefully loaded on to the pre-assembled casting trays using a pasture pipette and allowed to set for 15-20mins. A 5% acrylamide stacking gel was then added to overlay the separating gel and a 10-well comb was inserted and allowed to set.

2.8.3 *SDS polyacrylamide gel electrophoresis (SDS-PAGE)*

Gel electrophoresis was performed using the Electrophoresis cell apparatus for mini gels (Bio-Rad, UK). The units were assembled, and the middle chamber filled with running buffer (25mM Tris, 190mM glycine and 0.1% (w/v) SDS adjusted to pH 8.3). The comb was removed and 700µg of the protein samples were carefully loaded in each well. A pre-stained protein marker (NEB, UK) was also loaded to determine protein size. The outer chamber of the unit was also filled with the running buffer depending on the number of gels following the manufacturers' recommendation. The gels were run at 100V for 2 hours.

2.8.4 *Transfer of proteins on to membranes*

For protein transfer onto a membrane, gels were prised from the casting trays and the stacking gel removed. The separating gel, which contains the protein samples was assembled in a sandwich of sponges, filter papers, gel and polyvinylidene difluoride (PVDF) membrane on a cassette in the following order (Figure 2.4).

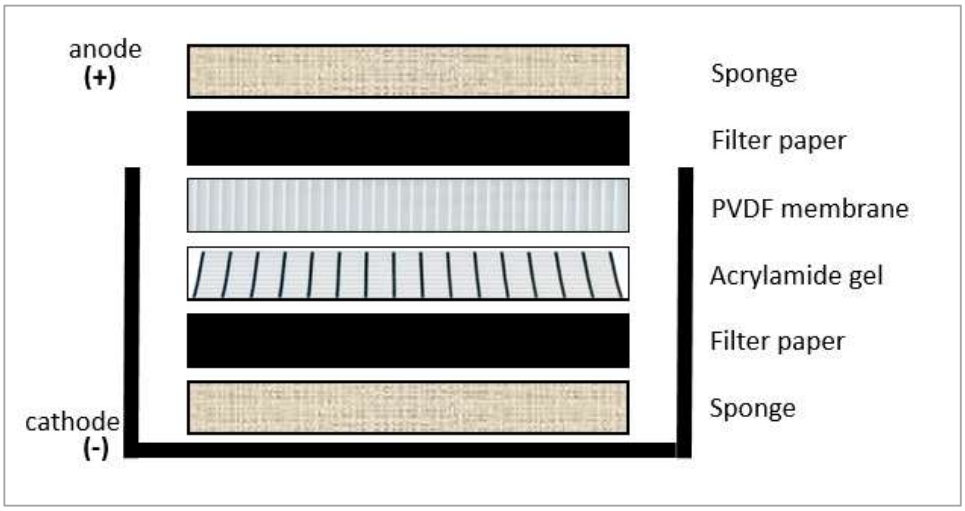


Figure 2.4: Diagram illustrating sandwich assembly for transfer of proteins to PVDF membrane.

Gels were transferred on to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific). The membrane was activated in absolute methanol for 20secs, thereafter, held in 1 x cold (4^{o}C) tris/glycine/methanol transfer buffer (25mM Tris, 190mM glycine and 20% methanol). Following assembly of the components on the cassette, the cassette was placed in the transfer tank containing cold transfer buffer. A magnetic stirrer and an ice pack were placed in the tank to maintain the cold temperature. Transfers were done in 4 hours.

2.8.5 *Immunoblotting*

Post-transfer, membranes were blocked in a 5% non-fat dry milk and 1xTBST solution for a minimum of 1 hour. This is to prevent unspecific binding of the antibody to membranes. Thereafter, the membrane was incubated at 4^{o}C overnight in the primary antibody of choice (Table 2.2).

Table 2.2: Antibodies used for western blotting. Sources and working dilutions are stated.

Antibody/ Specie	Concentration	Dilution used	Source	Secondary antibody used
Anti-β-actin (ab8226-Mouse)	1mg/ml	1:5000	Abcam	Horse anti-mouse IgG)
Anti-SRPK1 (EE-13-Mouse)	200µg/ml	1:1000	Santa Cruz Biotechnology	HRP-linked antibody (Cell Signalling)
Anti-SR (1H4) (sc-13509-Mouse)	200µg/ml	1:500	Santa Cruz Biotechnology	
Anti-SRSF1 (sc-33652-Mouse)	200µg/ml	1:1000	Santa Cruz Biotechnology	
Anti-CLK1 (G313-1-Mouse)	200µg/ml	1:2000	BD Biosciences	

Membranes were washed (3x) in appropriate volume of 1x tris-buffered saline with Tween-20 (TBST) (20mM Tris pH 7.5, 150mM NaCl, 0.1% Tween20). Each wash lasting 5mins each. This was followed by incubation with a horseradish peroxidase (HRP) linked secondary antibody (Cell signalling) at room temperature for 1 hour. Unbound secondary antibodies were removed by repeated washes (3x) in 1 x TBST, each wash lasting about 5mins. About 500µl of HRP-substrate (Millipore, UK) was dispensed on to the membrane for 2mins prior to image acquisition. Image acquisition from membranes was performed using the LI-COR Odyssey FC imaging system (USA) at chemi function for 2mins.

2.9 SRPK1 siRNA knockdown

2.9.1 Cell culture and preparation

All cell lines, TK6, K562 and Kasumi-1 cells were cultured in a T_{25} cell culture flask respectively for cells to be 80% confluence. Cells were then harvested and spun down to discard the growth media. For each cell line, $5x10^5$ cells were collected and washed in 1 x PBS followed by centrifugation at 106 x g for 5minutes. The supernatant was aspirated and discarded. Each cell line was re-suspended in 800µl of OptiMEM media (Gibco, UK) and transferred into a 6-well plate and incubated at 5% CO_2 at 37°C.

2.9.2 *siRNA Transfection*

A cocktail of Lipofectamine RNAiMAX (7.5µl) (Thermo Fischer Scientific, UK) and OptiMEM media (100µl) was prepared. One hundred microlitre of Lipofectamine/OptiMEM cocktail was added to the SRPK1 siRNA (Eurofins, Genomics, UK) which was reconstituted in OptiMEM media to a final concentration and volume of 100nM and 100µl, respectively. The entire mixture was incubated at room temperature (RT) for 20 minutes. Two hundred microlitre of siRNA/Lipofectamine/OptiMEM was added to each well and then left to incubate for 4 hours. Thereafter, each well was topped up with 1ml of appropriate tissue culture media and allowed to further incubate at 37^0C for 48hrs from the time of transfection.

A similar protocol was applied to controls using a scrambled sequence of siRNA, which had previously been described by Karakama *et al*., (2010) and listed in Table 2.3.

Table 2.3: siRNA sequences used for knockdown and target.
Complimentary sequence was determined by Eurofins Genomics, UK.

siRNA Targeted	Sequence	Source
SRPK1	5'-UUAAUGACUUCAAUCACUCCAUUGC-3'	Karakama *et al*., 2010
	5'UAAGAAAUCUGUGAAGCCAGCUGCC-3'	
Scrambled siRNA control	5'GCAGCAGCAGCAGCGGGACTT-3'	

2.10 Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 7.0. Error bars represent mean ± 95% confidence interval (CI). Number of experimental repeats equals three (n=3). Except where stated, one-way ANOVA was used for analysis. The Tukeys and Dunnett's test was employed for *posthoc* test within a treatment group where data assumes Gaussian distribution. Where distribution was not normal, Kruskal's Wallis test was used to test for the difference between two groups and the Student t-test for pair-wise comparison. Normality test was performed using the D'Agostino-Pearsons normality test which also computes the Skewness and Kurtosis, examining the symmetry

of the data relative to normal distribution. The confidence level was taken at 95%CI and P-value ≤0.05 was considered statistically significant.

CHAPTER 3

3 Effect of SRPK1 inhibition on leukaemic cell viability and growth

3.1 Introduction

The serine/arginine-rich protein kinase-1 (SRPK1) like other protein kinases interacts with other proteins in order to transmit cell signalling. SRPK1 phosphorylates the SR-protein family of splice factors; its most studied substrate is SRSF1 (Plocinik *et al*., 2011; Gonçalves *et al*., 2014). Phosphorylation of the SR-protein by SRPK1 plays an important role in the regulation of pre-mRNA splicing, translation and non-sense mediated RNA decay (Graveley *et al*., 1998; Plocinik *et al*., 2011;). Studies suggest that knockdown of SRPK1 or inhibition of its catalytic activities results in reduced phosphorylation of SRSF1 and other SR-protein, hence a shift in pre-mRNA splicing and changes in cell behaviour (Nowak *et al*., 2010; Goncalves *et al*., 2014; Mavrou and Oltean, 2016). SRPK1 interaction with SRSF1 has been shown to affect the alternative splicing of caspase 9 (Shultz *et al*., 2011) and vascular endothelial growth factor (VEGF-A), the latter controlling the ratio of pro and anti-angiogenic isoforms in a renal epithelial cell, colon and prostate cancer cells (Amin *et al*., 2011; Oltean *et al*., 2012; Mavrou *et al*., 2014). A previous study has also shown that SRPK1 interacts with activated protein kinase-B (Akt/PKB) through PHLPP, an Akt phosphatase and PI3 (Zhou *et al*., 2012). These pathways are upstream of several oncogenic signalling such as the mTOR and are known to regulate cell growth, upregulated proliferation, survival, differentiation, and protein synthesis. The PI3/Akt pathway through the mTOR-EGF signalling is known to affect pre-mRNA splicing by modulating the interaction between SRPK1 and Hsp70 and Hsp40 chaperone proteins (Zhou *et al*., 2012).

Interestingly, over-expression and dysregulation of SRPKs have been shown to promote cell proliferation in several cancers including leukaemia. In particular, overexpression of SRPK1 was found in acute lymphoblastic leukaemia (ALL) and chronic myeloid leukaemia (CML) (Siqueira *et al*., 2015). Inhibition of SRPK1 using SRPIN340 resulted in reduced cell viability (Siqueira *et al*., 2015). Additional research is needed to understand in greater details the exact role of SRPK1 in leukaemia.

The aim of this chapter is, therefore, to establish whether SRPK1 inhibition using SPHINX, a specific and more potent inhibitor (compared to an older analogue SRPIN340) of SRPK1 causes changes in cell growth and viability as well as on the alternative splicing of a panel of cancer-associated genes.

3.2 Results

3.2.1 Validation of SPHINX and effect of SRPK1 inhibition with SPHINX on TK6 cell viability and growth

First, the potency of SPHINX was determined by treating PC3 cell line with 10μM SPHINX for 48hrs. Similar experiment has been performed by Mavrou and colleagues (2014) suggesting that SRPK1 inhibition reduces tumour growth *in vivo* in orthotopic prostate cancer (PCa) mouse model. Result obtained from this experiment also showed that SRPK1 inhibition in PCa cells, PC3, reduces PC3 cell viability and growth *in vitro* (Figure 3.1A-B). It was observed that SRPK1 protein levels were significantly reduced as well as decreased phosphorylation of SR protein family (Figure 3.2A-B) following treatment with SPHINX.

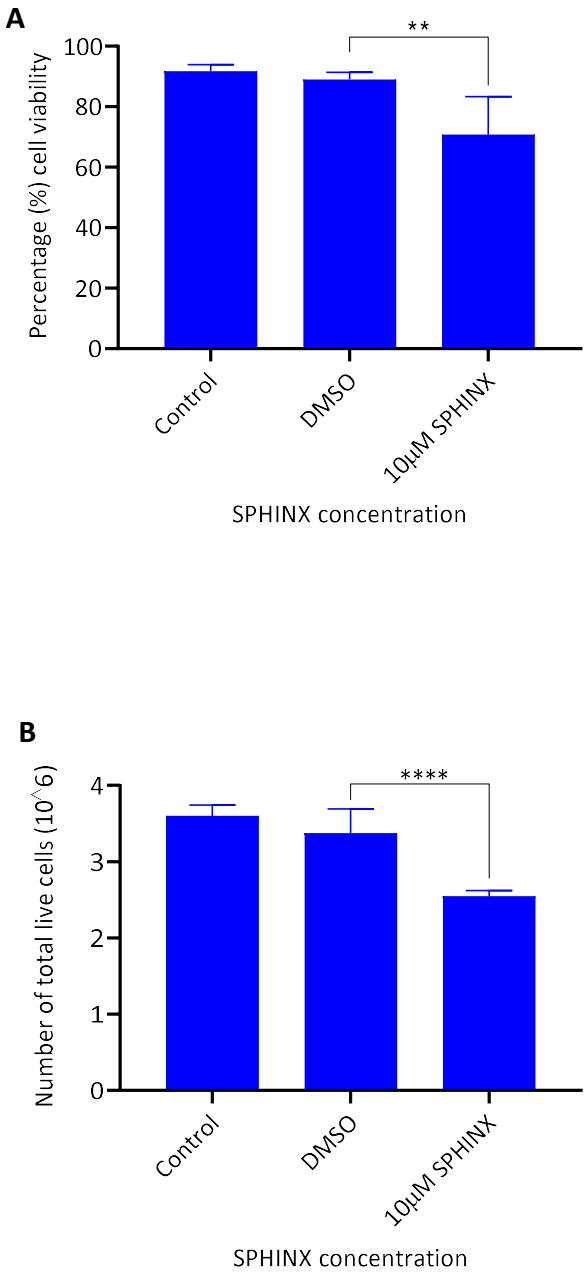


Figure 3.1. Effect of SRPK1 inhibition with SPHINX on PC3 cell viability and growth. PC3 cells treated with 10µM of SPHINX for 48hrs. **A**. Percentage cell viability in PC3 cells compared SPHINX treatment to DMSO (***P*=0.0096). **B**. Cell number significantly decreased in PC3 cell (*****P*≤0.0001) when SPHINX was compared to DMSO treatment (n=3).

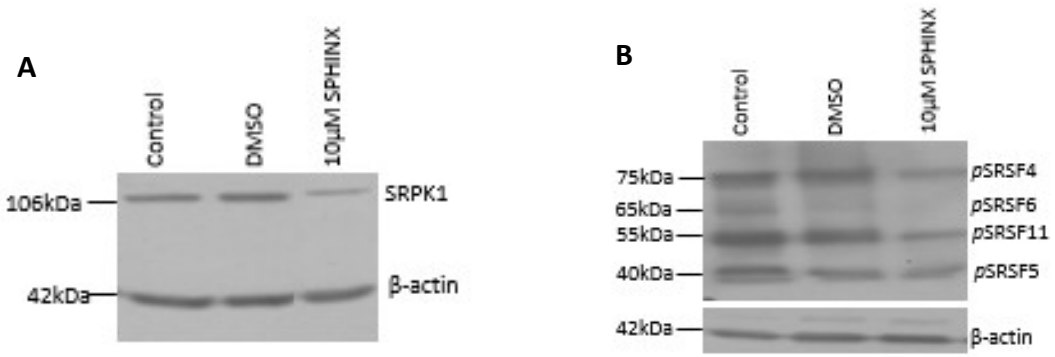


Figure 3.2. Levels of SRPK1 and phospho SR-proteins in PC3 cells. **A**. Immunoblot showing levels of SRPK1 in PC3 cells. **B**. Reduced phosphorylation of SR proteins when SPHINX treatment was compared to DMSO. n=3.

To investigate the effect of SRPK1 on the growth and viability of TK6 cells, 2 x 10^6 of the cells were seeded and treated with up to 10μM of SPHINX for up to 72hrs. Cells were counted at 24hr interval using trypan blue and an automated cell counting chamber and result compared to the DMSO control treatment.

Results showed a slight reduction in cell viability between the DMSO control and the SPHINX treatment at higher concentrations (1μM and 10μM). This was not found to be significant ($P \geq 0.05$). However, when the cell viability was compared between time points, there was a significant ($P=0.041$) apparent increases in cell viability between 48hrs and 72hrs (Figure 3.3A).

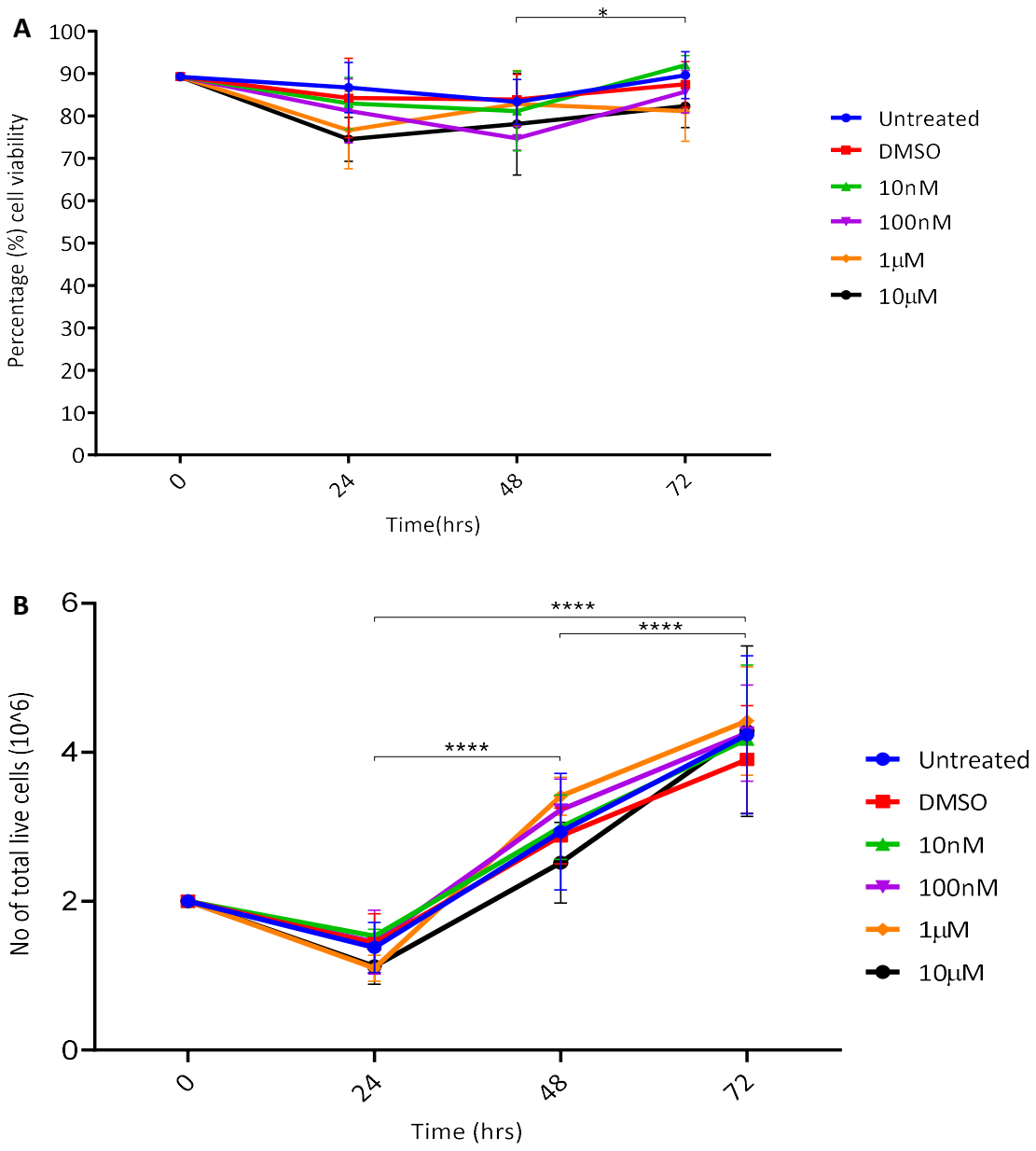


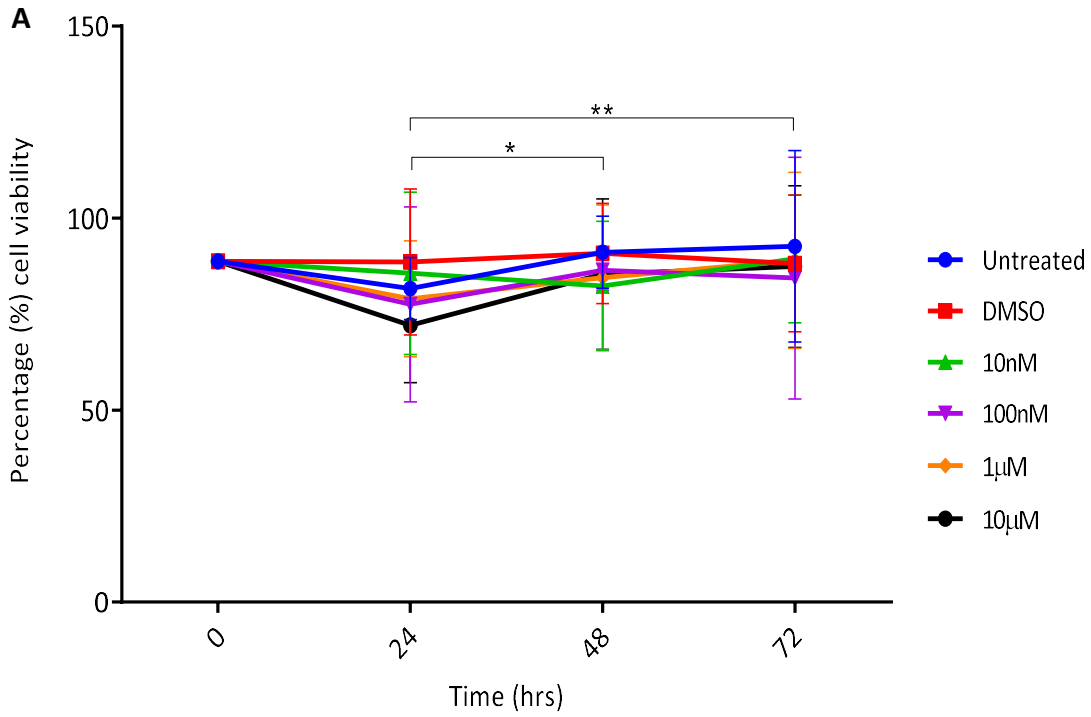
Figure 3.3. Effect of SRPK1 inhibition with SPHINX on TK6 cell viability and growth. TK6 cells were treated with increasing concentrations (10nM, 100nM, 1µM and 10µM) of SPHINX for up to 72hrs. **A.** Percentage cell viability was significant ($P \leq 0.041$) between 48hrs and 72hrs. Treatments were compared to DMSO for each time point ($n = 3$). **B.** TK6 cells showed significant increase in cell growth ($P < 0.0001$) between time points ($n = 3$).

Since no significant decrease in TK6 cell viability was observed, cell growth following SPHINX treatment was then examined. TK6 cells continued to grow with cell numbers doubling (up to 4 x 10^6) by 72hrs. There was also no difference when the treated group was compared to the DMSO control, proliferation rate significantly increased ($P<$ 0.0001) with time (Figure 3.3B). This suggests that SPHINX had no effect of TK6 cell growth and viability.

3.2.2 Effect of SRPK1 inhibition with SPHINX on K562 cell viability and growth

Different leukaemic cell lines will respond differently to treatment depending on their type and the inherent mutations they have acquired (Siqueira *et al.*,2015). Chronic myeloid leukaemia cell line, K562 cells was also investigated for the effect on viability and growth following SPHINX treatment with concentrations up to 10µM for 72hrs. Like TK6 cells, K562 cells were also counted every 24hrs to investigate changes in cell growth and viability. There was no significant percentage decrease in cell viability when the SPHINX treated cells were compared to the DMSO cells for each concentration (Figure 3.4A). Further analysis factoring in time showed an apparent percentage increase on cell viability between time points such that viability between 24hrs vs 48hrs and 24hrs vs 72hrs significantly increased *(P* ≤ 0.05).

Investigations into SPHINX effect on K562 cell growth also suggested there was no effect as K562 cell continued to grow and proliferate even at higher concentration of SPHINX (Figure 3.4B). K562 cell growth significantly increased with time suggesting that SPHINX does not reduce K562 cell viability and growth.



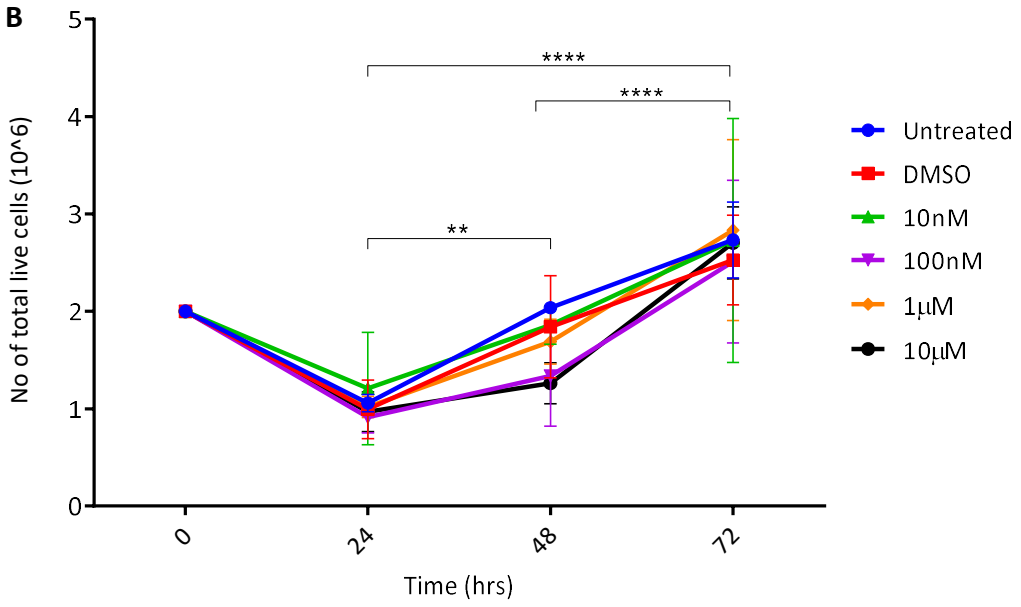


Figure 3.4. Effect of SRPK1 inhibition with SPHINX on K562 cell viability and growth. K562 cells were treated with increasing concentration (10nM, 100nM, 1µM and 10µM) of SPHINX for up to 72hrs. **A.** Represent percentage in cell viability. Significant differences and percentage reduction in cell viability are observed between 24 and 48hrs *(P* ≤ 0.05) and 24hrs and 72hrs *(P* ≤ 0.01). Treated cells were compared to DMSO for each time point. **B.** K562 cells showed significant increases in cell number (**=*P* ≤ 0.01 and ****=*P*< 0.0001) between time points. (*n* = 3).

3.2.3 Effect of SRPK1 inhibition with SPHINX on Kasumi-1 cell viability and growth

The effect of SRPK1 inhibition using SPHINX on Kasumi-1 (AML) cells was also investigated to see whether or not AML cells will react differently from the CML (K562) and lymphoblast (TK6) cell. Results showed significant percentage decrease in cell viability with Kasumi-1 cells at higher concentrations of 1µM and 10µM of SPHINX inhibition when compared to DMSO control (Figure 3.5A). Decrease in cell viability at 1µM was observed at 48hrs and 72hrs. At 10µM, Kasumi-1 cell viability continued to decrease with time. This suggests that Kasumi-1 cell viability may be affected by SRPK1 inhibition at higher concentrations.

Cell number in Kasumi-1 cells was also reduced at higher concentrations of 1µM and 10µM but only at 72hrs was this effect significant (Figure 3.5B). Having observed an apparent decrease in both cell viability and growth in Kasumi-1 cells suggests that Kasumi-1 cells are more sensitive to SRPK1 inhibition.

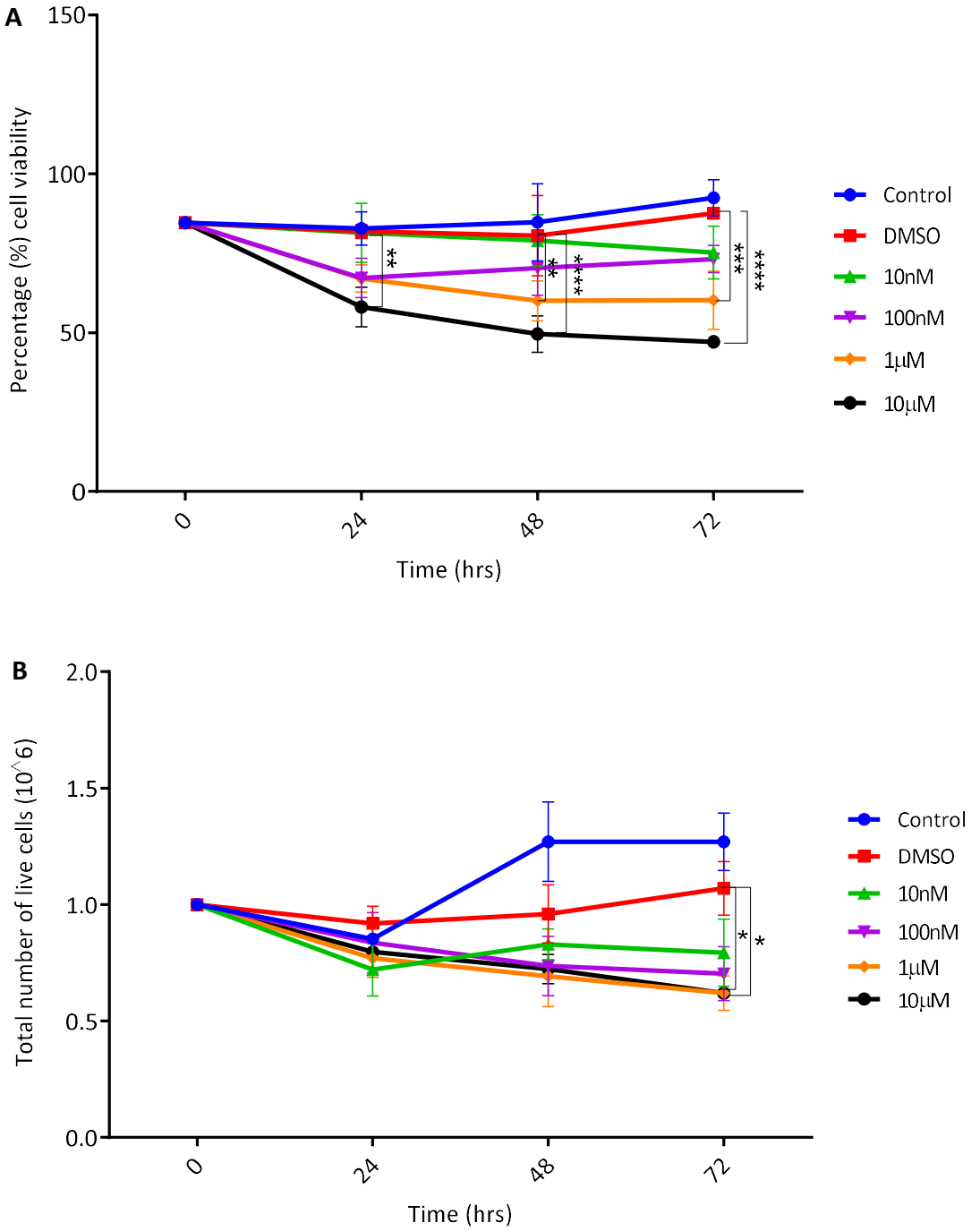
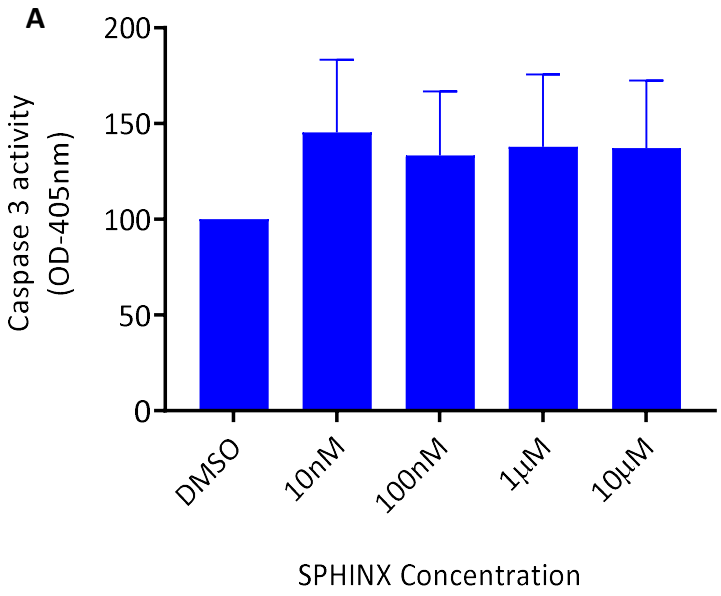


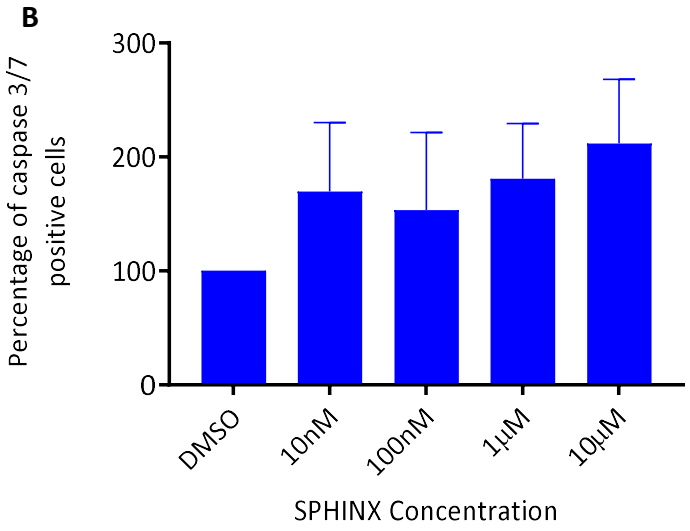
Figure 3.5. Effect of SRPK1 inhibition with SPHINX on Kasumi-1 cell viability and growth. Kasumi-1 cells were treated with increasing concentrations (10nM, 100nM, 1µM and 10µM) of SPHINX for up to 72hrs. Analysis compared DMSO treated cells with SPHINX treatment at each time point. **A.** A significant decrease in cell viability was observed at higher concentrations (1µM; ***P*=0.006 and ****P*=0.0002 respectively) and (10µM; **P*=0.013; ****P*<0.0001; 0.0001 respectively). **B.** Only at higher concentrations at 72hrs, was a reduced cell number observed (**P* ≤ 0.05) compared to time-matched control. *n* = 3.

3.2.4 Effect of SRPK1 inhibition on caspase 3/7 activity in leukaemic cells

3.2.4.1 Effect of SRPK1 inhibition on caspase activity in TK6 cells

To further confirm that inhibition of SRPK1 does not affect TK6 cell viability and growth, the activities and expression of cleaved caspase 3 were studied. Caspase 3 is known as the apoptosis executioner and its activation is a product of both the intrinsic and extrinsic pathway (Brentnall *et al*., 2013). Hence, the choice of its investigation. TK6 cells were treated with increasing concentrations of SPHINX for 72hrs. Post-treatment, cells were harvested separately and processed following the methods, which have already been described in chapter 2.5. TK6 cells showed no significant difference in caspase 3 activity with increasing concentration of SPHINX and when compared to DMSO control (Figure 3.6A).


A
Caspase 3 activity
(OD-405nm)
200
150
100
50
0
DMSO
10nM
100nM
1µM
10µM
SPHINX Concentration


B
Percentage of caspase 3/7
positive cells
300
200
100
0
DMSO
10nM
100nM
1µM
10µM
SPHINX Concentration

`

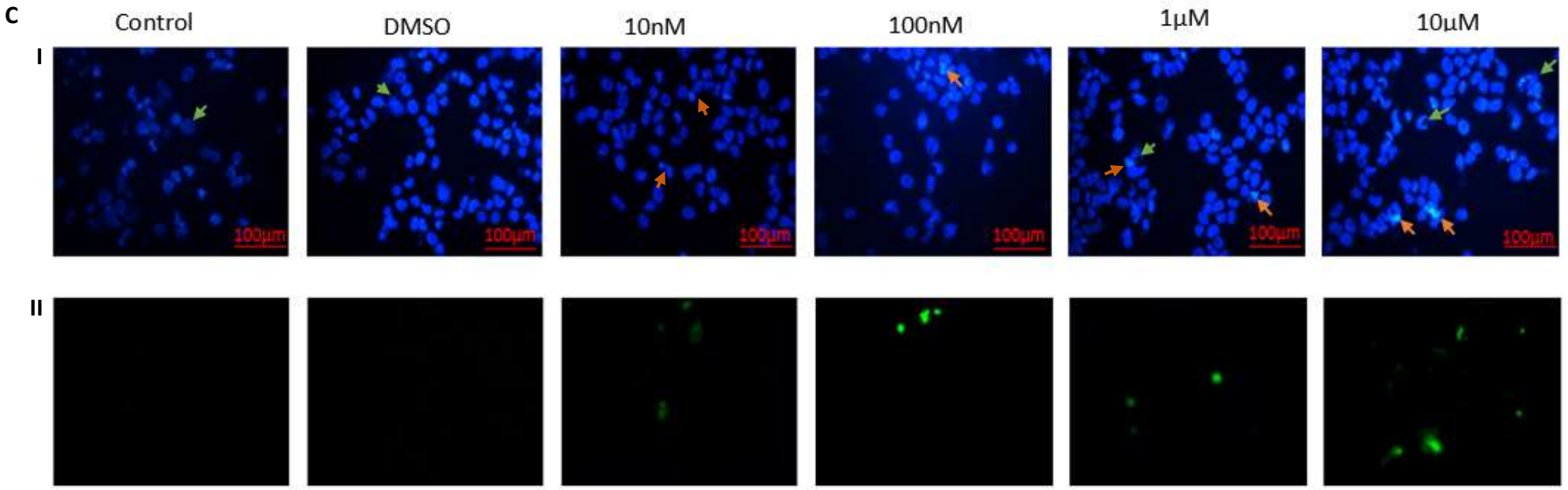


Figure 3.6. Measurement of caspase 3/7 activity in TK6 cells. (A) Cells were treated with increasing concentrations of SPHINX for 72hrs. **(B)** Percentages of apoptotic cells in TK6 cells. **(C)** Examination of TK6 cell morphology for caspase 3/7 activity. **I**. Micrograph represents Hoechst stain of the total cell at excitation between 420-495nm band width bypass. Arrows (green) represents necrotic cells marked by swelling of the cell and loss of cytoplasmic membrane. Apoptotic cells (orange). **II**. Corresponding image stained with cleaved caspase 3/7 fluorescent dye. *n*=3.

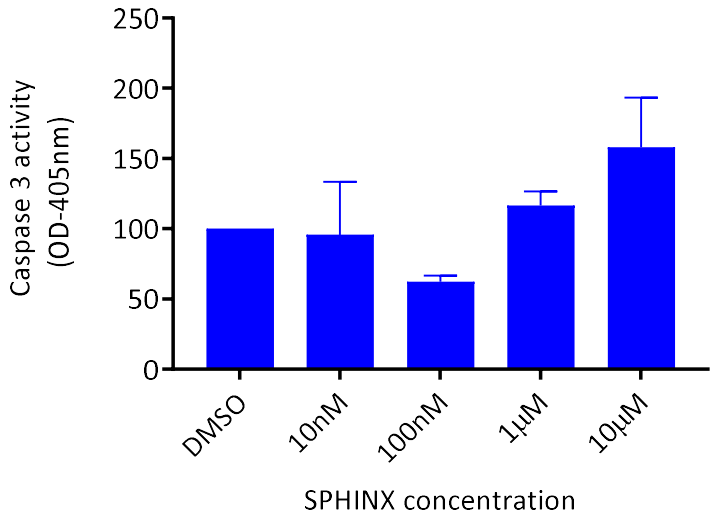
Lymphoblastic TK6 cells were stained with cleaved caspase 3/7 fluorescent dye and viewed under the microscope equipped with a fluorescent lamp. Since cells were in suspension culture, it was difficult to accurately view the same field. Therefore, cells were spun on to a microscope slide post staining with cleaved caspase 3/7 and counterstained using Hoechst fluorescent dye. Micrograph of the Hoechst dye (Figure 3.6 CI) represents the total cell found in a single field viewed per slide. Whereas Figure 3.6CII represent the total number of cells positive for caspase 3/7 in the corresponding field as the Hoechst. The percentage number of cells positive for caspase 3/7 relative to the total number of corresponding cells was calculated in TK6 cells and result found no apparent increase in caspase 3/7 positive cells with increasing concentration of SPHINX and when compared to the DMSO control (Figure 3.6B). This is further evidence that SPHINX has no effect on TK6 cell viability.

3.2.4.2 *Effect of SRPK1 inhibition on caspase activity in K562 cells*

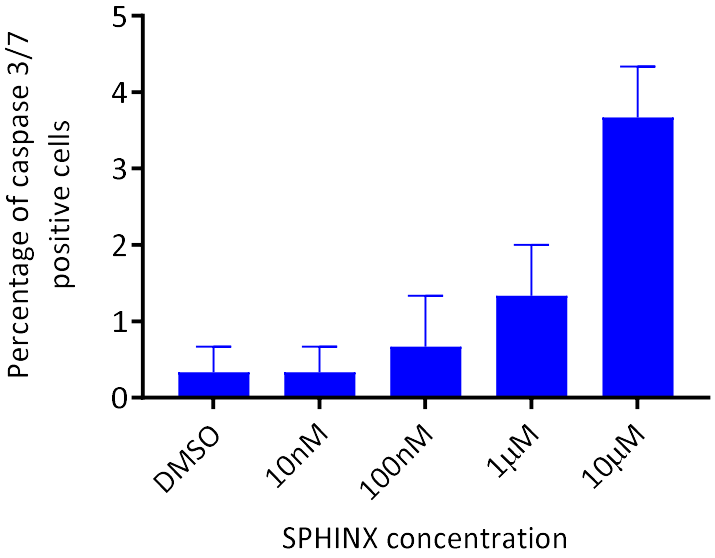
The CML cell line, K562 also did not display a change in cell viability measured via cell count using trypan blue. It was further investigated if the result is consistent by measuring the level of apoptotic activities in K562 cells treated with SPHINX. Spectrophotometric detection of the chromophore p-nitroaniline, which corresponds to the level of caspase 3 activity within the cell was found to be increased at the highest concentration of 10µM/ml, but this was not found to be statistically significant.

Examination of the cleaved caspase 3/7 fluorescent dyes (Figure 3.7C) showed the general cell morphology (I) and the caspase 3/7 positive cells (II). When the positive cells were counted, and percentages taken in relation to the total cell number per field view, there appeared to be an increase in the percentage of positive cells as SPHINX concentrations increased. This was also found not be significant when compared to the DMSO control (Figure 3.7B and C).

A


Caspase 3 activity
(OD-405nm)
250
200
150
100
50
0
DMSO
10nM
100nM
1μM
10μM
SPHINX concentration

B


Percentage of caspase 3/7
positive cells
5
4
3
2
1
0
DMSO
10nM
100nM
1μM
10μM
SPHINX concentration

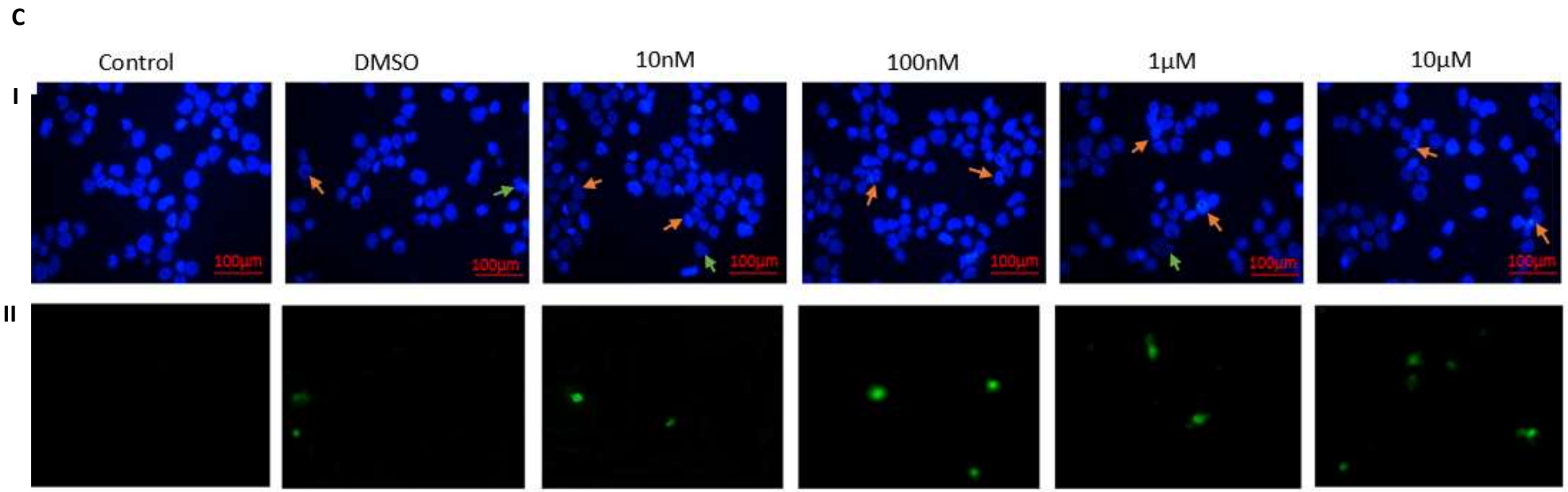
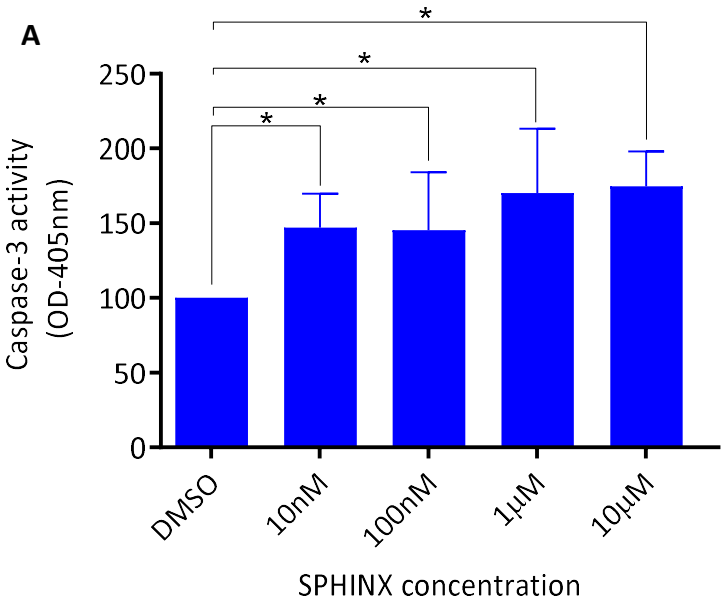
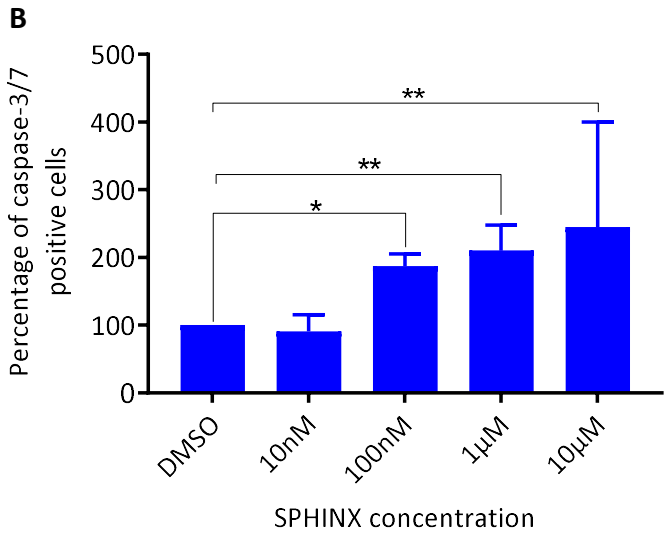


Figure 3.7. Measurement of caspase 3/7 activity in K562 cells. (A) Cells were treated with increasing concentration of SPHINX for 72hrs. (**B**) Percentages of apoptotic cells in K562 cells. **(C)** Examination of K562 cell morphology for caspase 3/7 activity. **I**. Micrograph represents Hoechst stained. Arrows (green) represents necrotic cells and (orange) apoptotic cells. **II.** Corresponding image viewed at a green (FITC) band width. *n*=3.

3.2.4.3 *Effect of SRPK1 inhibition on caspase activity in Kasumi-1 cells*

Kasumi-1 cells appeared to be more sensitive to SPHINX treatment and showed a decrease in cell viability and proliferation which was found to be significant at higher concentrations. To further investigate whether the decrease in Kasumi-1 cell viability is due to cells death via apoptosis, the level of caspase 3 activity was measured (Figure 3.8). A significant percentage increase in caspase 3 activity was observed. The level of activity was found to increase progressively with SPHINX concentration (Figure 3.8A).





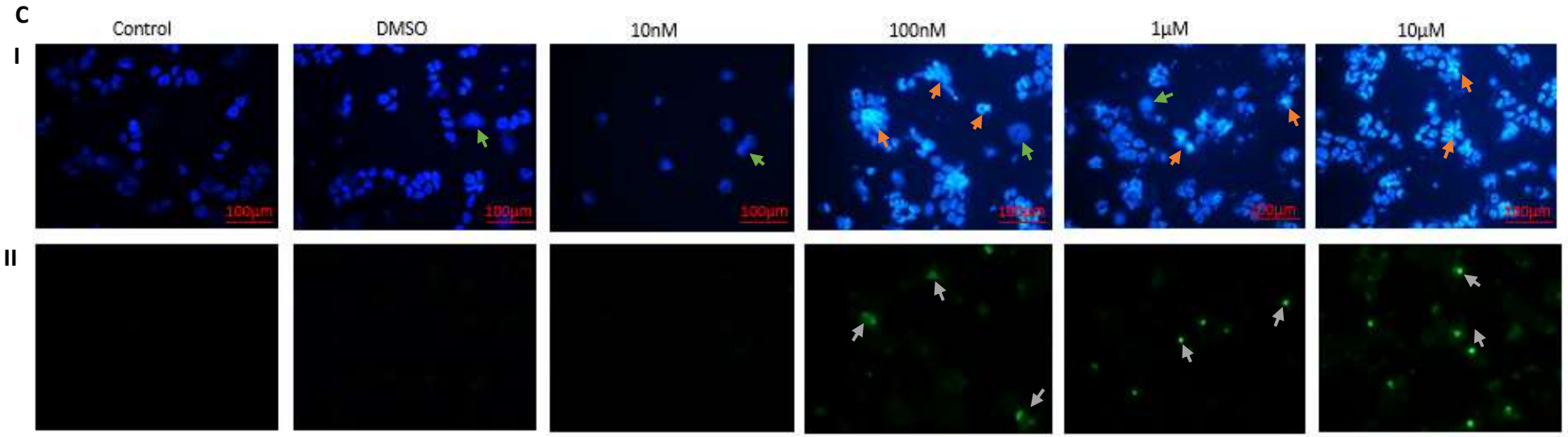


Figure 3.8. Measurement of caspase 3/7 activity in Kasumi-1 cells. (A) Cells were treated with increasing concentration of SPHINX for 72hrs. A significant difference in caspase 3 activity was found at concentrations of 10nm to 10µM (**P*= 0.044; 0.038; 0.024; 0.025 compared to DMSO. (**B**) Percentages of apoptotic cells. Significant increase in caspase 3/7 positive cells were observed at higher concentrations of 100nM and 10µM (**P*= 0.01; ***P*= 0.009; ***P*= 0.006) when SPHINX treated group were compared to the DMSO control. **(C)** Examination of Kasumi-1 cell morphology for caspase 3/7 activity. Cells treated with varying concentrations of SPHINX for 72hrs were stained cleaved caspase 3/7 fluorescent dye. **I**. Micrograph represents Hoechst stained. Arrows (green) represents necrotic cells and (orange) apoptotic cells. **II.** Corresponding image viewed at a green (FITC) band width. Arrows indicate caspase 3/7 positive (green). *n*=3.

The level of caspase 3 activity was further confirmed using fluorescent dye which was able to pick up cleaved caspase 3/7. Micrograph (Figure 3.8CI) of Kasumi-1 cells treated with increasing concentrations of SPHINX for 72hrs presented with cells with abnormal morphology, which appear to be distorted and hyper-fluorescent; an indication of chromatin fragmentation. Concentrations of 100nM- 10µM also showed fragments of particles, which are probably a product of the broken-down cells. When the percentage of caspase 3/7 positive cells were analysed in relation to the total number of cells per field view, the number of positive cells increased with concentration (Figure 3.8CII) when compared to the DMSO control. This result further suggests that the inhibition of SRPK1 in the AML cell, Kasumi-1, results in cell death by apoptosis and indicated that SRPK1 could be playing a crucial role in Kasumi-1 cell growth and viability and hence the propagation of the disease.

3.3 Effect of SPHINX on levels of SRPK1, VEGF and alternative splicing of a panel of apoptotic genes in TK6 cells

Studies have shown that nearly 95% of all human genes are alternatively spliced, and the process is disrupted in cancer cells (Wang *et al*., 2008). Aberrant splicing in most cancers is known to be responsible for cancer of progressive phenotype seen in prostate cancer, basal-like and triple negative breast cancer, which further determine the response of these cancer cells to treatment (Mavrou *et al*., 2014; van Roosmalen *et al*., 2015). Inhibition of SRPK1 has been shown to result in splicing in favour of the anti-angiogenic phenotype of VEGF165b in cancer cells, podocytes and vascular disease (Amin *et al*., 2011; Oltean *et al*., 2012; Mavrou *et al*., 2014). It has also been shown that SRPK1 through SRSF1 is able to alter splicing of caspase 9 favouring pro-apoptotic (9a) isoform (Shultz *et al*., 2011). Therefore, the effect of SRPK1 inhibition on splicing in leukaemic cells was investigated since inhibition was seen to affect cell viability and growth, in some cells.

3.3.1 Effect of SPHINX on SRPK1 expression

TK6 cells were treated with increasing concentrations of SPHINX for up to 48hrs and total RNA extracted. Complementary DNA (cDNA) was synthesised using the reverse transcription method and amplified by PCR using appropriate primers. Conditions for amplification are described in the method section (chapter 2.7.4).

To examine a potential auto-regulatory effect, levels of SRPK1 expression was measured. There was no significant change in SRPK1 levels when treatment was compared to control (Figure 3.9 A-C).

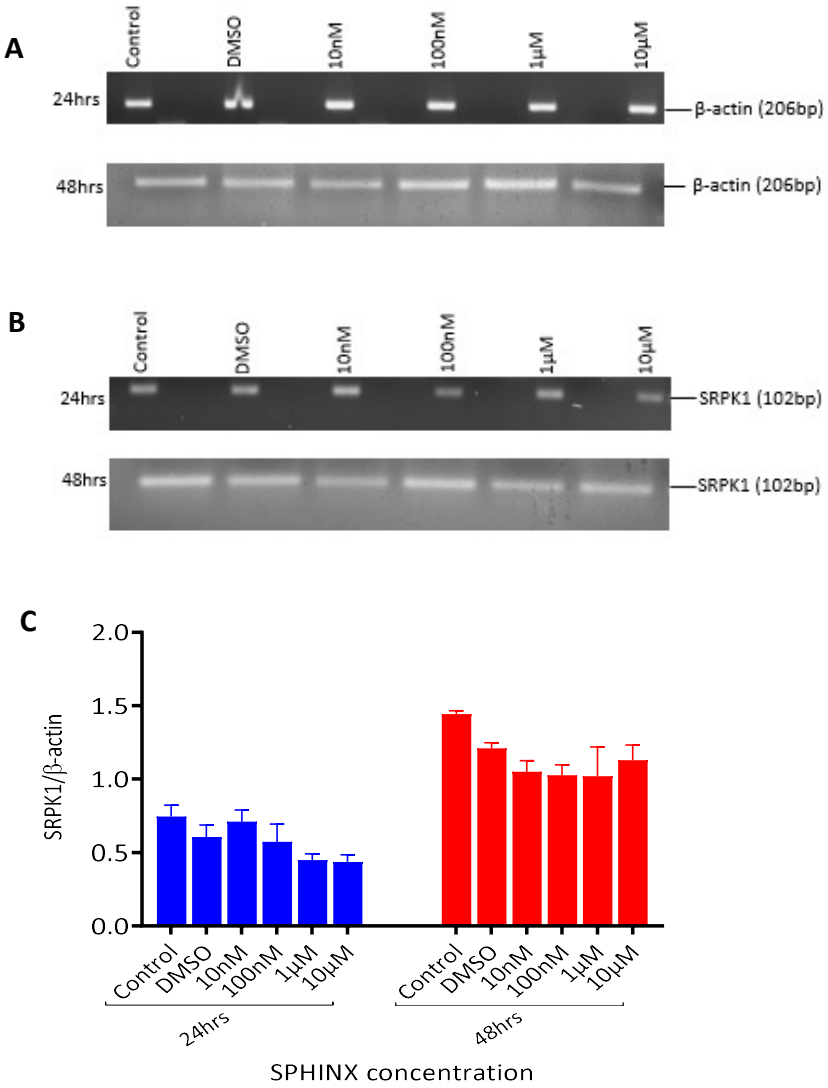


Figure 3.9. SRPK1 levels in SPHINX treated TK6 cells. Cells were treated with increasing concentration of SPHINX for 48hrs. PCR was performed using SRPK1 specific primers. (**A**) TK6 β-actin controls for 24hrs and 48hrs. Representative PCR result for 24hrs and 48hrs post-treatment (**B**). **C**. Densitometry of SRPK1 levels normalized to β-actin *n*=3.

3.3.2 Effect of SPHINX on VEGF alternative splicing

Previous work has shown that inhibition or knockdown of SRPK1 in cancer cells resulted in a change in VEGF splicing to a more anti-angiogenic isoform, VEGF165b. We investigated if the inhibition of SRPK1 in TK6 cells resulted in a switch in splicing. No switch in VEGF splicing was observed (Figure 3.10B-C).

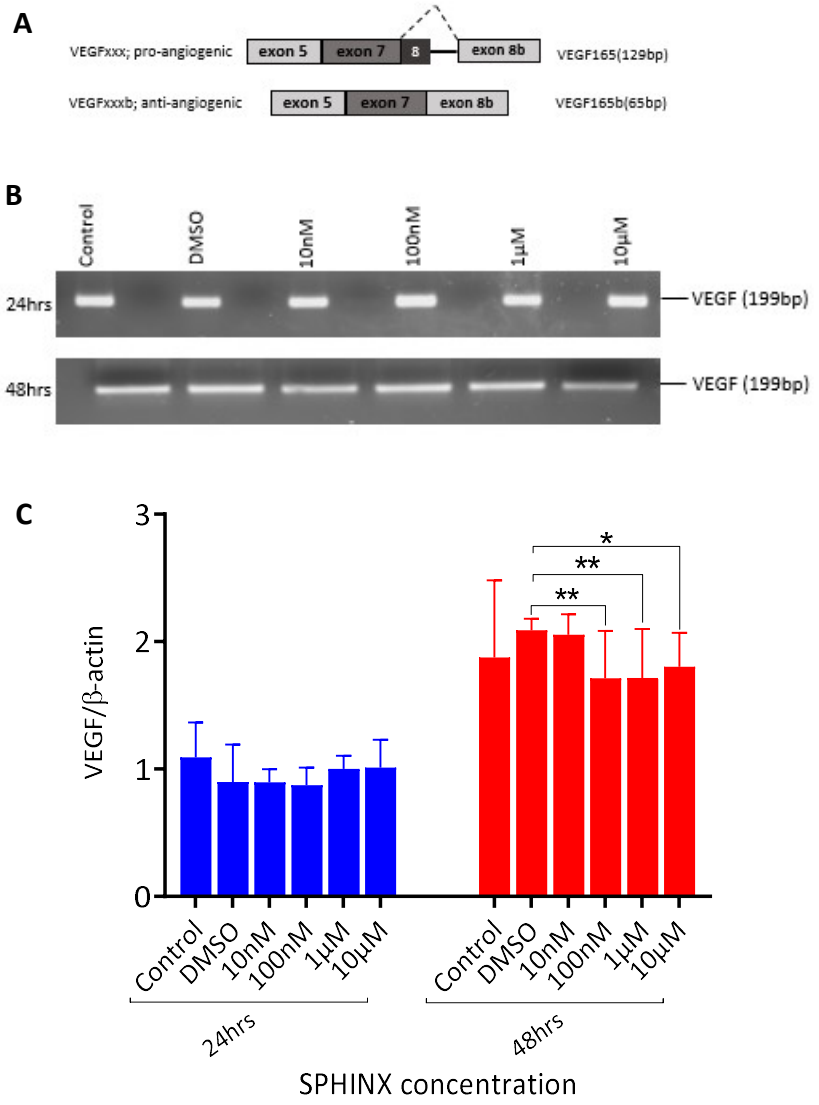


Figure 3.10. Alternative splicing of VEGF in SPHINX treated TK6 cells. Cells were treated with increasing concentrations of SPHINX for 48hrs. PCR was performed using VEGF specific primers. **B**. Representation of PCR amplicon sizes for 24hrs and 48hrs. **C**. Densitometry for VEGF amplicon normalized to β-actin. DMSO was compared to each SPHINX treated group at each time point. *n*=3.

3.3.3 Effect of SPHINX on Bclx alternative splicing

To confirm that inhibition of SRPK1 in TK6 cells with increasing concentration of SPHINX does not result in increased apoptosis, hence reduced cell viability, as previously observed, changes in Bclx splicing was investigated. No change in splicing of Bclx was observed. There was a higher level of anti-apoptotic Bcl-xl to pro-apoptotic phenotype Bcl-xs and this was not altered by SPHINX (Figure 3.11B-C).

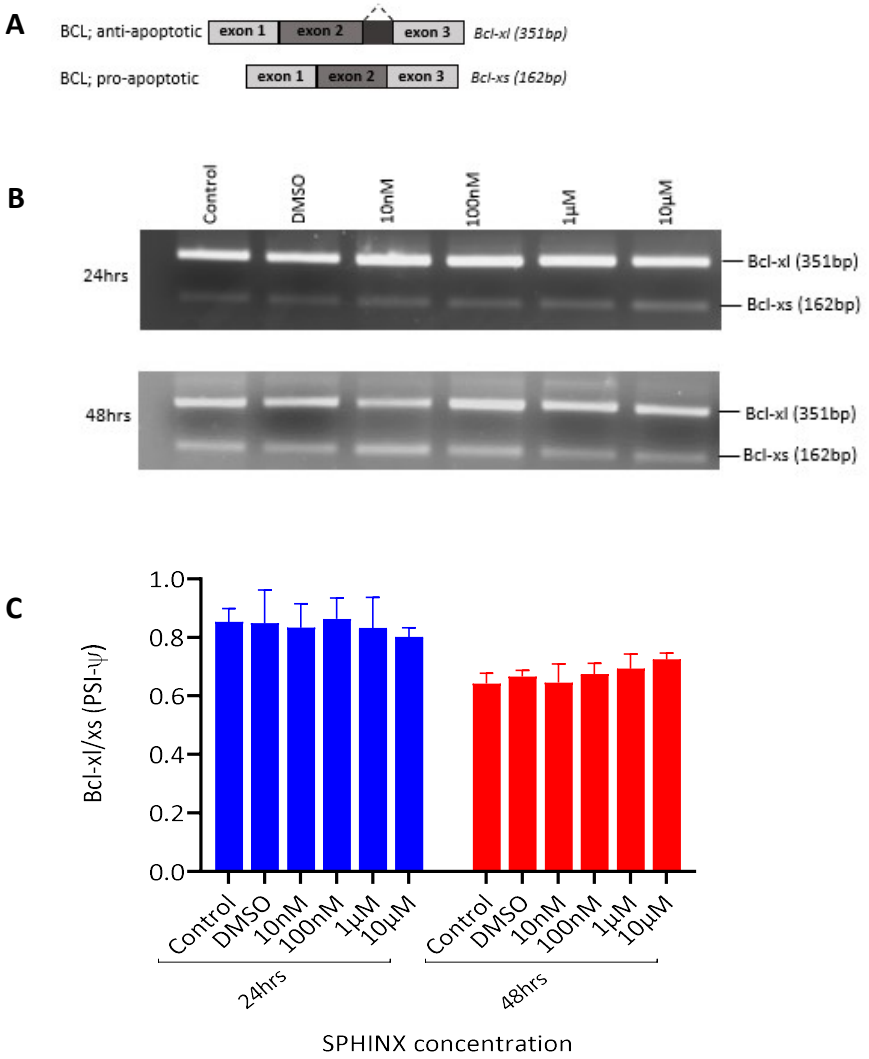


Figure 3.11. Alternative splicing of BCLx in SPHINX treated TK6 cells. Cells were treated with increasing concentrations of SPHINX for 48hrs. PCR was performed using Bclx specific primers. **B.** Representation of PCR amplicon sizes for 24hrs and 48hrs. **C**. PCR image quantification showing percentage of splice inclusion (PSI-Ψ) in Bcl-xl/xs isoform. Figures represent three experimental repeats, *n*=3.

3.3.4 Effect of SPHINX on Apaf-1 alternative splicing

Splice ratio of Apaf-1xl/1s showed no significant difference when treated cells were compared to the controls. Level of pro-apoptotic Apaf-1xl appears to be consistent between treated group and control (Figure 3.12B-C)

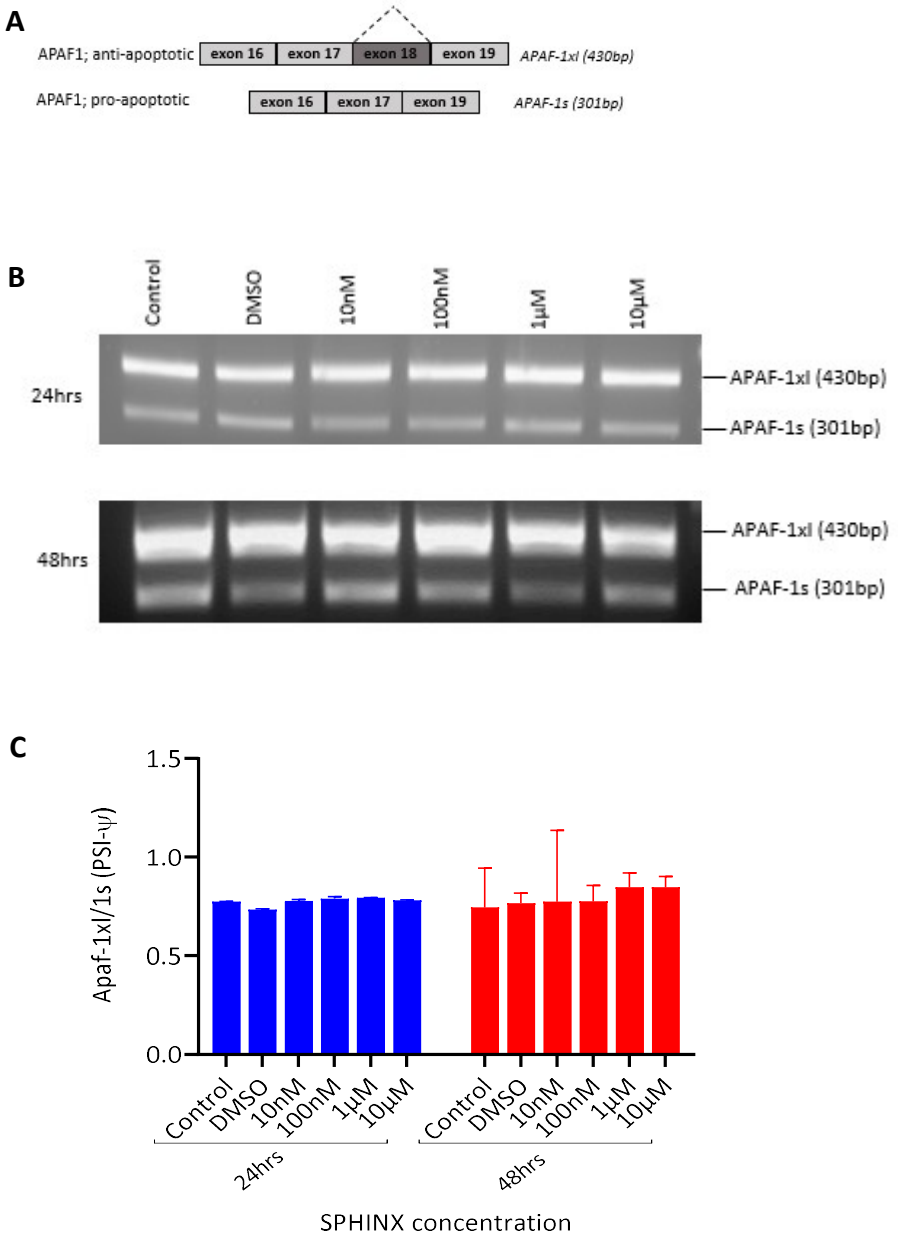


Figure 3.12. Alternative splicing of Apaf1 in SPHINX treated TK6 cells. Cells were treated with increasing concentrations of SPHINX for 48hrs. PCR was performed using Apaf1 specific primers. Representation of PCR amplicon sizes for 24hrs and 48hrs (**B**). **C**. Densitometry showing Apaf-1xl/1s PSI-Ψ values for 24hrs and 48hrs. *n*=3.

3.3.5 Effect of SPHINX on caspase 9 alternative splicing

When caspase 9 was investigated for a switch in splicing in TK6 cells following SRPK1 inhibition with SPHINX, there was also no change in splicing observed. There was an apparent increase in caspase 9a pro-apoptotic isoform at higher concentrations (100nM-10µM) at 48hrs. This was not found to be statistically significant when compared to the DMSO control (Figure 3.13B-C).

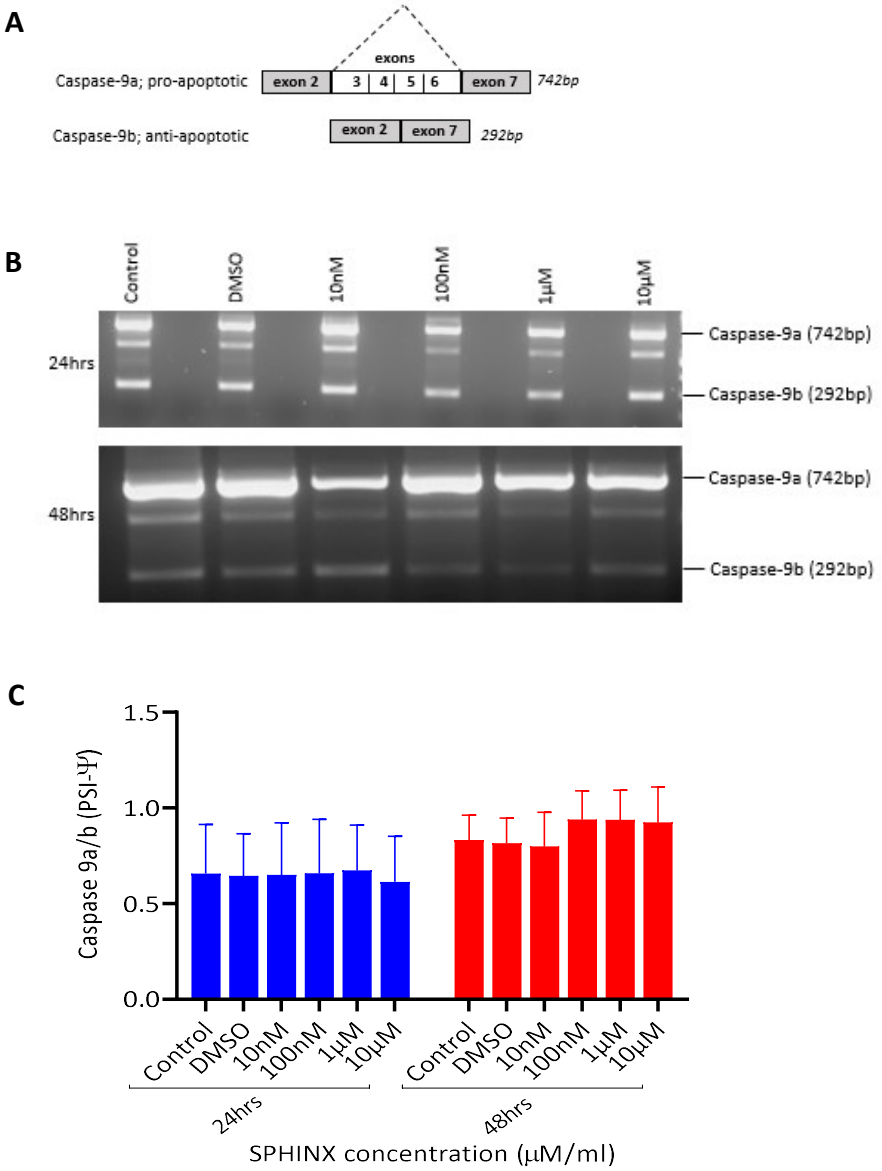


Figure 3.13. Alternative splicing of caspase 9 in SPHINX treated TK6 cells. Cells were treated with increasing concentrations of SPHINX for 48hrs. PCR was performed using caspase 9 specific primers. (**B**) PCR amplicon sizes for caspase 9 at 24hrs and 48hrs **C**. Densitometry showing caspase 9a/b PSI-Ψ values for 24hrs and 48hrs. DMSO vs treatment at 48hrs 100nM-10µM (*P*= 0.28; 0.29; 0.39). *n*=3.

3.4 Effect of SPHINX on protein levels of SRPK1, SRSF1 and on the phosphorylation of SR protein in TK6 cells.

3.4.1 *Effect of SPHINX on SRPK1 protein levels*

There was no change in SRPK1 levels when compared to DMSO control or between 24hrs and 48hrs (Figure 3.14) in TK6 cells.

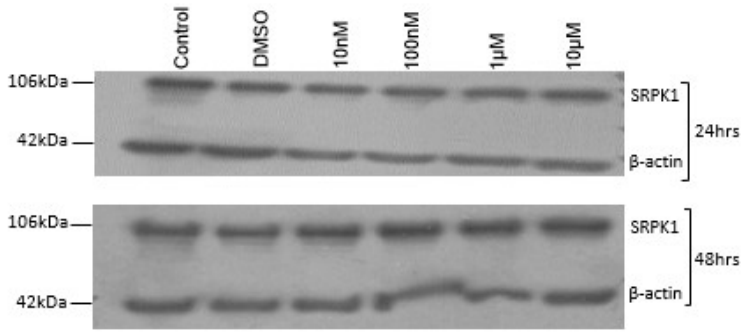


Figure 3.14 SRPK1 protein levels in SPHINX treated TK6 cells. Western blot images for SRPK1 in TK6 cells treated with SPHINX for up to 48hrs with corresponding β-actin. No significant difference in SRPK1 levels. *n*=3.

3.4.2 Effect of SPHINX on SRSF1 protein levels

SRSF1 is phosphorylated by SRPK1, however, it is also conceivable that the effect of inhibition of SRPK1 may (indirectly) alter total SRSF1 protein levels. To investigate this, an immunoblot for total SRSF1 was performed. At 48hrs, there seemed to be an apparent reduction in total SRSF1 protein level at the 1µM and 10µM concentrations which was found to be statistically significant when SPHINX treatment was compared to DMSO (Figure 3.15A-B).

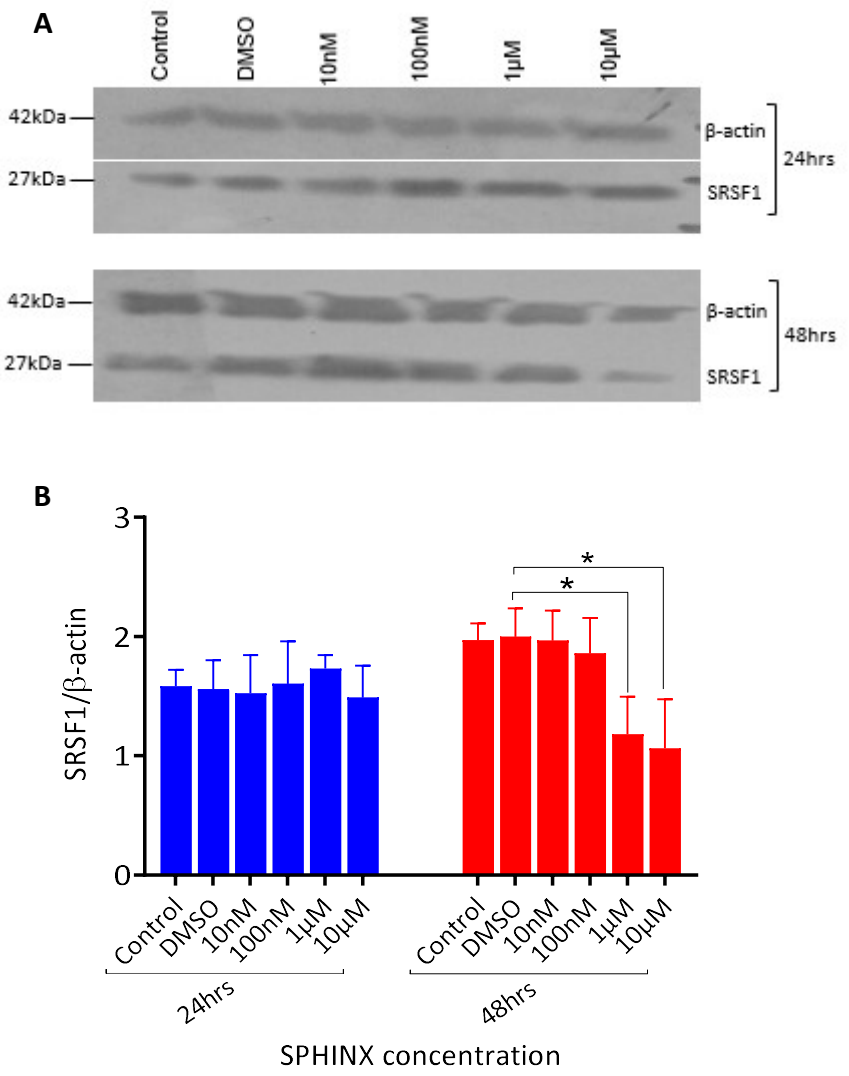


Figure 3.15 SRSF1 protein levels in SPHINX treated TK6 cells. A. Western blot images for SRSF1 in TK6 cells treated with SPHINX for up to 48hrs. **B**. Densitometry of SRSF1 protein level normalized to β-actin. Significant reduction in SRSF1 levels was found at higher concentrations of 1µM and 10µM at 48hrs (*P<0.05) when compared to DMSO. *n*=3.

3.4.3 Effect of SPHINX on SR protein phosphorylation

Since previous studies have shown that SRPK1 inhibition results in reduced phosphorylation of SR-proteins including SRSF1, phosphorylation of SR-proteins was examined in leukaemic cells. An immunoblot was performed using the phospho-antibody which pick up phospho-epitopes of SR-proteins. Levels of the SR-protein were not found to be significantly different in TK6 cells (Figure 3.16).

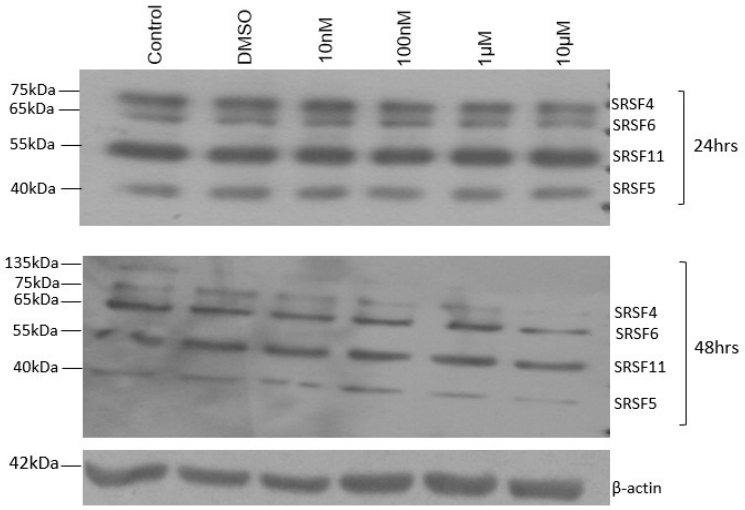


Figure 3.16 Protein levels of phospho-SR proteins in SPHINX treated TK6 cells. Representative western blot images for phospho-SR family protein in TK6 cells. *n*=3.

3.5 Effect of SPHINX on mRNA levels of SRPK1, VEGF and alternative splicing of a panel of apoptotic genes in K562 cells

3.5.1 *Effect of SPHINX on SRPK1 expression*

The effect of SPHINX on SRPK1 mRNA levels in K562 cells was observed following treatment. There were indications of significant changes in SRPK1 expression at higher concentrations of SPHINX at 48hrs compared to time-matched DMSO controls (Figure 3.17B-C).

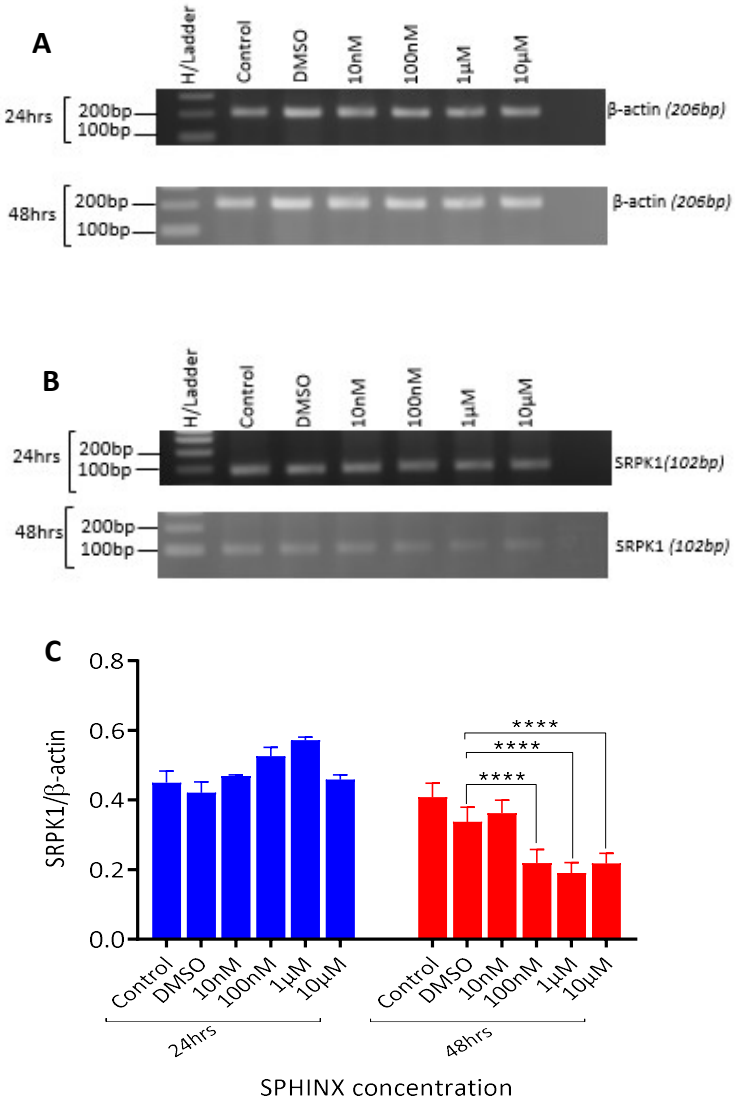


Figure 3.17. SRPK1 levels in SPHINX treated K562 cells. Cells were treated with increasing concentrations of SPHINX for up to 48hrs. PCR was performed using SRPK1 specific primers. (**A**) β-actin loading controls at 24hrs and 48hrs. (**B**) Amplicons of SRPK1 at for 24hrs and 48hrs **C**. Densitometry analysis of SRPK1 expression showing significant decrease (****$P \leq 0.0001$) on SRPK1 levels at higher concentrations at 48hrs, $n=3$.

3.5.2 Effect of SPHINX on VEGF alternative splicing

The Inhibition of SRPK1 had no effect on total levels of VEGF expression (Figures 3.18A-B).

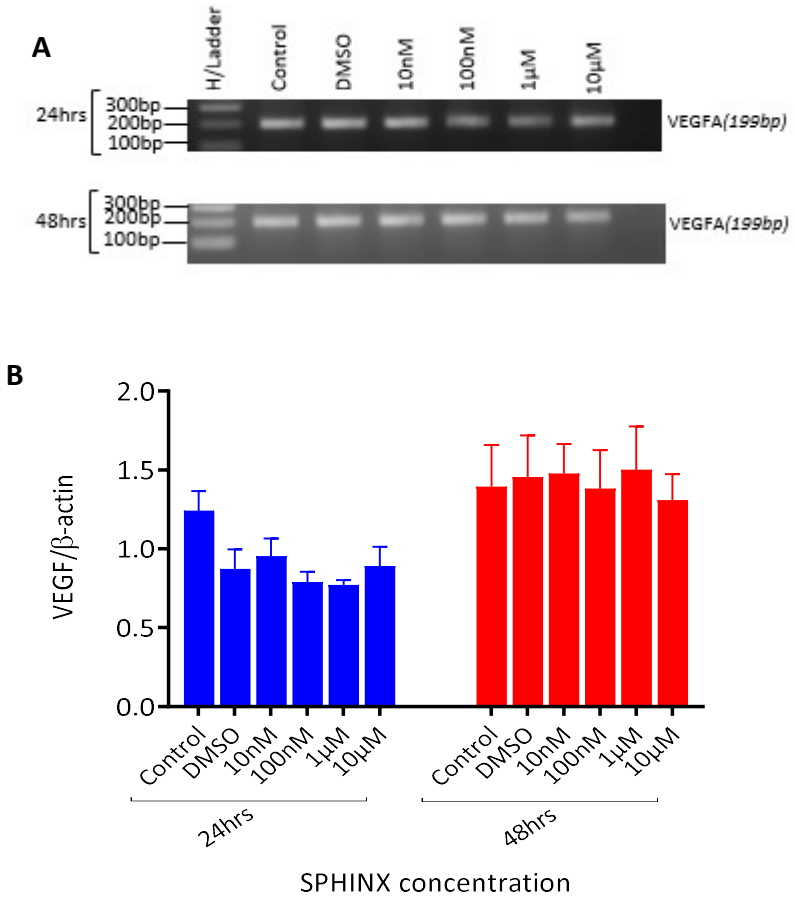


Figure 3.18. Alternative splicing of VEGF in SPHINX treated K562 cells. Cells were treated with increasing concentration of SPHINX for up to 48hrs. PCR was performed using VEGF specific primers. **A**. Representation of VEGF PCR amplicon sizes for 24hrs and 48hrs showing band intensity and size. **B**. Densitometry for VEGF amplicon normalized to β-actin. *n*=3.

3.5.3 *Effect of SPHINX on Bclx alternative splicing*

Result showed two distinct Bclx isoforms (xl and xs) with no switch in splicing to its anti-apoptotic, Bcl-xl or pro-apoptotic, Bcl-xs (Figure 3.19B).

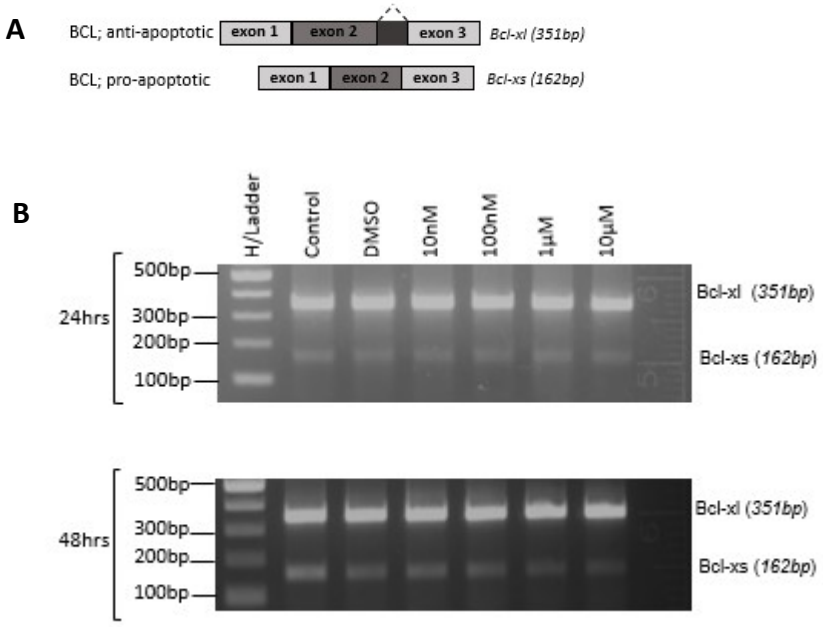


Figure 3.19. Alternative splicing of Bclx in SPHINX treated K562 cells. K562 cells were treated with increasing concentrations of SPHINX for up to 48hrs. **B**. Bclx PCR amplicon sizes for 24hrs and 48hrs showing band intensity and sizes. *n*=3.

3.5.4 *Effect of SPHINX on Apaf-1 alternative splicing*

There was a significant change in Apaf1 alternative splicing following SRPK1 inhibition in K562 cells at 24hrs when SPHINX treatment was compared to DMSO. No change in splicing was observed at both time points (Figure 3.20 B).

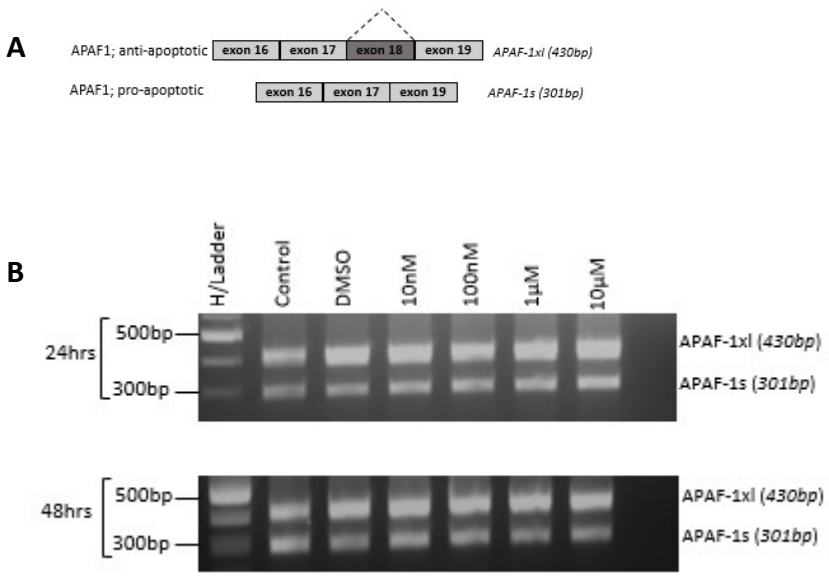


Figure 3.20. Alternative splicing of Apaf1 in SPHINX treated K562 cells. K562 cells were treated with increasing concentrations of SPHINX for up to 48hrs. **B**. Apaf1 PCR amplicon sizes for 24hrs and 48hrs showing band intensity and sizes. *n*=3.

3.5.5 Effect of SPHINX on caspase 9 alternative splicing

Two distinct caspase 9 isoforms (9a/b) are expressed in K562 cells. Following inhibition of SRPK1 in K562 cells, there was no switch in splicing. Levels of caspase 9a were significantly reduced in SPHINX treated group when compared to DMSO control (Figure 3.21C).

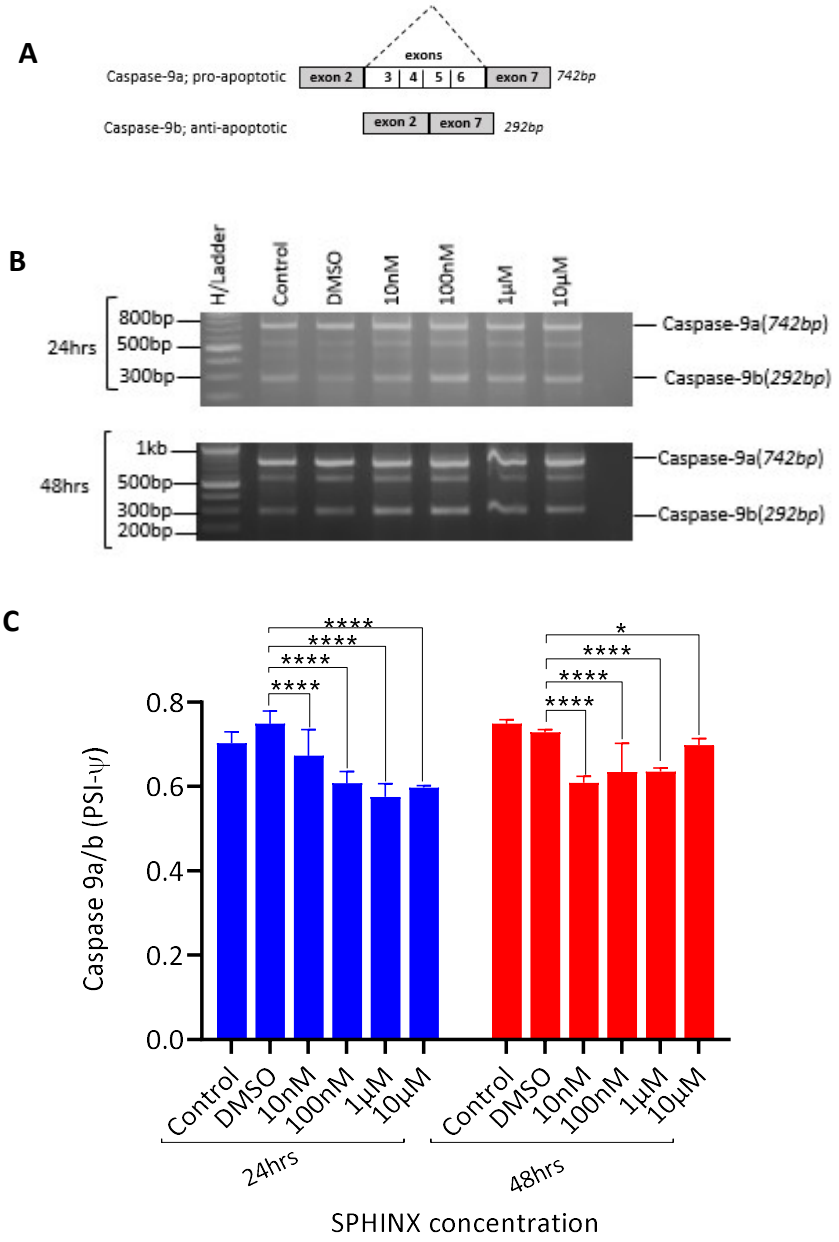


Figure 3.21. Alternative splicing of caspase 9 in SPHINX treated K562 cells. K562 cells treated with increasing concentrations of SPHINX for up to 48hrs. **B**. Representation of caspase 9 PCR amplicon sizes for 24hrs and 48hrs showing band intensity and sizes **C**. Percentage splice inclusion (PSI-Ψ) for caspase 9a/9b. SPHINX treatment compared to DMSO for 24hrs (****P<0.0001) and 48hrs (****P<0.0001 and *P=0.038). n=3.

3.6 Effect of SPHINX on protein levels of SRPK1, SRSF1 and on the phosphorylation of SR-protein in K562 cells.

3.6.1 *Effect of SPHINX on SRPK1 protein levels*

To investigate the effect of SRPK1 inhibition on SRPK1 protein levels and SR-protein phosphorylation, immunoblotting was performed. Protein levels of SRPK1 were found to be increased at concentrations of 10µM at 24hrs and 48hrs when compared to DMSO control (Figures 3.22A-C).

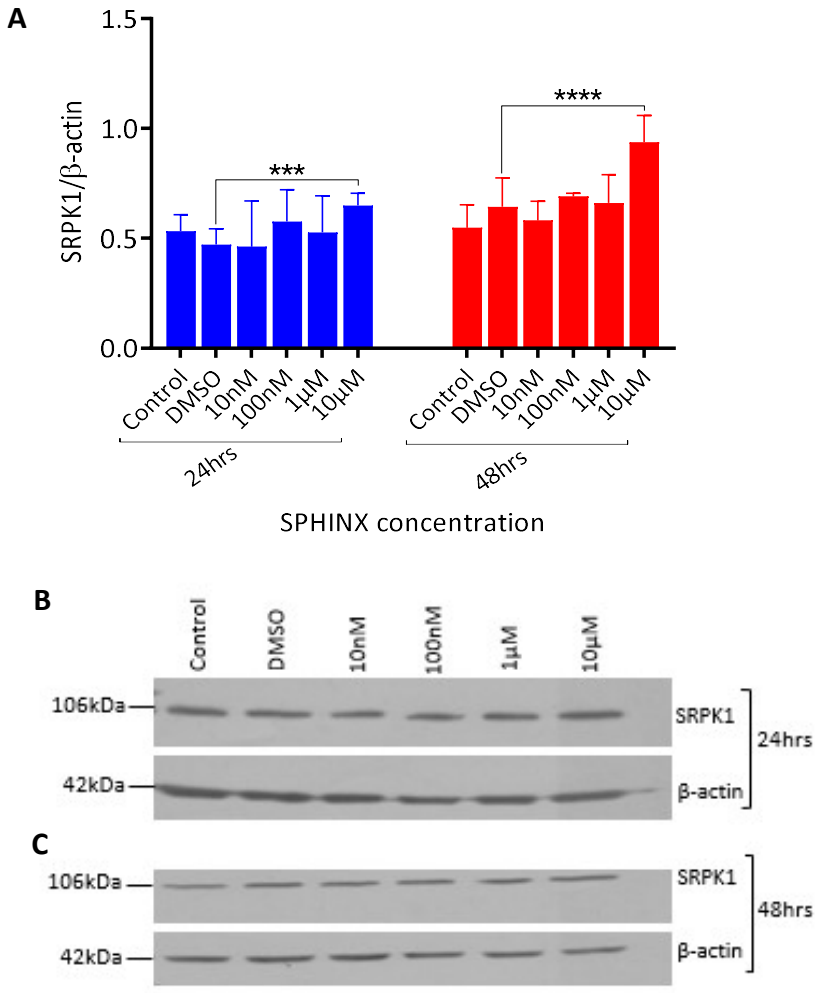


Figure 3.22 Protein levels of SRPK1 in SPHINX treated K562 cells. Densitometry for SRPK1 protein level normalized against β-actin. **A**. Result showing increased SRPK1 protein levels following higher concentrations of SPHINX for 24hrs and 48hrs (***P= 0.0008 and ****P<0.0001 respectively). **B**. and **C**. showing western blot images for SRPK1 with corresponding β-actin loading control. n=3.

3.6.2 Effect of SPHINX on SRSF1 protein levels

Protein levels of SRSF1 appeared to increase at higher concentrations of SPHINX inhibition at 24hrs but not at 48hrs. These differences were not found to be significant (Figures 3.23A-C).

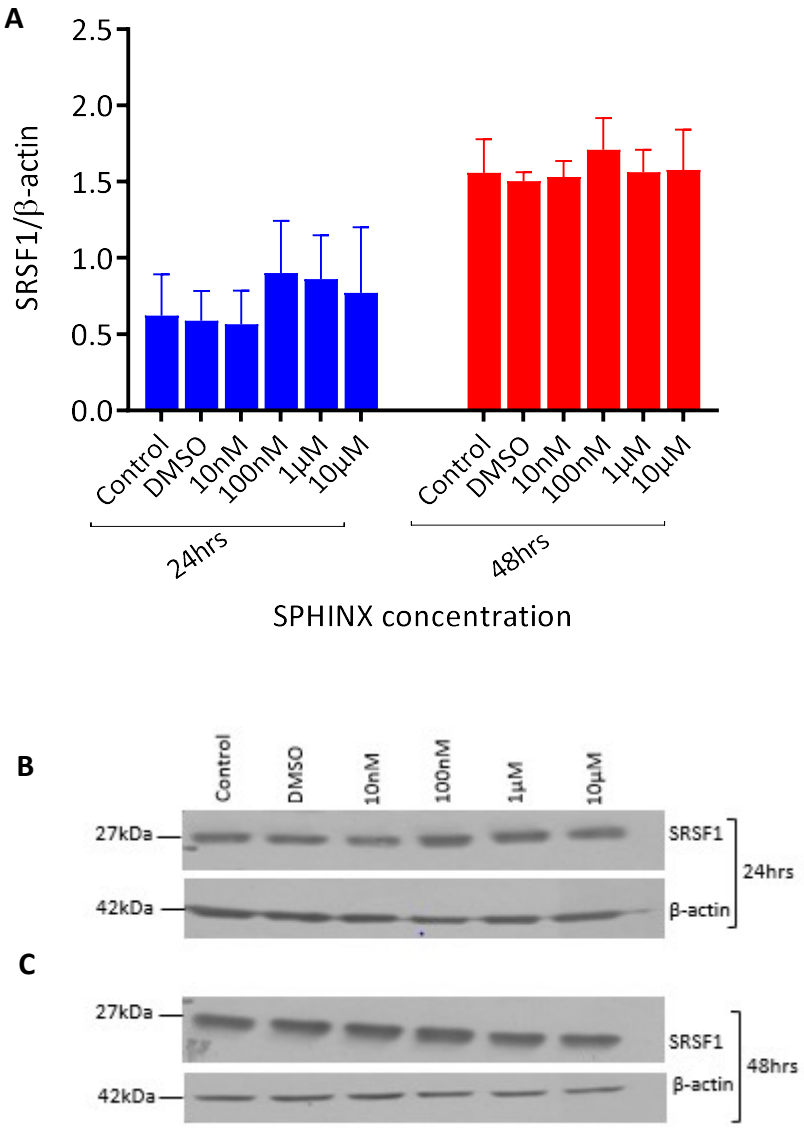


Figure 3.23 Protein levels of SRSF1 in SPHINX treated K562 cells. A. Densitometry for total SRSF1 protein level normalized to β-actin. **B**. and **C**. Western blot images for SRPK1 with corresponding β-actin loading control. *n*=3.

3.6.3 *Effect of SPHINX on SR protein phosphorylation*

An investigation into the effect of SPHINX on levels of SR-protein phosphorylation was also investigated in K562 cells. Interestingly, unlike the TK6 cells, levels of SRSF6 in K562 cell were nearly undetected both in the treatment group and controls (Figure 3.24). This might be an indication of the different abundance of each splice factor in different cell lines. Levels of phospho SRSF4 and SRSF5 were found to be reduced at higher concentrations at 48hrs with no change in phosphorylation observed in SRSF11 (Figure 3.24).

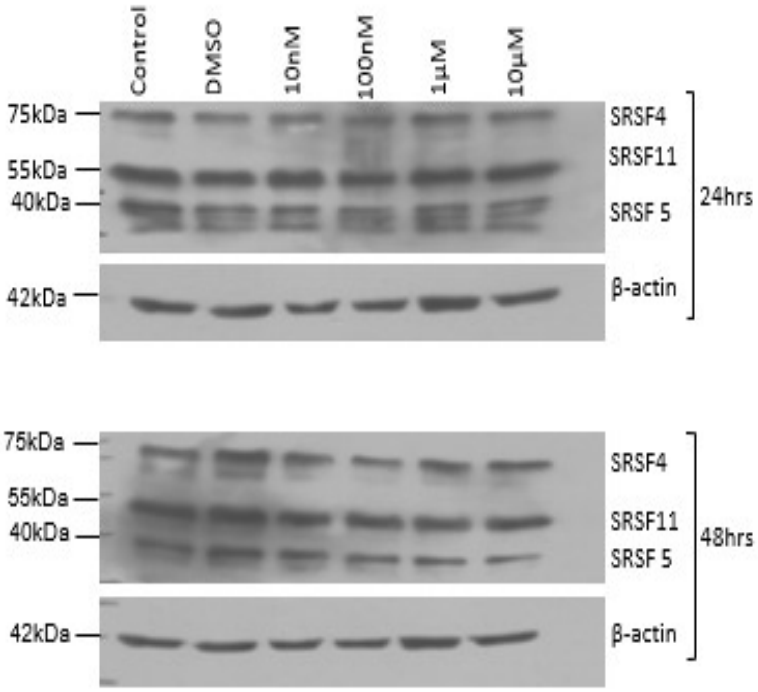
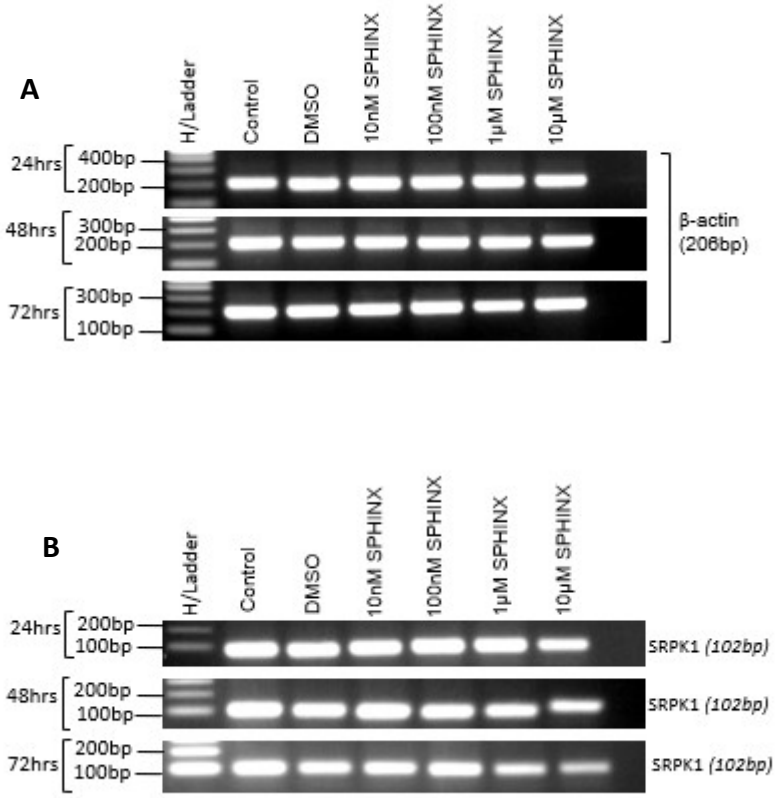


Figure 3.24 Protein levels of phospho-SR proteins in SPHINX treated K562 cells. Densitometry of phospho-SR protein level normalized with β-actin. *p*SRSF4 and *p*SRSF5 showing significant decreases in phosphorylation at 48hrs at higher concentrations *n*=3.

3.7 Effect of SPHINX on the level of SRPK1, VEGF RNA and alternative splicing of a panel of apoptotic genes in Kasumi-1 cells

3.7.1 *Effect of SPHINX on SRPK1 expression*

Results by Siqueira *et al*., (2015) indicates that each cell line responds differently to SRPK1 inhibition despite having a common origin from the white blood cell. This may be related to the unique mutations that each cell line has acquired, how quickly cells are able to get rid of factors, which are considered alien to the cells, and the functional importance of SRPK1 in the cell. In Kasumi-1 cells, SRPK1 levels appear to decrease with increasing concentration of SPHINX (Figure 3.25B-C).



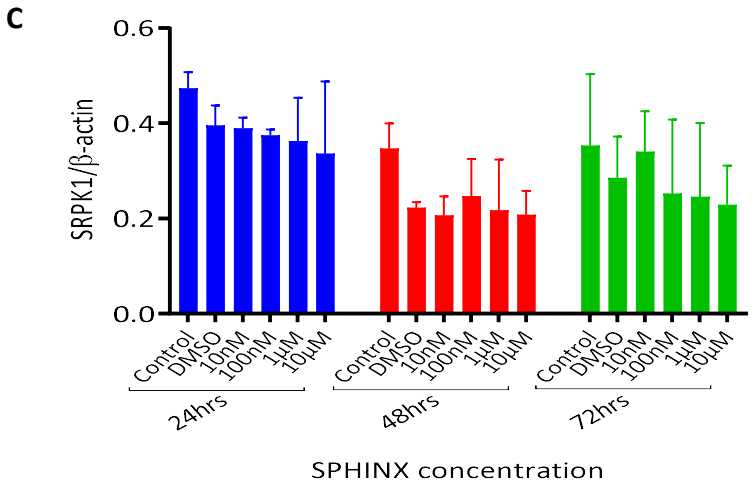


Figure 3.25. SRPK1 RNA levels in SPHINX treated Kasumi-1 cells. Cells were treated with increasing concentrations of SPHINX for up to 72hrs. PCR was performed using SRPK1 specific primers. **A**. Representation of β-actin PCR amplicon sizes at 24hrs, 48hrs and 72hrs. **B**. SRPK1 amplicons for 24hrs, 48hrs and 72hrs. **C.** Densitometry for SRPK1 amplicon normalized to β-actin, *n*=3.

3.7.2 Effect of SPHINX on VEGF alternative splicing

Levels of total VEGF was unchanged at 24hrs with increasing concentration of SPHINX inhibition. At 48hrs and 72hrs, total VEGF levels were significantly different from the controls with levels at 72hrs showing an apparent increase. This was not however dose dependent. No alternative splicing event was observed, specifically the anti-angiogenic VEGF165b isoform, which was not detected by standard PCR (Figure 3.26A-B).

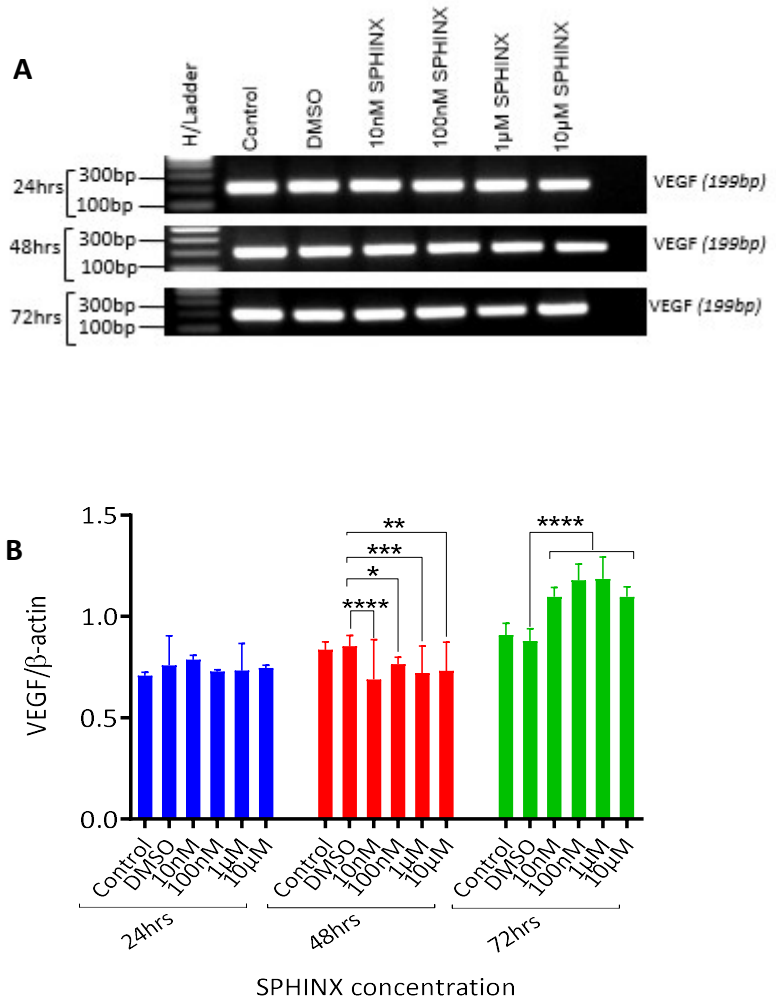


Figure 3.26. Alternative splicing of VEGF in SPHINX treated Kasumi-1 cells. Cells were treated with increasing concentrations of SPHINX for up to 72hrs. **A**. Representation of VEGF PCR amplicon sizes for 24hrs, 48hrs and 72hrs **B**. Densitometry for total VEGF normalized to β-actin. Significant changes were observed in VEGF at 48hrs (P<0.05) and 72hrs (****P<0.0001) when treated groups were compared to DMSO, n=3.

3.7.3 Effect of SPHINX of Bclx alternative splicing

Levels of Bclx showed an apparent change in splicing towards Bcl-xs pro-apoptotic isoform in treated groups when compared to controls (Figure 3.27B).

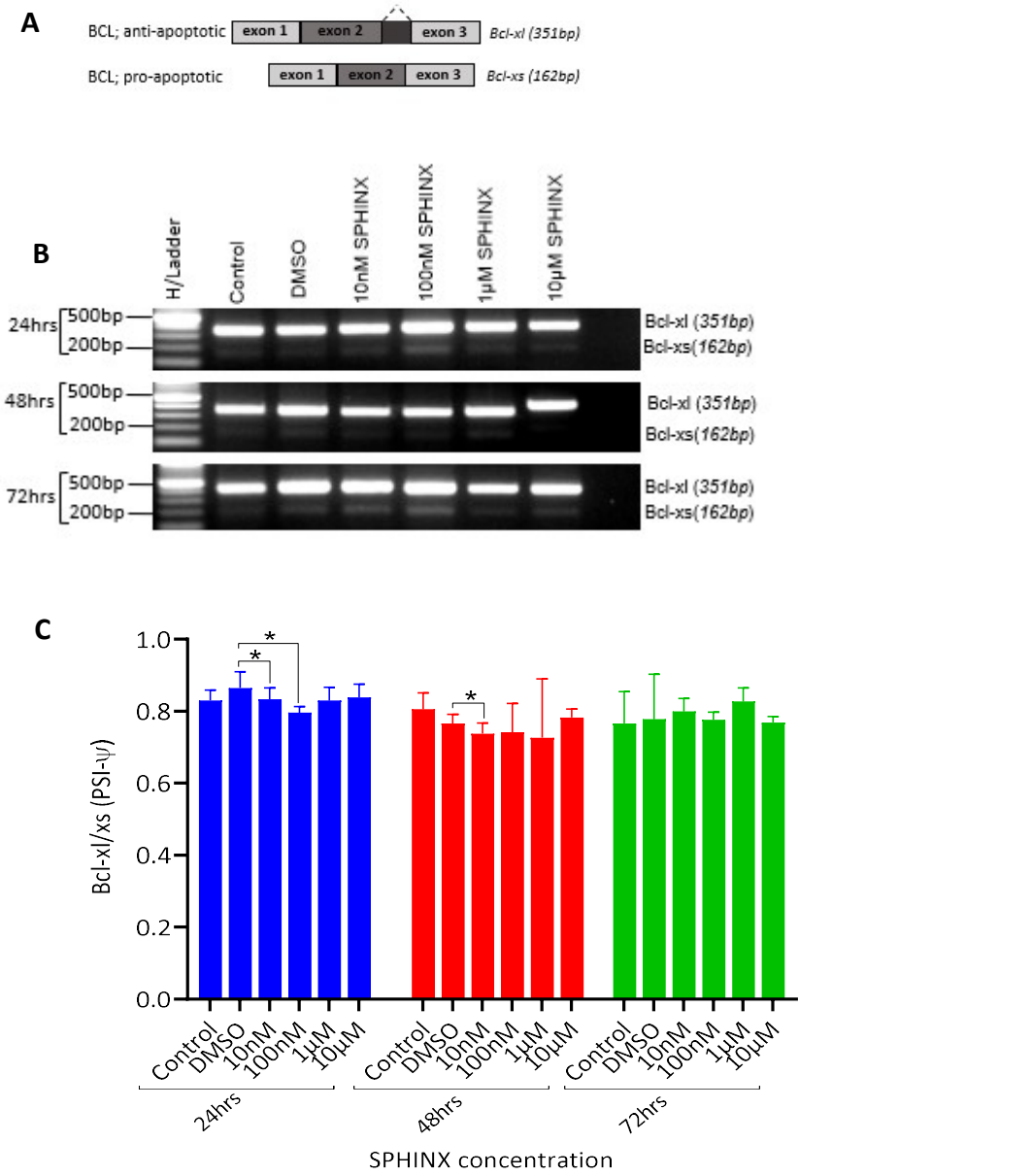


Figure 3.27. Alternative splicing of Bclx in SPHINX treated Kasumi-1 cells. Cells were treated with increasing concentrations of SPHINX for up to 72hrs. PCR was performed using Bclx specific primers. **B**. Representation of Bclx PCR amplicon sizes for 24hrs, 48hrs and 72hrs **C**. PSI-Ψ values for Bcl-xl/xs PSI-Ψ values. Statistical analysis for DMSO versus SPHINX for 24hrs (**P*<0.05), 48hrs (**P*<0.05) and 72hrs. *n*=3.

3.7.4 Effect of SPHINX on Apaf1 alternative splicing

There was no significant change in Apaf1 alternative splicing at 24hrs to 72hrs time points. Although, there was less expression of the pro-apoptotic isoforms at 24hrs and 48hrs especially at 10µM concentration. (Figure 3.28B-C).

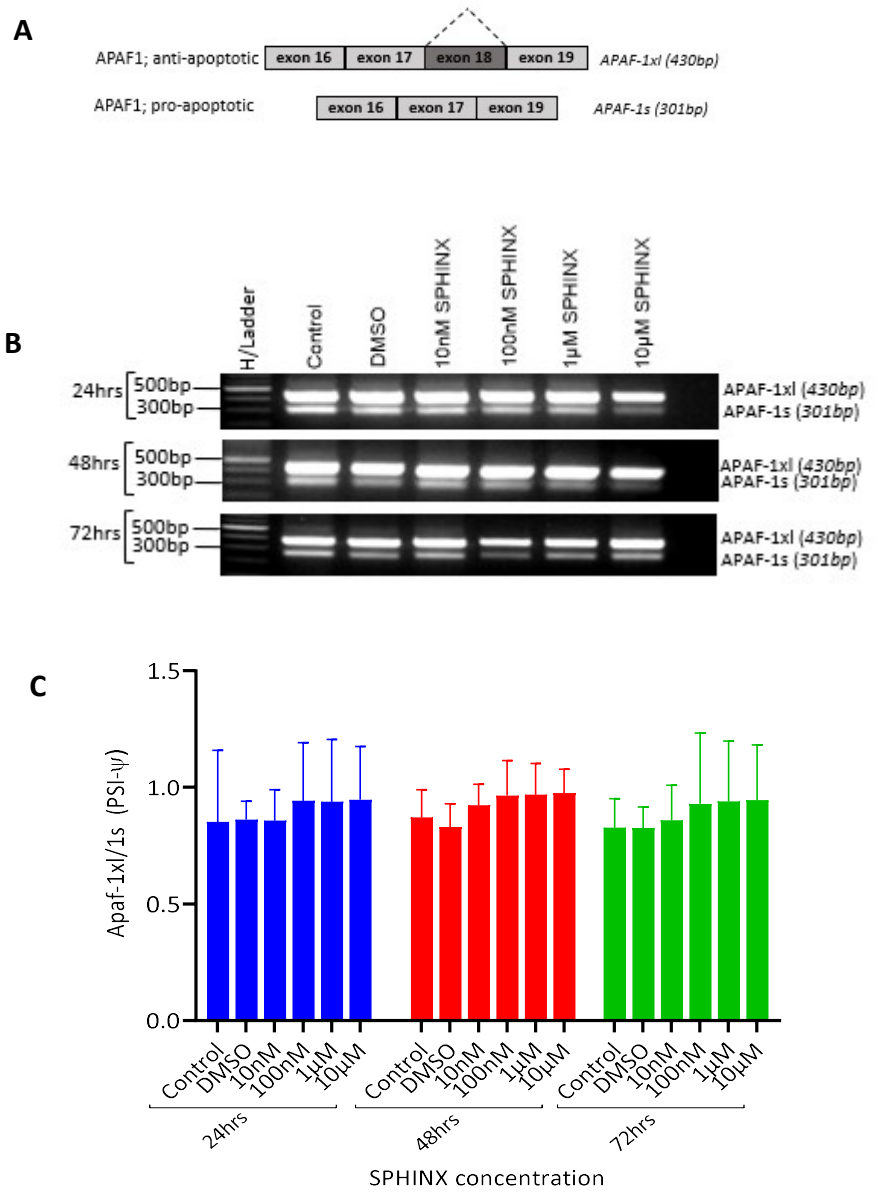


Figure 3.28. Alternative splicing of Apaf1 in SPHINX treated Kasumi-1 cells. Cells were treated with increasing concentrations of SPHINX for up to 72hrs. PCR was performed using Apaf1 specific primers. **B**. Representation of Apaf1 PCR amplicon sizes for 24hrs, 48hrs and 72hrs. **C**. Percentage splice inclusion (PSI-Ψ) for Apaf-1xl/1s for 24hrs -72hrs, n=3.

3.7.5 Effect of SPHINX on caspase 9 alternative splicing

Caspase 9 alternative splicing was affected by SRPK1 inhibition using SPHINX. Isoforms of caspase 9 were found to be decreased at higher contractions of SPHINX. Levels were found to be significantly different when compared to DMSO control (Figure 3.29B-C).

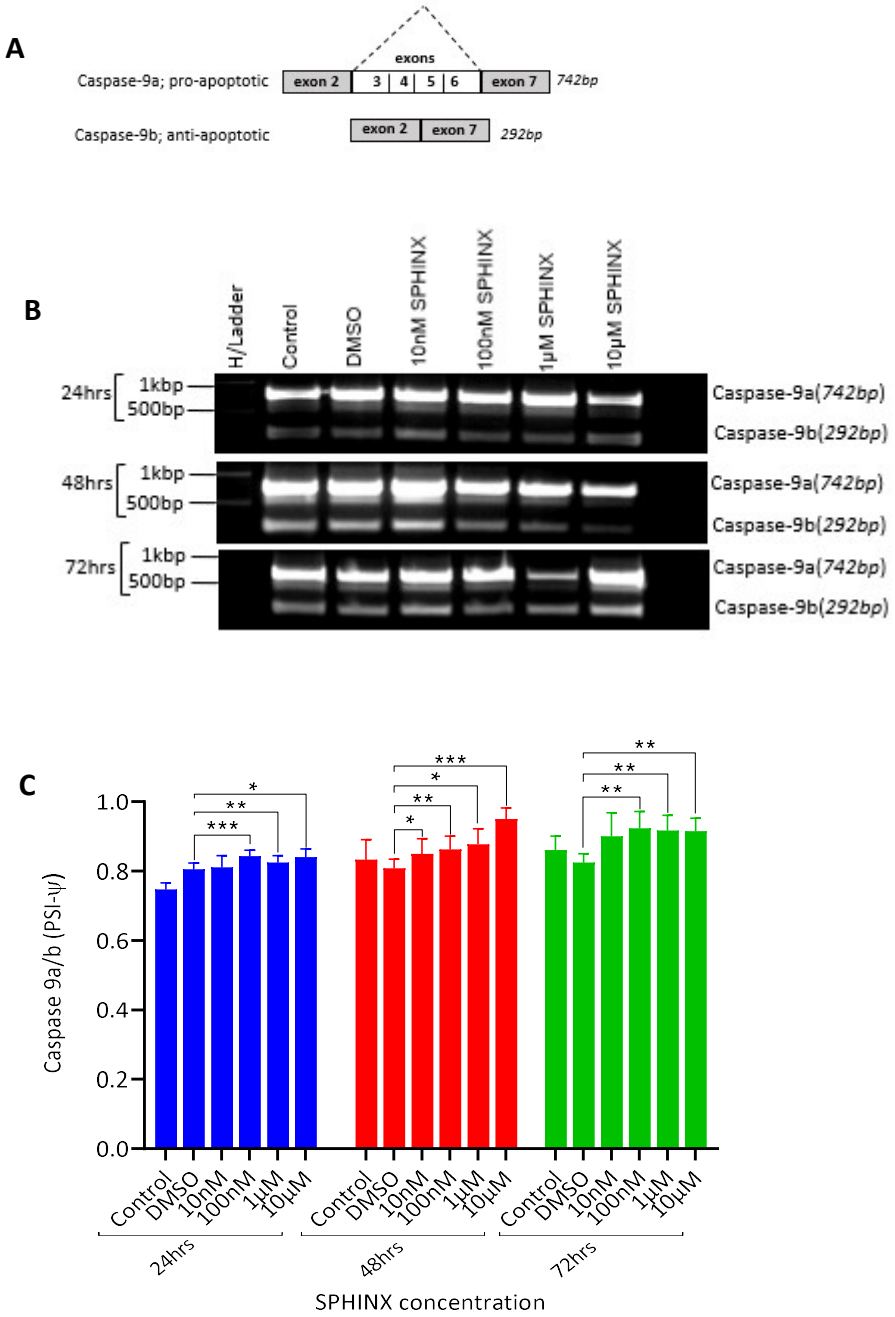


Figure 3.29. Alternative splicing of caspase-9 in SPHINX treated Kasumi-1 cells. Cells were treated with increasing concentrations of SPHINX for up to 72hrs. PCR was performed using caspase 9 specific primers. **B**. Representation of caspase 9 PCR amplicon sizes for 24hrs, 48hrs and 72hrs. **C**. Percentage splice inclusion (PSI-Ψ) for caspase 9a/9b. Statistics comparing DMSO and SPHINX treatment for 24hrs (***P=0.0004, *P=0.012; **P=0.005), 48hrs (*P=0.03; **P=0.008; *P=0.021, ***P=0.0007) and 72hrs (**P=<0.01), n=3.

3.8 Effect of SPHINX on protein levels of SRPK1, SRSF1 and on the phosphorylation of SR-protein in Kasumi-1 cells.

3.8.1 *Effect of SPHINX on SRPK1 protein levels*

Further studies were performed to understand what the effect of inhibiting SRPK1 in Kasumi-1 cells will be on the expression of SRPK1 at the protein level. There was no apparent change in SRPK1 protein levels in treated Kasumi-1 cells. Kasumi-1 cells appeared to express SPRK1 at a high level, owing to the high intensity of the bands (Figure 3.30).

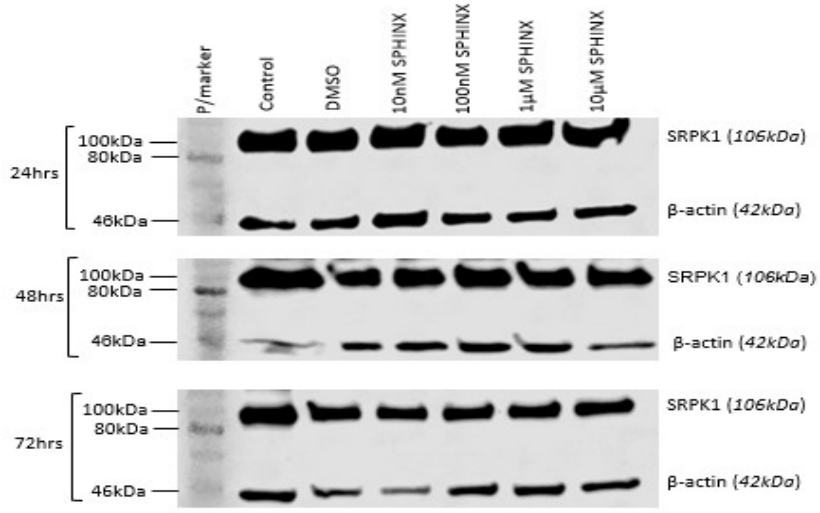


Figure 3.30. Protein levels of SRPK1 in SPHINX treated Kasumi-1 cells. Western blot analysis on Kasumi-1 protein extracts using anti-SRPK1 antibody. Image showing protein band visualized on PVDF membrane with corresponding β-actin loading control and band weight. *n*=3.

3.8.2 Effect of SPHINX on SRSF1 protein levels

Following the inhibition of SRPK1, levels of total SRSF1 in Kasumi-1 cells were measured. Total SRSF1 levels were increased across treatment and controls. Similar levels were found between SPHINX treatments and time matched DMSO controls. There was also no change observed between time points when compared (Figure 3.31A & B).

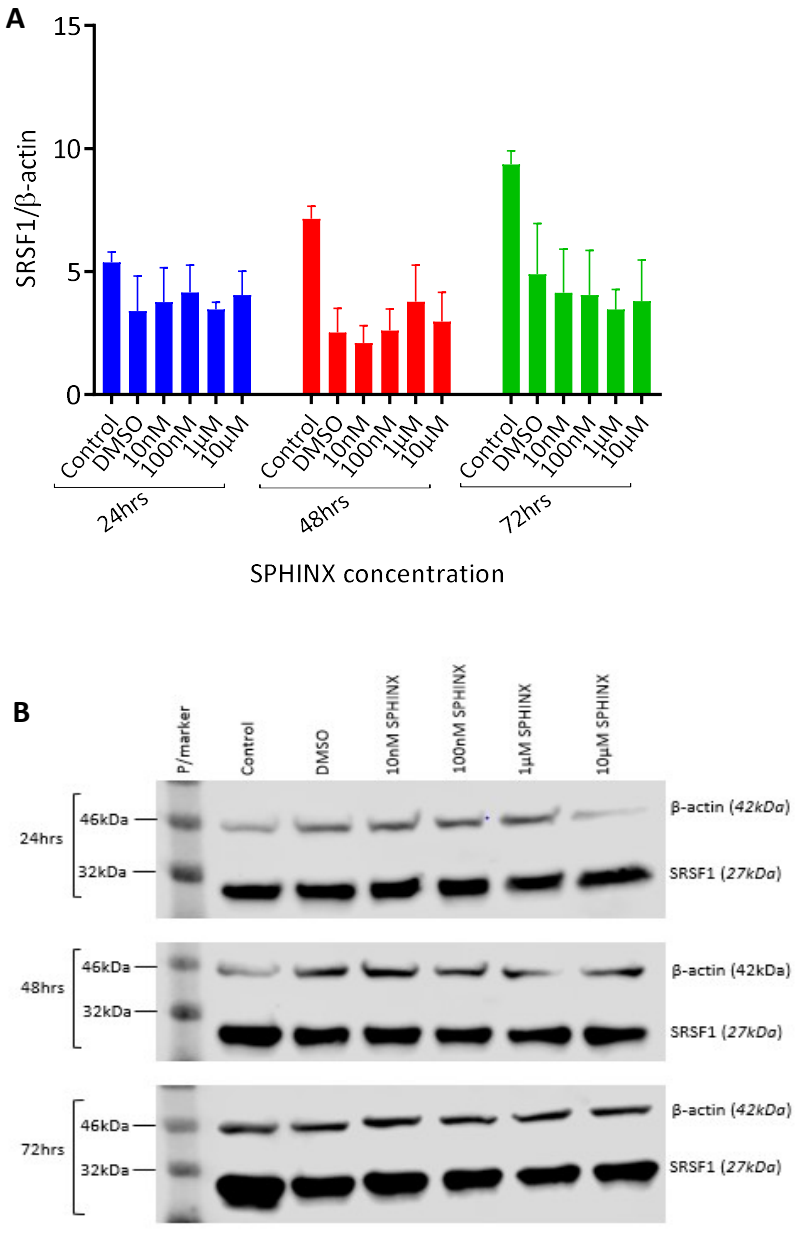


Figure 3.31. Protein levels of SRSF1 in SPHINX treated Kasumi-1 cells. Western blot analysis on Kasumi-1 protein extracts using anti-SRSF1 antibody. **A**. Densitometry showing protein levels of SRSF1 with no difference seen between treatment or time points. **B**. Image showing protein band visualized on PVDF membrane with corresponding β-actin loading control and band weight. *n*=3.

3.8.3 *Effect of SPHINX on SR-protein phosphorylation*

Phosphorylation of serine-arginine protein in Kasumi-1 was investigated following SRPK1 inhibition using SPHINX. The SR splice factors are a key substrate whose activities are dependent on the ability of SRPK1 to phosphorylate these proteins (Aubol *et al*.,2016). There was an apparent decrease in SRSF4 phosphorylation at higher concentrations of 1µM and 10µM when compared to time matched DMSO controls. Treatments at higher SPHINX concentration of 1µM and 10µM at 72hrs showed decrease phosphorylation in *p*SRSF6 levels. (Figure 3.32). Only with the 10µM concentration at 24hrs was the level of *p*SRSF11 found to be significantly reduced. Phosphorylation of *p*SRSF11 at 48hrs and 72hrs remained unchanged between treated group and controls. No significant differences were observed in *p*SRSF5 and *p*SRSF2 phosphorylation at 48hrs and 72hrs following SRPK1 inhibition.

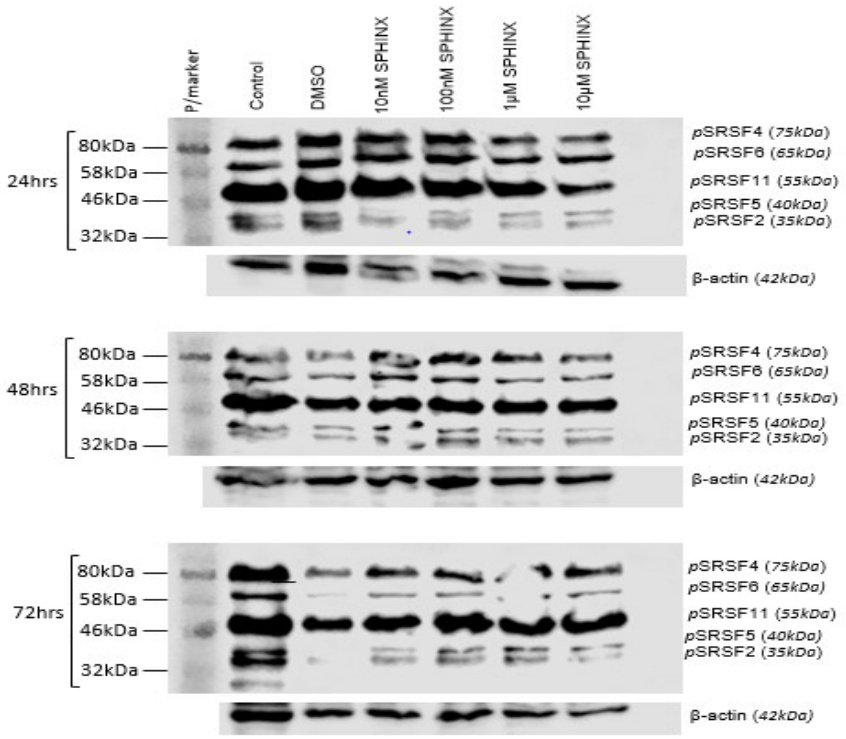


Figure 3.32. Protein levels of phospho-SR protein in SPHINX treated Kasumi-1 cells. Representative western blot image showing levels of SR protein phosphorylation, and time-matched β-actin loading control following inhibition with increasing concentration of SPHINX. *n*=3.

3.9 Discussion

3.9.1 *Effect of SPHINX on SRPK1 protein and SR protein phosphorylation in leukaemic cells*

All three cell lines used in this study appear to express SRPK1 (Figure 3.14;3.22 and 3.30). SRPK1 protein remained unchanged in treated TK6 and Kasumi-1 cells when compared to the DMSO control. However, in K562, increased SRPK1 protein level (Figure 3.22) were observed at concentrations of 10µM SPHINX without a corresponding increase observed in RNA levels (Figure 3.17). Studies which have investigated transcription to translation ratio have also found conflicting results. While Lundberg *et al*., (2010) and Wilhelm *et al*., (2014) found a correlation between transcriptomes and proteomes in cell lines and tissues respectively, others (Nagaraj *et al*., 2011; Edfors *et al*., 2016) have concluded that protein and RNA levels do not correlate, and either cannot serve as proxy to predict the abundance of the other. This, they have attributed to the complexity in regulation and post-modification changes of these -omics at different stages. Another study on colorectal cells found that knockdown of AKT2 and AKT3 affected expression of Rac1b only at the protein level and attributed differences in Rac1b RNA and protein to post-splicing effects on the *RAC1* gene (Goncalves *et al*.,2014). These modifications may account for the observed result. The unchanged protein levels in TK6 and Kasumi-1 and the increase observed in K562 suggests that the differential expression of SRPK1 could be cell line dependent. It was also observed that SPHINX altered levels of the splice factor SRSF1 as well, particularly in K562 cells (Figure 3.23). This was consistent with the report of Goncalves *et al*., (2014) which observed that inhibition of SRPK1 using 25µM SRPIN340, an earlier analogue of SPHINX on colorectal cancer cell line. significantly reduced protein levels of total SRSF1 and pSRSF1 but not SRPK1 protein levels when compared to the DMSO control. Thus, in evaluating the effects of SPHINX treatment going forward, it will be necessary to consider that it might, directly or indirectly alter the expression of its target and the target's substrates.

Levels of phosphorylation of SR-proteins varied between cell lines following SPHINX treatment. Levels of *p*SRSF4 decreased in TK6 and Kasumi-1. In K562 cells, it appeared significant only at higher concentrations. No apparent change in *p*SRSF6 levels was observed in TK6 whereas, in Kasumi-1 cells, it appeared to change at higher concentrations at 72hrs only. A significant reduction of *p*SRSF11 was observed in

Kasumi-1 cells at 24hrs. Levels of *p*SRSF5 were unaffected in TK6 and Kasumi-1 cells; a significant reduction was observed at higher concentrations of SPHINX at 48hrs. *p*SRSF2 could only be detected in Kasumi-1 cells with a variable degree of expression at different time points. With the caveat that the detection of phospho-SR proteins by western blotting is not strictly quantitative and therefore only indicative of changes, two conclusions are possible: one, that SPHINX broadly, and as expected, causes a reduction in levels of phospho-SR proteins; and two, the effectiveness of SPHINX on specific SR-protein may be cell type dependent.

The effect of SRPK1 inhibition on phosphorylation of SR protein has been widely studied, this study found consistent results with another study, which showed that SRPK1 inhibition following interaction with HPV viral protein E1^E4 (a fusion protein processed to form the E4 viral protein), results in variable reduction in the phosphorylation of SR proteins with the *p*SRSF4 being the most affected (Prescott *et al*., 2014). An earlier study reported differential phosphorylation of SR protein in sorbitol-treated HeLa cells with resultant alternative splice site selection in the conserved early region 1A (E1A) of adenovirus due to altered cellular distribution of SRPK1 following SRPK1 knockdown (Zhong *et al*., 2009). Increased phosphorylation of SR proteins with a corresponding increase in mRNA and protein levels of SRPK1 and CLK1 has been observed in hypoxic cells, an effect which was reversed with siRNA knockdown of both kinases (Jakubauskiene *et al*., 2015), suggesting the effect of SRPK1 on SR protein phosphorylation.

3.9.2 *Leukaemic cell growth, viability and apoptosis following SRPK1 inhibition*

Cancer therapeutic agents are generally compounds screened for their ability to increase cytotoxicity of human cancer cells. The overall aim is to develop therapeutic agents that can modulate or inhibit specific molecular targets identified as being essential for tumour growth (Oltersdorf *et al*., 2005; Tse *et al*., 2008). The aim of this chapter was to investigate the effect of SRPK1 inhibition with the compound SPHINX on leukaemic cancer cell lines. Lymphoblast cells (TK6), CML (K562) and AML cells Kasumi-1 were used as model cell lines for this experiment.

The inhibition of SRPK1 found no change in TK6 and K562 cell viability and growth (Figure 3.3 and 3.4). However, Kasumi-1 cell showed a significant percentage decrease at higher

concentrations of SPHINX, which were also time dependent (Figure 3.5). In terms of apoptosis, colorimetric assay and fluorescent examination in Kasumi-1 showed percentage increase in cleaved caspase 3 and caspase 7 activity (Figure 3.8). Although, K562 showed increased caspase 3/7 activity at the highest concentration of 10µM after 72hrs, this was not found to be significant (Figure 3.7). This suggests that sensitivity to drug could differ from one cancer to another, even though, they have a common origin (blood). It also further explains why certain drugs can be used for the management of one type of cancer but not the other.

Consistent with these findings is a study (Siqueira *et al*., 2015) investigating the effect of SRPK1 inhibition using SRPIN340 (an earlier version of SPHINX and less specific inhibitor of SRPK1) that showed that cells were sensitive to SRPK1 inhibition. It further reports that of all the representative cell lines, (i.e. cells of myeloid and lymphoid origin), the HL-60 cell line of myeloid origin was the most sensitive with increased apoptosis and reduced cell viability. Since both Kasumi-1 and HL-60 are cells derived from peripheral blood of acute leukaemic phases, this suggests that SRPK1 might be critical in the propagation of acute phases of leukaemia.

Published studies on other cell lines have found conflicting results on the effect of inhibiting SRPK1 in cells. While Mavrou *et al*., (2014) found no effect on cell growth, proliferation and migration in the prostate cancer cell lines PC3 and DU145, Gammons *et al*., (2016) reported a decrease in melanoma tumour growth *in vivo* but no effect on cell growth, proliferation or migration was observed on *in vitro* cell culture. This suggests a selective effect of the SRPK1 inhibitors on cell biology.

Yildiez (2018) in his experiment in hepatocellular cancers suggested that drug sensitivity is dependent on cell-specific genetic mutations and the effectiveness of the non-homologous end joining mechanism of DNA repair. It is, therefore, possible that the different mutations present in these cell lines, (Kasumi-1 t(8; 21); K562 t(9; 22)(q34; q11); (Kang *et al*., 2016) could be responsible for their sensitivity to SRPK1 inhibition and the selective effect of SRPK1 inhibitors on cell biology. Further to this, because this study was done on a population of cells, Nipel *et al*., (2017) suggested that heterogeneity in cell population has a significant impact on cell response as the assumption that cells in any given population react uniformly to any given concentration of a drug is not always true.

3.9.3 *Effect of SPHINX on alternative splicing of a panel of apoptotic genes*

Small molecule inhibitors are being developed to provide new avenues in cancer therapy. These small molecules are generally designed to target specific proteins and bind to extracellular, cell surface ligands as well as intracellular proteins. Most of these drugs inhibit critical cancer targets and block signal transduction pathways (Lavanya *et al*., 2014). Published work has shown that inhibition of SRPK1 resulted in altered VEGF splicing in podocytes in favour of its anti-angiogenic isoform, VEGF165b with a resultant decrease in angiogenesis (Nowak *et al*., 2010).

To further explore splicing patterns following SRPK1 inhibition, a panel of genes (VEGF-A, Bclx, Apaf1, and caspase 9) were investigated in leukaemic cell lines. This study determined whether SPHINX might alter the expression of SRPK1 itself (in a feedback loop). No effect on overall SRPK1 expression was observed (Figure 3.9). Whereas total VEGF in TK6 and Kasumi-1 at 48hrs significantly decreased at higher concentrations (Figure 3.10 & 3.26), levels in Kasumi-1 increased at 72hrs with no change seen in K562 upon treatment with SPHINX. This study was unable to detect the VEGF anti-angiogenic isoform which other study has detected in tumour growth in PC3 mouse xenografts and the PC3 cell line (Mavrou *et al*., 2015). It is possible that more sensitive assays, such as western blotting and ELISA, using a specific antibody targeting VEGF165b isoform and a suitable control such as podocyte cells or VEGF165b plasmid would give better indication of the VEGF165b isoform since PCR is semi-qualitative.

No clear effect on Bclx, Apaf1 and caspase 9 alternative splicing was observed in all cell lines with the dominant isoforms being highly expressed. When levels of these isoforms were compared to the DMSO control, an increase in Bcl-xs was observed in Kasumi-1 (Figure 3.27B). Also, a transient shift in Apaf1 splicing to Apaf-1xl isoform at 24hrs and 48hrs was observed at higher concentration of 1µM and 10µM in Kasumi-1 cells (Figure 3.28) but not in TK6 and K562 cells. The caspase 9a isoform was increasingly expressed in the SPHINX treated group in K562 and Kasumi-1 cells but not in TK6 when compared to the DMSO control. The increasing level of caspase 9a, a pro-apoptotic isoform of caspase 9 is consistent with increased caspase 3/7 activity in Kasumi-1 cells and at higher concentrations in K562 (Figure 3.7 & 3.8) which matched the decreased percentage in cell viability and growth (Figure 3.5). These findings suggest that the caspase pathway

could be a downstream target of SRPK1 inhibition and highlights the possible role of the caspase pathway in maintenance of AML clones.

The degree of mRNA processing is known to differ from cell to cell and depends partly on the rate of transcription which is not constant (Ingolia *et al*., 2011). There have been reports in which transcriptional changes feed back to regulate mRNA synthesis and structure (Namy *et al*., 2006; Yanagitani *et al*., 2011). Such transcriptional changes and the degree of mRNA processing may explain the differences in mRNA levels in these cell lines.

SRPK1 overexpression, which was also observed at protein levels in this experiment, has been reported in several cancers including colon and breast malignancy where elevated levels decrease cell apoptosis (Lin *et al*., 2014; Mavrou *et al*., 2015).

SRSF1 has been shown to regulate caspase 9 splicing in non-small lung cancer (NSCLC) and cell lines such as Hela cells, H838 and HBEC3-KT cells through interaction with RNA cis-element such as C9-I6/ISE and C9-E6/ESE that determines the choice of the splice site in caspase 9 (Shultz *et al*., 2011). It may be appropriate to infer that the levels of caspase 9a observed in this study is an indirect effect of SRPK1 inhibition since SRSF1 is a key substrate of SRPK1. The same study identified increased caspase 9a/b ratio in NSCLC following inhibition of phosphatidylinositol 3-kinase (PI3K/Akt), a pathway which has been shown to be activated by SRPK1 (Chang *et al*.,2015). Apaf1 binding to pro-caspase 9 is required for its activation in the intrinsic pathway (Anichini *et al*., 2006), studies have suggested that increased expression of Apaf-1xl isoform correlates to cancer cell survival and resistance to apoptosis (Shakeri *et al*., 2017). One study suggests that the balance between Apaf-1xl/s is required to overcome drug resistance and increase apoptosis in tumour cells (Benites *et al*., 2008). It has also been suggested that both hypo- and hyper-phosphorylation of SR proteins alter mRNA splicing significantly (Zhong *et al*., 2009). The variability in splicing observed remains inconclusive as to whether it is a direct effect of SRPK1 inhibition via SR protein phosphorylation. Assays such as qPCR and western blotting which are more quantitative and with a suitable control would give better indication to this result.

Chapter 4

4 Combining SRPK1 and CLK1 inhibition with standard chemotherapeutic drugs

4.1 Introduction

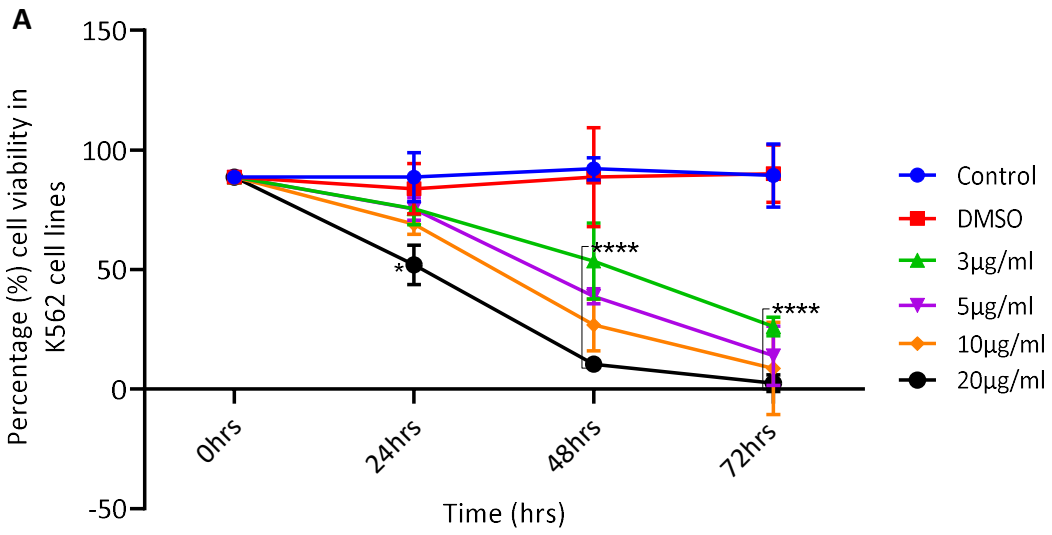
Treatment of blood cancers is a very challenging task. There is an ongoing need to develop new and effective drugs. The management of leukaemic conditions such as CML and AML continues to be challenging. Imatinib mesylate (Gleevec) is the first drug (first generation) developed for targeted therapy of BCR-ABL positive CML; and azacytidine, a hypo-methylation agent is the first drug of choice for the treatment of AML (O'Brien *et al*., 2003; Tallman *et al*.,2019). Several generations, up to the third generation of the likes of imatinib such as Ponatinib (Iclusig) have been developed (O'Hare *et al*., 2009). Even though each new generation of these drugs has increased potency and efficacy, they are known to have severe side effects which could lead to patients withdrawing from the treatments. Side effects can also lead to the development of secondary diseases such as embolism, diabetes, stroke, congestive heart failure and secondary cancers that could potentially kill the patient (Aichberger *et al*., 2011; Gugliotta *et al*., 2015). As newer generation of drugs becomes more focused, targeting specific mutations, unique to each of these cancers, it is still the case that targeting these pathways can be responsible for the severe side effects (Bagnyukova *et al*., 2010). Another challenge with chemotherapy regimen is the ability of patients to meet certain criteria such as presenting at a particular phase of the disease, never been exposed to certain drugs and having specific cytogenetic or chromosomal abnormalities to be eligible for a specific treatment. To overcome some of these therapy-related problems, it has been suggested that a combination of potential treatment regimen will increase treatment efficacy due to broad target and reduce side effects resulting from high dose (Cheng *et al*., 2011; Jabbour and Kantarjian, 2018).

The aim of this chapter is to evaluate the consequence of co-inhibition of the splice factor kinase, SRPK1 and CLK1 combined with conventional chemotherapeutic agents used in the treatment of CML and AML. It is hoped that combining this treatment could improve outcomes.

4.2 Results

4.2.1 Effects of Imatinib on K562 cell viability and growth

To understand the effect of imatinib on cell viability, K562 cells were treated with increasing concentrations of imatinib mesylate up to 20µg/ml for 72hrs using a method that has earlier been described in chapter 2.4. Treatment of K562 with imatinib show that imatinib can reduce K562 cell viability in a dose-dependent manner, with time, when compared to the DMSO control. (Figure 4.1A).



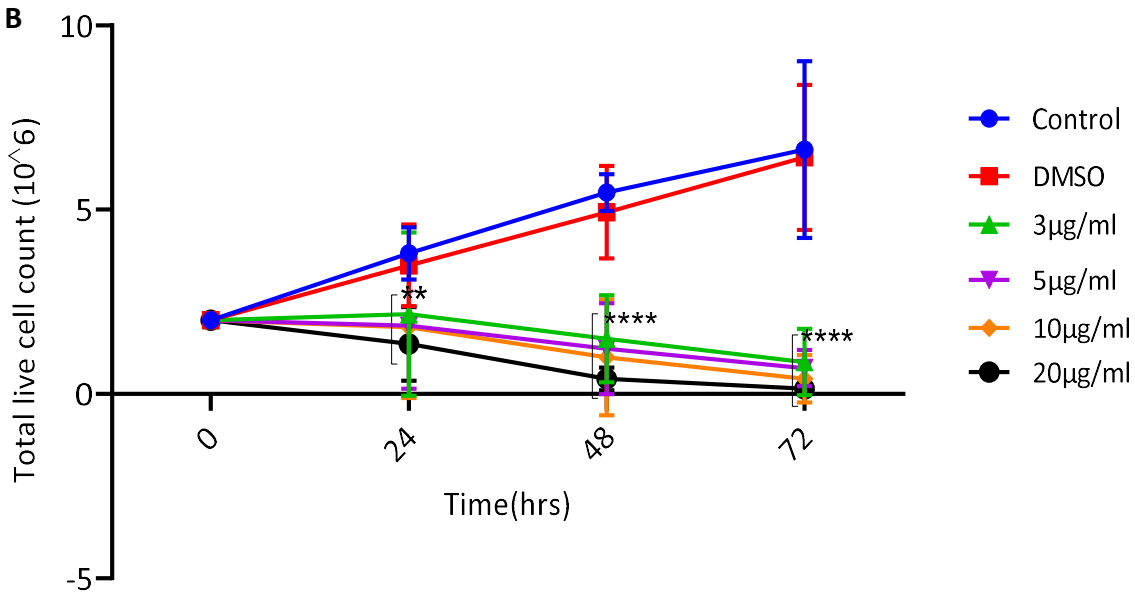


Figure 4.1. Effect of increasing concentration of imatinib mesylate on K562 cell viability and growth. Two million (2 x 10^6) cells were seeded over 72hrs with cell count performed every 24hrs. **A.** Percentage cell viability in K562 cells up to 72hrs. 20µg imatinib treatment at 24hrs (P=0.001). At 48hrs and 72hrs (P<0.0001). **B**. Reduction in cell growth/cell number was observed with 5µg/ml, 10µg/ml and 20µg/ml imatinib at 24hrs (P≤0.001). The cell number further reduced at all drug concentration for 48hrs and 72hrs (****P<0.0001) when treated group were compared to the DMSO control. n=3

Not only was cell viability decreased in a dose-dependent manner, a reduction in K562 cell proliferation, and hence, cell growth was also observed, which was highly significant (P<0.0001) especially at 48hrs and 72hrs (Figure 4.1B).

At 72hrs, for imatinib doses at 10µg/ml and 20µg/ml, less than 20% of the cells were viable and cell number decreased significantly. Therefore, these doses were considered very toxic to the cells and were not used in future experiments. Furthermore, considering that there was a need to extract RNA and proteins from these treated cells for further experiments, this would have been very difficult owing to the low cell number. For further experiments, cells were treated with no more than 5µg/ml of imatinib mesylate. This was considered as 3µg/ml is the clinically relevant dose and represent the peak serum concentration of imatinib when administered at FDA recommended a standard clinical dose of 400mg/day (Rezende *et al*., 2013).

4.2.2 *Effect of combined SRPK1 inhibition and imatinib on K562 cell viability and growth*

The effect of imatinib on K562 cell viability and an appropriate concentration range was confirmed. Further experiments were then aimed at combining imatinib treatment with SRPK1 inhibition using SPHINX at 10µM concentration. This aim was to investigate whether there is any added advantage to imatinib treatment in achieving increased cell death in CML at lower doses.

Therefore, cells were treated with either 10µM SPHINX, 3µg/ml and 5µg/ml imatinib alone or a combination of both for up to 72hrs. Results showed a significant decrease in cell viability when groups treated with SPHINX or in combination with imatinib were compared to DMSO controls (Figure 4.2A). However, further analysis to determine whether combined treatment with imatinib and SPHINX significantly differ from either SPHINX or imatinib alone found no significant changes in cell viability (Table 4.1A-B). Similar results were also obtained with the cell growth (Figure 4.2B) except for the 24hrs time point were only 5µg/ml imatinib and combination of imatinib with SPHINX differed from the controls.

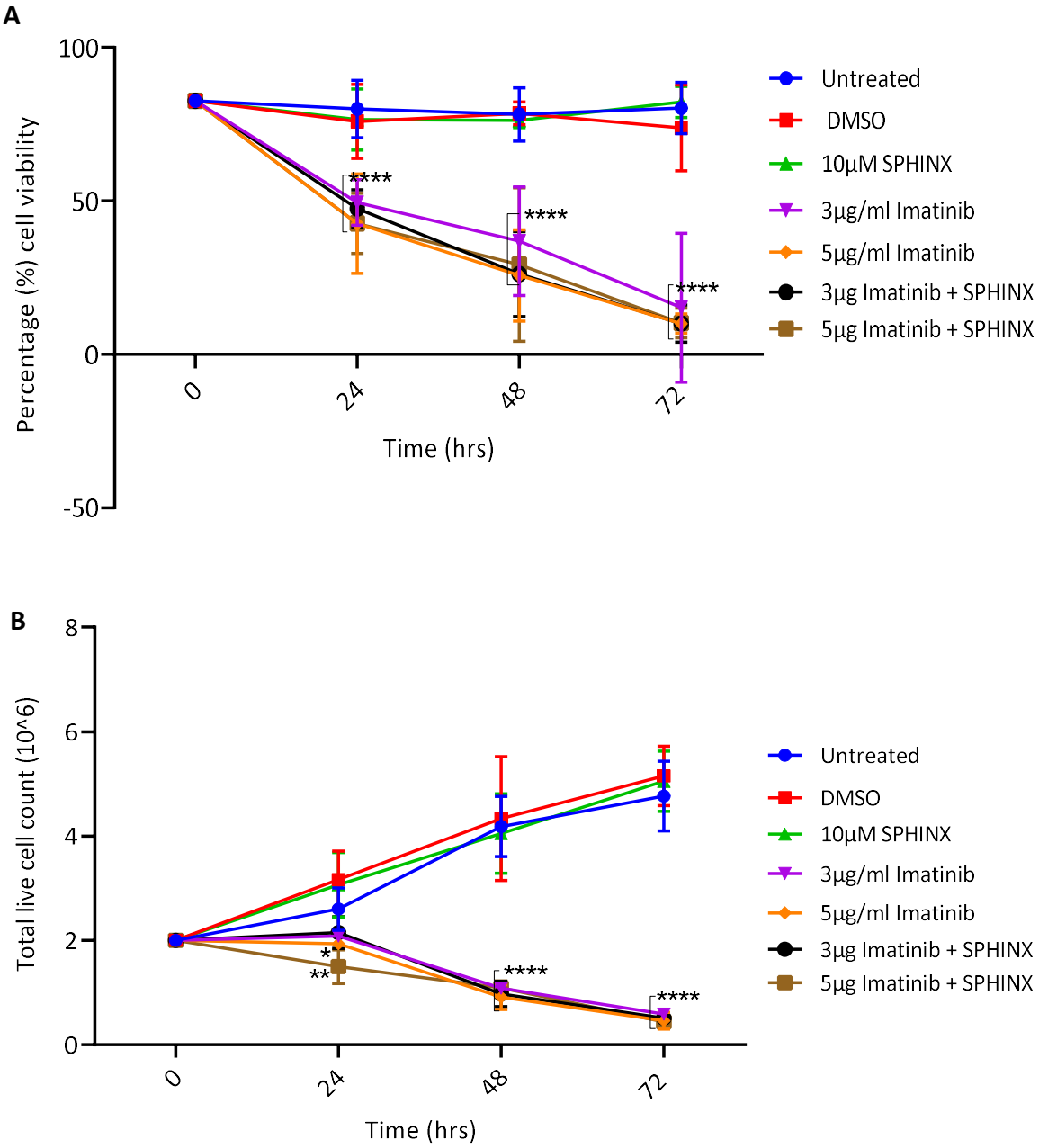


Figure 4.2. Effect of combined SPHINX and imatinib mesylate on K562 cell viability and growth. Two million (2 x 10^6) cells were seeded over 72hrs with cell count performed every 24hrs. **A.** Reduced in cell viability observed with imatinib and imatinib + SPHINX combined treatment at each time points when compared to DMSO control (****P<0.0001). **B**. Reduced cell growth/cell number was observed in 5µg/ml imatinib and imatinib + SPHINX at 24hrs (*P=0.03 and **P=0.002 respectively) and at 48hrs and 72hrs (****P<0.0001) respectively. Treated group were compared to time matched DMSO control, n=3.

Table 4.1A: K562 cell viability analysis for imatinib and imatinib combined with SPHINX

	N	Mean	StDev	SE Mean
3µg Imatinib	3	33.90	17.30	10.00
3µg Imatinib + SPHINX	3	27.90	18.80	10.80
Difference	3	5.94	4.39	2.53

Statistical analysis comparing cell viability on K562 cell treated with imatinib mesylate vs imatinib + SPHINX. Confidence interval (CI) at 95% for mean difference (-4.95, 16.84); T-value = 2.35; P = 0.144 (paired t-test). No significant statistical difference was observed in K562 cell viability when imatinib treated group was compared to imatinib and SPHINX combination.

Table 4.1B: K562 cell growth analysis for imatinib and imatinib combined with SPHINX

	N	Mean	StDev	SE Mean
3µg Imatinib	3	1.252	0.762	0.440
3µg Imatinib + SPHINX	3	1.212	0.845	0.488
Difference	3	0.040	0.094	0.0542

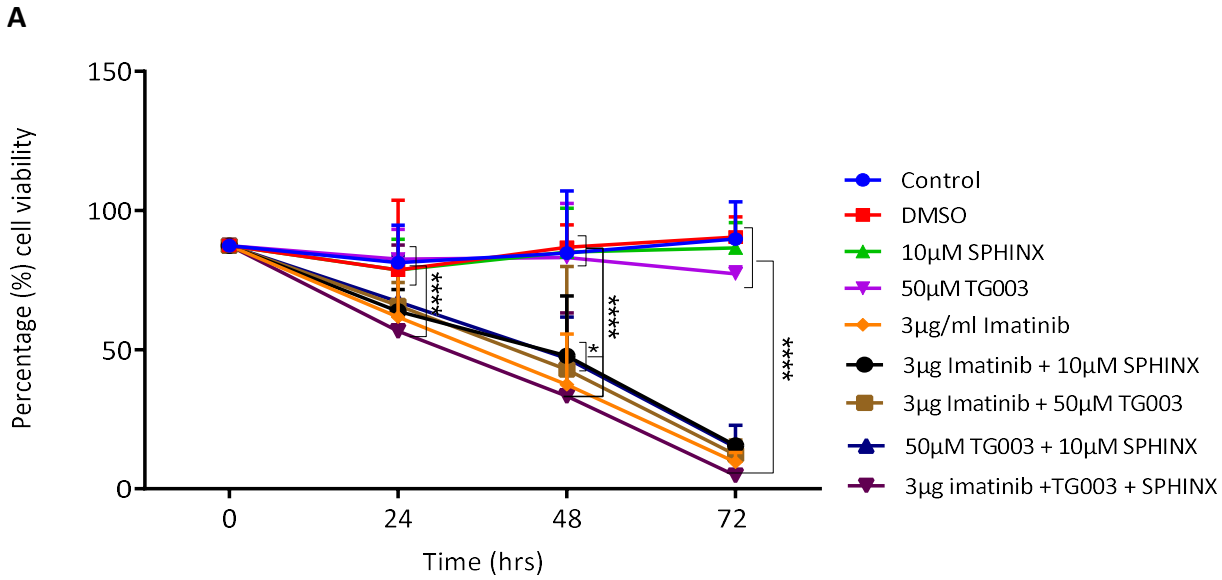
Statistical analysis comparing cell viability on K562 cell treated with imatinib mesylate vs imatinib + SPHINX. Confidence interval (CI) at 95% for mean difference (-0.1932, 0.2732); T-value = 0.74; P= 0.537 (paired t-test). No significant statistical difference was observed in K562 cell number when imatinib treated group was compared to imatinib and SPHINX combination.

4.2.3 *Effect of combined SRPK1, CLK1 inhibition with imatinib on K562 cell viability and growth*

It has been established that both SRPK1 and CLK1 are required for the phosphorylation of SR-protein. One study suggests that, whereas, SRPK1 which has both nuclear and cytoplasmic presence is responsible for phosphorylation dependent transport of SR protein from the cytoplasm to the nucleus, CLK1, which is nuclear localized, is responsible for SR protein mobilization from speckles to the site of active pre-mRNA splicing (Aubol *et al*., 2016). Further studies have also suggested that both kinases interact in the nucleus via the CLK1 N-terminal to complete the functional task of SR protein phosphorylation in the nucleus. This is such that nuclear SRPK1 is responsible for the release of SR proteins, which have been mobilized at active splice site by CLK1 which also regulates SRPK1 levels in the nucleus (Aubol *et al*., 2014; 2016). Thus, the

effect of inhibiting both SRPK1 and CLK1 in leukaemic cell line K562 was studied to see if inhibition of both kinases will increase the rate of cell apoptosis and if additional combined treatment with imatinib will have an added advantage. Results of the analysis showed that double kinase inhibition of SRPK1 and CLK1 in addition to imatinib treatment resulted in a significant decrease in cell viability when compared to either SRPK1 or CLK1 inhibition alone at all-time points except for 3µg/ml imatinib treatment. At 48hrs, all combined treatment also differed significantly from imatinib and SPHINX combination and TG003 (CLK1 inhibitor) and SPHINX combination respectively (P<0.05) (Figure 4.3A).

Similar results were also obtained when the effect on K562 cell growth was observed. The rate of K562 cell growth significantly reduced in all combined treatment than the single kinase inhibition/treatment or the controls. No significant change was observed when both kinases (SRPK1 and CLK1) were inhibited or with individual kinase inhibition combined with imatinib (Figure 4.3B).



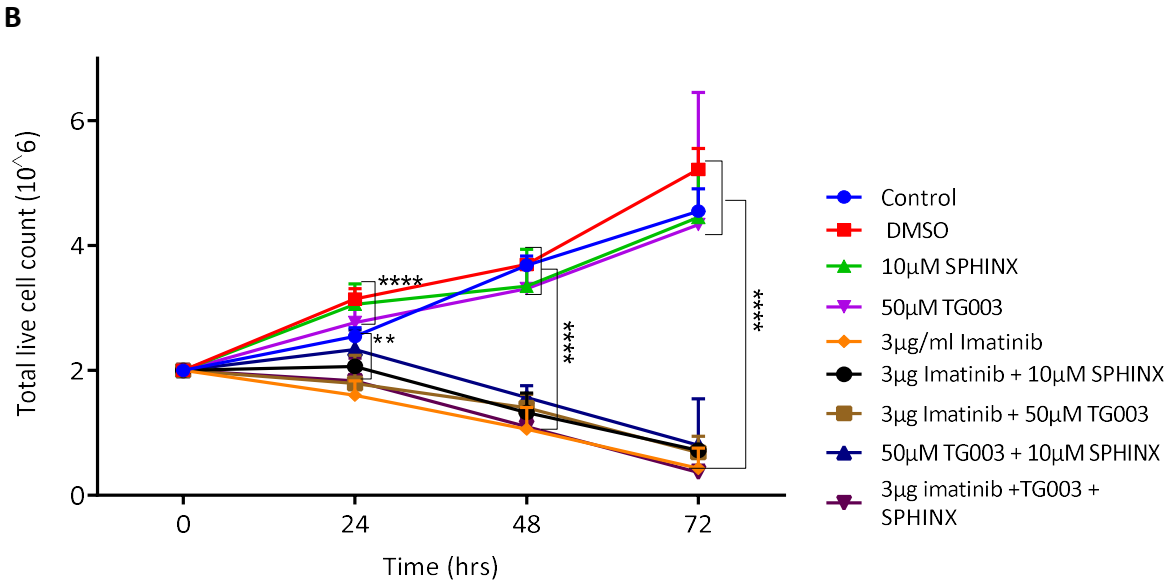


Figure 4.3. Effect of combined SPHINX and TG003 with imatinib mesylate on K562 cell viability and growth. Two million (2 x 10^6) cells were seeded over 72hrs with cell count performed every 24hrs. **A.** Cell viability in treated cells. Cell viability differs significantly between SPHINX+TG003+Imatinib treated group when compared to the single treatment (untreated, DMSO, SPHINX and TG003) (****P<0.0001) for 24hrs and 72hrs. At 48hrs, untreated, DMSO, SPHINX and TG003 also differ significantly (****P<0.0001) in addition to Imatinib + SPHINX treatment (P≤0.03). A comparative analysis of time points also showed significant difference (P≤0.01) between start point and other time points. **B**. Reduced cell growth/cell number was also observed between SPHINX+TG003+imatinib treated group and the single treatments (****P<0.0001) for 48hrs and 72hrs, and (**P=0.004, ****P<0.0001 respectively) for 24hrs. n=3

4.2.4 Effect of azacytidine on Kasumi-1 cell viability and growth

Kasumi-1 cells were also treated with azacytidine, a first line drug of choice in AML. FDA approved dose of 75mg/m^2 IV or IM results in a peak serum concentration of 750ng/ml (FDA, 2007), which was considered the clinically relevant dose in this experiment. However, doses up to 1.5µg/ml were used to determine the effect of azacytidine on Kasumi-1 viability. A dose and time-dependent decrease in Kasumi-1 cell viability was observed when compared to the treatment controls (Figure 4.4A).

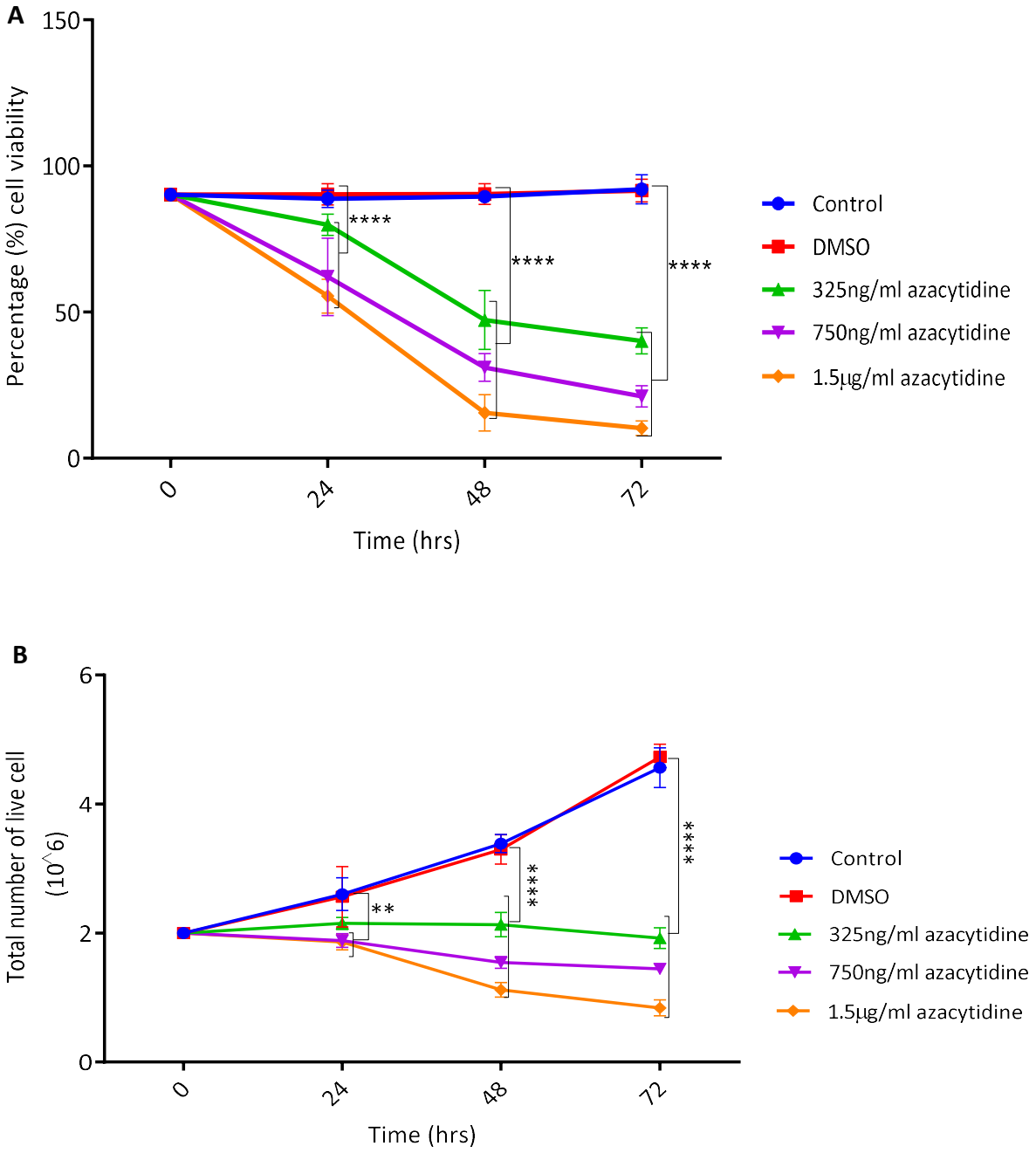


Figure 4.4. Effect of increasing concentration of azacytidine on Kasumi-1 cell viability and growth. Two million (2 x 10^6) cells were seeded over 72hrs with cell count performed every 24hrs. **A.** Result showing the percentage viability in Kasumi-1 cells up to 72hrs(****P<0.0001) when compared to the DMSO control. **B**. Time points also showed significant difference in cell number at 24hrs vs 72hrs and 24hrs vs 72hrs at ****P<0.0001 while at 48hrs vs 72hrs (***P=0.0004). n=3

When the effect of azacytidine on cell growth was observed, only at higher doses of 750ng/ml and 1.5µg/ml was there a significant decrease in cell growth at 24hrs. However, at 48hrs and 72hrs, all azacytidine treatment showed a significant decrease in cell growth and hence proliferation in a dose-dependent manner (Figure 4.4B).

4.2.5 *Effect of SPHINX and azacytidine on Kasumi-1 cell viability and growth*

To determine whether there is any beneficial effect in combining SRPK1 inhibition and azacytidine treatment in the management of AML patients, Kasumi-1 cells were treated with either 10µM SPHINX or 750ng/ml azacytidine or a combination of both. There were indications of a beneficial effect as cell treated with a combination of SPHINX and azacytidine showed a significant decrease in cell viability. This was compared to the untreated and DMSO controls or SPHINX only treatment at each time points. Only at 48hrs was azacytidine treatment significantly different from the combined treatment (Figure 4.5A). There was a difference between the combined treatment and the controls but not with either the SPHINX or azacytidine treatment (Figure 4.5B).

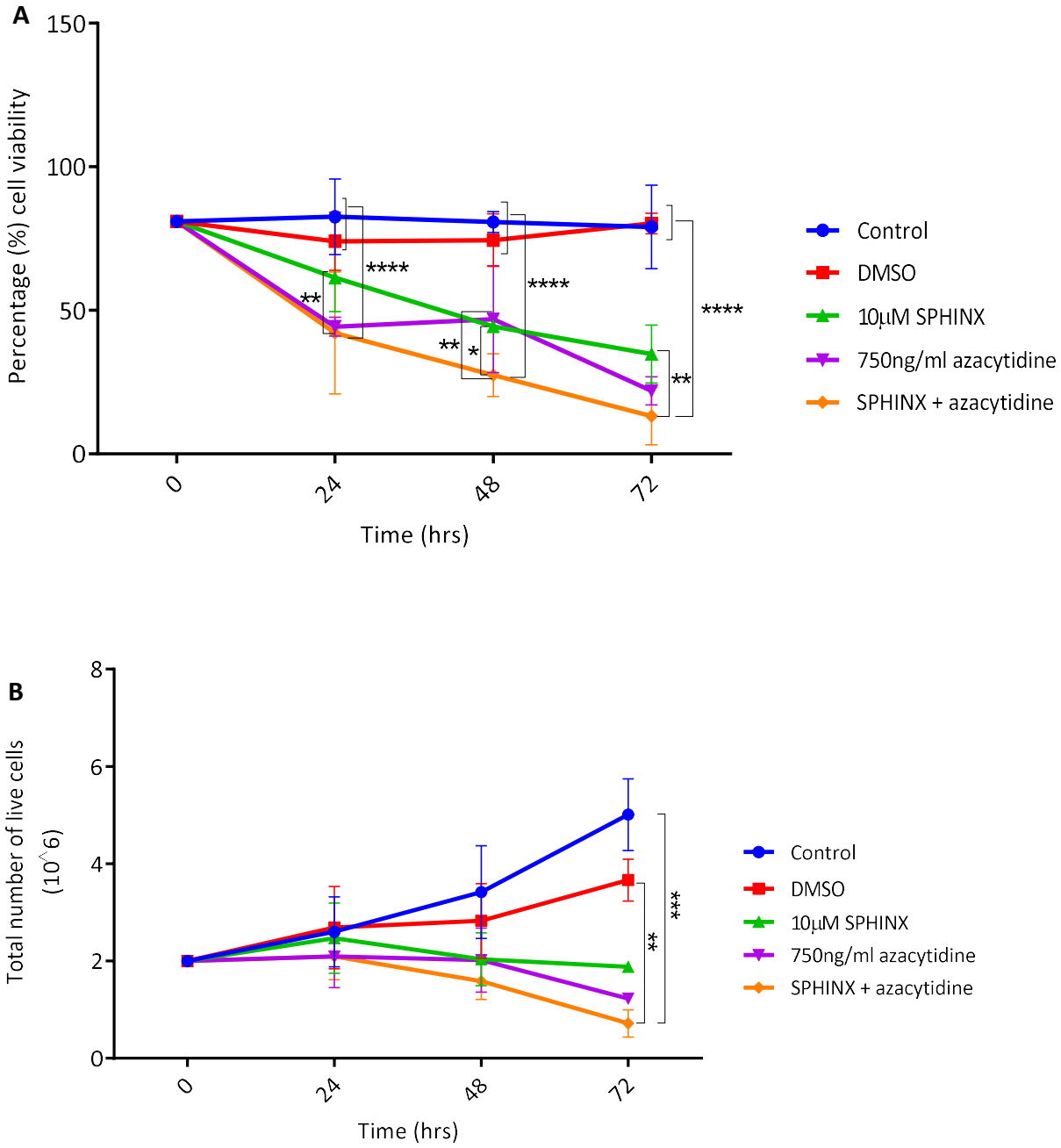


Figure 4.5. Effect of combined SPHINX and azacytidine on Kasumi-1 cell viability and growth. Cells were seeded over 72hrs with cell count performed every 24hrs. Cells were treated with SPHINX + azacytidine or SPHINX or azacytidine alone. **A.** Control, DMSO and SPHINX treated group showed increase in cell viability at 24hrs (****P<0.0001 and **P= 0.004) and 72hrs (****P<0.0001 and **P=0.004). Cells at 48hrs showed similar result in addition to the azacytidine treated group ****P<0.0001; *P= 0.01 and **P= 0.004). Values when time was compared were ****P<0.0001 for 24hrs vs 72hrs and *P= 0.02 48hrs vs 72hrs. **B**. Cell growth was observed to increase in controls when compared to the SPHINX+ azacytidine treatment (***P=0.0001 and **P= 0.007) at 72hrs. n=3

4.2.6 *Effect of combined SPHINX and TG003 with azacytidine on Kasumi-1 cell viability and growth*

The effect of combined CKL1, SRPK1 inhibition with azacytidine on Kasumi-1 cell viability was investigated to determine if there was a beneficial outcome in the response of an AML cell line. the combination of azacytidine with SPHINX and TG003 showed a significant decrease in cell viability against single treatments and azacytidine combined with individual kinase inhibitor except with cells treated with SPHINX and TG003 at 24hrs. At 48hrs, azacytidine and TG003 and SPHINX and TG003 were not statistically different whereas, at 72hrs, all single treatments, except azacytidine, showed no difference when compared to the combined treatment (Figure 4.6A). Cell growth was found to be increased in the untreated and DMSO controls only at 48hrs and 72hrs and this differed significantly when compared to cell growth in the azacytidine with SPHINX and TG003 combined treatment (Figure 4.6B).

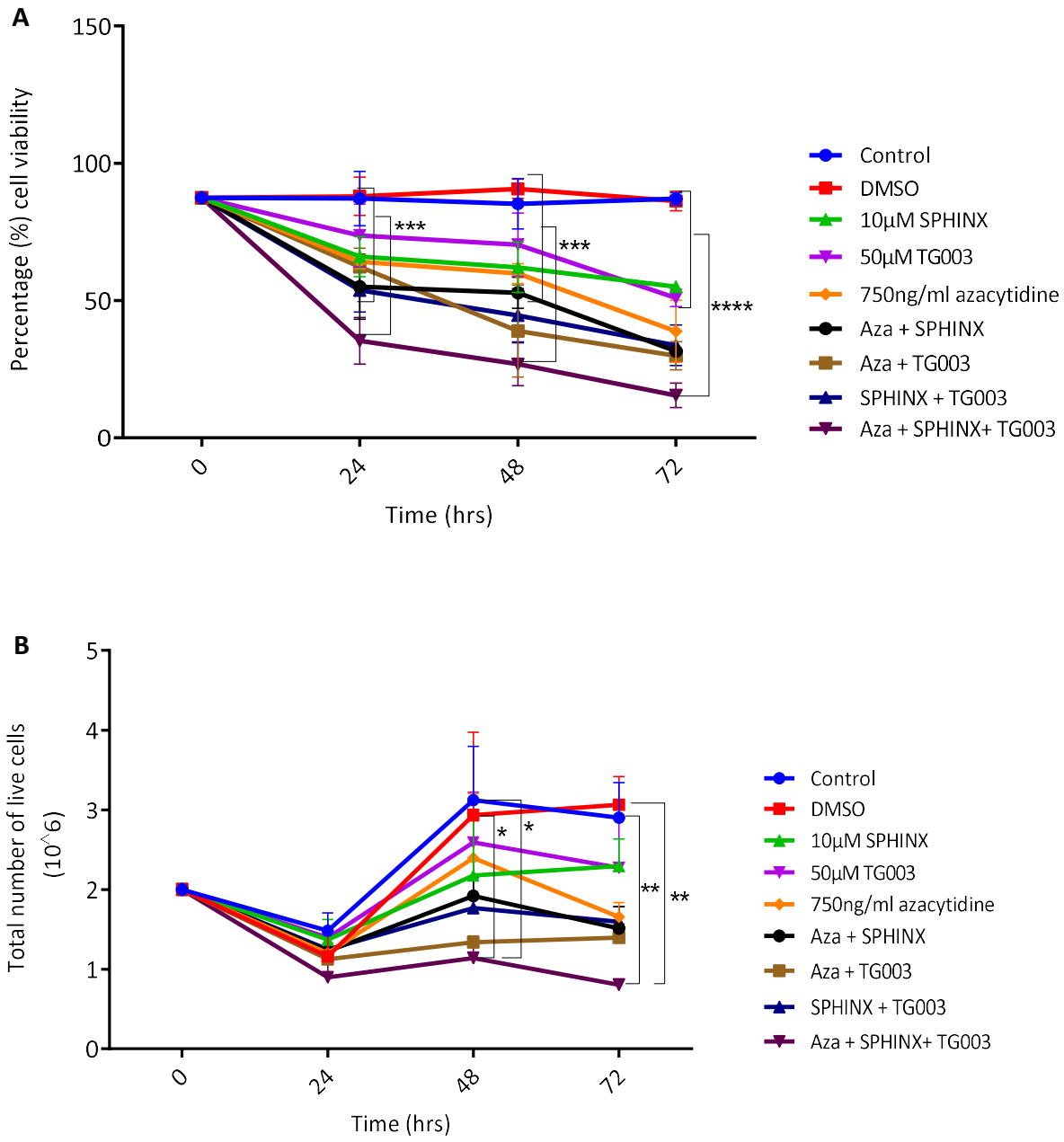
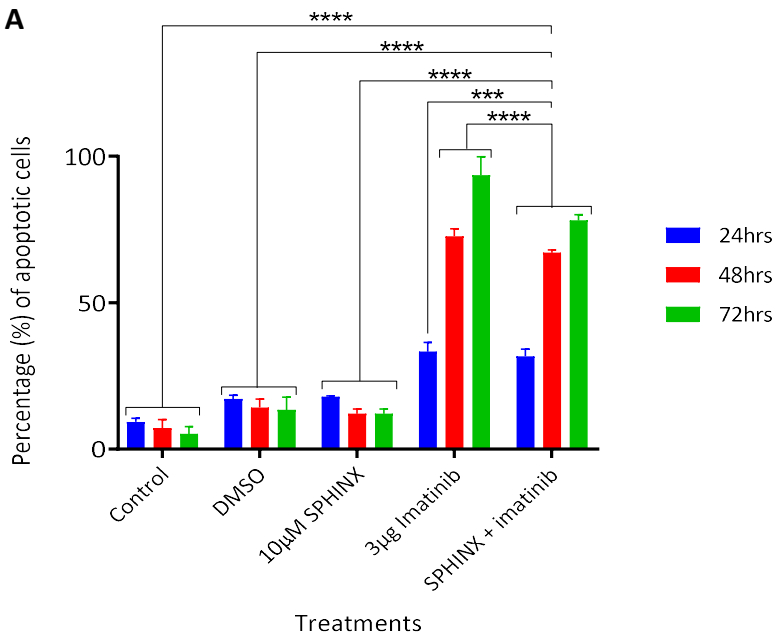


Figure 4.6. Effect of combined SPHINX and TG003 with azacytidine on Kasumi-1 cell viability and growth. Cells were seeded over 72hrs with cell count performed every 24hrs. Cells were treated with inhibitors (SPHINX or TG003) alone, inhibitor and azacytidine or in combination. **A.** At 24hrs, all treatment except SPHINX+TG003 showed a significant difference in cell viability when compared to the group treated with inhibitors and azacytidine (***$P \leq 0.0005$). Only azacytidine + SPHINX and SPHINX + TG003 did not differ significantly with inhibitors and azacytidine at 48hrs ($P \geq 0.05$) and at 72hrs, only the single treatment significantly differs from the inhibitors and azacytidine (****$P<0.0001$ and *$P= 0.01$ respectively). At time points, ****$P<0.0001$ for 24hrs vs 72hrs and *$P= 0.02$ 48hrs vs 72hrs. **B**. Cell growth was observed to increase in controls when compared to the inhibitors + azacytidine treatment (*$P<0.05$) at 48hrs and 72hrs. n=3

4.2.7 Effect of combined SPHINX and chemotherapeutic drug on leukaemic cell biology

4.2.7.1 Examination of the effect of combined SPHINX and imatinib on K562 cells

Further investigation on the effect of combined imatinib and SRPK1 inhibition using acridine orange, fluorescent dye, showed that combining imatinib with SPHINX significantly increased (P≤0.0001; 0.0014) cell apoptosis when compared to the controls and SPHINX only treatment, which was consistent with the cell viability and growth in K562 cells. Interestingly, it was observed that more cells were driven to apoptosis when K562 were treated with imatinib alone than when combined with SPHINX (Figure 4.7A-B).



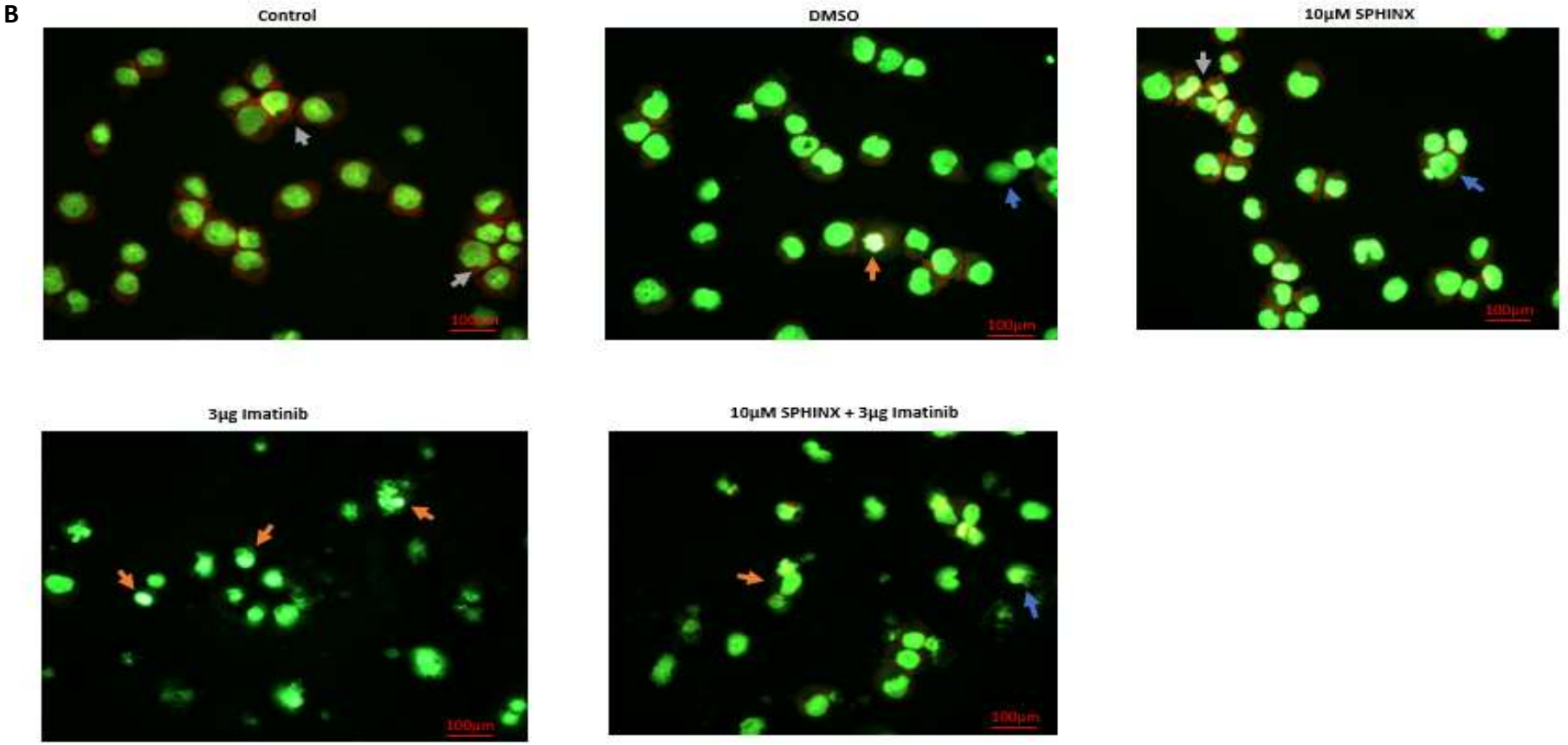
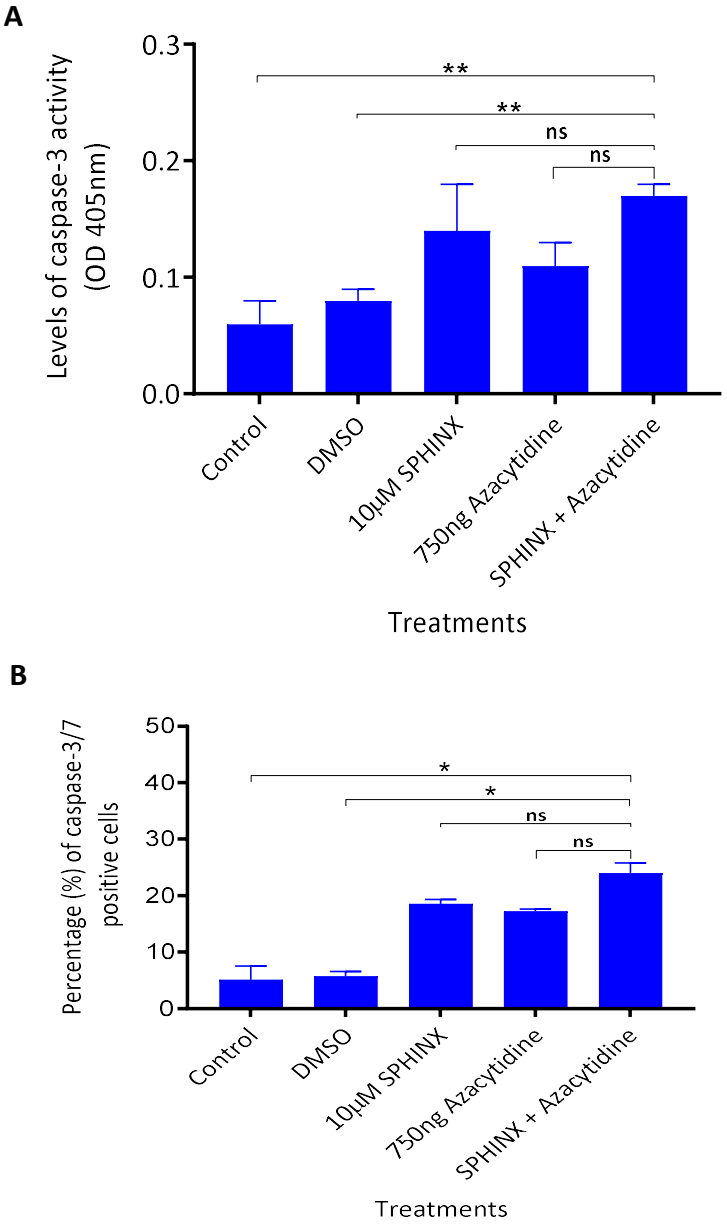


Figure 4.7. Effect of combined SPHINX and Imatinib on K562 cell morphology. Representative micrograph of K562 cells stained with acridine orange fluorescence dye. Cells were treated with either SPHINX, imatinib or in combination of both for up to 72hrs**. A.** Percentages of apoptotic cells. Significant decrease in cell apoptosis was observed in all group when compared to combined imatinib and SPHINX treated group (****$P \leq 0.0001$). **B.** Image viewed at a green (FITC) band width of about 510-560nm. Normal cells with intact nucleus and cytoplasm (grey arrow), apoptotic cell (orange) and necrotic cells (blue). $n=3$

4.2.7.2 *Effect of combined SPHINX and azacytidine on caspase 3/7 activity in Kasumi-1 cells*

Experiment was performed to further understand the effect of azacytidine and SPHINX combined treatment on Kasumi-1 cells by investigating caspase 3/7 activity. Increased level of caspase 3/7 activity was observed in all treatments except for the controls. The level of caspase-3 activity did not differ significantly when the combined SPHINX and azacytidine was compared to SPHINX or azacytidine group except for the controls. Suggesting no added benefit (Figure 4.8A).



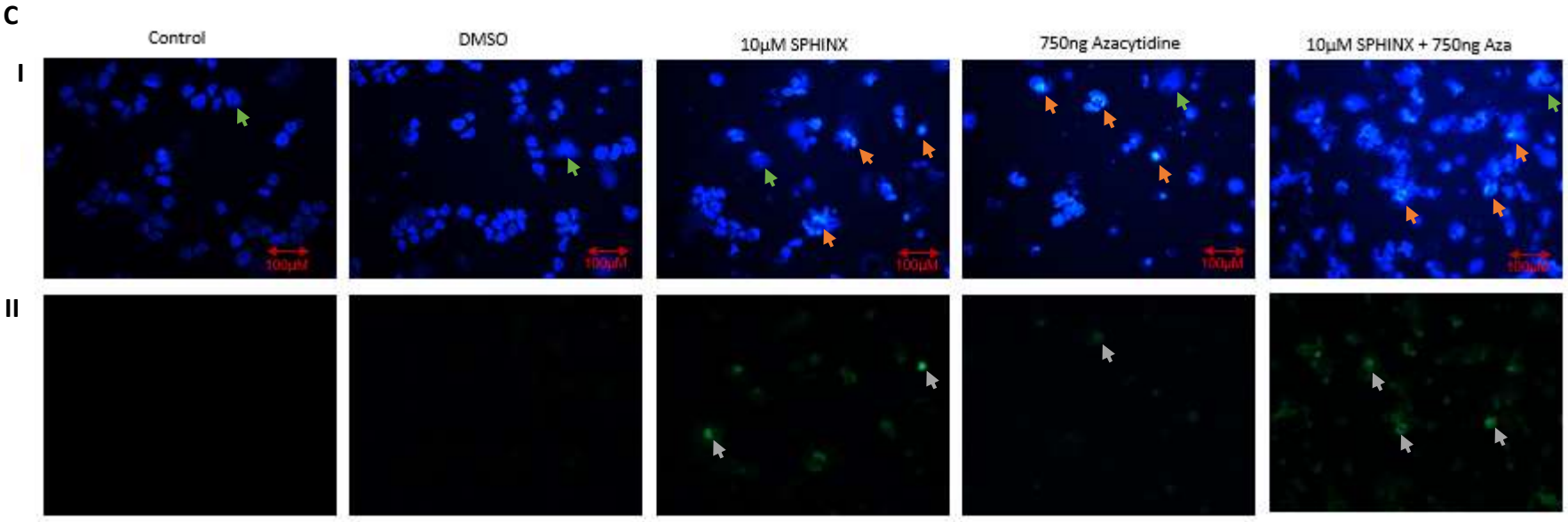


Figure 4.8. Examination of caspase 3/7 activity in Kasumi-1 cells. (**A**) Post-treatment, cells were labelled with a known substrate; DEVD-p-NA. Combined SPHINX + azacytidine cells significantly increased in caspase 3 activity when compared to untreated (***P*=0.006) and DMSO (***P*=0.008) control. (**B**) Percentage increase in apoptotic cells in combined SPHINX + azacytidine treatment compared to untreated (*P*=0.031) and DMSO (*P*=0.030) controls (**C**). **I**. Hoechst stain of the total cell at excitation between 330-380nm band width bypass. Arrows (green) represents necrotic cells and (orange) apoptotic cells. **II.** Corresponding image viewed at a green (FITC) band width of 490-590nm. Caspase 3/7 positive cell (grey arrow), *n*=3.

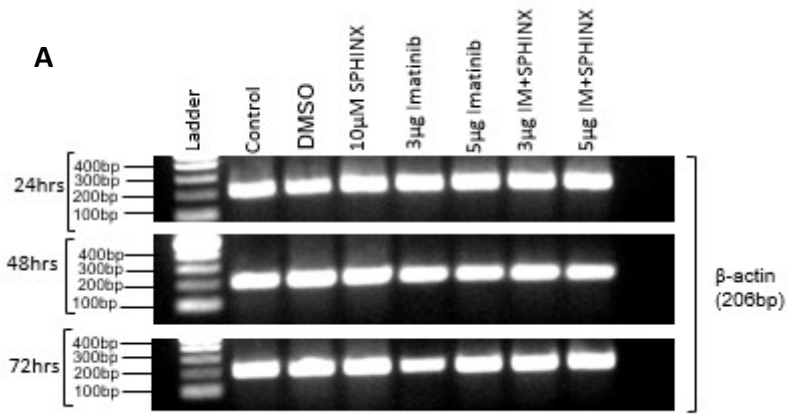
Examination of the activities of caspase 3/7 on Kasumi-1 cell (Figure 4.8B & C) showed results which were consistent with caspase 3 activity (Figure 4.8A) where only the controls significantly differed when compared to the SPHINX and azacytidine combined treatment. Although, there was an apparent increase in cell death in cells treated with combined SPHINX and azacytidine than SPHINX or azacytidine only. This was not found to be significant.

4.3 Effect of SPHINX and imatinib on levels of SRPK1, VEGF and alternative splicing of a panel of apoptotic genes in K562 cells

Experiments were performed to determine the effect of combined SPHINX and imatinib treatment on alternative splicing of genes. Hypothesis for this aspect of the study states that combining SPHINX and imatinib will lead to splicing in a panel of apoptotic genes in favour of pro-apoptotic isoforms than treatment with SPHINX or imatinib treatment alone. To test this hypothesis, PCR were performed using methods already described in chapter two.

4.3.1 Effect of combined SPHINX and imatinib on SRPK1 expression

Using appropriate primers, it was first observed whether combining SPHINX with imatinib will have any effect on SRPK1 mRNA levels. No significant changes in SRPK1 expression were observed (Figure 4.9B).



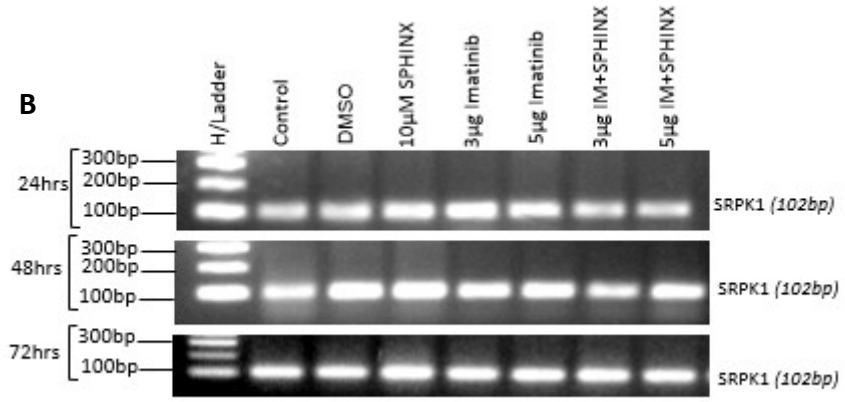


Figure 4.9. SRPK1 levels in combined SPHINX and imatinib treated K562 cells. Cells were treated with either SPHINX, Imatinib or a combination of both for up to 72hrs. **A**. Representation of β-actin amplicons for experimental time points;24hrs-72hrs. **B**. SRPK1 PCR amplicon (102*bp*) for 24hrs, 48hrs and 72hrs. *n*=3.

4.3.2 Effect of combined SPHINX and imatinib on VEGF alternative splicing

VEGF was amplified to check for alternative splicing event (specifically, expression of the pro- and anti-angiogenic VEGF arising from 3' splice site in exon 8) and levels of total VEGF. There was no difference in total VEGF level neither was there any splicing event observed. (Figure 4.10).

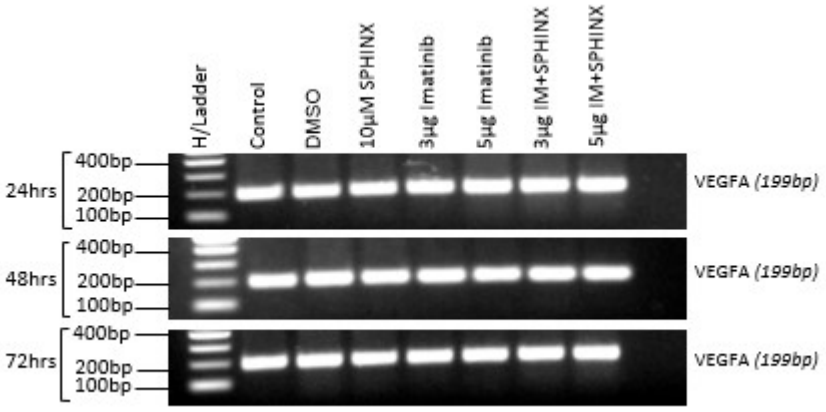


Figure 4.10. VEGF levels in combined SPHINX and imatinib treated K562 cells. Cells were treated with either SPHINX, Imatinib or a combination of both for up to 72hrs. Representation of VEGF PCR amplicon (199*bp*) for 24hrs, 48hrs and 72hrs. *n*=3.

4.3.3 Effect of combined SPHINX and imatinib on Bclx alternative splicing

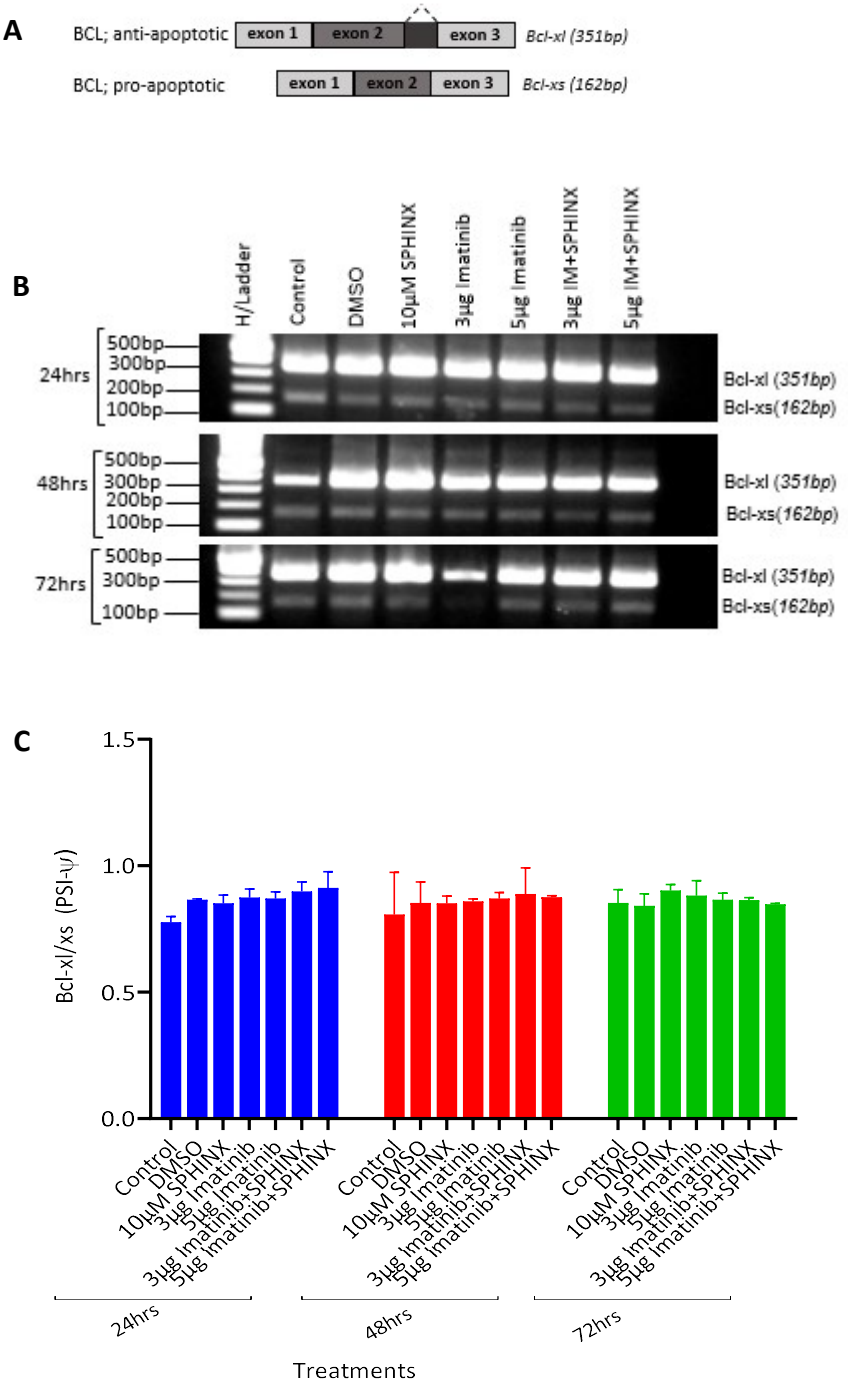
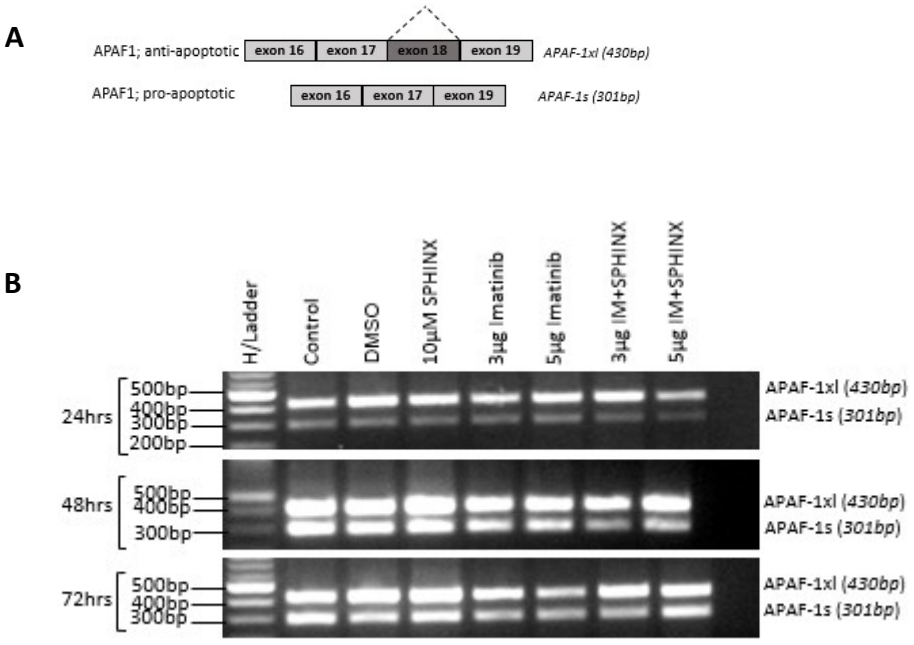


Figure 4.11. Alternative splicing of Bclx in combined SPHINX and imatinib treated K562 cells. Cells were treated with either SPHINX, Imatinib or a combination of both for up to 72hrs. **B**. Representation of PCR amplicon for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in Bcl-xl/xs, n=3.

Splicing events were studied by observing gene involved in the apoptotic pathway. Bcl-x was studied to check whether combined treatment with SPHINX and imatinib would result in a splice switch to the pro-apoptotic isoform Bcl-xs. There was no significant change in the splice ratio of Bcl-xl and Bcl-xs (Figure 4.11).

4.3.4 *Effect of combined SPHINX and imatinib on Apaf1 alternative splicing*

Apaf1 amplification suggests an alteration in alternative splicing. At 24hrs, levels of Apaf-1s, the pro-apoptotic isoform was found to be reduced compared to the pro-apoptotic isoform. Levels of the pro-apoptotic isoform were the same for both treatment and controls. However, at 48hrs and 72hrs, they were found to be the same in the controls while levels of Apaf-1s were also increased tending towards a switch to a pro-apoptotic phenotype when compared to images acquired at 24hrs (Figure 4.12B). However, quantification of PCR amplicons showed no change in splice ratio between Apaf-1xl/1s (Figure 4.12C).



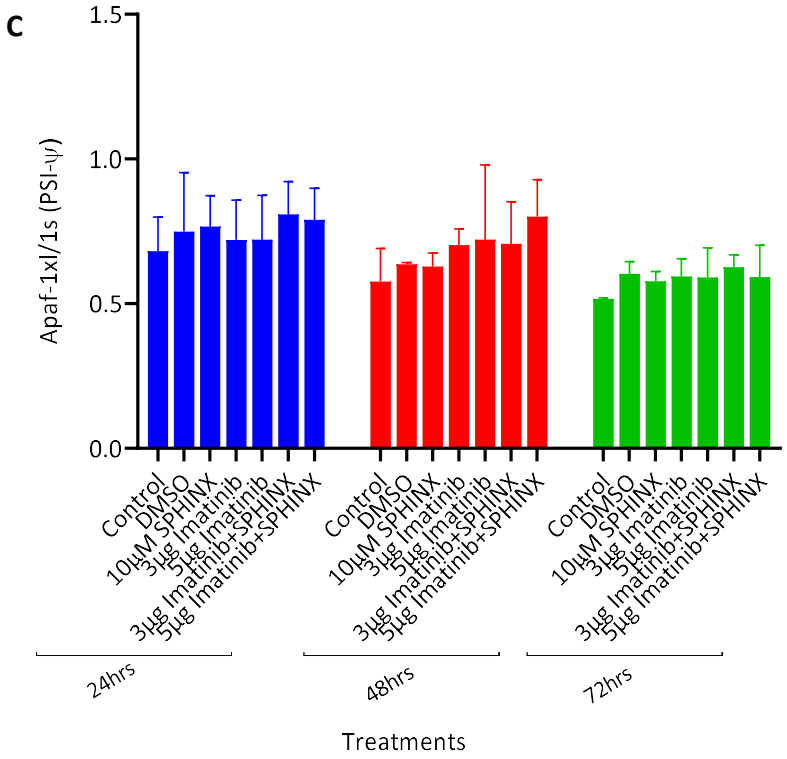


Figure 4.12. Alternative splicing of Apaf1 in combined SPHINX and imatinib treated K562 cells. Cells were treated with either SPHINX, Imatinib or a combination of both for up to 72hrs. **B**. Representation of PCR amplicon for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in Apaf-1x/1s. *n*=3.

4.3.5 Effect of combined SPHINX and imatinib on caspase 9 alternative splicing

Caspase 9 was examined to see if combining SPHINX with imatinib would lead to the expression of the pro-apoptotic caspase 9a isoform. Interestingly, unlike Apaf1, which did not show a complete switch in splicing, caspase 9 showed an apparent switch to pro-apoptotic caspase 9a at 48hrs and 72hrs (Figure 4.13B). Densitometry of the PCR amplicon also indicated a switch in splicing with more inclusion of caspase 9a isoform in treatment including imatinib (Figure 4.13C). Since this switch was observed in the imatinib and combined imatinib and SPHINX but not in the SPHINX only treatment, it is unlikely that this switch resulted from SPHINX alone. Hence, suggesting that a combination of SRPK1 inhibition (using SPHINX) and imatinib treatment may not be beneficial in driving more cells to apoptosis in a CML cell line model.

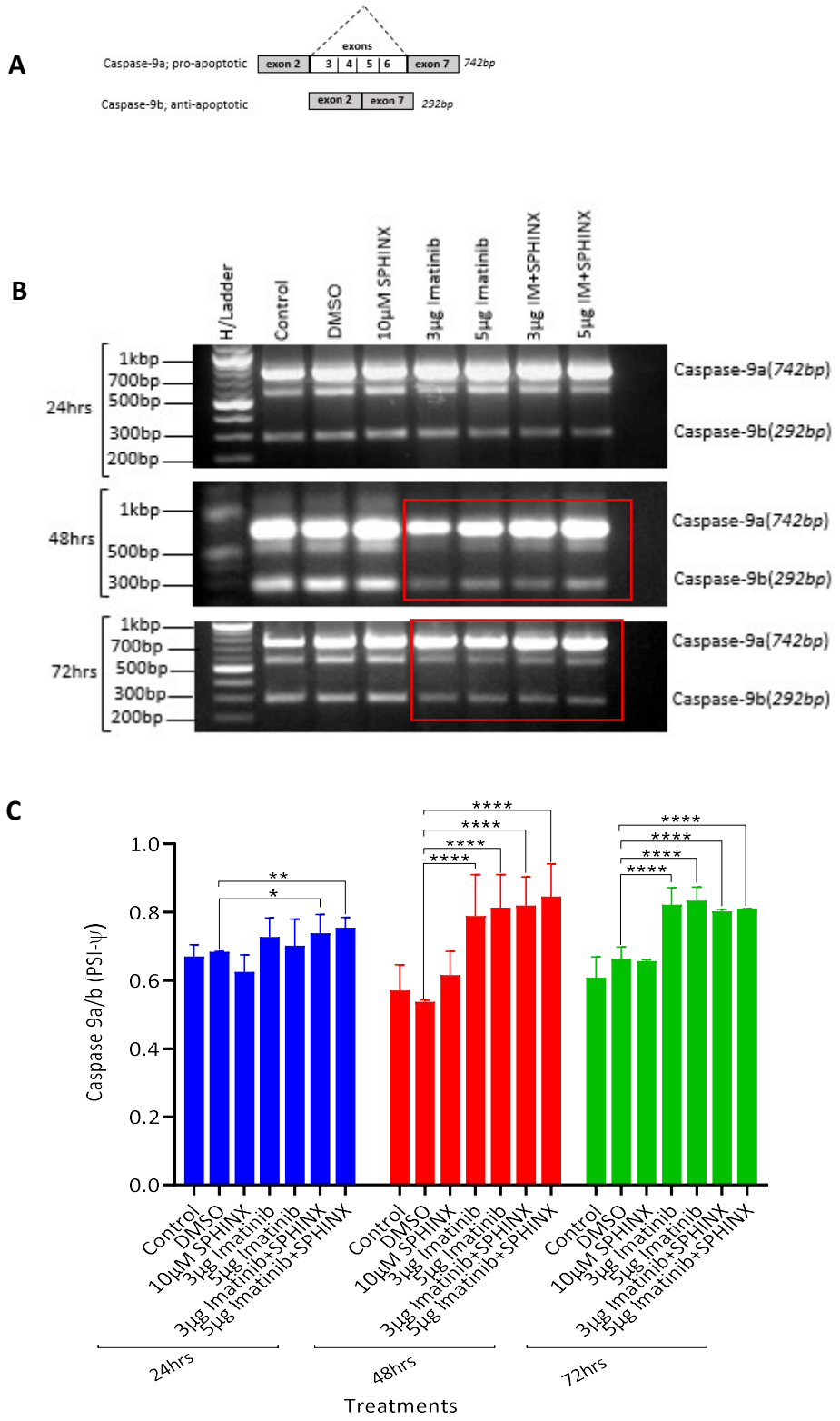


Figure 4.13. Alternative splicing of caspase 9 in combined SPHINX and imatinib treated K562 cells. Cells were treated with either SPHINX, Imatinib or a combination of both for up to 72hrs. **B**. Representation of PCR amplicon for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in caspase-9a/9b isoforms (**P*=0.04; ***P*= 0.006; *****P*<0.0001). *n*=3.

4.4 Effect of combined SPHINX and azacytidine on mRNA levels of SRPK1, VEGF and alternative splicing of a panel of apoptotic genes in Kasumi-1 cells

4.4.1 *Effect of combined SPHINX and azacytidine on SRPK1 expression*

Experiments were performed to also investigate the combined effect of SRPK1 inhibition using SPHINX and azacytidine on SRPK1 overall expression in Kasumi-1 cells. With the caveat that standard PCR is not quantitative, the data suggest no change in overall SRPK1 expression (Figure 4.14B).

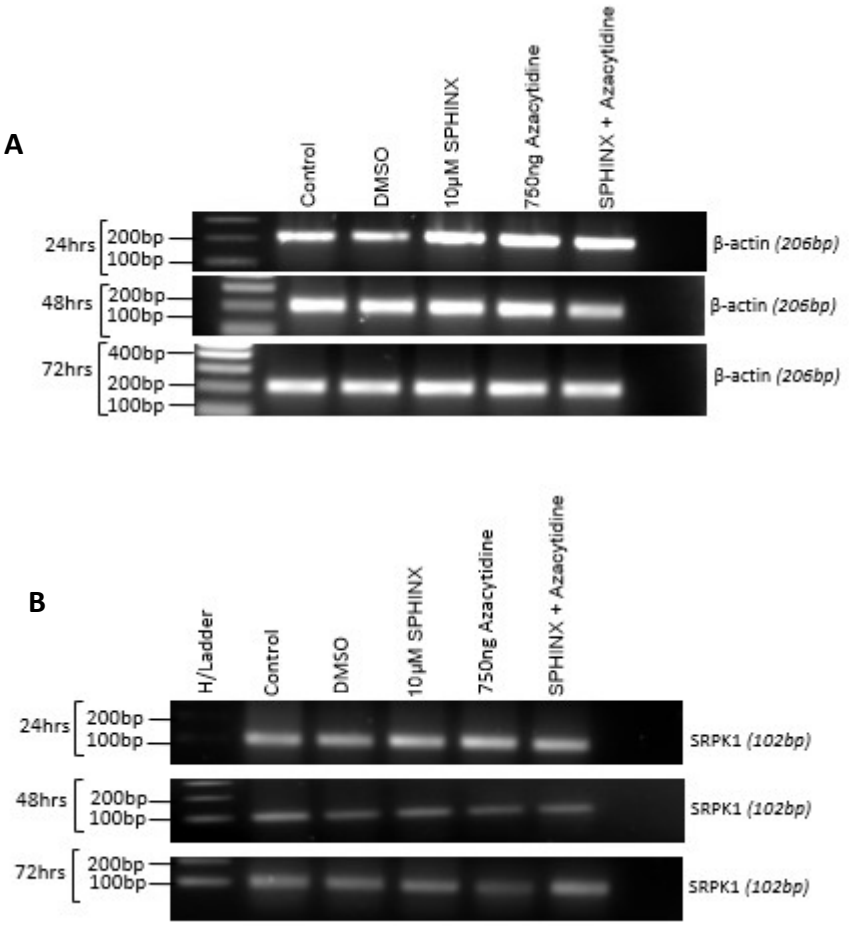


Figure 4.14. SRPK1 levels in combined SPHINX and azacytidine treated Kasumi-1 cells. Cells were treated with either SPHINX, azacytidine or a combination of both for up to 72hrs. **A**. Representative image for β-actin for experimental time points. **B**. SRPK1 PCR amplicon (102bp) for 24hrs, 48hrs and 72hrs. *n*=3.

4.4.2 Effect of combined SPHINX and azacytidine on VEGF alternative splicing

Alternative splicing event and levels of VEGF in Kasumi-1 cells indicate no change in VEGF expression level (Figure 4.15). At 48hrs, one experimental repeat showed an indication of a splicing event yielding a faint amplicon with a size of 65bp which corresponds to $VEGF_{165b}$ anti-angiogenic isoform. However, it was difficult to confirm this result as a repeat experiment aimed at reproducing the same result was not successful.

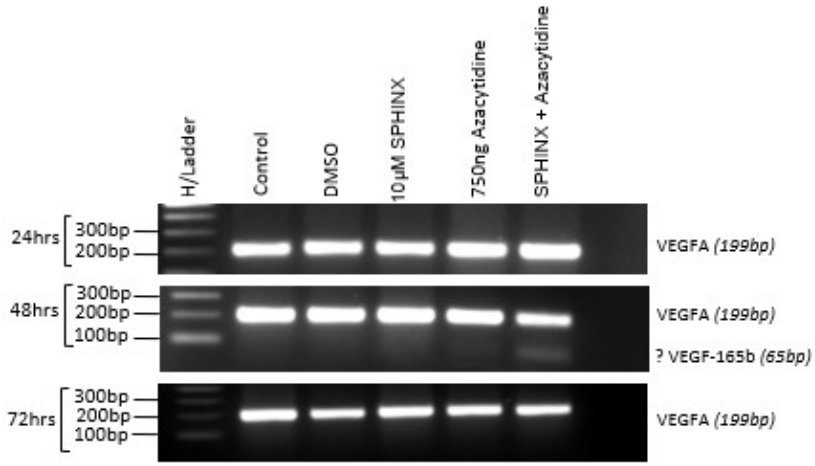


Figure 4.15. VEGF expression in combined SPHINX and azacytidine treated Kasumi-1 cells. Cells were treated with either SPHINX, azacytidine or a combination of both for up to 72hrs. Representation of total VEGF PCR amplicon (102bp) for 24hrs, 48hrs and 72hrs. *n*=3.

4.4.3 Effect of combined SPHINX and azacytidine on Bclx alternative splicing

The ratio of Bcl-xl to Bcl-xs was broadly unaffected. Levels of highly expressed anti-apoptotic Bcl-xl were predominant (Figure 4.16B). It was however interesting to note the very low levels of Bcl-xs in Kasumi-1 cells (Figure 4.16B). This provides further evidence that levels of gene expression differ from one cell type to another.

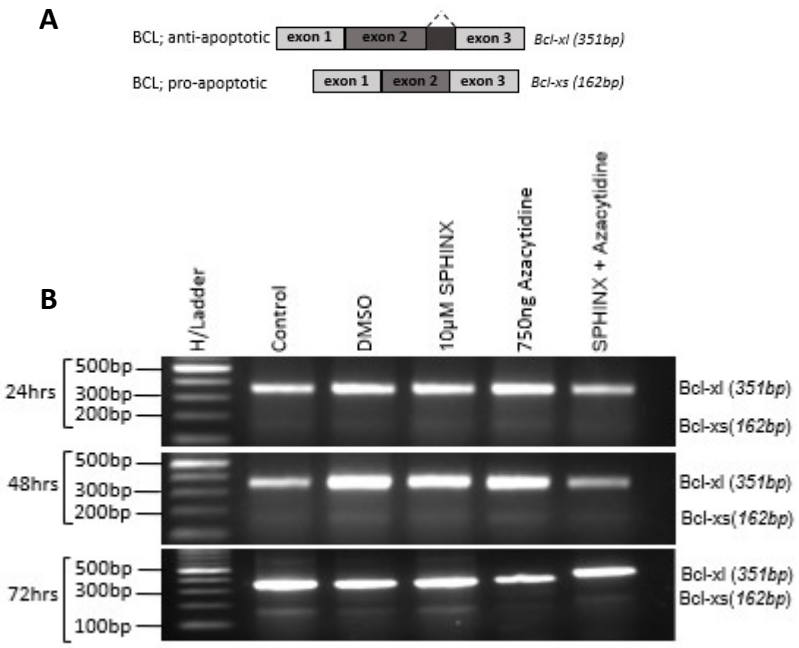


Figure 4.16. Alternative splicing of BCLx in combined SPHINX and azacytidine treated Kasumi-1 cells. Cells were treated with either SPHINX, azacytidine or a combination of both for up to 72hrs. **B**. Representation of PCR amplicon for 24hrs, 48hrs and 72hrs for Bcl-xl/xs isoform. *n*=3.

4.4.4 Effect of combined SPHINX and azacytidine on Apaf1 alternative splicing

There was no change in levels or alternative splicing events in Apaf1 when compared to the controls. This difference was not found to be statistically significant. (Figure 4.17B-C).

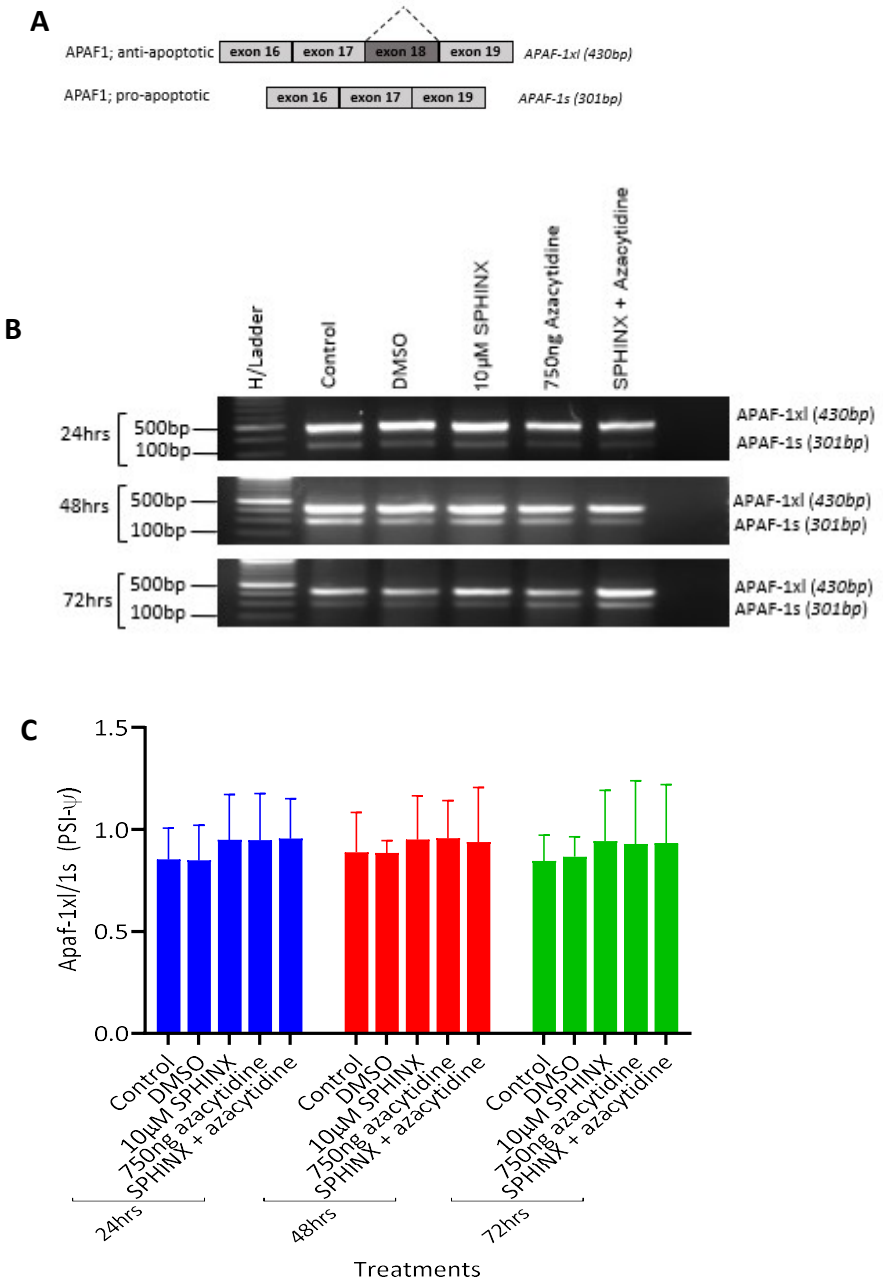


Figure 4.17. Alternative splicing of Apaf1 in combined SPHINX and azacytidine treated Kasumi-1 cells. Cells were treated with either SPHINX, azacytidine or a combination of both for up to 72hrs. **B**. Representation of PCR amplicon for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in Apaf-1x/1s isoform. *n*=3.

4.4.5 Effect of combined SPHINX and azacytidine on caspase 9 alternative splicing

Levels of caspase 9 appear unaffected with the highly expressed 9a isoform being consistent and showed no difference between the treatments and controls (Figure 4.18B-C).

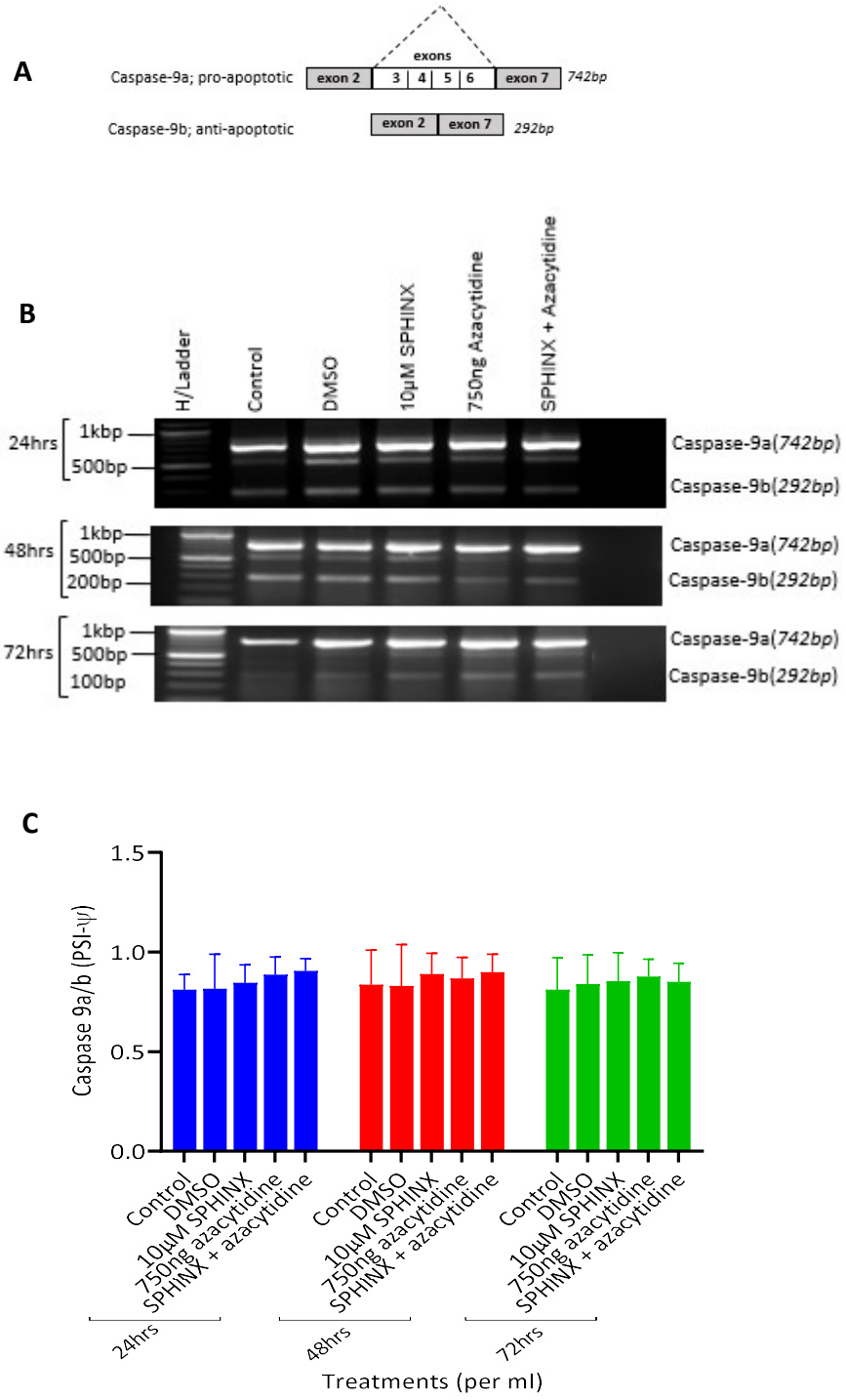
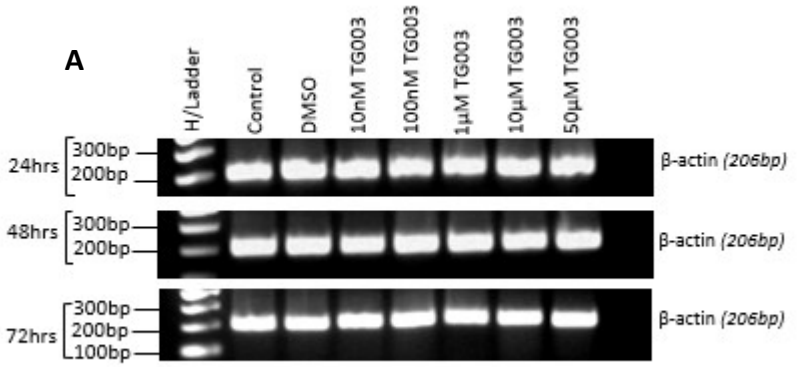


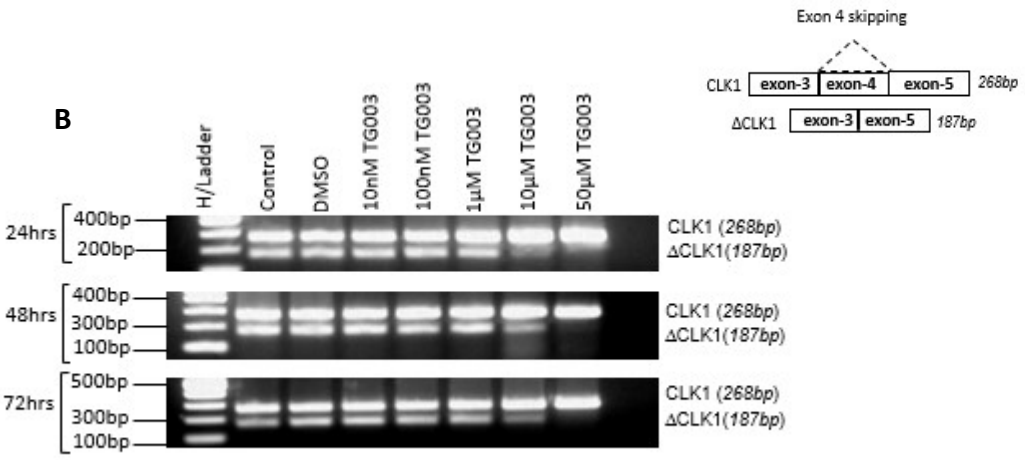
Figure 4.18. Alternative splicing of caspase 9 in combined SPHINX and azacytidine treated Kasumi-1 cells. Cells were treated with either SPHINX, azacytidine or a combination of both for up to 72hrs. **B**. Representation of PCR amplicon for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in caspase-9a/9b isoform. *n*=3.

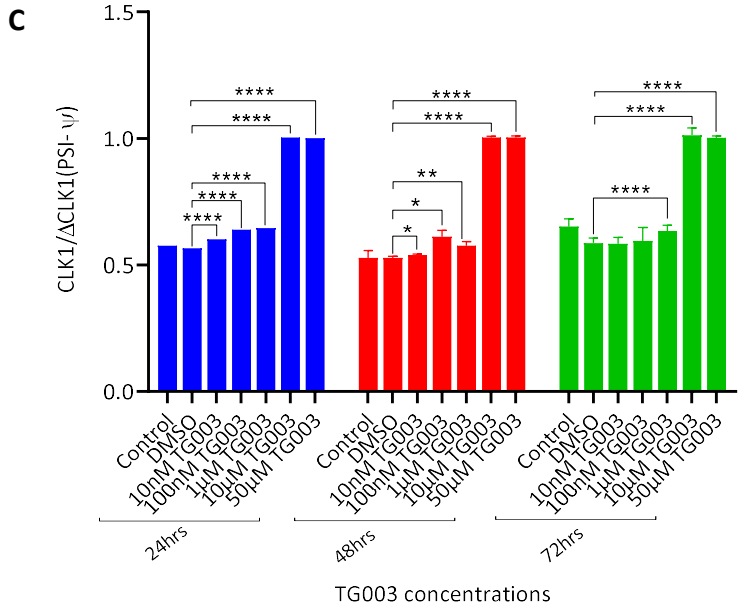
4.5 Effect of combined SPHINX and TG003 with imatinib mesylate on the expression of SRPK1, CLK1, VEGF and alternative splicing of a panel of apoptotic genes in K562 cells

4.5.1 *CLK1 alternative splicing following inhibition with TG003 in K562 cells*

As previously discussed, CLK1 may affect the function of SRPK1 as both kinases complement the activity of the other in the phosphorylation of SR proteins, their preferred substrate (Aubol *et al*., 2014). Therefore, the effect of inhibiting both kinases together was examined. Chronic myeloid leukaemic cells, K562 were treated with increasing concentration of TG003, a specific inhibitor of CLK1. The effect of CLK1 inhibition on its alternative splicing was then observed. Primers used were forward primers in exon 3 and exon 4 and two reverse primers on exon 5 of CLK1. A combination of these primers would detect alternative splicing events within these regions. Inhibition of CLK1 results in increased exon 4 retention at higher concentration of 10µM and 50µM (Figure 4.19B-C), with a significant difference when TG003 treatments were compared to DMSO control. This result was also matched with less intron 4 retention at the same higher concentration of 10µM at 24hrs and 10µM and 50µM at 48hrs and 72hrs respectively (Figure 4.19D-E).


A
H/Ladder
Control
DMSO
10nM TG003
100nM TG003
1µM TG003
10µM TG003
50µM TG003
24hrs
300bp
200bp
β-actin (206bp)
48hrs
300bp
200bp
β-actin (206bp)
72hrs
300bp
200bp
100bp
β-actin (206bp)


Exon 4 skipping
CLK1
exon-3
exon-4
exon-5
268bp
ΔCLK1
exon-3
exon-5
187bp
B
H/Ladder
Control
DMSO
10nM TG003
100nM TG003
1µM TG003
10µM TG003
50µM TG003
24hrs
400bp
200bp
CLK1 (268bp)
ΔCLK1(187bp)
48hrs
400bp
300bp
100bp
CLK1 (268bp)
ΔCLK1(187bp)
72hrs
500bp
300bp
100bp
CLK1 (268bp)
ΔCLK1(187bp)


C
CLK1/ΔCLK1(PSI- ψ)
1.5
1.0
0.5
0.0
****
**
*
Control
DMSO
10nM TG003
100nM TG003
1µM TG003
10µM TG003
50µM TG003
24hrs
48hrs
72hrs
TG003 concentrations

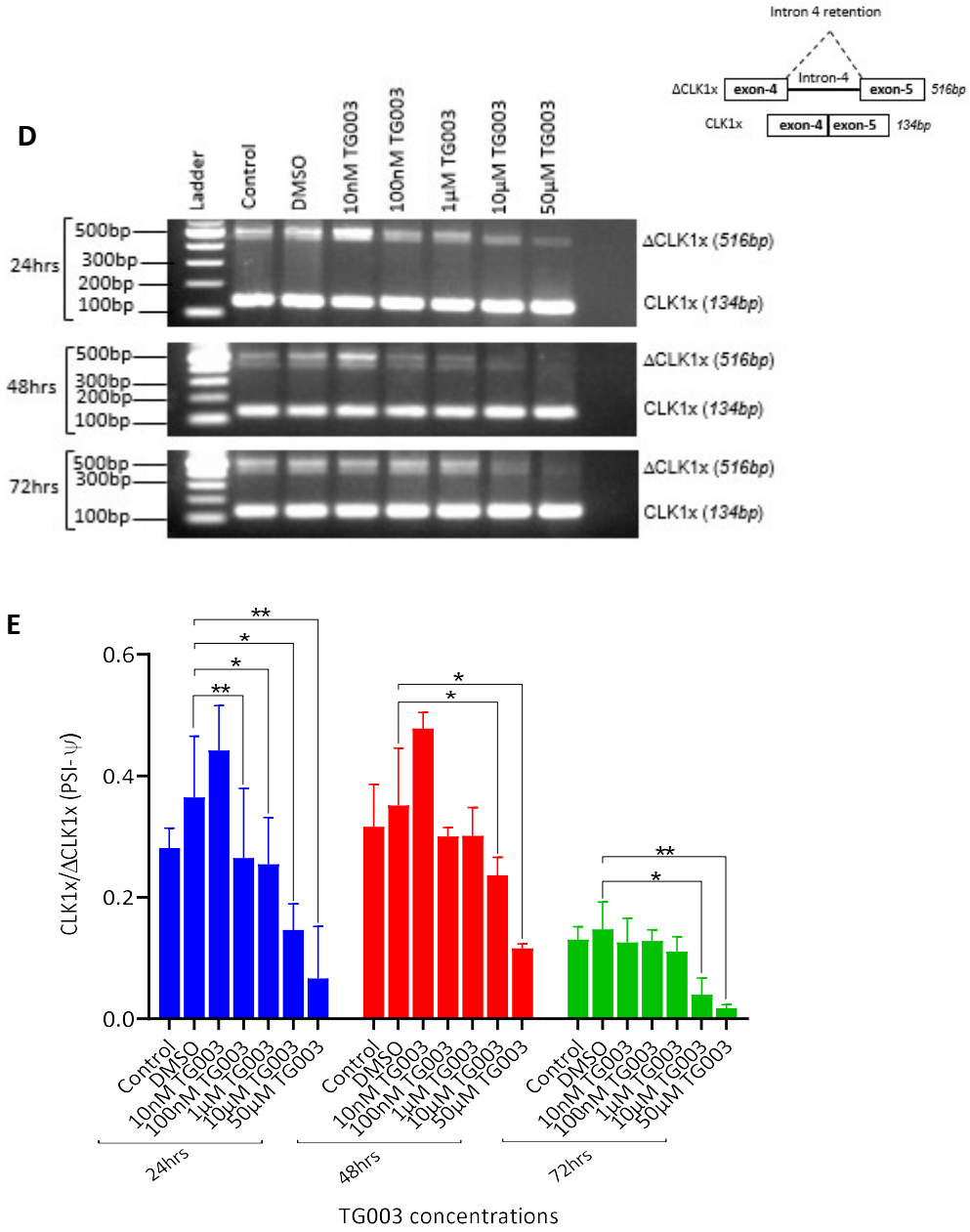


Figure 4.19. Alternative splicing of CLK1 following inhibition with TG003 in K562 cells. K562 cells were treated with increasing concentrations of TG003 for up to 72hrs. PCR was performed using CLK1 specific primers. **A**. representative image for β-actin for experimental time points. **B**. PCR amplicon for CLK1/ΔCLK1 exon 4 skipping at 24hrs, 48hrs and 72hrs and illustration of alternative splicing events in CLK1 showing retention of exon 4. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) for CLK1 exon 4. P-values for DMSO versus treatment at 24hrs (****P<0.0001), 48hrs (P=0.02; 0.01; 0.009; P<0.0001) and 72hrs(****P<0.0001). **D**. PCR amplicon for 24hrs, 48hrs and 72hr showing intron 4 retention and illustration of alternative splicing event in CLK1x showing retention of intron 4. **E**. Densitometry showing percentage of intron 4 retention for 24hrs-72hrs. DMSO was compared to TG003 concentrations at 24hrs (**P= 0.004; *P=0.01; *P=0.03; **P=0.003), 48hrs (*P=0.04; 0.02) and 72hrs (*P=0.017; **P=0.005). n=3.

4.5.2 Effect of combined SPHINX and TG003 with imatinib on SRPK1 expression in K562 cells

Levels of SRPK1, where both SRPK1 and CLK1 kinase was inhibited in addition to imatinib treatment was not found to be different from the controls or all other treatment (Figure 4.20B-C).

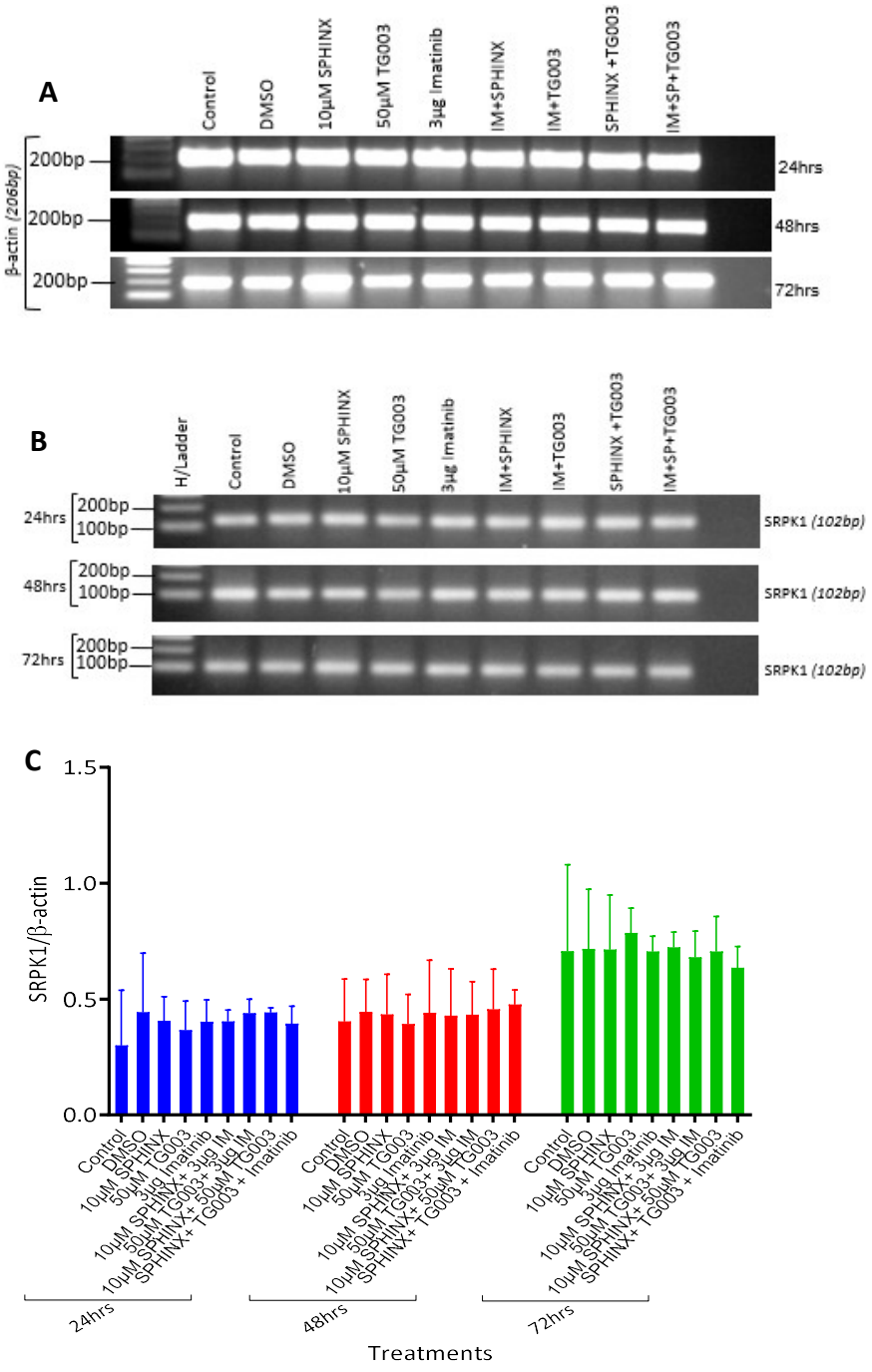
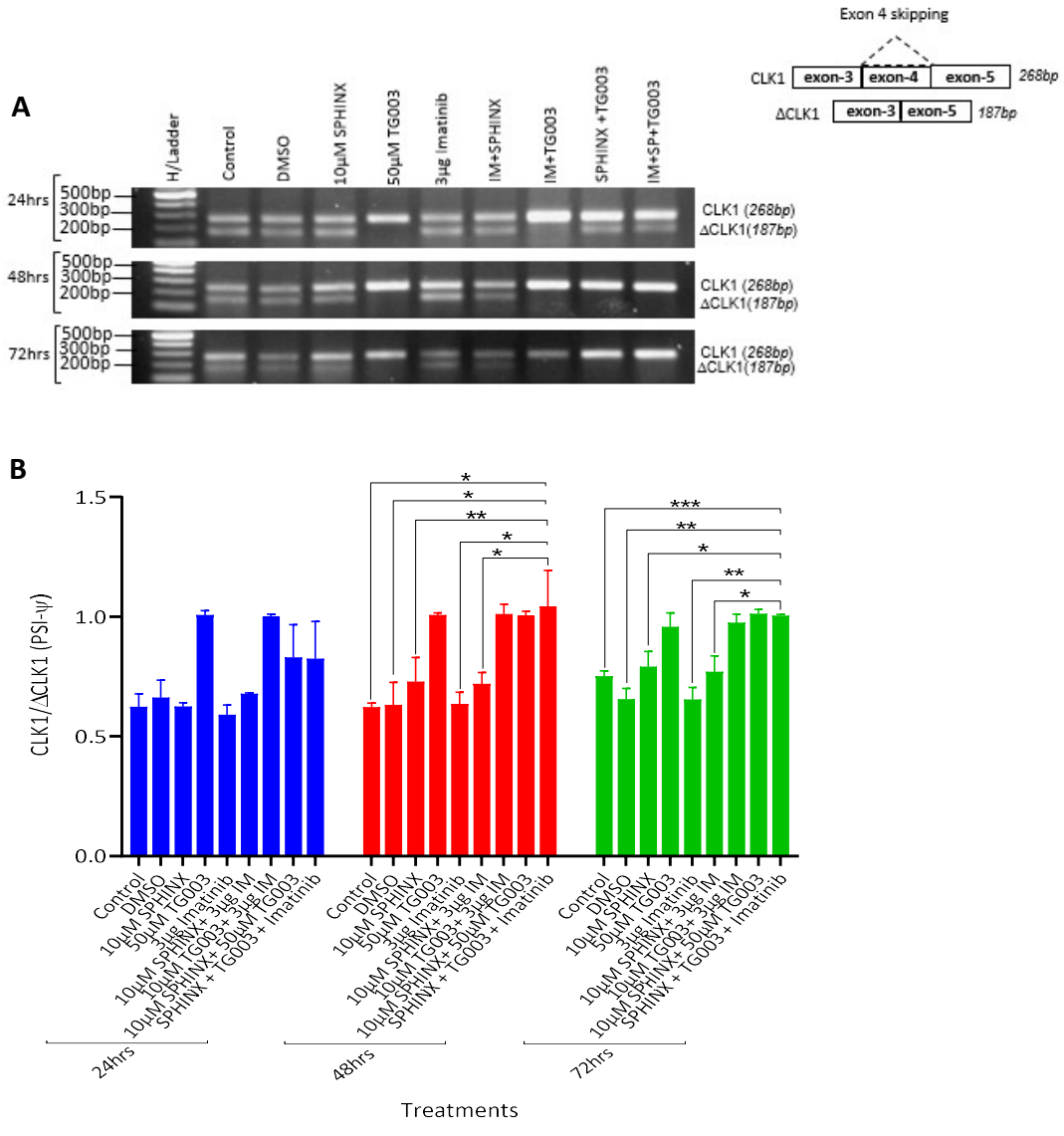


Figure 4.20. SRPK1 levels in combined SPHINX and TG003 with imatinib mesylate treated K562 cells. K562 cells were treated with either Imatinib, SPHINX or TG003 inhibitor, a combination of both inhibitors and all three treatments for up to 72hrs. **A**. Representative image for β-actin for the experimental time points. **B**. SRPK1 amplicons for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing levels of SRPK1 expression in K562 cells. *n*=3.

4.5.3 Effect of combined SPHINX and TG003 with imatinib on CLK1 alternative splicing K562 cells

Since the effect of CLK1 inhibition on its own splicing is known, experiments were then performed to see how CLK1 splicing is affected by other treatments including combining CLK1 and SRPK1 inhibition in addition to imatinib treatment. At 24hrs, there was more exon 4 retention in the TG003 and TG003 and imatinib treated cell. However, at 48hrs and 72hrs, all treated group with CLK1 inhibition using TG003 either singly or in combination with other treatment showed increased retention of exon 4 (Figure 4.21A-B). Further to this, a corresponding result was also observed with less intron 4 retention (Figure 4.21C-D). This suggests that inhibiting CLK1 and SRPK1 in addition to imatinib treatment may not have any added advantage to CLK1 activity in CML cell model.



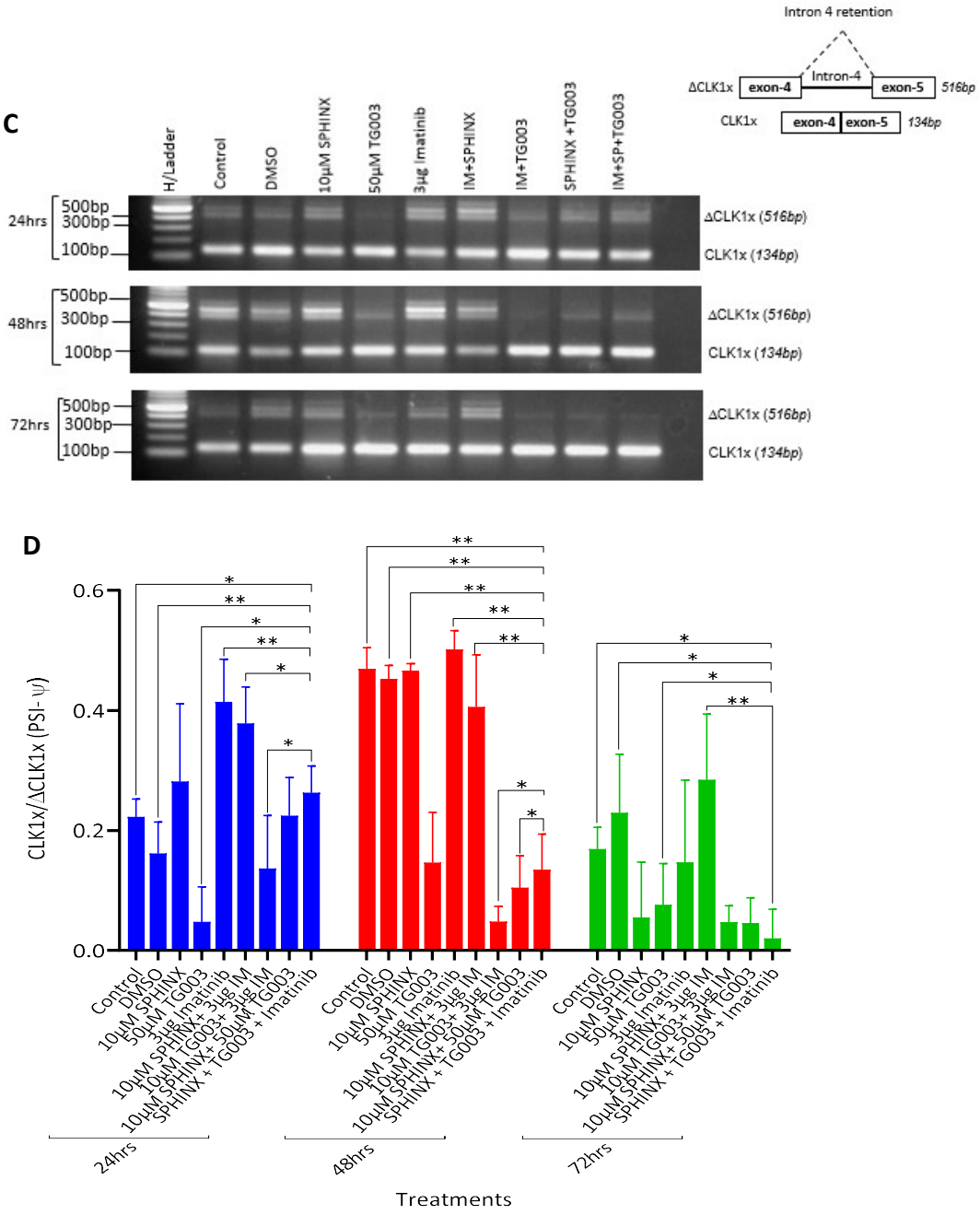


Figure 4.21. Alternative splicing of CLK1 in K562 cells following combined SPHINX and TG003 with imatinib mesylate treatment. K562 cells were treated with either Imatinib, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three treatments for up to 72hrs. **A**. Representative of PCR amplicon for 24hrs, 48hrs and 72hrs. illustration of alternative splicing events in CLK1 showing retention of exon 4. **B**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in CLK1 for exon 4 when other treatments were compared to combined SPHINX+TG003+Imatinib at 24hrs, 48hrs and 72hrs. **C**. Representation of PCR amplicon for 24hrs, 48hrs and 72hr showing levels of intron 4 retention and illustration of alternative splicing event in CLK1. **D**. Densitometry showing percentage of intron 4 retention in CLK1 and significant levels observed when SPHINX+TG003+Imatinib was compared to other treatment. *n*=3.

4.5.4 Effect of combined SPHINX and TG003 with imatinib on VEGF alternative splicing in K562 cells

There was also no difference in VEGF expression between treatments, neither was there any alternative splicing event observed (Figure 4.22).

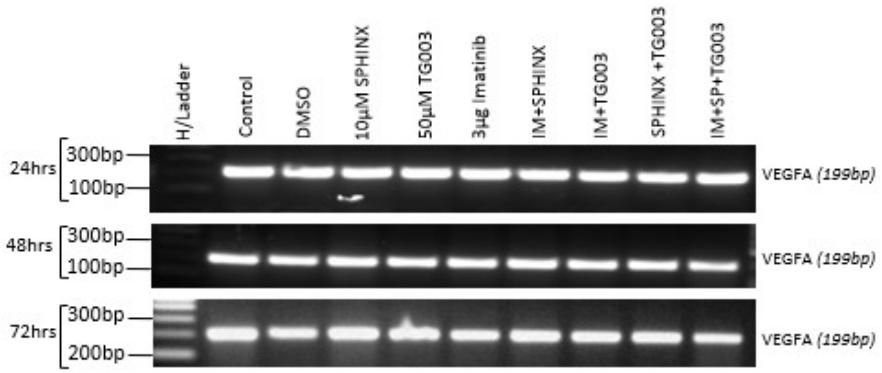


Figure 4.22. VEGF levels in combined SPHINX and TG003 with imatinib treatment in K562 cells. K562 cells were treated with either Imatinib, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three treatments for up to 72hrs. Representation of PCR amplicon (199bp) for 24hrs, 48hrs and 72hrs. *n*=3

4.5.5 Effect of combined SPHINX and TG003 with imatinib on Bclx alternative splicing in K562 cells

Alternative splicing in Bclx was studied using appropriate primers. There was no change in Bclx splicing across all treatments including the controls. The dominant isoform Bcl-xl was expressed.

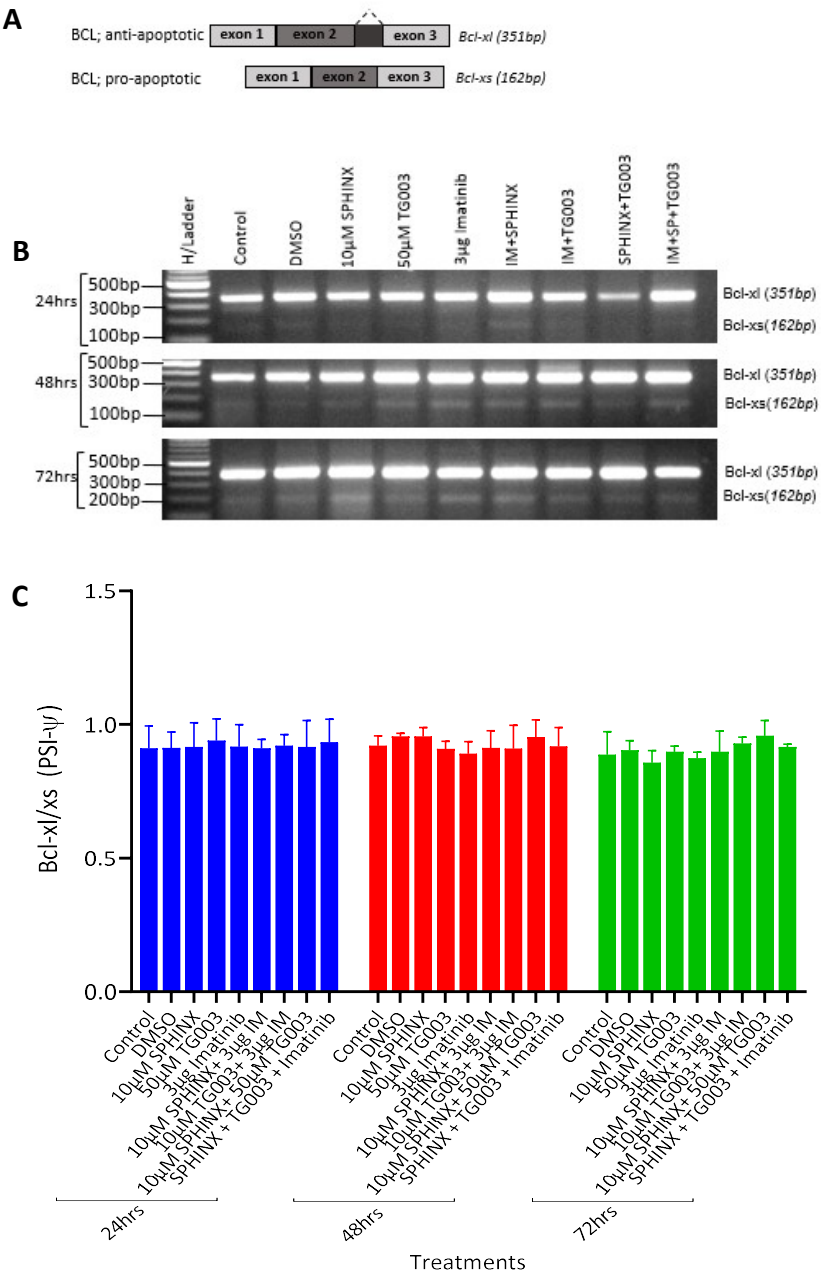
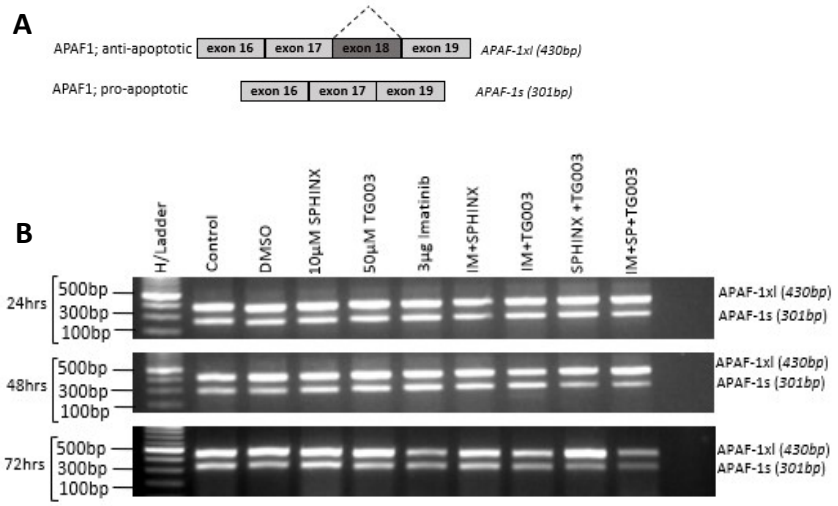


Figure 4.23. Alternative splicing of Bclx in K562 cells following combined SPHINX and TG003 with imatinib treatment. K562 cells were treated with either Imatinib, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three treatments for up to 72hrs. PCR was performed using Bclx specific primers. **B**. Representation of PCR amplicon (Bcl-xl, 351bp) and (Bcl-xs, 162bp) for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) of Bcl-xl isoform. *n*=3.

4.5.6 Effect of combined SPHINX and TG003 with imatinib on Apaf1 alternative splicing in K562 cells

PCR amplicons for Apaf1, on visual examination, appear to express equal level for both isoforms at 24hrs, but with a reduction in Apaf-1s level in the SPHINX and TG003 and its combination with imatinib at 48hrs. However, at 72hrs, the levels seem to vary with treatment, maintaining higher levels of Apaf-1xl and Apaf-1s levels in the controls, SPHINX and TG003 treated groups. Treatment with imatinib, Imatinib and TG003 and the combined SPHINX, TG003 and imatinib treatment showed a reduction but equal levels of both Apaf-1xl and Apaf-1s isoforms (Figure 4.24B). Image quantification of the bands showed varying ratio with each treatment at 48hrs and 72hrs. (Figure 4.24C).



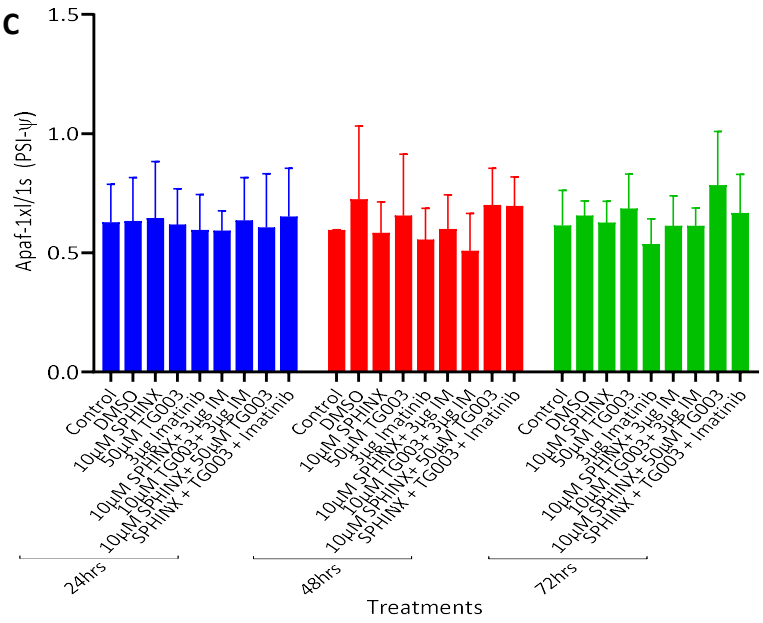


Figure 4.24. Alternative splicing of Apaf1 in K562 cells following combined SPHINX and TG003 with imatinib mesylate treatment. K562 cells were treated with either Imatinib, SPHINX or TG003, a combination of both inhibitor and all three treatments for up to 72hrs. **B**. Representative of PCR amplicons (Apaf-1xl, 430bp) and (Apaf-1s, 301bp) for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in Apaf-1xl/1s isoforms. *n*=3.

4.5.7 *Effect of combined SPHINX and TG003 with imatinib on caspase 9 alternative splicing in K562 cells*

The levels of caspase 9 were investigated to see whether inhibiting SRPK1 and CLK1 in addition to imatinib treatment would result in a complete switch in splicing to the pro-apoptotic caspase 9a since earlier treatments with imatinib alone resulted in a switch but non-significant change in splice ratio between 9a/9b (Figure 4.25B). Figure 4.25C shows levels of caspase 9a and 9b levels for 24hrs-72hrs. Reduced levels and an apparent change in splicing was observed at 48hrs and 72hrs in groups with imatinib. This result was consistent with the previous experiment indicating imatinib but not SPHINX or TG003 potentially being responsible for the induction of pro-apoptotic events observed in K562 cells.

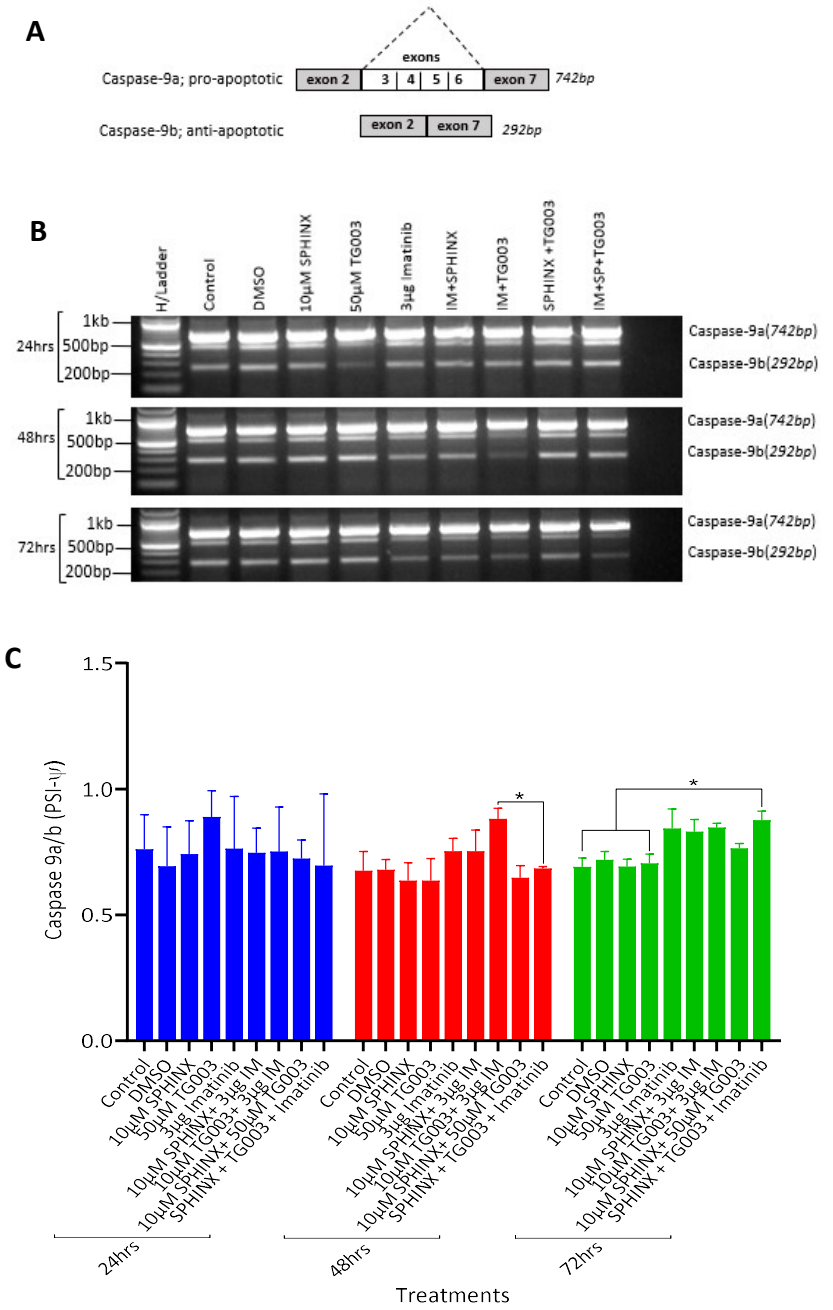
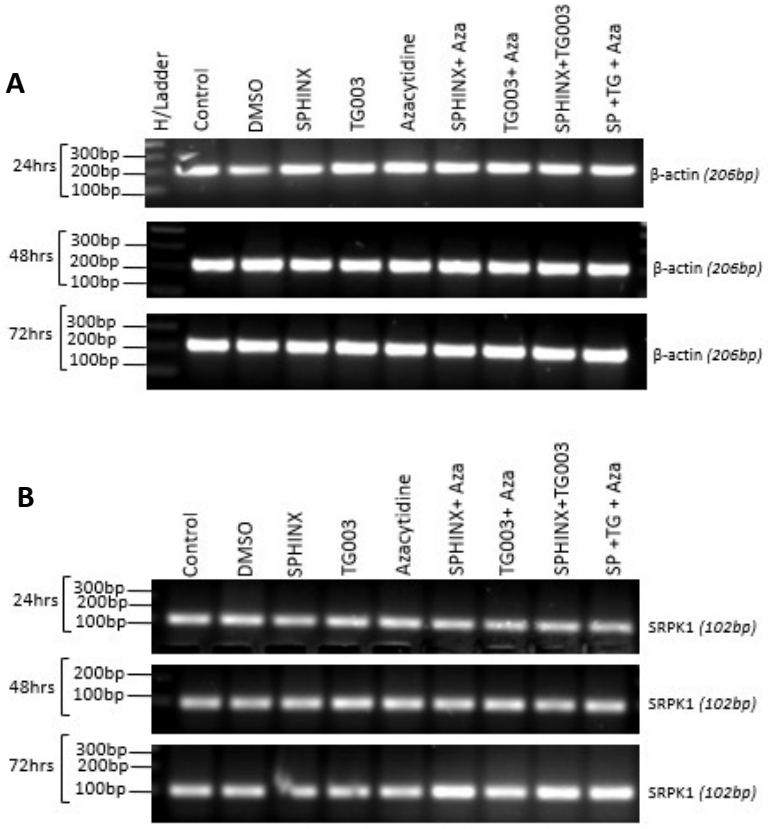


Figure 4.25. Alternative splicing of caspase 9 in K562 cells following combined SPHINX and TG003 with imatinib mesylate treatment. K562 cells were treated with either Imatinib, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three treatments for up to 72hrs. **B**. Representation of PCR amplicon (caspase 9a, 742bp) and (caspase 9b, 292bp) for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in caspase-9a/9b isoform. Statistics comparing SPHINX+TG003+Imatinib with other treatment was significant at 48hrs (**P*=0.01) and 72hrs (*P*<0.05), *n*=3.

4.6 Effect of combined SPHINX and TG003 with azacytidine on the expression of SRPK1, CLK1, VEGF and alternative splicing of a panel of apoptotic genes in Kasumi-1 cells

4.6.1 *Effect of combined SPHINX and TG003 with azacytidine on SRPK1 expression*

The effect of SRPK1 and TG003 inhibition in addition to treatment with azacytidine in Kasumi-1 was also investigated to see if combined treatment would influence RNA processing in AML treatment potentially indicating a better therapeutic outcome. Changes in levels of SRPK1 was examined to see if a combination of this treatment would result in any change in SRPK1 RNA when compared to the use of each inhibitor or drug alone. Results showed no change in SRPK1 levels (Figure 4.26B-C).



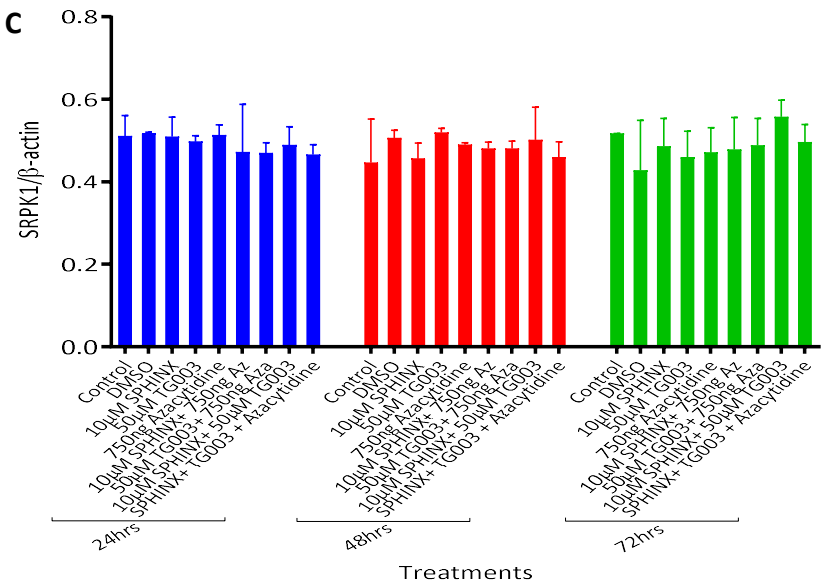


Figure 4.26. SRPK1 levels in combined SPHINX and TG003 with azacytidine treatment in Kasumi-1 cells. Kasumi-1 cells were treated with either azacytidine, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three molecules for up to 72hrs. **A**. Representation of loading control, β-actin (206*bp*) for 24hrs, 48hrs and 72hrs. **B**. SRPK1 PCR amplicon for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing levels of SRPK1 levels which showed no significant difference between treatments. *n*=3.

4.6.2 Effect of combined SPHINX and TG003 with azacytidine on VEGF alternative splicing in Kasumi-1 cells

Levels of total VEGF was also investigated for the changes and to see if treatment would result in a switch in splicing to VEGF anti-angiogenic isoform VEGF165b which has been shown to slow down disease progression through inhibition of neovascularization (Mavrou *et al*., 2014). There were no significant changes in total VEGF levels, neither was there any splicing event seen. (Figure 4.27).

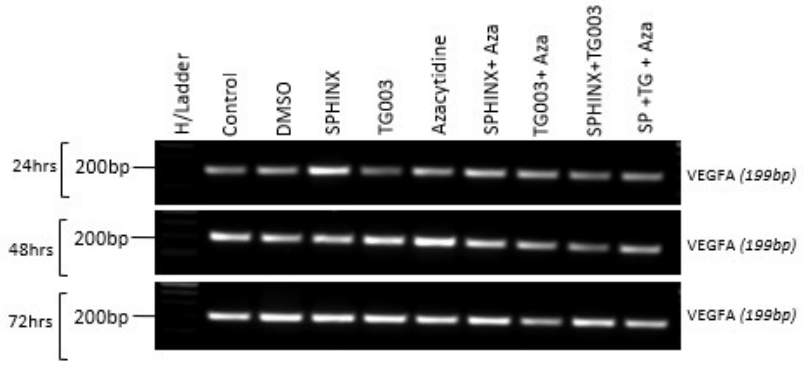


Figure 4.27. VEGF levels in combined SPHINX and TG003 with azacytidine treatment in Kasumi-1 cells. Kasumi-1 cells were treated with either azacytidine, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three treatments for up to 72hrs. Representation of VEGF PCR amplicon (199bp) for 24hrs, 48hrs and 72hrs.

4.6.3 Effect of combined SPHINX and TG003 with azacytidine on Bclx alternative splicing in Kasumi-1 cells

The apoptotic gene Bclx was studied to see if there was a change in splicing to the pro-apoptotic isoform Bcl-xs. The dominant isoform was seen to be highly expressed and no change in individual isoform level was observed with treatment or controls (Figure 4.28B-C).

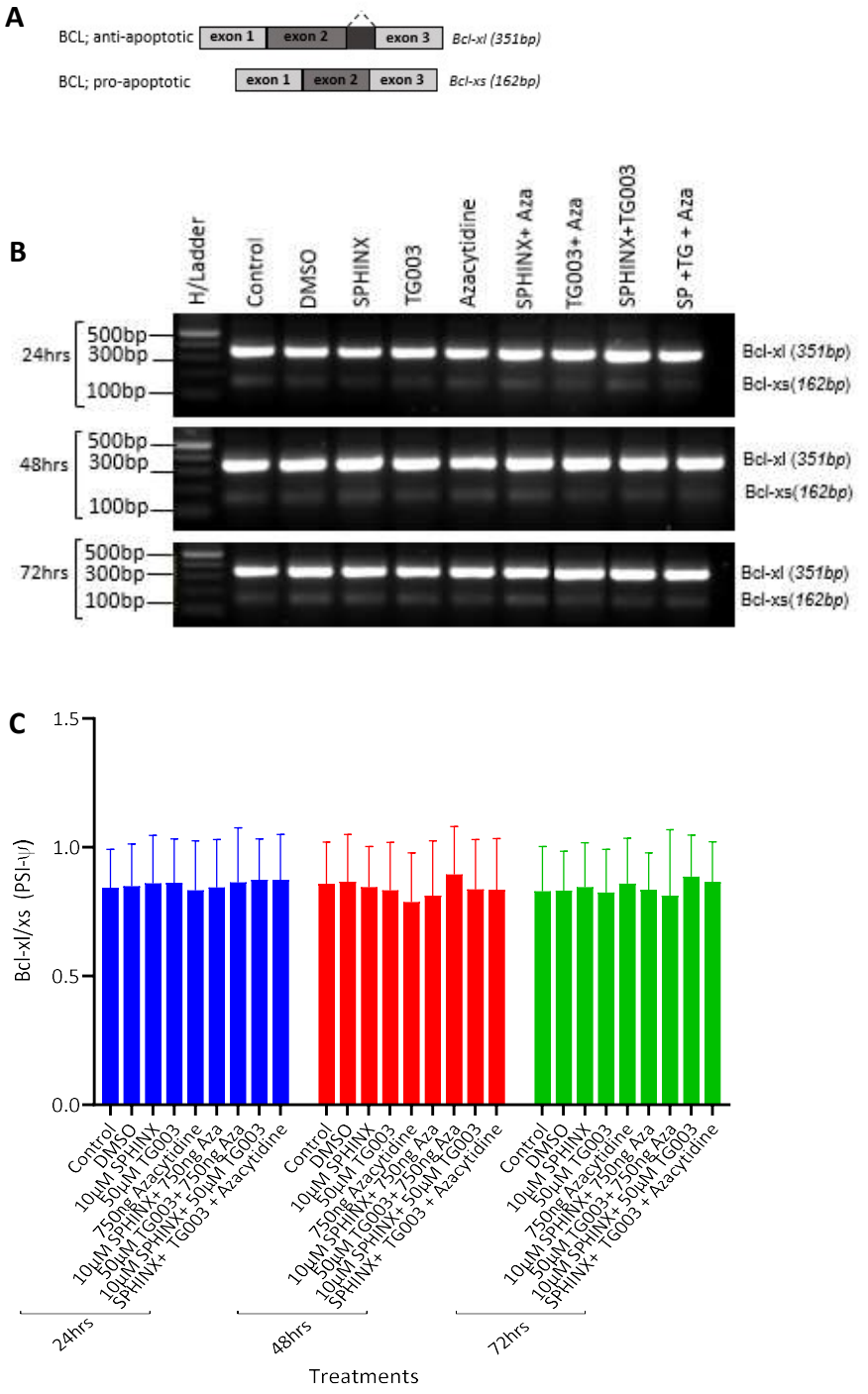


Figure 4.28. Alternative splicing of Bclx following combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Kasumi-1 cells were treated with either azacytidine, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three molecules for up to 72hrs. **B**. Representation of PCR amplicon (Bcl-xl, 351bp) and (Bcl-xs, 162bp) for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) of Bcl-xl isoform. *n*=3.

4.6.4 Effect of combined SPHINX and TG003 with azacytidine on Apaf1 alternative splicing

The levels of Apaf1 appear to be unaffected at 24hrs and 48hrs, anti-apoptotic isoform, Apaf-1xl seem to have a higher level of expression. While at 72hrs, cells treated with azacytidine, TG003 and azacytidine, SPHINX and TG003 and all three combined treatment showed reduced but equal levels of both isoforms.

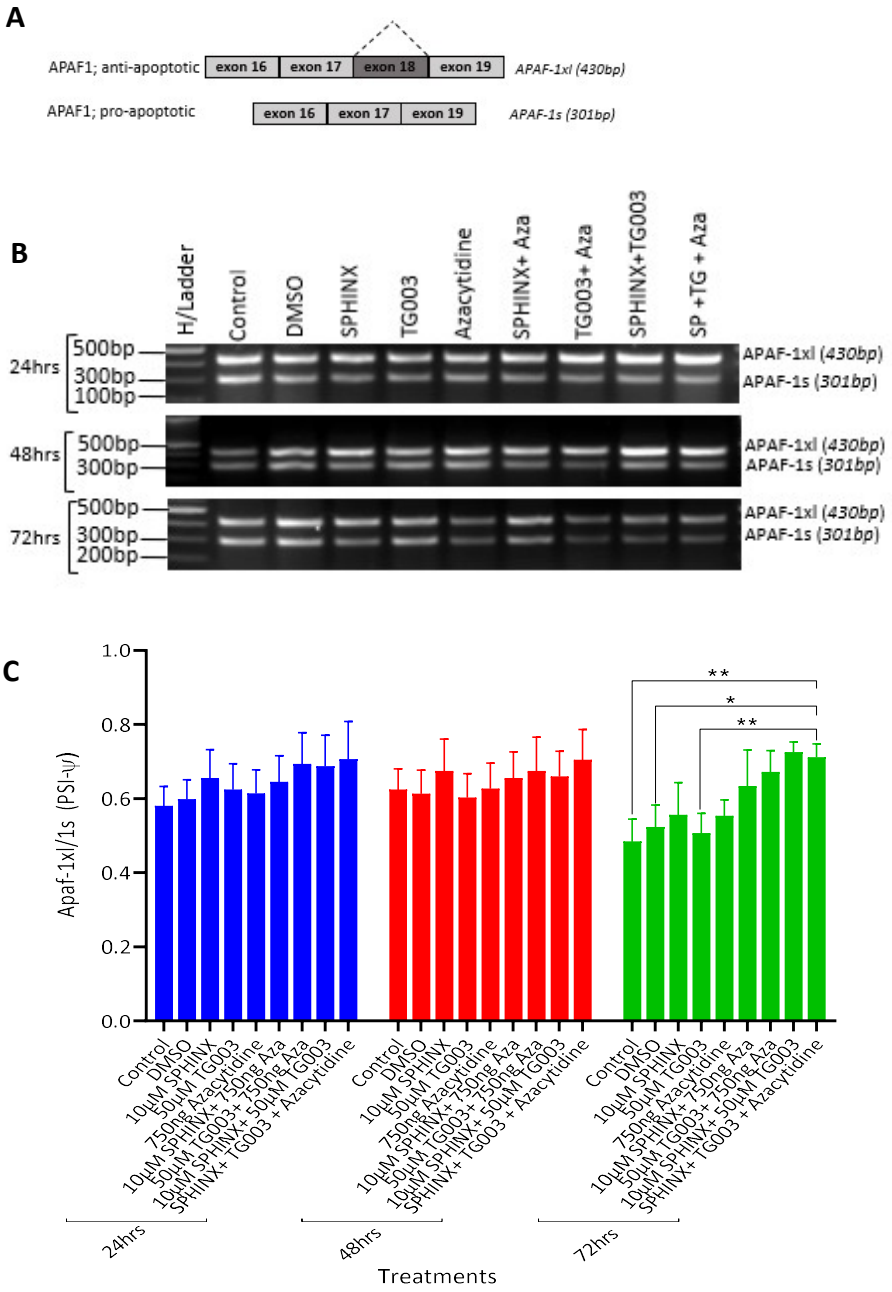


Figure 4.29. Alternative splicing of Apaf1 following combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Kasumi-1 cells were treated with either azacytidine, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three molecules for up to 72hrs. **B**. Representation of PCR amplicon (Apaf-1x, 430bp) and (Apaf-1s, 301bp) for 24hrs, 48hrs and 72hrs. **C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) of Apaf-1 isoform. *n*=3.

4.6.5 Effect of combined SPHINX and TG003 with azacytidine on caspase 9 alternative splicing in Kasumi-1 cells

Levels of caspase 9 appear unaffected across the treatment with caspase 9a isoform being more expressed (Figure 4.30B-C).

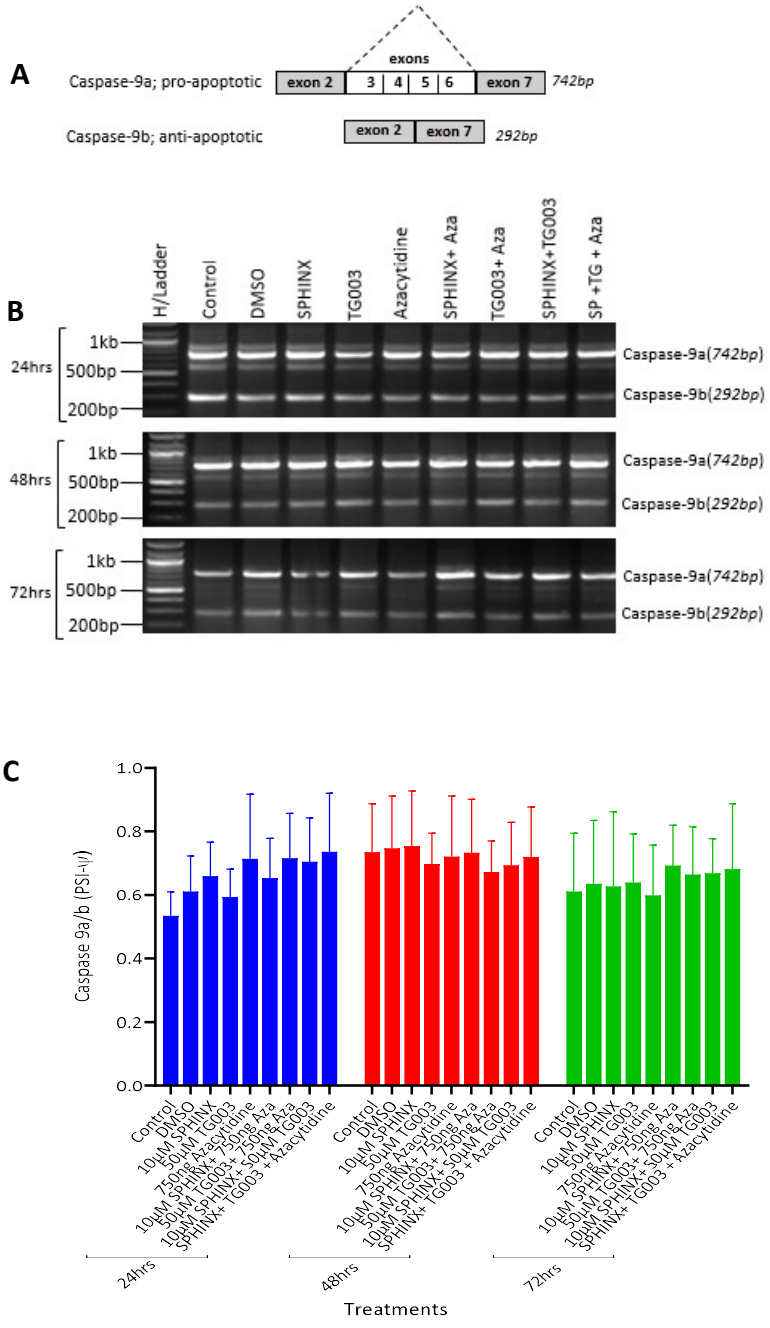
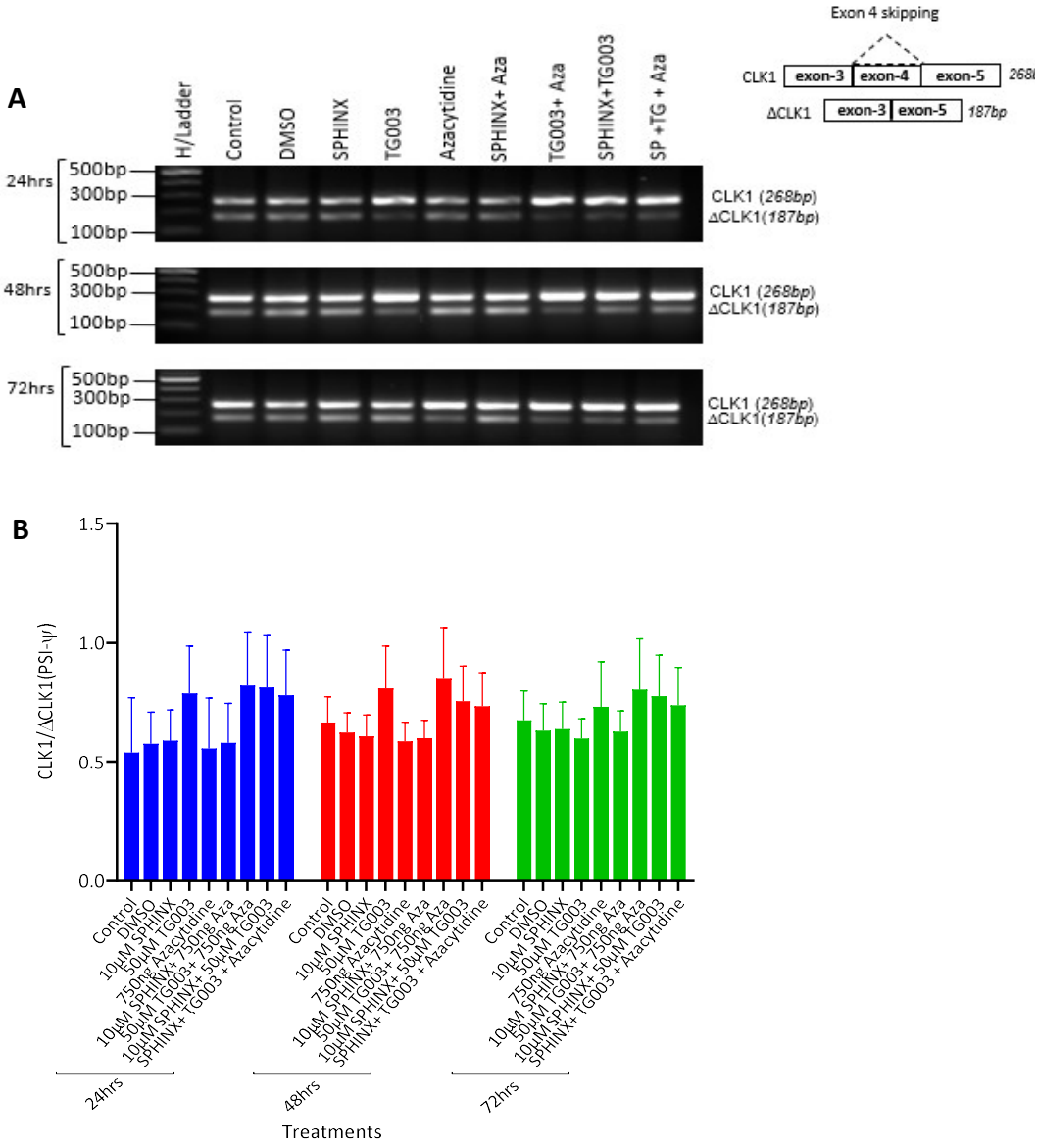


Figure 4.30. Alternative splicing of caspase-9 following combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Kasumi-1 cells were treated with either azacytidine, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three molecules for up to 72hrs. PCR was performed using caspase 9 specific primers. **B**. Representative PCR amplicon for caspase 9a, (742bp) and caspase 9b, (292bp) for 24hrs, 48hrs and 72hrs.**C**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in caspase 9. *n*=3.

4.6.6 Effect of combined SPHINX and TG003 with azacytidine on CLK1 alternative splicing in Kasumi-1 cells

Kasumi-1 cells showed more CLK1 exon-4 inclusion in all treatment involving TG003 at 24hrs and 48hrs. At 72hrs time point, except for the TG003 only treatment, all others involving TG003 in addition to the azacytidine only treatment had more exon 4 inclusion. (Figure 4.31A-B). Similarly, levels of CLK1 intron 4 retention varied between treatment (Figure 4.31C).



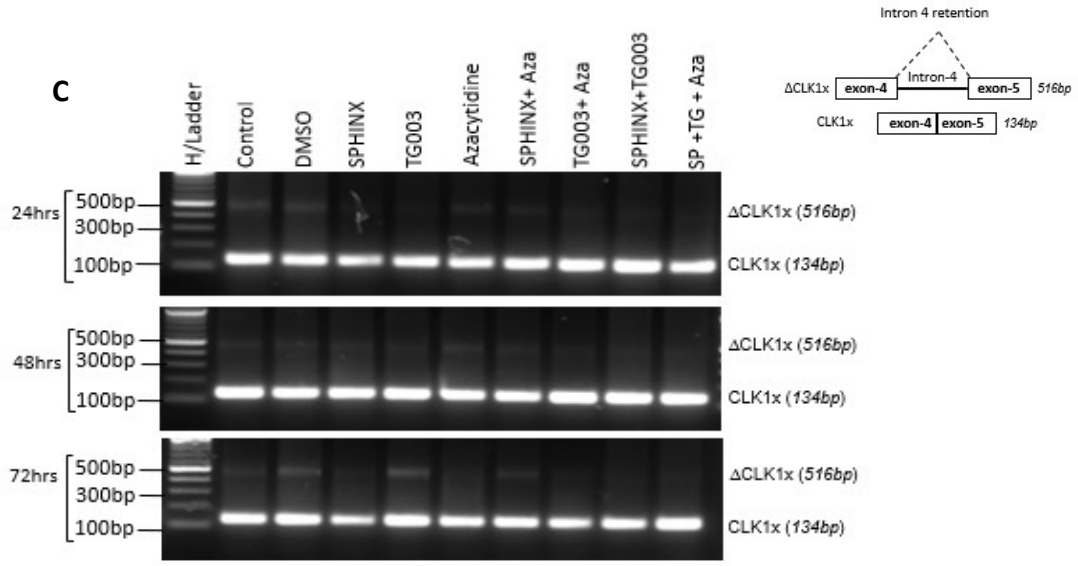
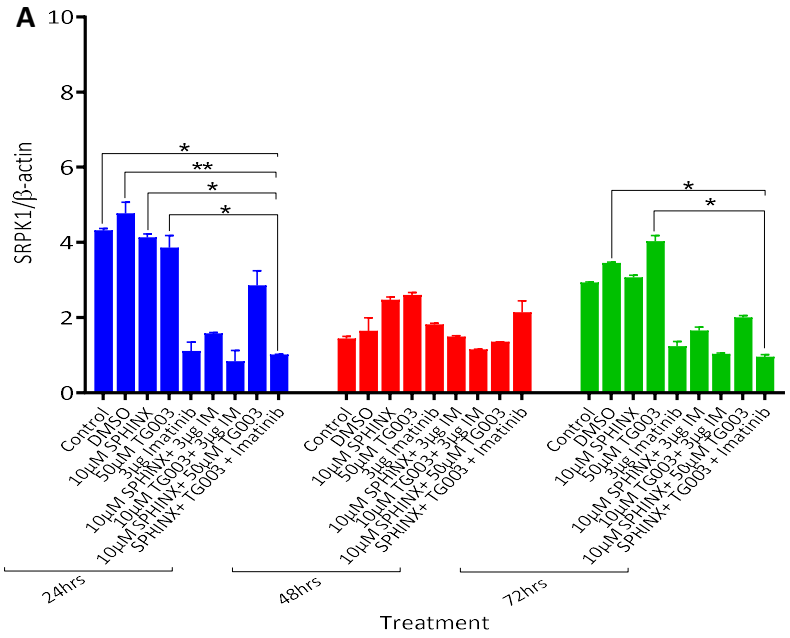


Figure 4.31. Alternative splicing of CLK1 following combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Kasumi-1 cells were treated with either azacytidine, SPHINX or TG003 inhibitor, a combination of both inhibitor and all three molecules for up to 72hrs. **A**. Representative PCR for (CLK1 268bp) and (ΔCLK1 187bp) for 24hrs, 48hrs and 72hrs. **B**. Densitometry showing percentage of exon 4 splice inclusion (PSI-Ψ) in CLK1. **C**. PCR showing levels of intron 4 retention following treatments. *n*=3.

4.7 Effect of combined SPHINX and TG003 with imatinib on SRPK1 expression and on SRSF1 and other SR-protein phosphorylation in K562 cells

4.7.1 Effect of combined SPHINX and TG003 with imatinib on SRPK1 protein expression in K562 cells

Studies into the effect of inhibiting SRPK1, CLK1 and imatinib treatment on K562s has shown that these have very little effect on the level and alternative splicing. We next examined the effect of the inhibitors on SRPK1 and CLK1 protein levels as there is evidence; at least in the case of CLK1 that expression of the splice factors themselves changes through presumed auto-regulatory mechanisms (Uzor *et al.*, 2018). Protein levels of SRPK1 were found to alter when a comparison was made between the cells treated with SPHINX, TG003 and imatinib to all other treatments, only the controls and single kinase inhibition was found to show a significant change in SRPK1 protein level at 24hrs and DMSO and TG003 at 72hrs (Figure 4.32A-B). This result was consistent with the data suggested by the standard PCR experiment.



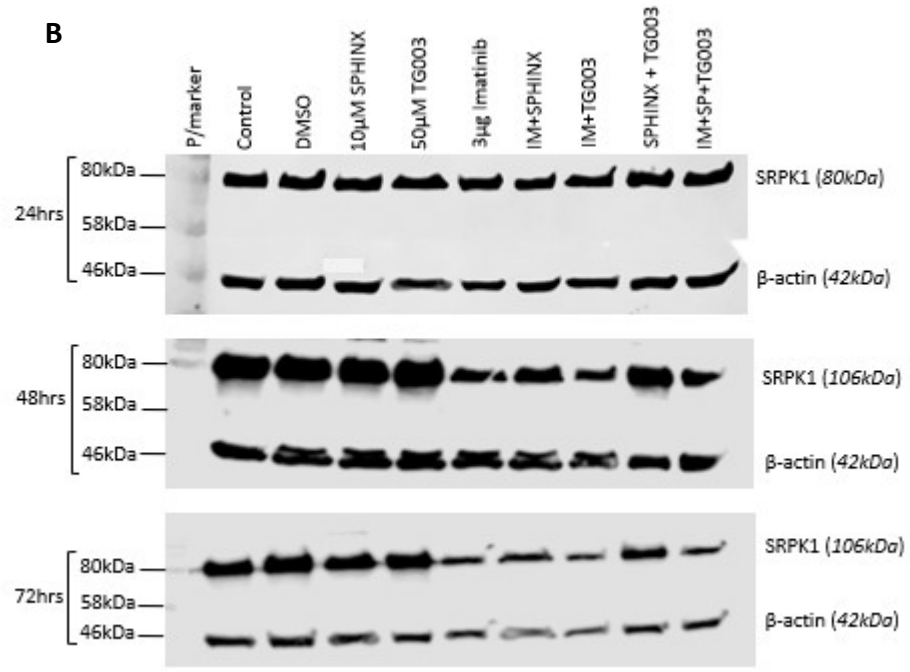


Figure 4.32. SRPK1 Protein levels in combined SPHINX and TG003 with imatinib in K562 cells. Western blot analysis on K562 protein extracts using anti-SRPK1 antibody. K562 cells were treated with either SPHINX; or TG003, a combination of both inhibitor and in addition to Imatinib. **A**. Densitometry showing protein levels of SRPK1 with increased SRPK1 protein level in the single treatment at 24hrs (*P*= 0.02, 0.006,0.04, and 0.03 respectively) and the DMSO and TG003 group at 72hrs (*P*<0.05) when compared to SPHINX+TG003+IM treated group. **B**. Image showing protein band visualized on PVDF membrane with corresponding β-actin loading control *n*=3.

4.7.2 *Effect of combined SPHINX and TG003 with imatinib on SRSF1 protein expression in K562 cells*

Like the levels of SRPK1, levels of total SRSF1 protein levels at 24hrs were found to be higher in the controls, SPHINX, TG003 and imatinib treatment than in the combination treatments. At 48hrs levels of the controls, combined SPHINX and imatinib and SPHINX and TG003 were higher compared to the combined treatment group. Whereas at 72hrs, SRSF1 levels were higher in the controls, SPHINX and TG003 treatment (Figure 4.33). It was expected that the total SRSF1 level will be unaffected by SRPK1 or CLK1 inhibition because both kinases are only known to act on the phosphorylated epitope.

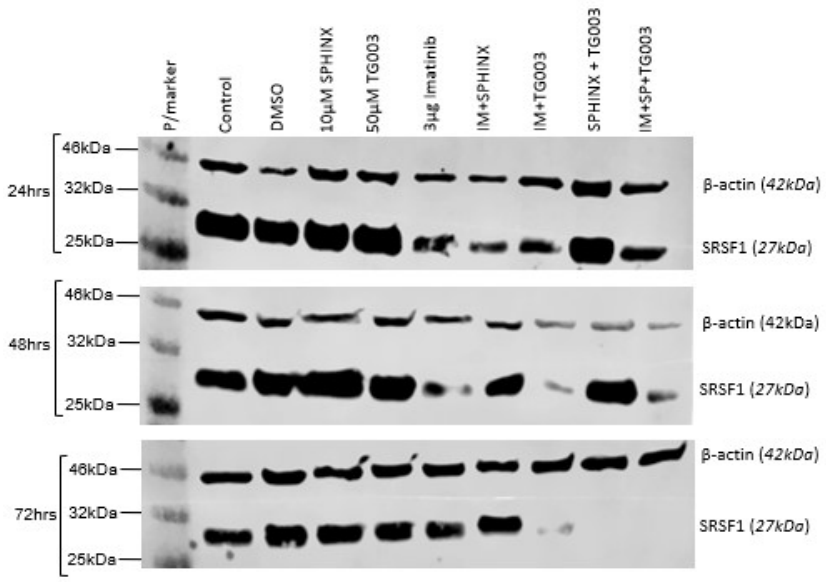


Figure 4.33. Total SRSF1 protein levels in combined SPHINX and TG003 with imatinib in K562 cells. Western blot analysis on K562 protein extract using anti-SRSF1 antibody. K562 cells were treated with either SPHINX or TG003, a combination of both inhibitor and addition to Imatinib. Image showing protein band visualized on PVDF membrane with corresponding β-actin loading control and band weight. *n*=3.

4.7.3 Effect of combined SPHINX and TG003 with imatinib on CLK1 protein expression in K562 cells

It was only at 72hrs did CLK1 levels in K562 cells treated with SPHINX, TG003 and imatinib significantly differ from the control but not with all other treatment (Figure 4.34A). Like with SRPK1, CLK1 proteins levels appear to be unaffected following CLK1 inhibition. In contrast, imatinib treatment results in a reduction in CLK1 protein levels as all treatment including imatinib showed an apparent decrease (Figure 4.34B). This was also an interesting finding knowing that imatinib could affect signalling pathway that results in changes in CLK1 expression. This was not the focus of this study; further investigation is necessary to understand what role CLK1 and changes in CLK1 levels in response to chemotherapy could be playing in CML progression and treatment outcome.

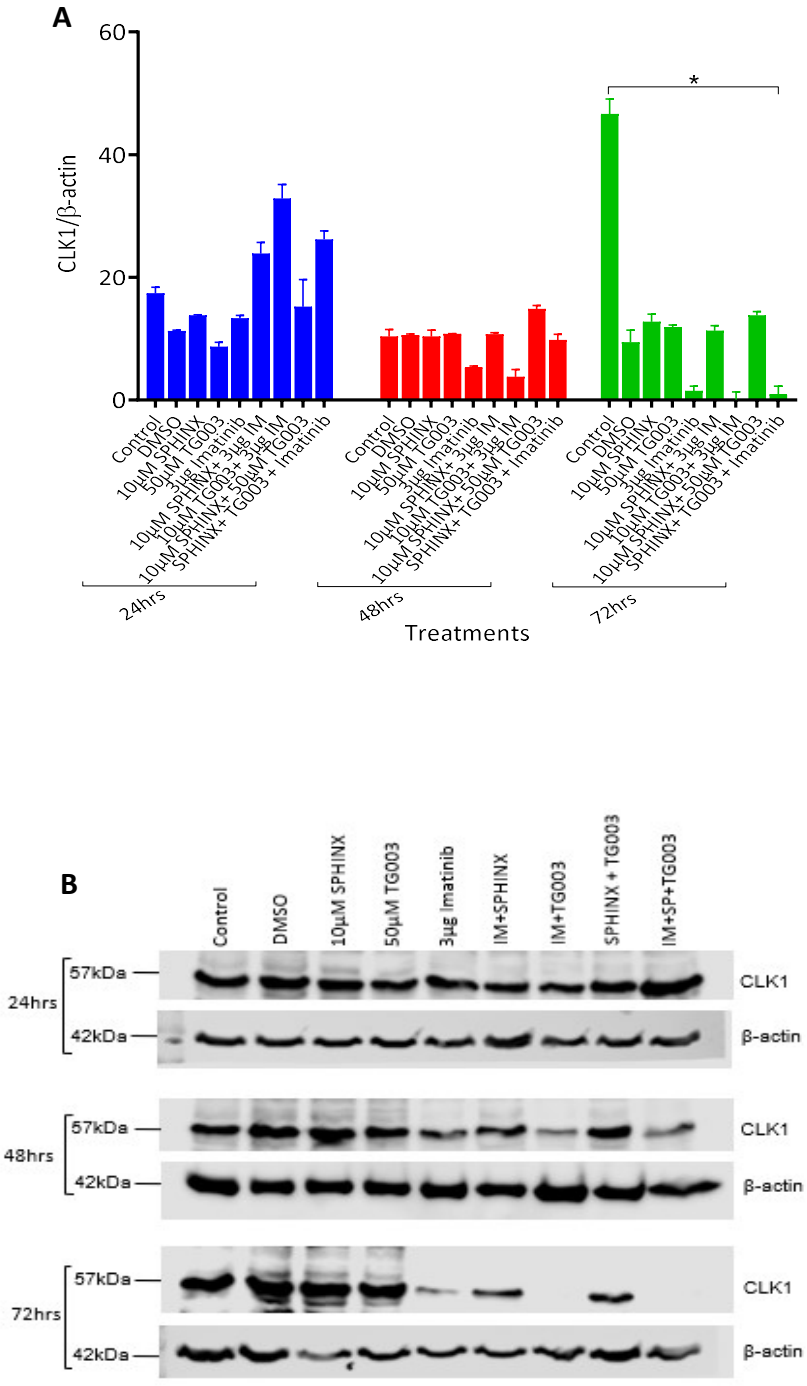


Figure 4.34. CLK1 protein levels in combined SPHINX and TG003 with imatinib in K562 cells. Western blot analysis on K562 protein extract using anti-Clk1 antibody. K562 cells were treated with either SPHINX or TG003, a combination of both inhibitor and in addition to Imatinib. **A**. Densitometry showing protein levels of CLK1 with only the control at 72hrs showing a significant difference (**P*=0.02). **B**. Image showing protein band visualized on PVDF membrane with corresponding β-actin loading control. *n*=3.

4.7.4 Effect of combined SPHINX and TG003 with imatinib on SR-protein phosphorylation in K562 cells

Examination of phosphorylation serine/arginine proteins in K562s showed varying levels of phosphorylation with each treatment measured using an antibody specific to phospho-SR epitopes. Representative image blots are shown in Figure 4.35 corresponding to each time point. There were indications of reduced phosphorylation in all treatments involving imatinib, and group treated with a combination of SPHINX and TG003 especially at 72hrs. Since SRPK1 and CLK1 are critical in the phosphorylation of SR proteins, it was expected that inhibition of either kinase will result in reduced expression of SR phospho-epitope. This was not the case, especially at 72hrs.

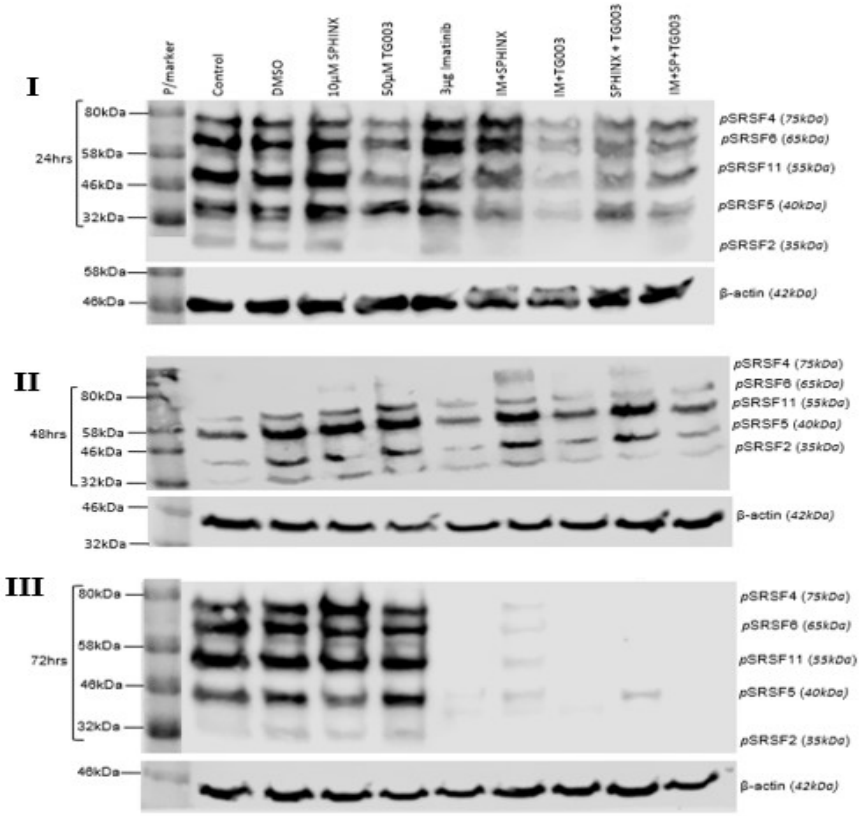
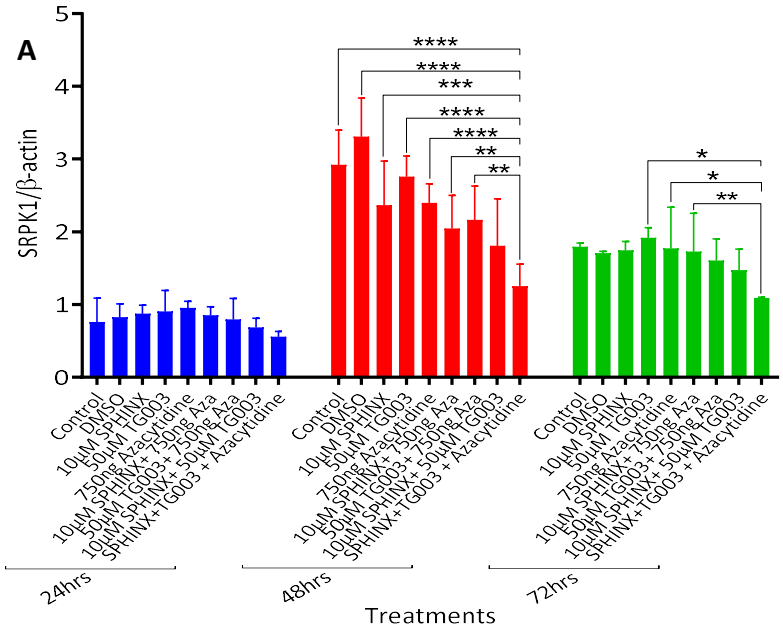


Figure 4.35. Protein levels of phospho-SR protein in combined SPHINX and TG003 with imatinib in K562 cells. Western blot analysis on K562 protein extract using 1H4 pan antibody. K562 cells were treated with either SPHINX or TG003 a combination of both inhibitor and in addition to Imatinib. Western blot image showing protein levels for phospho-SR family proteins at 24hrs **(I)**, 48hrs **(II)**, and 72hrs **(III).** Results are suggestive of reduced phosphorylation of SR proteins in treatments with imatinib and combined SPHINX with TG003. Images were visualized on PVDF membrane with corresponding β-actin loading control. *n*=3.

4.8 Effect of combined SPHINX and TG003 with azacytidine on SRPK1 expression and on SRSF1 and other SR-protein phosphorylation in Kasumi-1 cells

4.8.1 Effect of combined SPHINX and TG003 with azacytidine on SRPK1 protein expression in Kasumi-1 cells

Contrary to the effect of imatinib on SRPK1 in K562s, azacytidine has no effect on levels of SRPK1 protein expression in Kasumi-1 cells. Combining Inhibition of SRPK1 and CLK1 with azacytidine results in a significant reduction in SRPK1 levels at 48hrs when compared to other treatment. However, at 72hrs, levels only differed with TG003, azacytidine and combined SPHINX and azacytidine (Figure 4.36A&B). Even though the combination of SPHINX and TG003 with azacytidine was not significantly different from the SPHINX and TG003 combination, there are indications that azacytidine may have a potential effect on levels of the SRPK1 oncoprotein.



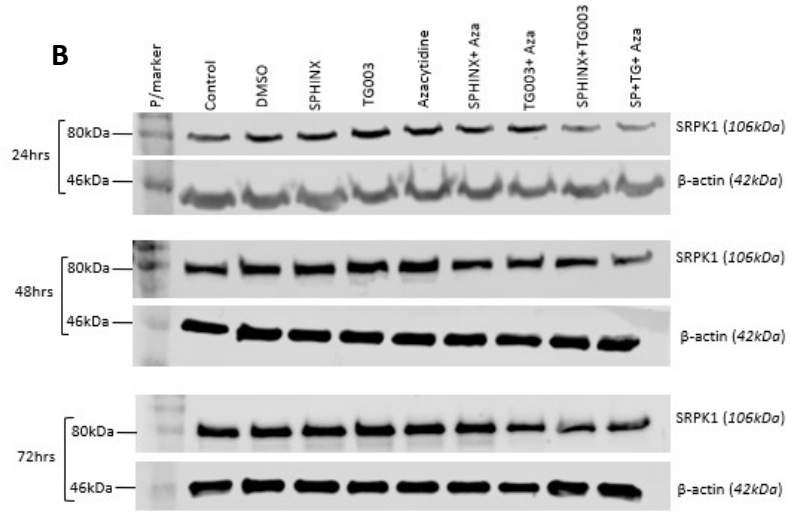


Figure 4.36. SRPK1 Protein levels in combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Western blot analysis on Kasumi-1 protein extract using anti-SRPK1 antibody. Kasumi-1 cells were treated with either SPHINX or TG003, a combination of both inhibitor and in addition to azacytidine. A. Densitometry showing increased protein level of SRPK1 at 48hrs in all treatment which differs significantly (****$P \leq 0.0001$; **$P=0.008$ and **$P=0.002$) from the SPHINX+TG003+azacytidine group except for SPHINX+TG003 treatment. At 72hrs (*$P<0.05$; **$P=0.007$ respectively) in the TG003, azacytidine and SPHINX+ azacytidine group. **B**. Images are representation of PVDF membrane showing protein levels with corresponding β-actin loading control. $n=3$.

4.8.2 *Effect of combined SPHINX and TG003 with azacytidine on SRSF1 protein expression Kasumi-1 cells*

Figure 4.37 are immunoblotting of total SRSF1 protein in Kasumi-1 cells following treatment with several compounds. Levels of total SRSF1 were found to be reduced at 24hrs treatment with combined SPHINX and azacytidine and combination of all three treatment. An apparent increase in total SRSF1 was observed in the controls, SPHINX, azacytidine and TG003 and azacytidine when compared to SPHINX, TG003 and azacytidine combination. At 48hrs and 72hrs, levels of SRSF1 appear to be reduction in total SRSF1 splice factor in combined TG003 and azacytidine, SPHINX and TG003 and SPHINX, TG003 with azacytidine combination which appears to be time dependent. The latter displayed the lowest levels at all time points.

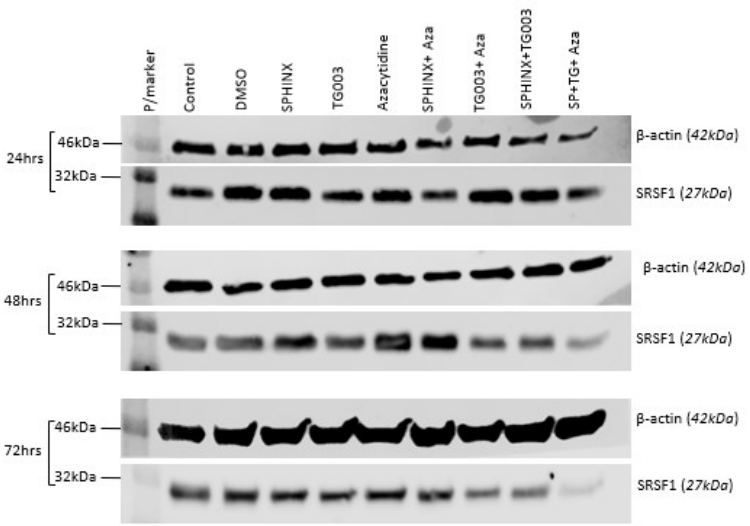


Figure 4.37. Total SRSF1 protein levels in combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Western blot analysis on Kasumi-1 protein extract using anti-SRSF1 antibody. Kasumi-1 cells were treated with either SPHINX or TG003, a combination of both inhibitor and in addition to azacytidine. Images are representation of protein levels with corresponding β-actin loading control. *n*=3.

4.8.3 *Effect of combined SPHINX and TG003 with azacytidine on CLK1 protein expression in Kasumi-1 cells*

Examining the levels of CLK1 in Kasumi-1 cells showed a trend which is both treatment and time dependent. There was an apparent decrease in CLK1 with the combined treatments at 24hrs and 48hrs. Furthermore, at 72hrs, combined SPHINX, TG003 and azacytidine resulted in low levels of CLK1, compared to all other single and combined treatment (Figure 4.38).

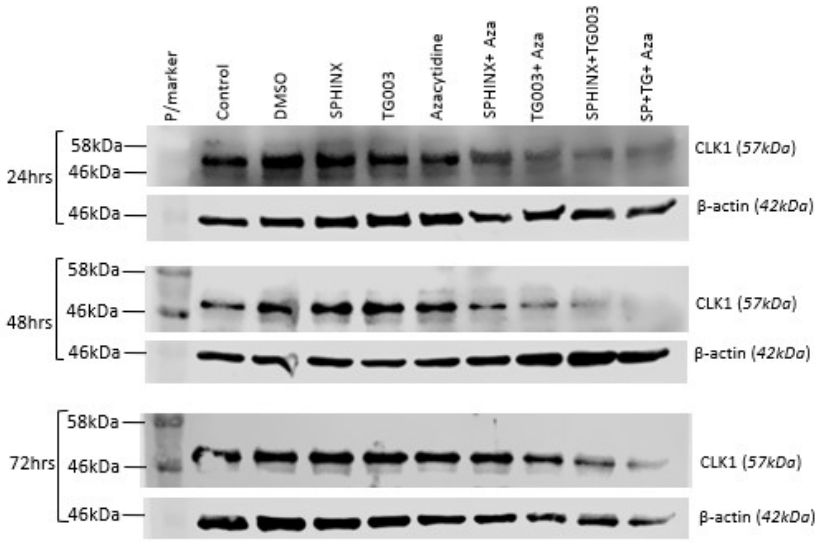


Figure 4.38. CLK1 protein levels in combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Western blot analysis on Kasumi-1 protein extract using anti-CLK1 antibody. Kasumi-1 cells were treated with either SPHINX or TG003, a combination of both inhibitor and in addition to azacytidine. Images shows level of CLK1 proteins with corresponding β-actin loading control. *n*=3.

4.8.4 *Effect of combined SPHINX and TG003 with azacytidine on SR-protein phosphorylation in Kasumi-1 cells*

Phosphorylation of the serine-rich splice factors is the key function of SRPK1 and CLK1. Protein blots for SR proteins showed significant decrease in phosphorylation in groups treated with TG003 and azacytidine, SPHINX and TG003 and a combination of all three compounds. These were more Phosphorylation of SRSF4 at 24hrs increased in the controls, SPHINX, TG003 and azacytidine but not in groups with combined treatment when compared to combined SPHINX, TG003 and azacytidine (Figure 4.39).

At 48hrs and 72hrs, reduced phosphorylation was observed in TG003 and azacytidine and SPHINX and TG003 combination of all three treatments. Phosphorylation of *p*SRSF11 for SPHINX and TG003 at 24hrs, TG003 and azacytidine and SPHINX and TG003 at 48hrs, and at 72hrs, SPHINX and TG003 was reduced.

Levels of *p*SRSF5 were different across each treatment. Except for SPHINX and TG003 treatment and a combination of all three treatments, levels were observed to rise after 24hrs. Levels of *p*SRSF2 were similar to those found in *p*SRSF5.

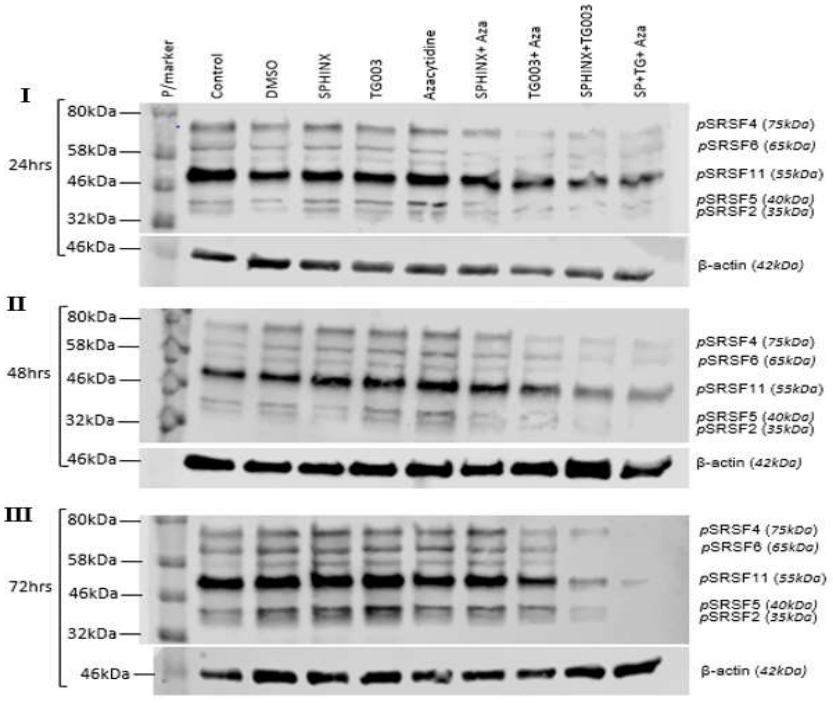


Figure 4.39. Protein levels of phospho-SR protein in combined SPHINX and TG003 with azacytidine in Kasumi-1 cells. Western blot analysis on Kasumi-1 protein extract using SR-antibody (1H4). For statistical analysis, all treatments were compared to the combined SPHINX+TG003+azacytidine treatment. Representative Western blot images for SR protein blot showing levels of phosphorylation of SR proteins and corresponding β-actin loading control (*n*=3).

4.9 Discussion

4.9.1 Effect of combined SPHINX and TG003 with imatinib on K562 cell viability and growth

CML is a myeloproliferative disorder characterized by a balanced translocation t(9;22) (q34; q11). This results in the formation of BCR-ABL oncoprotein, a constitutively active tyrosine kinase that promotes cellular growth and proliferation. Imatinib, a tyrosine kinase inhibitor, is a first-generation drug and a first line drug for the management of CML targeting the ATP-binding site of the fusion protein. Previous studies have shown that treatment of CML with imatinib results in decreased cell proliferation and cell death due to apoptosis (Husaini *et al*., 2017).

To observe first-hand the effect of imatinib on the K562 cell line, cells were treated with increasing concentrations of imatinib up to 20µg/ml (Figure 4.1). Results obtained were consistent with previous research which recorded increased cell death and reduced cell proliferation in CML cells treated with imatinib or its analogue, dasatinib (Roussidis *et al*., 2004; Lu *et al*., 2017). Contrary to this finding, an earlier study (Roseé *et al*, 2003) found no correlation between imatinib treatment and cell death in K562 possibly due to experimental design, as the cells used were synchronized and arrested at the G1/S phase and treated with 1µM imatinib for up to 48hrs. Another study has shown that imatinib is able to inhibit cell growth and proliferation in platelet derived growth factor receptor positive ($PDGFR^{+}$) ovarian cancer (Matei *et al*., 2004) and in colon cancer, with cell cycle arrest at G1/S phase (Samei *et al*., 2016). The increased cell death observed in K562 cells even at low doses of imatinib confirms the effectiveness of imatinib in CML treatment.

Combining imatinib treatment and SRPK1 inhibition using SPHINX in K562 cells showed no further decrease in cell viability and growth. However, the result was consistent with the reduced cell viability in imatinib only treated cells This finding was unexpected. However, with a further assay, using acridine orange fluorescent dye to study cell apoptosis in combined imatinib and SPHINX treatment, it was observed that there were fewer apoptotic cells in the combined treatment than imatinib treatment alone. This brings into question if there is an indirect effect of SPHINX on the targets of imatinib, since cells have more survival advantage when SPHINX was combined with imatinib.

A study has shown that both imatinib and SPHINX or its analogue (SRPIN340) are able to interact with the PI3/Akt pathway (Zhou *et al*., 2012; Wang *et al*., 2014). It is therefore possible that SPHINX interaction with PI3/Akt activates rather than inhibits downstream target pathways responsible for cell survival. This will require further study on the structure of both molecules to understand what relationship exists between them.

4.9.2 *Effect of combined SPHINX and TG003 with azacytidine on Kasumi-1 cell viability and growth*

The use of azacytidine (a cystidine analogue with DNA hypo-methylation properties) in the management of AML was suggested in patients with unfavourable cytogenetics or myelodysplasia-related changes who are ineligible for stem cell transplant (NICE, 2018). Treatment with azacytidine has a clear activity but treatment does not result in a significant increase in overall survival (OS) (Dombret *et al*., 2015).
Using the Kasumi-1 cell line as a model for AML, the effect of azacytidine on Kasumi-1 cell viability and growth was observed. With a clinically relevant dose of 750ng/ml of azacytidine, concentrations up to 1.5µg/ml of azacytidine were used. Decreasing cell viability and reduced cell growth was recorded.

Since treatment with azacytidine is not without side effects, which is a consequence of most chemotherapeutic agent, its combination with SPHINX was investigated with the thought that an increase in cell death would inform reduction in clinical doses. Kasumi-1 cell viability was decreased in the combined treatment when compared to controls and SPHINX treatment (Figure 4.5A). However, for cell growth, the combination treatment was only reduced relative to the untreated and DMSO controls but was not significantly reduced relative to SPHINX or azacytidine treatments alone (Figure 4.5B). Additional assays measuring caspase 3/7 activity showed increased caspase activity with the combined treatment but did not differ significantly with the SPHINX or azacytidine treatment alone. This study observed no change in cell viability and growth with additional inhibition of CLK1. Suggesting that combined treatment with azacytidine, SPHINX and TG003 may have no additional benefit in AML treatment than combining azacytidine and SPHINX or azacytidine and CLK1 alone.

With a lack of data comparing the effect of small molecules such as SPHINX and CLK1 inhibition with a conventional chemotherapeutic agent, it is unknown what to expect.

Research comparing the effect of combining azacytidine and romidepsin (AR) with interferon-α (IFN-α) on tumour progression and metastasis found that the addition of interferon potentiates the anti-proliferative, pro-apoptotic and metastasis activity of azacytidine and romidepsin alone in colorectal cancer (CRC) and CRC stem cells (CSCs) (Buoncervello *et al*., 2015). This combination was also known to affect the ERK1/2 and Akt pathway (Buoncervello *et al*., 2015). An earlier study has suggested a better outcome when immune therapy is combined with a conventional chemotherapeutic agent by induction of immunogenic cell death and increased T-cell recognition of tumour cells (Galluzzi *et al*., 2012).

4.9.3 *Effect of combined SPHINX and TG003 with imatinib on the expression of SRPK1, CLK1, VEGF and alternative splicing of a panel of apoptotic genes in K562 cells.*

The earlier results (Figure 3.17) demonstrated that inhibition of SRPK1 in K562 cells showed a fluctuating level of SRPK1 mRNA such that levels increased at 24hrs but decreased at 48hrs. Further experiments combining SRPK1 with imatinib treatment found no further change in levels of SRPK1, with levels remaining unaffected with additional inhibition of CLK1(Figure 4.20). This suggests that combined treatment offers no potential benefit where the aim is not just to inhibit the activity but also to reduce SRPK1 protein levels.

SRPK1 has been described as a constitutively active kinase and considered to be resilient to inactivation due to its activation loop not requiring intra-protein interaction to stay active (Ngo *et al*., 2007). This could explain the unaffected levels of SRPK1 in leukaemic cells as preliminary studies in this research carried out on the prostate cancer cell line PC3, observed a significant decrease in SRPK1 mRNA and protein levels following SRPK1 inhibition.

Results obtained were consistent with previous studies which showed that inhibition of CLK1 results in more exon 4 inclusion and less intron 4 retention in an auto-regulatory loop (Uzor *et al*., 2018). This change in CLK1 splicing was observed only at concentrations of 10µM and 50µM TG003. When CLK1 inhibition was combined with either SPHINX or imatinib treatment or a combination of all treatments, only treatment involving TG003 continued to show more exon 4 inclusion, whereas only at 72hrs did all treatments combined with TG003 result in less intron 4 retention. Other studies reported the

upregulation of CLK1 and its alternative splicing towards full-length CLK1 both in hypoxic conditions (Bowler *et al*., 2018), and in osmotic and heat shock conditions (Uzor *et al*., 2018). There are indications that most tissues express both full-length and truncated forms of CLK1. However, there is a shift in how much of each isoform is expressed in the presence of stress (Ninomiya *et al*., 2011; Uzor *et al*., 2018). Analysis of both exon 4 and intron 4 has shown that compared to intron 4, exon 4 is flanked by weak splice recognition sites and therefore will require strong ESE such as SRSF1 (Schwartz *et al*., 2008; Uzor *et al*., 2018). The need for effective splicing towards catalytically active full length CLK1 continues to suggest its auto-regulation as an adaptative mechanism to maintain its function in adverse and disease conditions.

Results presented here confirm that the autoregulation of CLK1 through its alternative splicing, observed following TG003 treatments in prostate cancer cell lines, can be reproduced in leukaemic cell lines. There is no strong evidence, however, of SPHINX causing a similar change to SRPK1 protein levels, and therefore, the effects measured are presumably due to an inhibitory effect on SRPK1 enzyme activity.

Compared to previous studies (Nowak *et al*., 2009; Mavrou *et al*., 2014; Batson *et al*., 2017) this study was unable to observe changes in VEGF splicing towards the anti-angiogenic isoform VEGF165b in three leukaemic cell lines, neither did any single kinase inhibition nor a combination of any two or more compounds find any change in splicing. This suggests that VEGF165b isoform could be selectively expressed in certain cell types. A study (Song *et al*., 2012) also suggests that the *BCR/ABL* mutation self-regulates VEGF expression through HIF-1α activation of STAT3 on the VEGF promoter in leukaemic cancer. This auto-regulatory ability of VEGF may account for the levels of total VEGF observed.

No change was observed in Bclx alternative splicing in combined imatinib and SPHINX treatment or in addition to CLK1 inhibition. Similarly, no change was observed in Apaf1 alternative splicing with increased PSI (Ψ) value indicating higher expression of Apaf-1xl anti-apoptotic isoform. Treatment with imatinib and imatinib TG003 combination expressed equal ratio of Apaf-1xl/1s at 72hrs. Like Apaf1, caspase 9 showed an apparent switch in splicing to caspase 9a upon treatment with imatinib or in combination with other inhibitory molecules. Combining imatinib with both kinase inhibitors had no potential additional benefit in CML treatment. The inference drawn from splice changes

in Apaf1 and caspase 9 continues to support the ability of imatinib to initiate alternative splicing and apoptosis in K562 cells (Liu *et al*., 2012 and 2015).

4.9.4 Effect of combined SPHINX and TG003 with imatinib on protein levels of SRPK1, SRSF1, CLK1, and on the phosphorylation of SR protein in K562

It has been observed that inhibition of SRPK1 using SPHINX had no effect on SRPK1 and total SRSF1 in the K562 CML cell line. The degree of the effect on SR protein phosphorylation in K562 varied from one SR protein to another, with reduced phospho-epitope as expected following increasing concentrations of SPHINX (Figure 3.24).

This study was expanded to investigate what effect a combination of SRPK1 and CLK1 inhibition, in addition to treatment with imatinib, would have on individual protein kinase expression and on phosphorylation of SR proteins. The research group confirms previous work (Uzor *et al*., 2018; Bowler *et al*., 2018) in which CLK1 is found to auto-regulate its expression in response to stress. Protein levels of CLK1 were elevated in treatments involving TG003 without imatinib in K562 cells. Interestingly, this increase is accompanied by an increase in SRSF1 supporting findings that suggest SRSF1 as the ESE required for CLK1 auto-regulation (Schwartz *et al*., 2008; Uzor *et al*., 2018); increased catalytically active CLK1 mRNA equals increased CLK1 protein. Furthermore, increased SRPK1 protein levels were observed even with a combination of TG003 with SPHINX which inhibits SRPK1. These results put together, suggests that CLK1 regulates SRPK1 levels, and both CLK1 and SRPK1 are responsible for SR protein function (Aubol *et al*., 2016 and 2018). It is also possible that SRPK1 undergoes auto-regulation, and a further complication is that its nuclear presence is controlled by CLK1 (Aubol *et al*., 2016). Interestingly, except for combined imatinib and SPHINX treatment, all other treatments with imatinib combinations showed a time-dependent decrease in protein levels of SRPK1, SRSF1 and CLK1 at 48hrs and 72hrs. Since imatinib is specific for targeting the tyrosine kinase BCR-ABL, it can be inferred that through a downstream signalling pathway of BCL-ABL such as mTOR/Akt, imatinib is able to regulate protein synthesis of SRPK1, CLK1 and SRSF1 (Kharas *et al*., 2008). One study suggests that Akt, which is a target of imatinib can induce auto-phosphorylation of SRPK1, increasing SRPK1 nuclear import and subsequent SR protein phosphorylation (Zhou *et al*., 2012) indicating a direct relationship between imatinib SRPK1, CLK1 and SRSF1. However, it was interesting to

observe that imatinib can reduce the levels of total SRSF1 through a mechanism, which is not known. This finding will need further investigation.

Examining the effect on SR protein phosphorylation, fluctuating levels of phosphorylation within the SR protein family was observed. An apparent reduction in phosphorylation was observed where treatments were combined at the 72hrs time point. This was consistent with the levels of SRPK1 and CLK1 levels earlier observed. Overall, combining imatinib treatment and both kinase inhibition suggests a potential in the regulation of SR protein kinases, SRPK1 and CLK1 (Figure 4.32 & 4.34) and their substrate, SRSF1 (Figure 4.33) in CML. This in the broad sense will regulate aberrant splicing and pathways such as the PI3K/Akt which are targets of the SRPK1-CLK1-SR protein axis necessary for progression of CML.

4.9.5 *Effect of combined SPHINX and TG003 with azacytidine on the expression of SRPK1, CLK1, VEGF and alternative splicing of apoptotic genes in Kasumi-1 cells*

Statistics have shown that many adult patients with AML will go into a refractory phase or relapse from the disease (Breems e*t al*., 2005). With the success achieved in the use of small molecule inhibitors for the treatment of multiple tumours such as gastrointestinal stromal tumour and CML (Luger, 2010; Demetri *et al*., 2002; O'Brien *et al*., 2003), the shift in research is the need to develop a therapeutic agent with a lasting positive outcome for patients in this category.

This study has shown that SPHINX, and likewise, azacytidine, affect Kasumi-1 cell viability and growth. Further study expanded on this to see how a combination of both molecules in addition to CLK1 inhibition affects mRNA levels and splicing. Levels of SRPK1 and VEGF were unaffected when SPHINX was combined with azacytidine or with additional inhibition of CLK1. At 48hrs in the SPHINX and azacytidine combination, a smaller amplicon band (65bp) was observed, suspected to be VEGF165b isoform (Figure 4.15). This was inconclusive as the result was not reproducible. CLK1 showed more exon 4 inclusion and less intron 4 retention in all groups involving TG003 treatment. Contrary to the levels of CLK1 intron 4 observed in K562, Kasumi-1 cells overall appear to express less of CLK1 intron 4 isoform. This observation was made considering the low levels observed in the untreated and DMSO controls. It does follow that different cells will express varying degrees of each isoform. It is also possible that the levels observed

might be common in acute phases of leukaemia as a previous study has observed similarly low levels of this isoform in MOLT 4, an acute lymphoblastic leukaemia cell line (Uzor *et al*., 2018). Choice of splice site involving binding of the ESE such as SR proteins on the alternative exons has been shown to promote exon inclusion (Han *et al*., 2011). This might be the case with Kasumi-1 mRNA splicing.

Furthermore, no change was observed in Bclx, Apaf1 and caspase 9 alternative splicing following combined SPHINX and azacytidine, or in addition to CLK1 inhibition. This can be accounted for by mutation and copy number changes in genes encoding spliceosome proteins such as *SRSF2, U2AF1* and *SF3B1* in AML shown to affect maintenance and stability of leukaemic stem cell (LSC) clones and propagation of AML (Crews *et al*., 2016; Papaemmanuil *et al*., 2016). These proteins interact with pre-mRNA, and when mutated, splice site recognition is altered, which results in more intron retention and exon skipping. This correlates with the splicing event observed in these genes as the larger isoforms were highly expressed. Results observed with Bclx also suggest that the cell death observed in Kasumi-1 cells may have resulted from pathways other than the intrinsic pathway in which Bclx is involved, while a reduced but equal ratio of both Apaf1 isoforms could be responsible for the induction of apoptosis rather than a complete switch from one isoform to the other. Since levels of Apaf1 in the combined treatment appear the same with azacytidine and with combined SPHINX and TG003 inhibition, it is unlikely that combining all three treatment may have an added advantage (Figure 4.29B-C)

4.9.6 *Effect of combined SPHINX and TG003 with azacytidine on of SRPK1, SRSF1, CLK1, protein expression and phosphorylation of SR protein in Kasumi cells*

A previous study has shown increased sensitivity of myeloid cells to SRPK1 inhibition using the small molecule inhibitor SRPIN340 when compared to leukaemic cancers of lymphoid origin (Siqueira *et al*., 2015), suggesting that Kasumi-1 cells, which belongs to the same category, could be more sensitive to treatment.

The result from this study has also suggested similar sensitivity with Kasumi-1 cells as a model when compared to K562 and TK6 cells where no effect on protein expression was observed following inhibition with SPHINX and TG003. Contrary to K562 cells, where decreased expression of SRPK1, SRSF1 and CLK1 appear to be affected by imatinib rather

than SPHINX or TG003, in Kasumi cells, a reduction was observed with treatment combination of TG003 and azacytidine, SPHINX and TG003 and a combination of both kinase inhibitor with azacytidine. Results were found to be consistent with time except for SRPK1 levels at 24hrs (Figure 4.36). Since SRPK1 and CLK1 phosphorylate SR-proteins, including SRSF1, it was expected that inhibition of both kinases would result in an increased expression of total SRSF1 rather than a decrease. The reduced expression of SRSF1 observed when SPHINX, TG003 and azacytidine were combined indicates a prospect of the potential benefit a combination of these compounds could play in the reduction of oncogenic splice factor SRSF1 in tumour cells. These results also suggest a cell-specific and cell-dependent effect of one kinase over another. Furthermore, the similarity in the protein levels of SRPK1 and CLK1 continue to confirm existing publications on the role SRPK1 and CLK1 in SR protein phosphorylation, and the regulation of SRPK1 by CLK1 (Zhou *et al*., 2012; Aubol *et al*., 2016).

Phosphorylation of SR proteins; *p*SRSF4, *p*SRSF6, *p*SRSF11, *p*SRSF5 and *p*SRSF2 appear to also follow the trend observed in SRPK1, SRSF1 and CLK1 when the cells were treated with a combination of TG003 and azacytidine, SPHINX and TG003 and a combination of both kinase inhibition with azacytidine. Reduced phosphorylation was observed when all three treatments were combined and consistent with time, especially at 48hrs and 72hrs. This confirmed the role of SRPK1 and CLK1 in SR protein phosphorylation. Also, since multiple SR proteins act on a set of ESEs, the degree of one SR protein expression over the other may result in competitive binding between SR proteins. The consequence which will either enhance or repress the binding of another SR protein (Pandit *et al*., 2013). The implication of this result outcome is that combined targeting of SRPK1 and CLK1 could be beneficial to augment existing AML therapies, subject of course to further research.

CHAPTER 5

5 Comparative assessment of the effect of SRPK1 inhibition and SRPK1 siRNA knockdown in leukaemic cell lines.

5.1 Introduction

The serine-arginine protein kinase-1 (SRPK1) plays an essential role in various cancer and its progression (Mavrou *et al*., 2014; Roosmalen *et al*., 2015). SRPK1 has been shown to be upregulated in several types of cancers (Han *et al*., 2017). It has been shown that knockdown of SRPK1 inhibited tumour growth in xenograft and altered splicing of VEGF to a more anti-angiogenic VEGFxxxb isoform (Gonćalves, *et al*., 2014; Mavrou *et al*., 2014). Other studies have shown that targeting SRPK1 using siRNA resulted in reduced cell proliferation and altered expression of key regulators of apoptosis (Hayes *et al*., 2007). In K562 and AML, SRPK1 knockdown has been shown to suppress cell growth, induce apoptosis and altered isoform levels of genes involved in leukaemogenesis and apoptosis such as *MYB*, *BRD4, VEGF-A and BCL2* (Tzelepis *et al*., 2018; Wang et al., 2018). This chapter was aimed at investigating the effect of SRPK1 knockdown using small interfering RNA (siRNA) on TK6, K562 and Kasumi-1 cell models.

5.1.1 *SRPK1 siRNA knockdown in leukaemic cell lines*

Using siRNA, SRPK1 was knocked down in leukaemic cell lines; TK6, K562 and Kasumi-1 cell. The target sequence of the siRNA has been previously used (Karakama *et al*., 2010) to successfully knockdown SRPK1 (described in chapter 2.9). To determine the effective concentration of siRNA for SRPK1 knockdown, leukaemic cell lines were transfected with increasing concentration of SRPK1 siRNA up to 100nM. Cell transfection time with the siRNA was four hours and total incubation time was 72hrs. Immunoblotting was used to determine the effective knockdown which showed significant SRPK1 knockdown in all cell lines. Concentration for siRNA knockdown was then maintained at 100nM for all cell lines in further experiments.

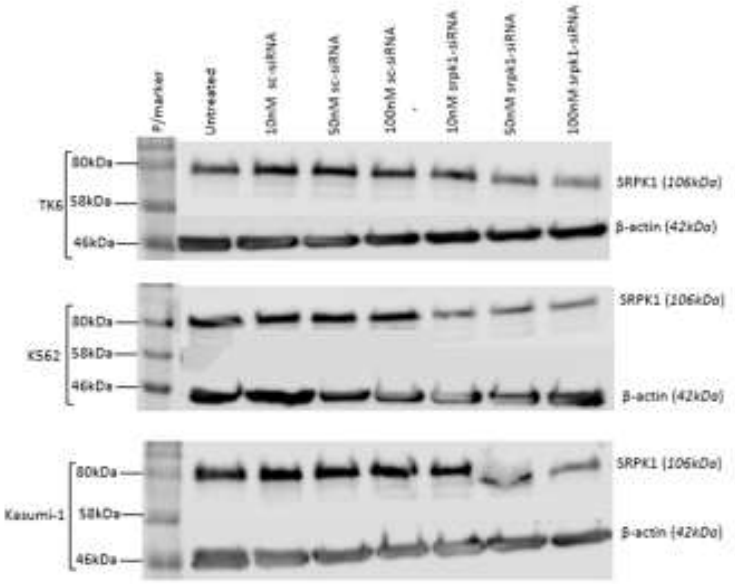


Figure 5.1. Knockdown at protein level of SRPK1 in leukaemic cells using small interfering RNA. Representative blots for TK6, K562 and Kasumi-1 cells showing knockdown efficiency of SRPK1. *n*=3

5.1.2 *Effect of siRNA knockdown on leukaemic cell viability and growth*

The effect of SRPK1 knockdown on leukaemic cell viability and cell growth was studied. This was done by transfecting TK6, K562 and Kasumi-1 cells with siRNA. Cell counts and viability assays were performed after 72hrs. Results of cell viability and growth in Knockdowns, scrambled control and SPHINX treatments were compared to DMSO. Results suggest no change in TK6 cell viability. In K562 and Kasumi-1 cells, SPHINX and knockdown cells significantly decreased when compared to DMSO (Figure 5.2A).

When cell growth for all cell line was investigated, TK6 cells showed changes in cell number even in the scrambled control whereas, K562 and Kasumi-1 cells showed a significant decrease in cell growth in SPHINX and knockdown cells when compared to DMSO. This indicates that knockdown of SRPK1 has an apparent effect on leukaemic cell growth and suggests that SRPK1 may be involved in regulating the growth and proliferation pathways of some of these cells (Figure 5.2B).

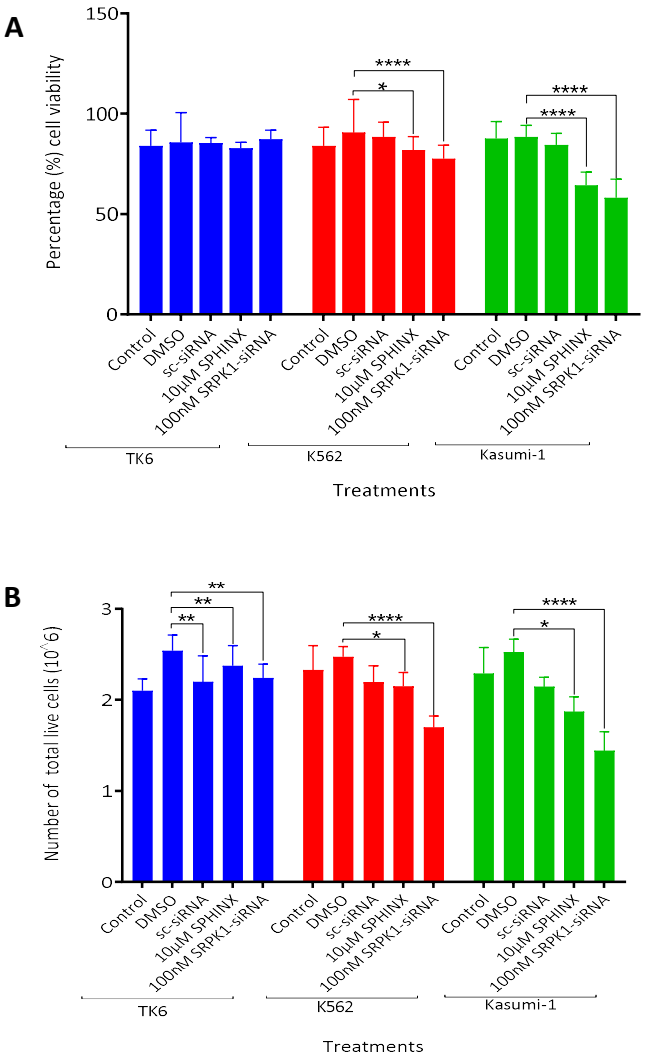


Figure 5.2. Comparative effect of SRPK1 inhibition and knockdown on leukaemic cell growth. Leukaemic cells were either treated with 10μM SPHINX or transfected with 100nM siRNA for 72hrs. **A**. TK6 cell viability were unaffected. SPHINX (*P*=0.012) and SRPK1-siRNA (*P*<0.0001) percentage viability differed with DMSO in K562 cells. Percentage decrease (****P<0.0001) in Kasumi-1 cell viability for SPHINX and SRPK1-siRNA when compared to DMSO. **B**. TK6 cells (**P= 0.007; 0.002; 0.002) showed changes in cell growth. Cell number was reduced (*P= 0.03; ****P<0.0001) when compared to DMSO in K562 cell. For Kasumi-1 cells, a reduction (*P=0.042; ****P<0.0001) in cell growth when compared to DMSO. n=3

5.2 Effect of SRPK1 siRNA knockdown on alternative splicing of a panel of genes in leukaemic cells

5.2.1 *Effect of siRNA knockdown on VEGF alternative splicing*

Levels of total VEGF showed no apparent difference between SPHINX treated cells and cells where SRPK1 were knocked down in TK6, K562 and Kasumi-1 cell. Both the treated group and control appear to be the same except for Kasumi-1 cells, where the VEGF in the SPHINX treatment and knockdown seem higher when compared to the controls. Alternative splicing of total VEGF to its anti-angiogenic isoform which has previously been reported (Mavrou and Oltean, 2016; Batson *et al*., 2017) following SRPK1 inhibition or knockdown was not detected (Figure 5.3B).

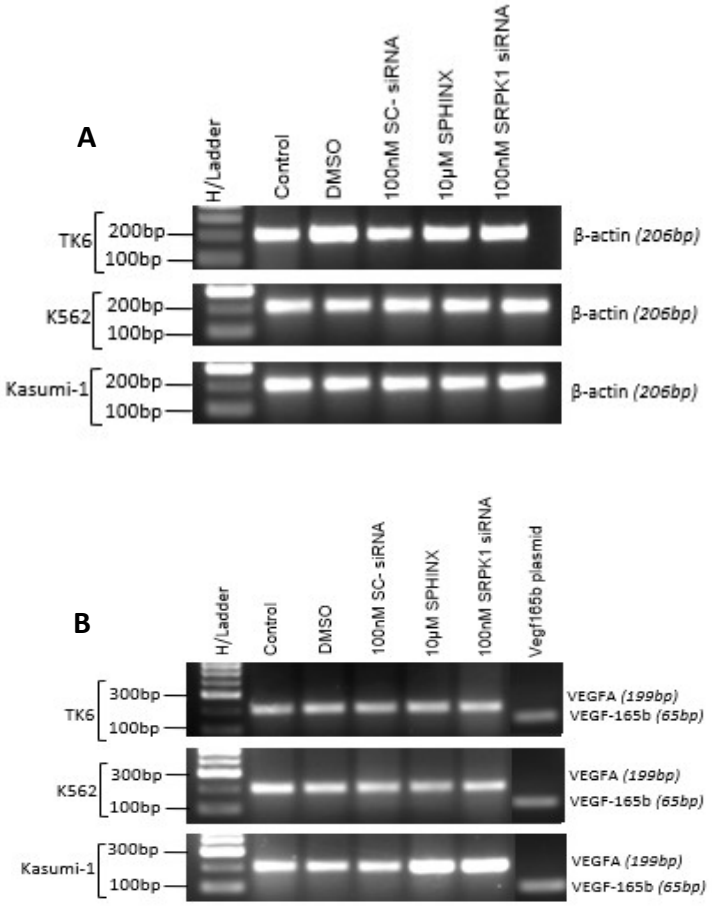


Figure 5.3. Comparative effect of SRPK1 inhibition and knockdown on VEGF alternative splicing. Leukaemic cells were either treated with 10μM SPHINX or transfected with 100nM siRNA. **A**. Actin control for all PCR amplification. **B**. Total VEGF PCR amplicon (199bp) and anti-angiogenic isoform, $VEGF_{165b}$ plasmid (65bp) in leukaemic cells. *n*=3

5.2.2 Effect of siRNA knockdown on Bclx alternative splicing

Ratios of Bclx isoforms (xl/xs) remained unchanged following SRPK1 knockdown in all three cell lines (Figure 5.4A-B). Bclx splicing levels in the knockdown were like the SPHINX treatment and controls with the more abundant anti-apoptotic isoform Bcl-xl (351bp) being more highly expressed.

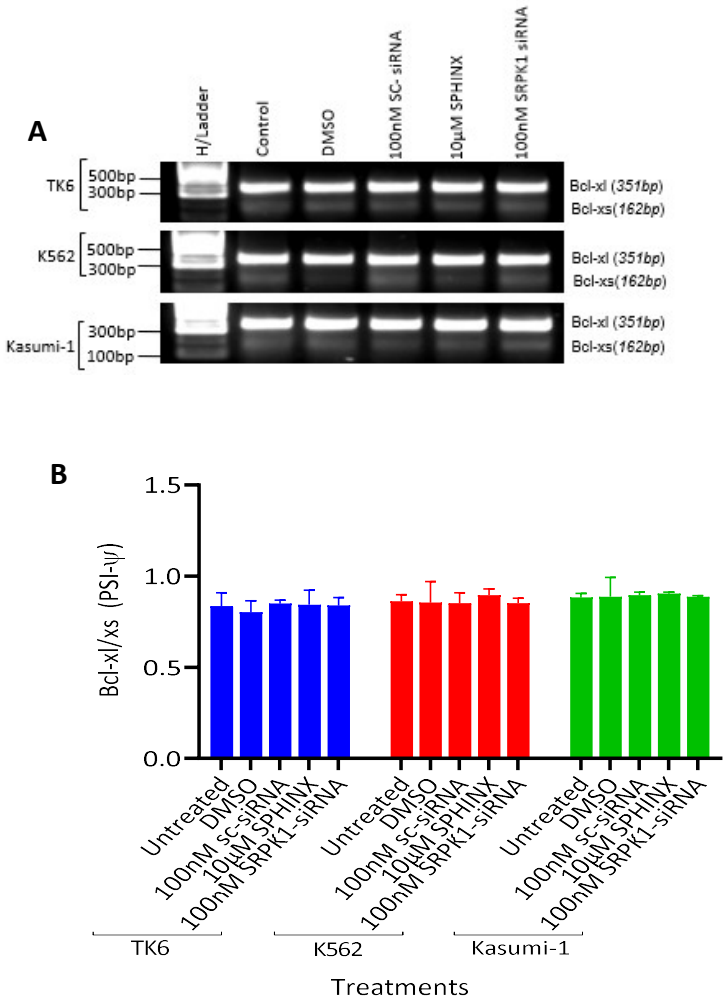


Figure 5.4. Comparative effect of SRPK1 inhibition and knockdown on Bclx alternative splicing. Leukaemic cells were either treated with 10μM SPHINX or transfected with 100nM siRNA or a scrambled control. **A**. PCR images for (Bcl-xl, 351bp) and (Bcl-xs, 162bp) for TK6, K562 and Kasumi-1. **B**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in Bcl-xl/xs isoform. *n*=3.

5.2.3 *Effect of siRNA knockdown on Apaf1 alternative splicing.*

Knockdown of SRPK1 showed no apparent effect on Apaf1 splicing (Figure 5.5).

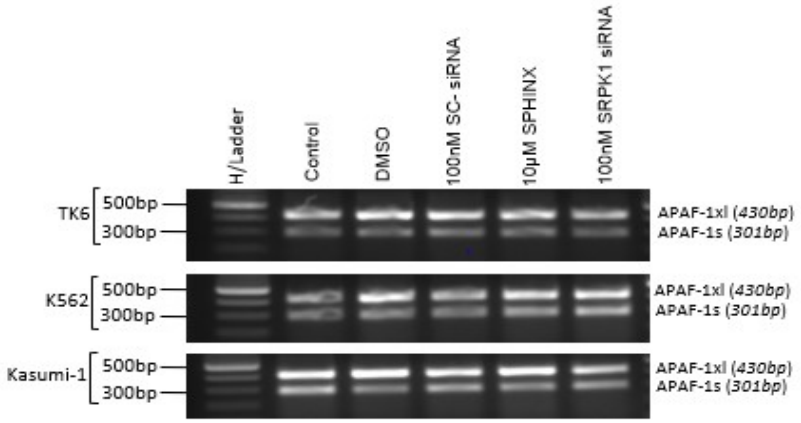


Figure 5.5. Comparative effect of SRPK1 inhibition and knockdown on Apaf1 alternative splicing. Leukaemic cells were either treated with 10μM SPHINX or transfected with 100nM siRNA or a scrambled control. Representative PCR image for Apaf-1xl (430bp) and Apaf-1s (301bp) for TK6, K562 and Kasumi-1. *n*=3.

5.2.4 Effect of siRNA knockdown on caspase 9 alternative splicing.

Knockdown of SRPK1 had no effect of caspase 9 alternative splicing. Quantitative analysis of gel electrophoresis indicates equal levels of both isoforms being expressed both in the controls and treated group (Figure 5.6A-B).

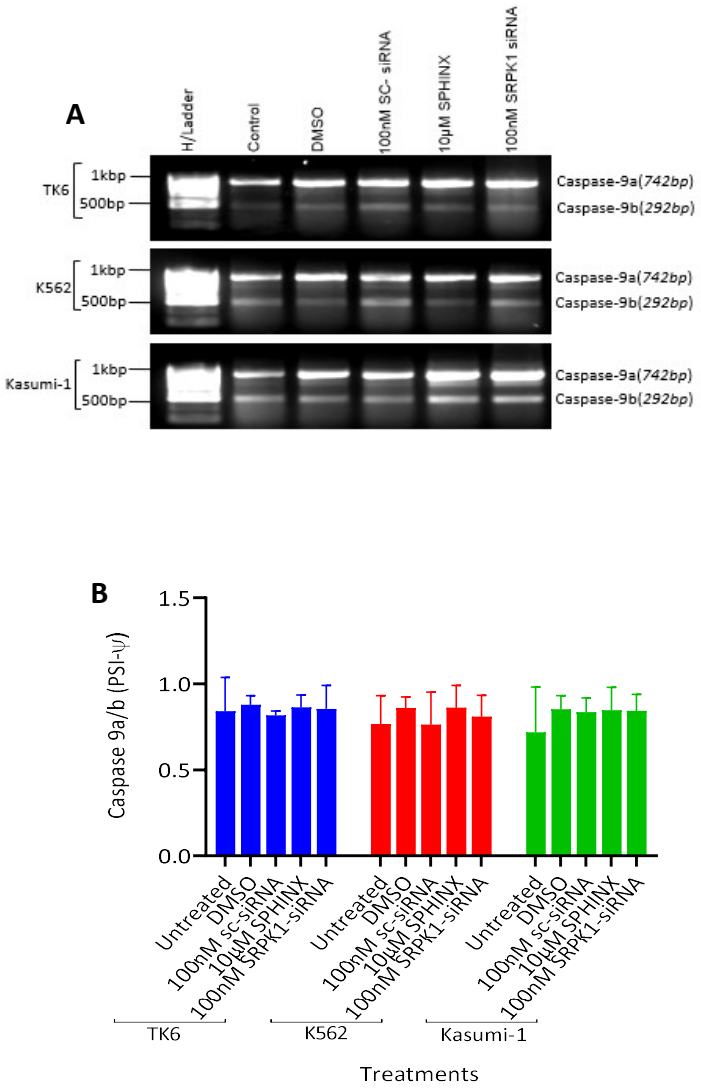


Figure 5.6. Comparative effect of SRPK1 inhibition and knockdown on caspase 9 alternative splicing. Leukaemic cells were either treated with 10μM SPHINX or transfected with 100nM siRNA or a scrambled control. **A**. Representation of PCR amplicon (caspase 9a, 742bp) and (caspase-9b, 292bp) for TK6, K562 and Kasumi-1. **B**. Densitometry showing percentage of splice inclusion (PSI-Ψ) in caspase-9a/b isoform. *n*=3.

5.3 Comparative assessment of SRPK1 inhibition and SRPK1 siRNA knockdown in leukaemic cells protein levels

5.3.1 Effect of siRNA knockdown on SRPK1 protein levels

Protein quantification indicated an effective knockdown of SRPK1 using siRNA. Levels of SRPK1 differed significantly between knockdown and DMSO (Figure 5.7A-B).

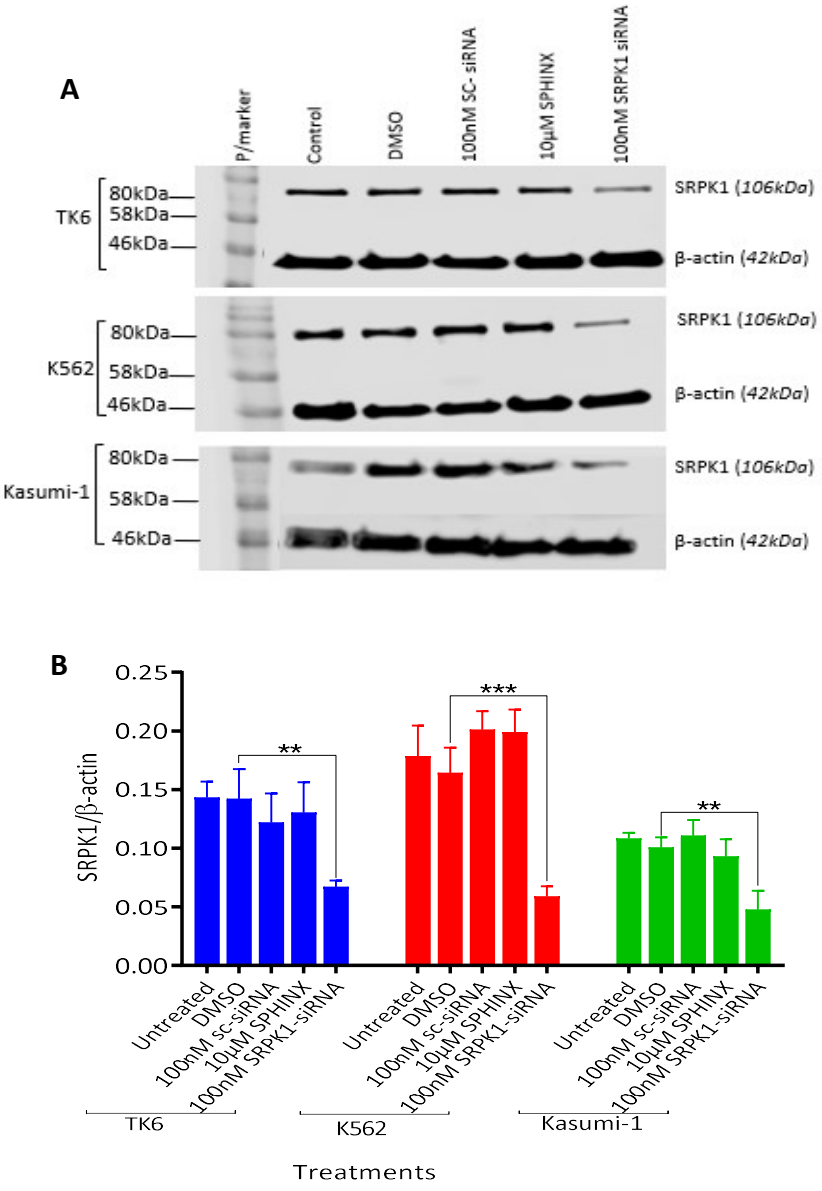


Figure 5.7. Comparative assessment of SRPK1 protein levels in SRPK1 inhibition and knockdown leukaemic cells. Cells were treated with either SPHINX or transfected with SRPK1 siRNA. **A**. Result showed significant knockdown of SRPK1 in the siRNA transfected cells for all cell lines. In TK6 (***P*= 0.006). K562 protein levels also differ (****P*=0.0002) compared to DMSO. Knockdown of SRPK1 in Kasumi-1 (***P*=0.003) when compared to DMSO. **B**. Images are representative of immunoblot with corresponding β-actin loading control. *n*=3.

5.3.2 *Effect of siRNA knockdown on SRSF1 protein levels*

Protein levels for total SRSF1 were found to be increased following knockdown of SRPK1 in K562 cells, when compared to the DMSO control. Kasumi-1 and TK6 cells showed no difference in both the knockdown and SPHINX when compared to DMSO control (Figure 5.8).

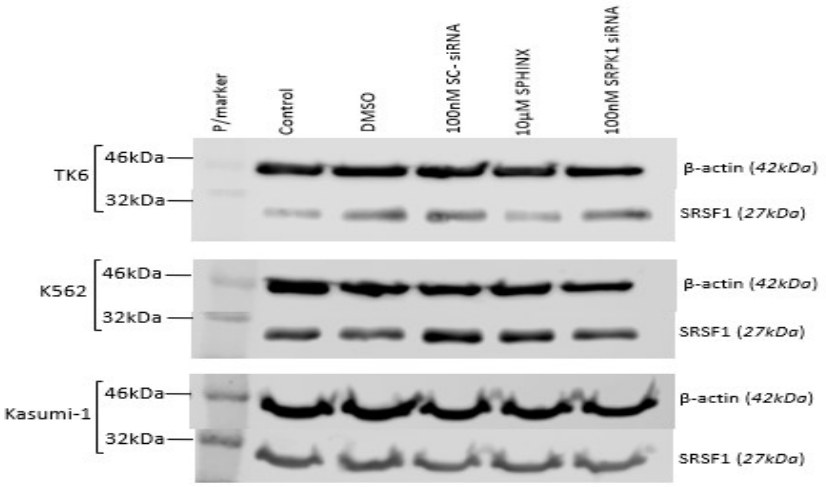


Figure 5.8. Comparative assessment of total SRSF1 protein levels in SRPK1 inhibition and knockdown leukaemic cells. Western blot analysis on leukaemic cells protein extract using anti-SRPK1 antibody. Cells were treated with either SPHINX or transfected with SRPK1 siRNA. Representative immunoblots with corresponding β-actin loading control. *n*=3.

5.3.3 *Effect of siRNA knockdown on SR protein phosphorylation*

Phosphorylation of SR protein following SRPK1 knockdown using siRNA was also investigated. Protein levels for *p*SRSF4 were found to be reduced in the knockdown group for all cell lines. In TK6 and K562 cells, reduced levels of phosphorylation significantly differed from the DMSO treated cells. Furthermore, in Kasumi-1 cells, both the SPHINX and knockdown cells showed a significant decrease in phosphorylation when compared to the DMSO.

Immunoblotting using the 1H4 monoclonal antibody designed to identify phospho-epitopes for the SR proteins showed that levels of *p*SRSF6 following SRPK1 knockdown was found to be reduced. Lower levels of phosphorylation were observed in the knockdown in TK6 and K562. While in Kasumi-1 cells, SPHINX and the knockdown cells significantly decreased *p*SRSF6 levels (Figure 5.9).

Levels of *p*SRSF11 appear to be affected by the knockdown in TK6 and K562 cells (I & II) when compared to the DMSO control. In Kasumi-1, reduced phosphorylation of *p*SRSF11 was observed in the SPHINX and knockdown cells when compared to DMSO (III).

Reduced levels of *p*SRSF5 were observed in knockdown cells in all three cell lines these were found to be significantly reduced compared to the increased level seen in the untreated, DMSO and scrambled group. Similar levels were also observed for Kasumi-1 in addition to the SPHINX treated cells which also showed a decreased level of SRSF5 phosphorylation.

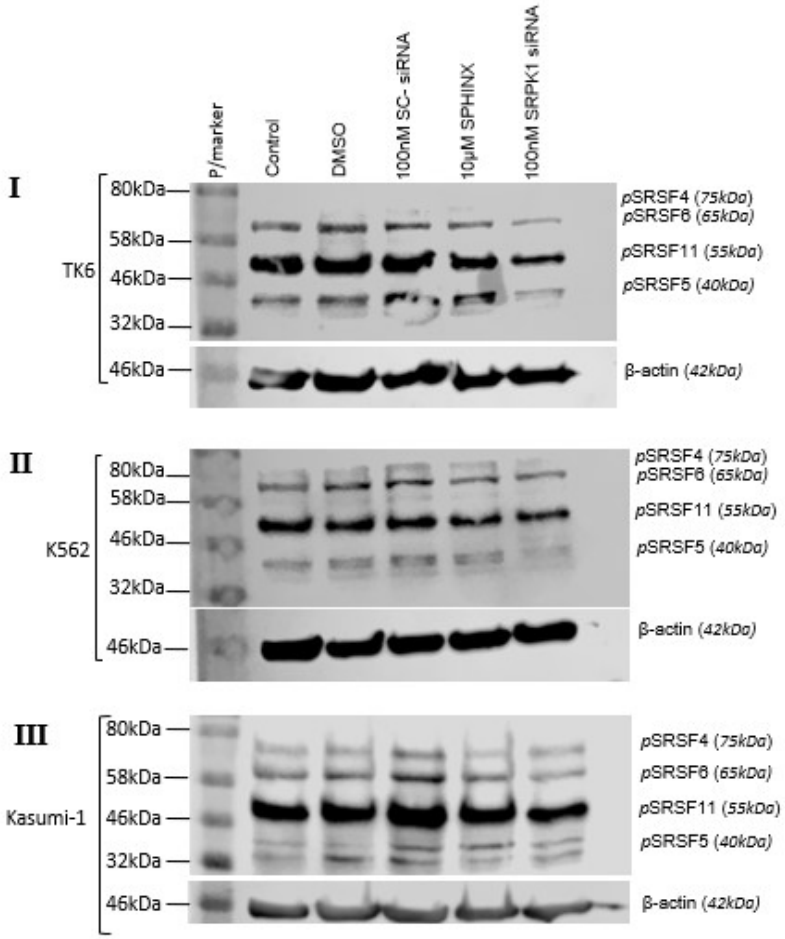


Figure 5.9. Comparative assessment of protein levels of phospho-SR protein in SRPK1 inhibited and knockdown leukaemic cells. Western blot analysis on leukaemic cells protein extract using anti-SRPK1 antibody. Cells were treated with either SPHINX or transfected with SRPK1 siRNA. Representative western blot images (**I-III**) for SR-proteins with corresponding β-actin loading control. (*n*=3).

5.4 Discussion

5.4.1 Effect of SRPK1 knockdown on leukaemic cell viability and growth

In a previous study, knockdown of SRPK1 in the CML cell line, K562 using siRNA has been shown to induce apoptosis and decrease K562 cell proliferation (Wang *et al*., 2018). Levels of apoptosis induction correlated with increased levels of cleaved caspase-3, cleaved PARP and p53 protein levels (Wang *et al*., 2018). A similar experiment carried out in renal cell carcinoma (RCC) and pancreatic tumour cells also observed growth and proliferation inhibition as well as suppression in the migratory and invasive ability of the cells (Hayes *et al*., 2007; Han *et al*., 2017).

A further experiment was carried out firstly to confirm that the effects observed with SPHINX was an SRPK1-dependent effect and secondly, to observe whether leukaemic cells will behave differently if SRPK1 was knocked down using siRNA rather than inhibited with small molecules. Apart from TK6 cells where no change was observed in the cell viability, a significant decrease in cell viability was observed in K562 and Kasumi-1 cells in SPHINX and knockdown cells when compared to DMSO. Furthermore, cell growth was significantly reduced in the SPHINX and knockdown cells when compared to DMSO controls in TK6, K562 and Kasumi-1 cells. This result is consistent with published work (Wang *et al*., 2018) and suggests that SRPK1 could be involved in CML and AML cell growth and disease progression.

5.4.2 Effect of SRPK1 knockdown on alternative splicing of VEGF and a panel of apoptotic genes

This study found no difference in VEGF levels between knockdown, SPHINX inhibition and controls. This suggests that knockdown of SRPK1 has no effect of VEGF in TK6, K562 and Kasumi-1 model cell lines. No change was also observed with splicing of apoptotic genes in all three cell lines, with the dominant isoforms being highly expressed. Other studies have found changes in VEGF alternative splicing following inhibition or knockdown of SRPK1 in several cancers (Gammons *et al*., 2014; Gonçalves *et al*., 2014; Hatcher *et al*., 2018). Previous studies have reported altered expression of *caspase* and *BCL2L* apoptotic genes following transfection with small interfering RNA targeting SRPK1 in leukaemic cells and other cancers (Hayes *et al*., 2007; Wang *et al*., 2018).

5.4.3 *Effect of SRPK1 knockdown on the levels of SRPK1, SRSF1 protein and on phosphorylation of SR protein in leukaemic cells*

Consistent with the established role of SRPK1 in phosphorylation of SR proteins, levels of total SRSF1 were increased, whereas phosphorylation for *p*SRSF4, *p*SRSF6, *p*SRSF11 and *p*SRSF5 were found to be reduced in knockdown cells. The levels of SR protein differed from the controls but not all SR protein showed a difference between the knockdown cells and the SPHINX treated cells. This suggests that there may not be a compensatory mechanism for SRPK1. So, whether inhibition or knockdown, the same effect will be observed on the phosphorylation of its substrate. Results suggest that targeting SRPK1 could be useful in controlling aberrant splicing via regulation of SR protein phosphorylation.

CHAPTER 6

6 Discussion of key findings, limitation of study and future work

6.1 Summary of key findings

Leukaemia is a malignant disorder that results from the abnormal proliferation of a clone of haematopoietic cells characterised by insensitivity of blood cells to growth regulation and apoptosis (Chu *et al*., 2012). According to CRUK, leukaemia is the 12th most common cancer in the UK with about 9,900 new cases of leukaemia reported in the UK each year (CRUK, 2018). The incidence rate is higher in males than in females. It is projected that the incidence rate of leukaemia will rise by 5% between 2014 and 2035 while the mortality rate will rise by 18%. Leukaemia prognoses differs significantly depending on whether it is acute or chronic, of myeloid or lymphoid cell type, age and time of diagnosis (CRUK). Despite advancements in understanding the molecular pathogenesis of leukaemia, and despite improvements in therapy and the introduction of novel drugs, most patients will relapse due to impaired haematopoiesis driven by clonal evolution of bone marrow stem cells (Martínez *et al*., 2005; Yilmaz *et al*., 2019).

The serine/arginine-rich protein splice factor kinase-1 (SRPK1) belongs to a family of protein kinases, which phosphorylate specific amino acids of proteins rich in serine/arginine repeats (RS-domain). A study has shown that following activation of EGF, SRPK1 can induce substantial changes in the alternative splicing landscape through phosphorylation of SR proteins (Zhou *et al*., 2012). It is apparent that the role and expression of SRPK1 in different cancers is heterogeneous and therefore, its role in cancer is not yet fully understood (Zhou *et al*., 2013; Bullock and Oltean, 2016).

A study in leukemic cells demonstrated that SRPK1 is highly expressed in erythroid and lymphoid cells and, there is an associated increased expression of SRPK1 to cell proliferation and tumour grade (Sanidas *et al*., 2010). Repression of SRPK1 in AML was shown to modify the choice of a splice site in VEGF and caspase 9 (Shultz *et al*., 2011; Oltean *et al*., 2012). In relation to VEGF, the mechanism is such that repression of SRPK1 results in hypo-phosphorylation of SRSF1 and hence, the selection of a distal 3' splice

site in exon 8 during VEGF pre-mRNA splicing resulting in expression of anti-angiogenic VEGF (Shultz *et al*., 2011; Oltean *et al*., 2012).

This thesis was aimed at targeting SRPK1 with a small molecular inhibitor as alternative mechanism in slowing the growth of leukaemic cells and augmenting existing conventional chemotherapeutic drugs.

6.1.1 *Effect of SPHINX on leukaemic cell viability and growth*

This study investigated what effect SRPK1 inhibition using SPHINX has on leukaemic cell viability and growth. It also investigated if combining SRPK1 inhibition with existing drugs will improve leukaemia treatment using three cell line models: the lymphoblastic cell line TK6, the chronic myeloid leukaemia cell line K562 and the acute myeloblastic leukaemia cell line Kasumi-1.

Results from this study showed that inhibition of SRPK1 using SPHINX had no effect on TK6 and K562 cell viability and cell number at experimental concentrations. Kasumi-1 cell viability and growth were significantly decreased and shown to be concentration dependent. The percentage decrease in cell viability observed was accompanied by an increase in caspase 3/7 activity, which are known to be indicators of apoptosis. When SRPK1 was knocked down using siRNA in all three cell lines, K562 and Kasumi-1 but not TK6 showed a decrease in both cell viability and growth. Peripheral blood cells of myeloid origin have been shown to be more sensitive to SRPK1 inhibition (Siqueira *et al*., 2015). This suggests that SRPK1 could play a role in leukaemic cell survival, but its effect could also be cell specific.

This study also confirms that both Imatinib mesylate (a tyrosine kinase inhibitor targeting the fusion protein Bcr/Abl in CML) and azacytidine (a DNA hypo-methylation agent used in AML therapy) are both effective in inducing cell death in CML and AML cell line models. However, when SPHINX was combined with either imatinib for K562 or with azacytidine for Kasumi-1, only in Kasumi-1 cells was an additive effect of increased cell death observed over cells treated with either SPHINX or azacytidine alone. Combining imatinib and SPHINX showed no additive effect in the percentage of cell death in K562 cells compared to imatinib treatment. A limitation to this aspect of the study is the lack of a suitable control in the microscopic investigation of apoptosis. A suitable control would have been cells treated with caspase 3/7 inhibitors such as isatin

sulfonamide which inhibits caspase 3/7 activity by competitively binding to the active binding site and catalytic cysteine residue respectively (Lee *et al*., 2000; Yoshimori *et al*.,2004). The established apoptotic inducer apoptolidin, has been shown to be selectively cytotoxic on several cancer cell lines and non-cytotoxic on normal cells (Ghidu *et al*.,2008) and would have served as a good positive control. These would have validated the observed result especially in the untreated and DMSO controls where no caspase activity was observed. Owing to this caveat, it is possible that some leukaemic cancers could benefit from therapies that include inhibiting SRPK1; this ought to be looked at in much further depth in future research.

6.1.2 *Effect of SPHINX on alternative splicing of CLK1 and a panel of apoptotic genes*

The results obtained showed that alternative splicing in RNA levels of *Bclx*, *Apaf1* and *caspase 9*, responded differently following inhibition of SRPK1 using SPHINX.
SPHINX inhibition of SRPK1 favoured the increased expression of the anti-apoptotic Bcl-xl isoform in all cell lines. Higher levels of Bcl-xl have been reported in several cancers including leukaemia where it confers a cell survival advantage in leukaemic cells (Boise *et al*., 1993; Takehara *et al*., 2001; Willimott *et al*., 2011).

Like with *BCL2L*, *APAF1* also encodes two isoforms; Apaf-1xl and 1s. Apaf-1xl which is the full-length isoform and anti-apoptotic in function was also found to be increasingly expressed in all cell lines except for Kasumi-1 cells at 10µM concentration at 24hrs. Studies have shown that an increased level of Apaf-1xl in cells prevents cell apoptosis and confers resistance to chemotherapeutic treatment (Benites *et al*., 2008). The study also suggests that an equal level of both isoforms rather than overexpression of either Apaf-1xl or 1s is required to initiate apoptosis in cells and overcome drug resistance (Benites *et al*., 2008).

On the alternative splicing of caspase 9, caspase 9a the larger of the caspase 9 isoforms which is also known to be pro-apoptotic (Shultz *et al*., 2011) in function was increasingly expressed in TK6 cells and Kasumi-1 cells at higher concentrations of SPHINX inhibition. Conversely, K562 showed decreased levels of the pro-apoptotic 9a isoform. This finding indicates that caspase 9a could be involved in the apoptosis of some leukaemia cells. Possible mechanisms may include inhibiting the attachment of caspase 9a to the

apoptosome and supressing (9b), or activation (9a) of the caspase enzyme cascade (Vu *et al*., 2013). A case-controlled study (Edathara *et al*., 2019) suggested that a polymorphism, *CASP9*-1263A>G, observed in a CML patient, enhanced the risk for developing CML while, Ex5 +32G>A exonic polymorphism, which results in the substitution of glutamine by arginine causes conformational changes in caspase 9 and therefore its affinity to Apaf-1. This change in caspase 9 affinity for Apaf-1 was observed to confer resistance to CML cells against apoptosis (Edathara *et al*., 2019). It also suggests that the choice of apoptotic pathway could be cell-specific since levels of caspase 9a matched the increased level of cell death in Kasumi-1 cells but not in the other two cell lines.

On combining SPHINX with either imatinib or azacytidine, CLK1 showed increased exon 4 retention and less intron 4 inclusion in treatments involving TG003. This shift in splicing is aimed towards higher expression levels of catalytically active CLK1 and its auto-regulation in order to maintain its activity. It was also observed that the level of retention of intron 4 was lower in Kasumi-1 cells as compared to K562. There was no additional effect of combining SPHINX and azacytidine in the alternative splicing of Bclx, Apaf1 and caspase 9 when these were compared with the single treatments in Kasumi-1 cells. Furthermore, in K562 cells, combining SPHINX and imatinib showed a change in alternative splicing towards increased expression of caspase 9a, an effect which could have been caused by imatinib since the same splicing event was not observed in the SPHINX only treatment. A previous study (Liu *et al*., 2012) had observed a switch in Bclx to a pro-apoptotic isoform (Bcl-xs) in K562 cells following treatment with imatinib. The concentration of imatinib used however, was higher than the concentration used in this thesis.

This aspect of the study was confronted with some challenges. First, PCR for the reference gene (β-actin) used as loading control and normalization of the gene of interest was performed on each batch of synthesized cDNA. This has the advantage of confirming the integrity of the cDNA after synthesis and a baseline for overall actin expression in the cell lines. It does have the disadvantage of not confirming the integrity of the cDNA in subsequent repeats. Even though cDNAs are known to be relatively stable, the cycle of freeze thawing, handling and temperature have an effect which was not accounted for, by not including a housekeeping gene in subsequent PCR assays. This

could be responsible for the second challenge, which is large variation in the confidence intervals observed during statistical analysis. As such, some of the PCR outputs were neither quantified nor normalized, and results presented were interpreted based on visual examination of the amplicon intensity, which could be subject to bias.

6.1.3 *Effect on protein levels of SRPK1 and SRSF1 and phosphorylation of SR-protein*

It is conceivable that the inhibition of SRPK1 might result in changes in its own expression as there are no published studies investigating the effect of SRPK1 inhibition on its own protein levels through such an auto-regulatory mechanism. Results from this thesis have shown that inhibition of SRPK1 in model cell lines using SPHINX suggest an increase in SRPK1 protein levels at higher concentrations in K562 but not in TK6 and Kasumi-1 cell lines. Exposing K562 cells to imatinib did appear to cause a reduction in SRPK1 levels, and this effect was not significantly augmented with SPHINX. In Kasumi-1 cells, there was an obvious advantage over combining SPHINX and TG003 with azacytidine as cells in this group expressed less SRPK1 protein overall. In summary there is some evidence that i) there may be an auto-regulatory mechanism that needs to be investigated further and ii) established drugs such as imatinib appear to influence SRPK1 expression presumably by interfering with regulatory or cell signalling pathways such as Rac signalling where it affects its splice selection of e3b with consequent decrease of Rac1b (Gonçalves *et al*., 2014) required to maintain SRPK1 expression.

Protein levels of total SRSF1 appeared to be reduced at 48hrs in TK6 at higher concentrations but were unaffected in K562 and Kasumi-1 cells. This suggests that SRPK1 activity might also influence the overall expression of its own substrates, perhaps through an alternative splicing mechanism. Protein levels of the splice factor kinase CLK1 were found to be reduced in K562 cells in treatment with imatinib, imatinib and TG003 and a combination of all three molecules. In Kasumi-1 cells, combining SPHINX and TG003 with azacytidine resulted in a significant decrease in CLK1 protein when compared to other treatments. This suggests that as well as affecting the expression of SRPK1 and SRSF1, treating leukaemic cell lines with these compounds also affects (reduces) CLK1 levels. The reduction of splice factor kinase levels could bring additional

benefit, since elevated expression of these splice factor kinases is generally associated with a more proliferative phenotype (Karni *et al*., 2007; Anczuków *et al*.,2012).

Phosphorylation of SR proteins was found to be significantly reduced and affected either in a concentration-dependent manner or at a time point in all three cell lines following SRPK1 inhibition with SPHINX. This was not surprising as these splice factors are SRPK1 substrates and required for their activation (Gui *et al*., 1994), and confirmed that SPHINX was having the desired effect. Knockdown of SRPK1 was shown to affect the level of SR protein phosphorylation. Combining SPHINX (to inhibit SRPK1) with TG003 (to inhibit CLK1) with imatinib or azacytidine resulted in decreased SR protein phosphorylation when compared to the single treatments in K562 especially at 72hrs. In the AML cell line, Kasumi-1, there was a marked decrease in SR protein phosphorylation in cells treated with SPHINX, TG003 and azacytidine. Increased SR protein phosphorylation and associated increases in SR protein activity is associated with a proliferative phenotype (Karni *et al*., 2007); therefore, the ability of SPHINX (especially in combination with other inhibitors) to reduce phosphorylation of SR proteins could prove beneficial in the treatment of AML. However, for an experiment lasting 72hrs, the half-life of the inhibitors and drugs used were not determined. Other factors such as drug handling, storage, light and temperature are known to affect shelf-life of drugs (Anderson and Scott, 1991; Singh *et al*., 2002). It is possible that some results which were not consistent were because of degradation of the drugs. For example in Figures 3.32, 4.36 and 4.39, where a decrease was observed at 24hrs while at 48hrs and or 72hrs, an increase was observed.

Taken together, the results presented in this thesis do suggest that there may be benefit in targeting the splice factor kinase SRPK1 with SPHINX, perhaps in combination with existing drugs, and in combination with CLK inhibitors. Further research is needed to address this hypothesis, including *in vivo* studies.

6.2 Future work

6.2.1 *Determine the broad effect of SRPK1 inhibition in leukaemic cells on the transcriptome*

Results presented in this thesis suggest that SRPK1 inhibition in the model cell lines (TK6, K562 and Kasumi-1) has a different effect on each cell line with Kasumi-1 cells being the most affected. It is possible that SRPK1 inhibition using SPHINX could influence other targets and pathways other than those investigated in this research. Tzepelis *et al*., (2016) have identified *SRPK1* as a gene that could be targeted in AML following a CRISPR screen and pharmacological validation. Previous studies (Zhou *et al*., 2012; Wang *et al*., 2014) have also found an effect of targeting SRPK1 on PI3/Akt pathways, which was not investigated in this study. As expected, we observed that SRPK1 inhibition affected phosphorylation of SR proteins, which are key in the splicing machinery. It would be of interest to determine the effect of SRPK1 inhibition, either alone or together with CLK inhibition, and together with established drugs on the transcriptome more widely. The most direct approach would be to use RNASeq and to examine the effect on the alternative splicing of a wide range of cancer-associated transcripts, associated with each of the hallmarks of cancer. As well as a broad analysis of alternative splicing, the alternative splicing of genes specifically associated with critical roles in each type of leukaemia could be examined. This type of research would also shed further light on the biological function of SRPK1.

6.2.2 *In vivo experiments to determine the effect of SRPK1 inhibition on tumour growth*

Siqueira *et al*., (2015) observed that peripheral blood cells of myeloid origin where Kasumi-1 cells belong are more sensitive to SRPK1 inhibition when compared to cells of lymphoid origin. Result obtained in this study were consistent with their findings with Kasumi-1 showing increased propensity to cell death following SPHINX inhibition and knockdown when compared to K562 and TK6 cells. It would be worth investigating if such a result could be replicated *in vivo* to confirm the previous reports of SRPK1 as a potentially druggable target in AML and, therefore, to move research forwards.

It is worth mentioning at this point that two *in vivo* study attempts were made during this project using K562 and Kasumi-1 cells to see if the *in vitro* experiment with cell lines

could be replicated *in vivo*, specifically in mouse xenograft experiments. However, due to technical issues experienced, viable tumours did not form and so, the experiment was not completed. This study was unable to achieve growth of tumours in all the mice at the end of the approved period for such work. Owing to the time limit of this project, it was not possible to make another attempt. This will be a primary objective in future research, perhaps using a different leukemic cell line model that is more suitable for xenografting.

6.2.3 *Investigating the potential of targeting CLK1 in leukaemic cells*

The effect of inhibiting CLK1 with TG003 on SR protein phosphorylation and on its own alternative splicing was investigated in this project even though it was not the focus of the research. It was observed that CLK1 splicing particularly in CML cells following inhibition with TG003 resulted in more exon 4 retention and intron 4 skipping; this was not apparent in Kasumi-1 cells. It was observed that CLK1 protein was increased following its inhibition with TG003; when imatinib was combined with TG003, an additive effect was observed. It would be of great interest to investigate the effect of CLK1 inhibition on leukemic cells, either on its own, or in combination with SRPK1 inhibition. It should be noted that TG003 is one of the first CLK inhibitors to be developed (in Masatoshi Hagiwara's laboratory); it also inhibits CLK4, which is closely related to CLK1. More specific CLK inhibitors are being developed that could supersede TG003. It is conceivable that a potential novel therapy will be focused on inhibiting both SRPKs and CLKs together. There are, furthermore, other splice factor kinases such as the DYRKs (Becker and Joost, 1999); their role and targetability in leukaemia should also be considered.

6.2.4 *Effect of combining SPHINX with imatinib*

This research also observed some interesting results with imatinib. It was observed that imatinib alone reduced cell viability as well as protein levels of CKL1, total SRSF1 and SRPK1 in CML. In addition, imatinib appeared to affect the alternative splicing of caspase 9 favouring pro-apoptotic caspase 9a. Interestingly, when imatinib was combined with SPHINX, the effect on SRPK1, CKL1 and total SRSF1 expression was less prominent. So, there are likely to be complex effects when SPHINX and imatinib are combined. In line with this, a study has reported that both SRPK1 and imatinib both interact with the

PI3/Akt pathway (Wang *et al*., 2014). Future work will address in more detail the consequences of combining SPHINX with imatinib in terms of effects on downstream targets.

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