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Title	Targeting aberrant kinase activity in myeloid leukaemias
Authors	Kelly, Kevin R.
Publication date	2013
Original Citation	Kelly, K.R. 2013. Targeting aberrant kinase activity in myeloid leukaemias. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-09-14 09:53:22
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Targeting aberrant kinase activity in myeloid leukaemias

A thesis submitted to the University College Cork in fulfilment of the requirement for the degree of

Doctor of Philosophy

By

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Date of submission:	**April 2013**

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I hereby declare that this thesis is my own work and effort and that it has not been
submitted anywhere for any award. Where other sources of information have been used,
they have been acknowledged.

Signature: …….

Date: …….

Acknowledgements

Firstly, I would like to thank Dr. Frank Giles, for giving me the opportunity to work and conduct research in the Institute for Drug Develpment. Your support and encouragement over the past three years has been an enormous asset. Thank you for taking me under your wing, supporting my work and introducing me to the world of science and developmental therapeutics. I sincerely appreciate all you have done for me.

A very special thank you to Drs. Jennifer Carew and Steffan Nawrocki. You both have inspired me from the day I entered the laboratory. You transformed me into the physician scientist I am today. You have supervised every aspect of this thesis and helped me along the way. I will remain always indebted to you both.

Thank you to Dr Mary Cahill for your advice, mentorship and support throughout this process. Thank you for your patience, feedback and guidance into preparing this thesis.

Thank you to Claudia and Ernest for your help with performing many of these experiments. Your skills are a tremendous asset to the lab.

And lastly and most importantly my family, Mum, Dad, Seamus, Neasa and Cliona. Thank you for your support through all this.

Abbreviations

ABL	Abelson
AKT	AKT8 virus oncogene cellular homolog
AML	Acute myeloid leukaemia
AMP	Adenosine monophosphate
Apaf-1	Apoptosis protease activating factor 1
APC/C	Anaphase promoting complex or cyclosome
APL	Acute promyeloyctic leukaemia
AIF	Apoptosis inducing factor
AP	Accelerated phase
ara-C	Cytarabine
ATCC	American type culture collection
ATF	Activating transcription factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
ATRA	All-trans retinoic acid
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2–associated X protein
BCL-2	B cell lymphoma-2
BCR	Breakpoint cluster region
BCL-xL	BCL extra long
BFU-E	Eythroid burst-forming unit
BIM	Bcl-2 interacting mediator
BID/tBID	Bcl-2 interacting protein (truncated)
BIRC6	Baculoviral IAP repeat-containing protein 6
BC	Blast crisis
BM	Bone marrow

BMF	BCL-2-modifying factor
BMSC	Bone marrow stromal cells
BMT	Bone marrow transplantation
BOK	BCL-2 related ovarian killer
Bub	Budding uninhibited by benzimidazoles
CALGB	Cancer and Leukemia Group B
CAMK	Ca2+/calmodulin-dependent protein kinases
Caspases	Cysteine proteases with aspartate specificity
CBFB	Core binding factor beta
CDC25	Cell division cycle 25
CDK	Cyclin dependent kinase
CDKN1B	Cyclin-dependent kinase inhibitor 1B (P27)
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEBPA	CCAAT/enhancer binding protein alpha
CFU	Colony Forming Units
CFU-Baso	CFU-basophil
CFU-Eo	CFU-eosinophil
CFU-GM	CFU-granulocyte–macrophage
CFU-GEMM	CFU-granulocyte–erythrocyte–macrophage–megakaryocyte
CHK	Checkpoint Kinase
CI	Combination Index
CKI	Cyclin dependent kinase inhibitor
CML	Chronic myeloid leukaemia
CML-AP	Accelerated phase chronic myeloid leukaemia
CML-BC	Blast crisis chronic myeloid leukaemia
CML-CP	Chronic phase chronic myeloid leukaemia
CPC	Chromosomal passenger complex
DASISION	Dasatinib versus Imatinib Study in Treatment-Naive CML-CP Patients
DDs	Death domains
DISC	Death inducing signalling complex

DIABLO	Direct inhibitor of apoptosis protein binding protein with low pI
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
4E-BP1	4E-binding protein 1
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal Growth Factor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
FAB	French-American-British
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
FGFR1	Fibroblast growth factor receptor 1
FLIP	FLICE-inhibitory protein
FLICE	FADD-like IL-1 beta-converting enzyme
FOXO	Forkhead box protein O
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H3K4	Histone H3 lysine 4
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylases
H&E	Haematoxylin and eosin
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplant
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hh	Hedgehog
HIF1α	Hypoxia-inducible factor1α
HRK	Harakiri BCL-2 interacting protein

HSP90	Heat shock protein 90
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
HtrA2	High temperature requirement A2
IAP	Inhibitor of apoptosis protein
IFNα	interferon-alpha
IL	Interleukin
INCENP	Inner centromere protein
IP	Intra peritoneal
JAK	Janus kinase
LC/MS	Liquid chromatography–mass spectrometry
LT-HSCs	Long-term HSCs
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen associated protein kinase
MAPs	Microtubule-associated proteins
MCAK	Mitotic centomere associated kinase
MDM2	Murine double minute 2
MDS	Myelodysplasia
MHC	Major histocompatibility complexes
MM	Multiple myeloma
MMR	Major molecular response
MRD	Minimal residual disease
mTOR	Mammalian target of rapamycin
MTT	2-(4, 5-dimethyltriazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MYC	Myelocytomatosis oncogene cellular homolog
NaCl	Sodium chloride
NaF	Sodium fluoride
Na_3VO_4	Sodium orthovanadate
N-CoR	Nuclear corepressors

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin's lymphoma
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
NP-40	Nonyl phenoxypolyethoxylethanol
NPM1	Nucleophosmin 1
OCT	Organic cation transporter
OH	Hydroxyl
OS	Overall survival
PARP	Poly ADP ribose polymerase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PFS	Progression free survival
Ph	Philadelphia chromosome
Ph+	Philadelphia chromosome positive
PMSF	Phenylmethanesulfonyl fluoride
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PlGF	Placental-derived growth factor
PIM	Proto-oncogene serine/threonine-protein kinase
PLK 1	Polo like kinase 1
PODs	PML oncogenic domains
PP2A	Protein phosphatase 2
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	Quantitative real time polymerase chain reaction
RARA	Retinoic acid receptor alpha

Ras	Rat sarcoma
RET	rearranged during transfection
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RUNX1	Runt-related transcription factor 1
RXR	Retinoid x receptor
S6K1	Ribosomal protein S6 kinase β1
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell derived factor 1
SD	Stable disease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering Ribonucleic acid
SMAC	Second mitochondrial activator of caspases
SRC	Sarcoma
STAT	Signal transducer and activator of transcription
TACC	Transforming acidic coiled-coil-containing protein
TKI	Tyrosine kinase inhibitor
TNFα	Tumour necrosis Factor-alpha
TRAF2	TNF Receptor-associated factor 2
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TUNEL	Terminal deoxyribonucleotide transferase–mediated nick-end labeling
TTC	2,3,5-triphenyltetrazolium chloride
UCE	Ubiquitin conjugating enzyme
VCR	Vincristine
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organisation

Thesis summary

The leukaemias are a group of diseases with differing aetiologies, presentations and treatments. Leukaemia can be divided into acute and chronic types and occur in cells of myeloid and lymphoid lineages. Acute leukaemias are characterised by the rapid accumulation of immature haematopoietic cells usually necessitating urgent treatment. Chronic leukaemias are characterised by the excessive build up of relatively mature, malignant, white blood cells and typically take months or years to progress. Acute myeloid leukaemia (AML) consists of a group of relatively well-defined haematopoietic neoplasms involving precursor cells committed to the myeloid line of cellular development. It is the most common form of acute leukaemia in adults and accounts for approximately 80% of cases in this group. The treatment of AML has remained largely unchanged for the past 30 years with the nucleoside analogue cytarabine (ara-C) remaining the backbone of therapy. While some patients are cured of the disease, most will relapse. Treatment of elderly patients with AML in particular represents a significant challenge as many patients in this group have adverse prognostic features, resistant disease, and a poor ability to tolerate intensive conventional chemotherapy. As such, there has been an enormous effort in recent years to develop novel targeted therapies for these patients.

Chronic myeloid leukaemia (CML) on the other hand represents a tremendous success story in the era of targeted therapy. CML is a myeloproliferative neoplasm characterized by the dysregulated production of mature granulocytes. CML is associated with the Philadelphia (Ph) chromosome t(9;22)(q34;q11) or a related translocation resulting in the breakpoint cluster region-Abelson (*BCR-ABL*) fusion gene. The constitutive activity of the BCR-ABL tyrosine kinase is the primary event that drives CML pathogenesis and thus, serves as an ideal target for therapy. Indeed,

targeted inhibition of BCR-ABL with tyrosine kinase inhibitors (TKI)s has changed the natural history of this disease and dramatically improved the survivorship of patients diagnosed with CML. However, significant challenges in CML therapy remain including the development of drug resistance due to BCR-ABL kinase domain mutations, the activation of secondary oncogenic signalling cascades in patients with advanced disease and disease persistance due to presence of CML stem cells. Combining BCR-ABL inhibitors with agents that target other key pathways may offer an opportunity to more effectively treat patients that do not achieve successful outcomes with TKI monotherapy.

In normal cells the cell cycle is a tightly regulated process that allows faithful inheritance of the genetic material from mother to daughter cells. Several kinase families including cyclin dependent kinases (CDKs), polo like kinases (PLKs) and Aurora kinases, tightly control cell cycle events. The Aurora family of serine/threonine kinases is essential for chromosome alignment, segregation, centrosomal maturation, mitotic spindle formation, and cytokinesis during mitosis. Their fundamental role in cell cycle regulation and their aberrant expression in a broad range of malignancies prompted the development of small molecules that selectively inhibit their activity. Recent studies have revealed new insights into the cellular effects of Aurora kinase inhibition. Moreover, early phase clinical studies have shown that these agents have therapeutic efficacy. Section 1.8 outlines the functions of Aurora kinases in normal cell division and in malignancy. The chapter focuses on recent preclinical and clinical studies that have explored the mechanism of action and clinical effect of Aurora inhibitors in cancer treatment.

One of the most exciting reports of clinical activity of the Aurora kinase inhibitors has been in the setting of refractory CML. Chapter 2 outlines the evaluation of the efficacy and mechanism of action of alisertib, a novel inhibitor of Aurora A kinase, in preclinical models of CML. We report that alisertib possessed equipotent activity against Ba/F3 cells and

primary CML cells expressing unmutated and mutated forms of BCR-ABL. Notably, this agent retained high activity against the T315I and E255K BCR-ABL mutations, which confer the greatest degree of resistance to standard therapy. Alisertib treatment disrupted cell cycle kinetics, induced apoptosis, caused a dose-dependent reduction in the expression of the large inhibitor of apoptosis protein Apollon, and produced a morphological phenotype consistent with Aurora A kinase inhibition. In contrast to other Aurora kinase inhibitors, alisertib did not significantly affect BCR-ABL activity. Moreover, inhibition of Aurora A with alisertib significantly increased the *in vitro* and *in vivo* efficacy of nilotinib. Targeted knockdown of Apollon sensitized CML cells to nilotinib-induced apoptosis, indicating that this is an important factor underlying alisertib's ability to increase the efficacy of nilotinib. Our collective data demonstrate that this combination strategy represents a novel therapeutic approach for refractory CML that has the potential to suppress the emergence of T315I-mutated CML clones.

Chapter 3 explores the activity of alisertib in preclinical models of AML. We investigate the preclinical efficacy and pharmacodynamics of alisertib in AML cell lines, primary AML cells, and mouse models of AML. We report that alisertib disrupted cell viability, diminished clonogenic survival, induced expression of the forkhead box O3 (FOXO3a) targets p27 and BCL-2 interacting mediator (BIM), and triggered apoptosis. A link between Aurora A expression and sensitivity to ara-C was established, suggesting that Aurora A inhibition may be a promising strategy to increase the efficacy of ara-C. Accordingly, alisertib significantly potentiated the anti-leukaemic activity of ara-C in both AML cell lines and primary blasts. Targeted FOXO3a knockdown significantly blunted the pro-apoptotic effects of the alisertib/ara-C combination, indicating that it is an important regulator of sensitivity to these agents. *In vivo* studies demonstrated that alisertib significantly augmented the efficacy of ara-C without affecting its pharmacokinetic profile and led to the induction of p27

and BIM. Our collective data indicate that targeting Aurora A with alisertib represents a novel approach to increase the efficacy of ara-C that warrants further investigation.

Many mechanisms of resistance to ara-C have been described including abnormal expression of transmembrane drug transporters and altered intracellular metabolism. Recently, aberrant kinase expression has emerged as a significant resistant mechanism to chemotherapy. In chapter 4 we investigate the role of the proto-oncogene serine/threonine-protein (PIM) kinases in resistance to ara-C in AML. We report that the novel small molecule PIM kinase inhibitor SGI-1776 disrupted cell viability and induced apoptosis in AML. We establish a link between ara-C resistance and PIM over-expression. Targeting PIM with SGI-1776 sensitized resistant cells to ara-C and significantly increased the efficacy of ara-C therapy in an AML mouse xenograft model. Collectively, our data demonstrate that antagonizing PIM activity represents a new strategy to increase the therapeutic efficacy of ara-C and possibly circumvent drug resistance. Further investigations aimed to define the role(s) of PIM kinases in AML pathogenesis and evaluate the therapeutic potential of PIM kinase inhibition are warranted.

Finally, chapter 5 explores how the preclinical work outlined in this thesis may be translated into clinical studies that may lead to novel therapeutic approaches for patients with refractory myeloid leukaemia. The problems with the use of Aurora and PIM kinase inhibitors in humans and the potential approaches to circumvent these problems are also discussed.

Chapter 1. Cancer biology, myeloid leukaemias, Aurora kinases and PIM kinases

1.1 Cancer

Cancer is a group of diseases characterized by the uncontrolled growth of cells and the spread of these cells from their site of origin to other parts of the body. The incidence of cancer is increasing worldwide with over 10 million new cases diagnosed each year with the number expected to increase to 20 million by 2020 (1). Our understanding of the molecular aberrations that characterize cancer cells has improved greatly in recent years heralding a new era of targeted therapy for cancer.

1.1.1 Myeloid leukaemia

The leukaemias are a group of diseases with differing aetiologies, presentations and treatments. There are four major types of leukaemia: acute lymphoblastic leukaemia (ALL), AML, chronic lymphoblastic leukaemia (CLL), and CML. Leukaemia is the tenth most common cancer in the Western countries with approximately 35,000 new cases diagnosed each year in the United States (2).

1.1.1.1 AML

AML consists of a group of relatively well-defined haematopoietic neoplasms involving precursor cells committed to the myeloid line of cellular development. It is the most common acute leukaemia in adults and accounts for approximately 80 percent of cases in this group (3). In the United States and Europe, the incidence has been stable at 3 to 5 cases per 100,000 persons (3, 4). It is the second most common haematological malignancy. The incidence increases with age with approximately 1.3 and 12.2 cases per 100,000 persons for those under or over 65 years, respectively. The male to female ratio is approximately 5:3.

AML is characterized by a clonal proliferation of myeloid precursors with a reduced capacity to differentiate into more mature cellular elements. As a result, there is an accumulation of leukaemic blasts or immature forms in the bone marrow (BM), peripheral blood (PB), and occasionally in

other tissues, with a variable reduction in the production of normal red blood cells, platelets, and mature granulocytes. The increased production of malignant cells along with a reduction in these mature elements results in a variety of systemic consequences including anaemia, bleeding, and an increased risk of infection.

1.1.1.2 Chronic myeloid leukaemia

Chronic myeloid leukaemia, (CML, also known as chronic myelocytic or chronic granulocytic leukaemia) accounts for approximately 15 to 20 percent of leukaemias in adults and is characterized by the dysregulated production of mature granulocytes, predominantly neutrophils, but also eosinophils and basophils (4). CML is associated with the Philadelphia (Ph) chromosome t(9;22)(q34;q11) or a related translocation resulting in the formation of the *BCR-ABL* fusion gene. Its encoded protein, BCR-ABL, functions as a constitutively active tyrosine kinase that drives disease pathogenesis. Over 90% of patients are diagnosed in the chronic phase of CML (CML-CP). If these patients are not treated with agents capable of affecting the natural course of the disease, the majority of these will progress through more advanced stages known as the accelerated phase of CML (CML-AP) and blast crisis (CML-BC).

1.1.2 Molecular characteristics of cancer cells

Cancer is fundamentally a genetic disease that is characterized by extreme complexity and heterogeneity. Despite the tremendous degree of diversity with respect to individual tumour types, Hanahan and Weinberg have proposed that there are six hallmarks features that are shared by all malignancies regardless of their tissue of origin (Figure 1.1) (5, 6). They hypothesized that these 6 “hallmarks” are acquired in a progressive manner over time during the evolution of a cell from a normal to a malignant state. Subsequent research has suggested that there are also two enabling characteristics that facilitate the acquisition of the 6 hallmark

features. Additionally, alterations in energy metabolism and immune evasion may represent two new candidate hallmarks that are critical to oncogenic transformation and disease progression (Figure 1.2) (6). Each of these 10 features is discussed below.

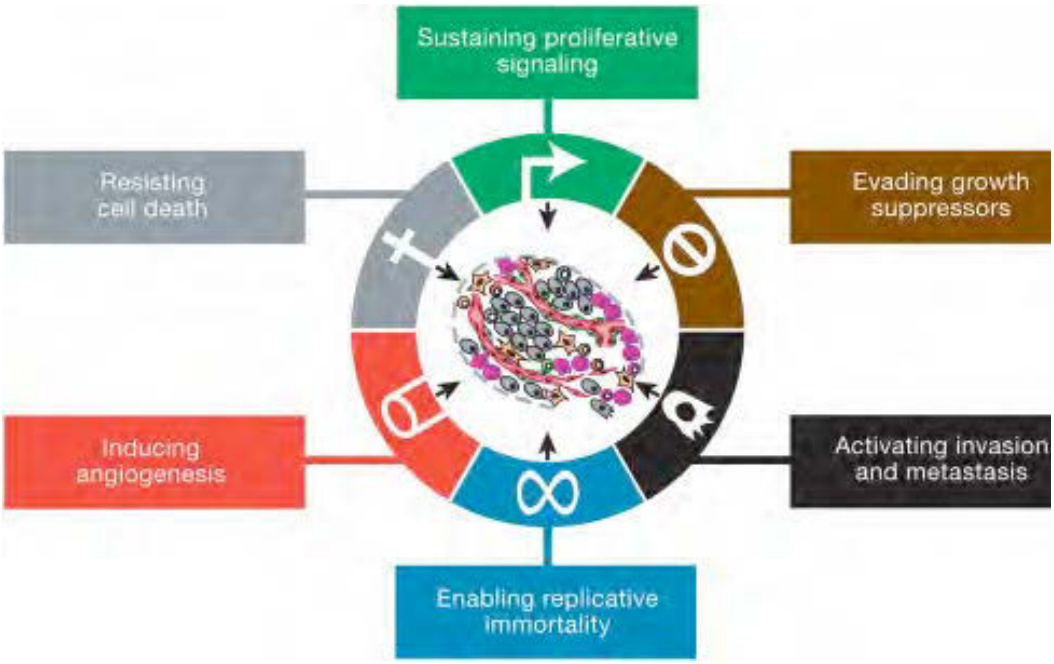

Figure 1.1 Acquired capabilities of cancer cells

Most if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies. Reproduced with permission from Hanahan et al, Cell. 2011 Mar 4;144(5):646-74 (6).

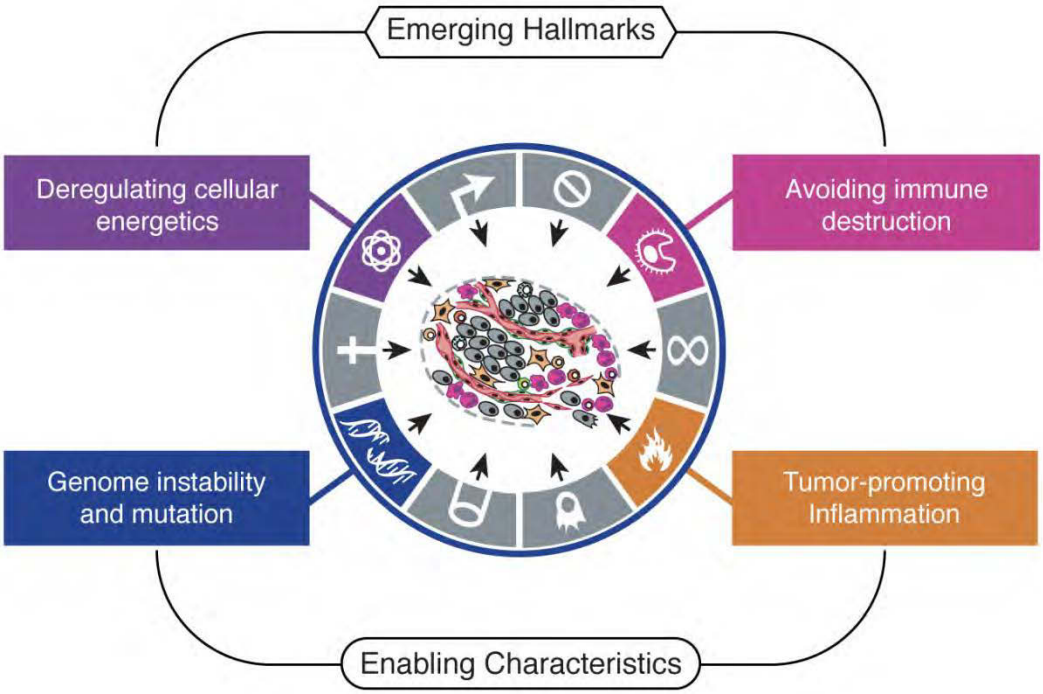

Figure 1.2 Emerging hallmarks and enabling characteristics of cancer cells

An increasing body of research suggests that two additional hallmarks of cancer are involved in the pathogenesis of some and perhaps all cancers. One involves the capability to modify, or reprogram, cellular metabolism to support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. Additionally, two consequential characteristics of neoplasia facilitate acquisition of both core and emerging hallmarks. Genomic instability creates genetic alterations that drive tumour progression. Inflammation by innate immune cells can inadvertently support multiple hallmark capabilities and promote elements of pathogenesis related to inflammatory responses. Reproduced with permission from Hanahan et al, Cell. 2011 Mar 4;144(5):646-74 (6).

1.1.2.1 Sustained proliferative signalling

The ability of malignant cells to continuously proliferate is one of the most critical traits acquired during the process of neoplastic transformation. Cell proliferation is normally tightly controlled and this is essential for the maintenance of cellular homeostasis and normal tissue function. Dysregulation of growth factor-mediated transduction pathways empowers cancer cells with the ability to control their own fates through increased proliferative capacity, enhanced survival potential, and altered energy metabolism. This can be achieved through several different mechanisms. Cells can establish autocrine loops for the synthesis and secretion of growth factors that stimulate their proliferation. In other cases, cancer cells may create paracrine signalling networks between themselves and their associated stroma to produce and secrete the growth factors they require (7, 8). A third possibility involves the over-expression or molecular/structural alteration of growth factor receptors, which renders them hypersensitive to their ligands or constitutively active in the absence of ligand-binding, respectively. Cancer cells can also lose the requirement of growth factor stimulation via the constitutive activation of downstream mediators of key signalling pathways.

In addition to direct and indirect hyperactivation of mitogenic cascades, the dysregulation of negative-feedback loops that normally serve as a mechanism to diminish proliferative signalling may also promote excessive proliferation in cancer (9-11). For example, the development of inactivating mutations in the phosphatase and tensin homolog (PTEN) tumour suppressor, which leads to elevated phosphoinositide 3-kinase (PI3K) activity, accelerates malignant pathogenesis, and may also promote drug resistance (12, 13).

1.1.2.2 Evading growth suppressors

The acquired ability to circumvent mechanisms of growth inhibition is nearly as important to a developing tumour as its need to sustain

proliferative signalling. One of the most common ways that this is achieved is through the loss of function of tumour suppressors. In particular, the loss of *RB* (retinoblastoma-associated) and/or *p53* antagonizes mechanisms of growth inhibition due to their critical and complementary roles in the regulation of the cell cycle and apoptosis (14, 15). Another notable difference between normal and transformed cells is their differential response to contact inhibition. Cell-cell contact normally signals cells to stop proliferating, but tumour cells are known to be insensitive to contact inhibition. This is thought to be due, in part, to the loss of moesin-ezrin-radixin-like protein (merlin), a prime regulator of contact inhibition (16) or the loss of function of the liver kinase B1 (LKB1) epithelial polarity protein (17).

1.1.2.3 Resistance to cell death

Apoptosis (discussed in section 1.5) functions as an intrinsic inhibitor of tumour development and progression. Therefore it is not surprising that tumour cells fundamentally evolve mechanisms to reduce their pro-apoptotic potential (18, 19). As discussed below cysteine proteases with aspartate specificity (caspases) are instrumental to the initiation and execution of apoptosis. The stimulation and execution of apoptosis is counterbalanced by anti-apoptotic members of the B cell leukaemia-2 (BCL-2) family of proteins (18). Tumour cells frequently upregulate their expression of these anti-apoptotic proteins as a means to prevent apoptosis that would normally be triggered by oncogenic or gentoxic stress. This phenomenon also plays an important role in conferring resistance to a wide variety of anti-cancer therapeutics. As mentioned earlier, the loss of *p53* tumour suppressor function is another important event that diminishes cellular apoptotic potential.

1.1.2.4 Infinite potential for replication

Most normal cells have a limited capacity for replication that is primarily

capped by the onset of senescence or crisis-stimulated cell death. In contrast, malignant cells exhibit an “immortal” phenotype that is characterized by a limitless ability to self-replicate. Telomere maintenance appears to be a critical factor underlying the ability of cells to infinitely replicate (20). In normal cells, telomeres become progressively shorter with each round of cell division and ultimately lose the capacity to protect DNA ends, which promotes genomic instability and cell death. Malignant cells are able to maintain the length of their telomeres through subsequent rounds of cell division by increasing their expression of telomerase, a DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA. Accordingly, telomerase activity confers resistance to senescence and crisis-driven apoptosis. Interestingly, an assessment of the major telomerase components, human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) in CD34+ cells from CML patients and normal controls revealed that CML samples exhibited a statistically significant increase in telomerase activity compared to normal samples. Further analysis suggested that telomere homeostasis is disrupted during the CML-CP (explained in section 1.6.3) and that this may impact disease progression (21). Recent studies have demonstrated that antagonizing telomerase activity significantly diminishes tumourigenesis in genetically engineered mouse models of cancer, suggesting that telomerase may be an attractive therapeutic target (22).

1.1.2.5 Angiogenesis

The generation of new vasculature, or angiogenesis, is imperative for developing tumours to gain consistent access to nutrients and oxygen and is a very early event in tumourigenesis. This process is accomplished by triggering normally quiescent vasculature to continuously bud new vessels that help sustain expanding neoplastic lesions (23, 24). Notably, the architecture of tumour vasculature is highly convoluted and can be readily distinguished from that of normal tissues (24).

Two of the best-characterized regulators of angiogenesis are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1). VEGF-A promotes angiogenic activity, whereas TSP-1, functions as an inhibitor of new vessel formation. VEGF expression is induced by hypoxia, oncogenic signalling and stromal interactions (25, 26). The discovery of TSP-1 and other endogenous inhibitors of angiogenesis along with their fundamental role in tumourigenesis provided the rational for the development of pharmacological inhibitors of angiogenesis for cancer therapy (26).

Although originally thought to be primarily important for the development and progression of solid tumours, increasing evidence has defined a role for angiogenesis in haematological malignancies. The BM microenvironment of patients with haematoligcal cancers is commonly more highly vascularised than that of healthy individuals. Additionally, a number of previous investigations have shown that BM-derived cells including macrophages, neutrophils, mast cells, and myeloid progenitors have critical functions in cancer-associated angiogenesis (27, 28). It has been shown that the levels of the VEGF homolog- placental-derived growth factor (PlGF) levels are elevated in CML and that the production of PlGF by BM stromal cells (BMSCs) increases the aggressiveness of this disease. Targeting PlGF significantly increases the survival of both imatinib-sensitive and -resistant CML mice and augments the anti-leukaemic activity of imatinib, suggesting that PIGF inhibition may represent a new therapeutic strategy for the treatment of imatinib-resistant CML (29).

1.1.2.6 Invasion and metastasis

The capacity for invasion and metastasis is a fundamental aspect of tumour progression. The loss of the cell-cell adhesion molecule e-cadherin through mutational inactivation or decreased expression is a well-characterized event that occurs in the invasion and metastasis of multiple

types of solid tumours (30). Intensive research has revealed that there are several distinct steps that occur during invasion and metastasis (31). This cascade is initiated by local invasion, followed by intravasation into nearby blood and lymphatic vessels, the journey of cancer cells through the haem-lymphatic systems, the extravasation of cancer cells into distant tissues, and the formation and expansion of metastases.

The epithelial-mesenchymal transition (EMT) program has been implicated as an essential regulator of invasion and metastasis (32). This program is controlled at the transcriptional level by several different transcriptional factors including Snail, Slug, Twist, and zinc finger E-box-binding homeobox 1/2 (Zeb 1/2). The tumour-specific mechanisms of regulation of these transcription factors remains unclear, but are likely influenced by interactions with stromal cells in the microenvironment (33).

1.1.2.7 An enabling characteristic: genomic instability

Genetic alterations underlie, at least in part, the ability of cancer cells to acquire all of the aforementioned 6 established hallmark features. Cancer cells often exhibit significantly higher rates of mutations than their normal counterparts. The breakdown of surveillance and repair pathways, loss of p53 function, and telomere maintenance are major causative factors in this phenomenon (34). It is likely that many of these genomic alterations confer growth and survival advantages to the tumour. Interestingly in preclinical models of CML it has been shown that BCR-ABL may directly induce karyotypic instability (35, 36). BCR-ABL-induced genetic instability may be via activation of STAT5 (37), increased homologous recombination mediated by RAD51 family members (38) or increased generation of reactive oxygen species (ROS) leading to DNA damage and double-strand DNA breaks (39).

1.1.2.8 An enabling characteristic: inflammation

Tumour-associated inflammation has been recognized as a potentially important aspect of the pathogenesis of cancers for more than 20 years (40). The overwhelming majority of human tumours are characterized by the infiltration of varying degrees of immune cells (41). Inflammation can promote the acquisition of multiple hallmark capabilities by directly supplying factors that stimulate growth, survival, angiogenesis, and invasion/metastasis to the tumour microenvironment (42, 43). Inflammatory cells can release mutagenic ROS and this may contribute to genetic instability (43).

1.1.2.9 An emerging hallmark: alterations in energy metabolism

The persistent cell proliferation that occurs as a hallmark of cancer requires changes in energy metabolism to create adequate sources of nutrition. This phenomenon was first reported by Otto Warburg who observed the preferential generation of adenosine-5'-triphosphate (ATP) through aerobic glycolysis in malignant cells in spite of its inferior bioenergetic efficiency compared to oxidative phosphorylation (44, 45). This appears to be partially accomplished through the upregulation of the glucose transporter 1 (GLUT1), which significantly increases cellular uptake of glucose (46). Oncogene activation, loss of tumour suppressors (particularly *p53*), and hypoxia also promote glycolytic metabolism (47). Rapidly proliferating malignant cells may be more heavily dependent upon glycolysis for ATP generation than those with lower proliferative indices. The potential role of the PIM kinases in facilitating tumour glycolysis is discussed in section 1.9.4.1. Activating mutations in the glycolytic enzymes isocitrate dehydrogenase (IDH) 1/2 have been reported in glioma and 20% of cases of AML (48, 49) in a manner that is associated with elevated oxidation and stability of the hypoxia-inducible factors (HIF-1) transcription factors (50). These findings have provided the rationale for the generation of novel IDH inhibitors for cancer therapy.

1.1.2.10 An emerging hallmark: evading immune destruction

The role of the immune system in oncogenesis and disease progression is complex and controversial. It is believed that immune surveillance is responsible for recognizing and eliminating the overwhelming majority of developing cancer cells and that progressing tumours have therefore acquired mechanisms of immune evasion. This theory is supported by the increased incidence of some virus-associated forms of cancer in immunocompromised people (51). Considering that more than 80% of all cases of cancer are not associated with viruses, there has been some doubt regarding how prominent of a role the immune system plays as a mechanism of tumour suppression. Recent evidence from mouse models suggests that the tumour suppressive functions of the immune system may be much more extensive than previously believed at least with respect to carcinogen-associated tumours (52). Correlative studies have shown that patients with colon and ovarian tumours that are heavily infiltrated with immune cells including natural killer cells and cytotoxic T lymphocytes have a better prognosis than those that lack such abundant killer lymphocytes (41). Preliminary evidence demonstrating the development of donor-derived tumours in some immunosuppressed organ transplant recipients suggests that the malignant cells may have been held in a dormant state in the donors by immune surveillance (53). At this time, the role of immune evasion in promoting malignancy remains to be fully elucidated. Additional research is required to determine how prevalent this phenomenon may be and whether it should be considered as a *bona fide* hallmark feature of cancer.

1.1.3 The genetic and molecular features of CML

1.1.3.1 The Philadelphia (Ph) chromosome

CML is associated with the Ph chromosome t(9;22)(q34;q11) (54, 55) which results in the formation of a unique gene product (BCR-ABL) a

constitutively active tyrosine kinase. This deregulated tyrosine kinase is implicated in the development of CML and has become a primary target for the treatment of this disorder. The fusion gene is created by juxtapositioning the *ABL* gene on chromosome 9 (region q34) to a part of the *BCR* ("breakpoint cluster region") gene on chromosome 22 (region q11). The *ABL* gene encodes a non-receptor protein-tyrosine kinase, c-ABL. The *BCR* gene on chromosome 22 spans a region of 135 kb and comprises 23 exons (56). Although ubiquitously expressed, *BCR* belongs to a family of genes whose functions remain unclear.

1.1.3.2 Leukaemogenesis

While the protein products of these the *BCR* and *ABL* genes have no intrinsic oncogenic properties by themselves, together they produce the BCR-ABL fusion protein that is essential for the development of CML. BCR-ABL promotes the development of CML by allowing uncontrolled proliferation of transformed cells, discordant maturation, escape from apoptosis and altered interaction with the cellular matrix. BCR-ABL is a constitutively active tyrosine kinase and induces tyrosine phosphorylation of a large number of cellular proteins in haematopoietic cells. As a result, a diverse group of intracellular signalling pathways is activated by BCR-ABL (57). A central role for the BCR-ABL tyrosine kinase in the pathogenesis of CML has been established by the therapeutic efficacy of small molecule inhibitors of the ABL tyrosine kinase (discussed in section 1.6.6). Signalling by BCR-ABL also manifests through kinase independent pathways. Support for this comes from studies that have shown leukaemia stem cells (LSCs) to be inherently resistant to TKIs (discussed in section 1.6.6.5) (58-60). In addition, mutations of BCR-ABL have been identified that impair leukaemogenesis despite preserving tyrosine kinase activity (61). DNA microarray analyses of CML cells and other studies have implicated many kinase independent pathways in the pathogenesis of CML including the Wnt/Beta-catenin (62), hedgehog (Hh) pathway (63) and FOXO pathway

(64).

1.1.3.3 Progression to CML-AP and CML-BC

The progression of CML-CP to CML-AP or CML-BC is a complex multistep process. While the Ph chromosome translocation may be the initiating event in CML, progression to CML-BC appears to require the acquisition of other chromosomal changes and/or dysregulation of differentiation-regulatory genes (65, 66). These include differentiation arrest, genetic instability, additional chromosomal abnormalities, and inactivation of tumour suppressor genes. Activation of the Wnt pathway and hedgehog pathways may be a central feature of disease progression enabling CML progenitors to acquire self-renewal properties (67, 68). CML-BC is characterized by a shift in the level of tumour differentiation to more immature forms. This failure of differentiation may be mediated through suppression of transcription factors necessary for myeloid differentiation such as CCAAT-enhancer-binding proteins alpha (CEBPA) (69, 70). BCR-ABL induces karyotypic instability facilitating the multiple genetic abnormalities during the progression from CML-CP to CML-BC. Some cases of CML-BC demonstrate loss of tumour suppressor genes. For example 20 to 30 percent of patients with CML-BC have deletions or rearrangements in the *p53* tumour suppressor gene (71).

1.1.4 The genetic and molecular features of AML

The collaboration of multiple molecular alterations in haematopoietic precursor cells culminates in the aberrant accumulation of immature myeloid cells in the BM and PB with an increased proliferative capacity and an inability to differentiate that is characteristic of AML. The specific genetic alterations underlying AML are complex and heterogeneous and involve the constitutive activation of established oncogenes and the loss of function of key tumour suppressors. Efforts to identify specific chromosomal anomalies and translocations that occur in AML have

significantly increased our understanding of the pathogenesis of this disease. Karyotype analyses have led to the identification of certain recurring cytogenetic abnormalities that are of prognostic significance and can be used to help guide treatment decisions for individual patients (see Appendix B) (72). A synopsis of what is currently known regarding the genetic features of AML is discussed below.

1.1.4.1 Alterations in genes that regulate DNA methylation

The methylation of cytosine or adenine residues within DNA silences gene transcription. DNA methylation is an important process in the transcriptional control of cellular differentiation and its dysregulation in AML is thought to be a significant factor underlying the inability of AML cells to properly differentiate into mature cells. The disruption of normal DNA methylation activity in AML has been attributed to mutations in DNA methyltransferases (DNMTs), which as their name implies are the primary enzymes responsible for the methylation of DNA. Activating mutations in DNMTs result in the inappropriate silencing of genes that would normally be expressed, whereas inhibitory mutations translate into the loss/reduction of methylation and the aberrant expression of genes that should be silenced (73-76).

1.1.4.2 Loss of tumour suppressor function

In contrast to the majority of other forms of cancer, a very small minority (approximately 7%) of AML patients exhibit inactivating mutations in the *p53* tumour suppressor gene at diagnosis (77, 78). However there are several other tumour suppressor genes whose functions are impaired in subsets of patients with AML. The Wilms' tumour suppressor gene 1 (*WT1*) encodes a transcriptional regulator for genes involved in cellular growth and maturation. Disruption of this gene may promote the proliferation of stem cells and disrupt cellular differentiation. Approximately 8 percent of

AML cases and 13 percent of patients with cytogenetically normal AML will harbour mutations in *WT1* (79, 80).

1.1.4.3 Mutational activation of RAS

Mutations of the rat sarcoma (*RAS*, *H-RAS*, *K-RAS* and *N-RAS*) proto-oncogenes that result in constitutive activation are a frequent event in many human malignancies. *RAS* mutations occur in approximately 25% of patients with AML and 35% of patients with MDS and seem to be disproportionately present in patients with monocytic morphology (81, 82). The prognostic significance of *RAS* mutations in AML is not completely understood, but may be associated with increased survival and a more favourable response to ara-C treatment (82).

1.1.4.4 Activation of FLT3

The fms-like tyrosine kinase-3 (FLT3) is a transmembrane tyrosine kinase receptor that stimulates cell proliferation when activated. Two main types of FLT3 mutations exist. The most common are internal tandem duplications (ITD) of different length that result in ligand-independent activation of the FLT3 receptor and a proliferative signal. Additionally point mutations in the activating loop of the kinase domain of FLT3 may result in tyrosine kinase activation of FLT3 (83, 84). Mutations/ITD of FLT3 are more prevalent in elderly AML patients and are associated with a poor prognosis and significantly shorter median survival (discussed in section 1.7.6.3.1), most likely due to the sustained pro-survival signalling by downstream effectors. The importance of FLT3-ITD as a prognostic indicator prompted the development of several FLT3-targeted inhibitors (discussed in section 1.7.16).

1.1.4.5 Chromosomal alterations and translocations

Chromosomal aberrations are a very frequent event in AML (see Appendix B). For example, the long arms of chromosomes 5, 7, and 20 are commonly lost in patients with therapy-related AML and AML that has

progressed from MDS, and are associated with a poor prognosis (85). Chromosomal translocations occur even more prevalently and have two primary consequences. In some cases, the juxtaposition of an intact transcriptional element from one chromosome to an enhancer element from a gene on another chromosome results in the overexpression of specific gene products. In other cases, chromosomal translocations can lead to the formation of chimeric fusion proteins such as BCR-ABL in CML. The majority of known chromosomal translocations that occur in AML are of the second variety that lead to the formation of fusion genes that are not expressed in normal cells. The investigation of fusion genes that have been identified in AML has significantly increased our understanding of the transcriptional regulation of haematopoiesis (86). Key genes that have been established to be involved in translocations in AML include the core binding factors (CBFs), mixed-lineage leukaemia (*MLL*), retinoic acid receptor (*RAR*), and homeobox (*HOX*) genes. In many cases these genes have been implicated in translocations with multiple partners, thus adding an additional layer of complexity to the genetics of AML. CBF transcription factors function as heterodimeric complexes that control an array of genes involved in differentiation (87).

The t(8;21)(q22;q22) was the first recurring chromosomal translocation identified in AML and is associated with the FAB-M2 subtype (discussed in section 1.7.5.1) (88). In AML with the t(8;21), the Runt-related transcription factor 1 (*RUNX1* (previously *AML1* or core binding factor alpha-2)) gene on chromosome 21 and the *RUNX1T1* (previously *ETO*) gene on chromosome 8 form a chimeric product that regulates the transcription of a number of genes that are vital for haematopoietic stem cell and progenitor cell growth, differentiation, and function (89-91).

The inv(16)(p13q22) and t(16;16)(p13;q22) occur primarily in patients with FAB-M4Eo AML and are both correlated with a favourable prognosis (92). These chromosomal alterations disrupt the CBF beta subunit of CBF and result in the formation of a fusion protein with smooth

muscle myosin heavy chain, MYH11, located at 16p13 (93).

The *MLL* histomethyltransferase gene is located on chromosome 11q23 and is frequently involved in translocations in both AML and ALL that are linked with a poor prognosis. MLL translocations produce fusion proteins that trigger the expression of several downstream effectors, (94-98).

Translocations of the retinoic acid receptor-alpha (*RARa*) locus on chromosome 17 are a hallmark feature of acute promyelocytic leukaemia (APL, FAB-M3) (99). The t(15;17)(q22;q11-12) is the most prevalently observed form of RAR translocation in APL patients. These translocations confer APL cells sensitive to treatment with retinoic acid, which is still utilized as standard therapy for patients with APL (discussed in section 1.7.9.2) (100).

The molecular complexity of AML creates many treatment challenges, but may provide a strong rationale for the development of personalized regimens comprised of targeted and conventional agents.

1.2 Normal haematopoeisis and LSCs

1.2.1 Introduction

Haematopoiesis refers to the tightly regulated process whereby the circulating blood cells are formed in BM. The BM has a huge production capability with an estimated 10^{10} erythrocytes and 10^{8} leukocytes produced per hour in the steady state. While production is maintained at a steady state in normal circumstances it can be greatly increased if needed. These circulating cells are immediate descendants of maturing precursors that arise from a smaller pool of progenitors. The progenitors in turn arise from an even smaller pool of haematopoietic stem cells (HSCs) that are believed to be mostly in a resting or non-dividing state and have the capacity to self-renew.

1.2.2 Haematopoetic stem cells

HSCs are multipotent and have the capacity to differentiate into the cells of all ten blood lineages — erythrocytes, platelets, neutrophils, eosinophils, basophils, monocytes, T and B lymphocytes, natural killer cells and dendritic cells (101-103). HSCs can be divided into a long-term subset (LT-HSC), capable of indefinite self-renewal, and a short-term subset (ST-HSC) that self-renew for a defined interval (Figure 1.3). The self-renewal capacity of HSC is associated with telomerase activity (discussed in section 1.1.2.4). Interestingly, recent studies have demonstrated that normal HSCs and cancer cells share the ability to self-renew and that many pathways classically associated with cancer also regulate normal stem cell development. For example the signalling pathways that have been shown to date to be involved in the regulation of HSC self-renewal, i.e., HOX genes and Notch, Sonic hedgehog (Shh), and Wnt signalling pathways, are also hypothesized to be associated with oncogenesis (104-108). For most cancers, the target cell of the transformation events is unknown, but evidence indicates that certain types of leukaemias arise from mutations that accumulate in HSCs.

The concept that sustained haematopoiesis originates from pluripotent stem cells arose from the findings that mice can be protected from the lethal effects of whole body irradiation by exteriorization and shielding of the spleen. This protective effect of shielding the spleen from radiation was then shown to be cell-mediated as the injection of spleen cells could initiate recovery and re-establish haematopoiesis in irradiated animals (109). Colonies of murine haematopoietic cells, referred to as colony forming units (CFU), were observed in the spleen of irradiated, transplanted recipients within 10 days after the transplant of spleen cells (110). Subsequent experiments using karyotypically marked donor cells confirmed the clonal origin of the differentiated cells in the colony, proving that a single pluripotent stem cell had given rise to these differentiated cells (111).

1.2.3 Identification of HSCs

Cell surface characteristics have been used to purify or define populations of cells that include stem cells and progenitors. HSCs are notably CD34+ and CD38- (112). The CD34 antigen is expressed on HSC and progenitors, and CD38 is expressed on a subset of more mature progenitors but not on stem cells. Other surface proteins expressed on HSCs include CD133, c-Kit and CD150 (113-115).

1.2.4 BM microenvironment and haematopoietic-stem-cell niches

Haematopoietic progenitor cells require both cellular and soluble growth factor support for development and differentiation. These cellular elements are provided by the surrounding bone, BM stroma, and the microenvironment (116-118). Fibroblastoid cells, endothelial cells, and macrophages comprise the stromal cells that support the stem and progenitor cells. In addition to providing an adhesive framework for developing cells the fibroblastoid cells produce haematopoietic growth factors essential for the proliferation, survival and differentiation of HSCs (119). A stem-cell niche can be defined as a spatial structure in which stem cells are housed and maintained by allowing self-renewal in the absence of differentiation (114, 120, 121).

1.2.5 Differentiation

In the first step towards the myeloid lineage of haematopoietic cell differentiation, HSC produce the common myeloid progenitor (CMP) which is capable of producing cells of all myeloid lineages and is also referred to as the CFU-GEMM (CFU-granulocyte–erythrocyte–macrophage–megakaryocyte) (122). Markers that can identify the CFU-GEMM include CD34 and HLA-DR. CD64 identifies a specific stem cell dedicated to granulocyte and monocyte development. This CD34, HLA-DR, and CD-64 positive stem cell is called CFU-GM (CFU-granulocyte–macrophage) (123, 124). Certain growth factors are required for maturing CFU-GEMM into

CFU-GM including stem cell factor (SCF, also called c-Kit ligand or Steel factor), interleukin (IL)-3, IL-6, FLT3 ligand and granulocyte-macrophage colony-stimulating factor (GM-CSF). CFU-GM gives rise to the more mature granulocyte and macrophage colony-forming units, CFU-G and CFU-M, respectively (125, 126).

Erythropoiesis begins with the differentiation of a small pool of pluripotent stem cells into the most primitive erythroid progenitors, which then develop into recognizable erythroid precursors and subsequently differentiate into mature erythrocytes. Erythropoietin is a growth factor essential for the amplification and terminal differentiation of erythroid progenitors and precursors. Progenitor cells committed to the megakaryocyte lineage differentiate into promegakaryoblasts, which in turn form mature megakaryocytes. Platelets are shed from megakaryocytes as ‘blebs’ formed of their cytoplasm. Thrombopoietin is a chief stimulatory player in thrombopoiesis.

B cells are generated in the BM from common lymphoid progenitor (CLP) cells (127). Immature B cells then move to the spleen where they differentiate into mature, but naive B cells in a process referred to as secondary B-cell development. Mature naive B cells then migrate to lymph nodes where they undergo further differentiation to become highly specialized plasma cells producing specific antibody in response to antigenic stimuli. The progeny of the CLPs cells destined to form T cells migrate to the thymus, which is critical for their further development and final commitment to the T-cell lineage.

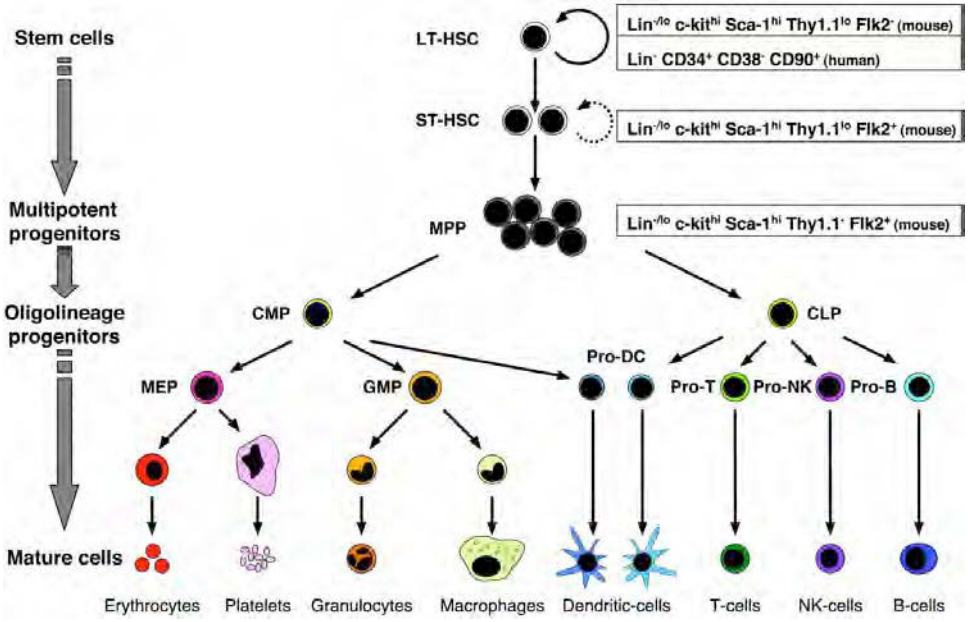

Figure 1.3 Haematopoietic and progenitor cell lineages

HSCs can be divided into LT-HSCs, highly self-renewing cells that reconstitute an animal for its entire life span, or ST-HSCs, which reconstitute the animal for a limited period. ST-HSCs differentiate into multipotent progenitor (MPPs), which do not or briefly self-renew, and have the ability to differentiate into oligolineage-restricted progenitors that ultimately give rise to differentiated progeny through functionally irreversible maturation steps. The CLPs give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells. The CMPs give rise to myelomonocytic progenitors (GMPs), which then differentiate into monocytes/macrophages and granulocytes, and to megakaryotic/erythroid progenitors (MEP), which produce megakaryocytes/platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells. All of these stem and progenitor populations are separable as pure populations by using cell surface markers. Reprinted with permission from Passegué et al Proc Natl Acad Sci U S A. 2003 Sep 30 (128).

1.2.6 LSCs

The notion of tumourigenic LSCs emerged from several studies including those published by Blair and colleagues (129) and Bonnet and Dick (130) that showed that most leukaemic cells were unable to proliferate extensively and only a small, defined subset of cells was consistently clonogenic. In these studies, LSCs for human AML were identified prospectively and purified as (Thy1- (Thymocyte differentiation antigen 1), CD34+, CD38-) cells from various patient samples. Although these cells represent a small and variable proportion of the totality of the AML cells (0.2-1% depending on the patient), they were the only cells capable of transferring AML from human patients to non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.

A given leukaemia can be viewed as a newly formed aberrant haematopoietic tissue initiated by tumourigenic leukaemic cells that have kept or reacquired the capacity for indefinite proliferation through accumulated mutations (Figure 1.4). This concept suggests that leukaemias are produced by a few LSCs that undergo an aberrant and poorly regulated process of organogenesis analogous to that of normal HSCs. Both cell types have extensive proliferative potential and the ability to give rise to new haematopoietic tissues, normal in the first case and abnormal in the second. For most leukaemias, as for most cancers, the target cell of transforming mutations is still unknown. Because normal stem cells and LSCs share the ability to self-renew, as well as various developmental pathways, it has been postulated that LSCs are HSCs that have become leukaemic as the result of accumulated mutations. An alternative theory is that LSCs could also be a more restricted progenitor or even a differentiated mature cell, which would have first to reacquire the stem cell capability for self-renewal before becoming tumourigenic to accumulate additional mutations (128).

Insight into the cell of origin of AML comes from observations that the expression of the AML1-ETO fusion transcript (discussed in section 1.1.4.5) can be detected in leukaemic blast cells and in normal HSCs obtained from AML patients in remission (131, 132). However, these prospectively isolated AML1-ETO-expressing stem cells and their progeny are not leukaemic and could differentiate into normal myelo-erythroid cells *in vitro* (131). This suggests that the translocation occurred originally in normal HSCs and that additional mutation in a subset of these HSCs or their progeny subsequently led to leukaemia.

The clonal nature of CML and other myeloproliferative neoplasms was confirmed in complementary clonality studies in women with CML utilizing isozymes of the X-chromosome linked enzyme glucose 6-phosphate dehydrogenase (G6PD) (133) or methylation-sensitive restriction fragment length polymorphisms in X-linked genes (134). The derivation of this clonal population from a pluripotent HSC was first supported by cytogenetic studies that demonstrated the Ph chromosome in granulocyte, monocyte, and erythroid precursors, megakaryocytes, and most B-lymphocytes from patients with CML (135). Subsequent findings supported the role of a pluripotent HSC progenitor as the cell of origin: BCR-ABL mRNA can be found in CD34+ (progenitor) subpopulations from patients with CML (136). Other studies showed that a population of quiescent LSCs could be isolated from the blood and BM of patients with untreated CML-CP using flow cytometry techniques (137). Taken together, these studies strongly support the hypothesis that CML is a clonal malignancy involving pluripotent HSCs.

Similar to AML, CML appears to be maintained by a pool of self-renewing malignant cells, as assessed by their ability to transfer the disease upon xenotransplantation into immunodeficient mice (130). In CML-CP, the Ph chromosome translocation occurs in a HSC with the capacity for multilineage differentiation and self-renewal. The size of this HSC compartment, as defined by cell surface antigen expression, is

normal, while committed myeloid progenitors and granulocyte-macrophage progenitors (CFU-GM) are significantly increased in number (67). Studies in mouse model systems demonstrate that BCR-ABL does not confer self-renewal capacity to committed myeloid progenitors, and sustained CML-like leukaemia cannot be produced by transplantation of BCR-ABL-expressing committed progenitors into recipient mice (138). This helps to explain why the Ph chromosome translocation is present in a pluripotent HSC even though CML predominantly affects the neutrophil lineage. It appears that stem cell origin is required for maintenance of the disease in the BM. Even though the BCR-ABL kinase is active in CML LSCs, CML LSCs exposed to TKIs are not dependent upon BCR-ABL kinase activity for survival but can revert to dependence on cytokines that normally mediate survival and proliferation (59, 139-142).

A number of studies have provided experimental evidence that LSCs are significantly less sensitive to conventional therapy than more differentiated malignant cells and based on this, it has been proposed that LSCs may be the ultimate cause of relapse and treatment failure (143, 144) (see section 1.6.6.5). Identifying new agents and therapeutic targets to specifically eradicate LSCs is an important challenge of increasing priority. Although myeloid leukaemias are heterogeneous in terms of phenotype, disease progression, prognosis, and response to therapy, our understanding of the general mechanisms underlying leukaemic transformation is improving. Future investigation of such deregulated mechanisms in the newly identified LSCs will lead to a considerable increase in our understanding of the molecular mechanisms and signalling pathways that are affected in a given type of leukaemia.

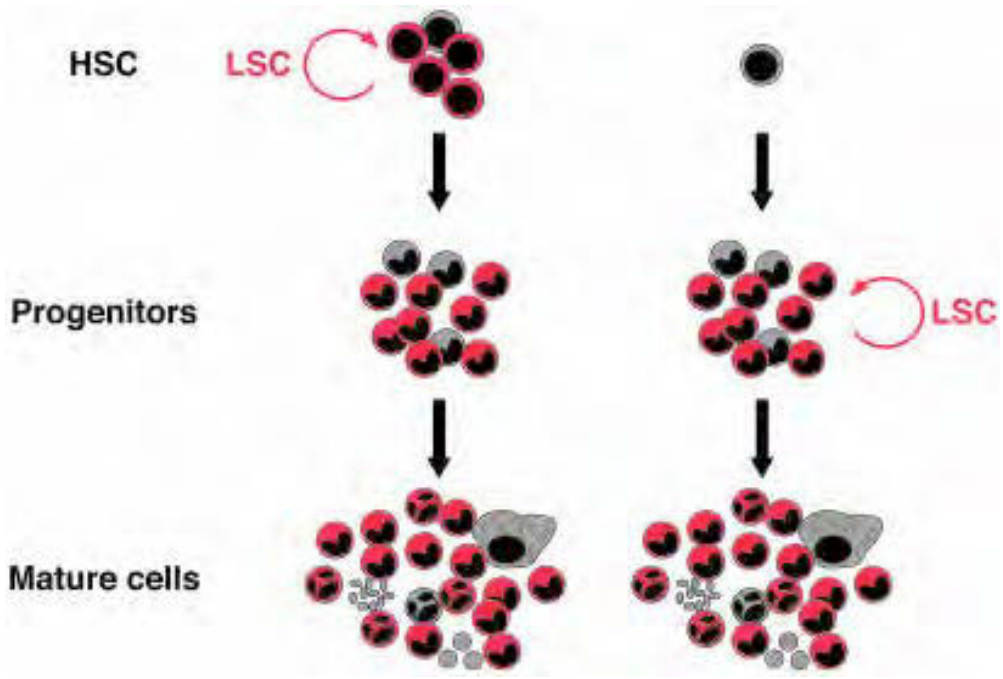

Figure 1.4 Origin of the LSC

A given leukaemia can be viewed as a newly formed abnormal haematopoietic tissue initiated by a few LSCs that undergo an aberrant and poorly regulated process of organogenesis analogous to that of normal HSCs. LSCs can either be HSCs, which have become leukaemic as the result of accumulated mutations (left), or more restricted progenitors (right), which have reacquired the stem cell capability of self-renewal. Regardless of their origin, both types of LSCs give rise to similar end-stage leukaemias. Reprinted with permission from Passegué et al Proc Natl Acad Sci U S A. 2003 Sep 30 (128).

1.3 The cell cycle in normal and cancer cells

1.3.1 Introduction

While cancer is characterized by abnormal cell proliferation, the cell cycle in normal cells is tightly regulated in order to maintain homeostasis and genomic integrity. The stages through which a cell passes between one cell division and the next are known as the cell cycle. It consists of an interphase and a mitotic (M) phase. Interphase is further subdivided into the G_1, S and G_2 phases. DNA replication occurs during the S phase, whereas the G_1 phase represents the gap between the end of mitosis and the beginning of the S phase and G_2 the interval between the S phase and the subsequent mitotic phase.

Normal mitosis is a complex process that is strictly regulated to preserve normal DNA content and organization. Mitosis is subdivided into prophase, prometaphase, metaphase, anaphase and telophase. At the onset of prophase, chromosomes condense to form sister chromatids that remain bound at the centromere by a protein complex known as the kinetochore. Concurrently, the centrosomes separate and migrate to opposite sides of the cell forming the mitotic spindle poles that act as the primary site of microtubule nucleation. The nuclear membrane degrades during prometaphase, and microtubules originating from each centrosome attach to the kinetochores of each chromosome. Subsequently, during metaphase the chromosomes convene along the metaphase plate, a line equidistant from the two mitotic spindle poles (145). The spindle assembly checkpoint (discussed in 1.3.3.5), ensures that sister chromatids are properly aligned to the metaphase plate before the onset of anaphase (146). During anaphase, the proteins at the centromeres that attach sister chromatids are degraded and the shortening of kinetochore microtubules leads to the poleward movement of the separated chromsomes (Figure 1.5). A reversal of the prophase and prometaphase events occurs during telophase when a new nuclear membrane forms around each set of

separated chromosomes and the cell completes division into two daughter cells.

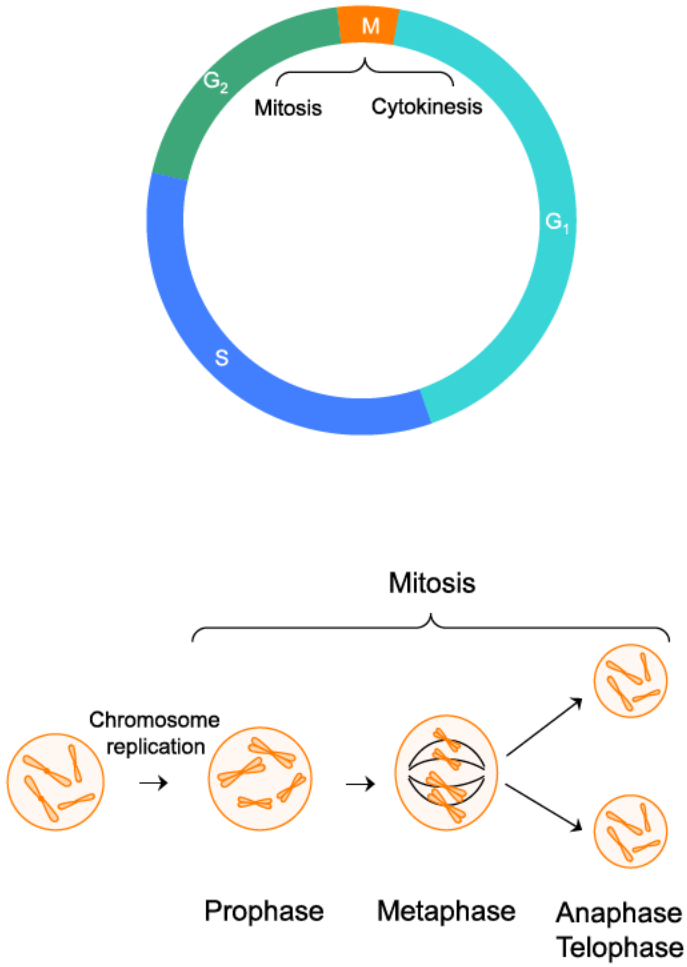

Figure 1.5 The normal cell cycle

Top. The cell cycle consists of an interphase and mitotic (M) phase. The interphase is subdivided into G_1 phase, S phase and G_2 phase. DNA replication occurs during the S phase whereas the G_1 phase represents the gap between the end of mitosis and the beginning of the S phase and G_2 the interval between the S phase and subsequent mitotic phase. Aurora A has a role in G_2-M transition. **Bottom.** Mitosis is further divided into prophase, metaphase, anaphase and telophase.

1.3.2 Cyclins and CDKs

Most cells in an adult organism are quiescent and enter an inactive period called G_0, outside of the cell cycle. In order for cells to re-enter the cell cycle, they must pass through a control point called the G_1 restriction point (RP) (Figure 1.6) (147). Prior to passage through the RP, cells are dependent on mitogens. After passage, cells are committed to progress through the cell cycle without the need for external growth factors. The progress through the multiple steps in the cell cycle is regulated by a set of proteins called cyclins and their associated CDKs. Cyclin levels are dynamic and are determined by the balance of gene transcription versus protein degradation. When cyclins bind to their highly specific CDK partner it induces a conformational change in the catalytic subunit of CDK, exposing its active site. Unlike cyclins, the levels of CDKs are regulated predominantly at the posttranslational level and do not vary during the cell cycle.

Various cyclin-CDK complexes are present a specific points in the cell cycle and regulate irreversible transitions to subsequent phases of the cell cycle. Cyclin D along with CDK 4/6 drives progression of the cell cycle through G_1. Additionally, cyclin D regulates the transcription of the cyclin E gene. Cyclin E plays a role in G_1 to S transition. Cyclin A-CDK2 is important for S phase progression. Cyclins A, B-CDK1 directs G_2 progression and the G_2 to M phase transition.

Cyclin-CDK complexes regulate a diverse set of proteins including transcriptional regulators, cytoskeletal proteins, nuclear pore, envelope proteins and histones proteins through phosphorylation of their substrates on serines and threonines. This, in turn, leads to multiple cell cycle events including chromosomal condensation, nuclear envelope breakdown, fragmentation of the Golgi apparatus, regulated gene expression and mitotic spindle assembly. Given that CDKs control progression through the cell cycle, the regulation of their activity is crucial and mediated by several

mechanisms: association with cyclins, association with CDK inhibitors (CKIs) and addition of phosphate groups that either stimulate or inhibit CDK activity.

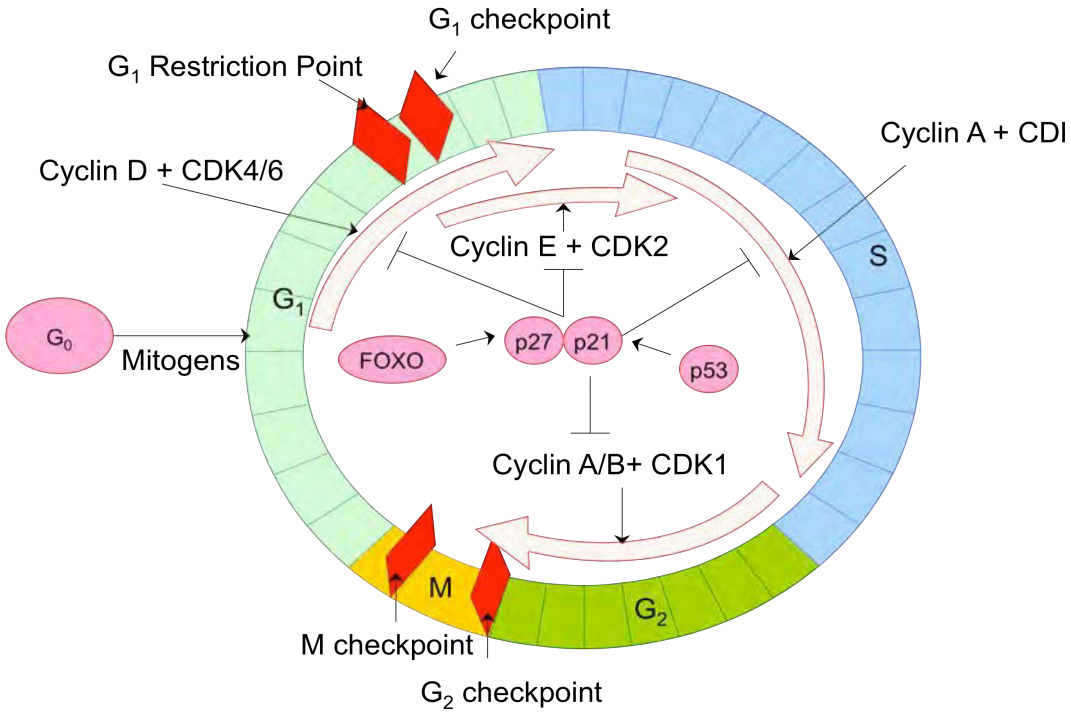

Figure 1.6 Pattern of cyclin-CDK activity during the cell cycle

Cyclin-CDK complexes (red bars) are the key regulators of the cell cycle phases and are involved in the checkpoint mechanisms at the transition of one cell cycle phase to the next. The inhibitory effect of the CKIs, p21, and p27, on the cyclin-CDK complexes is also shown.

1.3.3 Cell cycle checkpoints

1.3.3.1 Introduction

Cell cycle checkpoints ensure the fidelity of cell division in eukaryotic cells. These checkpoints verify that each phase of the cell cycle has been accurately completed before progression into the next phase. An important function of many checkpoints is to detect DNA damage. When damage is found, the checkpoint uses a signal mechanism to either stall the cell cycle until repairs are made or, if repairs cannot be made, to target the cell for destruction via apoptosis. All the checkpoints that assess DNA damage appear to utilize the same sensor-signal-effector mechanism. If the function of various checkpoints is disrupted, mutations and carcinogenesis can result.

1.3.3.2 G_1 checkpoint

The G_1 checkpoint leads to the arrest of the cell cycle in response to DNA damage ensuring that damaged DNA is not replicated during the S phase (148). The RB protein (Figure 1.7) is a major control point in the transition from the G_1 phase to S phase via its interactions with the E2 transcription factor (E2F) and histone deacetylases (HDACs) (149). In the absence of growth stimuli, RB is hypophosphorylated, which facilitates it's binding to E2F and HDACs. When E2F is bound to RB, E2F becomes sequestered, blocking its transactivation domain and preventing E2F transcriptional activity. Further repression of transcription occurs via RB recruitment of HDACs that lead to chromatin compaction. When it is time for a cell to enter S phase, the cyclin D/CDK4/CDK6 complex phosphorylates RB, inhibiting its activity (150). This initial phosphorylation causes a conformational change in RB that allows the transcription of cyclin E. Additional phosphorylations by the cyclin E-CDK2 complex lead to the release of E2F and subsequent expression of E2F target genes such as cyclin A, thymidylate synthase and dihydrofolate reductase, all of which are

important for S phase progression (151). RB remains phosphorylated throughout S, $G_{2,}$ and M phases.

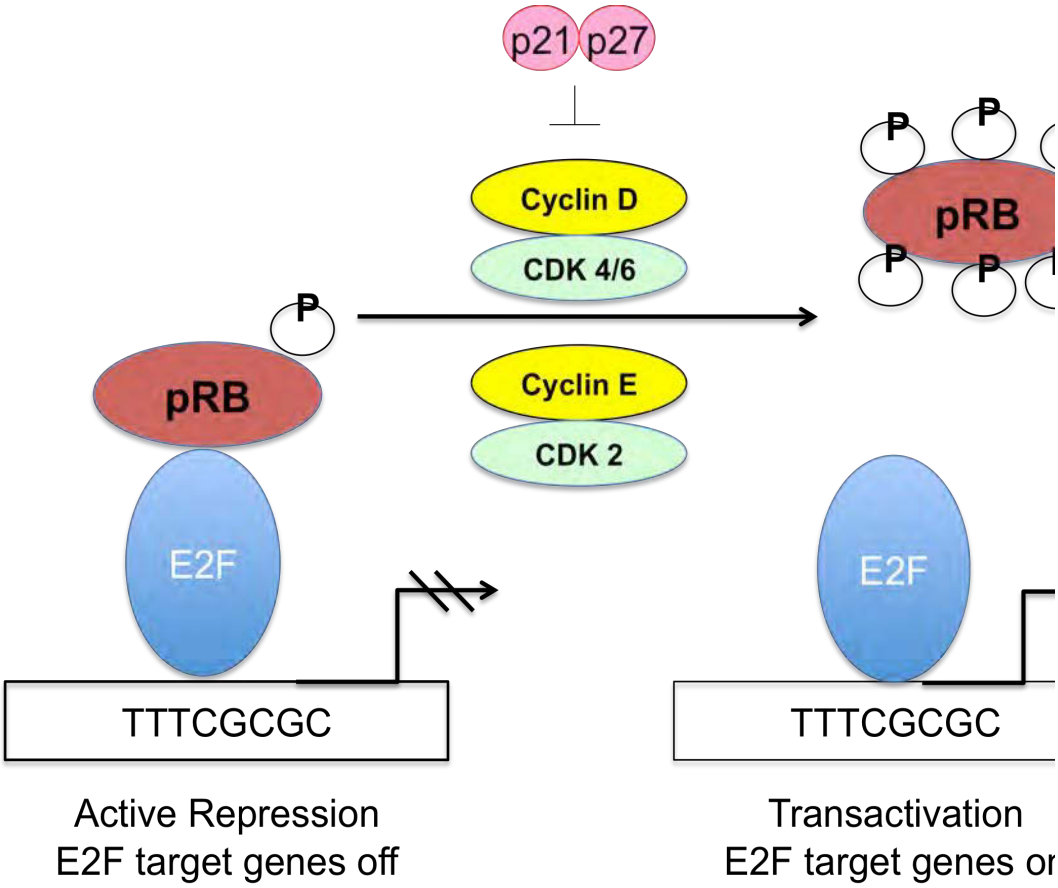

Figure 1.7 The retinoblastoma protein regulates the transcription factor E2F

E2F can bind to specific DNA sequences in the promoters of E2F responsive genes. In the hypophosphorylated form (which occurs early in G_1), RB binds to E2F and inactivates E2F as a transcription factor. The E2F/pRB complex can bind DNA, but instead of activating transcription, it acts as a dominant repressor complex that shuts the expression of E2F target genes off. Phosphorylation of pRB by cyclin/CDK complexes in mid to late G_1 causes pRB to lose its affinity for E2F. The free E2F transcription factor can now activate transcription of E2F target genes leading to expression of proteins necessary for S phase progression.

1.3.3.3 G_2 checkpoint

The G_2 checkpoint blocks entry into M phase when DNA damage has occurred or when the S phase has not been correctly completed (152). G_2/M progression is driven by the maturation-promoting factor (MPF), a complex of cyclin B1/CDK1 that forms during G_2 and is kept inactive by phosphorylation of CDK1 at Thr^{14} and Tyr^{15}. The entry into mitosis is triggered by translocation of the complex to the nucleus and activation by dephosphorylation by the cell division cycle 25 (CDC25) phosphatases (153). The role of Aurora A in the regulation of the progression from G_2 to M is discussed in section 1.8.2.

DNA damage triggers the G_2 checkpoint through activation one of two kinases, ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3 related (ATR) (154, 155) (Figure 1.8). These kinases then phosphorylate either checkpoint kinase 1 (CHK1) or CHK2 kinases, which results in inhibition of CDC25, which in turn prevents activation of CDK1, through phosphorylation at Ser^{216} (156-158). The important roles that CHK1 and CHK2 play in preventing mitotic catastrophe are discussed in section 1.5.2.

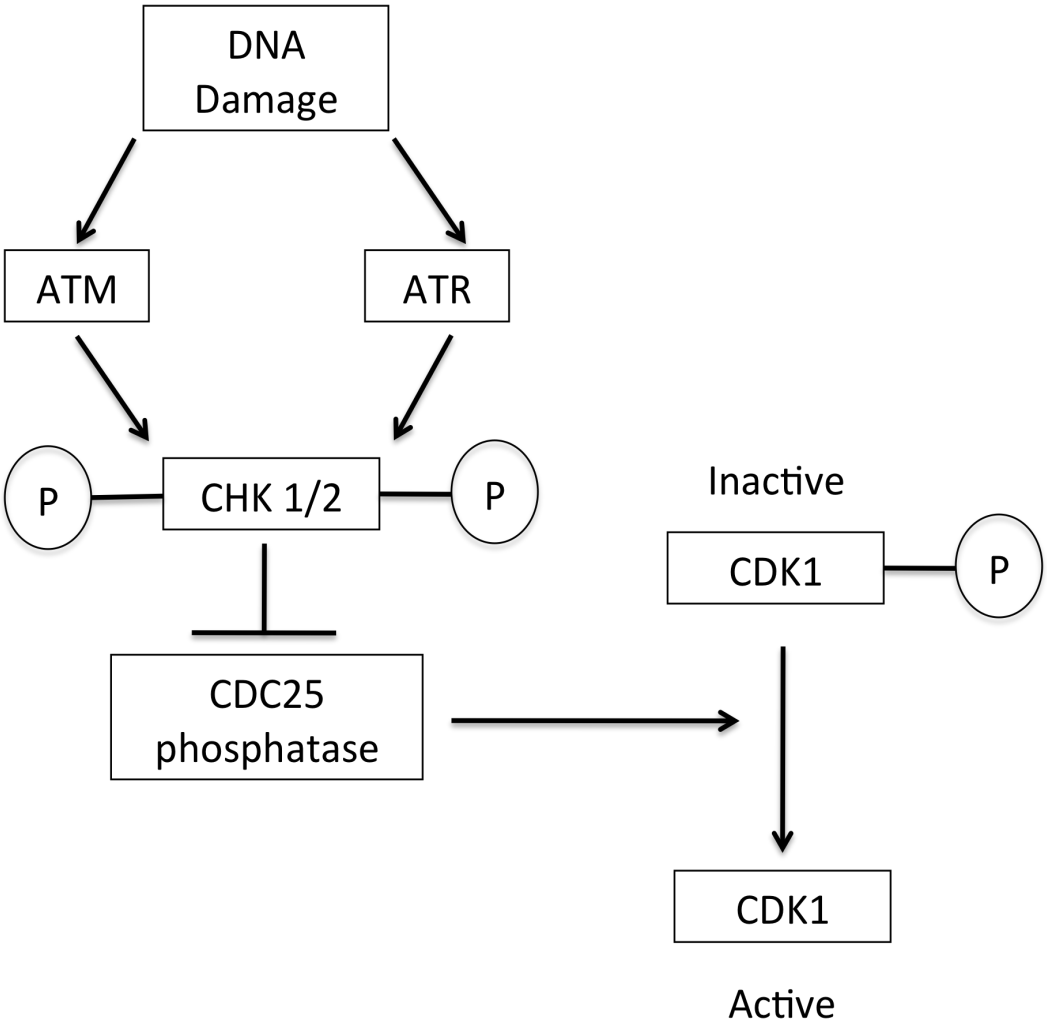

Figure 1.8 G_2 checkpoint

DNA damage activates, ATM or ATR. These kinases then phosphorylate either CHK1 or CHK2 kinases. CHK1/CHK2 kinases inhibit the CDC25 phosphatase. Inhibition of CDC25 tyrosine phosphatase prevents the removal of inhibitory phosphate groups from CDK1. This in turn prevents activation of CDK1.

1.3.3.4 S-phase checkpoint

The S checkpoint involves three distinct modes that share components and are mutually coordinated (159). The replication checkpoint is activated by stalling of replication forks due to nucleotide depletion or polymerase inhibition (160). The second is the intra-S-phase checkpoint that is activated when damaged DNA is detected during S-phase, outside of the replicons, and is therefore replication-independent. Finally, the S/M checkpoint prevents division before the cell has completely duplicated its genome (161).

1.3.3.5 Mitotic checkpoint

The mitotic checkpoint or spindle assembly checkpoint ensures correct chromosomal segregation during mitosis and the production of two genetically identical nuclei (162). Spindle microtubules attach to the centromere regions of the chromosomes during metaphase such that the sister chromatids can be pulled to opposite poles of during anaphase. The spindle assembly checkpoint ensures that cells will not enter anaphase, where chromosome segregation occurs, until all chromosomes are aligned at the equator and attached to the microtubules of the mitotic spindle. The role of Aurora B in detecting aberrant kinetochore to microtubule attachments is discussed in detail in section 1.8.3. Through activation of mitotic arrest deficient (MAD) 1/2 and other proteins, spindle defects or the presence of unattached kinetochores will result in inactivation of the anaphase promoting complex or cyclosome, (APC/C). This complex controls the proteasomal degradation of a number of proteins that regulate sister chromatid cohesion, spindle elongation, as well as cyclin B1. This is a prerequisite for progression of mitosis (163). Mitotic checkpoint failure can lead to cells exiting mitosis and entering the next S phase with a 4N DNA content, resulting in endoreduplication (see mitotic catastrophe discussed in section 1.5).

1.3.4 p53, p21 and p27

As discussed earlier, *p53* is a key tumour suppressor gene (164). The role of p53 as a tumour suppressor is to block cell cycle progression and/or to induce apoptosis, in response to cellular stresses such as DNA damage. Impaired p53 activity promotes the accumulation of DNA damage in cells, which leads to a cancer phenotype (165). Upstream activators of p53 include ATM, ATR, CHK2 and RAS. Expression of p53 following DNA damage, leads to the transcriptional induction of the *CDKN1A* gene (166, 167). Its protein product, p21, is a potent CKI that inhibits several cyclin-CDK complexes leading to a pause in both the G_1 to S and G_2 to M transition (Figure 1.6). In particular p21 inhibits the cyclin D–CDK4 complex through direct binding. The consequence of this inhibition of cyclin D–CDK4 is the absence of hyperphosphorylation of the RB protein leading to failure of E2F target gene expression and arrest of the cell in G_1 (Figure 1.6 and 1.7). p21 also promotes sustained G_2 arrest following DNA damage, through inhibition of the cyclin B1/CDK1complex (168).

Another p53 target gene involved in the G_2 arrest is the growth arrest and DNA damage 45 gene (*GADD45*). GADD45 has been shown to interact with and inhibit proliferating cell nuclear antigen (PCNA) and CDK1 (169-171). Studies also show that p53 plays a critical role in preventing aneuploidy by blocking endoreduplication of tetraploid cells that result from mitotic failure (172).

As discussed above the spindle assembly checkpoint ensures that cells will not enter anaphase, where chromosome segregation occurs, until all chromosomes are aligned at the equator and attached to the microtubules of the mitotic spindle. p53 plays a critical role in preventing aneuploidy by blocking endoreduplication of tetraploid cells that result from mitotic failure (172).

p27 is a CKI encoded by the cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene that regulates the G_0 to S phase transitions by binding to and regulating the activity of CDKs. It was discovered as an inhibitor of

cyclin E-CDK2 (173-175), but has since been shown to play dual roles to both promote and inhibit cell cycle progression. In G_0, p27 translation and protein stability are maximal as it binds and inactivates nuclear cyclin E-CDK2. In early G_1, p27 also promotes assembly and nuclear import of D-type cyclin-CDKs (176, 177). The progressive decrease of p27 in G_1 permits cyclin E-CDK2 and cyclin A-CDK2 to activate the G_1 to S transition (178). Importantly FOXO proteins activate p27 transcription, in response to cytokines or promyelocytic leukaemia protein (PML) and nuclear AKT signalling (179, 180).

1.3.5 Targeting the cell cycle for cancer therapy

The vinca alkaloids (vinblastine and vincristine (VCR)) and taxanes (paclitaxel and docetaxel) are the first cancer drugs developed to effectively target the mitotic phase of the cell cycle (181). These agents directly bind to tubulin and disrupt the assembly of spindle microtubules during mitosis, leading to mitotic arrest followed by cell death through multiple mechanisms. Cancer cells can acquire resistance to these microtubule damaging agents through a variety of means (182). An alternative approach for anti-mitotic therapy is to target key regulators of mitotic progression, including the mitotic kinases (183). The mitotic kinases are a diverse collection of serine/threonine kinases that tightly regulate each step of mitosis. Collectively, the mitotic kinases control the activity, localization and stability of a diverse array of effector proteins (184, 185). An important group of kinases within the family of mitotic kinases are the Aurora kinases, which are a discussed in section 1.8.

1.4 Apoptosis

1.4.1 Introduction

Apoptosis, a programmed form of cell death, is a highly regulated process that plays a role in developmental morphogenesis, control of cell numbers,

removal of damaged cells, and tumour suppression (186). It is characterized by cell shrinkage, membrane blebbing and budding, chromatin condenstaion, and precise fragmentation. Apoptotic cells are removed following phagocytosis by macrophages which recognize exposure of phosphatidylserine on the cell membrane.

Cells may be induced to undergo apoptosis by extracellular signals or by internal insults such as DNA damage or oxidative stress which activate a conserved family of aspartic acid-specific cysteine proteases called caspases (187). Thirteen mammalian caspases have been identified and can be divided into two functional groups based on their role in inflammation or apoptosis. The pro-inflammatory caspases are caspases -1, -4, -5, -11 and -12. The remainder are the pro-apoptotic caspases. The pro-apoptotic caspases are further subdivided into initiators (caspases -2, -8, -9 and -10) and effectors (caspases -3, -6, -7 and -14). Pro-apoptotic caspases are responsible for the dismantling of the cell during apoptosis (188) and their activation distinguishes this cell death programme from the others identified (189). Once active, these initiator caspases can cleave and activate downstream effector caspases (190-192). Effector caspases orchestrate the cell's demise through the cleavage of specific cellular substrates, resulting in the morphological characteristics of apoptosis.

1.4.2 Intrinsic apoptotic pathway

The intrinsic or mitochondrial apoptotic pathway is triggered in response to cellular stresses such as DNA damage, oxidative stress, ischemia or growth factor withdrawal. Disruption of the mitochondria results in mitochondrial membrane depolarization, loss of mitochondrial membrane integrity, leading to release of mitochondrial cytochrome c to the cytosol (Figure 1.9). In the cytosol cytochrome c binds to apoptosis protease activating factor 1 (APAF-1) and dATP/ATP, which triggers the formation of the apoptosome that activates pro-caspase-9, -3, -6 and 7 (193, 194)). Second Mitochondrial Activator of Caspases (SMAC) is another

apoptogenic protein released from mitochondria besides cytochrome c that binds to and inhibits inhibitor of apoptosis proteins (IAPs) (195, 196).

The intrinsic pathway is regulated by the B-cell lymphoma-2 (BCL-2) family of proteins of which pro- and anti-apoptotic members exist (Table 1.1) (197, 198). The BCL-2 family of proteins contain at least 20 members, all of which contain at least one of four conserved BCL-2 homology domains (BH1-BH4) that regulate protein-protein interactions. The pro-apoptotic BCL-2 proteins can be subdivided into the multi-domain (BCL-2–associated X protein (BAX) and BCL-2 homologous antagonist/killer (BAK)) and BH3-only members (BIM, BCL-2-associated death promoter (BAD), BH3 interacting-domain death agonist (BID), BCL-2-interacting killer (BIK), p53 upregulated modulator of apoptosis (PUMA) and NOXA (latin for damage)) (Table 1.1). The functions of the BCL-2 proteins are linked to their ability to localize to intracellular membranes and their capacity to homo- and hetero-oligomerise.

Pro-apoptotic BH3-only-proteins induce homo-oligomerization of BAX and BAK in the outer mitochondrial membrane leading to permeabilization of mitochondria (199, 200). Without BAX and BAK, BH3-only proteins are unable to induce apoptosis (201, 202). The anti-apoptotic BCL-2 family members, BCL-2, BCL-xL and myeloid cell leukaemia-1 (MCL-1) prevent cytochrome c release by inactivating BAX and BAK, preventing their activation of BH3-only proteins or by regulating permeability transition pore opening (203-207). As discussed in section 1.3.4, p53 promotes apoptosis in response to gentoxic stress by inducing the expression of key target genes (p21, p27, BAX, PUMA, NOXA, etc.) (208). Frequent p53 mutations, observed in cancer cells, may account for resistance to DNA damage inducing chemotherapeutic agents (197).

Anti-apoptotic members	Pro-apoptotic members	Pro-apoptotic members-BH3-only members
BCL-2	BAX	BAD
BCL-xL	BOK	BIK
BCL-w	BCL -xS	BID
A1	BAK	HRK
MCL-1	BCL-GL	BIM
		BMF
		NOXA
		PUMA

Table 1.1 BCL-2 family members

BOK: BCL-2 related ovarian killer; BMF: Bcl-2-modifying factor; HRK: Harakiri BCL-2 interacting protein.

1.4.3 Endoplasmic reticulum (ER) stress- induced apoptosis

The ER is another vital organelle from which apoptotic signals can originate. Key functions performed by the ER include, lipid biosynthesis, protein folding and Ca^{2+} storage. Prolonged ER stress can ultimately lead to cell death. By binding with the protein TNF Receptor-Associated Factor 2 (TRAF2), serine/threonine-protein kinase/endoribonuclease (IRE1) activates c-Jun N-terminal Kinase signalling, at which point human procaspase 4 is believed to cause apoptosis by activating downstream caspases (209-211) (212-214) (215, 216).

1.4.4 Extrinsic or death receptor-mediated pathway

The extrinsic pathway (also known as the death receptor-mediated apoptotic pathway) is activated following binding of death ligands to its cognate death receptor expressed on the cell surface. Death receptors include Fas (CD95), tumour necrosis factor (TNF) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Death ligands include soluble factors such as TNF or membrane bound ligands such as Fas ligand. When death ligands bind to cell surface death receptors, the receptors undergo a conformational change that exposes Death Domains (DDs) in the cytosol. This, in turn, leads to recruitment of adaptor molecules to form the Death Inducing Signalling Complex (DISC). The function of these adaptor proteins is to transduce the death signal from the receptor to caspases. The intrinsic and extrinsic pathways converge in the activation of these executioner caspases. Cross-talk between the extrinsic and intrinsic pathways occurs to amplify the death signal (217, 218).

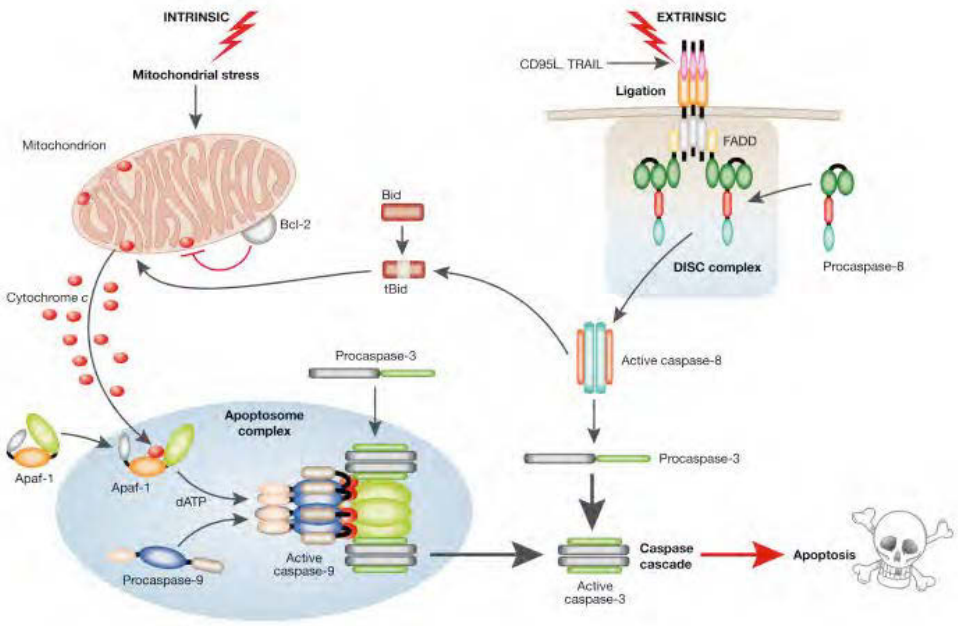

Figure 1.9 Apoptosis: the 'extrinsic' and 'intrinsic' pathways to caspase activation

Two major apoptotic pathways are illustrated: one activated via death receptor activation ('extrinsic') and the other by stress-inducing stimuli ('intrinsic'). Triggering of cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily, including CD95 and TRAIL-R1/-R2, results in rapid activation of the initiator caspase 8 after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). In the intrinsic pathway, stress-induced apoptosis results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the inter-mitochondrial membrane space. The release of cytochrome c, from mitochondria is regulated in part by BCL-2 family members, with anti-apoptotic (BCL-2/ BCL-X_L/MCL-1) and pro-apoptotic (BAX, BAK and tBID) members inhibiting or promoting the release, respectively. Once released, cytochrome c binds to apoptotic protease-activating factor 1

(APAF1), which results in formation of the APAF1–caspase 9 apoptosome complex and activation of the initiator caspase 9. The activated initiator caspases 8 and 9 then activate the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype. Reproduced with permission from MacFarlane et al, Apoptosis and disease: a life or death decision EMBO reports (2004) 5, 674 - 67 (219).

1.5 Mitotic catastrophe

1.5.1 Definition of mitotic catastrophe

Mitotic catastrophe (MC) has been defined as a mechanism of cell death characterized by the occurrence of aberrant mitosis with the formation of large cells that contain multiple nuclei, which are morphologically distinguishable from apoptotic cells (220-222). In recent years there has been considerable interest in inducing MC in cancer for several reasons. Firstly, many cancer cells are tetraploid or aneuploid, which renders them intrinsically more sensitive to mitotic aberrations and accordingly more sensitive to the induction of MC (223). Furthermore, many chemotherapeutic agents that induce cell cycle-independent cell death at high doses are very efficient at inducing MC at lower doses (224). Lastly, since cancer cells are frequently deficient in cell-cycle checkpoints, they may be particularly susceptible to the induction of MC.

There has been considerable controversy in the literature as to a broadly accepted definition of the term MC (225). Igor Roninson defined MC in morphological terms, that is, as a type of cell death resulting from abnormal mitosis, which results in the formation of large cells with multiple micronuclei and decondensed chromatin (226). Micronuclei often originate from chromosomes or chromosome fragments that have not been distributed evenly between daughter nuclei, whereas two or more nuclei with similar or heterogeneous sizes can occur following aberrant karyokinesis (227). However, as MC proceeds and is followed by apoptosis, necrosis or cell senescence many of the morphological traits that characterize these processes become prominent making MC difficult to recognize and classify morphologically (228). An alternative approach is to consider MC as an oncosuppressive signalling cascade that precedes cell death or senescence rather than a bona fide cell death executioner mechanism (227).

1.5.2 Molecular features of MC

The molecular events that accompany MC are not completely characterized and there appears to be a high degree of variability in the molecular cascades activated in distinct instances of MC. The levels of several proteins including CHK1/2, centromere protein A (CENP-A), MAD2 and budding uninhibited by benzimidazoles (BUB1) have been reported to be altered in drug-treated cells entering MC (224, 225, 229-231). These reports suggest that antitumour drugs may deplete important regulators of mitosis leading to abnormal nuclear division and subsequent cell death.

In order for MC to occur, DNA-damaged cells first halt in G_2/M, before subsequent entry into mitosis (220). Inhibition or absence of p53 and p21, is required to bypass the G_2/M checkpoint. Aberrant mitosis in irradiated or drug-treated cells may follow several pathways but the final step is usually the formation of nuclear envelopes around individual clusters of missegregated chromosomes (micronuclei) (232, 233). Aberrant mitotic entry, before the completion of DNA replication, can also result in MC. This requires the activation of CDK1, and it is thought that the premature entry of active CDK1/cyclin B1 complex (see Figure 1.6) into the nucleus causes premature chromatin condensation and apoptosis (234, 235). Increased nuclear cyclin B1 has been detected in examples of MC in human colorectal adenocarcinoma cell lines treated with fluorouracil (236) and in colon cancer cells treated with doxorubicin (237). CHK2 has been shown to negatively regulate MC and therefore the inactivation of CHK2 (and of its activating partners) can sensitize cells to this type of death. This raises the possibility that the inhibition of CHK2 (and similar enzymes) may sensitize cancer cells to MC induced by chemotherapy.

As discussed in section 1.3.4, p53 plays a key role in the polyploidy checkpoint. Interestingly the loss of p53 can sensitize cells to microtubule disrupting agents such as paclitaxel (238, 239). Following treatment with

spindle inhibitors, cells possessing an intact p53 system undergo a p21-mediated G_1 arrest making them relatively resistant to apoptosis induced by antimicrotubule agents. Cells deficient in p53 fail to undergo such a G_1 arrest (since they fail to upregulate p21) which allows endoreduplication of their DNA, leading to massive apoptosis (240).

1.5.3 MC and apoptosis

MC-induced apoptosis is accompanied by chromatin condensation, mitochondrial release of proapoptotic proteins (in particular cytochrome c), caspase activation and DNA degradation (225). Transfection with inhibitors of mitochondrial membrane depolarization such as BCL-2 and BCL-xL or knock out of BAX can prevent mitotic catastrophe (241-244). Conversely it has also been shown that inhibition of BCL-2 expression by antisense oligonucleotides can actually facilitate and amplify MC (245). The molecular links between abnormal mitosis and activation of the central executioner of apoptosis are beginning to be understood (Figure 1.10). In the situation of p53-dependent MC, the transcription of the proapoptotic p53 target genes BAX and PUMA may determine the induction of mitochondrial membrane depolarization (243, 244). In p53-independent MC, caspase-2 appears to be activated upstream of mitochondria (246). HDAC inhibition leads to the accumulation of cells with a 4N DNA content resulting in apoptosis, in a process that involves a primary caspase-2 activation that cannot be inhibited by BCL-2 (247). Exactly how MC leads to caspase-2 activation is unclear but procaspase-2 can be activated in the nucleus as a result of DNA damage (248-250).

1.5.4 Drugs that induce mitotic catastrophe

Many of the anti-cancer drugs currently utilized function by inducing MC. These include taxanes, vinca alkaloids, as well as recently developed compounds such as epothilones, which mimic the activity of taxanes yet bind to a distinct binding site on tubulin (251). In addition, there are several

inducers of MC that are currently being evaluated in pre-clinical and clinical settings, including inhibitors of Aurora kinases (252), CHK1 (253), polo-like kinases (PLKs) (discussed in section 1.8) (254, 255) and survivin (256).

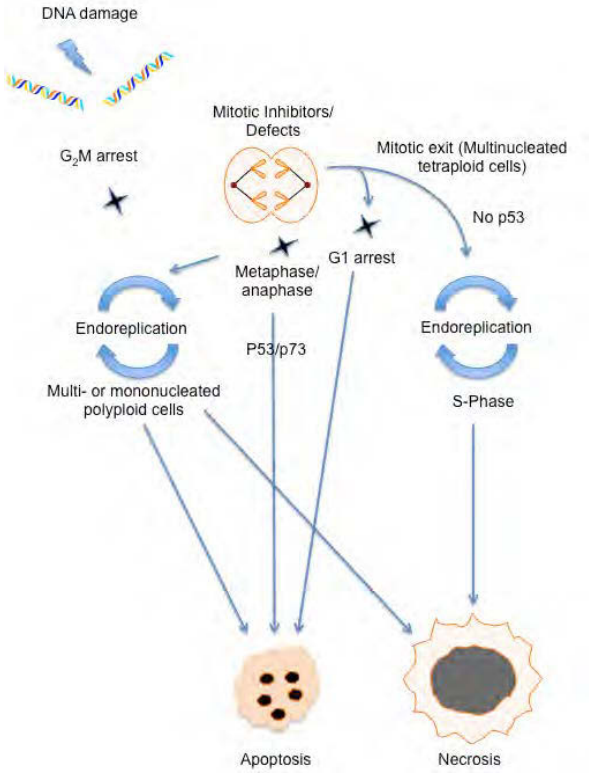

Figure 1.10 Schematic illustration of putative pathways leading from MC to cell death

Premature entry into mitosis as a result of abrogated G_2/M arrest in the presence of DNA damage or direct mitotic damage leads to arrest at the metaphase–anaphase transition due to spindle checkpoint and to catastrophic mitosis. MC cells can undergo endoreduplication and become polyploid. These cells can die by either necrosis or apoptosis. Cells being arrested at the metaphase–anaphase transition can escape mitosis through mitotic slippage and become tetraploid. Cells that cannot be arrested at the metaphase–anaphase transition due to defects in the spindle checkpoint also become tetraploid. These tetraploid cells either can arrest at G_1 and die through p53-dependent apoptosis or do not arrest at G_1 and enter S-phase (endoreplication) and die through necrosis. Modified and reprinted by permission from Nature Publishing Group: Vakifahmetoglu et al (2008).

1.6 Chronic myeloid leukaemia

1.6.1 Introduction

As discussed earlier CML is a myeloproliferative neoplasm characterized by the dysregulated production of mature granulocytes. It is characterized by the presence of the Ph chromosome t(9;22)(q34;q11), resulting in the *BCR-ABL* fusion gene and its unique gene product (BCR-ABL), a constitutively active tyrosine kinase that dysregulates apoptosis and promotes survival by modulating multiple downstream intracellular signalling pathways (see section 1.1.3).

1.6.2 Epidemiology

CML accounts for approximately 15 to 20 percent of leukaemias in adults (257). It has an annual incidence of 1 to 2 cases per 100,000, with a slight male predominance (4, 258). The median age at presentation is 50 years. Exposure to ionizing radiation is the only known risk factor (55, 259).

1.6.3 Clinical features

The clinical hallmark of CML is the uncontrolled production of maturing granulocytes (predominantly neutrophils), but also eosinophils and basophils. CML has a triphasic clinical course and progresses from CML-CP to CML-AP and to CML-BC. Over 90% of patients are diagnosed in the CML-CP. If these patients are not treated with agents capable of affecting the natural course of the disease, the majority of these will progress through the remaining two stages - the CML-AP and CML-BC.

1.6.4 Definition of accelerated and blast phase

Varying definitions have been applied to CML-AP (Table 1.2) (260). The most frequently used criteria are from the World Health Organization (WHO). CML-BC, is usually defined by the presence of one or more of the following findings: ≥20% PB or BM blasts (263) , large foci or clusters of blasts on the BM biopsy (264) or extramedullary blastic infiltrates (263).

MD Anderson*	Sokal et al•	WHOΔ
PB blasts ≥15 percent		
PB blasts + pro ≥30 percent	PB or BM blasts ≥5 percent	PB or BM blasts 10-19 percent
PB baso ≥20 percent	PB basophils ≥20 percent	PB basophils ≥20 percent
Platelets <100,000/μL not related to therapy	Thrombocytopenia, not related to therapy	Platelets <100,000/μL, unrelated to therapy
	Cytogenetic evolution	Cytogenetic evolution
	Platelets >1 million/μL despite adequate therapy	Platelets >1,000,000/μL unresponsive to therapy
	Marrow collagen fibrosis	
	Anemia, unrelated to Rx	
Cytogenetic evolution	Progressive splenomegaly	Progressive splenomegaly and increasing WBC unresponsive to therapy
	WBC doubling time <5 days	
	Unexplained fever	
	Pelger-Huett-like neutrophils, nucleated RBCs, megakaryocyte fragments	

Table 1.2 Accelerated phase CML criteria

WHO: World Health Organization; PB: peripheral blood; BM: bone marrow; pro: promyelocytes; baso: basophils; WBC: white blood cell count; RBC: red blood cells.

* Kantarjian, HM, et al. Cancer 1988; 61:1441.

• Sokal, JE, et al. Semin Hematol 1988; 25:49.

Δ Vardiman, JW, et al. Blood 2002; 100:2292. *For each of the three sets of criteria, the accelerated phase of CML is diagnosed if one or more of the listed features is present. Adapted from O'Dwyer, ME, et al. Blood 2002; 100:1628.*

1.6.5 Current challenges in CML therapy

Targeted BCR-ABL inhibitors (imatinib, nilotinib and dasatinib) have provided dramatic clinical benefit for Ph+ leukaemias (265). They competitively inhibit the inactive configuration of the BCR-ABL protein tyrosine kinase by blocking the ATP binding site and thereby preventing a conformational switch to the active form (266). Although they do not cure the disease, they result in long-term disease control in most patients and thus they have become standard of care. Second-generation TKIs have since also been developed. Despite the advances represented by these therapies, there remains considerable room for improvement in outcomes, especially in advanced disease.

1.6.6 Overview of treatment of CML

1.6.6.1 Monitoring response and response criteria

There are three principal strategies to assess response in CML (267, 268). Haematologic response (HR) is assessed by the white blood cell count, differential, and platelet count. Cytogenetic response (CyR) is assessed by BM biopsy. Conventional chromosomal cytogenetics or fluorescence in situ hybridization (FISH) (for patients with an inadequate number of metaphases) is used to evaluate the percentage of Ph positive cells present in the BM (269). Molecular response is assessed by quantitative real time polymerase chain reaction (qRT-PCR) of the PB. This technique is used to detect the presence or absence of malignant circulating cells that harbour the *BCR-ABL* gene. The definitions of responses are outlined in Table 1.3.

It is important to note that clinical trials in CML usually report best response level achieved at any time during follow-up. These cumulative response figures do not reflect the fact that many patients will achieve but then subsequently lose a response. Therefore, it has been suggested that a more informative indicator of long-term benefit would be to report on

responses maintained at particular landmark time-points, i.e. 3, 6, 12 and 18 months (270, 271). Based on this means of analysis, the 5-year probability of patients with newly diagnosed CML-CP being in a MCyR with imatinib is only 62.7% (270).

1.6.6.2 Failure, suboptimal response

Treatment failure is defined by the loss of a CHR or CCyR; the presence of BCR-ABL kinase mutations that are poorly sensitive to imatinib; clonal progression of disease; or the inability to reach the certain threshold levels after the initiation of therapy (272). These threshold levels are CHR by three months, any cytogenetic response by six months, PCyR by 12 months and CCyR by 18 months. Failure to achieve an optimal response occurs in up to 25 percent of CML-CP patients treated with imatinib (273). On the other hand secondary resistance (defined as loss of response in patients with an initial response to a TKI) occurs in approximately 8 percent of CML-CP patients treated with imatinib at two years (274) (275).

Response by type	Definitions
Haematological	
Complete (CHR)	WBC <10 x 10^9/L Basophils <5% No myelocytes, promyelocytes, myeloblasts in the differential Platelet count <450 x 10^9/L Spleen nonpalpable
Cytogenetic	
Complete (CCyR)	No Ph+ metaphases
Partial (PCyR)	1% to 35% Ph+ metaphases
Major (MCyR)	CCyR plus PCyR
Minor (mCyR)	36% to 65% Ph+ metaphases
Minimal (minCyR)	66% to 95% Ph+ metaphases
None (noCyR)	>95% Ph+ metaphases
Molecular	
Complete (CMR)	Undetectable BCR-ABL mRNA transcripts by real time quantitative and/or nested PCR in two consecutive blood samples of adequate quality (sensitivity >10^4)
Major (MMR)	Ratio of BCR-ABL to ABL (or other housekeeping genes) ≤0.1% on the international scale

Table 1.3 Response definitions in CML

CHR: complete hematologic response; CCyR: complete cytogenic response; PCyR: partial cytogenetic response; Ph+: Philadelphia chromosome positive; MCyR: major cytogenetic response; minCyR: minor cytogenetic response; noCgR: no cytogenetic response; CMR: complete molecular response; PCR: polymerase chain reaction; MMR: major molecular response. Reprinted with permission from: Baccarani, M, Cortes, J, Pane, F, et al. Chronic myeloid leukaemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol 2009; 27:604.

1.6.6.3 Imatinib

Imatinib is a 2-phenylaminopyrimidine derivative that functions as a specific inhibitor of a number of tyrosine kinases including ABL, c-Kit and platelet-derived growth factor receptor (PDGFR) (276, 277). Imatinib gained approval as the initial treatment of choice for CML-CP based on prospective clinical trials (278-280). These trials demonstrated more frequent and more durable cytogenetic responses when compared with interferon alpha (IFNα) plus ara-C, which was the previous standard of care for those unable to receive haematopoietic stem cell transplantation (HSCT). A detailed description of the pharmacological properties of imatinib is available in Appendix A.

1.6.6.4 Resistance in CML

While TKIs have dramatically improved CML treatment, resistance can develop due to BCR-ABL kinase domain mutations, *BCR-ABL* amplification and over-expression, clonal evolution (281, 282), excretion of imatinib from the cell by transmembrane transporters (283), or, most commonly, by the development of single nucleotide mutations in *BCR-ABL* which result in amino acid substitutions that change the conformation of the ATP binding site (P-loop), imatinib binding site, activation loop (controlling kinase activation) or catalytic domain. Kinase domain mutations can reduce BCR-ABL flexibility and destabilize the inactive form necessary for imatinib binding or interrupt critical contact points for drug binding (282, 284). More than 50 such mutations have been described to date and have been associated with both primary and secondary resistance. In one study, amino acid substitutions at seven residues (M244V, G250E, Y253F/H, E255K/V, T315I, M351T, and F359V) accounted for 85% of all resistance-associated mutations (285). P-loop and T315I mutations were particularly frequent in advanced phase CML and Ph+ ALL patients (286). Mutations leading to clinical resistance may

confer novel biological properties to the BCR-ABL kinase that may affect disease progression (287); their appearance often accompanies progression from CP to AP or BC (285). While some of these mutations confer only mild increases in the resistance to imatinib, others produce profound resistance *in vitro* and *in vivo*, which cannot be overcome by dose increases or by the use of newer TKIs such as dasatinib and nilotinib (288).

Although both dasatinib and nilotinib are active against most of the known BCR-ABL mutations, some of the mutations, especially a mutation known as T315I, are resistant to high concentrations of both of these newer agents (289, 290). However ponatinib (see Appendix A), a multikinase inhibitor that is effective in patients with the T315I mutation has now been approved by the United States Food and Drug Administration (FDA) (291).

Ongoing clinical trials are evaluating the mutational status of patients treated with nilotinib and dasatinib and it may be possible in the future to tailor the choice of second line therapy according to the specific mutation in a given patient. In other patients, resistance may be caused by activation of other, non BCR-ABL-mediated signalling pathways, such as the sarcoma (SRC) kinases. While imatinib and nilotinib do not directly inhibit members of the SRC kinase family, dasatinib and bosutinib inhibit both BCR-ABL and SRC kinases.

1.6.6.5 Disease persistence in CML

Early studies showed that a population of quiescent LSCs can be isolated from the blood and BM of patients with untreated CML-CP using flow cytometry techniques (137). These cells regenerate BCR-ABL-positive haemopoiesis in immunocompromised mice upon transplantation (292). *In vitro* studies show that while imatinib is antiproliferative for CML stem cells it does not induce apoptosis in CML stem cells (58). Non-proliferating Ph+ HSC are resistant to imatinib-induced apoptosis, despite achieving similar

intracellular levels of imatinib to more mature CML cells. *In vitro* studies of Ph+ HSC isolated from patients show that autophagy plays a critical role as a survival mechanism following treatment with TKI therapy (293).

In patients that achieve a CCyR with imatinib or IFN the persistence of malignant CD34+ cells, and CD34+/CD38- cells has been shown with the use of RT-PCR and FISH (60, 294-296). A recent study showed that prolonged imatinib exposure (at least 4 years) failed to eliminate BCR-ABL (+) stem cells (140). Another study showed that imatinib treatment resulted in a biphasic decrease in BCR-ABL transcript levels, with a rapid decrease during the first few months of treatment, followed by a more gradual decrease that often continues over many years. This long-term, gradual decrease in the BCR-ABL levels seen in most patients is consistent with a continual, gradual reduction of the LSC (297).

The French STIM (STop IMatinib) trial reported the results of imatinib cessation in patients with sustained undetectable molecular residual disease for more than 2 years. In this study most patients (59%) had a molecular relapse at 1 year following cessation of imatinib (298). A recent study showed that BCR-ABL-expressing marrow LSCs could be detected in patients with CML with undetectable molecular residual disease sustained for over 3 years following treatment with IFN-α, imatinib and dasatinib (299). This persistence of residual BCR-ABL expressing LSCs represents a theoretical risk of recurrence even in patients with sustained undetectable molecular residual disease after cessation of therapy.

1.6.7 Second generation TKIs

1.6.7.1 Nilotinib

Nilotinib (Tasigna®) is an orally administered TKI made by the Novartis Pharmaceuticals Corporation. Unlike dasatinib, which has a completely different structure to imatinib, nilotinib was methodically and rationally

designed to create a better topological fit in the ABL kinase domain of BCR-ABL resulting in enhanced BCR-ABL inhibition. Like dasatinib, its spectrum of inhibition also includes c-Kit and PDGFR (277). Similar to imatinib and dasatinib, nilotinib is unable to overcome the resistance of the T315I mutation (300). Nilotinib is approved by the FDA for the treatment of patients with newly diagnosed CML-CP or patients with CML-CP or CML-AP that are resistant to or intolerant of imatinib. A detailed description of nilotinib is available in Appendix A.

1.6.7.2 Dasatinib

Dasatinib (BMS-354825; Sprycel) was originally developed as an inhibitor of the SRC family of kinases (301). It is active against imatinib-resistant or intolerant CML and inhibits both the active and inactive conformations of the ABL domain (302-305). An *in vitro* study showed that dasatinib was 325 times more potent than imatinib in inhibiting wild type BCR-ABL and also has activity against BCR-ABL mutants with high levels of imatinib resistance, except for those with the T315I mutation (288). Dasatinb also inhibits a number of other important kinases including the proto-oncogene tyrosine-protein kinase, yes, c-Kit and PDGFR (305). Dasatinib is approved by the FDA for the treatment of patients with newly diagnosed CML-CP or patients with CML-CP, CML-AP, or CML-BC that are resistant to or intolerant of imatinib. A detailed description of dasatinib is available in Appendix A.

1.6.7.3 Bosutinib

Bosutinib (previously SKI-606) is a dual kinase inhibitor that targets both ABL and SRC pathways, but does not target c-Kit or PDGFR. In September 4, 2012, the FDA approved bosutinib for CML-CP, CML-AP or CML-BC in adult patients with resistance or intolerance to prior therapy. A detailed description of bosutinib is available in Appendix A.

1.6.7.4 Ponatinib

Ponatinib is an oral BCR-ABL inhibitor that has demonstrated activity against the native and mutated BCR-ABL proteins, including the T315I mutation (291). On December 14, 2012, the FDA granted accelerated approval to ponatinib for the treatment of adult patients with CML-CP, CML-AP, CML-BC or Ph+ALL that is resistant or intolerant to prior TKI therapy. The approval was based on the results of multicenter, international, single-arm phase 2 trial of 449 patients with disease that was resistant or intolerant to prior TKI therapy. Preliminary results reported MCyR in 54% of the with CML-CP. Importantly 75% of the CML-CP patients with T315I mutations had MCyR following ponatinib (306). A detailed description of ponatinib is available in Appendix A.

1.6.8 Choice of TKIs

1.6.8.1 Initial treatment of CML-CP

Patients with newly diagnosed CML-CP are generally treated with a TKI. Allogeneic HSCT may be a reasonable alternative in rare circumstances, such as that of a younger patient with an HLA matched sibling donor. The second generation TKIs, nilotinib and dasatinib, have shown improved clinical benefit over imatinib during the early treatment period. However with the current available evidence it is difficult at this time to recommend one TKI over another for individual patients. A choice among TKIs should take into consideration the drug side effect profiles and the patient's co-morbidities.

1.6.8.2 Choice of second line therapy

Patients who progress on frontline TKI therapy should be carefully evaluated. Those who appear to have resistant disease should be questioned carefully to assure that they are taking the prescribed TKI at the recommended dose and schedule and avoiding other medications or herbal supplements which may impair efficacy (307). If resistance is identified, the disease phase should be re-evaluated using a complete

blood count with differential and a BM biopsy with cytogenetics. In addition, mutational analysis of *BCR-ABL* should be performed. A newly acquired mutation in *BCR-ABL* may trigger a change in treatment (e.g. dose increase, change to another TKI or HSCT) depending upon the type of mutation found. Mutational analysis may provide useful information regarding the relative sensitivity of the clone to available alternate therapies. The presence of the T315I mutant indicates that therapy with imatinib, nilotinib or dasatinib is no longer appropriate. These patients may be considered for a HSCT, ponatinib (if available), omacetaxine (discussed in section 1.6.11) or a clinical trial with an agent known to be active against the T315I mutation. On the other hand patients with weakly resistant *BCR-ABL* kinase mutants, e.g. M351T, may benefit from imatinib dose escalation or switching to a second-line TKI. However the Y253H, E255K/V, and F359V/C/I mutations are resistant to imatinib and to a lesser degree to nilotinib but sensitive to dasatinib. The F317L/V/I/C, V299L, and T315A mutations are sensitive to nilotinib but show intermediate sensitivity to imatinib and dasatinib (308). Indeed this *in vitro* data has been borne out in clinical study findings. For instance, no CCyRs were observed in patients treated with nilotinib who had mutations in amino acids Y253, E255, T315, or F359 (309, 310). Similarly resistance to dasatinib has been associated with mutations at residues T315, F317 and V299L and these mutations are seen in patients developing dasatinib failure (311).

The differing side effect profiles of the available TKIs may lead certain agents to be more suitable for patients depending on their co-morbidities. For example nilotinib may be more suitable than dasatinib for patients than with significant respiratory or cardiac impairment or bleeding diatheses. On the other hand dasatinib than be more suitable than nilotinib for patients with liver dysfunction, pancreatitis and cardiac conduction abnormalities.

1.6.9 Potential toxicity of TKIs on normal haematopoietic precursors

All four currently approved TKIs inhibit c-Kit and PDGFR. As discussed earlier c-Kit is the receptor for SCF, a cytokine that is assumed to be critical for the expansion of immature human HSCs (312-314). In addition PDGF has been shown to be an effective cytokine for the *ex vivo* expansion of normal early stem and progenitor cells (315). However the inhibition of normal CD34+ cell expansion by imatinib exceeds that achieved by withdrawal of SCF or blocking of c-Kit by a monoclonal antibody suggesting that other mechanisms besides inhibition of c-Kit play a role in imatinib induced inhibition of normal progenitor cells (316).

1.6.10 Haematopoeitic stem cell transplant

While HSCT has been widely used in the past for the treatment of CML its use has declined with the emergence of TKIs. Using HLA-matched sibling donors, 50 to 75 percent of patients with CML transplanted in CML-CP achieve long-term remissions (317-321). The ability of allogeneic HSCT to cure CML is related to the anti-leukaemic effects of both the conditioning regimen and the graft versus leukaemia (GVL) effect of the donor lymphocytes. While myeloablative allogeneic HSCT offers potential cure to patients with CML it is associated with a high treatment-related mortality rate in older patients (322). Various reduced intensity conditioning (RIC) (323) or nonmyeloablative (324) HSCT regimens have been employed in fit older adults.

The prognosis for CML-AP or CML-BC is poor (particularly for patients previously treated with imatinib), as these phases tend to be relatively resistant to most forms of treatment (325-327). There is a significant relapse rate in patients in CML-AP or CML-BC even after successful treatment with imatinib, dasatinib or nilotinib and it is appropriate to consider transplantation in such individuals. As such, a reasonable plan is to initiate a search for a matched donor while attempting to return the patient to a second CML-CP (328) with suitable

candidates subsequently undergoing transplantation.

1.6.11 Omacetaxine

Omacetaxine mepesuccinate (previously known as homoharringtonine) is a protein synthesis inhibitor that has demonstrated activity in patients with CML-CP with a T315I mutation. It inhibits synthesis of proteins that regulate proliferation and cell growth (329) and has been approved by the FDA for the treatment of CM-CP or CML-AP with resistance or intolerance to two or more TKIs. Unlike TKIs, it has been shown to induce apoptosis in LSCs through down regulation of MCL-1 (330). A phase 2 trial investigated the use of omacetaxine in 62 patients with CML-CP resistant to an initial TKI who had an identified T315I mutation. Rates of CHR, MCyR, and CCyR were 77, 23, and 16 percent, respectively.

1.6.12 Treatment of CML-AP

Once a patient progresses from CML-CP to CML-AP their disease is much more difficult to control. Among patients who have not received imatinib, treatment options for CML-AP include TKIs and allogeneic HSCT.

1.6.13 Treatment of CML-BC

There are two major forms of CML-BC: lymphoid and myeloid. Myeloid blast crisis, which occurs in approximately 70 percent of cases, does not respond well to standard AML-induction regimens (331), although responses to TKIs, alone (332-334) or in combination with chemotherapy (335) have been noted. Lymphoid blast crisis with a B lineage immunophenotype accounts for approximately 30 percent of cases and often responds to chemotherapeutic programs used for ALL alone and in combination with a TKI. Since myeloid blast crisis is often refractory to chemotherapy, the preferred initial treatment is the use of a TKI followed by an allogeneic HSCT for eligible patients. Transplantation while the patient remains in CML-BC has poor results with less than 10 percent long-term survival (320). A reasonable plan is an attempt to return the patient to

an earlier phase of disease, with suitable candidates subsequently undergoing allogeneic HSCT.

1.6.14 Therapeutic relevance of CML stem cells

The development of TKIs has significantly improved the outcome for patients with CML. Furthermore the development of more potent second line inhibitors of BCR-ABL allow targeting of resistant disease. However few patients are cured of their disease probably due to the continued presence of reversibly quiescent Ph^+ HSC. Therefore targeting this population offers the potential of cure. However Ph^+ HSCs do not appear to undergo apoptosis in the setting of BCR-ABL inhibition with imatinib or second generation TKIs (143, 336, 337). Therefore alternative strategies to target this population are of interest (338). One strategy is to use growth factor stimulation to enhance the cycling of quiescent Ph+ HSC. Preclinical studies have shown that growth factor before imatinib treatment reduces the number of residual nondividing CML CD34(+) cells (339). This supports the potential efficacy of growth factor stimulation in reducing residual leukaemia progenitor population in imatinib-treated patients.

Similarly pretreatment with the protein kinase C inhibitor bryostatin-1 antagonises the anti-proliferative effect of imatinib, allowing greater efficacy against the non-cycling population (340). Other drugs that can potentially target Ph^+ HSCs include the farnesyl transferase inhibitors, lonafarnib and BMS-214662 (341, 342). Other potential strategies include targeting the hedgehog and the Wnt signalling pathways that regulate the self-renewal behaviour of HSCs (68, 343) or targeting autophagy as a potential resistance mechanism to TKIs (293). As mentioned earlier HSCT is a curative strategy in CML and effectively targets the Ph^+ HSC population.

1.6.15 Conclusion

While the development of TKIs in CML has been one of the most exciting advances in medicine in the last twenty years significant issues remain in CML. Resistance to TKI inhibitor therapy occurs through various mechanisms some of which can be overcome with second generation inhibitors. Efforts are underway to improve our understanding of the biology of Ph+ HSCs. Targeting this quiescent population of cells offers the potential for a cure for CML.

1.7 Acute myeloid leukaemia

1.7.1 Introduction

AML is characterized by a clonal proliferation of myeloid precursors with an impaired capacity to differentiate into more mature cellular elements. Consequently there is an accumulation of leukaemic blasts or immature myeloid precursors in the BM, PB, and occasionally in other tissues, with variable reduction in the production of normal red blood cells, platelets and mature granulocytes. The increased production of malignant cells along with a reduction in these mature elements results in a variety of systemic consequences including anaemia, bleeding and an increased risk of infection.

1.7.2 Epidemiology

As discussed in section 1.1 AML is the most common acute leukaemia in adults and accounts for approximately 80 percent of cases in this group (3, 257). In the United States and Europe, the incidence has been stable at 3 to 5 cases per 100,000 persons per year (4, 258, 344). The incidence increases with age with approximately 1.3 and 12.2 cases per 100,000 persons for those under or over 65 years, respectively. It is more common in males than females with a male to female ratio of approximately 5:3.

1.7.3 Clinical features

Patients with AML generally present with symptoms related to complications of pancytopaenia (eg, anaemia, neutropaenia, and thrombocytopaenia), including weakness and easy fatigability, infections of variable severity, and/or haemorrhagic findings such as gingival bleeding, ecchymoses, epistaxis, or menorrhagia (345). Skin involvement occurs in up to 13 percent of patients and is mostly found in cases with a prominent monocytic or myelomonocytic component (346).

1.7.4 Pathological features

1.7.4.1 Diagnosis

A presumptive diagnosis of AML can sometimes be made on the presence of circulating myeloblasts on the PB smear but an adequate BM aspiration and biopsy is usually necessary. Standard testing performed on the BM or PB includes Wright or Wright-Giemsa staining, cytochemical reactions, flow cytometric analysis for phenotyping and cytogenetic analysis. In the current WHO classification system, blast forms must account for at least 20 percent of the total cellularity. However the presence of certain genetic abnormalities are considered diagnostic of AML without regard to the blast count: AML with t(8;21), AML with inv(16) or t(16;16) or APL with t(15;17).

1.7.4.2 Immunonophenotyping

Flow cytometry of the PB or marrow aspirate identifies myeloblasts by characteristic patterns of surface antigen expression. Most cases will express CD34, HLA-DR, CD117, CD13, and CD33 (347) (Table 1.4). Flow cytometry helps distinguish APL from other subtypes of AML. In AML other than APL, expression of CD34 is seen in over 60% and expression of HLA-DR in over 85% of all cases. However less than 10% of cases of non-APL AML are negative for both CD34 and HLA-DR as opposed to over 80% of cases of APL (347). Among AML cases, CD2 coexpression is almost exclusively restricted to French-American- British (FAB (discussed below))

subtypes M3 variant and M4Eo. The most valuable markers to differentiate between myeloperoxidase-negative AML subtypes M0 and ALL are CD13, CD33, and CD117, typical of M0, and intracytoplasmic CD79a, intracytoplasmic CD3, CD10, and CD2, typical of B cell or T cell lineage ALL (348).

Marker	Lineage
CD13	Myeloid
CD33	Myeloid
CD34	Early precursor
HLA-DR	Positive in most AML, negative in APL
CD11b	Mature monocytes
CD14	Monocytes
CD41	Platelet glycoprotein IIb/IIIa complex
CD42a	Platelet glycoprotein IX
CD42b	Platelet glycoprotein Ib
CD61	Platelet glycoprotein IIIa
Glycophorin A	Erythroid
TdT	Usually indicates ALL however, may be positive in M0 or M1
CD11c	Myeloid
CD117 (c-Kit)	Myeloid/stem cell

Table 1.4 Immunophenotyping of AML Cells

1.7.4.3 Cytogenetic findings

All patients with suspected AML should undergo metaphase cytogenetic analysis of their BM biopsy specimen. Using standard banding techniques approximately 50 to 60 percent of patients with newly diagnosed AML will demonstrate cytogenetic abnormalities (Figure 1.11). Karyotype is a key determinant of prognosis in AML and is often used to choose the appropriate post-remission therapy. Identified cytogenetic abnormalities can also be used to monitor for minimal residual disease (MRD) after treatment if a RT-PCR or FISH probe is available for the abnormality. As such, the 2008 WHO classification of tumours of the haematopoietic and lymphoid tissues uses genetic findings in addition to morphologic, immunophenotypic, and clinical features to define distinct subtypes of AML.

The most common abnormalities seen on karyotype are t(15;17)(q24.1;q21.1), trisomy 8, t(8;21)(q22;q22), inv(16)(p13.1q22)/t(16;16)(p13.1;q22), and 11q rearrangements. A complete outline of the common cytogentic abnormalities in AML is available in Appendix B while their prognostic significance is discussed in section 1.8.7.2.

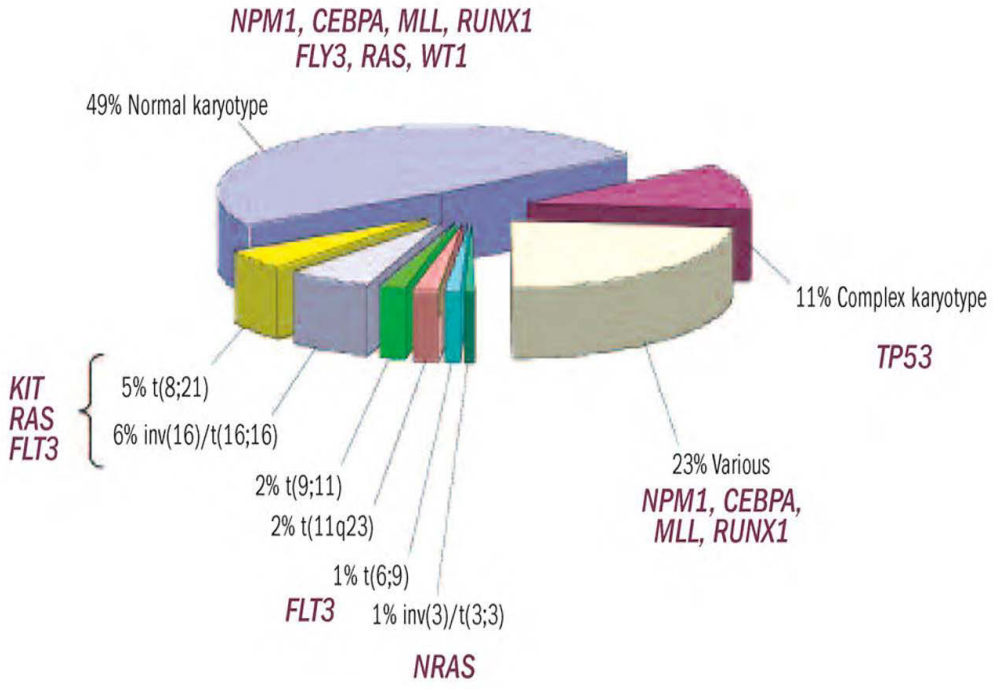

Figure 1.11 Major cytogenetic subgroups of AML (excluding acute promyelocytic leukaemia) and associated gene mutations

In the subgroup various, nucleophosmin (*NPM1*) mutations are frequently found in AML with 9q deletion and trisomy 8, *CEBPA* mutations in AML with 9q deletion, MLL mutations in AML with trisomy 11, and RUNX1 mutations in AML with trisomy 13 and trisomy 21. Frequencies of the cytogenetic subgroups are derived from 2,654 cytogenetically characterized adult (≥18 years) patients with *de novo* or secondary AML entered on five AML treatment trials. Reproduced with permission from Döhner K, et al. Haematologica 2008;93:976-982.

1.7.5 Classification of AML

1.7.5.1 FAB classification

The FAB classification system divides AML into eight subtypes, M0 through to M7, based on the type of cell from which the leukaemia developed and its degree of maturity (Table 1.5) (349). This is done by examining the appearance of the malignant cells with light microscopy and/or by using cytogenetics to characterize any underlying chromosomal abnormalities. The subtypes have varying prognoses and responses to therapy. Although the WHO classification (see below) may be more useful, the FAB system is still widely used.

1.7.5.2 WHO (World Health Organisation) classification

AML is currently classified using the WHO classification system based upon a combination of morphology, immunophenotype, genetics, and clinical features (350) (Table 1.6). It is recommended that patients with AML be classified according to this classification as it attempts to identify biologic entities with the intention that future work will elucidate molecular pathways that might be amenable to targeted therapies. There are four main groups of AML recognized in this classification system: AML with recurrent genetic abnormalities (11 percent), AML with MDS-related features (6 percent), therapy-related AML and MDS (2 percent) and AML, not otherwise specified (81 percent)

1.7.5.2.1 AML with recurrent genetic abnormalities

This WHO category contains AML variants that contain genetic abnormalities of prognostic significance including AML with t(8;21), inv(16)or t(16;16), t(15;17), t(9;11), t(6;9), inv(3) or t(3;3), t(1;22) (discussed in detail in Appendix B) and AML with mutated NPM1, CEBPA or FLT3.

1.7.5.2.2 AML with MDS-related features

The category of AML with MDS-related features (previously called AML with multilineage dysplasia) is defined by cases which fit the criteria for a diagnosis of AML (≥20 percent blasts); without a history of prior cytotoxic therapy for an unrelated disease with one or more of dysplastic neutrophils, megakaryocytes or erythrocytes (351) or MDS-related cytogenetic abnormalities (e.g., monosomy 5 or del(5q), monosomy 7 or del(7q), isochromosome 17p, etc). Patients who have a prior history of MDS or have MDS-related cytogenetic abnormalities have a poor outcome with conventional therapy.

1.7.5.2.3 Therapy-related AML and MDS

The diagnosis of therapy-related myeloid neoplasm (t-MN) is made when evaluation of the PB and BM demonstrates morphologic, immunophenotypic, and cytogenetic changes consistent with the diagnosis of AML or MDS in a patient with prior exposure to cytotoxic agents.

1.7.5.2.4 AML not otherwise specified

Cases of AML that do not meet the criteria for the categories described above are classified as AML, not otherwise specified (NOS). These cases are further sub classified by morphology that is similar to that used in the previous FAB classification system.

FAB subtype	Name	Adult AML patients (%)
M0	Undifferentiated acute myeloblastic leukaemia	5%
M1	Acute myeloblastic leukaemia with minimal maturation	15%
M2	Acute myeloblastic leukaemia with maturation	25%
M3	Acute promyelocytic leukaemia	10%
M4	Acute myelomonocytic leukaemia	20%
M4eos	Acute myelomonocytic leukaemia with eosinophilia	5%
M5	Acute monocytic leukaemia	10%
M6	Acute erythroid leukaemia	5%
M7	Acute megakaryocytic leukaemia	5%

Table 1.5 The French-American-British (FAB) classification system of AML

This classification system is based on morphology to define specific immunotypes.

AML and related neoplasms:

AML with recurrent genetic abnormalities
- AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
- APL with t(15;17)(q22;q12); PML-RARA
- AML with t(9;11)(p22;q23); MLLT3-MLL
- AML with t(6;9)(p23;q34); DEK-NUP214
- AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
- AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
- Provisional entity: AML with mutated NPM1
- Provisional entity: AML with mutated CEBPA

AML with myelodysplasia-related changes

Therapy-related myeloid neoplasms

AML, not otherwise specified
- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelomonocytic leukaemia
- Acute monoblastic/monocytic leukaemia
- Acute erythroid leukaemia
- Pure erythroid leukaemia
- Erythroleukaemia, erythroid/myeloid
- Acute megakaryoblastic leukaemia
- Acute basophilic leukaemia
- Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Myeloid proliferations related to Down syndrome
- Transient abnormal myelopoiesis
- Myeloid leukaemia associated with Down syndrome

Blastic plasmacytoid dendritic cell neoplasm

Table 1.6 WHO classification of WHO classification of myeloid neoplasms

1.7.6 Prognosis in AML

The response to treatment and overall survival (OS) of patients with AML is heterogeneous. A number of prognostic factors related to patient and tumour characteristics have been described for AML, including age, performance status, karyotype and the presence of absence of molecular abnormalities (Table 1.7) (352, 353).

1.7.6.1 Clinical risk factors

Older adults, variably defined as over age 55, 60, or 65 years have lower rates of achieving a complete response (CR (Table 1.8)) and shorter disease-free survival when compared with younger patients (354). The presence of comorbidities such as heart failure, renal insufficiency, concurrent infection and poor performance status status also predict early mortality and poor outcome.

1.7.6.2 Karyotype

Specific cytogenetic abnormalities in AML have considerable prognostic significance and affect treatment planning. Several large studies conducted by cooperative group efforts from the Medical Research Council (MRC), the Southwest Oncology Group/Eastern Cooperative Oncology Group (SWOG/ECOG), and the Cancer and Leukemia Group B (CALGB) have confirmed the prognostic relevance of pre-treatment karyotype (Figure 1.12) (92, 355-361). There is general agreement that t(8;21), inv(16), and t(15;17) predict a good outcome and occur in 16 percent of cases. Normal karyotype which occurs in approximately 40 percent and abnormalities not described in favourable or unfavourable (20 percent of cases) are considered intermediate risk (361). Adverse risk abnormalities include: del (5q); add (5q); del (7q); add (7q); monosomies 5 or 7; inv(3); t(3;3); t(6;11); t(10;11); t(9;22); 17p abnormalities; complex aberrant karyotypes described as at least 4 unrelated abnormalities; 11q23

abnormalities excluding t(9;11) and excluding t(11;19); or abnormalities of 3q excluding t(3;5). A "monosomal karyotype," defined as at least two autosomal monosomies or a single autosomal monosomy in the presence of one or more structural cytogenetic abnormalities is a better predictor of unfavourable risk disease than a complex karyotype (362).

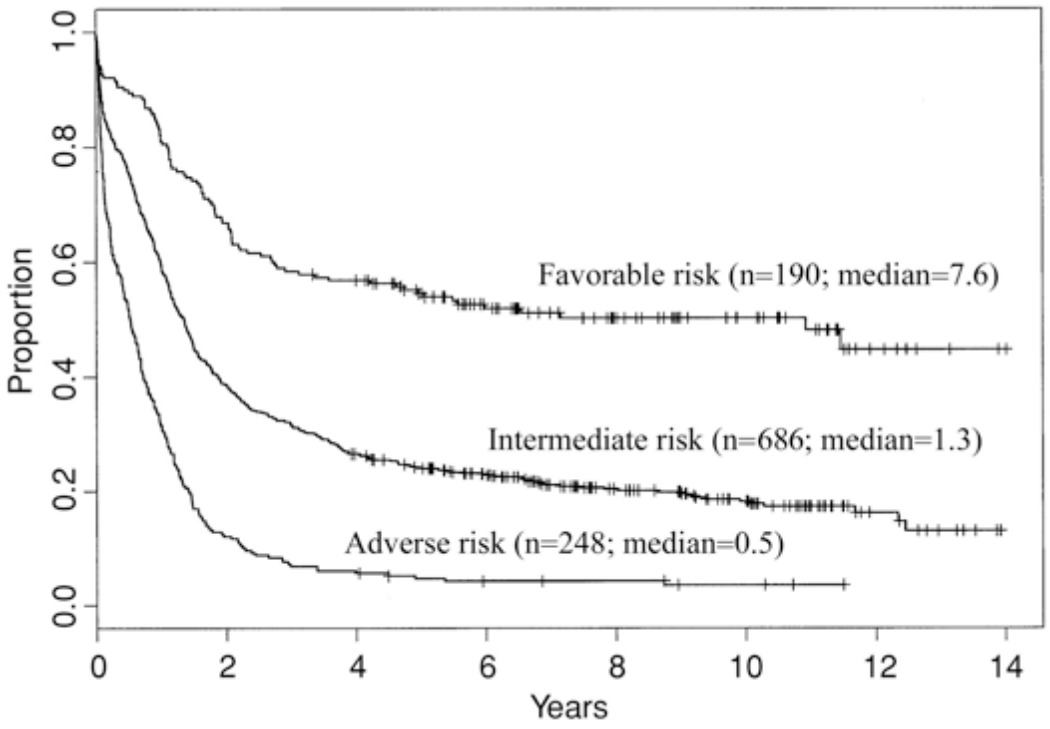

Figure 1.12 OS in AML patients categorized into favourable, intermediate, and adverse cytogenetic risk

For 5-year OS, the favourable risk group comprised 190 (17%) patients, the intermediate risk group 686 (61%) patients, and the adverse risk group 248 (22%) patients. The estimated probabilities (with 95% CI) of 5-year OS were 55% (47%-62%), 24% (21%-27%), and 5% (3%-8%), respectively. The differences in OS are depicted in the figure. Reprinted with permission from Byrd JC, et al. Blood 2002;100:4325-4336.

1.7.6.3 Gene mutations

As discussed earlier multiple AML is a heterogonous disease characterized by the presence of multiple molecular abnormalities. Abnormalities in certain genes (mutations in *FLT3*, *NPM1*, *KIT*) as well as gene expression profiles have prognostic significance in adult patients with AML (363). This is particularly important in patients with normal karyotypes. Some of these patients have better prognosis molecular abnormalities (e.g., mutations in CCAAT/enhancer binding protein alpha (*CEBPA*), mutations in the nucleophosmin (*NPM1*) gene in the absence of *FLT3/ITD* as well as others with an adverse prognosis (eg, *FLT3-ITD*) (Table 1.7).

1.7.6.3.1 *FLT3* gene

As discussed in section 1.1.4.4 *FLT3* mutations are common in AML, particularly in patients with normal karyotypes, and have been associated with poor survival in patients receiving intensive chemotherapy (364-367). It has been proposed that *FLT3/ITD* mutational status is the primary predictor of outcome among patients with intermediate-risk AML by karyotype analysis (79) and that *FLT3* mutation status may be considered in making recommendations about the use of HSCT in first remission (discussed in section 1.7.12).

1.7.6.3.2 Nucleophosmin gene

Abnormalities in the nucleophosmin (*NPM1*) gene are found in 25 percent of patients with de novo AML and 50 percent of de novo AML with normal karyotype AML. They are associated with improved outcomes in AML (368-370) but concurrent abnormalities in other genes, such as *FLT3*, may reduce their prognostic benefit.

1.7.6.3.3 *CEBPA* gene

The *CEBPA* gene encodes a transcription factor necessary for myeloid differentiation (371) and is found in 10 percent of patients with newly diagnosed AML (372). However 13 to 19 percent of patients with cytogenetically normal AML will have *CEBPA* mutations (373, 374). Patients with normal karyotype and *CEBPA* mutations have a significantly longer median OS that is independent of other high-risk molecular features.

1.7.6.3.4 *KIT* gene

Mutations of the *KIT* gene can be detected in approximately 6 percent of newly diagnosed AML and in 20 to 30 percent of patients with AML and either t(8;21) or inv(16) (79, 375, 376). Some studies suggest that *KIT* gene mutations confer a higher risk of relapse and adversely affect OS in those with inv(16) (375, 376) but other studies suggest that this negative prognostic effect is only seen among AML with t(8;21) (79). Screening for *KIT* mutations might also allow for use of TKIs such as imatinib or dasatinib, which have *in vitro* activity against some *KIT* mutations.

1.7.7 Gene expression profiling (GEP)

While not used routinely in practice there is interest in the use of gene expression profiling for the diagnosis, classification, and assessment of prognosis in AML. Some studies have analyzed leukaemia cells from patients with AML and have identified gene "signatures" that may be used to distinguish subsets with different outcomes (377, 378).

1.7.8 Tumour characteristics

Assessment of tumour characteristics can be used to assess patient outcome. Prognositc markers include the overexpression of drug efflux pumps, apoptosis inhibitors, or factors that lead to cell cycle progression (379-382).

Favourable factors	Unfavourable factors
Age <50	Age >60
Karnofsky score >60 percent	Karnofsky score <60 percent
MDR 1-negative phenotype	MDR 1-positive phenotype
No antecedent hematologic disorder or prior chemo/radiotherapy	Therapy-related AML, prior myelodysplastic syndrome, myeloproliferative or other hematologic disorder
t(8;21), inv(16)/t(16;16), t(15;17)	Complex karyotypic abnormalities, -5, -7, 3q26 aberrations, t(6;9), 11q23 aberrations except for t(9;11), "monosomal karyotype"
NPM1 mutation, *CEBPA* mutation	*FLT3/ITD* mutation

Table 1.7 Prognostic factors in AML

MDR 1: multidrug resistance protein 1

1.7.9 Treatment of AML

1.7.9.1 Introduction

The treatment of adult patients with AML has improved over the last several as evidenced by improved OS over time. However most of the observed progress has been among patients younger than 60 years of age, with smaller survival improvements among older patients (383). Much of the survival improvement may be attributable to enhancements in supportive care.

AML is a heterogeneous disease and the emerging prognostic knowledge concerning cytogenetic and molecular abnormalities are heralding a new era of individualized therapeutic approaches for patients. Identification of key molecular and cytogenetic abnormalities may allow for the addition of specific targeted small molecule inhibitors to cytotoxic chemotherapy. Moreover our appreciation of epigenetic aberrations in AML (see section 1.1.4.1) has led to the development of effective agents that target DNA methylation restoring the normal expression tumour suppressor genes (384). Hypomethylating agents such as azacitidine and decitabine are now approved for the treatment of AML and offer a less intensive approach for elderly patients (385, 386). Lastly our understanding of the properties of LSCs are now leading to new therapeutic approaches that target the quiescent AML stem cell population to prevent relapse (387, 388).

1.7.9.2 Treatment of APL

APL is a biologically and clinically distinct variant of AML (see Appendix B). It represents an emergency and without treatment patients have a median survival of less than one month (389). However, with modern therapy, APL is associated with the highest proportion of patients who are cured of their disease. A key component of the initial therapy is the use of all-trans retinoic acid (ATRA), which promotes the terminal differentiation of malignant promyelocytes to mature neutrophils. The traditional central

dogma in anti-cancer therapy has been to inhibit malignant cell proliferation or induce apoptosis with cytotoxic agents such as chemotherapy or radiotherapy. However, the finding of differentiation arrest, the accumulation of APL cells blocked at the promyelocytic stage of granulocytic differentiation, suggested the possibility of inducing cell differentiation as an alternative way to treat leukaemia. Early support for the rationale of this approach came from the finding that myeloid leukaemia cells could be induced by cytokines to resume normal differentiation and become non-dividing mature granulocytes or macrophages (390, 391). Later it was reported that retinoic acid (RA) and to a greater extent ATRA could induce terminal differentiation of human APL cells *in vitro* (392, 393). In a clinical trial 23 out of 24 (95.8%) APL patients treated with ATRA went into CR without developing BM hypoplasia or abnormalities of clotting (394). These findings were confirmed in larger randomized studies (395, 396).

The t(15;17) translocation in APL results in the generation of the fusion gene and protein, the promyelocytic leukaemia retinoic acid receptor alpha (PML-RARα), which plays a central role in APL pathogenesis (see Appendix B). The PML-RARα protein has several properties. Firstly it creates a complex with retinoid x receptor (RXR), nuclear corepressors (N-CoR), Sin3A, and histone deacetylase (HDAC) that represses the transcriptional expression of target genes (397). Secondly it acts in a dominant negative manner on the retinoic acid-signalling pathway which blocks the differentiation of myeloid cells (398); Therefore the normal function of PML as a growth inhibitor and regulator of apoptosis is disturbed when incorporated into PML-RARα complex.

ATRA has several mechanisms of action. The binding of ATRA to RAR receptors causes degradation of PML-RARα protein through the ubiquitin-protosome and caspase system, leading to restoration of terminal differentiation of promyelocytes (399, 400). Additionally, ATRA leads to disassociation of N-CoR from the repressive complex and recruitment of

CoA (coactivator) (397). As a result, the repression of transcriptional activation of target genes is relieved and the differentiation of promyelocytes is restored (Figure 1.13).

Arsenic trioxide (As_2O_3) is also effective in APL, exerting dual effects on APL cells. At high concentrations (0.5–1.0 μM), As_2O_3 induces apoptosis (401-403). However at lower concentrations, As_2O_3 can induce APL cells to partially differentiate along the granulocytic pathway. Degradation of PML-RARα in the presence of lower concentrations of As_2O_3 favours the release of differentiation arrest. In addition, acetylation of histones 3 and 4 probably contributes to the mechanism of the differentiation process (401).

ATRA must be combined with other agents since remissions induced by ATRA therapy alone are short-lived (404). The largest experience has been with ATRA combined with anthracycline-based chemotherapy but combination with arsenic trioxide can be considered for patients who are not candidates for anthracycline-based therapy (405).

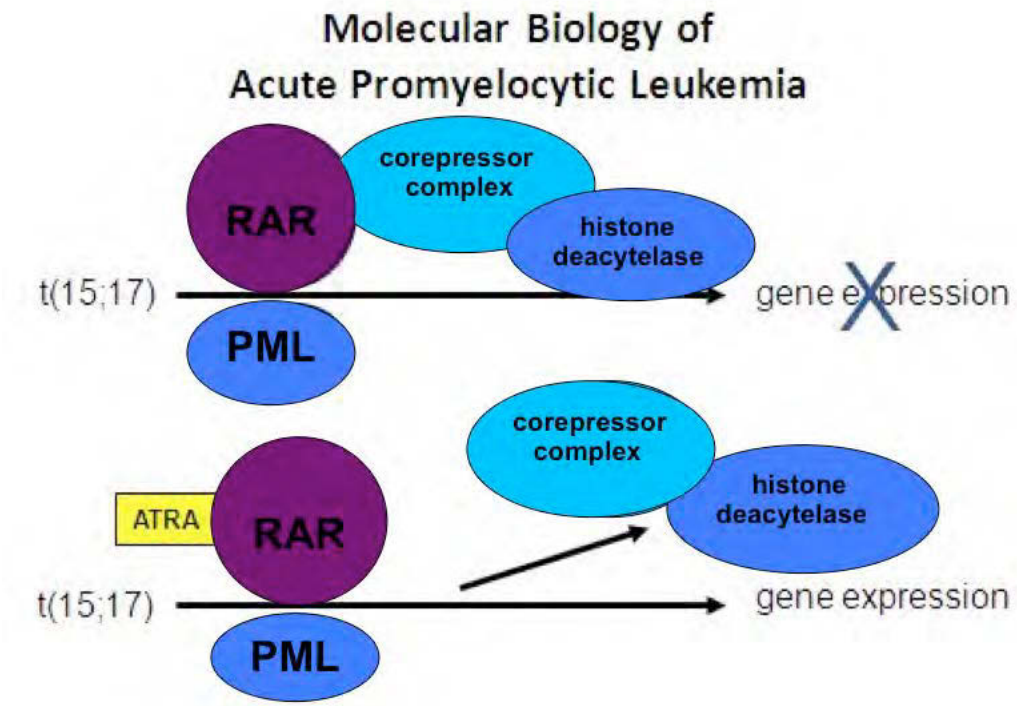

Figure 1.13 Molecular biology of APL

The *PML-RARα* fusion gene consists of the promyelocytic leukaemia gene *(PML)*, a tumour suppressor normally found in nuclear bodies, and the retinoid acid receptor alpha gene *(RAR)*, a transcription factor that is normally responsive to retinoid acid. The juxtaposition of these two genes produces a protein that does not respond to the usual form of retinoic acid. However, it does respond to all-trans retinoid acid, leading to restoration of gene transcription and thereby to cellular differentiation. Arsenic trioxide leads to a similar effect because it causes aggregation and degradation of the PML-RARA fusion protein, thereby promoting differentiation.

1.7.10 Induction therapy for AML

The goal of induction therapy is to rapidly restore normal BM function and to reduce the leukaemic cell burden from approximately 10^{12} to 10^{9} cells (below cytological detectable levels). Various acceptable induction regimens are available. The most common approach, "3 and 7," consists of 3 days of an anthracycline (idarubicin or daunorubicin) combined with ara-C for 7 days. Depending upon age and patient selection, 70 to 80 percent of younger adults achieve a CR with these regimens (406, 407). Most remissions come after a single course.

Higher dose daunorubicin may be more beneficial in younger patients. Two large randomized controllsed trials (RCTs) compared conventional-dose daunorubicin (45 mg/m^2/day for 3 days) or high-dose daunorubicin (90 mg/m^2/day for 3 days) in patients younger than 60 years with untreated AML (407, 408). These induction regimens were administered with ara-C. In both studies a higher CR rate was observed in the high-dose daunorubicin group (64 and 70.6%) relative to the conventional dose (54 and 57.3%). However, only one of the studies showed an improved OS.

1.7.11 Consolidation therapy

Without consolidation therapy most patients will eventually relapse. In patients aged 60 years or younger, treatment options for consolidation therapy include high-dose ara-C, autologous HSCT and allogeneic HSCT. In a landmark study performed by CALGB, 3 different doses of ara-C were evaluated in patients with AML who achieved remission after standard “3 and 7” induction chemotherapy (409). Patients received 4 courses of ara-C at one of the following dosages: (1) 100 mg/m^2/day by continuous infusion for 5 days, (2) 400 mg/m^2/day by continuous infusion for 5 days, or (3) 3 g/m^2 in a 3-hour infusion every 12 hours on days 1, 3, and 5. The probability of remaining in continuous CR after 4 years in patients aged 60

years or younger was 24% in the 100 mg group, 29% in the 400 mg group, and 44% in the 3 g group. The outcome in older patients did not differ. On the basis of this study, high-dose ara-C for 3 to 4 cycles is a standard option for consolidation therapy in younger patients.

1.7.12 Role of transplantation

Several large trials have compared allogeneic HSCT, autologous HSCT, and chemotherapy without HSCT in order to define the best postremission therapy for young patients. Outcome after autologous HSCT is considered at least as good as the use of postremission chemotherapy; however, there has been no evidence of an improvement in outcome. Autologous HSCT can be considered as an alternative option for postremission therapy in patients with favourable and intermediate-risk cytogenetics, whereas it cannot be recommended in patients with high-risk cytogenetics (410, 411).

Allogeneic HSCT as a postremission strategy is associated with the lowest rates of relapse. As is the case in CML, the benefit is attributable to both the high-dose therapy of standard conditioning regimens and a potent GVL effect (412). However, the advantages of allogeneic HSCT are offset by the high transplant related mortality. Single prospective trials have neither shown a definitive advantage nor disadvantage in OS of allogeneic HSCT for patients with AML in first CR (413-415). Meta-analyses of clinical trials that prospectively assigned allogeneic HSCT versus alternative consolidation therapies for AML in first CR on an intent-to-treat donor versus no-donor basis show that allogeneic HSCT offers significant OS benefit for patients with intermediate and high risk AML (416, 417). No advantage has been shown for autologous or allogeneic HSCT in frontline treatment of patients with good-risk cytogenetics (t(8;21) or inv(16))(359, 360, 416-419). Therefore these patients should be offered consolidation with high-dose ara-C as they have a good prognosis with this approach. Allogeneic HSCT transplantation should be reserved for patients who

relapse. A study by the German-Austrian AML Study Group (AMLSG) provided evidence that those AML patients whose molecular genetic profile predicts a favourable prognosis, such as cytogenetically normal AML with mutated *NPM1* without *FLT3*-ITD, may also not benefit from allogeneic HSCT (420).

On the other hand patients with high-risk cytogenetics findings are rarely cured with chemotherapy and should be offered transplantation in first CR. The best approach for patients with intermediate-risk cytogenetics findings is controversial. Studies using newer molecular markers, such as *FLT3*, *NPM1* and *CEBPA* are helping to define which patients with cytogenetically normal AML should receive standard consolidation therapy versus transplantation. For the remaining patients with intermediate-risk cytogenetics repetitive cycles of high dose ara-C are currently widely used but there is accumulating evidence that allogeneic HSCT is an attractive option for those patients who are at high risk of relapse. A beneficial effect has been shown for patients with normal cytogenetics and unfavourable molecular markers, that is, those who lack the favourable genotypes of mutated *NPM1* or mutated *CEBPA* without *FLT3*-ITD (420). In particular, although evidence from prospective trials is not available, allogeneic HSCT should be considered in patients whose leukaemic cells have *FLT3*-ITD (420).

1.7.13 Treatment of older adults

The management of older patients with AML (particularly those older than 75 years) is a difficult challenge. Compared to younger patients, older patients variably defined as > 55, >60 or > 65 have poorer performance status, higher incidence of multidrug resistance, lower percentage of favourable cytogenetics, higher percentage of unfavourable cytogenetics, higher treatment-related morbidity and mortality and higher incidence of treatment-resistant disease.

Some older patients do reasonably well with standard therapy. In an analysis of 998 older patients treated at MD Anderson Cancer Center, age greater than 75 years, poor performance status, previous antecedent hematologic disorder, unfavourable karyotype, renal insufficiency, and/or treatment outside of a laminar flow room were associated with an adverse outcome (421). Patients with none of these risk factors had a CR rate of 72% and median 2-year survival of 35%, whereas patients with 3 or more risk factors had a CR rate of 24% and a median 2-year survival of only 3%. Thus, some low-risk elderly patients can benefit from standard intensive chemotherapy.

The hypomethylating agents azacitidine and decitabine are also options for the treatment of elderly AML patients. A phase 3 randomized, open label study compared azacitidine with investigator selected therapy in 358 patients with higher risk MDS or AML (385). Compared with the patients treated at the discretion of their physicians, those assigned to receive azacitidine demonstrated an improved OS (median, 24.5 versus 15 months). Decitabine has shown promise in older patients with adverse cytogenetic features (386).

Occasionally, intensive treatment with the intent to achieve CR may be less advisable because of advanced patient age, debility, presence of significant co-existing medical problems and/or prior chemotherapy. These patients may be candidates for clinical trials with investigational agents that target typical AML molecular aberrations and that are less likely to cause severe cytopaenias. Some of these novel therapies such as alisertib or SGI-1776, discussed below, are particularly attractive for elderly patients in that they are available orally.

1.7.14 Remission criteria

As discussed above induction therapy for AML aims to achieve a CR in which the total body leukaemic cell population is reduced to below a cytologically detectable level. It is generally assumed that a substantial

burden of leukaemia cells persists undetected (i.e. MRD), leading to relapse within a few weeks or months if no further post-remission therapy (i.e. additional consolidation chemotherapy) were to be administered. CR is defined by the International Working Group criteria using morphologic and clinical data (Table 1.8). Ascertaining whether a patient in CR is destined to remain clinically disease-free after post-remission therapy is limited by the inherent insensitivity of routine tests on the BM for detecting residual leukaemia and the likelihood that the small area of BM examined does not reflect the much larger BM compartment. Techniques employing qRT-PCR and multiparameter flow cytometry techniques are significantly more sensitive for detecting the presence or absence of MRD than morphology or cytogenetics.

Category	Definition
Complete remission (CR)*	BM blasts < 5%; absence of blasts with Auer rods; absence of extramedullary disease; absolute neutrophil count > 1.0 × 10^9/L (1000/μL); platelet count > 100 × 10^9/L (100 000/μL); independence of red cell transfusions
CR with incomplete recovery (CRi)†	All CR criteria except for residual neutropaenia (< 1.0 × 10^9/L [1000/μL]) or thrombocytopaenia (< 100 × 10^9/L [100 000/μL])
Morphologic leukaemia-free state‡	BM blasts < 5%; absence of blasts with Auer rods; absence of extramedullary disease; no haematologic recovery required
Partial remission (PR)	Relevant in the setting of phase 1 and 2 clinical trials only; all hematologic criteria of CR; decrease of BM blast percentage to 5% to 25%; and decrease of pretreatment BM blast percentage by at least 50%
Cytogenetic CR (CRc)§	Reversion to a normal karyotype at the time of morphologic CR (or CRi) in cases with an abnormal karyotype at the time of diagnosis; based on the evaluation of 20 metaphase cells from BM
Molecular CR (CRm)‖	No standard definition; depends on molecular target
Treatment failure	
Resistant disease (RD)	Failure to achieve CR or CRi (general practice; phase 2/3 trials), or failure to achieve CR, CRi, or PR (phase 1 trials); only includes patients surviving ≥ 7 days following completion of initial treatment, with evidence of persistent leukaemia by blood and/or BM examination
Death in aplasia	Deaths occurring ≥ 7 days following completion of initial treatment while cytopaenic; with an aplastic or hypoplastic BM obtained within 7 days of death, without evidence of persistent leukaemia
Death from indeterminate cause	Deaths occurring before completion of therapy, or < 7 days following its completion; or deaths occurring ≥ 7 days following completion of initial therapy with no blasts in the blood, but no BM examination available
Relapse¶	BM blasts ≥ 5%; or reappearance of blasts in the blood; or development of extramedullary disease

Table 1.8 Response criteria in AML

* All criteria need to be fulfilled; marrow evaluation should be based on a count of 200 nucleated cells in an aspirate with spicules.

† The criterion of CRi is of value in protocols using intensified induction or double induction strategies, in which haematologic recovery is not awaited, but intensive therapy will be continued.
‡ This category may be useful in the clinical development of novel agents within phase 1 clinical trials, in which a transient morphologic leukaemia-free state may be achieved at the time of early response assessment.

§ Four studies showed that failure to convert to a normal karyotype at the time of CR predicts inferior outcome (422-425).

‖ As an example, in CBF AML low-level PCR-positivity can be detected in patients even in long-term remission. Normalizing to 104 copies of ABL1 in accordance with standardized criteria, transcript levels below 12 to 10 copies appear to be predictive for long-term remission (426, 427).

¶ In cases with low blast percentages (5-10%), a repeat marrow should be performed to confirm relapse. Appearance of new dysplastic changes should be closely monitored for emerging relapse. In a patient who has been recently treated, dysplasia or a transient increase in blasts may reflect a chemotherapy effect and recovery of hematopoiesis. Cytogenetics should be tested to distinguish true relapse from therapy-related MDS/AML.

1.7.15 Treatment of relapsed disease

Patients with relapsed AML have an extremely poor prognosis. Young patients who have not previously undergone transplantation should be referred for such therapy. The chances of obtaining a second remission with chemotherapy correlate strongly with the duration of the first remission. For example in one analysis, patients with initial CR duration of longer than 2 years had a 73% CR rate with initial salvage therapy while those with an initial CR duration of 1-2 years had a CR rate of 47% with initial salvage therapy (428).

1.7.16 Emerging treatments for AML

Patients failing all conventional drug protocols may elect to undergo investigational treatment with novel agents or protocols (Table 1.9). As discussed in section 1.1.5 the AML phenotype results from multiple genetic/epigenetic lesions affecting differentiation, proliferation, and apoptosis. Consequently, targeting of a single aberrant protein is unlikely to eradicate the leukaemic clone. Furthermore, although several molecularly targeted therapies have been shown to be active in AML, it is clear from early clinical studies that most of these novel agents will need to be used in combination with conventional cytotoxic therapy. A number of the most important novel agents in AML are discussed below.

Clofarabine is a novel purine nucleoside analogue structurally similar to fludarabine and cladribine, developed with the aim of avoiding the neurotoxicity that limits the maximal tolerated dose of old-generation purine analogues. Phase 2 trials of the nucleoside analogue clofarabine, with or without ara-C, in patients with relapsed/refractory AML have yielded CR rates of 28 to 42 percent (429, 430). A phase 3 study comparing clofarabine with traditional 7+3 as induction in newly diagnosed older patients with AML is currently recruiting.

Clinical trials with a number of different oral FLT3 inhibitors including lestaurtinib (CEP701), sunitinib malate (SU11248), sorafenib and midostaurin (PKC412) have been initiated (431-435). Most responses were incomplete and transient. However, lestaurtinib and midostaurin can be safely combined with standard AML chemotherapy and randomized trials comparing chemotherapy with or without these FLT3 inhibitors are underway. A report of a phase 3 trial of induction chemotherapy with or without lestaurtinib in 224 patients with FLT3 mutation and relapsed or refractory AML showed that the addition of lestaurtinib lestaurtinib failed to increase response rates or OS (436). However correlative studies found that FLT3 was not inhibited in many patients suggesting that a different dose or schedule or more potent agents may be necessary.

Gemtuzumab ozogamicin (GO) is a humanized, monoclonal antibody against CD33 that is covalently attached by a linker to calicheamicin, a potent toxin that binds to double-stranded DNA and causes breaks. Since CD33 is almost universally expressed on AML myeloblasts, the antibody delivers the toxin directly to the leukaemic blasts (437). GO was approved by the FDA for use in patients aged 60 or older with CD33+ AML in first relapse who are not considered candidates for cytotoxic chemotherapy (438-440). The FDA has since required that GO be removed from the market in the United States because subsequent randomized trials did not confirm a clinical benefit for newly diagnosed patients with AML when it was combined with standard chemotherapy. Further studies of GO in specific patient populations are continuing. Unconjugated anitbodies against CD33 have also been investigated. Lintuzumab is an unconjugated humanized murine monoclonal antibody directed against CD33. It was employed in a randomized phase 3 study in 191 patients with refractory or relapsed AML, which utilized combination chemotherapy (mitoxantrone, etoposide, cytarabine) with or without lintuzumab (441). While the addition of this agent to salvage chemotherapy

was safe, it did not result in significant improvement in response rate or survival.

An alternative approach is to modify the formulation of cytotoxic chemotherapy to improve its efficacy. For example, CPX-351 consists of a 5:1 molar ratio of ara-C to daunorubicin enclosed in a liposome. Preclinical studies showed that the 5:1 molar ratio is the most synergistic ratio of these 2 drugs in AML cell lines. In early phase clinical studies, CRs were seen in patients previously exposed to ara-C and daunorubicin (442). Elacytarabine is a lipophilic variation of ara-C that is independent of the human equilibrative nucleoside transporter 1 (hENT1) protein and is not a substrate of deoxycytidine deaminase. As both of these are mechanisms of resistance to ara-C, elacytarabine has been evaluated in the relapse setting (443).

Lastly several approaches have been undertaken to modify the microenvironment of LSC. Interfering with BM retention and mobilization of LSCs by disrupting the Stromal Cell-Derived Factor 1 (SDF-1)/ C-X-C chemokine receptor type 4 (CXCR4) axis has demonstrated to be promising for future therapies in patients with AML (444). CXCR4 belongs to the large superfamily of G protein-coupled receptors, and is directly involved in a number of biological processes including organogenesis, haematopoiesis, and immune response (445). Grafting of HSCs is a complex process regulated by several signalling pathways of which the SDF-1/CXCR4 ligand/receptor system plays a predominant role (446). Furthermore, high CXCR4 expression is a known negative prognostic marker in AML (447). Mounting evidence indicates that mobilization of HSC or LSC, from their protective niches, render them vulnerable to chemotherapeutic treatment (448). A phase 1/2 study of mitoxantrone, etoposide, and cytarabine (MEC) in combination with the CXCR4 antagonist, plerixafor, in 52 patients with relapsed and refractory AML reported a CR rate of 39 percent (449).

1.7.17 Conclusion

The treatment of AML has not changed significantly over the last 40 years. However recent progress in our understanding of the biology of this disease and identification of driver mutations has ushered in a new era of molecular therapeutics. A number of molecular markers and pathways have been identified and may serve as potential therapeutic targets. It is commonly accepted that the AML phenotype results from multiple genetic/epigenetic lesions affecting differentiation, proliferation, and apoptosis. Consequently, targeting of a single aberrant protein is unlikely to eradicate the leukaemic clone. Finally, although several molecularly targeted therapies have been shown to be active in AML, it is clear from early clinical studies that most of these novel agents will need to be used in combination with conventional cytotoxic therapy.

Mechanism of Action	Drug
FLT3 kinase inhibitors	Lestaurtinib, sorafenib, midostaurin
Modified Chemotherapy - Liposomal formulation	CPX-351
Modified Chemotherapy - Lipid conjugate	Elacytarabine
Conjugated monoclonal antibody	Gemtuzumab ozogamicin
Unconjugated monoclonal antibody	Lintuzumab
CXCR4 antagonist	Plerixafor
Epigenetic modulation	Azacytidine/decitabine/vorinostat
Nucleoside Analogues	Clofarabine

Table 1.9 New agents and strategies for AML

1.8 Aurora kinases

1.8.1 Introduction

The first Aurora kinase discovered, Aurora A, derived its name from a mutant form of the protein found in Drosophila melanogaster that caused the formation of monopolar spindles reminiscent of the aurora borealis (450). There are three known members of the Aurora family expressed in mammalian cells: Aurora A, B, and C. Aurora A and B are expressed in all proliferating cells, whereas Aurora C expression is mainly restricted to the testes and plays a role in spermatogenesis (451). Aurora A and B regulate a diverse array of events throughout mitosis. Aurora C functions similarly to Aurora B (452). The catalytic domain is highly conserved amongst all three Auroras, with 67-76% amino acid similarity. However, greater sequence diversity exists in their non-catalytic regions, allowing for variations in their subcellular localization, regulatory partners, and substrate specificity (453).

Aurora A and B are overexpressed in a wide variety of tumour types and their overexpression has been associated with tumourigenesis and a worse outcome in patients. These features, along with their vital roles in mitosis, make the Aurora kinases attractive targets for anti-cancer therapy. A number of small molecule inhibitors of Aurora kinases have demonstrated significant antiproliferative activity in preclinical models and have produced objective responses in early phase clinical trials (454-456).

1.8.2 Aurora A

In normal cells, the distribution and expression of Aurora A kinase is tightly regulated throughout the cell cycle with expression increasing during the late S phase and peaking during the G_2/M transition (Figure 1.14) (457). As discussed in section 1.3.2, G_2/M progression is triggered by translocation of the cyclin B-CDK1 complex to the nucleus and activation by dephosphorylation by CDC25. The regulation of the progression from G_2 to M by Aurora A has been proposed to occur, in part, through the relocalization of cyclin B1 to the nucleus and activation of CDKs.

Suppression of Aurora A expression leads to G_2 to M arrest, whereas ectopic expression abrogates the G_2/M checkpoint (453). During G_2, Aurora A forms a complex with its cofactor hBORA, and phosphorylates PLK1 at Thr^{210} (458). Subsequently, activated PLK1 phosphorylates CDC25 and WEE1, which induces activation of cyclin B-CDK1 complexes to promote mitotic entry (Figure 1.15) (459).

Aurora A kinase activity is also critical for the formation of a normal mitotic spindle. In the G_2 phase, Aurora A is localized to centrosomes and is activated by the LIM protein Ajuba (460). Once activated, Aurora A phosphorylates and recruits microtubule-associated proteins (MAPs) to the centrosome to promote centrosome maturation (453). Aurora A is necessary for the recruitment of several proteins essential to the spindle formation including transforming acid coiled-coil protein-3 (TACC3), a MAP that stabilizes centrosomal microtubules; kinesin 5, a motor protein involved in the formation of the bipolar mitotic spindle; and γ-tubulins, the base structure from which centrosomal microtubules polymerize (461, 462). Aurora A also assures proper organization and alignment of the chromosomes during prometaphase. It interacts directly with the kinetochore on each chromosome and phosphorylates and stabilizes HURP (hepatoma upregulated protein), which in turn stabilizes kinetochore fibers and ensures efficient kinetochore capture by the microtubules and normal inter-kinetochore tension (463, 464). Finally, an appropriate level of Aurora A expression is necessary to orchestrate an exit from mitosis by cytokinesis. Both overexpression and inhibition of Aurora A activity lead to failure of cytokinesis and multinucleation (450, 465-471).

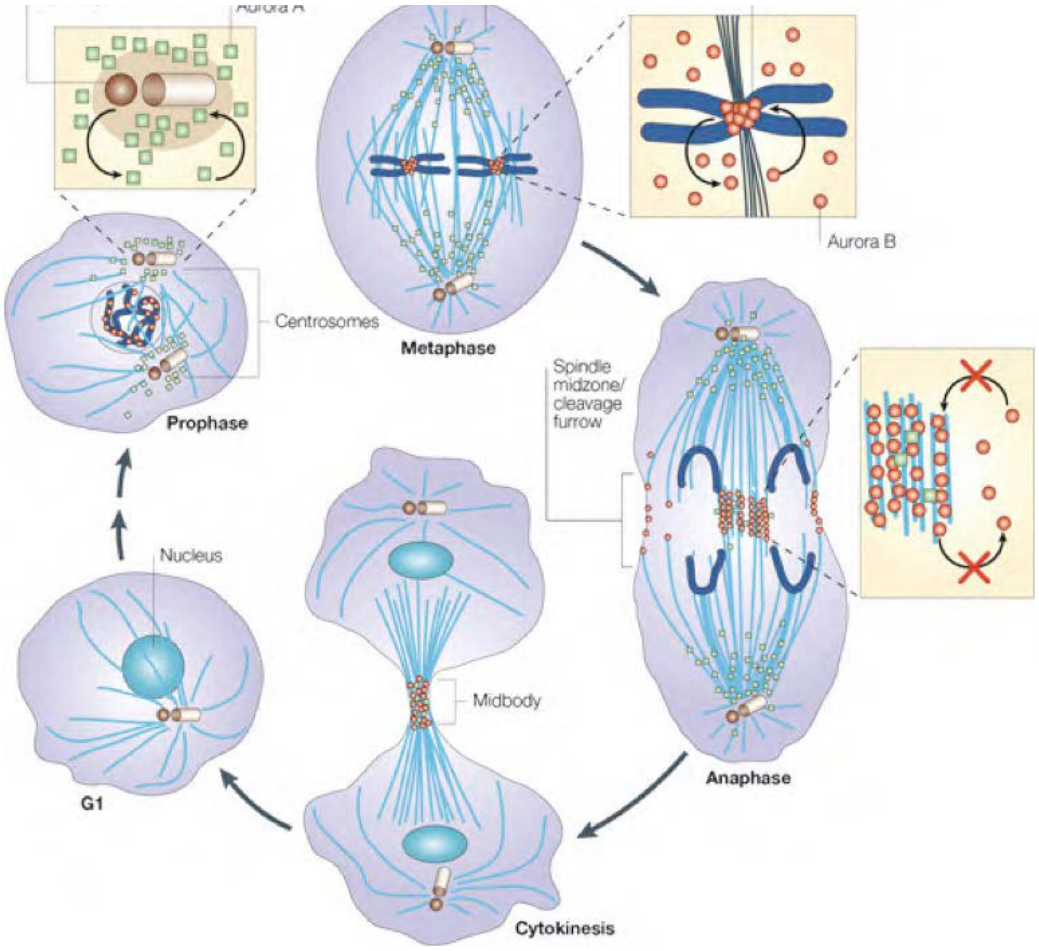

Figure 1.14 Cellular localization of Aurora A and B in mitosis

The level of both kinases is substantially reduced in G_1 cells. By prophase, Aurora A (green boxes) is concentrated around the centrosomes, whereas Aurora B (red circles) is nuclear. In metaphase, Aurora A is on the microtubules near the spindle poles, whereas Aurora B is located in the inner centromere. In anaphase, most Aurora A is on the polar microtubules, but some might also be located in the spindle midzone. Aurora B is concentrated in the spindle midzone and at the cell cortex at the site of cleavage-furrow ingression. In cytokinesis, both kinases are concentrated in the midbody. Modified with permission from Nature Publishing Group: Carmena and Earnshaw (2003) (472).

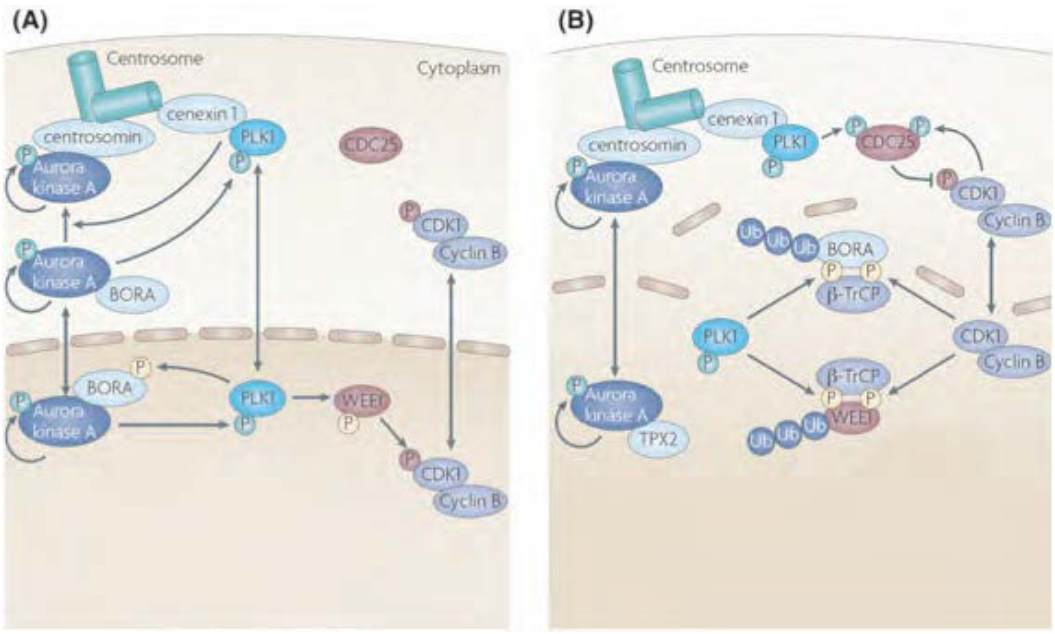

Figure 1.15 Functions of Aurora A during late G_2 and mitosis

(A) In late G_2, Aurora A autophosphorylates and also activates PLK1, which in turn promotes the binding of Aurora A to centrosomin. PLK1 is recruited to the centrosome by cenexin 1. While Aurora A can self-activate, its activity is enhanced by several cofactors, including targeting protein for TPX2 and BORA. Active PLK1 phosphorylates WEE1, a kinase that negatively regulates cyclin B-CDK1 complexes. The CDC25 phosphatases that antagonize WEE1 are largely inactive in G_2. (B) Mitotic entry is triggered by a steep increase in cyclin B-CDK1 activity. On the centrosome, Aurora A and PLK1 promote recruitment and activation of CDC25B, which in turn, activates cyclin B-CDK1. Cyclin B–CDK1 complexes further phosphorylate WEE1 and BORA, which are then recognized by the F-box protein β-transducin repeat containing (β-TrCP) that promotes polyubiquitylation (Ub) and degradation of WEE1 and BORA. Freed of BORA, Aurora A is then able to interact with TPX2, thus facilitating the role of the kinase in spindle assembly. Green phosphates (P) are activating, red phosphates are inhibitory and yellow phosphates prime a protein for degradation. Modified with permission from Nature Publishing Group: Lens et al (2010) (255).

1.8.3 Aurora B

Aurora B is critical for correct microtubule-kinetochore attachments, the establishment of the spindle assembly checkpoint and cytokinesis (473-476). Aurora B is the enzymatic core of the chromosomal passenger complex (CPC), so named for its precise movement from site to site at specific times. In the CPC, it is associated with multiple other proteins including inner centromere protein (INCENP), survivin, and borealin. INCENP forms the stabilizing core, survivin mediates binding of the CPC to the centromere, and borealin mediates binding of survivin to INCENP (452). These proteins help direct Aurora B's activity by allowing it to be in the right place at the right time. The CPC is associated with the centomeres from prometaphase to metaphase. After chromatid separation, the CPC travels to the midzone to complete cytokinesis.

Aurora B phosphorylates histone H3 at Ser^{10} and Ser^{28} and this may facilitate chromosome condensation and subsequent alignment during mitosis (477). Cells depleted of the Aurora B kinase show only partial chromosome condensation at mitosis (475). Correct chromosomal biorientation mediated in part by the attachment of microtubules emanating from opposite spindle poles to the kinetochores is required for accurate chromosome segregation and alignment. Aberrant kinetochore to microtubule connections such as syntelic (each kinetochore attaches to the same pole) or merotelic (each pole attaches to a single kinetochore) attachments can result in aneuploidy and chromosomal instability if left unfixed (473). Aurora B ensures accurate separation of chromatids in multiple ways. The mitotic centromere associated kinase (MCAK) enables microtubule attachment to the kinetochore (478). Aurora B temporally and spatially regulates MCAK activity by phosphorylation of MCAK at multiple sites. Phosphorylation of MCAK by Aurora B targets MCAK to the kinetochores where its microtubule depolymerase activity severs the kinetochore to microtubule attachment thereby relieving inappropriate kinetochore-microtubule attachments (Figure 1.16) (479, 480). Disruption

of Aurora B or MCAK activity leads to chromosomal misalignment and improper kinetochore to microtubule attachment (481). Secondly, Aurora B recruits spindle checkpoint proteins such as MAD2 during metaphase. The spindle assembly checkpoint complex (discussed in section 1.3.8) detects incorrect kineotchore to microtubule attachments and prevents the cell from entering anaphase if they are present (146, 453).

Aurora B also plays a critical role in cytokinesis and its depletion results in mitotic exit without cell separation, leading to cells in the G_1 portion of the cell cycle with a doubling in the size of the normal DNA complement at this phase of the cell cycle (453). Depending on other factors, these cells may undergo additional rounds of DNA synthesis, resulting in polyploidy. Aurora B-mediated phosphorylation of vimentin on Ser^{72} is necessary for normal cytokinesis as inhibition of this process results in long bridge-like intermediate filament structures between unseparated daughter cells (476). Figure 1.17 outlines the multiple functions of Aurora A and B in mitosis.

1.8.4 Aurora C

The expression of Aurora C is largely restricted to the testis and it regulates chromosome segregation during meiosis (482). Although Aurora C has been detected in some cancer cell lines, the involvement of Aurora C in the mitotic division of somatic cells or in oncogenesis is not clear.

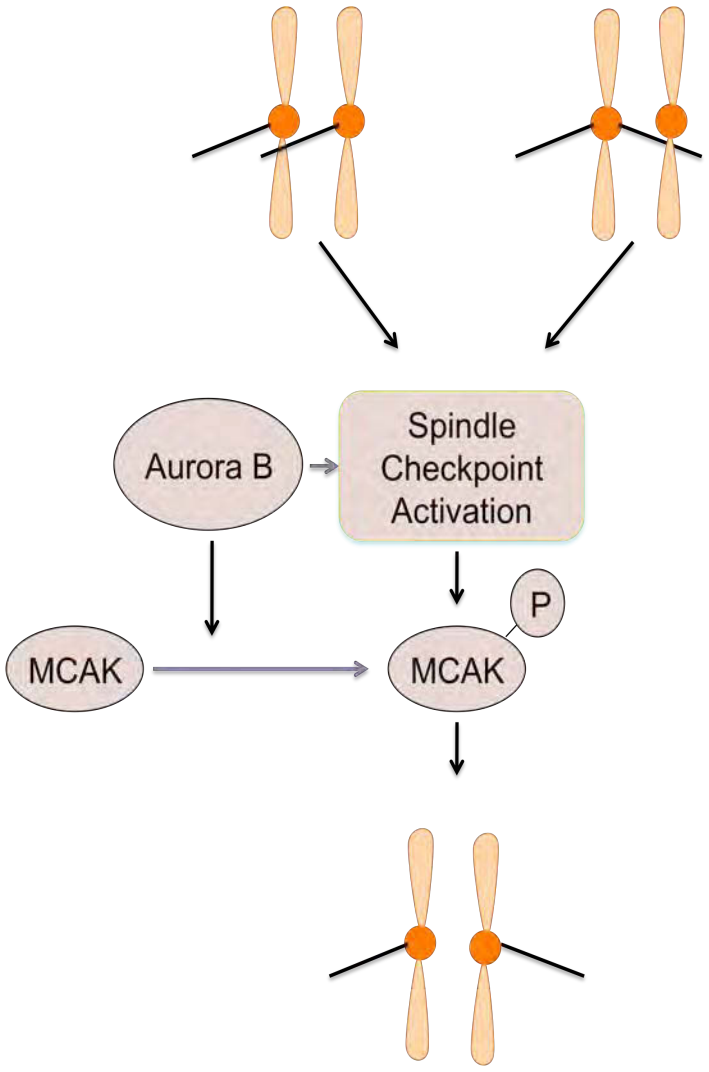

Figure 1.16 Aurora B corrects chromosome misalignments

Incorrect chromosome alignment can occur when microtubules from each kinetochore attach to the same pole (syntelic, **top left**) and when microtubules from each pole attach to a single kinetochore (merotelic, **top right**). Aurora B localizes the mitotic centromere associated kinase protein (MCAK) to the centromere where it removes incorrect kinetochore to microtubule attachments. Aurora B recruits checkpoint proteins to the spindle checkpoint that prevents cells with aberrant chromosome attachments form entering anaphase.

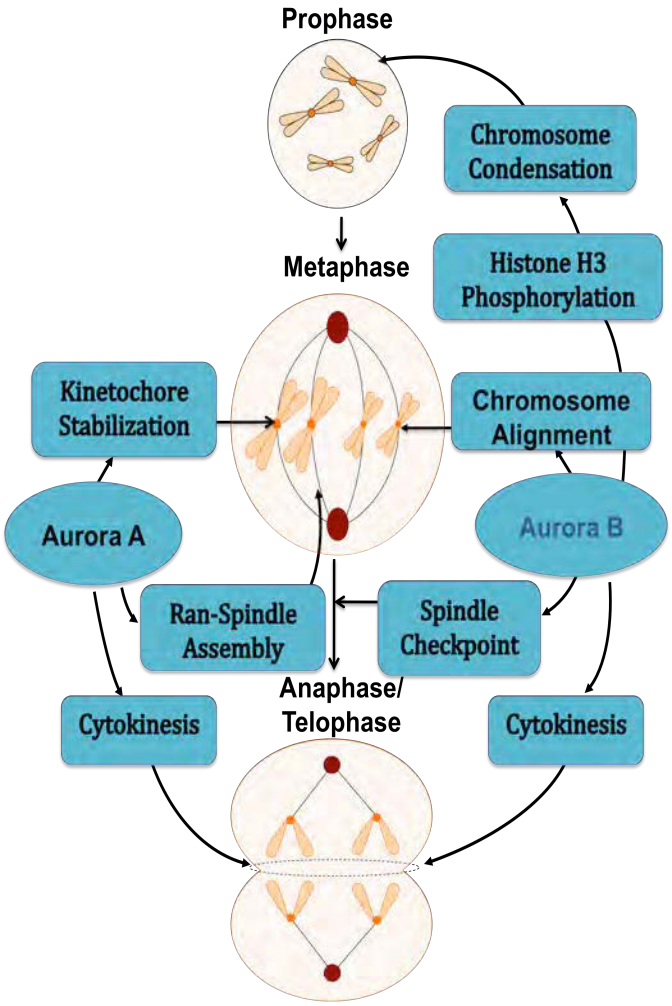

Figure 1.17 The multiple functions of Aurora kinases during mitosis

Aurora A helps maintain proper alignment and organization of the chromosomes during prometaphase. It also recruits spindle assembly proteins and plays a critical role in the spindle assembly process. At the end of mitosis, it has a role in cytokinesis. In the prophase, Aurora B facilitates chromosome condensation through phosphorylation of histone H3. During metaphase, Aurora B ensures accurate separation of chromatids and prevents the cell from entering anaphase if unattached kinetochores exist through recruitment of spindle checkpoint proteins. Finally, Aurora A and B play a critical roles in cytokinesis.

1.8.5 Aurora kinases in malignancy

Aurora kinases are aberrantly expressed in a variety of solid tumours including prostate, colon, breast, lung, prostate, head and neck and thyroid cancer as well as in most forms of leukaemia (483-488). High levels of Aurora A expression have been associated with amplification of the chromosomal region 20q13.2 which comprises the Aurora A kinase gene (*AURKA*) (489). Additional mechanisms leading to Aurora A overexpression in malignancy include transcriptional induction and post-translational stabilization (490, 491). In contrast to *AURKA*, the Aurora B kinase gene (*AURKB*) does not appear to be amplified in cancer. However, it is highly expressed in many tumour types including prostate as well as head and neck squamous cell carcinoma (486, 487).

There is evidence that Aurora A may function as an oncogene that transforms cells through the induction of genetic instability and enhanced survival signalling (483). Overexpression of Aurora A in mouse mammary epithelium led to genetic instability characterized by centrosome amplification, chromosome tetraploidization, premature sister chromatid segregation, and subsequently mammary tumour formation. These chromosomal abnormalities did not cause cell death possibly because increased Aurora A expression also induced pro-survival pathways such as AKT, mammalian target of rapamycin (mTOR), and nuclear accumulation of cyclin D1 (492). One potential mechanism of oncogenesis due to high Aurora A expression may be through Aurora A-mediated degradation of p53. As discussed in section 1.3.4, p53 is crucial for the post-mitotic checkpoint and induces growth arrest or apoptosis in cells exposed to stress. p53 function is frequently disrupted in cancers, often due to mutation or deletion. Aurora A has been shown to phosphorylate p53 at Ser^{315} which leads to its ubiquitination by murine double minute 2 (MDM2) and proteolysis (493). Therefore, ectopic expression of Aurora A may lead to down-regulation of the post-mitotic checkpoint response, inducing chromosomal instability and oncogenic transformation.

Aurora A may also play a role in preventing apoptosis in cancer cells by indirectly activating nuclear factor kappa B (NF-κB) through phosphorylation of its inhibitor IκB, which targets IκB for degradation (494). NF-κB, in turn, promotes the expression of its transcriptional target, BCL-2, a key anti-apoptotic protein (discussed in section 1.4.2). Overexpression of BCL-2 is associated with adverse prognosis in many forms of cancer as it diminishes cellular pro-apoptotic potential and consequently, confers drug resistance. Interestingly, it was recently shown that treatment with the Aurora kinase inhibitor VX-680/MK-0457 increases the BAX/BCL-2 ratio in AML cells helping to restore normal apoptosis (495).

Several studies including those in head and neck squamous cell carcinoma, ovarian cancer, neuroblastoma, and glioblastoma have shown that high levels of Aurora A messenger RNA in tumour specimens correlate with centrosome abnormalities, tumour progression and shortened survival (487, 496, 497). These findings support a role for Aurora kinases in oncogenesis. Similarly, high Aurora B expression has been correlated with adverse histological features and shortened survival in a variety of malignancies (486, 497). Ongoing clinical investigations are addressing the question of whether or not high Aurora kinase expression in tumours will serve as useful predictors of response to pharmacological inhibition with Aurora kinase inhibitors.

1.8.6 Aurora kinase inhibitors in development

The realization that Aurora kinases were aberrantly expressed in malignancies and that their overexpression was related to tumourigenesis spurred the development of Aurora kinase inhibitors for cancer therapy (Table 1.10). Many of these compounds are under investigation in clinical studies for cancer treatment. Early studies showed that microinjection of Aurora A antibodies and/or RNAi-mediated knockdown of Aurora A kinase led to accumulation of cells in the G_2/M phase followed by apoptosis and was associated with *in vitro* and *in vivo* growth inhibition (457, 460, 498).

Targeted knockdown of Aurora A expression was shown to disrupt multiple mitotic events, culminating in failure of centrosome separation, monopolar spindle formation, and incomplete cytokinesis (466). Inhibition of Aurora B expression with RNAi was associated with disruption of chromosomal biorientation, failure of cytokinesis and disruption of the mitotic checkpoint (476, 499). Abrogation of the mitotic checkpoint allows cells to go through multiple cycles of aberrant mitosis without cytokinesis resulting in massive polyploidy (473, 500). Considering this, Aurora kinase inhibitors would be predicted to be selectively toxic to rapidly dividing cancer cells over non-dividing cells.

1.8.6.1 Pan-Aurora kinase Inhibitors

1.8.6.1.1 Hesperadin

Hesperadin was one of the earliest compounds to demonstrate Aurora kinase inhibition. It is an indolinone derivative that was originally developed by Boehringer Ingelheim as a pan-kinase inhibitor. Subsequently, it was shown to inhibit Aurora B and induce a cellular and molecular phenotype consistent with Aurora B inhibition including a reduction in histone H3 phosphorylation and chromosome alignment and segregation defects (473). Crystallography analysis showed that the indolinone moiety of hesperadin binds to the catalytic cleft of Aurora B (501). Hesperadin never entered clinical studies, but served as a useful scientific tool to examine the function of Aurora kinases as mitotic regulators in normal and malignant cells.

1.8.6.1.2 ZM447439

ZM447439 is a quinazoline derivative developed by AstraZeneca that is an ATP competitive inhibitor of both Aurora A and B (474). ZM447439 was studied preclinically in myeloid and lymphocytic leukaemia cell lines where it induced growth inhibition, accumulation of polyploid cells and apoptosis (502). ZM447439 treatment resulted in defects in chromosome

condensation and alignment and impairment of the spindle checkpoint, phenotypes indicative of Aurora B inhibition (474, 503). Similarly, other small molecule pan-Aurora inhibitors such as MK-0457 and PHA-680632 induce a cellular phenotype consistent with Aurora B inhibition rather than Aurora A inhibition (504, 505). It has been shown that Aurora B is required for G_2 arrest induced by Aurora A inhibition and that therefore, Aurora B inhibition bypasses Aurora A inhibition (506). Consequently, it appears that dual inhibition of both Aurora A and B is comparable to inhibition of Aurora B alone. Additional studies are required to clarify this issue.

1.8.6.2 MK-0457 (VX-680)

The first Aurora kinase inhibitor to enter clinical evaluation was MK-0457 (VX-680), initially developed by Vertex and later by Merck Pharmaceuticals. MK-0457 is a pyrimidine derivative that has activity against all three Aurora kinases (0.6, 18, 4.6 nM against aurora A, B and C respectively) (504). It was shown to block the proliferation of pancreatic, leukaemia and colon cell lines *in vitro* and reduced tumour volume in human AML and pancreatic xenograft models. As with other pan-Aurora kinase inhibitors, MK-0457 induced a cellular phenotype consistent with Aurora B inhibition where cytokinesis was prevented, cells became polyploid, and phosphorylation of histone H3 on Ser^{10} was reduced.

MK-0457 was also found to have activity against wild type and T315I mutated BCR-ABL (507). It was shown to inhibit the proliferation of cells expressing BCR-ABL mutations with IC_{50} values of 100 to 200 nM. As discussed in section 1.6.6.4 mutations in BCR-ABL are a significant reason for failure of TKI therapy in CML (277, 302, 508). In particular, the “gatekeeper” T315I mutation at the base of the ATP binding pocket that accounts for 10-15% of mutations confers resistance to imatinib, nilotinib, and dasatinib (509). High-resolution crystallography demonstrated that in contrast to imatinib, MK-0457 binds to ABL in its inactive confirmation and in a manner that accommodates the substitution of isoleucine for threonine

at residue 315 explaining its ability to inhibit the T315I mutant (510). It was also found to have *in vitro* activity against a variety of other *BCR-ABL* mutations including the dasatinib resistant V229L mutation (511). Subsequently, it was reported to have disease activity in 3 patients with the T315I mutation, 2 with CML and one with Ph^+ ALL, all of whom were enrolled in a dose finding phase 1/2 study of MK-0457 for refractory haematological malignancies (509).

Many of the Aurora kinase inhibitors in clinical development have off target inhibition of other clinically relevant tyrosine kinases. MK-0457 was shown to be an inhibitor of FLT3 (discussed in section 1.1.4.4) (366, 504, 507). Several other Aurora kinase inhibitors including ZM447439 and AS703569 have also demonstrated off target inhibition of FLT3 *in vitro* (502, 512-514). Another potential beneficial off target inhibition by MK-0457 *in vitro* and *in vivo* is that of janus kinase 2 (JAK2). The Val617Phe point mutation in the *JAK2* gene leads to constitutive tyrosine phosphorylation activity and occurs in the majority of myeloproliferative disorders (515). Consistent with this, MK-0457 inhibits JAK2 *in vitro* and has normalized platelet counts and induced partial remission in patients with $JAK2^{V617F}$ positive MPD and AML transformed from MPD (509, 516, 517). Despite these promising and intriguing clinical responses the clinical development of MK-0457 was halted due to concerns regarding cardiac toxicity.

1.8.6.3 Danusertib (PHA-739358)

Danusertib is 3-aminopyrazole derivative developed by Nerviano/Pfizer (518). It is an ATP-competitive inhibitor of all three Aurora kinases (IC_{50} of 0.013, 0.079 and 0.061 for Aurora A, B, and C, respectively). As well as inhibiting the Aurora family of kinases, danusertib inhibits other tumour-related kinases such as fibroblast growth factor receptor 1 (FGFR1), transforming tyrosine kinase protein (TRKA), ABL and rearranged during transfection (RET) in the low nanomolar range. As is the case with other

pan-Aurora kinase inhibitors, cells treated with danusertib show endoreduplication and reduced phosphorylation of histone H3 (518). Danusertib has significant activity in preclinical models of cancer (519). Like MK-0457, danusertib inhibits BCR-ABL including T315I mutated BCR-ABL and synergistically increases the efficacy of imatinib (520).

Danusertib has been evaluated in a number of early phase clinical studies using various schedules (521, 522). The most common toxicity was neutropaenia that was typically of short duration. Non-haematological toxicities reported included nausea, mucositis and alopecia (523). Phase 1 single agent data in patients with advanced stage CML or Ph+ ALL resistant or intolerant of imatinib or second-generation TKI therapy was encouraging. The cohort of 23 resistant/relapsed patients treated (11 Ph+ ALL, 8 CML-BC and 4 CML-AP) showed 1 CCyR, 1 PCyR, 1 minCyR, 5 HRs and one clinical improvement (reduction in extramedullary disease mass) at the time of reporting (524). Further Phase 1 and 2 trials evaluating danusertib as single agent or in combination for both solid tumours and haematological malignancies are ongoing (514).

1.8.6.4 SNS-314

SNS-314 is an ATP-competitive pan-Aurora kinase inhibitor developed by Sunesis. It selectively inhibits Aurora A, B, and C with an IC_{50} value of 9 nM, 31 nM and 3.4 nM, respectively (85). It is synergistic when combined with gemcitabine and the tubulin disrupting agents, docetaxel and VCR *in vitro* and *in vivo* in preclinical models of colorectal carcinoma (86). It also has activity in mouse xenograft models of human prostate (PC-3), breast (MDA-MB-231), melanoma (A375) lung (H1299 and Calu-6) and ovarian (A2780) carcinomas (525). SNS-314 is currently being evaluated in early phase clinical trials. The compound appears to be well tolerated although objective responses have yet to be reported (526).

1.8.6.5 R763/AS703569

AS703569 (R763) is a pan-Aurora kinase inhibitor developed by Rigel Pharmaceuticals Inc that is orally available. (88). In addition to inhibiting Aurora A and B it also has activity against FLT3 kinase, VEGFR and BCR-ABL including T315I mutated BCR-ABL. At higher concentrations it has activity against JAK2 kinase. Oral administration of AS703569 markedly reduced tumour growth in xenograft models of breast, colon, pancreatic, lung and ovarian cancer. MV4-11, an AML cell line, which harbours the *FLT3*-ITD mutation, was particularly sensitive to AS703569 *in vitro* and *in vivo*. Consistent with Aurora kinase inhibition, AS703569 led to endoreduplication and an increase in the DNA content of the nuclei without subsequent cytokinesis. In addition, the inhibition of other non-Aurora kinases may enhance its anti-tumour effects. Interesting in a panel of cell lines representing *FLT3*-ITD AML, there was a trend toward the bypassing of endoreduplication and direct induction of apoptosis.

A phase 1 study of AS703569 has been completed in advanced haematologic malignancies evaluating different potential dosing schedules for the drug (527). Dose-limiting toxicities were mucositis/stomatitis, severe neutropaenia with infection, and diarrhea. There were some responses to monotherapy including 2 CRs among 54 patients with AML and 1 among 3 patients with ALL. PRs were seen in MDS, MPD, and CML. However in the expansion part of this study frequent toxicities were noted necessitating dose reductions and subsequent lack of efficacy at these reduced doses. As a result the clinical development of AS703569 was suspended.

1.8.6.6 AT9283

AT9283 is a pyrazole-benzimidazole compound that inhibits both Aurora A and B equally ($IC_{50} \approx$ 3 nM). It is being developed by ASTEX Pharmaceuticals. As well inhibiting Aurora A and B, AT9283 was also found to inhibit a number of other kinases including JAK2, FLT3, and ABL

(including T315I) (IC_{50} = 1−30 nM) (528). Consistent with its profile as a pan-Aurora kinase inhibitor, AT9283 treatment resulted in endoreduplication and reduction of histone H3 phosphorylation in tumour cells. AT9283 showed encouraging single agent activity in patients with refractory leukaemia (454, 529). One third of patients with refractory AML experienced a significant reduction in BM blasts following treatment with AT9283. Two patients with refractory CML had a CHR and one had a PCyR response after 4 cycles of treatment. Dose limiting toxicities (DLTs) included elevated transaminases, non-cardiac creatine kinase and lactate dehydrogenase rises, tumour lysis syndrome, myelosuppression and alopecia.

In a study using a panel of AML cell lines with or without mutations of *c-KIT*, *FLT3* and *RAS* two distinct phenotypes emerged. Those driven by the oncogenic mutations listed above showed accumulation of cells in G_2/M (4N) arrest followed by apoptosis characteristic of Aurora A inhibition. Those without these mutations developed >4N DNA content and polyploidy followed by apoptosis consistent with Aurora B inhibition. Authors went on to speculate that their findings indicated that patients with mutations in oncogenic signalling pathways, may prove more susceptible to inhibition by AT9283 due to the presence of an intact mitotic checkpoint and a dominant Aurora A phenotype (530).

1.8.7 Aurora A-Selective inhibitors

1.8.7.1 Alisertib (MLN8237)

Alisertib is a small molecule ATP competitive reversible inhibitor of Aurora A that is being developed for the treatment of advanced malignancies. Alisertib inhibits Aurora A with an inhibition constant (Ki) of 0.43 nM. Unlike many other Aurora kinase inhibitors undergoing clinical evaluation it is approximately 200-fold more selective for Aurora A than Aurora B (IC_{50} = 1534 nM) (531). Moreover, alisertib is selective for Aurora A kinase when compared to other kinases (at a minimum 250-fold more selective *in vitro*)

and receptors. Like its parent molecule, MLN8054, alisertib has a benzazapine scaffold. The clinical development of MLN8054 was stopped due to excess somnolence that patients experienced. Somnolence is significantly less of a problem with alisertib as it has less affinity for benzodiazapine receptors. As would be expected from its kinase inhibitory profile, alisertib treatment results in the formation of abnormal mitotic spindles, an accumulation of mitotic cells, induction of p53, p21, p27 and a decrease in the proliferation of a tumour cell lines grown in culture (531). Alisertib synergizes with docetaxol, another agent that disrupts mitosis in preclinical models of mantle cell lymphoma (532). Alisertib is currently being investigated in a large number of clinical studies in solid tumours and haematological malignancies. Several different formulations and dosing schedules have been evaluated in adult and paediatric patients.

Alisertib is rapidly absorbed with an overall median time to maximum plasma concentration (T_{max}) of 2 hours (455). The overall mean terminal half-life following multiple dosing is approximately 19 hours (533). The side effects of alisertib have been similar to those observed with other Aurora kinase inhibitors including myelosuppression, diarrhoea, and alopecia. However the use of a treatment-free period for BM and gastrointestinal tract recovery between each cycle of drug administration allows repeated treatment cycles over periods extending beyond 12 to 24 months (534, 535). Apart from alopecia, the predominant toxicities are largely reversible.

Objective responses to alisertib treatment have been observed across a broad range of malignancies including AML and lymphomas. In many cases these responses have been sustained over 6 months (536). In a phase 1 study of alisertib in 56 patients with advanced haematological malignancies, 4 patients with lymphoma and 1 patient with multiple myeloma had PR to therapy and a further 13 patients had prolonged SD (534). A phase 2 study of 48 patients with refractory aggressive non-Hodgkin's lymphoma (NHL) has been reported on. The overall response

rate was 32%, but 57% of patients with T cell lymphoma responded (537). These promising results have prompted a phase 1/2 study of alisertib in combination with rituximab and VCR in B cell lymphoma and a phase 3 registration study comparing alisertib with investigators choice in peripheral T cell lymphoma.

1.8.8 Aurora B-Selective Inhibitors

Barasertib (AZD1152)

Barasertib is a quinazoline prodrug that is converted to the active metabolite barasertib -HQPA in plasma. It is the first selective Aurora B inhibitor to be evaluated in clinical trials. It is 1000-fold selective for inhibition of Aurora B over Aurora A kinase activity (Ki of 0.36 nM and 1.7 μM for Aurora B and A respectively) (80). As expected, based on its inhibition of Aurora B, barasertib reduced histone H3 phosphorylation and induced the accumulation of polyploid cells in colorectal SW620 xenografts (538). Barasertib demonstrated activity in preclinical models of leukaemia (539). It suppressed the proliferation of leukaemia lines (MV4-11, PALL-2, NB4, HL-60, K562 and MOLM-13) with an IC_{50} ranging from 3 nM to 40 nM. Barasertib also enhanced the activity of the tubulin depolymerizing agent, VCR and the topoisomerase II inhibitor, daunorubicin. Barasertib has activity in preclinical models of myeloma and lymphoma, both of which have high levels of Aurora B expression (540, 541). VCR is an important component of lymphoma therapy. Notably, barasertib blocks the induction of phospho-Aurora B by VCR and synergistically enhances its activity in preclinical models of lymphoma (541). Barasertib has also been explored as a potential radiosensitizing agent in the preclinical setting (542, 543). Radiation treatment leads to micronucleated cells followed by MC (544). Activation of the spindle checkpoint inhibits this process (225). Barasertib-induced inhibition of Aurora-B attenuates the spindle checkpoint to enhance radiation-induced cell death in HCT-116 and A549 cells *in vitro* and in HCT-116 xenografts (542).

Barasertib is being investigated in a number of clinical trials in solid tumours and AML (545). In a phase 1 study of patients with refractory solid tumours, barasertib was administered as a 2-hour IV infusion given weekly. A DLT of grade 4 neutropaenia was observed and the treatment was associated with prolonged SD in 5 out of 13 patients (546). Thirty-two patients with AML received barasertib in a phase 1 study. Haematological responses were reported in 8 out of 32 patients. DLT was reported as grade 3 mucositis/stomatitis, consistent with Aurora kinase inhibition of proliferating epithelial cells (545). Barasertib is currently being investigated in combination with low dose ara-C in patients with newly diagnosed AML.

Compound	Company	Route	Targets	Clinical Trial	Tumour Types	Major Reported Toxicities	Responses
MK-0457	Merck	I.V.	Aurora A, B & C, FLT3, BCR-ABL	II	Solid Tumours and Leukaemia	Neutropaenia	CML and Ph+ ALL
PHA-739358	Nerviano/ Pfizer Italia	I.V.	Aurora A, B & C BCR-ABL Trk-A & Ret	II	CML & solid tumours	Neutropaenia, mucositis	SCLC, ovarian cancer & CML
SNS-314	Sunesis	I.V.	Aurora A, B & C	I	Solid Tumours	Neutropaenia	
AT9283	Astex	I.V.	Aurora A & B	I/II	Solid Tumours and Leukaemia	Neutropenia, Fatigue	NSCLC, CML & AML
R763/AS703569	Rigel/ Merck	Oral	Aurora A, B & C	I	Solid Tumours and leukaemia	Neutropaenia, mucositis	AML, CML and MPD
Barasertib (AZD1152)	AstraZeneca	I.V.	Aurora B & C	II	AML & solid tumours	Neutropaenia	AML
MLN8054	Millennium	Oral	Aurora A	I	Solid Tumours	Somnolence	SD in solid tumours
Alisertib (MLN8237)	Millennium	Oral	Aurora A	I/II/III	Solid Tumours, NHL, AML, MM & ALL	Neutropaenia	Ovarian & liposarcoma, AML, NHL and MM

Table 1.10 Aurora kinases inhibitors in clinical development

CML, chronic myeloid leukaemia; Ph+, Philadelphia chromosome positive; ALL, acute lymphoblastic leukaemia; SD, stable disease; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer: MM, multiple myeloma; NHL, non-Hodgkin's lymphoma.

1.9 PIM kinases

1.9.1 Introduction

An alternative strategy in the treatment of resistant myeloid leukaemia is to target pro-survival pathways such as the PIM kinase pathway. The *PIM* genes represent a small family of proto-oncogenes within the calcium/calmodulin kinases (*CAMK*) super-family and encode three different serine/threonine protein kinases (547). All three kinases have an ATP binding pocket, an active site and a kinase domain (547). The first of these proteins (PIM-1) was discovered in 1984 when cloning of retroviral integration sites in murine Moloney leukaemia virus induced lymphomas led to the identification of the *PIM* (Proviral Integration Moloney virus) gene locus (548). PIM-1 and PIM-2 are 61% identical at the amino acid level whereas PIM-1 and PIM-3 are 77% identical (549, 550). *PIM-2* was identified as a gene activated in secondary transplants of virus induced lymphomas (551). Of note, myelocytomatosis oncogene cellular homolog (*c-myc*) transgenic mice lacking PIM-1 had compensatory activation of PIM-2. PIM-2 is expressed with highest levels in brain and lymphoid cells (552). PIM-3 was identified as a PIM-1 and PIM-2 related kinase and subsequently as a gene overexpressed in lymphomas developing in *c-myc* transgenic mice lacking *PIM-1* and *PIM-2* (553). The *PIM-3* gene is located on chromosome 22q and is expressed with highest levels in kidney, breast, and brain (554). Selective activation of PIM-3 occurs in tumours of *c-myc* transgenic mice lacking *PIM-1* and *PIM-2* suggesting that PIM-3 can substitute for PIM-1 and PIM-2 (553).

The PIM kinases are positive regulators of cell proliferation and survival and consequently have the potential to significantly reduce the efficacy of chemotherapeutic agents. The major pro-survival pathways that are regulated by the PIM kinases include the Wnt pathway and the signal transducer and activator of transcription (STAT) 3 signalling pathway (555, 556). Additionally PIM has also been identified as a positive regulator of

the NFκB pathway (557). As well as being implicated in drug resistance due to increased expression of pro survival pathways, PIM has also been implicated as an activator of drug efflux pumps (558, 559).

The importance of PIM kinases in normal body growth was underscored by the finding that PIM-1, PIM-2 and PIM-3 compound knockout mice had significant growth retardation. However these compound mice were viable and fertile suggesting that this family of serine/threonine kinases is important but dispensable for growth factor signalling (560). This may also indicate that there may be other, yet to be identified PIM kinases or that there is some degree of functional redundancy between the PIM kinases and other important cell signalling pathways.

1.9.2 The importance of PIM kinases in cancer

The oncogenic potential of PIM-1 and PIM-2 is evidenced by the findings that transgenic mice overexpressing these kinases in the lymphoid system develop lymphomas (561). However these tumours developed slowly indicating PIM kinases are weak oncogenes. In murine BM, enforced expression of PIM-1 leads to increased cell turnover, prolonged survival (562), protection from toxin induced cell death (563) and IL-3 independent cell survival (564). While PIM kinases function as weak oncogenes by themselves their oncogenic potential is significantly enhanced in co-operation with the c-MYC oncogene. Double transgenic mice cross-bred in order to express both PIM-1 and c-myc under control of the immunoglobulin heavy chain enhancer developed T cell lymphomas around birth (565). Therefore it appears that PIM-1 co-operates with c-MYC in the formation of lymphoid tumours. Additional data supporting the co-operation between PIM and c-MYC comes from pre-clinical data in prostate cancer. Significant overexpression of PIM-1 is noted in *c-myc* transgene driven prostate tumours in mice (566). Additionally, grafting normal mouse prostate epithelial cells expressing exogenous PIM-1 and c-

myc under the subrenal capsule of SCID mice results in large tumours compared to grafting epithelial cells expressing only PIM-1, an inactive kinase mutant PIM-1 or c-myc (567). Correspondingly, PIM-1 is overexpressed in clinical cases of lymphoma (568) and leukaemia (569). Overexpression has also been documented in several solid tumours including prostatic adenocarcinoma, bladder and oral cancers (570). Expression of the transmembrane serine protease hepsin and PIM-1 together, correlate with measures of clinical outcome in prostate cancers (571). PIM-2 is largely expressed in both solid (adenocarcinoma and squamous cell carcinoma of the lung) and haematological malignancies (AML and ALL) whereas PIM-3 expression seems to be restricted to solid tumours (melanoma, pancreatic adenocarcima, gastric cancers and hepatomas) (570).

1.9.3 Activation of PIM kinases

PIM kinases are constitutively active and the regulation of PIM kinase activity is largely at the transcriptional and translational levels (572, 573). Binding of several ligands activates a complex network of signalling pathways that results in upregulation of PIM-1 mRNA (Figure 1.18). Because PIM-1 translation is initiated by the STAT3 and STAT5 protein, its production is regulated by the cytokines that regulate the STAT pathway, or STAT factors. These include interleukins (IL-2, IL-3, IL-5, IL-6, IL-7, IL-12 and IL-15), prolactin, TNFα, epidermal growth factor (EGF) and IFNγ (547). Interestingly, PIM-1 itself can bind to negative regulators of the JAK/STAT pathway, resulting in a negative feedback loop. Additionally, hypoxia has been shown to induce PIM-1 expression, in a HIF1α-independent manner (574, 575). PIM-1 also binds to heat shock protein 90 (HSP90), which in turn protects it from proteasomal degradation (576). Furthermore, the serine/threonine protein phosphatase 2A (PP2A) has been shown to degrade PIM-1 (577, 578). In addition it appears that autophosphorylation and/or phosphorylation by as yet unknown kinases

might play key roles in the determining PIM kinase function. Most of the experimental data regarding regulation of PIM kinase expression has been generated using PIM-1 and little is known about the regulation of PIM-2 and PIM-3.

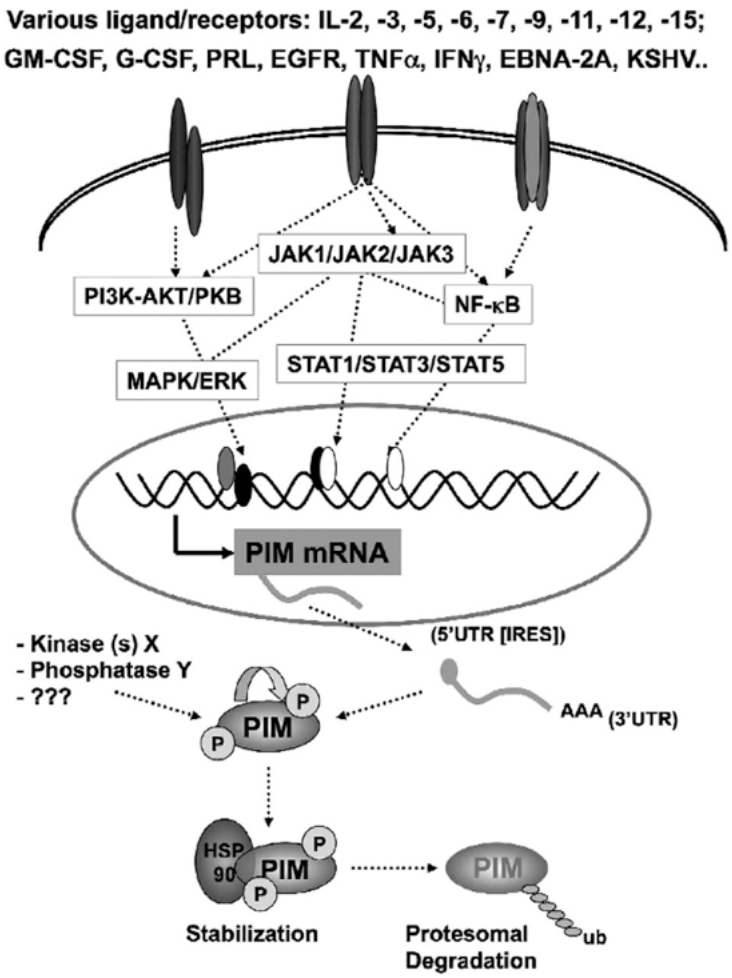

Figure 1.18 Regulation of PIM-1 expression

Binding of several ligands leads to activation of a complex network of signalling pathways that results in upregulation of PIM-1 mRNA. Binding of PIM-1 to HSP90 protects from proteasomal degradation. Most experimental data has been generated using PIM-1; very little is known about regulation of PIM-2 and PIM-3. There is increasing evidence for modification of PIM kinases through as yet unkown protein kinases and/or phosphatases. Reproduced with permission from Brault et al, Haematologica. 2010 Jun;95(6):1004-15.

1.9.4 Activities of PIM kinases

1.9.4.1 Prevention of apoptosis

Pro-survival signalling by PIM kinases is thought to be necessary for its oncogenic properties and is the primary reason for the efforts to therapeutically target this kinase (Figure 1.19) (579). An important finding was that PIM-1 co-localized with and physically interacted with the pro-apoptotic protein BAD (discussed in section 1.4.2). This results in a phosphorylation of BAD on Ser^{112}, which is a gatekeeper site for BAD inactivation. This suggested a direct functional role of PIM-1 in preventing cell death since the inactivation of BAD can enhance BCL-2 activity, thereby promoting cell survival (580). PIM also phosphorylates BAD on Ser^{136} and Ser^{155}, which contribute to the inactivation of BAD (581, 582).

The pro-apoptotic function of BAD is regulated by growth factor induced signalling survival kinases that result in the phosphorylation of three serine residues (Ser^{112}, Ser^{135}, and Ser^{155}). All three PIM kinase family members have been shown to phosphorylate BAD at Ser^{112} (572, 580, 581, 583). This is consistent with the known ability of PIM kinases to promote cell proliferation and prevent apoptosis. The three PIM isoforms variably phosphorylate serine residues on BAD. For example, PIM-1 and PIM-2 can phosphorylate BAD on Ser^{112}, Ser^{136}, and Ser^{155}, with predominance for Ser^{112}. Conversely, PIM-3 is the least selective for Ser^{112}, and mostly phosphorylates BAD on Ser^{136} and Ser^{155} (582). Phosphorylation of BAD on Ser^{112} and Ser^{136} induces 14-3-3 binding, disassociation from BCL-2 family members and transfer of BAD from the mitochondria to the cytosol (584). The phosphorylation of BAD on Ser^{112} and Ser^{136} is required for phosphorylation on Ser^{155}, which is instrumental for the dissociation of BAD from BCL-2. PP2A binds to and dephosphorylates PIM-1 leading to its ubiquitylation and proteasomal degradation (577). Additional mechanisms by which PIM may inhibit apoptosis include the phosphorylation of the proline-rich AKT substrate 1

and impairment of the activity of the apoptosis signalling kinase 1 (ASK1) (585, 586).

Lastly PIM kinases may promote tumour cell survival by enhancing glycolysis. As discussed in 1.1.2.9, tumour cells are characterized by aerobic glycolysis (587). Phosphorylation of BAD on Ser ^{155} induces glucokinase binding and enhances glycolysis (588). Since the PIM family members phosphorylate BAD, it is possible that PIM-1 and PIM-3 induced BAD phosphorylation may enhance glycolysis.

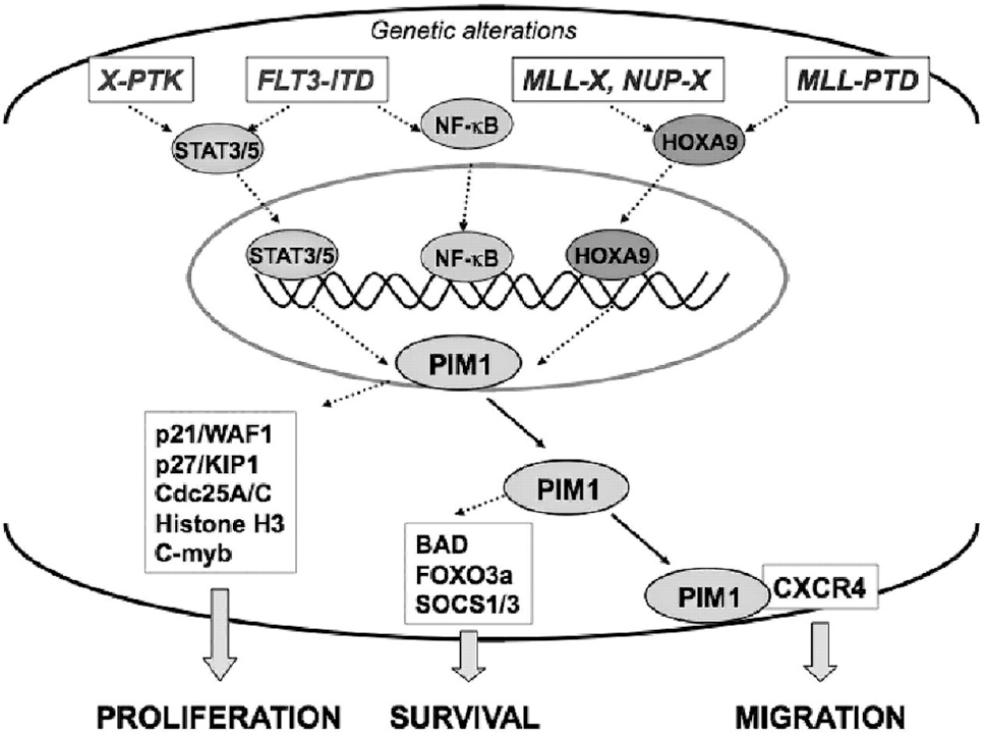

Figure 1.19 Potential downstream substrates of over-expressed PIM-1 in haematologic malignancies

Functionally collaborating genetic alterations such as mutated protein tyrosine kinases and fusion genes involving transcriptional regulators such as mixed lineage leukaemia (MLL) lead to constitutive activation of major signalling mediators, like STAT5 or HOXA9, shown to be transcriptional activators of PIM-1. Elevated PIM-1 levels support cellular proliferation through modification of cell cycle regulators, survival through modification of regulators of apoptosis, as well as homing and migration through modification of the CXCR4 chemokine receptor. Reproduced with permission from Brault et al, Haematologica. 2010 Jun;95(6):1004-15.

PTK: protein tyrosine kinase; SOCS: suppressor of cytokine signalling; NUP: nucleoside permease; HOXA9 homeobox protein A9; PTD: partial tandem duplication; myb: myeloblastosis; FOXO3a: forkhead box O3a.

1.9.4.2 Cell cycle regulation

PIM kinases regulate multiple aspects of cell cycle progression and in transgenic mouse models, PIM overexpression leads to enhanced cell proliferation (589, 590). As discussed earlier p21 and p27 are key CKIs that regulate cell cycle progression. The phosphorylation of p21 is directly induced by PIM-1 at Thr^{145} and indirectly on Thr^{146}. This results in the transfer of p21 to the cytoplasm and increased p21 protein stability leading to enhanced cell proliferation (591). All three PIM family members bind to and phosphorylate p27 at Thr^{157} and Thr^{198}, which leads to enhanced binding of p27 to 14-3-3 leading to its nuclear export and proteasome-dependent degradation (592). Consistent with this, ectopic expression of PIM kinases can overcome the G_1 arrest mediated by p27. Additionally, PIM kinases suppress p27 transcription through phosphorylation and inactivation of the forkhead transcription factors, FOXO1a and FOXO3a (592). In keeping with these observations is the finding that pharmacological inhibition of PIM kinases induces cell cycle arrest in tumour cell lines, and is associated with decreased CDK2 activity and p27 nuclear accumulation (593, 594).

CDC25A cell cycle phosphatase, a direct transcriptional target for c-MYC, is a substrate for PIM-1 kinase and functions as an effector for PIM-1, leading to activation of the cyclin D1-associated kinases, cell cycle progression, enhanced cellular transformation and apoptosis (595). As mentioned earlier CDC25C regulates the G_2/M transition. PIM kinases seem not only to interfere with G_1-S (through modification of CDC25A, p21 and p27) but also with the G_2-S transition of the cell cycle by phosphorylating CDC25C phosphatase (596, 597).

1.9.4.3 Regulation of Myc transcriptional activity

MYC protein is a transcription factor that activates expression of a great number of genes through binding on consensus sequences (enhancer box

sequences (E-boxes)) and recruiting histone acetyltransferases (HATs) (598). It is thought to regulate the expression of approximately 11% of all human genes increasing up to 15% depending on cell type and MYC expression levels (599). Both PIM-1 and PIM-2 stabilize c-MYC *in vivo* dramatically increasing its transcriptional activity. This effect is partially mediated by phosphorylation of c-MYC by PIM kinase on Ser^{329}.

1.9.4.4 Regulation of protein transcription

PIM-2 has a role in the regulation of cap-dependent protein translation in parallel to and independently of the PI3K–AKT pathway. Upon growth factor signalling and nutrient availability, the mammalian target of rapamycin complex 1 (mTORC1) regulates protein synthesis and growth (600). Growth factors activate receptor tyrosine kinases that then activate both the PI3K–AKT pathway and the JAK–STAT pathway. AKT activation in turn leads to activation of the mTORC1 complex while STAT transcription factors will induce expression of PIM-2 (601). mTORC1 regulates translation by phosphorylating 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase β1 (S6K1) (602). PIM-2 activity induces phosphorylation of 4E-BP1 through an unknown intermediate (572, 603). Upon phosphorylation, 4E-BP1 disassociates from eukaryotic translation initiation factor 4E (EIF4E) and allows the recruitment of the ribosomal 40S subunit and the translation initiation machinery. This is the rate-limiting step in cap-dependent mRNA translation. Increased activity of EIF4E selectively increases the translation of mRNAs that are involved in cell cycle, cellular growth, angiogenesis, survival and malignancy (e.g. cyclin D1, c-MYC, VEGF, matrix metalloprotease 9) (600).

As discussed below AML blast cells frequently display high levels of PIM-2 expression. PIM-2 has been identified as being mainly responsible for 4E-BP1 phosphorylation on the Ser^{65} residue and subsequent translation control in AML (604). This suggests that PIM-2 plays a role in an mTORC1-independent deregulation of oncogenic proteins synthesis in

human myeloid leukaemogenesis. Therefore inhibiting PIM-2 in AML may result in direct inhibition of the translation-initiating complex and represents an attractive therapeutic approach.

1.9.5 PIM kinases in AML

Early studies showed that PIM-1 is highly expressed in haematopoietic malignancies. In an analysis of 51 primary patient samples and 19 cell lines, PIM-1 was overexpressed in approximately 30% cases, particularly in AML and ALL. The level of PIM-1 overexpression was not related to any stage of cellular differentiation and was not due to gene rearrangement or amplification (569). PIM-1 and PIM-2 play key roles downstream of oncogenic protein tyrosine kinases such as BCR-ABL, FLT3-ITD, or the JAK2 V617F mutant (549). These effects are probably mediated through aberrant JAK-STAT signalling. BCR-ABL increases the expression of PIM-1 leading to increased cell proliferation and protection from apoptosis (605).

FLT3-ITD is constitutively active, associated with poor prognosis and plays an important role in leukaemogenesis in AML (see section 1.1.4.4). Constitutively activated FLT3 up-regulates PIM-1 expression in leukaemia cells and PIM-1 expression is significantly down-regulated upon FLT3 inhibition (606). Increased expression of PIM-1 leads to resistance to the FLT3 inhibitor, lestaurtinib (see section 1.7.16), -mediated cytotoxicity and apoptosis whereas decreased PIM-1 expression sensitizes AML cells to cytotoxicity in response to FLT3 inhibition. As well as through activation of FLT3, PIM-1 expression may also occur through aberrant activation of HOXA9, a direct transcriptional regulator of PIM-1 (607). PIM-1 expression is also upregulated in AML harbouring alterations of the MLL gene such as the MLL/ENL or MLL/AF9 fusion genes (608).

As discussed in section 1.7.16, CXCR4 is important in haematopoiesis, and grafting of HSCs (446). Furthermore, high CXCR4 expression is known negative prognostic marker in AML (447). Experiments using PIM knockout cells have shown that transplantation of

wild-type or PIM-2 −/− BM retrovirally expressing the FLT3-ITD mutant led to induction of typical lympho-myeloproliferative disease (609). In contrast, PIM-1 −/− BM cells were not able to reconstitute lethally irradiated recipients and showed a significant defect for homing to the BM and spleen. Furthermore, blocking PIM-1 activity with siRNAs or by a small molecule inhibitor impairs CXCR4 expression. PIM-1 may regulate CXCR4 by direct phosphorylation of the Ser^{339} residue, which in turn may affect receptor internalization and surface re-expression. These observations suggested that PIM-1 (but not PIM-2) regulate homing and migration of leukaemic cells through modification of surface CXCR4 expression.

Similar to PIM-1, PIM-2 levels have been found in to be increased in primary blasts from AML patients (604, 610). As mentioned earlier PIM-2 phosphorylates 4E-BP1 resulting in mTOR-independent translational control in AML cells highlighting a potential important role for PIM-2 in AML.

1.9.6 PIM kinases as anti-cancer targets

The over-expression of PIM kinases in various types of malignancies as well as their roles in the regulation of important cancer pathways have made PIM kinases attractive targets for novel anti cancer agents (611). Another attraction of targeting the PIM kinases is that the effects of PIM kinase inhibition in normal cells appears to be limited because triple PIM-1, PIM-2, and PIM-3 knock-out mice are viable and show only a limited number of phenotypic abnormalities including a reduction in body weight and slight changes in haematopoietic cell signalling (560).

Both PIM-1 and PIM-2 assume an active conformation in the reported crystal structures (573, 612). In contrast to most kinases, PIM kinases are catalytically active in the absence of phosphorylation and the crystal structures of PIM provides an explanation for this constitutive activity. The unphosphorylated activation segment forms a large number of polar interactions with the lower kinase lobe that stabilizes the observed

active conformation. Although PIM kinases do autophosphorylate, the functional consequences of these post-translational modifications are not known (613). Lastly the involvement of PIM kinases in the regulation of drug resistance suggests that PIM kinase inhibitors may be combined with currently available therapies to overcome resistance.

1.9.7 Small molecule PIM kinase inhibitors

PIM kinase inhibitors can be grouped into two main classes based on their binding mode (549).The first class of inhibitors are ATP-mimetic compounds that form a hydrogen bond with the hinge region of PIM-1. These compounds include the broad-spectrum kinase inhibitor staurosporine and its analogue K252, bisin-doyl maleinimides (BIM) and the PKC inhibitor LY333531 (613-616). Also flavonoids are potent inhibitors of PIM kinases through their multiple polar interactions with the hinge backbone region (617, 618).

The second class of PIM inhibitors does not form classical hydrogen bonds with the hinge region of PIM and can therefore be considered as ATP competitive inhibitors rather than ATP mimetic inhibitors. These include the imidazo[1,2-b]pyridazines, pyrazolo[1,5-a]pyrimidines and LY294002 (617). K00135 is an imidazo[1,2-b]pyridazines that has been evaluated in preclinical models of leukaemia. K00135 impairs the survival human AML cell lines, human primary AML cells and murine Ba/F3 cells that are cytokine independent due to overexpression of PIM. Consistent with the inhibition of PIM, K00135 inhibits the phosphorylation of known PIM downstream targets, such as BAD and 4E-BP1 (619).

1.9.7.1 SGI-1776

SGI-1776 is an imidazo [1,2-b]pyridazine compound, developed by SuperGen Inc., Dublin, CA, USA and later by Astex Pharmaceuticals, Dublin, CA, USA. It is a potent ATP competitive inhibitor of the PIM kinases with IC_{50} concentrations for PIM-1, PIM-2, and PIM-3 of 7, 363, and 69 nM, respectively (620). While it is relatively specific for PIM kinases it has some

activity against FLT3 (IC_{50} 44nM) and the haspin group of mitotic kinases (IC_{50} 34nM).

SGI-1776 has shown preclinical activity against leukaemia and solid tumour cell lines with IC_{50} values of 0.005–11.68 μM. It is orally available and in testing conducted by the Paediatric Preclinical Testing Program the median relative IC_{50} value paediatric cancer cell lines was 3.1 μM, with a range from 0.3 (Kasumi-1 (an AML cell line)) to 4.5 μM (Ramos (a Burkitt's lymphoma cell line)). Other sensitive cell lines included, CHLA-9 an Ewing sarcoma cell line, but most solid tumour and ALL cell lines were relatively insensitive to SGI-1776. In the same study SGI-1776 was tested against a large number of solid tumour xenografts using a dose of 74 mg/kg administered by oral gavage daily for 5 days/week for 3 weeks. SGI-1776 induced CRs in AML xenograft models but paediatric models of solid tumours and ALL are relatively less sensitive to SGI-1776 (620).

SGI-1776 has shown significant activity in prostate cancer models. PIM-1 is known to be upregulated in human prostate cancer clinical samples and in animal models of prostate cancer (566, 571). Dose-dependent reduction in phosphorylation of PIM substrates apoptosis such as p21 and BAD were observed following treatment of prostate cancer cell lines with SGI-1776 (559). Consistent with this, SGI-1776 induced G_1 cell cycle arrest and triggering of apoptosis. SGI-1776 marginally sensitized prostate cancer cell lines to taxane-based therapies and was active in a multidrug resistance 1 (MDR1) protein based taxane-refractory prostate cancer cell line. Interestingly, SGI-1776 was able to resensitize chemoresistant cells to taxane-based therapies by inhibiting MDR1 activity. Based on these promising findings a phase 1, dose escalation study of SGI-1776 was initiated in refractory prostate cancer.

SGI-1776 also has significant activity in preclinical models of CLL. PIM kinases and in particular PIM-2, are known to be overexpressed in CLL (621). SGI-1776 treatment of CLL cells caused a concentration-dependent induction of apoptosis (622). Probably due to the fact that CLL

cells do not replicate *in vitro*, SGI-1776 did not affect the phosphorylation of PIM-1 targets such as BAD and histone H3. However SGI-1776 did reduce levels of total c-MYC as well as phospho-c-MYC (Ser^{62}) and the inhibitor of apoptosis, MCL-1.

Given the central role of MYC in renal cell carcinoma (RCC), SGI-1776 has been evaluated as a therapeutic agent in preclinical models of RCC (623). Treatment of RCC cell lines with SGI-1776 led to a decrease in phosphorylated and total c-MYC levels, which resulted in the modulation of c-MYC target genes (624). Sunitinib potentiated SGI-1776 activity leading to a further reduction in c-MYC levels and enhanced anti-cancer activity *in vitro* and *in vivo*.

A DLT of cardiac QTc prolongation was identified in the phase 1 study of SGI-1776 in patients with refractory prostate cancer and lymphoma. Additional detailed cardiac and pharmacokinetic data evaluation of SGI-1776 in this trial has failed to demonstrate a safe therapeutic window to prudently continue clinical development of this molecule. The QTc prolongation was probably due to SGI-1776 interaction with the hERG (human Ether-à-go-go-Related Gene) potassium channel. These findings resulted in the halting of the clinical development of SGI-1776 and Astex Pharmaceuticals are now developing a second-generation PIM kinase inhibitor, SGI-9481.

1.10 Thesis objectives

Despite the recent advances in the treatment of CML, resistance and disease persistence continues to be problematic. The Aurora kinase inhibitors represent a novel class of anti cancer therapy that have shown promise in resistant CML. Alisertib is an orally available Aurora A inhibitor undergoing evaluation in multiple tumour types and therefore remains an attractive agent for early phase investigation for patients with resistant CML.

While our understanding of the molecular aberrations in AML have improved greatly recent years the standard induction treatments have remained unchanged. The elderly and patients with multiple co-morbid conditions represent a significant challange. Given their roles in cell cycle progression and oncogenesis the Aurora and PIM family of kinases represent attractive therapeutic targets in AML. Targeting aberrant kinase expression with small molecule inhibitors in these patients represent an attractive therapeutic stategy.

Objective 1: To investigate the efficacy and mechanism of action of alisertib in preclinical models of CML that are sensitive or refractory to standard TKI therapy.

Objective 2: To evaluate the activity and pharmacodynamic effects of alisertib in preclinical models of AML and assess its ability to augment the efficacy of ara-C.

Objective 3: To evaluate the activity and pharmacodynamic effects of SGI-1776 in preclinical models of AML and assess its ability to augment the efficacy of ara-C.

2 Chapter 2: An investigation of the activity and mechanism of action of alisertib in CML

2.1 Introduction

As discussed earlier imatinib and the second generation TKIs, nilotinib and dasatinib target the constitutively active BCR-ABL tyrosine kinase in CML and have become standard treatment based on excellent responses achieved in clinical trials. However, resistance can occur through several mechanisms including BCR-ABL kinase domain mutations, amplification, overexpression, and clonal evolution (see section 1.6.6.4) (281). Successful strategies to overcome resistance include dose escalation or the use of second-generation BCR-ABL kinase inhibitors including nilotinib, dasatinib, bosutinib or ponatinib (277, 302, 508). However, with the exception of ponatinib none of these agents are effective in CML cells harbouring the "gatekeeper" T315I mutation at the base of the ATP binding pocket, which occurs in up to 20% of imatinib resistance cases (509).

As discussed in section 1.8, Aurora A kinase is a central mitotic regulator necessary for mitotic entry, mitotic spindle assembly, and accurate chromosome separation (453, 466, 468). The discovery that Aurora kinases were abnormally expressed in malignancies including leukaemia prompted the development of agents that inhibit their activity (502, 504, 625). The pan-Aurora kinase inhibitors, MK-0457 and PHA-739358 have shown pre-clinical and clinical activity against CML harbouring the BCR-ABL T315I mutation (504, 520, 626, 627). The anti-leukaemia efficacy of MK-0457 in CML was originally attributed to direct inhibition of BCR-ABL kinase activity (628, 629). However, a recent study demonstrated that the efficacy of MK-0457 at clinically relevant doses in BCR-ABL+ cells was primarily due to inhibition of Aurora, rather than BCR-ABL, kinase activity (630). The development of MK-0457 was ceased due to problems with cardiac toxicity observed in some patients during early phase clinical trials with the compound. In spite of this, the clinical responses achieved by MK-0457 in refractory CML patients have served to maintain interest in targeting Aurora kinases for CML therapy and a

significant effort is currently being put forth to develop new agents that inhibit Aurora kinase activity and lack undesired cardiac side effects.

As discussed in section 1.8.7.1 alisertib is a competitive and reversible inhibitor of Aurora A kinase with an *in vitro* inhibition constant (Ki) of 0.43 nM (631). We hypothesized that alisertib -mediated inhibition of Aurora A kinase activity would abrogate the growth and survival of CML cells in a manner independent of BCR-ABL mutation status. Our results indicate that alisertib impairs growth, disrupts cell cycle kinetics, induces a cellular phenotype consistent with Aurora A kinase inhibition, and triggers apoptosis in CML cell lines and primary human resistant CML cells including those bearing the drug resistance conferring T315I mutation. Furthermore, alisertib significantly increases the anti-cancer activity of the standard agent nilotinib through a mechanism involving downregulation of the apoptotic and mitotic regulator, Apollon. Our collective data demonstrate that alisertib is a promising novel agent for the treatment of refractory CML that warrants further investigation.

Project hypothesis

Targeting Aurora A kinase with alisertib will lead to apoptosis and growth inhibition in preclinical models of CML and will potentiate the anti-leukaemic activity of nilotinib.

Project aims

I. To determine if alisertib has *in vitro* and *in vivo* anti-leukaemia activity in models of CML that are sensitive and resistant to frontline TKI therapy.
II. To investigate whether inhibition of Aurora kinase A activity with alisertib can be used as a strategy to increase the anti-leukaemia activity of nilotinib in preclinical models of CML.
III. To elucidate the mechanism of action of alisertib and identify key pharmcodynamic regulators of sensitivity to alisertib in CML.

2.2 Project summary

Alisertib is a novel inhibitor of Aurora A kinase currently under investigation in multiple phase 1, 2 and 3 studies. Here we report that alisertib possessed equipotent anti-cancer activity against Ba/F3 cells and primary CML cells from patients expressing unmutated and mutated forms of BCR-ABL, including T315I and E255K. Notably, the efficacy of alisertib was unaffected by impairment of p53 function through RNA interference. Alisertib treatment disrupted cell cycle kinetics, induced mitochondrial-dependent apoptosis, caused a dose-dependent reduction in the expression of the large inhibitor of apoptosis Apollon, and led to a morphological phenotype consistent with Aurora A kinase inhibition. In contrast to other Aurora kinase inhibitors, alisertib did not significantly affect BCR-ABL activity. Moreover, inhibition of Aurora A with alisertib significantly increased the *in vitro* and *in vivo* efficacy of nilotinib in CML cell lines and immunodeficient mice bearing K562 xenografts. Targeted knockdown of Apollon sensitized CML cells to nilotinib-induced apoptosis, indicating that this aspect of the mechanism of action of alisertib is an important factor underlying its ability to increase the efficacy of nilotinib. Our collective data demonstrate that this combination strategy represents a novel therapeutic approach for refractory CML that has the potential to suppress the emergence of T315I mutated CML clones.

2.3 Materials and methods

2.3.1 Cells and cell culture

Ba/F3 cells engineered to express comparable levels of wild type (p210) BCR-ABL with and without stable shRNA p53 knockdown and T315I, E255K, H396P, Y253F, M351T and Q252H mutant forms of BCR-ABL, LAMA-84 and K562 cells were maintained in RPMI 1640 medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) in a humidified incubator at 37°C with 5% CO_2, 50 U/ml penicillin and 50 mg/ml streptomycin. LAMA-

84 and K562 cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA). Ba/F3 wild type and mutated cells were kindly provided by Dr. Brian Druker (Oregon Health & Science University, Portland, OR, USA). Primary human CML cells were obtained from the PB of imatinib-resistant CML patients after obtaining informed consent in accordance with an approved institutional review board (IRB) protocol. PBMCs from healthy donors were purchased from Stem Cell Technologies (Vancouver, Canada).

2.3.2 Chemicals and reagents

Reagents were obtained from: alisertib (Millennium Pharmaceuticals, Cambridge, MA), nilotinib (Novartis, Basel, Switzerland), c-ABL, anti-actin, anti-active caspase-3, anti-phospho-Aurora A, anti-Aurora A antibodies phospho-BCR antibodies (Cell Signalling, Beverly, MA), anti-β tubulin (Sigma, St. Louis, MO), anti-Apollon antibody (Bethyl Laboratories, Montgomery, TX), and sheep anti-mouse-HRP and donkey anti-rabbit-HRP antibodies (Amersham, Pittsburgh, PA).

2.3.3 Enzyme assays

Alisertib was screened against a subset of Invitrogen's SelectScreen^{TM} kinase panel at concentrations ranging between 10 to 0.00051 mM in three fold serial dilutions. The Z´-LYTE® biochemical assay employs a fluorescence-based, coupled-enzyme format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage. The peptide substrate is labeled with two fluorophores—one at each end—that make up a (fluorescence resonance energy transfer) FRET pair. A known inhibitor control standard curve, 10 point titration, is run for each individual kinase on the same plate as the kinase to ensure the kinase is inhibited within an expected IC_{50} range previously determined. The enzymes screened included ABL1, ABL1

E255K, ABL1 G250E, ABL1 T315I, ABL1 Y253F, ABL12 and Aurora A, each at the respective apparent ATP Km.

2.3.4 Analysis of cell cycle effects and apoptosis

Propidium iodide (PI) staining were used in conjunction with flow cytometry (BD Biosciences, San Jose, CA) to examine the cell cycle effects of drug treatment and to measure drug-induced apoptosis by calculating the percentages of cells containing sub G_0/G_1 (fragmented apoptotic). Briefly, cells were treated with alisertib, nilotinib or both drugs as indicated. Following drug exposure, cells were washed twice in phosphate buffered saline (PBS) and re-suspended in a PI solution comprised of 50 µg/mL PI, 0.1% Triton X-100 and 0.1% sodium citrate. Cells were stained in the dark for 1 hour and PI fluorescence was also quantified by flow cytometry (BD FACSCanto II). The percentages of apoptotic (sub G_0/G_1) cells were determined for each experimental condition.

2.3.5 MTT assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were cultured in 96-well plates at a density of 10,000 cells per well. Cells were treated with alisertib, nilotinib or the combination for 96 hours. Following drug treatment, MTT (0.5 mg/ml) was added to cells and incubated for 3 hours at 37ºC. The reaction was stopped by addition of dimethysulfoxide (DMSO). The purple formazan precipitate generated was allowed to dissolve for 1 hour on an orbital shaker. The colour intensity was measured at 550 nm on a BioTek (Winooski, VT) microplate reader. Cell viability was expressed relative to the absorbance of untreated control cells, which was taken as 100% viable. IC_{50} was determined by calculating the concentration of alisertib or nilotinib that caused a 50% loss of viability measured by the MTT assay.

2.3.6 Cell morphology

Cell suspensions (100 µl) were cytospun in a Shandon Cytospin 2 Cytocentrifuge (Thermo Scientific, Cheshire, UK) at 400 r.p.m. for 2 minutes, air-dried and stained using RapiDiff (Biotech Sciences Ltd). Slides were visualized using Olympus BX51 system DP71 camera with Olympus DP3.2 software (Melville, New York, USA).

2.3.7 Colony assays

K562 and LAMA-84 cells were treated for 24 hours with the indicated concentrations of alisertib, nilotinib or both and then washed twice in PBS. The cells were incubated in Methocult methylcellulose-containing medium (Stem Cell Technologies, Vancouver, Canada) for 14 days in a humidified incubator at 37°C with 5% CO_2. Colonies were washed in PBS and stained with 2,3,5-triphenyltetrazolium chloride (TTC). Colonies were then scored using an Alpha Innotech (San Leandro, CA) gel documentation system.

2.3.8 Immunoblotting

CML cells were incubated with alisertib, nilotinib or the combination for 24 hours following which the cells were collected. Cells were collected by centrifugation and lysed in ice with a 1% Triton-X-100 lysis buffer containing 50 mM Tris–HCL pH 7.4, 1% nonyl phenoxypolyethoxylethanol (NP-40), 150 mM sodium chloride (NaCl), 1 mM EDTA, 0.25% Na-deoxycholate, 1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM sodium fluoride (NaF) a complete Mini protease inhibitor tablet (Roche, Indianapolis, IN, USA). Lysates were mixed with reducing buffer and heated to 95ºC for 5 minutes. The protein concentrations of each sample were determined by Bradford reagent (BioRad Laboratories, USA). Approximately 50 µg of total cellular protein from each sample were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels or precast 7.5–12% gradient gels (BioRad Laboratories, USA). Proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk (BioRad

Laboratories, USA) in a Tris-buffered saline solution containing 0.1% Tween-20 for 1 hour. The blots were then probed overnight with the relevant primary antibodies (dilution at 1:1000), washed, and probed with species-specific secondary antibodies. All secondary antibodies were horseradish peroxidase-conjugated and used at 1:1000 (Amersham, Pittsburgh, PA). Immunoreactive material was detected by enhanced chemiluminescence (West Pico, Pierce, Inc., Rockville, IL) on X-ray film (Agfa). Membranes were re-probed with anti-tubulin antibody 1:1000 (Sigma, St. Louis, MO).

2.3.9 *In vivo* evaluation of alisertib and nilotinib

K562 and Ba/F3 cells were harvested, washed in PBS, and suspended in a 50:50 mixture of Hank's Buffered Salt Solution (HBSS) and Matrigel (BD BioSciences, San Jose, CA). An *in vivo* model of CML was generated by injecting 10^7 K562 or Ba/F3 cells expressing wild type (p210) or T315I mutant forms of BCR-ABL into the flanks of female nude mice (BALB/c background) from Harlan (Indianapolis, IN). After tumour growth reached 150 mm^3, mice were randomly assigned to receive alisertib 20 mg/kg BID (n=10), nilotinib 50 mg/kg QID (n=10), vehicle control (n=10) or both alisertib and nilotinib (n=10) for 14 days. Mice were monitored daily and tumour volumes were measured twice weekly. The two longest perpendicular axes in the x/y plane of each xenograft tumour were measured to the nearest 0.1 mm by three independent observers. The depth was assumed to be equivalent to the shortest of the perpendicular axes, defined as y. Measurements were made using a digital vernier caliper while mice were conscious and were calculated according to equation: xenograft volume = $xy^2/2$ (632). At the completion of the study, tumours were excised, formalin-fixed and paraffin-embedded for immunohistochemical analysis.

2.3.10 Haematoxylin and eosin (H&E) staining

Paraffin-embedded tumour sections (4–6 µ m thick) were mounted on slides. Sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H_2O (v/v)] and rehydrated in PBS (pH 7.5). Slides were then stained with haematoxylin in a staining dish for 1 minute. Haematoxylin was then washed off with distilled H_2O and rinsed in 95% ethanol for 10 seconds. Fifty µl eosin was pipetted onto each slide for 25-30 seconds after which the slides were rinsed in ethanol, washed in xylene and air-dried on racks for 5 minutes.

2.3.11 Terminal deoxyribonucleotide-transferase–mediated dUTP nick-end labeling (TUNEL) assay

DNA fragmentation was analyzed using a colorimetric terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assay kit (Promega, Madison, WI). This method detects DNA fragmentation by labeling the terminal end of nucleic acids. The TUNEL System measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3´-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail. The 12-dUTP-labeled DNA can then be visualized directly by microscopy. The assay was carried out according to the manufacturer's instructions. Images were obtained with an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20X objective. Percentages of TUNEL-positive cells were determined by manual counting of 5 random high-power fields per section by two investigators blinded to the treatment arms. The average percentage of TUNEL positive cells per high-power field was calculated for comparison. Image-Pro Plus software Version 6.2.1 (MediaCybernetics, Bethesda, MD) was used for image acquisition.

2.3.12 shRNA knockdown of p53

BCR-ABL p210 Ba/F3 cells were infected with a retrovirus encoding a short hairpin RNA (shRNA) sequence specific for the knockdown of murine

p53 or an empty vector control (Santa Cruz Biotechnology). Infected cells were selected with constant culture in 1 µg/ml puromycin, and p53 knockdown was confirmed by immunoblotting.

2.3.13 siRNA transfection

Apollon and Aurora kinase A SMARTpool or siCONTROL siRNA directed at luciferase were obtained from Dharmacon, Lafayette, CO. Cells were transfected with 100 nM of each siRNA according to the manufacturer's protocol using the Nucleofector II according to the manufacturer's instructions (Amaxa Inc., Gaithersburg, MD) (633). Transfected cells were incubated at 37°C for 24 hours and then treated with alisertib, nilotinib, or the combination for 48 hours. Efficiency of RNAi was measured at 48 hours by immunoblotting using an anti-apollon or Aurora A antibody. Drug-induced apoptosis was quantified by PI/FACS as described above.

2.3.14 Statistical analyses

Statistical significance of differences observed between samples was determined using the Student's t test. Differences were considered significant in all experiments at $p < 0.05$.

2.3.15 Combination of alisertib and nilotinib

The interaction between alisertib and nilotinib was analyzed with the CompuSyn software (ComboSyn Inc., NJ, USA). Data from cell viability assays (MTT) were expressed as fraction of cells affected by the dose in drug-treated cells compared with untreated cells (control). This program is based on the Chou-Talalay method (634) according to the following equation: $CI = (D)_1/(Dx)_1 + (D)_1(D)_2/(Dx)_1(Dx)_2$, where $(D)_1$ and $(D)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone, and CI is the combination index. $CI < 1.0$ indicates synergism; $CI \cong 1.0$ indicates an additive effect; and $CI > 1.0$ corresponds to an antagonistic effect. K562 and LAMA-84 cells were treated for 72 hours with

combinations of alisertib and nilotinib at a nonconstant ratio and MTT assays were done as previously described. CI values for each drug combination were obtained from three different experiments fulfilling experimental prerequisites of the CompuSyn program.

2.4 Results

2.4.1 Alisertib inhibits Aurora A

Alisertib is a potent inhibitor of the Aurora A enzyme (IC_{50} = 2 nM) and demonstrated approximately 50 to 700 fold selectivity against various ABL isoform enzyme assays (Figure 2.1). We determined the inhibitory effects of alisertib on Aurora A kinase. Exposure of cultured K562 cells to 30 nM alisertib reduced the kinase activity of Aurora A kinase as evidenced by reduced phosphorylation of Aurora A at Thr^{288} within its kinase activation loop without affecting the total levels of Aurora A (Figure 2.1). A dose of 30 nM alisertib was chosen, as this was the lowest dose that induced significant growth inhibition and apoptosis of K562 or LAMA-84 cells. This dose is significantly higher than 2 nM, the IC_{50} of alisertib in the cell free kinase assay. A significantly higher dose of alisertib appears to be required to inhibit intracellular Aurora A kinase compared to that required to inhibit Aurora A in the cell free kinase assay.

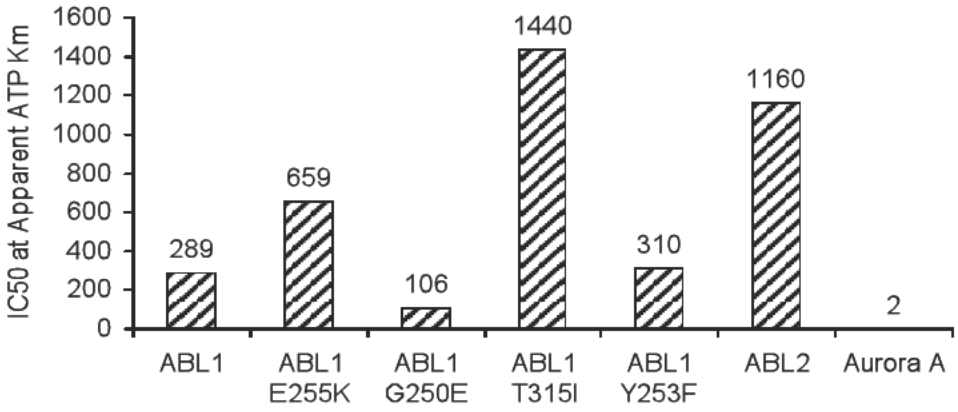

Figure 2.1 Effects of alisertib on the activity of selected kinases

Alisertib was screened against a kinase panel as described in the materials and methods. The ABL1 and the related ABL2 cytoplasmic tyrosine kinases share 89% sequence identity and have some overlapping functions, but are distinct in that ABL1 fuses with BCR to form the Ph chromosome while ABL2 does not.

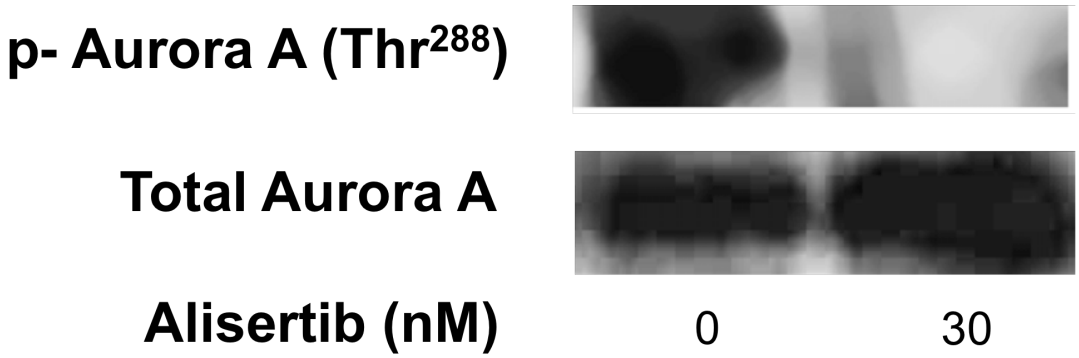

Figure 2.2 Alisertib reduces Aurora kinase A phosphorylation

K562 cells were treated with 30 nM alisertib for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with phospho- Aurora A (Thr^{288}) and Aurora A antibodies.

2.4.2 Alisertib impairs growth and induces apoptosis in CML cell lines

The effects of alisertib treatment on cellular viability were assessed using MTT assays in human CML cell lines. Alisertib inhibited the *in vitro* growth and survival of K562 and LAMA-84 cell lines with IC_{50} values less than 100 nM (Figure 2.3). Pharmacokinetic analyses of alisertib concentrations in preclinical and phase 1 clinical studies have demonstrated that micromolar concentrations of this drug are achievable in plasma (455, 533). Thus, the concentrations of alisertib that we utilized in our experiments are relevant and biologically achievable. Similarly alisertib inhibited the growth and survival of the APL cell line, HL-60, but had little effect on normal PBMCs. Inhibition of the Aurora kinases results in mixed outcomes, including polyploidy and G_2/M growth arrest. We therefore assessed the cell cycle distribution and apoptotic fraction (sub G_0/G_1) of PI-stained K562 and LAMA-84 cells by flow cytometry after treatment with alisertib for 48 and 72 hours. Alisertib treatment disrupted cell cycle kinetics as evidenced by the accumulation of cells in G_2/M phase and cells with >4N DNA prior to the onset of apoptosis (sub G_0/G_1) in a dose- and time-dependent manner (Figure 2.4). At 72 hours compared to 48 hours, the proportion of apoptotic cells (<2N DNA) increased whereas the percentage of cells with polyploid nuclei (>4N DNA) decreased.

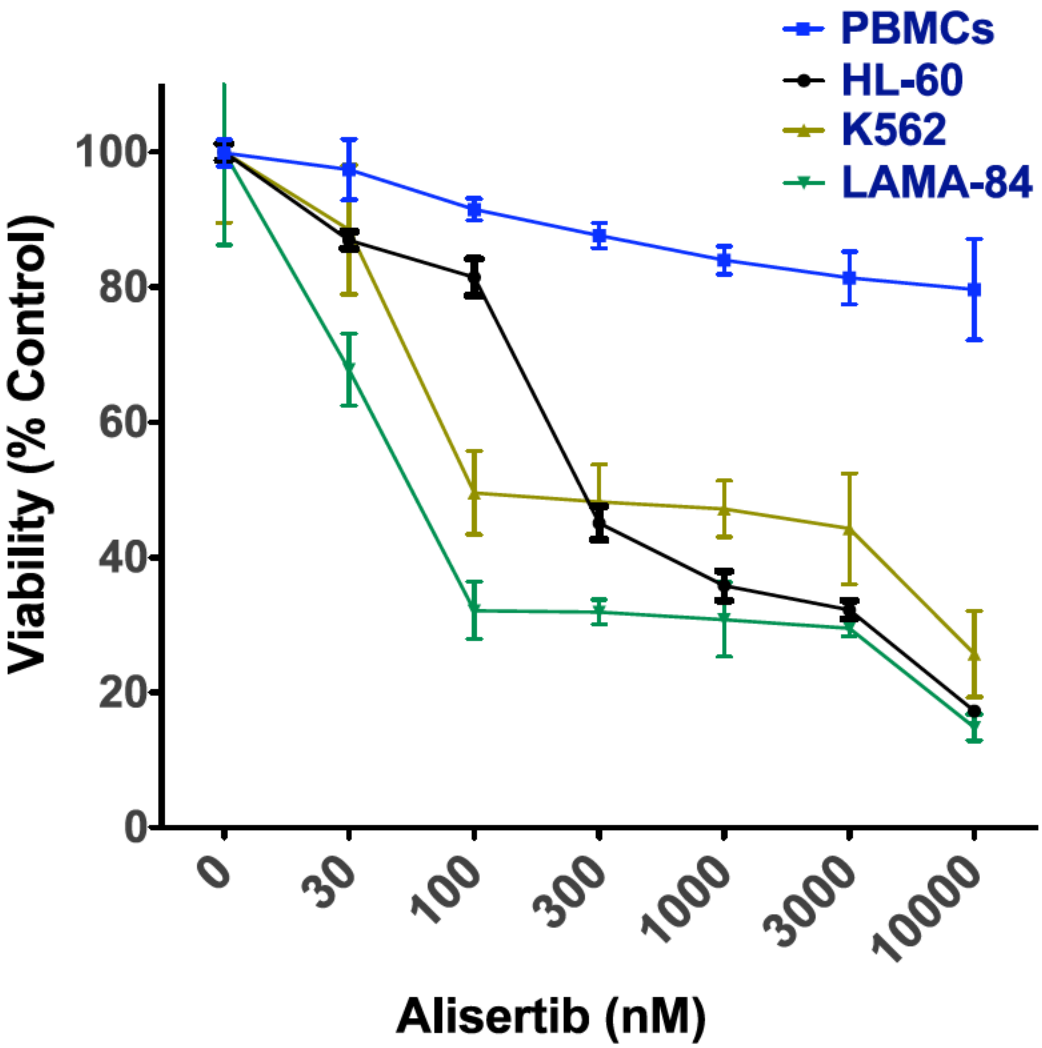

Figure 2.3 Alisertib impairs growth in CML cell lines

Effects of alisertib on the *in vitro* growth and survival of K562 and LAMA-84 human CML cell lines. Cells were treated with the indicated concentrations of alisertib for 96 hours and viability was assessed by MTT assay. n = 3 ± SD. HL-60 cells and peripheral blood mononuclear cells (PBMCs) were used as Ph negative and non-transformed controls respectively.

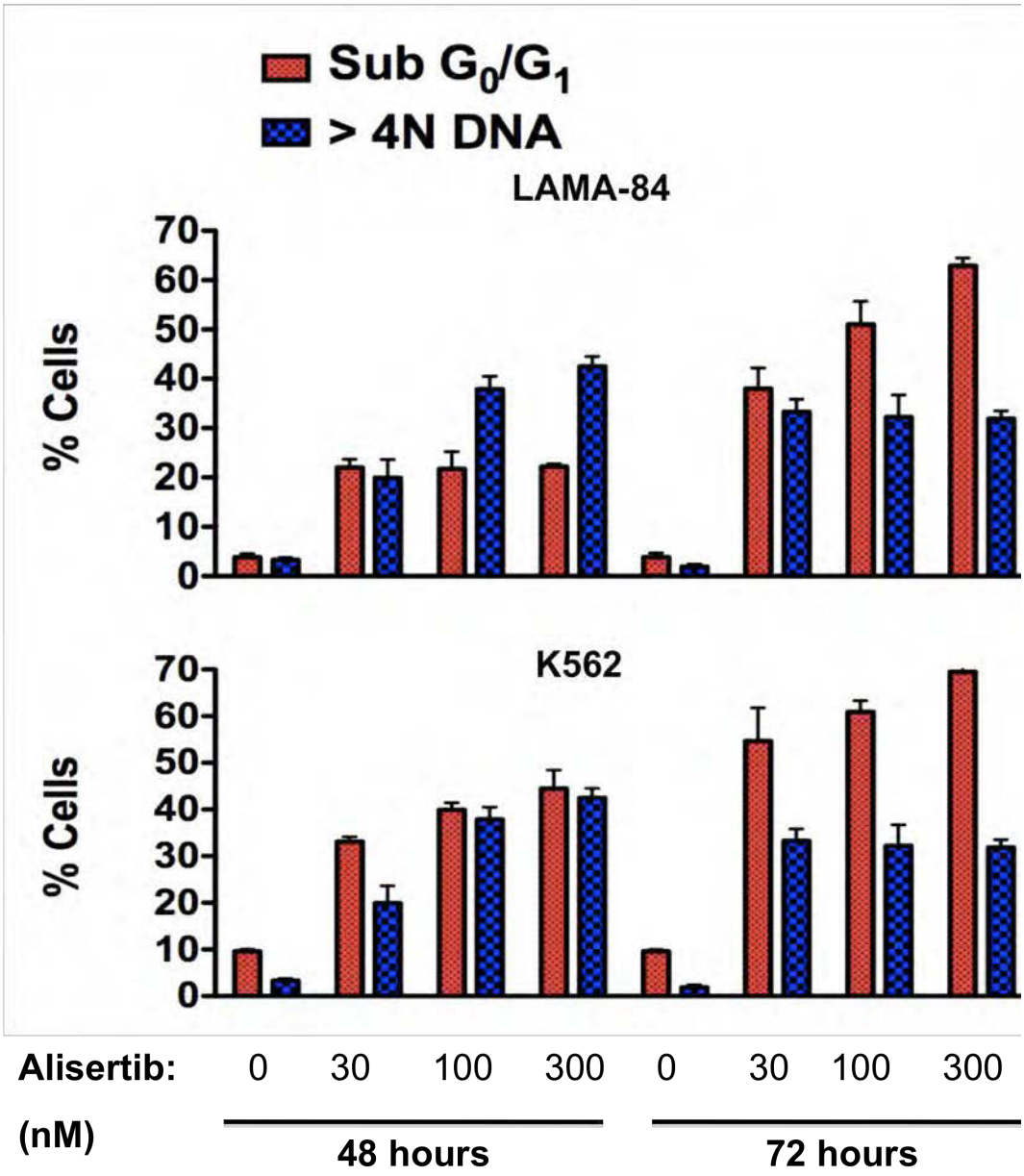

Figure 2.4 Alisertib disrupts cell cycle kinetics causes time-dependent induction of DNA fragmentation

LAMA-84 and K562 cells were treated with 30, 100 or 300 nM alisertib for 48 hours and 72 hours. Percentages of cells with sub G_0/G_1DNA and > 4N DNA were determined by PI/FACS. n = 3 ± SD.

2.4.3 Morphological analysis of CML cells after 48 hours of alisertib treatment

Considering that the cell cycle analysis showed that alisertib could induce polyploidy and that previous studies have shown that Aurora kinase inhibition results in MC we examined the morphology of alisertib-treated cells. As discussed in section 1.5, MC is currently defined primarily by morphology (635). Figures 2.5 and 2.6 show representative examples of both treated and untreated K562 and LAMA-84 cells respectively. After 48 hours of treatment, cells with distinct chromatin images suggestive of a monopolar spindles and duplicated but unseparated chromosomes were visible. In addition large cells with multiple micronuclei were seen. These features are consistent with MC. In addition to features of MC, the alisertib-treated CML cell lines also showed a subpopulation of cells with clear classical apoptosis morphology: chromatin condensation, cytoplasm shrinkage, and the formation of apoptotic bodies.

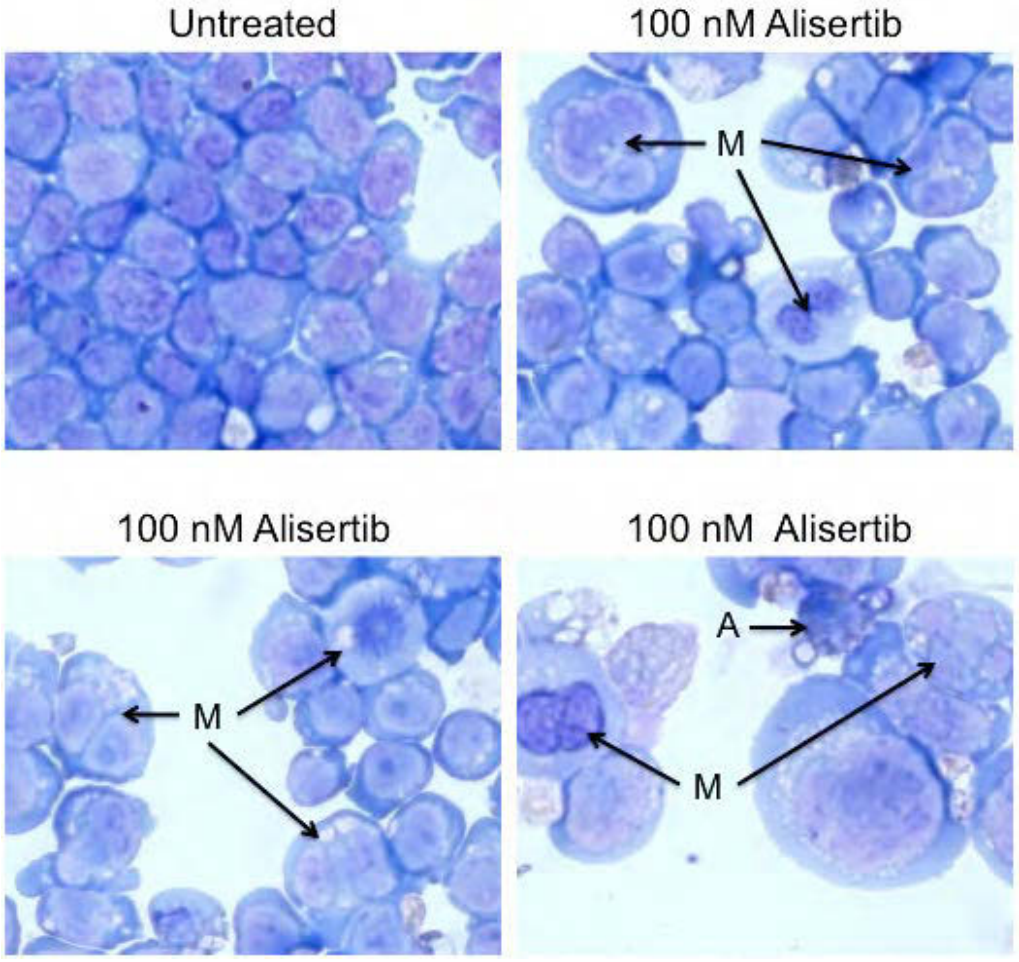

Figure 2.5 Morphology of alisertib-treated K562 cells

Cell morphology was visualised using RapiDiff staining by light microscopy after treatment with 100 nM of alisertib for 48 hours. Typical cytospin images for untreated and alisertib-treated K562 cell lines are shown. Alisertib treatment (100 nM) produced a distinct morphology suggestive of monopolar spindles, duplicated but unseparated chromosomes centrally located in the cell and large cells with multiple micronuclei (M). These features are consistent with mitotic catastrophe. In addition to mitotic catastrophe, K562 cells treated with alisertib show a population of cells with membrane blebbing and budding and chromatin condensation, features consistent with apoptotic morphology (A).

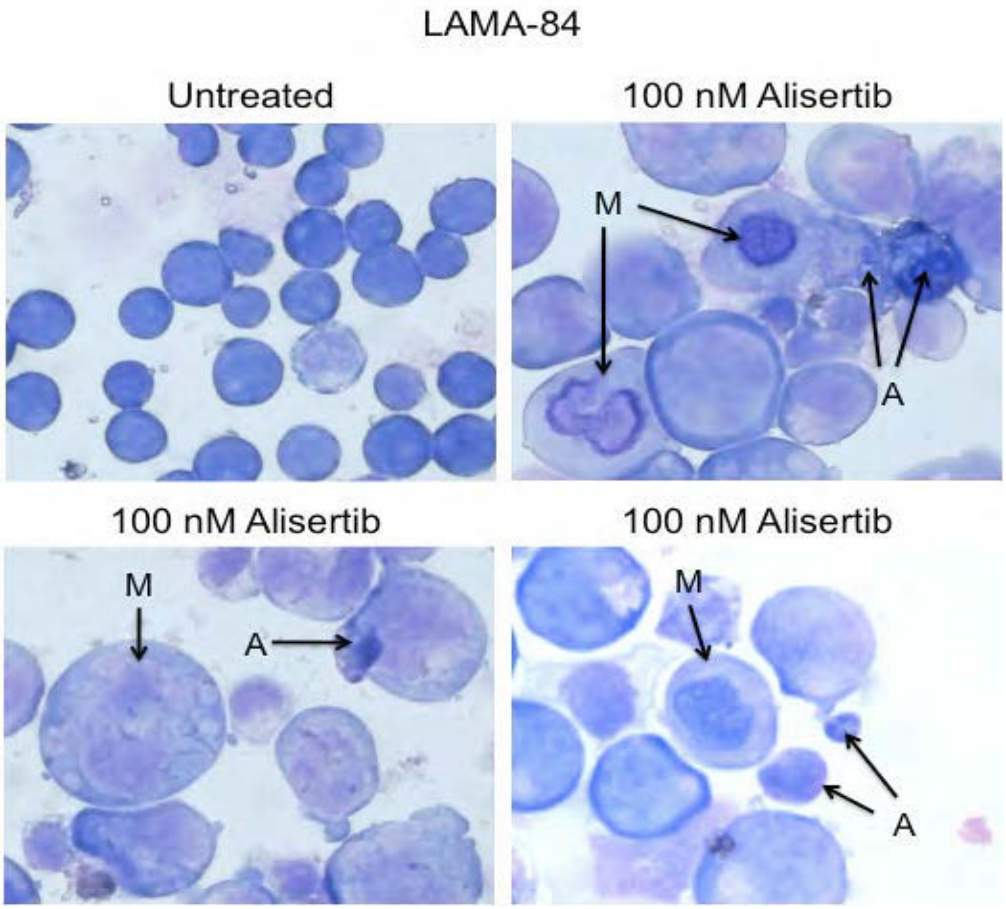

Figure 2.6 Morphology of alisertib-treated LAMA-84 cells

Cell morphology was visualised using RapiDiff staining by light microscopy after treatment with 100 nM of alisertib for 48 hours. Typical cytospin images for untreated and alisertib-treated LAMA-84 cell lines. Alisertib treatment (100 nM) produced a distinct morphology suggestive of duplicated but unseparated chromosomes centrally located in the cell and large cells with multiple micronuclei (M). These features are consistent with mitotic catastrophe. In addition to mitotic catastrophe, LAMA-84 cells treated with alisertib show a population of cells with membrane blebbing and budding and chromatin condensation, features consistent with apoptotic morphology (A).

2.4.4 Alisertib activity is unaffected by impairment of p53 function

Loss or mutation in the tumour suppressor gene *p53* occurs in over 30% of cases of CML-BC (636). Inactivation of p53 has been shown to impede the response to tyrosine kinase inhibition and represents a mechanism of resistance to targeted therapy in advanced phase CML (637). The potential impact of loss of p53 function on cellular sensitivity to alisertib was evaluated by achieving stable shRNA-mediated p53 knockdown in Ba/F3 cells expressing p210 BCR-ABL. Cells were treated with the chemotherapeutic agent VCR and immunoblotting analyses of the expression of p53 and its direct transcriptional target, p21, were conducted to confirm functional p53 knockdown (Figure 2.7). Impairment of p53 function did not affect the anti-cancer activity of alisertib, indicating that it may be an effective agent for patients with p53 defects (Figure 2.8).

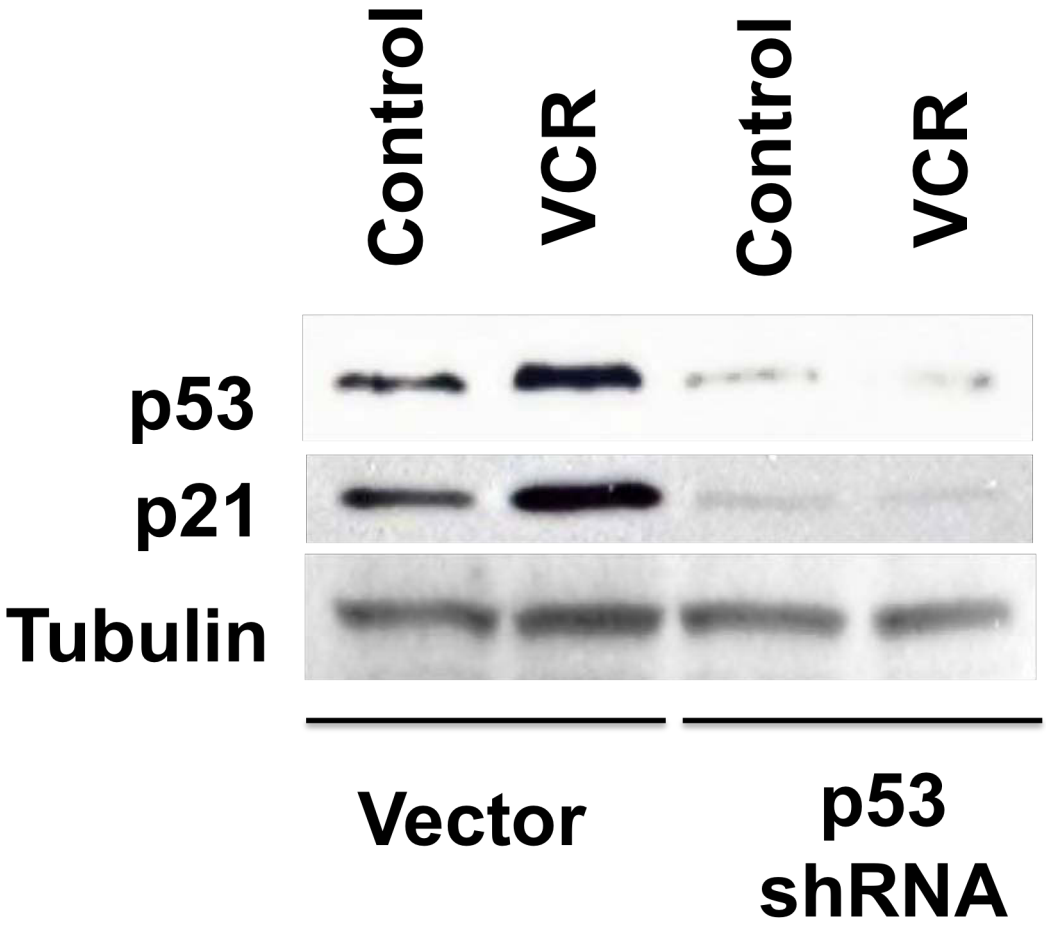

Figure 2.7 Establishment of BCR-ABL cells with stable p53 knockdown Ba/F3 p210 BCR-ABL cells were stably infected with p53 shRNA or vector control. These cells were then treated with 100 nM VCR for 24 hours and subjected to immunoblotting for p53 and p21 to confirm functional knockdown efficiency of the knockdown. Tubulin documented equal loading.

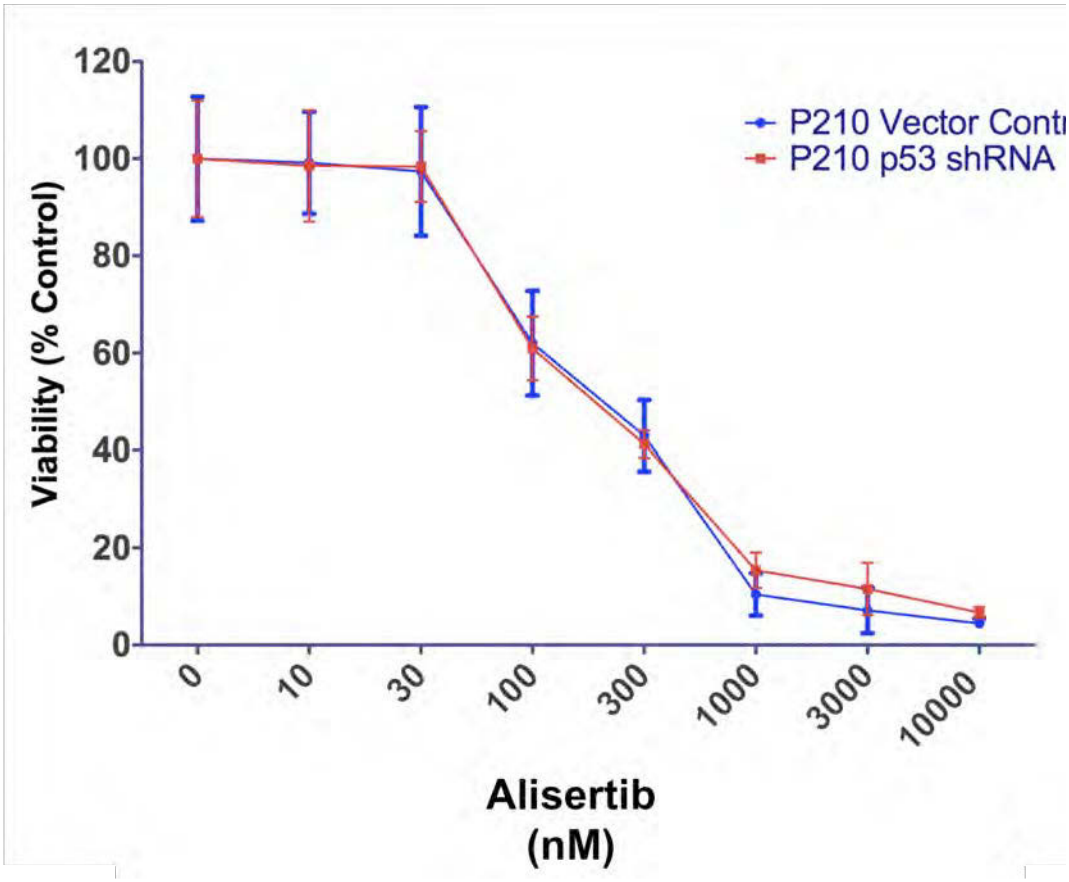

Figure 2.8 Alisertib is unaffected by impairment of p53 function

Ba/F3 p210 BCR-ABL cells stably infected with p53 shRNA or vector control were treated with the indicated concentrations of alisertib for 96 hours and viability was assessed by MTT assay. n = 3 ± SD.

2.4.5 Alisertib is active in Ba/F3 cells expressing mutated BCR-ABL

To investigate the potential impact of imatinib resistance on the efficacy of alisertib, we treated Ba/F3 expressing unmutated BCR-ABL and the clinically relevant TKI resistant BCR-ABL mutants T315I, E255K, H396P, Y253F, M351T, and Q252H as well as K562 cells that are sensitive and resistant to imatinib due to differential expression of unmutated BCR-ABL with this agent for 96 hours. Notably, alisertib inhibited the viability of Ba/F3 cells expressing unmutated BCR-ABL and mutated BCR-ABL (Figure 2.9) and imatinib-sensitive and -resistant K562 cells at similar concentrations (Figure 2.10).

We next created an animal model of T315I mutated CML by injecting Ba/F3 cells expressing T315I mutated BCR-ABL into the flanks of nude mice to investigate the *in vivo* efficacy of alisertib against CML cells bearing the T315I mutation. Tumour bearing mice were randomized into groups of 10 and treated orally with 20 mg/kg alisertib twice daily or vehicle control for 14 days. Consistent with our *in vitro* data, alisertib possessed equipotent *in vivo* activity against xenografts of Ba/F3 cells expressing wildtype and imatinib, nilotinib and dasatinib resistant T315I-mutated forms of BCR-ABL (Figure 2.11).

We subsequently determined the antileukaemic effects of alisertib against primary CML cells from three imatinib refractory patients, (one each from a patient in CML-CP, CML-BC and CML-BC harbouring the T315I mutation) and primary leukaemia cells from a patient with Ph+ ALL. As primary cells do not tend to actively proliferate in culture, higher concentrations of alisertib were needed to inhibit their viability compared to CML cell lines (534). However alisertib concentrations of up to 10,000 nM are achievable in the plasma of patients recieving alisertib (533). Normal PBMCs cultured under the same conditions were less susceptible to the effects of alisertib compared to the primary CML cells (Figure 2.12). Collectively, these results suggest that the activity of alisertib in CML cells is unaffected by BCR-ABL mutational status or impairment of p53 function.

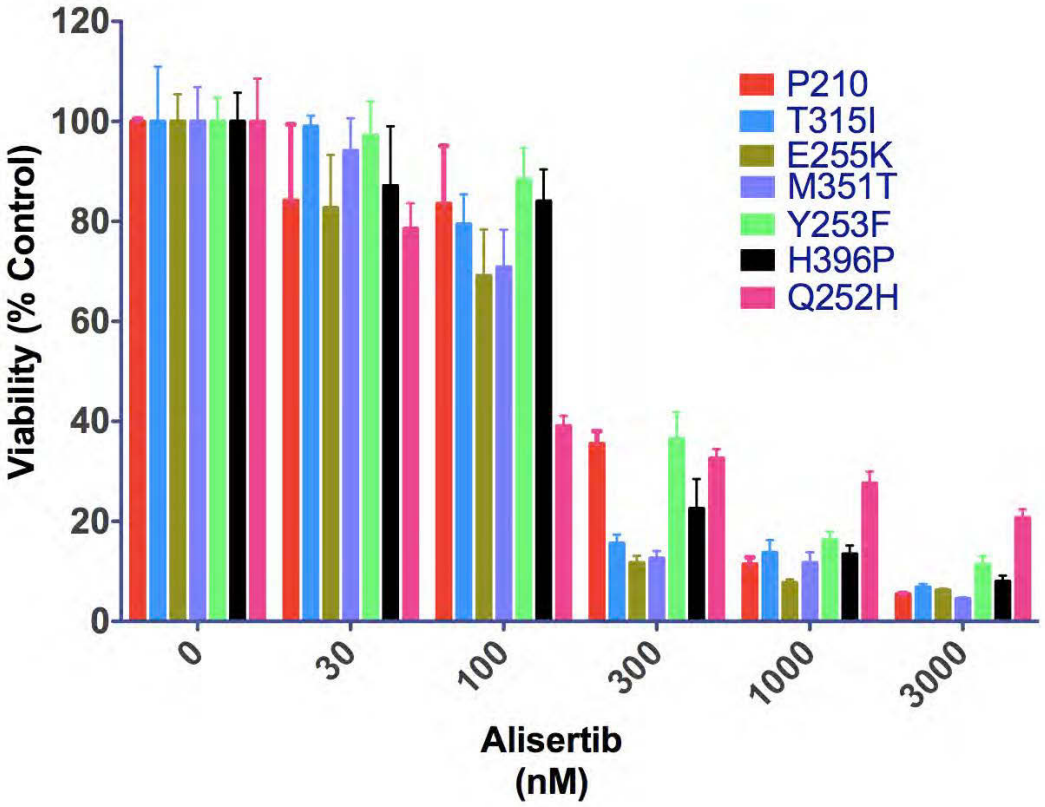

Figure 2.9 Alisertib has activity in cells expressing unmutated and mutated BCR-ABL

Ba/F3 cells expressing p210 (unmutated) and T315I, E255K, H396P, Y253F, M351T and Q252H mutant forms of BCR-ABL were treated with the indicated concentrations of alisertib for 96 hours and viability was assessed by MTT assay. n = 3 ± SD.

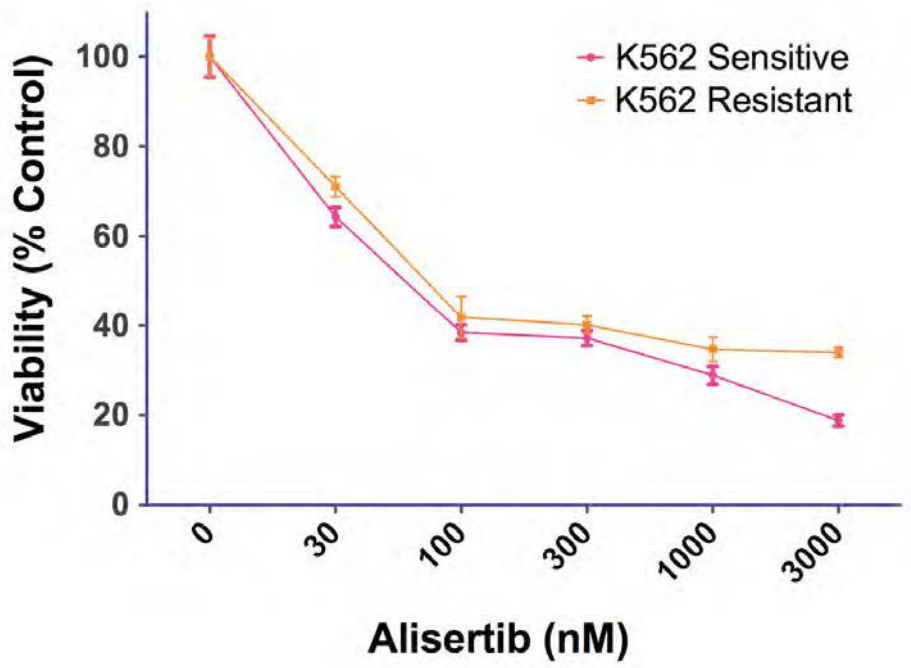

Figure 2.10 Alisertib has activity in imatinib sensitive and resistant K562 cells

Imatinib-sensitive and imatinib–resistant K562 cells were treated with the indicated concentrations of alisertib for 96 hours and viability was assessed by MTT assay. n = 3 ± SD.

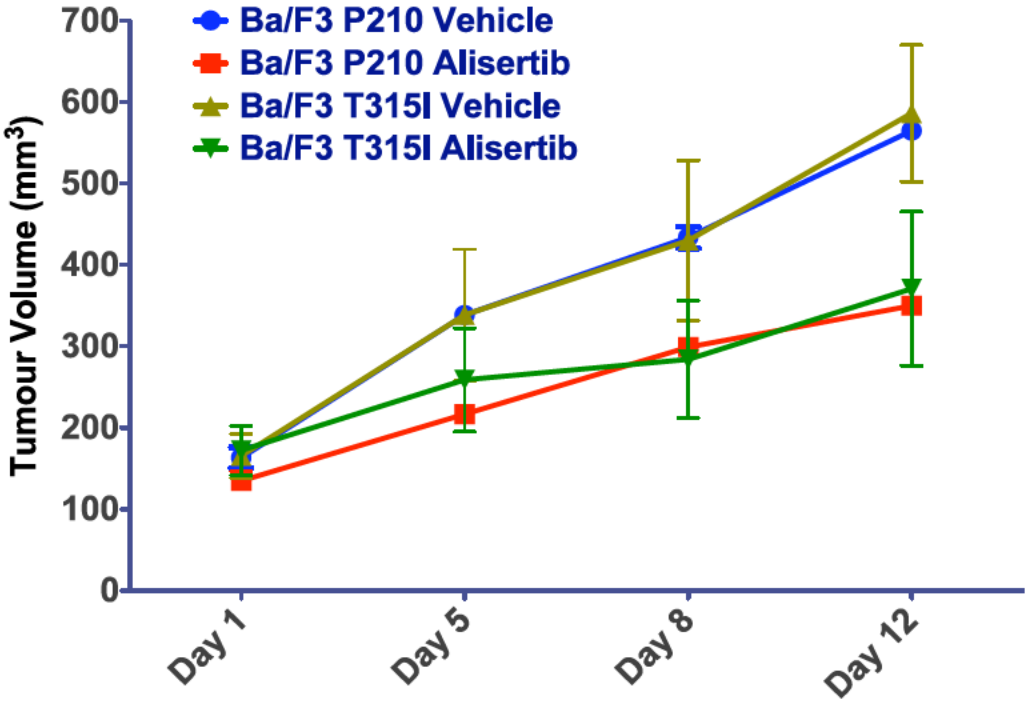

Figure 2.11 *In vivo* efficacy of alisertib

Ba/F3 cells expressing unmutated p210 BCR-ABL or T315I mutated BCR-ABL were injected into the flanks of nude mice to investigate the *in vivo* efficacy of alisertib against CML cells bearing the T315I mutation. Tumour bearing mice were randomized into groups of 10 and treated orally with 20 mg/kg alisertib twice daily or vehicle control for 14 days. n = 10 ± SD.

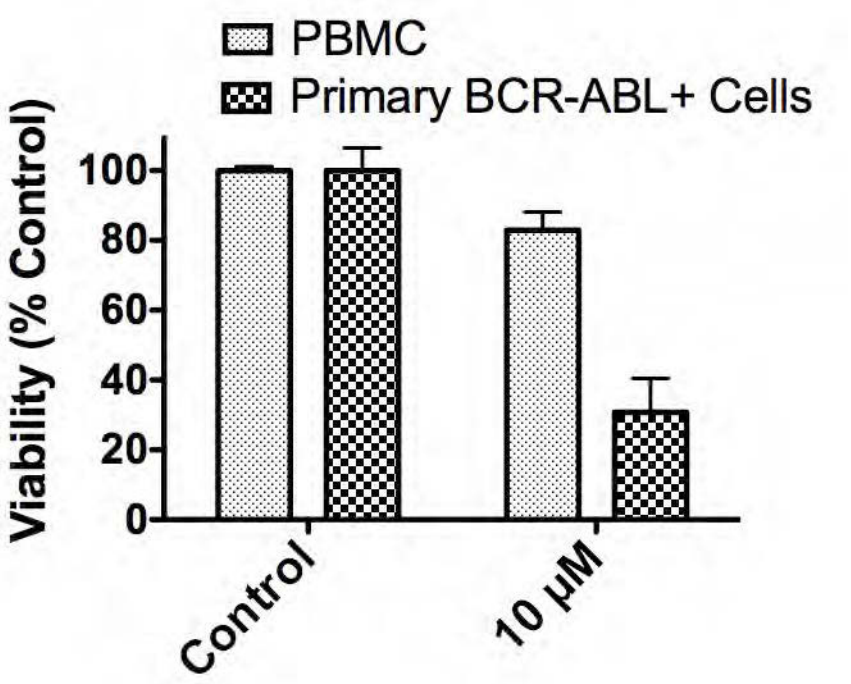

Figure 2.12 Activity of alisertib in primary CML cells and normal PBMCs

Cells from healthy donors or patients (4) with BCR-ABL+ leukaemia including 1 patient each with: unmutated BCR-ABL, T315I-mutated BCR-ABL, CML-BC, and Ph+ ALL and normal PBMCs were treated with alisertib for 96 hours and cell viability was assessed by MTT assay.

2.4.6 Alisertib inhibits Aurora A without significantly affecting BCR-ABL activity

In contrast to MK-0457, alisertib is a potent inhibitor of the Aurora A enzyme and is approximately 50 to 700 fold more selective against Aurora A compared to various ABL isoforms. As mentioned earlier, there has been some controversy regarding whether the anti-CML activity of MK-0457, which is no longer being clinically developed, was due to inhibition of Aurora kinases, BCR-ABL, or both. In an *in vitro* enzyme activity assay, MK-0457 has been shown to potently inhibit both wild type ABL (IC_{50} value 10 nM) and ABL (T315I) (IC_{50} value 30 nM) (507). To confirm that efficacious concentrations of alisertib do not significantly affect BCR-ABL activity, we evaluated its effect on BCR-ABL autophosphorylation and phosphorylation of the BCR-ABL direct substrate CRKL, which are accurate predictors of BCR-ABL kinase activity. Consistent with the *in vitro* enzyme assay data, immunoblotting analysis demonstrated that treatment of LAMA-84 and K562 cells with alisertib did not have a significant effect on the total levels of BCR-ABL or the levels of phospho-BCR-ABL at its Tyr^{177} autophosphorylation site (Figure 2.13). This indicates that alisertib induces growth inhibition and apoptosis in CML cells through a BCR-ABL-independent mechanism. Primary CML cells isolated from patients with CML are non-cycling and therefore much less sensitive to the growth inhibitory effects of alisertib. Consequently much higher doses of alisertib are needed to inhibit their growth (Figure 2.11). Therefore a dose of 10 µM was used in the western blot analysis that confirmed the lack of effect of alisertib on BCR-ABL activity on primary CML cells (Figure 2.14).

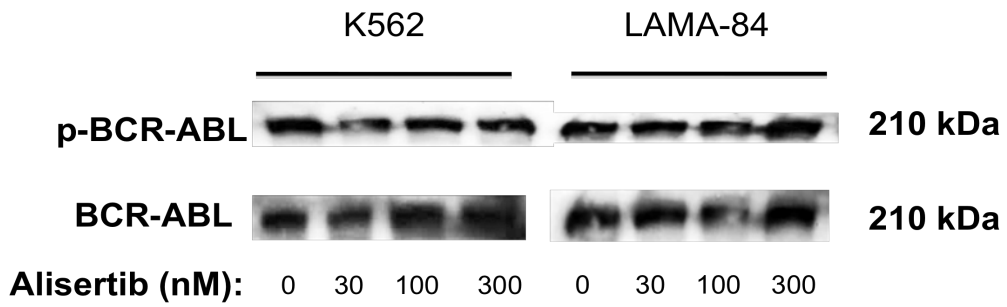

Figure 2.13 Alisertib does not significantly affect BCR-ABL activity in CML cell lines

K562 and LAMA-84 cells were treated with alisertib for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with phospho-BCR (Tyr^{177}) (Cell Signalling) and c-ABL (Cell Signalling) antibodies.

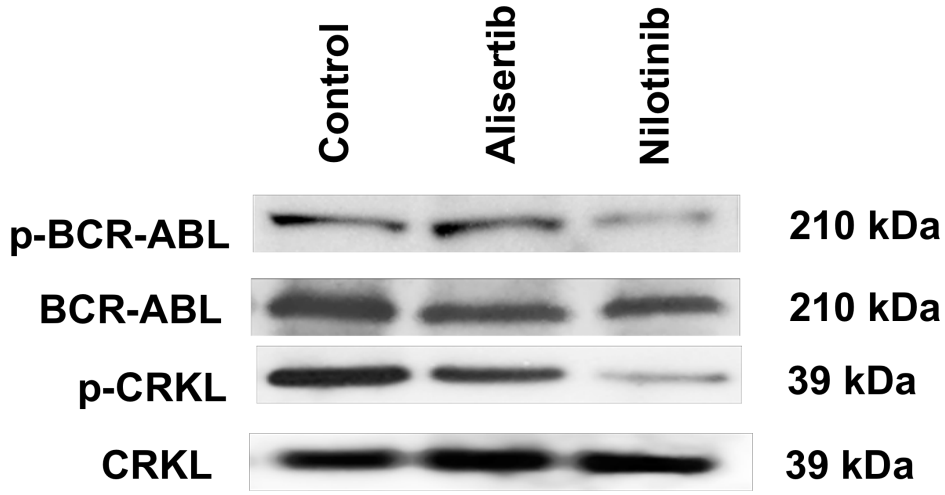

Figure 2.14 Alisertib treatment does not significantly affect BCR-ABL kinase activity in primary CML cells

Primary CML cells obtained from a patient with unmutated BCR-ABL were treated with 10 µM alisertib for 24 hours. BCR-ABL autophosphorylation and CRKL phosphorylation were assessed by immunoblotting and probing with phospho-Bcr (Tyr^{177}) (Cell Signalling) and phospho-CRKL (Tyr^{207}) (Cell Signalling) antibodies. c-ABL and total CRKL antibodies were also purchased from Cell Signalling, Beverly, MA. Nilotinib was used as a positive control for BCR-ABL inhibition.

2.4.7 Co-treatment with alisertib and nilotinib is significantly more effective than either agent alone

Patients with advanced stage CML have been hypothesized to benefit from non cross-resistant combinations of TKIs and agents effective against CML cells harbouring the T315I and E255K mutations (308, 638). Considering that alisertib is active against cells expressing the E255K and T315I mutations, we investigated whether alisertib augmented the activity of nilotinib, an FDA approved BCR-ABL inhibitor that is used in CML therapy. K562 and LAMA-84 cells were treated with 30 nM alisertib, 10 nM nilotinib, or the combination for 48 hours. Percentages of cells with sub G_0/G_1 DNA were quantified by PI/FACS. Co-treatment with alisertib and nilotinib resulted in significantly greater levels of apoptosis as determined by accumulation of sub G_0/G_1 cells (Figure 2.15). Immunoblotting analysis showed that the combination of these two agents induced mitochondrial-dependent apoptosis as evidenced by processing of caspases-9 and -3 to their active forms indicating that they cooperate to induce mitochondrial-dependent apoptosis (Figure 2.16).

The cytotoxic effects of the combination were also assessed by MTT assay. LAMA-84 and K562 cells were treated with alisertib, nilotinib, or the combination for 96 hours. Inhibition of growth and survival were significantly increased by combination treatment in both cell lines (Figure 2.17). Clonogenic assays were performed to evaluate the prolonged *in vitro* effects of alisertib and nilotinib on the growth and survival of both LAMA-84 and K562 cells (Figure 2.18). As expected, alisertib enhanced the ability of nilotinib to inhibit clonogenic survival. Formal synergy analysis according to the Chou-Talalay method (described in section 2.3.15) in LAMA-84 cells determined that combinations of 30 nM alisertib and 10 nM nilotinib combined with various doses of nilotinib (10 nM - 1 µM) or alisertib (30 nM - 3 µM) all produced CIs lower than 1 (range = 0.01 - 0.4), thus demonstrating the synergistic anti-cancer activity of this combination (Table 2.1. and Figure 2.19). Similarly in K562 cells, combinations of 100

nM alisertib and 0.1 nM nilotinib combined with various doses of nilotinib (10 nM - 1 μM) or alisertib (30 nM - 3 μM) all produced combination indices (CI) lower than 1 (range = 0.03 - 0.97) (Table 2.2 and Figure 2.20). Collectively, our findings suggest that alisertib significantly enhances the anti-leukaemia activity of nilotinib in human CML cells.

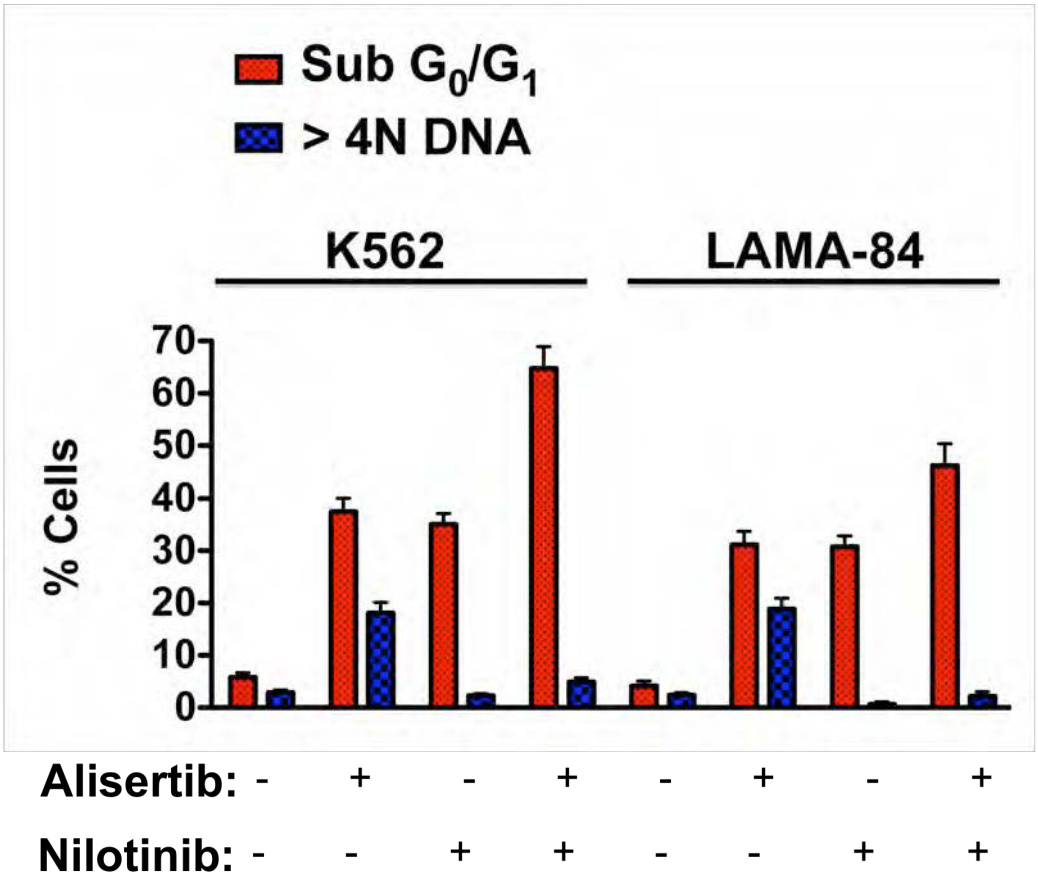

Figure 2.15 Alisertib significantly increases the efficacy of nilotinib

Alisertib potentiates the pro-apoptotic effects of nilotinib. K562 and LAMA-84 cells were treated with 30 nM alisertib, 10 nM nilotinib or the combination for 48 hours. Percentages of cells with sub G_0/G_1 DNA were determined by PI/FACS. n = 3 ± SD.

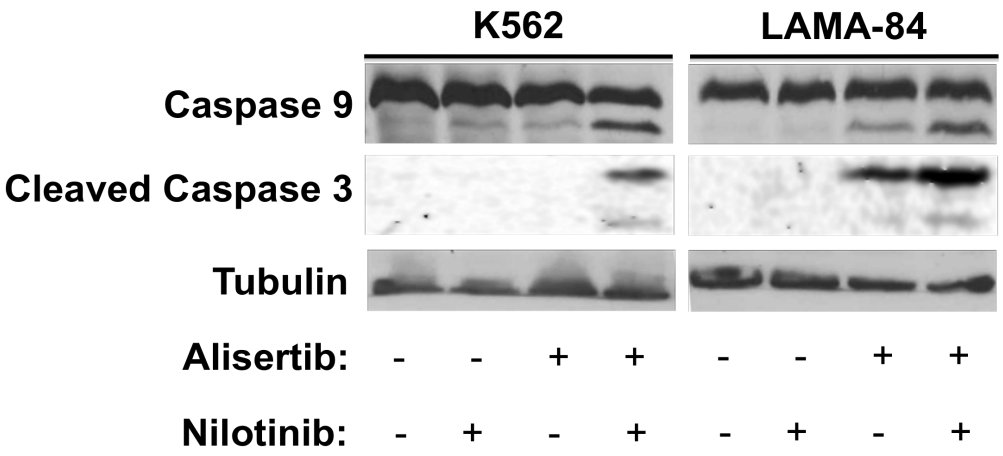

Figure 2.16 The combination of alisertib and nilotinib induces mitochondrial dependent apoptosis

K562 and LAMA-84 cells were treated with 100 nM alisertib, 30 nM nilotinib, or both for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with active caspase-3 and caspase-9 antibodies. Anti β-tubulin was used as loading control.

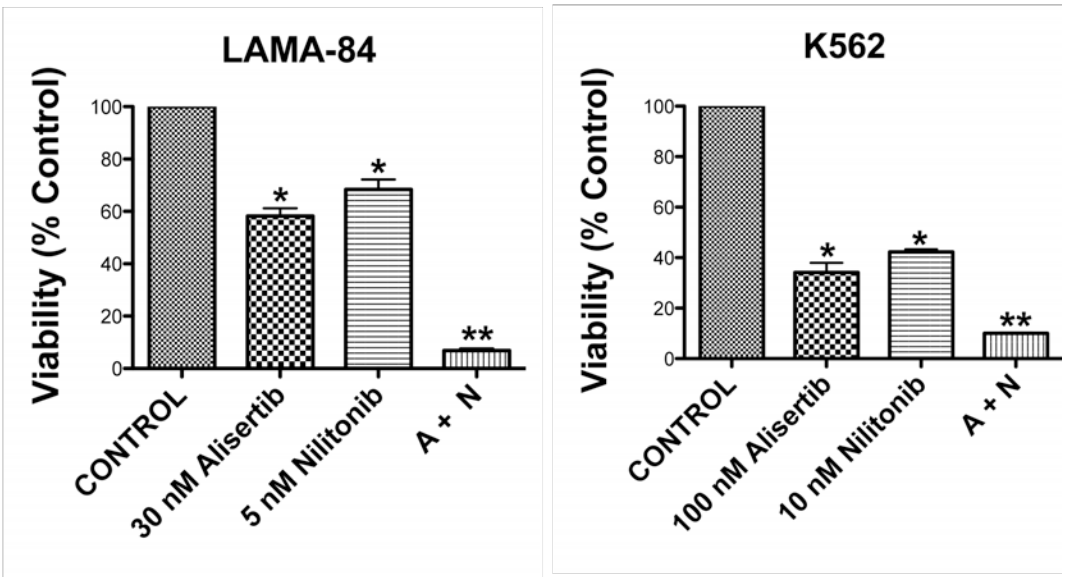

Figure 2.17 Co-treatment with alisertib and nilotinib results in significantly greater growth inhibition and reduction in survival than that achieved by either agent alone

Cells were treated with the indicated concentrations of alisertib for 96 hours and viability was assessed by MTT assay. Error bars indicate the SD. *p < 0.05 (controls vs. single agents), **p < 0.05 (single agents vs. combination).

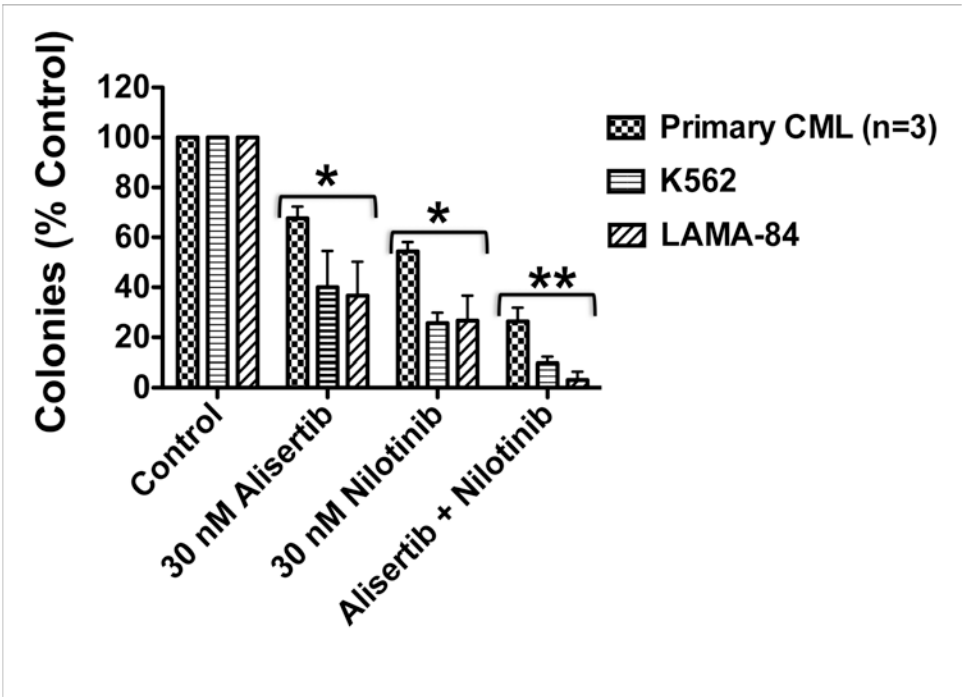

Figure 2.18 Effects of alisertib and nilotinib on clonogenic survival

Primary CML cells from patients in BC (n = 3), K562, and LAMA-84 cells were treated with alisertib, nilotinib, or both drugs for 24 hours. Cells were plated and scored as described in the materials and methods. Error bars indicate the SD. *$p < 0.05$ (controls vs. single agents), **$p < 0.05$ (single agents vs. combination).

Alisertib (nM)	Nilotinib (nM)	Viability (% Control)	CI
30	10	14.3	0.01
30	30	12.9	0.02
30	100	12.6	0.04
30	300	12.4	0.11
30	1000	11	0.22
100	10	16.9	0.04
300	10	16.2	0.09
1000	10	15	0.23
3000	10	12	0.40

Table 2.1. Evaluation of the combination of alisertib with nilotinib in LAMA-84 cells

LAMA-84 cells were treated with the indicated concentrations of alisertib and nilotinib for 72 hours following, which MTT assays performed. Calculated CIs of different double combinations for the Lama 84 cell lines are indicated in the table. The CI values were calculated for 3 independent experiments. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism of the 2 agents, respectively.

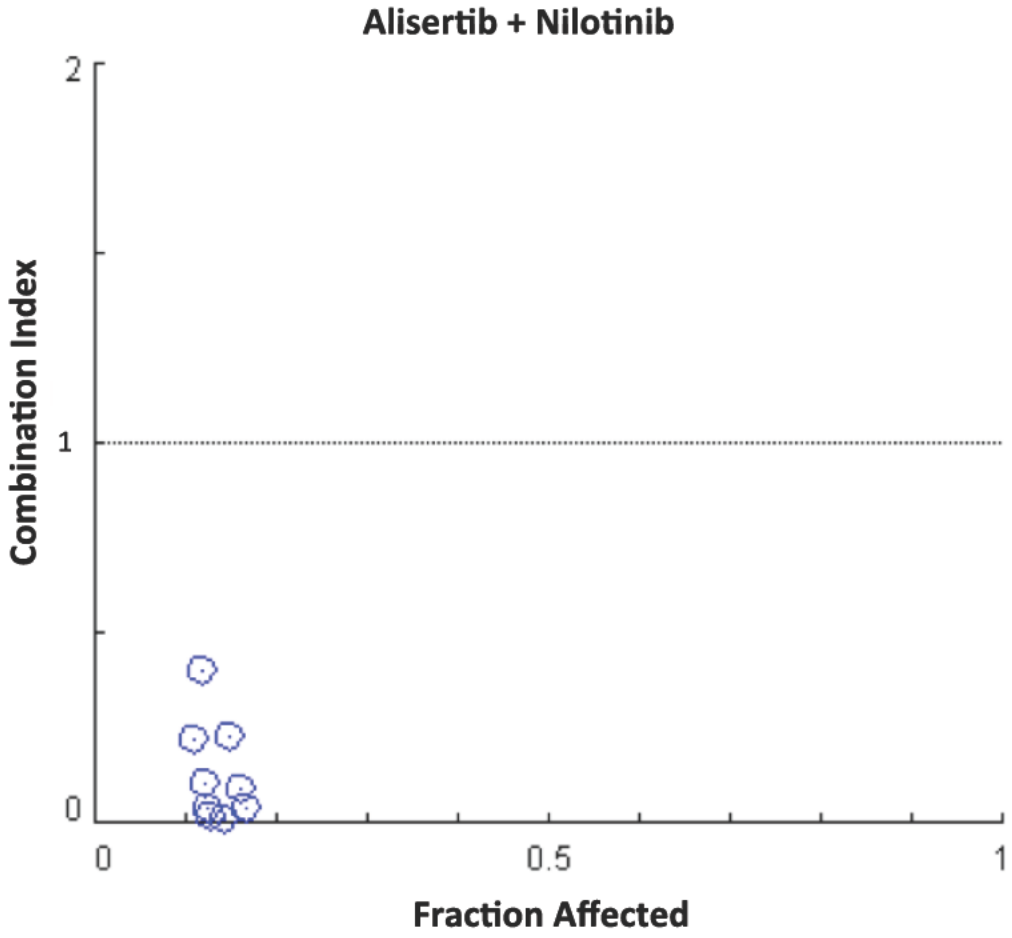

Figure 2.19 Evaluation of the combination of alisertib with nilotinib in LAMA-84 cells

LAMA-84 were treated with the indicated concentrations of alisertib and nilotinib for 72 hours following which MTT assays performed. Using CompuSyn software (Biosoft), the analysis of the dose-effect relationship for alisertib and nilotinib was performed according to the median effect method of Chou and Talalay (634). A fraction affected-CI plot is provided.

Alisertib (nM)	Nilotinib (nM)	Viability (% Control)	CI
100	0.1	71.2	0.97
100	0.3	69	0.82
100	1.0	64	0.55
100	3.0	57	0.31
100	10.0	46	0.12
30	0.1	73	0.40
300	0.1	3.22	0.08
1000	0.1	2	0.07
3000	0.1	1	0.04

Table 2.2. Evaluation of the combination of alisertib with nilotinib in K562 cells

K562 were treated with the indicated concentrations of alisertib and nilotinib for 72 hours following, which MTT assays performed. Using CompuSyn software (Biosoft), the analysis of the dose-effect relationship for alisertib and nilotinib was performed according to the median effect method of Chou and Talalay (634). CIs of different double combinations for the K562 cell lines are indicated in the table.

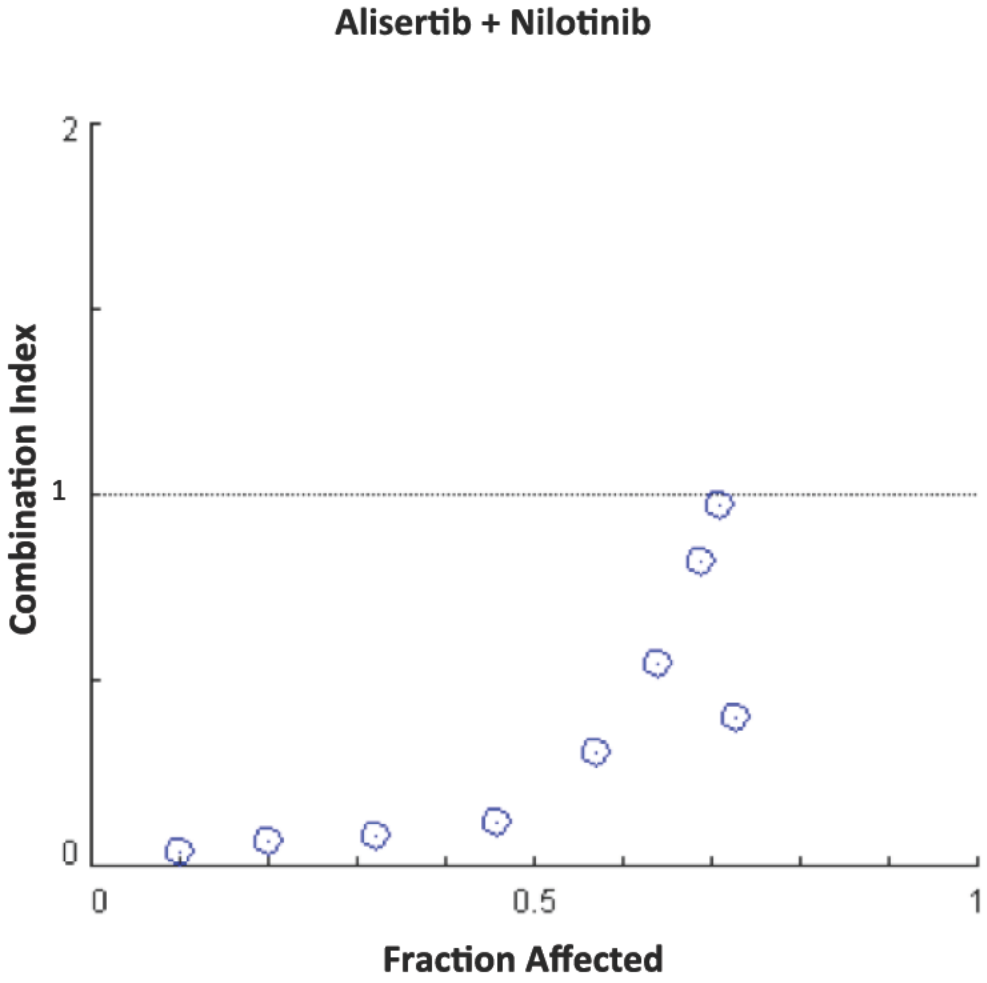

Figure 2.20 Evaluation of the combination of alisertib with nilotinib in K562 cells

K562 were treated with the indicated concentrations of alisertib and nilotinib for 72 hours following, which MTT assays performed. Using CompuSyn software (Biosoft), the analysis of the dose-effect relationship for alisertib and nilotinib was performed according to the median effect method of Chou and Talalay (634). A fraction affected-CI plot is provided.

2.4.8 Alisertib cooperates with nilotinib to reduce tumour burden in K562 xenografts

K562 xenograft studies were carried out to investigate the *in vivo* therapeutic potential of the combination of alisertib and nilotinib. Both agents had substantial effects on tumour burden and the combination resulted in significantly greater tumour growth inhibition than was achieved by either agent alone (Figure 2.21). Furthermore, the combination was well tolerated and only a modest loss in body weight was observed in the treated groups (Figure 2.22). Notably, the single agent *in vivo* effects of alisertib were more impressive in this K562 experiment than that we observed in our studies with the murine Ba/F3 models (Figure 2.11). Given that human and murine Aurora genes share 79% sequence homology and that alisertib was specifically designed to target human Aurora A kinase activity, it is possible that species specific differences in the potency of alisertib-mediated kinase inhibition could have contributed to this phenomenon.

H&E staining was used to visualize the architecture of tumours from each treatment group and revealed substantial differences in the morphology of single agent and combination-treated tumours. In particular, the tumours treated with the combination of alisertib and nilotinib displayed evidence of stromal disruption and high levels of cell death with very few intact CML cells remaining (Figure 2.23). This suggests that remaining tumours from combination treated mice were largely comprised of matrigel and non-viable cells/tissue and also highlights the potential therapeutic benefit provided by the combination over single agent treatments.

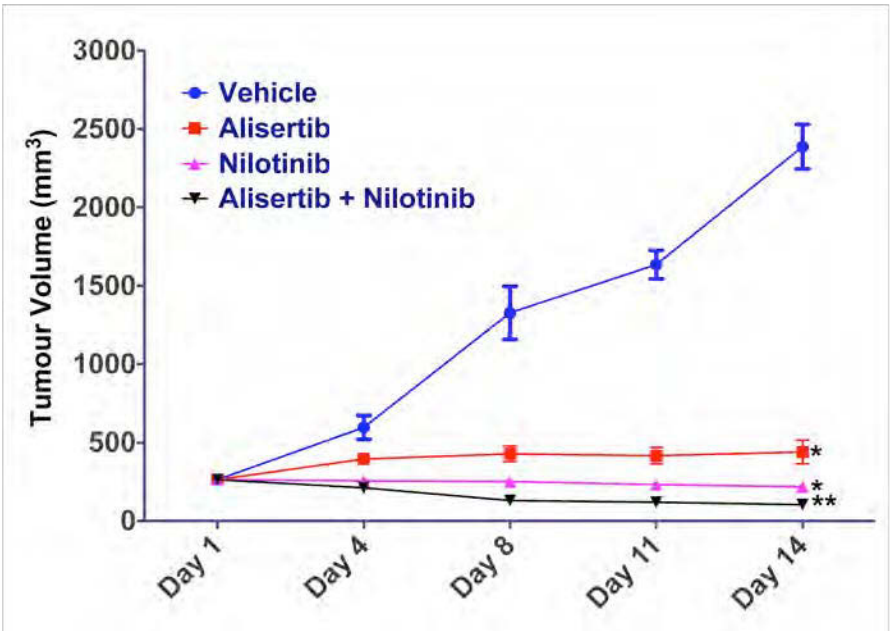

Figure 2.21 *In vivo* efficacy and tolerability of alisertib and nilotinib

K562 cells were injected into the flanks of nude mice. Vehicle, alisertib, nilotinib or both were administered for 14 days. After tumour growth reached 150 mm^3, mice were randomly assigned to receive alisertib 20 mg/kg BID (n=10), nilotinib 50 mg/kg QID (n=10), vehicle control (n=10) or both alisertib and nilotinib (n=10) for 14 days. Mice were monitored daily and tumour volumes were measured twice weekly. N = 10 ± SD. *p < 0.05 (controls vs. single agents), **p < 0.05 (single agents vs. combination).

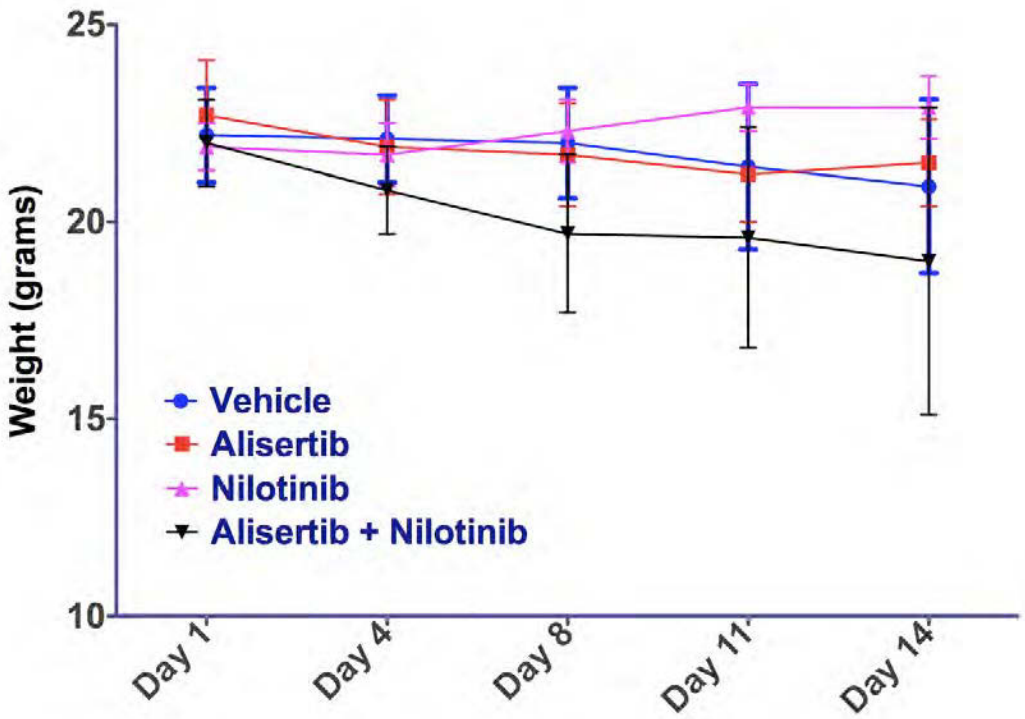

Figure 2.22 *In vivo* tolerability of alisertib and nilotinib

K562 cells were injected into the flanks of nude mice. Vehicle, alisertib, nilotinib or both were administered for 14 days. After tumour growth reached 150 mm^3, mice were randomly assigned to receive alisertib 20 mg/kg BID (n=10), nilotinib 50 mg/kg QID (n=10), vehicle control (n=10) or both alisertib and nilotinib (n=10) for 14 days. Animal weights were measured biweekly. n = 10 ± SD.

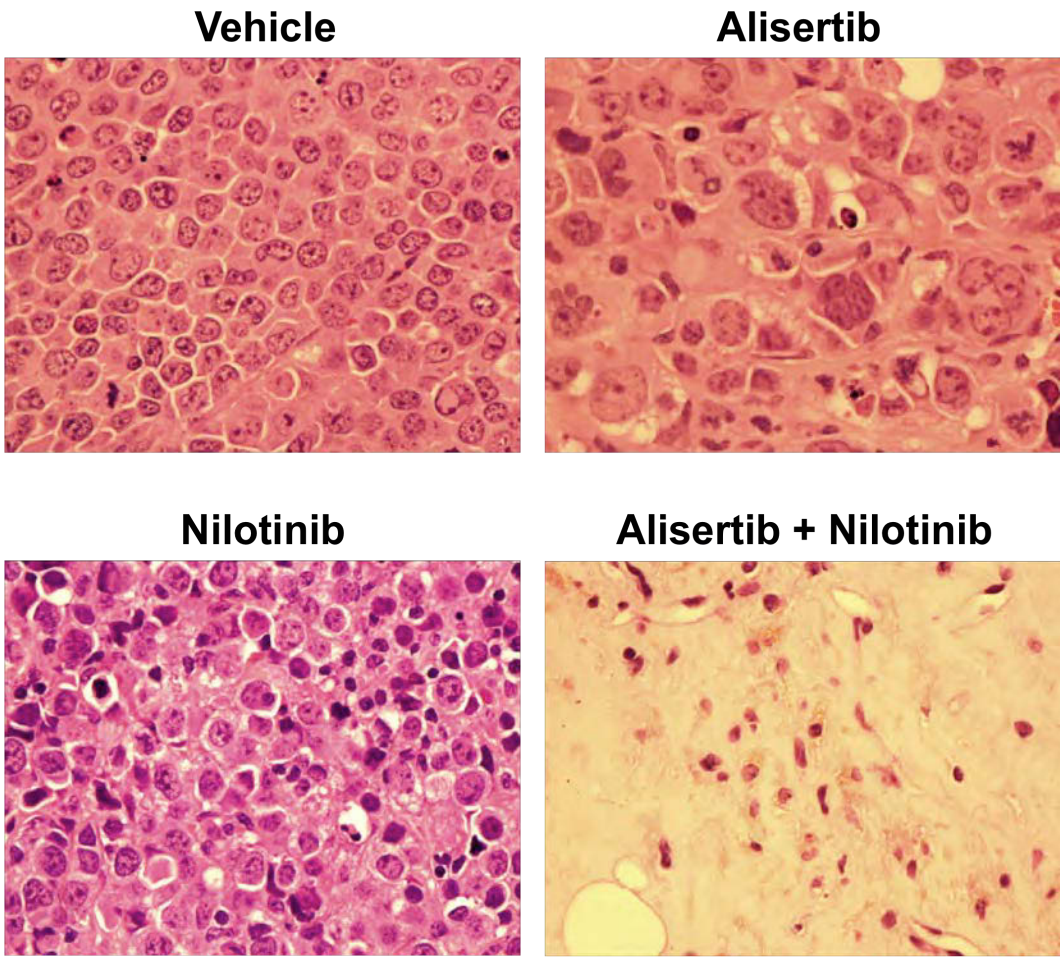

Figure 2.23 Immunohistochemistry

Tumours were stained with H&E as described in materials and methods. Representative images are shown from each treatment group.

2.4.9 Treatment with alisertib leads to a morphological phenotype consistent with Aurora A inhibition

The inhibition of Aurora A causes defects in centrosome segregation, spindle pole organization, and chromosome congression, which can ultimately lead to tumour cell death via the development of deleterious aneuploidy (468). H&E staining of tumours obtained at the completion of treatment was carried out to examine the morphological changes in K562 cells caused by treatment with alisertib. Consistent with the high number of *in vitro* aneuploid cells observed with PI/FACS analysis, the number of multinucleated cells visible in the alisertib-treated tumours was increased as compared with vehicle control indicating that alisertib causes cells to exit mitosis without completing cytokinesis, a process known as mitotic slippage (Figure 2.24 top right). Although this outcome is primarily associated with inhibition of Aurora B, cytokinesis failure can occur upon inhibition of Aurora A as well (466). Other functional consequences of Aurora A inhibition that were prominent in the alisertib-treated tumours included an increased number of cells with monopolar mitotic spindles (Figure 2.24, bottom right), an elevated number of cells with chromatin bridging (Figure 2.24, top left), and cells with internuclear bridging (Figure 2.24, bottom left). Taken together, these findings indicate that treatment with alisertib results in morphological changes in CML cells that are consistent with Aurora A kinase inhibition and indicative of deleterious aneuploidy and MC.

2.4.10 Alisertib augments the *in vivo* pro-apoptotic effects of nilotinib

TUNEL assays were carried out on xenograft tumour sections after completion of treatment to assess the degree of apoptosis induced by alisertib and nilotinib *in vivo*. Both agents induced apoptosis as indicated by TUNEL positivity, but the percentage of TUNEL positive cells was significantly greater in the combination group compared to treatment with

either agent alone indicating that the two agents cooperate to provoke apoptosis *in vivo* (Figure 2.25 and 2.26).

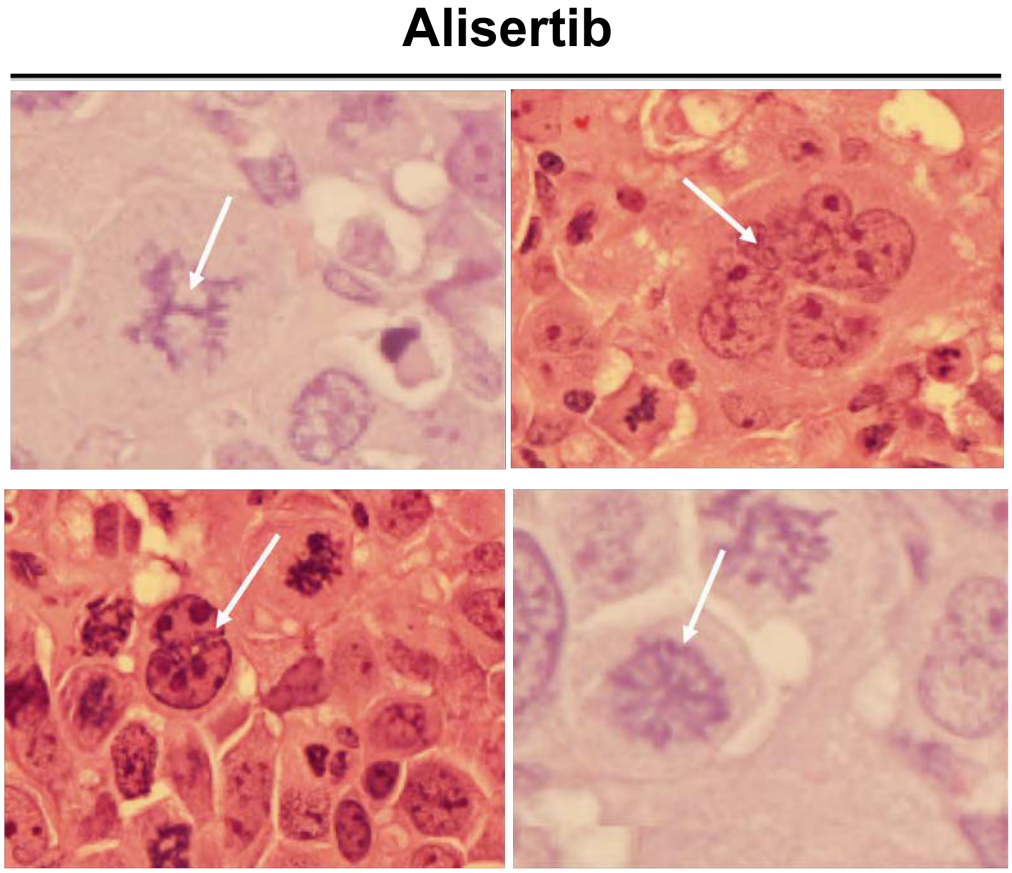

Figure 2.24 Treatment with alisertib leads to a morphological phenotype consistent with Aurora A kinase inhibition

Tumours were stained with H&E. Representative images from the alisertib treatment group are shown. Arrows indicate the following: an elevated number of cells with chromatin bridging (top left), large cells with multiple micronuclei (top right), cells with internuclear bridging (bottom left), and monopolar mitotic spindles (bottom right).

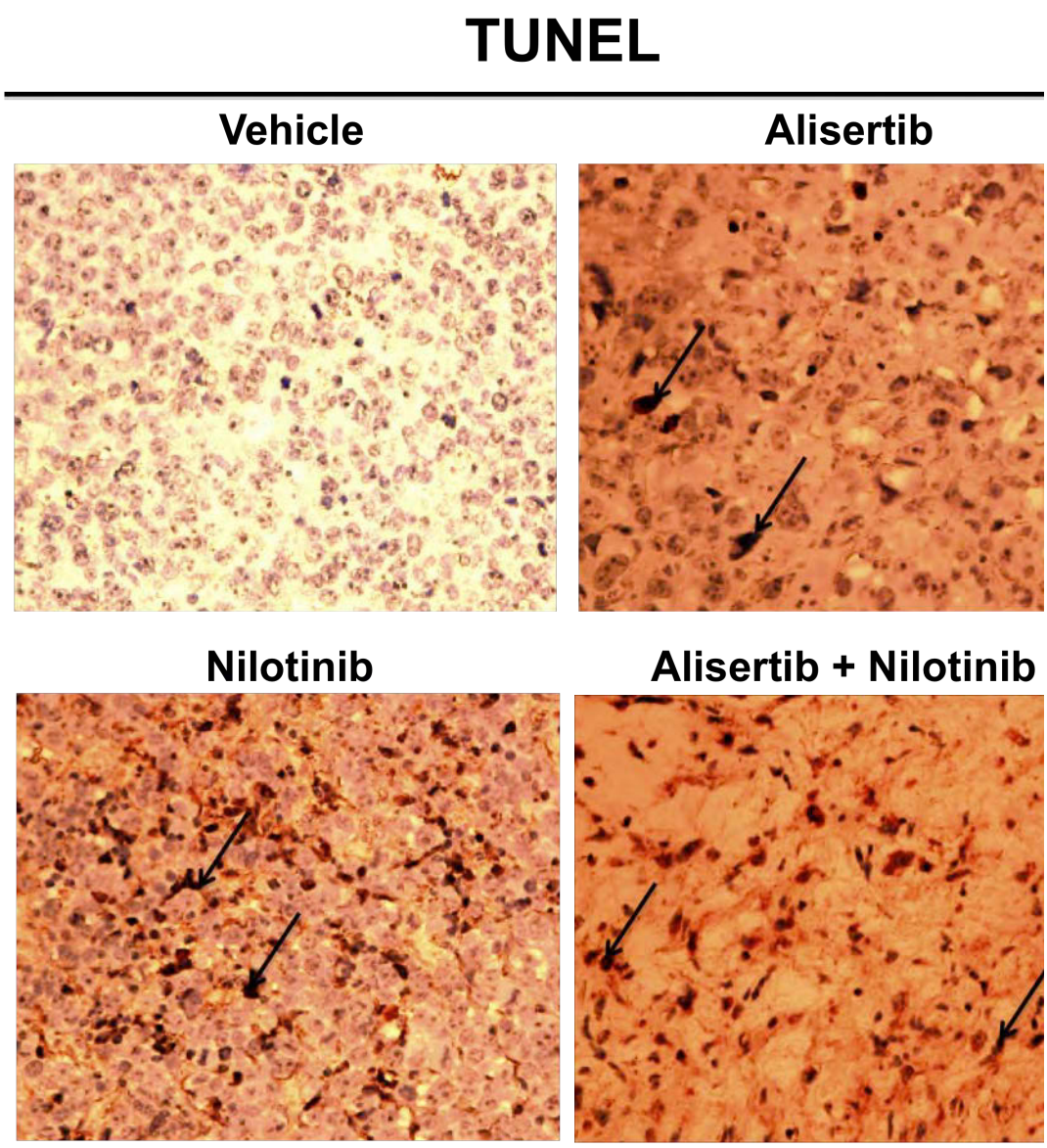

Figure 2.25 Assessment of TUNEL-positive cells

DNA fragmentation was analyzed using a colorimetric terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assay kit (Promega, Madison, WI). The assay was carried out according to the manufacturer's instructions. Images were obtained with an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20X objective. Arrows indicate representative TUNEL positive cells.

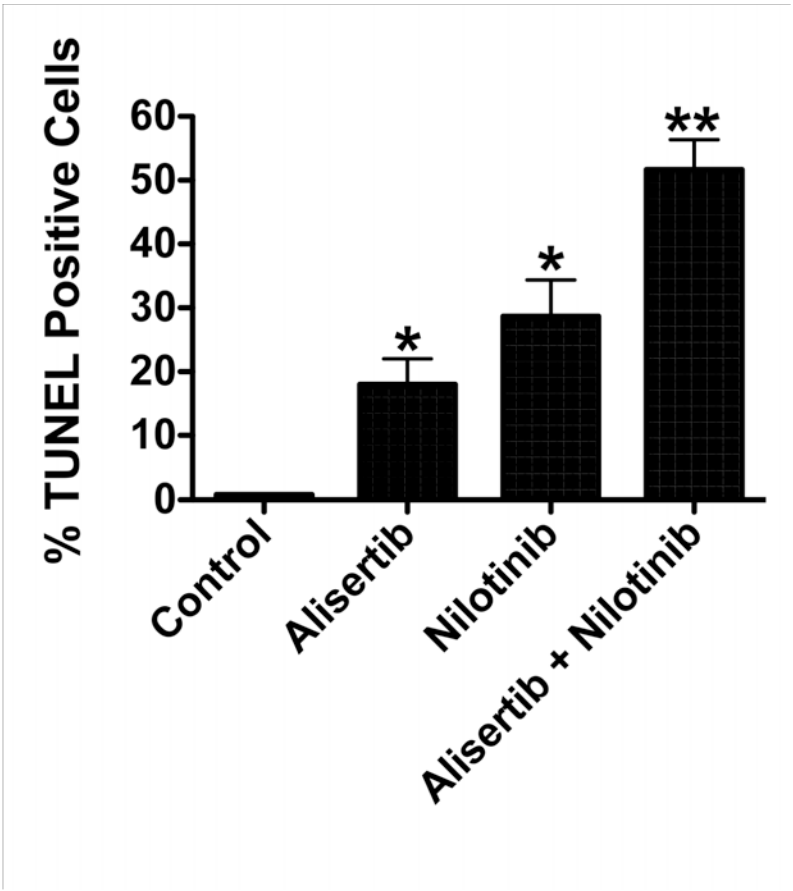

Figure 2.26 Quantification of TUNEL-positive cells

Percentages of TUNEL-positive cells were determined by manual counting of 5 random fields per section. Image-Pro Plus software Version 6.2.1 (MediaCybernetics, Bethesda, MD) was used for image acquisition. Positive cells were scored manually. Percentages of TUNEL-positive cells were determined by manual counting of 5 non-overlapping random high-power fields per section by two investigators blinded to the treatment arms. The average percentage of TUNEL positive cells per high-power field was calculated for comparison. Mean ± SD, n = 5. *$p < 0.05$ (controls vs. single agents), **$p < 0.05$ (single agents vs. combination).

2.4.11 Inhibition of Aurora A activity leads to reduced expression of Apollon

Apollon (also known as BRUCE or BIRC6, baculovirus inhibitor of apoptosis protein (IAP) repeats (BIR)-containing protein 6) is a very large (528 kDa) IAP that can be distinguished from other IAP family members by the presence of its ubiquitin conjugating enzyme (UCE) domains, which are not contained within any other IAPs (639). The UCE domains within Apollon allow it to reduce the pro-apoptotic potential of cells by targeting SMAC and caspase-9 for proteasomal degradation in addition to directly binding to caspases and other pro-apoptotic molecules via its BIR domain to prevent apoptosis in a manner similar to other IAPs (640, 641). Apollon is overexpressed in leukaemia and other cancers and has been linked with resistance to chemotherapy (639, 642). A recent investigation demonstrated that Apollon also has important functions during mitosis. It coordinates multiple events in cytokinesis and moves to the midbody ring during cell division where it serves as a platform for the membrane delivery machinery and mitotic regulators including the Aurora kinases (643). Apollon depletion causes defective abscission and cytokinesis-associated apoptosis (643).

Considering that Apollon is associated with Aurora kinases and has roles in cell division and inhibition of apoptosis, we investigated whether abrogation of Aurora A kinase activity would affect Apollon expression. Immunoblotting analysis showed that alisertib treatment resulted in a dose-dependent reduction in the expression of Apollon and increased expression of the pro-apoptotic Apollon substrate, SMAC (Figure 2.27). To confirm that this reduction in Apollon expression is a direct consequence of Aurora A kinase inhibition, we used siRNA to knockdown Aurora A expression. This led to a very significant reduction in the levels of Apollon (Figure 2.28). This effect does not appear to be a general feature of mitotic disruption as treatment with the microtubule disrupting agents, nocodazole

and VCR, did not significantly decrease Apollon expression and therefore rather suggests a link between Aurora A activity and Apollon expression (Figure 2.29).

2.4.12 Knockdown of Apollon sensitizes CML cells to nilotinib

To determine whether inhibition of Apollon played a role in sensitizing CML cells to nilotinib, we knocked down Apollon expression in LAMA-84 cells using siRNA (Figure 2.30). LAMA-84 cells transfected with Apollon targeted siRNA and non-targeted siRNA were treated with alisertib, nilotinib or the combination for 48 hours and the percentages of apoptotic cells were determined by assessing the degree of DNA fragmentation by PI/FACS analysis. Nilotinib induced significantly greater levels of apoptosis in LAMA-84 cells treated with Apollon-targeted siRNA compared to non-targeted controls (Figure 2.30). However alisertib and the combination of alisertib and nilotinib induced only minor increases in the levels of apoptosis in LAMA-84 cells treated with Apollon-targeted siRNA compared to non-targeted controls. This data suggests that inhibition of Apollon expression caused by alisertib treatment sensitizes CML cells to nilotinib-induced apoptosis and provides a rationale for the combination of these two agents in CML.

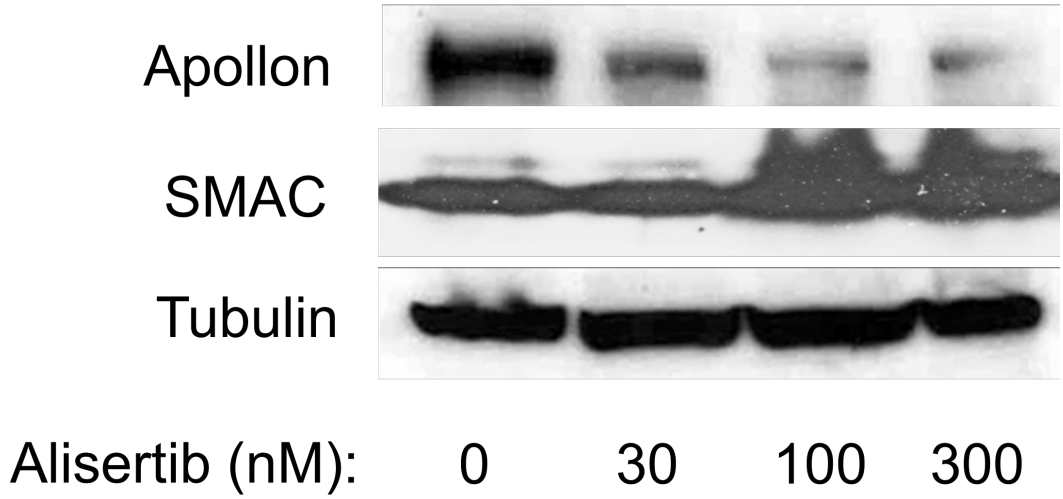

Figure 2.27 Alisertib treatment results in a dose-dependent reduction in the large inhibitor of apoptosis protein, Apollon and increased expression of its substrate, SMAC

LAMA-84 cells were treated with indicated doses of alisertib for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with Apollon and SMAC antibodies. Tubulin documented equal loading.

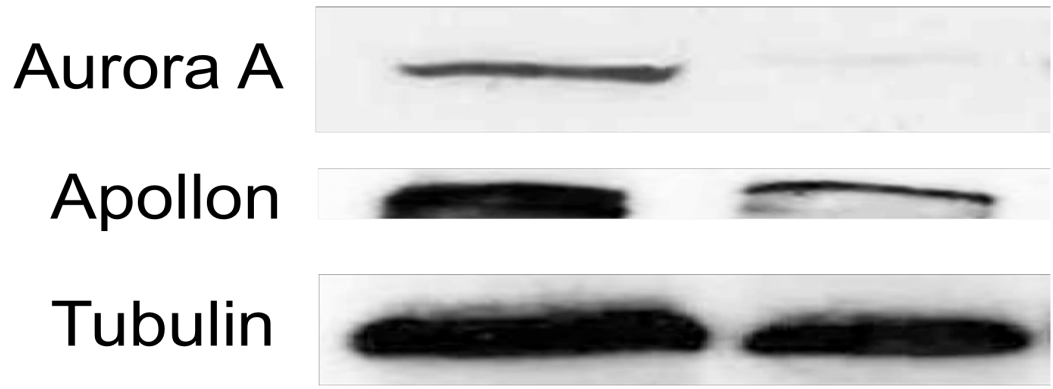

Figure 2.28 siRNA of knockdown Aurora A expression leads to a significant reduction in the levels of Apollon

Aurora A SMARTpool or siCONTROL siRNA directed at luciferase were transfected into LAMA-84 cells using the Nucleofector II. Protein lysates were subjected to SDS-PAGE, blotted, and probed with Apollon and Aurora A antibodies. Tubulin documented equal loading.

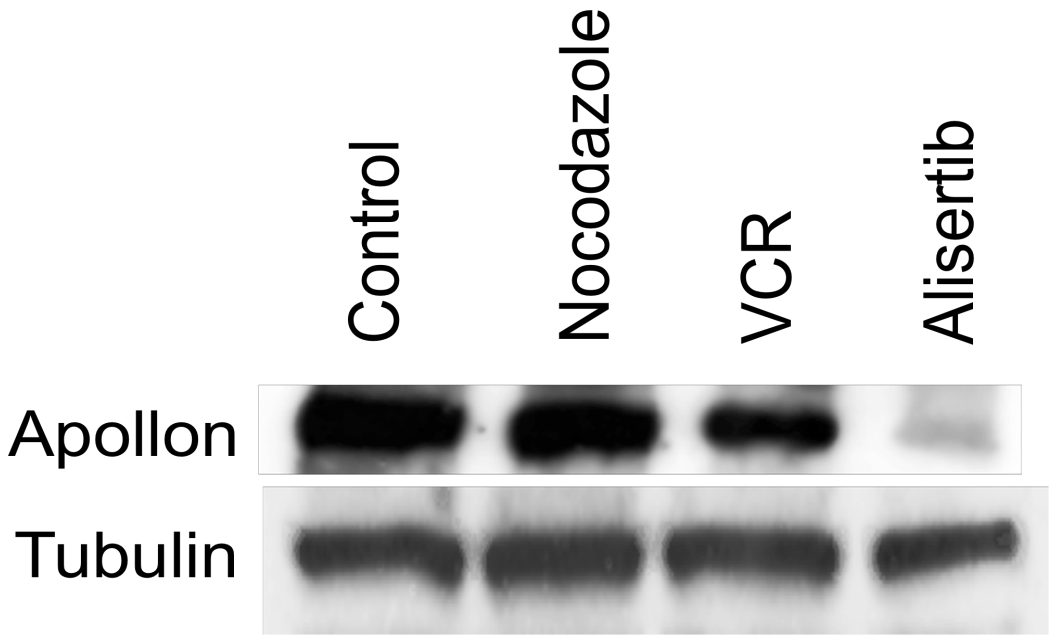

Figure 2.29 General disruption of mitosis does not significantly effect Apollon expression

LAMA-84 cells were treated with nocodazole, VCR, or alisertib for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with an Apollon antibody. Tubulin documented equal loading.

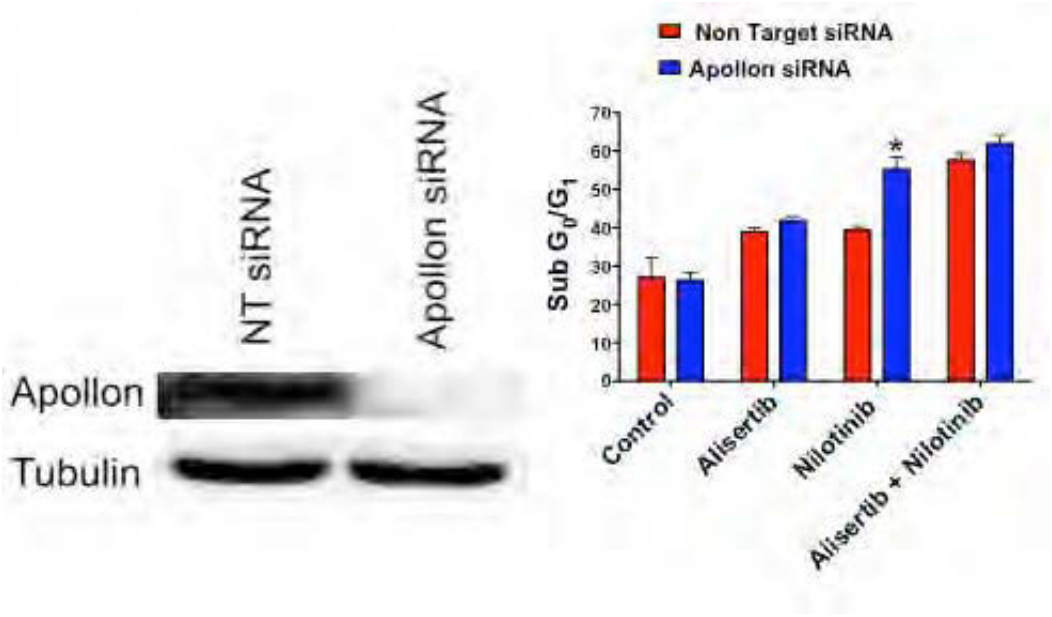

Figure 2.30 siRNA knockdown of Apollon sensitizes CML cells to nilotinib

Apollon SMARTpool or siCONTROL siRNA directed at luciferase were transfected into LAMA-84 cells using the Nucleofector II. Tubulin was used as a loading control. LAMA-84 cells transfected with Apollon-targeted siRNA and non-targeted siRNA were treated with 30 nM alisertib, 10 nM nilotinib or both for 48 hours and the percentage of apoptotic cells were determined by PI/FACS analysis. n =3 ± SD, *p < 0.05.

2.5 Discussion

Resistance to TKI therapy in CML continues to be a significant problem. In particular, the T315I and E255K mutations in BCR-ABL confer cross-resistance to imatinib, dasatinib, and nilotinib (308). The pan-Aurora kinase inhibitor MK-0457 has shown clinical activity against CML cells harbouring the T315I mutation (626). Early *in vitro* competition binding assays revealed that MK-0457 bound to wild type ABL1 and T315I ABL1 This observation led to the hypothesis that the anti-leukaemic efficacy of MK-0457 was due to inhibition of BCR-ABL, rather than Aurora activity (628, 629). However, a more recent investigation revealed that the efficacy of clinically relevant concentrations of MK-0457 was primarily due to inhibition of Aurora kinase activity (644). How significantly Aurora kinase inhibition contributes to the activity of MK-0457 in CML remains somewhat controversial. This matter may not be definitively resolved for MK-0457 as its development has been stopped due to issues with cardiac toxicity observed in some early phase clinical trials and will have to be addressed in studies with other Aurora kinase inhibitors.

Alisertib is a highly selective inhibitor of Aurora A and demonstrates little inhibition of various ABL isoforms in enzyme assays. Our data suggests that alisertib does not directly inhibit BCR-ABL activity, indicating that Aurora A kinase is a valid therapeutic target in CML. The biological consequences of Aurora A kinase inhibition have been intensively investigated in recent years. Ultimately, cells treated with Aurora A kinase inhibitors undergo cell death through the development of deleterious aneuploidy (468). Our data shows that alisertib treatment initially results in a significant degree of aneuploidy before cell death ensues (Figure 2.4).

Notably, alisertib-induced apoptosis was associated with significant decreases in the expression levels of Apollon. Apollon has several functional domains, multiple binding partners, and plays important roles in

the regulation of apoptosis and cell division (643, 645). As Apollon over-expression has been associated with an unfavourable outcome and resistance to chemotherapy in leukaemia, we were particularly interested in examining whether the ability of alisertib to reduce Apollon expression could potentially sensitize CML cells to the standard of care agent nilotinib (642). Our results show that targeted knockdown of Apollon significantly augments the pro-apoptotic effects of nilotinib, suggesting that suppression of Apollon expression by alisertib may contribute to its ability to heighten the anti-cancer activity of nilotinib. Furthermore, it is likely that the reduction in Apollon expression associated with alisertib is a direct consequence of Aurora inhibition as targeted knockdown of Aurora A kinase by siRNA was associated with a significant reduction in Apollon expression. These findings represent a novel mechanistic approach for improving the efficacy of TKI therapy in CML.

There are two potential important clinical translations of the results of the current study. We have shown that alisertib is effective against cell lines and primary patient CML cells expressing unmutated and mutated forms of BCR-ABL including the highly resistant T315I mutation and that it has activity independent of p53 function. Therefore, alisertib may be clinically active as a single agent in the setting of T315I and E255K mutations for which the currently available TKIs are ineffective. Moreover, the combination of alisertib and nilotinib is effective and well tolerated in preclinical models of CML and represents a novel therapeutic strategy for advanced phase CML that is orally active and has the potential to suppress the emergence of CML clones expressing a range of resistant mutations including T315I and E255K.

The current treatment strategy for resistant CML involves sequential administration of TKIs, which is associated with the development of compound mutations in BCR-ABL with increased oncogenic potency (638). Patients with imatinib resistance have heightened genomic instability and in this setting combination treatment with an agent effective against cells

harbouring the T315I mutation and a BCR-ABL kinase inhibitor could possibly prevent resistance caused by kinase domain mutations in CML (308, 646).

In conclusion, this study show the Aurora A inhibitor, alisertib, has significant activity in preclinical models of CML. Notably, the efficacy of alisertib is unaffected by impairment of p53 function or the presence of clinically relevant BCR-ABL kinase domain mutations. Alisertib treatment leads to a reduction in the expression of the large inhibitor of apoptosis, Apollon and a morphological phenotype consistent with Aurora A kinase inhibition. Moreover, inhibition of Aurora A with alisertib significantly increases the *in vitro* and *in vivo* efficacy of nilotinib in CML cell lines and immunodeficient mice bearing K562 xenografts. These data provide rationale for a novel therapeutic approach targeting Aurora A and BCR-ABL in CML.

3 Chapter 3: Targeting Aurora A in preclinical models of AML

3.1 Introduction

As discussed earlier AML accounts for 80 percent of adult acute leukaemia and while outcomes have improved for younger patients the elderly are particularly less likely to respond to therapy (3, 354, 647, 648). Improvements in outcome in this age group depend on the development of agents targeted to the molecular abnormalities in this disease with minimal off target side effects (649-651). These agents on their own produce transient responses only but will likely improve outcome as individualized targeted therapy in combination with conventional treatment. A recent study demonstrated that elderly patients with good or intermediate risk cytogenetics that received therapy with low-dose ara-C had a significant survival advantage over patients that received supportive care. Despite this, no patients with unfavourable cytogenetics achieved CRs on this study (652). It is unclear whether elderly patients benefit from standard induction chemotherapy. One approach in experimental therapeutics that has shown promise is to selectively target the aberrant expression of Aurora kinases in malignancies. Given the overexpression of Aurora kinases in myeloid malignancies and their high proliferation rate, targeting of these key regulators of cell cycle progression appears to be an attractive therapeutic option.

High Aurora A and to a lesser extent Aurora B expression has been shown in AML cells in several studies of large numbers of patient samples (see section 1.8.5). As discussed in section 1.8.6, Aurora kinase inhibitor therapy in AML is currently being evaluated in phase 1 and 2 studies. The widely demonstrated *in vitro* effects of Aurora B (reduction in histone H3 phosphorylation) and Aurora A inhibition (caspase activation) have been replicated in patient samples and modest disease activity in AML patients refractory to standard therapy has been demonstrated (529). To date, the pan-Aurora kinase inhibitor MK-0457/VX-680, the Aurora B selective inhibitor AZD1152, and the multi-kinase inhibitor with anti-Aurora effects KW-2449 have shown pre-clinical activity in models of AML (512, 539, 629,

653-655). Aurora A selective inhibitors have not been previously evaluated in preclinical models of AML.

Considering the dual role of Aurora A in regulating cell cycle progression and programmed cell death and the high basal expression of Aurora A in AML cells, we hypothesized that AML cells would be particularly sensitive to alisertib (502). To test this hypothesis, we investigated the efficacy and pharmacodynamic effects of alisertib in AML cell lines, primary AML blasts, and mouse models of AML.

Project hypothesis

Targeting Aurora A kinase with alisertib will lead to apoptosis and growth inhibition in preclinical models of AML and will potentiate the anti-leukaemic activity of ara-C.

Project aims

i. To investigate the therapeutic potential of targeting Aurora kinase A activity with alisertib in preclinical models of AML and primary blasts from patients with AML.
ii. To evaluate the relationship between Aurora A expression/activity and cellular sensitivity to ara-C.
iii. To elucidate the mechanism of action of alisertib in AML and identify essential regulators of sensitivity to the combination of alisertib and ara-C.

3.2 Project summary

Novel therapies are urgently needed to improve clinical outcomes for patients with AML. We investigated the preclinical efficacy and pharmacodynamics of alisertib in AML cell lines, primary AML cells, and mouse models of AML. Alisertib disrupted cell viability, diminished clonogenic survival, induced expression of the FOXO3a targets p27 and BIM, and triggered apoptosis. A link between Aurora A expression and sensitivity to ara-C was established, suggesting that Aurora A inhibition may be a promising strategy to increase the efficacy of ara-C. Accordingly, alisertib significantly potentiated the anti-leukaemic activity of ara-C in both AML cell lines and primary blasts. Targeted FOXO3a knockdown significantly blunted the pro-apoptotic effects of the alisertib/ara-C combination, indicating that it is an important regulator of sensitivity to these agents. *In vivo* studies demonstrated that alisertib significantly augmented the efficacy of ara-C without affecting its pharmacokinetic profile and led to the induction of p27 and BIM. Our collective data indicate that targeting Aurora A with alisertib represents a novel approach to increase the efficacy of ara-C that warrants further investigation.

3.3 Materials and methods

3.3.1 Cells and cell culture

MV4-11, PL-21, and MOLM-13 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmBH, Braunschweig, Germany). HL-60, OCI-AML2, SH2, SKM-1, NOMO-1 and KG-1 cells were obtained from the ATCC. Normal PBMCs from healthy donors were purchased from Stem Cell Technologies (Vancouver, Canada). Primary human AML cells were obtained from the BM of patients after obtaining informed consent in accordance with an approved IRB protocol. Paired HL-60 cells that are resistant and sensitive to ara-C were kindly provided by Dr. Kapil Bhalla (University of Kansas Cancer Center,

Kansas City, Kansas, USA). All cells and cell lines were maintained as described in section 2.3.1.

3.3.2 qRT-PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen Inc., Valencia, CA) and treated with TURBO DNA-free™ Kit (Ambion Inc., Foster City, CA). First-strand cDNA synthesis was performed using the high-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). *AURKA* transcripts were amplified using a TaqMan® Gene expression assay (Applied Biosystems, Foster City, CA). Relative gene expression was calculated with the $2^{-\Delta Ct}$ method using *GAPDH* as a housekeeping gene (656).

3.3.3 Chemicals and reagents

Reagents were obtained from: alisertib (MLN8237, Millennium Pharmaceuticals, Cambridge, MA, USA), ara-C (CTRC pharmacy), anti-tubulin, anti-Aurora A, anti-Aurora B, anti-phospho-FOXO3a, anti-FOXO3a, anti-BIM, and anti-p27 antibodies (Cell Signalling, Beverly, MA, USA).

3.3.4 Analysis of cell cycle effects and apoptosis

Please refer to section 2.3.4.

3.3.5 Analysis of drug-Induced apoptosis by caspase-3 assay

The Active Caspase-3 Mab Apoptosis FITC kit was used in conjunction with flow cytometry to measure drug-induced cell death (BD Biosciences, San Jose, CA). Cells were treated with various concentrations of alisertib or ara-C for the indicated times. Following drug treatment, cells were fixed with 500 µl of CytoFix/CytoPerm Solution (BD Biosciences) and were incubated for 20 minutes in ice. After incubation, pellets were washed twice in 1X Perm/Wash buffer and stained with a FITC-conjugated antibody directed against the active form of caspase-3 for 30 minutes in the dark. Cells were washed twice with 1X Perm/Wash to remove unbound antibody and cellular fluorescence was analyzed using the FL-1H channel of a

FACSCanto II flow cytometer (BD Biosciences). The percentage of cells containing the active caspase-3 fragment was quantified using BD FACSDiva software.

3.3.6 MTT assay

Cell viability was assessed by MTT as described in section 2.3.5**.** Cells were cultured in 96-well plates at a density of 10,000 cells per well. Cells were treated with alisertib, ara-C or the combination for 96 hours. IC_{50} was determined by calculating the concentration of alisertib or ara-C that caused a 50% loss of viability measured by the MTT assay.

3.3.7 Cell morphology

Please refer to section 2.3.6.

3.3.8 Colony assays

AML cells were treated for 24 hours with the indicated concentrations of alisertib, ara-C or both and colony assays were performed as described in section 2.3.7.

3.3.9 Immunoblotting

AML cells were incubated with alisertib, ara-C or the combination for 24 hours. Cells were collected and then lysed. For protein lysate preparation and Western blot method please refer to 2.3.8. Densitometric quantification of p-FOXO3a, FOXO3a, BIM_{EL}, BIM_L, p27 and tubulin was performed using FluorChem HD2 software (ProteinSimple). P-FOXO3a, FOXO3a, BIM_{EL}, BIM_L and p27 expression values were normalised to tubulin expression. Results are presented as the mean of ± SD of 3 independent experiments.

3.3.10 RNA interference

AURKA SMARTpool, *AURKB* SMARTpool or siCONTROL siRNA directed at luciferase (Dharmacon, Lafayette, CO) were transfected into AML cells as previously described using the Nucleofector II according to the

manufacturer's instructions (Amaxa Inc., Gaithersburg, MD, USA) (633). Immunoblotting was utilized to assess knockdown efficiency. Transfected cells were treated with the indicated concentrations of ara-C for 48 hours. Drug-induced apoptosis was quantified by PI/FACS as described above. FOXO3a and control shRNA lentiviral particles were introduced into MV4-11 cells according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Stable clones were selected following puromycin treatment.

3.3.11 Measuring alisertib and ara-C plasma levels

Female naive CB-17 SCID mice were given a single dose of alisertib, 20 mg/kg orally and ara-C, 50 mg/kg IP alone or in combination and sacrificed at various time points (1, 2, 4, 6, 8, 16 and 24 hours). Blood was drawn using cardiac puncture and plasma was isolated. Extractions were performed separately for the analysis of alisertib and ara-C, and alisertib and ara-C plasma concentrations were determined by liquid chromatography–mass spectrometry (LC/MS). For alisertib, 25 µL of each sample was mixed with 50 µ L of internal standard solution [$^{13}CD_3{}^{15}N_2$] alisertib. The prepared samples were then extracted by solid phase extraction (Isolute C8 50 µm). The samples were washed, evaporated, and reconstituted. Ten µL of the sample solution was loaded onto a 5 µm Sunfire C8, 2.1 mm internal diameter x 50 mm, HPLC column (Waters, Milford, MA, USA). For ara-C, 25 µL of each sample was mixed with 150 µL of internal standard solution ara-C $^{13}C_3$. The prepared samples were vortexed and then eluted from the plate. Ten µL of the sample solution was loaded onto a 5 µm Hypersil Silica, 3 mm internal diameter x 50 mm, HPLC column (Thermo Fisher, Hanover Park, IL, USA). An API 4000 LC/MS/MS (MDS Sciex) was used for detection of alisertib (*m/z* 519.1→328.1), [$^{13}CD_3{}^{15}N_2$]alisertib (m/z 526.1→329.1), ara-C (*m/z* 244.2→112.1), and ara-C $^{13}C_3$ (*m/z* 247.1→115.1). Quantification was based upon integrating peaks corresponding to elution of the drug and

internal standard in the extracted product ion chromatograms. The lower limit of quantitation (LLQ) for alisertib and ara-C was 5 ng/mL.

3.3.12 Pharmacokinetics analysis

Pharmacokinetic analysis of the plasma concentration data was performed using WinNonlin® Professional, Version 4.0 (Pharsight Corp., Mountain View, CA, USA). Kinetic parameters were estimated using a non-compartmental model.

3.3.13 *In vivo* evaluation of alisertib and ara-C

MOLM-13 and KG-1 cells were harvested, washed in PBS, and suspended in a 50:50 mixture of HBSS and Matrigel (BD Biosciences, San Jose, CA, USA). An *in vivo* model of AML was generated by injection of 10^7 MOLM-13/KG-1 cells into the flanks of female nude mice. After tumour growth reached 150 mm^3, mice were randomly assigned to receive alisertib 20 mg/kg BID orally (n=10), ara-C 75 mg/kg three times a week (TIW) Intraperitoneally (n=10), vehicle control (n=10) or both alisertib and ara-C (n=10) for 14 days. Mice were monitored daily and tumour volumes and body weights were measured twice weekly as described in section 2.3.9. At the completion of the study, tumours were excised, formalin-fixed and paraffin-embedded for immunohistochemical analysis.

3.3.14 Immunohistochemistry

Paraffin-embedded tumour sections (4–6 μ m thick) were mounted on slides. Sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H_2O (v/v)] and rehydrated in PBS (pH 7.5). Heat-induced epitope retrieval was performed by microwaving slides in a citrate buffer for 5 minutes. The slides were allowed to cool and endogenous peroxides were blocked with a 3% hydrogen peroxide solution for 10 minutes. Slides were then incubated in a protein block solution (5% horse and 1% goat serum in PBS) for 20 minutes. Primary antibodies were diluted in the protein block solution and

placed at 4 °C overnight. After washing with PBS, slides were incubated in appropriate secondary antibodies (PCNA, BIM and p27 (Cell Signalling Technologies, Beverly, MA, USA)) for 1 hour at ambient temperature. Positive reactions were visualized by immersing the slides with stable 3,3'-diaminobenzidine diaminobenzidine (Research Genetics, Huntsville, AL) for 10–20 minutes. The sections were rinsed with distilled water, counterstained with Gill's haematoxylin (Sigma, St. Louis, MO), and mounted with Universal Mount (Research Genetics, Huntsville, AL). Images were captured using an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20X objective. Image-Pro Plus software Version 6.2.1 (MediaCybernetics, Bethesda, MD) was used for image acquisition and quantification by densitometric analysis of BIM and p27 expression in five random high-power fields containing viable tumour cells. Percentages of PCNA-positive cells were determined by manual counting of 5 non-overlapping random high-power fields per section by two investigators blinded to the treatment arms. The average percentage of PCNA positive cells per high-power field was calculated for comparison.

3.3.15 Statistical analyses

Statistical significance of differences observed between samples was determined using the Student's *t* test. Differences were considered significant in all experiments at $p < 0.05$.

3.4 Results

3.4.1 Targeting Aurora A with alisertib disrupts the viability of AML cells.

In order to investigate the therapeutic potential of targeting Aurora A activity in AML, we first assessed the relative expression of the *AURKA* gene in normal PBMCs and a panel of 9 human AML cell lines using a qRT-PCR method. *AURKA* expression levels were significantly higher in all 9 AML cell lines than in normal PBMCs (Figure 3.1). Our findings are consistent with a recent study that demonstrated prevalent *AURKA*

overexpression in AML cells from patients (657). However normal PBMCs would not be expected to express high levels of Aurora A as they are not actively proliferating. Using alternative controls such as CD34 positive mononuclear cells isolated from the BMs of healthy donors may have yielded different results. We next treated MV4-11 cells with three concentrations of alisertib to examine the effects of this agent on Aurora A autophosphorylation as a measure of Aurora A activity. Our results showed that alisertib caused a dose-dependent reduction in the phosphorylation of Aurora A on its Thr^{288} auto-phosphorylation site. Our findings are consistent with other recent preclinical studies with alisertib and demonstrate that this agent effectively inhibits Aurora A activity in AML cells (Figure 3.2) (658, 659). We next treated 9 AML cell lines and normal PBMC controls with various concentrations of alisertib and quantified the consequential effects on cell viability by MTT assay. Clinically achievable concentrations of alisertib preferentially inhibited the *in vitro* growth and survival of AML cell lines as compared with normal PBMCs, indicating that this agent may have therapeutic selectivity (Figure 3.3). Importantly, *in vitro* treatment with alisertib also diminished the viability of primary blasts obtained form patients with AML (Figure 3.4) and disrupted the clonogenic survival of AML cells (Figure 3.5). Interestingly Aurora A expression as measured by qRT-PCR did not appear to correlate with sensitivity to alisertib as assessed by MTT assay. For example MOLM-13 cells appear to have relatively low expression of Aurora A (Figure 3.1), but remain very sensitive to alisertib treatment as assessed by MTT assay. This is consistent with a recent study which showed that Aurora A expression is a poor predictor of response to Aurora kinase inhibitors (660).

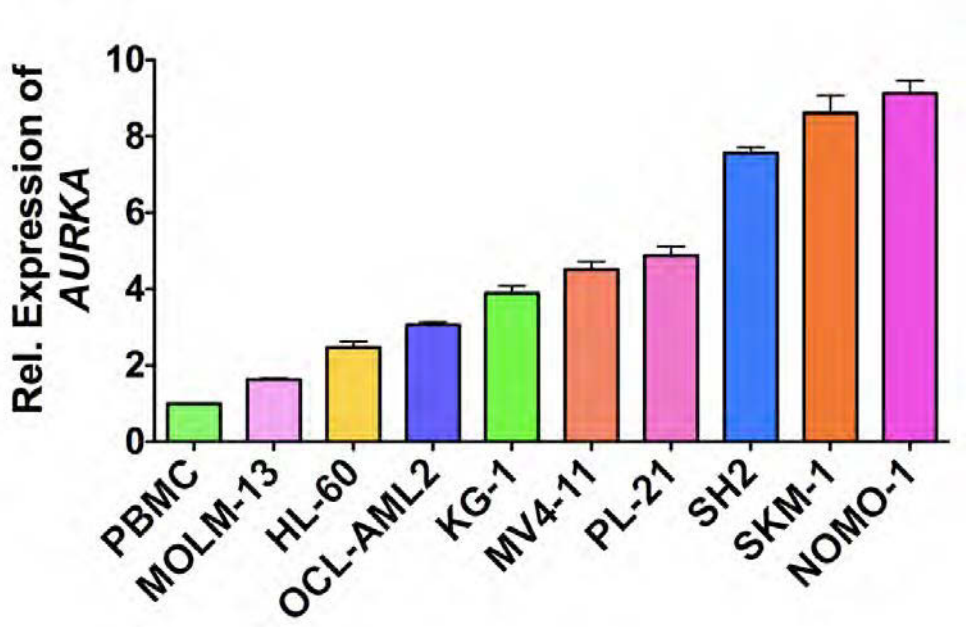

Figure 3.1 Relative expression of *AURKA* in normal PBMCs and AML cell lines

The basal expression levels of *AURKA* were quantified by qRT-PCR. n = 3 ± SD.

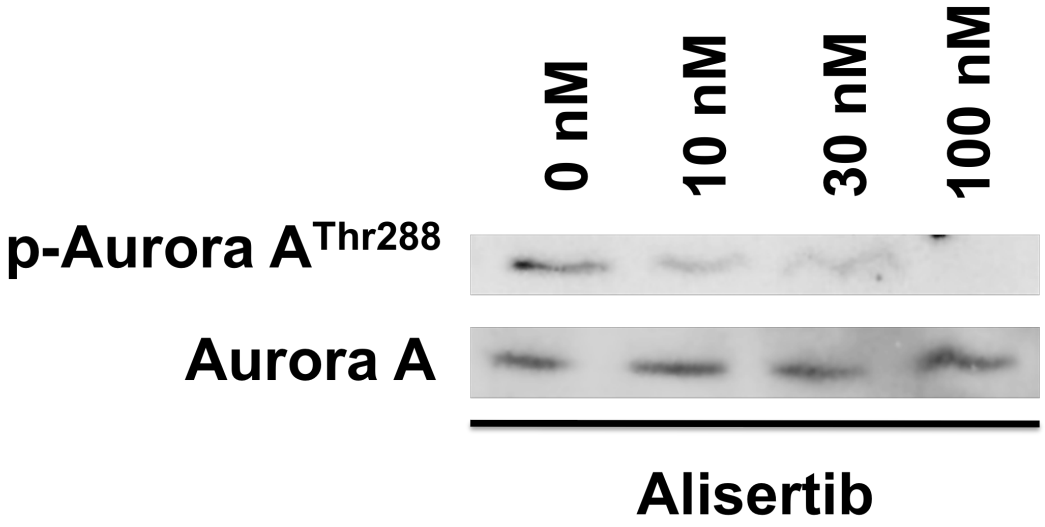

Figure 3.2 Alisertib treatment abrogates Aurora kinase A autophosphorylation

MV4-11 AML cells were treated with the indicated concentrations of alisertib for 24 hours. The levels of phospho-Aurora A (Thr^{288}) and total Aurora A were assessed by immunoblotting.

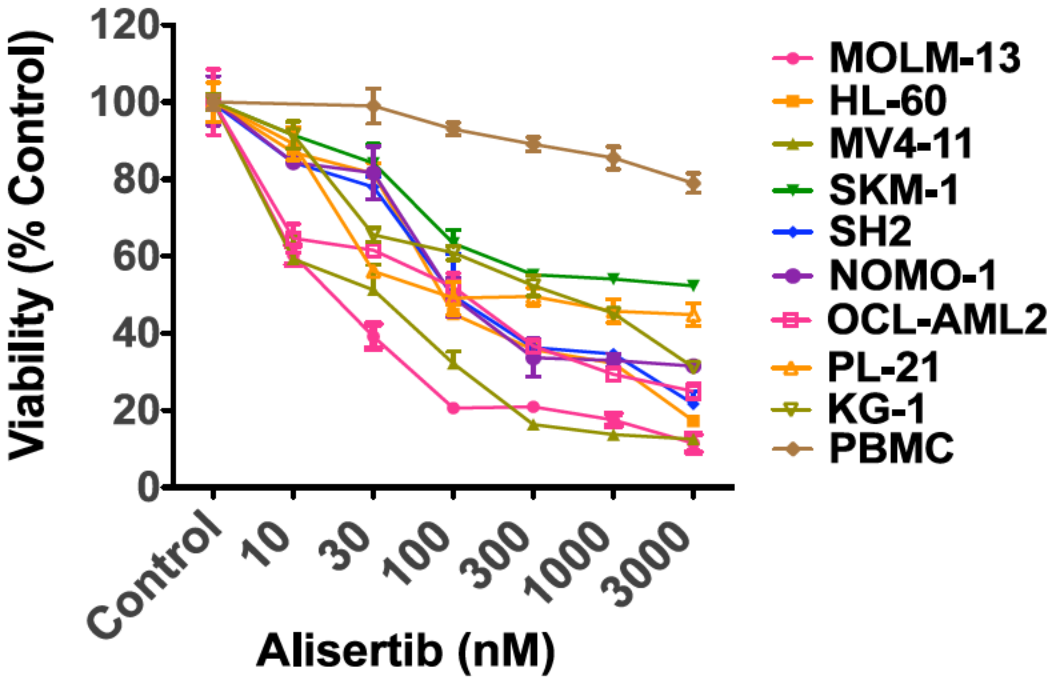

Figure 3.3 Alisertib selectively diminishes the viability of AML cell lines

Normal PBMC and a panel of 9 human AML cell lines were treated with the indicated concentrations of alisertib for 72 hours. Cell viability was measured by MTT assay. n = 3 ± SD.

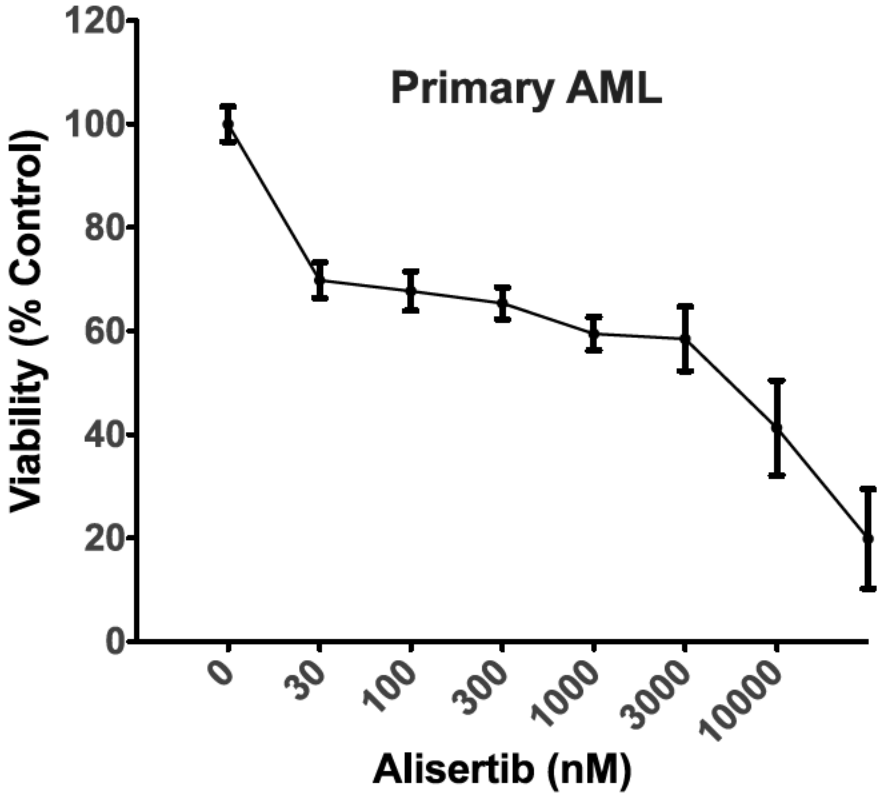

Figure 3.4 Alisertib has activity against primary AML cells

Primary cells from 3 different patients with AML were treated *ex vivo* with alisertib for 72 hours. Cell viability was measured by MTT assay. n = 3 ± SD.

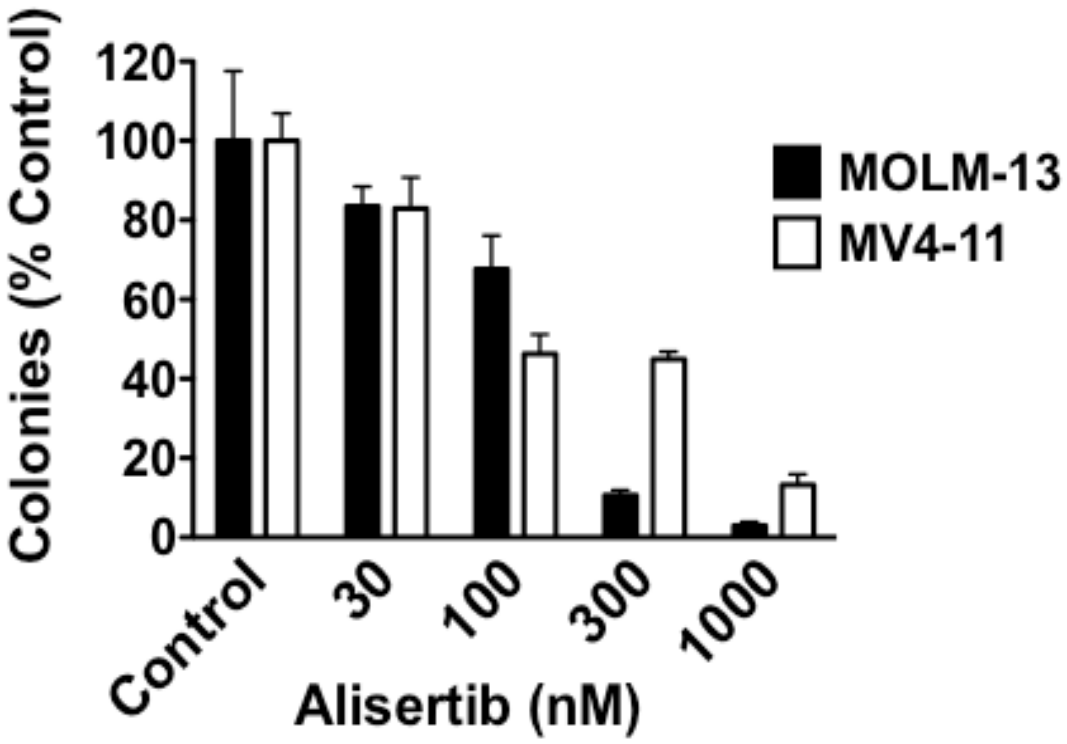

Figure 3.5 Treatment with alisertib inhibits clonogenic survival

MOLM-13 and MV4-11 AML cells were treated with the indicated concentrations of alisertib for 24 hours. Drug was washed away and cells were seeded in Methocult. Colonies were scored on day 14. n = 3 ± SD.

3.4.2 Morphological analysis of AML cells after 48 hours of alisertib treatment

Considering that the cell cycle analysis showed that alisertib could induce polyploidy and that previous studies have shown that Aurora kinase inhibition results in MC we examined the morphology of alisertib-treated cells. As discussed in section 1.5, MC is currently defined primarily by morphology (635). Figure 3.6 and 3.7 show representative examples of both treated and untreated MOLM-13 and MV4-11 cells respectively. After 48 hours of treatment, cells with distinct chromatin images suggestive of a monopolar spindles and duplicated but unseparated chromosomes were visible. These features are consistent with MC. In addition to features of MC, the alisertib-treated AML cell lines also show a subpopulation of cells with clear classical apoptosis morphology: chromatin condensation, cytoplasm shrinkage, and the formation of apoptotic bodies.

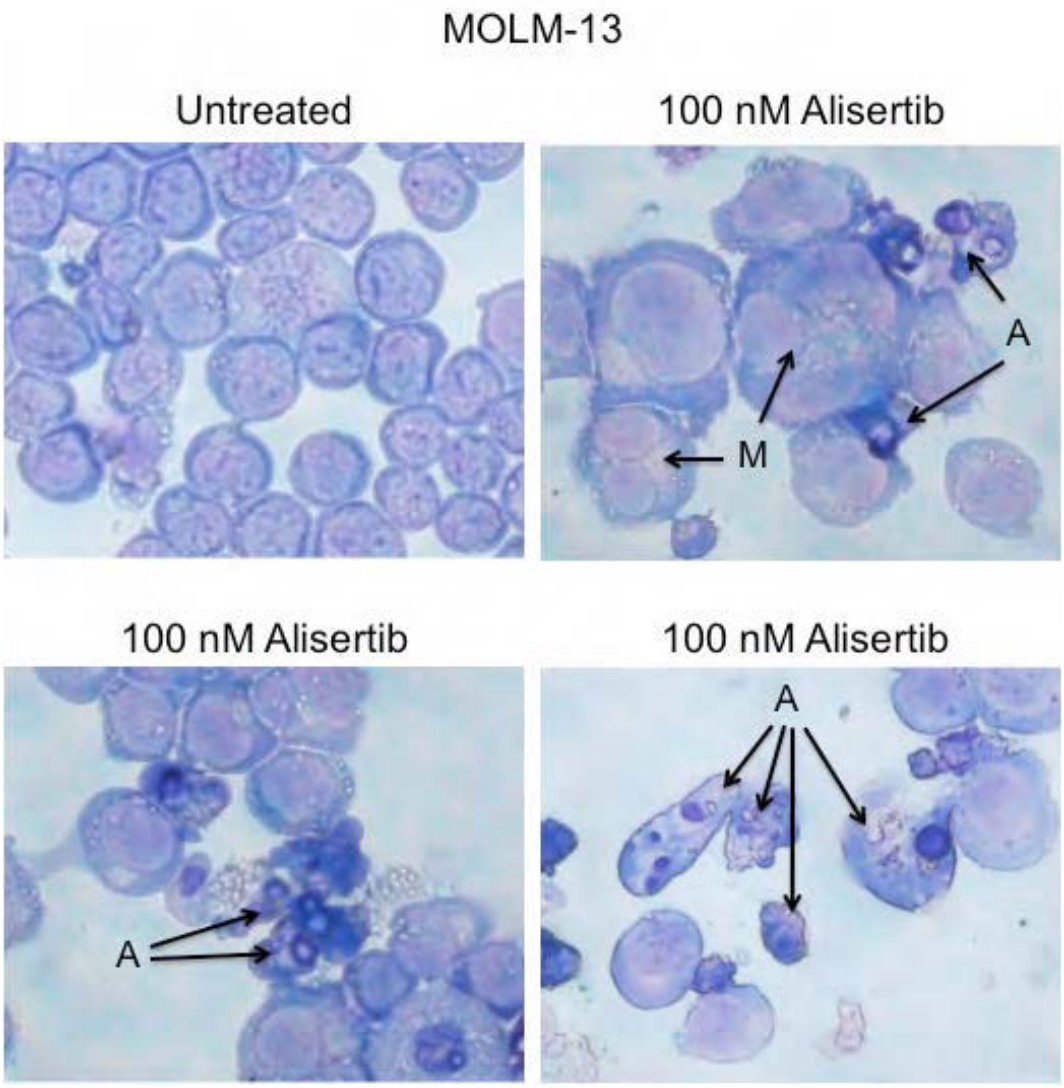

Figure 3.6 Morphological features of alisertib-induced cell death in MOLM-13 cell lines

Cell morphology was visualised using RapiDiff staining by light microscopy after treatment with 100 nM of alisertib for 48 hours. Typical cytospin images for untreated and alisertib-treated MOLM-13 cell lines are shown. Alisertib treatment (100 nM) produced a distinct morphology with large cells with multiple micronuclei (M). These features are consistent with mitotic catastrophe. In addition to mitotic catastrophe, MOLM-13 cells treated with alisertib show a population of cells with membrane blebbing and budding and chromatin condensation features consistent with apoptotic morphology (A).

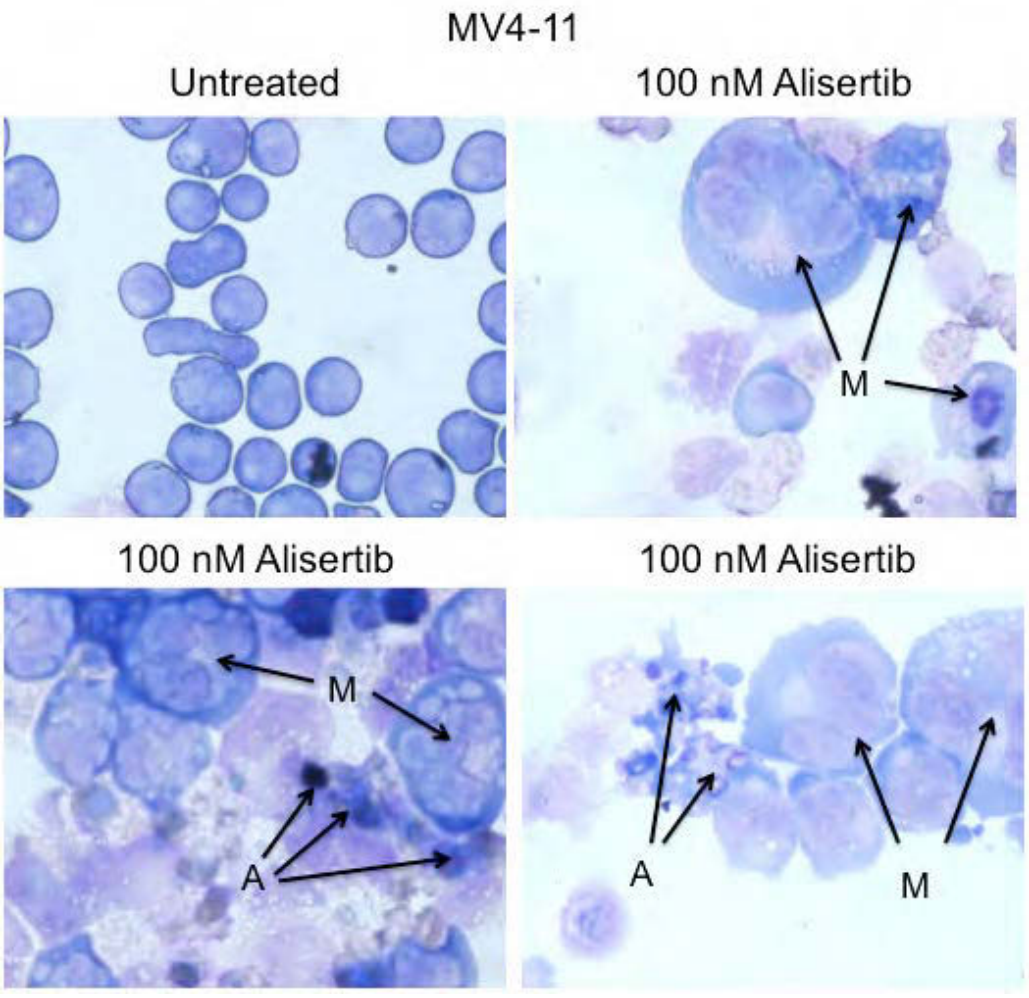

Figure 3.7 Morphological features of alisertib-induced cell death in MV4-11 cell lines

Cell morphology was visualised using RapiDiff staining by light microscopy after treatment with 100 nM of alisertib for 48 hours. Typical cytospin images for untreated and alisertib-treated MV4-11 cell lines are shown. Alisertib treatment (100 nM) produced a distinct morphology suggestive of monopolar spindles, duplicated but unseparated chromosomes centrally located in the cell and large cells with multiple micronuclei (M). These features are consistent with mitotic catastrophe. In addition to mitotic catastrophe, MV4-11 cells treated with alisertib show a population of cells with membrane blebbing and budding and chromatin condensation features consistent with apoptotic morphology (A).

3.4.3 Alisertib modulates cell cycle distribution and induces apoptosis

Genetically or pharmacologically antagonizing the activity of Aurora kinase A has been reported to produce mixed effects on cell cycle dynamics including polyploidy and G_2/M growth arrest (457, 466, 661, 662). In order to assess the cell cycle-related effects of alisertib, we treated MOLM-13, MV4-11, HL-60 and KG-1 cells with different concentrations of alisertib for 48 hours and quantified the effects on cell cycle distribution using PI/FACS analysis (Figure 3.8 a, b, c, and d). These analyses showed dose-dependent increases in the percentage of MOLM-13, MV4-11, HL-60 and KG-1 cells with ≥4N DNA content (Figure 3.9 a) and with sub G_0/G_1 (apoptotic) DNA (Figure 3.9 b). Considering that DNA fragmentation (sub G_0/G_1 DNA) is a hallmark feature of apoptosis, we quantified the effects of alisertib on caspase-3 activation as a second measure of apoptosis to confirm the pro-apoptotic effects of this agent. Treatment with alisertib for 48 hours led to a concentration-dependent increase in the percentages of cells expressing the active form of caspase-3, indicating that the induction of apoptosis significantly contributes to the activity of this agent (Figure 3.10 a, b, and c). Notably, MOLM-13 cells displayed significantly greater sensitivity to alisertib in MTT and colony formation assays than in assays that directly measured apoptosis. This suggests that the unique genetic background of individual cell types may be important in determining the cellular response to Aurora A inhibition (growth inhibition, apoptosis, or both) and that suppression of cell proliferation and stimulation of apoptosis both underlie the anti-cancer activity of alisertib.

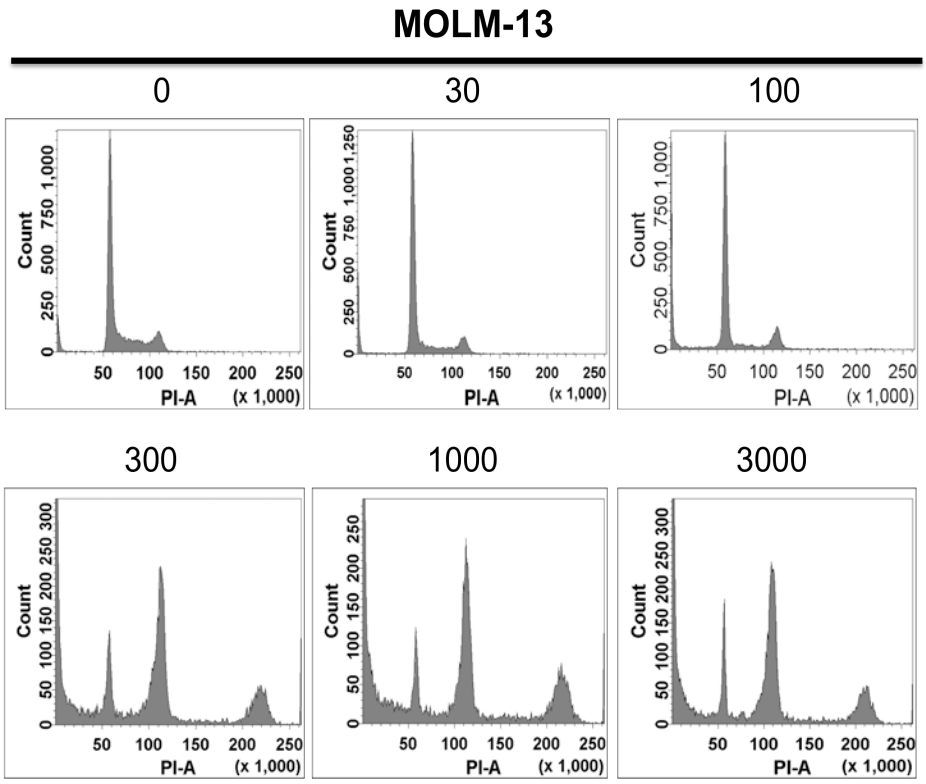

Figure 3.8 (a) Alisertib disrupts cell cycle dynamics and induces apoptosis

MOLM-13 cells were treated with the indicated concentrations of alisertib for 48 hours. PI/FACS was utilized to assess drug-related effects on cell cycle distribution. Representative histograms are shown.

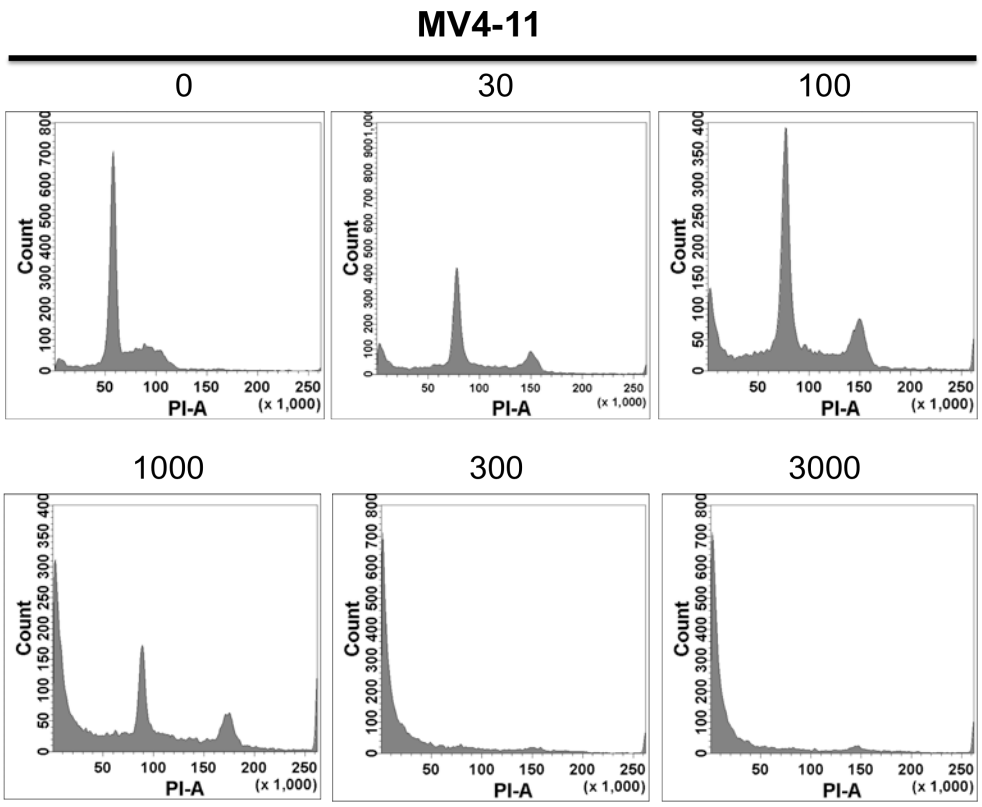

Figure 3.8 (b) Alisertib disrupts cell cycle dynamics and induces apoptosis

MV4-11 cells were treated with the indicated concentrations of alisertib for 48 hours. PI/FACS was utilized to assess drug-related effects on cell cycle distribution. Representative histograms are shown.

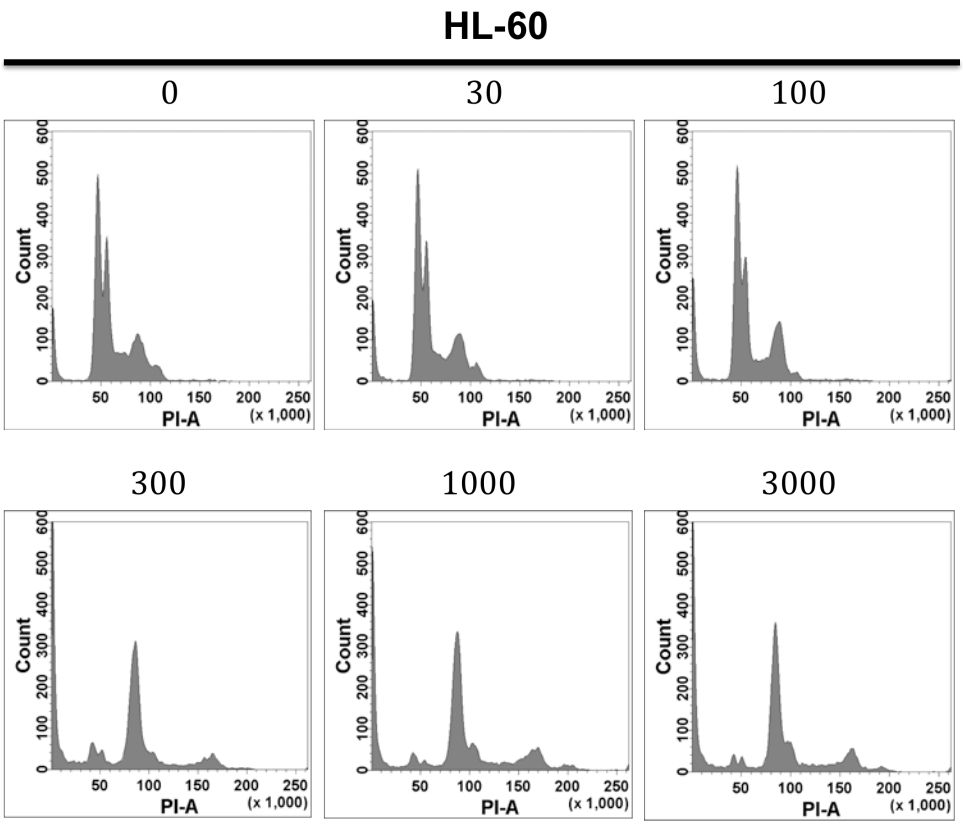

Figure 3.8 (c) Alisertib disrupts cell cycle dynamics and induces apoptosis

HL-60 cells were treated with the indicated concentrations of alisertib for 48 hours. PI/FACS was utilized to assess drug-related effects on cell cycle distribution. Representative histograms are shown.

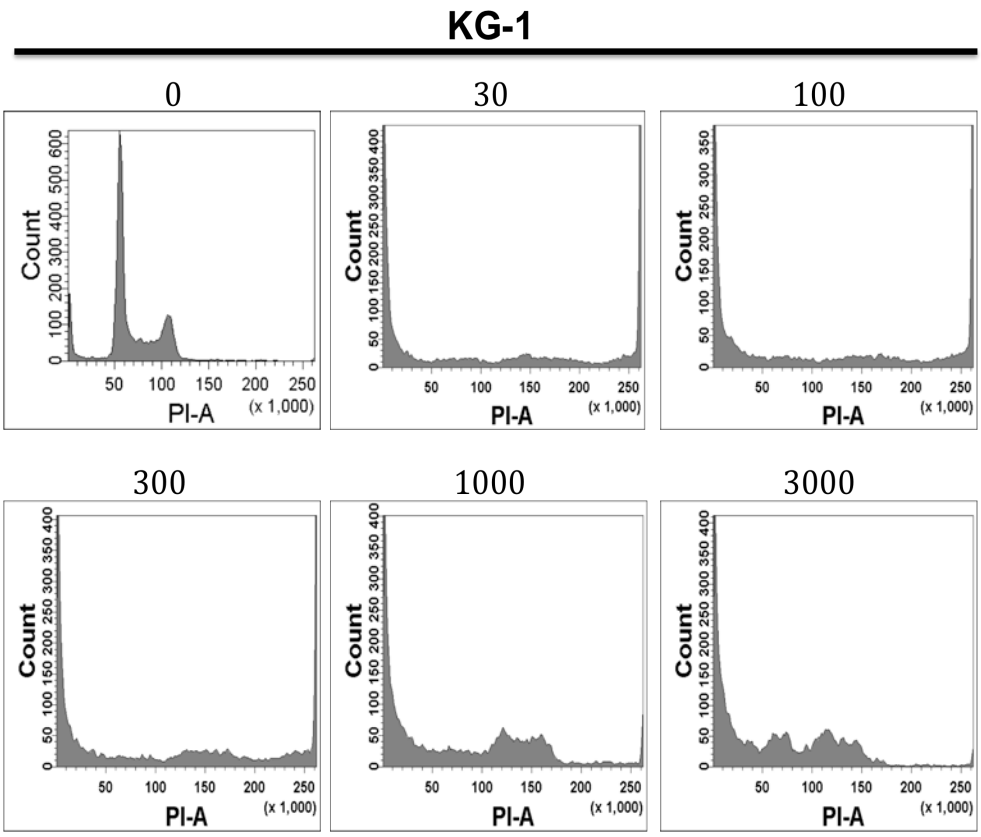

Figure 3.8 (d) Alisertib disrupts cell cycle dynamics and induces apoptosis

KG-1 cells were treated with the indicated concentrations of alisertib for 48 hours. PI/FACS was utilized to assess drug-related effects on cell cycle distribution. Representative histograms are shown.

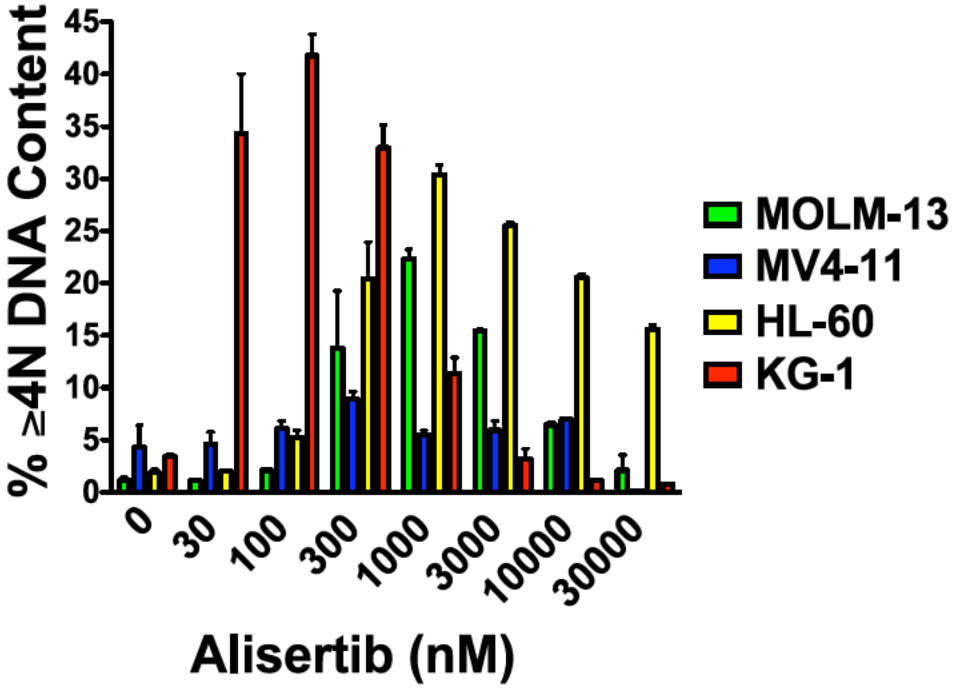

Figure 3.9 (a) Alisertib disrupts cell cycle dynamics and induces apoptosis

MOLM-13, MV4-11, HL-60, and KG-1 cells were treated with the indicated concentrations of alisertib for 48 hours. PI/FACS was utilized to assess drug-related effects on cell cycle distribution. The percentages of cells with ≥4N DNA content are quantified. n = 3 ± SD.

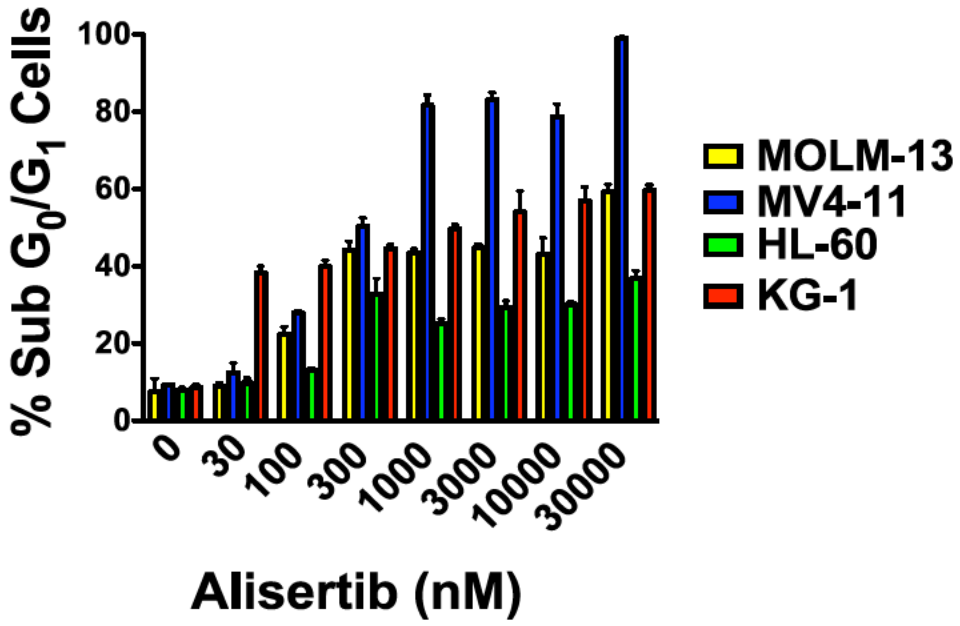

Figure 3.9 (b) Alisertib disrupts cell cycle dynamics and induces apoptosis

MOLM-13, MV4-11 and HL-60, and KG-1 cells were treated with the indicated concentrations of alisertib for 48 hours. PI/FACS was utilized to assess drug-related effects on cell cycle distribution. The percentages of cells with sub G_0/G_1 DNA content are quantified. n = 3 ± SD.

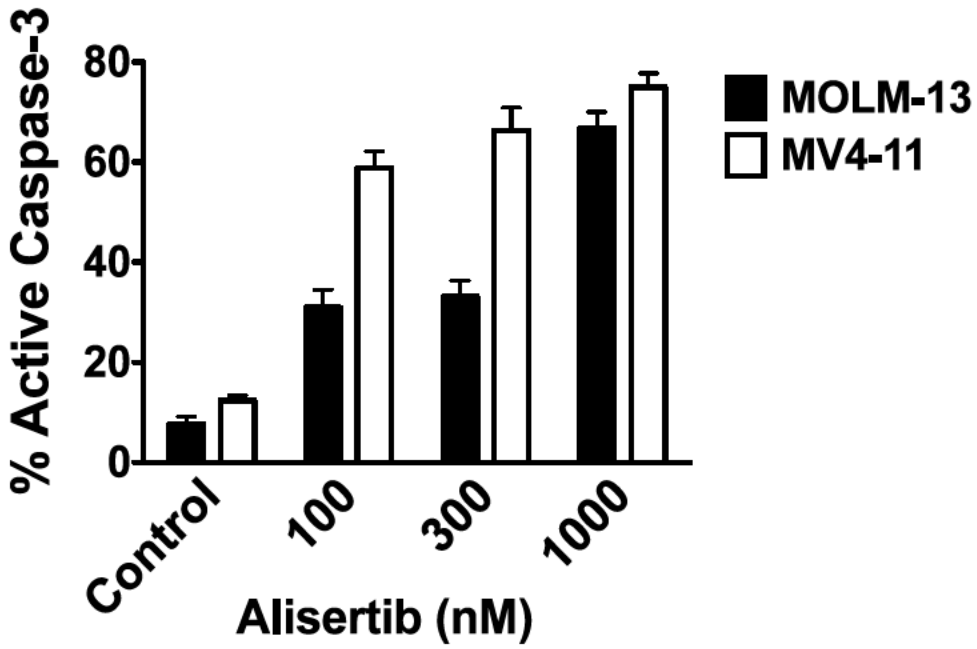

Figure 3.10 (a) Alisertib activates caspase-3

MOLM-13 and MV4-11 cells were treated with the indicated concentrations of alisertib for 48 hours. The percentages of cells expressing active caspase-3 were quantified using a FACS-based method as detailed in the materials and methods. n = 3 ± SD.

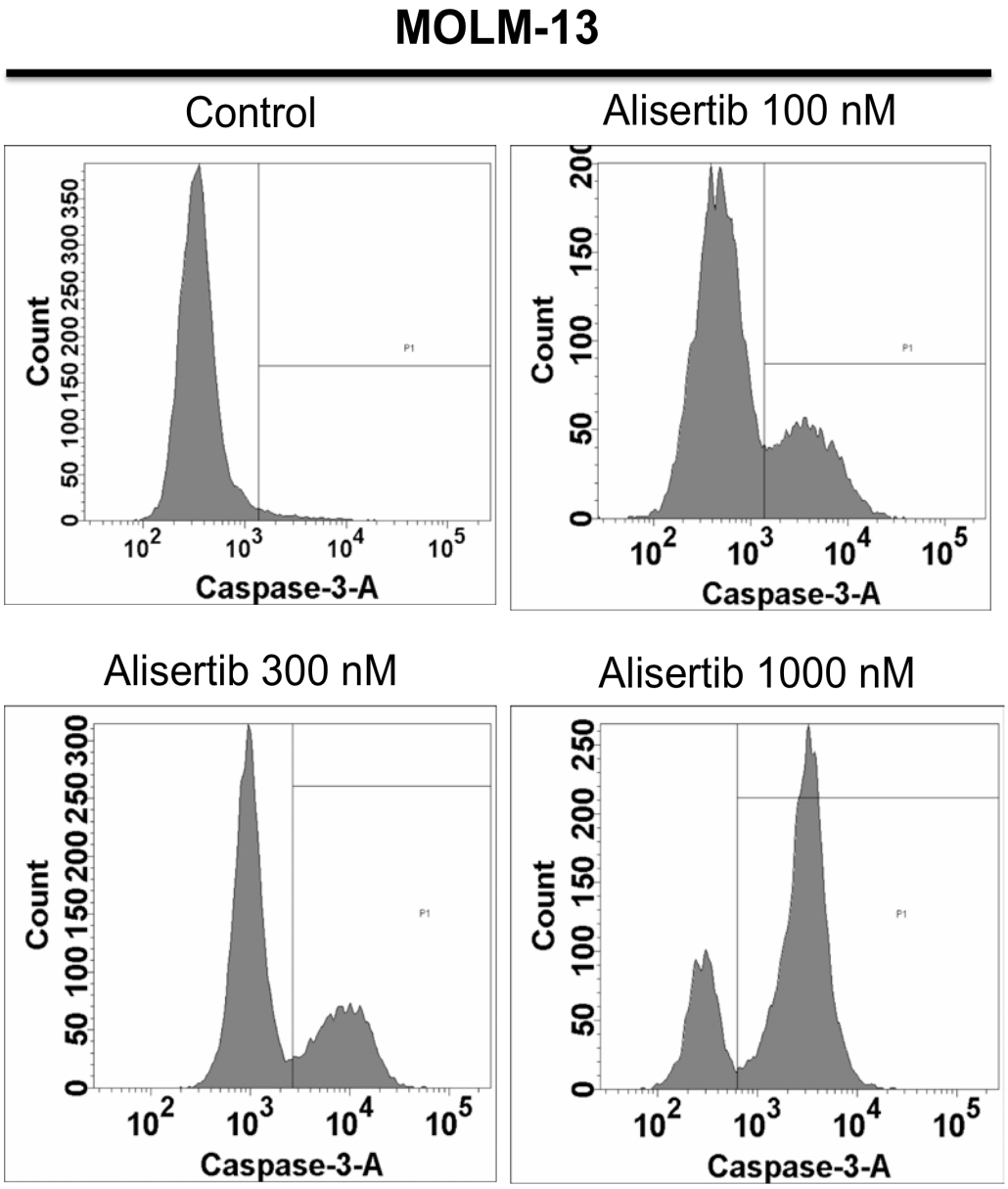

Figure 3.10 (b) Alisertib activates caspase-3

MOLM-13 cells were treated with the indicated concentrations of alisertib for 48 hours. Active caspase-3 was quantified using a FACS-based method as detailed in the materials and methods. Representative Histograms are shown.

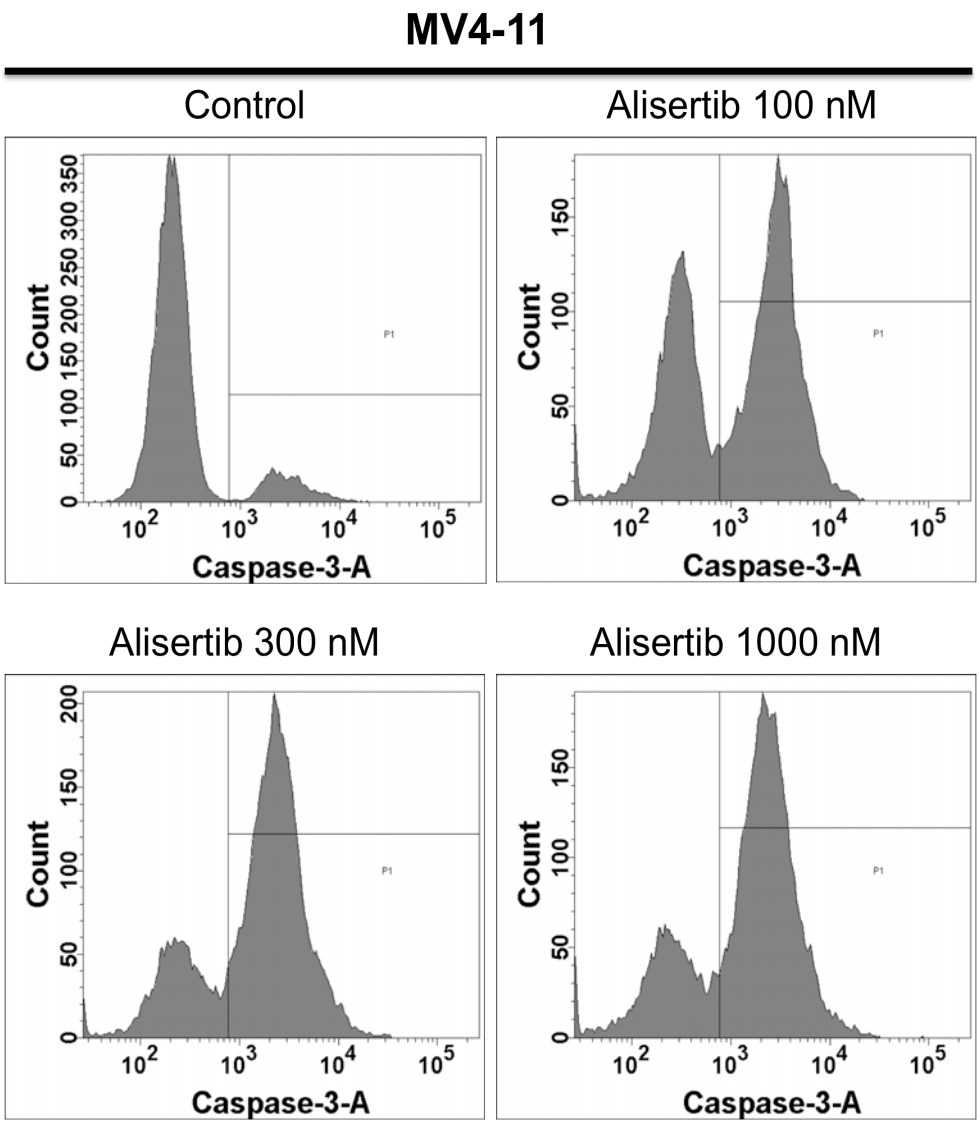

Figure 3.10 (c) Alisertib activates caspase-3

MV4-11 cells were treated with the indicated concentrations of alisertib for 48 hours. Active caspase-3 was quantified using a FACS-based method as detailed in the materials and methods. Representative Histograms are shown.

3.4.4 Alisertib induces the expression of the FOXO3a targets p27 and BIM

The combined effects of growth inhibition/cell cycle disruption and apoptosis that we observed in our FACS analyses were supported by immunoblotting analyses, which revealed that alisertib treatment led to a significant rise in the levels of the CKI inhibitor, p27, and of the microtubule-associated BH3-only apoptotic regulator, BIM, in MV4-11 cells. The increased levels of p27 and BIM were correlated with a significant reduction in the transcriptionally inactive, cytosolically localized (phosphorylated) form of FOXO3a, which is an important transcriptional regulator of both p27 and BIM expression (Figure 3.11) (663, 664). Densiometry analysis shows that alisertib treatment is associated with a modest rise in total FOXO levels perhaps through stabilization of FOXO as a result of its migration from the cytosol (where it can be subject to ubiquitination and degradation) to the cell nucleus (Figure 3.12).

In order to investigate whether Aurora A and B inhibition have similar effects on FOXO3a phosphorylation status, we utilized siRNA to knockdown *AURKA* and *AURKB* expression, respectively. Our results showed that *AURKA* siRNA led to a significantly greater reduction in phospho-FOXO3a levels than *AURKB* siRNA (Figure 3.13). This suggests that Aurora A may play a more prominent role in the regulation of FOXO3a phosphorylation status than Aurora B.

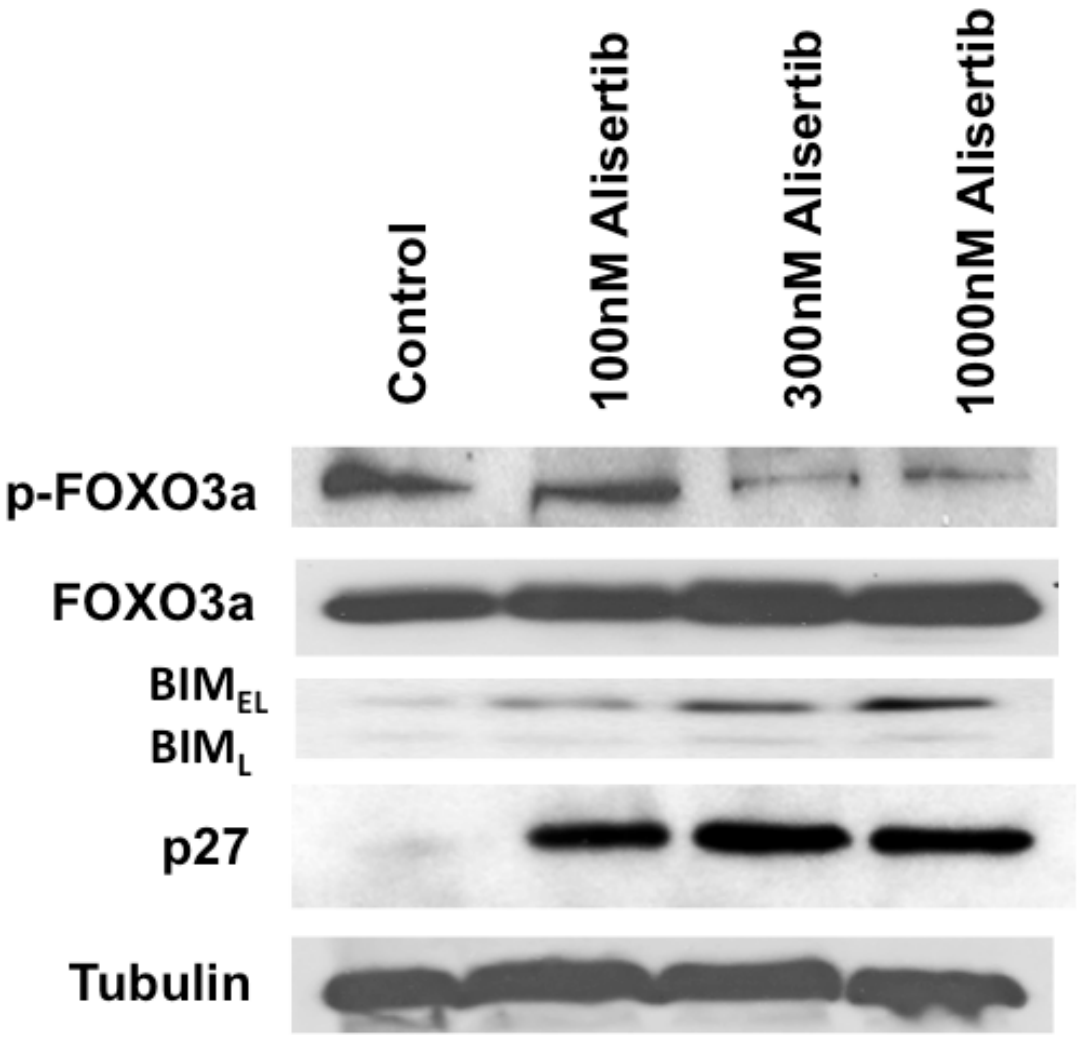

Figure 3.11 Alisertib induces the expression of the FOXO3a targets p27 and BIM

Cells were treated with the indicated concentrations of alisertib for 24 hours. Protein lysates were subjected to SDS-PAGE and membranes were probed with antibodies as described in the materials and methods. Tubulin documented equal protein loading.

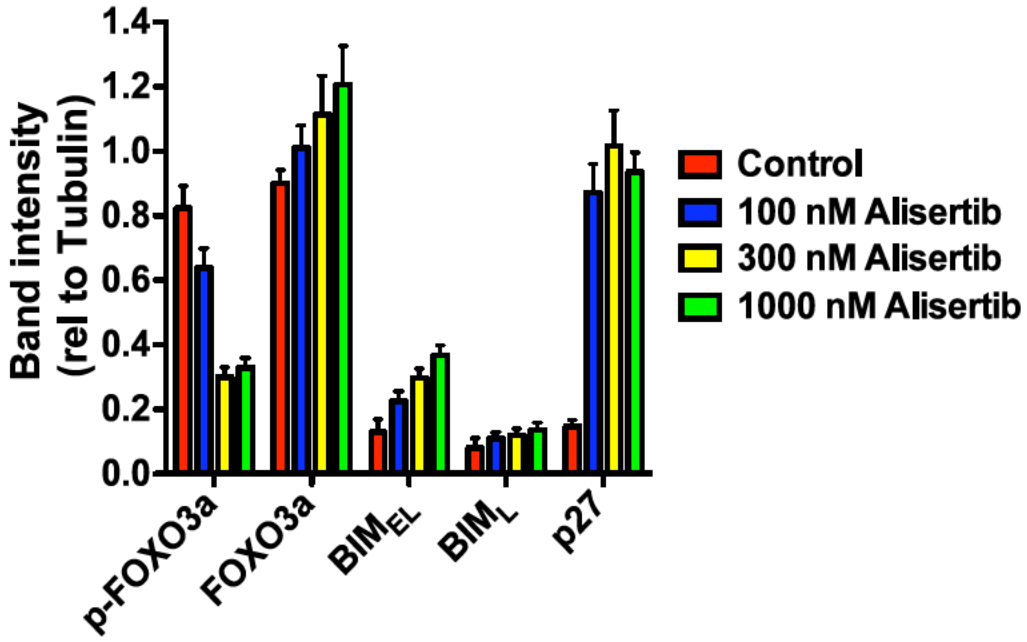

Figure 3.12 Densitometric quantification of p-FOXO3a, FOXO3a, BIM_{EL}, BIM_L and p27 using FluorChem HD2 software (ProteinSimple)

Cells were treated with the indicated concentrations of alisertib for 24 hours. Protein lysates were subjected to SDS-PAGE and membranes were probed with antibodies as described in the materials and methods. Tubulin documented equal protein loading. Densitometric quantification of p-FOXO3a, FOXO3a, BIM_{EL}, BIM_L, p27, and tubulin was performed using FluorChem HD2 software (ProteinSimple). P- FOXO3a, FOXO3a, BIM_{EL}, BIM_L, and p27 expression values were normalised to tubulin expression.

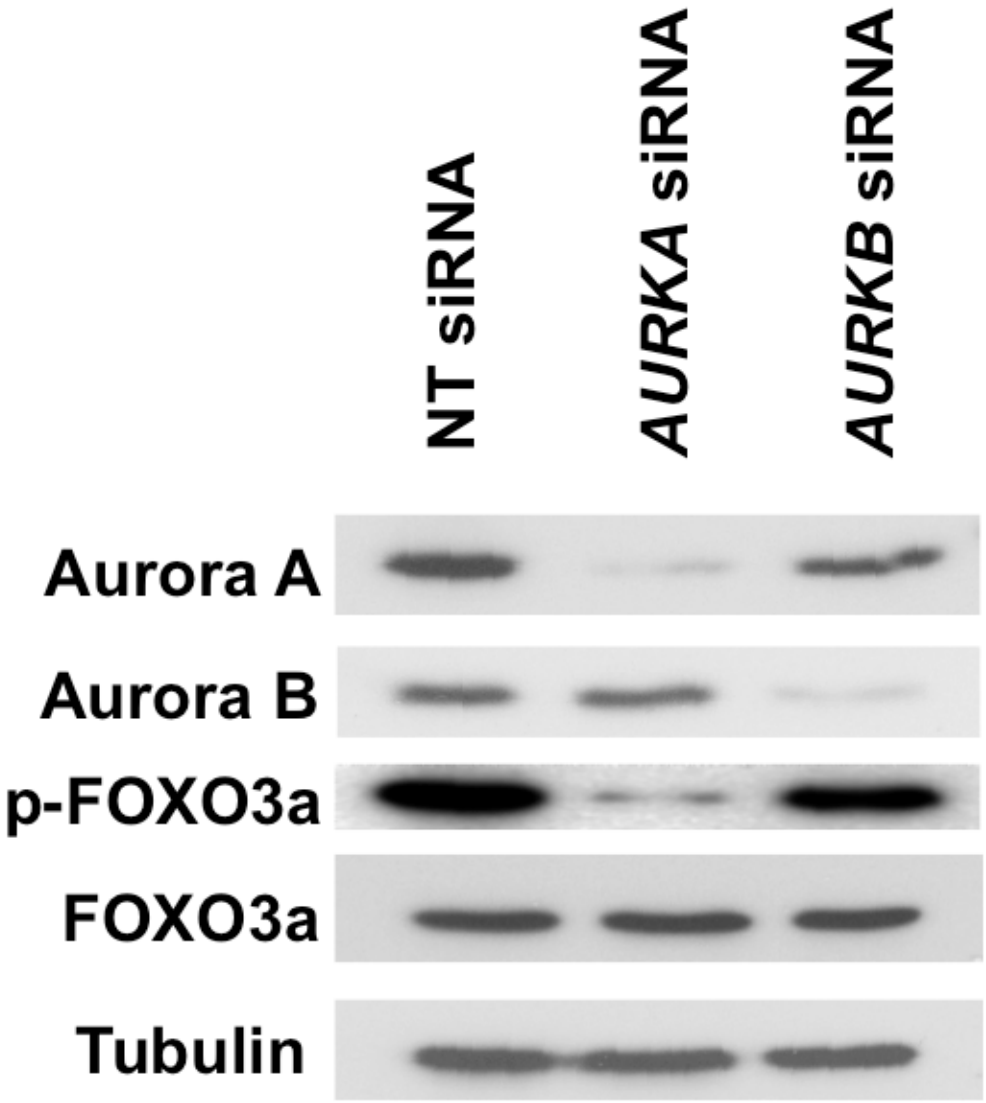

Figure 3.13 Aurora A preferentially regulates FOXO3a phosphorylation levels

MV4-11 cells were transfected with non-targeted (NT) control siRNA, *AURKA* siRNA, or *AURKB* siRNA using the Nucleofector II. The effects of targeted Aurora A and Aurora B knockdown on the levels of p-FOXO3a were assessed by immunoblotting. Tubulin documented equal protein loading.

3.4.5 ara-C induces the expression of Aurora A

A number of studies have reported a link between Aurora kinase expression and resistance or reduced sensitivity to anti-cancer agents (665-670). However, the mechanistic basis for this correlation has not been well investigated. We hypothesized that the stress response induced by cytotoxic drugs such as ara-C, a frontline agent in AML therapy, could result in increased expression of *AURKA*. In order to explore this possibility, we treated AML cell lines (HL-60, MV4-11, and MOLM-13) and primary blasts from patients with AML (n = 3) with ara-C for 24 hours and quantified the effects on the expression of *AURKA* by qRT-PCR and immunoblotting (Figure 3.14 and 3.15). Our results showed that acute exposure to ara-C was sufficient to trigger a significant increase in *AURKA* expression in both established cell lines and primary AML cells. The exact mechanism by which ara-C induces *AURKA* expression is not known. Ara-C typically induces S phase arrest (671, 672) and Aurora A levels are low in G_1 and S phase and peak in the G_2/M phase of the cell cycle (457). One possibility is that Aurora A levels increase in leukaemia cells following ara-C treatment as a compensatory mechanism to overcome S phase arrest.

We next compared the expression of *AURKA* by qRT-PCR in paired ara-C sensitive and resistant cell lines. Resistance to ara-C in AML cells has been attributed to cytokinetic factors as well as reduced intracellular metabolism of ara-C. A HL-60 subline that is highly resistant to ara-C has been generated that is capable of proliferating in the presence of ara-C concentration exceeding 1 µmol/L. In contrast, the parental HL-60 line exhibits an ara-C IC_{50} of approximately 5 nmol/L (673). These resistant cells were generated by prolonged culture in increasing doses of ara-C and share numerous biological and biochemical features with the parent line, including: morphology; rate of growth; cloning characteristics; karyotype; rates of DNA, RNA, and protein synthesis. Of note they exhibit decreased activity of the pyrimidine salvage pathway enzyme - deoxycytidine kinase (dCK), a common mechanism for clinical resistance

to ara-C. However other mechanisms of ara-C resistance are also like to be present in these cells. Interestingly the HL-60 ara-C resistant cell line had significantly higher levels of *AURKA* expression as measured by qRT-PCR compared to paired ara-C sensitive HL-60 cells (Figure 3.16) suggesting that chronic exposure to ara-C is also associated with an increase in *AURKA* expression.

We next utilized siRNA to knockdown *AURKA* expression in MV4-11 cells to assess whether the expression of Aurora A significantly impacted cellular sensitivity to ara-C (Figure 3.17, top). Direct comparison of the pro-apoptotic effects of ara-C in cells transfected with non-targeted control siRNA and Aurora A-targeted siRNA revealed that ara-C was nearly twice as effective at inducing apoptosis when Aurora A expression was diminished, suggesting that targeting Aurora A activity may be an effective strategy to increase the therapeutic efficacy of ara-C (Figure 3.17 bottom).

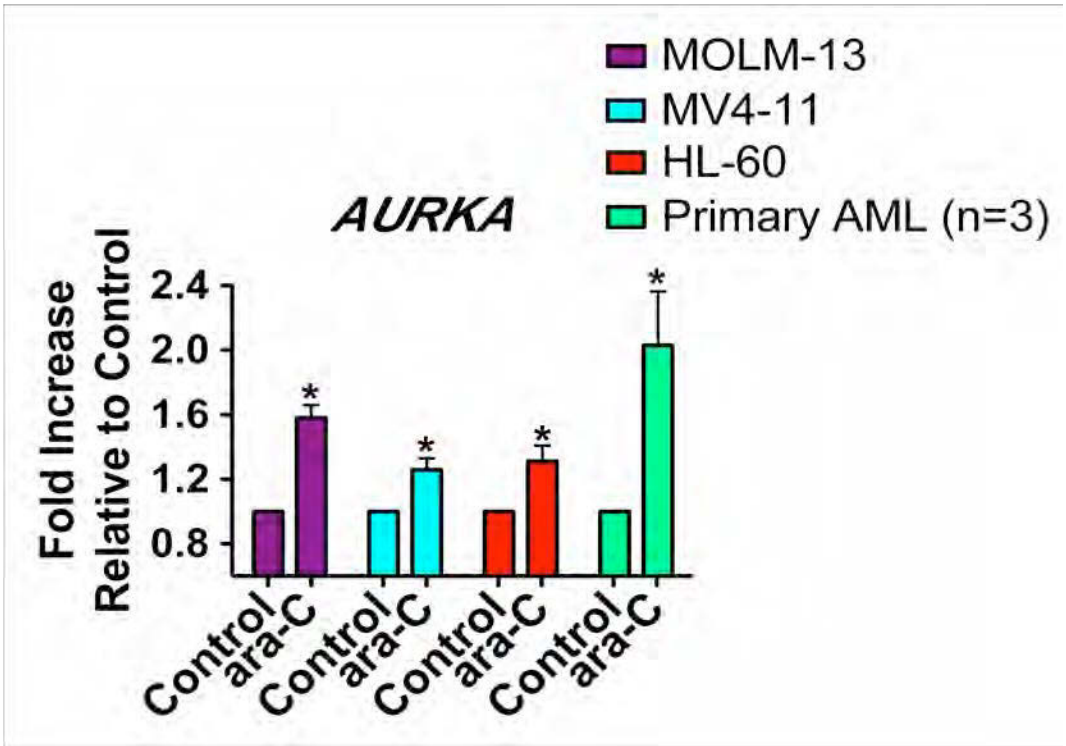

Figure 3.14 Effects of ara-C treatment on *AURKA* expression

Human AML cell lines (MOLM-13, MV4-11, and HL-60) and primary AML cells (n=3) were treated with ara-C for 24 hours. qRT-PCR was utilized to quantify the impact of drug treatment on the relative expression of *AURKA*. n = 3 ± SD, *p ≤ 0.05.

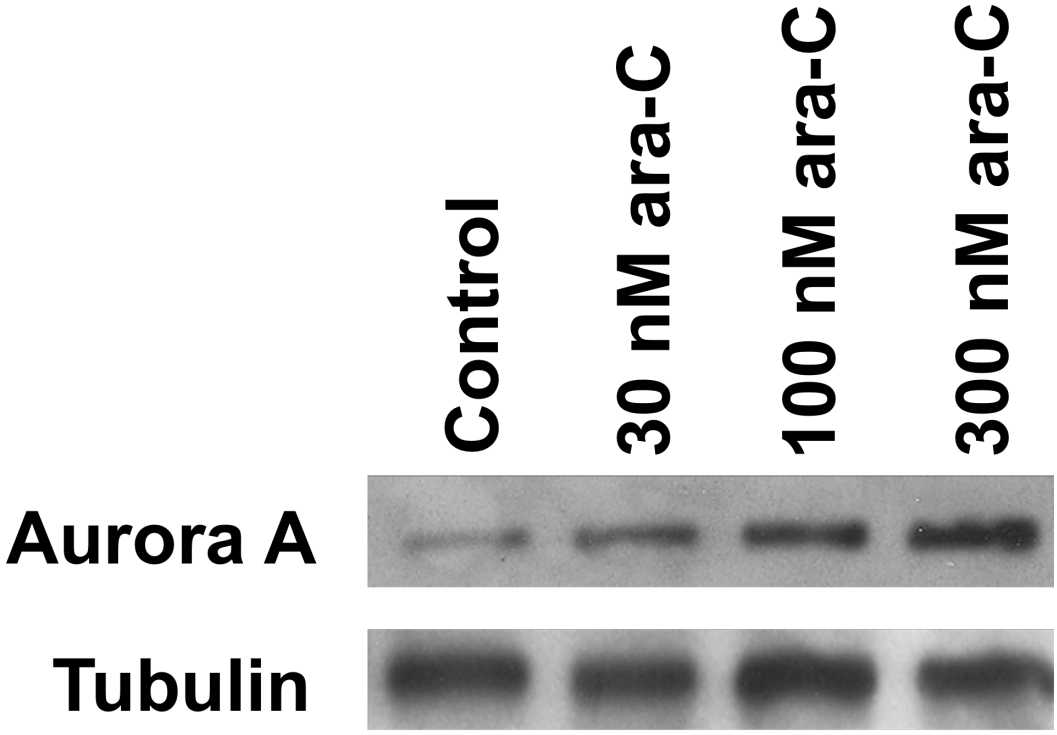

Figure 3.15 Ara-C causes a dose-dependent increase in Aurora A levels

MV4-11 cells were treated with the indicated concentrations of ara-C for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with an Aurora A specific antibody. Tubulin documented equal protein loading.

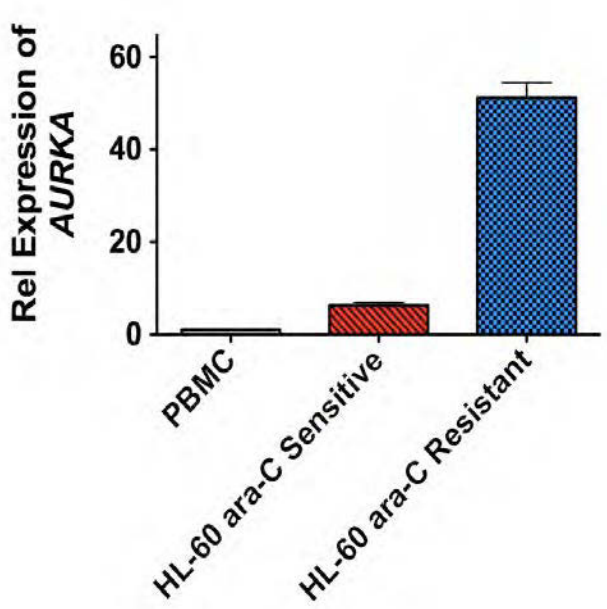

Figure 3.16 Aurora A is overexpressed in ara-C resistant cells

The relative expression levels of *AURKA* were evaluated in paired HL-60 cells that are sensitive and resistant to ara-C and PBMCs by qRT-PCR.

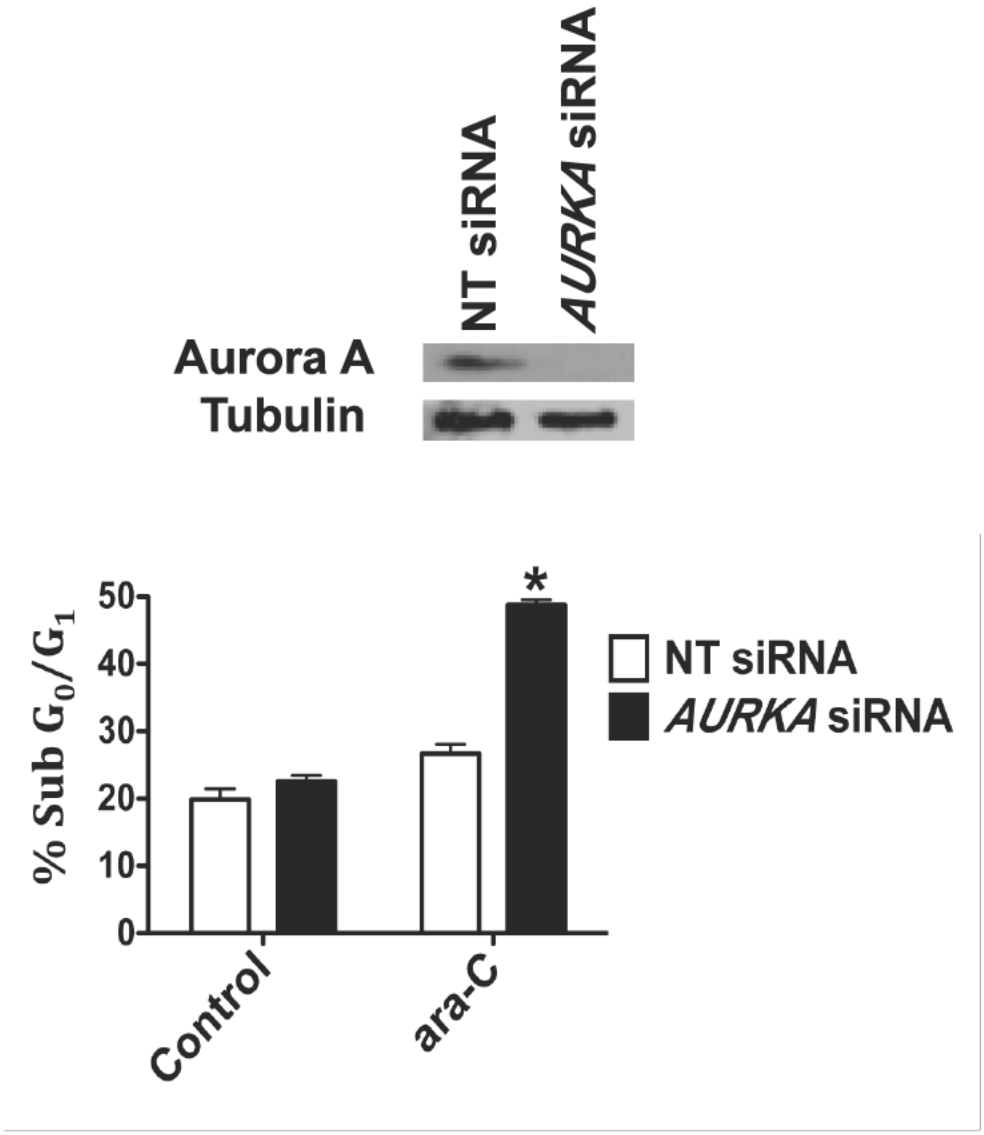

Figure 3.17 Targeting Aurora A increases the pro-apoptotic effects of ara-C

MV4-11 cells were transfected with non-target control or *AURKA*-targeted siRNA as described in the materials and methods. Immunoblotting was utilized to assess knockdown efficiency **(Top)**. Cells transfected with non-target control and Aurora A siRNA were treated with ara-C for 48 hours.

Drug-induced apoptosis was quantified by PI/FACS **(Bottom)**. n = 3 ± SD, *p ≤ 0.05.

3.4.6 Alisertib significantly augments the *in vitro* activity of ara-C

Based on our data demonstrating that targeted knockdown of Aurora A significantly increased the sensitivity of MV4-11 cells to ara-C, we hypothesized that inhibition of Aurora A activity with alisertib would potentiate the anti-cancer effects of ara-C. We tested our hypothesis by treating AML cell lines (MV4-11, MOLM-13, HL-60, and KG-1) with alisertib, ara-C, or both drugs for 72 hours. Quantification of cell viability by MTT assay showed that alisertib cooperated with ara-C to reduce AML cell viability (Figure 3.18). Investigation of the effects of these agents in primary AML blasts confirmed that alisertib significantly increased the ability of ara-C to disrupt AML cell viability (Figure 3.19). Colony formation assays demonstrated that inhibition of Aurora A activity with alisertib significantly enhanced the ability of ara-C to suppress the clonogenicity of AML cells (Figure 3.20).

Considering that earlier investigations have shown that Aurora A can function to inhibit apoptosis, we next tested whether antagonizing Aurora A activity with alisertib could potentiate the pro-apoptotic effects of ara-C. Quantification of the percentages of cells with active caspase-3 (Figure 3.21) and with sub G_0/G_1 DNA content (Figure 3.22) following 48 hours treatment with alisertib, ara-C, or both drugs revealed that the level of apoptosis induction was significantly greater in cells treated with alisertib + ara-C than with either drug alone. These data indicate that targeting Aurora A with alisertib may be an effective strategy to increase the anti-leukaemic activity of ara-C.

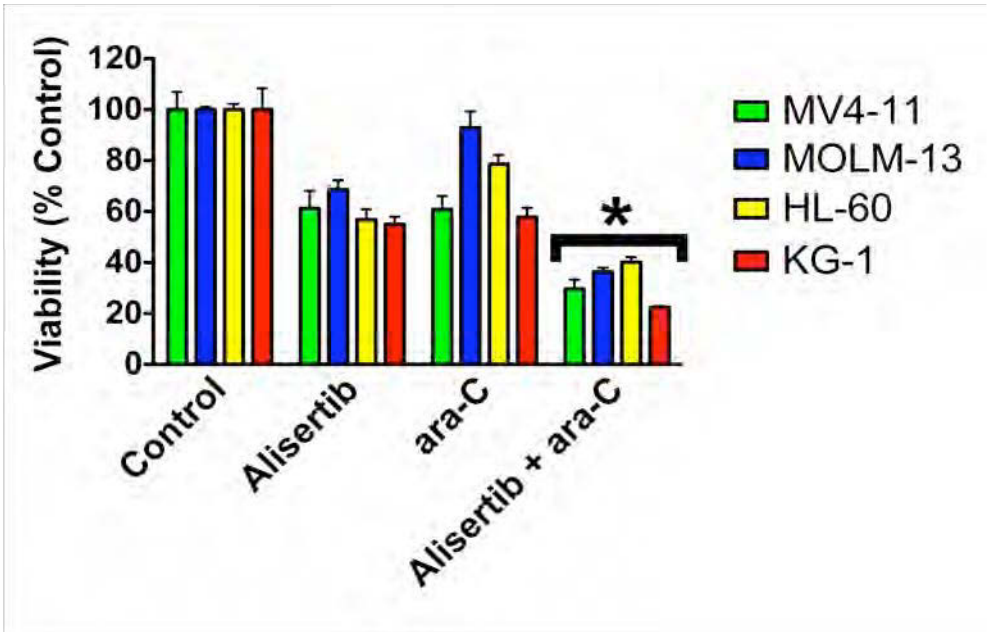

Figure 3.18 Alisertib potentiates the anti-leukaemic effects of ara-C
Human AML cell lines (MV4-11, MOLM-13, Hl-60, and KG-1) were treated with 100 nM alisertib, 100 nM ara-C, or the combination for 72 hours. Percentages of viable cells were determined by MTT assay. n = 3 ± SD, *p ≤ 0.05

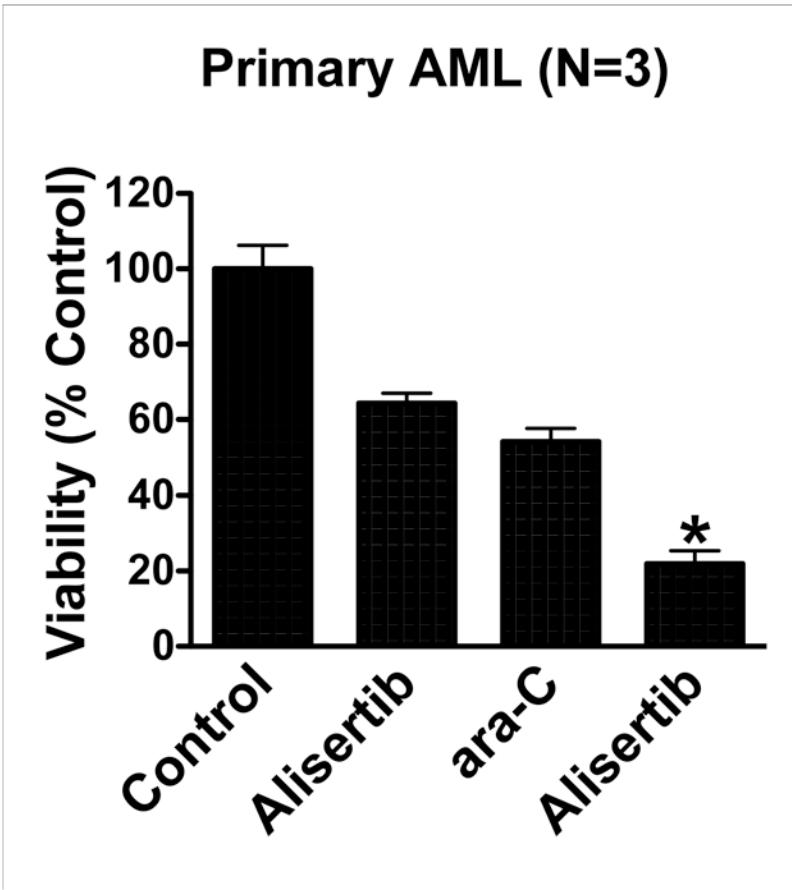

Figure 3.19 Alisertib potentiates the anti-leukaemic effects of ara-C
Primary AML cells were treated with 100 nM alisertib, 100 nM ara-C, or the combination for 72 hours. Percentages of viable cells were determined by MTT assay. n = 3 ± SD, *p ≤ 0.05.

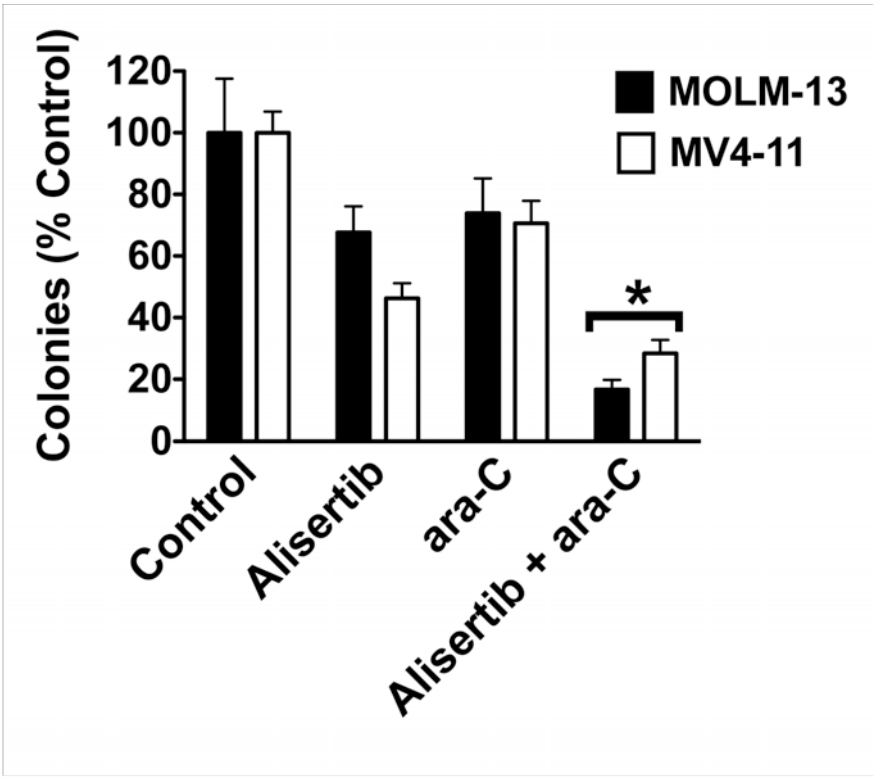

Figure 3.20 Alisertib and ara-C cooperate to disrupt clonogenic survival

MOLM-13 and MV4-11 cells were treated 100 nM alisertib, 100 nM ara-C, or both for 24 hours. Drugs were washed away and cells were plated in methocult. Colonies were scored on day 14. n = 3 ± SD, *p ≤ 0.05.

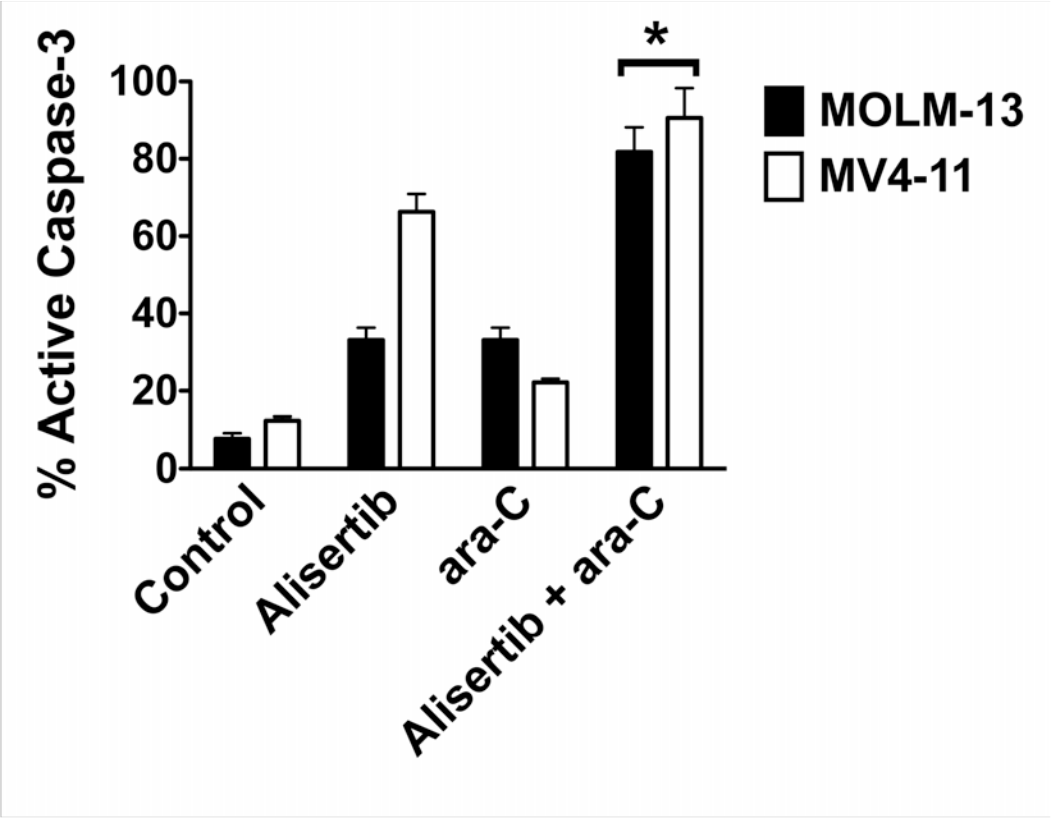

Figure 3.21 (a) Alisertib augments ara-C-mediated caspase-3 induction

Cells were treated with alisertib, ara-C, or both drugs for 48 hours. Drug-induced apoptosis was quantified by active caspase-3. The percentages of cells with caspase-3 positivity are quantified. n = 3 ± SD, *p ≤ 0.05.

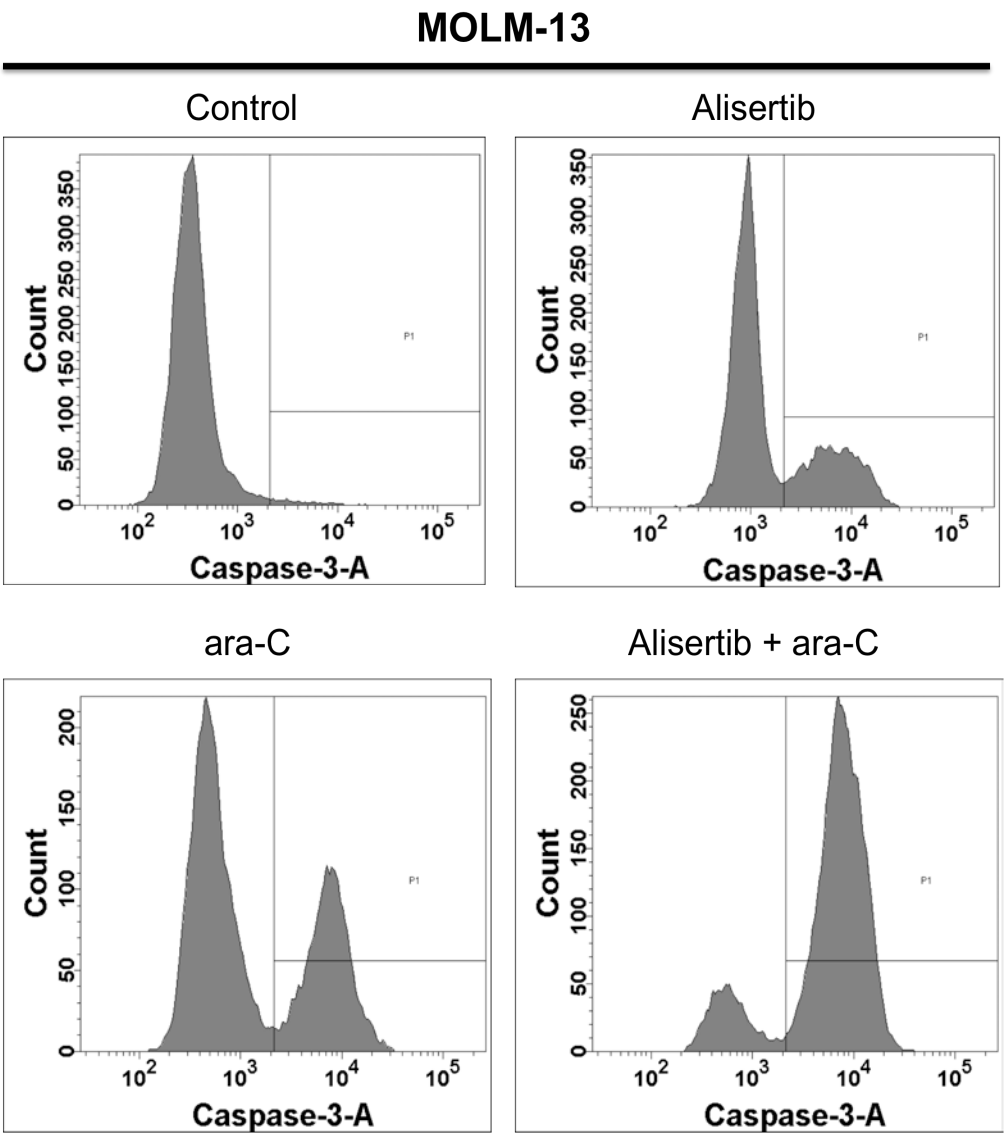

Figure 3.21 (b) Alisertib augments ara-C-mediated caspase-3 induction

MOLM-13 cells were treated with alisertib, ara-C, or both drugs for 48 hours. Drug-induced apoptosis was quantified by active caspase-3. Representative histograms are shown.

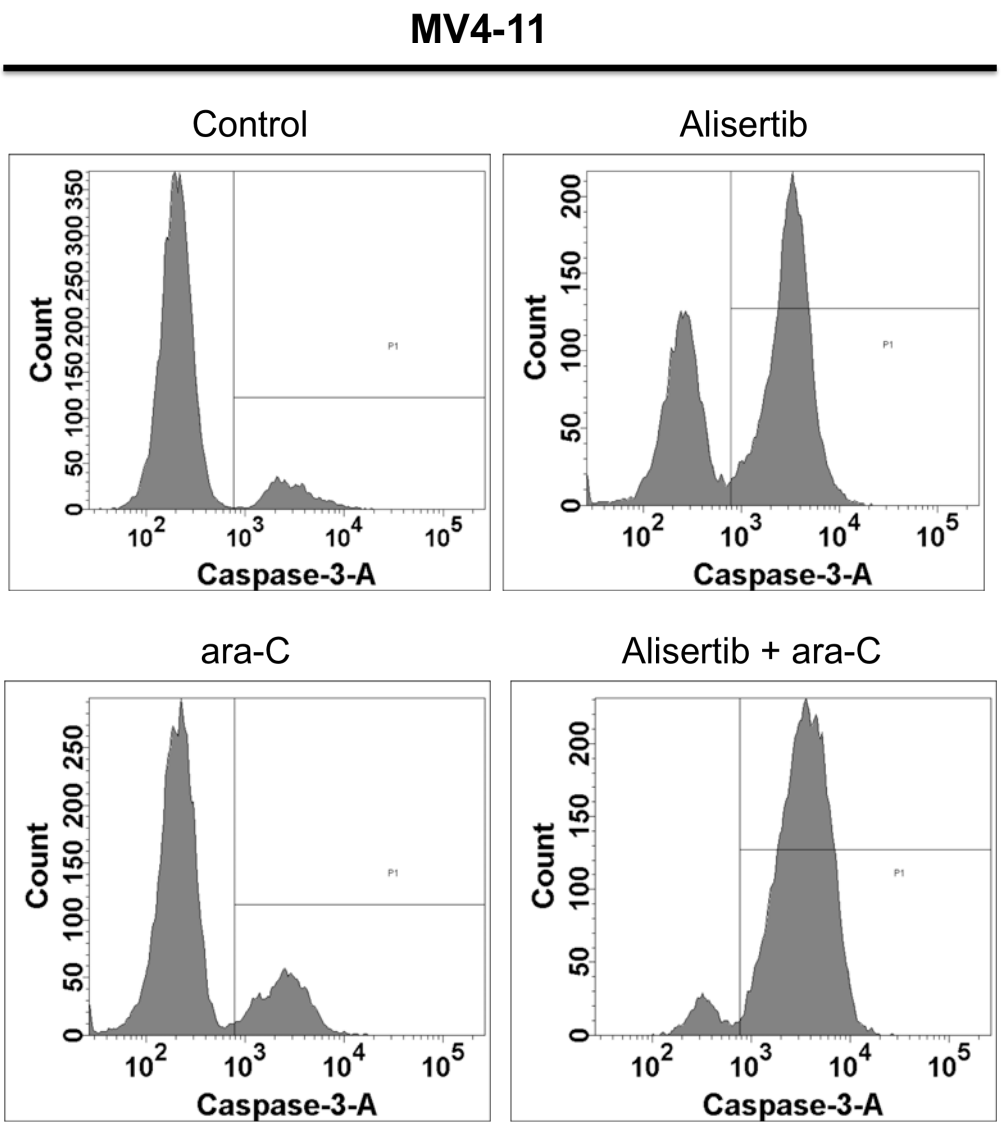

Figure 3.21 (c) Alisertib augments ara-C-mediated caspase-3 induction

MV4-11 cells were treated with alisertib, ara-C, or both drugs for 48 hours. Drug-induced apoptosis was quantified by active caspase-3. Representative histograms are shown.

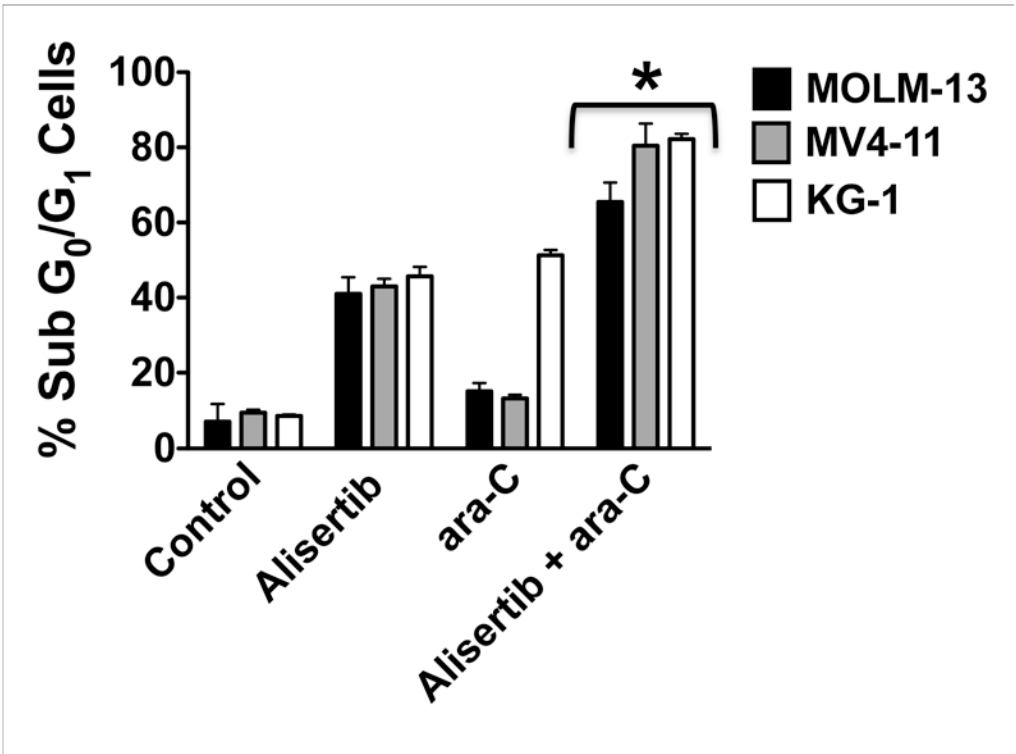

Figure 3.22 (a) Alisertib augments ara-C-mediated apoptosis

MOLM-13, MV4-11 and KG-1 cells were treated with alisertib, ara-C, or both drugs for 48 hours. Drug-induced apoptosis was quantified by PI/FACS. The percentages of cells with sub G_0/G_1 DNA content are quantified in the bottom panels. Representative histograms are shown. n = 3 ± SD, *$p \leq 0.05$.

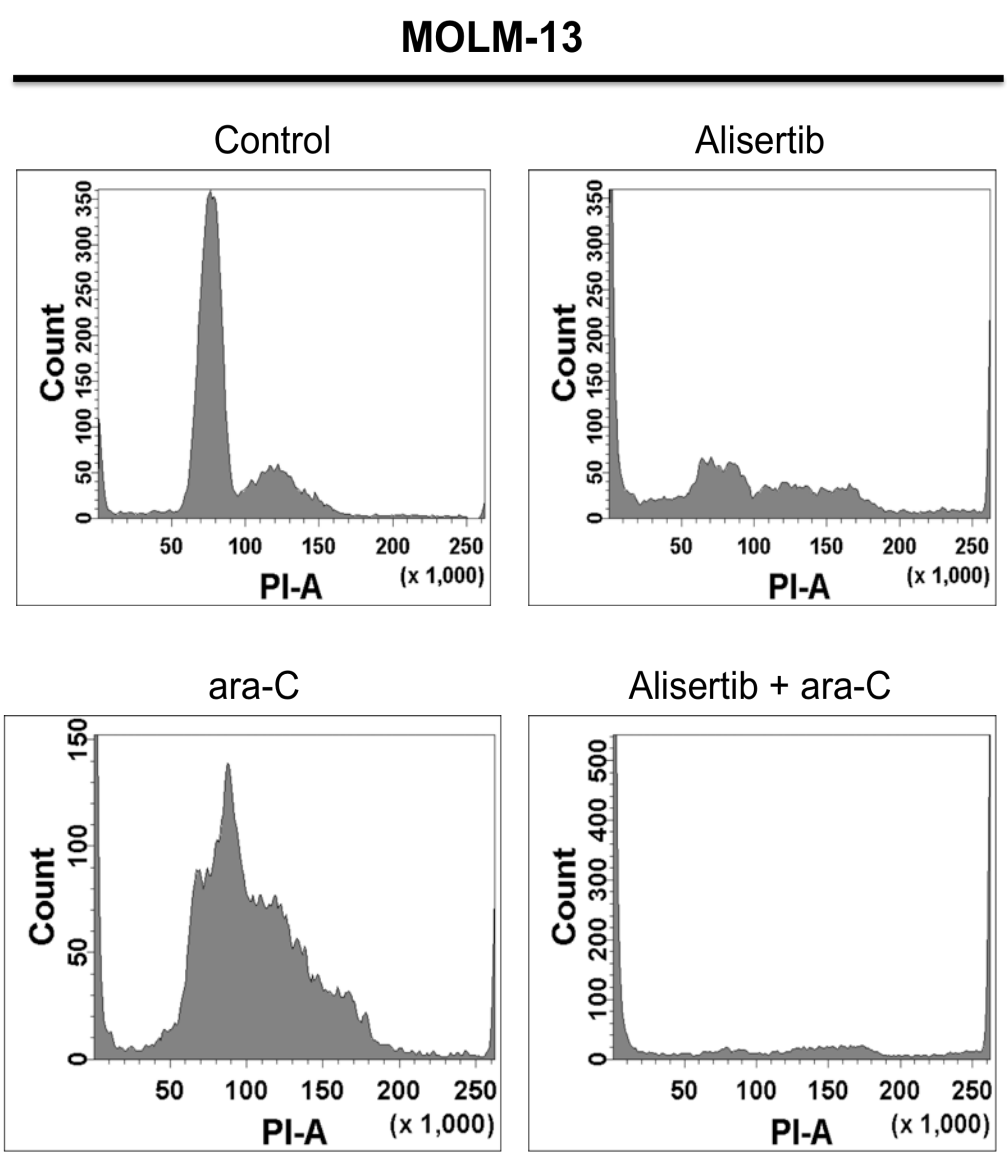

Figure 3.22 (b) Alisertib augments ara-C-mediated apoptosis

MOLM-13 cells were treated with alisertib, ara-C, or both drugs for 48 hours. Drug-induced apoptosis was quantified by PI/FACS. Representative histograms are shown. n = 3 ± SD, *p ≤ 0.05.

3.4.7 FOXO3a is a critical regulator of cellular sensitivity to alisertib and ara-C

FOXO3a is a member of the forkhead family of transcription factors and regulates the expression of a large number of target genes with critical roles in processes that are essential for oncogenic transformation and malignant pathogenesis. Phosphorylation of FOXO3a sequesters it in the cytosol and consequentially inhibits its transcriptional activity. Upon its dephosphorylation, FOXO3a translocates to the nucleus where it can initiate the transcription of its target genes (663, 664). Given that treatment with alisertib led to a significant reduction in the levels of phosphorylated (transcriptionally inactive) FOXO3a and the induction of the FOXO3a targets p27 and BIM (Figure 3.11), we hypothesized that these downstream effects of Aurora A inhibition may contribute to the ability of this agent to increase the efficacy of ara-C. In order to investigate this possibility, we first conducted immunoblot analyses of the levels of total and phosphorylated FOXO3a, p27, and BIM following treatment with alisertib, ara-C, or the combination of these drugs. Our results showed that the levels of phospho-FOXO3a and its targets p27 and BIM were more profoundly changed by treatment with both alisertib and ara-C as compared with the effects produced by either single agent treatment (Figure 3.23 and Figure 3.24). This suggested that the combined effects of these drugs on FOXO3a play an important role in regulating sensitivity to these agents. To determine whether FOXO3a is required for the induction of p27 and BIM expression in response to treatment with alisertib and ara-C, we used a lentiviral approach to generate MV4-11 cells with stable shRNA-mediated knockdown of FOXO3a (Figure 3.25).

We next treated cells expressing non-targeted control shRNA and FOXO3a shRNA with alisertib, ara-C, and the combination of these drugs and assessed the consequential effects on the expression of p27 and BIM by immunoblotting. Treatment with either single agent or the combination of both agents led to significant increases in the expression of p27 and BIM. In contrast, cells expressing FOXO3a-targeted shRNA displayed only

minor changes in the levels of p27 and BIM following single agent and combination treatments (Figure 3.26). Our results demonstrate that FOXO3a is required for maximal induction of p27 and BIM by alisertib and ara-C.

We next evaluated the potential role of FOXO3a as a regulator of the efficacy of the alisertib/ara-C combination. MV4-11 cells stably expressing non-targeted or FOXO3a shRNA were treated with alisertib, ara-C, or both drugs for 48 hours and drug-induced apoptosis was quantified by PI/FACS. Targeted knockdown of FOXO3a blunted the pro-apoptotic effects of alisertib/ara-C by more than 50% of what was observed on cells expressing control shRNA (Figure 3.27). Collectively, these data support a role for FOXO3a as a critical mediator of the therapeutic efficacy of this combination.

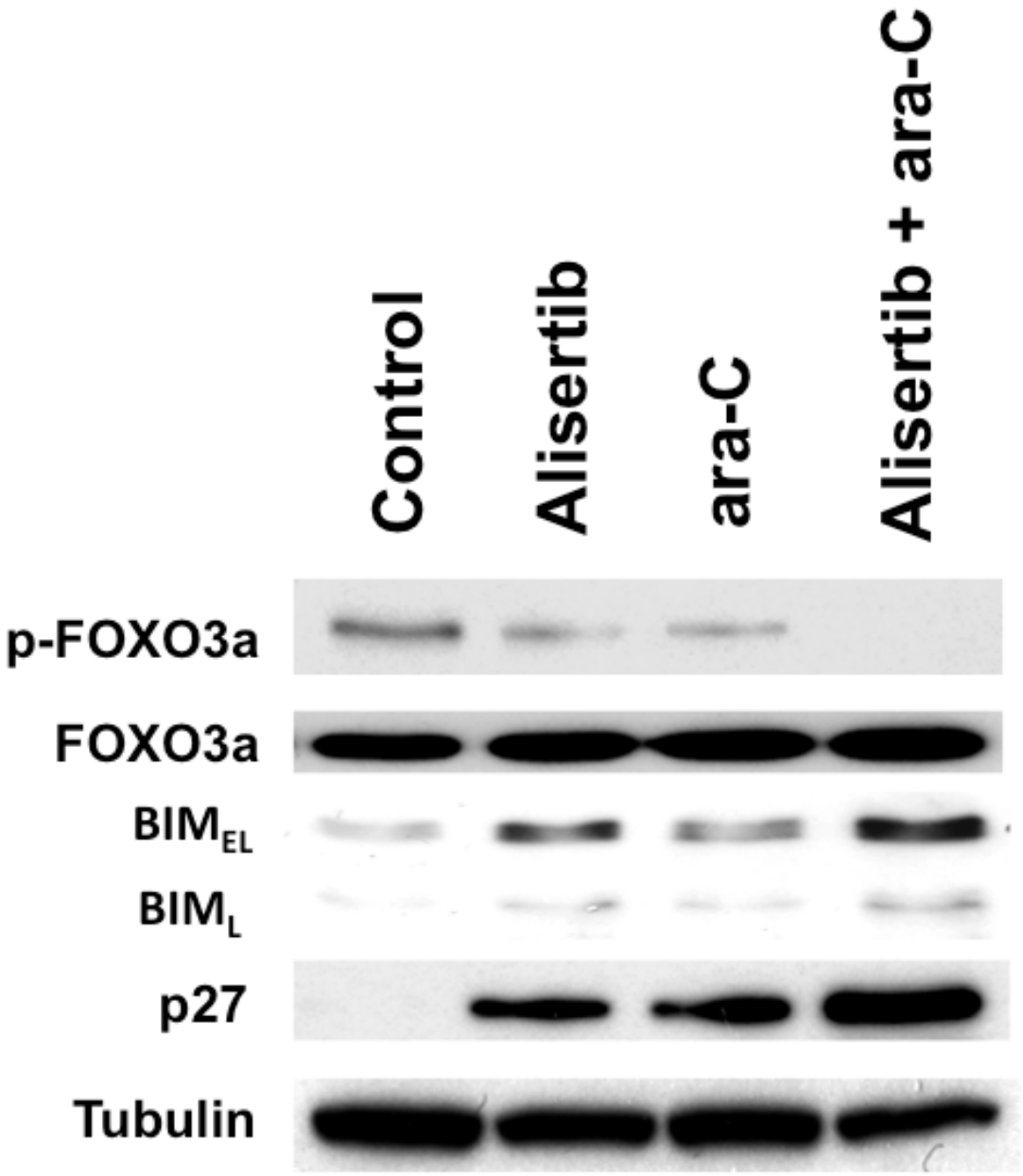

Figure 3.23 FOXO3a regulates sensitivity to the alisertib/ara-C combination

Alisertib and ara-C cooperate to induce expression of the FOXO3a targets BIM and p27. Cells were treated for 24 hours with alisertib, ara-C, or both agents. Immunoblotting was utilized to assess the effects of drug treatment on the levels of phospho-FOXO3a, total FOXO3a, BIM, and p27. Tubulin documented equal protein loading.

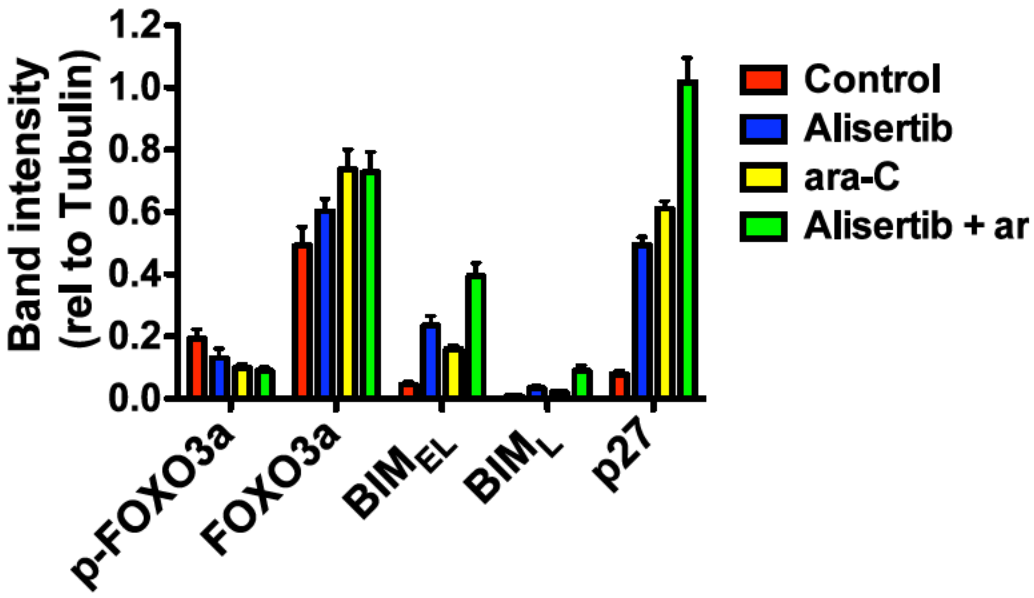

Figure 3.24 Densitometric quantification of p-Foxo3a, Foxo3a, BIM_{EL}, BIM_L and p27 using FluorChem HD2 software (ProteinSimple)

Cells were treated with the alisertib, ara-C or both for 24 hours. Protein lysates were subjected to SDS-PAGE and membranes were probed with antibodies as described in the materials and methods. Densitometric quantification of p-Foxo3a, Foxo3a, BIM_{EL}, BIM_L, p27 and tubulin was performed using FluorChem HD2 software (ProteinSimple). P-Foxo3a, Foxo3a, BIM_{EL}, BIM_L and p27 expression values were normalised to tubulin expression.

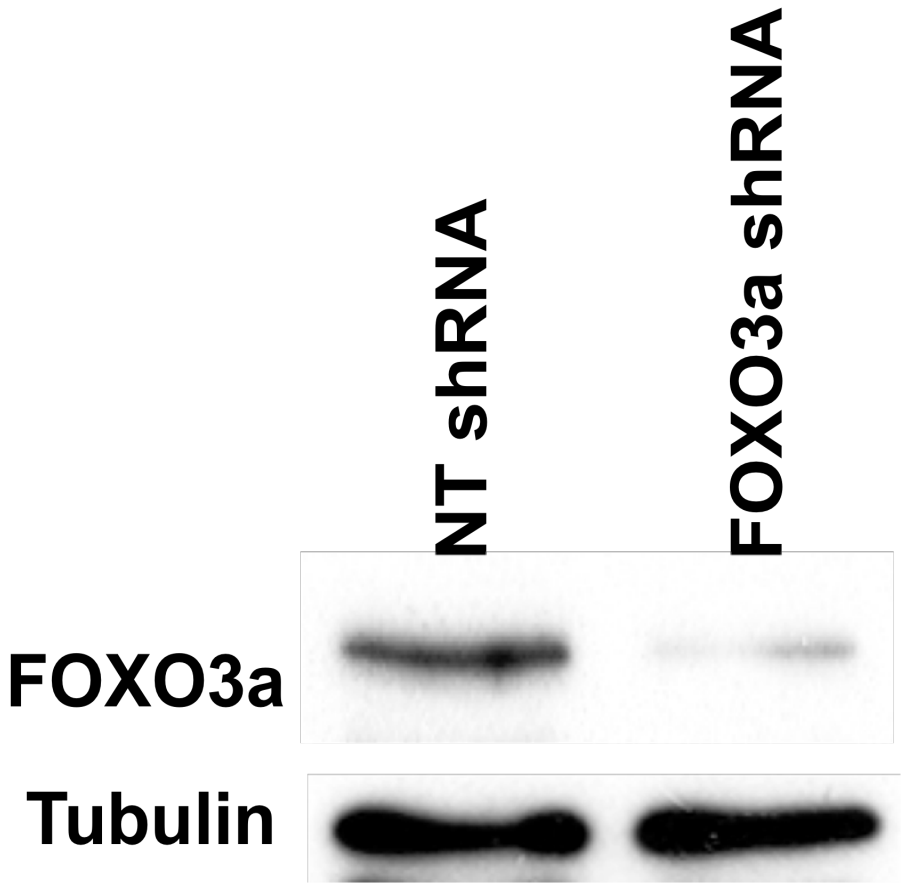

Figure 3.25 Generation of MV4-11 cells with stable FOXO3a knockdown

Cells were infected with non-targeted control or FOXO3a-targeted shRNA using a lentiviral approach. Stable cell lines were selected with puromycin treatment. Immunoblotting was utilized to assess knockdown efficiency.

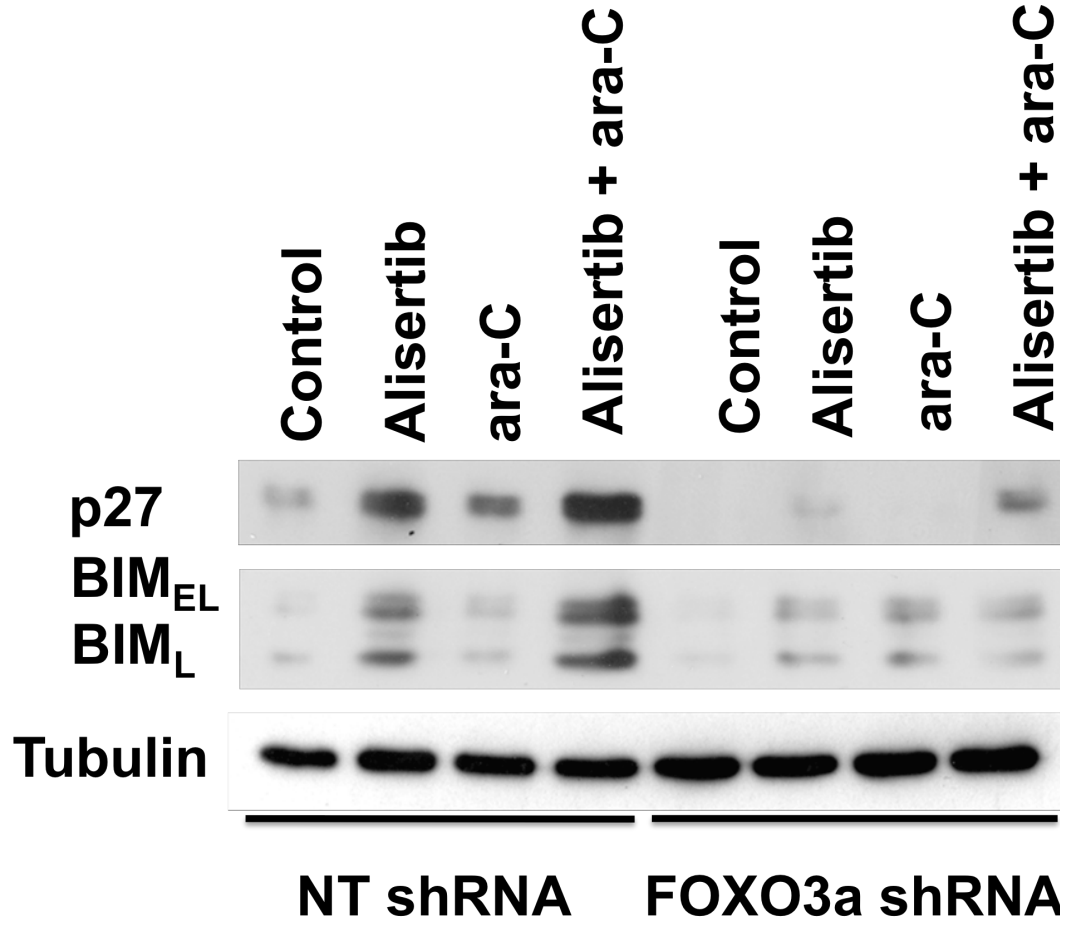

Figure 3.26 FOXO3a is required for maximal induction of p27 and BIM by alisertib/ara-C

MV4-11 cells expressing control or FOXO3a-targeted shRNA were treated with alisertib, ara-C, or both drugs for 24 hours. Protein lysates were subjected to SDS-PAGE and the levels of p27 and BIM were evaluated by immunoblotting. Tubulin documented equal protein loading.

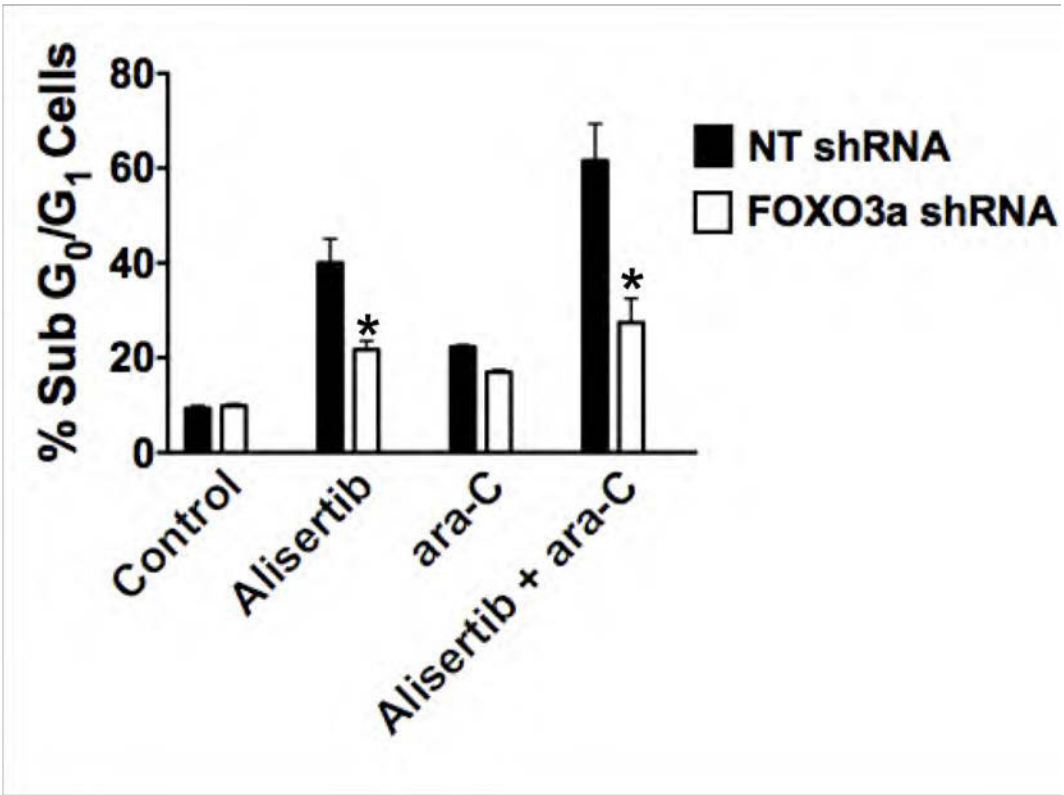

Figure 3.27 Targeted knockdown of FOXO3a blunts the pro-apoptotic effects of the alisertib/ara-C combination

MV4-11 cells expressing control or FOXO3a-targeted shRNA were treated with alisertib, ara-C, or both drugs for 48 hours. Drug-induced apoptosis was quantified by PI/FACS. The percentages of cells with sub G_0/G_1 DNA content are quantified. n = 3 ± SD, *p ≤ 0.05.

3.4.8 Alisertib potentiates the *in vivo* efficacy of ara-C

Our *in vitro* data demonstrated that targeting Aurora A with alisertib enhanced the efficacy of ara-C in AML cell lines and primary blasts from patients with AML. In order to further investigate the therapeutic potential of this novel strategy, we first conducted a pharmacokinetic experiment to ascertain any possible effects that co-administration of alisertib may have on the PK profile of ara-C. Mice were given a single dose of alisertib (20 mg/kg orally) and ara-C (50 mg/kg by intraperitoneal (IP) injection) alone or in combination and sacrificed at various time points. Plasma concentrations of ara-C and alisertib were determined using a LC/MS method. Pharmacokinetic parameters were estimated using a non-compartmental model. Our results demonstrate that the addition of either agent did not significantly affect the pharmacokinetics of the other (Figure 3.28).

We next conducted xenograft studies to investigate the *in vivo* therapeutic potential of the combination of alisertib and ara-C. MOLM-13 and KG-1 cells were injected subcutaneously into the flanks of immunodeficient nude mice. Vehicle, alisertib, ara-C or the combination of alisertib and ara-C were administered to mice for 14 days. In both KG-1 and MOLM-13 AML models, both alisertib and ara-C had substantial effects on tumour burden. The combination of both agents was well tolerated without a significant impact on animal body weight and resulted in significantly greater tumour growth inhibition than what was achieved by either agent alone (Figure 3.29 to 3.32). These findings indicate that inhibition of Aurora A activity with alisertib may be an attractive strategy to heighten the anti-leukaemic activity of ara-C.

Our earlier *in vitro* assays demonstrated that FOXO3a is an important regulator of the therapeutic activity of the alisertib/ara-C combination. We utilized an immunohistochemical approach to quantify the impact of *in vivo* administration of these agents on the expression of the FOXO3a targets p27 and BIM and on proliferating cell nuclear antigen

(PCNA) as a general measure of proliferative activity. Consistent with our *in vitro* observations, the alisertib/ara-C combination was significantly more effective at inducing the expression of p27 and BIM and also at globally diminishing tumour cell proliferation than either alisertib or ara-C alone (Figure 3.33 to 3.38). Our collective findings indicate that inhibition of Aurora A activity with alisertib represents a novel approach to increase the growth inhibitory and pro-apoptotic effects of treatment with ara-C.

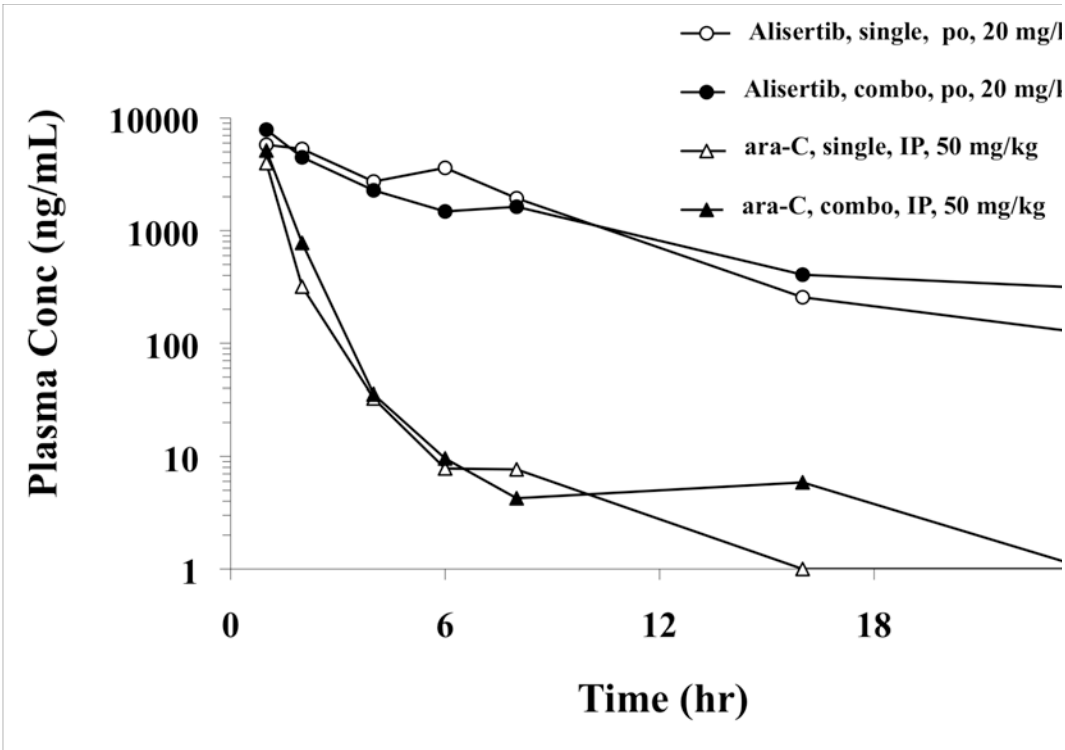

Figure 3.28 Alisertib does not significantly impact the pharmacokinetic profile of ara-C

Female naive CB-17 SCID mice were given a single dose of alisertib (20 mg/kg orally) and ara-C (50 mg/kg IP) alone or in combination and sacrificed at various time points (1, 2, 4, 6, 8, 16, and 24 hours). Blood was drawn using cardiac puncture and plasma was isolated. Extractions were performed separately for the analysis of alisertib and ara-C, and alisertib and ara-C plasma concentrations were determined by LC/MS/MS.

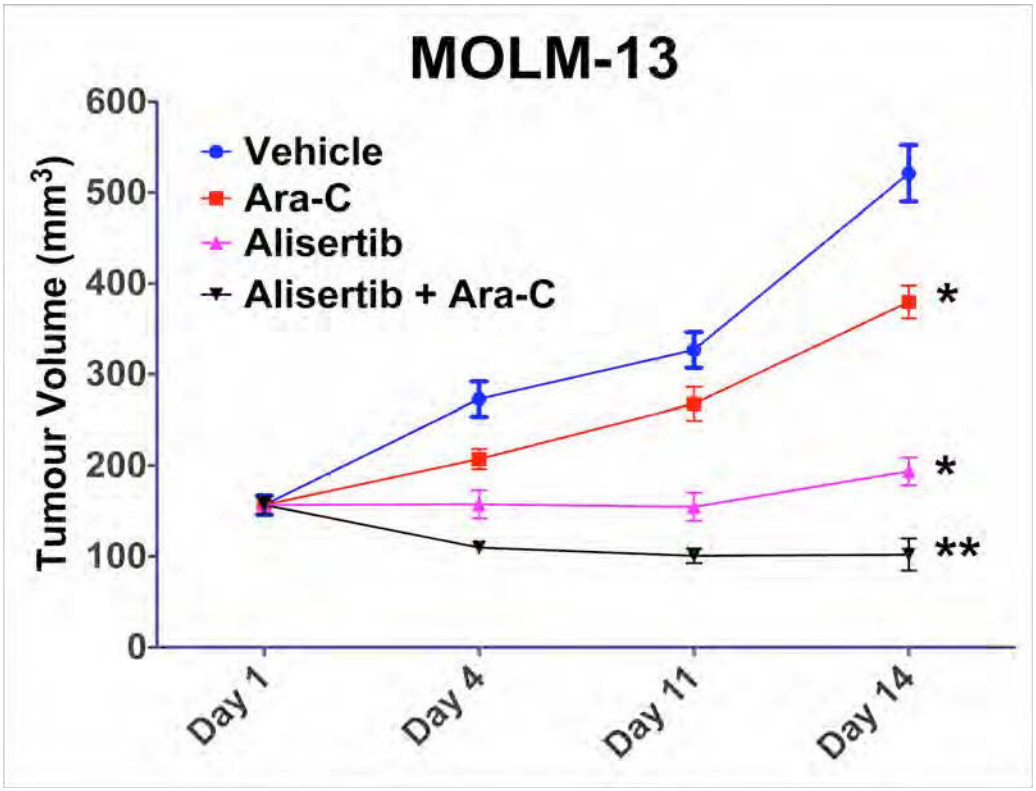

Figure 3.29 Alisertib augments the *in vivo* activity of ara-C

MOLM-13 cells were injected into the flanks of nude mice. Vehicle, alisertib (20 mg/kg po BID), ara-C (75 mg/kg IP three times a week) or both drugs were administered for 14 days. Tumour volume was measured biweekly. n = 10 ± SD, *p ≤ 0.05 (controls vs. single agents, **p ≤ 0.05 (single agents vs. combination).

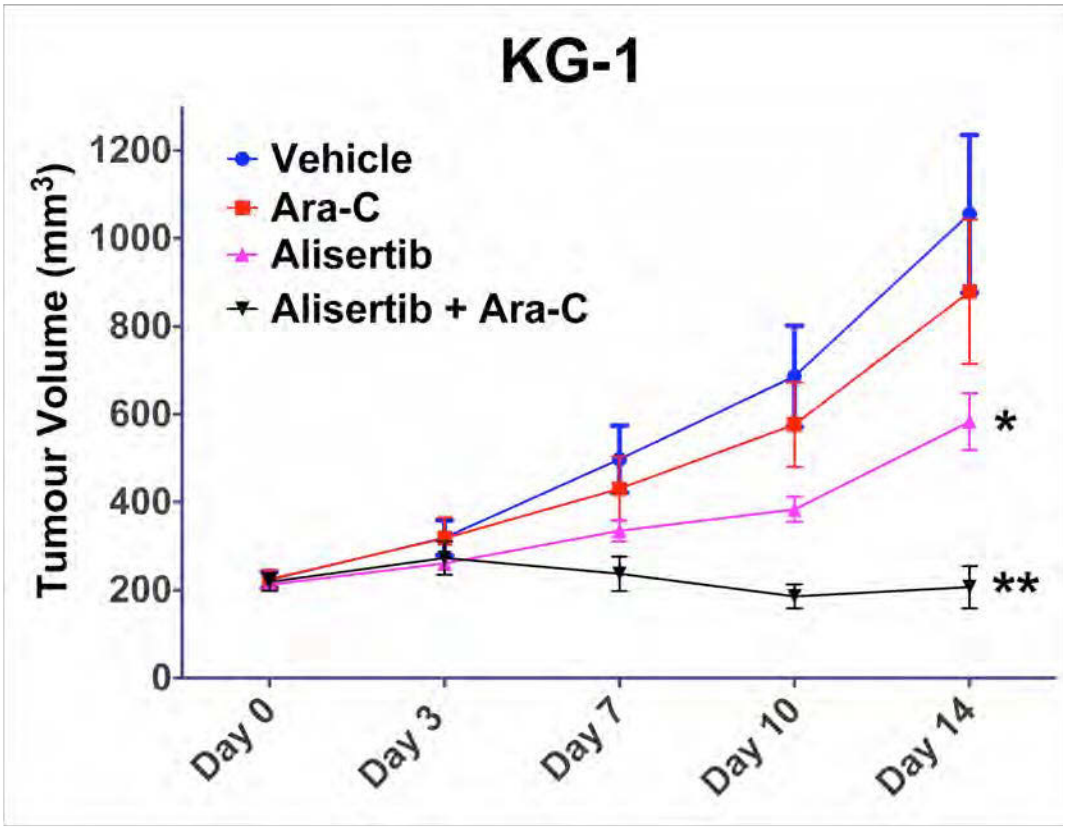

Figure 3.30 Alisertib augments the *in vivo* activity of ara-C

KG-1 cells were injected into the flanks of nude mice. Vehicle, alisertib (20 mg/kg po BID), ara-C (50 mg/kg IP QD for 5 days per week) or both drugs were administered for 14 days. Tumour volume was measured biweekly. n = 10 ± SD, *p ≤ 0.05 (controls vs. single agents, **p≤ 0.05 (single agents vs. combination).

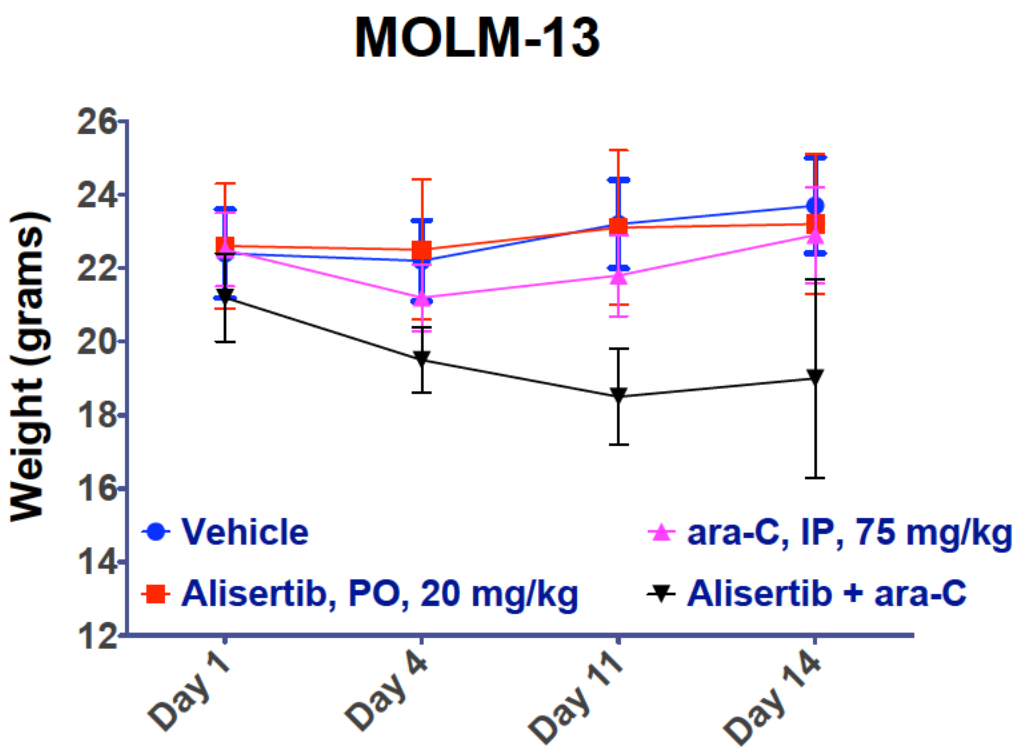

Figure 3.31 *In vivo* tolerability of alisertib and ara-C

MOLM-13 cells were injected into the flanks of nude mice. Vehicle, alisertib (20 mg/kg po BID), ara-C (75 mg/kg ip TIW for MOLM-13, 50 mg/kg IP QDx5) or both drugs were administered for 14 days. Animal weights were measured biweekly. n = 10 ± SD.

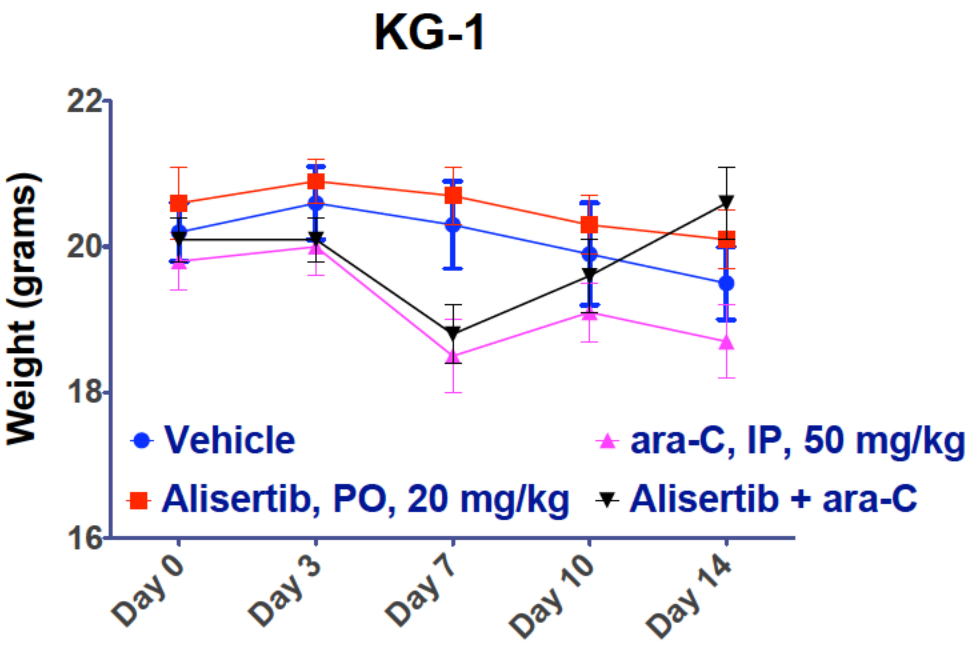

Figure 3.32 *In vivo* tolerability of alisertib and ara-C

KG-1 cells were injected into the flanks of nude mice. Vehicle, alisertib (20 mg/kg po BID), ara-C (75 mg/kg IP TIW for MOLM-13, 50 mg/kg IP QDx5) or both drugs were administered for 14 days. Animal weights were measured biweekly. n = 10 ± SD.

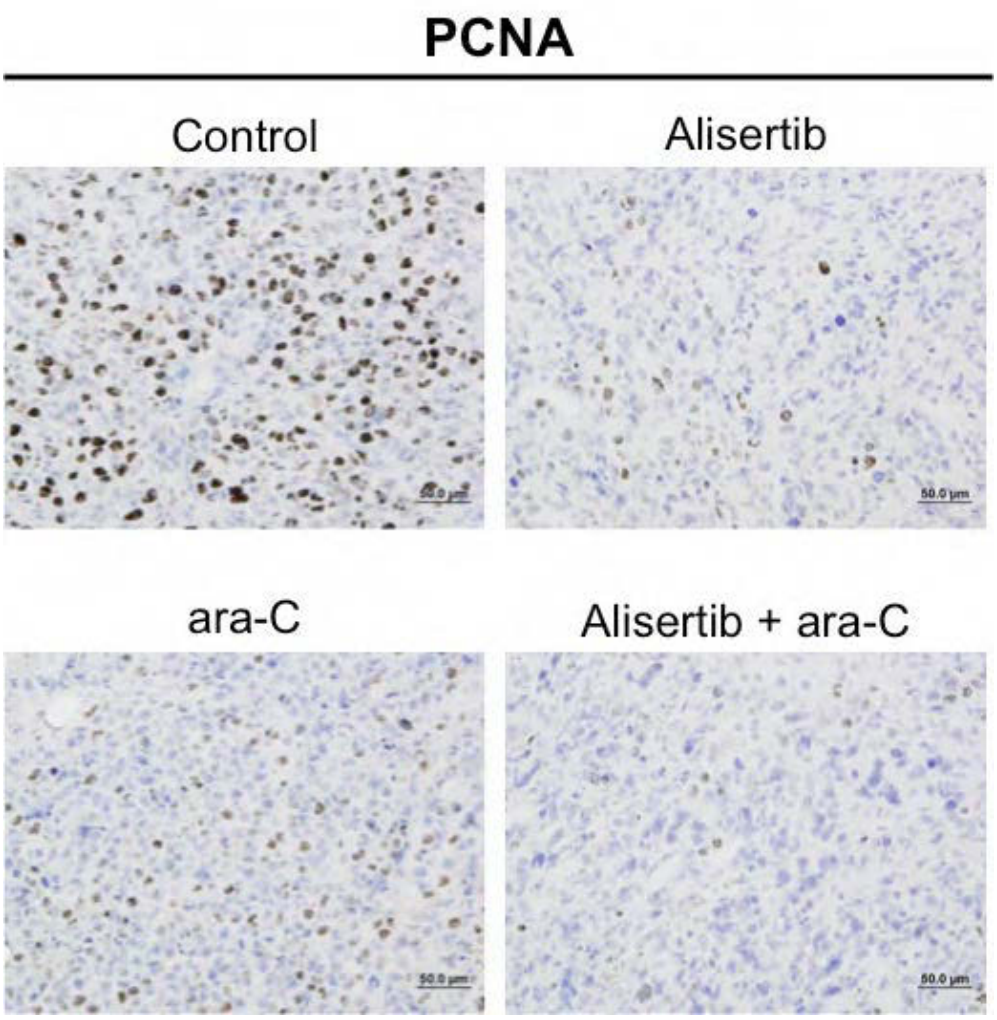

Figure 3.33 Alisertib and ara-C combine to reduce cell proliferation *in vivo*

Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment. Cells were stained with PCNA was used as a general measure of tumour cell proliferation. Representative images are shown from each treatment group. Positive cells were scored manually under 20X magnification.

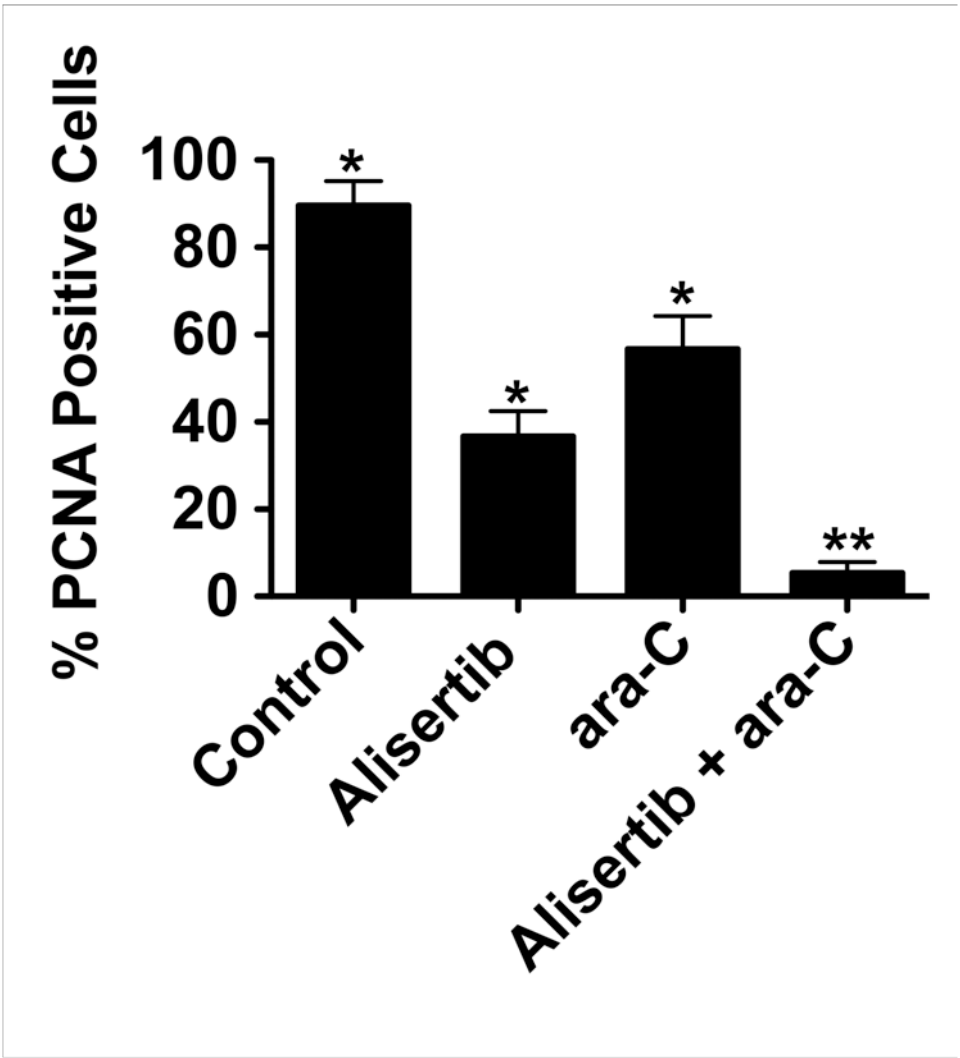

Figure 3.34 Alisertib and ara-C combine to reduce cell proliferation *in vivo*

Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment. Cells were stained with PCNA as a general measure of tumour cell proliferation. Percentages of PCNA-positive cells were determined by manual counting of 5 non-overlapping random high-power fields per section by two investigators blinded to the treatment arms. The average percentage of PCNA positive cells per high-power field was calculated for comparison. Mean ± SD, n = 5. *$p < 0.05$ (controls vs. single agents), **$p < 0.05$ (single agents vs. combination).

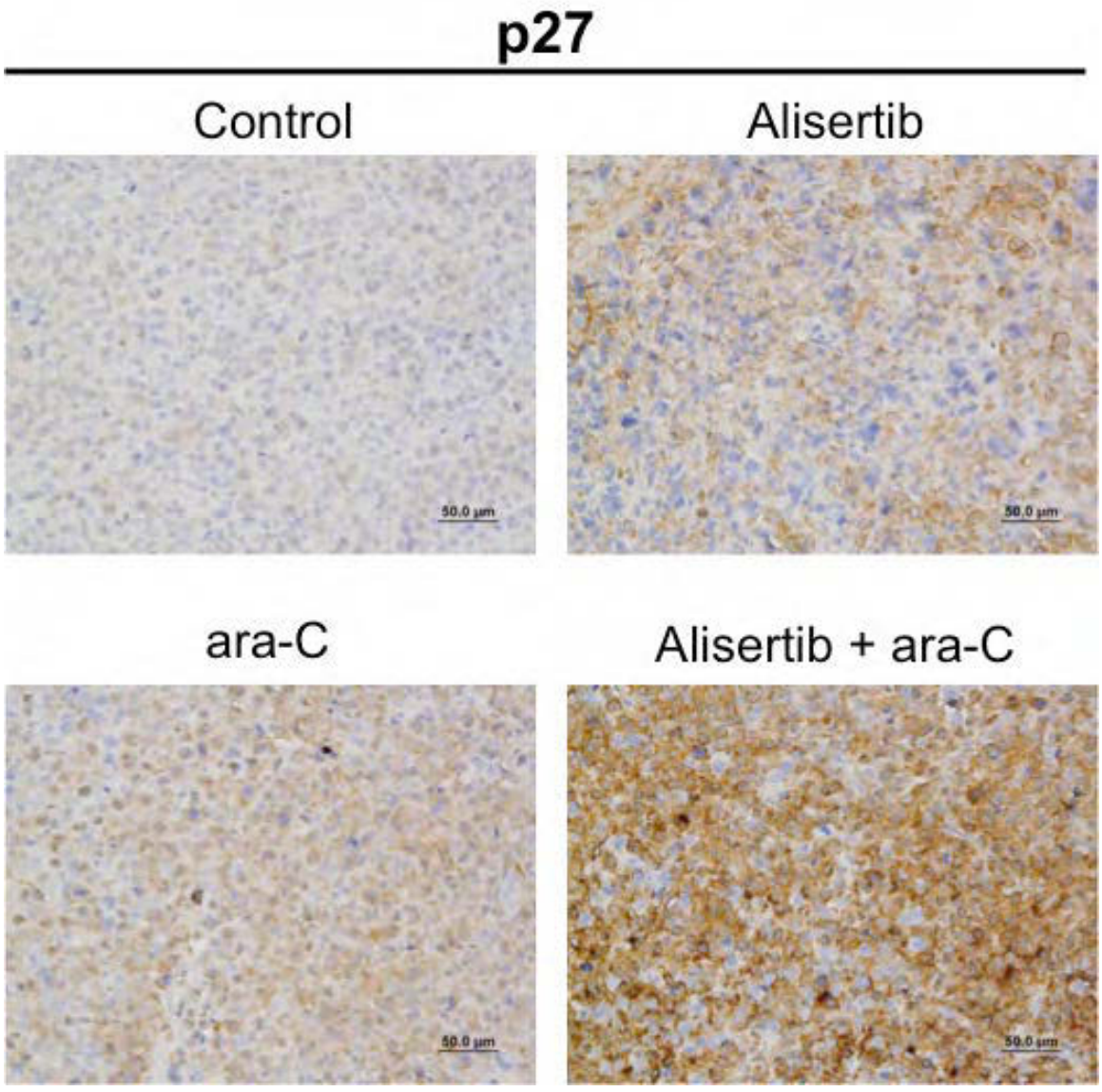

Figure 3.35 The alisertib/ara-C combination induces the expression of p27 *in vivo*

Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment on the expression of the FOXO3a target p27. Representative images are shown from each treatment group.

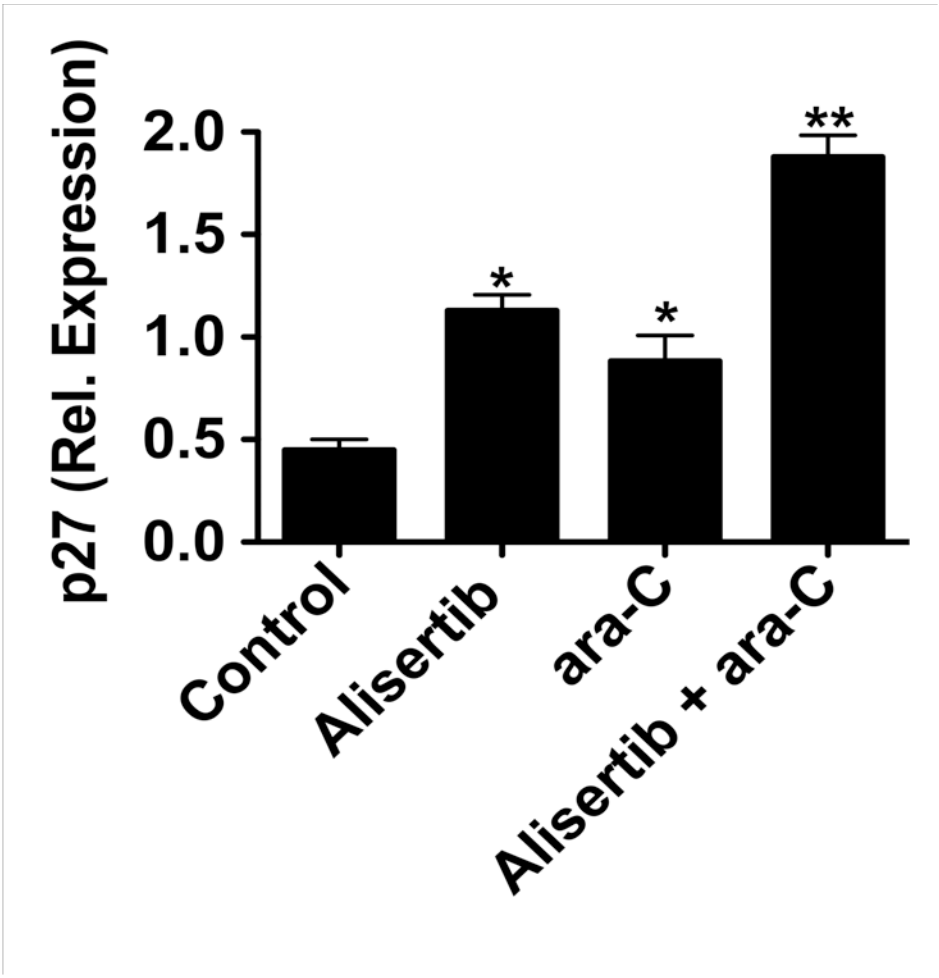

Figure 3.36 The alisertib/ara-C combination induces the expression of p27 *in vivo*

Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment on the expression of the FOXO3a target p27. The relative intensity of p27 expression was measured using Image-Pro Plus software Version 6.2.1 by densitometric analysis of five random high-power fields containing viable tumour cells by two independent investigators blinded to the treatment arms. Mean ± SD, *n*=5 Mean ± SD, n = 5, *p≤ 0.05 (controls vs. single agents, **p≤ 0.05 (single agents vs. combination).

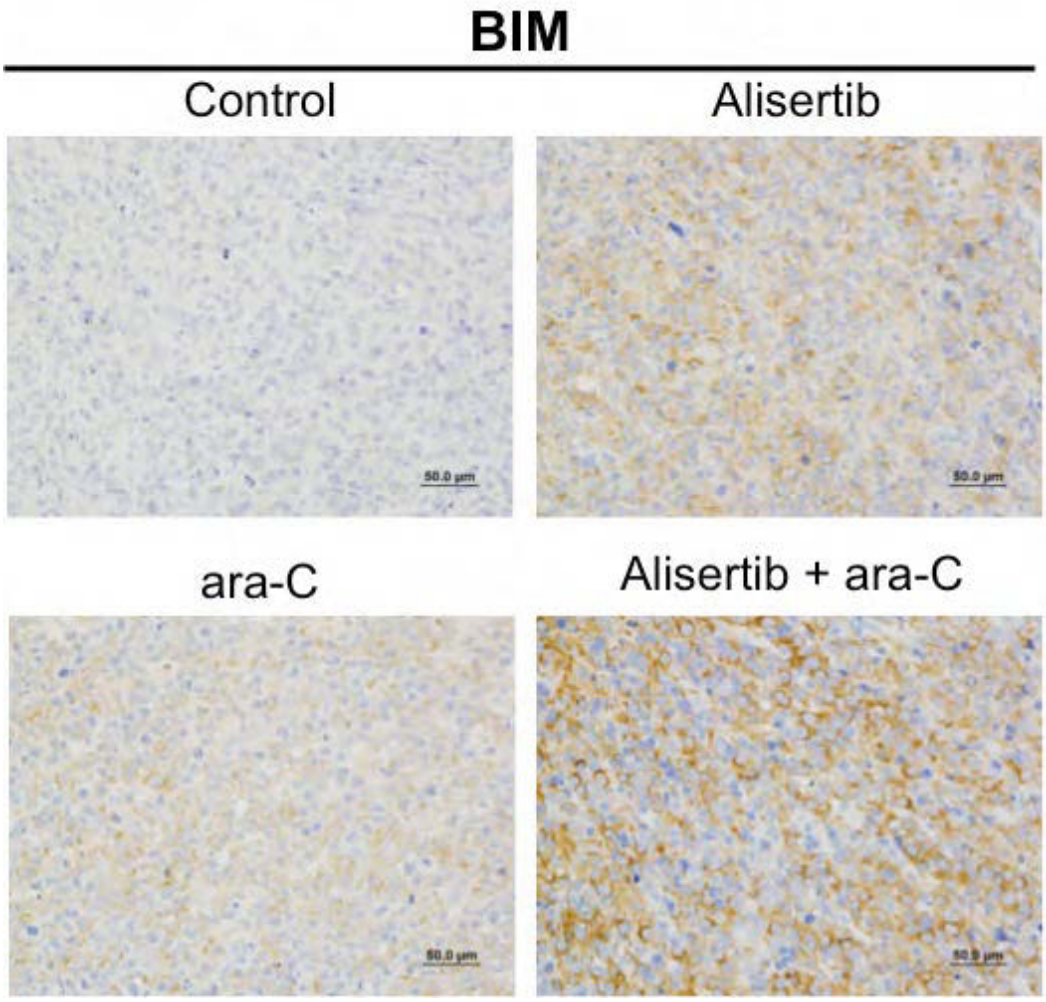

Figure 3.37 The alisertib/ara-C combination induces the expression of BIM *in vivo*

Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment on the expression of the FOXO3a target BIM. Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment on the expression of the FOXO3a target BIM. Representative images are shown from each treatment group.

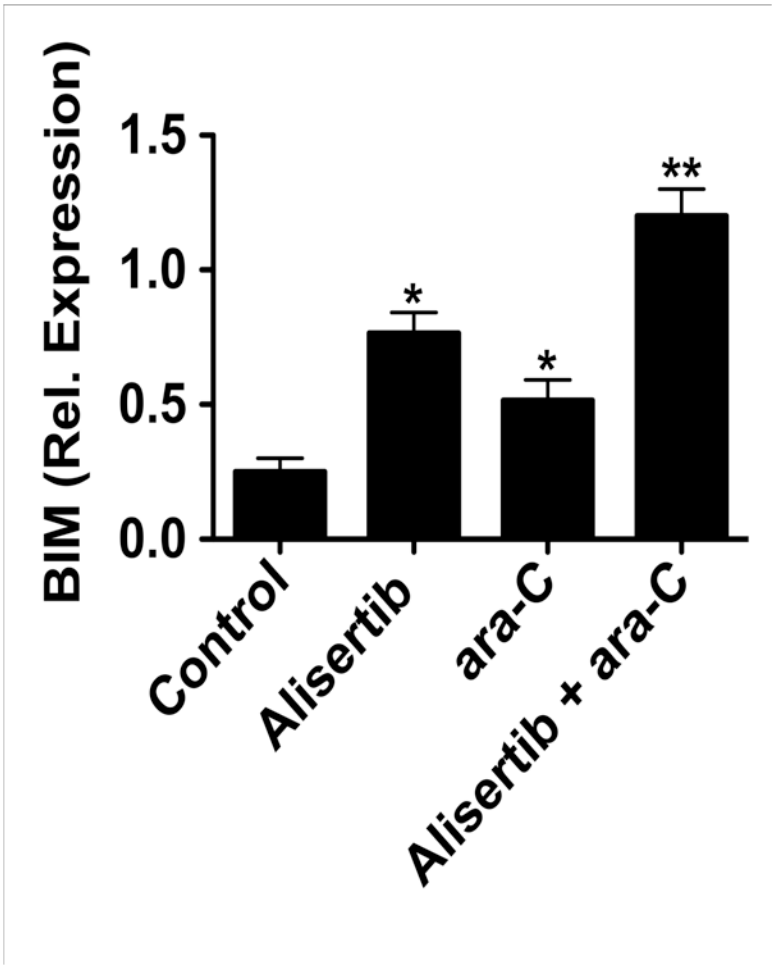

Figure 3.38 The alisertib/ara-C combination induces the expression of BIM *in vivo*

Immunohistochemistry was carried out on tumour specimens from mice in each treatment group to quantify the effects of drug treatment on the expression of the FOXO3a target BIM. The relative intensity of BIM expression was measured using Image-Pro Plus software Version 6.2.1 by densitometric analysis of five random high-power fields containing viable tumour cells by two independent investigators blinded to the treatment arms. Mean±SD, n=5 Mean ± SD, n = 5, *p≤ 0.05 (controls vs. single agents, **p≤ 0.05 (single agents vs. combination).

3.5 Discussion

Given its intrinsic overexpression in cancer, essential functions in the regulation of mitosis, and potential roles in promoting drug resistance and disease progression, Aurora kinase A is an attractive target for cancer therapy. As discussed in section 1.8.6, a number of Aurora kinase inhibitors that have varying degrees of activity against Aurora A are currently in development (674). We hypothesized that the prevalent overexpression of Aurora A in AML and highly proliferative nature of this malignancy would render it particularly sensitive to alisertib. We conducted a series of preclinical experiments with the aim to ascertain the antileukaemic activity and pharmacodynamic effects of targeting Aurora A with alisertib in AML cell lines, primary AML blasts, and mouse xenograft models of AML.

Our *in vitro* assays demonstrated that alisertib has pleiotropic effects in AML cells. Exposure to alisertib diminished cell viability and clonogenic survival, disrupted cell cycle dynamics, and induced apoptosis. Interestingly, our data demonstrated that cellular sensitivity to alisertib was not directly correlated with Aurora A expression levels in AML cells. These findings are consistent with those from early phase clinical trials with alisertib, which have also failed to show a direct relationship between Aurora A expression levels and sensitivity to alisertib (677). Similarly in a preclinical investigation of 87 cancer cell lines the expression of Aurora A and B were found to be weak predictors of response to Aurora kinase inhibitors (660). The reason for this phenomenon is unknown. It is possible that alisertib may achieve similar degrees of Aurora A inhibition in cells with relatively low, intermediate, and high basal Aurora A expression. If this were the case, sensitivity to alisertib would not appear to be directly linked to Aurora A levels. Pharmacodynamic studies from ongoing and planned phase 2 and phase 3 clinical trials with alisertib may help to clarify this issue.

FOX proteins are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity (678). The defining feature of FOX proteins is the forkhead box, a sequence of 80 to 100 amino acids forming a motif that binds to DNA. They are grouped into subclasses (FOXA-FOXS) based on sequence conservation (679). There are four members of the class O: FOXO1, FOXO3, FOXO4 and FOXO6. They have roles in metabolism, cellular proliferation and stress tolerance. The activity of FOXO is tightly regulated by post-translational modifications, including phosphorylation, acetylation and ubiquitylation. FOXO1 plays important roles in regulation of gluconeogenesis, glycogenolysis by adipogenesis (680). FOXO3a functions as a trigger for apoptosis through upregulation of BIM and PUMA (681) or downregulation of anti-apoptotic proteins such as FADD-like IL-1beta-converting enzyme (FLICE)-inhibitory protein (FLIP). It is FOXO3a's role in apoptosis that most likely mediates a portion of the therapeutic effect of the alisertib and ara-C combination in this study.

The growth inhibitory and pro-apoptotic effects we observed in AML cells treated with alisertib were associated with significantly increased expression of the FOXO3a transcriptional targets p27 and BIM. Given that p27 is a critical component of the G_2/M cell cycle transition and that BIM is a microtubule-associated pro-apoptotic factor, the observed induction of p27 and BIM expression is a predictable consequence of Aurora A inhibition. These findings are consistent with those of earlier investigations conducted in other cancer models that also demonstrated elevated p27 and BIM levels following treatment with Aurora kinase inhibitors (628, 661).

Several prior studies have established a link between Aurora A overexpression and resistance to therapeutic agents, supporting a role for Aurora A in the regulation of chemosensitivity (665-670). However, it is not completely clear at this time whether this is an intrinsic or acquired relationship. We postulated that treatment with conventional cytotoxic agents such as ara-C could promote elevated levels of Aurora A during the

genotoxic stress response. Indeed, our data demonstrate that *in vitro* treatment with ara-C leads to increased expression of Aurora A in AML cell lines and primary blasts from patients. In order to investigate the potential therapeutic implications of ara-C mediated induction of Aurora A expression, we first utilized siRNA as a proof of principle to demonstrate that targeted knockdown of Aurora A significantly increased the pro-apoptotic effects of ara-C. This suggested that Aurora A may play a chemoresistance role with respect to ara-C and that targeting its activity could be of therapeutic benefit.

We further investigated the potential impact of targeting Aurora A activity with alisertib on the efficacy of ara-C in a series of *in vitro* experiments in AML cell lines and primary AML cells. These assays demonstrated that alisertib significantly increased both the growth inhibitory and pro-apoptotic effects of ara-C. Interestingly, the combination of both drugs led to enhanced induction of the FOXO3a targets p27 and BIM compared to either single agent treatment. Our targeted knockdown assays demonstrated that FOXO3a expression was required for maximal alisertib/ara-C mediated induction of these specific targets and consequently, for the combination to most effectively trigger apoptosis. To our knowledge, this is the first report demonstrating a link between Aurora A kinase inhibition and FOXO3a activity. Our subsequent pharmacokinetic and mouse xenograft studies validated the potential therapeutic benefit of combining alisertib and ara-C for AML therapy. Additionally, immunohistochemical assays conducted with specimens obtained from mice treated with alisertib/ara-C established p27 and BIM as pharmacodynamic effectors of these agents.

FOXO3a has several other intriguing functions. In non haematopoietic cells activation of FOXO blocks cellular proliferation and drives cells into a quiescent state (682). Deregulation of FOXO3a is involved in tumourigenesis, for example translocation of this gene with the MLL gene is associated with secondary ALL (683). PTEN is inactivated in

many tumours (684) and results in AKT activation. FOXO3a can reduce the level of cellular oxidative stress by directly increasing mRNA and protein levels of manganese superoxide dismutase (MnSOD) and catalase (682, 685). Reintroduction of FOXO in PTEN-negative tumours results in cell cycle arrest and apoptosis (686). Recent findings have also pointed to a role for FOXO in the growth of tumour stem cells (687). Furthermore simultaneous loss of FOXO1, FOXO3A and FOXO4 results in HSC exhaustion, possibly through an increase in oxidative stress (688). Therefore FOXO is important in restricting HSC proliferation and protecting against proliferative exhaustion.

Multiple layers of post-translational modifications that include phosphorylation, acetylation and ubiquitylation regulate the activity of FOX factors (689). These modifications determine the cellular localization and activity of FOX factors; nuclear FOX proteins act as transcriptional regulators whereas cytoplasmic FOX proteins are inactive and often subject to proteasomal degradation. FOXO3a proteins are phosphorylated by several upstream kinases including AKT (690), JNK (691), a dual specificity tyrosine-phosphorylated and regulated kinase (DYRK1A) (692) and IkappaB (692, 693). FOXO3a is inactivated through phosphorylation by AKT at Thr^{24}, Ser^{256} and Ser^{319}, which results in nuclear export and inhibition of transcription factor activity (694). Forkhead transcription factors can also be inhibited by the deacetylase sirtuin (SIRT1) (694).

The FOXO3a-related effects that occur downstream of Aurora A inhibition by alisertib are very interesting. The exact mechanism by which alisertib induces the expression of FOXO3a transcriptional targets remains to be fully elucidated. I was unable to co-immunoprecipitate Aurora A and FOXO3a, which indicates that FOXO3a is unlikely to be a direct Aurora A phospho-substrate. A previous study conducted in models of multiple myeloma demonstrated that alisertib treatment leads to activation of PP2A (662). Considering that PP2A has been shown to de-phosphorylate FOXO3a to its nuclear-localized transcriptionally active form, it is possible

that the effects of alisertib on PP2A may contribute to the FOXO3a-mediated increased expression of p27 and BIM that we observed in response to alisertib treatment in our study. Alternative mechanisms for alisertib-induced activation of FOXO3a exist. Aurora A is known to activate AKT and inhibition of Aurora A leads to a reduction in phosphorylation of AKT (666). As discussed above, FOXO3a is phosphorylated and in turn inhibited by AKT (695). Therefore alisertib induced inhibition of Aurora A may lead to inhibition of AKT which in turn leads to reduced phosphorylation of FOXO3a. Additional studies are required to clarify this relationship mechanistically. However, considering that FOXO3a is a critical regulator of cell death, the ability of alisertib to increase FOXO3a activity may contribute to its ability to potentiate the efficacy of a broad range of anti-cancer agents.

As discussed earlier, alisertib has been evaluated in several studies in solid tumours and haematological malignancies (696-698). Notably, alisertib has also been evaluated as a single agent in a phase 2 study of 57 patients with advanced AML or MDS (697). While objective responses (13% of the population) including one CR were observed in this study, it is likely that combination therapy with cytotoxic agents such as ara-C would allow greater initial disease control facilitating sustained Aurora kinase A inhibition.

Our collective data demonstrate that the combination of alisertib and ara-C is effective in preclinical models of AML. Based on this promising preclinical data, a phase 1/2 study is warranted to investigate the safety and activity of the alisertib/ara-C combination in patients with AML who would not be expected to benefit from conventional therapy.

4 Chapter 4 Targeting PIM kinase in preclinical models of AML

4.1 Introduction

As discussed in section 1.1.4, AML is heterogeneous disease with variable outcomes in response to treatment depending on a number of prognostic factors. The molecular and biochemical complexity of AML cells creates tremendous treatment challenges.

The PIM kinases (*PIM-1*, *PIM-2*, *PIM-3*) are a small family of proto-oncogenes that have essential roles in the regulation of signal transduction cascades that promote cell survival, proliferation, and drug resistance (see section 1.9) (550, 579, 699, 700). They are frequently over-expressed in a wide array of malignancies (569, 621, 701, 702). A separate investigation demonstrated that PIM-1 directly phosphorylates the ABC transporter, BCRP/ABCG2, which promotes its multimerization and consequently confers drug resistance in human prostate cancer cells (558).

As discussed in section 1.1.4, FLT3-ITD is present in over 30% of cases of AML and is associated with poor outcome (703). Interestingly, FLT3 has also been shown to be a potent inducer of PIM-1 expression (606). PIM-1 can also directly phosphorylate FLT3 in a positive feedback loop, creating a feedback cycle that maintains high levels of PIM-1 expression (704). Considering that constitutive FLT3 activity is exhibited by more than 30% of patients with AML and is associated with resistance to chemotherapy, its downstream effects on PIM-1 may promote the phenotype of excessive proliferation and hyperactive anti-apoptotic pathways that have been linked to FLT3 signalling in cancer. Their fundamental roles in the regulation of processes that accelerate malignant pathogenesis and their prevalent overexpression in cancer make the PIM kinases attractive targets for therapeutic inhibition. A number of specific inhibitors of PIM kinase activity are currently in various stages of development and are discussed in section 1.9.7.

Although there is sufficient data in the literature establishing key roles for PIM kinases in multiple aspects of oncogenesis and cancer

progression, their potential regulation of cellular sensitivity to standard agents utilized in AML therapy remains to be elucidated. As discussed in section 1.9, SGI-1776 is novel small molecule inhibitor of PIM kinase activity that has demonstrated preclinical activity in cancer models and has entered phase 1 clinical trials (559, 622). Considering the high basal expression of PIM kinases in AML cells and the functions of PIM signalling in the control of cell survival and proliferation, we hypothesized that SGI-1776 would possess significant anti-leukaemic activity in AML models (606).

Project Hypothesis

Targeting PIM kinases with SGI-1776 will lead to apoptosis and growth inhibition in preclinical models of AML and will potentiate the anti-leukaemic activity of ara-C.

Project Aims

I. To investigate the the efficacy and mechanism of action of the PIM kinase inhibitor SGI-1776 in preclinical models of AML.
II. To evaluate the relationship between PIM kinase expression and cellular sensitivity to ara-C
III. To investigate the therapeutic potential of PIM kinase inhibition as a novel strategy to increase the efficacy of ara-C therapy in preclinical models of AML.

4.2 Project Summary

Here we report that the novel small molecule PIM kinase inhibitor SGI-1776 disrupted AML cell viability, impaired clonogenic survival, and induced apoptosis. Subsequent experiments established a link between ara-C resistance and PIM overexpression. Targeting PIM with SGI-1776 sensitized resistant cells to ara-C and significantly increased the efficacy of ara-C therapy in an AML mouse xenograft model. Immunohistochemical analyses of tumour specimens revealed that SGI-1776 diminished BAD

phosphorylation and cooperated with ara-C *in vivo* to promote activation of caspase-3 and inhibit tumour cell proliferation. Collectively, our data demonstrate that antagonizing PIM activity represents a new strategy to increase the therapeutic efficacy of ara-C and possibly circumvent drug resistance.

4.3 Materials and methods

4.3.1 Cells and cell culture

HL-60, KG-1, SKM-1, SH2, NOMO-1, and OCI-AML2 cells were obtained from ATCC (Manassas, VA). MV4-11, MOLM-13, and PL-21 cells were obtained from DSMZ (Braunschweig, Germany). Paired HL-60 cells that are resistant and sensitive to ara-C (described in section 3.4.5) were kindly provided by Dr. Kapil Bhalla. All cells and cell lines were maintained as described in section 2.3.1.

4.3.2 Chemicals and reagents

Reagents were obtained from: SGI-1776 (SuperGen, Inc., Dublin, CA); ara-C (Clinical Pharmacy at the Cancer Therapy & Research Center, San Antonio, TX); anti-tubulin, anti-phospho- and total AKT, anti-phospho- and total BAD antibodies (Cell Signalling, Beverly, MA); anti-FLT3, anti-PIM-1, and anti-PIM-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PIM-2 (R&D Systems, Minneapolis, MN); and sheep anti-mouse-HRP and donkey anti-rabbit-HRP antibodies (Amersham, Buckinghamshire, UK)**.**

4.3.3 Cell Viability Assay

Cell viability was assessed by the MTT assay as described in section 2.3.5***.***

4.3.4 Analysis of cell cycle effects and apoptosis

Please refer to section 2.3.4.

4.3.5 Detection of caspase-3 activation

Please refer to section 3.3.5.

4.3.6 Colony assays

AML cells were treated for 24 hours with the indicated concentrations of SGI-1776, ara-C or the combination and colony assays were performed as described in section 2.3.7.

4.3.7 Immunoblotting

AML cells were incubated with SGI-1776, ara-C or the combination of both drugs for 24 hours after which the cells were collected by centrifugation and washed in PBS. For protein lysate preparation and Western blot method please refer to 2.3.8.

4.3.8 qRT-PCR

Total RNA was isolated, cDNA was synthesised and *PIM-1*, *PIM-2*, and *PIM-3* transcripts were amplified as described in section 3.3.2.

4.3.9 *In vivo* evaluation of SGI-1776 and ara-C

MOLM-13 human leukaemia cells were harvested, washed in PBS, and suspended in a 50:50 mixture of HBSS and Matrigel (BD Biosciences, San Jose, CA). An *in vivo* model of AML was generated by injecting 10^7 MOLM-13 cells into the flanks of female nude mice (BALB/c background) from Harlan (Indianapolis, IN). After tumour growth reached 150 mm^3, mice were randomly assigned to receive vehicle, SGI-1776 (100 mg/kg orally administered 5 days for 21 days), ara-C (75 mg/kg IP injection 3 days per week for 21 days), or both drugs for 21 days. Mice were monitored daily and tumour volumes were measured twice weekly. Tumour growth and animal toxicity was assessed as described in 2.3.9 (520). At the completion of the study, tumours were excised, formalin-fixed and paraffin-embedded for immunohistochemical analyses.

4.3.10 Immunohistochemistry

Please refer to section 3.3.14.

4.3.11 TUNEL assay

Please refer to section 2.3.11.

4.3.12 shRNA interference

FLT3 and control shRNA lentiviral particles were introduced into MV4-11 cells according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Stable clones were selected following puromycin treatment.

4.4 Results

4.4.1 SGI-1776 impairs growth and induces apoptosis in AML cell lines

In order to assess the potential therapeutic benefit achieved by inhibiting PIM kinase activity in AML cells, we first investigated the *in vitro* efficacy of SGI-1776 in a panel of 9 human AML cell lines (MV4-11, MOLM-13, HL-60, KG-1, SH2, PL-21, NOMO-1, OCI-AML2, and SKM-1). Inhibition of PIM activity with SGI-1776 led to a dose-dependent reduction in cell viability in all cell lines tested as determined by MTT assay (Figure 4.1). Interestingly, cell lines expressing the FLT3-ITD (MV4-11 and MOLM-13) exhibited a greater level of sensitivity (lower IC_{50}s) than other AML cell lines in the panel that are FLT3-ITD negative. The reduction in cell viability that we observed in response to treatment with SGI-1776 could be caused by a reduction in cell proliferation, cell death, or both. In order to better understand the effects of SGI-1776 on AML cells, we next conducted Methocult colony formation assays to quantify the impact of acute exposure to SGI-1776 on clonogenic survival. Three AML cell lines (MOLM-13, MV4-11, and HL-60) were treated for 24 hours with 100 nM, 300 nM, or 1000 nM SGI-1776. The drug was washed away and cells were seeded in Methocult and incubated for 14 days. Quantification of the

number of colonies present under each experimental condition revealed that SGI-1776 significantly impaired the ability of AML cells to form colonies (Figure 4.2). To determine whether drug-induced apoptosis contributes to the mechanism of action of SGI-1776, we treated MOLM-13, MV4-11, and HL-60 cells with a range of concentrations of SGI-1776 for 48 hours and assessed the effects of drug treatment on cell cycle distribution and percentages of cells with sub G_0/G_1 (apoptotic) DNA content by PI/FACS analysis. Our results showed that SGI-1776 disrupted cell cycle kinetics in a manner that was characterized by the accumulation of cells with G_1 DNA content (Figure 4.3, 4.4 and 4.5). Quantification of the cells with sub G_0/G_1 fragmented DNA for each experimental condition showed that SGI-1776 caused a dose-dependent increase in apoptotic cells (Figure 4.6). In order to confirm the pro-apoptotic effects of SGI-1776, we tested the ability of SGI-1776 to activate the effecter caspase-3 using a flow cytometric method. These experiments demonstrated that SGI-1776 treatment significantly increased the percentages of cells expressing active caspase-3 (Figures 4.7 and 4.8).

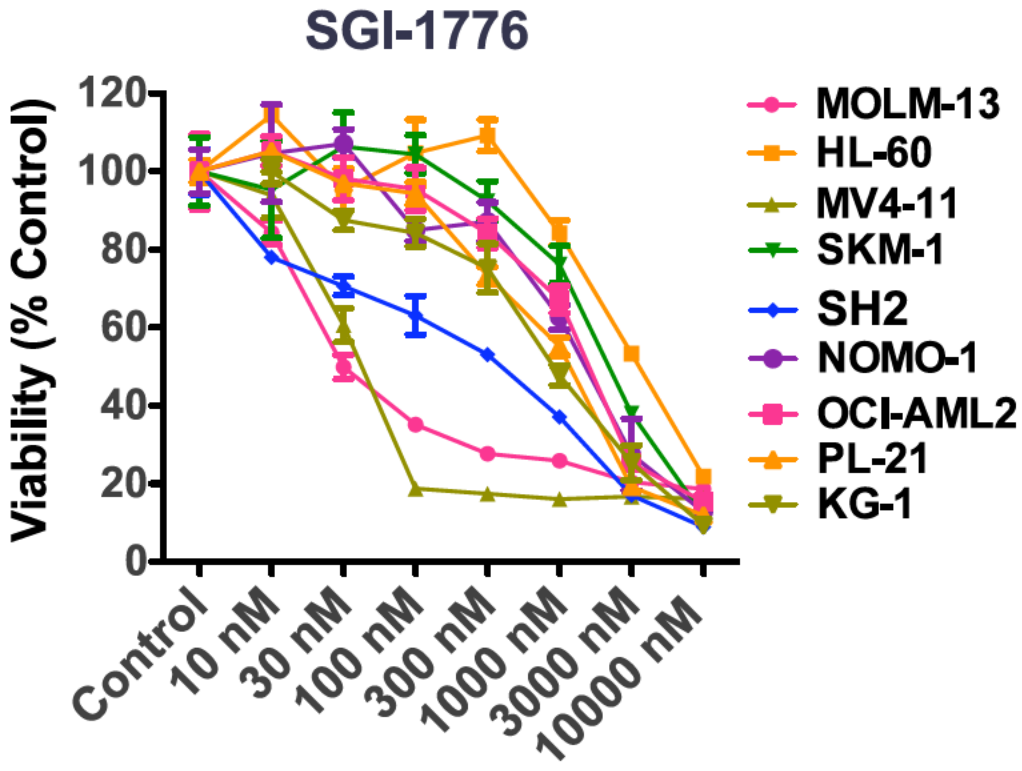

Figure 4.1 SGI-1776 causes a dose-dependent reduction in AML cell viability

9 human AML cell lines were treated with the indicated concentrations of SGI-1776 for 72 hours. Cell viability was determined by MTT assay. n = 3, mean ± SD.

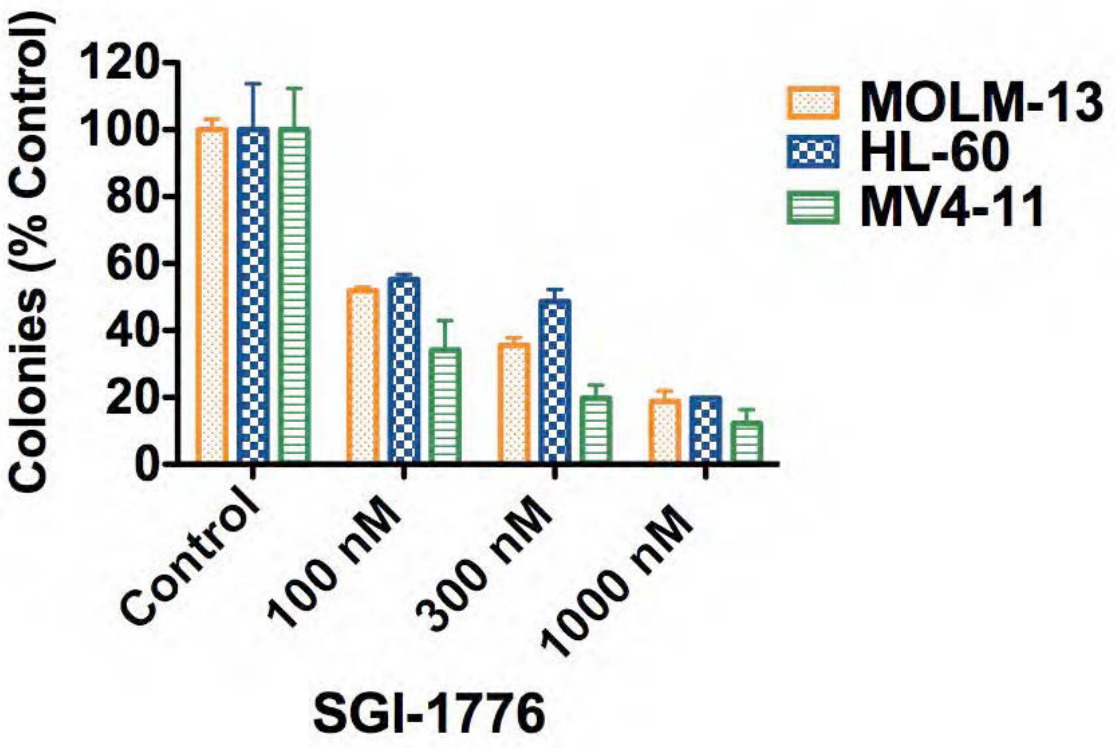

Figure 4.2 SGI-1776 diminishes clonogenic survival in a dose dependent manner

Human AML cell lines (MOLM-13, HL-60, and MV4-11) were treated with the indicated concentrations of SGI-1776 for 24 hours. Drug was washed away and cells were plated in MethoCult methylcellulose-containing medium. Colonies were scored 14 days later with the assistance of an Alpha Innotech imaging system. n = 3, mean ± SD.

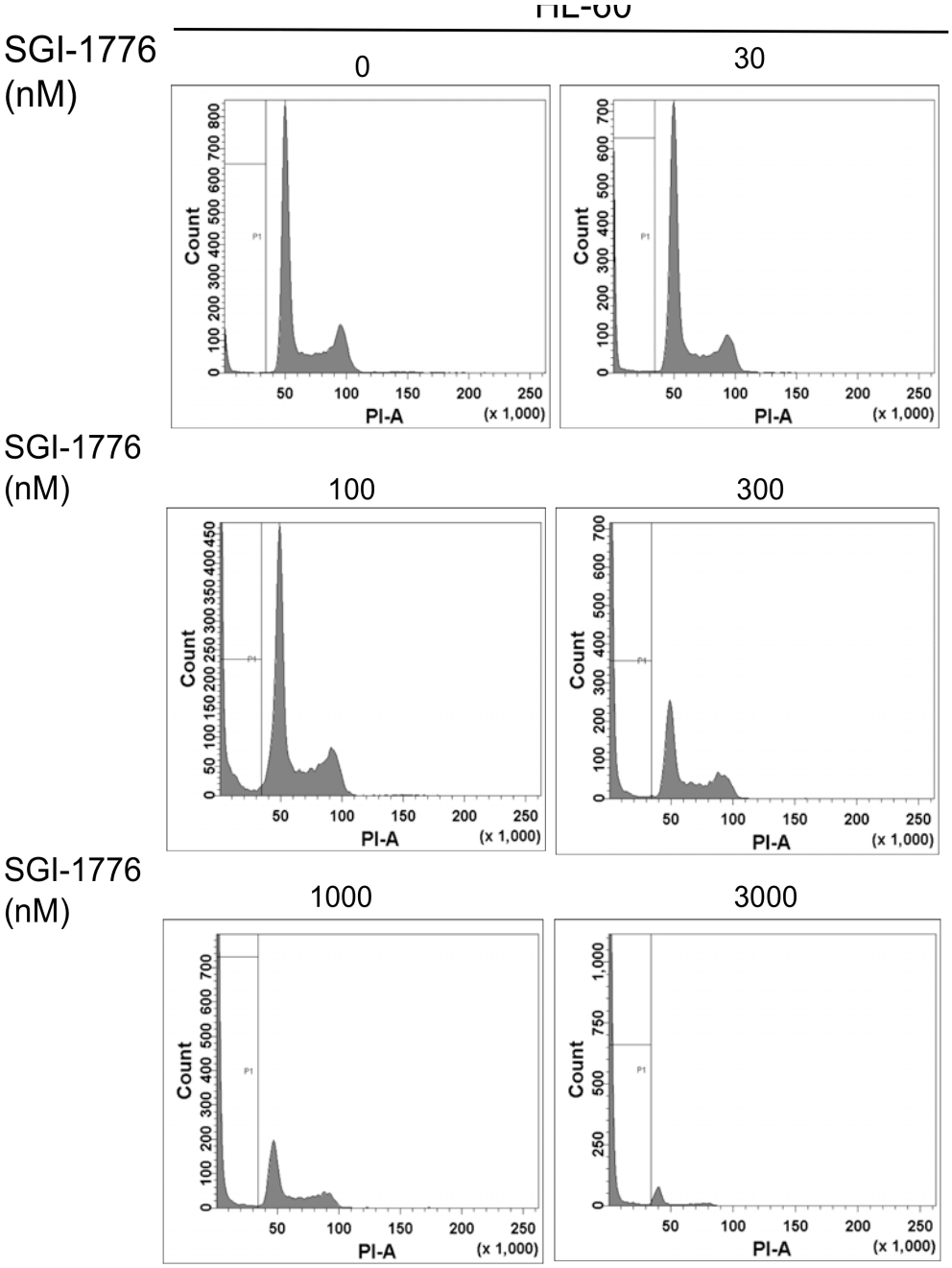

Figure 4.3 SGI-1776 disrupts the cell cycle kinetics of HL-60 cells

HL-60 Cells were treated with the indicated concentrations of SGI-1776 for 48 hours. Cell cycle distribution was assessed by PI/FACS analysis. Representative histograms are shown.

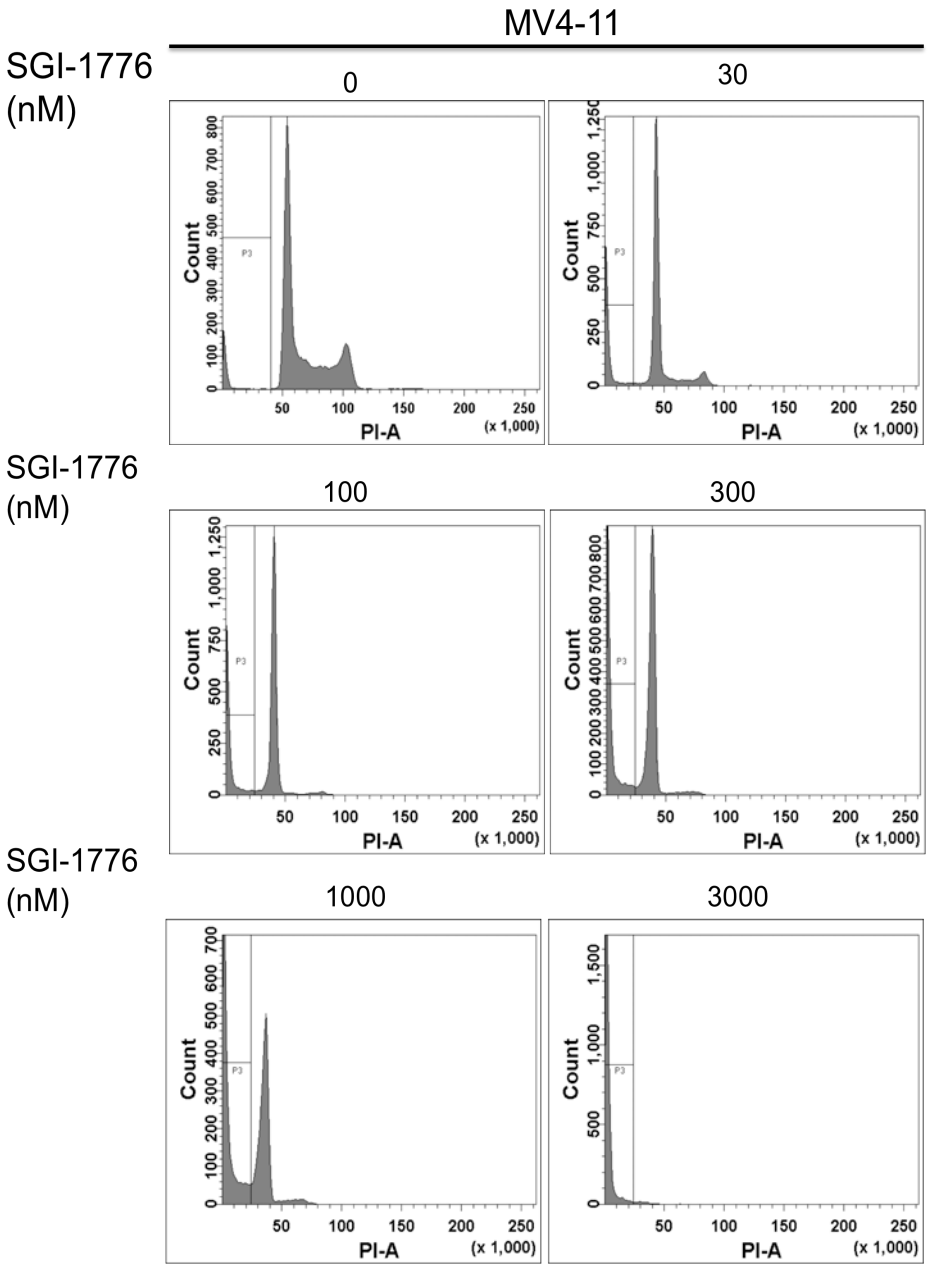

Figure 4.4 SGI-1776 disrupts the cell cycle kinetics of MV4-11 cells

MV4-11 Cells were treated with the indicated concentrations of SGI-1776 for 48 hours. Cell cycle distribution was assessed by PI/FACS analysis. Representative histograms are shown.

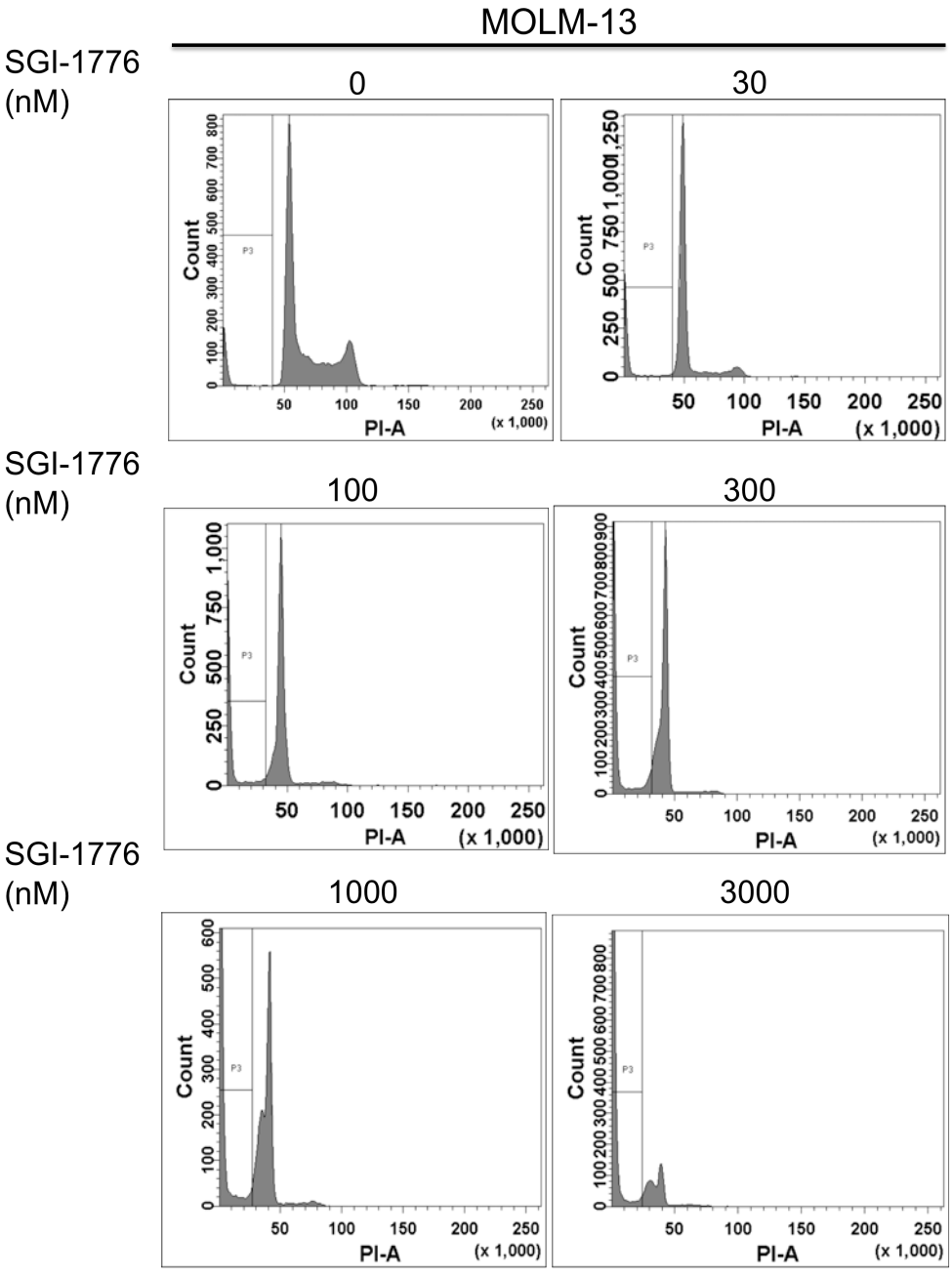

Figure 4.5 SGI-1776 disrupts the cell cycle kinetics of MOLM-13 cells

MOLM-13 cells were treated with the indicated concentrations of SGI-1776 for 48 hours. Cell cycle distribution was assessed by PI/FACS analysis. Representative histograms are shown.

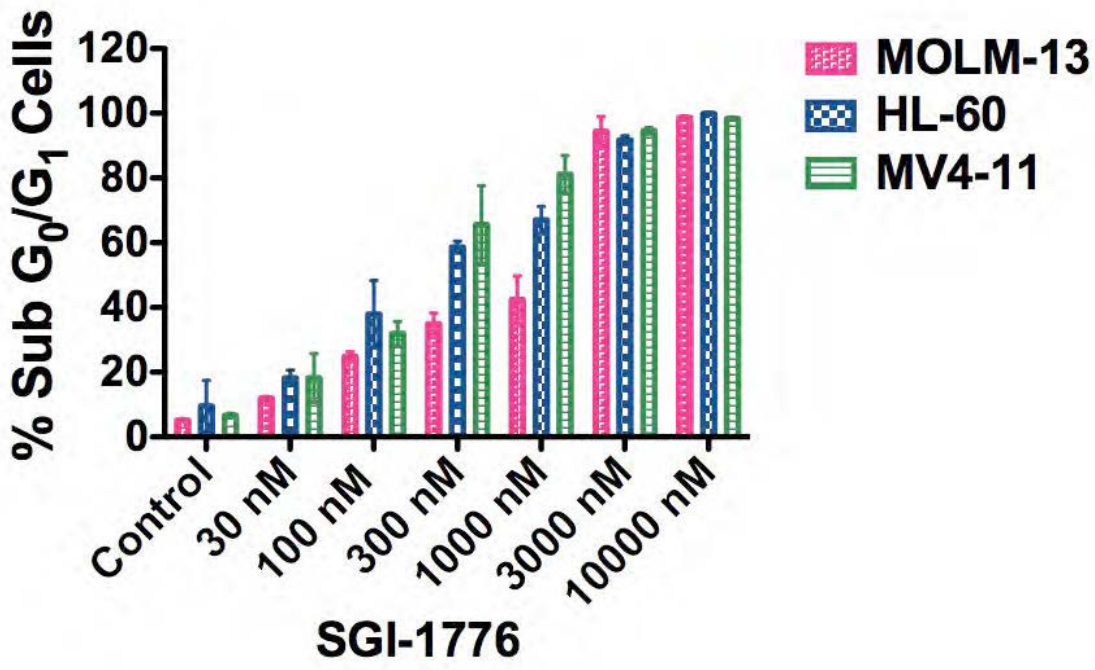

Figure 4.6 SGI-1776 induces dose dependent apoptosis in AML cells
Cells were treated with the indicated concentrations of SGI-1776 for 48 hours. Apoptosis was quantified by PI/FACS analysis of cells with fragmented (Sub G_0/G_1) DNA content. n = 3, mean ± SD.

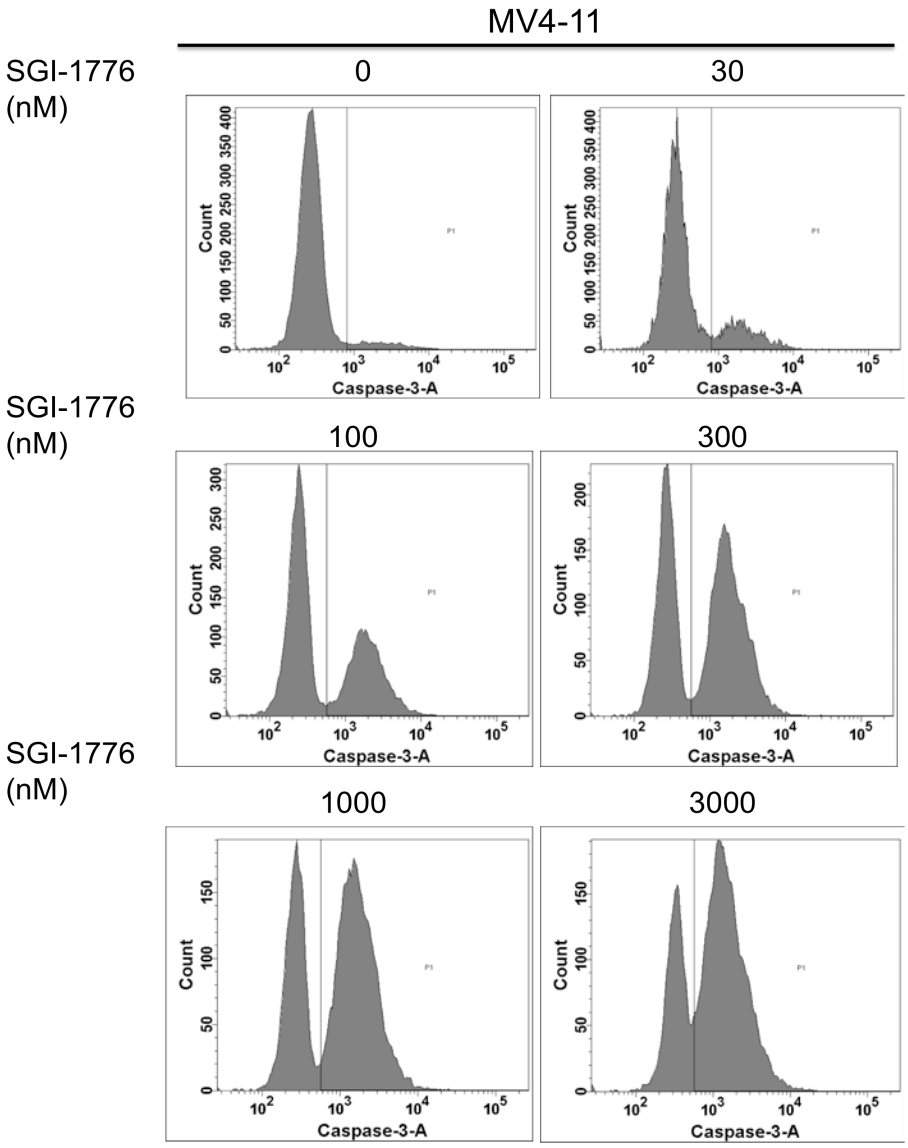

Figure 4.7 SGI-1776 induces caspase-3 activation

AML cells were treated with the indicated concentrations of SGI-1776 for 48 hours. The effects of SGI-1776 on caspase-3 activation were determined using the BD Biosciences Active Caspase-3 Mab Apoptosis kit followed by flow cytometry. Representative histograms of MV4-11 treated with SGI-1776 are shown. n = 3, mean ± SD.

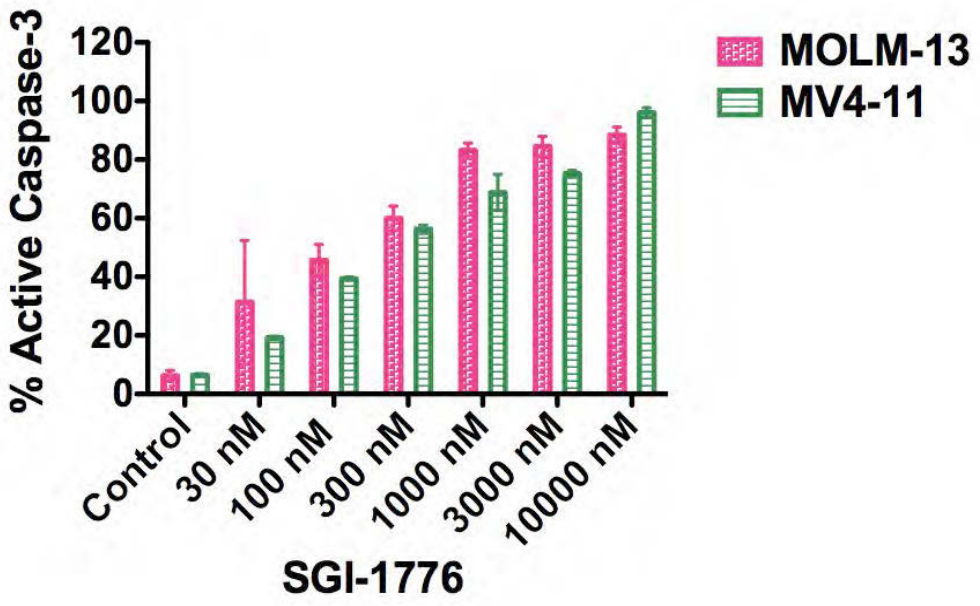

Figure 4.8 Quantification of the effects of SGI-1776 on caspase-3 activation

Cells were exposed to SGI-1776 for 48 hours. The percentages of cells expressing the active form of caspase-3 were determined using the BD Biosciences Active Caspase-3 Mab Apoptosis kit followed by flow cytometry. n=3 Mean ± SD.

4.4.2 SGI-1776 abrogates phosphorylation of the BH3-only protein BAD

As discussed in section 1.9.4.1, earlier studies demonstrated that one of the mechanisms by which PIM kinases function to reduce the apoptotic potential of malignant cells is through the direct phosphorylation of the BH3-only apoptotic regulator BAD on its Ser^{112} residue. This inhibitory phosphorylation event neutralizes the pro-apoptotic properties of BAD and has been linked to drug resistance in cancer cells. In order to investigate whether disruption of PIM kinase activity with SGI-1776 impacted the levels of BAD phosphorylation, we treated MV4-11 AML cells, which express high basal levels of phospho-BAD with a range of concentrations of SGI-1776 and assessed the levels of total and phospho-BAD-Ser^{112} by immunoblotting. Our results showed that exposure to SGI-1776 led to a significant reduction in the phosphorylation of BAD on its Ser^{112} residue (Figure 4.9). Considering that AKT has also been shown to phosphorylate BAD on its Ser^{112} residue, we also assessed the impact of SGI-1776 treatment on the levels of total and phospho-AKT (Thr^{308}) as a negative control. Notably, this drug-related reduction in BAD phosphorylation did not appear to be due to alterations in AKT activity as SGI-1776 treatment did not significantly affect the phosphorylation of AKT (Thr^{308}) in MV4-11 cells, which have constitutive AKT activity (Figure 4.9).

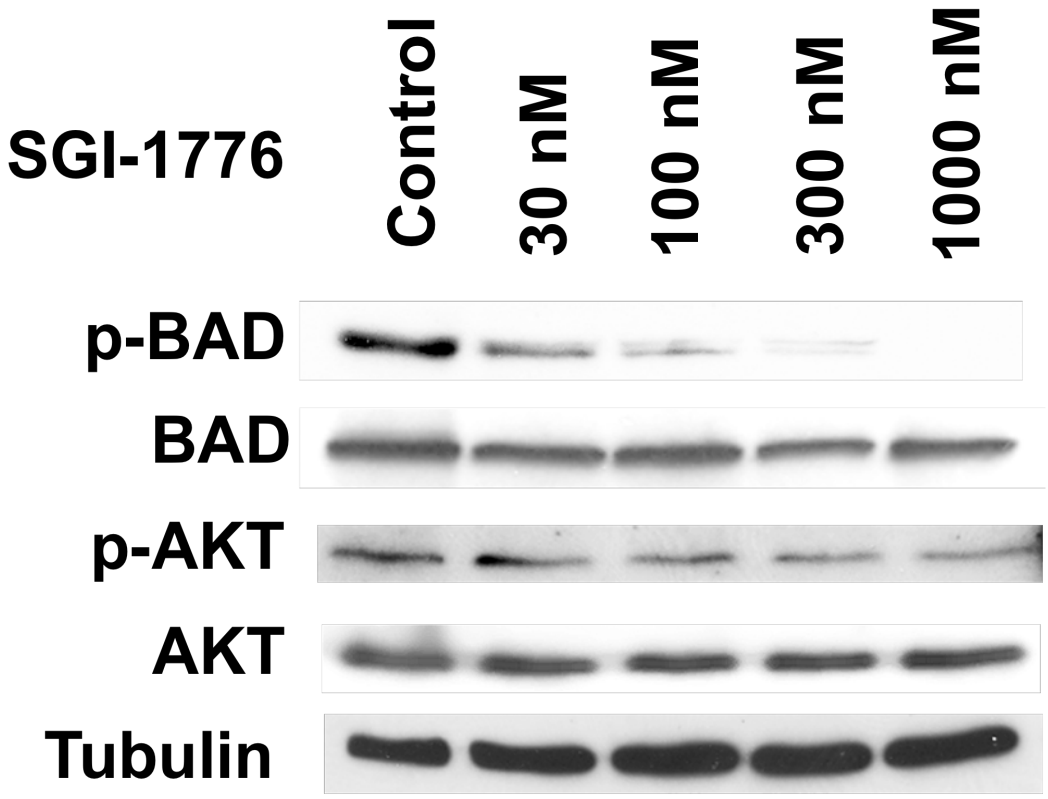

Figure 4.9 SGI-1776 abrogates phosphorylation of the BH3-only protein BAD (Ser^{112})

MV4-11 cells were treated with SGI-1776 for 24 hours. Protein lysates were subjected to SDS-PAGE analysis and the impact of drug treatment on the relative expression levels of phosphorylated BAD (Ser^{11}), total BAD, phosphorylated AKT (Thr^{308}), and total AKT were evaluated by immunoblotting. Tubulin served as a loading control.

4.4.3 Inhibition of FLT3 contributes to the efficacy of SGI-1776 in AML

Comprehensive *in vitro* kinase inhibition profiling in an earlier investigation of the preclinical efficacy of SGI-1776 revealed that in addition to its effects on the activity of PIM kinases, SGI-1776 displayed off-target effects on the activity of FLT3 (622). These off-target effects on FLT3 activity are most likely due to the structural similarities between the ATP binding sites of the PIM kinases and FLT3. As discussed earlier, approximately 30% of patients with AML have constitutive FLT3 activity due to internal tandem duplication (ITD) or activating mutations of the tyrosine kinase domain. Therefore we next attempted to determine whether FLT3 inhibition represents a significant component of the mechanism of action of SGI-1776. We utilized lentiviral shRNA to stably knockdown FLT3 expression in the FLT3-ITD+ MV4-11 cells. Knockdown efficiency was assessed by immunoblotting (Figure 4.10). Cells expressing control and FLT3-targeted shRNA were exposed to the indicated concentrations of SGI-1776 for 72 hours and the impact of differential FLT3 expression on cell viability was determined by MTT assay. Our results revealed that FLT3 knockdown caused a modest reduction in sensitivity to SGI-1776 (Figure 4.10). This suggests that although FLT3 inhibition is not the primary mechanism of action in AML of SGI-1776, this effect does contribute to its efficacy and suggests that AML patients with FLT3 activation may potentially derive a greater therapeutic benefit from this agent over patients with unmutated FLT3.

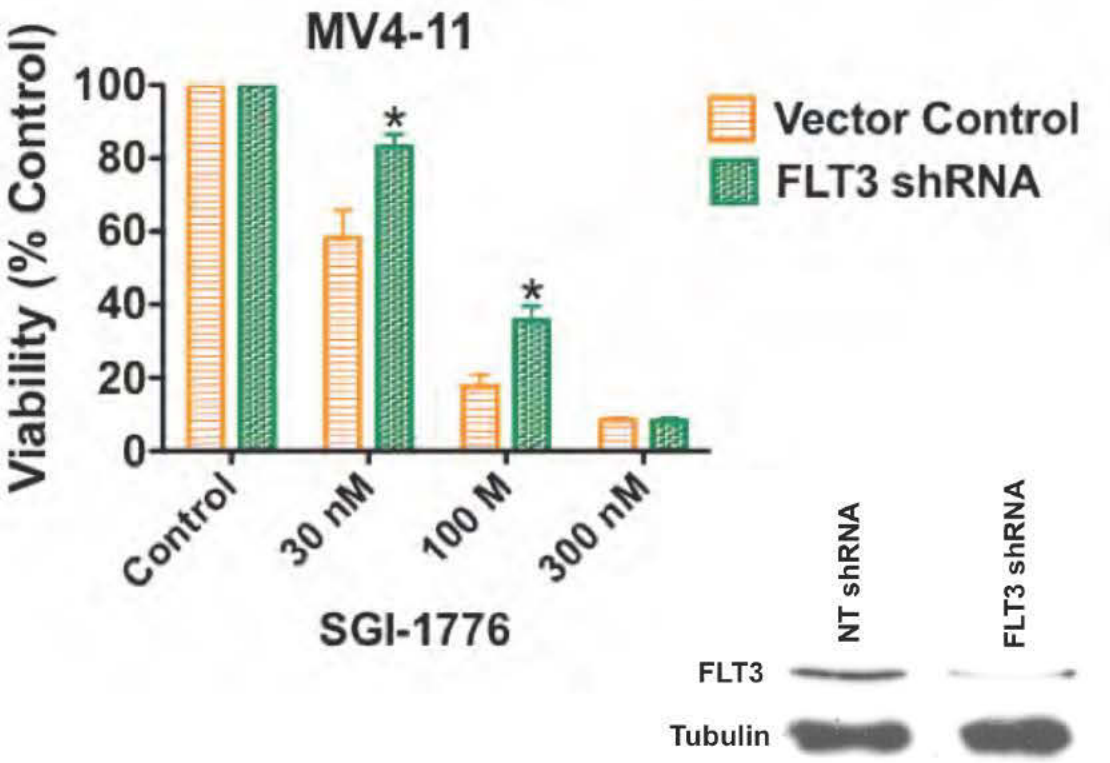

Figure 4.10 Inhibition of FLT3 contributes to the efficacy of SGI-1776 in AML

Left. MV4-11 cells with and without stable shRNA-mediated FLT3 knockdown were treated with the indicated doses of SGI-1776 for 72 hours and the consequential impact on cell viability was determined by MTT assay. n = 3, mean ± SD, *p < 0.05. **Right.** Lentiviral shRNA was used to stably knockdown FLT3 expression in the FLT3-ITD+ MV4-11 cells. Knockdown efficiency was assessed by immunoblotting. Tubulin was used as a loading control.

4.4.4 Treatment of AML cells with ara-C induces PIM-1 and PIM-3 expression

Although PIM kinases have been shown to contribute to oncogenesis and disease progression through a number of different mechanisms, one of the most interesting potential roles of the PIM kinases within the context of cancer biology is its potential ability to promote drug resistance. Several recent studies have suggested a mechanistic link between aberrant expression of PIM kinases and reduced sensitivity to certain anti-cancer agents (705, 706). We were interested in further investigating this potential aspect of PIM function in a manner relevant to AML therapy. We first evaluated the basal expression levels of PIM-1, PIM-2, and PIM-3 in paired HL-60 cells that are sensitive and resistant to ara-C by immunoblotting and qRT-PCR. Interestingly, our results showed that the levels of PIM-1 and PIM-3, but not PIM-2, were significantly higher in ara-C-resistant HL-60 cells (Figures 4.11 and 4.12). Given that the acquired resistance of these HL-60 cells was achieved by chronic exposure to ara-C, our results suggested that perhaps treatment with ara-C alone is sufficient to trigger increased expression of PIM kinases. In order to test this possibility, we treated MOLM-13 AML cells with a range of concentrations of ara-C for 24 hours and then assessed the expression levels of PIM-1, PIM-2, and PIM-3 by immunoblottting. Consistent with our hypothesis based on our initial findings regarding PIM expression in ara-C resistant HL-60 cells, acute treatment with ara-C led to a significant increase in the levels of PIM-1 and PIM-3 (Figure 4.13) We confirmed these effects by treating MOLM-13 cells with ara-C and utilizing qRT-PCR to assess PIM kinase expression (Figure 4.14).

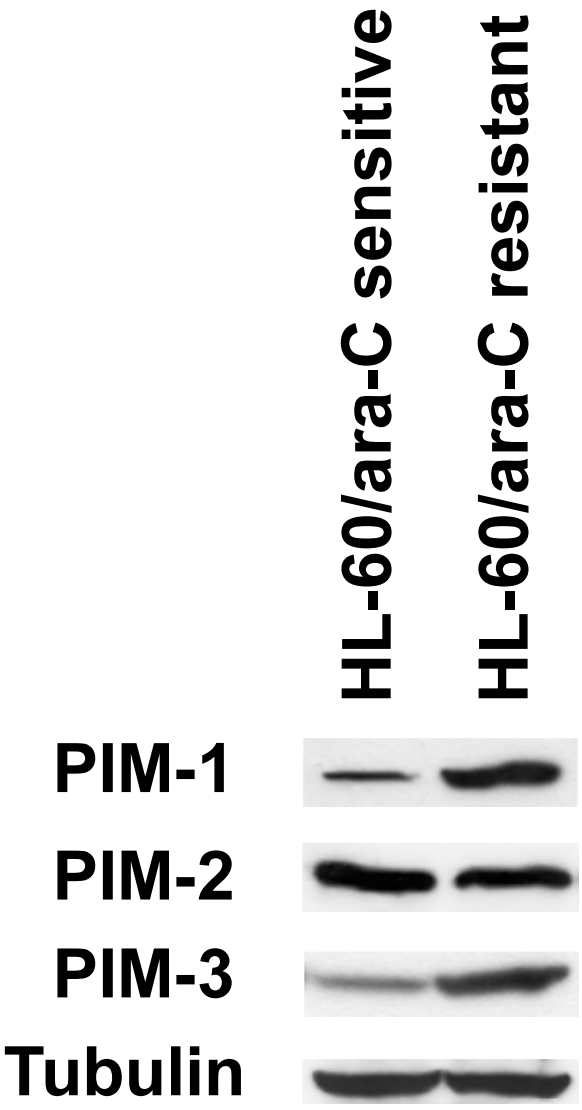

Figure 4.11 Ara-C resistance is linked to overexpression of PIM-1 and PIM-3

The relative expression levels of PIM-1, -2, and -3 were evaluated in paired HL-60 cells that are sensitive and resistant to ara-C by immunoblotting. Tubulin documented equal loading.

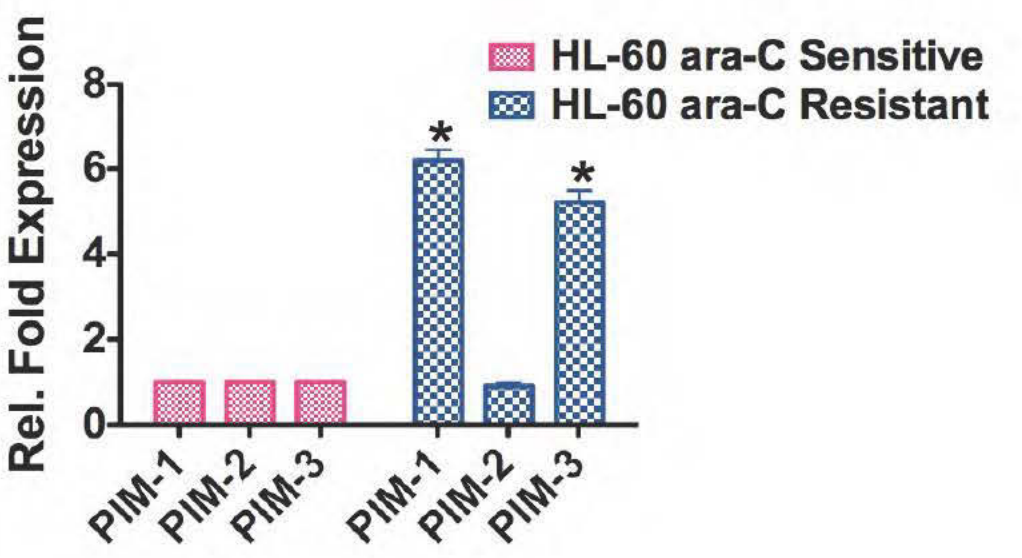

Figure 4.12 Relative expression levels of PIM kinases in HL-60 ara-C sensitive and –resistant cells

The relative expression levels of PIM-1, -2, and -3 were evaluated in paired HL-60 cells that are sensitive and resistant to ara-C by qRT-PCR. *Indicates a significant difference compared to controls, $p < 0.05$.

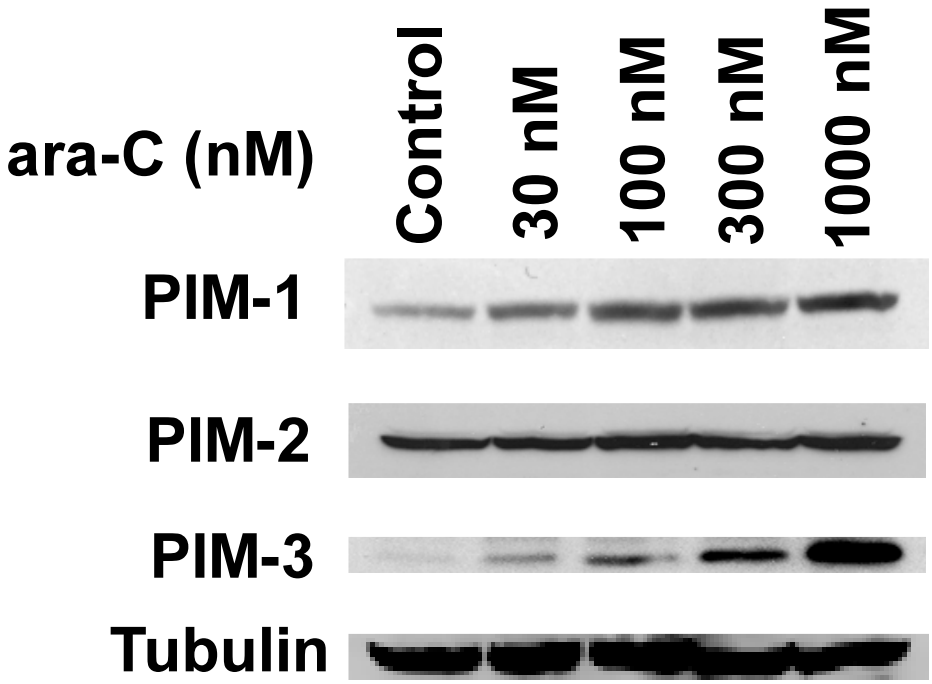

Figure 4.13 Treatment with ara-C induces PIM-1 and PIM-3 expression

Cells were treated with ara-C for 24 hours. Protein lysates were subjected to SDS-PAGE. Immunoblotting was utilized to quantify the levels of PIM-1, -2, and-3. Tubulin documented equal loading.

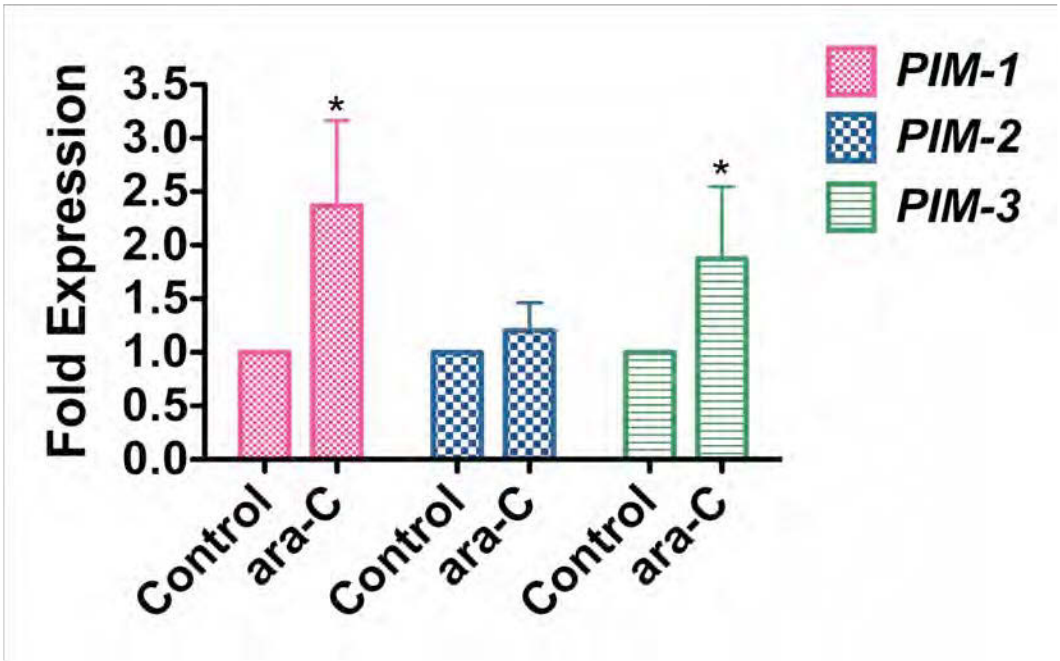

Figure 4.14 Impact of ara-C treatment on PIM expression

MOLM-13 cells were treated with ara-C for 24 hours. qRT-PCR was used to assess *PIM-1*, *-2*, and *-3* expression levels. *Indicates a significant difference compared to controls, $p < 0.05$.

4.4.5 SGI-1776 increases the *in vitro* anti-cancer activity of ara-C

Considering the link between ara-C treatment and upregulation of PIM kinase expression that we observed in AML cells and the established roles of PIM activity in malignant pathogenesis, we hypothesized that inhibition of PIM kinase activity may be a novel approach to heighten the sensitivity of AML cells to ara-C. To test this hypothesis, we treated AML cells with ara-C, SGI-1776, or the combination of ara-C and SGI-1776 for 72 hours and quantified the impact of drug treatment on cell viability by MTT assay. Our results showed that cells treated with both agents displayed significantly lower viability than cells treated with either single agent, suggesting that PIM inhibition may increase the efficacy of ara-C (Figure 4.15). Similar to what we observed in our MTT experiments, acute exposure (24 hours) to the combination of SGI-1776 and ara-c was significantly more effective than either drug alone with respect to inhibiting clonogenic survival (Figure 4.16). Since our initial experiments with SGI-1776 showed that this agent induces apoptosis and disrupts cells cycle kinetics in AML cells and ara-C is also known to have similar properties, we conducted PI/FACS analysis to assess the effects of SGI-1776, ara-C, and the combination of these agents on cell cycle distribution and drug-induced apoptosis. Our data demonstrated that SGI-1776 promoted the accumulation of cells with G_1 DNA content (Figure 4.17). The combination of SGI-1776 and ara-C abrogated the classical S-phase accumulation caused by ara-C treatment and significantly augmented the proportion of apoptotic (sub G_0/G_1) cells compared to either single agent treatment (Figure 4.18).

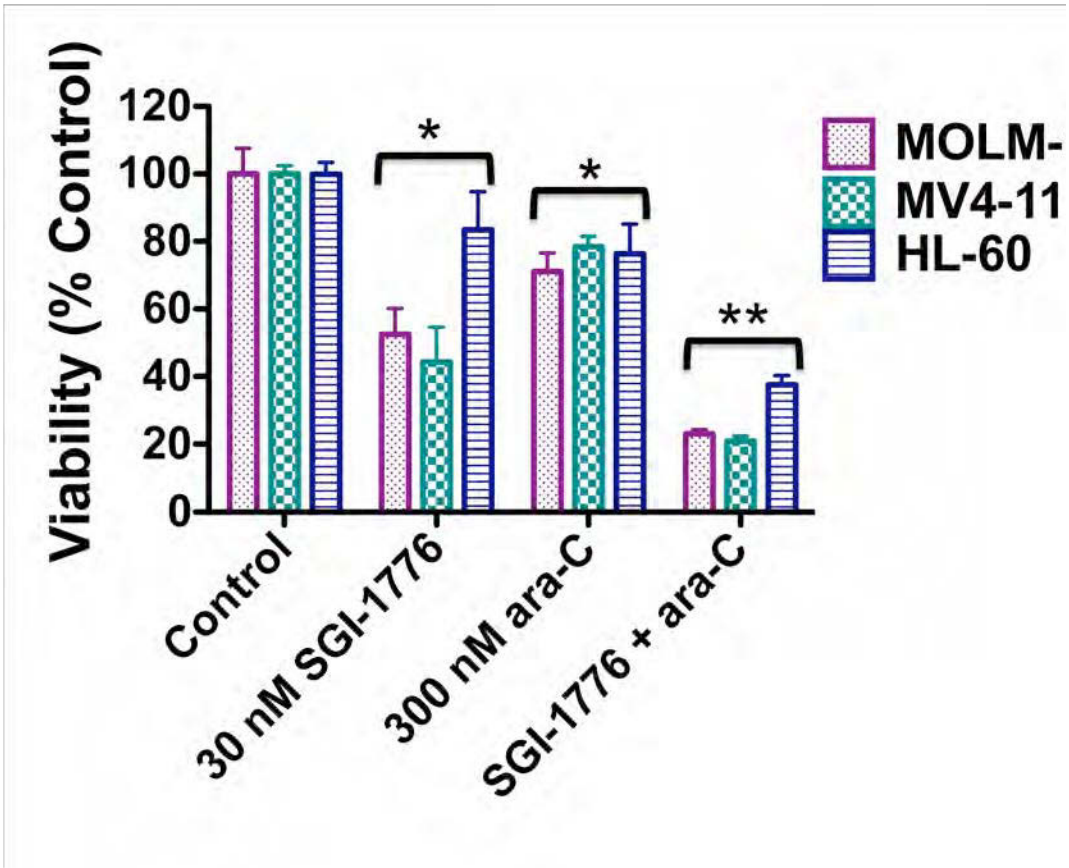

Figure 4.15 SGI-1776 significantly increases the *in vitro* anti-cancer activity of ara-C

Cells were treated with the indicated concentrations of SGI-1776, ara-C, or both for 72 hours. Cell viability was determined by MTT assay. *Indicates a significant difference compared to controls or **single agent treatment, $p < 0.05$.

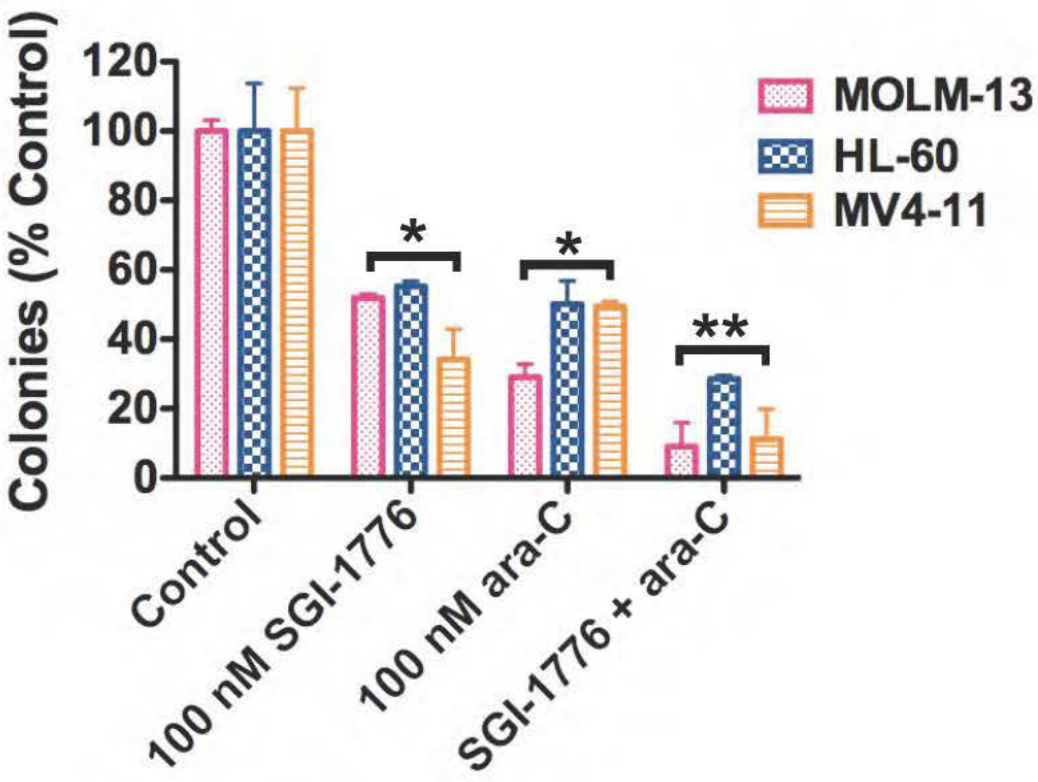

Figure 4.16 SGI-1776 and ara-C cooperate to antagonize clonogenic survival

Cells were treated with SGI-1776, ara-C, or both for 24 hours Drug-containing medium was washed away and cells were plated in Methocult. Colonies were scored 14 days later with the assistance of an Alpha Innotech imaging system. Mean ± SD, n = 3. *Indicates a significant difference compared to controls or **single agent treatment, $p < 0.05$.

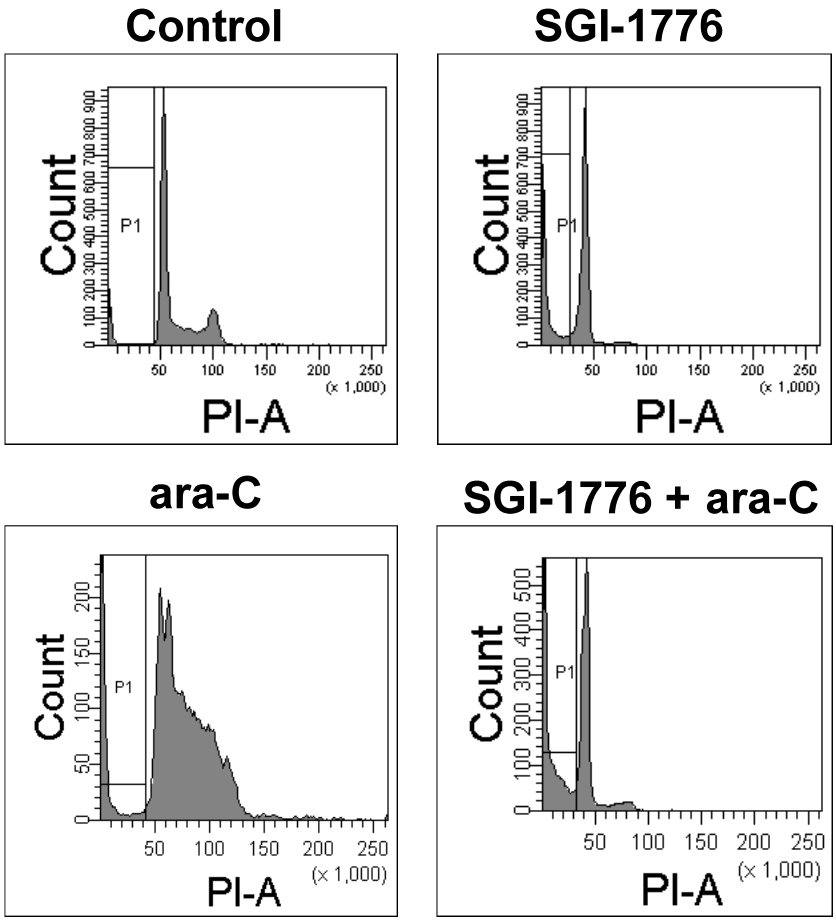

Figure 4.17 SGI-1776 and ara-C work together to disrupt cell cycle kinetics and induce apoptosis

MOLM-13 cells were treated with SGI-1776, ara-C, or the combination of both for 48 hours. PI/FACS was used to evaluate drug-related effects on cell cycle distribution and apoptosis (cells with sub G_0/G_1 DNA content, gated by marker P1). Representative histograms are shown.

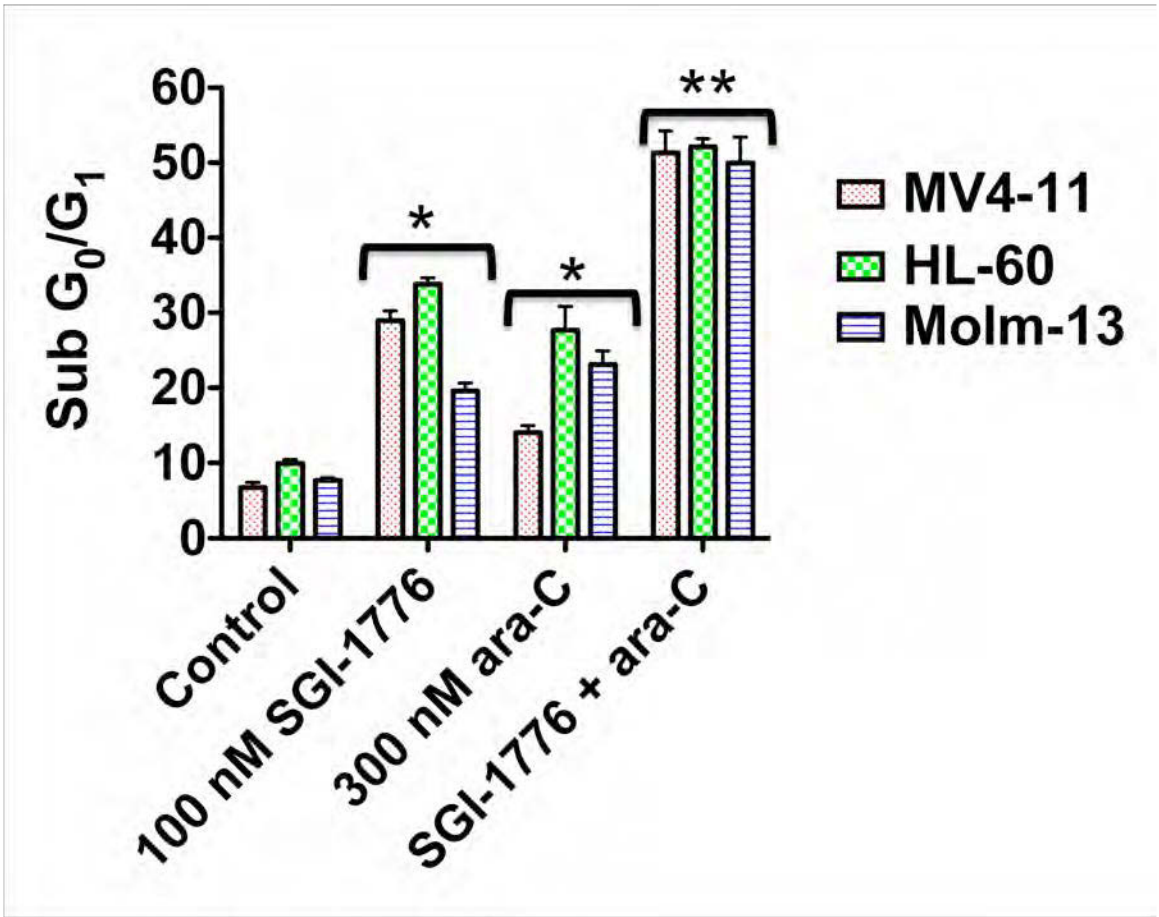

Figure 4.18 Quantification of the combined pro-apoptotic effects of ara-C and SGI-1776 in AML cells

MOLM-13 cells were treated with SGI-1776, ara-C, or the combination of both for 48 hours. PI/FACS was used to evaluate drug-related effects on cell cycle distribution and apoptosis, n = 3, mean ± SD. *Indicates a significant difference compared to controls or **single agent treatment, $p < 0.05$.

4.4.6 SGI-1776 partially restores the sensitivity of ara-C resistant cells to ara-C treatment

Given our *in vitro* findings regarding SGI-1776 and ara-C along with the link between PIM activity and drug resistance, we wondered whether targeting PIM kinase activity with SGI-1776 could be used as a strategy to overcome intrinsic ara-C resistance. In order to investigate this, we treated HL-60 ara-C sensitive and resistant cells with SGI-1776, ara-C, or both drugs for 72 hours and quantified the effects of drug exposure on cell viability by MTT assay. Our results showed that SGI-1776 partially restored the sensitivity of ara-C resistant cells to ara-C (Figure 4.19). These findings indicate that ara-C resistance is a multifaceted problem with multiple underlying mechanisms including PIM overexpression. Additionally, our data demonstrate that abrogating PIM kinase activity could possibly be utilized as a novel approach to improve the therapeutic efficacy of ara-C that may retain utility in circumstances of intrinsic ara-C resistance.

4.4.7 SGI-1776 increases the *in vivo* anti-cancer activity of ara-C

In order to further investigate the potential therapeutic benefit of PIM kinase inhibition as a new approach for the treatment of AML, we generated a xenograft mouse model of AML by injecting MOLM-13 human AML cells into the flanks of nude mice. Tumour-bearing mice were randomized into groups of 10 and were administered vehicle control, ara-C, SGI-1776, or ara-C and SGI-1776 were for 21 days. Treatment with the combination of these two agents was well tolerated and significantly increased the efficacy of single agent ara-C therapy (Figures 4.20 and 4.21). Immunohistochemical analyses of tumours from mice revealed that SGI-1776 significantly diminished BAD phosphorylation and cooperated with ara-C *in vivo* to promote activation of caspase-3 and inhibit tumour cell proliferation (PCNA expresssion) (Figures 4.22 through 4.27).

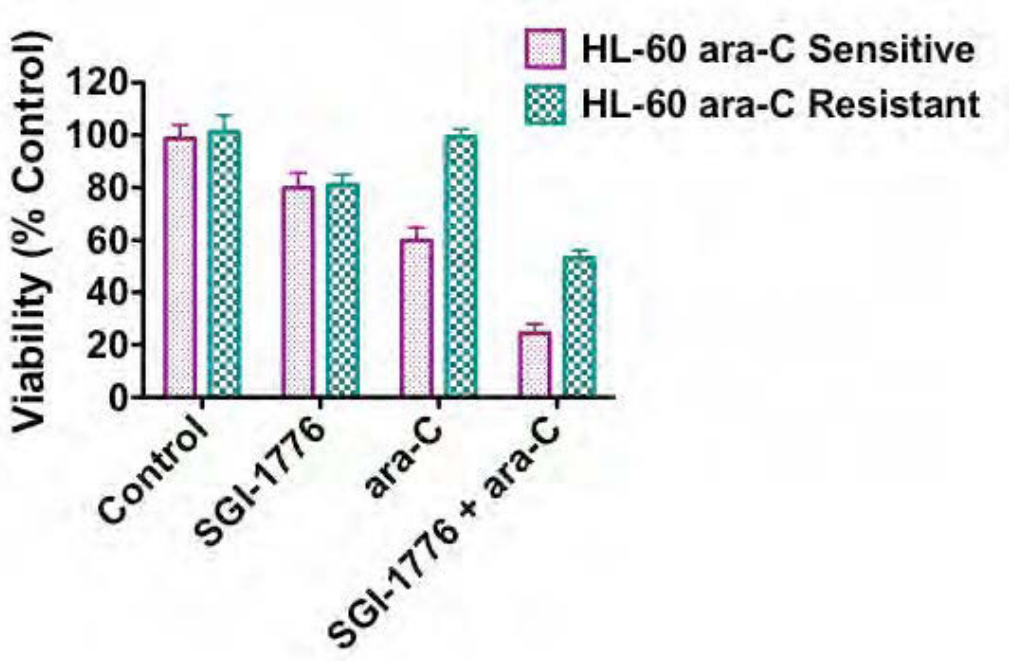

Figure 4.19 SGI-1776 partially restores the sensitivity of ara-C resistant cells to ara-C treatment

Paired HL-60 cells that are sensitive and resistant to ara-C were treated with SGI-1776, ara-C, or the combination for 72 hours. The effects of drug treatment on cell viability were quantified by MTT assay. n = 3, mean ± SD.

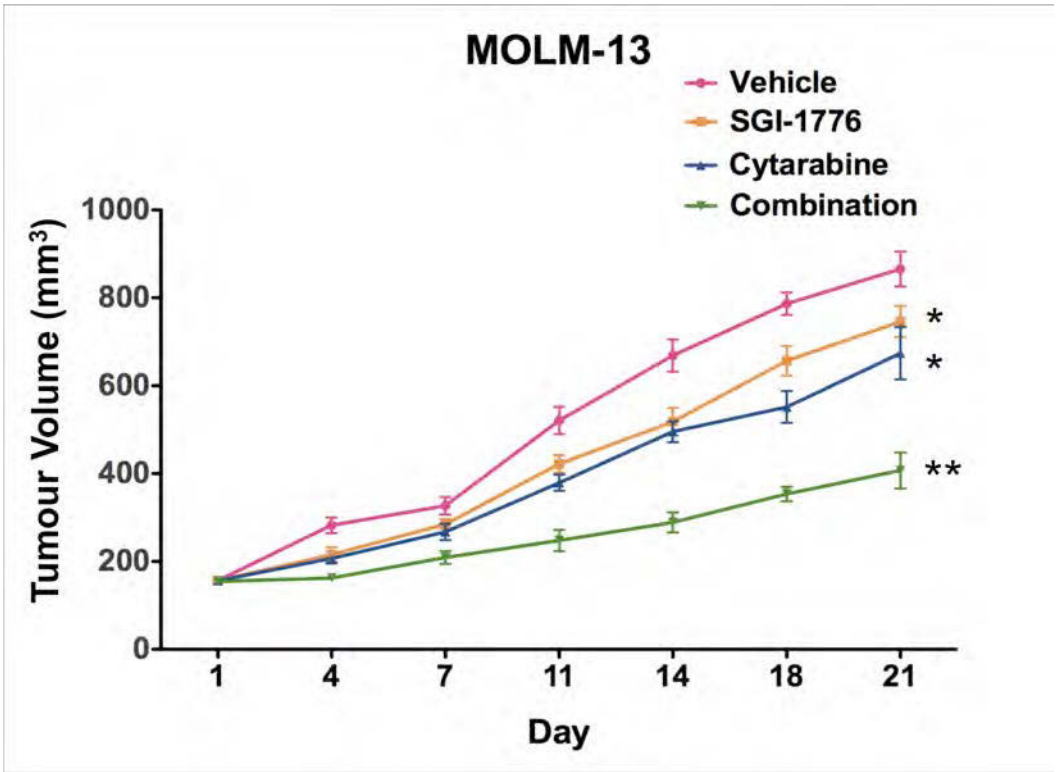

Figure 4.20 Effects of drug treatment on tumour growth

MOLM-13 AML cells were implanted subcutaneously into the flanks of nude mice. Mice with palpable tumours were randomized into groups of 10 and treated with vehicle, SGI-1776 (100 mg/kg orally administered 5 days per week for 3 weeks), ara-C (75 mg/kg IP injection 3 days per week for 3 weeks), or both drugs for 21 days. Tumour volume was monitored with calliper measurements. *Indicates a significant difference compared to controls or **single agent treatment, $p < 0.05$.

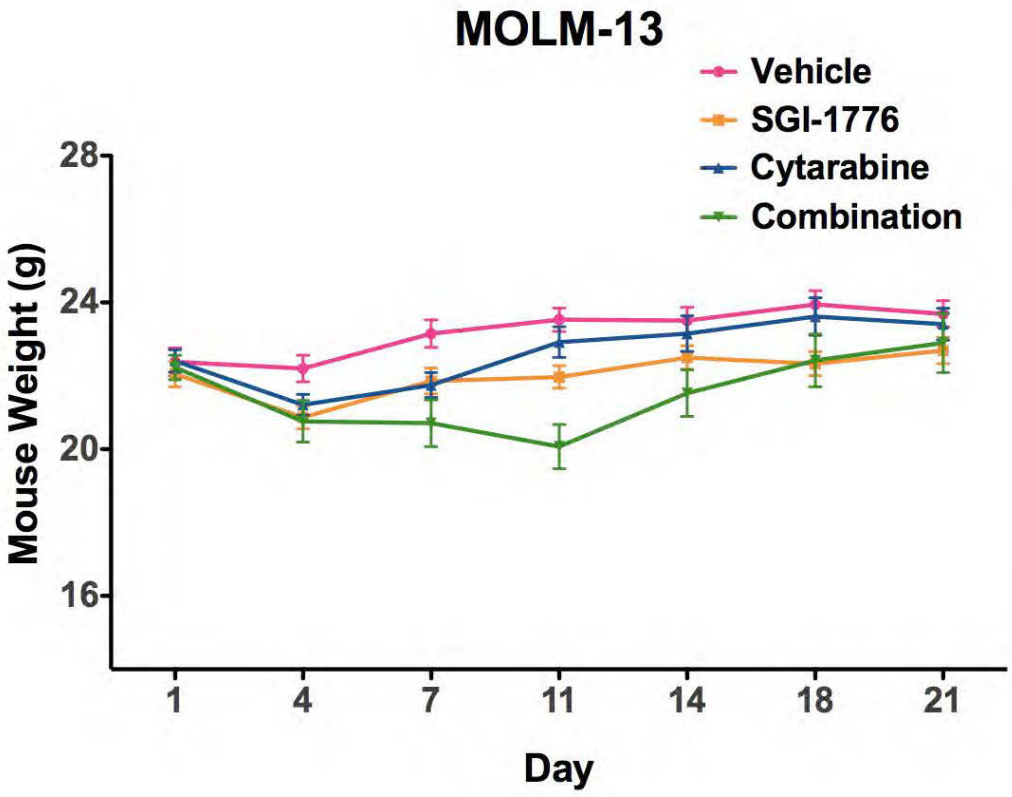

Figure 4.21 Treatment with SGI-1776 and ara-C is well tolerated

Mouse weight (g) was monitored twice weekly throughout the 21-day treatment regimen.

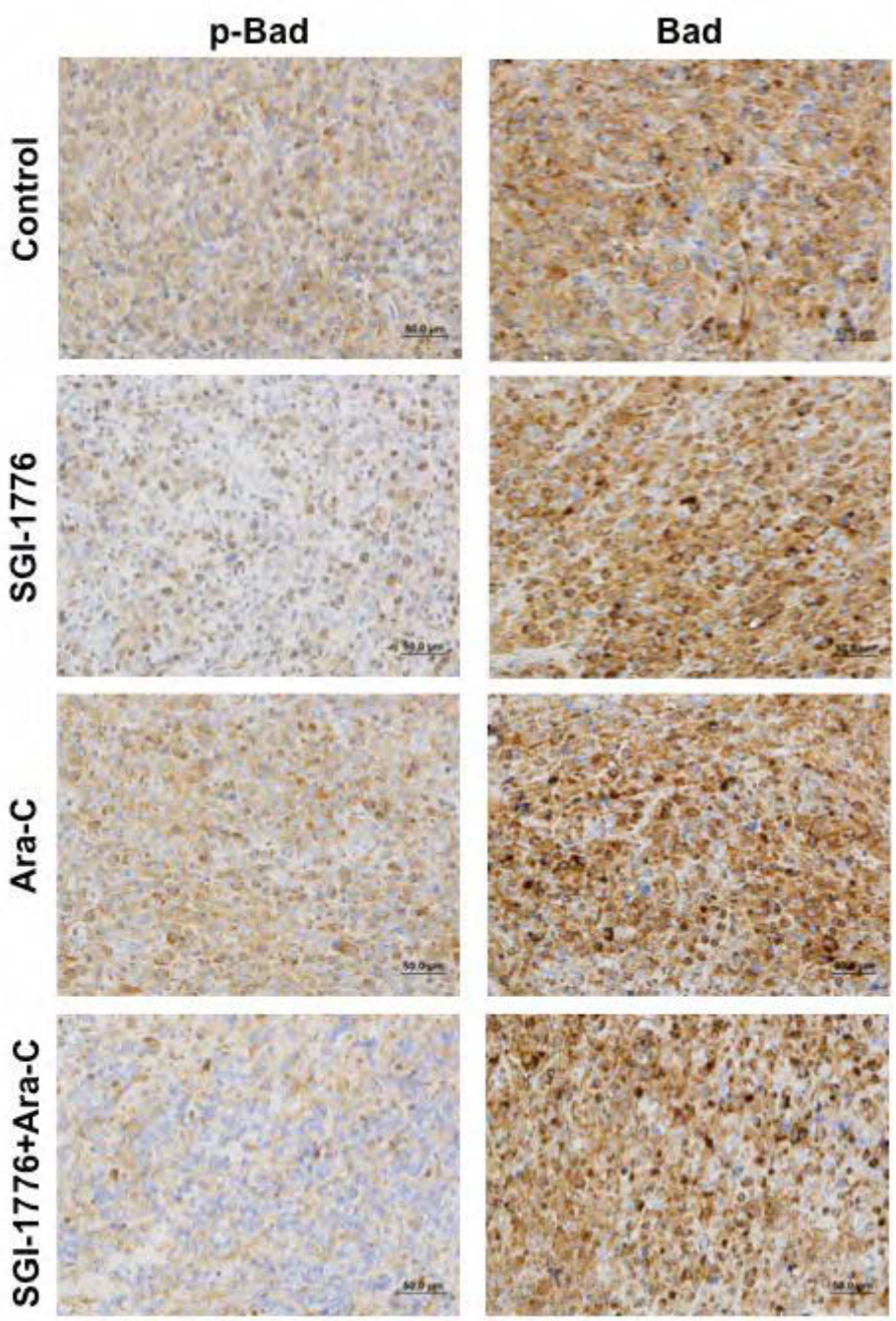

Figure 4.22 SGI-1776 and ara-C diminish BAD phosphorylation (Ser^{112}) Immunohistochemistry was utilized to assess the relative levels of phospo-BAD and total BAD in tumour sections obtained from animals in all treatment groups. Images were captured using an Olympus microscope with a DP71 camera and a 20X objective.

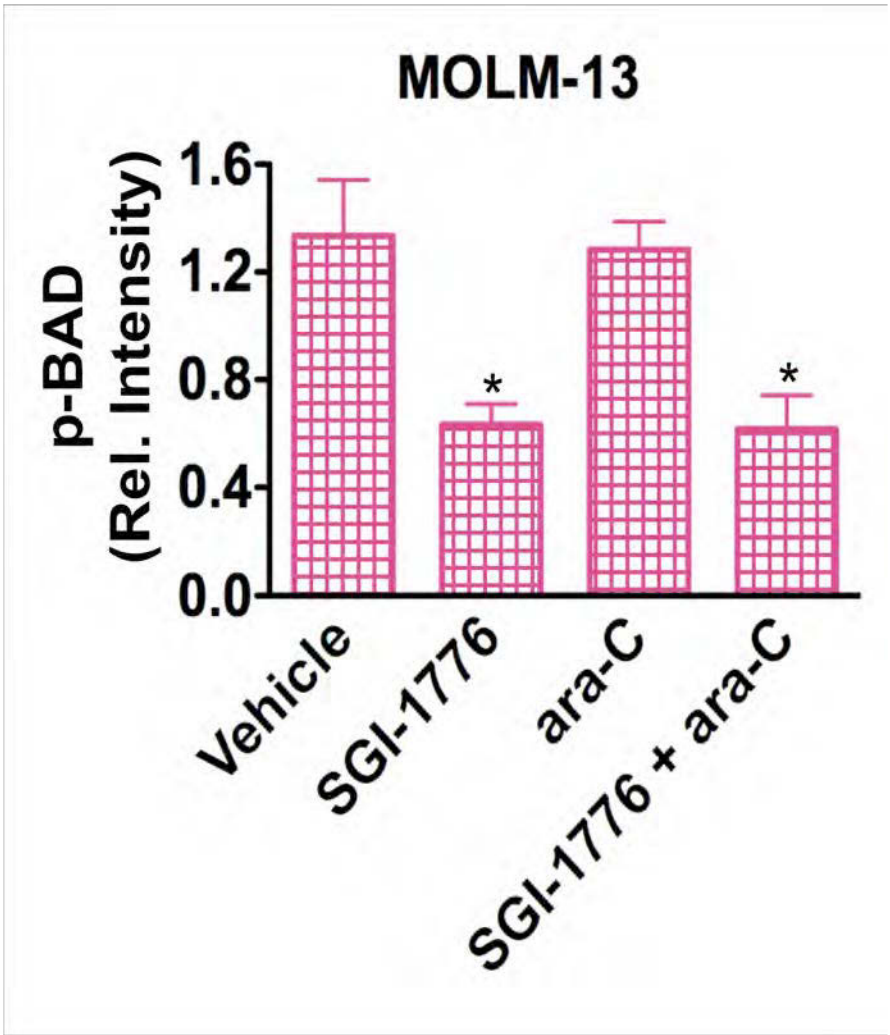

Figure 4.23 Quantification of the relative intratumoural expression levels of phospho-BAD

Tumours were stained with either an antibody to phospho (Ser^{112}) or total Bad and analysed by immunohistochemistry. The relative intensity of phospho-Bad expression was measured using Image-Pro Plus software Version 6.2.1 by densitometric analysis of five random high-power fields containing viable tumour cells. Mean ± SD, *n*=5. *Indicates a significant difference compared with controls. *P*<0.05. No significant differences were observed in total Bad expression in the different treatment groups.

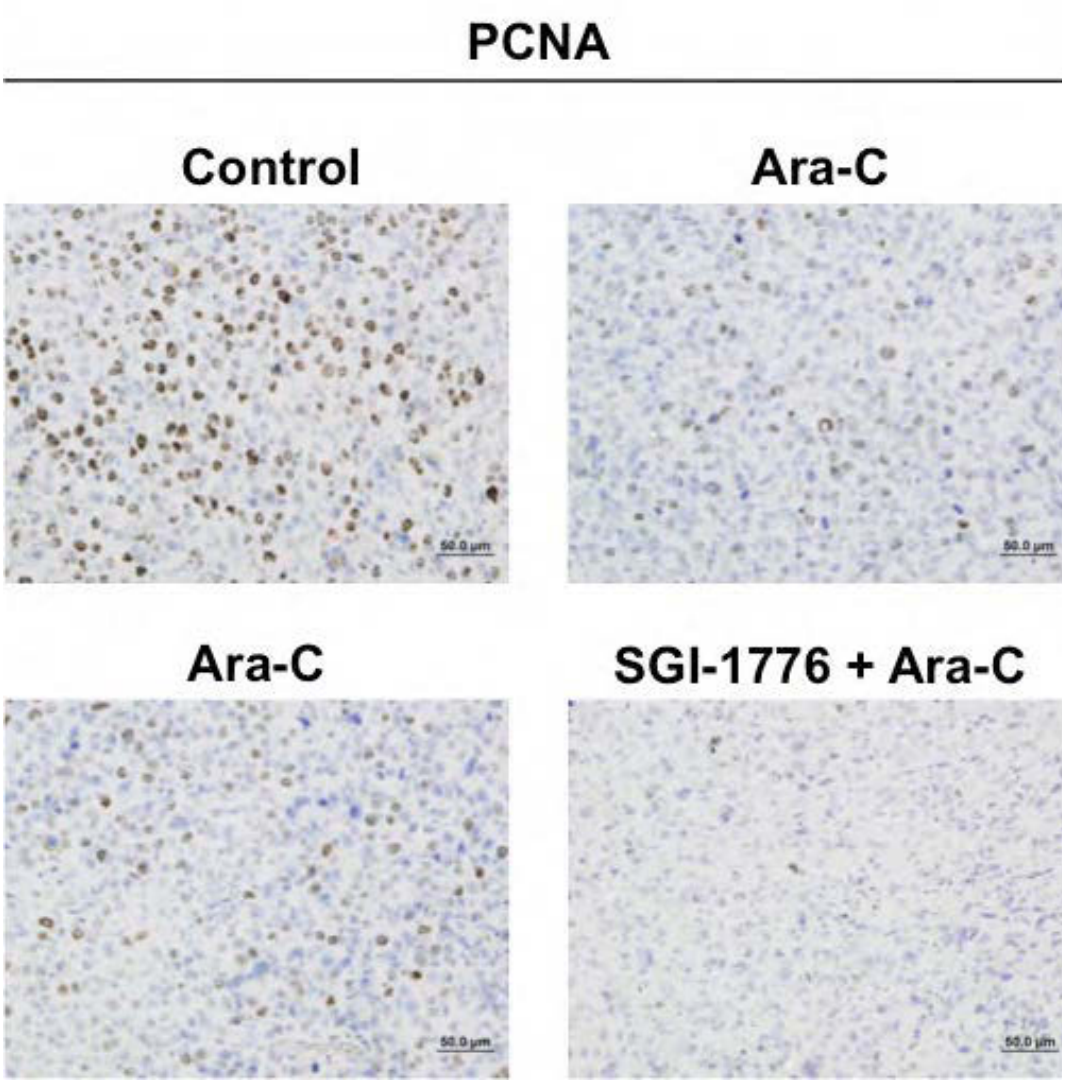

Figure 4.24 SGI-1776 and ara-C inhibit tumour cell proliferation Immunohistochemistry was utilized to assess the relative levels of PCNA in tumour sections obtained from animals in all treatment groups. Images were captured using an Olympus microscope with a DP71 camera and a 20X objective.

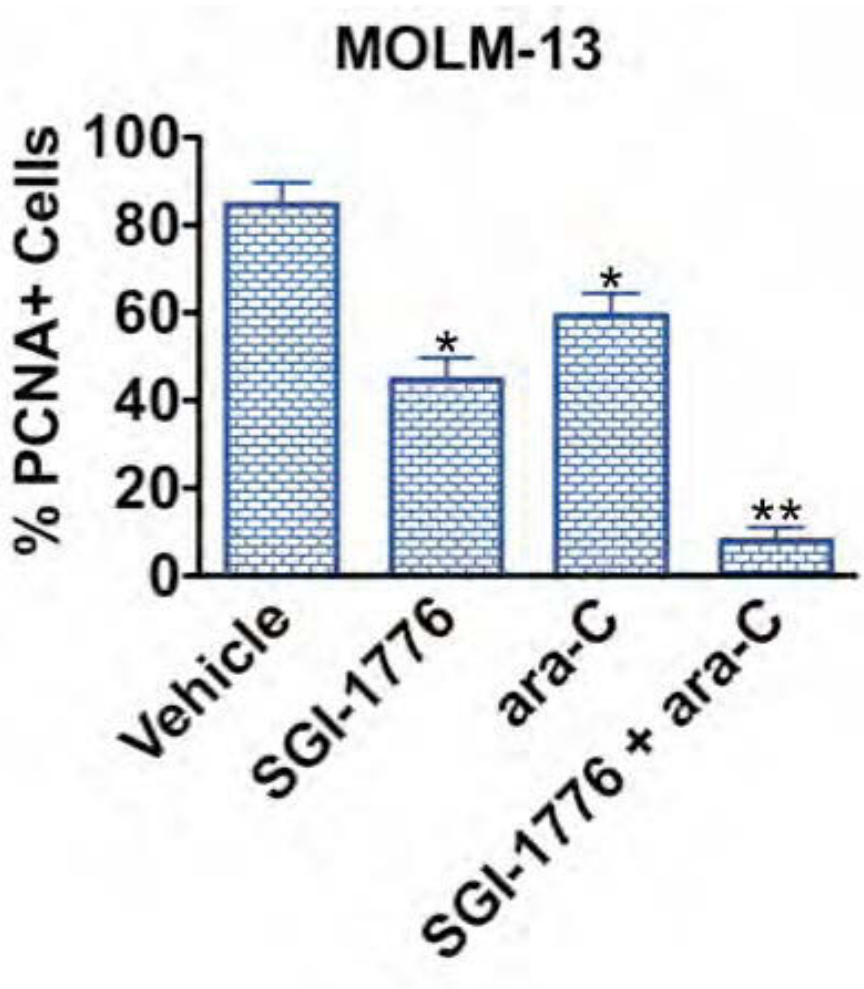

Figure 4.25 Quantification of the relative intratumoural expression levels of PCNA

The percentage of PCNA cells was scored manually. Percentages of positive cells were determined by manual counting of 5 random high-power fields per section by two investigators blinded to the treatment arms. The average percentage of positive cells per high-power field was calculated for comparison. Mean ± SD, n = 5. *Indicates a significant difference compared to controls or **single agent treatment, $p < 0.05$.

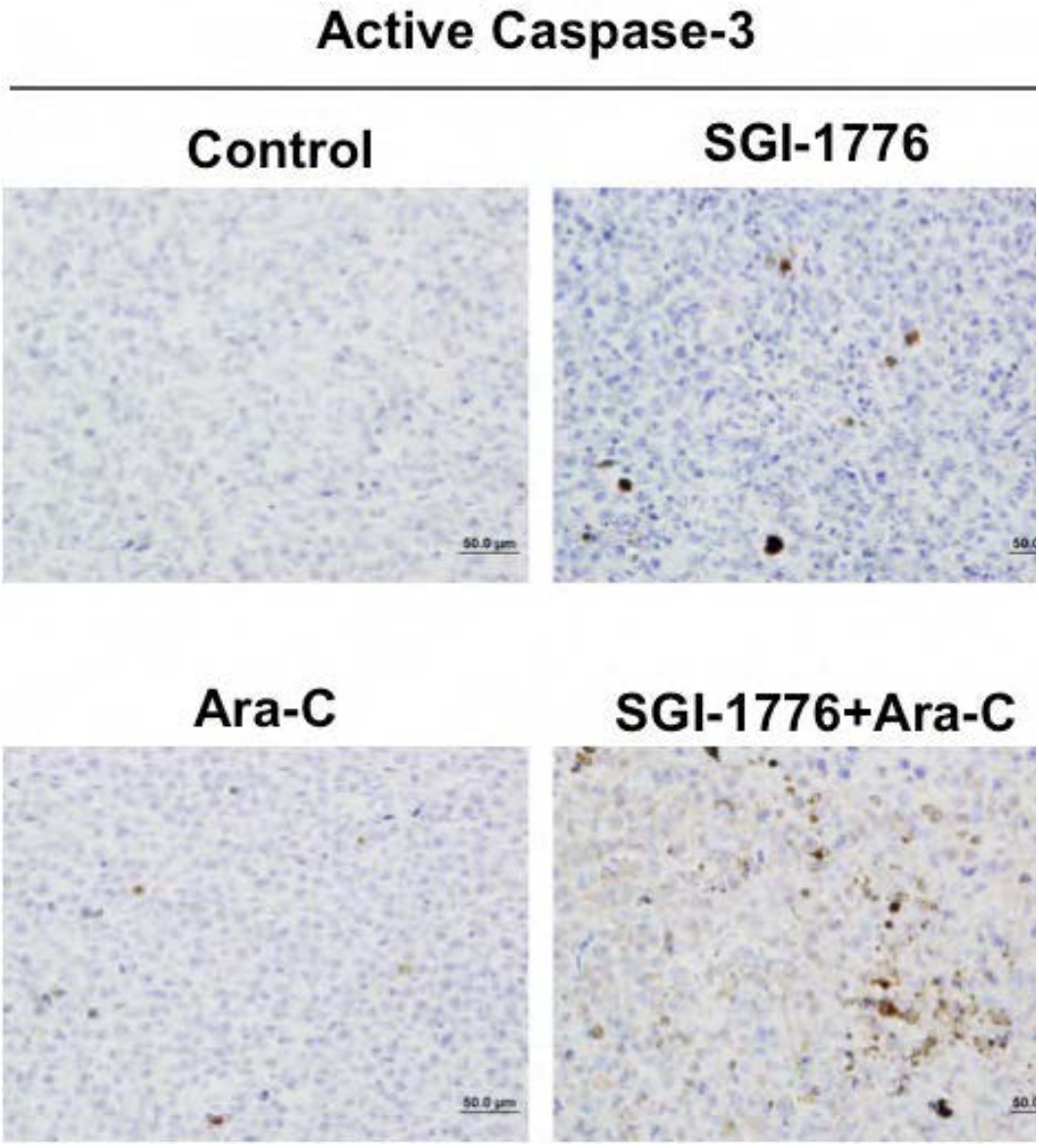

Figure 4.26 SGI-1776 and ara-C activate apoptosis

Immunohistochemistry was utilized to assess the relative levels of active caspase-3 in tumour sections obtained from animals in all treatment groups. Images were captured using an Olympus microscope with a DP71 camera and a 20X objective.

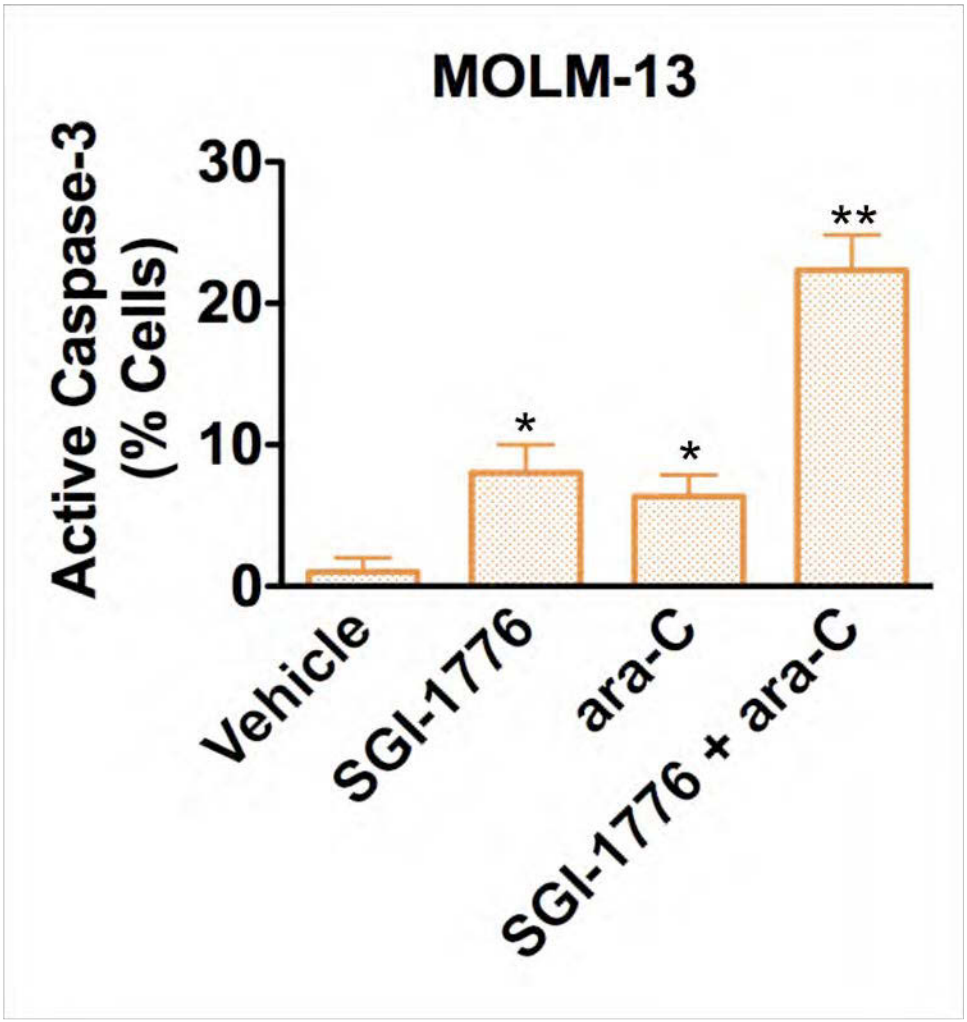

Figure 4.27 Quantification of active caspase-3 positive cells Percentages of caspase-3 positive cells were determined by manual counting of 5 random high-power fields per section by two investigators blinded to the treatment arms. The average percentage of positive cells per high-power field was calculated for comparison. Mean ± SD, n = 5. *Indicates a significant difference compared to controls or **single agent treatment, $p < 0.05$.

4.5 Discussion

The PIM kinases have received increasing attention over the last decade as potential therapeutic targets for many forms of cancer due to their prevalent overexpression and regulation of cell proliferation and pro-survival signal transduction pathways. These attributes arm PIM kinases with the potential ability to reduce the efficacy of many classes of chemotherapeutic agents. In addition to promoting drug resistance by activating downstream pro-survival/anti-apoptotic transduction cascades, PIM has also been recently shown to directly stimulate drug efflux pumps (558, 559).

Based on the high expression of PIM kinases in AML cells and their established functions in cancer biology, we hypothesized that antagonizing PIM activity may be an effective approach for AML therapy. We tested our hypothesis by investigating the efficacy and mechanism of action of a novel, small molecule pan-PIM kinase inhibitor, SGI-1776, in AML cell lines and a xenograft mouse model.

Our results demonstrated that SGI-1776 has a multifaceted mechanism of action in AML cells that is characterized by both anti-proliferative and pro-apoptotic effects. Based on an earlier report demonstrating significant off-target inhibition of FLT3 by SGI-1776 (622), we utilized FLT3-targeted shRNA to quantify the potential contribution of FLT3 inhibition to the efficacy of SGI-1776 in AML cells. Our data demonstrated that FLT3 knockdown reduced, but did not completely abrogate the sensitivity of MV4-11 cells to SGI-1776, suggesting that FLT3-related effects do contribute to the anti-AML activity of this agent (Figure 4.8). This observation is further supported by our initial screening of the effects of SGI-1776 on the viability of a panel of 9 AML cells lines, which showed that the FLT3-ITD+ cell lines MOLM-13 and MV4-11 exhibited noticeably greater sensitivity to this drug than the FLT3-ITD- cell lines included in our analyses (Figure 4.1).

Considering that FLT3 is constitutively active in more than 30% of patients with AML and this phenomenon is associated with inferior disease-free survival and increased relapse rate, this aspect of SGI-1776's mechanism merits further study. In fact, several investigational FLT3 inhibitors have been developed as potential agents for the treatment of AML and other disorders that display high levels of FLT3 activity. Early clinical trials with these experimental FLT3 inhibitors have been largely disappointing due to the development of drug resistance and lack of sustained FLT3 inhibition as discussed in section 1.7.16. Notably, an earlier study suggested that PIM-1 is an important factor underlying resistance to FLT3 inhibitor as it can directly phosphorylate and activate FLT3 in a feedback loop (606). Considering these collective findings, PIM kinase inhibitors irrespective of their potential off-target effects on FLT3, may be effective for the treatment of FLT3+ AML.

Although, PIM kinases have been implicated in processes that promote drug resistance, their potential role as a regulator of sensitivity to standard ara-C therapy had not been previously investigated. Our study established a new link between ara-C treatment and elevated levels of PIM expression, suggesting that PIM kinases may be induced in response to the therapeutic stress associated with ara-C treatment in a pro-survival capacity. Our analyses showed that ara-C-resistant HL-60 cells overexpress PIM-1 and PIM-3, establishing a correlation between elevated PIM expression and resistance to ara-C. Of note, the sensitivity of these drug-resistant cells to ara-C was partially restored by co-treatment with SGI-1776. Our MOLM-13 xenograft experiment further validated the ability of SGI-1776 to augment the efficacy of ara-C and showed significant therapeutic benefit achieved by targeting PIM activity. This strategy was very well tolerated and was associated with decreased tumour cell proliferation, increased apoptosis, and a significant reduction in the levels of phospho-BAD (Ser^{112}). These findings suggest that PIM inhibitors may have utility as an addition to standard AML therapy and that drugs of this

class may be effective for patients that are clinically refractory to ara-C or in elderly patients where low dose ara-C can be used as a single agent. Additional studies are warranted to further explore this possibility.

The preclinical activity of SGI-1776 in AML models was recently confirmed in an investigation conducted by an independent group. This study showed that SGI-1776 treatment disrupted the phosphorylation of the established PIM kinase targets, c-Myc (Ser^{62}) and 4E-BP1 (Thr^{36}/Thr^{47}). A significant reduction in the levels of the anti-apoptotic protein MCL-1 was observed and this was correlated with inhibition of global RNA and protein synthesis. A xenograft experiment conducted with mice bearing MV4-11 tumours demonstrated that SGI-1776 possessed single agent therapeutic activity that was associated with a significant reduction in MCL-1 expression (704).

SGI-1776 is the first PIM kinase inhibitor to enter phase 1 clinical trials for cancer therapy. Since its initial development, several other investigation PIM kinase inhibitors have emerged and are currently being evaluated in preclinical studies and phase 1 trials. One of the most advanced of these newer compounds is LGH447, which is being developed by Novartis Oncology and recently entered a phase 1 trial for patients with multiple myeloma. Another PIM inhibitor that has advanced into phase 1 trials is AZD-1208. This agent is being developed by Astra Zeneca and is currently being evaluated in two separate phase 1 studies for AML and advanced solid tumours. Preclinical studies with LGH447 and AZD-1208 have not yet been published. It will be vey interesting to learn how their mechanisms of action compare and contrast with that of SGI-1776.

Collectively, our data, demonstrate that inhibiting PIM kinase activity represents a promising new strategy to augment the therapeutic efficacy of ara-C. Further investigations aimed to define the role(s) of PIM kinases in AML pathogenesis and evaluate the therapeutic potential of PIM kinase inhibition as a strategy to circumvent drug resistance are warranted.

5 Chapter 5 Conclusions and Future Directions

AML and CML represent two haematological malignancies with very distinct pathophysiological characteristics. Whereas CML-CP is caused by a single molecular aberatation (BCR-ABL), AML is a very heterogenous disease characterized by numerous of possible genetic abnormalities. The importance of BCR-ABL in the pathogenesis of CML is underscored by the tremendous success of targeted inhibitors of BCR-ABL. While the development of targeted therapy with TKIs represents one of the most exciting stories of translational research in recent years, most of these agents are not optimally effective in the setting of resistant BCR-ABL kinase domain mutations, in particular the T315I mutation nor do they successfully eliminate the CML stem cell population. New approaches are required for this patient population and for patients that progress to the advanced phases of CML. In contrast, there has been little change in our standard approach to AML therapy in the past 30 years despite the enormous improvements in our understanding of the molecular biology of this disease and novel therapeutic approaches are needed for patients that fail or are unfit for standard therapy.

Aberrant signalling by Ser/Thr kinases is a unifying feature of both CML and AML. Novel targeted therapies that inhibit specific kinases that are dysregulated in leukaemia have the potential to disrupt disease pathogenesis and augment standard therapy for patients with these haematological malignancies. The work outlined in this thesis explores the efficacy and mechanisms of action of two investigational small molecular inhibitors of Ser/Thr kinases (alisertib, a small molecule Aurora A kinase inhibitor; SGI-1776, a small molecule PIM kinase inhibitor) in human leukaemia cell lines, mouse models, and primary patient specimens.

Our preclinical investigations demonstrated that inhibition of Aurora kinase A activity with alisertib diminishes the viability of both CML and AML cells irrespective of their sensitivity to conventional therapeutic agents. Notably, treatment with alisertib significantly antagonized disease

progression in multiple mouse models of CML and AML irrespective of their specific genetic backgrounds. This work has now been published in the *Journal of Cellular and Molecular Medicine* and the *International Journal of Cancer* and is available in Appendices C and D. It has been hypothesized that leukaemias may be hypersensitive to agents that inhibit Aurora kinases due to their rapid rates of proliferation and high number of cells undergoing mitosis. Indeed, numerous objective responses including CRs have been observed in patients with AML in early phase clinical trials of Aurora kinase inhibitors. However, it is becomng increasingly clear that the direct disruption of mitosis does not account for all of the anti-neoplastic effects associated with inhibition of Aurora activity. Our data suggest that alisertib has a multifaceted mechanism of action in leukaemia cells that involves the disruption of cell cycle kinetics as well as the induction of apoptotic cell death. The mechanistic links between drug-induced MC and apoptosis with respect to alisertib have not been fully elucidated.

Our preliminary results have suggested a role for the protein phosphatase PP2A, which is induced by alisertib treatment and is known to dephosphoylate FOXO3a allowing it to enter the nucleus and transcribe for its target genes including, BIM and p27. Impairment of PP2A function has been reported to be associated with an adverse prognosis in AML (707-709) and the restoration of PP2A activity is an attractive therapeutic goal (710). Indeed, we have shown that treatment of AML cell lines with TAK-901 (a pan Aurora kinase inhibitor) is associated with significantly increased expression of p27 and the BH3-only pro-apoptotic protein PUMA. Chromatin immunoprecipitation (ChIP) assays revealed that the elevation in the expression of these genes caused by administration of TAK-901 was due to increased FOXO3a transcriptional activity. Performing similar experiments with alisertib treated AML cell would help confirm that alisertib treatment results in increased transcriptional activity of FOXO3a.

Our data indicates that the bulk of the pro-apoptotic effects of alisertib are triggered after mitosis has been disrupted. I plan to more rigorously investigate the relationship between these events in a follow up study. In particular, it would be extremely interesting to determine how the effects of alisertib on the anti-apoptotic factor Apollon may be linked to MC, FOXO3a activation, and the execution of apoptosis. We also plan to investigate a potential functional relationship between Apollon and PP2A, which has not been previously described in the literature. Given its role as an inhibitor of apoptosis and intrinsic overexpression in leukaemias, the development of novel agents targeting Apollon activity is of great interest.

Our data indicate that the complex mechanisms of action of Aurora kinase inhibitors should be carefully considered when designing clinical studies to assess their safety and efficacy. The cytostatic potential of Aurora kinase inhibitors may yield significant clinical benefit if used optimally. For example, despite the modest effects on tumour regression observed in some clinical studies with Aurora inhibitors, their potential ability to induce prolonged disease stabilization could potentially translate into improved survival. It also appears from the available literature that most dose limiting toxicities reflect the effect of Aurora kinase inhibition on normal cells that have a high proliferation rate such as those of the gastrointestinal and BM tissues. To overcome this problem, various dosing schedules have evolved that allow for a short period of Aurora kinase inhibition followed by a rest period to allow for normal tissue recovery. An alternative strategy is to allow concurrent supportive care such as colony stimulating factors. Lastly, it may be possible to combine Aurora kinase inhibitors with agents that would not be predicted to have cross intolerance such as targeted BCR-ABL inhibitors like nilotinib.

Another important avenue for future investigation is a more comprehensive dissection of the specific role that Aurora A plays in promoting resistance to standard AML therapy. We showed that ara-C treatment is associated with increased expression of Aurora A and that

targeted knockdown of Aurora A significantly increases the efficacy of ara-C. This suggests that Aurora A may promote resistance/reduced sensitivity to ara-C and thus, inhibition of Aurora A activity may be an effective strategy to augment the anti-leukaemic activity of ara-C. Indeed, combined treatment with alisertib and ara-C is associated with a greater anti-leukaemia effect than either agent alone (chapter 3). Defining the mechanistic details regarding how ara-C, which is primarily active in the S phase of the cell cycle, promotes elevated level of a mitotic kinase (Aurora A) is a very challenging and clinically relevant endeavour that I plan to pursue.

Although Aurora kinase inhibitors have significant clinical activity, their utility as single agents may be limited. Accordingly, a number of studies investigating their use in combination with standard agents have been recently initiated. In particular, the combination of alisertib and ara-C may represent an attractive treatment strategy for AML. However, one potential limitation to this approach may be the combined cytotoxic effect of ara-C and alisertib on normal tissues. The combination of these agents was well tolerated in mouse models of AML, but phase 1 studies are required to more definitively assess the safety and tolerability of this therapeutic approach in patients with AML. While there is certainly great potential for the use of alisertib in myeloid leukaemias, this drug also appears to have significant activity in lymphoid malignancies. A safe and effective dosing schedule has been determined and a registration study is currently underway to determine its benefit in refractory peripheral T cell lymphoma.

An alternative novel target that is of significant interest in developmental therapeutics is the PIM family of kinases. Considering their functional contributions to the malignant phenotype and their overexpression in AML we investigated the role of a novel inhibitor of PIM kinases, SGI-1776, in preclinical models of AML. In chapter 5, we showed that SGI-1776 disrupts AML cell viability, impairs clonogenic survival, and

induces apoptosis. Given the role of PIM kinases in the development of drug resistance, we investigated whether ara-C resistance was linked to the overexpression of PIM. Targeting PIM with SGI-1776 sensitized ara-C resistant cells to ara-C and significantly increased the efficacy of ara-C therapy *in vivo*. This suggests that antagonizing PIM activity may increase the therapeutic efficacy of ara-C and possibly help overcome drug resistance. This work has now been published in the *British Journal of Haematology* and the manuscript is available in Appendix E. Previous studies have shown that PIM-1 phosphorylates MDR-1 (a transmembrane glycoprotein that transports drugs across the cell membrane) at Ser^{683} which results in protection of MDR-1 from ubiquitination and proteasomal degradation (706). This and other mechanisms may link PIM overexpression to ara-C resistance. Further experiments would be needed to show if overexpression of PIM in AML cell lines would lead directly to ara-C resistance. Comparison of the expression of PIM kinases in a large number of patients with newly diagnosed AML who have not been exposed to ara-C and patients who are clinically refractory to ara-C therapy would also be informative. Unfortunately, the clinical development of SGI-1776 has been halted due to concerns regarding potential cardiac toxicity. However, a number of other small molecule inhibitors of PIM are in early phase clinical development including a second-generation compound, SGI-9481, developed by SuperGen Inc. and LGH447 being developed by Novartis. An attractive therapeutic strategy would be to investigate the safety and activity of one of these newer PIM kinase inhibitors in combination with ara-C in AML patients.

Our collective findings have shown that both Aurora A and PIM kinases represent promising therapeutic targets for the treatment of myeloid leukaemias. Inhibition of either target yields significant benefit on its own in preclinical models, augments the efficacy of standard therapy, and antagonizes mechanisms of resistance to conventional therapy. Considering that our data demonstrate that the downstream

pharmacodynamic effects of inhibition of PIM and Aurora A and have several commonalities, it is possible that there may be some degree of previously unreported functional redundancy or pathway convergence between these kinases. PIM kinases are constitutively active when expressed and have the theoretical capability to phosphorylate the sites within Aurora A that control its enzymatic activity. We are currently investigating the possibility that PIM kinases may promote increased Aurora A activity through direct phosphorylation and are studying the potential effects of PIM inhibition on mitotic progression.

As our understanding of the biology of myeloid leukaemias has improved in the past decade so too has our appreciation of the role that aberrant kinase expression plays in their malignant pathogenesis. In addition to the logical avenues for future investigation discussed above, a number of other important global questions remain to be fully answered. Whether optimal therapeutic efficacy is achieved through the inhibition of Aurora A, Aurora B, or both kinases simultaneously is still unclear and is the subject of considerable debate. One study attempted to address this question by evaluating the consequences of Aurora A and Aurora B inhibition using antisense oligonucleotides in pancreatic cancer cells (711). No obvious advantages to targeting both Aurora kinases concurrently were observed, but targeting Aurora A alone appeared to have some potential advantage over targeting Aurora B alone due to rapid induction of apoptosis and mitotic arrest. In another preclinical study, colon cancer cells were found to be more sensitive to Aurora B inhibition compared to Aurora A inhibition (712). Ongoing trials may provide new insights regarding whether there are any advantages to selectively targeting individual Aurora isoforms (625). Preliminary findings suggest that there is no simple answer to this question and the benefit derived from targeting individual or pan-Aurora kinases is likely to vary depending upon the unique biology of individual forms of cancer.

It would also be very informative to determine whether alisertib can induce apoptosis in myeloid stem cells. Targeting these quiescent stem cells offers the potential for a cure in CML and may also significantly improve the outcome in AML. A preliminary investigation of the activity of alisertib in AML stem cells has been presented in abstract form (713). This study found that freshly isolated CD34+/CD38- cells from individuals with AML expressed a greater amount of *AURKA* than their CD34+/CD38+ counterparts, as measured by real time qRT-PCR. Alisertib treatment significantly inhibited proliferation and induced apoptosis of CD34+/CD38- AML cells, as assessed by the clonogenic assay and detection of the cleaved form of poly (ADP-ribose) polymerase by Western blot analysis, respectively. Importantly alisertib impaired engraftment of CD34+/CD38- AML cells in severely immunocompromised mice and prolonged their OS compared with vehicle treated mice. Additional studies would be required to investigate whether alisertib has significant activity in CML stem cells.

There are over 500 kinases in the human genome. Many of the clinically effective kinase inhibitors were initially developed to inhibit a single target, but were subsequently shown to inhibit a much broader range of kinases. It is therefore plausible that the toxicities, pharmacodynamic activity and clinical responses of both Aurora and PIM kinase inhibitors observed in clinical studies are complemented by inhibition of other kinases. This is an important area of ongoing investigation. Although this thesis contributes to the expanding knowledge base of the utility and mechanism of action of Aurora and PIM kinase inhibitors in myeloid malignancies, more focused research is required in order to maximize their clinical potential. It is particularly important that we increase our understanding of how cancer cells become resistant to these agents and that we elucidate additional biomarkers predicitve of responsiveness to therapy. Despite the unanswered questions, the collective findings of this thesis research provide preclinical rationale for the development of effective combination stategies that will be evaluated in

early phase clinical trials that aim to improve outcome in myeloid leukaemias. It is hoped that these concerted efforts in Aurora and PIM kinase research will translate into novel anti-cancer strategies that will ultimately improve outcomes for patients with a broad range of malignancies.

Appendix A: Tyrosine kinase inhibitors in CML

Imatinib

Imatinib is a 2-phenylaminopyrimidine derivative that functions as a specific inhibitor of a number of tyrosine kinases including ABL (IC_{50} 194 nM), c-Kit (IC_{50} 96 nM) and PDGFR (IC_{50} 74 nM), but does not affect closely related kinases such as c-Fms, kinase insert domain receptor (KDR), FLT-1, TEK, and FLT -3 (Table I) (276, 277). Inhibition of c-Kit has led to the development of imatinib in tumours where c-Kit activation is important such gastrointestinal stromal tumours and aggressive systemic mastocytosis (714, 715).

Imatinib gained approval as the initial treatment of choice for CML-CP based on prospective clinical trials (278-280). These trials demonstrated more frequent and more durable cytogenetic responses when compared with IFNα plus ara-C, which was the previous standard of care for those unable to receive HSCT. Comparative survival benefits have not been reported; this is principally because of the large number of patients who crossed over to imatinib from the other treatment arm.

	Imatinib (IC_{50} in nM)		Nilotinib (IC_{50} in nM)	
Kinase (cell type)	**Autophosphorylation**	**Proliferation**	**Autophosphorylation**	**Proliferation**
p210 BCR-ABL (32D)	194 ± 7, n = 94	334 ± 37, n = 23	20 ± 1, n = 53	9.2 ± 0.3, n = 3
BCR-ABL (K562)	470 ± 59, n = 15	272 ± 27, n = 21	43 ± 15, n = 3	12 ± 2, n = 3
BCR-ABL (Ku-812F)	466 ± 59, n = 7	80 ± 35, n = 13	60 ± 19, n = 5	8 ± 2, n = 6
p210 BCR-ABL (Ba/F3)	220 ± 36, n = 12	649 ± 52, n = 18	21 ± 2, n = 5	25 ± 2, n = 49
p190 BCR-ABL (Ba/F3)	122 ± 15, n = 3		33 ± 4, n = 3	
PDGFR-α + PDGFR-β (A31)	74 ± 11, n = 11		71 ± 7, n = 20	
PDGFR-β (Tel Ba/F3	NA	39 ± 4, n = 8	NA	57 ± 7, n = 18
c-Kit exon13 mutant (GIST882)	96 ± 12, n = 7	120 ± 6, n = 15	200 ± 13, n = 19	160 ± 12, n = 17
c-Kit del 560-561 (Ba/F3)	27, n = 1	27 ± 2, n = 2	27 ± 3, n = 3	26 ± 1, n = 4
VEGFR-2 (CHO-VEGFR2)	>10000, n = 6		3720 ± 920, n = 5	NA
c-erbB-2 (BT-474)	>10000, n = 4		>10000, n = 2	>10000, n = 6
c-erbB-2 (Ba/F3-erbB-2)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
FLT3-ITD (Ba/F3-NPOS)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Ret (Ba/F3-PTC-3)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Met (Ba/F3-Tpr-Met)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
IGF-1R (NWT-21)	>10000, n = 1		>10000, n = 4	
Ins-R (A14)	>10000, n = 1		>10000, n = 4	
FGFR-1 (Ba/F3-Bcr-FGFR1)		$>3000^{AB}$, n = 3		$>3000^{AB}$, n = 2
JAK-2 (Ba/F3-JAK-2)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Ras (Ba/F3-H-Ras-G12V)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
NPM-Alk (Ba/F3-NPM-Alk cl. 1)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Akt (Ba/F3-MyrAkt, cl. 21)		>3000, n = 1		>3000, n = 2

Table I Comparison of imatinib and nilotinib for effects on autophosphorylation and proliferation in cells

The influence of imatinib and nilotinib on kinase autophosphorylation or cell viability was calculated as percentage inhibition. Dose-response curves were used to calculate IC_{50} values, expressed as mean ± SEM, n = number of experiments. The antiproliferative activity was assessed using either the ATPlite assay kit (Perkin-Elmer) or, where indicated (AB), the Alamar Blue assay kit (Biosource International Inc.). wst, WST-1 reagent (Roche), as previously described (Azam et al., 2003), was used to generate IC_{50} values. *, values as reported previously for imatinib (Azam et al., 2003) (716). Reproduced with permission from Weisberg et al, Cancer Cell, 2005 (277).

Efficacy of imatinib

The IRIS trial (International Randomized study of IFN and STI571) was a phase 3 randomized, open-label, multicenter, crossover trial of imatinib (400 mg/day by mouth) versus IFNα (5 million units/m^2 per day) plus ara-C (20 mg/m^2 per day for 10 days/month) in 1106 patients with newly diagnosed CML-CP (273). On an intention-to-treat analysis, imatinib was significantly better than IFNα plus ara-C for all early endpoints studied, with the following 18-month estimates: CHR, 97% versus 69%, MCyR, 87% versus 35% (717), CCyR, 76% versus 14% and MMR 39% versus 2% (717). Updated results from the IRIS trial with a median follow-up of 60 months have shown that the results with imatinib treatment have been durable (265). Patients receiving imatinib as initial therapy for CML-CP had overall and event-free survivals of 89% and 83%, respectively. The annual rate of progression to CML-AP or CML-BC was 0.6% in the fifth year of therapy, which was lower than that seen during the first four years of treatment (1.5%, 2.8%, 1.6%, and 0.9%, respectively). Patients who achieved a CCyR by 12 months after initiation of imatinib had better OS without progression to CML-AP or CML-BC (97% versus 81%) compared with patients who did not achieve at least a MCyR by 12 months.

However it is important to note that while cumulative incidences of CCyR on imatinib are high the 5-year probability of remaining in MCyR while still receiving imatinib was only 62.7% in 204 consecutive adult patients with newly diagnosed CML-CP received imatinib (270). Similarly in a study of 276 patients with CML-CP on imatinib therapy the cumulative incidence of CCyR was 91% but the incidence of CCyR at 48 months into therapy was only 78%.

Side effects are mild or moderate. The most common adverse effects are oedema, nausea, muscle cramps, rash, and diarrhoea, occurring in approximately 60%, 55%, 50%, 30%, and 30% of patients, respectively (718). There is no universal definition of intolerance. In general a patient is considered to be imatinib intolerant when a non-haematologic toxicity of at least grade three recurs despite appropriate dose reductions and optimal symptomatic

management (273). Grading of toxicities is based on the United States National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE).

Nilotinib

Nilotinib (Tasigna®) is an orally administered TKI made by the Novartis Pharmaceuticals Corporation. Unlike dasatinib, which has a completely different structure to imatinib, nilotinib was methodically and rationally designed to create a better topological fit in the ABL kinase domain of BCR-ABL resulting in enhanced BCR-ABL inhibition. It is an aminopyrimidine derivative of imatinib, structurally changed to eliminate two energetically unfavourable hydrogen bonds with the replacement of the N-methylpiperazine ring of imatinib by a trifluoromethyl-substituted phenyl group (277, 719). Like dasatinib, its spectrum of inhibition also includes c-Kit (IC_{50} 200 nM) and PDGFR (IC_{50} 71 nM) (Table I) (277). However, unlike dasatinib, it only binds the inactive conformation of ABL and it does not inhibit SRC kinases (277). Unlike imatinib, nilotinib uptake does not involve organic cation transporter 1 (OCT-1) (720). Altered OCT-1 expression has been reported to be a mechanism of resistance to imatinib. Nilotinib's various characteristics make it 20-30 fold more potent against BCR-ABL expressing cells (IC_{50} < 30 nM) with well-documented activity against 32 of 33 imatinib-resistant BCR-ABL mutants. However, like imatinib and dasatinib, nilotinib is unable to overcome the resistance of the T315I mutation (300).

Nilotinib is orally bioavailable and has a half-life of approximately 15 hours. It should be taken 2-hours before or 1-hour after meals as food consumption increases its drug bioavailability. It is metabolized by hepatic oxidation reactions, mainly involving the CYP3A4 pathway and therefore has potential to interact with CYP3A4 inhibitors and inducers (300).

Efficacy of nilotinib in relapsed disease

A phase 2, open-label study evaluated the efficacy and safety of nilotinib in 321 patients with CML-CP who were imatinib resistant (69%) or intolerant. An updated analysis, with a minimum follow-up of 24 months, reported that of the

321 patients, 124 (39%) continue on nilotinib treatment (721). Overall, 59% of patients achieved a MCyR while 44% achieved a CCyR. Of patients achieving CCyR, 56% achieved MMR. Cytogenetic responses were durable, with 84% of patients who achieved CCyR maintaining response at 24 months. The OS at 24 months was 87%. Common grade 3/4 side effects included thrombocytopaenia, neutropaenia, elevated lipase, hyperglycemia, hypophosphataemia, and QT prolongation.

The ENACT (Expanding Nilotinib Access in Clinical Trials) study was a large international open-label expanded access trial in 1422 patients with CML-CP and imatinib resistance (60%) or intolerance. Patients were treated with nilotinib 400 mg twice daily. Nilotinib demonstrated significant efficacy, with CHR and CCyR achieved in 43% and 34% of patients, respectively. With regard to cytogenetic response, 45% of patients achieved a MCyR, with the majority of these responses (approximately 75%) being CCyR (34% overall). At 18 months, the PFS was 80%. Most patients achieved planned dosing of 400 mg twice daily and maintained the dose for greater than 12 months. Common adverse events included rash (28%), headache (25%), and nausea (17%). Complete efficacy data collection was performed in the subset of ENACT patients (n = 168) from France. The number of French patients achieving MCyR (54%) and CCyR (45%) was higher than in the overall patient population (45% and 34%, respectively). French patients were also monitored for molecular response by qRT-PCR at baseline and every 3 months thereafter. Of the French subset, 37% of patients achieved MMR by 12 months.

Nilotinib is also approved for CML-AP. A phase 1 dose-escalation study of nilotinib included 46 patients with imatinib-resistant CML-AP and showed haematologic and cytogenetic response rates of 33% and 22%, respectively (722). A phase 2 study examined the use of nilotinib in 119 patients with CML-AP and imatinib resistance (81%) or intolerance (723). Patients received nilotinib 400 mg twice daily with escalation to 600 mg twice daily for inadequate response. At 24 months follow-up the rate of CHR, MCyR and CCyR was 31%,

32% and 21% respectively. Notably, 66% of patients who achieved a MCyR maintained this response at 24 months (310).

Nilotinib is also effective in patients with CML following imatinib and dasatinib failure. A phase 2 study, evaluating the safety of nilotinib 400 mg BID in CML-CP, -AP, and –BC who either failed or were intolerant of both imatinib and dasatinib, demonstrated clinical activity in these patients (724). In a single institution study, long term follow up for 14 patients receiving nilotinib following imatinib and dasatinib failure showed 2 patients achieving CCyR, 1 patient with PCyR, 5 patients with MCyR, 4 patients with CHR and 2 with no response (725).

Efficacy of nilotinib as frontline therapy

In a phase 2 pilot study evaluating the efficacy and safety of nilotinib 400 mg BID as first line therapy for patients with CML-CP, among 51 patients who had been followed for at least 3 months, CCyR and MMR are 98% and 76% respectively (726). The GIMEMA CML Working Party investigated the efficacy of nilotinib 400 mg BID in patients with early CML-CP in a multicenter phase 2 trial. In 72 patients, after 2 years, there was only one patient with T315I mutation who progressed to CML-AP. Patients responded very rapidly with CCyR and MMR of 78% and 52% at 3 months.

In the phase 3, randomized, open-label multicenter ENESTnd (Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed Patients) study, 846 patients with CML-CP were randomized in a 1:1:1 ratio to receive nilotinib 300 mg twice daily, nilotinib 400 mg twice daily or imatinib 400 mg once daily. In the original IRIS study, none of the patients who achieved an MMR at 12 months with imatinib progressed to CML-AP or CML-BC. In light of this, attainment of a MMR at 12 months was chosen as the primary endpoint in the ENESTnd study. The rate of CCyR by 12 months was a secondary endpoint. At 12 months, CCyR was 80%, 78% and 65% in these three groups respectively and MMR was 44%, 43% and 22%. This similar pattern of response was observed in a subset of patients with high Sokal risk; CCyR in this group was 74%, 63% and 49% and the MMR was 41%, 32% and 17% respectively. No

patient achieving a MMR had progression to CML-AP or CML-BC. At the time of the cutoff date, <1% of the patients on nilotinib had progression to AP or BC while progression occurred in 4% of patients on imatinib (727). Based on this promising data the FDA granted accelerated approval to nilotinib for the treatment of adult patients with newly diagnosed CML-CP on June 17, 2010. The recommended nilotinib dose for this indication is 300 mg orally twice daily. The 300 mg twice-daily dosing regimen was chosen, as its safety profile appeared more favourable than that of the 400 mg twice-daily regimen, while the efficacy appeared comparable.

The data from the ENESTnd study after a minimum follow-up of 24 months was recently published (728). By 24 months, significantly more patients had a MMR with nilotinib than with imatinib (71% with nilotinib 300 mg twice daily, 67% with nilotinib 400 mg twice daily, and 44% with imatinib). Significantly more patients in the nilotinib groups achieved a CMR at any time than did those in the imatinib group (26% with nilotinib 300 mg twice daily, 21% with nilotinib 400 mg twice daily, and 10% with imatinib). There were fewer progressions to CML-AP or CML-BC on treatment, including clonal evolution, in the nilotinib groups than in the imatinib group (two with nilotinib 300 mg twice daily, five with nilotinib 400 mg twice daily, and 17 with imatinib).

Efficacy of nilotinib in patients with BCR-ABL kinase domain mutations

In patients with baseline kinase domain point mutations, the *in vitro* sensitivity of the mutant clone correlates with response. Baseline mutation data has been evaluated in 288 patients with CML-CP from the phase 2 nilotinib registration trial. After 12 months of therapy, those with highly *in vitro* nilotinib-sensitive mutations (cellular $IC_{50} \leq$ 150 nM) showed the best responses with MCyR, CCyR and MMR of 60%, 40% and 29% respectively, which were equivalent to those without baseline mutations. These include M244V, L248V, G250E, Q252H, E275K, D276G, F317L, M351T, E355A, E355G, L387F and F486S (729, 730). The patients who had less sensitive mutations (cellular IC_{50} 201-800 nM) had less favourable responses to nilotinib and none achieved CCyR.

These mutations include Y253H, E255K/V, and F359C/V (729-731). Patients with the T315I mutation, which has IC_{50} > 10,000 nM (300), remain highly resistant to nilotinib.

Safety of nilotinib

Nilotinib is generally well tolerated. The most common haematologic adverse events are neutropaenia and thrombocytopaenia. The rates of neutropenia and thrombocytopaenia were 10 to 12% for patients taking nilotinib in the frontline setting, 27% to 30% in the 2nd line setting, 44 to 51% following imatinib and dasatinib failure, 27% to 30% in patients with CML-AP and 62% to 67% in patients with CML-BC. As would be expected, these cytopaenias correlate with disease phase and number of prior lines of therapy. The most common non-haematologic adverse events are rash, headache, nausea, and pruritus. The most common laboratory abnormalities include increased total bilirubin, increased alanine aminotransferase (ALT), increased aspartate aminotransferase (AST), decreased phosphate, increased glucose, increased lipase and increased amylase.

Dasatinib

Introduction

Dasatinib (BMS-354825; Sprycel) was originally developed as an inhibitor of the SRC family of kinases (IC_{50} < 0.5 nM) (301) (Table II). It is active against imatinib-resistant or intolerant CML and inhibits both the active and inactive conformations of the ABL domain (IC_{50}<1.0 nM) (302-305). An in vitro study showed that dasatinib was 325 times more potent than imatinib in inhibiting wild type BCR-ABL and also has activity against BCR-ABL mutants with high levels of imatinib resistance, except for those with the T315I mutation (288). In addition dasatinib has limited activity against the F317L mutation (302). Dasatinb also inhibits a number of other kinases important kinases including the proto-oncogene tyrosine-protein kinase, yes (IC_{50} < 0.50 nM), c-Kit (IC_{50} < 5.0 nM) and PDGFR (IC_{50} 28 nM) (305).

Kinase	Enzyme IC_{50}, nM	kinase	Enzyme IC_{50}, nM
BCR-ABL	<1.0	MEK	1700
SRC	0.50	VEGFR-2	>2000
lck	0.40	CDK2	>5000
yes	0.50	IKK	>10000
c-Kit	5.0	AKT	>50000
PDGFRß	28	FAK	>50000
p38	100	IGF-1R	>50000
Her1	180	IR	>50000
Her2	710	MK2	>50000
FGFR-1	880	PKC α,δ,τ,ζ	>50000

Table II. Kinase selectivity profile of dasatinib

Dasatinib evaluated in a kinase selectivity panel and Ki values were determined for both SRC and Bcr-Abl kinase. Dasatinib was confirmed to be a highly potent, ATP competitive inhibitor of both SRC and BCR-ABL, with measured Ki values of 16 ± 1.0 pM and 30 ± 22 pM, respectively and also demonstrated significant activity against c-Kit and PDGFRβ. Reproduced with permission from Lombardo et al J Med Chem. 2004

Efficacy of dasatinib in relapsed disease

Three studies have evaluated the effectiveness of dasatinib in patients in CML-CP with resistance or intolerance to prior therapy. The reported CHR and MCyR rates were approximately 90% and 60%, respectively. These rates are comparable to those observed with nilotinib.

A series of phase 2 studies known as START (SRC/ABL Tyrosine kinase inhibition Activity Research Trials) have evaluated dasatinib in various phases of CML. The START-C study examined the use of dasatinib 70 mg twice daily in 387 CML-CP patients with imatinib resistance or intolerance (732). After follow up for a median of 15 months, rates of CHR, MCyR, and CCyR were 90%, 52%, and 40%, respectively. Among patients with imatinib intolerance, these same levels of response were seen in 94%, 80%, and 75%, respectively. Dasatinib was well tolerated and only 9% had to discontinue therapy due to adverse events. START-A, -B and -L looked at the efficacy of dasatinib in CML-AP, myeloid and lymphoid CML-BC, respectively. All three studies showed that dasatinib was effective in imatinib resistant or intolerant patients with advanced phase CML (733, 734).

A randomized phase 3 study investigated different dosing schedules of dasatinib in 662 patients with CML-CP who were resistant (74%) or intolerant to imatinib (735). Patients were randomly assigned to four dosing strategies (50 mg twice daily, 100 mg daily, 70 mg twice daily, or 140 mg daily). There was no significant difference in CHR or MCyR rates among the four treatment arms. CHR was achieved in 86% to 92% of patients and CCyR was achieved in 41 to 45% of patients. However, when compared with 70 mg twice daily, 100 mg daily dosing resulted in significantly less grade 3/4 thrombocytopaenia, fewer pleural effusions, fewer dose reductions or interruptions, and fewer discontinuations due to toxicity. Lastly, a randomized phase 2 trial showed better response rates with dasatinib compared with increased dose imatinib in patients who had failed

imatinib therapy (736). Taken together these studies support the use of dasatinib for the treatment of non-transplant candidates with CML-CP after the failure of imatinib therapy.

Efficacy of dasatinib in frontline therapy

A randomized phase 3 trial called DASISION (Dasatinib versus Imatinib Study in Treatment-Naïve CML-CP Patients) involved 518 patients with previously untreated CML-CP. It compared dasatinib 100 mg daily with imatinib 400 mg daily. At 12 months of follow-up, patients assigned to dasatinib therapy had significantly higher rates of confirmed CCyR (77% versus 66%) and MMR (46% versus 28%) (737). However a subsequent report demonstrated that the difference in CCyR had lost statistical significance by 24 months (85 versus 82%), but that the improvement in rates of MMR (64% versus 46%) remained (738). Additionally there was no significant difference in PFS (94% versus 92%), failure-free survival (91% versus 88%), or OS (95% for both). Consistent with earlier experience, dasatinib therapy was associated with lower rates of nausea, vomiting, rash, and myalgia, but higher rates of thrombocytopaenia and pleural effusions than imatinib. In summary when compared with imatinib in the frontline setting in CML-CP, dasatinib produces faster, deeper responses with a different side effect profile. Dasatinib has not been directly compared with nilotinib in the frontline setting. Dasatinib at 100 mg once daily has been granted accelerated approval by the FDA for the treatment of newly diagnosed CML.

Bosutinib

Bosutinib (previously SKI-606) is a dual kinase inhibitor that targets both ABL and SRC pathways, but does not target c-Kit or PDGFR. In preclinical in vitro testing, bosutinib retained activity in cells where resistance to imatinib was caused by BCR-ABL gene amplification as well as in CML cell lines carrying the Y253F, E255K, and D276G mutations (508).

Bosutinib (500 mg once daily) was evaluated in a phase 1-2 trial in 288 patients with CML-CP with imatinib resistance (69%) or intolerance (739). After

a median follow-up of 24 months, rates of CHR, MCyR, and CCyR were 86%, 53%, and 41%, respectively. Of the patients achieving a CCyR, 64% attained a MMR. The most common severe (CTCAE grade 3/4) toxicities included diarrhea (9%), rash (9%), and vomiting (3%).

A subset analysis of this trial evaluated the 118 patients with CML-CP who had been initially treated with imatinib followed by dasatinib and/or nilotinib prior to treatment with bosutinib (740). A MCyR was seen in 32% and a CCyR in 24% of patients. After a median follow-up of 28.5 months, estimated two-year rates of PFS and OS were 73 and 83%, respectively. In summary this study demonstrates that more than half of patients with resistance or intolerance to imatinib will attain a CCyR after treatment with bosutinib but response rates are lower among patients who have previously received two TKIs.

Ponatinib

Ponatinib (formerly called AP24534) is a TKI that was developed using chemical modification of a purine scaffold (741). A carbon–carbon triple bond extends from the purine scaffold allowing the molecule to take up a position with no steric hindrance attributable to the T315I mutation. The substructure beyond the triple bond is quite similar to that of imatinib. Ponatinib was tested *in vitro* and inhibited a variety of tyrosine kinases, including SRC and ABL. Moreover cell-based mutagenesis screens showed that ponatinib, when administered at pharmacologically realistic drug levels, suppressed the growth of all BCR-ABL mutant subclones (742).

In a phase 1 study ponatinib at higher dose levels induced CCyRs and MMRs in patients with CML whose disease was resistant to earlier treatment with two or more TKIs. In particular patients whose resistance could be attributed to the presence of a T315I subclone also had a response. In most such patients, the response seemed to be durable. Of 12 patients who had CML-CP with the T315I mutation, 100% had a CHR and 92% had a MCyR. Of 13 patients with CML-CP without detectable mutations, 100% had a CHR and 62% had a MCyR. Responses among patients with CML-CP were durable. Of

22 patients with CML-AP or CML-BC or Ph-positive ALL, 36% had a MHR and 32% had a MCyR. The major side effect of this drug was dose-related pancreatitis, which was manageable in all cases.

The phase 1 study led to a recommended clinical dose of 45 mg daily. It was followed by the PACE (Ponatinib for CML evaluation and Ph+ ALL) trial, a multicenter, international, single-arm clinical trial of 449 patients with disease that was resistant or intolerant to prior TKI therapy. The primary endpoints were MCyR for patients with CML-CP and major haematologic response (MaHR) for patients with CML-AP, CML-BC or Ph+ALL. MaHR was defined as CHR or no evidence of leukaemia (NEL). Preliminary results of a phase 2 trial of ponatinib reported MCyR in 54% of the patients with CML-CP. Importantly 75% of the CML-CP patients with T315I mutations had MCyR following ponatinib (306). The efficacy results demonstrated a 54% MCyR rate in patients with CML-CP. For patients with CML-AP, CML-BC and Ph+ ALL, the MaHR rates were 52%, 31% and 41%, respectively. On December 14, 2012, the FDA granted accelerated approval to ponatinib for the treatment of adult patients with CML-CP, CML-AP, CML-BC or Ph+ALL that is resistant or intolerant to prior TKI therapy.

Lastly ponatinib functions as a multikinase inhibitor with activity against other tyrosine kinases, including Kit, PDFGRA, FGFR1, and FLT3 (743), and could therefore be useful in treating patients with these gene mutations.

Appendix B: Cytogenetic abnormalities in AML

AML with t(8;21)

AML with the t(8;21)(q22;q22); RUNX1-RUNX1T1 (previously AML1-ETO) is seen in approximately 7% of adults with newly diagnosed AML and is the most common cytogenetic abnormality in children with AML (Figure 1.12) (361). This translocation is most common in patients with AML-M2 but is also found in AML-M4 (88, 744). Myeloblasts of AML with the t(8;21) have a morphologically distinct phenotype with indented nuclei, basophilic cytoplasm, prominent paranuclear hof and easily identifiable auer rods (745).

In AML with the t(8;21), the *RUNX1* (previously AML1 or core binding factor alpha-2) gene on chromosome 21 and the *RUNX1T1* (previously *ETO* or *MTG8*) gene on chromosome 8 form a chimeric product that regulates the transcription of a number of genes that are vital for haematopoietic stem cell and progenitor cell growth, differentiation, and function (89-91). Leukaemogenesis by *RUNX1-RUNX1T1* probably results from both altered transcriptional regulation of normal RUNX1 target genes and activation of new target genes that block apoptosis and cellular differentiation pathways (746).

The median age in adults with t(8;21) is approximately 25 to 30 years, significantly younger than that for the total group of adults with AML (747, 748). The CR rate is uniformly high and with intensive post remission consolidation chemotherapy, the expected DFS exceeds two years, after which time relapses are uncommon (92, 361).

t(15;17) translocation in APL

APL is a unique clinicopathological entity characterized by the infiltration of the BM by promyelocytes in association with clinical or laboratory evidence of disseminated intravascular coagulation and fibrinolysis. It is characterized by the balanced translocation t(15;17)(q24.1;q21.1) (749). This rearrangement is seen in 13% of newly diagnosed AML and is highly specific for APL (749). On the

other hand it is not found in patients with other leukaemias or solid tumours, although there are rare cases of t(15;17) occurring in CML, coincident with development of a promyelocytic blast crisis.

The breakpoint on chromosome 17 occurs within the first intron of the *RARA* gene in most patients, whereas the break on chromosome 15 occurs within the *PML* gene (99, 398). The translocation results in a *PML/RARA* fusion gene that contains most of the *PML* coding sequences, and the DNA binding and ligand binding domains of the *RARA* gene. The PML/RARA fusion protein shows reduced sensitivity to retinoic acid in terms of dissociation of N-CoR, a ubiquitous nuclear protein that mediates transcriptional repression (398, 750). This leads to persistent transcriptional repression, thereby preventing differentiation of promyelocytes.

Inv(16) and t(16;16)

Abnormalities of chromosome 16 are seen in approximately 7% of adults with newly diagnosed AML (Figure 3.1) (361). Patients with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) have a favourable prognosis with standard therapy, whereas those with other abnormalities of chromosome 16 do not (361). AML with inv(16) typically demonstrates monocytic and granulocytic differentiation with abnormal eosinophils in the BM (361, 751, 752). Different abnormalities in chromosome 16 were identified in these reports including a pericentric inversion of chromosome 16, inv(16)(p13.1q22) or a reciprocal translocation involving both chromosome 16 homologs, t(16;16)(p13.1;q22)(752, 753).

The inversion breakpoint at 16q22 occurs near the end of the coding region of the core binding factor beta (*CBFB*) gene (93), which encodes one subunit of the heterodimeric *RUNX1*/*CBFB* transcription factor (754). As described above, this transcription factor binds directly to an enhancer core motif that is present in the transcriptional regulatory regions of a number of genes that are critical to myeloid cell growth, differentiation, and function. A smooth muscle myosin heavy chain gene (*MYH11*) is interrupted by the breakpoint on 16p. A fusion protein is produced containing the 5' region of

CBFB (165 of 182 amino acids), including the domain that heterodimerizes with *RUNX1*, fused to the 3' portion of *MYH11* (93, 755). The CBFB/MYH11 fusion protein appears to act by disrupting the function of the *RUNX1/CBFB* transcription factor, resulting in the repression of transcription (756). Patients with inv(16) or t(16;16) have a good response to intensive chemotherapy (92, 361, 757). For example in one study patients with inv(16) or t(16;16) had CR rates and 10-year survival of 92 and 55%, respectively (361).

Rearrangements of 11q23

Rearrangements of 11q are seen in approximately 6% of young adults with newly diagnosed AML and up to 12% of children with AML (361). These rearrangements of 11q are common (35%) in patients with AML-M5 particularly those with poorly differentiated monoblasts (AML-M5a) (758). This association with M5a is particularly strong in children (759). Translocations of 11q23 involve the *MLL* gene (myeloid/lymphoid, or mixed-lineage, leukaemia) (760-763). The MLL protein has homology to the Drosophila trithorax gene product, a transcription factor that regulates embryonic development and tissue differentiation in this organism. MLL is a DNA-binding protein that methylates histone H3 lysine 4 (H3K4), and positively regulates gene expression by binding to open chromatin structures at the active promoter regions of various genes, including multiple HOX genes, that are important in haematopoietic and lymphoid cell development, including myelomonocytic differentiation (764-766). An important feature of MLL fusion proteins is their ability to efficiently transform haematopoietic cells into LSCs (767, 768). In addition, a molecular rearrangement leading to a partial tandem duplication of the MLL gene has been found in 11% of patients with AML and a normal karyotype and in approximately 90% of adults with AML who have trisomy 11 as the only karyotypic abnormality (769). In general, leukaemia patients with 11q23/MLL rearrangements, have a very dismal prognosis (770). In contrast, patients with t(9;11)(p22;q23) which occurs in 1% of AML cases tend to have an intermediate response to standard therapy (361). Balanced translocations occur in therapy

related AML post DNA topoisomerase II inhibitors most often involve the MLL gene at 11q23 (771).

t(3;3) and inv(3)

The t(3;3) and inv(3) account for approximately 1% of AML cases (361). This abnormality is seen in de novo AML and in therapy-related MDS/AML. Cytogenetic abnormalities of 3q are associated with thrombocytosis in the PB and increased atypical megakaryocytes in the BM of patients with AML (772-774). The specific cytogenetic abnormalities involve bands 3q21 and 3q26.2 simultaneously, and they include the inv(3)(q21q26.2), t(3;3)(q21;q26.2), and the ins(5;3)(q14;q21q26.2) (insertion of chromosomal material from 3q into 5q).In a study of 6515 adults with newly diagnosed AML enrolled in prospective trials 3q abnormalities were detected in 4.4% of cases (775). Of these, t(3;3)/inv(3), t(3q26.2), t(3q21), and other 3q abnormalities accounted for 32, 18, 7, and 43%, respectively. Abnormalities of 3q are associated with a poor prognosis with rates of CR, OS and relapse-free survival at five years of 31, 6, and 4%, respectively. The t(3;3) and inv(3) abnormalities seen in AML result in the activation of the *EVI1* (*MECOM*) gene, located at 3q26.2 (776). EVI1 can act as a transcriptional activator to promote the proliferation of HSCs (eg, when bound to GATA2) or as a transcriptional repressor inhibiting erythroid differentiation (eg, when bound to GATA1).

t(6;9)(p23;q34)

AML with t(6;9)(p23;q34), *DEK-NUP214*, is seen in approximately 1% of patients with newly diagnosed AML (361). The pathological features of AML with the t(6;9) include basophilia, single or multilineage dysplasia, variable FAB morphology, a CD13+, CD33+, CD38+, CD45+, and HLA-DR+ phenotype, and a high incidence (71%) of FLT3-ITD. (777). The translocation results in the juxtaposition of *DEK* gene on chromosome 6 with *NUP214* (also known as CAN) on chromosome 9. This results in the creation of a nucleoporin fusion protein that acts as a transcription factor and also alters nuclear transport.

Patients with the t(6;9)(p23;q34) typically have a poor outcome with standard therapy.

Chromosomal gain and loss

In addition to chromosomal translocations, there is a recurring pattern of gain or loss of specific chromosomes in patients with AML. Any chromosome can be affected, but with variable frequency. Some chromosomes are more likely to have a gain or loss. The most common abnormalities are gain of chromosome 8 (trisomy 8, 13%), loss of chromosome 7 (9%), and loss of chromosome 5 (6%). Abnormalities of chromosomes 5 and 7 are particularly characteristic of therapy-related AML induced by alkylating agents and/or radiation therapy and are associated with a poor prognosis (362).

Appendix C

J. Cell. Mol. Med. Vol XX, No XX, 2010 pp. 1-14

The novel Aurora A kinase inhibitor MLN8237 is active in resistant chronic myeloid leukaemia and significantly increases the efficacy of nilotinib

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Received: May 20, 2010; Accepted: October 12, 2010

Abstract

Novel therapies are urgently needed to prevent and treat tyrosine kinase inhibitor resistance in chronic myeloid leukaemia (CML). MLN8237 is a novel Aurora A kinase inhibitor under investigation in multiple phase I and II studies. Here we report that MLN8237 possessed equipotent activity against Ba/F3 cells and primary CML cells expressing unmutated and mutated forms of BCR-ABL. Notably, this agent retained high activity against the T315I and E255K BCR-ABL mutations, which confer the greatest degree of resistance to standard therapy. MLN8237 treatment disrupted cell cycle kinetics, induced apoptosis, caused a dose-dependent reduction in the expression of the large inhibitor of apoptosis protein Apollon, and produced a morphological phenotype consistent with Aurora A kinase inhibition. In contrast to other Aurora kinase inhibitors, MLN8237 did not significantly affect BCR-ABL activity. Moreover, inhibition of Aurora A with MLN8237 significantly increased the *in vitro* and *in vivo* efficacy of nilotinib. Targeted knockdown of Apollon sensitized CML cells to nilotinib-induced apoptosis, indicating that this is an important factor underlying MLN8237's ability to increase the efficacy of nilotinib. Our collective data demonstrate that this combination strategy represents a novel therapeutic approach for refractory CML that has the potential to suppress the emergence of T315I mutated CML clones.

Keywords: apoptosis • cancer • BCR-ABL • CML • aurora kinase A • kinase inhibitors

Introduction

Imatinib targets the constitutively active BCR-ABL tyrosine kinase in chronic myeloid leukaemia (CML) and has become standard treatment based on excellent responses achieved in clinical trials [1–3]. However, imatinib resistance can occur through several mechanisms including BCR-ABL kinase domain mutations, amplification, overexpression and clonal evolution [4]. Successful strategies to overcome imatinib resistance include dose escalation or the use of second-generation BCR-ABL kinase inhibitors including nilotinib, dasatinib or bosutinib [5–7]. However, none of these agents are effective in CML cells harbouring the 'gatekeeper' T315I mutation at the base of the ATP binding pocket, which occurs in up to 20% of imatinib resistance cases.

The discovery that Aurora kinases were abnormally expressed in malignancies including leukaemia prompted the development of agents that inhibit their activity [8–10]. The pan-Aurora kinase inhibitors, MK-0457 and danusertib (PHA-739358) have shown pre-clinical and clinical activity against CML harbouring the BCR-ABL T315I mutation [10–13]. The anti-leukaemia efficacy of MK-0457 in CML was originally attributed to direct inhibition of BCR-ABL kinase activity [14, 15]. However, a recent study demonstrated that the efficacy of MK-0457 at clinically relevant doses in BCR-ABL$^+$ cells was primarily due to inhibition of Aurora, rather than BCR-ABL, kinase activity [16]. The development of MK-0457 was ceased due to problems with

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doi:10.1111/j.1582-4934.2010.01218.x

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cardiac toxicity observed in some patients during early phase clinical trials with the compound. In spite of this, the clinical responses achieved by MK-0457 in refractory CML patients have served to maintain interest in targeting Aurora kinases for CML therapy and a significant effort is currently being put forth to develop new agents that inhibit Aurora kinase activity and lack undesired cardiac side effects.

Aurora A kinase is a central mitotic regulator necessary for mitotic entry, mitotic spindle assembly and accurate chromosome separation [17–19]. The therapeutic potential of specifically targeting Aurora A kinase activity as an anticancer strategy has not been rigorously investigated because all of the agents previously designed to target Aurora kinases have significant off-target effects on other family members and/or BCR-ABL kinase activity. MLN8237 is a novel, highly selective ATP-competitive and reversible inhibitor of Aurora A kinase with an *in vitro* inhibition constant (Ki) of 0.43 nM [20]. It has a benzazepine core scaffold and is orally available. It is approximately 200-fold more selective for Aurora A kinase than the structurally related family member, Aurora B kinase. Moreover, MLN8237 is selective for Aurora A kinase when compared to most other kinases and receptors. It has shown broad-spectrum anticancer activity in preclinical models and is currently undergoing early clinical evaluation in solid tumours and hemelymphatic malignancies.

We suggested that MLN8237-mediated inhibition of Aurora A kinase activity would abrogate the growth and survival of CML cells in a manner independent of BCR-ABL mutation status. Our results indicate that MLN8237 impairs growth, disrupts cell cycle kinetics, induces a cellular phenotype consistent with Aurora A kinase inhibition and triggers apoptosis in CML cell lines and primary human resistant CML cells including those bearing the drug resistance conferring T315I mutation. Furthermore, MLN8237 significantly increases the anticancer activity of the standard agent nilotinib through a mechanism involving down-regulation of the apoptotic and mitotic regulator, Apollon. Our collective data demonstrate that MLN8237 is a promising novel agent for the treatment of refractory CML that warrants further investigation.

Materials and methods

Cells and cell culture

Ba/F3 cells with wild-type (p210) BCR-ABL with and without stable shRNA p53 knockdown and T315I, E255K, H396P, Y253F, M351T and Q252H mutant forms of BCR-ABL, LAMA 84, K562 cells and imatinib-resistant K562 cells were maintained as previously described [21, 22]. Primary human CML cells were obtained from the peripheral blood of imatinib-resistant CML patients after obtaining informed consent in accordance with an approved institutional IRB protocol. Normal $CD34^+$ bone marrow cells were purchased from Stem Cell Technologies (Vancouver, British Columbia, Canada).

Chemicals and reagents

Reagents were obtained from: MLN8237 (Millennium Pharmaceuticals, Cambridge, MA, USA), nilotinib (Novartis), anti-actin, anti-active caspase-3, anti-phospho-Aurora A, anti-Aurora A phospho-BCR, phospho-CRKL, and CRKL antibodies (Cell Signaling), anti-β tubulin (Sigma), anti-Apollon antibody (Bethyl Laboratories) and sheep antimouse-HRP and donkey anti-rabbit-HRP antibodies (Amersham).

Enzyme assays

MLN8237 was screened against a subset of Invitrogen's SelectScreen™ kinase panel at concentrations ranging between 10 and 0.00051 μM in 3-fold serial dilutions. The enzymes screened included ABL1, ABL1 E255K, ABL1 G250E, ABL1 T315I, ABL1 Y253F, ABL12 and Aurora A, each at the respective apparent ATP Km.

Analysis of cell cycle effects and apoptosis

Apoptosis was evaluated by PI/FACS analysis of sub-G_0/G_1 DNA content as previously described [21, 23].

MTT assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [21].

Colony assays

K562, LAMA 84, normal $CD34^+$ bone marrow or primary CML cells were treated for 24 hrs with the indicated concentrations of MLN8237 and nilotinib and then washed twice in PBS, seeded in Methocult methylcellulose containing medium (Stem Cell Technologies), incubated and scored as previously described [24].

Immunoblotting

CML cells were incubated with MLN8237, nilotinib or the combination for 24 hrs. Cells were then lysed and subjected to SDS-PAGE as previously described [24].

In vivo evaluation of MLN8237 and nilotinib

K562 and Ba/F3 cells were harvested, washed in PBS, and suspended in a mixture of HBSS and Matrigel (BD BioSciences). An *in vivo* model of CML was generated by injecting K562 or Ba/F3 cells expressing wild-type (p210) or T315I mutant forms of BCR-ABL into the flanks of female nude mice. After tumour growth reached 150 mm^3, mice were randomly assigned to receive MLN8237 20 mg/kg BID ($n = 10$), Nilotinib 50 mg/kg once daily ($n = 10$), vehicle control ($n = 10$) or both MLN8237 and Nilotinib ($n = 10$) for 14 days. Mice were monitored daily and tumour

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volumes were measured twice weekly. At the completion of the study, tumours were excised, formalin-fixed and paraffin-embedded for immunohistochemical analysis.

Immunohistochemistry

Paraffin-embedded tumour sections (4–6 μm thick) were mounted on slides and stained with haematoxylin and eosin as previously described [25].

Terminal deoxyribonucleotide-transferase–mediated dUTP nick-end labeling assay (TUNEL)

TUNEL staining and quantification were performed as previously described [25].

shRNA knockdown of p53

Ba/F3 p210 cells were infected with a retrovirus encoding a short hairpin RNA (shRNA) sequence specific for the knockdown of murine p53 or an empty vector control as previously described [26].

siRNA transfection

Apollon and Aurora kinase A SMARTpool or siCONTROL siRNA directed at luciferase (Dharmacon) were transfected into CML cells as previously described using the Nucleofector II according to the manufacturer's instructions (Amaxa, Inc.) [26]. Transfected cells were treated with the indicated concentrations of MLN8237 and nilotinib for 48 hrs. Drug-induced apoptosis was quantified by PI/FACS as described above.

Statistical analyses

Statistical significance of differences observed between samples was determined using the Tukey–Kramer comparison test or the Student's t-test. Differences were considered significant in all experiments at $P < 0.05$.

Results

MLN8237 impairs growth, disrupts cell cycle kinetics and induces apoptosis in CML cell lines

Treatment with MLN8237 inhibited the *in vitro* growth and survival of the human K562 and LAMA 84 CML cell lines with IC_{50} values less than 100 nM (Fig. 1A). Considering that inhibition of the Aurora kinases results in mixed outcome, including polyploidy and G_2/M growth arrest, we assessed the cell cycle distribution and apoptotic fraction (sub-G_0/G_1) of cells treated with MLN8237 by propidium iodide staining and flow cytometry. MLN8237 treatment disrupted cell cycle kinetics as demonstrated by the accumulation of cells in G_2/M phase and cells with >4N DNA prior to the onset of apoptosis (sub-G_0/G_1) in a dose- and time-dependent manner (Fig. 1B, C).

MLN8237 has *in vitro* and *in vivo* antiproliferative effects in imatinib-resistant cells and its activity is unaffected by impairment of p53 function

Loss or mutation of the tumour suppressor gene *TP53* occurs in over 30% of cases of CML blast crisis and can impede the response to therapy [27–29]. We evaluated the potential impact of loss of p53 function on cellular sensitivity to MLN8237 by achieving stable shRNA-mediated p53 knockdown in Ba/F3 cells expressing p210 BCR-ABL. Cells were treated with the chemotherapeutic agent vincristine and immunoblotting analyses of the expression of p53 and its direct transcriptional target, p21, were conducted to confirm functional p53 knockdown. Impairment of p53 function did not significantly affect the anticancer activity of MLN8237, indicating that it may be an effective agent for patients with p53 defects (Fig. 2A). To investigate the potential impact of imatinib resistance on the efficacy of MLN8237, we treated Ba/F3 expressing unmutated BCR-ABL and the clinically relevant tyrosine kinase inhibitor resistant BCR-ABL mutants T315I, E255K, H396P, Y253F, M351T and Q252H as well as K562 cells that are sensitive and resistant to imatinib due to differential expression of unmutated BCR-ABL with this agent for 96 hrs. Notably, MLN8237 inhibited the viability of Ba/F3 cells expressing unmutated BCR-ABL and mutated BCR-ABL and imatinib-sensitive and –resistant K562 cells at similar concentrations (Fig. 2B) [22]. We next created an animal model of unmutated and T315I-mutated CML by injecting Ba/F3 cells expressing p210 or T315I-mutated BCR-ABL into the flanks of nude mice to investigate the *in vivo* efficacy of MLN8237 against CML cells bearing the T315I mutation. Consistent with our *in vitro* data, MLN8237 possessed equipotent *in vivo* activity against xenografts of Ba/F3 cells expressing unmutated and imatinib, nilotinib and dasatinib resistant T315I-mutated forms of BCR-ABL (Fig. 2C). We subsequently determined the anti-leukemic effects of MLN8237 against primary CML cells from three imatinib refractory patients, (one each from a patient in chronic phase CML, blast crisis CML and a patient in blast crisis harbouring the T315I mutation) and primary leukaemia cells from a patient with Philadelphia chromosome positive acute lymphocytic leukaemia. MLN8237 equally inhibited the viability of primary human CML cells from patients with unmutated and T315I-mutated BCR-ABL (Fig. 2D). As primary cells do not tend to actively proliferate in culture, higher concentrations of MLN8237 were needed to inhibit their viability compared to CML cell lines. Normal peripheral blood mononuclear cells cultured under the same conditions were less susceptible to the effects of MLN8237 compared to the primary CML cells, thus demonstrating the therapeutic selectivity of this agent (Fig. 2D). Collectively, these results suggest that the activity of MLN8237 in CML cells is unaffected by BCR-ABL mutational status or impairment of p53 function.

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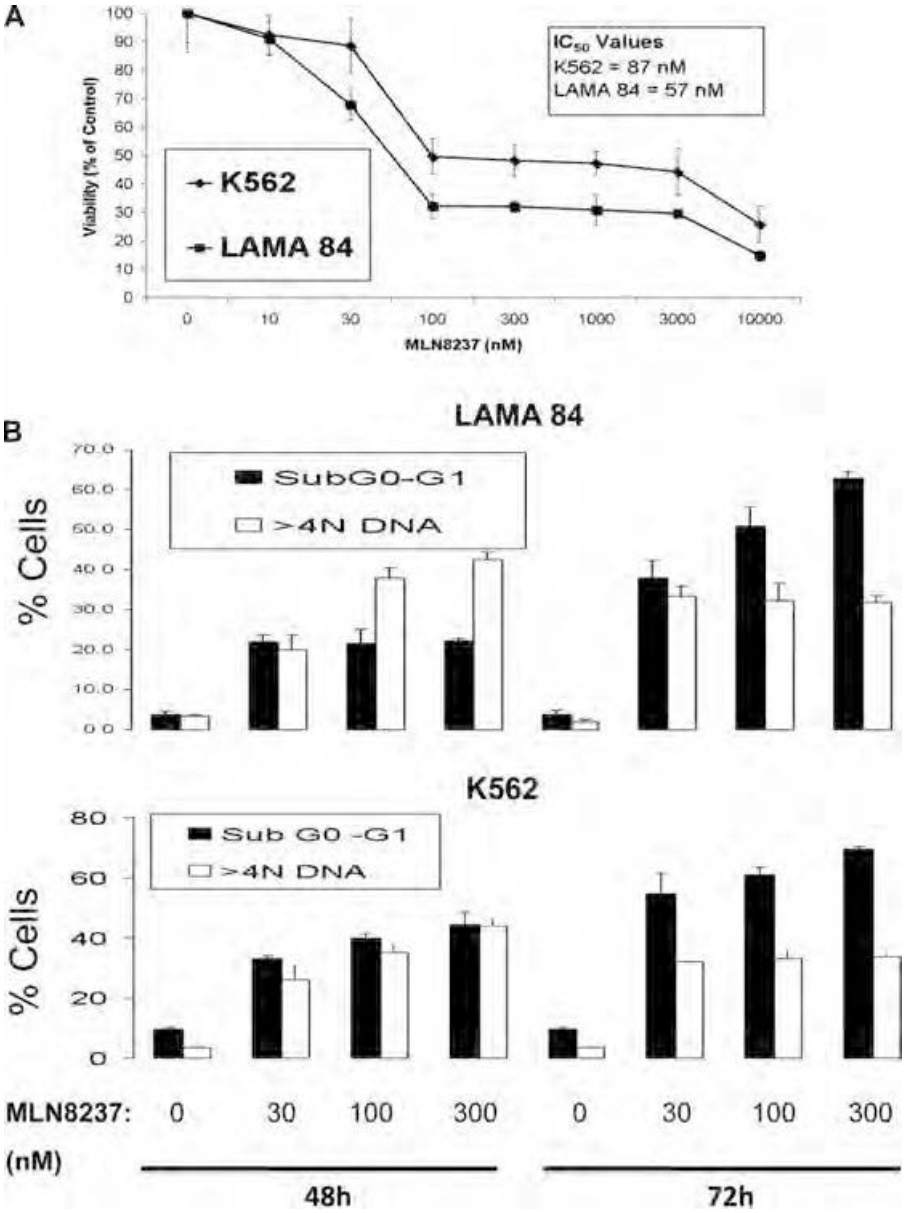

Fig. 1 MLN8237 impairs growth, disrupts cell cycle kinetics and induces apoptosis in CML cell lines. (**A**) Effects of MLN8237 on the *in vitro* growth and survival of K562 and LAMA 84 human CML cell lines. Cells were treated with the indicated concentrations of MLN8237 for 96 hrs and viability was assessed by MTT assay. $n = 3 \pm$ S.D. (**B**)–(**C**) Time-dependent induction of DNA fragmentation. LAMA 84 and K562 cells were treated with 30, 100 or 300 nM MLN8237 for 48 hrs and 72 hrs. Percentages of cells with sub-G_0-G_1 DNA and >4N DNA were determined by PI/FACS. $n = 3 \pm$ S.D.

MLN8237 inhibits autophosphorylation of Aurora A without affecting BCR-ABL activity

We next determined the *in vitro* inhibitory effects of MLN8237 on Aurora A kinase. Exposure of cultured K562 cells to 30 nM MLN8237 reduced the phosphorylation of Aurora A kinase as demonstrated by reduced phosphorylation of Aurora A at Thr288 within its kinase activation loop without affecting the total levels of Aurora A (Fig. 3A). As mentioned earlier, there has been some controversy regarding whether the anti-leukemic activity of MK-0457, which is no longer being clinically developed, was due to inhibition of Aurora kinases, BCR-ABL or both. In contrast to MK-0457, MLN8237 is a potent inhibitor of the Aurora A enzyme ($IC_{50} = 2$ nM) and demonstrated approximately 50–700-fold selectivity against various ABL isoform enzyme assays (Fig. 3B). To confirm that efficacious concentrations of MLN8237 do not significantly affect BCR-ABL activity, we evaluated its effect on BCR-ABL autophosphorylation and phosphorylation of the BCR-ABL direct substrate CRKL, which are accurate predictors of BCR-ABL kinase activity. Consistent with the *in vitro* enzyme assay data, immunoblotting analysis demonstrated that treatment of LAMA 84 and K562 cells with MLN8237 did not have a significant effect on

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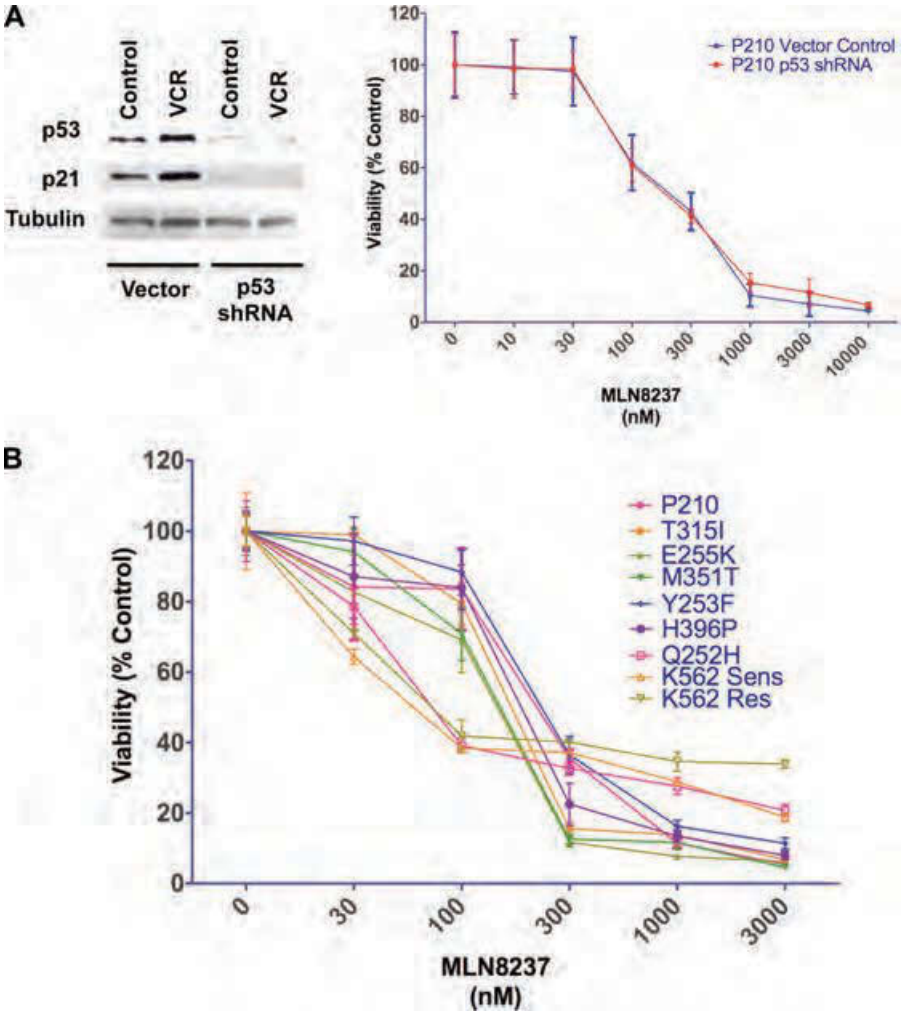

Fig. 2 MLN8237 has *in vitro* and *in vivo* antiproliferative effects in Ba/F3 cells expressing unmutated and mutated BCR-ABL and its activity is unaffected by impairment of p53 function. (**A**) Left, Ba/F3 p210 BCR-ABL cells stably infected with p53 shRNA or vector control were treated with 100 nM vincristine (VCR) for 24 hrs and subjected to immunoblotting for p53 and p21 to confirm functional knockdown efficiency. Tubulin documented equal loading. Right, Ba/F3 p210 BCR-ABL cells stably infected with p53 shRNA or vector control were treated the indicated concentrations of MLN8237 for 96 hrs and viability was assessed by MTT assay. $n = 3 \pm$ S.D. (**B**) MLN8237 has activity in cells expressing unmutated and mutated BCR-ABL. Ba/F3 cells expressing p210 (unmutated) and T315I, E255K, H396P, Y253F, M351T and Q252H mutant forms of BCR-ABL and imatinib-sensitive and –resistant K562 cells were treated with the indicated concentrations of MLN8237 for 96 hrs and viability was assessed by MTT assay. $n = 3 \pm$ S.D. (**C**) *In vivo* efficacy of MLN8237. Immunodeficient mice bearing xenografts of P210 and T315I BCR-ABL expressing Ba/F3 cells were administered MLN8237 (20 mg/kg BID) daily or vehicle control. $n = 10 \pm$ S.D. (**D**) Activity of MLN8237 in primary CML cells and normal peripheral blood mononuclear cells. Cells from healthy donors or patients (4) with BCR-ABL$^+$ leukaemia including 1 patient each with: unmutated BCR-ABL, T315I-mutated BCR-ABL, blast crisis CML and Ph+ ALL were treated with MLN8237 for 96 hrs and cell viability was assessed by MTT assay.

the total levels of BCR-ABL or the levels of phospho-BCR-ABL at its Tyr177 autophosphorylation site (Fig. 3C). We conducted similar experiments to also confirm that the concentration of MLN8237 that we utilized for our experiments with primary patient cells did not significantly affect BCR-ABL kinase activity (Fig. 3D).

Co-treatment with MLN8237 and nilotinib results in significantly greater apoptosis, growth inhibition and reduction in clonogenic survival than what is achieved by either agent alone

Patients with advanced stage CML have been suggested to benefit from non-cross-resistant combinations of tyrosine kinase inhibitors and agents effective against CML cells harbouring the T315I and E255K mutations [30, 31]. Considering that MLN8237 is active against cells expressing the E255K and T315I mutations, we investigated whether MLN8237 augmented the activity of nilotinib, an FDA-approved BCR-ABL inhibitor that is used in CML therapy. LAMA 84 and K562 cells were treated with 30 nM MLN8237, 10 nM nilotinib or the combination for 48 hrs. Percentages of cells with sub-G_0-G_1 DNA were quantified by PI/FACS. Co-treatment with MLN8237 and nilotinib resulted in significantly greater levels of apoptosis as determined by accumulation of sub-G_0-G_1 cells (Fig. 4A). Immunoblotting analysis showed that the combination of these two agents induced mitochondrial-dependent apoptosis as demonstrated by processing of caspases-9 and -3 to their active forms (Fig. 4B). The cytotoxic effects of the combination were also assessed by MTT assay, which demonstrated that inhibition of growth and survival were significantly increased by combination treatment (Fig. 4C). Finally, clonogenic assays were performed to evaluate the prolonged *in vitro* effects

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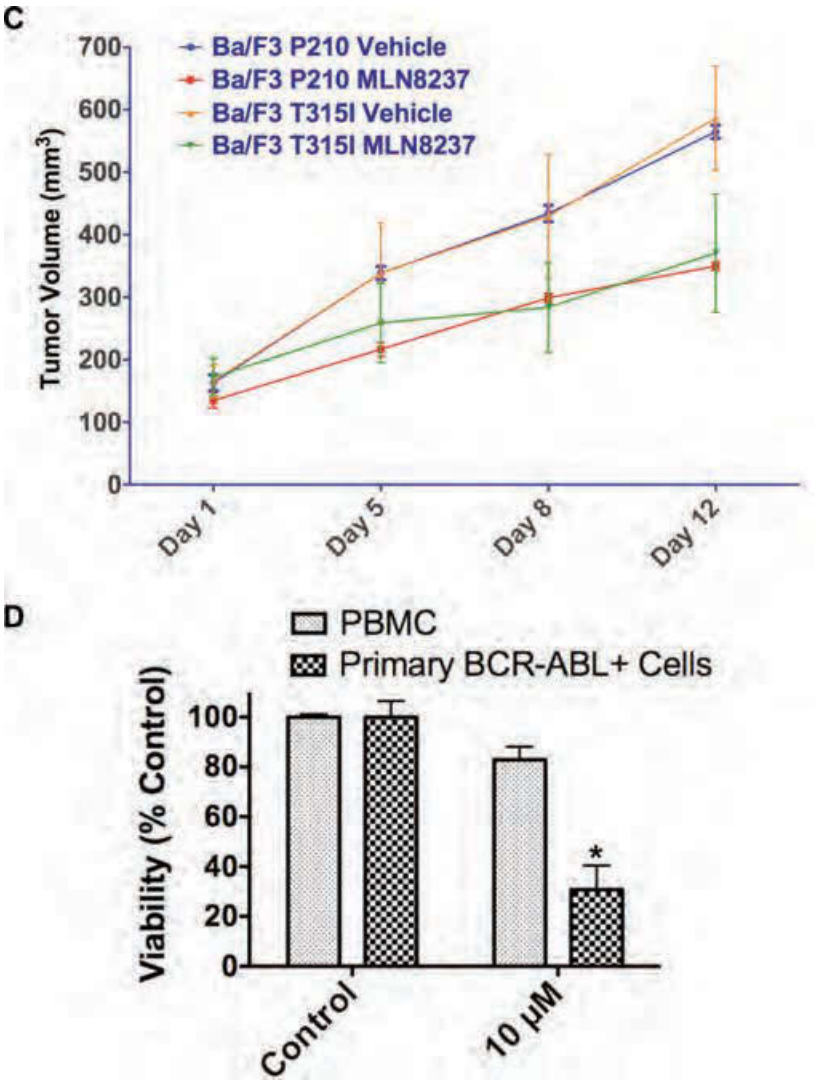

Fig. 2 Continued

of MLN8237 and nilotinib on the growth and survival of normal $CD34^+$ bone marrow cells, primary CML cells from patients in blast crisis, LAMA 84 and K562 cells (Fig. 4D). As expected, MLN8237 enhanced the ability of nilotinib to inhibit the clonogenic survival of CML cells, but had little effect on normal $CD34^+$ cells. Collectively, these data suggest that MLN8237 significantly and selectively enhances the anti-leukaemia activity of nilotinib in human CML cells.

MLN8237 cooperates with nilotinib to reduce tumour burden in K562 xenografts

K562 xenograft studies were carried out to investigate the *in vivo* therapeutic potential of the combination of MLN8237 and nilotinib. Both agents had substantial effects on tumour burden and the combination resulted in significantly greater tumour growth inhibition than what was achieved by either agent alone (Fig. 5A). Furthermore, the combination was well tolerated and only a modest, statistically insignificant loss in body weight was observed in the treated groups (Fig. 5A). Notably, the single agent *in vivo* effects of MLN8237 were more impressive in this K562 experiment than what we observed in our studies with the murine Ba/F3 models (Fig. 2C). Given that human and murine Aurora A genes share 79% sequence homology and that MLN8237 was specifically designed to target human Aurora A kinase activity, it is possible that species-specific differences in the potency of MLN8237-mediated kinase inhibition could have contributed to this phenomenon.

Haematoxylin and eosin staining was used to visualize the architecture of tumours from each treatment group and revealed

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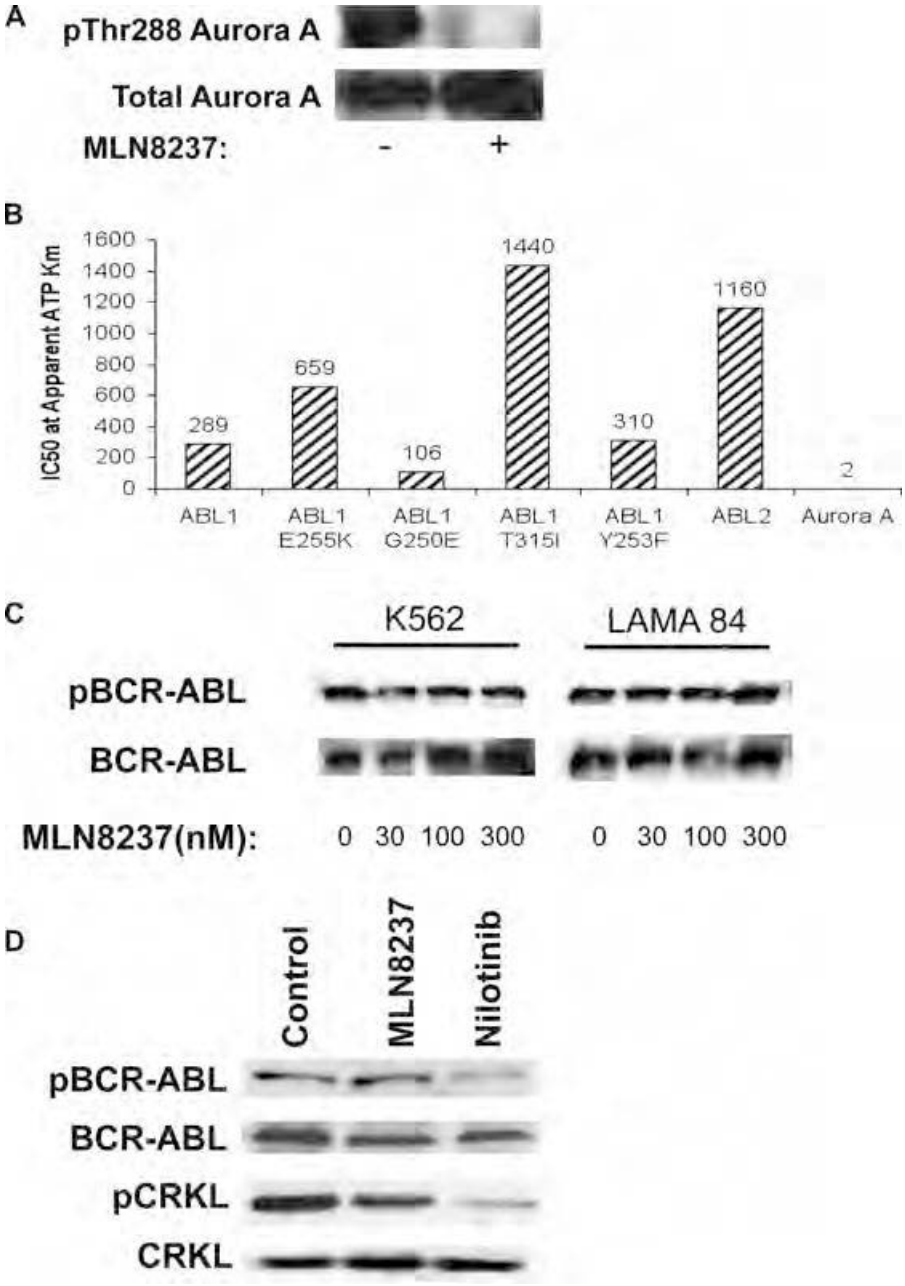

Fig. 3 MLN8237 reduces autophosphorylation of Aurora A without significantly affecting BCR-ABL activity. (**A**) MLN8237 reduces Aurora kinase A phosphorylation. K562 cells were treated with 30 nM MLN8237 for 24 hrs. Protein lysates were subjected to SDS-PAGE, blotted, and probed with phospho-Aurora A (Thr288) and Aurora A antibodies. (**B**) Effects of MLN8237 on the activity of selected kinases. MLN8237 was screened against a kinase panel as described in 'Materials and methods'. The ABL1 and the related ABL2 cytoplasmic tyrosine kinases share 89% sequence identity and have some overlapping functions, but are distinct in that ABL1 fuses with BCR to form the Philadelphia chromosome while ABL2 does not. (**C**) K562 and LAMA 84 cells were treated with MLN8237 for 24 hrs. Protein lysates were subjected to SDS-PAGE, blotted, and probed with phospho-BCR and c-Abl antibodies. (**D**) MLN8237 treatment does not significantly affect BCR-ABL kinase activity in primary CML cells. Primary CML cells obtained from a patient with unmutated BCR-ABL were treated with 10 μM MLN8237 for 24 hrs. BCR-ABL autophosphorylation and CRKL phosphorylation were assessed by immunoblotting. Nilotinib was used as a positive control for BCR-ABL inhibition.

substantial differences in the morphology of single agent and combination-treated tumours. In particular, the tumours treated with the combination of MLN8237 and nilotinib displayed evidence of stromal disruption and high levels of cell death with very few intact CML cells remaining (Fig. 5B). This suggests that remaining tumours from combination-treated mice were largely comprised Matrigel and non-viable cells/tissue and also highlights the potential therapeutic benefit provided by the combination over single agent treatments.

Treatment with MLN8237 *in vivo* leads to a morphological phenotype consistent with inhibition of Aurora A kinase

Aurora A inhibition causes defects in centrosome segregation, spindle pole organization and chromosome congression. This can ultimately lead to tumour cell death *via* the development of

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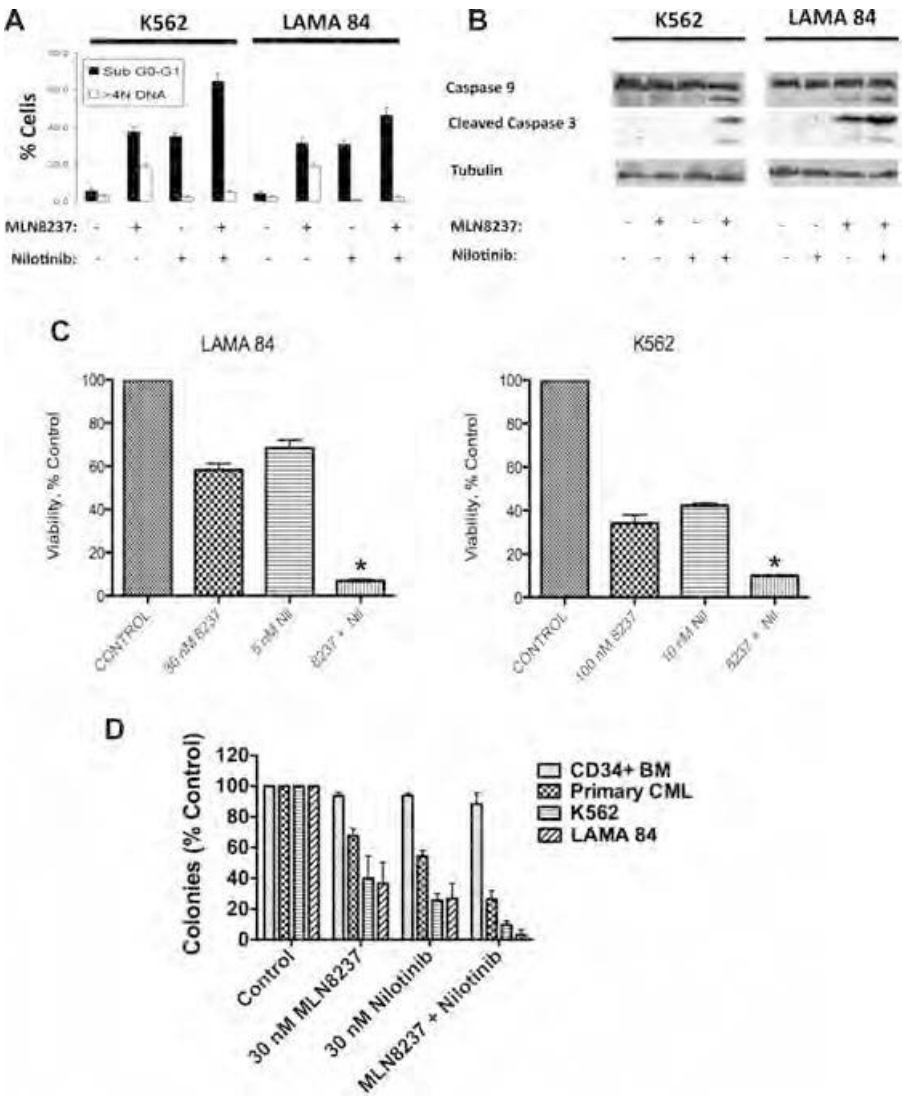

Fig. 4 MLN8237 significantly increases the efficacy of nilotinib. (**A**) MLN8237 potentiates the pro-apoptotic effects of nilotinib. LAMA 84 and K562 cells were treated with 30 nM MLN8237, 10 nM nilotinib or the combination for 48 hrs. Percentages of cells with sub-G_0-G_1 DNA were determined by PI/FACS. $n = 3 \pm$ S.D. (**B**) The combination of MLN8237 and nilotinib induces mitochondrial-dependent apoptosis. K562 and LAMA 84 cells were treated with 100 nM MLN8237, 30 nM nilotinib or both for 24 hrs. Protein lysates were subjected to SDS-PAGE, blotted, and probed with active caspase-3 and caspase-9 antibodies. Anti β-tubulin was used as a loading control. (**C**) Co-treatment with MLN8237 and nilotinib results in significantly greater growth inhibition and reduction in survival than that achieved by either agent alone. Cells were treated with the indicated concentrations of MLN8237 for 96 hrs and viability was assessed by MTT assay. Error bars indicate the S.D. $^*P < 0.05$ (**D**) Effects of MLN8237 and nilotinib on clonogenic survival. $CD34^+$ normal bone marrow ($n = 3$), primary CML from patients in blast crisis ($n = 3$), K562 and LAMA 84 cells were treated with MLN8237, nilotinib or both drugs for 24 hrs. Cells were plated and scored as described in the 'Materials and methods'.

deleterious aneuploidy [19]. Consistent with our *in vitro* data, the number of multi-nucleated cells visible in the MLN8237-treated tumours stained with haematoxylin and eosin was increased as compared with vehicle control indicating that MLN8237 causes cells to exit mitosis without completing cytokinesis, a process known as mitotic slippage (Fig. 5C, second from the left). Although this outcome is primarily associated with inhibition of Aurora B, cytokinesis failure can occur upon inhibition of Aurora A as well [18]. Other functional consequences of Aurora A inhibition that were prominent in the MLN8237-treated tumours (Fig. 5C) included an elevated number of cells with chromatin bridging (far left), mitotic slippage (second from left), cells with internuclear bridging (second from right) and monopolar mitotic spindles (far right). Taken together, these findings indicate that treatment with MLN8237 results in morphological changes in CML cells that are consistent with Aurora A kinase inhibition and indicative of deleterious aneuploidy.

MLN8237 augments the *in vivo* pro-apoptotic effects of nilotinib

TUNEL assays were carried out on xenograft tumour sections after completion of treatment to assess the degree of apoptosis induced by MLN8237 and nilotinib *in vivo*. The percentage of $TUNEL^+$ cells was significantly greater in the combination group compared to treatment with either agent alone indicating that the two agents cooperate to provoke apoptosis *in vivo* (Fig. 5D).

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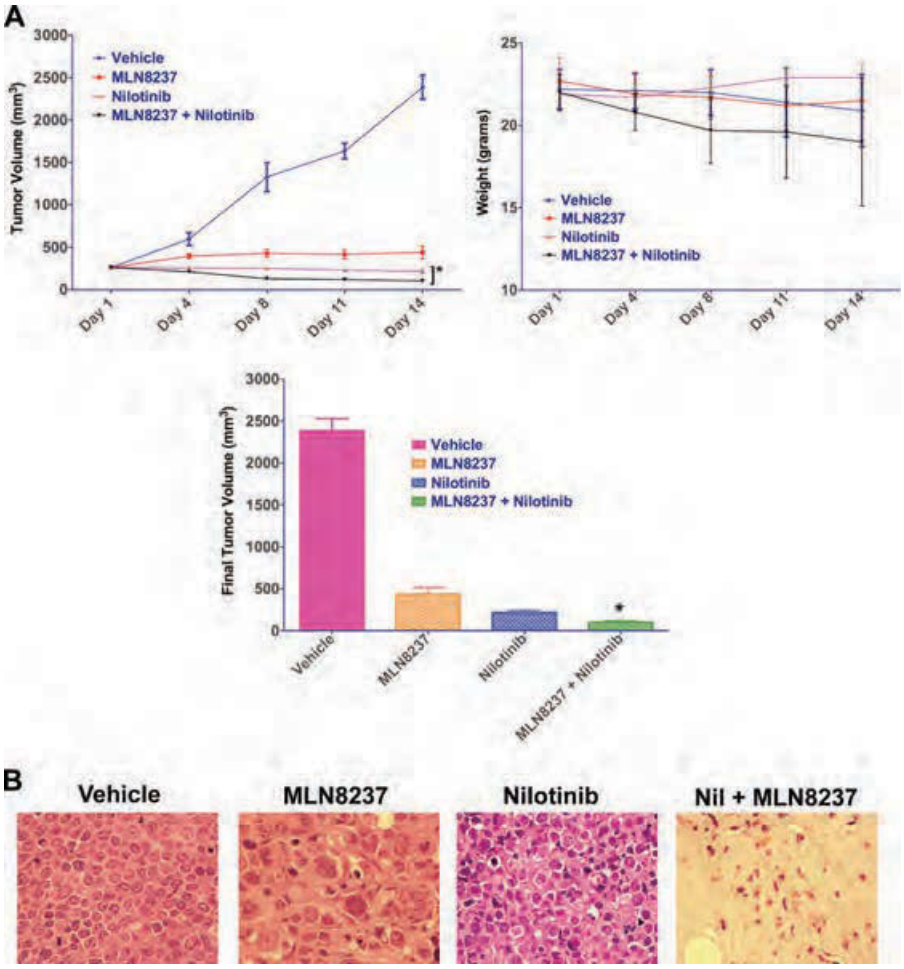

Fig. 5 *In vivo* efficacy and tolerability of MLN8237 and nilotinib. (**A**) K562 cells were injected into the flanks of nude mice. Vehicle, MLN8237, nilotinib or both were administered for 14 days. $n = 10 \pm$ S.D. $^*P = 0.00028$. (**B**) Immunohistochemistry. Tumours were stained with haematoxylin and eosin as described in 'Materials and methods'. Representative images are shown from each treatment group. (**C**) Treatment with MLN8237 leads to a morphological phenotype consistent with Aurora A kinase inhibition. Tumours were stained with haematoxylin and eosin. Representative images from the MLN8237 treatment group are shown. Arrows indicate the following: an elevated number of cells with chromatin bridging (far left), mitotic slippage (second from left), cells with internuclear bridging (second from right) and monopolar mitotic spindles (far right). (**D**) Quantification of TUNEL$^+$ cells. Positive cells were scored manually under 20× magnification. Mean ± S.D., $n = 5$. $^*P < 0.05$.

Inhibition of Aurora A activity leads to reduced expression of the IAP and mitotic regulator Apollon and this effect sensitizes cells to nilotinib-induced apoptosis

Apollon (also known as BRUCE or BIRC6, baculovirus inhibitor of apoptosis protein (IAP) repeats (BIR)-containing protein 6) is a very large (528 kD) IAP that can be distinguished from other IAP family members by the presence of its ubiquitin conjugating enzyme (UCE) domains, which are not contained within any other IAPs [32]. The UCE domains within Apollon allow it to reduce the pro-apoptotic potential of cells by targeting Smac and caspase-9 for proteasomal degradation in addition to directly binding to caspases and other pro-apoptotic molecules *via* its BIR domain to prevent apoptosis in a manner similar to other IAPs [33, 34]. Apollon is overexpressed in leukaemia and other cancers and has been linked with resistance to chemotherapy [32, 35]. A recent investigation demonstrated that Apollon also has important functions during mitosis. It coordinates multiple events in cytokinesis and moves to the midbody ring during cell division where it serves as a platform for the membrane delivery machinery and mitotic regulators including the Aurora kinases [36]. Apollon depletion causes defective abscission and cytokinesis-associated apoptosis [36].

Considering that Apollon is associated with Aurora kinases and has roles in cell division and inhibition of apoptosis, we investigated whether abrogation of Aurora A kinase activity would affect Apollon expression. Immunoblotting analysis showed that MLN8237 treatment resulted in a dose-dependent reduction in the expression of Apollon and increased expression of the pro-apoptotic Apollon substrate, Smac (Fig. 6A). To confirm that this reduction in Apollon expression is a direct consequence of Aurora A kinase inhibition, we used siRNA to knockdown Aurora A expression. This led to a very significant reduction in the levels of Apollon (Fig. 6B). This effect does not appear to be a general feature of mitotic disruption as treatment with nocodazole and vincristine did not significantly decrease Apollon expression and therefore rather suggests a link between Aurora A activity and Apollon expression (Fig. 6C).

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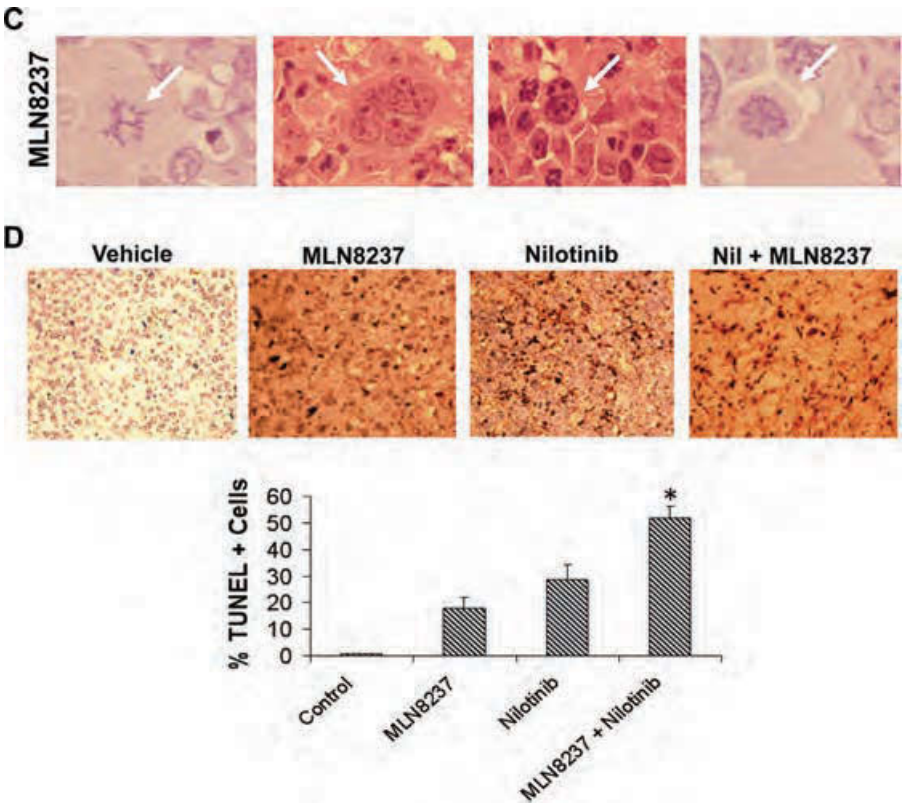

Fig. 5 Continued

To determine whether inhibition of Apollon by MLN8237 significantly contributes to its ability to sensitize CML cells to nilotinib, we knocked down Apollon expression in LAMA 84 cells using siRNA (Fig. 6D). Nilotinib induced significantly greater levels of apoptosis in LAMA 84 cells treated with Apollon-targeted siRNA compared to non-targeted controls (Fig. 6D). These data suggest that inhibition of Apollon expression caused by MLN8237 treatment sensitizes CML cells to nilotinib-induced apoptosis and provides a rationale for the combination of these two agents in CML.

Discussion

Resistance to tyrosine kinase inhibitor therapy in CML continues to be a significant problem. In particular, the T315I and E255K mutations in BCR-ABL confer cross-resistance to imatinib, dasatinib and nilotinib [30]. Several investigational agents have demonstrated preclinical efficacy in T315I-mutated CML cells. The pan-Aurora kinase inhibitor MK-0457 has shown clinical activity against CML cells harbouring the T315I mutation [11]. Early *in vitro* competition binding assays revealed that MK-0457 bound to wild-type ABL1 and T315I ABL1 [37, 38]. This observation led to the hypothesis that the anti-leukemic efficacy of MK-0457 was due to inhibition of BCR-ABL, rather than Aurora, activity [14, 15]. However, a more recent investigation revealed that the efficacy of clinically relevant concentrations of MK-0457 was primarily due to inhibition of Aurora kinase activity [16]. How significantly Aurora kinase inhibition contributes to the activity of MK-0457 in CML remains somewhat controversial. This matter will not be definitively resolved for MK-0457 as its development has been stopped due to issues with cardiac toxicity observed in some early phase clinical trials and will have to be addressed in studies with other Aurora kinase inhibitors. MLN8237 is a highly selective inhibitor of Aurora A ($IC_{50} = 2$ nM) and demonstrates little inhibition of various ABL isoforms in enzyme assays. Our data suggest that MLN8237 does not directly inhibit BCR-ABL activity, indicating that Aurora A kinase is a valid therapeutic target in CML.

The biological consequences of Aurora A kinase inhibition have been intensively investigated in recent years. Aurora A kinase plays an essential role in the assembly and function of the mitotic spindle and disruption of its activity affects spindle pole organization, centrosome separation and chromosome congression [39]. Ultimately, cells treated with Aurora A kinase inhibitors undergo cell death through the development of deleterious aneuploidy [19]. Our data show that MLN8237 treatment initially results in a significant degree of aneuploidy before cell death ensues (Figs 1B, C, 5C). The collective effects of MLN8237 in BCR-ABL$^+$ cells are summarized in Table 1.

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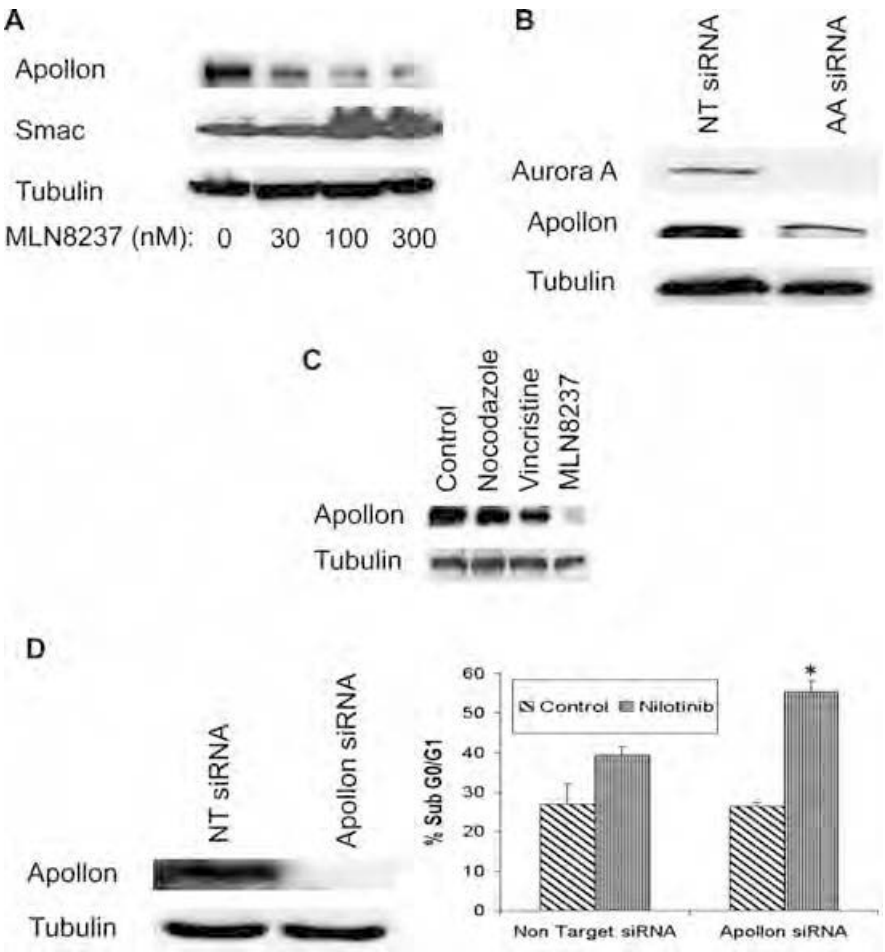

Fig. 6 Targeting Apollon expression sensitizes CML cells to nilotinib-induced apoptosis. (**A**) MLN8237 treatment results in a dose-dependent reduction in the large IAP, Apollon and increased expression of its substrate, Smac. LAMA 84 cells were treated with MLN8237 for 24 hrs. Protein lysates were subjected to SDS-PAGE, blotted, and probed with Apollon and Smac antibodies. Tubulin documented equal loading. (**B**) Aurora A SMARTpool or siCONTROL siRNA directed at luciferase were transfected into LAMA 84 cells using the Nucleofector II. (**C**) General disruption of mitosis does not significantly affect Apollon expression. LAMA 84 cells were treated with nocodazole, vincristine or MLN8237 for 24 hrs. Protein lysates were subjected to SDS-PAGE, blotted, and probed with an Apollon antibody. Tubulin documented equal loading. (**D**) Apollon SMARTpool or siCONTROL siRNA directed at luciferase were transfected into LAMA 84 cells using the Nucleofector II. Tubulin was used as a loading control. LAMA 84 cells transfected with Apollon-targeted siRNA and non-targeted siRNA were treated with nilotinib for 48 hrs and the percentage of apoptotic cells were determined by PI/FACS analysis. $n = 3 \pm$ S.D., $^*P <$ 0.05. (**E**) Schematic depicting the multiple anti-leukaemia properties of the MLN8237. MLN8237 inhibits Aurora A kinase leading to deleterious aneuploidy, inhibition of the IAP, Apollon and cell death.

Table 1 Effects of MLN8237 in BCR-ABL$^+$ cells

Apoptosis	Cell cycle
Activation of caspases-9 and -3	Accumulation of aneuploid cells
Decreased expression of Apollon	Inhibition of colony formation
Increased expression of Smac	Mitotic slippage
Induction of TUNEL$^+$	Monopolar mitotic spindles
DNA fragmentation	Chromatin bridging

Notably, MLN8237-induced apoptosis was associated with significant decreases in the expression levels of Apollon. Apollon has several functional domains, multiple binding partners and plays important roles in the regulation of apoptosis and cell division [36, 40]. Apollon can be distinguished from other IAP family members by its high molecular weight (528 kD) and unique E2/E3 ubiquitin conjugating/ubiquitin ligase functions. As Apollon overexpression has been associated with an unfavourable outcome and resistance to chemotherapy in leukaemia, we were particularly interested in examining whether the ability of MLN8237 to reduce Apollon expression could potentially sensitize CML cells to the standard of care agent nilotinib [35]. Our results show that targeted knockdown of Apollon significantly augments the pro-apoptotic effects of nilotinib, suggesting that suppression of Apollon expression by MLN8237 may contribute to its ability to heighten the anticancer activity of nilotinib. Furthermore, it is likely that the reduction in Apollon expression associated with MLN8237 is a direct consequence of Aurora inhibition as targeted knockdown of Aurora A kinase by siRNA was associated with a significant reduction in Apollon expression. These findings represent a novel mechanistic approach for improving the efficacy of tyrosine kinase inhibitor therapy in CML (Fig. 6E).

There are two potential important clinical translations of the results of the current study. We have shown that MLN8237 is effective against cell lines and primary patient CML cells

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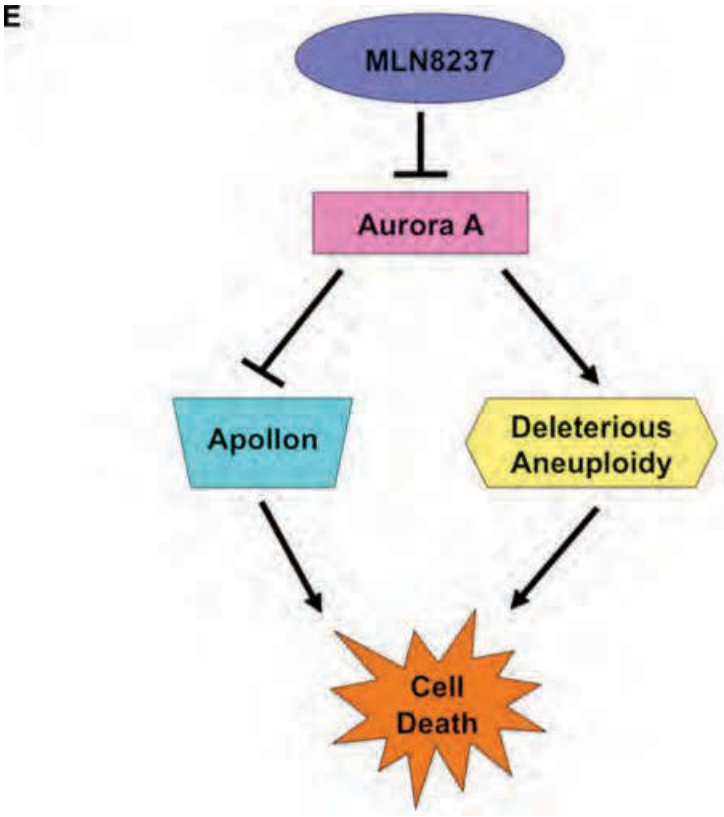

Fig. 6 Continued

expressing unmutated and mutated forms of BCR-ABL including the highly resistant T315I mutation and that it has activity independent of p53 function. Therefore, MLN8237 may be clinically active as a single agent in the setting of T315I and E255K mutations for which the currently available tyrosine kinase inhibitors are ineffective. Moreover, the combination of MLN8237 and nilotinib is effective and well tolerated in preclinical models of CML and represents a novel therapeutic strategy for advanced phase CML that is orally active and has the potential to suppress the emergence of CML clones expressing a range of resistant mutations including T315I and E255K.

The current treatment strategy for resistant CML involves sequential administration of tyrosine kinase inhibitors, which is associated with the development of compound mutations in BCR-ABL with increased oncogenic potency [31]. Patients with imatinib resistance have heightened genomic instability and in this setting combination treatment with an agent effective against cells harbouring the T315I mutation and a BCR-ABL kinase inhibitor could possibly prevent resistance caused by kinase domain mutations in CML [30, 41]. The combination of these two agents has the potential to eliminate BCR-ABL kinase domain mutation as a mechanism of resistance in CML, suppressing resistant disease and leading to sustained remissions in the vast majority of patients. Based on this promising preclinical data, a phase I/II study is warranted to investigate the safety and activity of MLN8237 in refractory CML.

Acknowledgements

The authors thank Dr. Charles L. Sawyers (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center) and Dr. Brian Druker (Howard Hughes Medical Institute, Oregon Health Sciences University) for providing Ba/F3 cells expressing wild-type and mutant forms of BCR-ABL, and Dr. James Griffin (Dana-Farber Cancer institute) for providing imatinib-sensitive and imatinib-resistant K562 cells for this study. This work was supported by grants from LeukemiaTexas (J.S.C and F.J.G.), the CTRC at UTHSCSA Experimental and Developmental Therapeutics Program (J.S.C.), the Owens Biomedical Research Foundation (J.S.C.) and The AT&T Foundation (F.J.G.).

Conflict of interest

J.E. is an employee of Millennium Pharmaceuticals. The other authors have no conflicts of interest to declare.

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