INHIBITION OF THE PHOSPHATIDYLINOSITOL-3-KINASE PATHWAY IS SELECTIVELY CYTOTOXIC TO PRIMITIVE AND MATURE ACUTE MYELOID LEUKEMIA PROGENITOR CELLS WHILE SPARING THEIR NORMAL COUNTERPARTS

by

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Abstract

Acute myeloid leukemia (AML) is a disorder of the hematopoietic system in which there is an overproduction of immature myeloblast (blast) cells in the bone marrow and suppression of normal hematopoiesis. Like normal hematopoiesis, AML is structured hierarchically and is maintained by rare progenitor cells capable of self-renewal and production of large numbers of leukemic blasts. These rare cells, leukemia-initiating cells (L-IC), are capable of engraftment and proliferation in immunocompromised mice. Dysregulation of signal transduction pathways are frequent occurrences in AML, leading to enhanced cell proliferation and resistance to apoptosis. Pathways which are frequently constitutively active in AML blasts include the phosphatidylinosityl-3-kinase (PI3K) and FMS-like tyrosine kinase 3 (FLT3) receptor signalling pathways.

Constitutive PI3K activation, as measured by Akt phosphorylation, was detected in the majority of the 36 AML patient samples tested. The PI3K signalling intermediate integrin linked kinase (ILK) was ubiquitously expressed, and FLT3 abundantly expressed although at variable levels. Furthermore, in the five samples tested, ILK and FLT3, as well as PI3K pathway activation, were also detected in $CD34^{+}CD38^{-}$ sorted and quiescent AML cells, populations enriched for L-IC.

siRNA inhibition of ILK caused a decrease in AML colony forming cell (CFC) production. To further test the effects of ILK inhibition on AML, the small molecule inhibitor QLT0267 was used. *In vitro* kinase assays showed that QLT0267 inhibits both ILK and FLT3 kinases to a similar degree. Treatment of AML blasts with QLT0267 caused dose-dependent killing of AML-CFC, with AML samples containing FLT3

mutations being more sensitive to the inhibitor, although some samples with wildtype FLT3 were also effectively killed. Treatment of AML cells with QLT0267 also decreased survival of both primitive leukemic progenitor cells and L-IC in a dose-dependent fashion. In contrast, QLT0267 showed little toxicity toward normal bone marrow progenitors detected in the same assays. When combined with cytarabine or daunorubicin, QLT0267 worked synergistically to kill AML-CFC from a proportion of AML samples tested. Thus, FLT3, ILK, and constitutive PI3K activation are present in L-ICs, and these candidate leukemic stem cells can be eliminated by inhibition of these pathways, while their normal hematopoietic counterparts are spared.

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List of Abbreviations

AML	Acute Myeloid Leukemia
APC	Allophycocyanine
Ara-C	Cytosine arabinoside
ATRA	All-trans retinoic acid
B2M	β-microglobulin
BM	Bone marrow
BSA	Bovine serum albumin
CFC	Colony forming cell
CFU	Colony forming unit
CI	Combination Index
CML	Chronic myeloid leukemia
CREB	cAMP-responsive element-binding protein
DMSO	Dimethyl sulfoxide
DNR	Daunorubicin
FAB	French American British
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLT3	Fms-like tyrosine kinase 3
FL	Fms-like tyrosine kinase 3 ligand\
FoxO	Forkhead box
G-CSF	Granulocyte-colony stimulating factor
GM	Granulocytes/monocytes
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GSK3	Glycogen synthase kinase 3
HFN	Hank's balanced salt solution plus 2% fetal bovine serum and 0.04% sodium azide
Hst	Hoechst 33342
HSC	Hematopoietic stem cell

IC	Inhibitory concentration
IL	Interleukin
IL2Rko	interlukin-2 receptor γ
ILK	Integrin Linked Kinase
IMDM	Iscove's modified Dulbecco's medium
ITD	Internal tandem duplication
L-IC	Leukemia-initiating cell
LSC	Leukemic stem cell
LTC-IC	Long-term culture-initiating cell
MDR1	Multidrug resistance-protein 1
mTOR	Mammalian target of rapamycin
NBM	Normal bone marrow
NK	Natural killer
NOD/SCID	Non-obese diabetic severe combined immunodeficient
NOD/SL-IC	NOD/SCID leukemia-initiating cell
NPM1	Nucleophosmin
IL2RKo	Interlukin 2 receptor knockout
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependant kinase-1
PE	Phycoerythrin
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PtdIns	Phosphatidylinositol
Py	Pyronin Y
qRT	Quantitative reverse transcriptase
RAR	Retinoic acid receptor
RBC	Red blood cell

RTK	Receptor tyrosine kinase
SC-IC	Suspension culture-initiating cell
SCID	Severe combined immunodeficient
SCF	Stem cell factor
SDF-1	Stromal-cell-derived factor 1
SFM	Serum free media
SHIP	SH2-containing inositol phosphatise
siRNA	Short interfering RNA
VLA	Very late antigen
WBC	White blood cell
WHO	World Health Organization

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Chapter 1

Introduction

1.1. Normal and Malignant Hematopoiesis

1.1.1. Normal Hematopoiesis

1.1.1.1. Normal Hematopoiesis

Hematopoiesis is a tightly regulated system in which billions of blood cells are produced, circulate for a finite lifespan and die, or are lost due to infection or injury [1,2]. These cells reside primarily in the bone marrow (BM) and peripheral blood, but depending on their function or stage of development can also be found in other tissues such as the liver or lymphoid tissues.

This balance in blood cell homeostasis is maintained by a remarkably small number of cells, 'hematopoietic stem cells' capable of long-term self-renewal as well as producing every different hematopoietic cell type [1]. These cells give rise to progeny that become progressively more differentiated, and lineage restricted. Eventually this process ends in the production of terminally differentiated non-proliferating hematopoietic cells such as platelets, red blood cells, neutrophils, monocytes, B cells, T cells, and natural killer (NK) cells that live for a few hours or days (neutrophils) to a few months (memory T cells).

Hematopoiesis is regulated by a number of mechanisms. Cytokines and growth factors, small proteins that mediate cellular processes such as proliferation, quiescence, differentiation, and apoptosis, bind to receptors found on the cell surface triggering the activation of signal transduction pathways [2]. Many cytokines and growth factors such

as interleukins, Fms-like tyrosine kinase 3 (FLT3) ligand, stem cell factor (SCF), erythropoietin, granulocyte (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) are involved in the regulation of hematopoiesis [2-4].

The microenvironment in which the hematopoietic cells reside is also important in the regulation of hematopoiesis, particularly for hematopoietic stem cells (HSCs). HSCs primarily reside in the BM close to the endosteal surface where they come in contact with osteoblasts. This microenvironment provides cytokines such as SCF and stromal-cell-derived factor 1(SDF-1), and adhesion molecules such as Tie-2, N-Cadherin, and very late antigen 4 (VLA-4) all of which are thought to recruit HSCs to their BM niche and promote their maintenance and quiescence[5]. Cytokines such as G-CSF, and enzymes such as matrix metalloproteinase-9 (MMP-9) promote hematopoietic cell growth and differentiation by signalling hematopoietic cells to leave their BM niche [5,6].

1.1.1.2. Hematopoietic Hierarchy and Assays

1.1.1.2.1. Mature Progenitor Cell

The majority of hematopoietic cells are short-lived and have little proliferative capacity. When plated into semisolid media, such as methylcellulose media, and supplemented with appropriate cytokines and growth factors, after 10-14 days in culture most of the cells die out. However, a few cells survive and give rise to clonogenic colonies of cells suspended in the media [1]. This progenitor cell is termed a colony-forming cell (CFC), or colony-forming unit (CFU), and is at a frequency of approximately 1 per 1000 nucleated hematopoietic cells [7]. Four different types of colonies can be observed based on size and morphology, representing progenitors of the

different myeloid cell types present in hematopoiesis. They are the granulopoietic, erythropoietic, megakaryocytic, and multilineage CFC.

1.1.1.2.2. Primitive Progenitor Cell

There exist primitive hematopoietic progenitor cells responsible for producing and maintaining CFCs. These primitive progenitors, when placed in long-term cultures containing a supportive stromal cell feeder layer and cytokines, can be kept alive for months *in vitro* and still produce CFCs [1,8,9]. For this reason these rare hematopoietic progenitor cells are termed long-term culture-initiating cells (LTC-ICs). LTC-ICs are found primarily in the less than 1% of BM cells that stain positive for CD34 and negative for CD38 cell surface markers ($CD34^{+}CD38^{-}$) [10]. These primitive progenitor cells are important in the long-term survival and function of the hematopoietic system as they produce the CFCs that give rise to the mature hematopoietic cells. Although they are rare, studying primitive progenitors is informative in understanding the hematopoietic system and the long-term effects caused by changes in the system such as perturbances in signalling pathways.

1.1.1.2.3. Stem Cell

Hematopoiesis is ultimately regulated by the HSC, which is capable of both self-renewal and reconstitution of all the cell lineages. When needed, the HSC gives rise to the various progenitor cells, but otherwise remains quiescent.

In vivo assays have been developed using xenogenic hosts such as immunocompromised severe combined immunodeficient (SCID) mice for lymphomyeloid human hematopoietic cell engraftment. Before they are used, SCID mice, which have impaired B and T cell functions due to the presence of a homozygous mutation in prkdc (protein kinase DNA activated catalytic polypeptide), are sublethally irradiated to eliminate any residual B, T and NK cells [11-14]. When human hematopoietic cells are injected into these mice, a small portion of the cells are capable of migrating to the BM and engrafting in the mice, producing both human myeloid and lymphoid (primarily B lineage) cell types. This assay was improved by the use of the non-obese diabetic (NOD)/SCID mouse, which contains additional host NK cell impairment, allowing for better and longer human cell engraftment [15].

The cells capable of long-term engraftment share properties found in both mouse HSCs, and human LTC-ICs, suggesting they are human HSCs [14,16]. Although there is overlap in properties of the cells detected in LTC-IC and NOD/SCID mouse assays, there are differences between the two. First, the LTC-IC assay is inefficient at detecting lymphoid lineage cells. Thus, any event affecting lymphoid cells would go unnoticed in this assay. Secondly, although LTC-IC can be kept in culture for months, they eventually expire due to differentiation to more mature cell types. This limitation may be due to suboptimal culture conditions. Cells engrafted in xenograft transplants can be maintained for much longer periods of time, and are often capable of secondary and tertiary transplants. LTC-ICs are also more frequent and heterogeneous in cell cycle status than stem cells, suggesting that stem cells may make up only a fraction of all LTC-IC [14,17].

1.1.2. Acute Myeloid Leukemia

1.1.2.1. Definition

The term acute myeloid leukemia (AML) encompasses a group of malignant disorders affecting the hematopoietic system and is characterized by the overproduction of undifferentiated myeloblast (blast) cells in the BM [18,19]. This rapid production of non-functional blast cells, which are arrested in differentiation, crowds the BM, suppresses normal hematopoiesis, and spills into the peripheral blood. This suppression of normal hematopoiesis causes the majority of symptoms displayed in the disease which generally present as fatigue, weakness, and weight loss due to anemia; bleeding, bruising, and hemorrhage caused by thrombocytopenia; and infection because of reduced granulocytes. In the World Health Organization's (WHO) classifications, myeloid neoplasms are considered to be acute myeloid leukemia if more than 20% of the cells in the BM are myeloblasts [18].

1.1.2.2. Classifications

Since AML is such a heterogeneous disease a number of different methods have been employed to characterize and classify AML patients into subsets based on various features of the disease and prognosis. One of the first classification systems used was derived from a cooperative of haematologists from France, America, and Britain (FAB) in the late 1970's. This system categorizes AML based on the morphology and cytochemistry of the blast cells present, grouping cases according to the degree of maturation of the blasts, with the categories ranging from M0 (undifferentiated) to M7 (megakaryoblastic) [20]. Although the FAB system partially defines prognostic groups,

it has modest clinical relevance. Aside from the promyelocytic leukemia subgroup, it does not adequately define biologic and treatment groups [21].

Approximately 50% of AML samples have one or more detectible cytogenetic abnormalities such as chromosome translocations, inversions, duplications, or deletions. These chromosomal abnormalities produce aberrant gene products, or cause abnormal gene expression. Cytogenetics can be used to distinguish subsets of AML samples that share similar clinical features, and can be used as an independent prognostic factor to group AML samples into three categories, favourable, intermediate, and poor prognosis (Table 1.1)[22,23].

The favourable subgroup contains the 15-20% of patients who have one of the following reciprocal translocations: t(15;17) which creates the PML-RARα fusion product, t(8;21) which creates the AML1-ETO fusion product, or inv(16) which creates the CBFB-MYH11 fusion product [24]. Both t(8;21) and inv(16) disrupt the core binding factor (CBF), a transcription factor complex important for normal hematopoiesis [25]. The fusion protein PML-RARα created by the translocation t(15;17) causes dysregulation of retinoic acid receptor α, a nuclear hormone receptor and transcription factor important for cellular differentiation. The PML-RARα fusion protein is unresponsive to physiological concentrations of retinoic acid and acts as a universal repressor of its target genes. This effect can be reversed by the addition of ATRA (all trans retinoic acid) or arsenic trioxide, two agents currently used in the treatment of patients with t(15;17) [26].

The intermediate subgroup contains patients with a normal karyotype, various small chromosomal deletions, or with trisomies 8, 11, 13 or 21. This category contains

more than half of all patients, indicating that another system is necessary to further refine this prognostic group.

The poor subgroup contains patients with unbalanced karyotypes including gain or loss of whole chromosomes, aberrations of chromosome 3, and complex karyotypes including three or more chromosome aberrations. The relapse rates of patients in this category can be upwards of 80% [27].

A number of genetic lesions that occur in AML are on a scale too small to be detected by cytogenetics. These lesions often occur as deletions, additions, or duplications of single or multiple DNA base pairs in genes and lead to dysregulated gene expression or function. These mutations confer leukemogenic properties to the AML samples they are present in, whether it is a growth advantage in the absence of cytokines, resistance to apoptosis, or inhibition of differentiation [21]. One such mutation is internal tandem duplication (ITD) of the FLT3 receptor which leads to constitutive activation of the receptor and downstream signalling pathways (described in detail later). FLT3 mutations are associated with both high white blood cell counts and marrow blast percentage, a higher risk of relapse and early relapse, making FLT3 mutations a poor prognostic factor [24].

Another prognosis indicator in AML is the presence of a mutation, most often a four base pair repeat, in nucleophosmin (NPM1), a nucleocytoplasmic shuttling protein [28]. These mutations cause aberrant cytoplasmic sequestering of the protein. Mutations in NPM1 occur in approximately 50-60% of cytogenetically normal AML and are prognostically favourable when not accompanied by a FLT3 mutation.

Mixed lineage leukemia (MLL) is a transcription factor that is found to be dysregulated by a partial duplication (MLL-PTD) in approximately 7-8% of normal karyotype AML and up to 47% of AML with trisomy 11[29]. The presence of MLL-PTD in AML patients is associated with shortened remission, increased rates of relapse, shortened overall survival, and is a poor prognostic factor. MLL mutations are very prominent in infant AML, and acute lymphoid leukemia.

Most cases of AML are sporadic. However some hematological disorders such as myeloproliferative and myelodysplastic syndromes, and congenital disorders such as Down syndrome and Fanconi's anemia increase the risk of AML development [30-33]. There is also a risk of developing AML after receiving treatment with alkylating agents, radiation, or topoisomerase II inhibitors, often used during the treatment of other neoplasms [18]. AML arising from chemotherapy represents a distinct group of cases with poorer prognosis as compared to *de novo* AML with otherwise similar characteristics. The WHO categorizes AML based on all of these properties as well as other factors such as immunophenotype, and clinical features, accounting for the great heterogeneity within the disease **(**Table 1.2**)** [18].

1.1.2.3. Treatment of AML

With standard chemotherapeutic agents patients are able to achieve a remission (<5% myeloblast cells in the BM) 50 to 85% of the time [34,35]. However, only 20 to 30% of patients are able to achieve long-term disease-free survival (>5 years after remission). The remainder of patients relapse with a more aggressive and often drug-resistant form of the disease, and die within 2 years of remission. These outcomes are

even worse in patients over 60 years of age where only 40 to 55% ever achieve a complete remission. Considering that the median age of presentation of the disease is 65-70 years of age, and rate incidence increases with age, this represents a large population of AML patients[36,37].

Standard induction chemotherapy typically consists of an antimetabolic agent cytosine arabinoside (Ara-C) and an anthracyclin such as daunorubicin (DNR), which work primarily on rapidly dividing cells [34,35]. Ara-C, once in the cell, becomes phosphorylated and converted to arabinoside triphosphate by endogenous deoxycytidine kinase (dCK). Activated Ara-C acts on the cells by two mechanisms, both of which involve DNA replication [38,39]. During DNA synthesis Ara-C competes with deoxycytidine triphosphate (dCTP) for incorporation into the newly synthesized DNA. Once incorporated into DNA Ara-C leads to termination of DNA synthesis. Ara-C also directly inhibits DNA polymerase, preventing DNA from being synthesized. Daunorubicin, when taken up by cells, disrupts DNA synthesis by intercalating and distorting DNA, as well as inhibiting topoisomerase II and RNA polymerase [40].

For patients where standard chemotherapy is not enough to eradicate the disease, other agents such as the purine analogs chlorodeoxyadenosine or fludarabine, or the topoisomerase II inhibitor etoposide may be used [41]. Antibody-based compounds such as Gemtuzumab, an anti-CD33 ab conjugated to the cytotoxic agent calicheamicin, or a diphtheria toxin-IL3 fusion protein are also undergoing clinical testing, as are a large number of small molecule inhibitors targeting signalling pathways such as PKC412 and CEP-701, which inhibit both wildtype and mutant FLT3 receptor kinase activity, farnesyl transferase inhibitors such as Tipifarnib, histone deacetylase inhibitors, and multidrug

resistance inhibitors [41-44]. Allogenic or autologous BM transplants may also be performed. However, transplants come with high mortality rates of their own, making them unsuitable options for many patients, in particular the elderly [34,37]. Furthermore, although autologous BM transplants improve disease-free survival, it is not clear whether there is an overall survival advantage compared to standard therapy [34]. Thus novel therapies are necessary in the treatment of AML for patients where standard chemotherapy has failed and who are unable to undergo BM transplants.

1.1.2.4. Mutations Dysregulating Hematopoiesis in AML

A variety of mutations detected in AML blasts dysregulate normal cell growth and differentiation resulting in a competitive advantage for the leukemic cells over their normal hematopoietic cell counterparts. Mutations in receptor or non-receptor tyrosine kinases, which allow for activation of signalling pathways in the absence of an appropriate stimulus, include mutations in FLT3, c-Kit, and FMS receptors, or BCR-ABL, TEL-JAK2, and BCR-JAK2 fusion proteins [23]. Another mechanism for dysregulation of receptor signalling is increased expression of the receptor, ligand, or both, which also commonly occurs with the FLT3 and c-Kit receptors [45]. Mutations in downstream signalling pathways also occur, circumventing the need for initial stimuli to activate the pathways [46-49]. These mutations confer a proliferative advantage to the cells, overcoming negative regulators or lack of positive regulators.

In most cases a single mutation is not sufficient to cause AML. Expression of FLT3-ITD, PML-RARα, or AML-ETO, all common mutations in AML, affect differentiation or proliferation of hematopoietic cells but do not directly cause acute

leukemia, suggesting cooperating mutations are necessary for disease progression [50-53]. Two classes of mutations have been proposed to collaborate in the development of AML: one enhances proliferation and/or survival of hematopoietic cells while the other alters/blocks cellular differentiation [23,32]. Mutations enhancing the proliferation and survival of hematopoietic cells often involve receptor tyrosine kinases such as FLT3 or Kit, or the RAS signalling pathway. Mutations blocking differentiation work primarily through aberrant expression of transcription factors and commonly include the PML/RARα, AML1/ETO, or HOX gene fusions [32,51,52]. For instance the PML/RARα fusion, a result of t(15;17), acts to silence the retinoic acid receptor α (RARα) transcription factor target genes, which are important for differentiation. Suppression of the dominant negative PML/RARα with ATRA reverses these effects, allowing for normal cell differentiation [54].

1.1.2.5. Leukemic Stem Cell

AML, like normal hematopoiesis, is structured hierarchically. While in AML most cells have little to no proliferative capacity, a rare population of cells is capable of long-term survival in culture or engrafting in immunocompromised mice [55,55-57]. This cell is referred to as a leukemic stem cell (LSC). Much like the HSC, the LSC is the cell capable of both self-renewal and producing all the leukemic cell types present in the disease.

In the early 1990's John Dick's group demonstrated in AML, using an assay to detect normal HSCs, the presence of a LSC capable of engrafting in SCID mice [57]. Engrafted cells exhibited many morphologic, and immunophenotypic features seen in the original

patients that the samples came from. Further work in this area relied, and for the most part still relies, primarily on using the NOD/SCID mouse model which is superior to the SCID mouse model for both leukemic and normal hematopoietic cell engraftment due to the non-obese diabetic (NOD) background which results in low NK cell activity and macrophage function [58,59]. New mouse strains derived from NOD/SCID mice are also being used such as those with β-microglobulin (NOD/SCID B2M), or interlukin-2 receptor γ (NOD/SCID IL2Rko) knocked out, which further impair the host immune system and allow for better human cell engraftment [60,61].

The frequency of LSCs, also referred to as leukemia-initiating cells (L-ICs, or NOD/SL-ICs) because of their ability to engraft for long periods of time in NOD/SCID mice, is approximately 1 per 10^4 to 10^6 total blast cells. This varies from sample to sample, and approximately half of AML samples are unable to achieve engraftment in NOD/SCID mice [62,63]. Cells harvested from mice engrafted with human AML can give rise to both LTC-IC and CFC, demonstrating the ability of L-ICs to give rise to the various cell types found in leukemia [62]. Like normal HSCs, L-ICs are often found in the $CD34^+CD38^-$ and $CD34^+CD71^-$ HLA-DR^- populations [58,63]. However, this phenotype is not without controversy. First, it has been reported that in rare cases $CD34^-$ AML cells are capable of engrafting in NOD/SCID mice, implying that $CD34^-$ cells can contain L-ICs [63]. More interestingly, Dominique Bonnet's group recently demonstrated that commonly used anti-CD38 antibodies have inhibitory effects on the engraftment of both normal hematopoietic and AML cells in NOD/SCID mice [64]. Inhibition is due to the Fc portion of the antibody receptor which elicits an immune response in the mice. This effect can be reversed by either treating the mice with

immunosuppressive antibodies, or injecting cells directly into the mouse BM. This allows for engraftment of both $CD34^+CD38^-$ and $CD34^+CD38^+$ cells in immunocompromised mice for a large proportion of AML samples, which suggests not only that L-ICs can have a phenotype other than $CD34^+CD38^-$, but also that the L-IC frequency may be greatly underestimated. More work will need to be done to further characterize this finding.

Although L-ICs and HSCs share expression patterns for many cell surface markers such as CD34, CD38, and CD71, there are some differences that can be used to distinguish them. Two markers which are expressed on normal HSCs and are often, but not always, lacking on L-ICs are CD90 and CD117(c-Kit) [65-67]. One marker that is expressed on leukemic but not hematopoietic stem cells is IL-3 receptor α (CD123) [68]. This unique expression of the IL-3 receptor α on L-ICs has been exploited in the development of monoclonal antibody-toxin fusion proteins to specifically kill LSCs [69]. CD33 is a cell surface marker found on committed myeloid progenitors, but not normal HSC [70]. It is also widely expressed on AML cells, including the stem cell population, and is the target of monoclonal antibodies such as Gemtuzumab [42,43].

The L-IC is difficult to kill as it is resistant to therapeutic agents. A number of properties of the L-IC contribute to making the cells drug-resistant. One such property is overexpression of adenosine triphosphate binding cassette (ABC) transporters, in particular MDR1 (multidrug resistance-protein 1, or P-glycoprotein) and BCRP1 (breast cancer resistance-protein 1) on the L-ICs. This allows the cells to export toxic agents such as chemotherapeutic agents from the cell, preventing damage to the cell [71,72]. Another property is the upregulation of anti-apoptotic signalling pathway proteins such as

NFκB or anti-apoptotic Bcl-2 family members, which skews the balance of anti- and pro-apoptotic signals in the cell [22,73]. Upregulation of these prosurvival signals allow the L-IC to survive under circumstances that would normally trigger apoptosis. The L-IC is also for the most part quiescent, and not actively cycling. This quiescence protects the L-IC from chemotherapeutic agents and therapies that mainly target cells actively replicating [74].

The clinical leukemic stem cell is believed, but not proven to be responsible for producing and maintaining all of the characteristics of the disease in patients, and for causing relapse of the disease after remission has been achieved. Since we are unable to study clinical LSCs, as they grow in humans, we infer their properties from the knowledge gained in studying L-ICs in xenograft transplant models (Figure 1.1).

The L-IC is the most relevant cell to study in the context of developing new therapies for AML as it is thought to share the properties of the clinical LSC, the cell responsible for maintaining the disease in patients. By eradicating the LSC, it is believed that the leukemia would be unable to persist and therefore be eliminated.

There are challenges that come with studying L-ICs, many of which involve the use of immunocompromised mice to assay for L-ICs. As the mice used are highly immunocompromised, they are more susceptible to infections and illnesses than other mouse strains. Since these experiments are carried out for long periods of time, it can be a challenge maintaining the health of the mice, especially once they have been sublethally irradiated. There can also be great variability in the engraftment rates of AML cells in mice, even when patient samples and cell numbers are constant. This may reflect technical issues, such as whether all the cells were successfully injected into each mouse,

or biological issues such as how many L-ICs actually make it from the blood stream to the BM and successfully engraft in a mouse. As well, since the L-IC frequency is not known for most AML samples, the number of cells injected into mice is based on estimation of the L-IC frequency. When measuring the effects of perturbations on L-ICs it is desirable to inject low numbers of L-ICs into a mouse so that each L-IC represents a significant proportion of AML cells engrafted in the mouse. If there is variability in the actual number of L-ICs injected into each mouse, even just by one or two, this may be reflected by a large change in AML engraftment. Also, human L-ICs have only been enriched to approximately one in 10^3 to 10^4 cells by using cell surface markers [75] leaving a tremendous amount of background cells in L-IC-enriched populations. This complicates studies which characterize phenotypic properties of the L-IC.

1.1.2.6. Primitive Leukemic Progenitor Cell

The frequency of AML LTC-ICs and long-term suspension culture-initiating cells (SC-ICs, which are grown in the absence of a feeder cell layer), as defined by their ability to produce CFC after 5 or more weeks in culture, also varies from 1 per 10^4 to 4 per 10^6 total blast cells [56,76]. Their frequencies decrease when the cells are cultured for longer periods of time. The CFCs produced per SC-IC after 8 weeks in culture or per LTC-IC after 5 weeks in culture vary from 3 to 25 CFC/SC-IC, and 1.6 to 33 CFC/LTC-IC, respectively [56]. These primitive progenitor cells are enriched in the population of cells that are $CD34^+CD38^-$ and $CD34^+CD71^-$[56]. Although the cells that read out in these *in vitro* assays share long-term growth and cell surface properties with the L-IC, it has not been demonstrated that an AML SC-IC or LTC-IC can repopulate a NOD/SCID mouse,

the true measure of a L-IC. It is uncertain whether this observation is because L-ICs are more rare than LTC-ICs or whether this is a technical issue regarding the LTC-IC assay, as culture conditions *in vitro* likely lack all the essential requirements for the primitive cells to be maintained long-term, causing them to eventually expire. L-ICs are also less frequent than LTC-ICs for a given sample [62].

Studying primitive progenitor cells can be informative as this population of cells exhibits many of the qualities the L-IC possesses. As well they are cultured *in vitro*, eliminating many of the issues associated with the NOD/SL-IC assay. Although the long-term culture assays still require 8 weeks to perform, this is only half the length of time needed for assaying L-ICs.

1.1.2.7. Mature Leukemic Progenitor Cell

The frequency of AML cells capable of producing colonies of blast cells when plated in semisolid media such as methylcellulose for two weeks is approximately 1 per 10^2 to 10^5 bulk blast cells [77,78]. These colonies can be pooled together and replated generating secondary and occasionally tertiary colonies, although the secondary plating efficiencies are usually <1% and tertiary plating efficiencies are even less. This demonstrates that these CFCs have limited self-renewal capacity [79,80]. In contrast to L-ICs and primitive progenitor cells, the majority of CFCs arise from cells that are $CD34^-$ [63].

The major advantage of studying mature progenitor cells is the short length of time necessary to perform the assay. Although this cell type has little in common with the L-IC, it is more relevant than studying the total blast population of a sample, since

99% of the cells in an AML sample have little to no proliferative capacity, whereas AML-CFCs do have some, albeit short-lived, proliferative capacity. Another benefit from a technical standpoint is that the readout of the CFC assay is cell survival as opposed to cell death. When working with primary AML samples the initial viability of cells in some samples can be quite poor. For these samples in particular it is difficult to measure response of total blast cells to a compound using cell death markers such as propidium iodide (PI) as the background cell death is high.

1.1.2.8. AML Cell of Origin

It is currently unclear which normal cell type becomes transformed in the initial formation of AML. Because of the similarities in functional as well as immunological properties between normal and leukemic stem cells, many suggest the normal HSC as a candidate cell in which the leukemic transformation occurs [81,82]. Both the HSC and L-IC possess the ability to self-renew and proliferate, and share the $CD34^+CD38^-CD71^-$ phenotype. Another reason is that normal HSCs are long-lived and thus have a greater opportunity to accumulate the transforming mutations required to become leukemic.

It is also possible that a cell other than an HSC, such as a committed hematopoietic progenitor cell, can gain the ability to self-renew and have disrupted differentiation, thus becoming a L-IC and initiating AML. There is evidence that this may be the case in at least some instances of AML. As mentioned previously, in rare cases of AML, cells other than those in the more primitive $CD34^+$ fraction are capable of engraftment in mice. As well, recently it has been shown that $CD34^+CD38^+$ cells have an underestimated engraftment potential [63,64]. The fusion gene product MLL-ENL (mixed lineage

leukemia-eleven nineteen leukemia) has also been shown to transform both HSCs and common myeloid progenitors, demonstrating that the leukemic cell of origin need not be a HSC [83].

1.2. Signal Transduction Pathways Dysregulated in AML

1.2.1. FMS-like Tyrosine Kinase 3 Pathway

1.2.1.1. Normal Signalling

FLT3 is a class III receptor tyrosine kinase (RTK) activated by FLT3 ligand (FL), and is involved in the maintenance and proliferation of hematopoietic cells. It is also found in the placenta, central nervous system and liver [84,85]. The FLT3 gene, located on chromosome 13q12, is composed of 24 exons and encodes a 993 amino acid protein which results in three products 160, 140 and 130 kDa in size [86]. The FLT3 receptor contains 5 extracellular immunoglobulin-like domains, a transmembrane region, a juxtamembrane domain, and two kinase domains located in the cytoplasm. The receptor is primarily membrane-bound. In normal hematopoiesis FLT3 expression is restricted to $CD34^+$ cells and subsets of dendritic cells [87,88,88], and generally decreases with increased differentiation of progenitor cells. In its inactive state FLT3 is found as a monomer on the plasma membrane, in a conformational structure that inhibits activation of its tyrosine kinase domain [84]. When bound by its ligand, FLT3 undergoes a conformational change, forming a homodimer and exposing its tyrosine kinase domain. As a homodimer bound to its ligand, the conformation of FLT3 is stable and its tyrosine kinase domain becomes autophosphorylated and activated [84]. Shortly after FLT3

activation the receptor-ligand complex is internalized and degraded, which acts as a negative regulatory mechanism to limit downstream pathway activation.

Once phosphorylated, human FLT3 interacts with a number of proteins including GRB2, GAP2, CBL, SHC and SHIP. This leads to activation or regulation of the PI3K and RAS signalling cascades and a number of downstream targets (Figure 1.2). Activation of FLT3 alone in hematopoietic cells leads to a mild stimulation of proliferation and promotes monocytic differentiation [89]. Stimulation of FLT3 in conjunction with cytokines or other growth factor receptors (e.g. IL-3R, G-CSFR, GM-CSFR, erythropoietinR, or c-Kit) synergizes to produce a much stronger proliferative signal. FLT3 also cooperates with IL-3, IL-6, and IL-7 to stimulate lymphoid cell proliferation and differentiation [90,91].

1.2.1.2. Signalling in AML

FLT3 is mutated in approximately 30% of AML patients. The most common mutation of FLT3 is internal tandem duplication (ITD) of the juxtamembrane region (exons 14 and 15) [92,93]. The length of this duplication varies from 3 to ≥400 base pairs, with the reading frame of the transcript always being maintained [84]. Other less common mutations of the FLT3 receptor primarily include missense point mutations in the tyrosine kinase domain, such as D835 mutations which involve the conversion of aspartic acid at position 835 to another amino acid such as tyrosine, valine or histidine [94]. All these mutations lead to conformational changes allowing for ligand-independent activation of the FLT3 receptor and downstream signalling pathways such as Ras, STAT, and PI3K signalling pathways, and β-catenin, and FoxO proteins [47,95-98].

Wildtype FLT3 is also expressed in AML and may be used by the cells for growth and proliferation [99,100]. Overexpression of FLT3, regardless of mutation status leads to activation of the tyrosine kinase domain, and downstream pathways [101]. However, FLT3-ITD signalling may affect different downstream targets to that of wildtype FLT3, such as members of the Bcl-2 family of proteins, and myeloid transcription factors [95,102]. Although wildtype and mutant FLT3 alleles are usually coexpressed in FLT3 mutated samples, in some leukemic clones the wildtype FLT3 allele is lost, likely due to homologous recombination, resulting in loss of heterozygosity and the expression of only mutant FLT3 [103,104].

FLT3 mutation status has also been shown to change in patients between diagnosis and relapse. In some cases FLT3 mutations are not present at diagnosis and appear only in relapse, while in other cases FLT3 mutations are present at diagnosis and undetectable in relapsed AML. This suggests that FLT3 mutations are not the initial mutations driving the disease but may be occurring in only a subset of leukemic clones or in later stages of leukemia development [105,106].

In experiments designed to determine the response of hematopoietic cells to the FLT3-ITD mutation *in vivo,* FLT3-ITD transduced cells were injected into recipient mice [107]. This led to a myeloproliferative disease in the mice, demonstrating that FLT3 mutations confer a proliferative advantage to hematopoietic cells. In another study mice with an ITD mutation at the FLT3 locus were bred. These mice developed a myeloproliferative disease resembling chronic myelomonocytic leukemia but not an acute leukemia [108]. Altogether these data indicate that on their own FLT3-ITD

mutations are not sufficient to cause leukemia, and other mutations are necessary for full transformation.

Nevertheless, the presence of a FLT3-ITD mutation is clinically an unfavourable prognostic factor associated with leukocytosis (likely due to the proliferative advantage this mutation exerts on cells), and correlating with a decrease in complete remission rate and overall survival, and an increase in relapse risk in patients [109]. A number of small molecule inhibitors targeting FLT3 have been created, some of which are being tested in clinical trials for AML. Compounds such as PKC412, CEP-701, and SU11248 inhibit both the wildtype and mutant FLT3 kinase domain, which leads to inhibition of downstream targets. These compounds cause leukemic cell death both *in vitro* and *in vivo*, and have shown some clinical activity in AML patients with FLT3 activating mutations [110-114]. These inhibitors also lead to a slight decrease in normal lymphoid and myeloid progenitor cell numbers [87].

1.2.2. Phosphatidylinositol 3-Kinase Signalling Pathway

1.2.2.1. Normal Signalling

1.2.2.1.1. Phosphatidylinositol 3-Kinase

The phosphatidylinositol 3-kinase (PI3K) pathway is a key signal transduction pathway involved in regulation of cell growth, proliferation, survival, differentiation, motility and intracellular trafficking. Its deregulation has been linked to a number of human diseases and disorders including inflammation, allergy, heart disease, and cancer [115]. PI3K is a dual specificity kinase capable of phosphorylating serine and threonine residues as well as catalyzing the addition of a phosphate on the 3′-position of

phosphatidylinositol (PtdIns) on the inner cell membrane. It converts PtdIns , PtdIns(4)P, PtdIns(5)P, and PtdIns(4,5)P_2 to PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 respectively, with PtdIns(4,5)P_2 being the most common substrate [116]. Phosphorylated PtdIns act as binding sites for pleckstrin homology (PH) domain-containing proteins such as PDK1, Akt, and ILK, sequestering them to the cell membrane [117]. PI3K can also activate members of the MAPK cascade via its serine/threonine kinase domain [118]

PI3K consists of a family of 9 distinct catalytic subunits, which can be broken down into class I, II and III based on protein domain structure and substrate specificity, with class I PI3K thought to be the most important of the three [116]. The class I PI3Ks form heterodimers, with a regulatory subunit binding to one of 4 different catalytic subunits, p110α, p110β, p110δ or p110γ, all of which share a common structure and similar substrate specificities. PI3Ks are found in the cytosol in an inactive state until activation by a stimulus and are then recruited to the membrane, their primary site of function. PI3Kα, β and δ are activated upon phosphorylation of the p85 regulatory subunit by RTKs, RAS, and signalling intermediates such as insulin receptor substrates (IRS1 and 2) (Figure 1.3) [119].

1.2.2.1.2. Akt

The central PH domain-containing protein downstream of PI3K is Akt, also known as protein kinase B (PKB). Akt exists in three isoforms (Akt1, 2 and 3), each of which contain an N-terminal PH domain, a catalytic domain, and a C-terminal regulatory

domain [120,121]. There is some evidence to suggest that the different isoforms may have different functions, although this has not yet been thoroughly examined [122-124].

Once bound to PtdIns(3,4,5)P_3 Akt undergoes a conformational change revealing its activating loop and exposing residue threonine 308 [121]. This allows for phosphorylation of threonine 308 in the activation loop and serine 473 in the C-terminal hydrophilic motif, both necessary steps for Akt activation [118,120]. Phosphorylation on residue threonine 308 occurs by the phosphoinositide-dependant kinase-1 (PDK1) [118,125]. There are several kinases thought to play a role in phosphorylation of Akt on serine 473 including integrin linked kinase (ILK), the mammalian target of rapamycin (mTORC2)-complex containing Rictor, PKCβII, and DNA-dependant protein kinase (DNA-PK). Autophosphorylation by Akt has also been suggested as a mechanism [121,126].

Once activated, Akt can leave the cell membrane to phosphorylate and regulate downstream targets (Figure 1.3): glycogen synthase kinase 3 (GSK3) becomes inactivated leading to an increase of glycogen synthesis; eukaryotic initiation factor-4E binding protein (4EBP) becomes inactivated lifting repression of mRNA translation; pro-apoptotic Bcl-2 family member BAD becomes inactivated preventing it from binding to and inhibiting anti-apoptotic Bcl-2 family members, leading to protection from apoptosis; and the mTORC1 complex, a critical regulator of translation initiation and ribosome biogenesis as well as many others, becomes activated leading to activation of p70S6 kinase and inhibition of eukaryotic translation initiation factor-binding protein. This activates translational machinery, and allows cells to transition from the G_1 to S phase of cell cycle. Akt can also regulate transcription factors such as NFκB, cAMP-responsive

element-binding protein (CREB), and forkhead transcription factors [126-129]. Akt can activate IκK, which in turn inhibits IκB, allowing NFκB to enter the nucleus where it can regulate transcription of a large number of genes involved in immune, inflammatory and survival responses [130,131]. Akt causes the phosphorylation and activation of CREB, which regulates the transcription of numerous genes including those involved in cellular growth and proliferation [132]. Akt can also directly phosphorylate and inhibit FoxOs which when unphosphorylated can activate transcription of proapoptotic signals such as TRAIL, and Bim, and cause cell cycle arrest through activation of p27 and p21, and repression of cyclin D [133,134].

In times of nutrient abundance when PI3K is activated through insulin receptor (IRS-1) signalling, Akt also inhibits tuberous sclerosis 2 (TSC2), a GTPase-activating protein (GAP) which in its active state associates with TSC1 to inactivate Rheb. When TSC2 is inhibited, GTP-bound Rheb accumulates and activates the mTORC1-complex containing Raptor, which regulates protein translation via 4EBP and p70 ribosomal S6 kinase ($p70^{S6K1}$) [127,128]. S6K1 (activated by $p70^{S6K1}$) can then inhibit IRS-1, creating a negative feedback loop regulating PI3K activity. This Akt-TSC2-mTORC1 signalling is also disrupted in conditions of low glucose, amino acid, or oxygen levels, and is inhibited by rapamycin. Under conditions of low nutrients, mTOR is inhibited, and this feedback loop is disrupted, leading to a strengthened Akt signal [128].

1.2.2.1.3. PI3K Pathway Regulation

Akt can be regulated by a number of mechanisms including negative feedback loops as described above, and dephosphorylation of serine 473 or threonine 308 by

phosphatases. Specific dephosphorylation of Akt on serine 473 occurs by the phosphatases PHLPP and PHLPP2 (PH domain leucine-rich repeat protein phosphatise), each of which have an Akt isoform bias [135,136]. By far the most well studied mechanism for PI3K pathway inactivation is by removal of the PH domain binding site by PTEN and SHIP [126]. PTEN (phosphatase and tensin homolog deleted on chromosome ten) converts PtdIns(3,4,5)P_3 back into PtdIns(4,5)P_2 by hydrolyzing the 3´-phosphate, directly opposing the effect of PI3K [119,137]. Conversion of this phospholipid removes the binding site of PH domain-containing proteins, including Akt, preventing them from localizing at the inner cell membrane. PTEN is widely expressed amongst different tissue types, and is commonly downregulated or mutated in a broad range of cancers including breast, prostate, colon, lung, and glioma, to name a few [138,139]. Homozygous PTEN knockout mice are embryonic lethal, and heterozygous knockout mice have increased incidences of tumour development [140].

SHIP1 (SH2-containing inositol 5´ phosphatase), is a hematopoietic lineage restricted phosphatase that hydrolyzes the 5´-phosphate from inositol(1,3,4,5)P_4, and PtdIns(3,4,5)P_3, once again removing PH domain binding sites [141,142]. Upon cytokine stimulation SHIP becomes recruited to the cell membrane and tyrosine phosphorylated, creating more protein-binding domains [143]. Since phosphorylation of SHIP does not appear to affect its activity it is believed that localization of SHIP is important for its regulation. Increased SHIP expression leads to a decrease in PI3K pathway activity and inhibition of downstream targets [144]. SHIP can bind to Grb2, shc, DOK1 and DOK2, and might also play a role in Ras inhibition [143]. SHIP knockout mice overproduce

granulocytes and macrophages, and are hyper-responsive to multiple cytokines and growth factors [145].

1.2.2.1.4. Mammalian Target of Rapamycin

Mammalian target of rapamycin (mTOR) is a 289 kD highly conserved serine/threonine kinase involved in RNA translation, cell cycle progression, and activation of transcriptional activators [146-149]. mTOR activation can have different effects depending on which complex it is found in; when bound to Raptor (mTORC1) the complex is downstream of Akt and is sensitive to rapamycin, when bound to Rictor (mTORC2) the complex works upstream of Akt. mTOR affects Akt phosphorylation by one of two mechanisms previously mentioned. Briefly, the first involves a negative feedback loop created when the mTORC1 complex activates S6K1, which in turn inhibits IRS-1 an activator of the PI3K pathway, leading to Akt inhibition. The second mechanism, involves the mTORC2 complex which has been shown to either directly, or with additional proteins, phosphorylate Akt on serine 473, and is insensitive to rapamycin [150,151].

1.2.2.2. Aberrant Signalling in AML

1.2.2.2.1. Phosphatidylinositol 3-Kinase and Akt

A number of groups have shown that the PI3K pathway or specific compounds in the pathway are constitutively active in a large proportion of AML samples [46,152-154]. Inhibition of this pathway with compounds such as LY294002, or rapamycin derivatives lead to apoptotic cell death, demonstrating that PI3K plays a role in AML survival.

Mutations in the genes encoding PI3K or Akt are very uncommon in AML, excluding these as potential mechanisms for constitutive PI3K pathway activation [155]. However, RTKs such as FLT3 and c-Kit are commonly mutated or overexpressed in AML, and are known to activate the PI3K pathway [156-159]. Inhibition of or mutations in the negative regulators PTEN and SHIP allow for activation of Akt and phosphorylation of downstream targets. Although mutations in PTEN and SHIP are rare in AML they do exist [160-162].

PI3K is not only active in blast cells, but is also found to be important for the survival of primitive progenitor cells in AML as well. In one study the $CD34^{+}CD38^{-}$ fraction of AML cells, which is enriched for candidate L-ICs, was treated with LY294002 for 24 to 48 hours, after which a marked decrease in cell viability was observed when compared to DMSO controls [152]. In another study purified $CD34^{+}CD38^{-}$ cells were exposed to LY294002 for 16 hours and then injected into NOD/SCID mice [46]. AML engraftment levels in the mice were measured as an indication of stem cell survival. LY294002 treated cells had lower engraftment than DMSO treated or untreated cells. Furthermore, it has been demonstrated that *in vivo* conditional activation of Akt in a murine model promotes a myeloid leukemia-like disease [158]. Altogether, this suggests that PI3K is important in AML stem cell survival, and inhibition of this pathway may be cytotoxic to the L-ICs.

There is no clear consensus as to whether activation of the PI3K pathway is a favourable or unfavourable prognostic factor in AML. However, the majority of the groups suggest it is unfavourable [154,163-165].

1.2.2.2.2. PI3K Pathway Regulation

In 2006 two groups independently published their results using murine model systems to examine the role of PI3K in hematopoiesis [166,167]. In their systems PTEN was conditionally deleted from hematopoietic cells in mice and a number of experiments were performed to look at HSC survival. Both studies found that in the short term the number of HSCs increased, but in the long term the number of HSCs dropped dramatically compared to in control mice. One group treated the mice with rapamycin during PTEN knockdown and found this was sufficient to prevent the short-term increase and long-term decrease in HSCs [166]. Altogether this indicates regulation of the PI3K pathway is important in HSC maintenance. In both studies a number of PTEN knockout mice also developed acute leukemias including AML. This AML was transplantable into recipient mice. Mice treated with rapamycin during the initial knocking out of PTEN had no evidence of neoplasia both at 4 weeks post knockout and in secondary transplants. However, treatment of mice engrafted with established AML with rapamycin prolonged survival of the mice but did not provide a cure. These experiments imply distinct roles for the PI3K pathway in hematopoiesis and leukemia. In hematopoiesis, regulation of the PI3K pathway appears to be related to the maintenance of normal HSCs, while in leukemia its activation contributes to transformation and preservation of the L-IC.

1.2.2.2.3. Mammalian Target of Rapamycin

mTORC1 is inhibited by rapamycin, an immunosuppressant used clinically, primarily during organ transplantation [168]. There has been much interest as of late in testing rapamycin and rapamycin analogs therapeutically for AML. Rapamycin binds to

immunophillin FK506 binding protein 12 (FKBP12), inhibiting formation of the mTORC1 (Raptor) complex [146].

Several groups have found that rapamycin has little effect on the survival of short lived AML blast cells, but more pronounced affects on the leukemic progenitor cells [169,170]. However, inhibition of mTORC1 by rapamycin can lead to increased PI3K activity due to removal of the negative feedback loop used to control PI3K activity. Consistent with this finding, rapamycin or rapamycin analogues are more effective in killing AML cells when working in conjunction with inhibition of other targets [170-174].

Some rapamycin derivatives are capable of inhibiting both the mTORC1 and mTORC2 complexes [175]. This would lead to inhibition of both mTOR and PI3K signalling, and may explain some of the differences observed in the literature regarding the effects of mTOR inhibition on AML survival [175]. In clinical trials, treatment with rapamycin or rapamycin derivatives has led to some partial responses in patients [169,176,177].

1.2.3. ILK

Integrin-linked kinase (ILK) is a serine/threonine kinase-containing adaptor protein. It connects cell adhesion molecules to the actin cytoskeleton by interacting with a number of proteins such as PINCH (particularly interesting new cysteine-histidine rich protein), ILKAP (ILK-associated phosphatase), α and β-parvin, Rictor, and integrin β subunits [178-180]. By doing so, ILK is involved in regulating a number of cellular processes such as anchorage-dependent cell growth, survival, migration, and cell cycle progression

[181-186]. The ILK protein contains 4 N-terminal ankyrin repeats, a PH-like domain, and a C-terminal kinase domain. ILK binds to PtdIns P_3 via its PH-like domain allowing for regulation of ILK by PI3K. Activation of PI3K has been shown to stimulate ILK kinase activity [187]. ILK also interacts with, and may be regulated by growth factor receptors through the adaptor protein PINCH which binds to Nck-2 (non-catalytic (region of) tyrosine kinase adaptor protein 2), which in turn can bind to phosphorylated tyrosine residues (Figure 1.4) [178,186]. Interactions with α- and β-parvin, as well as paxillin link ILK to the actin cytoskeleton.

The kinase activity of ILK has not been completely characterized. However, studies have shown that GSK3β serine 9 and Akt serine 473 are substrates of the ILK kinase domain [183,187,188]. Conversely some groups suggest that this phosphorylation is through indirect mechanisms [186,189,190]. McDonald *et al* reveal that ILK interacts with Rictor during its phosphorylation of Akt on serine 473 [191]. As well, it has been demonstrated that inhibition of ILK leads to a decrease in Akt phosphorylation [188,191,192]. Regardless of mechanism, it appears that ILK is important for the phosphorylation of Akt and GSK3. ILK can also be localized to the nucleus through phosphorylation of threonine 173, and serine 246 by p21-activated kinase1 (PAK1), where it can then interact with gene-regulatory chromatin, and regulate mitotic spindle organization [193,194].

β-parvin, DOC-2 (differentially-expressed in ovarian carcinoma-2), and ILKAP are negative regulators of ILK, as are negative regulators of the PI3K pathway such as PTEN which disrupt PtdInsP_3, the binding site for the ILK PH-like domain [183,188,195].

ILK is overexpressed or constitutively active in many solid tumours including colon, pancreas, glioblastoma, and breast cancer [181,196], and has been correlated to tumour grade or survival for a number of these cancers [185]. As well, inhibition of ILK is toxic to many of these tumours or inhibits their migration, working at least in part through PI3K dependent mechanisms [197-199].

Very little is known about ILK in the context of leukemia. Aside from our work there are only two papers examining ILK in AML. The first, by *Hess et al* in 2007 focused on the role of ILK in radiation-induced apoptosis. They found that overexpression of ILK in the HL60 cell line increased apoptosis after irradiation, particularly when cultured with fibronectin. This could be inhibited by treating the cells with siRNA targeting ILK [200]. In the second paper by *Tabe et al* in 2007, QLT0267, a small molecule inhibitor of ILK was used to disrupt stroma-leukemia cell interactions and downstream signalling. This led to a decrease in stromal cell mediated PI3K activity in the leukemic cell lines, and some primary AML samples. This disruption reversed the protective advantage exerted on the leukemic cells by the stroma, and increased apoptosis in leukemic blast cells [201].

1.2.4. Strategies to Block PI3K Activation

1.2.4.1. RNA Interference

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism first identified in plants and *Caenorhabditis elegans* [202,203]. It has since been characterized in mammals, and is a very powerful tool for selectively inhibiting the translation of mRNA into proteins [204-206]. During RNAi double stranded RNA,

whether produced endogenously as a hairpin loop and processed or introduced exogenously, becomes processed by a cytoplasmic ribonuclease III-like protein, Dicer, which cleaves the dsRNA into small RNA duplexes of 19-25 base pairs with 3′-dinucleotide overhangs, referred to as short interfering RNA (siRNA). The siRNA duplex is unwound and the strand containing a sequence complementary or near-complementary to the mRNA target interacts with the RNA-induced silencing complex (RISC), which contains an ATP-dependant helicase. Once bound to mRNA the RISC complex can repress translation by one of two mechanisms: it can cleave the target mRNA 10 base pairs upstream of the nucleotide paired with the 5′-nucleotide of the RNA in the RISC complex, or it can bind to the mRNA and physically block translation.

siRNA mediated mRNA degradation occurs quickly, in as little as 18 hours, but the effects on protein levels are more variable and dependent on the turnover rate of the specific protein [204]. RNAi can be a powerful tool for elucidating gene function and when designed properly is very specific, demonstrating little off target effects. The main limitation to using RNAi is introducing the synthetic siRNA into the desired tissue or cells in sufficient quantity using techniques which are not toxic to the target cells [205].

1.2.4.2. Small Molecule Inhibitors

Protein kinases catalyse the transfer of a phosphate from an ATP molecule to a protein substrate, most often on a serine, threonine, or tyrosine residue. This phosphorylation is a necessary process in almost every signal transduction pathway. Small molecule kinase inhibitors most often function by competing for the ATP-binding

pocket of a kinase, preventing the kinase access to the necessary phosphate of ATP in a process that is usually reversible [207,208].

Kinase inhibitors have gained in popularity as potential cancer therapies in part due to the success of imatinib, a small molecule tyrosine kinase inhibitor that targets amongst other things, BCR-ABL, the fusion product of the chromosome translocation (9:22)(q34:q11). This translocation, which causes the joining of the BCR regulatory region to the ABL kinase, is found in 95% of chronic myeloid leukemia (CML) cases, as well as in a number of other hematologic malignancies, and is crucial to the progression of the disease [209]. Due to its ability to effectively inhibit BCR-ABL, which causes death of the leukemic cells, imatinib has become the frontline therapy for CML [210-212]. Many groups have since been looking for small molecule inhibitors of oncogenic kinases for the treatment of other malignancies including the FLT3-ITD mutation in AML [84,112,114]. However, this success has been difficult to replicate, possibly because unlike CML, AML has multiple signalling pathways disrupted. Another potential complication with using small molecule kinase inhibitors is that since kinase inhibitors are generally designed to target the ATP-binding domain, and these domains are highly conserved amongst kinases, most inhibitors inhibit multiple targets to varying degrees [213-215].

1.3. Cell Cycle

In AML the majority of cells are actively cycling, which differs from normal hematopoiesis where there is a large quiescent population [17,216-218]. This partially explains the proliferative advantage leukemic cells have over normal cells. In studies

examining the cycling status of various leukemic progenitor cell populations it was found that they were mostly proliferating, although there was much more heterogeneity than in normal hematopoietic cells and a fraction of cells remained quiescent [17]. *In vitro* studies have demonstrated that quiescent leukemic cells tend to spontaneously enter cell cycle, even in the absence of serum or cytokines which stimulate cells [17]. In contrast to this, normal BM cells remain quiescent under similar conditions, indicating that in AML achieving or maintaining quiescence may be an active process.

The leukemic cells most capable of engraftment in NOD/SCID mice are in the G_0 stage of the cell cycle, demonstrating that at least some candidate L-ICs directly isolated from patients are quiescent [17]. Consistent with these results, *Guzman et al* found that $CD34^+CD38^-$ AML cells, the fraction most enriched for L-ICs, were primarily non-cycling [219].

Culturing of AML cells on a stromal cell layer allows for the maintenance of a portion of cells in G_0, demonstrating that interaction with the stromal cell layer also plays an important role in maintaining leukemic cell quiescence [220,221]. These quiescent leukemic cells show resistance to both standard chemotherapeutic agents, which commonly exert their effects by disrupting DNA synthesis, and tyrosine kinase inhibitors, which also tend to work best on proliferating cells, possibly by activation of the PI3K pathway [74]. This makes these cells particularly difficult to eliminate in patients.

Mitotic cell division is the process by which a cell divides producing a genetically identical daughter cell. Mitosis is comprised of 4 distinct phases: G_1, a 'gap' phase where the cell is metabolically active in preparation; S, the phase where DNA is synthesized; G_2, a second 'gap' phase; and M, the mitotic phase where all the cellular

components of the cell, including the DNA, is partitioned into two daughter cells [222]. There is an additional state which the cell can enter when it is not actively proliferating known as G_0 or quiescence [223-225].

Mitosis is a tightly coordinated process with checkpoints set up to ensure each step has been properly completed (Figure 1.5)[222,223,226]. Cyclins and cyclin-dependent kinases (CDKs), serine/threonine kinases that become activated upon binding to appropriate cyclins, regulate these checkpoints. Entry from G_0 to G_1 and into S is regulated by CDKs 2, 4 and 6, as well as cyclins D1, D2, and D3, and E. CDK 4/6 when bound to cyclin D, and then CDK 2 when bound to cyclin E, phosphorylate and inactivate retinoblastoma protein (RB), which normally binds to and represses E2F/DP-1 transcription factors during G_0 and G_1. Once inhibition is lifted on E2F/DP-1, transcription of genes necessary for DNA replication can occur. The G_2-M checkpoint prevents cells with damaged DNA from entering mitosis. Mitosis begins when Cyclins A and then B bind to CDK1(cdc2), causing phosphorylation of necessary targets. This whole process is regulated by signal transduction pathways such as GSK3, p53, RAS, and PI3K, which monitor growth factor and nutrient levels, as well as DNA damage.

1.4. Rationale and Thesis Objectives

At least some of the abnormal cell proliferation in AML can be attributed to dysregulation of signal transduction pathways. The PI3K pathway has been shown to give cells a proliferative advantage, and has been reported to be upregulated in AML patients. Based on this, I hypothesize that the PI3K pathway gives leukemic cells a

growth and survival advantage over normal cells and that targeting PI3K or its pathway intermediates may be effective in eradicating leukemia.

In the first part of my thesis I sought to determine how frequently the PI3K pathway was activated in AML. Western blotting was used to semi-quantitatively detect levels of Akt, phosphorylated Akt as a measure of PI3K pathway activation, ILK, and FLT3, a commonly mutated receptor that signals in part through the PI3K pathway. As described in chapter 2, Akt and other PI3K pathway intermediates were in fact constitutively active in a large proportion of primary AML samples, and ILK in particular was ubiquitously expressed.

Based on these results, I targeted ILK as a means to downregulate the PI3K pathway in AML. Using siRNA to specifically inhibit ILK, I demonstrated in CFC assays that this PI3K pathway intermediate is important for AML cell survival. Since using siRNA to inhibit targets in primary AML samples is currently quite difficult, and transduction efficiencies inconsistent, I decided to use a small molecule inhibitor, QLT0267, first characterized for its ability to inhibit ILK, to disrupt the PI3K pathway in primary AML samples. Exposure of the cells to QLT0267 killed both total blast cells as well as mature progenitor cells detected in CFC assays, particularly those harbouring the FLT3-ITD mutation, implicating this mutation as a possible target of the compound. Although further testing of QLT0267 using kinase assays confirmed this compound inhibits both ILK and FLT3 to a similar degree, some AML samples lacking FLT3 mutations were sensitive to QLT0267. To further strengthen our hypothesis that ILK is an important PI3K intermediate in AML, QLT0267 was directly compared to PKC412, a small

molecule inhibitor of FLT3 currently in use in the clinic. QLT0267 was more effective than PKC412 in causing leukemic cell death in both FLT3 mutant and wildtype samples.

Normal BM progenitors, when treated with QLT0267, showed much less toxicity to QLT0267 than AML progenitors despite ILK being present and Akt phosphorylated in these samples. This further supported the notion that the PI3K pathway is particularly important for AML cell survival.

Cure of AML ultimately requires elimination of the rare L-IC. In chapter 3, I further characterized the effects of PI3K inhibition on the L-IC-enriched fraction from 5 AML samples using QLT0267. First, cells were sorted into various fractions based on CD34 and CD38 cell surface markers and the targets of QLT0267 were measured. ILK and FLT3 were detected and GSK3, a surrogate marker for ILK and PI3K activity, was phosphorylated in these fractions, including the $CD34^{+}CD38^{-}$ fraction enriched for L-IC. The presence and activation of these PI3K intermediates provided a rationale for further testing of QLT0267 on primitive leukemic progenitors.

The quiescent AML cell fraction, which is enriched for L-ICs, was also analyzed for PI3K pathway activation. The presence of ILK, FLT3, and pGSK3 was documented in both quiescent and cycling populations from the 5 AML samples characterized above. This indicates that regardless of cell cycle status the PI3K pathway is to some degree active.

Thus, PI3K and ILK are present and active in stem cell enriched fractions of leukemic cells including $CD34^{+}CD38^{-}$ and quiescent cells. To determine whether inhibition of the PI3K pathway is toxic to leukemic or normal stem cells, AML cells and normal BM were treated with QLT0267 and placed into either long term suspension

cultures or NOD/SCID xenograft assays. In both assays QLT0267 was far more toxic to the leukemic cells than normal cells. *In vivo* treatment of mice with QLT0267 also demonstrated reduction of AML engraftment. Therefore, inhibition of the PI3K pathway through targeting of ILK and FLT3 by the small molecule inhibitor QLT0267 is toxic to L-ICs, but not normal HSCs. Altogether this demonstrates the PI3K pathway is active in AML and targeting this pathway, particularly in L-ICs, may be effective at eliminating the disease in patients.

Table 1.1: Classification of acute myeloid leukemia based on cytogenetics

	Favorable risk	Intermediate risk	Unfavorable risk
Chromosomal aberration	t(15;17)(q22;q12-21) t(8:21)(q22;q22) inv(16)(p13q22)/ t(16;16)(p13;q22)	Normal karyotype t(9;11)(p22;q23) del(7q) del(9q) del(11q) del(20q) −Y +8 +11 +13 +21	Complex karyotype inv(3)(q21q26)/t(3;3)(q21;q26) t(6;9)(p23;q34) t(6;11)(q27;q23) t(11;19)(q23;p13.1) del(5q) −5 −7

From Shipley 2009 [34].

Table 1.2: WHO classification of acute myeloid and related leukemias

ACUTE MYELOID LEUKEMIA AND RELATED NEOPLASMS
- Acute myeloid leukemia 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*
- Acute myeloid leukemia with myelodysplasia-related changes
- Therapy-related myeloid neoplasms
- Acute myeloid leukemia, not otherwise specified
  - AML with minimal differentiation
  - AML without maturation
  - AML with maturation
  - Acute myelomonocytic leukemia
  - Acute monoblastic/monocytic leukemia
  - Acute erythroid leukemia
    - Pure erythroid leukemia
    - Erythroleukemia, erythroid/myeloid
  - Acute megakaryoblastic leukemia
  - Acute basophilic leukemia
  - Acute panmyelosis with myelofibrosis
- Myeloid Sarcoma
- Myeloid Proliferations related to Down Syndrome
  - Transient abnormal myelopoiesis
  - Myeloid leukemia associated with Down syndrome
- Blastic Plasmacytoid Dendritic Cell Neoplasm

ACUTE LEUKEMIAS OF AMBIGUOUS LINEAGE
- Acute undifferentiated leukemia
- Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*
- Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged
- Mixed phenotype acute leukemia, B/myeloid, NOS
- Mixed phenotype acute leukemia, T/myeloid, NOS
- *Provisional entity : Natural killer (NK) cell lymphoblastic leukemia/lymphoma*

From Vardiman 2009 [18].

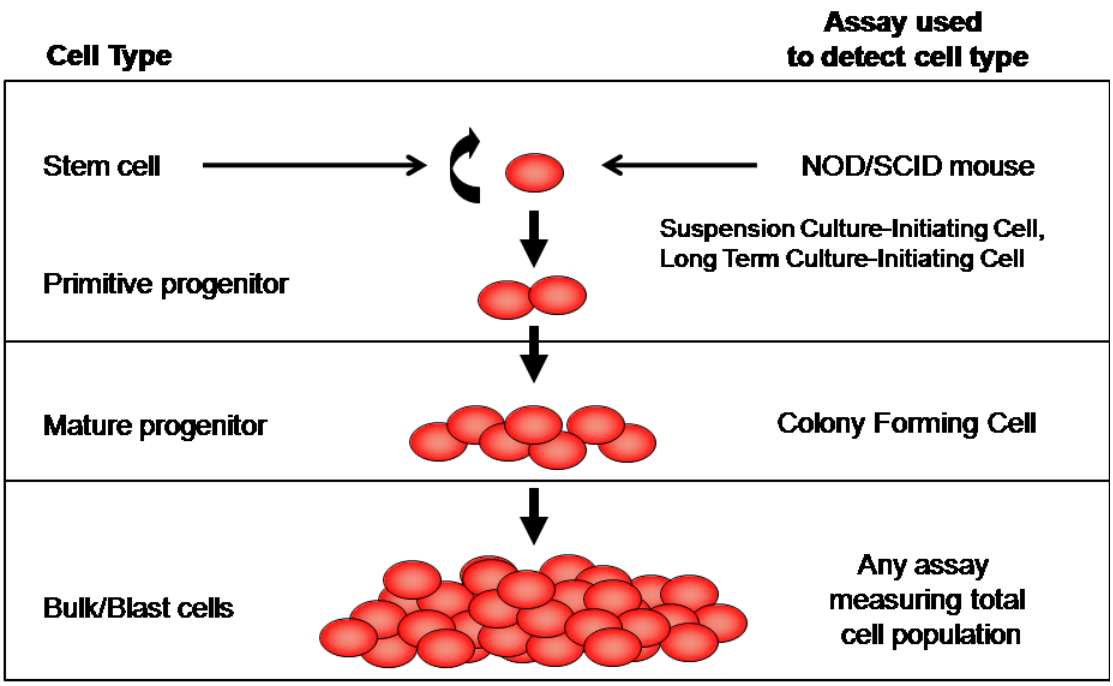

Figure 1.1. The hematopoietic and leukemic hierarchies and the assays used to detect different progenitor cell types.

AML, like normal hematopoiesis, is structured hierarchically. The majority of the blast cells in AML have little to no proliferative capacity. However, there are infrequent cells with varying degrees of proliferative potential. Mature progenitor cells are capable of producing cells over a 14 day period in culture. If grown in semisolid media, these mature progenitors form colonies of cells, and are thus termed colony-forming cells. Primitive progenitor cells, which are less frequent than mature progenitor cells, are capable of producing cells in culture over a 6 week period, some of which are mature progenitor cells. The rarest AML cell type is the leukemic stem cell, capable of both self-renewal and reconstitution of the features of the disease. These cells can be detected using immunocompromised mice, such as NOD/SCID mice.

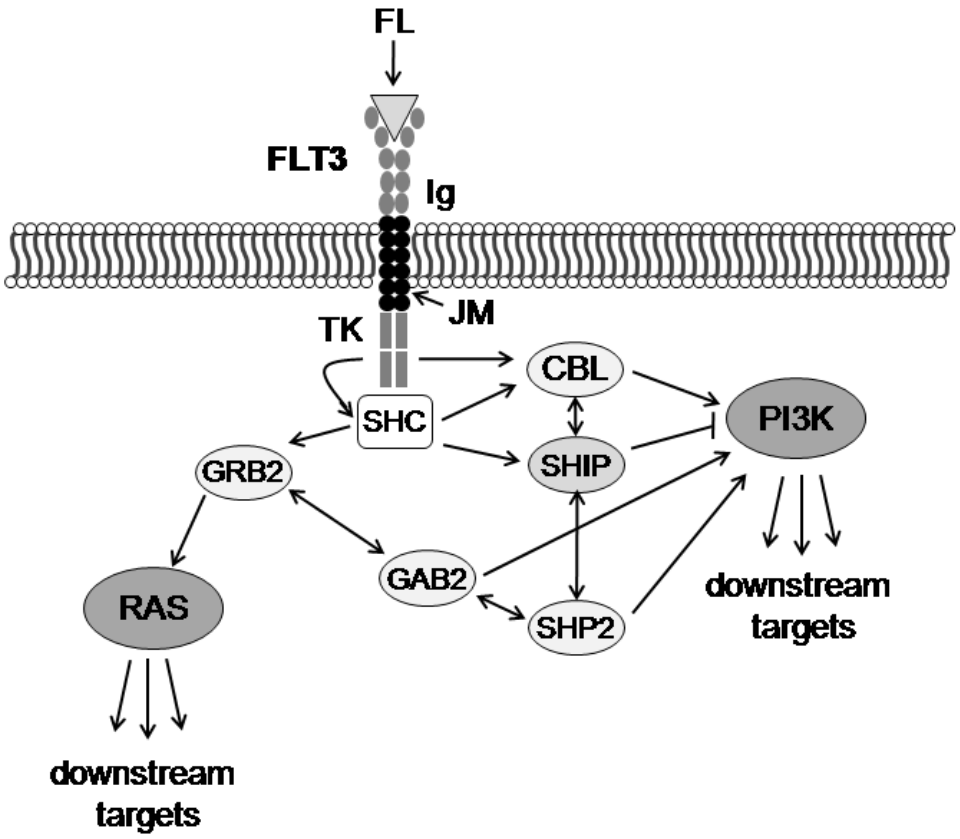

Figure 1.2. The FMS-like tyrosine kinase 3 (FLT3) signalling pathway.

FLT3, a receptor tyrosine kinase (RTK) expressed on hematopoietic cells, is involved in cell maintenance and proliferation. When bound to FLT3 ligand (FL), FLT3 homodimerizes, activating the cytoplasmic tyrosine kinase domain, which leads to phosphorylation of a number of proteins and activation of signalling pathways such as RAS and PI3K. FLT3 is mutated in approximately 30% of AML patients, the most common mutation being an ITD of the juxtamembrane region, although point mutations in the tyrosine kinase domain also occur. These mutations cause constitutive activation of the tyrosine kinase domain and downstream signalling pathways regardless of whether FL is present.

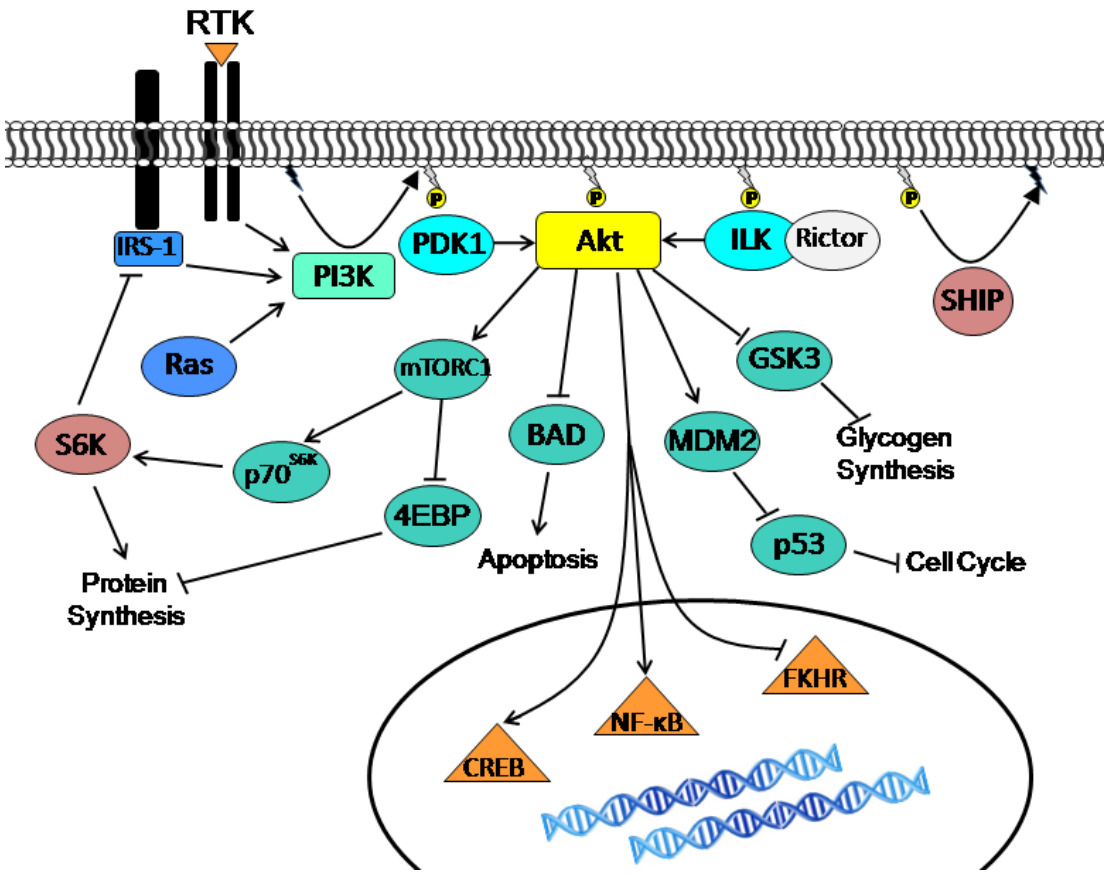

Figure 1.3. The Phosphatidylinositol 3-kinase (PI3K) signalling pathway.

PI3K becomes phosphorylated by upstream RTKs, RAS, and insulin receptor substrates. Upon activation PI3K phosphorylates PtdIns in the inner cell membrane, creating a docking site for PH-domain containing proteins such as PDK1, and Akt, as well as ILK. Once localized to the cell membrane, activated PDK1 and ILK phosphorylate Akt on threonine 308 and serine 473 respectively, causing activation of Akt. Other proteins, for instance DNA-PK, mTORC2, PKC, and Akt itself, may also play a role in Akt phosphorylation on serine 473. Akt in turn acts on a number of downstream targets including GSK3, BAD, mTORC1, and transcription factors such as NFκB, CREB, and Forkhead transcription factors, all of which leads to regulation of cell growth, survival, and differentiation.

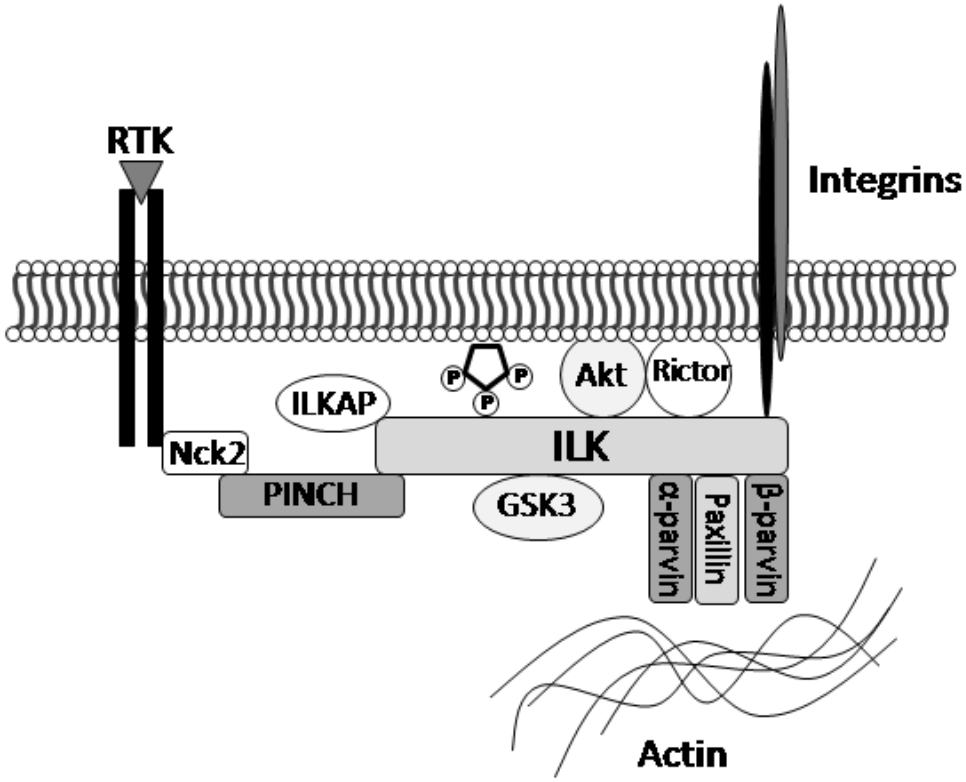

Figure 1.4. Integrin linked kinase (ILK) protein interactions.

Once recruited to the inner cell membrane, ILK interacts with phosphorylated PtdIns via its PH-like domain, as well as with integrins via its C-terminal domain. Growth factors and cytokines, as well as integrin binding, stimulate the kinase activity of ILK which works through a PI3K-dependant manner to phosphorylate Akt, GSK3, and a number of other proteins. α- and β-parvin, and paxillin link ILK to the actin cytoskeleton. Phosphatases such as ILKAP and PTEN inhibit ILK signalling.

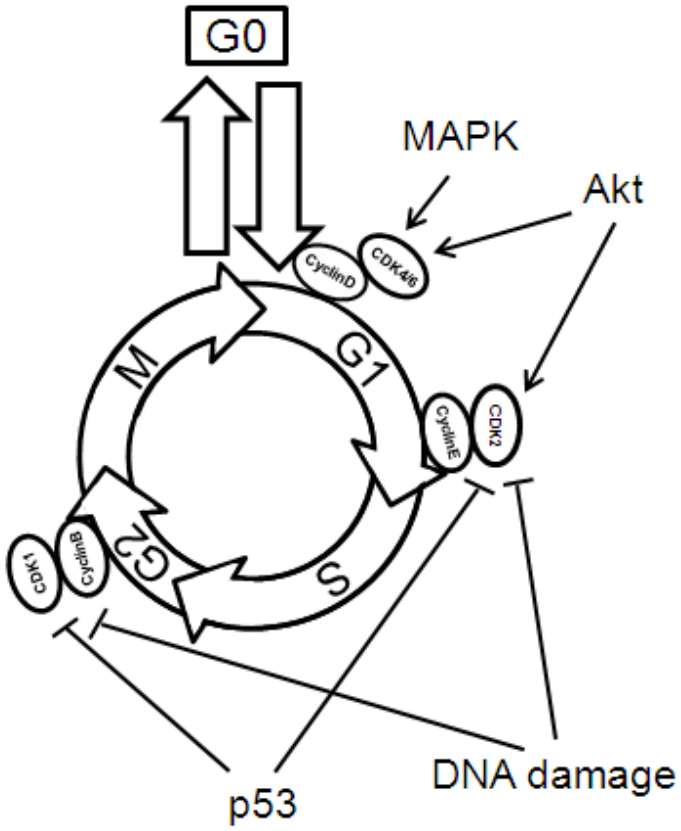

Figure 1.5. Mitosis and regulators of mitotic cell cycle.

Mitotic cell division is a highly ordered process regulated primarily by cyclins and cyclin-dependant kinases (CDKs). CDKs are serine/threonine kinases that become activated by cyclins at specific stages of cell cycle, and in turn phosphorylate a number of proteins to regulate cell cycle progression. Cyclin D binds to CDK 4/6 to facilitate progression from G_0 to G_1; Cyclin E binds to CDK 2 to regulate progression from G_1 to S; Cyclin A and then B bind to CDK1 to regulate progression into mitosis. These processes are all regulated by upstream signals from pathways such as p53, PI3K, and MAPK.

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Chapter 2

Combined Inhibition of Integrin Linked Kinase and FMS-Like Tyrosine Kinase 3 is Cytotoxic to Acute Myeloid Leukemia Progenitor Cells[†]

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2.1 Introduction

Acute myeloid leukemia (AML) is a heterogeneous disorder characterized by aberrant proliferation and differentiation of the malignant cells. Factors contributing to the loss of responsiveness to normal growth controls exhibited by the leukemic clone include factor independent activation of signal transduction pathways critical to normal cell proliferation and survival [1-4].

The phosphatidylinositol-3-kinase (PI3K)-dependent pathway regulates cellular proliferation, differentiation and apoptosis. An important downstream target of the PI3K pathway is the serine/threonine kinase Akt which becomes phosphorylated/activated by phosphoinositide-dependent kinases 1 and 2 and in turn phosphorylates a number of proteins which increase survival, proliferation and/or differentiation depending on the cell type [5,6]. Constitutive activation of Akt has been described in many types of human cancer including AML [2,3,7,8]. Inhibition of PI3K activity in AML blasts results in apoptotic cell death suggesting this pathway is important for the survival of malignant cells from at least some patient samples. A variety of mechanisms have been implicated in PI3K activation in AML including FLT3 and c-Kit gene mutations, dominant negative mutations of SHIP-1 and autocrine growth factor stimulation [1,4,9,10].

Integrin linked kinase (ILK) is an ankyrin-repeat containing serine/threonine kinase involved in both cell interactions with the extracellular matrix and signal transduction [11,12]. ILK plays an essential role in connecting integrins to the actin cytoskeleton and in regulating actin polymerization [13]. In addition, through its kinase activity ILK activates a range of signalling pathways. ILK is implicated in the regulation of anchorage-dependent cell growth and survival, cell cycle progression, epithelial-

mesenchymal transition, invasion, migration and tumour angiogenesis [14]. PI3K activation stimulates ILK activity and recent data suggest that ILK, in combination with Rictor (a component of the mTORC2 complex) promotes cell survival by stimulating the phosphorylation of Akt on ser473, a necessary step for maximal Akt activation [15-17]. Conversely, inhibition of ILK downregulates the phosphorylation of Akt and inhibits cell survival. The expression and activity of ILK are increased in a variety of solid tumours. Small molecule inhibitors of ILK activity have been identified and shown to inhibit tumour growth, invasion and angiogenesis [18,19]. However, little was known about the expression of ILK in human AML or how such expression might contribute to the signalling abnormalities that have been observed in leukemic blasts.

FMS-like tyrosine kinase 3 (FLT3) is a well characterized membrane-bound receptor tyrosine kinase expressed on hematopoietic cells [20-24]. FLT3 mutations are found in approximately 30% of AMLs, the majority being internal tandem duplications of the juxtamembrane region (FLT3-ITD) of the receptor, although less common point mutations also occur [25-27]. These mutations lead to ligand-independent constitutive activation of the receptor, which in turn leads to activation of a number of downstream signalling pathways including PI3K/Akt [28-30]. FLT3 is also found to be constitutively active in AML by other mechanisms that are not as well characterized such as autocrine stimulation [24,31]. A number of inhibitors have been developed to target aberrant FLT3 activity in AML [32,33], and although many have been shown to be cytotoxic to AML cells *in vitro* and *in vivo* the clinical usefulness of these agents has yet to be determined [34,35].

QLT0267 is a potent second generation kinase inhibitor which was originally characterized for its ability to inhibit ILK but not a variety of other kinases including Akt, PDK-1, DNA-PK and GSK3 [18]. A dose dependent inhibition of ILK substrate phosphorylation was demonstrated in both tumorigenic and nontumorigenic cell lines with concentrations of 10 μM reducing ILK kinase activity by ≥50% in all cell types tested [18]. This agent has also been shown to have ~30% oral bioavailability with efficacy and little detectable toxicity in murine solid tumour models at doses up to 100 mg/kg (personal communication QLT Inc.).

The data presented here demonstrate the expression of ILK as well as FLT3 and PI3K activation in malignant cells from patients with newly-diagnosed AML. Specific inhibition of ILK using siRNA was cytotoxic to an AML cell line leading to further experiments to explore the effects of its down regulation in primary AML samples. Additional characterization of QLT0267 demonstrated that it was equally effective in inhibiting the activity of FLT3 and ILK. The combined inhibition of 2 pathways involved in mediating the survival and chemotherapy resistance in AML cells with one molecule seemed worth investigating, particularly in light of emerging data suggesting that inhibition of a multiple signalling pathways may be synergistic in eradicating leukemic progenitors [36-38]. Clinical data also suggests that targeted disruption of the activity of a single kinase such as FLT3 may not be as effective clinically in AML as it has been in chronic myeloid leukemia [39,40].

The data presented here demonstrate cytotoxicity of QLT0267 against AML progenitors. Although both ILK and FLT3 expression were also detected in normal bone marrow cells, CFCs from this source were less sensitive to the inhibitor than their

malignant counterparts. These results suggest further exploration of ILK as a possible therapeutic target in AML and the possible benefit of combined inhibition of ILK and FLT3 in this context.

2.2 Materials and Methods

AML and normal cells. Peripheral blood (PB) cells and bone marrow samples were obtained from 36 newly-diagnosed AML patients and 5 normal bone marrow donors after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. AML patients from whom blood was obtained consisted of 20 male and 16 female individuals with a median age of 56.5 years (range 28 to 78 years). The median presenting peripheral blood white blood cell count was $179x10^9$/L (range 49 to $457x10^9$/L). Diagnostic evaluation of bone marrow samples from these patients revealed a variety of morphological subtypes (French, American, British subtypes M0, M1, M2, M4 and M5) [41]. Bone marrow metaphase cytogenetic analysis was successful in 31 of 36 cases and demonstrated intermediate risk characteristics in 25 (17 with a normal karyotype), good risk in 4 (inv(16) in all 4) and poor risk in 2 AML samples according to Medical Research Council (UK) criteria [42]. An internal tandem duplication (ITD) of the FLT3 gene was detected in 17 of these 36 samples, and a D835 point mutation in the FLT3 kinase domain was found in one (sample 37), while the remaining 18 showed no evidence of these mutations [27]. As predicted by the poor prognostic features of this patient group, the median survival from diagnosis of AML was 5 months (range 1 day to >92 months). There were 11 deaths within 1 month of diagnosis, 7 induction failures and 4 patients who required 2 cycles of chemotherapy to

achieve complete remission. Only 3 of 36 patients remain alive > 50 months from diagnosis. Blood cells were Ficoll separated to obtain the mononuclear cell population, which were then cryopreserved in Iscove's Modified Dulbecco's Medium with 50% fetal calf serum (StemCell Technologies, Inc, Vancouver, Canada) and 10% dimethyl sulfoxide (DMSO) and stored at -135^{o}C. Prior to cryopreservation normal bone marrow was enriched for $CD34^{+}$ cells using an immunomagnetic column (Easysep, StemCell Technologies, Inc). The growth factor dependent leukemic cell line TF-1 [43] was obtained from ATCC, Manassas, VA, USA.

Suspension Cultures. All cells were cultured at 37°C in a humidified incubator with 5% CO_2. Thawed AML and normal $CD34^{+}$ bone marrow cells were resuspended in IMDM with 10% FCS at $1x10^{6}$ cells/mL ($1x10^{5}$ cells/mL for normal bone marrow cells) and then cultured for 48 hours with concentrations of QLT0267 (QLT Inc. Vancouver, Canada, dissolved in DMSO) varying from 0.3-25 μM, the PI3K inhibitor LY294002 (Calbiochem, San Diego, CA, USA) at 10 μM, PKC412 at 100nM (Novartis Pharmaceuticals, Basel, Switzerland) or equivalent concentrations of DMSO as a control. A second dose of each inhibitor was added after 24 hours to ensure sustained inhibition of the target. After 48 hours cells were washed with PBS (StemCell Technologies, Inc) and analyzed by flow cytometry for the presence of apoptotic cells or placed into methylcellulose-based colony assays. TF-1 cells were cultured in RPMI 1640 supplemented with 10% FCS and 2ng/mL GM-CSF (StemCell Technologies, Inc.).

AML and normal Colony forming cell (CFC) Assays. After 48 hours in culture AML or normal cells were washed with PBS. AML cells were then plated at 0.05-2x10^5 cells/mL in methylcellulose-based culture media (StemCell Technologies, Inc) containing 30% FCS, 2 mmol/L L-glutamine, 10^{-4} mol/L β-mercaptoethanol, 1% bovine serum albumin, 15.2 mL IMDM, supplemented with: 3 U/mL erythropoietin, 10 ng/mL of each IL-3 and GMCSF, 50 ng/mL of each FLT3 ligand and Stem Cell Factor in 35 mm culture dishes [44]. Normal bone marrow cells were plated at 1-1.67x10^3 cells/mL in complete methylcellulose culture media (Methocult^{TM} GF H4434; StemCell Technologies, Inc) [45]. After 12-15 days in culture at 37°C in a humidified incubator with 5% CO_2 colonies were scored using an inverted microscope. Percent kill was calculated using the following formula:

Percent $kill_{treatment}$ = ($colonies_{control}$ - $colonies_{treatment}$)/$colonies_{control}$ x100.

Western Blotting. After thawing and the indicated treatments cells were washed twice in PBS, centrifuged, lysed in 1X SDS sample buffer containing β-mercaptoethanol and boiled for 2 minutes. For the samples used in measuring baseline protein levels cells were lysed directly after thaw and one wash in PBS. For all other samples protein extracted from 2x10^6 cells was applied to each lane of the gels used for blotting. Sample volumes were normalized according to cell numbers, separated on 10% polyacrylamide gels, transferred to a nitrocellulose membrane and incubated with the appropriate antibodies at 4°C overnight (anti-phospho-Akt (Ser473), anti-ILK1, anti-phospho-GSK3β (Ser9), anti-FLT3 from Cell Signaling Technologies, Beverly, MA; anti-Akt-1/PKBα, anti-phosphotyrosine clone 4G10 from Upstate Cell Signaling Solutions, Lake Placid, NY;

anti-GAPDH from Research Diagnostics Inc., Flanders, NJ) [46]. Blots were then incubated with peroxidase conjugated affinity purified anti-Rabbit IgG (Rockland Inc., Gilbertsville, PA) for 1 hour at room temperature followed by Western Lightning Chemiluminescence Reagent (PerkinElmer, Wellesley, MA) and visualized on X-ray film. Band intensities were measured using densitometry.

Densitometry. X-ray film images were scanned into computer images using FotoLook32 V3.60.0 (AGFA Canada, Toronto, Canada). Band intensities were quantified using ImageQuant (Molecular Dynamics Inc., Sunnyvale, CA, USA) and normalized to account for differences in protein loading between samples by dividing this value by the value obtained for the GAPDH loading control for that sample. Differences in film exposures amongst different Western blots were accounted for by comparing band intensities to a MO7E cell line sample that was run on each Western Blot.

Immunoprecipitation and Kinase Assays. The ILK kinase assay was carried out as described previously [18], and the FLT3 kinase assay as described below. Briefly, cells were lysed using 1% NP40 in 50 mM Hepes, and 150 mM NaCl in the presence of protease inhibitors (complete protease inhibitor cocktail, Roche Applied Science, Laval, Canada) and 1 mM of both Sodium orthovanadate and Sodium fluoride (Sigma-Aldrich, Oakville, Canada) for phosphatase inhibition. Cell lysates were then incubated with either an anti-ILK antibody or anti-FLT3 antibody (anti-ILK, anti-FLT3 C-20, Santa Cruz Biotechnology), or anti-myc tag antibody as a control (Upstate Cell Signaling Solutions) overnight at 4°C. Protein G sepharose beads (Santa Cruz Biotechnology) were added to

the cell lysates, which were then rotated at 4°C for 1 hour. For the ILK kinase assays the beads were washed twice with a 1% NP40 lysis buffer containing 750 mM NaCl, and twice with ILK kinase reaction buffer (50 mM HEPES, 85 mM KCl, 10 mM EGTA, 10 mM DDT, 0.1% Tween 80, 1 mM $NaVO_4$, 10 mM $MgCl_2$), and then incubated with kinase reaction buffer, containing 5µg LC_{20} peptide (a gift from Dr. Walsh, Calgary, Canada) [47]. For the FLT3 kinase assays the beads were washed once with 1% NP40 lysis buffer and once with FLT3 kinase reaction buffer (60 mM HEPES, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM Na_3VO_4, 1.25 mM DTT) and then incubated with kinase reaction buffer containing 1.2 mM gastrin precursor biotinylated peptide from Cell Signaling Technologies. Both kinase assays commenced with the addition of 0.5 µCi [γ-^{32}P]ATP (Amersham Biosciences Corp., Piscataway, NJ) and in some cases QLT0267 and were incubated at 30°C for 25 minutes. The reaction was stopped by placing the tubes onto ice. SDS was added to the samples, and ILK kinase assay samples were separated on 10% polyacrylamide gels, while FLT3 kinase assay samples were separated on 10-18% polyacrylamide step gels. The gels were then separated between the substrate and protein band and phosphorylated substrate was visualized by autoradiography. Individual substrate bands were then cut out and the radioactivity was measured by liquid scintillation counting. The portion of the gel containing the protein was transferred to a membrane (Immobilon-P^{SQ} membrane, ISEQ00010, Mississauga, Canada), blocked for 1 hour (Odyssey blocking buffer, LI-COR Biosciences, Lincoln, Nebraska, USA), probed for either ILK or FLT3 and then IRDye 800-conjugated anti-rabbit secondary antibody (Rockland Inc.) and visualized using the Odyssey infrared imager (LI-COR Biosciences).

siRNA inhibition of ILK expression

TF-1 cells were transfected with siRNA using electroporation. Cells were taken at the log phase of cell growth and resuspended in fresh media containing 1.25% DMSO at $1x10^7$ cells/mL. For each electroporation $5x10^6$ cells were added to a 4 mm cuvette (Bio-Rad, Mississauga, Canada). An siRNA oligomer specifically targeting the ILK integrin binding domain (ILK-A, sequence 5′-GACGCTCAGCAGACATGTGGA-3′) [48] was added at a concentration of 50 µg. A non-silencing siRNA oligomer (sequence 5′-AATTCTCCGAACGTGTCACGT-3′) was used as the control (Qiagen Inc., Mississauga, Canada). Electroporation was performed using 960 µF and 290 V in a Bio-Rad GenePulser Xcell electroporator. After electroporation TF-1 cells were plated at $1x10^3$ cells/mL in methylcellulose-based culture media (StemCell Technologies, Inc) containing 30% FCS, 1.2% methylcellulose, 1% BSA, 1% glutamine, 2ng/mL GMCSF and IMDM. Colonies were scored after 7-10 days in culture at 37°C. Experiments were performed in triplicate.

Statistical analysis

Correlations between ILK expression and kinase activity were determined in Excel (Microsoft, Seattle, WA, USA). Correlations between AML-CFC percent kill and FLT3-ITD mutation status, and FLT3 and ILK kinase activity in response to QLT0267 were determined using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The significance of the correlation co-efficients and differences between mean CFC kills were determined using the Student T-test. $P<0.05$ was considered significant.

2.3 Results

Akt activation, FLT3 and ILK expression in AML blasts.

Phosphorylation of Akt on serine 473 was detected in 31 of 36 (86%) AML blast samples analyzed by Western blotting, indicating activation of the PI3K pathway in the majority of these leukemias (Figure 2.1). Bands were quantified using densitometry and normalized to GAPDH as described in the materials and methods. Samples with a p-Akt:Akt ratio of ≤0.35 were considered negative for phosphorylation of Akt and include samples 3, 8, 24, 27 and 31.

FLT3 protein was detected on most AML samples although the amount varied significantly. Samples 1, 2, 7, 12, 13, 16, 29, 35, 38, 39 and 41 had particularly high levels of FLT3 as determined by visual inspection (Figure 2.1). Stripping and reprobing of the same Western blots demonstrated that ILK was ubiquitously expressed amongst these samples. $CD34^+$ cells from 2 normal bone marrow samples were also analyzed (Figure 2.1) and both of these expressed ILK, FLT3 and p-Akt.

FLT3 activation and ILK kinase activity in AML blasts.

For 8 FLT3 wild-type (WT) and 8 FLT3-ITD AML samples FLT3 was immunoprecipitated from cell lysates and analyzed for phosphorylation of FLT3 tyrosine residues by Western blotting to detect constitutive activation of the kinase. As expected 7 of 8 FLT3-ITD AML samples showed high levels of FLT3 autophosphorylation. However, interestingly this was also detected in 4 of 8 FLT3-WT samples (Figure 2.2A).

ILK kinase activity was detected in all 12 AML samples tested (Figure 2.2B). ILK kinase activity and ILK protein expression (as detected from the same gel) were

highly correlated suggesting that the kinase function of ILK was constitutively and ubiquitously active in AML samples ($r=0.74$, $p<0.01$, Student t-test, Figure 2.2C).

siRNA inhibition of ILK expression causes cell death in an AML cell line.

In preliminary experiments to assess the effect of ILK inhibition on the viability of AML cells the AML cell line TF-1 was studied. TF-1 cells express high levels of ILK and p-Akt and undergo dose dependent cell death when exposed to the kinase inhibitor QLT0267 (TF-1 cell kill after exposure to 3, 10 and 25 μM was 83, 93 and 98%, respectively). To assess the specificity of this cell death response to ILK inhibition TF-1 cells were transfected with ILK siRNA or control oligonucleotide. As shown on Figure 2.3A, Western blotting performed on lysates of transfected cells show a decrease in ILK protein in cells transfected with siRNA targeting ILK but not with the control. In parallel studies, colony formation by TF-1 cells was also greatly reduced after transfection with the ILK siRNA as compared with transfection of the control (Figure 2.3B). The mean % colony kill for 3 experiments was 57% and 19% for cells transfected with the ILK siRNA and control, respectively. These results suggested that ILK inhibition might be toxic to patient AML cells. Since QLT0267 has previously been shown to be cytotoxic to malignant cells from a variety of solid tumours where both ILK and p-Akt were highly expressed this compound was tested against AML blasts and CFC [18].

QLT0267 treatment causes dose and time-dependent inhibition of p-Akt expression in AML blasts.

To determine if QLT0267 treatment was successful in inhibiting activation of the PI3K pathway in leukemic blasts AML samples were cultured with this inhibitor and then analyzed for expression of p-Akt and its downstream target p-GSK3β by Western blotting. Cells from 2 AML samples cultured with various doses of QLT0267 for 6 hours each demonstrated p-Akt ser473 and p-GSK3β inhibition (Figure 2.4A), while cells from 1 AML sample cultured with 10 or 25 μM QLT0267 showed inhibition of phosphorylation after as little as 4 hours of drug exposure with the degree of inhibition increasing to 8 hours (Figure 2.4B).

QLT0267 is cytotoxic to leukemic blasts and AML-CFC.

In initial experiments leukemic blasts from 8 AML patients were cultured in various concentrations of QLT0267 for 48 hours and then analyzed by FACS for the presence of apoptotic cells. Exposure to the inhibitor resulted in a significant proportion of PI and/or Annexin-V positive cells from most patient samples (Appendix Figure A.1). When direct comparison was made between FACS analysis and inhibition of AML-CFC colony growth from the same patient samples treated with QLT0267 a direct correlation was seen ($r=0.88$, $p<0.005$, Student t-test, Appendix Figure A.2). Subsequently, a total of 27 AML patient samples which have been characterized for expression of p-Akt and the presence of FLT3 mutations were cultured in the presence of QLT0267 or the PI3K inhibitor LY294002 and placed into AML-CFC assays (Figure 2.5A). The median % kill was 22, 77 and 100% for 3, 10 and 25 μM QLT0267 respectively compared to 43% for

10 μM LY294002. Greater than or equal to 90% AML-CFC kill was achieved with 10 μM QLT0267 for 9 of 27 (33%) samples tested. The addition of the growth factors IL-3 (20 ng/mL), Steel factor (100 ng/mL) and FLT3 ligand (100 ng/mL) to the 48 hour culture did not significantly change the % AML-CFC kill observed with either inhibitor (Appendix Table A.2).

AML samples harbouring the FLT3-ITD mutation were more susceptible to QLT0267 induced cell death than those with wild-type FLT3 with the mean % kill being 41 and 84% for FLT3-ITD samples compared to 18 and 59% for FLT3-WT AML samples achieved with 3 and 10 μM QLT0267 respectively ($p=0.056$, $p< 0.01$ Student t-test, Figure 2.5A). However there was considerable overlap between the ITD positive and negative AML samples with respect to their QLT0267 sensitivity with some samples carrying this mutation being relatively insensitive while others without this abnormality showing high levels of AML-CFC kill. Interestingly, AML FLT3-ITD samples were also more susceptible to PI3K inhibitor LY294002 with a mean % kill of 60% versus 30% for FLT3-WT samples.

Comparison of AML-CFC kill with QLT0267 to that obtained with PKC412, another small molecule kinase inhibitor known to be active against FLT3 and which is currently undergoing clinical evaluation for treatment of AML (Appendix Figure A.3) [32,34], revealed QLT0267 to achieve higher percent kills in both FLT3 ITD positive and negative samples (mean % kill 57 and 85% with 10 μM QLT0267 compared to 14 and 29% with 100 nM PKC412 for FLT3-WT and FLT3-ITD AML samples, respectively, $p<0.05$ Student t-test, Figure 2.5B).

There was no apparent relationship between expression of ILK, p-Akt, or AML-CFC kill by QLT0267 and other clinical characteristics of the AML patients including bone marrow cytogenetic abnormalities, presenting white blood cell count or response to induction chemotherapy.

QLT0267 inhibits both ILK and FLT3.

To test the possibility that QLT0267 might inhibit FLT3 as well as ILK both ILK and FLT3 kinase assays were performed in the presence of QLT0267 on 3 AML samples, one FLT3-WT (Figure 2.6A) and two containing FLT3-ITD mutations (Figure 2.6B). In all 3 samples a decrease in kinase activity was detected for both ILK and FLT3 with increased concentrations of QLT0267, indicating direct inhibition of not only ILK but FLT3 kinase activity as well. The ratio of phosphorylated substrate to protein was plotted and no significant difference was detected for any of the 3 AML samples tested between ILK and FLT3 kinase inhibition with regard to sensitivity to QLT0267.

Comparison of the effect QLT0267 on normal bone marrow and AML CFC.

$CD34^+$ cells from 5 normal bone marrows and blasts from 5 AML samples which were highly sensitive to QLT0267 were cultured with or without the same concentrations of the inhibitor to allow comparison of the effect on normal and AML-CFC. Direct comparison of the dose response for CFC kill achieved with QLT0267 against normal and sensitive AML progenitors determined that the IC_{50} was 10 and 3 μM, respectively (Figure 2.5B and C). At 5 μM QLT0267 the range of % CFC kill for normal bone marrow and AML samples was 0-19% and 48-93% respectively, while at 10 μM

QLT0267 none of the normal bone marrow samples demonstrated ≥75% CFC kill whereas this was obtained for all 5 AML samples.

2.3 Discussion

As described previously and confirmed in this report, both the FLT3 and PI3K pathways are frequently constitutively activated in malignant blasts from poor prognosis AML patients [2,8,20,23]. Mutations in FLT3 lead to an increase in cell proliferation and activation of downstream signalling pathways including the PI3K pathway, and may contribute to leukemogenesis. Other factors that may lead to activation of PI3K include activating mutations of c-Kit and possibly SHIP-1 and autocrine growth factor production [1,9,10,49]. None of these abnormalities are thought to be capable of independently resulting in leukemic transformation. Nevertheless, tyrosine kinase inhibitors and other drugs which target these abnormalities have been shown to be cytotoxic to AML cells and may ultimately prove to be useful in AML treatment [33,39]. This study was focused on AML blasts and progenitors from patients with poor prognosis AML who are rarely cured with conventional chemotherapy regimens and therefore are most in need of novel therapeutic approaches.

ILK is a PI3K-dependant kinase and binds to PIP_3, a product of PI3K. Thus a number of upstream proteins, including FLT3, which can induce PI3K activation may be responsible for ILK activation in AML blasts and progenitors. Through phosphorylation of downstream targets such as Akt and GSK-3β ILK promotes malignant transformation by induction of anti-apoptotic pathways and cell cycle progression [15-17]. Inhibition of ILK results in apoptosis in cancer cells where over expression is detected [18,19]. The

data presented here demonstrate that ILK is ubiquitously expressed in AML samples and that the kinase function is active in these cells. In addition, specific inhibition of ILK using siRNA resulted in cell death and inhibition of colony formation in the AML cell line TF-1. This suggested that at least some AML blasts and progenitors might be dependent on ILK for their survival [50].

In subsequent experiments primary AML samples were treated with QLT0267, a kinase inhibitor which had previously been shown to inhibit ILK leading to cell death and tumour regression in various solid tumour model systems [51]. As shown here, QLT0267 effectively inhibited ILK activity in AML blasts and mediated a dose-dependant down regulation of p-Akt ser473 expression. However, although ILK was ubiquitously expressed in AML blasts and p-Akt was detected in the large majority, there was considerable variability among samples in the sensitivity of AML-CFC to killing by the inhibitor. This suggested that QLT0267 might be acting on another relevant molecular target in these cells. When the responses of AML-CFCs to the inhibitor was compared between samples with and without the FLT3-ITD the former were found to be more sensitive suggesting FLT3 might be such a target. This possibility was confirmed in subsequent experiments where similar inhibition of FLT3 as compared to ILK kinase activity was demonstrated in AML samples. Furthermore, in the three AML samples tested the inhibitor appeared to be equally effective against wild-type or mutant FLT3.

The presence of both ILK and FLT3 activation rendered AML-CFC particularly sensitive to killing by QLT0267. However, the response to QLT0267 from the AML progenitors was variable even when FLT3 mutations were present and at least 1 AML sample (sample 33 Figure 2.1) had no FLT3 expression detected and yet had over 75%

kill with 10 μM of QLT0267. Thus, it appears that the presence of FLT3 activation is not always either necessary or sufficient to render cells sensitive to the inhibitor. In cells without FLT3 mutations sensitivity to QLT0267 might be explained if the cells were uniquely sensitive to ILK inhibition or if the inhibitor was acting on yet another molecular target which collaborated with ILK inhibition to kill the cells. QLT0267 has been tested against a large number of kinases and has shown no activity against c-Kit, c-abl, c-fms and other kinases known to be involved in human leukemias. In addition, it does not target other components of the PI3K pathway such as GSK3β, PDK1 and Akt subunits (personal communication QLT Inc.). Nevertheless, it is possible that unknown targets in addition to ILK or FLT3 are responsible for some of the effects seen. On the other hand, when QLT0267 was relatively ineffective against AML-CFC in samples where both ILK and FLT3 activation could be demonstrated it is possible that alternative pathways which the inhibitor does not affect such as the ERK/MAPK pathway could be maintaining cell viability. Interestingly FLT3-ITD AML samples also responded better to the PI3K inhibitor LY294002 than FLT3 wild-type samples. Whether this is due to off target effects which have been documented for this inhibitor [52] or solely due to inhibition of PI3K in these samples is unknown.

The near ubiquitous expression of FLT3 and ILK in AML with the activation of ILK in all samples tested and FLT3 in many suggests that the target specificity of QLT0267 may allow it to be particularly effective against AML cells. Others have shown that inhibiting FLT3 does not lead to inhibition of constitutive activation of Akt in all AML samples [53], and that FLT3 can activate a number of different signalling pathways [28-30], adding further rationale to inhibiting both kinases simultaneously.

Interestingly, PKC412, which inhibits FLT3 as well as a variety of other kinases, appeared less effective than QLT0267 in eliminating AML-CFC in our experiments providing further evidence that the combined inhibition of ILK and FLT3 may be advantageous.

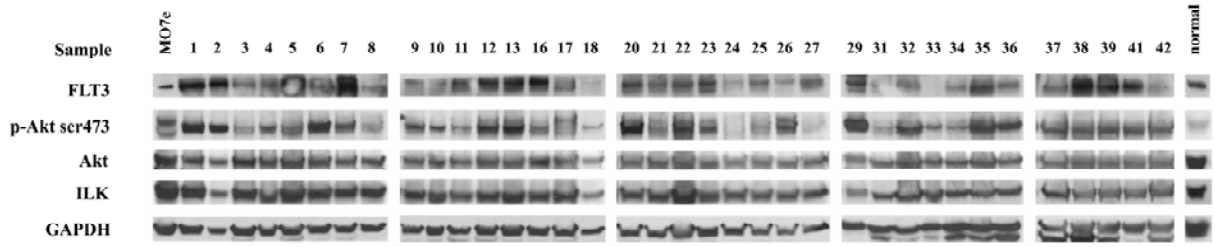
Interaction with stromal cells has been shown to induce relative chemoresistance in AML blasts suggesting the bone marrow microenvironment as a site of minimal residual disease and eventual relapse [54,55]. However, culturing AML cells on a stromal cell layer has also been shown to activate the PI3K pathway and to increase the sensitivity of leukemic cells to killing with QLT0267 [56]. Although the contribution of FLT3 to these effects was not examined, these results suggest that agents such as QLT0267 might overcome resistance to conventional chemotherapy drugs.

Approximately one third of AML samples were substantially more sensitive to the QLT0267 inhibitor, as measured by AML-CFC kill, than normal bone marrow (Figures 2.5C and D) although both normal and leukemic cells express ILK and FLT3. This suggests that at least some AML cells are more dependent than analogous normal progenitors on these pathways for their survival, a finding that is similar to that observed for breast cancer cells versus normal breast epithelial cells [18]. The high cell kill seen when both normal and malignant cells were exposed to very high concentrations of QLT0267 was likely due in part to off target inhibition which is well described for other kinase inhibitors including those in clinical use [57].

The treatment of AML has not improved substantially for several decades and the majority of patients with this diagnosis still die of their disease. From the long list of molecular abnormalities described in the leukemic cells from AML patients a number

have been identified as potential targets for therapeutic intervention. These may ultimately provide new therapies that are both specific for the malignant clone and cytotoxic through mechanisms of action that are distinct from those of conventional chemotherapy agents. The data presented here suggest further evaluation of the therapeutic potential of agents such as QLT0267 which inhibit more than one relevant target in AML. Furthermore, the results suggest one of these relevant targets may be ILK.

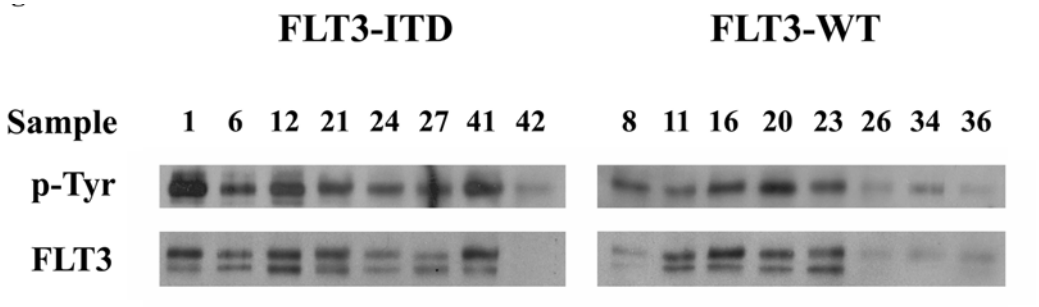
Figure 2.1: FLT3, ILK and p-Akt protein are detected in AML blasts and normal bone marrow cells.



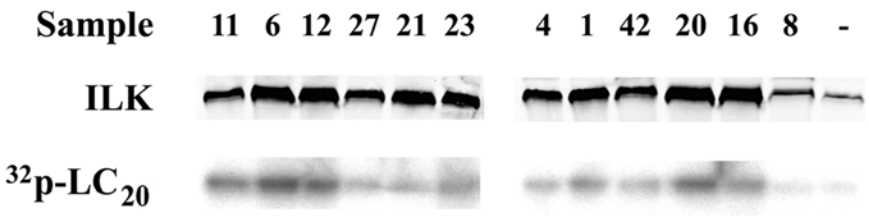
Western blots showing 36 AML patient samples, the AML cell line MO7E, and $CD34^{+}$ cells from one of two normal bone marrows analyzed. Samples were processed and blots prepared as described in the Methods. Western blots were probed with anti-FLT3, anti-phospho-Akt ser473, anti-Akt, anti-ILK and anti-GAPDH as a loading control.

Figure 2.2: FLT3 and ILK proteins are expressed, and kinases are active in AML samples.

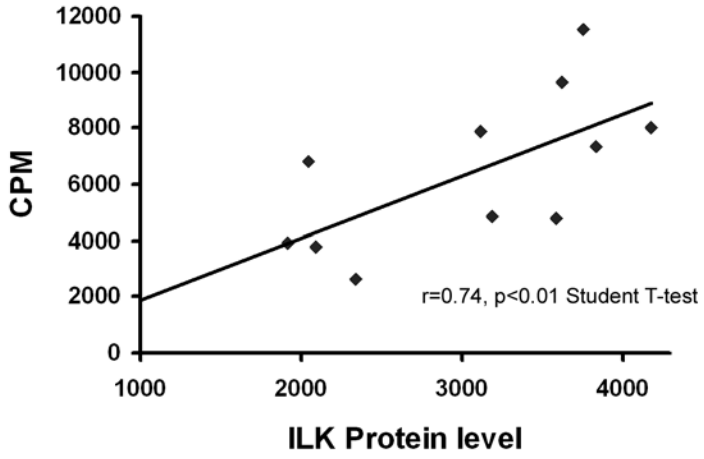
A



B



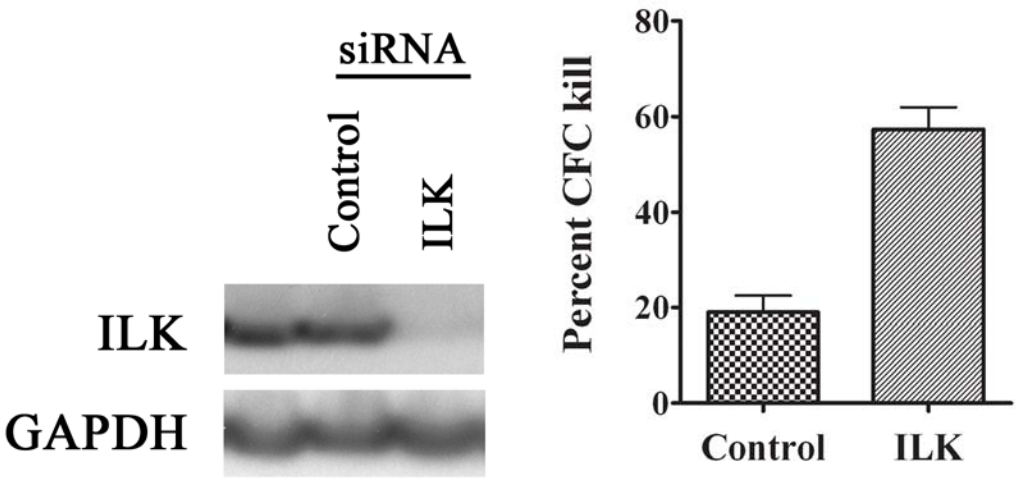
C



A) Western blots of FLT3 protein isolated from 8 wildtype and 8 FLT3-ITD AML sample cell lysates. Detection of tyrosine phosphorylation (p-Tyr) is a measure of kinase activation. B) ILK protein was isolated from 12 AML patient sample cell lysates and kinase activity determined as described in materials and methods. The amount of ILK protein as detected by Western blotting in the top portion of the figure is compared to the amount of phosphorylated ILK substrate, LC_{20}, as detected by audioradiography in the lower half of the figure. For each sample the substrate band was excised and ILK kinase activity quantified using liquid scintillation to measure radioactivity. C) For each sample ILK expression was plotted against ILK kinase activity and a linear trend line was fitted to the points.

Figure 2.3: TF-1 cells transfected with siRNA targeting ILK downregulate ILK protein production and show reduced CFC growth compared to cells transfected with control siRNA.

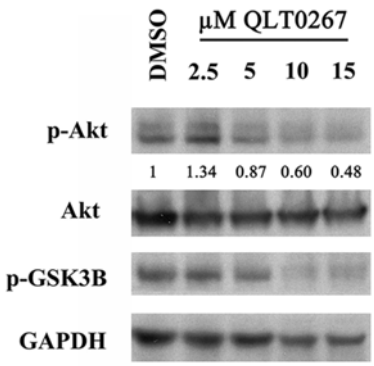
A B



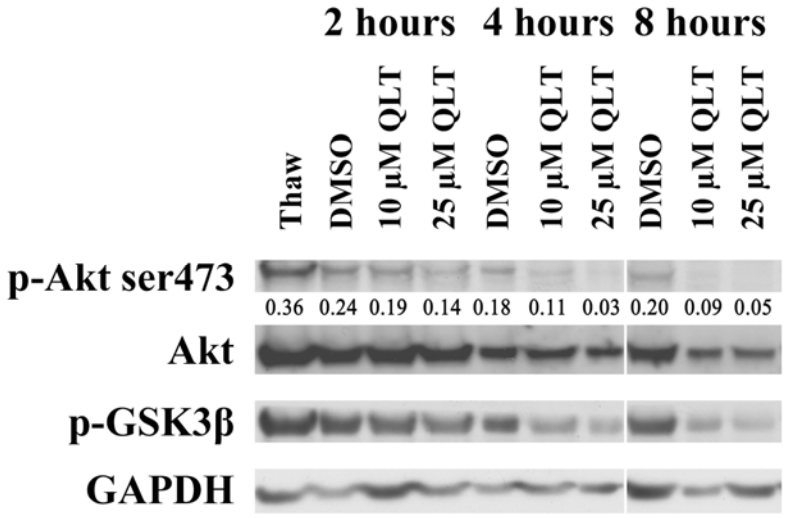
TF-1 cells were transfected with siRNA oligonucleotide targeting ILK or non-silencing control oligonucleotide and a portion of the cells were (A) plated in suspension cultures for 48 hours, and then harvested for Western blotting. ILK protein expression is inhibited in cells transfected with ILK siRNA but not control. The remaining cells were plated into methylcellulose media to detect AML-CFC (B). The mean % kill (± standard deviation) for cells transfected with ILK siRNA or control siRNA in 3 independent experiments was 57.3 (±9.0)% for ILK siRNA, and 19 (±5.5)% for control siRNA.

Figure 2.4: QLT0267 results in time and concentration dependent down regulation of Akt phosphorylation on serine 473 in AML blasts.

A

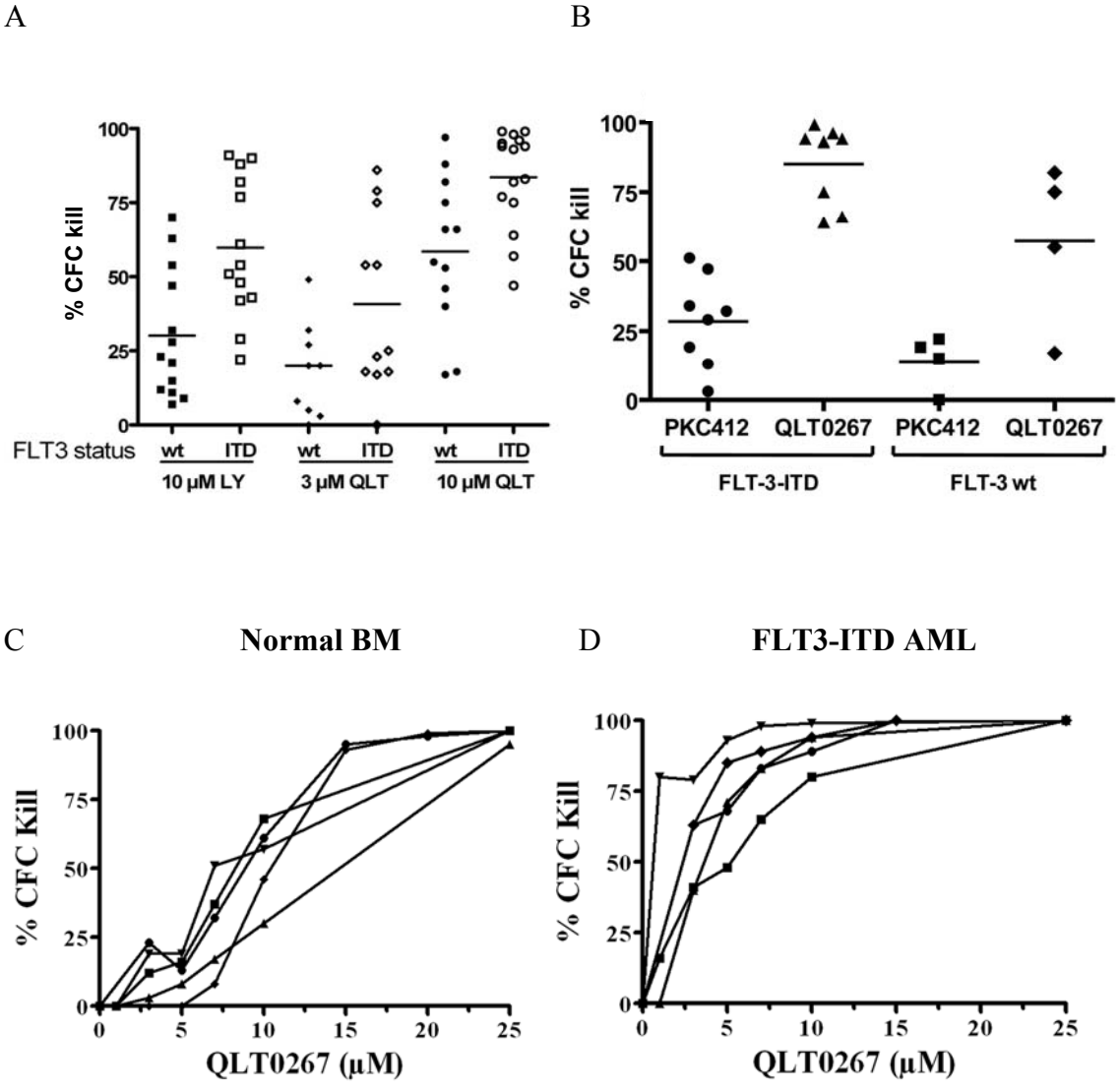


B



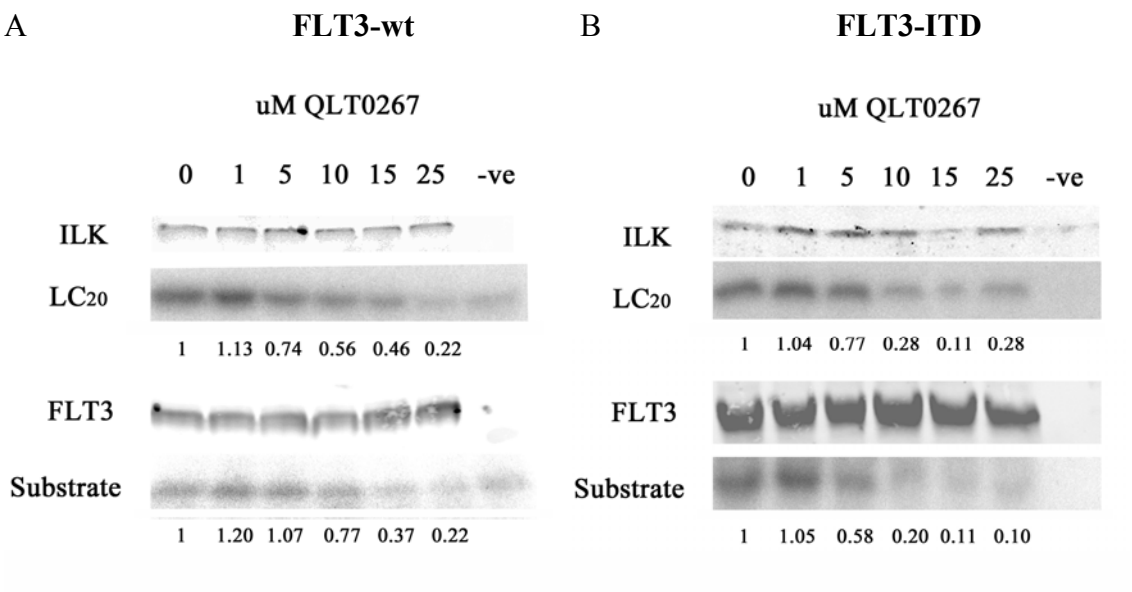
Western blot analysis of AML blasts from A) patient sample 26 treated for 6 hours with increasing concentrations of QLT0267, and B) patient sample 23 incubated with 10 or 25 μM QLT0267 or DMSO control for various time intervals. Phosphorylated and total Akt levels were quantified for each sample using densitometry and the ratio of p-Akt:total Akt is displayed as a numerical value between the bands. Both p-Akt ser473 and its downstream target p-GSK3β decrease in the QLT0267 treated samples with increased concentrations of QLT0267, and with increased time intervals.

Figure 2.5: AML and normal CFC cell death after exposure to QLT0267.



AML blasts from 27 patient samples were incubated for 48h with the PI3K inhibitor LY294002, QLT2067 at the indicated concentrations in suspension cultures and then plated in methylcellulose assays for detection of AML-CFC. A) The values shown are the percent reduction of AML-CFC numbers as compared to DMSO control treated cultures. Horizontal bars indicate the means of the % kill for each different drug treatment. Sample response was compared between wild-type and mutated FLT3 AML. Mean % kill was 18 and 59% for FLT3-WT AML samples, and 41 and 84% for FLT3-ITD samples with treatment of 3 and 10 μM QLT0267 respectively ($p=0.056$, $p< 0.01$ Student t-test comparing ITD positive and negative samples). B) Cells from 8 FLT3-ITD and 4 FLT3 wildtype AML samples were cultured for 48h in the presence of 100 nM PKC412 , 10 μM QLT0267, or DMSO and placed into CFC assays. The mean % kill was 29 vs. 85% for FLT3-ITD samples, and 14 vs. 57% for FLT3 wildtype AML samples treated with 100 nM PKC412 or 10 μM QLT0267 respectively. In preliminary experiments maximum AML-CFC kill (from samples both with and without the FLT3 ITD) was obtained with PKC412 at a concentration of 100 nM (data not shown). C) Cells from 5 normal patient bone marrow samples enriched for $CD34^+$ cells and (D) 5 FLT3-ITD AML samples responsive to QLT0267 were incubated for 48h in suspension cultures with various concentrations of QLT0267 and then plated in methylcellulose assay for detection of CFC. The percent kill of CFC was calculated by comparison to assays of DMSO-treated (control) cells. The IC_{50}s for QLT0267 against normal and AML CFC are 10 and 3 μM, respectively, indicating that these AML progenitors are more sensitive than analogous normal bone marrow cells to this drug.

Figure 2.6: QLT0267 inhibits both ILK and FLT3 kinases.



ILK and FLT3 kinase assays were performed on 3 AML samples as described in the Methods, in the presence of increasing concentrations of QLT0267. Shown are A) FLT3-WT and B) one of two FLT3-ITD AML samples. Protein and kinase activity levels as measured by substrate phosphorylation, were quantified using densitometry and displayed as a ratio of the two values.

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Chapter 3

Targeting Phosphatidylinositol-3 Kinase Pathway Activation Leads to Selective Killing of Acute Myeloid Leukemia Stem Cells[†]

[†]A version of this chapter has been submitted. Muranyi, A.L., Dedhar, S., and Hogge, D.E. Targeting Phosphatidylinositol-3 Kinase Pathway Activation Leads to Selective Killing of Acute Myeloid Leukemia Stem Cells.

3.1 Introduction

Among the malignant blast cells in patients with acute myeloid leukemia (AML) are progenitors that exhibit the capacity for self renewal of their own numbers and a much greater proliferative capacity than the majority of leukemic cells [1]. These AML progenitors can be demonstrated by their capacity to engraft and proliferate in immunodeficient mice (NOD/SCID mouse leukemia-initiating cells or NOD/SL-IC) and to initiate long-term malignant hematopoiesis in tissue culture (long-term suspension culture-initiating cells or SC-IC) [2-4]. Cell surface phenotypes which enrich for progenitors that are detected in both assays are often similar to each other and to those exhibited by primitive normal progenitors [3-5]. It seems possible that such progenitors are important for maintenance of leukemia in patients.

Aberrant cell signalling is thought to play a role in maintenance of the leukemic clone in AML by providing a proliferative advantage to malignant cells and allowing them to escape mechanisms that lead to cell death or apoptosis. The FMS-like tyrosine kinase 3 (FLT3) and phosphatidylinositol-3-kinase (PI3K)-dependent pathways are two candidate signalling pathways thought to play such roles [6-8].

The FLT3 receptor is a member of the type III receptor tyrosine kinase (RTK) subfamily which also includes c-Kit, c-FMS and PDGF [9]. Activation of FLT3 normally occurs when FLT3 ligand binds to the receptor, inducing formation of a homodimer which in turn activates the kinase domain of the receptor, resulting in signalling to downstream pathways such as Ras, and PI3K [10-12]. FLT3 is mutated in approximately one third of AML samples, the majority of these mutations being internal tandem duplications (ITD) of the juxtamembrane region of the receptor although other

mutations exist which can, like the ITD, lead to aberrant and constitutive activation of the receptor tyrosine kinase and downstream signalling pathways [13,14].

The PI3K-dependent signalling pathway controls cell growth and proliferation via activation of Akt and downstream targets, and is activated in a large number of AML samples including those with poor prognostic features [8,15]. Inhibition of this pathway by compounds targeting various intermediates is cytotoxic to AML blasts. Integrin linked kinase (ILK) is an ankyrin-repeat containing serine/threonine kinase involved in phosphorylation of Akt (ser473). ILK is overexpressed or constitutively active in a large number of cancers, and is ubiquitously expressed in AML blast samples [16]. Inhibition of ILK by siRNA-mediated approaches or small molecule inhibitors in solid tumours results in apoptosis and/or impaired invasion of the cancer cells [17-19].

Here we demonstrate that specific inhibition of ILK using siRNA is toxic to primary human AML-CFC progenitors, suggesting ILK as a relevant target for AML therapy. In addition, the expression of FLT3, ILK and phosphorylated GSK3 (a marker of activation of the PI3K pathway) was measured in subpopulations of AML blasts enriched for NOD/SL-IC and SC-IC as well as quiescent and cycling leukemic cells. Furthermore, inhibition of these targets with QLT0267, a small molecule inhibitor of both ILK and FLT3 [16], is shown to inhibit the survival of both SC-IC and NOD/SL-IC but to have relatively little effect on normal bone marrow SC-IC, and NOD/SCID repopulating cells (RC). The combined effect on AML-CFC of QLT0267 with conventional chemotherapy drugs often used in the treatment of AML (cytarabine or daunorubicin) was also investigated. In total, these data suggest that ILK and FLT3 expression are more important to the maintenance of leukemia-initiating cells in AML

than to normal primitive progenitors and that their combined inhibition may be useful in eradicating the leukemic clone while sparing normal hematopoiesis.

3.2 Materials and Methods

AML and normal cells. Peripheral blood (PB) cells and bone marrow samples were obtained from 11 newly-diagnosed AML patients and normal bone marrow donors after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia (Table 1). Blood cells were Ficoll separated to obtain the mononuclear cell population and cryopreserved as described [20]. Prior to cryopreservation normal bone marrow from multiple donors was pooled and was enriched for $CD34^+$ cells using an immunomagnetic column (Easysep, StemCell Technologies Inc., Vancouver, Canada).

Cell culture. Thawed AML and normal $CD34^+$ bone marrow cells were resuspended in IMDM with 10% FCS at $1x10^6$ cells/mL ($1x10^4$ cells/mL for $CD34^+$ normal bone marrow) and then cultured for 24 hours (unless otherwise stated) with varying concentrations of the inhibitor QLT0267 (QLT Inc. Vancouver, BC, Canada, dissolved in DMSO), Ara-C, daunorubicin, or equivalent concentrations of DMSO as a control. Cultured cells were washed with IMDM and plated in methylcellulose-based colony forming cell (CFC) assays as previously described[16], placed into long-term suspension-culture initiating cell assays, or injected into sublethally-irradiated immunodeficient mice.

For long-term suspension culture-initiating cell assays weekly half media changes were carried out during the 6 week culture period after which the entire culture was harvested, washed once in IMDM, counted and placed into CFC assays to measure progenitor cell output [21].

FACS isolated quiescent and cycling AML cells were cultured in serum free media (SFM) with or without QLT0267 for 24 hours [22].

QLT0267. QLT0267 is a potent small molecule second generation kinase inhibitor which was originally characterized for its ability to inhibit ILK but not a variety of other kinases including Akt, PDK-1, DNA-PK and GSK3 [19]. More recently this compound has also been shown to inhibit both wildtype and mutant FLT3 with equivalent potency to that seen against ILK [16]. Treatment of AML blasts and CFCs with QLT0267 leads to inhibition of the PI3K pathway, and cell death [16].

siRNA inhibition. RNA inhibition experiments using Accell siRNA delivery system were performed on primary AML samples as instructed by the manufacturer (Dharmacon, Lafayette CO, USA). Briefly, cells were cultured for 1 to 2 hours in IMDM with 10% FCS before being placed into Accell siRNA delivery media (B-005000-100. Dharmacon) supplemented with 100 ng/mL stem cell factor, 100 ng/mL FLT3 ligand, and 20 ng/mL IL-3 (StemCell Technologies), in the presence of either siRNA targeting ILK or non-targeting siRNA as control. After 72 hours some cells were placed into CFC assays, while RNA from the remainder were analyzed by qRT-PCR for mRNA knockdown.

Fluorescence Activated Cell Sorting (FACS). Freshly-thawed AML cells were resuspended in Hanks' balanced salt solution modified (StemCell Technologies) with 2% FCS, and 0.04% sodium azide (HFN) with 5% human serum and then incubated with CD34-APC (clone 8G12, StemCell Technologies) and CD38-PE (clone HB-7, StemCell Technologies) antibodies on ice for 30 minutes, washed with HFN, followed by HFN with 2 μg/mL propidium iodide (PI), and resuspended in HFN. Cells were sorted into 3 populations ($CD34^-$, $CD34^+CD38^+$, and $CD34^+CD38^-$) and collected, using the FACSVantage SE or FACSVantage DiVa cell sorters (Becton Dickinson, San Jose CA), after setting gates based on single antibody control stained cells.

For cell sorting based on cell cycle status AML samples were cultured overnight in SFM and then washed twice in HFN prewarmed to 37°C. Cells were then incubated for 45 minutes at 37°C in the dark in 3 μg/mL Hoechst 33342 (Hst). Pyronin Y (Py) was then added at 1 μg/mL for 45 minutes at 37°C. Cells were then pelleted at 4°C, washed with HFN with Hst, Py, and PI (1 μg/mL), and resuspended in HFN with Hst and Py. Two populations of cells were isolated; quiescent (Hst and Py dull) and cycling (Py bright) (Appendix Figure B.2) [23].

Intracellular staining for FACS analysis. Cells were resuspended in HFN and prepared for intracellular staining as per manufacturer's instructions (Phosflow Perm Buffer III, Phosflow Fix Buffer I, BD Biosciences). Cells were resuspended in 100 μL wash/stain solution, to which pGSK3α/β Ser21/9, ILK1, or rabbit IgG isotype control (AlexaFluor-488 Conjugate)(37F11, 3862, and 4340 respectively, Cell Signaling) antibodies were added and incubated on ice for 30 minutes. Cells were washed and anti-pGSK3, and ILK

treated cells were incubated with AlexaFluor-488 conjugated secondary Ab (A11070, Invitrogen) for a further 30 minutes on ice, followed by washing and resuspension in wash/stain solution and analysis on a FACScan flow cytometer using CellQuest™ software (BD Biosciences). Protein levels were quantified by calculating geographic mean fluorescent intensity (MFI) of the various cell stainings using FlowJo software (Tree Star Inc, Ashland, OR USA).

Quantitative RT-PCR. RNA was isolated from cells using the Arcturus PicoPure RNA isolation kit (KIT0202, MDS Analytical Technologies, Sunnyvale CA, USA), and transcribed into cDNA using the superscript III first-strand synthesis system (Invitrogen, Burlington ON, Canada) as per manufacturer's instructions. Control reactions were performed without RNA present.

Quantitative PCR was performed on the cDNA in MicroAmp Fast Optical 96-Well Reaction Plates (#4346906, Applied Biosystems) using an Applied Biosystems 7500 Real-Time PCR System under the following conditions. Reaction mixture: 3 μL cDNA, 1x SYBR Green PCR Master Mix (#4309155, Applied Biosystems, Foster City, CA), 375 nM forward and reverse primers, ddH_2O to 25 μL. cDNA was quantified using a two-step PCR reaction running 42 cycles with the cDNA of interest being measured relative to GAPDH. siRNA-mediated mRNA knockdown was measured relative to mRNA in untreated cells. Primers used: FLT3 forward (CCGCCAGGAACGTGCTTG), FLT3 reverse (ATGCCAGGGTAAGGATTCACACC); GAPDH forward (CCATCACCATCTTCCAGGAG), GAPDH reverse (TGAAGACGCCAGTGGACTC);

ILK forward (GAATTCGTATGGACGACATTTTCACTCAGTGC), ILK reverse (CTCGAGCTACTTGTCCTGCATCTTCTCAAG).

Real-time PCR and data analysis were performed on a 7500 Fast Real-Time PCR system, using 7500 Fast Sequence Detection Software (Applied Biosystems). The relative quantification data of ILK and FLT3 in comparison to a reference gene (GAPDH) was generated on the basis of a mathematical model for relative quantification in real-time RT-PCR as described [24-26].

PCR to detect FLT3-ITD in AML-CFC. Colonies were plucked from CFC assays using a glass pipette, and washed once in PBS. DNA was extracted from cell pellets using the Arcturus Pico Pure DNA extraction kit (KIT0103, MDS Analytical Technologies) as per manufacturer's instructions. PCR was performed on the DNA under the following conditions: 45 mM Tris-HCl (pH 8.8), 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM β-mercaptoethanol, 4.4 mM EDTA (pH 8.0), 1 mM each dATP, dCTP, dGTP, dTTP, 113 μg/mL BSA, 12.5 pM each forward and reverse primers, 0.375 μL Platinum Taq DNA Polymerase High Fidelity (Invitrogen) to a total volume of 25 μL. PCR program: 95°C 3 minutes, 35x (95°C 30 seconds, 53°C 1 minute, 72°C 2 minutes), 72°C 7 minutes. The PCR products were run on a 3% agarose gel and stained with ethidium bromide to allow the different sized products associated with the wild type and ITD FLT3 to be discriminated. Blast cell DNA from the patient was amplified and run as a positive control and H_2O as a negative control. Primers used: FLT3 forward (GCAATTTAGGTATGAAAGCCAGC), FLT3 reverse (CTTTCAGCATTTTGACGGCAACC).

Immunodeficient mice. NOD.CB17-*Prkdc*^{scid}/J (NOD/SCID) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NOD/SCID IL2Rko) mice [27,28] were bred and maintained under sterile conditions in the British Columbia Cancer Research Centre Animal Research Facility under conditions approved by the Animal Care Committee of the University of British Columbia.

NOD/SCID mouse Leukemia-Initiating Cell Assay. NOD/SCID mice 8 to 10 week old were irradiated with 3 cGy (2.85 cGy for NOD/SCID IL2Rko) from an X-ray source and within 24 hours of irradiation injected via the tail vein with 10^7 AML cells (10^6 cells for NOD/SCID IL2Rko). AML cells were cultured for 24 hours in the presence of QLT0267 (dissolved in DMSO) or equivalent concentrations of DMSO and washed before injection. Femoral bone marrow (BM) aspirations were performed on the mice at 4, 8, and 12 weeks and experiments were terminated 16 weeks post injection of AML cells[29]. Mice were killed by CO_2 inhalation, and BM harvested from the 4 long bones by flushing with IMDM media containing 50% FCS, and analyzed for human AML engraftment using a FACScan flow cytometer (BD Biosciences). Human AML cells were detected by co-expression of human CD45 (FITC-conjugated antibody clone 9.4, Terry Fox Laboratory), and CD33 (PE-labelled antibody clone P67.6, BD Biosciences).

Human Lympho-Myeloid Repopulating Cell (RC) Assay. To measure the frequency of normal hematopoietic progenitors with both lymphoid and myeloid potential limiting dilution assays were performed in NOD/SCID mice as previously described [21]. Normal $CD34^+$ BM cells were taken from pools of 53, and 15 donors (samples 1 and 2

respectively) to obtain enough cells to perform these experiments. To detect cells of lymphoid and myeloid lineages CD45-PE (clone HI30), CD15-FITC (clone HI98), CD19-APC (clone SJ25C1), CD20-APC (clone L27, BD Biosciences), CD71-PE (clone OKT9, eBioscience), CD66b-FITC (clone G10F5), and CD34-PE (clone 8G12, StemCell Technologies) antibodies were used. RC frequencies and confidence intervals were calculated using Poisson statistics and L-calc software (StemCell Technologies).

Statistical Analysis. Mean fluorescent intensities (MFI) of proteins were calculated using FlowJo software (Tree Star Inc, Ashland, OR USA), and normalized to total blast cells. Correlations between ILK mRNA knockdown and AML-CFC percent kill was determined in Excel (Microsoft, Seattle, WA, USA). The significance of the correlation co-efficients, and differences in MFI or mRNA levels between AML populations were determined using the Student t-test. Differences in AML engraftment levels in mice between treatments were determined using GraphPad Prism (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significant. IC_{50}'s, IC_{75}'s, IC_{90}'s, and combination indices (CI) were calculated using Calcusyn software (Calcusyn, Biosoft, Great Shelford, UK). Calculation of CI is a method to numerically quantify drug synergism based on the multiple drug-effect equation of Chou-Talalay derived from enzyme kinetic models [30]. A $CI < 0.9$ shows synergism; a $0.9 < CI < 1.1$ indicates additivity; a $CI > 1.1$ indicates antagonism.

3.3 Results

siRNA inhibition of ILK is toxic to primary human AML-CFC

Previous investigation had determined that ILK was ubiquitously expressed in AML blasts [16]. siRNA targeting ILK was used to determine if downregulation of this expression would affect AML-CFC growth from four patient samples (Figure 3.1A). 72 hour exposure to ILK siRNA reduced AML-CFC growth, as compared to control, nontargeting siRNA, from all 4 patient samples. ILK mRNA expression was quantified using qRT-PCR and ILK knockdown was determined as compared to ILK expression in untreated cells. The proportion of ILK mRNA knockdown correlated with the percent AML-CFC kill for the 4 AML samples treated with siRNA targeting ILK (R^2=0.84, 0.1>p>0.05 Student t-test, Figure 3.1B). Thus, reduced ILK expression inhibits the survival of AML-CFC.

ILK, pGSK3, and FLT3 Expression in Subpopulations of AML cells enriched for Malignant Progenitors

In most AML samples $CD34^-$ blasts are depleted of progenitor activity while $CD34^+CD38^-$ cells contain the majority of leukemia-initiating cells (L-IC) capable of engrafting in immunodeficient mice or initiating long-term growth in culture [3,5]. To determine if evidence of PI3K activation, as measured by pGSK3 expression, as well as ILK and FLT3 could be detected in subpopulations of AML cells enriched for L-IC five AML blast samples were sorted based on CD34 and CD38 expression (Appendix Figure B.1). The relative amounts of pGSK3, and ILK present in $CD34^+CD38^-$, $CD34^+CD38^+$, and $CD34^-$ cells as compared to total unsorted blast cells were quantified (Figures 3.2A-

C). As shown in Figure 3.2B, pGSK3 was detected in each population for all 5 AML samples although $CD34^+CD38^-$ cells on average contained less pGSK3 than the other two populations ($p=0.04$ and $p=0.02$, Student t-test $CD34^+CD38^-$ vs $CD34^-$, and $CD34^+CD38^+$, respectively). ILK was also ubiquitously expressed but significantly lower in $CD34^+CD38^-$ as compared to $CD34^-$ cells ($p=0.02$, Student t-test). qRT-PCR analysis showed that FLT3 was expressed in each of the sorted cell populations at similar levels relative to total blasts ($p>0.05$) (Figure 3.2D). These data indicate that the PI3K pathway was activated and could potentially be targeted through ILK and/or FLT3 in L-IC.

ILK and FLT3 are expressed, and GSK3 is phosphorylated in both cycling and quiescent AML cells

Quiescent AML cells are enriched for progenitors capable of engrafting in immunodeficient mice [23]. Hoechst and Pyronin staining and FACS were used to isolate cells in G_0 and cells in active cell cycle from 5 AML samples (Appendix Figure B.2). These cells were then further analyzed for pGSK3 and ILK and FLT3 expression by FACS and qRT-PCR, respectively (Table 3.2). There was no substantial difference in the expression of either pGSK3 or ILK between cycling and quiescent cells in any of the 5 AML samples. However, although FLT3 RNA could be detected in both populations, in 3 cases the relative amount was less in quiescent as compared to cycling cells (Table 3.2).

QLT0267 effects on cycling and quiescent AML cells

Quiescent and cycling cells from 3 AML samples were cultured in varying concentrations of QLT0267 to determine the relative sensitivity of AML-CFC from these populations to this drug (Table 3.2). In two cases (samples 1 and 2) there was no significant difference in the IC_{50}'s and IC_{75}'s between AML-CFC derived from cycling and quiescent cells. However, in the third case (sample 5) CFCs from the G_0 population were relatively resistant to QLT0267 and showed a colony morphology that was quite different from that seen among the cycling cells (Appendix Figure B.3). Interestingly, when colonies were plucked from these assays to allow PCR for the FLT3-ITD to be performed, none of the 14 colonies from the quiescent fraction showed this abnormality while 3 of 7 colonies from the cycling fraction were FLT3-ITD positive ($p<0.01$, chi-square test). These data indicate that the relative resistance of quiescent cells from sample 5 to QLT0267 may have been caused by a substantial proportion of normal CFC in this fraction which we have shown to be more resistant than AML-CFC to this inhibitor [16] (Table 3.3 below).

QLT0267 is cytotoxic to AML Suspension Culture-Initiating Cells (SC-IC), but spares normal bone marrow SC-IC

To determine the effects of ILK and FLT3 inhibition on AML progenitors that initiate long-term malignant hematopoiesis in culture, 5 AML samples were placed into both CFC and long-term suspension culture initiating cell assays after 24h culture with or without QLT0267. The median percents kill seen among SC-IC-derived CFC were 63 and 100 % for 20 and 50 μM QLT0267, respectively, which was similar to the median

AML-CFC kills of 94 and 100% for the same patient samples and inhibitor concentrations with the exception of Sample 5 which grew rather poorly in suspension culture (Table 3.3).

The sensitivity to QLT0267 of normal CFC and SC-IC derived from two pools of $CD34^+$ cell-enriched normal bone marrow (NBM) was also tested in similar experiments and demonstrated far less toxicity (Table 3.3). Thus, AML SC-IC as well as CFC are more sensitive to killing with QLT0267 than their normal counterparts.

AML cells which engraft in NOD/SCID mice are killed following exposure to QLT0267

To assess the sensitivity of NOD/SL-IC to QLT0267 AML blasts from 4 patient samples (1, 2, 4, and 5) were cultured for 24 hours with or without this drug and then injected into cohorts of 3 to 6 NOD/SCID mice. Sixteen weeks after AML cell injection the mean percents engraftment for control cells were 90, 88, 38, and 32% for samples 1, 2, 4 and 5, respectively, while cells treated with 20 μM had mean engraftment rates of 1, 51, 1, and 1%, respectively. Samples 2, 4 and 5 were also treated with 50 μM QLT0267 and showed 2, 1, and 0% engraftment, respectively (Figure 3.3A). For two AML samples cells were also injected in NOD/SCID mice, and allowed to engraft before the mice were treated with QLT0267 orally (Appendix Figure B.4).

Normal bone marrow lymphomyeloid repopulating cells (RC) survive treatment with QLT0267

Two normal $CD34^+$ bone marrow pools were also cultured for 24 hours with 20 μM QLT0267 or DMSO, and then injected into cohorts of NOD/SCID mice at limit dilution. The frequencies of lymphomyeloid RC in the cells injected into mice and

detected 8 and 12 weeks later were not significantly changed by QLT0267 treatment (Figure 3.3B). Similarly, the output of $CD45^+$ human cells detected per RC in mouse bone marrow was not reduced by QLT0267 exposure and, in fact, appeared somewhat higher for QLT0267-treated than DMSO-treated cells from sample 1 (mean ± SD % CD45 cells detected 12 weeks after injection of cells containing an equivalent of 1.25 RCs was 0.43 ± 0.27 vs 20.5 ± 33.4, and 0.69 ± 0.39 vs 0.39 ± 0.21 for DMSO and QLT0267 treated cells from samples 1 and 2 respectively). Thus, normal lymphomyeloid bone marrow RC survive QLT0267 treatment which eliminates NOD/SL-IC activity from at least some AML samples.

Combinations of QLT0267 with Ara-C or daunorubicin

To investigate whether or not QLT0267 could enhance the effects of chemotherapeutic agents commonly used in the treatment of AML, 6 AML patient samples were cultured with Ara-C, daunorubicin (DNR), QLT0267 or one of the chemotherapy drugs in combination with QLT0267 at various doses based on the IC_{50} determined for each drug and sample individually. The schedule of the addition of each compound was also varied so that QLT0267 was added 24 hours before, at the same time as, or 16 hours after the addition of Ara-C or DNR to the cells. Cultured cells were plated in CFC assays to allow calculation of the IC_{90} for AML-CFC kill for each drug alone and in combination, and the combination index (CI) as a measure of drug interaction. Overall, the schedule adding QLT0267 first followed by the addition of Ara-C or DNR resulted in slightly lower CI(90)s than the other schedules tested ($p<0.05$, $p=0.15$, for CI(90) of QLT0267 1^{st} Ara-C 2^{nd} vs QLT0267+ Ara-C, and vs Ara-C 1^{st}

QLT0267 2nd respectively, Student t-test, Appendix Table B.1). The results of this schedule of drug administration are shown on Table 3.4. Analysis of drug interaction varied from sample to sample with 2 samples (1 and 11) showing synergy between QLT0267 and both Ara-C and DNR, 2 showing an additive effects (5 and 6) and 2 showing antagonism (9 and 10).

3.4 Discussion

The PI3K pathway is frequently constitutively active in many human malignancies including AML[8,15,31-34]. Although this pathway is necessary for normal cell proliferation, differentiation and survival its down-regulation has shown selective toxicity for malignant rather than normal cells [15,16]. It has been suggested that this differential sensitivity is a form of 'oncogene addiction' in which malignant cells become abnormally dependent on pathways that provide them with a competitive growth advantage [35]. Such findings have also lead to the investigation of PI3K inhibitors as potential cancer therapeutics.

Although mutations in components of the PI3K pathway are rarely found in AML, the PI3K pathway is downstream of a number of common mutations found in AML including those in receptor tyrosine kinases such as FLT3, c-Kit, or insulin-like growth factor receptor, Ras and others [10,36-42]. This suggests that PI3K activation may be a relevant therapeutic target in AMLs with various molecular pathogeneses. One of the key regulators of this pathway is ILK, which we previously demonstrated to be expressed and active in AML [16]. Here we show that specific downregulation of ILK expression in these cells reduces AML-CFC growth (Figure 3.1). It seemed likely that

targeting this molecule might also be toxic to malignant progenitors with greater proliferative and self-renewal properties i.e. leukemia-initiating cells (L-IC) detected in mice and long-term culture.

To establish further rationale for investigating this possibility, FACS analysis was performed which demonstrated ILK expression and a marker of PI3K activation (pGSK3) in subpopulations of AML blasts enriched for L-IC, although at somewhat lower levels than detected in other cell fractions. Interestingly, AML blasts which are not in active cell cycle (G_0) also express both pGSK3 and ILK indicating that PI3K signalling may be necessary in these cells for functions such as prevention of apoptosis [23,43-45]. Although the majority of AML blasts are in active cell cycle those in the $CD34^+CD38^-$ subpopulation and/or capable of engrafting in NOD/SCID mice are largely quiescent [22,23,46]. This property renders such cells relatively resistant to chemotherapeutic agents such as Ara-C which preferentially act on cycling cells [47]. In contrast, as shown on Table 3.2 quiescent AML-CFC can be effectively targeted by PI3K inhibition.

FLT3 was detected in the various blast cell subpopulations isolated from a sample that had no detectable FLT3 mutation as well as from those with the FLT3-ITD. The presence of ILK, FLT3 and PI3K activation in the L-IC-enriched subpopulations from all 5 AML samples studied suggested that combined inhibition of these targets would be toxic to these progenitors.

QLT0267 is a small molecule inhibitor which has been previously shown to target both ILK and FLT3 and to down regulate PI3K activation in AML blasts [16,19]. Dose dependent killing of AML-CFC was also demonstrated in these earlier studies. Although FLT3-ITD positive AML-CFC were, in general, more sensitive to killing with QLT0267

than those with wildtype FLT3 the latter could also be successfully targeted in some patient samples [16].

The current experiments extend these observations to candidate 'leukemic stem cells' detected in mice and long-term suspension culture. As shown on Table 3.3 SC-IC from 4 of 5 AML patient samples tested were killed by QLT0267 at concentrations similar to those that also eliminated AML-CFC from the same sample. In contrast, very little kill of SC-IC from 2 pooled normal bone marrow samples was observed with the same drug concentrations and these progenitors even appear much less sensitive to QLT0267 than normal CFC. The relative selectivity of the inhibitor for killing of AML rather than normal SC-IC is even greater than that demonstrated for CFC. Similarly, when QLT0267-treated AML cells were injected into sublethally-irradiated immunodeficient mice the L-IC activity detected in untreated cells was largely eliminated (Figure 3.3A) while the same treatment had little effect on normal bone marrow lymphomyeloid repopulating cells (Figure 3.3B). Thus, downregulation of PI3K activation through combined inhibition of FLT3 and ILK kills candidate AML stem cells while sparing similar normal progenitors.

PI3K or its downstream targets have been shown to be activated by AML blasts in response to chemotherapeutic agents, possibly to avoid apoptosis [48-51]. Thus, such inhibitors might render them more susceptible to conventional chemotherapy agents. To test the possibility that PI3K/FLT3 inhibition with QLT0267 would enhance the sensitivity of AML progenitors to conventional chemotherapy drugs typically used in the treatment of AML, QLT0267 was combined with Ara-C or daunorubicin. In 4 of 6 AML samples it was possible to demonstrate a synergistic or additive effect with combined as

compared to single drug treatments. Inhibition of the PI3K pathway with QLT0267 may disrupt prosurvival signals leaving leukemic cells, including quiescent progenitors where NFκB is upregulated, more vulnerable to chemotherapeutic agents [46]. It is unlikely that treatment of AML with receptor tyrosine kinase (RTK) inhibitors, including those which target FLT3, will be as effective in AML as it has been in CML [52] given the relative complexity and variety of the molecular abnormalities seen in AML and the aggressive clinical course of the disease [53,54]. It seems more probable that regimens that combine such agents with conventional therapy will be effective. The data shown here supports that possibility, at least for some AML patients.

Thus, in summary the results presented show that the PI3K pathway intermediate ILK, PI3K activation, and FLT3 can be detected in L-ICs as well as total blasts from the same patient samples. The relevance of ILK as a target in AML has been demonstrated by siRNA studies in primary human leukemias. A small molecule inhibitor which targets both ILK and FLT3 was used to demonstrate the importance of these pathways to the survival of candidate leukemic stem cells detected in long-term culture and in mice. Selectivity for killing of malignant rather than normal progenitors was also demonstrated as was the potential synergistic interaction with conventional chemotherapy drugs. Together, these results suggest further exploration of agents such as QLT0267 for the treatment of AML.

Table 3.1: AML patient sample characteristics

Patient	Age	Sex	FAB	Cytogenetics	FLT3 status	WBC	Response	Status
1	58	F	M4	47, XX+13 (38%)	ITD+	370	IF	D 6 mos
2	60	M	M5b	47,XY+8	ITD+	175	ED	D 1 wk
3	49	M	M4eo	46,XY inv(16)(p13;q22)[4], 49idem +15, +22, +mar [cpl7]	wt	126	CR	D 30 mos
4	59	M	M4	46, XY	ITD+	101	CR	D 9 mos
5	45	M	M4	46, XY	ITD+	175	CR	D 7 mos
6	28	F	M4	46, XX	ITD+	306	IF	D 27 mos
7	52	M	M5b	46, XY	wt	60	CR	D 11 mos
8	51	M	M5 post MDS	46,XY	wt	52	CR	A 84 mos
9	72	M	M2	46,XY	wt	167	IF	D 6 mos
10	48	F	M5b	46, XX	wt	205	IF	D 3 mos
11	34	F	M2	46, XX t(7;11)(p15;p15)	ITD+	78	CR	D 11 mos

FAB, French, American, British classification; ITD, internal tandem duplication; wt, wild type FLT3; WBC, peripheral blood white blood cell count $x10^9$/L at diagnosis; Response, response to induction chemotherapy; IF, induction failure; ED, early death; CR, complete remission; Status, patient status at last follow-up and duration of follow-up; D, dead; A, alive.

Table 3.2: pGSK3, ILK and FLT3 levels, and response to QLT0267 in cycling and non-cycling cells

		Ratio of expression in quiescent/cycling cells			IC_{50}		IC_{75}	
Sample	% G_0	pGSK3	ILK	FLT3	Cycling	Quiescent	Cycling	Quiescent
1	16.4	1.24	0.80	1.85	14.3	18.8	35.4	29.1
2	11.2	0.96	0.85	0.16	23.2	26.5	28.1	30.7
3	9.7	0.70	1.09	0.10	ND	ND	ND	ND
4	6.3	1.02	0.75	0.63	ND	ND	ND	ND
5	12.2	0.77	0.60	0.37	11.3	21.8	17.6	38.1

Values for pGSK3 and ILK are expressed as the ratio of MFI detected in G_0 vs cycling cells isolated based on Hoechst and Pyronin staining. For FLT3 qRT-PCR was performed on RNA from the same cell populations and the expression relative to GAPDH for quiescent and cycling cells expressed as a ratio. The concentrations of QLT0267 (μM) in a 24h culture required to kill 50% (IC_{50}) or 75% (IC_{75}) of AML-CFC from 3 patient samples are also shown. For samples 3 and 4 this experiment was not performed.

Table 3.3: AML and normal bone marrow CFC and SC-IC cell death after 24 hour exposure to QLT0267

		CFC		SC-IC-derived CFC	
Sample	Treatment	per 10^5 cells[a]	% kill	per 10^5 cells[a]	% kill
1	DMSO	2360		1312	
	20 μM QLT	260	89	493	62
	50 μM QLT	100	100	1	99.9
2	DMSO	7920		5941	
	20 μM QLT	460	94	0	100
	50 μM QLT	60	99	33	99
3	DMSO	105		206	
	20 μM QLT	6	94	76	63
	50 μM QLT	0	100	0	100
4	DMSO	2800		106	
	20 μM QLT	80	97	0	100
	50 μM QLT	0	100	0	100
5	DMSO	925		26	
	20 μM QLT	150	84	19	28
	50 μM QLT	15	98	10	61
NBM 1	DMSO	357		5544	
	20 μM QLT	342	4	8160	0
	50 μM QLT	129	64	6385	0
NBM 2	DMSO	672		5676	
	20 μM QLT	378	44	4834	15
	50 μM QLT	132	80	4175	26

AML blasts from 5 samples and $CD34^+$ cells from 2 pools of normal bone marrow cells were cultured for 24h with and without QLT0267 and then placed in CFC or SC-IC assays. To detect SC-IC the entire contents of 6 week old cultures were harvested and plated in CFC assays. CFC numbers are expressed per 10^5 cells ($5x10^3$ cells for normal bone marrow) initially cultured with QLT0267

[a]For normal $CD34^+$ cells CFC and SC-IC-derived CFC are per $5x10^3$ cells.

Table 3.4: QLT0267 shows variable ability to enhance AML-CFC cell death with cytarabine or daunorubicin

		IC_{90}				IC_{90}		
Sample	CI(90)[a]	QLT[b]	AraC[c]	Combined	CI(90)[a]	QLT[b]	DNR[c]	Combined
11	0.73	28.9	201	7.8:93	0.59	28.9	56.8	6.2:21.6
1	0.81	29.7	374	14.5:121	0.65	29.7	140.3	12.6:31.4
5	0.96	20.1	227	11.5:88	1.08	20.1	44.4	10.7:24.6
6	0.97	14.5	>1000	11.8:167	0.98	14.5	54.8	7.6:25.2
10	1.23	28.9	>1000	22.5:563	1.68	23.2	49.0	20.5:38.4
9	1.28	45.8	434	25.8:309	9.28	53.1	79.8	185:463

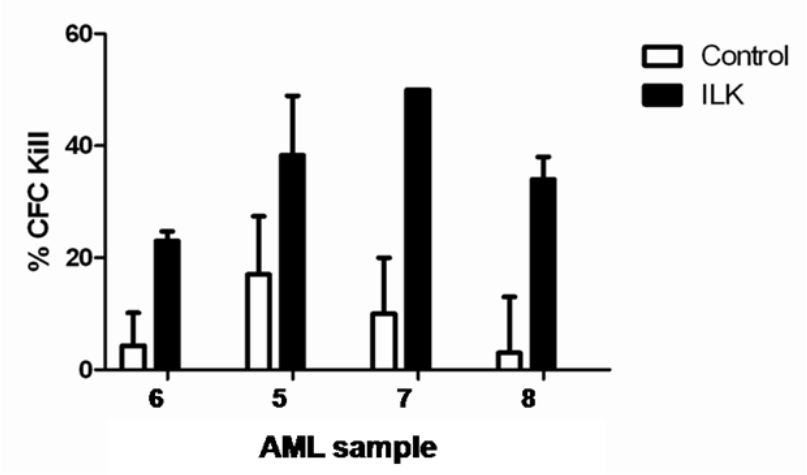
[a]Combination index (CI) measuring drug interactions at 90% kill: <0.90 = synergy, 0.90 ≤ X≤ 1.10 = additive, >1.10 = antagonistic.

[b]Concentration of QLT0267 in μM.

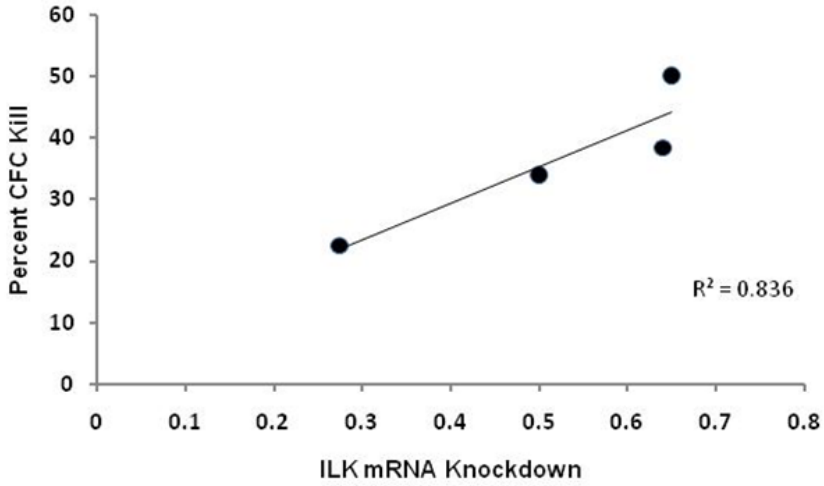
[c]Concentrations of Ara-C and DNR in ng/mL.

Figure 3.1: Inhibition of ILK is toxic to AML-CFC

A



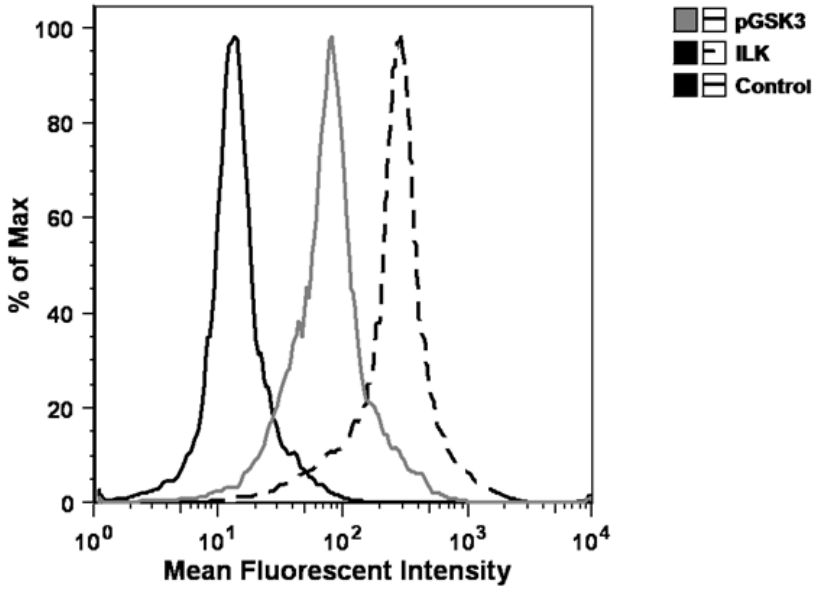
B



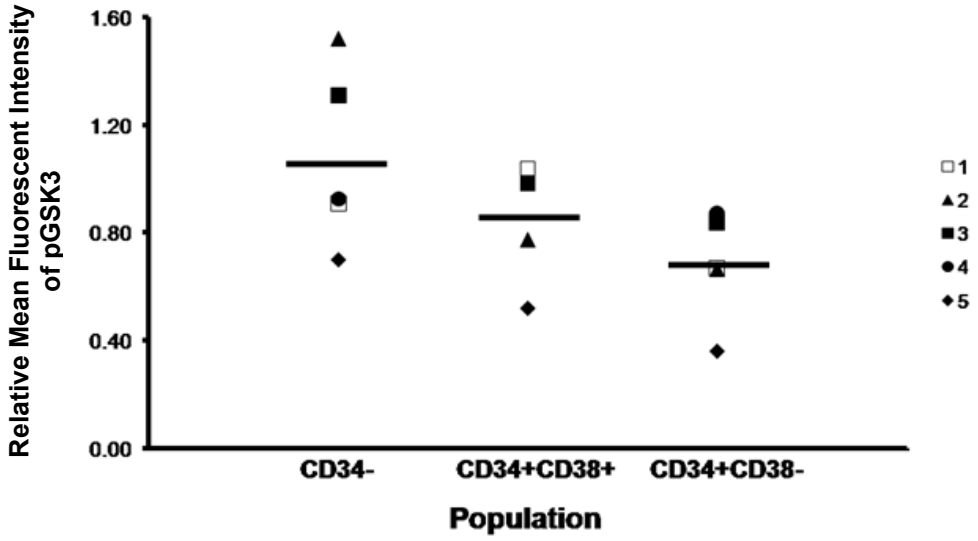
Four AML samples were cultured for 72 hours in the presence of control or ILK targeting siRNA, after which A) a portion of the cells were plated in CFC assays and percent kill calculated based on comparisons to untreated cells (n=3). B) RNA was extracted from the remaining cells and ILK knockdown measured compared to untreated cells, and plotted against CFC percent kill with ILK siRNA. There was a correlation between CFC kill and ILK knockdown (R^2=0.84, 0.1>p>0.05 Student t-test).

Figure 3.2: pGSK3, ILK, and FLT3 are present in $CD34^{+}CD38^{-}$ AML cells

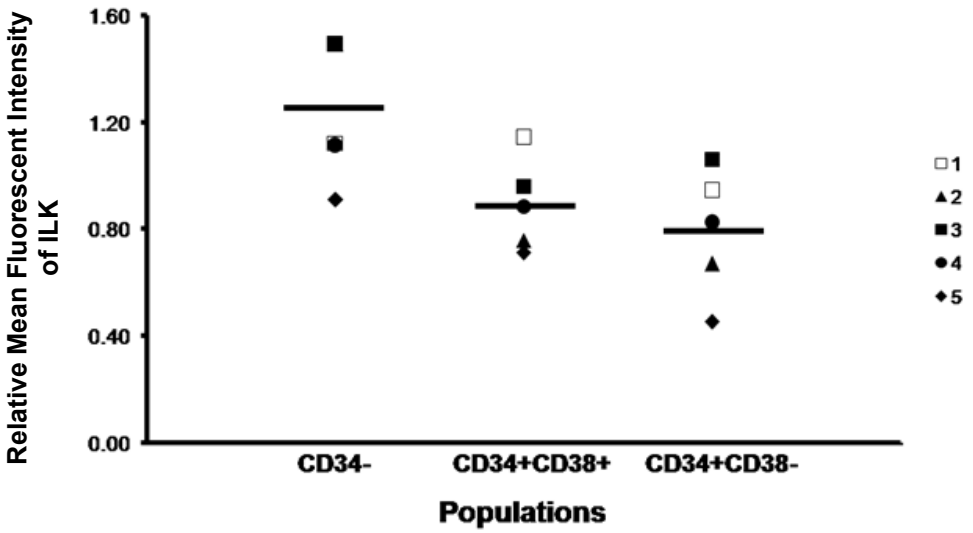
A



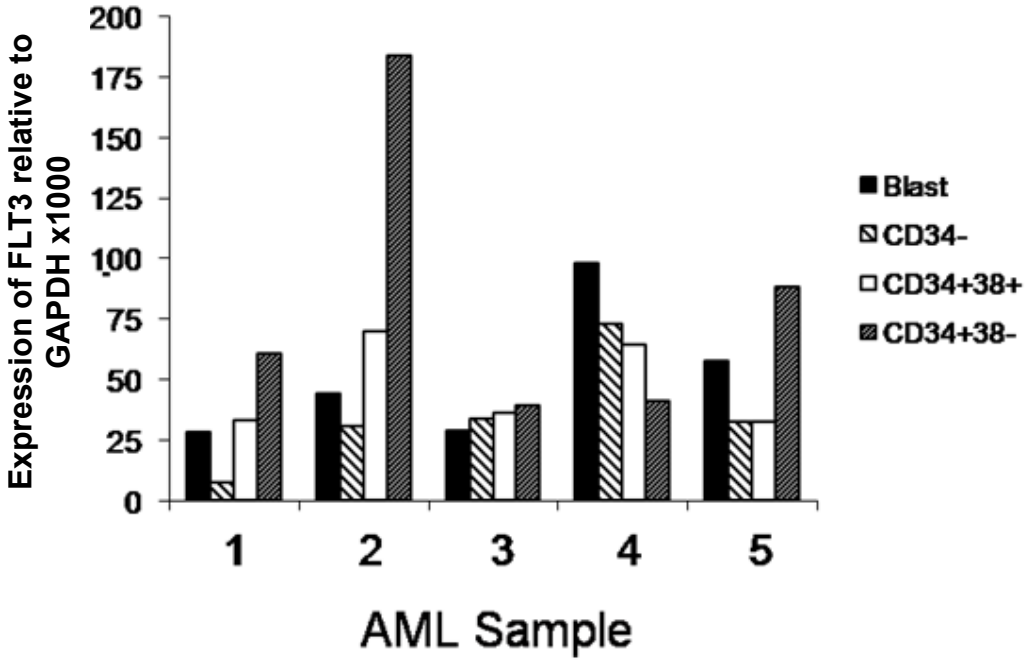
B



C


Relative Mean Fluorescent Intensity of ILK
1.60
1.20
0.80
0.40
0.00
CD34-
CD34+CD38+
CD34+CD38-
Populations
1
2
3
4
5

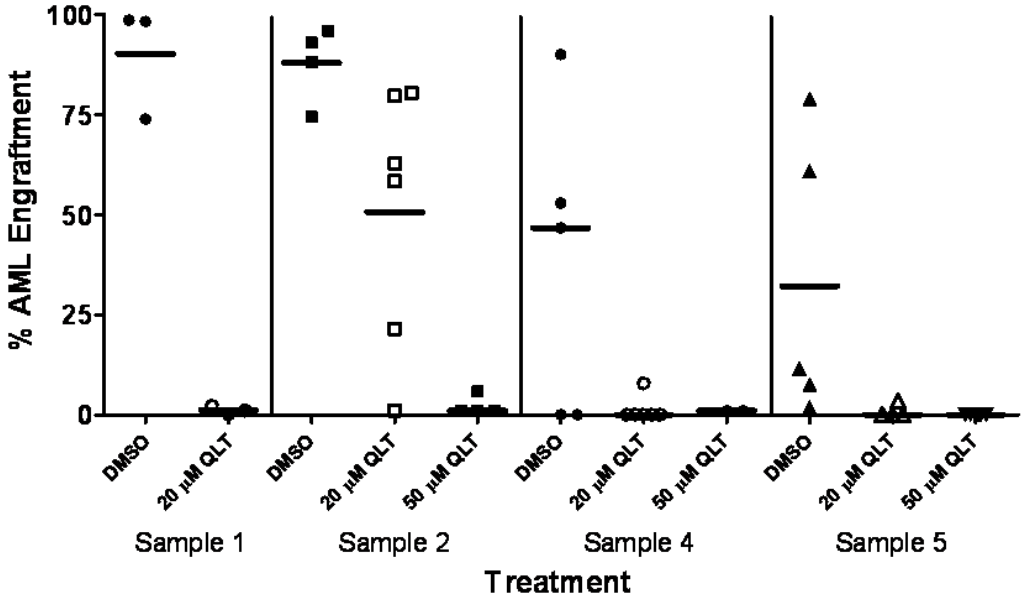
D


Expression of FLT3 relative to GAPDH x1000
200
175
150
125
100
75
50
25
0
1
2
3
4
5
AML Sample
Blast
CD34-
CD34+38+
CD34+38-

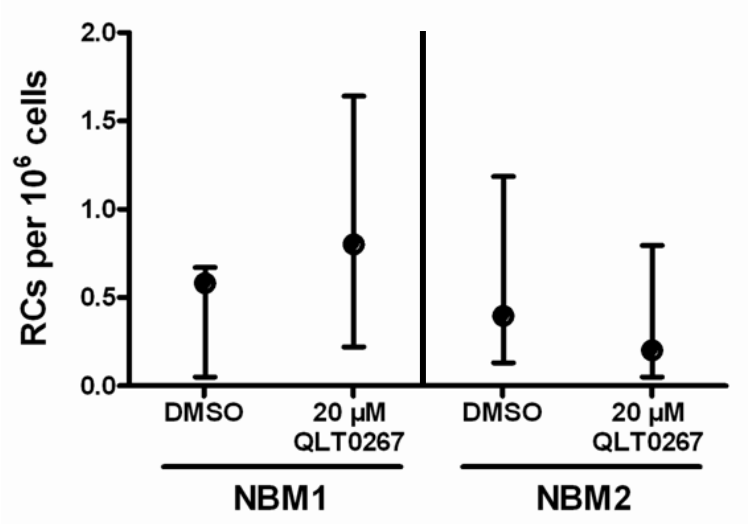
Five AML samples were sorted into $CD34^-$, $CD34^+CD38^+$, and $CD34^+CD38^-$ populations, with the percent AML blasts ranging from 46.5-98.8%, 0.7-49.4%, and 0.4-6.4% for the three populations respectively. A portion of these cells were stained for pGSK3 and ILK, and analysed by flow cytometry for MFI of the positive cells. A) Representative FACS histogram of total blast cells from AML sample 5 showing pGSK3 and ILK expression. B) and C), MFI in subpopulations are expressed relative to the MFI of total blast cells from the same patient sample – B, pGSK3; C, ILK. The absolute MFI for total blast cells from the 5 samples varied from 38 to 129 for pGSK3, and from 79 to 232 for ILK. $CD34^+CD38^-$ on average contained less pGSK3 ($p=0.04$ and $p=0.02$, Student t-test $CD34^+CD38^-$ vs $CD34^-$, and $CD34^+CD38^+$, respectively), and ILK ($p=0.02$ and $p>0.05$, Student t-test $CD34^+CD38^-$ vs $CD34^-$, and $CD34^+CD38^+$, respectively) than the other subpopulations. D) The remainders of the sorted cells were used to extract RNA, and qRT-PCR was performed to quantify FLT3 expression relative to GAPDH (set at 1000). No significant differences were observed amongst the populations.

Figure 3.3: QLT0267 is toxic to NOD/SL-IC

A



B



A) 4 AML samples and B) 2 CD34-enriched pools of normal bone marrow (NBM) cells were cultured for 24 hours in the presence of QLT0267 or equivalent doses of DMSO control prior to injection into sublethally irradiated NOD/SCID mice (NOD/SCID IL2Rko mice for AML sample 1). A). The percentage of $CD45^{+}CD33^{+}$ human AML cells in mouse BM 16 weeks after injection. Each symbol represents an individual mouse, horizontal lines indicate the mean. Statistically significant differences ($p<0.05$, Student t-test) were found between DMSO and QLT0267 treated cells in all cases with the exceptions of sample 4, 50 μM QLT0267, and sample 5, 20 μM QLT0267 treatments. B) The frequency of NBM lymphomyeloid repopulating cells per 10^6 cells injected into mice 12 weeks earlier determined at limit dilution. Error bars indicate 95% confidence intervals.

3.5 References

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Chapter 4

Discussion and Conclusions

A great deal of focus has been placed on studying and characterizing L-ICs with the belief being that if differences between normal and leukemic stem cells are identified they can be exploited to treat AML. Many different approaches have been employed in trying to eradicate L-ICs based on this principle, including targeting cells based on surface receptor expression using cytotoxic antibodies, inhibiting prosurvival signalling pathways and transcription factors using small molecules, inhibiting multidrug resistance transport receptors found overexpressed in AML, and disruption of the L-IC microenvironment [1-4]. I have chosen to target the PI3K pathway in my pursuit to eliminate L-ICs as a potential treatment for the disease.

The PI3K pathway is a complex pathway activated by a number of upstream stimuli such as cytokines, growth factors, and other signalling pathways [5-7]. It also has numerous downstream targets, and affects many cellular processes. Despite these numerous stimuli and targets, the PI3K pathway encounters a 'bottleneck' of sorts where PI3K signalling leads to the activation of Akt, a central protein in the pathway, after which the signal diverges to numerous downstream targets (Figure 1.3). Because of the structure of the PI3K pathway there are advantages and disadvantages to targeting particular proteins in this pathway.

A number of proteins have been targeted in attempts to achieve inhibition of PI3K signalling in AML, including receptors such as FLT3, and c-Kit, intermediates such as

mTOR, PDK1, Akt, and isoform specific inhibition of PI3K itself [8-13]. To this list I have added ILK as a valid target for the inhibition of the PI3K pathway in AML.

In my pursuit to inhibit the PI3K pathway in AML, a number of small molecule compounds that target the PI3K pathway upstream of Akt were initially tested. These include LY294002, an inhibitor PI3K, USO-03012, an inhibitor of PDK1, PCK412, an inhibitor of FLT3, 16A, an activator of SHIP, and QLT0267, an inhibitor of ILK [14-17]. These compounds were judged on their ability to inhibit the PI3K pathway, and cause leukemic blast and CFC progenitor cell death at concentrations that show little toxicity to normal hematopoietic progenitor cells, as well as being a compound that could potentially be used clinically. Based on the results of preliminary experiments, I began to focus on ILK and the compound QLT0267.

ILK was chosen as a target for PI3K pathway inhibition because it lies directly upstream of Akt, with the rationale being that if Akt is inhibited the majority of end targets of PI3K signalling will be affected as well. Many mutations present in AML can activate the PI3K pathway including those affecting RAS, FLT3 and c-Kit, as can aberrant autocrine and paracrine cytokine signalling [8-13]. Inhibition of the PI3K pathway directly upstream of Akt could effectively shut down the pathway regardless of the originating stimulus.

It is possible that because PI3K affects so many processes, inhibition of this pathway could create unacceptable toxicity to normal cells. However, when ILK and FLT3 are reversibly inhibited by the small molecule inhibitor, QLT0267, although there is some toxicity to normal hematopoietic cells, it is much less than that seen with AML cells (Chapters 2 and 3). This creates a therapeutic window of use for this compound

where leukemic cells can be killed while sparing their normal counterparts. Leukemic cells may be more affected by PI3K inhibition because they can become reliant on pathways such as this for growth and proliferation, a term referred to as 'oncogene addiction'. This refers to the phenomenon where cancer, despite being a potentially lengthy multistage process involving many mutations and epigenetic abnormalities, can be abnormally dependant on aberrant expression of one or more pathways for proliferation and survival. This renders the malignant cells profoundly susceptible to inhibition of one or a few oncogenes [18,19]. Normal cells are not nearly as affected by these perturbances, making cancer cells uniquely susceptible to this inhibition. Added to this, is the fact that normal hematopoietic cells are not expanding as rapidly as leukemic cells [20-23], so short term inhibition of this proliferative pathway is more likely to be tolerated.

To try and minimize the toxicity of PI3K inhibition to normal cells even more, one approach may be to selectively target proteins downstream in the PI3K signalling cascade. A few of these downstream targets appear to be particularly important for AML survival.

One such group is the forkhead box (FoxO) family of transcription factors. FoxO transcription factors regulate cell cycle, proliferation, and survival, are important in the maintenance of HSCs, and are inhibited by Akt [24-26]. FoxO3a has been shown to be regulated by FLT3-ITD through an Akt-dependant manner [27-29]. In one experiment treatment of a FLT3-ITD cell line with inhibitors of FLT3 led to a decrease in Akt phosphorylation, an increase in FoxO3a activation, and expression of proapoptotic protein Bim [29]. Overexpression of FoxO3a in this cell line led to an increase in

apoptosis. This effect however, may not be specific to FoxO3a. *Tothova et al* demonstrated both the importance and redundancy of FoxO family members in hematopoiesis [30]. They showed that deficiency of one or two FoxO family member did not result in a change in phenotype in hematopoietic cells. However, when FoxO1, 3, and 4 were deleted the HSC population had an increased proportion of cells in active cell cycle, and increased rates of apoptosis, leading to defects in HSC function.

Another potential target important for AML cell survival is NFκB [31]. NFκB is constitutively active in leukemic but not normal stem cells, and may be used as a survival mechanism by the leukemic cells [32-34]. Treatment of leukemic cells with agents that inhibit NFκB leads to apoptosis of LSCs while sparing their normal hematopoietic counterparts. Furthermore, it has been shown that NFκB activation correlates with FLT3 activation in primary leukemic cells, furthering the case for the importance of NFκB in AML survival [35].

As described in Chapter 2, the PI3K pathway intermediate ILK is ubiquitously expressed in the unstimulated leukemic blast cells from all 36 AML samples tested. As well, ILK kinase activity correlates to ILK expression in unstimulated cells. It is interesting then that Akt is not ubiquitously phosphorylated in these cells as would be expected since ILK is involved in Akt phosphorylation.

It is likely that there are multiple proteins involved in the phosphorylation of Akt. As mentioned previously, there are many proposed activators of Akt, such as PKCβII, DNA-dependant protein kinase, and mTORC2 [36,37]. Thus ILK may be only partly responsible for Akt phosphorylation. As well, negative regulators of PI3K signalling such as SHIP, PTEN, or PHLPP may be overactivated in cells where low Akt

phosphorylation is observed, which would cause downregulation of the PI3K pathway and decreased phosphorylation of Akt [38-41].

As described in Chapter 1, class I PI3Ks consist of 4 different catalytic subunits (p110α, p110β, p110δ or p110γ) [42]. *Canobbio et al* demonstrated that in platelets, integrin binding leads to signalling through the p110β isoform of the catalytic domain of PI3K [43]. If leukemia cells also follow this principle, ILK might be regulated by the PI3K signalling fraction controlled by p110β. p110β can be expressed at low levels in some AML samples [44]. If so, one might also expect that ILK-mediated Akt phosphorylation would also be low.

There appear to be contradictions in the literature regarding the importance of individual signalling pathways downstream of FLT3. Some groups demonstrate that RAS is the major pathway downstream of FLT3 while others show that PI3K signalling is essential [28,45-48]. It is possible that both are correct in individual cases. Most experiments designed to determine the importance of downstream targets of FLT3 signalling in AML rely either on cell lines expressing FLT3 or FLT3 mutations, or transforming cell lines with the FLT3-ITD mutation [10,27,28,47,49]. Since cell lines are immortalized they contain not only the introduced FLT3-ITD mutation but others as well, some of which may not be known or characterized. The introduced gene is also artificially overexpressed which could affect downstream signalling. Therefore it is difficult to directly compare these results to what is happening in primary AML, which may also contain undetected or uncharacterized mutations. Because of this, it is likely that in different patients the pathway downstream of FLT3 important for leukemic cell survival and proliferation vary.

In the quantification of pAkt expression in 36 AML samples performed in Chapter 2 there was no significant difference in pAkt levels between FLT3 wildtype and ITD samples (Figure 2.1). However, because the majority of AML samples tested demonstrated Akt activation regardless of FLT3 mutation status, one cannot draw conclusions from these experiments as to the importance of PI3K signalling downstream of FLT3 mutations versus wildtype FLT3 as PI3K is active in a large portion of AML samples with wildtype FLT3.

It would be interesting to compare the effects of direct PI3K or Akt inhibition against that of ILK inhibition in these primary AML samples. However, this would be a complicated experiment to perform, since to specifically target these proteins RNAi would need to be used to avoid the off-target effects small molecule kinase inhibitors have. Both PI3K and Akt have several isoforms [42,50], which means efficient inhibition of either target would require targeting multiple isoforms. Furthermore, with method of siRNA transfection used in Chapter 3 the transfection efficiency of siRNA into rare LSC, and primitive leukemic progenitor cell populations is unknown and likely low, making interpretation of any results obtained very difficult.

It is unlikely that activation of the PI3K pathway is sufficient on its own to cause leukemia, and that cooperation with other mutations such as those affecting cell differentiation is necessary [51,52]. In murine models, hematopoietic cells with the FLT3-ITD mutation introduced or PTEN knocked down develop into a myeloproliferative disease which then *may* progress to AML [53-56]. Since the PI3K pathway primarily generates proliferative and anti-apoptotic signals and does not greatly affect cell differentiation, this result is not surprising. At some point these rapidly

expanding cells might acquire one or more additional mutations, disrupting differentiation and transforming these cells to generate full blown leukemia in a proportion of mice.

In Chapter 3 I show that the PI3K pathway, as measured by GSK3 phosphorylation, is active in $CD34^{+}CD38^{-}$ cell populations which are enriched for L-ICs, albeit at lower levels compared to more mature cell populations. This is consistent with the literature that indicates that PI3K is active in, and important for L-IC survival [57,58]. My results also demonstrate that ILK expression is increased in more mature leukemic progenitor populations (Chapter 3). This is consistent with the work performed by *Haase et al* which shows that ILK levels are increased in more differentiated cells [59].

Somewhat surprisingly, the PI3K pathway is also active in quiescent AML cells, with no discernable difference observed when compared to levels in cycling cells (Table 3.2). Usually PI3K is associated with cell proliferation, and is not expected to be active in quiescent cells. Perhaps the PI3K pathway has additional functions in quiescent AML cells. PI3K also produces anti-apoptotic signals, and activates proteins such as NFκB which, as mentioned previously, have been shown to be expressed in both L-IC-enriched and quiescent cell populations [32]. The results of FACS analysis of GSK3 phosphorylation are based on measurement of MFI in positive cells and are semi-quantitative. Thus, it is possible that subtle differences in PI3K activation between cycling and quiescent cells were not detected. However, the fact that quiescent AML cells were equally susceptible to QLT0267-induced cell death as cycling cells in two of three AML samples tested suggests that PI3K activation was quite actively measured by

flow cytometry. Together this suggests that PI3K is active to some degree in quiescent AML cells.

The experiments in Chapter 3 describing the effects of QLT0267 on quiescent and cycling AML cells demonstrated for one FLT3-ITD AML sample the presence of cells lacking the FLT3 mutation (Appendix Figure B3). There are a couple of possible explanations for this observation. The first and most likely for a number of reasons is that these colonies arose from normal hematopoietic progenitors present in the sample. The morphology of the colonies present in the CFC assays of quiescent cells resembled colony types expected in normal hematopoietic rather than leukemic CFC assays. This included the presence of what appeared to be mixed lineage colonies usually only found in normal hematopoiesis. In the other two samples tested this difference in colony morphology was not observed. It has been described in the literature that normal hematopoietic progenitors can be detected in AML CFC and LTC-IC assays [60-63]. Studies were performed where AML cells with known cytogenetic abnormalities were placed into CFC or LTC-IC assays. Fluorescence in situ hybridization was then performed on the colonies that arose from these assays to detect the chromosomal abnormality and it was found that a proportion of colonies from both assays were cytogenetically normal [62]. In further studies which exploited the highly polymorphic nature of the androgen receptor found on the X chromosome, it was shown that a portion of these cytogenetically normal colonies were also polyclonal [63]. This differs from the blast cells from these samples which were shown to be highly monoclonal, suggesting these blast cells arouse from a single leukemic clone. Lastly, the literature shows that a much larger proportion of normal hematopoietic progenitors are found to be quiescent

than leukemic progenitors [20-23]. Altogether, this supports the hypothesis that these CFCs arose from normal hematopoietic progenitor cells [22].

A second possible explanation for this observation is that the FLT3-ITD negative colonies present in the quiescent cell CFC assays are from a leukemic subclone lacking the FLT3 mutation. The FLT3 mutation can arise as a secondary mutation in a portion of AML cells [64,65]. This clonal evolution can provide FLT3-ITD positive cells with an extra proliferative advantage making them the dominant subclone of the disease in these patients. Because of this proliferative advantage, sorting out quiescent cells could isolate other leukemic clones present which lack this mutation. To test this one could analyze cycling and quiescent cells for another genetic abnormality present in the leukemic blasts. However, the sample studied has a normal karyotype and did not lend itself to this analysis.

In the studies involving treatment of leukemic stem and progenitor cells with QLT0267 (Chapter 3) the effects observed in the *ex vivo* treatment of AML cells placed into NOD/SCID mice were similar to that of the *in vitro* studies using SC-IC assays (Figure 3.3, Table 3.3). However, this varied from the effects observed in the *in vivo* treatment of AML engrafted mice with QLT0267 (Appendix Figure B.4), even though the same samples were tested. This variation can be attributed to differences in experimental design between the two sets of experiments. In the *ex vivo* experiments, cells were grown in suspension cultures and exposed to QLT0267 dissolved in DMSO before being injected into NOD/SCID mice. In these experiments there were few influences on the cells other than culture conditions, length of exposure to, and concentration of QLT0267. The effects QLT0267 exerted on the L-ICs occurred in this

short initial culture period. In the *in vivo* experiments, cells were engrafted into NOD/SCID mice first, and allowed to grow for 14 days. The mice were then treated with the QLT0267 compound dissolved in PTE. Although in these *in vivo* experiments the length of treatment with the compound (14-28 days) was much longer than the *ex vivo* experiments, the results were far more modest.

There are many potential explanations for this observation. The stability of QLT0267 in the two different vehicles (DMSO and PTE) is likely quite different from each other. In mice, a dose of 200 mg/kg QLT0267 in PTE administered by oral gavage results in maximum plasma concentrations of 134 μM, with a T_{max} of 30 minutes, and $T_{1/2}$ of approximately 3 to 4 hours. However, in the *in vivo* experiments it is uncertain what actual concentration of QLT0267 the cells engrafted in the mouse BM were exposed to because of factors such as the route of administration, and binding of plasma proteins to the compound. This likely means that in the *in vivo* experiments AML cells were repeatedly exposed to higher concentrations of QLT0267, for much shorter periods of time than in the *in vitro* experiments. The cells directly engrafted in mice are likely more robust than those grown *in vitro* because of their contact with the BM microenvironment and exposure to cytokines and growth factors, even if they are of a murine source [62,66,67]. This would also affect their response to QLT0267, or any compound for that matter. Thus direct comparison of *ex vivo* and *in vivo* results of drug treatment on AML cells is difficult to perform.

The *in vivo* dose of QLT0267 tested on mice was limited by the apparent toxicity of the compound to normal mouse tissues in our immunocompromised NOD/SCID mouse assay. In initial experiments, mice were treated with QLT0267 by oral gavage at

200 mg/kg per day, a dose previously tolerated in other murine tumour models [68,69]. Although, on average, a reduction in the BM burden of AML cells was achieved in the treated mice, malignant cells were not eliminated. It is possible that longer treatment or higher doses of QLT0267 would have produced better results. However, the latter was not tolerated in the immunodeficient mice used for these studies, as after 14 days of treatment mice receiving 200 mg/kg QLT0267 began to die. In studies that followed, lower concentrations were used and the mice were able to continue treatment for the full 28 days tested, with a partial reduction of engrafted AML cells being observed in these mice.

There is emerging evidence that the interaction between leukemic cells and stromal cells is important for AML progenitor survival [70,71]. This interaction occurs primarily via β-1 and β-2 integrins, activates the PI3K pathway and anti-apoptotic signals providing resistance to chemotherapeutic agents, and can to some degree maintain leukemic cell quiescence [70,72]. It has been demonstrated that blockage of VLA-4 ($\alpha_4\beta_1$ integrin) with antibodies reversed the anti-apoptotic effects observed when cells bind to fibronectin [70]. VLA-4 expression also correlated negatively to complete remission and positively to rate of relapse. Since QLT0267 disrupts the PI3K pathway through inhibition of ILK, which also interacts with integrins, this compound may be particularly relevant in the context of leukemia/stroma interactions. It would then be interesting to examine the affects of QLT0267 on leukemic progenitor or stem cell survival while in the presence of a stromal cell layer.

A number of groups have been trying to exploit this leukemia/stroma interaction for the treatment of AML. Antagonists to CXCR4 (chemokine (C-X-C motif) receptor

4), a chemokine receptor important in HSC homing to the BM commonly found overexpressed in AML, are being used to mobilize leukemic cells from the BM where they are protected from chemotherapeutic agents [73-75]. Inhibition of CD44, a cell adhesion molecule often elevated on leukemic cells, with a monoclonal antibody *in vivo* leads to a decrease of leukemic stem cell homing and engraftment and an increase in differentiation of leukemic cells [1]. Normal hematopoietic cells are not affected by this inhibition. As well, expression of a variant of CD44 correlates with poor survival in AML [76].

Tabe et al demonstrated that stromal cells did not protect bulk leukemic blast cells from QLT0267 induced apoptosis, even though spontaneous apoptosis was suppressed by interaction with stromal cells [77]. Since primitive leukemic cells are known to reside in the BM where they are in contact with stromal cells, and the leukemia/stromal cell interaction activates the PI3K pathway, one might expect these cells, especially the L-ICs, to be most affected by inhibition of this pathway. This would be particularly applicable in the context of studying drug combinations where is has been shown that upregulation of the PI3K pathway is a mechanism for leukemia cell resistance to chemotherapeutic agents [70,75,78-81].

As mentioned before, most (if not all) small molecule kinase inhibitors have more than one target since kinase inhibitors are generally designed to block the ATP-binding domain, which is loosely conserved amongst kinases [82-84]. This can be both beneficial and problematic. On the one hand, when studying biological processes and the role specific kinases have on these processes it is desirable to have one target. When more than one kinase is inhibited it is difficult to distinguish the roles of each individual kinase.

In the literature it is not uncommon to find studies where conclusions are drawn on the function of proteins solely based on findings of experiments that rely on the use of small molecule inhibitors [44,85]. These studies do not accurately characterize the function of these proteins because the effects observed may be due to inhibition of other, often unknown, targets of the inhibitor. In circumstances where biological functions of a kinase are being studied it is desirable to use a system specific to individual targets such as siRNA inhibition [86,87].

On the other hand, when choosing small molecules for clinical development the inhibition of multiple targets may be advantageous. Compounds that inhibit multiple targets can be beneficial in the treatment of diseases such as cancer where many proteins may potentially be dysregulated. This is particularly true for AML, a heterogeneous disease in which each leukemia typically harbours a variety of genetic mutations. Unlike Chronic myelogenous leukemia (CML) which is predominantly driven by the presence of the BCR-ABL fusion product causing dysregulation of the ABL kinase [88,89], and in which small molecule kinase inhibitors such as imatinib have been dramatically successful in therapy [90-92], AML has no single common mutation dominant in the patient population. This is likely to be a reason why the treatment of AML has been so difficult. With the exception of one notable subset of AML, acute promyelocytic leukemia, which as mentioned in Chapter 1 is driven by the PML-RARα fusion product causing dysregulation of the retinoic acid receptor, and is treated with ATRA or arsenic trioxide [93], therapies inhibiting one target may have little use in this field.

Compounds such as QLT0267, a small molecule inhibitor of both ILK and FLT3, may be useful in the treatment of AML because they target both FLT3 a gene commonly

mutated in AML, and ILK, a PI3K pathway intermediate. These pathways share considerable overlap, and both of these targets are found to be either mutated or constitutively active in a large number of AML patients.

4.1 Concluding Remarks

As of late, there has been great interest in targeting signal transduction pathways for the treatment of AML. One pathway that stands out, the PI3K pathway, is involved in cell growth, regulation of apoptosis, and interactions with the leukemia microenvironment [5,70,72]. A number of PI3K intermediates have been studied in AML. My focus has been on ILK, an integrin-binding protein and activator of Akt. ILK is expressed in AML cells, both primitive and mature, and is important for AML progenitor cell survival.

Inhibition of the PI3K pathway using QLT0267, a small molecule inhibitor of ILK and FLT3, is toxic to various primary human AML progenitor cell types, the most relevant of these being the L-IC, while showing minimal toxicity towards normal hematopoietic progenitor and stem cells. The data presented here are consistent with the body of literature indicating that the PI3K pathway is active in a large portion of AML samples, and is important for L-IC survival [10,55-58]. In combination with cytarabine and daunorubicin, two chemotherapeutic agents commonly used in the treatment of AML, PI3K inhibition yields synergistic or additive effects in a portion of samples. Thus, targeting the PI3K pathway with compounds such as QLT0267 may be of great therapeutic benefit in the treatment of patients with AML.

4.2 References

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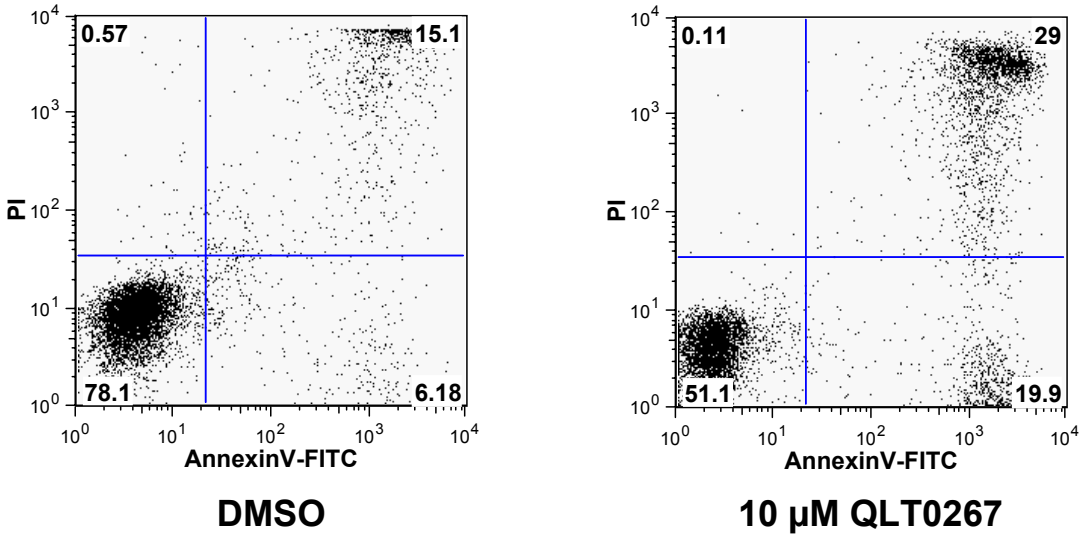
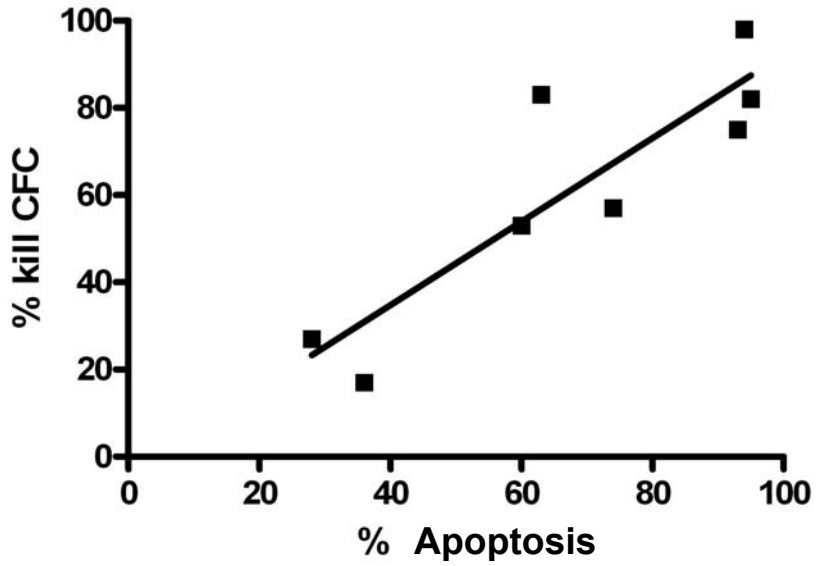
Appendix A

Figure A.1. QLT0267 is cytotoxic to AML blast cells.



Representative plot of AML sample 16 cultured for 48 hours in the presence of 10 µM QLT0267, or DMSO as control. Cells were then washed and stained with Annexin V and Pi to stain for viable (Annexin V low, Pi low), dying (Annexin V high, Pi low), and dead (Pi high) cells and percent blast cell kill was calculated.

Figure A.2. AML blast and CFC cells respond similarly to QLT0267



AML cells were cultured for 48 hours in the presence of 10 μM QLT0267 and then plated into CFC assays or stained for Annexin V and Pi to measure apoptosis. Percent apoptosis and CFC kill were compared for the 8 samples and were found to be similar ($r=0.88$, $p<0.002$ Student t-test).

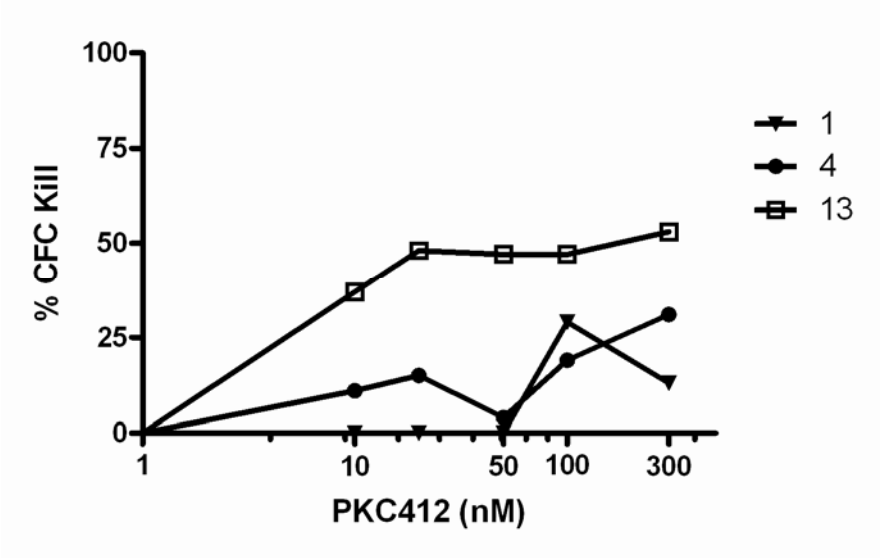
Table A.1. The addition of cytokines and growth factors does not enhance QLT0267-induced CFC death

	% CFC kill			
	3 μM QLT0267		10 μM QLT0267	
sample	+GF*	-GF	+GF*	-GF
1	0	0	77	95
4	32	25	96	94
5	35	31	99	99
23	84	52	96	83
41	23	18	63	57

Cells were cultured for 48 hours in the presence of QLT0267 with or without growth factors (GF), and then plated in CFC assays. There was no significant difference in CFC kill between cells treated with and without cytokines and growth factors during exposure to QLT0267 ($p=0.142$, Student t-test).

*20 ng/mL interlukin-3, 100 ng/mL steel factor, 100 ng/mL FLT3 ligand

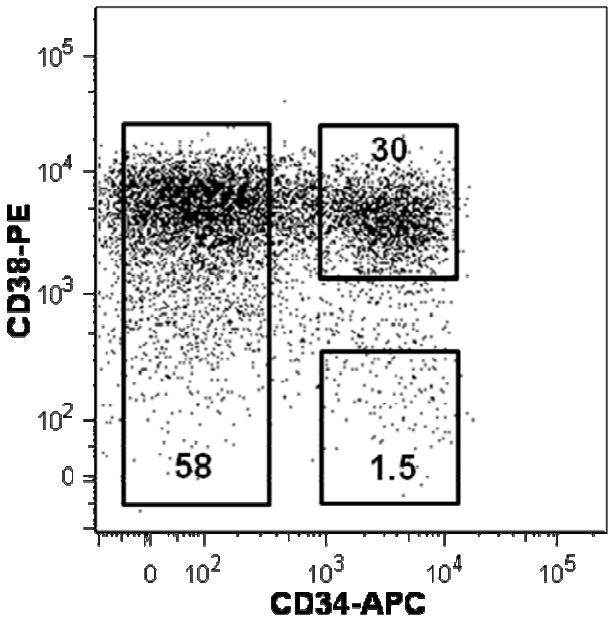
Figure A.3. Determination of the optimal concentration of PKC412 to use for inhibitor comparison experiments.



To determine optimal concentrations of PKC412, a small molecule inhibitor of FLT3, for use in subsequent experiments 3 FLT3-ITD AML samples were cultured for 48 hours in the presence of varying concentrations of PKC412 chosen based on the literature. Cells were then washed and placed in CFC assays, and percent CFC kill was calculated. At concentrations of PKC412 > 100 nM there was no significant difference in CFC kill ($p=0.97$, Student t-test between 100 nM and 300 nM PKC412). Based on this, the optimal concentration of PKC412 was determined to be 100 nM.

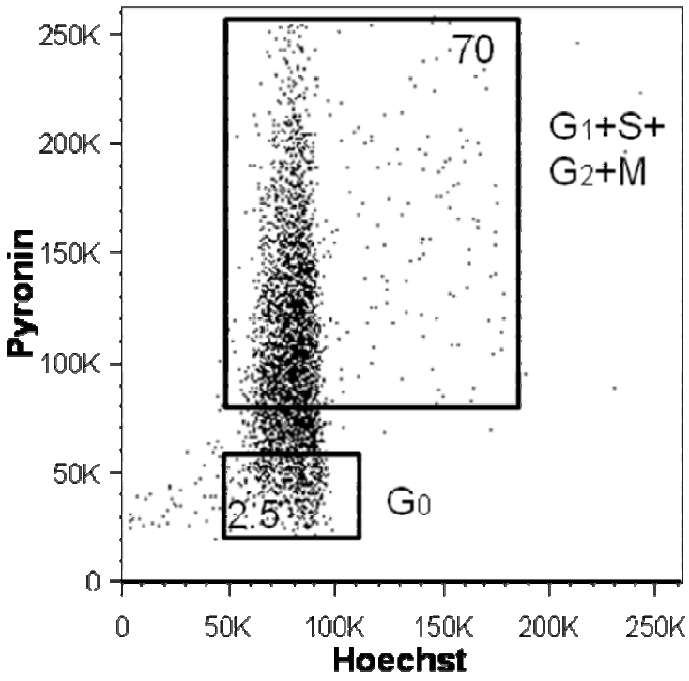
Appendix B

Figure B.1. AML samples sorted based on CD34 and CD38 cell surface makers.



Representative FACS plot of 1 of 5 samples (sample 5) sorted based on CD34 and CD38 expression into $CD34^-$, $CD34^+CD38^+$, and $CD34^+CD38^-$ populations. Numbers indicate the percent of the total population in the specific gates. Sorted cells were then prepared for intracellular staining to measure pGSK3 and ILK expression levels, or mRNA was extracted and FLT3 expression measured using qRT-PCR.

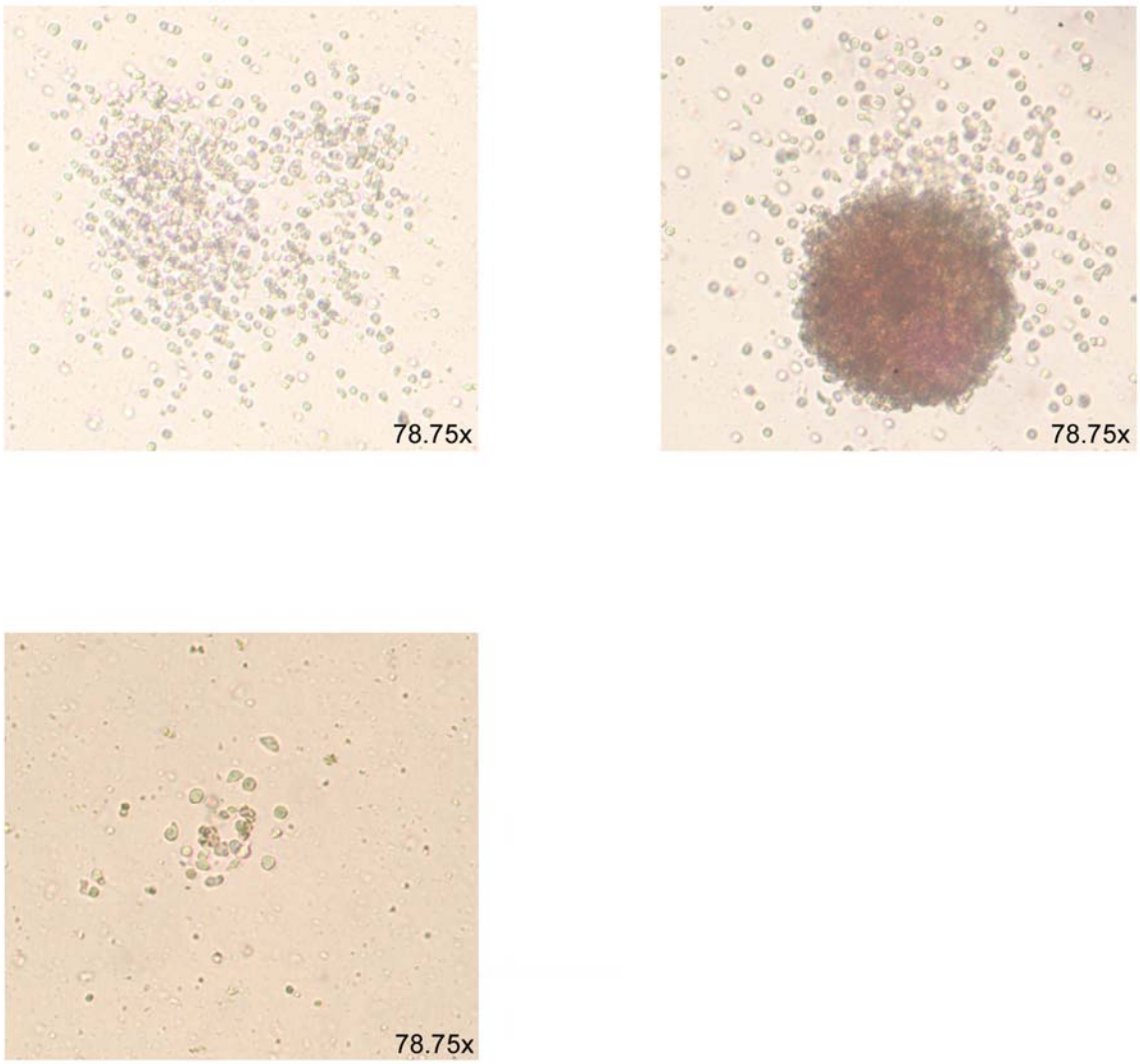
Figure B.2. AML samples sorted into quiescent and cycling populations.



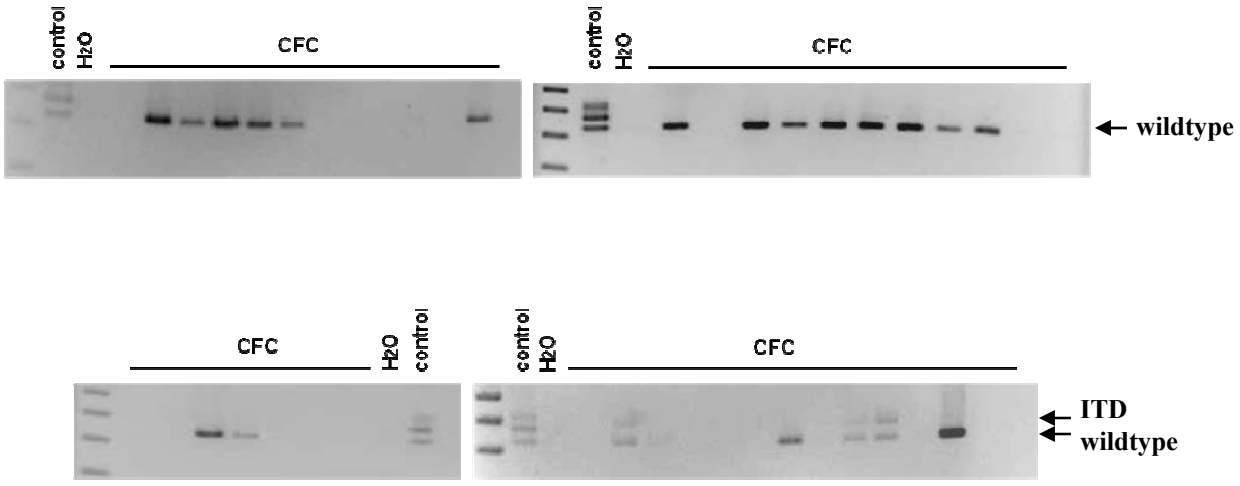
Five AML samples were sorted based on Hoechst and Pyronin staining (staining DNA and RNA respectively) into quiescent and cycling cells. Representative FACS plot of Hoechst and Pyronin staining for cycling (Pyronin high, indicating active mRNA production) and quiescent (Pyronin low, Hoechst low, indicating low levels of mRNA production and no DNA replication) populations from sample 4. Sorted cells were then prepared for measurement of pGSK3, ILK, and FLT3 as described previously, or placed into SFM for 24 hours in the presence of QLT0267 or DMSO as control.

Figure B.3. Quiescent cells are enriched for normal hematopoietic cells in some AML samples.

A

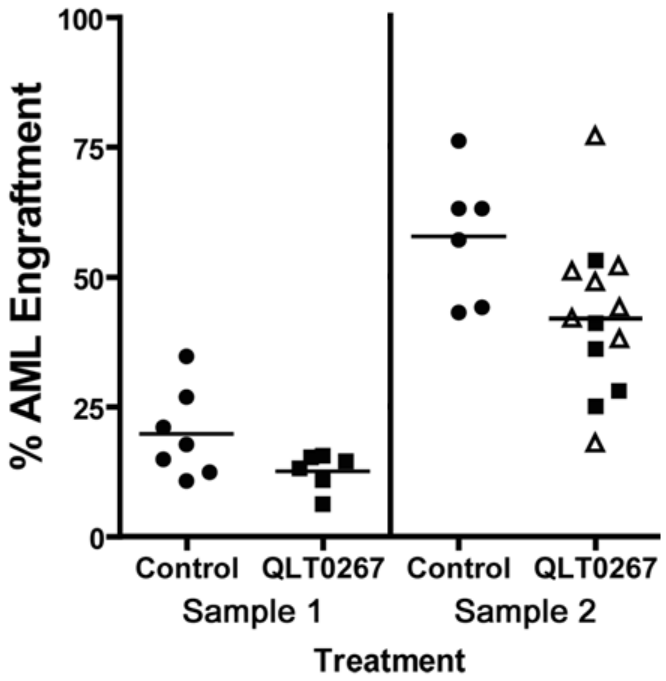


B and C



A) Upper left panel represents a typical colony present in CFC assays from quiescent cells of sample 5. Upper right panel represents a colony that resembles a mixed lineage colony typically only found in normal hematopoietic CFC assays, but also appeared in quiescent CFC assays from sample 5. Bottom panel represents a typical colony present in CFC assays from cycling cells from sample 5. B and C) In total 22 colonies were plucked from quiescent and cycling cell populations respectively and DNA was extracted. As sample 5 is known to contain a FLT3-ITD mutation, PCR amplification of the FLT3 gene was used as a marker to distinguish whether colonies were derived from cells of normal or leukemic origin. DNA from 14 colonies derived from quiescent cells PCR amplified and were negative for the FLT3 mutation, while 3 of 7 colonies derived from cycling cells that PCR amplified contained the FLT3-ITD mutation ($p<0.01$, chi-square test). Altogether, this demonstrates that the quiescent population of cells from this sample is enriched for normal hematopoietic progenitor cells.

Figure B.4. Treatment of mice engrafted with human AML stem cells with QLT0267.



Cells from two AML samples were injected into NOD/SCID or NOD/SCID IL2ko mice via the tail vein. Two weeks later, mice received oral treatment of QLT0267 (sample 1: 200 mg/kg QLT0267 for 14 days, sample 2: 100 or 150 mg/kg QLT0267 for 28 days). QLT0267 was dissolved in PTE (PEG_{300}/ethanol/Tween 80/citrate (63:29:7.8:0.2 w/v/w/w)). Each symbol represents the percent human AML cells in mouse marrow for a single mouse, with the bars indicating the mean percent engraftment for a treatment arm. For sample 2 ■ represents 100 mg/kg and Δ represents 150 mg/kg QLT0267.

After 14 days of treatment mice engrafted with sample 1 showed a decrease in the proportion of AML cells in mouse bone marrow after QLT0267 treatment as compared to control treated animals (12.6% ± 3.5 vs 19.7% ± 8.5 for QLT0267 and control treatments, respectively, p=0.04, Student t-test). However, there was apparent toxicity with mouse deaths, particularly after QLT0267 treatments, shortly after this measurement. In a subsequent experiment using AML sample 2, lower doses of QLT0267 were tested and the mice were treated for 28 days. These doses were well-tolerated and QLT0267-treated mice were again found to have lower AML cell engraftment as compared to controls (42.6 ± 15.0 vs 57.7 ± 12.6 for QLT0267 and control treatments respectively, $p < 0.05$, Student t- test).

Table B.1 Scheduling of QLT0267 with cytarabine or daunorubicin has some effect on AML-CFC cell death.

sample	CI(90)			CI(90)		
	QLT+Ara-C	QLT 1st Ara-C 2nd	Ara-C 1st QLT 2nd	QLT+DNR	QLT 1st DNR 2nd	DNR 1st QLT 2nd
1	0.84	0.81	0.92	0.38	0.65	0.62
5	1.56	0.96	1.17	1.58	1.08	0.94
6	1.24	0.97	1.25	1.13	0.98	1.15
9	1.41	1.28	1.20	9.84	9.28	5.33
10	1.44	1.23	1.14	2.67	1.68	2.87
11	1.14	0.73	1.01	0.76	0.59	0.69

Cells were cultured in the presence of four concentrations of QLT0267, cytarabine (Ara-C), daunorubicin (DNR), or QLT0267 with Ara-C or DNR, based on the estimated IC_{50}'s of each compound for each individual sample. DMSO was used as a control. The scheduling of each compound also varied so that cells received QLT0267 and Ara-C or DNR together for 24 hours, QLT0267 for 24 hours then Ara-C or DNR for 16 hours, or Ara-C or DNR for 16 hours then QLT0267 for 24 hours, after which the cells were harvested and placed into CFC assays. IC_{90}'s and combination indices (CIs), as a measure of drug interaction, were calculated for each sample and drug combination. The schedule of QLT0267 first followed by the addition of Ara-C or DNR resulted in slightly lower CI(90)s than the other schedules tested ($p<0.05$ and $p=0.15$, for CI(90) of QLT0267 1st Ara-C 2nd vs QLT0267+ Ara-C, and vs Ara-C 1st QLT0267 2nd respectively, Student t-test).

Appendix C

University of British Columbia - British Columbia Cancer Agency Research Ethics Board
(UBC BCCA REB)

BC Cancer Agency
CARE & RESEARCH

UBC BCCA Research Ethics Board
Vancouver Centre – Rm. 4113
600 West 10th Avenue
Vancouver, B.C. V5Z 4E6
Tel: (604) 877-6284 Fax: (604) 708-2132
www.bccancer.bc.ca

CERTIFICATE OF FULL BOARD APPROVAL

BCCA PRINCIPAL INVESTIGATOR	BCCA PI CENTRE	BCCA DEPARTMENT	UBC BCCA REB NUMBER
SMITH, Clayton	BCCA – Vancouver Centre + VCHA-VGH	Hematology/TFL	New #: R04-1292 Prev.# : R04-0292

PRINCIPAL INVESTIGATOR FOR EACH ADDITIONAL PARTICIPATING BCCA CENTRE IF APPLICABLE: (NAME & CENTRE)

N/A

PROJECT TITLE:

Collection of Blood or Bone Marrow from Normal Donors & Persons with Hematologic Malignancies or Other Hematologic Disorders for Research on the Development & Treatment of Blood Diseases

SPONSORING AGENCIE(S) AND COORDINATING GROUP(S):

University of British Columbia

The **full UBC BCCA Research Ethics Board** has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval for a period of one year from the approval date. This approval expires one year from the approval date.

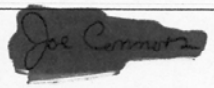
APPROVAL DATE	TERM
29 August 2004	One (1) Year

DOCUMENTS INCLUDED IN THIS ACTION:

1) Protocol (SOP) Version 1: August 8, 2004
2) Appendix 1: List of Investigators
3) Consent Document (Normal Cells) - Version 2 dated July 22, 2004
4) Consent Document (Abnormal Cells)- Version 2 dated July 22, 2004
5) Appendix 3: Sample Wording (re: Verbal re-consent)
6) Appendix 4: SOP - # CS-301 Logging in of Subjects and Samples - Version1 : 30 May 2001
7) Appendix 5: SOP - #LT-302 Processing and Storing of Cells - Version 1: 19 November 1999
8) SOP #LT-303 - CFC Assay of Bone Marrow - Version 1: 19 November 1999
9) SOP #LT-306 - (Freezing Vials) - Version #3 - 25 June 2001

Certification

1. The membership of the UBC BCCA REB complies with the membership requirements for research ethics boards defined in Division 5 of the Food and Drug Regulations of Canada.
2. The UBC BCCA REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC BCCA REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the principal investigator named above. This review and the associated minutes of the UBC BCCA REB have been documented electronically and in writing.



Approval of the full UBC BCCA Research Ethics Board verified by the above signature of one of the following:

Dr. Joseph Connors, Chair
Dr. Lynne Nakashima, first Vice-Chair
Dr. Ken Swenerton, Second Vice-Chair

If you have any questions, please call:

Bonnie Klimek, BCCA Research Ethics Coordinator, 604-877-6284 or email: bklimek@bccancer.bc.ca
Dr. Joseph Connors, Chair, 604-877-6000–ext. 2746 or email: jconnors@bccancer.bc.ca
Dr. Lynne Nakashima, first Vice-Chair, 604-877-6000–ext.2241 or email: lnakas@bccancer.bc.ca
Dr. Ken Swenerton, second Vice-Chair, 604-877-6000–ext. 2365 or email: kswener@bccancer.bc.ca
All Correspondence by fax or mail should be sent to the UBC BCCA Research Ethics Board office above.

University of British Columbia - British Columbia Cancer Agency Research Ethics Board (UBC BCCA REB)

UBC BCCA Research Ethics Board
Fairmont Medical Building (6th Floor)
614 - 750 West Broadway
Vancouver, BC V5Z 1H5
Tel: (604) 877-6284 Fax: (604) 708-2132
E-mail: reb@bccancer.bc.ca
Website: http://www.bccancer.bc.ca > Research Ethics
RISe: http://rise.ubc.ca

Certificate of Expedited Approval: Annual Renewal

PRINCIPAL INVESTIGATOR:	INSTITUTION / DEPARTMENT:	REB NUMBER:
Clayton Smith	BCCA/BCCA/Terry Fox Lab (BCCA)	H04-61292

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Vancouver Coastal Health (VCHRI/VCHA)	Vancouver General Hospital
Providence Health Care	St. Paul's Hospital

Other locations where the research will be conducted:
N/A

PRINCIPAL INVESTIGATOR FOR EACH ADDITIONAL PARTICIPATING BCCA CENTRE:

Vancouver:	N/A	**Vancouver Island:**	N/A
Fraser Valley:	N/A	**Southern Interior:**	N/A
Abbotsford Centre:	N/A		

SPONSORING AGENCIES AND COORDINATING GROUPS:
University of British Columbia

PROJECT TITLE:
Collection and Tissue Banking of Blood, Bone Marrow Material, Peripheral Blood Stem Cells or Leukapheresis Product and Collection of Clinical Data from Persons with Hematologic Malignancies or Other Hematologic Disorders and (Persons with Normal Blood, Bone Marrow Material and/or Peripheral Blood Stem Cells) for Research on the Development & Treatment of Blood Diseases.

APPROVAL DATE:	EXPIRY DATE OF THIS APPROVAL:	PAA#: H04-61292-A019
August 11, 2009	**August 11, 2010**	

CERTIFICATION:

1. The membership of the UBC BCCA REB complies with the membership requirements for research ethics boards defined in Division 5 of the Food and Drug Regulations of Canada.
2. The UBC BCCA REB carries out its functions in a manner fully consistent with Good

Clinical Practices.

3. The UBC BCCA REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the provincial investigator named above. This review and the associated minutes of the UBC BCCA REB have been documented electronically and in writing.

The UBC BCCA Research Ethics Board has reviewed the documentation for the above named project. The research study as presented in documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC BCCA REB.

UBC BCCA Ethics Board Approval of the above has been verified by one of the following:

Dr. George Browman, Chair
Dr. Lynne Nakashima, Second Vice-Chair

If you have any questions, please call:

Bonnie Shields, Manager, BCCA Research Ethics Board: 604-877-6284 or e-mail: reb@bccancer.bc.ca
Dr. George Browman, Chair: 604-877-6284 or e-mail: gbrowman@bccancer.bc.ca
Dr. Lynne Nakashima, Second Vice-Chair: 604-707-5989 or e-mail: lnakas@bccancer.bc.ca