STRATEGIES FOR IMPROVING THE THERAPEUTIC AND DIAGNOSTIC APPROACHES TO ACUTE LEUKEMIAS

by

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Abstract

Acute leukemia (AL) represents a hematological cancer originated from malignantly transformed myeloid or lymphoid precursors. Steady improvement in the prognosis of patients with AL has been observed over the last 30 years. However, the duration of remission has been still short and the overall survival of AL remains poor. Therefore, novel therapeutic strategies are necessary to enhance the cure rate and the quality of life.

Although initial remissions can be obtained with tyrosine kinase inhibitor (TKI) in Ph+ AL, drug resistance develops and the responses are short-lived. I chose to study DT388IL3 because the target IL-3 receptor is expressed on most Ph+ AL blast samples and the action mechanism of DT388IL3 is different from that of the TKIs. I studied cytotoxicity against malignant progenitors from patients with Ph+ AL and demonstrated a synergistic interaction between both TKIs and the fusion protein. Normal progenitors were relatively resistant to these drugs alone or in combination.

The enhanced selective cytotoxicity of CPX-351 (a liposomal formulation of a fixed ratio combination of cytarabine and daunorubicin) for AML progenitors as compared to normal hematopoietic cells is described. AML cells were shown to be more sensitive to CPX-351 than normal cells. Moreover, it was demonstrated that normal hematopoietic cells and progenitors were less sensitive to CPX-351 in comparison with conventional cytarabine:daunorubicin. These data suggest that CPX-351 might overcome drug resistance in AML without increasing toxicity to normal blood cells.
The ability to reliably predict the likelihood of induction failure in a timely fashion would be useful for optimizing treatment plans for leukemia patients. Thus, I developed a flow cytometry-based assay that could predict the outcome of induction chemotherapy. The assay is based on the efflux of mitoxantrone. The median fluorescence intensity (MFI) for AML blasts incubated with mitoxantrone was measured with or without the ABC transporter inhibitor, cyclosporine A, and a ratio between the inhibited and uninhibited MFI was calculated (MFIR). A high MFIR value was shown to be highly predictive of induction failure.

In conclusion, these findings may play an important role in developing better therapeutic and diagnostic approaches for AL.
Preface

A version of chapter 2 has been published. Kim HP, Frankel AE, Hogge DE 2010 A diphtheria toxin interleukin-3 fusion protein synergizes with tyrosine kinase inhibitors in killing leukemic progenitors from BCR-ABL positive acute leukemia. Leukemia Research 34;1035-1042. I conducted all the experiments and wrote most of the manuscript. As a co-author, Dr. Frankel provided technical support such as materials and comments. As a supervisor, Dr. Hogge provided overall ideas and discussions in this manuscript.

A version of chapter 3 has been published. Kim HP, Gerhard B, Harasym TO, Mayer LD, Hogge DE 2011 Liposomal encapsulation of a synergistic molar ratio of cytarabine and daunorubicin enhances selective toxicity for acute myeloid leukemia progenitors as compared to analogous normal hematopoietic cells. Experimental Hematology 39;741-750. I conducted all the experiments except daunorubicin accumulation assay (HPLC analysis) and wrote most of the manuscript except HPLC analysis part. As a co-author, B. Gerhard assisted in performing the CFC assay. Drs. Harasym and Mayer provided materials, the data from HPLC analysis and comments. As a supervisor, Dr. Hogge provided overall ideas and discussions in this manuscript.

A version of chapter 4 has been prepared to submit. Kim HP, Bernard L, Berkowitz J, Nitta J, Hogge DE. A Flow Cytometry, Mitoxantrone Efflux-Based Assay for Predicting
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABL</td>
<td>Abelson murine leukemia viral oncogene homolog</td>
</tr>
<tr>
<td>AL</td>
<td>Acute leukemia</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
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<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster regio</td>
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<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>BP</td>
<td>Blast phase</td>
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<tr>
<td>CA</td>
<td>Cyclosporine A</td>
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<tr>
<td>CBF</td>
<td>Core binding factor</td>
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<tr>
<td>CEBPA</td>
<td>CCAAT/enhancer-binding protein alpha</td>
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<tr>
<td>CFC</td>
<td>Colony forming cell</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease free survival</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>Fms-like tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>FLT3</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HFN</td>
<td>Hank’s balanced salt solution plus 2% fetal bovine serum and 0.04% sodium azide</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic progenitor cell</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>Hst</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>IF</td>
<td>Induction failure</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
</tr>
<tr>
<td>IL-3R</td>
<td>Interleukin-3 receptor</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>ITD</td>
<td>Internal tandem duplication</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemic stem cell</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>Long-term culture-initiating cell</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi drug resistance</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
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<tr>
<td>MFIR</td>
<td>Median fluorescence intensity ratio</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>MNC</td>
<td>Mononuclear cell</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance-associated protein</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>NK</td>
<td>Normal karyotype</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic severe combined immunodeficient</td>
</tr>
<tr>
<td>NOD/SL-IC</td>
<td>NOD/SCID leukemia-initiating cell</td>
</tr>
<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
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<tr>
<td>NR</td>
<td>Non respondent</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia chromosome</td>
</tr>
<tr>
<td>Ph+</td>
<td>Philadelphia chromosome positive</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>PY</td>
<td>Pyronin Y</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RARA</td>
<td>Retinoic acid receptor alpha</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>SCT</td>
<td>Stem cell transplantation</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SL-IC</td>
<td>Suspension culture-initiating cell</td>
</tr>
<tr>
<td>SP</td>
<td>Side population</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1. Introduction

1.1. Acute leukemias

1.1.1. Acute myeloid leukemia

1.1.1.1. Overview

Acute myeloid leukemia (AML) is a broad variety of leukemias that results from the malignant transformation of early hematopoietic stem cells or immature myeloid cells and is characterized by the clonal growth of abnormal progenitor cells [1-4]. The phenotypic diversity of AML is attributed to the cell stage when transformation occurs and the level of cell maturation [5-7]. The occurrence of AML escalates with age, and the median age at diagnosis is around 65 years [8]. AML is the most frequent adult leukemia, however, the survival rate of the disease is the lowest among all leukemia [9]. Patients with AML have leukemia cells inside their bone marrow, which grow rapidly and replace normal blood cells with resulting anemia, thrombocytopenia and leukopenia. Therefore, patients with AML become more susceptible to infectious disease and have higher risks for anemia and bleeding. AML arises in the bone marrow, but it quickly enters the peripheral blood in most cases. AML advances rapidly and is usually lethal within several months when left untreated. AML is diagnosed when more than 20% of leukemic blasts exist in the bone marrow, which differentiates the disease from other related blood disorders [10-13].
The collaboration of two major genetic aberrations is thought to cause AML. The first is described by alterations in myeloid transcription factors governing hematopoietic differentiation, which may be caused by direct modification of transcription factors from gene fusions, including PML-RARA positive AML or core-binding-factor (CBF)-leukemias or by indirect disturbance of transcription such as MLL-rearrangements. The second corresponds to activating mutations of signal transduction that cause unlimited cell proliferation for example by deregulated tyrosine kinases such as Fms-related tyrosine kinase 3 (FLT3) and C-KIT, a small GTPase Ras, and phosphatidylinositol 3-kinase (PI3 kinase). In fact, approximately 50% of primary AML samples have both aberrant and constitutive activation of signal transduction molecules [14-16].

Over the past two decades, some enhancements have been made in the treatment and prognosis of AML patients. However, there has been little advance in overall survival (OS). Combination therapy of cytarabine and anthracyclines is still the primary treatment for AML patients because many trials to improve this therapy have been failed. In addition, the treatment outcome of relapsed or refractory disease in both young and elderly patients is still disappointing as well.

Therefore, in this thesis I have tried to explore better therapeutic and diagnostic approaches for improving existing ones. In the following, I briefly reviewed current classifications, diagnosis, and therapies for AML.
1.1.1.2. Classifications

The classification of AML includes morphological, cytochemical, cytogenetic, molecular, and immunophenotypic characterization of the leukemic blasts [17].

Currently there are two classifications. The French-American-British (FAB) classification has been generally used for more than 30 years and the recent World Health Organization (WHO) classification have been used to classify AML in minute detail.

1.1.1.2.1. The French-American-British (FAB) classification of AML

AML was classified by a group of French, American, and British leukemia experts in 1970s into 8 subtypes (M0 through M7), based on how the leukemia cells look when stained and examined under a microscope to characterize any underlying morphological abnormalities. Cytogenetic studies were not part of the FAB classification, however, M3 and M4Eo subtypes were found to be associated with recurring cytogenetic aberrations. These subtypes have a favourable prognosis. Generally the morphologic categories of the FAB classification do not represent distinct biological and clinically significant disease subtypes except in the case of FAB M3 and M4Eo.

Now the WHO classification is generally accepted for classifying AML, however, the FAB system is still occasionally used [18].

1.1.1.2.2. World Health Organization (WHO) classification of AML

The FAB classification system has been largely replaced by the WHO classification which takes many of the prognostic factors into account. In the WHO
system, all obtainable information such as genetic, immunophenotypic, biologic, and clinical features as well as morphologic findings are utilized to define specific disease entities. The WHO classification system categorizes AML as diverse subgroups [19](Table 1.1). While at least 20% blasts in the blood or bone marrow is the criterion for the diagnosis of AML in WHO classification, at least 30% of blasts is the requisite in the FAB scheme.

1.1.1.3. Prognostic factors

A comprehensive understanding of the individual prognostic factors is very critical to determine treatment options in clinical practice. Current treatment of patients with AML is established based on several parameters including white blood cell count, age, and the cytogenetic/molecular genetic characteristics. These prognostic factors are important to work out treatment strategy, to identify potential targets for molecular therapies, and to decide on allogeneic transplantation [20].

1.1.1.3.1. Age

Age is highly associated with the outcome of AML. In general, the age of 60 years is used as a criterion for older patients. Young and middle-aged adults have approximately a 70% probability of achieving a first complete remission (CR) and about a half of them survive 5 years or more [21-24]. On the other hand, elderly patients have only a 50% probability of obtaining CR and the 5 year survival rate is less than 10% [25, 26]. No notable improvement in treatment outcome has been seen among elderly AML
patients, however, the treatment outcome has enhanced steadily in younger patients for the last 2 decades thanks to more intensive chemotherapy, stem cell transplantation, and advanced supportive care [27, 28]. The adverse clinical outcome of elderly patients may be correlated with a higher rate of unfavourable cytogenetics, a greater frequency of drug resistance phenotypes, and a greater probability of having antecedent hematologic malignancies. Additionally, more frequent co-morbid illness in older patients compared to younger persons makes it difficult for them to tolerate intensive induction therapy [20].

1.1.1.3.2. Karyotype

Approximately 60% of AML cases have cytogenetic abnormalities which are good indicators to predict response to chemotherapy and probability of relapse. Among several prognostic factors including age, karyotype, subtype of AML, and white blood cell count, the karyotype represents a key predictor of outcome and an index for current risk-stratified treatment approaches. Therefore, karyotype is indispensable for accurate diagnosis and classification [29]. In general, AML is divided into three categories: favorable, intermediate, and unfavorable based on the karyotype in the diagnostic bone marrow sample.

The translocations t(8;21), inv16 and t(15;17), are more frequent in younger patients with AML, and generally carry a relatively favorable prognosis. If patients have these karyotypes or the consequent molecular abnormalities such as AML1-ETO (consisting of the N-terminal DNA-binding domain of AML1, a transcription factor essential for definitive hematopoiesis, and almost all of ETO, a protein thought to
function as a corepressor for a variety of transcription factors), CBFβ-MYH11 (core binding factor beta gene-smooth muscle myosin heavy chain gene), and PML-RARα (promyelocytic leukemia- retinoic acid receptor alpha) respectively, the probability of response to induction chemotherapy would be around 80% and the relapse rate is only 30%-40% with 5 years survival rate between 60% and 90%. In contrast, patients who have deletions of 5q [del(5q)], aberrations of 3q [abn(3q)], monosomies of chromosome 5 and/or 7 (-5/-7) or complex karyotype display a much worse prognosis with regards to the probability of complete response (60%), relapse (80%) or 5 year survival (15%). Thus, cytogenetics is currently considered as the most powerful prognostic factor for rates of complete remission (CR), relapse risk and OS [30-32] (Table 1.2).

1.1.1.3.3. Molecular genetics

Although the prognostic value of the many cytogenetic abnormalities is critical, a substantial proportion of AML patients (40%-50%) has a normal karyotype. Patients with normal karyotype are classified with the intermediate risk group but include subgroups with variable prognosis. The presence of mutations in specific genes has been used for predicting outcome in normal karyotype AML. For example, mutations of Fms-related tyrosine kinase 3 (FLT3), CCAAT/enhancer binding protein alpha (CEBPA) and nucleophosmin 1 (NPM1) has proven to be most critical [33].

FLT3 is a receptor tyrosine kinase normally expressed on the surface of hematopoietic progenitor cells and has a role in cell survival, proliferation and differentiation. FLT3 mutations happen in approximately 30% of AML. FLT3-internal
tandem duplication (ITD) mutations are usually characterized by an in-frame insertion of 3 to >400 base pairs within the coding sequence of the juxtamembrane region, which leads to spontaneous dimerization and constitutive enzyme activation even without ligand binding. FLT3-ITD mutations have been related to confer a poor prognosis in all AML patients regardless of the presence of other prognostic factors [34, 35]. NPM1 gene mutations, which cause delocalization of NPM1 from the nucleus to the cytoplasm, are the most frequent genetic aberration in adult AML. NPM1 mutations are observed in approximately 35% of all cases and up to 60% of patients with normal karyotype [36]. Interestingly, a NPM1 mutation usually co-exists with FLT3-ITD in normal karyotype AML, which suggests they may collaborate in creating the leukemic phenotype [37]. This mutation generally offers more favorable prognosis with better CR rate and lower relapse risk. CEBPA is an essential transcription factor for normal myelopoiesis. About 15%-20% of AML cases have CEBPA mutations in the N- or C-terminal region. The mutation produces a truncated protein which blocks DNA binding and impedes granulocytic differentiation. CEBPA mutations have been correlated with a relatively favorable prognosis, including fewer relapses and longer overall survival (OS) [38].

1.1.1.3.4. White blood cell count

A higher white blood cell count (hyperleukocytosis, more than $100 \times 10^9/$l) at diagnosis predicts a poor prognosis. Hyperleukocytosis is more frequent in acute leukemias compared with chronic leukemias. Approximately 5% to 13% of adult AML and 10% to 30% of adult acute lymphoblastic leukemia (ALL) have hyperleukocytosis.
Patients having hyperleukocytosis show a worse complete response rate and a greater relapse rate [39, 40]. The WBC count can be used as a practical parameter to predict treatment outcome in patients with favorable cytogenetics. Naturally, patients with unfavorable cytogenetics exhibit a grave prognosis when they also have high numbers of WBC (20 × 10⁹/l or more) [41, 42].

1.1.1.3.5. Secondary leukemia and advanced disease

Having an antecedent hematological disease such as a myelodysplastic syndrome or myeloproliferative disorder or AML related to chemotherapy or radiotherapy for a previous cancer is also associated with poor prognosis, and these types of AML are usually difficult to treat [43-45]. Relapsed AML after initial induction therapy is usually fatal. On the whole, less than 10% chance of long term disease free survival. These patients may be advised to undergo stem cell transplant or, if this option is not available, they may be recommended for experimental or palliative therapy [46-48].

1.1.1.4. Treatment

1.1.1.4.1. Standard treatment

The standard treatment of patients with AML comprises one course (or more) of intensive induction chemotherapy. A generally accepted form of induction therapy is composed of a continuous infusion of standard dose cytarabine (100 to 200 mg/m²) for 7 days and 3 days of daunorubicin (45 to 60 mg/m²) intravenously (the 7+3 induction regimen). This therapy has been reported to induce CR in 50% to 75% of patients with
AML. The probability of long-term disease-free survival after standard induction therapy followed by consolidation with similar treatment is approximately 30%, with most patients ultimately dying from relapsed, chemotherapy refractory leukemia [49, 50].

As no new drugs have been developed that showed superior efficacy to cytarabine and daunorubicin, clinicians have focused mainly on the amendment of the standard regimen. Efforts to improve the efficacy of this standard induction therapy have included cytarabine intensification, substituting different anthracyclines, and adding other drugs such as fludarabine, etoposide, or cladribine. In spite of theoretical benefits, those trials have failed to show a survival advantage compared to standard 7 + 3 induction therapy [51].

1.1.1.4.2. Consolidation therapy

Consolidation therapy is given following induction of CR to maximize the duration of remission and, when possible, cure the leukemia. There are 2 main options for consolidation therapy; additional chemotherapy or autologous or allogeneic stem cell transplantation (SCT). The choice of therapy typically depends on the age of the patient, cytogenetic risk group, comorbidities, and accessibility of a suitable hematopoietic stem cell donor [52].

Consolidation chemotherapy comprises additional courses of cytarabine-based treatment for most patients. If the patient is young and has good health status, high dose cytarabine at doses of 3 g/m² every 12 hours on days 1, 3, and 5 are typically given [53, 54]. The mean disease-free survival for patients who receive the induction therapy only is
4 to 8 months. However, 35 to 50% of younger patients receiving high dose cytarabine consolidation treatment survive 2 to 3 years [55, 56]. Patients who have favorable cytogenetic risk obtain the most benefit from this intensive consolidation. Those in the intermediate and unfavorable risk groups show less benefit therapy and are often considered for allogeneic stem cell transplantation. However, elderly patients frequently are not considered as candidates for stem cell transplantation due to the higher incidence of treatment related toxicity [22].

1.1.1.4.3. Relapsed and refractory disease

The treatment of patients with relapsed or refractory AML remains a major challenge. Generally 20% to 40% of patients do not attain CR with standard induction chemotherapy, and 50% to 70% of patients who achieve initial CR are expected to relapse within 3 years [57, 58]. The prognosis for patients with refractory or relapsed AML is generally poor. However, some relapsed patients who achieve a second CR and have an available donor may be cured by SCT [59]. Although there is no single standard regimen for refractory and relapsed AML, extra chemotherapy is given in the hope of achieving CR for most patients including the use of high (2-3 g/m²) dose cytarabine in combination with other drugs. The duration of first remission in relapsed patients is the best predictor for achieving a second CR and survival. If the first CR was less than 12 months, the possibility for the second CR is only 10 to 20%. If the first CR was longer than 12 months, achievement for the second CR increases to 40 to 50% [60].
1.1.1.5. Leukemic stem cell (LSC)

Human hematopoiesis is organized in a hierarchy which is ultimately maintained by a small number of pluripotent hematopoietic stem cells (HSCs) which are long-lived, cell cycle quiescent, and capable of self-renewal. HSCs can produce every downstream lineage found in the hematopoietic system including erythrocytes, platelets, and various lymphoid and myeloid cells. Hematopoiesis is an ingeniously designed process which controls blood cell production without loss or excess. The most definitive method to detect and characterize HSC is long-term in vivo hematopoietic repopulation of lethally irradiated recipients. Advances in technologies such as the development of stem cell markers, immunomagnetic selection method, and multicolor FACS analysis have significantly helped to purify mouse HSC [61]. HSCs comprise a very rare population representing only 0.001 to 0.01% of bone marrow cells, which generates successive generations of lineage-restricted and differentiated progenitors which ultimately produce huge numbers of completely differentiated cells. HSC self-renewal is considered to be symmetrical or asymmetrical. Symmetrical self-renewal produces two identical HSCs and asymmetrical produces a progenitor with limited self-renewal capacity and an identical HSC [62]. Progenitors have a tendency to differentiate into a specific lineage of mature cell.

The study of human HSC has been advanced greatly since the development of non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) and NOD/SCID-β2 microglobulin -/- xenotransplantation assays in mice which detect primitive human hematopoietic cells with lympho-myeloid repopulating capacity. These model systems
have played an important role in identifying and characterizing HSC. The self-renewal capacity of these HSCs was demonstrated by serial transplantation in which HSCs transplanted into lethally irradiated primary recipients are reisolated and injected into secondary recipients [63-65]. This experiment exhibited that HSCs are long-lived and possess critical replicative potential, consistent with self-renewal activity.

To explain the cells of origin in AML, two major models of leukemic cell growth have been proposed. In the stochastic model, all cells within leukemic population have a same probability of extensive proliferation and the potential to act as a stem cell. However, only a few cells that maintain self-renewal capacity may be able to sustain leukemia growth. In contrast, the stem cell model proposes that leukemic cells are organized in hierarchical fashion similar to normal hematopoiesis and that only a small population of defined primitive cells steadily has the ability to initiate leukemia and reproduce the cells that comprise leukemia (Figure 1.1). In 1994, John Dick and colleagues reported that only a small portion (0.1 to 1%) of leukemic blasts in AML having the cell-surface phenotype CD34\(^+\)CD38\(^-\) was capable of engrafting in NOD/SCID mice and initiating AML [66]. In addition, these SCID leukemia-initiating cells (SL-ICs) had self-renewal capacity in serial transplantation experiments, and could be considered as AML stem cells (LSC). These findings showed that AML is organized as a hierarchy of progenitors and blasts and sustained by a rare population of LSCs like the normal hematopoietic system. LSCs are thought to retain a limited or aberrant differentiation program, however, the defects in cellular machinery typically obstruct their differentiation into mature cells morphologically and phenotypically. Accordingly the
whole leukemic population is composed of leukemic cells with undifferentiated and variably differentiated status. This and related observations suggested the cancer stem cell hypothesis in which many cancers are continued by a small portion of stem-like cancer cells that retain self-renewal capacity.

Previous studies have reported that LSC seems to share many cell surface markers with normal HSC. For instance, both HSC and LSC express CD34, CD71 and HLA-DR but not CD38. In contrast, c-kit (CD117) and Thy-1 (CD90) are expressed on normal HSCs only [67, 68]. Although CD34^-CD38^- subpopulation has been generally considered as the phenotype of LSC, some recent studies have argued that not all LSC have the CD34^-CD38^- phenotype. Taussig et al. reported that the CD34^+CD38^+ subpopulation seemed to have more LSC than the CD34^-CD38^- subpopulation in some patient samples [69]. They also found LSC in both CD34^-CD38^- and CD34^-CD38^+ subpopulations of NPM1 mutated patient samples [70]. Sarry et al. validated these results. They also identified LSC activity in CD34^-CD38^+ and CD34^-CD38^- fractions [71]. These studies suggest some novel possibilities that LSC phenotype may be variable and not always be predicted by CD34^-CD38^-, and that LSC can be found in more than one LSC subpopulation. They also underlined the need for advanced methods for isolating LSC as CD34 and CD38 surface markers displayed their limitations to identify LSC in all patient samples. Functional properties of LSC such as dye exclusion and their cell cycle status would be alternative approaches to enrich LSC. Both normal HSCs and LSCs have high expression levels of the ATP-binding cassette (ABC) transporters proteins such as MDR1 (multi-drug resistance 1), MRP1 (MDR-related protein 1) and BCRP (breast cancer
resistance protein), which leads to the active efflux of many dyes and cancer therapy
drugs from cells contributing to drug resistance of LSCs [72, 73]. Moreover, most LSCs
are non-cycling, quiescent cells and can be detected by flow cytometric measurement of
DNA and RNA content [7, 74-76]. These quiescent LSCs are resistant to standard
chemotherapies that typically target actively cycling populations including the leukemic
blasts as well as the leukemic progenitors such as AML colony-forming units (CFUs) and
long-term culture-initiating cells (LTC-ICs) [77]. Therefore, the development of new
strategies to eradicate these drug resistant LSC should be a primary goal of therapy in
AML.

1.1.2. Acute lymphoblastic leukemia
1.1.2.1. Overview

Acute lymphoblastic leukemia (ALL) is a malignant disease deriving from the
accumulation of multiple genetic lesions of hematopoietic stem cells and lymphoid
progenitors. ALL affects both children and adult, however, its greatest prevalence is in
children under 10 years of age [78]. In adults ALL is less common than AML and
chronic lymphocytic leukemia (CLL). The steady improvement of treatment approaches
has led to cure rates of greater than 80% for children, but the cure rate of adults is still
discouraging with only 30%-40% survival [79].

Most ALL occurs as de novo disease, with only rare cases rising as secondary
neoplasms. Although various genetic and environmental factors have been related to ALL,
the precise pathogenic events causing development of ALL are not clarified yet. Less
than 5% of ALL cases are associated with inherited chromosomal disorders, such as Down’s syndrome, Bloom’s syndrome, and ataxia-telangiectasia or with ionizing radiation or exposure to some cancer chemotherapeutic drugs. Chemotherapy and BMT are the most widespread and effective treatments for ALL [80, 81].

It has been known that adult ALL patients have a worse prognosis compared to children due to differences in disease biology. Adult cases have relatively higher incidence of specific karyotypic abnormalities connected with poor outcomes. A representative example is the Philadelphia (Ph) chromosome which exists in 20 to 30% of adult ALL patients, but less than 5% of children with ALL [82]. In this thesis, I evaluated the efficacy of combination therapy with DT$_{388}$IL3 and tyrosine kinase inhibitors which are specific for BCR-ABL, a product of Ph chromosome.

1.1.2.2. Classification

Identifying morphology and immunophenotypic features are essential to classify ALL. The French American British (FAB) Group classified ALL into three subtypes (L1, L2, and L3) based on cell size, prominence of nucleoli, degree of cytoplasmic basophilia, amount of cytoplasm and vacuolation. The morphologic distinction between L1 and L2 does not have prognostic significance because L1 and L2 morphologies do not predict immunophenotype, genetic aberrations, or clinical outcome. The L3 morphology is a feature of mature B-cell ALL characterized by a high rate of cell turnover.

The newer WHO classification established that more than 20% blasts in the diagnostic BM are sufficient for the diagnosis of ALL. The WHO classification identifies
two diagnostic entities: precursor B-cell ALL and precursor T-cell ALL, both of which cover a range of cytogenetic, immunophenotypic, morphologic, clinical, and molecular features [83, 84] (Table 1.3).

1.1.2.3. Philadelphia chromosome-positive acute leukemias

The Philadelphia (Ph) translocation is observed in 95% patients with chronic myeloid leukemia (CML) and about 25%-30% adult patients with ALL. The Ph translocation [t(9;22)(q34;q11)], an exchange of DNA between chromosome 9 and 22, results in the chimeric BCR-ABL gene. The aberrant BCR-ABL is essential to the pathogenesis of a subset of ALL and almost all CML. The BCR-ABL oncoprotein is a constitutively activated tyrosine kinase (TK) which interferes with various signal transduction pathways causing increased cell proliferation, perturbation of cell adhesion, growth factor independence, and alterations of DNA repair [85, 86]. Two different sized proteins can be generated by the translocation - p190 and p210 (Figure 1.2). The p190 protein is present in approximately 90% of pediatric cases and in 20-50% of adults of Ph positive (Ph+) ALL, while p210 is mainly associated with CML. CML may undergo transformation to a more aggressive blast crisis (CML-BP), which resembles acute leukemia (lymphoid in one third of patients and myeloid in two thirds of patients) [87-89].

The Ph chromosome is the commonest chromosomal abnormality detected in adult patients with ALL. The frequency of Ph rises with age, and it happens in more than a half of patients older than 50 years. Regardless of age, both adult and pediatric patients with Ph+ ALL generally show a higher rate of induction failure and relapse than patients
without this cytogenetic abnormality [90]. The development of molecularly targeted therapy using a specific BCR-ABL TK inhibitor, imatinib mesylate, has changed the therapeutic paradigm and improved outcomes in this high-risk subset of leukemia [91].

1.1.2.4. Treatment

The treatment of ALL involves intensive induction chemotherapy (using high-dose methotrexate, cyclophosphamide, cytarabine, dexamethasone or prednisone, vincristine, L-asparaginase, and/or an anthracycline). This is followed by intensification or consolidation therapy and prolonged maintenance to eliminate residual leukemia, prevent or eradicate central nervous system leukemia, and guarantee prolongation of remission [92].

The aim of induction therapy is to eradicate the initial leukemic cell burden as much as possible and to recover normal hemopoiesis and healthy status. A three-drug regimen including glucocorticoid, vincristine, and L-asparaginase induction appears to be sufficient to induce remission for most standard-risk cases of children’s ALL. Children with high-risk ALL and almost all adult cases undergo treatment with four drugs or more for remission induction. Intensive consolidation treatment followed by several years of oral maintenance therapy is commonly used to reduce the risk of relapse [93].

Although chemotherapy alone is used to treat most ALL patients, allogeneic SCT clearly benefits several subgroups of patients with high-risk ALL, especially for patients with Ph+ and with a poor initial response to induction chemotherapy. However, this
therapy is limited to only younger patients with good health status and without significant complications [94].

1.1.2.5. Specific molecular targeted therapy for Ph+ acute leukemia

Before the introduction of imatinib mesylate (IM), the treatment outcome of Ph+ ALL patients was extremely poor. Although conventional chemotherapy regimens induce CR in more than 70%, the median CR duration was considerably shorter than that seen in patients with Ph-negative disease, leading to very few long-term survivors. Median survival ranged from 8-16 months due to relapse-related mortality.

The enzymatic activity of tyrosine kinases (TK) is critical to signal transduction and growth of cells. Constitutively higher TK activity has been correlated with leukemic transformation. ABL is a nonreceptor TK, and the activity of ABL is tightly controlled by N-terminal motifs in its normal state. However, loss of this region in BCR-ABL leads to high constitutive TK activity [95]. IM (Gleevec; Novartis Pharma, Basel, Switzerland) is an oral specific inhibitor of BCR-ABL. Several phase I through phase III trials have shown promising efficacy with moderate toxicity [96]. IM functions by occupying the ATP binding site of BCR-ABL competitively thus blocking access to ATP, and inhibiting the enzyme activity of the protein [97] (Figure 1.3). In clinical trials, imatinib has been shown to have a potent anti-leukemic activity against CML and relapsed or refractory Ph+ ALL. Currently imatinib is the standard treatment for newly diagnosed CML and is included in combination chemotherapy with other agents for Ph+ ALL [98]. However, clinical resistance to imatinib occurs when the drug is used as a single agent for ALL.
Point mutations in the TK domain of BCR-ABL select or develop resistant leukemic subclones that lose their binding affinity to imatinib. Most mutations (about 70%) are found in the ATP binding pocket and the T315I is the second most frequent (about 17%) location for mutations. The T315I mutation has been associated with resistance to imatinib and to the second-generation TK inhibitors as well [99].

Dasatinib (Sprycel; Bristol-Myers Squibb, New York, USA), a novel dual SRC/BCR-ABL kinase inhibitor, exhibits 30 to 50-fold more potency than imatinib in vivo and has been approved by US FDA for the treatment of imatinib mesylate (IM)-resistant CML and Ph+ ALL. A superior benefit of dasatinib over imatinib is that it has greater affinity for BCR-ABL with ability to bind both inactive and active conformations. Due to its flexible binding conditions, dasatinib has displayed improved efficacy against imatinib-resistant mutations except T315I and F317L [100, 101]. Novel BCR-ABL mutations have been described in Ph+ ALL following successive treatment with dasatinib after imatinib [102, 103]. Moreover, although dasatinib inhibits most of the BCR-ABL mutants, it still does not induce durable remission in resistant CML and Ph+ ALL patients [104].

1.1.2.6. ALL leukemic stem cell

Leukemia stem cells are quite well described for AML, but their existence and relevance for ALL is less obvious. Identification and characterization of putative ALL LSCs would be important to the development of novel strategies for eradicating leukemia. Recent studies propose that some lymphoid diseases may be caused by malignant
stem/progenitor cells. Like AML, the blast population of ALL has a restricted capacity for proliferation suggesting the existence of LSCs perpetuating the malignancy. Functional studies have defined LSC population for Ph+ ALL. Cobaleda et al. demonstrated that only the phenotypically primitive CD34+CD38- subfraction of Ph+ ALL could engraft NOD/SCID mice and recapitulate the disease in vivo [105]. Overall, these findings have supported a hypothesis that ALL may also originate from a primitive stem cell population similar to AML [105-107].

1.1.3. Interleukin-3 receptor (IL-3R) expression in leukemias

1.1.3.1. Overview

The main biological function of IL-3 is enhancing the survival, proliferation and differentiation of hematopoietic progenitor cells (HPCs), especially the myeloid, erythroid, and megakaryocyte lineage. The potential clinical role of IL-3 is to expand HPC population either alone or in combination with G-CSF or GM-CSF [108-110].

The IL-3 receptor (IL-3R) consists of α and β subunits. The α subunit is associated with specificity and ligand binding. The common β (βc) subunit is in charge of signaling and shared by GM-CSF receptor and interleukin-5 receptor [14-17]. Co-expression of α and β chains allows formation of a high-affinity IL-3R heterodimer that can bind IL-3 and transduce signals for cell survival, proliferation, and differentiation [111-113] (Figure 1.4).

Initial studies based on IL-3 binding to membrane receptors and the induced biological responses demonstrated that most human AML blasts expressed the IL-3R and
proliferated in response to IL-3. IL-3 stimulated proliferation in over 85% of AML samples and its proliferative activity was stronger than other hemopoietic growth factors [96, 114, 115]. In addition, the IL-3R is expressed on over 80% of AML blasts [116, 117]. Other studies showed that the IL-3R was expressed in CD34+ HPCs during normal hemopoietic differentiation, and its expression was continued throughout all stages of monocytic and granulocytic differentiation, whereas it was maintained only during the early stages of megakaryocyte and erythroid differentiation and then disappeared [118].

IL-3R and GM-CSFR are often co-expressed on AML blasts [119, 120]. Moreover, specific IL-3 binding was detected in approximately a half of B-cell ALL [121, 122]. IL-3R expression on AML LSC was also studied. In this study a putative LSC (CD34+CD38-) subpopulation expressed high levels of IL-3R, whereas normal HSC had low level of the receptor. Therefore, this study proposed that IL-3R might be a unique biomarker for AML LSC [123]. Among the receptors that share the common βc, in AML only IL-3Rα is overexpressed but both the GM-CSFRα and the IL-5Rα chains are not [124].

Furthermore, studies on cell lines have demonstrated that the overexpression of IL-3R elicits oncogenic effects by promoting cell proliferation with suboptimal concentrations of IL-3 or even without IL-3 [125]. Consequently, it was concluded that the deregulated expression of IL-3R may confer a growth advantage to leukemic blasts under conditions where the cytokine is limited. In addition, elevated levels of IL-3R expression is correlated with negative prognosis [126].
1.1.3.2. Development of therapeutic approaches tailored to the IL-3R

Current induction and consolidation therapy for AML elicit long periods of pancytopenia, substantial morbidity and occasional mortality because they have low selectivity for AML vs normal cells. Hence, the development of a cytotoxic agent with a different action mechanism compared to conventional drugs that cause drug resistance and have low selectivity could greatly improve AML therapy.

To take advantage of the expression of IL-3R in AML and ALL [127], a fusion toxin which links the catalytic domain of diphtheria toxin (DT) to human IL-3 was created [128, 129] (Figure 1.5). The basic concept of this approach is that the fusion protein binds specifically to the IL-3R expressed at high level on leukemic blasts and exerts its cytotoxic effects through the diphtheria toxin moiety [130-132].

DT is a single polypeptide chain of 535 amino acids which consists of two polypeptide fragments, A and B, which are linked by disulfide bridges [133, 134]. The A-fragment is made up of the catalytic domain, whereas the B-fragment consists of the receptor binding domain and the transmembrane domain. After binding to the cell surface receptor, DT enters the cytoplasm by receptor mediated endocytosis. In the cytoplasm the catalytic domain is cleaved and becomes an active enzyme that inhibits the ADP-ribosylation of elongation factor 2, which blocks protein synthesis, thereby killing cells [135]. The target specificity of DT can be changed by substituting the B-fragment with a sequence such as a growth factor which will bind to a cell surface molecule. Various DT-growth factor fusion proteins with specific cytotoxicity to cells expressing the relevant receptor have been developed [136]. For instance, DT linked to GM-CSF (DT_{388}GMCSF)
kills most AML CFCs and some AML LTC-ICs and NOD/SI-IC if the samples expressed GM-CSF receptors. However, DT_{388}GMCSF showed some toxicity against normal CFCs and LTC-ICs in vitro and displayed liver toxicity in a phase I clinical trial [137].

Therefore, an alternative and more selective diphtheria fusion toxin, DT_{388}IL3 was designed and developed. In previous studies, DT_{388}IL3 has been effective in killing AML CFCs, AML LTC-ICs, and NOD/SI-IC from many patient samples while sparing analogous normal bone marrow cells [138-140]. Mutagenesis studies by several laboratories determined that the K116W variant of DT_{388}IL3 in which lysine at position 116 was substituted with tryptophan had enhanced binding affinity to IL-3R [141]. In the current Phase 1b/2 trial of DT_{388}IL3 in patients with relapsed and refractory AML, early evidence of anti-leukemic activity and acceptable toxicity has been observed [142].

1.2. Liposome-encapsulated cytarabine:daunorubicin

1.2.1. Overview

Although the development of effective chemotherapeutic drugs has greatly advanced over several decades, fundamental problems of the drugs such as non-specificity, adverse effects and drug resistance still remain [143]. Liposomes, the original models of nanoparticulate drug carrier (20-200nm diameter), were discovered in the 1960s. Liposomes have been used extensively to deliver anticancer agents and other therapeutic drugs more efficiently. They are spherical envelopes of phospholipid bilayer membranes surrounding an aqueous interior and able to deliver both hydrophilic and
hydrophobic compounds by carrying them in the internal aqueous core or in the lipid bilayer. The composition of lipid bilayer membranes can be manipulated to target a specific tissue, to be sensitive to temperature, or to control the rate of drug release [144-147]. For example, drug incorporation in a liposome could result in the change of pharmacokinetics, which can modify the drug exposure profile of tissues, increase the effect of drug to kill tumor more specifically, and reduce toxicity to normal cells [148, 149]. In addition, liposomes are generally considered non-toxic, non-immunogenic and biodegradable.

Owing to the ability of liposomes to encapsulate a variety of compounds such as water soluble, lipid soluble, proteins, oligonucleotides, and small molecular drugs, many attempts have been done to develop single liposome formulations co-encapsulating two or more therapeutic agents and modulating pharmacokinetics (PK) of drugs after administration. Several commercial liposomal formulations based on drug-in-liposome have already been developed and introduced on the pharmaceutical market [147]. For instance, liposomal formulations encapsulating doxorubicin (Doxil) and daunorubicin (DaunoXome) are used for the treatment of certain types of cancers.

However, liposomal formulations of combined drugs do not always show improved therapeutic outcome over single drug formulations. For example, Abraham et al. reported that the efficacy of liposomal formulation co-encapsulated with doxorubicin and vincristine was not improved compared to liposomal vincristine alone [150]. In vitro cytotoxicity assays for doxorubicin:vincristine combination revealed the strong antagonism for this drug combination [151]. To solve this problem, specific liposomes
which can deliver combined agents at synergistic drug ratio have been developed recently and shown dramatic improvements in the therapeutic activity of the drug combinations [152].

1.2.2. Drug combination

Combination chemotherapy using multiple drugs has played a pivotal role in many forms of cancer chemotherapy for more than 30 years. Regimens incorporating multiple drugs are developed based on the maximum tolerated dose (MTD) of individual agents. Although the MTD concept of anticancer combination chemotherapy is theoretically rational, it does not always elicit the best treatment outcome. This is due to interactions between the anticancer drugs and the different PK of individual drugs.

Recently several studies suggested that the efficacy of drug combinations appears to be enhanced by selecting the ratio of drug concentrations which is most synergistic [148, 149, 152]. Synergistic drug combinations are commonly evaluated in vitro because the dose and duration of drug administration can be controlled accurately. When anticancer drugs are administered to patients in chemotherapy mixtures, each drug is processed independently in the body, which results in various distributions of the individual agents to the tumor and potentially suboptimal or ineffective drug ratios [152-154]. In vivo, maintenance of the synergistic drug:drug ratio is attained by using nanoparticle drug delivery systems such as liposomes, which can improve the efficacy of the combination and avoid the possibility of exposure to an antagonistic drug ratio.
1.2.3. CPX-351

The combination of cytarabine and daunorubicin has been used as a standard induction chemotherapy for AML for over 35 years. Although several approaches to enhance this regimen have been tried including increasing dose of each drug, prolonging exposure time of cytarabine, and adding a third drug to this combination, all of them failed to advance the rate of CR or OS. Based on the findings about drug ratio-dependent antitumor activity, previous studies found the cytarabine:daunorubicin 5:1 molar ratio displayed the greatest synergistic effect in vitro, while other ratios of the same combination were additive or even antagonistic. However, this synergistic ratio is difficult to maintain in vivo due to the dissimilar distribution, metabolism and elimination properties of the each drug in the conventional free drug combination. One efficient strategy to maintain the synergistic ratio of drug combinations is the use of a liposomal drug delivery system. By manipulating the composition of lipid membrane and internal buffer, liposomes are able to deliver a synergistic ratio to tumor site [148, 155].

CPX-351(Celator Pharmaceutical company, Vancouver, BC) is a liposomal formulation which maintains the fixed 5:1 molar ratio of cytarabine:daunorubicin in vivo for more than 24h following intravenous administration. The lipid membrane composition of CPX-351 is distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), and cholesterol (CHOL) in a 7:2:1 molar ratio. The lipid film of CPX-351 was hydrated to form liposomes with 50mM copper gluconate and 110 mM triethanolamine. CPX-351 has shown improved and enhanced selective killing of leukemia in preclinical tumor models [154, 155]. For example, CPX-351 will
eradicate leukemia in mice while a free drug cocktail (a mixture of the two drugs without liposome) failed to achieve long-term cures even with higher drug doses. Two Phase II clinical trials of CPX-351 are ongoing in elderly AML patients and patients with relapsed AML. In this thesis, I evaluated the selectivity of CPX-351 for AML progenitors compared to normal counterparts.

1.3. ATP-binding cassette (ABC) transporters

1.3.1. Overview

Chemotherapy can be unsuccessful due to the development of cancer cell resistance to multiple drugs, also known as multidrug resistance (MDR) [156, 157]. Thus, establishing the mechanisms of inherent or acquired MDR is crucial to developing more effective therapies. There are many causes of MDR including decreased drug uptake, increased drug efflux, defective apoptotic pathways, enhanced detoxification mechanisms, and modification of target proteins [158, 159]. Among them, one important mechanism of MDR in cancer cells is high expression of cell-membrane bound molecules which can transport a wide spectrum of chemotherapeutic agents out of cells, thereby, reducing their intracellular concentration. The efflux pump proteins mediating MDR in human cancers belong to the ABC superfamily [160-162].

ABC transporters belong to a broader group of ATP-dependent drug efflux pumps which contribute to drug resistance. In humans, ABC transporters constitute one of the biggest groups of transmembrane proteins [163, 164]. The 49 ABC proteins have been identified in almost all human cells. They transport hydrophilic and hydrophobic compounds across cell and intracellular membranes. Some ABC transporters derive their
compound specificity based on substrate chemical structure and composition but most are capable of extruding many compounds with diverse structures. In normal physiology, ABC transporters play intrinsic and critical roles such as transferring toxins, nutrients and biological materials across the blood-brain blood-placenta and blood-testis barriers. In addition, ABC transporter-mediated processes notably affect the absorption, distribution, metabolism and excretion of drugs. These transporters utilize energy derived from the hydrolysis of ATP for the active efflux of various compounds against their concentration gradients across cell membranes [165-173].

Similar gene structure in the ATP-binding domains (nucleotide-binding folds) results in identifying and subdividing the ABC transporters into seven subfamilies (from ABCA to ABCG). Both malignant and normal cells express these ABC transporters [164]. ABC transporters exist in all living species from prokaryotes to human with an evolutionary well-conserved structure. The functional ABC proteins are usually comprised of 12 transmembrane domains (TMDs) and 2 ATP binding domains (Figure 1.6). In full transporters, these domains exist within one polypeptide, while half transporters have these domains in two separate proteins. In the latter case, the dimerization of specific half-transporters is necessary to form functionally active ABC transporters [167, 174]. ABC transporters are known to be associated with drug efflux and resistance in cancers. Therefore, in this thesis, I developed a flow cytometry based assay for predicting responsiveness to chemotherapy in AML patients by measuring the function of ABC transporters.
1.3.2. ABC transporters and MDR in cancer

Resistance to anticancer drugs represents one of the major impediments to the successful cancer chemotherapy. Cancer cells having MDR are cross resistant to various chemotherapeutic drugs, even chemically and functionally different ones [175]. The MDR phenomenon arises from the overexpression of ABC transporters which pump various chemotherapeutic drugs out of the cells. As a result, MDR cancer cells can escape from the cytotoxic effect of the drugs. While as many as 15 ABC transporters have been associated with chemotherapeutic drug transport and resistance, ABCB1, ABCC1, and ABCG2 are the three major multidrug resistance proteins in cancer cells [176] (Table 1.4).

Discovered over 30 years ago by Ling and co-workers, P-glycoprotein (Pgp; MDR1; ABCB1), was the first mammalian ABC protein to be identified [177]. Pgp is present at low levels in many tissue types in humans and rodents but is generally expressed at higher levels on epithelial cell surfaces throughout the body [178]. Indeed, knockout mice lacking one or both drug-transporting Pgp proteins appear normal in every way, are fertile, and have a normal life span. However, when challenged with amphipathic drug substrates, these compounds accumulate in the brain to a much greater extent than in wild-type mice, resulting in neurotoxicity [179]. The presence of Pgp in hematopoietic progenitor cells of the bone marrow protects these vital cells from toxic drugs during chemotherapy. Thus, one important role of Pgp in the body is probably protection of tissues from toxic compounds [180].

Pgp is highly expressed in cancers arisen from tissues that normally express Pgp and causes resistance to some chemotherapeutic drugs. In some cancers, the expression of
Pgp is low before chemotherapy, but can be induced during or after chemotherapy [181, 182]. The expression of Pgp in cancer cells often correlates with a weak response to chemotherapeutic compounds and poor clinical prognosis for the patient. For example, in treatment of ovarian cancer with paclitaxel, Pgp overexpression was reported to correlate inversely with probability of survival [183].

Studies on human cancer cells having MDR without Pgp overexpression and reports that failed to detect Pgp in a range of MDR cancers strongly implied the existence of other MDR related proteins. In 1992, Cole and co-workers observed elevated level of a novel gene, MRP1 (MDR related protein; ABCC1) in an MDR small cell lung cancer cell line without Pgp. MRP1 is expressed at low levels in many normal tissues and cell types in the body, but it is more highly expressed in the adrenal gland, bladder, choroid plexus, colon, erythrocytes, kidney, lung, placenta, spleen, stomach, testis, helper T-cells, and muscle (both skeletal and cardiac) [184]. MRP1 knockout mice are viable and fertile, however, drug sensitivity in some tissues that normally express high levels of MRP1 is increased [185]. MRP1 appears to export its substrates either in the form of glutathione conjugates or with co-transported reduced glutathione (GSH) [172]. MRP1 expression has been detected in various cancer types, ranging from solid tumors such as gastrointestinal, breast, and kidney cancers to hematological malignancies including AML [186].

Recently, Ross and Doyle cloned a newer ABC transporter involved in MDR from a drug-resistant breast cancer cell line MDF-7/AdrVp. They named this transporter breast cancer resistance protein (BCRP; ABCG2). BCRP has a somewhat different
structure. It is a half-transporter and functions as a homodimer or homomultimer. mRNA analysis indicates that BCRP is most highly expressed in the placenta, with high levels also found in the brain, liver, prostate, and small and large intestine [187]. Additionally, the expression of BCRP is highly increased in the mammary gland during lactation, where it transports chemotherapeutic drugs into the milk of humans, cows, and mice. BCRP can efflux a narrow range of drugs compared with Pgp which includes anthracyclines, topotecan derivatives, mitoxantrone etoposide and bisantrene [188].

Other ABC transporters may also be involved in MDR. For example, ABCA2 is amplified in an ovarian cancer cell line and creates resistance to estraustine. ABCC11 confers some resistance to 5-FU and methotrexate in breast and kidney cancer cell lines [176, 189, 190].

1.3.3. ABC transporters and MDR in AML

MDR can be detected at diagnosis intrinsically or at relapse after chemotherapy in patients with AML. Flow cytometry has shown expression of Pgp in up to 50% of clinical AML samples. Levels of the protein were increased in samples from patients whose leukemia was refractory to chemotherapeutic treatment and on AML recurrence, and the expression correlated well with poor clinical prognosis [191, 192]. Others have shown that Pgp expression is more common in older AML patients who typically show a poor response to treatment [193].

MRP1 has been correlated with poorer response to chemotherapy in AML [194, 195]. Other transporters in the MRP subfamily including MRP2 to MRP7 have been
investigated but have not been proven to function as an efflux pump in AML. Early studies indicated that the expression level of MRP1 mRNA increased in AML cells together with disease progression and that cells from relapsed AML had higher mRNA expression compared with cells at diagnosis [196]. On the other hand, some studies failed to show any prognostic value of MRP1 in AML patients [197, 198].

In approximately 30% of high-risk AML cases, BCRP mRNA is highly expressed regardless of the expression of Pgp or MRP1, which suggests a potential independent role of BCRP in MDR in AML [199]. One study reported a significantly higher expression (1.7-fold) of BCRP mRNA at the time of relapse than at initial diagnosis. There were similar findings in pediatric AML cases including much higher expression level of BCRP mRNA at the time of relapse and in cases of induction failure. Other studies also showed that the BCRP expression level correlated with overall survival independent of cytogenetic abnormalities [200, 201].

1.3.4. ABC transporters in normal hematopoietic and leukemia stem cells

Generally human HSCs and stem cells of other tissues efficiently efflux fluorescent dyes such as Hoechst-33342 and rhodamine-123. Thus, enrichment for stem cells has been achieved by isolating cells with poor retention of these fluorescent dyes. Rhodamine-123/Hoechst-33342-dull bone marrow cell fractions are able to engraft after injection into immunocompromised mice and regenerate hematopoiesis. By contrast, most non-stem cells accumulate these fluorescent dyes. The molecular mechanism of this phenotype has been unknown. However, recent studies showed that the efflux pump
activity of ABC transporters is responsible for the phenotype of HSCs [72, 73, 179, 202-204]. Also, a specific population of cells, called side population (SP) cells, can be sorted by flow cytometry with Hoechst-33342. SP cell is revealed by analysis with Hoechst 33342 dye at 2 wavelengths. SP cells express transport proteins that allow them to actively pump out the dye from cells. The dye is excited by UV wavelengths (395 nm) and emits in the blue (450 nm). However, the emission wavelength shifts to the red end of the spectrum when the intracellular dye concentration is high. In a dot plot profile, SP cells appear at the side of the main population cells. The majority of HSCs are found in this SP cell fraction [205-207]. ABCA1, ABCB1 (Pgp), ABCB2, ABCC1 (MRP1), ABCD4, and ABCG2 (BCRP) are mainly expressed in the immature CD34−CD38− subpopulation and are decreased on more differentiated CD34−CD38+ subpopulation [208].

Despite the broad range of ABC transporters expression in HSC, the exact role of ABC transporters for HSC physiology is not recognized well. One possible role of ABC transporters for normal HSC is to efflux various toxic compounds that may cause genetic damage [72, 209]. The protective role of ABCB1 for HSC was mentioned in a previous report. In this study, the higher expression of ABCB1 is confirmed in normal hematopoietic progenitor cells, and rhodamine-dull cells from these progenitors were less sensitive to daunorubicin and adryamycin compared with rhodamine bright cells. These results implied that ABCB1 may be critical for protection of hematopoietic progenitors against anthracycline toxicity [210]. The substantial protective effect of ABCG2 on HSC was exhibited in a knock-out mouse model. An increased cytotoxicity of mitoxantrone to
bone marrow was observed in ABCG2 knock-out mice compared to wild-type mice [211]. In a study with ABCC1 knock-out mice, bone marrow cells displayed increased sensitivity to vincristine and slow hematopoietic recovery [212]. Since a recent study revealed that the expression of all 15 members of ABC transporters correlated with drug resistance were detected in human CD34⁺CD38⁻ hematopoietic cells the protective effect of ABC transporters in normal HSC against xenobiotics may result from the concurrent activity of many minor as well as major transporters [213].

In AML cells, ABCB1 and ABCG2 have been broadly studied, however, their role in drug resistance of LSC has not been definitively addressed. Inhibition of ABCB1 hindered mitoxantrone efflux in normal CD34⁺CD38⁻ cells but not in leukemic counterparts probably owing to redundant ABC transporters [214]. ABCG2 is highly expressed on leukemic CD34⁺CD38⁻ cells and makes a contribution to mitoxantrone efflux in these cells [215]. However, specific inhibition of ABCG2-mediated drug extrusion using KO143, the most powerful ABCG2 inhibitor currently available [216], did not increase sensitivity of leukemic CD34⁺CD38⁻ cells to mitoxantrone in most cases. These findings suggest that selective inhibition of only one or two major ABC transporters is not enough to overcome the drug resistance of LSC. Modulation of ABC transporter activity by broad spectrum inhibitors may be required to increase chemosensitivity of LSC and to eradicate them in AML. However, this strategy is likely to increase hematological toxicity because most ABC transporters in LSCs are also expressed in normal HSCs [217, 218]. Moreover, nonhematological toxicity is expected
as well since many ABC transporters normally play essential roles in detoxification processes of other tissues.

1.3.5. Overcoming ABC transporter-mediated drug efflux

Many studies have been performed to overcome MDR by modulating ABC transporter expression [219-221]. The strong prognostic value of ABCB1 expression in AML suggested the idea that this MDR phenotype could be an important therapeutic target for the treatment of AML. Although in theory quite promising, the clinical trials with the first generation ABCB1 inhibitors including verapamil, cyclosporine A and quinidine showed low effectiveness and excessively high toxicity [219]. To address these issues, second-generation modulators were developed. However, in large multicenter randomized trials, chemotherapy with or without a second generation MDR1 modulators such as PSC-833 (a cyclosporine A derivative) had no clinical benefit [222, 223]. Furthermore, clinical trials using the latest ABC transporter inhibitors that have more specificity and less toxicity have also failed to show clinical benefit thus far [159, 224]. Because normal cells also express various ABC transporters, these inhibitors may increase the toxicity of cytotoxic agents against normal cells and tissues. Thus, MDR inhibitors have failed to enhance the therapeutic efficiency of chemotherapeutic agents.

1.3.6. Prognostic value of ABC transporter expression

It is widely known that the cytogenetics at diagnosis is the most powerful prognostic factor in AML. However, a highly variable prognosis exists even within an
AML patient group having the same cytogenetic risk. Nowadays MDR expression is considered to be another prognostic factor for treatment failure in AML patients. Thus, more accurate prediction of treatment outcome may be possible by considering both karyotype and MDR status [200, 225, 226]. Del Poeta and co-workers reported that Pgp expression had a negative prognostic significance within the same cytogenetic risk classes in AML [191, 192]. The expression of BCRP and MRP3 are also independent prognosis factors of clinical or biological factors such as age, WBC count, performance status, and cytogenetics [227]. Previously, Benderra et al. have shown that the coexpression of Pgp and MRP1 was a poorer prognostic factor than the expression of only one of these proteins [200]. In recent studies, the negative role of Pgp and BCRP overexpression in patients with normal karyotype AML has been observed [227]. Ho et al. also demonstrated the correlation between ABC transporter gene expression in candidate leukemic stem cells and chemotherapy response [228]. Therefore, these studies emphasized the importance of ABC transporters as an important prognostic determinant in AML. Thus, based on previous data, I tried to develop a novel FACS-based functional assay for predicting chemotherapy refractoriness in newly-diagnosed AML patients.

1.4. Rationale and Thesis Objectives

Acute leukemia encompasses a heterogeneous group of diseases that have different clinical and prognostic characteristics. Steady advances have been achieved over the last three decades in the treatment and survival of patients with acute leukemia. However, the management of acute leukemia is still a challenge to hematologists. Thus,
the overall aim of this thesis was to improve the therapeutic and diagnostic approach to acute leukemia including AML and ALL.

In chapter 2, I describe the synergistic interaction of the tyrosine kinase inhibitors (TKI) imatinib and dasatinib with DT\textsubscript{388IL3} for killing of malignant progenitors from patients with Ph+ acute leukemia. 10 Ph+ AL samples were tested to see whether they expressed IL-3R, which is the primary requirement to be a target for DT\textsubscript{388IL3}. Both α and β subunits of IL-3R were expressed at various levels as detected by FACS and QRT-PCR. These results showed that DT\textsubscript{388IL3} might be effective in killing these cells. Cells were treated with imatinib, dasatinib and DT\textsubscript{388IL3} individually or in combination, and synergistic killing was observed on malignant CFC from patients with Ph+ AL, but not CFC from normal blood. In subsequent experiments, I tested the effectiveness of this combination therapy against subpopulations of Ph+ AL cells that might contain reservoirs of chemotherapy resistant leukemic stem cells such as those in the quiescent phase of the cells cycle or more primitive progenitors detected in long-term suspension culture (SC-IC). Synergy between DT\textsubscript{388IL3} and each TKI was again observed in all experimental groups. This further supported the notion that DT\textsubscript{388IL3} synergizes with TKIs in killing Ph+ AL and candidate LSCs due to their expression of the IL-3R while sparing normal progenitors.

In chapter 3, I demonstrate the enhanced selective cytotoxicity of CPX-351 for AML progenitors as compared to normal hematopoietic cells. Previously selective uptake and cytotoxicity of CPX-351 in leukemia was reported in a mouse leukemia model. Thus, our hypothesis was that CPX-351 has an improved selectivity against AML cells
compared to normal hematopoietic progenitors. I tested the efficacy of CPX-351 against leukemic cells from CR or IF AML patients, and normal PB or BM progenitors. In addition, I compared cytotoxicity of CPX-351 against normal progenitors compared with free drug cocktail. My coworkers in the company analyzed drug accumulation in leukemic cells by HPLC and sub-cellular drug distribution using confocal microscopy as well. The data presented in this chapter demonstrated equivalent potency for CPX-351 to free drugs in killing AML progenitors and candidate LSC population. Moreover, CPX-351 showed enhanced selectivity for AML rather than normal progenitors. These data support the putative improved efficacy of CPX-351 compared to conventional cytarabine/daunorubicin induction therapy.

In chapter 4, I develop a new flow cytometry based mitoxantrone efflux assay for predicting response to induction chemotherapy in AML patients. In this project, I hypothesized that higher expression of ABC transporters in AML is correlated with a low CR rate and that the cells from these AML patients efflux anthracyclines more efficiently and are more sensitive to ABC transporter inhibitors than cells from patients with low level ABC transporter expression and a higher CR rate. If so, I will be able to predict the response to intensive chemotherapy, which would be beneficial to both patients and clinicians. The aim of this chapter was to develop a flow cytometry-based assay that would predict patients’ response to induction chemotherapy in newly-diagnosed AML. The reason I chose flow cytometry for this assay is that the technology is routinely available in all clinical hematology laboratories and already used in the pre-treatment analysis of leukemia cells from newly-diagnosed AML patients. In this assay, the
median fluorescence intensity (MFI) for AML cells incubated with mitoxantrone (a substrate for ABC transporters) in the presence or absence of cyclosporine A (a broad-spectrum inhibitor for ABC transporters) was measured. An MFI ratio (MFIR) between the inhibited and uninhibited MFI was calculated. The mean MFIR for CR patients was lower than that obtained for chemotherapy refractory patients. In addition, an MFIR of 2.45 was determined by logistic regression analysis to be the most reliable threshold value to discriminate CR and induction failure (IF) patients. Among all tested patients, 81% of patients with MFIR ≤ 2.45 achieved CR, but only 25% patients with MFIR > 2.45 obtained CR. This finding was more prominent in patients with normal karyotype or over 60 years of age. Therefore, I think this assay is potentially a rapid and reliable pretreatment assessment for predicting response to induction chemotherapy in AML.
### Table 1.1 AML classification

<table>
<thead>
<tr>
<th>WHO classification</th>
<th>FAB classification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AML with recurrent cytogenetic translocations</strong></td>
<td>M0: minimally differentiated</td>
</tr>
<tr>
<td>AML with t(8;21)(q22,q22) AML 1 CBFalpha/ETO</td>
<td>M1: myeloblastic leukemia without maturation</td>
</tr>
<tr>
<td>Acute promyelocytic leukemia:</td>
<td>M2: myeloblastic leukemia with maturation</td>
</tr>
<tr>
<td>AML with t(15;17)(q22;q12) and variants PML/RARgamma</td>
<td>M3: hypergranular promyelocytic leukemia</td>
</tr>
<tr>
<td>AML with abnormal bone marrow eosinophils inv(16)(p13q22)</td>
<td>M4: myelomonocytic leukemia</td>
</tr>
<tr>
<td>vayy (16;16)(p13q22) CBFbeta/MYH1</td>
<td>M4Eo: variant, increase in marrow eosinophils</td>
</tr>
<tr>
<td>AML with 11q23 MLL abnormalities</td>
<td>M5: monocytic leukemia</td>
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<tr>
<td><strong>AML with multilineage dysplasia</strong></td>
<td>M6: erythroleukemia (DiGuglielmo's disease)</td>
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<tr>
<td>With prior MDS</td>
<td>M7: megakaryoblastic leukemia</td>
</tr>
<tr>
<td><strong>AML with myelodysplastic syndrome, therapy related</strong></td>
<td></td>
</tr>
<tr>
<td>Alkylating agent related</td>
<td></td>
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<tr>
<td>Epipodophyllotoxin related</td>
<td></td>
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<tr>
<td><strong>Other types</strong></td>
<td></td>
</tr>
<tr>
<td><strong>AML not otherwise categorized</strong></td>
<td></td>
</tr>
<tr>
<td>AML minimally differentiated</td>
<td></td>
</tr>
<tr>
<td>AML without maturation</td>
<td></td>
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<tr>
<td>AML with maturation</td>
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<tr>
<td>Acute myelomonocytic leukemia</td>
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<tr>
<td>Acute monocytic leukemia</td>
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<tr>
<td>Acute erythroid leukemia</td>
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<tr>
<td>Acute megakaryocytic leukemia</td>
<td></td>
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<tr>
<td>Acute basophilic leukemia</td>
<td></td>
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<tr>
<td>Acute panmyelosis with myelofibrosis</td>
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Table 1.2 Prognostic genetic characteristics in acute myeloid leukemia

<table>
<thead>
<tr>
<th>Favorable risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;21)(q22;q22)</td>
</tr>
<tr>
<td>inv(16)(p13;q22) t(16;16)(p13;q22)</td>
</tr>
<tr>
<td>t(15;17)(q22;q12-21)</td>
</tr>
<tr>
<td>NPM1 mutation with normal karyotype and without FLT3 ITD</td>
</tr>
<tr>
<td>CEBPA mutation</td>
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<table>
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<tr>
<th>Intermediate risk</th>
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<tbody>
<tr>
<td>Normal karyotype</td>
</tr>
<tr>
<td>t(9;11)(p22;q23)</td>
</tr>
<tr>
<td>del(7q), del(9q), del(11q), or del(20q)</td>
</tr>
<tr>
<td>-Y</td>
</tr>
<tr>
<td>+8, +11, +13, or +21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unfavorable risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex karyotype (≥3 abnormalities)</td>
</tr>
<tr>
<td>inv(3)(q21;q26) or t(3;3)(q21;q26)</td>
</tr>
<tr>
<td>t(6;9)(p23;q34)</td>
</tr>
<tr>
<td>t(6;11)(q27;q23)</td>
</tr>
<tr>
<td>t(11;19)(q23;p13.1)</td>
</tr>
<tr>
<td>del(5q)</td>
</tr>
<tr>
<td>-5</td>
</tr>
<tr>
<td>-7</td>
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</table>

Modified from Gulley ML et al. 2010 [32]
Figure 1.1 Models of tumor cell proliferation

(a) Cancer cells are phenotypically and functionally heterogeneous, but they are biologically equivalent to proliferate extensively and to form new cancers. According to this model, all cells in the cancer can lead the development and progression of malignancy. (b) Although cancer cell are heterogeneous, only a small portion of cancer cells can start new tumor growth. In this model, cancer stem cells have biologically and functionally distinctive features compared to the bulk of cancer cells.
### Table 1.3 Classifications of ALL

<table>
<thead>
<tr>
<th>WHO</th>
<th>FAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute lymphoblastic leukemia/lymphoma Synonyms:</td>
<td>ALL-L1: small uniform cells</td>
</tr>
<tr>
<td>Former Fab L1/L2</td>
<td>ALL-L2: large varied cells</td>
</tr>
<tr>
<td>i. Precursor B acute lymphoblastic leukemia/lymphoma</td>
<td>ALL-L3: large varied cells with vacuoles (bubble-like features)</td>
</tr>
<tr>
<td>Cytogenetic subtypes</td>
<td></td>
</tr>
<tr>
<td>t(12;21)(p12;q22)</td>
<td></td>
</tr>
<tr>
<td>t(1;19)(q23;p13)</td>
<td></td>
</tr>
<tr>
<td>t(9;22)(q34;q11)</td>
<td></td>
</tr>
<tr>
<td>t(V;11)(V;q23)</td>
<td></td>
</tr>
<tr>
<td>ii. Precursor T acute lymphoblastic leukemia</td>
<td></td>
</tr>
<tr>
<td>Burkitt's leukemia/lymphoma Synonyms:Former FAB L3</td>
<td></td>
</tr>
<tr>
<td>Biphenotypic acute leukemia</td>
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</tbody>
</table>

Figure 1.2 Two representative fusion genes of the BCR-ABL

The size of fusion genes depends on the specific BCR gene breakpoint. Squares of ABL and BCR means exons. Modified from Deininger MW et al. Blood. 2000 [87]
Figure 1.3 The inhibition mechanism of imatinib to BCR-ABL fusion protein
Imatinib binds close to the ATP binding pocket semi-competitively and inhibits the enzyme activity of BCR-AML.
Figure 1.4 Structure of IL-3R complex

The human interleukin-3 receptor (IL-3R) is a heteromeric complex of a specific α-subunit and a common βc-subunit. The expression of both IL-3Rα and βc is necessary for triggering signalling and cellular proliferation in response to IL-3.

Modified from Reddy EP. et al. Oncogene. 2000 [229]
Figure 1.5 DT\textsubscript{388} and IL-3 fusion protein

The binding domain of the DT\textsubscript{388}IL3 fusion protein binds to IL3 receptors on cell surface. After that, the DT\textsubscript{388}IL3 enters the cell by receptor-mediated endocytosis. In the cytosol, the catalytic domain inhibits the function of elongation factor 2, which results in the blocking of protein synthesis. The affected cells undergo apoptotic cell death.
Table 1.4 ABC transporters associated with the efflux of chemotherapeutical agents and drug resistance

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Drug substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA2</td>
<td>Estramustine</td>
</tr>
<tr>
<td>ABCA3</td>
<td>Daunorubicin</td>
</tr>
<tr>
<td>ABCB1 (MDR1, P-glycoprotein)</td>
<td>Anthracyclins, etoposide, imatinib, taxanes, mitoxantrone, vinca alkaloids</td>
</tr>
<tr>
<td>ABCB4 (MDR2)</td>
<td>Paclitaxel, vinblastine</td>
</tr>
<tr>
<td>ABCB5</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ABCB11 (BSEP)</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>ABCC1 (MRP1)</td>
<td>Anthracyclins, etoposide, methotrexate</td>
</tr>
<tr>
<td>ABCC2 (MRP2/eMOAT)</td>
<td>Cisplatin, doxorubicin, etoposide, methotrexate, mitoxantrone, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC3 (MRP3)</td>
<td>Cisplatin, doxorubicin, etoposide, methotrexate, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC4 (MRP4)</td>
<td>Methotrexate, thiopurines</td>
</tr>
<tr>
<td>ABCC5 (MRP5)</td>
<td>6-Mercaptopurine, 6-thioguanine</td>
</tr>
<tr>
<td>ABCC6 (MRP6)</td>
<td>Anthracyclins, etoposide, teniposide</td>
</tr>
<tr>
<td>ABCC10 (MRP7)</td>
<td>Docetaxel, paclitaxel, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC11 (MRP8)</td>
<td>Purine and pyrimidine nucleotide analogues</td>
</tr>
<tr>
<td>ABCG2 (BCRP/MXR)</td>
<td>Mitoxantrone, methotrexate, topotecan, SN-38, imatinib, flavopiridol, anthracyclins</td>
</tr>
</tbody>
</table>

Modified from Raaijmakers MHGP, Leukemia. 2007 [163]
Figure 1.6 Structural representation of an ABC transporter

Generally 12 transmembrane domains (TD) and 2 ATP binding sites constitute an ABC transporter.
Chapter 2. A diphtheria toxin interleukin-3 fusion protein synergizes with tyrosine kinase inhibitors in killing leukemic progenitors from BCR-ABL positive acute leukemia

2.1. Introduction

The Philadelphia chromosome (Ph) is originated from the swapping of DNA between the q arms of chromosomes 9 and 22. The resulting fusion protein, BCR-ABL, has constitutively elevated tyrosine kinase activity which causes reduced apoptosis, uncontrolled cell proliferation, and impaired cell adhesion. The BCR-ABL abnormality is essential to the pathogenesis of CML [89, 230-232]. The Ph may also be seen in patients with precursor B-cell ALL where the overall frequency is 20% to 30%, with the incidence rising to more than a half in older patients (> 50 years) [90]. The acute or blast phase of CML (CML-BP) (which may be lymphoid or myeloid) is typically associated with genetic changes in addition to BCR-ABL [233-235]. Both Ph positive (Ph⁺) ALL and CML-BP carry a very poor prognosis characterized by relative resistance to conventional chemotherapy and short survival. Targeting of the BCR-ABL abnormality with the tyrosine kinase inhibitor imatinib mesylate has dramatically improved the therapy of chronic phase CML [236]. Imatinib is also active in the treatment of Ph⁺ acute leukemias. However, in contrast to the results in chronic phase CML, responses are often incomplete and the duration of any remission obtained brief [100, 237, 238]. Relapse is characterized by drug resistant disease often correlated with mutations in the ABL kinase
domain [239, 240]. An alternate kinase inhibitor, dasatinib, has shown activity against imatinib resistant disease [241, 242]. Unlike imatinib, dasatinib binds to both the active and inactive configuration of the BCR-ABL kinase and the majority of ABL mutants [239, 240]. Although dasatinib is much more potent than imatinib, resistance to this drug can also develop, particularly in patients with advanced phase CML or Ph⁺ acute leukemia [243].

The heterodimeric interleukin-3 receptor (IL-3R) is expressed at high levels on the blasts from most patients with AML with the α subunit (CD123) expressed at particularly high levels [118, 120]. Analysis of subpopulations of AML blasts has also shown that this high level IL-3R expression is seen on candidate AML stem cells as well as the majority population of blasts. Similar studies of B lineage ALL cells have demonstrated specific IL-3 binding in about a half of cases and on both myeloid and lymphoid blasts from patients with blast phase CML [114, 121].

A fusion protein which links a truncated version of diphtheria toxin to human interleukin-3 (DT₃₈₈IL3) has been developed as potential therapy for AML [128]. In preclinical studies this molecule was displayed to kill AML colony forming cells (AML CFC), long-term culture-initiating cells (AML LTC-IC) and NOD/SCID mouse leukemia-initiating cells (N/S-L-IC) from many patient samples while sparing analogous normal cells [130, 136, 244]. A Phase 1b/2 trial is currently evaluating the efficacy of this drug in patients with refractory or relapsed AML and myelodysplasia [142].

The cytotoxicity of diphtheria toxin is mediated through inhibition of protein synthesis and thus the mechanism of action of DT₃₈₈IL3 is different from that of
conventional chemotherapy drugs [245]. DT$_{388}$IL3 has been shown to synergize with cytarabine in killing AML cells [246]. We hypothesized that DT$_{388}$IL3 might also synergize with tyrosine kinase inhibitors (TKIs) in killing leukemic blasts and progenitors that express both the IL-3R and BCR-ABL. Autocrine production of IL-3 by Ph$^+$ leukemia cells or normal bone marrow cells engineered to express BCR-ABL has been shown to contribute to the uncontrolled proliferation and relative growth factor independence of these cells [247, 248]. Thus, it seemed possible that targeting of the IL-3R on Ph$^+$ cells with DT$_{388}$IL3 would be cytotoxic. Ten Ph$^+$ acute leukemia (AL) samples were studied for their expression of the IL-3R subunits and for their sensitivity to DT$_{388}$IL3, imatinib, dasatinib or DT$_{388}$IL3 in combination with a TKI. Synergy between DT$_{388}$IL3 and each of the TKIs was observed against leukemic colony forming cells (CFC), including those derived from the quiescent (G$_0$) fraction of leukemic blasts, and the more primitive progenitors that elicit long-term suspension culture. In contrast, normal progenitors were relatively resistant to these drugs alone or in combination. Thus, the combination of inhibitors targeting two different abnormalities in leukemic cells enhances the elimination of malignant progenitors including those with high proliferative capacity and characteristics associated with chemotherapy drug resistance. Such combinations may ultimately be useful clinically.
2.2. Materials and Methods

2.2.1. Leukemia and normal samples

Peripheral blood (PB) or bone marrow (BM) cells were obtained from 10 patients with Ph⁺ acute leukemia (AL) and normal donors after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. Morphologic assessment, immunophenotyping and cytogenetic analysis were performed on patient bone marrow at initial diagnosis and the leukemia categorized according to World Health Organization criteria [19] (Table 2.1). G-CSF-mobilized PB was obtained from normal individuals donating blood progenitor cells for clinical allogeneic transplantation. Normal and leukemic mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden) and cryopreserved in Iscove’s modified Dulbecco’s medium (IMDM) with 50% fetal calf serum (FCS) (StemCell Technologies, Vancouver, Canada) and 10% dimethylsulfoxide. Thawed cells were washed twice in IMDM containing 10% FCS and used for the experiments described below.

2.2.2. Drugs

DT₃₈₈IL3 was constructed by joining the human IL3 cDNA to a truncated DT sequence that lacks the native binding site via an intervening (G₄S)₂ linker. Recombinant protein was prepared, purified, and stored as described previously [128]. This material was found to kill IL-3R-positive cell lines (TF1 and the TF1 derivatives TF1/Bcl2, TF1 MEK_LNL6, and TF1 MEK_Bcl2) at an inhibitory concentration (IC₅₀) of 1–28 pM.
(0.06–1.7 ng/ml), whereas no kill of IL-3R-negative cell lines was seen. Similar results were obtained from primary AML samples, where expression of high affinity IL-3R was necessary to obtain AML-CFC kill [130]. In addition, the cytotoxicity of DT\textsubscript{388}IL3 was blocked in a dose-dependent competitive fashion by prior incubation of IL-3R-positive cells with native human IL-3 or anti-IL3R antibodies [136]. Imatinib (Novartis, Basel, Switzerland) was dissolved in PBS (pH 5.0 with acetic acid) to a final concentration of 10 mM before being filter sterilized and stored at −70°C. Dasatinib (Bristol Myers Squibb, New York, NY) was dissolved in DMSO to a concentration of 10mg/ml and stored at −20°C. The specified dilutions of imatinib and dasatinib in IMDM medium were freshly prepared prior to each experiment. The ranges of drug concentrations tested were 0-20 μM of imatinib, 0-600 nM of dasatinib, and 0-500 ng/ml DT\textsubscript{388}IL3, respectively.

### 2.2.3. Cultures of leukemic and normal PB cells

AL and normal mononuclear cells were incubated for 24h at 1 × 10^6 cells/ml in IMDM with 10% FCS with or without imatinib, dasatinib, and DT\textsubscript{388}IL3. Assays for leukemic CFCs were performed by plating cells at 0.2 to 1.0 × 10^5 cells/ml in growth factor-supplemented methylcellulose medium and scored for the presence of colonies after 14 days as described previously [249]. Normal PB CFCs were detected by plating cells in methylcellulose medium (Methocult H4330; StemCell) supplemented with 3 units/ml human erythropoietin (StemCell), 20 ng/ml each of interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF) and
Flt-3 ligand (FL). Granulopoietic, erythroid, and mixed colonies detected after 16 days of incubation at 37°C were scored as described [130]. For suspension culture-initiating cell (SC-IC) assays, 4 Ph⁺ AL samples were cultured in serum-free medium (SFM) containing 10⁻⁴M β-mercaptoethanol, 2 mM glutamine, 20% BIT (10% bovine serum albumin, 50 μg/ml insulin, and 1000 μg/ml transferrin; StemCell) in IMDM with imatinib, dasatinib, or DT₃₈₈IL3 in single and combination treatments for 24h. Cultures were maintained in IMDM supplemented with 10 ng/ml each of human IL-3, IL-7, Flt-3, IL-6, GM-CSF, G-CSF, and 50 ng/ml SCF for 5 weeks with weekly half-media changes [250]. After 5 weeks, cells were assessed for their CFC content as above [130]. Data were expressed as CFC generated per 10⁵ cells initially plated in suspension culture and the different drug treatments compared to untreated controls.

2.2.4. FACS analysis for detecting IL-3R expression in Ph⁺ acute leukemic cells

Analysis of IL-3R subunit expression was performed by labeling cells with biotinylated antibodies recognizing CD123 (IL-3Rα) and CD131 (IL-3Rβc) followed by incubation with streptavidin-PE or -allophycocyanin (APC) (Becton Dickinson, Franklin Lakes, NJ). Cells were analyzed on the basis of fluorescence intensity on a dual laser FACSaria instrument (Becton Dickinson). Gates were set to exclude nonviable, propidium iodide positive (PI⁺) cells, and 100% of cells labeled with an irrelevant isotype control antibody. For analysis of IL-3R subunit expression the mean fluorescence intensity (MFI) and percentage of total AML blasts labeled with anti-CD123 and anti-CD131 were calculated using Flowjo software (Tree Star Inc., Ashland, OR).
2.2.5. Quantitative RT-PCR analysis

Total RNA was extracted from Ph^+ cells using the Absolutely RNA, Microprep, or Nanoprep kits (Stratagene, La Jolla, CA). The RT reaction was performed in 20 µL with superscript III reverse transcriptase (Invitrogen, Burlington, Canada) using random hexamer oligonucleotides (Amersham Pharmacia, Piscataway, NJ). Real-time PCR was performed using 12.5 µL SYBR Green PCR Master Mix ( Applied Biosystems, Foster City, CA), 1 µL of 20 pM-specific primers, 1 to 2 µL cDNA, and water to a final volume of 25 µL. Specific forward and reverse primers to produce approximately 100-bp amplicons for optimal amplification in real-time PCR of reverse-transcribed cDNA for human IL-3Rα were 5’-GACCTGTACTTGAAACGGTCC and 5’-GAAACGACACCACCCGATACGTGT, for human IL-3Rβ were 5’-GCAGCATGTCGCGCTTCACACTA and 5’-GTCCCGGAATCCTACAGGGAA, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were (5’-CCCATCACCATCTTCCAGGAG and 5’-CTTCTCCATGGTGTTGAAAGACG. Real-time PCR and data analysis were performed on an iCycler iQ system, using iCycler iQ Real-time Detection Software (Bio-Rad, Hercules, CA). The relative quantification data of human IL-3Rα and IL-3Rβ compared with a reference gene (GAPDH) was generated on the basis of a mathematical model for relative quantification in real-time RT-PCR as described [132, 251]. The level of expression of the IL-3R subunits was expressed relative to the expression of GAPDH set at 1000.
2.2.6. Analysis of individual AL colonies for BCR-ABL expression

Individual colonies from methycellulose assays were visualized under an inverted light microscope and plucked using a sterile micropipette tip into 20 µL phosphate-buffered saline. Total RNA was isolated using PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer’s instructions. cDNA synthesis was performed 10 µL with superscript III reverse transcriptase (Invitrogen) using random hexamer oligonucleotides. PCR was performed as a 5-min denaturation at 95°C, 40 cycles at 94°C for 30 sec, 57°C for 1 min,72°C for 1 min, and a final elongation at 72°C for 7 min using the following oligonucleotide primers [252].

For p210 bcr-abl sequences
CML A 5'-TGG AGC TGC AGA TGC TGA CCA ACT CG-3'
CML B 5'-ATC TCC ACT GGC CAC AAA ATC ATA CA-3'

For p190 bcr-abl sequences
ALL A 5'-AGA TCT GGC CCA ACG ATG GCG AGG GC-3'
ALL B 5'-ATC TCC ACT GGC CAC AAA ATC ATA CA-3'

The PCR products were separated on 2% agarose gel.

2.2.7. Hoechst 33342 (Hst) and pyronin Y (PY) staining for cell cycle analysis

Previously described methods were used with minor modifications [74]. Cryopreserved cells were thawed and incubated overnight in SFM to reactivate RNA synthesis and then incubated at 37°C for 45 minutes in Hanks buffered solution with 2% fetal calf serum and 0.02% sodium azide (HFN) and Hst at 3 µg/mL. PY was then added
to a final concentration of 1 µg/mL and incubation continued for another 45 minutes.
Cells were washed in HFN with the same concentration of Hst and PY, and 2 µg/mL PI.
Finally, cells were resuspended in HFN with Hst and PY and sorted by BD
FACSVantage™ SE Cell Sorter (Becton Dickinson Immunocytometry Systems [BDIS],
San Jose, CA). Sorted quiescent and cycling cells from each Ph+ AL sample were
incubated for 24h in SFM containing 10^{-4}M β-mercaptopethanol, 2 mM glutamine with or
without one of the 3 drugs alone or with one of the TKIs in combination with DT_{388}IL3
and then plated in CFC assay as performed previously for unsorted blast cells.

2.2.8. Statistical analysis

Combination index (CI) values were calculated using CalcuSyn software
(CalcuSyn, Biosoft, Great Shelford, UK). Calculation of CI is a method to numerically
quantify drug interaction based on the multiple drug-effect equation of Chou-Talalay
derived from enzyme kinetic models [253]. A CI < 1 shows synergism; a CI = 1 indicates
additivity; a CI > 1 displays antagonism. The mean differences of drug effect and
correlation coefficients were determined in Excel (Microsoft Corp., Seattle, WA). A level
of P < 0.05 using Student’s t test was considered statistically significant.

2.3. Results

2.3.1. Expression of IL-3 receptor subunits on Ph+ leukemic blasts

Previous data have shown that the sensitivity of leukemic blasts and progenitors
to DT_{388}IL3 correlates with the level of expression of the IL-3R subunits as measured by
QRT-PCR or FACS analysis [132, 133]. To determine if the receptor subunits could be detected on Ph⁺ AL cells, blasts from each of the 10 AL samples listed on Table 2.1 were analyzed by both techniques. As shown on Table 2.2, QRT-PCR detected both subunits in each of the samples but the values (expressed relative to GAPDH expression set at 1000) varied by more than 100-fold among samples. There was a significant (p<0.02) correlation (r=0.75) between the level of the expression of the two subunits on these samples. On average the α subunit was expressed at higher levels than the β subunit but this difference did not reach statistical significance.

FACS analysis for expression of CD123 (IL-3R α subunit) and CD131 (β subunit) was expressed for each sample as both the geometric mean fluorescence intensity (MFI) and the % of positive cells (Table 2.2). Both these values were higher for the IL-3Rα subunit than for the β subunit and these differences were highly significant (p<0.001, paired T test). There were weak correlations between the levels of IL-3Rα and IL-3Rβ expression detected by QRT-PCR and mean fluorescence intensity (MFI) (r=0.48 and 0.52 respectively) and percentage of positive cells (r=0.43 and 0.51 respectively) detected by FACS for CD123 and CD131 but these did not reach statistical significance.

The three measurements of IL-3R subunit expression on AL blasts (QRT-PCR, MFI, and % FACS positive cells) were assessed for their ability to predict the sensitivity of malignant CFC from that sample to DT₃₈₈IL3. As shown on Table 2.2B quantification of IL-3Rα subunit expression by FACS using either MFI or % positive cells was significantly correlated (r= -0.75, p= 0.013 and r= -0.78, p= 0.008, respectively) with the IC₅₀ of DT₃₈₈IL3 against leukemic CFC from that sample while QRT-PCR was not.
None of the 3 measurements of IL-3R β subunit expression predicted CFC kill with DT$_{388}$IL3.

2.3.2. DT$_{388}$IL3 synergizes with tyrosine kinase inhibitors in killing CFC from Ph$^+$ AL patients

In initial experiments the concentration of DT$_{388}$IL3, imatinib and dasatinib as individual drugs in 24h suspension culture necessary to produce 50% kill (IC$_{50}$) of leukemic and normal CFC was determined for each of 10 AL samples and 3 G-CSF-mobilized normal PB samples (Figure 2.1, Table 2.3). Figure 2.1 shows representative dose response curves testing the 3 drugs individually and combinations of DT$_{388}$IL3 with one of the TKIs against one normal and one Ph$^+$ AL sample. As illustrated by this example and expected from previous investigations [130, 254], normal CFCs were relatively insensitive to all 3 drugs and the combinations as compared to leukemic CFC. The IC$_{50}$s for imatinib, dasatinib and DT$_{388}$IL3 ranged from 0.21 to 1.16 µM, 4.9 to 22 nM, and 35 to 256 ng/ml, respectively, for the 10 Ph$^+$ AL samples and were >10 to >100-fold higher for the TKIs and as much as 15-fold higher for DT$_{388}$IL3 when tested on normal CFC (Table 2.3B). There was no difference in drug sensitivity between erythroid and nonerythroid CFC detected in cultures of normal blood.

RT-PCR for the BCR-ABL abnormality verified the leukemic origin of individual colonies from the CFC assays of Ph$^+$ AL samples (Figure 2.2). For samples 116-67 and 105-98 15 of 15 (100%) and 14 of 15 (93%) colonies, respectively, with
successful amplification of cDNA as indicated by GAPDH positivity were also BCR-ABL positive.

Combining imatinib or dasatinib with DT_{388IL3} at a fixed ratio based on the IC_{50} of each drug lowered the drug concentrations of each needed to kill Ph^{+} AL CFC (Table 2.3, Figure 2.1). Calculation of a combination index (CI) to quantify interaction between the two drugs demonstrated a synergistic interaction (CI < 1) for each sample for the combinations of both imatinib and dasatinib with DT_{388IL3} at both the IC_{50} and IC_{90} (Calcusyn, Biosoft) (Table 2.3A). In contrast, no synergy could be detected against normal blood CFC (Table 2.3B).

2.3.3. Both quiescent and cycling Ph^{+} AL CFC are killed effectively by synergistic drug combinations

Hoechst (Hst) and pyronin (Py) staining with FACS was used to isolate quiescent (G_{0}) and cycling (G_{1} + S/G_{2}/M) cells from 3 Ph^{+} AL patient samples so that their drug sensitivity could be compared (Figure 2.3). Cells in G_{0} have diploid DNA and low RNA content and therefore low staining with both Hst and PY [74]. The 3 samples showed similar proportions of G_{0} cells (3.0%, 2.8%, and 3.4%).

Aliquots of quiescent and cycling cells from each Ph^{+} AL sample were treated for 24h with one of the 3 drugs alone or one of the TKIs in combination with DT_{388IL3} and then plated in CFC assay as performed previously for unsorted blast cells. As shown in Table 2.4, imatinib, dasatinib, and DT_{388IL3} killed both cycling and quiescent cells in a dose-dependent manner. Although, G_{0} cells were relatively resistant to all 3 drugs, as
shown by the higher IC$_{50}$s for these cells as compared to cells which were actively cycling (Table 2.4), this effect was most prominent for dasatinib (mean 166% increase IC$_{50}$ in G$_0$ cells compared to cycling cells), less for imatinib (mean 80% increase) and least for DT$_{388}$IL3 (mean 40% increase in IC$_{50}$ in G$_0$ vs cycling cells).

The combination of a TKI with DT$_{388}$IL3 reduced the concentration of both drugs required to eliminate leukemic CFC in both cycling and quiescent subpopulations (Table 2.4). This interaction was synergistic for both cycling and quiescent cells as shown by the CI (all < 1) calculated for both the IC$_{50}$ and IC$_{90}$ of combinations using either imatinib or dasatinib (Table 2.4).

2.3.4. TKIs and DT$_{388}$IL3 show synergy in eliminating Ph$^+$ AL progenitors that initiate long-term suspension cultures

To test cooperative drug effects on malignant progenitors with a greater proliferative capacity than CFC, 4 Ph$^+$ AL samples were placed in long-term (5 week) suspension culture after they had been treated for 24h with or without the 3 drugs singly or in combination as described for the CFC assays above. Comparison of the leukemic CFC content of the 5-week-old suspension cultures revealed that all 3 drugs were effective in eliminating malignant progenitors (Table 2.5). However, the IC$_{50}$s and IC$_{90}$s of imatinib, dasatinib, and DT$_{388}$IL3 were somewhat higher for long-term suspension culture-derived CFC than they were for CFC assayed directly from the same sample (Tables 2.3 and 2.5). As previously observed for leukemic CFC, the combination of a TKI with DT$_{388}$IL3 was synergistic at both IC$_{50}$s and IC$_{90}$s against suspension culture-
initiating cells for all 4 samples tested regardless of whether dasatinib or imatinib was used in the combination (Table 2.5).

2.4. Discussion

Targeting the BCR-ABL rearrangement has been remarkably successful in improving the treatment and prognosis for patients with chronic myeloid leukemia (CML) with chronic phase disease. There are now many patients who have been in continuous complete cytogenetic remission for more than 5 years while receiving imatinib [236]. More recent data suggest that dasatinib and other second generation tyrosine kinase inhibitors (TKI) are equally efficacious and will successfully treat most of the minority of patients whose disease fails to respond optimally to imatinib [100]. However, even in chronic phase CML, in most cases TKI therapy is not curative at least partly due to the relative resistance to TKIs of a reservoir of quiescent CML progenitors [255, 256]. Although TKIs have also proven to be active in the treatment of advanced phase CML and Ph+ acute leukemia, remissions are obtained less frequently and those that are achieved are typically not durable [100, 237, 238]. Mechanisms that have been implicated in disease relapse and resistance include mutations in the ATP-binding site of BCR-ABL, amplification of the BCR-ABL gene, rapid drug efflux, and BCR-ABL independence from secondary transforming events [239, 257, 258]. These diseases are also difficult to eradicate with conventional chemotherapy [259]. More recently, the addition of imatinib to standard chemotherapy regimens appears to be improving on the results obtained with either therapy used alone [259]. However, despite this improvement
in initial response rates most patients with Ph+ AL eventually relapse and die with drug resistant disease unless they are candidates to receive intensive consolidation with allogeneic stem cell transplantation [260].

DT₃₈₈IL₃ was developed as an agent to target the IL-3R on human leukemia cells. In particular, it is well-documented that this receptor is expressed at high levels on the majority of acute myeloid leukemia (AML) blasts and progenitors. The α subunit is usually expressed at much higher levels than those seen on normal cells including hematopoietic progenitors [133]. In addition, as detected using QRT-PCR, the expression of the α subunit is at least as high on subpopulations of AML blasts enriched for candidate leukemic stem cells as it is on the majority populations of AML blasts [133]. As in CML, many of these AML progenitors are quiescent [74]. In preclinical studies DT₃₈₈IL₃ was shown to kill AML progenitors including those detected in CFC assays, long-term suspension cultures and immunodeficient mice [130, 136]. The effectiveness of this fusion protein showed a direct correlation with the amount of IL-3R expression on AML blasts [133, 134, 261]. DT₃₈₈IL₃ is now undergoing Phase 1b/2 evaluation as a potential therapeutic agent for AML [142]. More recently a CD123-specific monoclonal antibody has been shown to selectively target and kill AML progenitors including leukemia-initiating cells detected in mice providing further validation of the IL-3R as a therapeutic target in this disease [262].

The IL-3R is also expressed on malignant cells from a proportion of ALL patients [121, 125]. As shown on Table 2.1, 9 of the 10 patient samples studied in this report had a pre-B lymphoid cell immunophenotype including 2 of the 3 CML blast crisis
samples. These Ph⁺ AL samples expressed both subunits of the IL-3R at various levels as detected by either QRT-PCR or FACS suggesting that DT₃₈₈IL₃ might be effective in killing these cells. Subsequent experiments confirmed that this drug could kill malignant progenitors from these samples to a degree that was predicted by the amount of expression of the IL-3R α subunit as detected by FACS. Similar data have been reported for the DT₃₈₈IL₃ sensitivity of AML samples [133, 134]. As compared to the same analysis previously performed on AML blasts [132] the level of expression of the IL-3Rβ subunit was similar (median expression level assessed by QRT-PCR on Ph⁺ AL 2.9 versus 4.7 on AML cells). However, the median IL-3Rα subunit expression was only 6.1 compared to the median of 39.9 previously detected on AML blasts [132]. Consistent with these results the IC₅₀ of DT₃₈₈IL₃ against CFC from Ph⁺ AL samples (mean ± SD, 150±81 ng/ml) was generally higher than what we have reported for AML samples where doses less than 50 ng/ml were often effective [132]. Although the IC₅₀ for DT₃₈₈IL₃ against normal CFC was even higher than that for Ph⁺ AL CFC (608±77 ng/ml), these data suggest that DT₃₈₈IL₃ may not be effective as a single agent in most patients with Ph⁺ AL even when the IL-3R is present on target cells.

The mechanism of action of DT₃₈₈IL₃ is distinct from that of TKI as well as conventional chemotherapy drugs. After binding to the cell surface and internalization through receptor-mediated endocytosis, the fusion protein translocates to the cytosol where it blocks protein synthesis through inhibition of elongation factor 2 leading to apoptotic cell death [263, 264]. Diphtheria toxin fusion proteins have been shown to synergize with cytarabine in killing AML progenitors [246, 265]. This observation and
the unique mechanism of action of DT$_{388}$IL3 suggested that combining it with a TKI might also be synergistic.

As shown on Table 2.3 and Figure 2.1, imatinib, dasatinib and DT$_{388}$IL3 were able to kill malignant CFC from patients with Ph$^+$ AL. As expected the IC$_{50}$ for both imatinib and dasatinib was greater than 10-fold lower for the Ph$^+$ AL samples than for normal G-CSF mobilized blood CFC (mean IC$_{50}$±SD Ph$^+$ AL vs normal PB 0.63±0.30 μM vs 18.0±2.2 μM for imatinib and 13.2 ± 5.9 nM vs 493±200 nM for dasatinib).

The IC$_{50}$ of DT$_{388}$IL3 against Ph$^+$ AL CFC was 3 to >10-fold lower (mean IC$_{50}$ ±SD 45±28 ng/ml and 34±25 ng/ml) when used in combination with imatinib and dasatinib, respectively, than when used as a single agent. Similarly, the IC$_{50}$ of imatinib and dasatinib against Ph$^+$ AL CFC was also much reduced in these combinations (mean IC$_{50}$ ±SD 0.19 ± 0.12 μM and 3.9 ± 2.1 nM, respectively) and a striking synergy between the TKIs and DT$_{388}$IL3 was demonstrated (Table 2.3A). On the other hand, there was little change in the IC$_{50}$ of any of the 3 drugs when used the combinations were tested against normal PB CFC (Table 2.3B) and no synergy was demonstrable. Thus, a combination of 2 agents which target different abnormalities in Ph$^+$ AL cells and have different mechanisms of action has greater selectivity for malignant rather than normal progenitors than any of the 3 agents used singly.

In subsequent experiments the effectiveness of this combination therapy was tested against Ph$^+$ AL cells that are likely to contain reservoirs of chemotherapy- and TKI-resistant cells i.e. quiescent leukemic cells and cells which have long-term proliferative capacity in culture.
Quiescent malignant cells are relatively resistant to both conventional chemotherapy drugs and TKIs [255, 256]. As shown on Table 2.4 the IC\textsubscript{50} for all 3 drugs was higher for quiescent that for cycling Ph\textsuperscript{+} AL CFC from all 3 samples tested. Even DT\textsubscript{388}IL3 which acts through inhibition of protein synthesis required higher concentrations to kill cells that were not actively cycling. This is consistent with the probability that protein synthesis is relatively inactive in quiescent cells, including leukemic stem cells [266, 267]. However, when tested in combination with DT\textsubscript{388}IL3 the IC\textsubscript{50} and IC\textsubscript{90} for both TKIs were markedly reduced for both cycling and quiescent cells as were the IC\textsubscript{50} and IC\textsubscript{90} for DT\textsubscript{388}IL3 and synergy was demonstrated. For each of the 3 patient samples the IC\textsubscript{50}s and IC\textsubscript{90}s for all 3 drugs tested in the combinations against quiescent cells were less than the IC\textsubscript{50}s and IC\textsubscript{90}s for the individual drugs tested against cycling cells suggesting that the use of the combination overcame at least some of the drug resistance of the quiescent population.

Previous data have shown that ALL or AML blasts which initiate long-term suspension culture or engraft in immunodeficient mice are enriched in small subpopulations of cells which can be isolated by their cell surface phenotype [250, 268]. These suspension culture-initiating cells (SC-IC) and NOD/SCID mouse leukemia-initiating cells are capable of self-renewal and of producing a large number of more differentiated blast cells and progenitors [7, 268]. Thus, they are candidate ‘leukemic stem cells’ that may be capable of maintaining malignant clone in leukemia patients and are important targets for therapeutic intervention. Many of these leukemic stem cells are quiescent [74, 247]. To determine if the synergistic interaction between the TKIs and
DT$_{388}$IL3 that we had seen with Ph$^+$ AL CFC could also be demonstrated against more primitive SC-IC 4 patient samples were studied in long-term suspension culture. As shown on Table 2.5, although the IC$_{50}$ for both the individual drugs and the combinations tended to be higher against SC-IC than it had been against CFC from the same patient sample, a synergistic interaction could still be demonstrated, particularly for the IC$_{90}$. Thus, the combination of a TKI and DT$_{388}$IL3 shows synergy in killing Ph$^+$ AL progenitors including those which are not in active cell cycle and which have long-term proliferative potential.

In previous studies DT$_{388}$IL3 has been shown to synergize with cytarabine in killing AML progenitors including those that engraft in mice [246]. Thus, the current data are consistent with these prior results in providing evidence that the novel mechanism of action of this fusion protein may make it useful in combination therapies. The possibility that DT$_{388}$IL3 may enhance the effectiveness of TKIs in Ph$^+$ AL where short response durations and survival are expected unless the patient is a candidate for allogeneic stem cell transplantation is particularly appealing. Early evidence of anti-leukemic activity and acceptable toxicity has been seen in the current Phase 1b/2 trial of DT$_{388}$IL3 in patients with relapsed and refractory AML [142]. If these results are confirmed it is feasible that evaluation of the combination of a TKI and this fusion protein might be tested clinically.
Figure 2.1 Effect of imatinib, dasatinib, and DT\textsubscript{388}IL3 as single treatments or in combination on Ph\textsuperscript{+} AL and normal CFC. Normal cells (A, C) and Ph\textsuperscript{+} AL cells (B, D) were incubated with various concentrations of tyrosine kinase inhibitor or DT\textsubscript{388}IL3 or with combination of both drugs. Imatinib (♦) or DT\textsubscript{388}IL3 (■) or with combination of both drugs (▲) (A, B), and dasatinib (●) or DT\textsubscript{388}IL3 (■) or with combination of both drugs (Δ) (C, D). Y axis indicates the percent kill of CFC obtained as compared with untreated control cultures. Error bars represent standard deviation. Asterisk in combination treatment indicates P < 0.05 when it was compared with each single treatment.
Figure 2.2 Detection of BCR-ABL mRNA expression in AL samples. mRNA from colonies of representative samples were extracted and amplified. p210 transcripts (234 bp) were detected in colony of patient # 105-98 and p190 transcripts (196 bp) were detected in patient # 116-67. (-) means BCR-ABL negative AML sample. GAPDH (105 bp) was used for internal control.
Figure 2.3 Isolation of quiescent and cycling Ph⁺ AL cells by Hoechst 33342 (Hst) /Pyronin Y (PY) -staining and FACS. A representative Hst/PY dot profile of Ph⁺ AL cells is shown. Numbers in the plots indicate the percentage of cells in each gate. Quiescent (G₀) cells have diploid DNA and low RNA content and, therefore, they show the lowest Hst and PY staining. Although G₁ cells have no change in DNA content, their RNA synthesis and PY staining augment significantly. S/G₂/M cells synthesize both DNA and RNA, so stain with both Hst and PY brightly.
Table 2.1 Patient characteristics

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age/Sex</th>
<th>Type</th>
<th>Cytogenetics</th>
<th>Blast Immunophenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>118-94</td>
<td>71/M</td>
<td>ALL</td>
<td>46, XY, t(9;22) (q34;q11)</td>
<td>CD10, CD19, CD34, TdT, HLA-DR, CD2 (partial and weak)</td>
</tr>
<tr>
<td>116-67</td>
<td>57/F</td>
<td>ALL</td>
<td>45, XX, del(9) t(9;22) (q34;q11), t(4;5) (q31;q4), del(9), del(19)(q13) der(20) t(9;22) del(22) t(9;22)</td>
<td>CD10, CD19, CD20, CD34, TdT, HLA-DR</td>
</tr>
<tr>
<td>105-98</td>
<td>31/M</td>
<td>ALL</td>
<td>46, XY, t(9;22) (q34;q11), del(9) (p22)</td>
<td>CD10, CD19, CD20, CD34, TdT, HLA-DR, CD13, CD33</td>
</tr>
<tr>
<td>41-04</td>
<td>57/F</td>
<td>ALL</td>
<td>52, XX, +X, +8, +8, t(9;22) (q34;q11)</td>
<td>CD10, CD19, TdT</td>
</tr>
<tr>
<td>130-01</td>
<td>68/M</td>
<td>ALL</td>
<td>46, XY, t(9;22) (q34;q11), t(2;14) (q22;q34)</td>
<td>CD10, CD19, CD20 (dim), CD34, TdT, HLA-DR, cytoplasmic CD79A and CD22, CD13 (dim)</td>
</tr>
<tr>
<td>105-78</td>
<td>49/M</td>
<td>ALL</td>
<td>46, XY, t(9;22) (q34;q11)</td>
<td>CD10, CD19, CD20, CD34, TdT, HLA-DR, CD13 (partial)</td>
</tr>
<tr>
<td>102-56</td>
<td>31/M</td>
<td>ALL</td>
<td>45, XY, der(9) t(9;22) (q34;q11) t(7;9) (p10;q10), -7, del(22) t(9;22) (q34;q11)</td>
<td>CD10, CD19, CD20, CD34, HLA-DR, TdT (weak)</td>
</tr>
<tr>
<td>150-80</td>
<td>55/M</td>
<td>CML-BP lymphoid</td>
<td>46, XY, t(9;22) (q34;q11)</td>
<td>CD10, CD19, CD20 (partial), CD34, CD79a (dim), TdT (dim), HLA-DR</td>
</tr>
<tr>
<td>131-48</td>
<td>43/F</td>
<td>CML-BP lymphoid</td>
<td>46, XX, t(9;22) (q34;q11), t(2;14) (p20;q32.3)</td>
<td>CD10, CD19, CD20, CD22, CD34, CD79a, TdT, HLA-DR, CD13 (dim), CD33 (dim)</td>
</tr>
<tr>
<td>113-36</td>
<td>55/F</td>
<td>AML</td>
<td>49, XX, t(9;22) (q34;q11), +10, +19</td>
<td>CD13, CD33, CD34, CD64 (partial), MPO (partial), TdT, CD2, CD7</td>
</tr>
</tbody>
</table>

ALL, pre-B cell acute lymphoblastic leukemia; CML-BP lymphoid, chronic myeloid leukemia in lymphoid blast phase; AML, acute myeloid leukemia; TdT, terminal deoxynucleotidyl transferase; MPO, myeloperoxidase
Table 2.2 IL-3R subunit quantification on AL blasts by FACS (positive cells and MFI) and QRT-PCR (A), and correlation between FACS and QRT-PCR with the IC$_{50}$ of DT$_{388}$IL3 against leukemic CFC (B)

(A)

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Positive cells (%)</th>
<th>Mean fluorescence intensity (MFI)</th>
<th>mRNA level*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-3R$\alpha$</td>
<td>IL-3R$\beta$</td>
<td>IL-3R$\alpha$</td>
</tr>
<tr>
<td>118-94</td>
<td>85.3</td>
<td>1.8</td>
<td>40.1</td>
</tr>
<tr>
<td>116-67</td>
<td>32.1</td>
<td>0.9</td>
<td>25.1</td>
</tr>
<tr>
<td>105-98</td>
<td>73.5</td>
<td>1.3</td>
<td>38.4</td>
</tr>
<tr>
<td>41-04</td>
<td>91.2</td>
<td>1.4</td>
<td>45.2</td>
</tr>
<tr>
<td>130-01</td>
<td>66.5</td>
<td>1.1</td>
<td>33.5</td>
</tr>
<tr>
<td>105-78</td>
<td>80.2</td>
<td>0.8</td>
<td>39.9</td>
</tr>
<tr>
<td>102-56</td>
<td>78.9</td>
<td>1.3</td>
<td>39.1</td>
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<td>150-80</td>
<td>88.0</td>
<td>1.5</td>
<td>44.3</td>
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<td>131-48</td>
<td>42.1</td>
<td>2.4</td>
<td>35.1</td>
</tr>
<tr>
<td>113-36</td>
<td>75.4</td>
<td>1.2</td>
<td>40.4</td>
</tr>
</tbody>
</table>

* expressed relative to expression of GAPDH set at 1000

(B)

|          | IL-3R | r=  | p=*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>QRT-PCR</td>
<td>$\alpha$</td>
<td>-0.08</td>
<td>NS</td>
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<tr>
<td></td>
<td>$\beta$</td>
<td>0.26</td>
<td>NS</td>
</tr>
<tr>
<td>MFI</td>
<td>$\alpha$</td>
<td>-0.75</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>0.27</td>
<td>NS</td>
</tr>
<tr>
<td>% +ve</td>
<td>$\alpha$</td>
<td>-0.78</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>0.30</td>
<td>NS</td>
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</tbody>
</table>

*Student T test; NS, not significant
Table 2.3 Comparisons between single and combination treatments and combination indices (CI) for Ph+ AL (A) and normal (B) G-CSF mobilized blood CFC

(A)

<table>
<thead>
<tr>
<th>Patient #</th>
<th>IM(μM)</th>
<th>DA(μM)</th>
<th>DT(nmol/ml)</th>
<th>IM+DT</th>
<th>DA+DT</th>
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</thead>
<tbody>
<tr>
<td>118:04</td>
<td>0.69</td>
<td>2.51</td>
<td>20.1</td>
<td>73.2</td>
<td>169</td>
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<tr>
<td>113:36</td>
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<td>16.0</td>
<td>75.3</td>
<td>79.9</td>
</tr>
<tr>
<td>150:80</td>
<td>0.26</td>
<td>1.02</td>
<td>8.3</td>
<td>33.2</td>
<td>135</td>
</tr>
<tr>
<td>116:67</td>
<td>0.56</td>
<td>2.80</td>
<td>10.4</td>
<td>92.3</td>
<td>256</td>
</tr>
<tr>
<td>105:08</td>
<td>0.48</td>
<td>2.14</td>
<td>4.9</td>
<td>36.8</td>
<td>33.3</td>
</tr>
<tr>
<td>41:04</td>
<td>0.21</td>
<td>1.52</td>
<td>6.4</td>
<td>15.8</td>
<td>58.9</td>
</tr>
<tr>
<td>131:48</td>
<td>0.73</td>
<td>2.69</td>
<td>12.7</td>
<td>56.4</td>
<td>367</td>
</tr>
<tr>
<td>102:56</td>
<td>0.88</td>
<td>3.23</td>
<td>22.1</td>
<td>58.2</td>
<td>134</td>
</tr>
<tr>
<td>130:01</td>
<td>0.92</td>
<td>3.65</td>
<td>19.2</td>
<td>45.2</td>
<td>228</td>
</tr>
<tr>
<td>105:78</td>
<td>1.16</td>
<td>4.23</td>
<td>12.1</td>
<td>38.3</td>
<td>115</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Normal Samples</th>
<th>IM(IC50)</th>
<th>DA(IC50)</th>
<th>DT(IC50)</th>
<th>IM+DT(IC50)</th>
<th>DA+DT(IC50)</th>
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</thead>
<tbody>
<tr>
<td>120-48</td>
<td>17.5</td>
<td>524</td>
<td>675</td>
<td>15.2+482</td>
<td>501+556</td>
</tr>
<tr>
<td>120-93</td>
<td>20.5</td>
<td>675</td>
<td>624</td>
<td>20.1+559</td>
<td>642+603</td>
</tr>
<tr>
<td>121-43</td>
<td>16.1</td>
<td>278</td>
<td>523</td>
<td>13.5+485</td>
<td>226+467</td>
</tr>
</tbody>
</table>

Note: Ph+ AL cells (A) and G-CSF-mobilized normal blood cells (B) were incubated for 24h with imatinib (IM), dasatinib (DA), and DT388IL3 (DT) at various concentration to determine the IC50 and IC90 for each drug and sample. Based on the IC50 of single drugs, the concentrations for co-treatment was determined. Cells were then plated in CFC assays and scored 14 days later as described in materials and methods. A CI less than 1 indicates synergism; 1 additivity; and more than 1 antagonism.
Table 2.4 Comparisons between single drugs and combination treatments and combination indices (CI) for quiescent and cycling Ph⁺ AL cells

<table>
<thead>
<tr>
<th>Pt #</th>
<th>Cell Cycle</th>
<th>IM(μM)</th>
<th>DA(μM)</th>
<th>DT(h210)</th>
<th>IM+DT</th>
<th>DA+DT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC₅₀</td>
<td>IC₅₀</td>
<td>IC₅₀</td>
<td>IC₅₀</td>
<td>IC₅₀</td>
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<tr>
<td>105-78</td>
<td>cycling</td>
<td>1.05</td>
<td>3.89</td>
<td>10.1</td>
<td>35.1</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>quiescent</td>
<td>1.81</td>
<td>4.91</td>
<td>29.1</td>
<td>36.4</td>
<td>125</td>
</tr>
<tr>
<td>170-67</td>
<td>cycling</td>
<td>0.47</td>
<td>2.69</td>
<td>11.2</td>
<td>82.6</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>quiescent</td>
<td>0.92</td>
<td>4.12</td>
<td>28.6</td>
<td>112</td>
<td>422</td>
</tr>
<tr>
<td>150-80</td>
<td>cycling</td>
<td>0.35</td>
<td>0.95</td>
<td>7.4</td>
<td>27.5</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>quiescent</td>
<td>0.51</td>
<td>1.36</td>
<td>19.0</td>
<td>38.3</td>
<td>232</td>
</tr>
</tbody>
</table>

Note: Ph⁺ AL cells were stained with Hst/PY and sorted into 2 subpopulations. Cycling cells are Hst and/or PY bright cells in G₁ and S/G₂+M while quiescent cells are Hst/PY dull cells in G₀. FACS isolated cells were treated with drugs for 24h as described in the methods and the legend for Table 2.3 and then plated in CFC assay.
Table 2.5 Imatinib (IM) and dasatinib (DA) synergize with DT<sub>388</sub>IL3 (DT) for killing of Ph<sup>+</sup> AL long-term suspension culture-initiating cells

<table>
<thead>
<tr>
<th>Patient #</th>
<th>IM (nM)</th>
<th>DA (nM)</th>
<th>DT (ng/ml)</th>
<th>IM-DT</th>
<th>DA-DT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;S&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;C&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;S&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;C&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;S&lt;/sub&gt;</td>
</tr>
<tr>
<td>133-36</td>
<td>0.79</td>
<td>2.15</td>
<td>20.6</td>
<td>85.6</td>
<td>136</td>
</tr>
<tr>
<td>116-67</td>
<td>1.21</td>
<td>3.54</td>
<td>22.1</td>
<td>112</td>
<td>278</td>
</tr>
<tr>
<td>150-80</td>
<td>0.82</td>
<td>3.89</td>
<td>20.5</td>
<td>79.5</td>
<td>205</td>
</tr>
<tr>
<td>195-78</td>
<td>1.78</td>
<td>5.12</td>
<td>28.5</td>
<td>81.2</td>
<td>121</td>
</tr>
</tbody>
</table>

Note: After 24 h incubation with single drugs or combinations, Ph<sup>+</sup> AL cells were cultured for 5 weeks in IMDM supplemented with 10% FCS and growth factors and then plated into methylcellulose assay to detect SC-IC.
Chapter 3. Liposomal encapsulation of a synergistic molar ratio of cytarabine and daunorubicin enhances selective toxicity for acute myeloid leukemia progenitors as compared to analogous normal hematopoietic cells

3.1. Introduction

Modern induction chemotherapy for acute myeloid leukemia (AML) has been used for over three decades. Approximately 50% to 75% of adults with AML achieve complete remission (CR) with the deoxycytidine analog cytarabine and an anthracycline antibiotic, such as daunorubicin or idarubicin which inhibit the enzyme topoisomerase IIa, however, only 20% to 30% of patients achieve long-term disease-free survival [269]. The majority of patients die of their disease, primarily due to persistent or relapsed disease [49]. Standard induction chemotherapy typically consists of cytarabine 100 mg/m² by continuous infusion for 7 days and daunorubicin 45 mg/m² intravenously for 3 days [270, 271]. To improve CR and survival rates, higher doses of cytarabine, higher doses of anthracyclines, and new agents combined with cytarabine and daunorubicin were tested. However, none of these trials has shown improved overall survival rates compared to standard therapy [272-275].

Previous studies have shown that anticancer drug combinations interact synergistically, additively or antagonistically against cancer cells depending on the ratios
of the individual agents [253, 276, 277]. Maintaining an optimal ratio of drug combinations should be coordinated to avoid the harmful effects of exposing cancer cells to antagonistic drug ratios [148, 149]. However, the different pharmacokinetics of the individual agents prevents adequate control of the drug ratio with conventional free-drug formulations [278]. We previously reported that the drug ratio can affect the efficacy of combinations of cytarabine and daunorubicin and found that a 5:1 (cytarabine:daunorubicin) molar ratio is the most synergistic [154, 279].

Liposomes are spherical structures of amphipathic lipid that surrounds an internal aqueous core. The composition of the lipid envelope can be manipulated for controlling the drug release rate. Liposomes have been used broadly as the delivery vehicles of anticancer agents and other drugs [144, 145]. CPX-351 (cytarabine/daunorubicin HCl liposome injection) is a liposome that contains cytarabine and daunorubicin HCl, which retains the synergistic 5:1 molar drug ratio in vivo. CPX-351 has more enhanced therapeutic activity than the free cytarabine plus daunorubicin cocktail with retention of the optimal 5:1 molar ratio in vivo and promising antileukemic activity in Phase I and Phase II trials with AML patients [153, 277, 278, 280, 281].

In this study we performed a direct comparison of the ability of CPX-351 and free cytarabine and daunorubicin to kill leukemic blasts and progenitors from patients with AML including subpopulations of malignant cells enriched for candidate leukemic stem cells (LSC). Furthermore, this comparison was extended to analogous populations of normal mobilized peripheral blood and bone marrow cells. The results demonstrate equivalent killing of AML colony forming cells and sorted subpopulations of blasts
enriched for LSC with CPX-351 and the free drug combination. In contrast, normal cells and progenitors were relatively resistant to CPX-351. These data help provide a possible explanation for the putative improved efficacy observed with CPX-351 as compared to conventional ‘7+3’ AML induction therapy.

3.2. Materials and methods

3.2.1. Liposome preparation and drug encapsulation

Liposomes were prepared by hydration of dried lipid films by conventional methods. Briefly, a lipid film of DSPC/DSPG/Chol (7:2:1 mol ratio) containing the lipid marker 3H-cholesteryl hexadecyl ether (CHE) was hydrated with 100mM copper gluconate, pH adjusted to 7.4 using triethanolamine. The liposomes were extruded through 100 nm polycarbonate filters using an extruder apparatus (Northern Lipids, Vancouver, BC, Canada) until the mean diameter of the formulations were 100±20 nm as determined by quasi-elastic light scattering. The encapsulation of cytarabine was carried out by mixing solutions of cytarabine and liposomes in the above buffers and incubating the resultant mixture beyond the phase transition temperature (55°C) until a drug:lipid ratio of 0.5 was achieved (overall encapsulation efficiency = 5%). To determine encapsulated cytarabine, 100μL aliquots of the loading mixture were removed at appropriate timepoints and applied to a Sephadex G-50 spin column to remove the unencapsulated cytarabine. The columns were prepared by adding glass wool to a 1mL syringe and Sephadex G-50 beads hydrated in sucrose phosphate buffer (SUP) at neutral pH. The columns were packed by spinning at 290 g for 1min. Following addition of the
sample to the column, the liposome fraction was collected in the void volume by centrifuging at 515 g for 1 min. The drug:lipid ratio at each time point was obtained by determining the concentration of cytarabine and lipids in the spin column eluents using high performance liquid chromatography (HPLC). To incorporate daunorubicin into the cytarabine-containing liposomes daunorubicin was first dissolved in sucrose phosphate ethylenediaminetetraacetic acid (EDTA) buffer (SPE) at neutral pH, warmed and then incubated with the cytarabine-loaded liposomes for 10 min to achieve the desired daunorubicin:cytarabine (1:5 mol/mol) ratio. The unencapsulated drug was removed by exchanging the external liposomal buffer into sucrose phosphate buffer at neutral pH using tangential flow chromatography. The concentration of daunorubicin in the liposomes was determined by HPLC [153].

3.2.2. Leukemia and normal samples

Peripheral blood (PB) or bone marrow (BM) blast cells were obtained at diagnosis (pre-treatment) from 7 AML patients who subsequently achieved complete remission (CR) after standard cytarabine and daunorubicin-containing induction therapy, and 6 AML patients whose leukemia was refractory to this induction chemotherapy (NR). Six G-CSF-mobilized PB and 1 BM samples were obtained from 7 normal individuals donating blood or marrow cells for clinical allogeneic stem cell transplantation. All patient and donor cells were obtained after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. Normal and leukemic mononuclear cells were isolated by Ficoll-Hypaque density gradient
centrifugation (Pharmacia, Uppsala, Sweden) and cryopreserved in Iscove’s modified Dulbecco’s medium (IMDM) with 50% fetal calf serum (FCS) (StemCell Technologies, Vancouver, Canada) and 10% dimethylsulfoxide. Thawed cells were washed twice in IMDM containing 10% FCS and used for the experiments described below.

3.2.3. Drug Treatments

AML and normal mononuclear cells were cultured for 24h at 1 × 10^6 cells/ml in IMDM containing 10% FCS with or without cytarabine:daunorubicin as free drugs or in liposomes at a fixed 5:1 molar ratio at four different concentrations which had previously been shown to be cytotoxic to primary human AML blasts. The ranges of drug concentrations tested were 13-1023 ng/ml of cytarabine and 5.6-450 ng/ml of daunorubicin. After drug exposure cells were washed and plated in colony assays or used for flow cytometry experiments as described below.

3.2.4. Colony forming cell (CFC) assays

Assays for leukemic CFCs (AML CFC) were performed by plating AML cells at 0.2 to 1.0 × 10^5 cells/ml in growth factor-supplemented methylcellulose-containing medium and scoring for the presence of colonies 14 days later as described previously [282]. Normal PB or BM CFCs were detected by plating cells in semi-solid medium (Methocult H4330; StemCell) supplemented with 3 units/ml human erythropoietin (StemCell), 20 ng/ml each of interleukin-3 (IL-3), IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF) and Flt-3 ligand (FL). Total
granulopoietic, erythroid, and mixed colonies detected after 14 days of incubation at 37°C were scored as described [249]. The percent kill of AML or normal CFC at different drug concentrations was calculated by comparison of colony numbers with untreated control cultures. All assays were performed in duplicate.

### 3.2.5. Flow cytometry

To determine the proportion of viable cells in drug-treated or control CD34⁺CD38⁻, CD34⁺CD38⁺ and CD34⁻ subpopulations of AML and normal samples, cells were labelled with APC/Cy7-conjugated anti-CD34 (BioLegend, San Diego, CA) and APC-conjugated anti-CD38 (BioLegend) for 30 min and then stained with annexin V-FITC and PI (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences Pharmingen, San Jose, CA), according to the manufacturer’s protocol. Mouse IgG antibodies conjugated with each fluorochrome were used as isotype controls. The proportion of viable, Annexin V-/PI-negative cells, in each subpopulation defined by CD34 and CD38 expression was determined by flow cytometry (FACScalibur; Becton-Dickinson) and quantified by Flowjo software (Tree Star Ing., Ashland, OR, USA). The percent kill of drug-treated cells was calculated using the formula (% viable untreated control – % viable drug-treated)/% viable untreated control × 100%. Representative FACS plots and gates used to isolate subsets of CD34 and CD38-labelled AML and normal cells are shown in Figure 3.1.
3.2.6. Daunorubicin accumulation in AML blasts and normal cells (HPLC)

For daunorubicin analysis, 1 part cell sample was mixed with 4 parts of acidified methanol (acetic acid:methanol = 1:5, v/v) by vortexing. The mixture was centrifuged at 3200g for 10 minutes and 20 μL of supernatant was injected onto the HPLC for quantification. Daunorubicin was evaluated using a Phenomenex Luna C18(2) reverse phase analytical column with a multi λ fluorescence detector set at excitation/emission wavelengths of 480/560 nm. The mobile phase was 1 mL/min 25 mM ammonium acetate:acetonitrile (67.5:32.5, v/v at pH 4.8). The HPLC method used was suitable for separating daunorubicin from the active metabolite daunorubicinol.

3.2.7. Statistical analysis

The significance of differences in mean drug effect between CPX-351 and free drug cocktail-treated cells was calculated using the Student t-test with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). A P value <0.05 was considered significant. The IC₅₀s of cytarabine and daunorubicin were calculated using CalcuSyn software (Biosoft, Great Shelford, UK).

3.3. Results

3.3.1. CPX-351 and free cytarabine:daunorubicin kill AML CFC with equivalent potency.

As shown on Figure 3.2 the mean AML CFC kills for the 7CR samples was lower than that obtained for the 6 NR samples after exposure to either CPX-351 or
equivalent concentrations of free cytarabine:daunorubicin (P<0.002 for all comparisons). However, the percentage AML CFC kill was not significantly different between CPX-351 and the free drugs regardless of whether the progenitors were obtained from CR or NR patients. Similarly, the calculated cytarabine:daunorubicin IC50s for CPX-351 and the free drugs, were very similar for the CR and NR samples (Table 3.1).

3.3.2. CPX-351 exhibits reduced cytotoxicity against normal CFC as compared with free daunorubicin:cytarabine.

To compare the ability of CPX-351 and free cytarabine:daunorubicin to kill normal CFC, 6 G-CSF mobilized normal peripheral blood and 1 bone marrow mononuclear cell samples were cultured under the same conditions that had been used for the AML samples and then plated in CFC assays. Figure 3.3 shows that normal CFCs were relatively resistant to killing with CPX-351 as compared to the free drugs with statistically significant differences at all except the lowest drug concentration. The IC50 for cytarabine:daunorubicin was 5.2-fold higher for CPX-351 than that for free drug cocktail (P=0.0004) (Table 3.1). When comparing the IC50 obtained for normal and AML CFC treated with free cytarabine:daunorubicin the IC50 of normal CFC was 2-fold higher and slightly lower as compared to AML CFC from CR and NR samples, respectively (P=0.003 and 0.03, respectively). In contrast, this difference was much greater with CPX-351; 7.8-fold and 2.4-fold higher when comparing normal CFC to AML CFC in CR and NR samples, respectively (p≤ 0.001 for both comparisons). Thus, selective cytotoxicity for AML as compared to normal CFC could be demonstrated for CPX-351 even for
progenitors from chemotherapy refractory patients while such selectivity was not apparent with the free drugs.

3.3.3. HPLC analysis demonstrates greater AML blast-selective drug accumulation for CPX-351 compared to free drugs at equivalent concentrations.

To explore the possibility that differences in intracellular drug accumulation between normal and AML cells might explain the differences in cell kill seen with CPX-351, intracellular daunorubicin was compared in 3 AML and 3 normal PB mononuclear cells (PBMC) that were exposed to CPX-351 or free drug using HPLC. Figure 3.4 shows the intracellular concentration of daunorubicin (ng) per 100,000 cells. Only daunorubicin accumulation was examined by HPLC as its intracellular chemical stability afforded efficient extraction and quantification. In contrast, cytarabine rapidly (i.e., within 15 minutes) converts to the mono-, di-, and then tri-phosphorylated active metabolites; the latter subsequently incorporates into DNA and thus precludes its determination using HPLC. However, we have observed that detecting intracellular cytarabine and its metabolites using radiolabelled drug yielded total drug-related uptake consistent with a 5:1 cytarabine:daunorubicin intracellular molar ratio, although in this case the relative amounts of active vs inactive metabolites cannot be resolved [154].

Daunorubicin accumulation was significantly higher in AML blasts than in normal PBMC after exposure to CPX-351 (1.76±0.10 ng and 0.89±0.19 ng, respectively, P<0.01). In contrast, there was no difference between AML and normal PBMC after treatment with free drug cocktail (1.09±0.21 ng and 1.13±0.11 ng, respectively, P=0.85).
Additionally, CPX-351 treated AML cells maintained significantly higher concentrations of intracellular daunorubicin (1.76±0.10 ng) than free drugs treated cells (1.09±0.21 ng). The difference in drug uptake by normal PBMC treated with CPX-351 and free drugs was not statistically significant.

3.3.4. Subpopulations of AML blasts enriched for candidate leukemic stem cells are killed by CPX-351 and free cytarabine:daunorubicin.

Subpopulations of leukemia blasts expressing the CD34⁺CD38⁻ phenotype can be identified from many AML patient samples (Figure 3.1) and are often capable of sustaining malignant hematopoiesis for many weeks in immunodeficient mice. Elimination of these candidate leukemic stem cells (LSC) is thought to be necessary to cure patients with AML. However, LSC may be relatively chemotherapy drug resistant due to their quiescent cell cycle status and high level expression of genes such as p-glycoprotein [163]. To determine the relative drug sensitivity of LSC as compared to the majority of AML blasts, cells from 3 CR and 3 NR patients were treated with CPX-351 or free cytarabine:daunorubicin and then analyzed by flow cytometry for their expression of CD34 and CD38 and staining with annexin V/propidium iodide as an indicator of cell death. As shown in Figure 3.5, the % kill of total blasts from the NR samples was lower than that seen with the CR samples. The same observation held true when comparing the different sorted subpopulations with CD34⁺CD38⁺, CD34⁻ and CD34⁺CD38⁻ cells all showing less drug sensitivity when isolated from NR then from CR patients. In particular, CD34⁻CD38⁻ cells from NR samples exhibited an average of 55% kill at the highest
CPX-351 concentration tested vs 90% for the same population from CR patients (P=0.003). The abilities of CPX-351 and free cytarabine:daunorubicin to kill these different subpopulations from CR or NR patients were similar.

3.3.5. CPX-351 is less toxic to subpopulations of normal blood and marrow cells than free cytarabine:daunorubicin

To study the effects of drug treatments on normal PB and BM cells with stem/progenitor cell properties similar to the AML cells studied in Figure 3.5, mononuclear cells from two G-CSF-mobilized PB and one normal BM sample were treated with CPX-351 or free cytarabine:daunorubicin and then stained and analyzed by flow cytometry as described above for AML cells (Figure 3.1). As shown in Figure 3.6, total normal mononuclear cells as well as CD34+CD38-, CD34+CD38+, and CD34- normal cells were less sensitive to killing with CPX-351 than with the free drugs with statistically significant differences documented for all of the cell populations. The IC50 for CPX-351 was 5.1- to 5.8-fold higher than that for the free drugs for total mononuclear cells as well as all the subpopulations tested (p<0.04 for all comparisons). When comparing the IC50s of subpopulations of normal and AML cells treated with free cytarabine:daunorubicin no significant differences were observed. In contrast, the IC50 for subpopulations of AML cells treated with CPX-351 was always lower than that obtained for the same subpopulation of normal cells. In particular, among CD34+CD38- cells which are enriched for both normal and AML stem cells, the IC50 was 7.6- and 1.9-
fold lower when comparing CR and NR AML samples, respectively, to normal cells (p=0.006 and 0.05 Table 3.2).

3.4. Discussion

CPX-351 is a liposomal formulation of a 5:1 molar ratio combination of the antineoplastic drugs cytarabine and daunorubicin HCl which is designed to deliver both drugs in a synergistic ratio to the bone marrow in patients with leukemia [278]. Maintenance of the synergistic drug ratio in vivo is accomplished by the use of liposomes which encapsulate and retain the two drugs [155, 283]. Previous studies have demonstrated that CPX-351 has improved efficacy in mice engrafted with leukemia cell lines as compared to an antagonistic molar ratio of cytarabine:daunorubicin in liposomes, or a 5:1 molar ratio combination of free cytarabine:daunorubicin where the drugs were rapidly and independently cleared in vivo [153, 154]. A more recent study using immunodeficient mice engrafted with a human acute T cell lymphoblastic leukemia cell line showed that the superior antitumor activity of CPX-351 is likely due to prolonged maintenance of synergistic cytarabine:daunorubicin ratios in the marrow and selective killing through direct liposome-leukemia cell interactions [279]. This and other preclinical data provided the rationale for a Phase 1 trial of CPX-351 which demonstrated anti-leukemic activity in patients with advanced AML. More recently, evidence of improved survival for CPX-351 compared to 7+3 treatment was reported for a randomized Phase II trial in newly diagnosed elderly AML patients [281].
The experiments described here are the first to compare the in vitro cytotoxicity of CPX-351 and conventional cytarabine:daunorubicin against leukemia cells directly isolated from AML patients and normal hematopoietic progenitors. The goal was to determine if the enhanced anti-leukemic efficacy and/or selectivity of CPX-351 that had been seen against cell lines and in mouse models could be documented against these primary human cells. As expected, AML CFC from patients with chemotherapy refractory leukemia (NR patients) were less sensitive to killing with free cytarabine:daunorubicin than AML CFC from patients who achieved complete remission (CR) [228]. Treatment with CPX-351 produced very similar AML CFC kills to those obtained with the free drugs for both CR and NR patient samples (Figure 3.2). Given the in vitro conditions which should not have disturbed the molar ratio of the free drug substantially over the relatively short-term exposure of the AML cells, these results are not surprising. However, the equivalence of CPX-351 to free drugs in killing AML progenitors at least argues for effective uptake of the liposomal drug in relevant target cells. The daunorubicin cell uptake studies further demonstrate that CPX-351 has selectivity against AML over normal PBMC whereas the free drug cocktail does not and that liposomal encapsulation is compatible with efficient drug uptake and cytotoxicity in these cells (Figure 3.4).

CPX-351 and free cytarabine:daunorubicin were also tested against normal CFC from G-CSF mobilized peripheral blood and bone marrow cells. In contrast to the data obtained from testing of AML CFC, normal progenitors proved to be much less sensitive to CPX-351 than the free drugs (Figure 3.3). Furthermore, although the IC_{50}s for AML
and normal CFC treated with the free drugs were very similar, the same comparison for CPX-351 revealed AML CFC from both CR and NR patients to be significantly more sensitive to the liposomal formulation than normal progenitors (Table 3.1). Daunorubicin uptake was also lower in normal than in AML cells treated with CPX-351 (Figure 3.4). These latter results are similar to those reported in a previous study which showed that cytarabine and daunorubicin concentrations in leukemic cells were higher than those in normal BM cells of leukemia bearing mice [279].

Comparison of cytotoxicity and drug accumulation values between AML and normal cells were consistent within each treatment type. Specifically, increased drug uptake and cell kill was observed for CPX-351 in AML compared to normal progenitors whereas the free drug cocktail displayed comparable drug uptake and cell kill levels in the two cell types. However, intracellular drug levels were higher for CPX-351 exposed to AML cells compared to free drug cocktail, despite the fact that the two treatments yielded comparable IC50 values (compare results with AML cells in Table 3.1 and Figure 3.4). This observation may be attributable to the fact that intracellular drug accumulation for CPX-351 occurs via active uptake of intact liposomes into cytoplasmic vacuoles [279]. Release of the encapsulated drugs after internalization is therefore required for the agents to be bioavailable to their intracellular targets. Consequently, processing of CPX-351 liposomes within endocytic/lysosomal bodies may be less than 100% efficient due to incomplete drug release and/or drug degradation within these vacuoles [284]. This CPX-351 uptake phenomenon also occurs, albeit to a lesser extent, in normal bone marrow
cells [279] so similar considerations apply when comparing CPX-351 and free drug cocktail results in normal progenitors.

The in vitro drug doses tested here are well within the range of those which can be obtained therapeutically. In fact, the plasma drug concentrations obtained in patients 24 hours after a single dose of CPX-351 administered at the maximum-tolerated dose (MTD) of 101 units/m² (1 unit = 1 mg cytarabine + 0.44 mg daunorubicin) were 1700-fold to 22-fold higher than the in vitro drug concentrations tested in this study (13-1,023 ng/ml of cytarabine and 5.6-450 ng/ml of daunorubicin). The mean plasma concentrations of cytarabine and daunorubicin attained in 13 patients 24 hours after treatment were 23,000 ng/ml and 10,000 ng/ml, respectively [280]. As well, greater plasma concentrations were obtained with the induction treatment schedule (treatments on day 1, 3, and 5) where $C_{\text{max}}$ values of 64,600 ng/ml for cytarabine and 30,200 ng/ml for daunorubicin where obtained and detectable levels were present 7 days after administration.

In total, these findings suggest that CPX-351 might be used at doses high enough to overcome intrinsic chemotherapy drug resistance in AML without intolerable toxicity to normal hematopoiesis. Twenty percent of AML patients fail to achieve CR with conventional chemotherapy induction regimens. It is conceivable that CPX-351 may be useful in this setting. In fact, complete remissions were documented in AML patients receiving CPX-351 whose AML had previously proven refractory to standard ‘7+3’ induction [285]. In addition, older individuals with AML have a grim prognosis in part because they tolerate aggressive induction and consolidation chemotherapy less well than
younger individuals. Concern over potential treatment-related toxicities may result in undertreatment of the leukemia [286-288]. The ability to deliver effective therapy with more acceptable toxicity using CPX-351 may also improve outcomes in this group.

Both normal and malignant hematopoiesis in AML are organized as a hierarchy of progenitor cells in which a rare population of cells with a large capacity for proliferation, and self-renewal known as ‘stem cells’ maintain long-term production of more differentiated cells. Rare cells with the phenotype CD34+CD38- are found both in normal bone marrow and among the blasts from many patients with AML. These cells exhibit stem cell properties when grown in long-term culture or injected into immunodeficient mice while CD34+CD38+ cells or cells lacking CD34 expression are typically devoid of stem cell activity [268, 289]. Normal stem cells are required to maintain lifelong lymphomyeloid hematopoiesis while leukemic stem cells (LSC) are believed to be responsible for producing and maintaining all the characteristics of the disease in patients, and for causing relapse of the disease after remission has been achieved [5, 66, 175]. The large proportion of AML patients who relapse suggests that existing therapies are not effectively targeting the LSC [74, 90, 290, 291]. To assess the relative ability of CPX-351 and free cytarabine:daunorubicin to kill AML and normal stem cells, leukemic blasts and normal blood or marrow cells were treated with drugs and then sorted according to their expression of CD34 and CD38 and staining with Annexin V/PI. As shown on Figure 3.5, the relative ability of CPX-351 and free drugs to kill the CD34+CD38- fraction of LSC-enriched AML blasts was not significantly different. This fraction of cells from NR patients was relatively resistant to both CPX-351 and free drugs as we had
already observed for AML CFC. The AML data contrast with those obtained with normal marrow and blood cells where CPX-351 was found to cause less toxicity than the free drug and the IC$_{50}$s calculated were higher with the liposomal drug when sorted subpopulations of cells, including the CD34$^+$CD38$^-$ fraction, were analyzed (Figure 3.6, Table 3.2). If the data obtained here with a small number of patient samples is typical of AML in general, it is conceivable that the relatively selective targeting of leukemic as compared to normal stem cells and progenitors with CPX-351 will result in more durable remissions and reduced hematopoietic toxicity than has been seen with conventional cytarabine:daunorubicin induction regimens. The mechanism behind the selective toxicity of CPX-351 for AML cell is unclear although mechanisms involving molecules such as the class B scavenger receptor and/or the low density lipoprotein receptor which could provide specific binding and uptake of anionic liposomes have been proposed [279]. Additionally, it has been reported that those receptors were highly expressed in leukemic cells of AML patients [279].

In summary, CPX-351 is a novel liposomal formulation of cytarabine and daunorubicin at synergistic concentrations. The data presented here demonstrated equivalent potency for CPX-351 to free drugs in killing AML progenitors and candidate LSC from patients with both chemotherapy sensitive and refractory disease. Importantly, CPX-351 is relatively nontoxic to normal hematopoietic progenitors as compared to conventional cytarabine:daunorubicin. Ongoing clinical trials of CPX-351 in elderly AML patients and those with leukemia in first relapse will ultimately determine if these laboratory data accurately predict clinical benefit from this promising new agent.
Figure 3.1 Isolation of CD34−, CD34+CD38−, and CD34+CD38+ cells from total mononuclear cells

Representative FACS plots of an AML (left) and a normal (right) sample are shown. Cells were labelled with APC/Cy7-conjugated anti-CD34 and APC-conjugated anti-CD38. Apoptotic cells in each subpopulation were measured by annexin V/PI staining. Numbers in the plots indicate the percentage of cells in each gate.
Figure 3.2 Effect of CPX-351 and free drug cocktail on AML CFC of CR and NR patient samples

AML cells were incubated with various concentrations of drugs for 24 hours and then plated in CFC assay. Plotted values display the mean AML CFC kills for 7 different CR patient and 6 NR patient samples as compared to untreated controls. Error bars represent standard deviations. CR samples treated with CPX-351 (●) or free drug cocktail (■); NR samples treated with CPX-351 (○) or free drug cocktail (□). Concentration of drugs (cytarabine:daunorubicin, ng/ml) were -1(13:5.6), -2(39:17), -3(114:50), and -4(341:150), respectively.
Figure 3.3 Comparison of drug sensitivity between CPX-351 and free-drug cocktail in normal progenitors

Normal blood or marrow cells were incubated with the indicated drug concentrations for 24 hours followed by plating in CFC assay. Plotted values represent the mean CFC kills from individual experiments with 7 different normal mononuclear cell samples as compared to untreated controls. Error bars represent standard deviations. Normal samples treated with CPX-351 (●) or free drug cocktail (■). Concentration of drugs (cytarabine:daunorubicin, ng/ml) were -1(13:5.6), -2(39:17), -3(114:50), and -4(341:150), respectively. P values indicate significant differences between CPX-351 and free drug (Student t-test).
Figure 3.4 Intracellular drug accumulation for CPX-351 compared to free drug cocktail at equivalent concentrations

After completion of 24-hour drug incubation with 341 ng cytarabine and 150 ng daunorubicin as either free drugs (Free) or CPX-351 (CPX) ~1x10⁶ cells, were evaluated for the presence of daunorubicin using HPLC. Y-axis indicates intracellular concentration of daunorubicin (ng) in 100,000 cells for each of 3 normal and 3 AML samples. P value is displayed if there is a statistically significant difference (T test).
Figure 3.5 Comparison of apoptosis induction with CPX-351 and free drug cocktails in AML subpopulations from CR and NR samples

After 24 hours incubation with or without drug exposure cells were labelled with CD34- APC/Cy7 and CD38-APC antibodies and quantified for Annexin V and propidium iodide staining by flow cytometry. The percent cell kill as compared to untreated control cultures was assessed in three representative CR and NR patient samples. CR samples treated with CPX-351 (●) or free drug cocktail (■); NR samples treated with CPX-351 (○) or free drug cocktail (□). Total blasts (A), CD34- (B), CD34+CD38- (C), CD34+CD38+ (D). Concentrations of drugs (cytarabine:daunorubicin, ng/ml) were -1(39:17), -2(114:50), -3(341:150), and -4(1023:450), respectively.
Figure 3.6 Comparison of apoptosis induction with CPX-351 and free drug cocktails in normal mononuclear cell subpopulations

After 24 hours incubation with or without drug exposure, cells were labelled with CD34-APC/Cy7 and CD38-APC antibodies and quantified for Annexin V and propidium iodide staining by flow cytometry. The percents cell kill were assessed in three representative normal samples as compared to untreated controls. Horizontal lines represent the mean of each group. Normal samples treated with CPX-351(●) or free drug cocktail (■), Total mononuclear (A), CD34− (B), CD34+CD38− (C), CD34+CD38+ (D). Concentration of drugs (cytarabine:daunorubicin, ng/ml) were -1(39:17), -2(114:50), -3(341:150), and -4(1023:450), respectively. P values are indicated if they are statistically significant (T-test).
Table 3.1 Comparisons of IC50s for AML and normal CFC treated with CPX-351 or free-drugs

<table>
<thead>
<tr>
<th>Sample</th>
<th>N=</th>
<th>CPX-351*</th>
<th>Free drugs*</th>
<th>P = CPX vs free†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML CR</td>
<td>7</td>
<td>38±11 : 17±5</td>
<td>28±7 : 12±3</td>
<td>0.06</td>
</tr>
<tr>
<td>AML NR</td>
<td>6</td>
<td>126±33 : 55±14</td>
<td>86±21 : 37±9</td>
<td>0.03</td>
</tr>
<tr>
<td>Normal</td>
<td>7</td>
<td>298±75 : 129±33</td>
<td>57±17 : 25±7</td>
<td>0.0004</td>
</tr>
<tr>
<td>P= AML vs normal</td>
<td></td>
<td>CR, 0.0003; NR, 0.001</td>
<td>CR, 0.003; NR, 0.03</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± standard deviation (SD); †Student t test.
Table 3.2 Cytarabine:daunorubicin IC₅₀s (ng/ml) for CD34⁺CD38⁻ AML or normal cells exposed to CPX351 or free drugs

<table>
<thead>
<tr>
<th>Sample</th>
<th>N=</th>
<th>CPX-351*</th>
<th>Free drugs*</th>
<th>P = CPX vs free†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML CR</td>
<td>3</td>
<td>199±62:86±27</td>
<td>306±92:133±40</td>
<td>NS</td>
</tr>
<tr>
<td>AML NR</td>
<td>3</td>
<td>767±189:333±82</td>
<td>1047±505:455±219</td>
<td>NS</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>1487±419:644±181</td>
<td>272±70:118±30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

P= AML vs normal†: CR, 0.006; NR, 0.05

*Mean ±SD; †Student t test. ; NS, not significant
Chapter 4. A flow cytometry, mitoxantrone efflux-based assay for predicting chemotherapy refractoriness in newly-diagnosed acute myeloid leukemia

4.1. Introduction

Although majority of patients with acute myeloid leukemia (AML) achieve complete remission (CR) with standard induction chemotherapy at least 20% of younger patients and as many as 50% of those over the age of 60 years have leukemia that is chemotherapy refractory at diagnosis [60, 292]. Conventional AML induction chemotherapy typically consists of cytarabine in combination with an anthracycline in doses sufficient to induce temporary marrow aplasia [293]. This therapy causes considerable morbidity and occasional mortality particularly in elderly patients and those with co-morbid illness. If CR is not obtained with initial induction, survival is typically short especially in those not fit enough to undergo even more aggressive salvage treatment. Many factors have been shown to have prognostic impact in AML. The most powerful of these are cytogenetic and/or molecular abnormalities detected in the diagnostic bone marrow sample [214, 294, 295]. However, 40%-50% of AML patients have a normal karyotype (NK) at diagnosis. Although NK AML is considered to have an intermediate prognosis overall a wide variability in response to induction therapy and survival is seen in this group [191, 296]. A number of molecular abnormalities with prognostic significance are now routinely assessed in the NK group [297]. These include
mutations in the fms-like tyrosine kinase 3 (FLT3), CCAAT/enhancer binding protein α (CEBPA) genes, and nucleophosmin 1 (NPM1) [35, 298, 299]. However, some NK patients have none of these abnormalities. Detection of cytogenetic and/or molecular abnormalities is extremely useful for planning of post remission therapy. However, in many centers the results of this relatively labor intensive testing is not available for a number of days after the diagnosis of AML has been made and initial induction therapy should be initiated. The ability to reliably predict the presence of chemotherapy refractory AML at diagnosis would be clinically useful for many patients [300]. For example, the overall results of induction chemotherapy for AML patients over age 70 are very poor [301]. If the futility of induction chemotherapy could be predicted it could be avoided along with the associated toxicity. On the other hand, if achievement of CR was predicted to be highly likely elderly patients or those with significant co-morbidities could be somewhat reassured that the risk associated with therapy should be justified by the benefit obtained [226, 302, 303].

As discussed above, resistance to conventional chemotherapy drugs, whether primary or acquired, is a major obstacle to successful treatment and a main cause of death in AML. There are 49 human genes in the ATP-binding cassette (ABC) transporter superfamily encoding transmembrane proteins which mediate drug efflux via ATP-dependent pumps [158, 159, 164, 166, 167]. Among the ABC transporters, expression of multi-drug resistance protein 1 (MDR1 or P-glycoprotein), and breast cancer resistance protein (BCRP) have been associated with chemotherapy resistance in AML in some studies [161, 192, 304-308] although not in all[221, 222, 309-311]. One possible reason
for the conflicting prognostic relevance suggested for ABC transporter expression in AML is the variety of methods used to assess that expression in previous studies [312]. The results of molecular or immunological testing would not necessarily be comparable to each other or to assays measuring transporter function. Testing which assesses ABC transporter function by measuring drug efflux allows the simultaneous assessment of the activity of more than one of these proteins which may provide an advantage in predicting drug resistance over more targeted assessment of individual genes or proteins [191, 313].

Previous work from our center used quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) to study MDR1 and BCRP expression in AML blasts and subpopulations of AML cells highly enriched for candidate leukemic stem cells (LSC) [228]. The latter were studied since the persistence of LSC is thought to be a mechanism of chemotherapy refractoriness in AML [163]. In this study expression of both MDR1 and BCRP was higher in AML cells from chemotherapy refractory patients than in cells from those who achieved CR when the CD34⁺CD38⁻ fraction enriched for LSC was studied [228]. Although this data strongly suggested the prognostic relevance of quantifying ABC transporter expression in AML the technique used required both cell sorting and QRT-PCR making it unlikely that a clinical laboratory would adopt this for routine testing of AML patients. Thus, the aim of the current study was to develop a flow cytometry-based assay that would predict response to induction chemotherapy in newly-diagnosed AML patients with a rapid turnaround that would allow the results to be available before initial treatment decisions are necessary. Flow cytometry was chosen because the technology is widely-available in clinical hematology laboratories and
routinely used for immunophenotyping of AML blasts at diagnosis. In the assay described, the median fluorescence intensity (MFI) for AML blasts incubated with mitoxantrone (an ABC transporter substrate) is measured with or without co-incubation with cyclosporine A (a non-specific inhibitor of ABC transporter function). A ratio between the inhibited and uninhibited MFI is then calculated (MFIR). This assay was applied to both the total blast cell population and the CD34⁺CD38⁻ LSC-enriched fraction from newly-diagnosed patients for whom the outcome of induction therapy was known. The data show that the MFIR value strongly predicts the outcome from initial induction chemotherapy in AML patients including those with normal karyotype and beyond 60 years of age. The predictive value of the MFIR assay for this endpoint was also independent of the influence of other known prognostic variables in AML suggesting that this testing could have clinical value.

4.2. Materials and methods

4.2.1. AML patients and samples

Peripheral blood (PB) or bone marrow (BM) leukemia blast cells were obtained at diagnosis from newly diagnosed AML patients. All samples were obtained after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. The diagnosis of AML was established on presentation bone marrow biopsy and aspirate using World Health Organization (WHO) criteria (WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 2008). Cytogenetic risk was assigned using MRC (UK) criteria [314]. Mononuclear cells
(MNCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden) and cryopreserved in Iscove’s modified Dulbecco’s medium (IMDM) with 50% fetal calf serum (FCS) (StemCell Technologies, Vancouver, Canada) and 10% dimethylsulfoxide (Sigma-Aldrich, Oakville, Ontario, Canada). Thawed cells were washed twice in IMDM containing 10% FCS and used for the experiments described below.

4.2.2. Induction chemotherapy

Induction chemotherapy was cytarabine and anthracycline-based for all patients. Among the 174 AML patients whose clinical data were used for the logistic regression analysis 83 received one or two courses of conventional dose cytarabine (200 mg/m²/d for 7 days) with daunorubicin 45 mg/m² for 3 days (7+3). The remaining 91 patients received intermediate to high dose cytarabine (at least 6 gm/m² total dose) in combination with daunorubicin 45 mg/m² for 3 days or mitoxantrone 12 mg/m² for 3 days plus one dose of etoposide 800 mg/m². Complete remission (CR) was defined as described by Cheson et al [315, 316]. One hundred twenty-seven (73%) of patients achieved CR. Twenty-two of these required two cycles of chemotherapy to achieve CR; in 9 cases the additional chemotherapy was a second cycle of 7+3, in 5 high dose cytarabine with daunorubicin and in 6 cases 2.4 gm/m² etoposide with cytoxan 2 gm/m² × 3 [317]. Forty-seven (27%) of patients failed to achieve CR. In twenty-nine cases induction failure (IF) was determined after one or two courses of 7+3 chemotherapy, while the remaining 18 IF patients received high dose cytarabine with daunorubicin or mitoxantrone/etoposide
as the first cycle of induction therapy followed by salvage chemotherapy with 2.4 gm/m² etoposide with cytoxan 2 gm/m² × 3 [317] (n=14), a second course of high dose cytarabine with an anthracycline (n=2) or gemtuzumab ozogamicin (n=2). Overall survival (OS) was defined as the interval from diagnosis to death independently of the cause.

4.2.3. Quantitative RT-PCR (QRT-PCR) analysis

Total RNA was extracted from 1 × 10⁶ unsorted AML blast cells, CD34⁺CD38⁺, and CD34⁺CD38⁻ AML cells with Absolutely RNA Miniprep, Microprep, or Nanoprep kits (Stratagene, La Jolla, CA, USA). The RT reaction was performed in 20 µL with superscript III reverse transcriptase (Invitrogen, Burlington, Canada) using random hexamer oligonucleotides (Amersham Pharmacia, Piscataway, NJ, USA). Real-time PCR was performed using 12.5 µL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL of 20 pM-specific primers, 1 to 2 µL cDNA, and water to a final volume of 25 µL. Specific forward and reverse primers to produce approximately 100-bp amplicons for optimal amplification in real-time PCR of reverse-transcribed cDNA for human MDR1 were

5’-GGCCTAATGCGAACACATT-3’ (forward) and 5’-AGGCTCAGTCCCTGAAGCACPACACAT-3’ (reverse), for human BCRP were 5’-CCAGGCGAAGGTGTTCAC-3’ (forward) and 5’-TGCGACTGTAGTTATAGGTGACA-3’ (reverse), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5’-CCCATCACCATCTTCCAGGAG-3’ (forward) and
5’-CTTCTCCATGGTGAGACGACG-3’ (reverse). Thermal cycling conditions were 50°C for 2 minutes and 95°C for 5 minutes, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C. Real-time PCR and data analysis were performed on an iCycler iQ system, using iCycler iQ Real-time Detection Software (Bio-Rad, Hercules, CA, USA). Fold-expression relative to GAPDH was calculated by: \((1 + \text{AE})^{-\Delta\text{Ct}}\) where AE is the amplification efficiency of the specific gene and \(\Delta\text{Ct} = (\text{Ct of Gene X}) – (\text{Ct of GAPDH})\).

4.2.4. Mitoxantrone efflux for assessment of ABC transporter function

ABC transporter function was assessed with flow-cytometry using mitoxantrone (Sigma-Aldrich) as a fluorescent substrate and cyclosporine A (CA) (Sigma-Aldrich) as an inhibitor of ABC transporter function. ABC transporter-mediated mitoxantrone efflux was quantified by determining the MFI of AML cells incubated with this drug alone as compared to the MFI measured in cells incubated with mitoxantrone plus CA [214, 215]. MNCs isolated from patients’ PB were washed with Hanks buffered solution with 2% fetal calf serum and 0.02% sodium azide (HFN) and titrated to a cell count of 5×10^5/ml in pre-warmed IMDM media with 10% FCS. Mitoxantrone (0.1ug/ml) was added with or without CA (10uM), and cells were incubated for 1 hour at 37°C. Subsequently, cells were washed and incubated for an additional 1 hour in mitoxantrone-free 10% FCS containing IMDM with or without CA to allow time for mitoxantrone efflux. In some experiments cells were then washed in HBS and stained with anti-CD34-PE and anti-CD38-FITC for 30 minutes at 4°C (Becton Dickinson, Franklin Lakes, NJ, USA) to
allow analysis of subpopulations of cells enriched for progenitor cells or anti-CD3-APC-Cy7 and CD19-APC-Cy7 (Becton Dickinson) to allow exclusion of B and T lymphocytes. FACS analysis was performed with a FACScalibur flow cytometer (Becton Dickinson) (excitation wavelength of 635 nm and emission wavelength of 661 nm). MFI was calculated using Flowjo software (Tree Star Ing., Ashland, OR, USA) for cells stained with mitoxantrone with or without CA after machine calibration using unstained control cells. The MFI in the presence of CA divided by the MFI in the absence of CA was calculated for each AML sample and cell population and reported as the MFIR in the results reported below. In experiments where cells were stained with anti-CD34/CD38 or anti-CD3/CD19, mouse IgG antibodies conjugated with each fluorochrome were used as isotype controls. Gates were set to exclude nonviable, propidium iodide positive (PI+) cells, and cells labeled with the irrelevant isotype control antibody. The MFI for cells incubated with mitoxantrone ± CA was calculated for subpopulations defined by their expression of CD34 and CD38 using a gating strategy previously described [318] and for cells negative for CD3 and CD19 by comparison to isotype controls.

4.2.5. Statistics

The correlation between mRNA expression level of ABC transporters and MFIR for the same patient sample was estimated using Spearman’s rank order correlation coefficient. Comparison of mean MFIR values between patient groups was performed using the Student t-test. The predictive value of MFIR with respect to CR rate was determined using logistic regression analysis. Clinical parameters independently
associated with response were determined in multivariate logistic regression analysis. P values \( \leq 0.05 \) were considered statistically significant.

4.3. Results

4.3.1. MDR1 and BCRP expression in leukemic blasts and subpopulations from AML patients varies with response to induction chemotherapy

Previous work has shown that expression of the major ABC transporters, MDR1 and BCRP is higher in AML blasts from patients who fail to achieve CR with remission induction chemotherapy (NR patients) than in blasts from patients who successfully enter CR, particularly when the CD34\(^+\)CD38\(^-\) subpopulation enriched for candidate leukemic stem cells (LSC) is analyzed [228]. In the current study a similar analysis was performed using QRT-PCR to study MDR1 and BCRP expression in total blasts, CD34\(^+\)CD38\(^+\), and CD34\(^+\)CD38\(^-\) subpopulations from 28 newly-diagnosed AML patients whose subsequent response to chemotherapy was known (14 CR and 14 NR). Consistent with the previous results and as shown on Figure 4.1, although there was interpatient variability, the expression of both MDR1 and BCRP was on average higher in NR than CR patient samples. Although the absolute difference between NR and CR patients was highest for the CD34\(^+\)CD38\(^-\) subpopulation, significant differences were also seen for CD34\(^+\)CD38\(^+\) cells and total blasts (\( p < 0.04 \) for all comparisons). Thus, high expression of two ABC transporters in leukemic blasts at diagnosis predicts clinical drug resistance in these AML patients.
4.3.2. Assessment of ABC transporter function by flow cytometry

The data shown on Figure 4.1 and our previously published work have shown consistent differences in QRT-PCR values for MDR1 and BCRP expression between CR and NR patient samples. To develop a flow cytometry-based assay to measure ABC transporter function mitoxantrone was chosen as a fluorescent ABC transporter substrate and cyclosporine (CA) as a nonspecific inhibitor of ABC transporter protein function, including that of MDR1 and BCRP. As described in the Methods, the MFI of AML cells incubated with both mitoxantrone and cyclosporine is divided by the MFI of cells incubated with mitoxantrone alone to create a ratio (MFIR).

As shown on Figure 4.2, comparison of the MFIR calculated for total blasts from the same 28 AML patient samples that were analyzed by QRT-PCR (Figure 4.1) revealed a direct and close correlation between the MFIR and the QRT-PCR results for both MDR1 ($r = 0.78$, $p < 0.0001$) and BCRP ($r = 0.63$, $p < 0.0004$). Comparison of the MFIR values from total blasts and CD34$^+$CD38$^-$ cells between NR and CR patient samples revealed highly significant differences with the mean MFIR in the NR group being substantially higher ($\text{mean} \pm \text{SD}; 2.179 \pm 0.548$ and $2.386 \pm 0.591$ for total blasts and CD34$^+$CD38$^-$ cells, respectively) than the same mean MFIR values for CR patients ($1.443 \pm 0.403$ and $1.636 \pm 0.470$, respectively) ($p < 0.001$ for both comparisons between NR and CR patients) (Figure 4.3).

Thus, among this small group of AML patient samples where treatment outcome was known calculation of the MFIR appeared to have similar predictive value for chemotherapy responsiveness as QRT-PCR for MDR1 and/or BCRP.
4.3.3. Comparison MFIR between PB and BM, and between fresh and frozen samples

The above analysis was performed using cryopreserved PB samples from patients with high circulating blast counts. To determine if blasts from the PB and BM would give similar results paired PB and BM samples from 12 patients were tested. As shown in Table 4.1A, MFIRs of PB and BM blasts were very similar for all patients (mean ± SD, 1.7 ± 0.6 and 1.7 ± 0.7, respectively). Similarly as described in previous studies, when the MFIR from cryopreserved and fresh blood and marrow cells were compared for 3 patient samples no significant difference was observed (Table 4.1B) [214, 319].

4.3.4. MFIR predicts response to induction chemotherapy in 174 AML patients

To attempt to confirm the value of the MFIR assay for predicting the outcome of induction chemotherapy and to further analyze its possible prognostic utility for independently predicting this and other outcomes a larger group (n=174) of AML patient samples was analyzed. To minimize the risk of investigator bias treatment outcome was unknown at the time the MFIR was determined for these samples.

The characteristics of AML patients used for this analysis are listed in Table 4.2. Among 174 patients who received remission induction therapy, 105 (60%) achieved CR after 1 cycle of induction therapy (CR1), 22 (13%) required 2 cycles to achieve CR (CR2), and 47 (27%) were refractory to induction therapy (NR). The relationship between CR status and MFIR in total PB MNCs from these newly-diagnosed patients is
shown on Table 4.3. In addition, to determine if the predictive value of the test could be improved by focusing on the subpopulation enriched for LSC or MNC from which T and B lymphocytes had been depleted CD34⁺CD38⁻ and CD3⁻CD19⁻ cells from the some of these same samples were also analyzed. The mean MFIR for the CR patients was significantly lower than that obtained for both the CR2 and NR patients and the mean MFIR for CR2 patients was intermediate between the means for CR and NR patients when total MNCs, CD34⁺CD38⁻ or CD3⁻CD19⁻ cells were compared (P < 0.001 for all comparisons). Among the 3 cell populations studied the difference between CR and NR samples was greatest for CD3⁻CD19⁻ cells. The mean MFIRs for CD34⁺CD38⁻ cells were, on average, higher for all patients regardless of their remission status. However, restricting analysis to this cell population did not enhance the ability to discriminate CR from NR patients.

On univariate analysis the MFIR did not vary with cytogenetic risk group, presenting WBC count or the presence of the FLT3 ITD but was higher in patients greater than 60 years of age as compared to younger patients (see below).

4.3.5. Determination of threshold MFIR values predicting failure of remission induction

To determine the MFIR value above which failure of remission induction could be most reliably predicted, a logistic regression analysis was performed using data from the 152 patients from Table 4.3 who were in the CR or NR categories. This analysis determined 2.45 as the MFIR threshold above which achievement of CR was unlikely.
Although the overall CR rate for these 152 patients was 69%, 97 (81%) of the 120 patients with MFIR $\leq 2.45$ calculated from total MNC achieved CR as compared with only 8 (25%) of the 32 patients with MFIR $> 2.45$ ($P < 0.0001$) (Table 4.4).

4.3.6. MFIR values predict induction failure in normal karyotype AML

Cytogenetic abnormalities detected in diagnostic BM samples are the most powerful prognostic factor in newly-diagnosed AML. However, at least 40% of AML patients present with a normal karyotype (NK) and are classified as having intermediate risk disease [296]. To determine if MFIR analysis would retain its prognostic significance in NK AML, a subgroup analysis was performed using data from the 73 NK patients. As shown on Table 4.5, 5 (29%) of 17 patients with an MFIR $> 2.45$ obtained CR as compared to 49 (88%) of 56 patients with MFIR $\leq 2.45$ ($p < 0.0001$) demonstrating that this measurement in total MNC retained its predictive value in the NK group.

4.3.7. The MFIR predicts outcome of remission induction chemotherapy in elderly AML patients

Increasing age has a negative prognostic impact in AML with lower overall remission rates and higher treatment related complications in the elderly [300]. Consistent with the relative chemotherapy resistance seen in older AML patients, in the current group of 174 AML patients the mean total MNC MFIR was higher among patients above 60 years of age than among younger patients (mean ± SD 2.17 ± 1.04 and
1.78 ± 0.74 for older and younger patients, respectively, \( p < 0.004 \). The ability to predict the outcome of remission induction would be particularly relevant in elderly AML patients. Thus, further analysis was performed restricted to the 69 patients greater than age 60 at diagnosis of AML. Among 49 patients with \( \text{MFIR} \leq 2.45 \), 42 (86\%) achieved CR, while CR was obtained for only 4 (20\%) of 20 patients with higher MFIR values (\( p < 0.0001 \), Table 4.6) indicating that the predictive value of the MFIR is retained in this elderly patient group.

4.3.8. Further refinement of the MFIR by restricting analysis to CD3⁺CD19⁻ cells

Because the analysis of mean MFIR values shown on Table 4.3 had suggested that restricting analysis to CD3⁺CD19⁻ cells would enhance the predictive power of this test, samples from 36 NK and 63 elderly AML patients where testing had been done on CD3⁺CD19⁻ cells were analyzed separately. Table 4.7A shows the CR rate of 93\% in NK AML patients with \( \text{MFIR} \leq 2.45 \) which was much higher than the 11\% CR rate seen with patients whose MFIR was above this threshold (\( p = 0.001 \)). Similar results were seen among the 63 elderly AML patients where those with the low MFIR had a much higher probability of CR as compared to the high MFIR group (89\% vs 13\%, \( p < 0.0001 \), Table 4.7B). Thus, exclusion of lymphocytes may improve the predictive value of the MFIR assay when performed on PB samples.
4.3.9. MFIR predicts overall survival (OS) but not CR duration

Additional logistic regression analysis was performed to determine if the MFIR could predict either CR duration or overall survival (OS). No predictive value for this measurement was seen for CR duration greater than or less than one year among the 126 CR patients (p = 0.15) However, an MFIR value ≤ 1.91 was able to predict patients who would achieve survival of > 1 year (the median survival for the entire group of 174 patients) (p < 0.0001, Table 4.8).

4.3.10. MFIR is an independent predictor of CR and NR

Finally, to determine if the MFIR determination was an independent predictor of the probability to achieve CR with induction chemotherapy a stepwise multiple logistic regression analysis was performed. In this analysis the variables considered in addition to the MFIR were, cytogenetics (good, intermediate or poor risk by MRC (UK) criteria [314]), age at diagnosis (≥ or < 60 years), presenting white blood cell count (≥ or < the median of 30 × 10⁹/L), and the presence or absence of FLT3 ITD. Only the MFIR value determined using either total MNC or CD3⁻CD19⁻ cells showed independent predictive value in this model (p < 0.0001 for both MFIR determinations, p ≥ 0.4 for all other variables).

4.4. Discussion

Although several ABC transporter genes (ABCA2, ABCA3, ABCB1, ABCB4, ABCB5, ABCB11, ABCC1~6, and ABCG2) are associated with chemotherapeutic drug
resistance, MDR1 and BCRP are the principal multidrug resistance (MDR) proteins that have been identified in AML blasts including leukemia stem cells (LSCs) [175, 200, 227, 320, 321]. In our own previous work QRT-PCR was used to measure expression of all 49 human ABC transporters thus far identified in AML blasts from newly diagnosed patients [228]. However, only MDR1 and BCRP expression in AML cells could be clearly associated with chemotherapy refractoriness [228] (Figure 4.1). Although this previous work clearly implicates ABC transporter function in the MDR phenotype so often seen in AML, the use of such data to enhance assessment of prognosis has not been widely adopted, likely at least partly due to the difficulty with clinical implementation and standardization of the techniques used.

In previous studies a variety of assays have been used to determine ABC transporter expression including QRT-PCR and flow cytometry using monoclonal antibodies specific for individual transporters [228, 305, 322, 323]. In the present analysis, flow cytometry was used to assess ABC transporter function. Mitoxantrone was selected as the ABC transporter substrate since its efflux from AML cells can be mediated by various transporters including both MDR1 and BCRP. In addition, our early comparison of mitoxantrone with daunorubicin and other reports suggested assays using the former drug would be more sensitive for measurement of drug efflux [215, 324]. Cyclosporine A was chosen as the transporter inhibitor since it inhibits the function of both MDR1 and BCRP. Thus, the combined use of mitoxantrone and cyclosporine A allows the assay to give a more global assessment of ABC transporter function than measurement of individual gene products. Consistent with this assumption, Figure 4.2
shows the good correlation observed between the MFIR efflux assay and QRT-PCR for MDR1 and BCRP. The MFIR assay requires small numbers of cells from clinical PB or BM samples that are routinely available from newly-diagnosed AML patients and technology (flow cytometry) which is widely available and frequently used in clinical hematology laboratories.

The goal of developing this assay was to produce a test that had strong predictive value for assessing the likely outcome of induction chemotherapy. As shown by Tables 4.3-4.6 this goal was accomplished for a large group of unselected newly-diagnosed AML patients. Importantly, when the analysis was restricted to patients with NK AML or over 60 years of age, two groups where additional prognostic information would be particularly useful, the predictive value was retained. Most of the samples analyzed in this retrospective study were cryopreserved PB cells from patients with relatively high circulating blast counts. In clinical practice it is expected that many AML patients would present with few circulating leukemia cells or a mixture of blasts and normal hematopoietic elements in PB and that fresh rather than cryopreserved samples would be analyzed. Thus, a series of experiments were done to compare MFIR values obtained from PB and BM or fresh and cryopreserved cells from the same patient. As shown in Table 4.1, no significant difference was seen between PB and BM samples suggesting that blasts from either source will give comparable results in the MFIR assay. Similarly as reported by others, cryopreservation did not change the MFIR value obtained demonstrating that samples routinely available for clinical diagnostics could be successfully used for testing [214, 319].
Because of concerns raised by previous reports suggesting that contaminating lymphocytes could increase the apparent ABC transporter expression in AML peripheral blood [325] and to ensure that the majority of PB cells used to calculate the MFIR were AML blasts, further experiments were done in which the MFIR was determined after gating out CD3^+ and CD19^+ T- and B- cells. As shown on Table 4.7, the MFIR retained its predictive value and may even have been improved when CD3^-CD19^- cells were analyzed with less than 15% of NK or elderly AML achieving CR when the MFIR was > 2.45 while the CR rate was approximately 90% in those with values \( \leq 2.45 \).

Considerable data have accumulated demonstrating that malignant hematopoiesis in AML is organized as a hierarchy with a primitive subpopulation of leukemia-initiating or ‘stem’ cells which maintain the leukemic clone [5, 7]. These candidate leukemia stem cells (LSC) display properties of relative chemotherapy resistance including cell cycle quiescence, over expression of ABC transporters and intrinsic mitoxantrone and daunorubicin efflux capacity [74, 163, 228]. Thus, LSC likely contribute to failure of induction chemotherapy and relapse after CR is obtained. In many AML samples the CD34^-CD38^- subpopulation of blasts is greatly enriched for LSC [5]. Thus, experiments were done to determine if the MFIR assessed in these cells would have enhanced value for predicting chemotherapy outcomes. Figure 4.1 shows that, consistent with our previous report, QRT-PCR values for MDR1 and BCRP appeared to show somewhat greater difference between CR and NR patient samples when the analysis was restricted to CD34^-CD38^- cells as compared to total blasts or CD34^-CD38^+ cells. Nevertheless, significant differences between chemotherapy responsive and refractory patient samples
were seen for all 3 cell populations. This difference between total blasts and CD34⁺CD38⁻ cells was less apparent when the MFIR was calculated for these populations (Figure 4.3, Table 4.3). This suggests that LSCs transmit at least some of their MDR phenotype to their progeny and that the MFIR assessed on total blast cells can be a good predictor of outcome to initial chemotherapy.

The logistic regression analysis was extended to determine if the MFIR could predict other treatment outcomes in addition to achievement of CR. As would be expected from the effect of the MFIR on CR rates, a significant association between high MFIR values and short OS was seen (Table 4.8). However, among 126 CR patients the MFIR did not predict remission duration. This result is consistent with the likelihood that relapse is mediated by subclones of drug resistant leukemia cells that emerge after the large majority of chemotherapy sensitive cells have been eliminated. Although we did not have the opportunity to measure the MFIR at relapse in this retrospective study, prior reports have documented increased ABC transporter expression in blasts from relapsed AML patients [196, 326, 327].

Lastly, to determine if the MFIR provided value for predicting the outcome of induction chemotherapy that was independent of other established prognostic variables in AML a stepwise multiple logistic regression analysis was performed. In this model which included cytogenetic risk group [314], age, WBC count, and FLT3 ITD as well as the MFIR as variables only the MFIR determined using either total MNC or CD3⁻CD19⁻ PB cells retained statistical significance. Although this result may appear surprising given the large number of studies validating the listed prognostic variables in AML it is
likely explained by the specific endpoint that was evaluated (achievement of initial CR rather than overall or disease free survival) and the relatively small number of patients in this study. In any case, the data validate the MFIR as determined in this study as a strong predictor of the likelihood of successful induction chemotherapy.

Thus, we believe that this rapid assessment of ABC transporter function is potentially a relevant assay to include in the work-up of newly-diagnosed AML patients. The MFIR test could be particularly useful for selecting therapy for patients where the toxicity of induction chemotherapy is predictably high; e.g. AML patients who are elderly or who have severe co-morbid illness, and may also be helpful for patients where other prognostic markers are either unavailable or unhelpful e.g. NK AML. A prospective evaluation of this MFIR testing using clinical laboratory services and a larger cohort of AML patients is planned.
Figure 4.1 mRNA expression levels for MDR1 (A) and BCRP (B) in AML patient samples

Expression levels in total PB mononuclear cells (blasts), CD34^+CD38^+, and CD34^+CD38^- cells from 14 CR (●) and 14 NR (■) patients were determined using QRT-PCR for MDR1 (A) and BCRP (B). Expression levels are set relative to GAPDH (set at 10^6). Horizontal lines represent the mean value for each group. P-values comparing CR and NR samples were determined using the Student t-test.
Figure 4.2 Correlation between QRT-PCR for ABC transporter expression and the median fluorescence intensity ratio (MFIR)

mRNA expression values as determined by QRT-PCR (normalized to GAPDH set at $10^6$) for MDR1 (A) and BCRP (B) in total mononuclear cells (blasts) from 28 AML samples are plotted against the MFIRs for the same samples (determined by calculating mitoxantrone-induced fluorescence in the presence of cyclosporine A divided by fluorescence in the absence of cyclosporine A as described in the Methods). Correlation co-efficients ($r$) and p-values (t-test) are indicated.
Figure 4.3 The MFIR in newly-diagnosed AML patients varies with response to induction chemotherapy

MFIRs of total PB mononuclear cells (blasts) and CD34⁺CD38⁻ cells from 14 complete remission (CR, ●) and 14 chemotherapy refractory (NR ■) newly-diagnosed AML patients. Values for individual patient samples and the means (horizontal lines) are shown for each group. P-values (t-test) comparing CR and NR patient samples are shown.
Table 4.1 Comparison of MFI ratio determined from marrow or peripheral blood (A) or fresh vs cryopreserved cells (B)

(A)

<table>
<thead>
<tr>
<th>Patient #</th>
<th>MFI ratio</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB</td>
<td>BM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
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<td></td>
</tr>
<tr>
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<td>2.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>1.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.2</td>
<td>2.1</td>
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</tr>
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<td>8</td>
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<td>1.6</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.4</td>
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<tr>
<td>12</td>
<td>1.1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>mean±SD</td>
<td>1.7±0.6</td>
<td>1.7±0.7</td>
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</table>

(B)

<table>
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<tr>
<th>Patient #</th>
<th>MFI ratio</th>
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<th>Frozen</th>
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<td>1.9</td>
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<td></td>
<td>BM</td>
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<td>1.6</td>
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<td>1.1</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

(A) The MFIR was determined from total MNCs in paired cryopreserved peripheral blood (PB) and bone marrow (BM) samples from 12 newly-diagnosed AML samples. (B) Fresh PB and BM samples from 3 patients were divided into two aliquots and the MFIR was measured immediately from one aliquot (fresh) while the second aliquot was cryopreserved, left frozen for several days and then thawed for analysis (frozen).
Table 4.2 AML patient characteristics (n=174)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs) Med (range)</td>
<td>57 (17 – 78)</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>89/85</td>
</tr>
<tr>
<td>Cytogenetics* number (% of total)</td>
<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>16 (9)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>131 (75)</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>18 (10)</td>
</tr>
<tr>
<td>Normal</td>
<td>80 (46)</td>
</tr>
<tr>
<td>Unknown/failed</td>
<td>9 (5)</td>
</tr>
<tr>
<td>FLT3 ITD** +/- (% +)</td>
<td>36/138 (21)</td>
</tr>
<tr>
<td>WBC (X10^9/L) at diagnosis Med (range)</td>
<td>30.6 (0.5 – 431)</td>
</tr>
<tr>
<td>Response to Induction number (% of total)</td>
<td></td>
</tr>
<tr>
<td>CR – 1 cycle</td>
<td>105 (60)</td>
</tr>
<tr>
<td>CR – 2 cycles</td>
<td>22 (13)</td>
</tr>
<tr>
<td>Induction failure</td>
<td>47 (27)</td>
</tr>
</tbody>
</table>

*prognostic groupings according to MRC (UK) criteria.

**Fms-like tyrosine kinase-3 internal tandem duplication.
Table 4.3 The MFIR is lower in AML patients achieving CR with one cycle of chemotherapy than in those requiring two cycles to enter CR or those refractory to induction chemotherapy

<table>
<thead>
<tr>
<th></th>
<th>Total MNC*</th>
<th>MFIR mean ± SD</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>N=174</td>
<td>CD34^+CD38^-</td>
<td>N=174</td>
<td>CD3^-CD19^-</td>
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<tr>
<td>CR (1 cycle)</td>
<td>1.62 ± 0.53</td>
<td>1.96 ± 0.80</td>
<td>1.62 ± 0.50</td>
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<tr>
<td>CR (2 cycles)</td>
<td>2.22 ± 1.29</td>
<td>2.21 ± 1.20</td>
<td>2.99 ± 1.47</td>
<td></td>
</tr>
<tr>
<td>Induction failure</td>
<td>2.59 ± 0.98</td>
<td>3.01 ± 1.30</td>
<td>3.15 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>P &lt; (T-test)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

*Total PB mononuclear cells (blasts).
Table 4.4 The MFIR Predicts Induction Failure in 152 AML patients

<table>
<thead>
<tr>
<th></th>
<th>Induction Failure (n=)</th>
<th>Complete Remission (n=)</th>
<th>% CR</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFIR &gt; 2.45</td>
<td>24</td>
<td>8</td>
<td>25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MFIR ≤ 2.45</td>
<td>23</td>
<td>97</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>47</td>
<td>105</td>
<td>69</td>
<td></td>
</tr>
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</table>

Peripheral blood mononuclear cell MFIR.
22 patients who required 2 cycles of chemotherapy to achieve CR were excluded from this analysis.
Table 4.5 The MFIR Predicts Induction Failure in 73 normal karyotype AML patients

<table>
<thead>
<tr>
<th></th>
<th>Induction Failure (n=)</th>
<th>Complete Remission (n=)</th>
<th>% CR</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFIR &gt; 2.45</td>
<td>12</td>
<td>5</td>
<td>29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MFIR ≤ 2.45</td>
<td>7</td>
<td>49</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>19</td>
<td>54</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

MFIR determined from total PB MNC.
Table 4.6 The MFIR Predicts Induction Failure among 69 AML patients ≥ 60 years old at diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Induction Failure (n=)</th>
<th>Complete Remission (n=)</th>
<th>% CR</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFIR &gt; 2.45</td>
<td>16</td>
<td>4</td>
<td>20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MFIR ≤ 2.45</td>
<td>7</td>
<td>42</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>23</td>
<td>46</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

MFIR determined from total PB MNC.
Table 4.7 MFIR determined on CD3^-CD19^- AML PB cells predicts outcome in NK and elderly AML patients

<table>
<thead>
<tr>
<th></th>
<th>Induction Failure</th>
<th>Complete Remission</th>
<th>% CR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFIR &gt; 2.45</td>
<td>8</td>
<td>1</td>
<td>11</td>
<td>0.001</td>
</tr>
<tr>
<td>MFIR ≤ 2.45</td>
<td>2</td>
<td>25</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>10</td>
<td>26</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

B) Patients ≥ 60 years (n=63)

<table>
<thead>
<tr>
<th></th>
<th>Induction Failure</th>
<th>Complete Remission</th>
<th>% CR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFIR &gt; 2.45</td>
<td>14</td>
<td>2</td>
<td>13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MFIR ≤ 2.45</td>
<td>5</td>
<td>42</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>19</td>
<td>44</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.8 The MFIR predicts overall survival (OS) ≥ or < 1 year

<table>
<thead>
<tr>
<th></th>
<th>OS &lt; 1 yr N = (% of total)</th>
<th>OS ≥ 1 yr N = (% of total)</th>
<th>Total</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFIR &gt; 1.91</td>
<td>44 (70)</td>
<td>19 (30)</td>
<td>63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MFIR ≤ 1.91</td>
<td>43 (39)</td>
<td>68 (61)</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>87 (50)</td>
<td>87 (50)</td>
<td>174</td>
<td></td>
</tr>
</tbody>
</table>

Logistic regression analysis was used to determine the MFIR value (from total MNC) that was most predictive of OS from diagnosis ≥ one year.
Chapter 5. Discussion and conclusions

Acute leukemia is a hematological malignancy that can be subdivided into various subgroups that have different clinical and prognostic features. In the past decades, there has been much improvement in the treatment of adult AL [328]. However, the overall survival rate of adult AL remains very low particularly for elderly patients (over 60 years of age) who comprise the majority of adult patients with AL [329]. Although the prognosis for younger and middle-aged adults has greatly improved with intensified chemotherapy during the past 3 decades, these approaches have provided little benefit to older adults (OS < 10%) [330]. Conventional intensive chemotherapy is an option for elderly patients only if they are fit. Moreover, resistance to chemotherapy represents a major cause of treatment failure in this patient population. Approximately 30% of these patients fail to enter remission because of early death or treatment-induced death [331]. In addition, elderly patients who experience significant adverse side-effects during the first cycle of induction chemotherapy are usually ineligible for additional treatment [332].

Cytogenetics and other biological or clinical features provide useful information in predicting the likelihood of CR and relapse and can be used in the development of risk-stratified treatment approaches [32]. However, the management of AL still stands for a major challenge to hematologists.

In this thesis, I attempted to provide new therapeutic and diagnostic strategies that could potentially change the standard of care for leukemia patients.
ALL, a malignancy of lymphoid cells, is characterized by excess lymphoblasts and occurs at any age. The overall incidence of ALL in the U.S. is 1.5 per 100,000 people with peak incidences at ages 3 to 5 and after 70 years. Ph+ ALL occurs less often in children (5%) than in adults (20%) [333]. The presence of the Ph chromosome confers a poorer prognosis compared to other cytogenetic or molecular abnormalities [334]. Single TKI agents (eg. imatinib and dasatinib) can give response rates between 20% and 30% but the response duration is short due to the emergence of drug-resistant leukemic subclones [256, 257]. Combinations of TKIs with conventional chemotherapeutic agents have shown more potential but the long-term success rate of combination therapy has yet to be described [335].

Therefore, in chapter 2, I evaluated the possibility of a synergistic effect between DT\textsubscript{388}IL3 and TKIs. The action mechanism of DT\textsubscript{388}IL3 is different from that of conventional chemotherapy drugs. DT\textsubscript{388}IL3 translocates to the cytosol after binding specifically to IL-3R and inactivates protein synthesis causing apoptotic death [136]. Although DT\textsubscript{388}IL3 was developed as potential therapy for AML (by exploiting the high IL-3R expression on AML blasts) [125, 130], it can also be used to treat Ph+ ALs that express IL-3R. DT\textsubscript{388}IL3 has been shown to synergize with cytarabine in targeting AML progenitors [246]. This observation inspired the hypothesis that combination DT\textsubscript{388}IL3 with a TKI may also be synergistic.

Firstly, it was necessary to determine if the IL-3R could be detected on Ph+ AL samples. IL-3R expression is a pre-requisite for treatment with DT\textsubscript{388}IL3, so blasts from 10 Ph+ AL samples were analyzed by FACS and QRT-PCR to determine if the receptor
subunits could be detected. These Ph+ AL samples expressed both subunits of the IL-3R at various levels suggesting that DT$_{388}$IL3 might target these cells effectively. Based on these results, subsequent experiments were performed to determine whether synergism could occur between DT$_{388}$IL3 and TKIs. Imatinib, dasatinib, and DT$_{388}$IL3 could all kill leukemic CFCs from patients with Ph+ AL. When used in combination with imatinib or dasatinib, the IC$_{50}$ of DT$_{388}$IL3 against Ph+ AL CFC was 3 or $>10$ fold lower, respectively, than the IC$_{50}$ for DT$_{388}$IL3 alone. The IC$_{50}$ of imatinib and dasatinib against Ph+ AL CFC was also $>3$ fold lower in these combinations demonstrating a striking synergy between the TKIs and DT$_{388}$IL3. By contrast, no synergy was observed against normal PB CFCs as the IC$_{50}$s of any of the 3 drugs did not change significantly. These results suggested that the combination of 2 therapeutic agents that have different mechanism of actions exhibits a greater selectivity for leukemic rather than normal progenitor cells.

Previous data have shown that rare subpopulations of ALL or AML blasts are able to initiate long-term growth in suspension cultures or engraft and proliferate in immunodeficient mice. These cells are considered as LSCs [63, 336-338]. LSCs are usually quiescent [74]. Because leukemia is sustained by the rare LSCs, which are resistant to therapeutic agents, this small population needs to be targeted rather than the majority of blast cells that have very limited proliferative ability [337].

The effectiveness of this combination therapy was tested against both quiescent and SC-IC sub-fractions of Ph+ AL samples. The IC$_{50}$s for all three drugs were higher for quiescent CFCs compared to cycling CFCs. However, the IC$_{50}$s and IC$_{90}$s for all drugs
administered in combination were markedly decreased in both quiescent and cycling cells suggesting that the synergistic combination can more efficiently eliminate quiescent stem cells than each drug acting alone. Additionally, 4 Ph+ AL samples were tested in long-term suspension culture to determine if the synergistic interaction could also be demonstrated in more primitive SC-ICs. Synergy could also be demonstrated for both the IC50 and IC90 of the drug combination against these primitive progenitors. In summary, the results in this chapter demonstrate the possibility that DT388IL3 may enhance the effectiveness of TKIs in Ph+ ALs that otherwise have a dismal prognosis. In previous studies, DT388IL3 has been shown to synergize with cytarabine to eliminate AML progenitors. Thus, these data were consistent with prior reports that support the rationale that the unique mechanism of DT388IL3 makes combination therapy more powerful than conventional chemotherapy [142, 246]. The data were clearly demonstrated the potential synergism between DT388IL3 and TKIs, however, sample numbers were too small, and all results were from in vitro experiments. Thus, future studies with more samples and in vivo models are necessary to reproduce these data and to reinforce the concept of this regimen.

The treatment of AML patients includes at least one course of intensive induction chemotherapy or more. A standard form of induction therapy includes the use of continuous infusion cytarabine administered intravenously for 7 days combined with daunorubicin for 3 days [292]. Initial remission rates following standard induction chemotherapy range from 50% to 75% in AML. However, most patients relapse within 2 years and the long term disease-free survival is less than 30%. Although there have been
many efforts to improve the OS rate including the use of alternative anthracyclines or anthracenediones, cytarabine intensification, or addition of other drugs such as etoposide, fludarabine or cladribine, none of them have enabled improved outcomes [60, 339].

In previous studies, the idea that the efficacy of drug combinations could be affected by the ratio of the drug concentrations was introduced. A 5:1 cytarabine:daunorubicin molar ratio was found to be the most synergistic for AML both in vitro and in vivo [148, 154]. In the free cytarabine:daunorubicin cocktail, each drug is metabolized differently in vivo, resulting in suboptimal or ineffective drug ratios at tumor sites. However, the synergistic 5:1 cytarabine:daunorubicin ratio could be maintained for at least 48 hours in blood plasma if engineered liposomes encapsulating the two drugs at the synergistic ratio were administered [153, 278]. CPX-351 is a liposomal preparation of a fixed combination of cytarabine and daunorubicin, which is developed according to the concept of ratiometric dosing.

In chapter 3, I compared the in vitro cytotoxicity of CPX-351 and of the conventional cytarabine:daunorubicin cocktail against primary leukemia cells from AML patients and normal hematopoietic progenitors in collaboration with the Celator pharmaceutical company. Peripheral blood leukemia blast cells were obtained from 7 AML patients who achieved CR following standard induction chemotherapy and 6 AML patients (NR) who did not respond to induction therapy.

Although the NR samples were less sensitive than CR samples to CPX-351 and the conventional free cocktail of drugs, CPX-351 produced similar AML CFC kills to those obtained with free drugs for both CR and NR AML samples. This result was
different from \textit{in vivo} results which showed that CPX-351 had a greater efficiency than the free cocktail [153, 154]. This difference is likely due to the different test system as \textit{in vitro} conditions may not affect the molar ratio of free cocktails within the short time the AML cells are exposed. This result also confirmed that liposomal encapsulation did not reduce the efficacy of cytarabine and daunorubicin.

Next, I evaluated the selectivity of CPX-351 in leukemic and normal cells in response to a previous report showing that intracellular daunorubicin accumulation was higher in leukemic cell lines engrafted in mice than in normal murine BM cells after treatment with CPX-351 [279]. I treated 6 G-CSF mobilized normal PB and 1 BM samples with the same concentration of CPX-351 and free cocktail drugs. The percent kill of normal CFCs was lower in the CPX treated group compared to the free cocktail treated group at each equivalent concentration and the \( IC_{50} \) was >5 fold higher for CPX-351. These results were in agreement with those of the previous report [279]. To support these results, intracellular daunorubicin accumulation was measured by HPLC in AML and normal cells. There was not a significant difference between daunorubicin accumulation in AML versus normal cells treated with free cocktail. However, there was a large difference between AML and normal cells treated with CPX. These findings suggest that CPX-351 is superior to free cocktail in terms of selectivity to malignant cells which has been a major hurdle to overcome in cancer therapy. Thus, CPX-351 may be used at higher doses to overcome drug resistance in AML without toxicity to normal blood cells. This is important especially for approximately 20\% of chemotherapy
refractory patients and for older patients who may not tolerate aggressive induction therapy.

As I mentioned earlier, LSCs comprise a relatively chemotherapy drug resistant cell type, partially due to their quiescence state and high level activity of drug efflux, and may be the source of leukemia relapse [74, 337, 340]. Thus, eradication of LSCs is the key for therapy leading to a complete cure. A common phenotype for LSC has not been identified due to the heterogeneity in cell surface protein expression. However, the cells that initiate leukemia in immunodeficient mice generally have a CD34⁺CD38⁻ or CD34⁺CD38ₕₖₖ phenotype for most AML subtypes [74, 336-338]. Accordingly, I compared the sensitivity to CPX-351 and free cocktails between the different subpopulations of AML blasts including the LSC-enriched CD34⁺CD38⁻ subpopulation. All subpopulations, including CD34⁺CD38⁻, CD34⁻ and CD34⁺CD38⁻, from NR patients showed less drug sensitivity than those from CR patients which is consistent with the clinical chemotherapy refractoriness seen in NR patients. However, all subpopulations from both CR and NR patients had similar drug sensitivity to CPX and free cocktail which was consistent with the results from the CFC assay. Subsequently, I examined the same subpopulations from normal PB and BM cells to investigate whether there is a difference in toxicity between CPX and free drugs in normal cells. All 3 subpopulations and total mononuclear cells were significantly less sensitive to CPX-351 compared to the free drug cocktail and the IC₅₀ for CPX-351 was >5 fold higher than that of the free drug cocktail in all subpopulations. Due to limited number of samples tested, these results should be verified with more samples in the future. If these observations are valid, CPX-
351 may be administered in the clinic with expectations of prolonged remissions and less hematopoietic toxicity compared to conventional cytarabine:daunorubicin induction therapy.

The mechanism of action explaining CPX-351’s selectivity for AML has not been elucidated so far. However, certain receptors that are highly expressed in AML cells, such as the class B scavenger receptor or low density lipoprotein receptors were reported to be associated with the binding and uptake of anionic liposomes [266, 279, 341]. As CPX-351 is an anionic liposome, these receptors are candidate receptors for CPX-351. Future study is needed to define the relevance of these receptors in the uptake of CPX-351 and the selectivity for leukemia cells.

In conclusion, the data presented in this chapter decisively shows that CPX-351 has the same efficacy as a free drug cocktail in killing AML progenitors and LSCs regardless of their responsiveness to induction chemotherapy. Interestingly, CPX-351 elicited lower toxicity to normal hematopoietic progenitors while the free drug cocktail killed normal progenitors as well as leukemic progenitors. Current clinical trials of CPX-351 in elderly and relapsed AML patients will help confirm whether or not the in vitro data can predict the effectiveness of this promising new agent for AML therapy.

AML is a complicated and heterogeneous group of clonal disorder and is one of the most common types of adult leukemia [292]. Currently, the prognosis for patients with AML is predicted by the number of parameters including cytogenetics, age, molecular abnormalities, white blood cell count, performance status, and presence of previous hematological disorder. Among these parameters, abnormal cytogenetics is
considered the most powerful predictors of the response to therapy and clinical outcome in AML [294, 342, 343]. However, 40-50% patients who do not have an abnormal karyotype are classified in intermediate-risk group but wide variability in response to induction therapy and survival has been observed in this group [191, 296, 344]. Thus, more precise prognostic distinctions are needed.

Over 50% of patients with AML are over 60 years of age. Elderly patients have a poorer prognosis than younger adults and have a different set of disease features including a higher incidence of expression of unfavorable cytogenetics, a high rate of multidrug resistance, increased rate of treatment-related complications, and higher mortality after initiation of induction therapy [51, 295]. For this reason, pre-treatment assessment is especially important for elderly patients. Based on this assessment, customized treatment approaches could determine whether standard induction chemotherapy, molecularly targeted therapy, or palliative treatment is most appropriate for each elderly AML patient. Accurate prediction of disease prognosis is ultimately useful for clinicians in therapeutic decision-making [345].

One of the most promising prognostic factors in predicting treatment outcome is the degree of responsiveness to induction therapy. MDR, intrinsic or acquired resistance of the cancer cells to functionally and structurally different anticancer agents, has emerged as a negative prognostic factor for predicting the outcome of leukemia patients [158, 346]. There are many mechanisms of MDR, including increased expression of drug transporters, enhanced detoxifying mechanisms, alterations in DNA repair processes, activation of anti-apoptotic pathways and changes in cell cycle regulation. Among these,
ABC transporter mediated drug efflux, which causes a decrease in cellular drug accumulation and toxicity may be a critical mechanism of drug resistance in AML patients [161, 303]. However, the clinical importance of ABC transporters still remains controversial [313]. Some reports found that ABC transporter expression was highly associated with treatment outcome [215, 228, 323, 347-349] while others did not [197, 350, 351]. One of the main reasons of the inconsistency in published data is the shortage of a standardized, specific and reliable detection method.

In chapter 4, I applied a functional flow cytometric drug efflux assay for prediction of outcome from initial treatment in AML patients. The aim of this study was to develop a quick, clinically applicable method to predict patients’ responsiveness to chemotherapy prior to the availability of the results of other conventional laboratory tests such as cytogenetics. Thus, QRT-PCR was not a good choice for prediction of MDR due to the complexity of the procedure and the broad variety of ABC transporter genes expressed in AML. Hence, I focused on the functional flow cytometry assay. This assay is based on the efflux of mitoxantrone, a chemotherapeutic drug in clinical use, and a substrate for principal multidrug resistance proteins such as MDR1, BCRP1 and MRP1 [214]. I also evaluated several inhibitors of ABC transporter function including verapamil, PSC-833, fumitremorgin C, and CA. Among them, CA treatment resulted in the biggest difference in the MFI between cells treated with mitoxantrone with and without an inhibitor (Figure 5.2). This result may be explained by the fact that CA is a broad-spectrum inhibitor of ABC transporters such as MDR1, BCRP, and MRP1 while verapamil and PSC-833 are specific inhibitors for MDR1, and fumitremorgin C has
specificity for BCRP only. The median fluorescence intensity of mitoxantrone-stained cells was measured with and without CA and a ratio calculated between these 2 values (MFIR) (Figure 5.3). This functional assay is more useful than a molecular or immunological test because it evaluates the ABC transporter activity directly by measuring the drug retention or efflux of cells. I predicted that the NR patient samples would express ABC transporters at a high level and thus be more sensitive to the inhibitor so that the MFIR values would be higher in this group than in CR patients.

To determine if the MFIR value would be vary between AML blasts from CR and NR patients I chose 28 AML samples for which the treatment outcome was already known (14 CR and 14 NR). A QRT-PCR test for MDR1 and BCRP and the MFIR analysis were performed on each sample. As predicted, significant differences between CR and NR samples were observed using both techniques [192, 215, 226, 227, 269, 319, 323, 347, 348]. I also observed a significant correlation between QRT-PCR for MDR1 or BCRP and the MFIR.

In total, I determined the MFIR in 174 patients. As expected, CR1 patients (achieved CR after 1 cycle of induction therapy) showed low MFIR, CR2 patients (required 2 cycles to achieve CR) intermediate and NR patients high MFIRs. Accordingly, I recognized that different MFIRs among patients with different outcomes could be used to predict patients’ responsiveness before starting induction therapy. A previous report that used a similar method to evaluate the function of ABC transporters in AML cells did not demonstrate a correlation with intracellular drug accumulation and response to chemotherapy [352]. This may be caused by some differences between this assay and
mine. First, they used daunorubicin as a substrate for ABC transporters, but my preliminary results and data from others [187, 215, 324] showed that mitoxantrone elicited a sharper difference of fluorescence between cells treated with mitoxantrone alone and mitoxantrone plus an ABC transporter inhibitor compared to daunorubicin (Figure 5.1). That is the reason I used only mitoxantrone as a substrate for efflux in subsequent experiments. Second, they incubated cells longer for drug efflux (16 hours), which might cause more drug efflux even in the CR patient samples and decrease the difference between the CR and NR groups. Third, they compared only the final fluorescence values (drug + modulator) between CR and NR groups. However, in my experience, the basal fluorescence level without modulator (drug only) is heterogeneous in patient samples. Usually, NR samples have lower basal levels than CR samples because of their higher ABC transporter activity. Thus, comparison of fluorescence with or without a modulator in the same sample would be a more precise way to measure ABC transporter activity. Lastly, their patient number was too small (22 CR and 20 NR patients, respectively), which made their statistics less powerful.

Logistic regression is a technique in statistics for making predictions. In logistic regression, there are one or more independent variables (predictors) that predict the dichotomous dependent variable (outcome or response). Logistic regression analysis is used for predicting the category of outcome for individual cases in medical science [353]. In line with this, a logistic regression model was performed in collaboration with a statistician to determine whether the MFIR (predictor) could predict the achievement of CR (outcome). A threshold of 2.45 that could predict the outcome of initial induction
chemotherapy in AML was calculated using a total of 152 AML samples (CR1 and NR patients). Patients under the threshold level usually achieved CR (81%) while only 25% patients over threshold obtained CR (P < 0.001). This indicates that MFIR may predict the responsiveness to induction chemotherapy in AML patients with statistical significance.

As mentioned earlier, about 50% of AML patients have a normal cytogenetics making outcome prediction using cytogenetic information difficult. Therefore, the predictive value of MFIR is more important for these patients. 88% of this group of patients with MFIR ≤ 2.45 achieved CR, but only 29% of patients with MFIR > 2.45 obtained CR. These results indicate that MFIR may be clinically meaningful and could be used to stratify patients where the karyotype is less useful. Several reports also mentioned the role of ABC transporters in the prognosis of AML patients with normal karyotype [192, 296, 354].

Prediction of CR in elderly AML patients using MFIR was of interest to me because the majority of AML patients are elderly. Furthermore, elderly patients have an increased frequency of unfavorable cytogenetics, a higher rate of cellular MDR, and a greater frequency of mortality after initiation of induction chemotherapy. All of this contributes to the observed extremely poor treatment outcome when compared to younger patients [300]. Thus, predicting the responsiveness to conventional chemotherapeutic regimens is critical in elderly patients as less aggressive therapies or palliative care may be employed if a patient is unlikely to response to standard induction chemotherapy. The probability of CR was predicted quite accurately with the MFIR
assay in these patients so this assay can be regarded as a promising predictive test for elderly AML.

This assay functions as a screening test therefore minimizing false positive and negative results needs to be addressed. MFIR values from total MNC, CD34⁺CD38⁻ and CD3⁻CD19⁻ cells were compared to determine which subpopulation gave the assay superior predictive power. AML is a stem cell disease and LSC enriched CD34⁺CD38⁻ subpopulation express various ABC transporters at higher level when compared to more differentiated subpopulation and total blasts [163, 175]. Thus, I predicted that the MFIR calculated in this subpopulation may have enhanced predictive power. In fact, CD34⁺CD38⁻ cells displayed a similar difference of MFIR between CR and NR when compared with total MNC and the subpopulation was deemed unlikely to improve the predictive power of the test. Generally, it has been demonstrated that CD34⁺CD38⁻ populations have enhanced expression of several ABC transporters which are down-regulated on differentiation to the CD34⁺CD38⁺ subpopulation [305, 355]. However, the proportion of this subpopulation is very small so the initial responsiveness to chemotherapy depends mainly on the responsiveness of more committed cells, the majority of total blasts.

Contaminating lymphocytes in the total MNC population may affect the overall MFIR so analysis with CD3⁻CD19⁻ cells was expected to improve this assay. The MFIR determined on AML samples excluding CD3⁺ and CD19⁺ lymphocytes increased the probability of predicting induction failure in both normal karyotype and elderly AML patients. This suggests that lymphocytes may have a different level of ABC transporter
expression which affects the MFIR of total MNC and interferes with precise analysis of AML blasts [325].

While CR duration was not predicted by the MFIR, OS was predicted by this assay. This finding is consistent with previous studies [192, 356, 357] which showed the prognostic value of ABC transporter expression in AML for this endpoint.

Finally, only MFIR was determined to be an independent predictor of CR in stepwise multiple logistic regression analysis that included cytogenetics, age, WBC count, and FLT-3 mutation as well as MFIR as variables [27, 28, 358-361]. This result may appear to be inconsistent with previous studies which demonstrated significance for the listed prognostic variables. However, the specific aim of this study (for predicting initial CR rather than the duration of CR, overall survival, or disease free survival) and the relatively small number of patients analyzed here may explain this contradiction.

In conclusion, the assay presented in this chapter could be an improved method for predicting initial responsiveness to chemotherapy in AML and may be beneficial in assessing the likely benefit of this treatment, particularly for elderly patients and those with a normal karyotype. However, this study was done by myself, therefore, future large-scale studies are necessary to verify the reproducibility, reliability and usefulness of this test in clinical practice.

5.1. Concluding remarks

Overall, my work achieved 3 objectives: to examine the synergetic effect of DT388IL3 with TKIs against Ph+ AL, to compare the efficacy and selectivity of CPX-351
with free cytarabine and daunorubicin cocktails in primary human AML cells and to develop a clinically applicable method for predicting treatment outcome in AML patients.

Based on my studies, the following conclusions are reached. Firstly, DT₃₈₈IL3 has a synergistic effect with imatinib and dasatinib in Ph+ AL cells expressing the IL-3 receptor, but not in normal hematopoietic progenitors. Secondly, CPX-351 has a similar effect to a free drug cocktail and is selective in killing leukemic rather than normal hematopoietic cells. Thirdly, MFIR calculated from a functional flow cytometric assay measuring mitoxantrone efflux is a promising predictor of treatment outcome in AML patients.

Although my studies reported in this thesis were completed individually, it was part of one unified aim of improving treatment outcome in both ALL and AML. Thus, I hope these findings play an important role in developing better therapeutic measures for acute leukemia.
Figure 5.1 Comparison of the sensitivity of fluorescence detection between daunorubicin and mitoxantrone with or without CA
Figure 5.2 Comparison of ABC transporters inhibitors
Figure 5.3 The principle of MFIR assay
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