Targeting Susceptible Signaling Pathways in Chronic Lymphocytic Leukemia

by

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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University of Manitoba
Winnipeg, Manitoba

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Thesis Summary

Chronic lymphocytic leukemia (CLL) is a cancer of B cells and is the most common leukemia in North America. Current therapies are fraught with challenges, and drug resistance and disease relapse remain common occurrences. Therefore, novel therapies and novel therapeutic strategies are needed to improve CLL therapy. Better yet, therapies targeted at specific weaknesses of CLL cells will ensure maximum efficacy and minimum adverse toxicity.

To this end, this thesis focuses on targeting the susceptible BCR pathway and lysosome-mediated cell death pathway using gefitinib and lysosomotropic agents, respectively.

Firstly, the novel use of the tyrosine kinase inhibitor gefitinib was explored. This drug was most effective in aggressive ZAP-70+ CLL cells and cell lines. A similar inhibitor, erlotinib, had no effect in CLL. Gefitinib inhibited phosphorylation of Syk and ZAP-70, prevented downstream kinase activation, and suppressed the pro-survival BCR response. ZAP-70 is implicated in the mechanism of action of gefitinib as introduction of ZAP-70 into a B cell line increased their sensitivity to gefitinib.

Secondly, the novel strategy of targeting lysosomes was explored. The lysosomotropic drugs siramesine, nortriptyline, desipramine, mefloquine, and tafenoquine were all found to induce cytotoxicity and lysosome permeabilization. Lysosome permeabilization was accompanied with lipid peroxidation and followed by loss of mitochondrial membrane potential. Compared with healthy B cells, CLL cells were more sensitive to this cell death pathway. This was potentially due to the overexpression of SPP1 and overproduction of sphingosine, which destabilized lysosomes.

Lastly, this thesis explored the clinical utility of these targeted therapies. Both gefitinib and siramesine were more effective in CLL cells than patient T cells. Furthermore, they retained efficacy amid protective stromal cells. Clinical correlations revealed that gefitinib and siramesine
were effective in CLL cells with poor prognostic features. Siramesine was more effective in male cells and in previously-treated cells. Gefitinib was most effective in young patients.

Overall, work presented herein demonstrates the efficacy of the tyrosine kinase inhibitor gefitinib and lysosomotropic agents in primary CLL cells. This work investigates the altered biology of the BCR pathway and lysosomes in CLL cells, and takes advantage of these weaknesses using targeted therapies.
Dedication

This thesis is dedicated to my family who have blessed me with genuine love and support.

My father Richard- you showed me how to work hard and make my own opportunities.

My mother Christine- you showed me that it was possible to work and raise a family.

My sister Jocelyn- you showed me the importance of doing what I love.

My husband Tyler- you showed me that life is better with a partner.

My son Seth- you showed me that family is above all else.

Lastly, I dedicate my work to all cancer patients and their families. Cancer sucks.

I hope research like that presented in this thesis is a source of hope.
Acknowledgements

I thank my supervisor Dr. Spencer Gibson for his guidance and support. Under his supervision, I learned so much and grew more independent.

I thank the Gibson lab manager Liz Henson for always sharing my excitement when results were good and listening to my complaints when results were bad.

I thank all the past and present members of the Gibson lab for giving me experimental advice and creating a welcoming and stimulating work environment.

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I thank the members of my graduate committee Drs. Aaron Marshall, Versha Banerji, and Abdel Soussi Gounni. Your advice and support were invaluable.

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I acknowledge the employees in the Manitoba Tumor Bank and CancerCare Manitoba for their work in acquiring, processing, and organizing CLL patient blood samples.

I thank all of the people who donated blood for my study. My research would not have been done without these samples.
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<td>AAD</td>
<td>Aminoactinomycin D</td>
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<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>AIF</td>
<td>Apoptosis-Inducing Factor</td>
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<td>ALL</td>
<td>Acute Lymphocytic Leukemia</td>
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<td>APAF-1</td>
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<td>Antigen-Presenting Cell</td>
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<td>APRIL</td>
<td>A Proliferation-Inducing Ligand</td>
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<td>ASM</td>
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<td>ATG</td>
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<td>β2M</td>
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<td>BLNK</td>
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<td>CDR</td>
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<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>Erk</td>
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<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<td>Fas-Associated protein with Death Domain</td>
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<td>FC</td>
<td>Fludarabine Cyclophosphamide</td>
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<td>FCR</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FDA</td>
<td>Federal Drug Administration</td>
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<td>FISH</td>
<td>Fluorescence In-Situ Hybridization</td>
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<td>Abbreviation</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>cyclin-G Associated Kinase 1</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>Grb2</td>
<td>Growth Factor receptor-Bound protein 2</td>
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<td>GVHD</td>
<td>Graft Versus Host Disease</td>
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<td>GWAS</td>
<td>Genome-Wide Association Study</td>
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<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<td>HEL</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>Horse Radish Peroxidase</td>
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<td>Inhibitor of Apoptosis Protein</td>
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<td>IC50</td>
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<td>Immunoglobulin Variable Heavy chain</td>
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<td>Immunoreceptor Tyrosine-based Activation Motif</td>
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<td>Lymphocyte Kinase</td>
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<td>LMP</td>
<td>Lysosome Membrane Permeabilization</td>
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<td>Monoclonal B cell Lymphocytosis</td>
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<td>MLKL</td>
<td>Mixed Lineage Kinase domain-Like</td>
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<td>mTOR</td>
<td>Mammalian Target Of Rapamycin</td>
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<td>Nonidet P-40</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
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<td>NZB</td>
<td>New Zealand Black</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PD-L1</td>
<td>Programmed Death Ligand 1</td>
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<td>PE</td>
<td>Phycoerythrin</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
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<tr>
<td>PLCγ</td>
<td>Phospholipase C γ</td>
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RAG  Recombination-Activating Genes
RBC  Red Blood Cell
RIP  Receptor-Interacting Protein kinase 1, 2, or 3
ROR-1  Receptor tyrosine kinase-like Orphan Receptor 1
ROS  Reactive Oxygen Species
S1P  Sphingosine-1-Phosphate
SCF  Surviving Cell Fraction
SDF-1  Stromal cell-Derived Factor 1
SDS  Sodium Dodecyl Sulfate
SFM  Serum-Free Medium
SH2  Src Homolog 2
Shc  SH2 domain Containing
SK  Sphingosine Kinase
SKI  Sphingosine Kinase Inhibitor
Smac  Second Mitochondrial-derived Activator of Caspase
SPP1  Sphingosine-1-Phosphate Phosphatase 1
Syk  Splenic Tyrosine Kinase
TCR  T Cell Receptor
TBS-T  Tris-Buffered Saline with Tween
TFEB  Transcription Factor EB
TLR  Toll-Like Receptor
TNFR1  Tumor Necrosis Factor Receptor 1
ULK1  Unc-51-Like autophagy activating Kinase 1
UVRAG  UV Radiation resistance-Associated Gene
VEGF  Vascular Endothelial Growth Factor
ZAP-70  Zeta chain Associated Protein 70
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Chapter 1

1. Introduction

1.1. Cancer

Cancer is a disease that 2 of every 5 Canadians will fight in their lifetime, and 1 of every 4 Canadians will fight and lose. Cancer is the leading cause of death for Canadians. Research has defined cancer as a group of diseases characterized by un-controlled cell growth. It is the only disease known that can affect any cell at any time.

A cancer of blood cells is termed a leukemia. White blood cells of both myeloid and lymphoid origin can transform into a leukemia, and these can be acute or chronic in nature. An acute leukemia has rapid onset and progression. In contrast, a chronic leukemia has slower progression. There are four major types of leukemia: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL). AML includes myeloblastic leukemia, promyelocytic leukemia, myelomonocytic leukemia, monocytic leukemia, erythroleukemia, and megakaryocytic leukemia. CML can include chronic myelomonocytic leukemia and chronic granulocytic leukemia. Rare leukemia include large granular lymphocyte leukemia, prolymphocytic leukemia, and hairy cell leukemia. Of all these leukemia, chronic lymphocytic leukemia (CLL) is the most common.

1.2. Chronic Lymphocytic Leukemia

Chronic Lymphocytic Leukemia (CLL) is a cancer of B lymphocytes and is the most common leukemia in North America and Europe. In 2010 approximately 2,195 Canadians were diagnosed with this disease; this is a rate of approximately 6 in every 100,000 Canadians. However, a higher incidence rate has been noted in some provinces, such as Manitoba. This
disease is predominant in the elderly population of European descent and is twice as common in males as in females. The median age of diagnosis can vary depending on the center and the country and ranges from 65 to 73 years of age \(^5,7\). The annual cost per American patient can range up to 44,000 US dollars \(^8,9\), and thus CLL presents a large economic burden to the health care system. Furthermore, CLL patients have a significantly lower survival and health-related quality of life as compared to age-matched population \(^6,8\)–\(^10\).

CLL is defined by a clonal B cell population of at least \(5 \times 10^9\) B cells per litre of peripheral blood \(^4\). These B cells are shown by diagnostic flow cytometry to express markers of activated and antigen-experienced B cells such as the typical B cell surface proteins CD19, CD23, and CD20 and the T cell surface protein CD5 \(^11,12\). These cells are determined by flow cytometry to be monoclonal by the presence of the same immunoglobulin light chain \(^13\). In the case where a clonal population of B cells is observed in an enlarged lymph node but has not exceeded \(5 \times 10^9\) cells/litre in the peripheral blood, this disease is termed Small Lymphocytic Leukemia (SLL). Many consider SLL and CLL to be the same entity, clinically \(^4,14\).

CLL is divided into different stages based on its presentation. These stages are termed Rai stages after Dr. Kanti Rai who developed the staging system in North America in 1975 \(^15\). Rai stage 0 is the least advanced stage and is defined by only high lymphocyte count, termed lymphocytosis. Rai stage I is defined by lymphocytosis and enlarged lymph nodes. Rai stage II is defined by lymphocytosis, enlarged spleen, and possibly enlarged lymph nodes and liver. Rai stage III is defined by lymphocytosis, anemia, and possibly enlarged lymph nodes, spleen, and liver. Rai stage IV is the most advanced stage and is defined by lymphocytosis; thrombocytopenia; enlarged spleen, lymph nodes, and liver; and possibly anemia. Upon physical examination, patients with lymph nodes of greater than 5 cm in diameter and spleen greater than
6 cm below the costal margin are said to have bulky disease. A similar system using the Binet stages A, B, and C was developed in 1981 and is used in Europe. Of note, the Rai stages define anemia as < 11 g/dl of hemoglobin while the Binet stages define anemia as < 10 g/dl of hemoglobin. Both staging systems define thrombocytopenia as a platelet count of <100 x 10^9/L. These staging systems are summarized in Table 1.1.

Table 1.1. Risk, clinical characteristics, and median survival of Rai stages 0, I, II, III, and IV and Binet stages A, B, and C in CLL, adapted from 18.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Risk</th>
<th>Clinical Characteristics</th>
<th>Median Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rai - 0</td>
<td>Low</td>
<td>Lymphocytosis</td>
<td>&gt; 10 years</td>
</tr>
<tr>
<td>Rai - I</td>
<td>Intermediate</td>
<td>Lymphocytosis, lymphadenopathy</td>
<td>6-7 years</td>
</tr>
<tr>
<td>Rai - II</td>
<td>Intermediate</td>
<td>Lymphocytosis, hepatomegaly/splenomegaly</td>
<td></td>
</tr>
<tr>
<td>Rai - III</td>
<td>High</td>
<td>Lymphocytosis, anemia</td>
<td>2-3 years</td>
</tr>
<tr>
<td>Rai - IV</td>
<td>High</td>
<td>Lymphocytosis, anemia, and thrombocytopenia</td>
<td></td>
</tr>
<tr>
<td>Binet - A</td>
<td>Low</td>
<td>Lymphocytosis and &lt; 3 areas involved</td>
<td>&gt; 10 years</td>
</tr>
<tr>
<td>Binet - B</td>
<td>Intermediate</td>
<td>Lymphocytosis and &gt; 3 areas involved</td>
<td>5-6 years</td>
</tr>
<tr>
<td>Binet - C</td>
<td>High</td>
<td>Lymphocytosis, anemia, and thrombocytopenia</td>
<td>3-4 years</td>
</tr>
</tbody>
</table>

i – Areas are defined as lymph nodes, spleen, and liver.

ii – Data obtained from reference 18

CLL was once thought to be a disease where the cells resisted cell death and thus accumulated. However, a recent study has shown that CLL is indeed a proliferative disease. All CLL patients studied had at least 10^9 new CLL cells born each day. Birth rates varied from 0.1 to over 1.0% of the CLL clone each day. With proliferation, CLL cells can acquire new features and evolve. This clonal evolution can occur in 17% to 19% of CLL cases and this frequency increases in drug-treated cohorts. Furthermore, it is not rare for CLL cases to harbour more than one CLL clone; small cohort studies have found that 13-14% of CLL cases are not monoclonal. Researchers have proposed that CLL subclones compete and can become the dominate clone with time and after treatment intervention. These clones were identified as CLL but harboured distinct immunoglobulin variable heavy chain genes, and thus
were distinct clones. These small CLL subclones can harbour adverse features and negatively correlate with patient overall survival 29.

In 5-10% of CLL cases, the leukemia can transform into an aggressive lymphoma. Of these cases, 80% of the aggressive lymphoma are derived from the CLL clone, whereas 20% are distinct 30. This transformation, called Richter’s transformation, is frequently a transformation from CLL to Diffuse Large B Cell Lymphoma (DLBL or DLBCL). Rarely, CLL transforms into Non-Hodgkin’s lymphoma 31.

CLL can present with various comorbidities, additional cancers, and autoimmune diseases. At diagnosis, up to 89% of CLL patients present with at least one comorbidity. The most common comorbidities are coronary artery disease/peripheral vascular disease, hypertension, heart disease, diabetes mellitus, chronic obstructive pulmonary disorder, gastrointestinal complications, joint problems, and an additional cancer 32. These are expected due to the elderly age of patients. The most common second cancer is non-melanoma skin cancer, followed by gastrointestinal cancers, breast cancer, prostate cancer, and lung cancer 32,33. Even when compared with another similar disease, follicular lymphoma, CLL patients had a nearly two-fold increased risk of second cancers. Treatment further increased the risk 33. In 5-10% of CLL cases autoimmune diseases are present. These include autoimmune hemolytic anemia, immune thrombocytopenia, pure red cell aplasia, and autoimmune granulocytopenia. These demonstrate further breakdown of the immune system, particularly of immune tolerance. The primary causes of death to CLL patients are progressive disease, second cancers, and infections 7,33–35.

Approximately one third of CLL patients will require treatment upfront, while others may never require treatment at all 36. A meta-analysis of trials investigated immediate or delayed
treatment intervention and found no advantage to early treatment versus delayed treatment for early stage CLL. Signs that treatment should begin include disease progression characterized by anemias, massive/symptomatic organ and lymph node involvement, increase in serum lactate dehydrogenase, and other disease-related symptoms including unintentional weight loss, extreme fatigue, and fevers. The choice of treatment is made based on a number of different factors such as fitness, disease aggressiveness, and the quality-adjusted life years (QALYs). Fitness can be assessed using the Cumulative Illness Rating Scale (CIRS) for comorbidities and Eastern Cooperative Oncology Group (ECOG) system for performance status. Disease aggressiveness is indicated by advanced Rai stage, lymphocyte doubling time of less than 6 months, and the presence of poor prognostic markers. QALY captures the health gains by cancer treatments and can be used for cost-effectiveness analysis. For relatively young and fit patients, treatment with fludarabine + cyclophosphamide + rituximab (FCR) is the standard approach. For unfit patients, treatment includes a different anti-CD20 antibody in combination with chlorambucil. For elderly patients, treatment includes an anti-CD-20 antibody and bendamustine. Upon relapse, patients can be treated with a different combination therapy, perhaps including novel agents such as kinase inhibitors. Patients with refractory disease that has relapsed multiple times may be candidates for an allogeneic stem cell transplant. These treatments will be discussed in detail in sections 1.11 and 1.12.

1.3. CLL Etiology

CLL is preceded by Monoclonal B cell Lymphocytosis (MBL, Figure 1.1), which is defined when a clonal population of B cells exists but is less than $5 \times 10^9$ B cells per litre of peripheral blood. In a landmark prospective study, MBL was found in blood samples months to years prior to the diagnosis of CLL in 44 of the 45 CLL cases. The prevalence of MBL is
3.5% of adults over the age of 40 \(^{42,43}\), and 5.5% in adults over the age of 60 \(^{43}\). However, a more sensitive flow cytometry technique found a MBL frequency of 12% over the age of 40 \(^{44}\).

Depending on gene expression, MBL cells can harbour a classic CLL phenotype, an atypical CLL phenotype, or an non-CLL phenotype \(^{42,45}\). An MBL clone can harbour many genetic features of CLL \(^{46}\). The rate of transformation from MBL into CLL is 1-2% each year. The majority of individuals with MBL will never develop a hematological malignancy \(^{45}\).

Figure 1.1. Development of CLL. Within the bone marrow, a hematopoietic stem cell (HSC) can give rise to a common lymphoid progenitor (CLP) which can give rise to B cells, T cells, and NK cells. CLL develops from the malignant transformation of B cells. First, B cells within 3-12% of adults transform into monoclonal B cell lymphocytosis (MBL). Next, 1-2% of MBL cases transform into CLL cells annually. In 17-19% of CLL cases, the CLL clone can change and evolve. Finally, 5-10% of CLL cases can transform into more aggressive lymphomas, such as diffuse large B cell lymphoma (DLBL or DLBCL).

The cause of MBL and CLL is unknown, although genetic factors are implicated because up to 5% of CLL patients have a family history of leukemia \(^{47,48}\). However, to date no genetic study has found any polymorphism or mutation to explain all familial CLL. Early candidate gene association studies created much debate as some studies found positive genetic links between a gene or mutated gene and risk of CLL, which did not hold true in subsequent studies. One such
study identified chromosome 2q21.2 as being associated with inherited CLL, but no causative genes at this locus have been elucidated. In addition to chromosome 2q21.2, chromosomes 6p22.1 and 18q21.1 were also statistically significant. These correspond to major histocompatibility (MHC) locus and SMAD family member genes, respectively. The first Genome-Wide Association Study (GWAS) in CLL identified 6 loci that were associated with the risk of developing this disease: 2q13, 2q37.1, 6p25.3, 11q24.1, 15q23, and 19q13.32. A follow-up study added another 4 loci: 2q37.3, 8q24.21, 15q21.3, and 16q24.1. The products of only 4 of these 10 loci are known: SP140, IRF4 PRKD2, and FARP2. A later GWAS found risk loci that corresponded to ACTA2/FAS, BCL2, C11orf21, LEF1, CASP10/8, CDKN2B-AS1, PMAIP1, BMF, QPCT, and ACOXL. A recent meta-analysis of GWAS, the largest to date, found additional risk loci including those that corresponded to EOMES, SERPINB6, LPP, BCL2L11, BANK1, and CSRNP1. In addition to the identification of risk alleles, additional studies have performed whole-exome sequencing and deep sequencing to identify putative CLL cancer drivers: DDX3X, IKZF3, MYD88, NOTCH1, POT1, RPS15, SF3B1, XPO1, among others. Much work remains to be done to assess the role of the protein products in the development and progression of CLL. All of these genetic studies demonstrate that CLL genetics are not simple, and no single gene accounts for CLL heredity or risk.

1.4. B cell and CLL cell Immunobiology

Within the bone marrow, hematopoietic stem cells (HSCs) can differentiate into a common myeloid progenitor or a common lymphoid progenitor (CLP; Figure 1.1). Exposure of a CLP to IL-7 generates a pro-B cell. This cell expresses B220/CD45R. Upon rearrangement of the immunoglobulin heavy chain genes, as discussed later, the pro-B cell becomes a pre-B cell. This pre-B cell expresses the immunoglobulin heavy chain on its surface with a surrogate light chain, and signaling through this complex is requirement for further B cell development.
Upon rearrangement of the immunoglobulin light chain genes, this pre-B cell becomes an immature B cell. If a rearrangement does not result in a productive protein product, the genes on the second allele are rearranged. If the first rearrangement is productive, allelic exclusion ensures that the second allele is silenced thus ensuring that each B cell only expresses one BCR. Provided that each rearrangement is productive and the cell receives pro-survival BCR signals, this immature B cell can exit the bone marrow and migrate throughout the periphery into the spleen, lymph nodes, and elsewhere. Initially, the immature B cell only expresses IgM on its surface. However, upon exit from the bone marrow the mature B cell also expresses IgD. A mature B cell can develop into either a memory B cell or antibody-secreting plasma cells. Mature B cells can be subdivided into different innate-like B cell subsets in mice such as marginal zone B cells, follicular B2 cells, and CD5+ B1a and CD5- B1b cells. Within these subsets, there are also IL-10-secreting B regulatory cells. In humans, these subdivisions have yet to be fully defined particularly in the case of B1 cells, as differences in protein expression and isolation difficulties have made this task a challenge.

This B cell development is altered in CLL. The alterations begin at the HSC. One group found CLL-driver mutations in multipotent hematopoietic progenitors. Indeed, isolated hematopoietic stem cells (HSCs) from CLL patients produce monoclonal or oligoclonal B cells when engrafted into mice. It is still an open question as to whether the HSCs become altered after CLL progression, or if this is an initiating step in tumorigenesis. Another study found higher frequencies of pro-B cells in the bone marrow of 12 out of 13 CLL patients. CLL cells alter two key B cell immunobiological functions: the ability to process and present antigen; and the ability to select unmutated, stereotyped, and poly-reactive immunoglobulins.
1.4.1. CLL Antigen Presentation

Antigen presentation involves the presentation of a peptide antigen in the groove of an MHC molecule to a T cell receptor (TCR). All nucleated cells in the human body, except for neurons, express MHC class I and present antigens to a TCR on a CD8+ T cell. However, only antigen-presenting cells (APCs) express MHC class II and present antigens to a TCR on a CD4+ T cell. Professional APCs are dendritic cells, macrophages, and B cells. The least efficient of these is the B cell. B cells are able to re-activate T cells but unlike dendritic cells, B cells are unable to present antigen and activate naïve T cells \cite{67,68}. Compared with dendritic cells, B cells have a lower density of MHC class II protein and costimulatory molecules \cite{67}. CLL cells are even further inefficient due to the decreased expression of costimulatory molecules such as B7 \cite{69} and the increased expression of inhibitory ligands such as CD200, CD270, CD274, and CD276 \cite{70}. CD40 stimulation of CLL cells can improve their antigen presentation ability by increasing the expression of co-stimulatory molecules and adhesion molecules \cite{71}. Furthermore, while CLL cells have normal expression of the Fc receptors CD32 and CD23 \cite{72} and the lipid antigen presentation molecule CD1d \cite{73}, they lack expression of the BCR \cite{72} which may limit their ability to bind and endocytose soluble antigens for the purpose of antigen presentation. CLL cells have less than 10% of the surface BCRs found on normal B cells \cite{72}. Despite this deficiency, a well-designed experiment using anti-light chain antibodies showed that CLL cells and normal B cells could uptake and present the same amount of soluble antigen by their BCR \cite{72}. CLL cells can also present various different types of antigens including HSV antigen \cite{74}, Rh protein \cite{75}, and the most common antigen in autoimmune hemolytic anemia: erythrocyte protein band 3 \cite{76}. However, CLL cells cannot present and stimulate tetanus toxoid-specific T cells \cite{69}. Healthy B cells and healthy T cells can bind and communicate by forming an effective immune synapse. However, CLL T
cells with healthy B cells or healthy T cells with CLL B cells both form defective immune synapses. Overall, CLL cells are not inefficient antigen presenters, but are defective communicators.

1.4.2. CLL Immunoglobulin

An immunoglobulin is expressed on the surface of a B cell as the B cell receptor (BCR) or secreted as an antibody. The immunoglobulin is unique to each B cell clone. It dictates the antigen reactivity of that clone. As each B cells develops they combine a unique set of BCR genes, employ numerous mechanisms to create diversity, and then induce somatic hypermutations in a process of affinity maturation. Each immunoglobulin has two chains: heavy chain and light chain. Each chain is composed of a variable domain and a constant region.

The variable domain on the heavy chain is composed of a V gene, D gene, and J gene. The variable region on a light chain is composed of a V gene and a J gene. This V(D)J recombination is mediated by Recombination-Activating Genes 1 and 2 (RAG1 and 2). The heavy chain genes are on chromosome 14 in the human. The light chain has two flavours: kappa genes found on chromosome 2 and lambda genes found on chromosome 22. As with normal B cells, CLL cells use kappa light chain more frequently than lambda. Within these gene groups, there are numerous different V, D, or J genes to choose from. This selection itself creates diversity. Additional diversity is generated by the imprecise joining of these regions; random deletions, insertions, or palindromic additions can be made at the D and J junction, and the V and D junction. In 20-30% of CLL cases, certain immunoglobulin genes are more frequently used and thus those BCRs are considered stereotyped. The largest report to date found 952 subsets; however, only 19 of these were deemed major subsets which defined 41% of the stereotypes.
In addition to stereotypy of heavy chain genes, there is also stereotypy of light chain genes in CLL \(^78\) (Figure 1.2).

Aside from gene rearrangement, diversity can be further achieved in a process termed affinity maturation. Following antigen exposure, a B cell can somatically hypermutate the variable region of its immunoglobulin genes. This somatic hypermutation is used to increase affinity for the target antigen and occurs within germinal centers in secondary lymphoid organs \(^81\). Approximately 40\% of CLL patients harbour clones that do not somatically hypermutate their BCR, and are thus termed unmutated. Interestingly, somatic hypermutation decreases with age \(^82\), which may help explain why CLL is only a disease of adults. Un-mutated CLL BCRs are poly-reactive, and when mutated BCRs are reverted back to their germline sequence, they are also poly-reactive \(^83\). Several groups have shown that CLL BCRs can bind to internal epitopes \(^84,85\), instead of external antigens, thus creating a model where the BCR is autoreactive and can crosslink with other BCRs on the surface of the same CLL cell or on neighbouring CLL cells. Normally, autoreactive B cells can edit and mutate their receptor, die, or become anergic \(^86-89\).

The sites with concentrated hypermutation are termed complementarity-determining regions (CDRs). The V genes encodes CDR1 and CDR2, while the V-D-J joining creates CDR3 (Figure 1.2). The heavy chain CDRs and light chain CDRs form the antigen-binding site \(^90\). Two different types of CDR3 sequence patterns are present in CLL. The first type is termed “mainly combinatorial” and is encoded by unmutated sequences in the D region and the J region. This CDR3 pattern is present in stereotype subset 8. The second is termed “combinatorial and junctional” and is encoded by mutated sequences in the D region, but similar sequences in the non-templated (N) regions between V-D and D-J regions. This CDR3 pattern is seen in stereotype subset 4 \(^80\).
Constant domains dictate the class of antibody be it IgM, IgD, IgG, IgA, or IgE. Each B cell begins with IgM and IgD, and can then class switch to any other immunoglobulin class. This class switch is a permanent change, as the other possible constant regions are deleted in the process. Class switch recombination and somatic hypermutation are both mediated by Activation-Induced cytidine Deaminase (AID, Figure 1.2). Some CLL cells express AID, and while some researchers have found a correlation between AID expression and mutational status, others have not. However, AID expression is associated with class switch recombination. CLL cells express IgM or IgM and IgD, and can class switch to IgA or IgG.

Figure 1.2. Immunoglobulin gene rearrangements and CLL alterations. V(D)J genes are rearranged to produce the heavy and light chains of an immunoglobulin. CLL cells alter this process by using selective V(D)J genes (stereotypy) and avoiding somatic hypermutations (yellow lines), which results in poly-reactive and auto-reactive immunoglobulins. These alterations are shown in red.
CLL cells resemble mature B cells, not the antibody factories that are plasma cells. Therefore, there is a low expectation of CLL cells to secrete antibody. CLL cells can secrete antibody upon stimulation with the toll-like receptor (TLR) 9 ligand CpG, interferon (IFN)-α, IFN-γ, phorbol ester, CD40 + TLR7 ligand, CD40 + TLR9 ligand, normal allogeneic helper T cells, and pokeweed mitogen. CLL cells can secrete anti-erythrocyte antibodies, but this does not account for all cases of autoimmune hemolytic anemia. In addition, CLL cells have been shown to secrete poly-reactive antibodies that can bind to double stranded DNA, single stranded DNA, histones, cardiolipin, constant domain of IgG, and B-(1,6)-glucan of yeast and fungi. Both IgVH mutated and unmutated CLL cases can secrete antibodies. CLL cells have been shown to differentiate into plasma cells in vivo.

1.5. Cell of Origin

The cell of origin for MBL and CLL is not known. Some believe the cell of origin of CLL is a memory B cell due to their gene expression and methylation patterns; however, one study used CD27 as a marker of memory cells which is also a marker for marginal zone B cells. Others believe the cell of origin is splenic marginal zone B cells or B1 cells due to their poly-reactive BCRs and antibodies. Likewise, the autoreactive BCRs may indicate that the CLL cell of origin is a pre B cell, as 55-75% of these B cells produce autoreactive immunoglobulins. Still others believe that CLL cells originated from regulatory B cells due to their expression of IL-10. The expression of the BAFF receptor on CLL cells indicates that these cells may arise from immature or mature B cells which express BAFF receptor (BAFFR), and not pre B cells which lack BAFFR. One study found that CLL cells have numerous different methylation signatures, similar to the continuum found in different B cells types.
Therefore, CLL cells may have different cells of origin. There is also the theory that unmutated CLL and mutated CLL derive from different B cell types. Overall, there is much debate over the cellular origin of CLL and this requires significant further study.

1.6. B Cell Receptor Pathway

The B cell receptor (BCR) pathway (Figure 2.1) is a series of signaling events initiated by the BCR. This pathway results in either apoptosis, anergy, or activation and proliferation. Its function is to regulate B cell fate and to activate the B cell in response to its target antigen. The pro-survival BCR response is mediated through PI3K and Akt, Erk, and NFκB. The pro-cell death BCR response is mediated by phosphatase and tensin homolog (PTEN) and involves mitochondrial dysfunction and caspase-dependent apoptosis. This cell death occurs during negative selection which removes auto-reactive B cells, or during activation-induced cell death which ensues after prolonged BCR crosslinking without T cell help. A basal low level of BCR signaling is required for B cell maintenance; however, the role of antigen in this process is debated and requires further investigation.

The BCR pathway is initiated by ligation of the BCR by antigen. The BCR can bind to soluble antigens, antigens that are tethered by complement, or presented by macrophages. Upon crosslinking, the BCRs are recruited to lipid rafts where the co-receptors CD79a and CD79b, also known as Igα and Igβ, and CD19 are phosphorylated by the lipid-anchored kinase Lyn. A single BCR is associated with a single Igα/Igβ heterodimer. This phosphorylation occurs on tyrosine residues within Immunoreceptor Tyrosine-based Activation Motifs (ITAMs). These tandem phosphorylated tyrosines provide docking sites for the SH2 domain of the kinase Syk, which can then autophosphorylate and phosphorylate other targets such as the adaptor proteins BLNK/SLP65, Shc, Grb2, and BCAP. Src kinases such as Lyn can also
phosphorylate adaptor proteins such as Bam32. These events serve to amplify the signaling cascade by providing numerous binding sites for other adaptors and enzymes. BLNK recruits the tyrosine kinase Btk, the lipid kinase PLCγ which leads to calcium mobilization, and the adaptor Vav which induces actin reorganization. She binds Grb2, and Grb2 binds the Ras guanine nucleotide exchange factor Sos which leads to activation of the Ras-Raf-Mek-Erk pathway. BCAP and the membrane protein CD19 recruit PI3K. Bam32 leads to Erk activation, and Rac and cdc42 activation which induces cytoskeletal rearrangements. Activated Erk, NFκB, and NFAT translocate into the nucleus to activate gene transcription.

CLL cells highjack this BCR pathway to induce constitutive activation and prolong their survival (Figure 2.1). This is accomplished by alterations in the B cell receptor and in its downstream signaling kinases. CLL cells ectopically express kinases such as Lck and ZAP-70. CLL cells overexpress the adaptor protein BLNK and the kinase Lyn. In addition, CLL cells have been shown to overexpress the kinase Syk, but this has been disputed. However, Syk is constitutively phosphorylated on tyrosine. Moreover, CLL cells can mutate IκBe, an inhibitor of NFκB, rendering NFκB more active.

Therefore, CLL cells alter the BCR and several downstream adaptors and kinases (Figure 1.3). The numerous alterations downstream of the BCR are not necessarily redundant in function and serve to amplify different aspects of the BCR pathway, and also serve to amplify other pathways such as those responding to cytokines and growth signals. This highlights the BCR pathway as a therapeutic target in CLL.
In a normal B cell, engagement of the BCR on the plasma membrane with antigen results in crosslinking and recruitment into a lipid raft. There, Lyn phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) on the co-receptors CD79a and CD79b. These phosphorylates tyrosines provide docking sites for Syk, which can then in turn autophosphorylate and phosphorylate downstream adaptors such as BLNK and kinases such as Btk. The membrane protein CD19 is also phosphorylated by Lyn, and provides docking sites for the lipid kinase PI3K. The pathway is amplified and results in the downstream activation of the transcription factors Erk, NFAT, and NFκB. CLL cells alter this pathway by expressing BCRs that are often stereotyped and/or unmutated. In addition, they ectopically express kinases Lck and ZAP-70, and over-express and constitutively activate many downstream kinases and adaptors, resulting in tonic signaling.
1.7. Zeta chain-associated protein 70

Zeta chain-associated protein 70 (ZAP-70) is a tyrosine kinase and homolog of Syk. This kinase is normally expressed in T cells, Natural Killer (NK) cells\textsuperscript{144,145}, and in pro/pre-B cells. These early B cell precursors express ZAP-70 which is later replaced by Syk in mature B cells\textsuperscript{146–148}. However, some small mature B cell subsets in the tonsils and spleen can express ZAP-70\textsuperscript{148,149}, and this expression can increase with stimulation of the BCR\textsuperscript{150}, CD40L, and CpG stimulation of TLR9\textsuperscript{149}. In addition, knockdown of Syk does not remove all B cells; knockdown of both Syk and ZAP-70 is required to prevent all B cell development in mice\textsuperscript{146,148}. This shows that although Syk is the dominant kinase in B cells, ZAP-70 still may play a role.

The role of ZAP-70 in CLL cells has been debated. ZAP-70 associates with CD79b after BCR stimulation\textsuperscript{151} and increases tyrosine phosphorylation and BCR signaling\textsuperscript{151–153}; however, one group showed that expression of ZAP-70 with or without its kinase domain both increased BCR signaling\textsuperscript{154}. Furthermore, ZAP-70 is inefficiently activated in CLL cells as compared to Syk, but its ability to recruit downstream mediators such as PI3K and Shc is intact\textsuperscript{155}. Moreover, ZAP-70 can prolong the activation of kinases such as ERK and Akt\textsuperscript{155}. Thus, the kinase function of ZAP-70 may not mediate the increased BCR signaling; instead it is possible that ZAP-70 could play the role of an adaptor protein.

In addition to promoting BCR signaling, ZAP-70 expression in CLL can promote migration. ZAP-70+ CLL cells are more responsive to chemokines signals, such as CXCL12\textsuperscript{156}. ZAP-70+ CLL cells migrate and adhere better to stromal cells\textsuperscript{157,158}. The role of the ZAP-70 kinase domain in the migration response has yet to be elucidated.
1.8. Cell Death Pathways in CLL

Not only do CLL cells depend on the BCR pathway for survival, but they alter many different cell death pathways to prolong their survival. These cell death pathways include apoptosis, necroptosis, and autophagic cell death (Figure 1.4).

**Figure 1.4.** CLL cells alter apoptosis, necroptosis, and autophagic cell death pathways. CLL cells alter apoptosis by overexpressing Bcl-2 and IAPs while downregulating Smac. CLL cells alter necroptosis by overexpressing IAPs and downregulating CYLD and RIP3. CLL cells alter autophagy by overexpressing ATG4, UVRAG, and ULK1. For illustrative purposes, upregulations are shown in green and downregulations are shown in red.

### 1.8.1. Apoptosis

Apoptosis is the most common and well-studied form of programmed cell death. This form of cell death is energy-dependent and involves distinct morphological and biochemical features. During apoptosis, the morphology of the cell changes as it begins to shrink and chromatin condenses and fragments. This eventually results in membrane blebbing and the
production of apoptotic bodies. There are three main biochemical pathways that initiate apoptosis: the extrinsic pathway involving death receptors, the intrinsic pathway involving mitochondria, and the perforin/granzyme pathway.\textsuperscript{159,160}

The extrinsic pathway begins with ligation of a death receptor and subsequent activation of caspase 8. Death receptors include Fas ligand (FasL), tumor necrosis factor receptor 1 (TNFR1), and death receptors 3, 4, 5, and 6.\textsuperscript{161,162} The protein Fas-associated protein with death domain (FADD) recruits pro-caspase 8 to the receptor for the death-induced signaling complex.\textsuperscript{163} This allows for dimerization, proteolytic cleavage, and activation of caspase 8.\textsuperscript{164} Activated caspase 8 can then in turn cleave numerous substrates within the cell, such as the executioner caspase 3, and initiate loss of mitochondrial membrane potential. The anti-apoptotic inhibitor of apoptosis proteins (IAPs) bind and inhibit caspases.\textsuperscript{159}

The intrinsic pathway begins with loss of mitochondrial membrane potential induced by reactive oxygen species, growth factor withdrawal, irradiation, or therapeutic agents. Pro-apoptotic proteins such as Bad, Bax, and Bid oligomerize in the outer mitochondrial membrane and form pores. This can be inhibited by Bcl-2 family members. As mitochondrial membrane potential is loss, proteins such as cytochrome C, Smac, and apoptosis-induced factor (AIF) are released into the cytoplasm.\textsuperscript{165} Cytochrome C facilitates the binding of APAF-1 and procaspase 9, which allows for the cleavage and activation of caspase 9.\textsuperscript{166} Caspase 9 can proceed to cleave many substrates within the cell, such as caspase 3. Smac inhibits IAPs, preventing their inhibition of caspases.\textsuperscript{167} AIF enters the nucleus and degrades DNA.\textsuperscript{168} Therefore, intrinsic apoptosis can depend on the actions of either caspases or AIF.

Lastly, apoptosis can be initiated by perforin and granzymes released by Natural Killer (NK) cells or cytotoxic CD8+ T cells. Perforin introduces pores within membranes, allowing the
entry of granzymes into the cell. Like caspases, granzymes are proteases which cleave numerous cellular substrates including Bid and caspases \(^\text{169,170}\).

CLL cells are more susceptible to apoptosis due to the altered expression of numerous apoptotic machineries. The anti-apoptotic proteins Bcl-2 and IAPs are up-regulated \(^\text{171,172}\), while the pro-apoptotic protein Smac is downregulated in CLL cells compared to healthy B cells \(^\text{173}\) (Figure 3.1). Bcl-2 can bind and inhibit pro-apoptotic proteins that are responsible for forming pores in the mitochondrial membrane, causing loss of mitochondrial membrane potential. IAPs can inhibit the pro-apoptotic proteases caspases. Smac is a protein released from permeabilized mitochondria which inhibits IAPs.

### 1.8.2. Necroptosis

Necroptosis is a modified and regulated form of necrosis. By definition, it requires either receptor interacting protein kinase 1 (RIP1) or RIP3 and results in morphological changes including rounding of the cell, swelling of the cytoplasm, and rupture of the plasma membrane \(^\text{174,175}\).

Like the extrinsic apoptotic pathway, necroptosis is initiated by the ligation of FasL or TNFR1. Unlike apoptosis, necroptosis can only ensue if caspase 8 is inhibited, deleted, or not functional. IAPs ubiquitinate RIPK1, while cylindromatosis (CYLD) removes the ubiquitin chains \(^\text{176}\). RIP3 phosphorylates and binds RIP1 thus forming the necrosome \(^\text{174}\). This complex phosphorylates and activates the mixed lineage kinase domain-like (MLKL), which plays a pivotal in mediating calcium influx during necroptosis \(^\text{177–179}\).

CLL cells may be capable of undergoing necroptosis, a regulated form of necrosis that functions in the absence or inhibition of caspase 8. It has been shown that PI3K promotes survival of CLL cells by preventing caspase 8 activation \(^\text{180}\). However, CLL cells may avoid
necroptosis by down-regulating two key components: RIP3 and CYLD. CYLD deubiquitinates RIP1 allowing it to bind with RIP3 forming the necrosome. The formation of the necrosome is vital to phosphorylating and activating MLKL.

1.8.3. Autophagy

Autophagy is the cellular process of self-digestion. In moderation, autophagy helps the cell to recycle and reuse components. However, excess autophagy can destroy vital cellular resources and result in cell death. Autophagy can be stimulated by starvation, reactive oxygen species, endoplasmic reticulum stress, or inhibition of mammalian target of rapamycin (mTOR) or class I phosphatidylinositol 3-kinase PI3K. Autophagy initiation requires UNC51-like kinase (ULK1), autophagy-related protein 13 (ATG13), ATG101, and FAK family kinase interacting protein of 200 kDa. Further nucleation around the cytoplasmic contents targeted for degradation involves the class III PI3K complex, Beclin-1, and Barkor. This is blocked if Bcl-2 binds to Beclin-1. The elongating phagophore membrane requires the ATG5-ATG12 complex, which recruits LC3-I, which is converted to LC3-II by ATG3. LC3-II, or LC3-PE, is formed when the soluble cytosolic LC3-I is lapidated with phosphatidylethanolamine (PE). Fusion of the autophagosome with a lysosome accomplishes the degradation of the autophagosome contents.

CLL cells express all the required machineries for functional autophagy, and in some instances this can promote drug resistance. CLL cells alter the pathway of autophagy by overexpressing three vital component of autophagy: ATG4C, ULK1, and UVRAG (Figure 3.1). ATG4 cleaves LC3 producing LC3I thus priming it for lipidation. ULK1 phosphorylates Beclin-1 and promotes autophagosome formation. UVRAG binds to phosphorylated Beclin-1.
and promotes autophagosome maturation. Furthermore, high expression of PIK3C3, PIK3R4, and Beclin-1 is associated with poor patient prognosis and aggressive disease.

Thus, CLL cells have devised many ways to avoid apoptosis and necroptosis, and they may upregulate autophagy-related genes as a way to use this pathway for survival and drug resistance (Figure 1.4). One form of cell death that has yet to be investigated in CLL is lysosome-mediated cell death.

1.9. Lysosome Biology

Lysosomes are the most acidic vesicles within the cell. This acidic pH is maintained by the action of a proton pump which hydrolyzes ATP to ADP in order to pump an H+ ion into the lumen of the lysosome. The lysosomal membrane consists of a lipid bilayer and membrane proteins. The most abundant lysosomal membrane proteins are lysosome-associated membrane proteins 1 and 2 (LAMP1 and 2). The inner lumen of these proteins is highly glycosylated and protects the lysosomal membrane from the digestive enzymes within. These enzymes can digest DNA, RNA, sugars, lipids, and proteins. Among these enzymes is the diverse cathepsin protease family. Cathepsins A and G are serine proteases, meaning that their active site contains a vital serine. Cathepsins B, C, F, H, K, L, O, S, V, X, and W are cysteine proteases. Cathepsins D and E are aspartic protease. Cysteine cathepsins are most stable and active at an acidic pH. Like caspases, cathepsins have a wide range of cellular substrates. Cystatins, thyropins, and serpins prevent cathepsin substrates from binding and are thus endogenous inhibitors of cathepsins.

Lysosome biogenesis is controlled by master regulators transcription factor EB (TFEB) and microphthalmia-associated transcription factor (MITF). These proteins receive cues in the cytoplasm and translocate into the nucleus to induce the transcription of lysosome biogenesis
genes\textsuperscript{196–198}. TFEB and MITF are phosphorylated by mTOR in the cytoplasm and retained there by binding 14-3-3 proteins\textsuperscript{199}. The cues that initiate their nuclear translocation are autophagy and lysosome permeabilization, but the complex signaling biology is not completely understood.

The function of the lysosome is dictated by its contents: digestive enzymes. Lysosomes can fuse with endosomes to degrade material bound by receptors. Lysosomes can fuse with phagosomes or autophagosomes to degrade extracellular or intracellular material, respectively. Lysosomes can fuse with the plasma membrane to deliver enzymes to degrade the surrounding matrix. Lysosomes can also be permeabilized and lead to cell death\textsuperscript{200,201}.

Lysosome Membrane Permeabilization (LMP) involves either the slight or the complete permeabilization of the lysosome. This permeabilization can cause lipid peroxidation and a partial or complete release of lysosomal contents. Cell death can be mediated by the reactive oxygen species and/or lysosomal cathepsins\textsuperscript{202–204}. Various cellular components can protect the lysosome from permeabilization such as cholesterol\textsuperscript{205}, lysosomal-localization of heat shock protein 70\textsuperscript{206}, and lipid peroxidation scavengers. Tocopherols are endogenous inhibitors of lipid peroxidation. Among tocopherols is \(\alpha\)-tocopherol, otherwise known as vitamin E\textsuperscript{207,208}.

Cancer cells are sensitive to LMP by a variety of mechanisms. Cell lines transformed with oncogenic Src and Ras display altered lysosome localization and decreases in LAMP-1 and LAMP-2\textsuperscript{209}. Decreases in the LAMP proteins prime cells for LMP. Other cancer cells increase lysosome biogenesis\textsuperscript{198,210,211} and alter heat shock protein 70 (HSP-70) localization creating destabilized lysosomes\textsuperscript{206}. Due to these numerous alterations to this pathway, LMP is an effective way to kill many different cancer cell types. These include breast cancer\textsuperscript{207,212,213}, ovarian cancer\textsuperscript{212}, cervical cancer\textsuperscript{212}, colon cancer\textsuperscript{209,212–214}, prostate cancer\textsuperscript{212}, lung cancer\textsuperscript{213,215,216}, bone cancer\textsuperscript{212}, skin cancer\textsuperscript{213}, and AML\textsuperscript{210}. 

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LMP can be induced by numerous different stimuli that are collectively called lysosomotropic agents. Characteristics of a chemical that can cause lysosomotropism include a neutral partition coefficient and a basic pKa between 6.5 and 11. Lysosomotropic agents include metal nanoparticles, kinase inhibitors ML-9, and numerous different types of pharmaceuticals. Pharmaceutical lysosomotropic agents include the anti-depressants siramesine, nortriptyline, desipramine, imipramine, and clomipramine; anti-malarials mefloquine and chloroquine; anti-allergy terfenadine; the stilbenoid antioxidant pterostilbene, and anti-psychotics chlorpromazine, thioridazine, and aripiprazole. The use of these agents is summarized in Table 1.2. Aside from chloroquine, none of these lysosomotropic agents have been investigated in lymphoid cancers. The use of chloroquine in cancer has been reviewed elsewhere and was not included in Table 1.2.
Table 1.2. The use of lysosomotropic agents as therapeutics in cancer. A literature search of lysosomotropic agents tested in different *in vitro* and *in vivo* models at different doses.

<table>
<thead>
<tr>
<th>Lysosomotropic Agent</th>
<th>Model</th>
<th>Effective Doses</th>
<th>References</th>
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NOD: non-obese diabetic
SCID: severe combined immunodeficiency
wk: week
1.10. Disease Biomarkers and Prognostic Indicators

CLL is not a uniform disease and has a heterogeneous clinical course. Aside from the clinical Rai stage, CLL can be divided into aggressive and indolent categories based on biomarkers. These biomarkers include cytogenetics, serum proteins, plasma membrane proteins, kinase expression, and immunoglobulin status.

Cytogenetics are determined by Fluorescent In-Situ Hybridization (FISH) which detects chromosomal abnormalities in interphase nuclei. Chromosomal abnormalities can involve an entire chromosome, or just the long (q) arm or short (p) arm. These are present in up to 85% of patients. The most common cytogenetic abnormality in CLL cells is the loss of 13q14 which occurs in 45-55% of cytogenetically-abnormal cases. The next most common aberration is the loss of 11q23 which occurs in 15-18% of patients and results in a deletion of the DNA repair enzyme ATM gene. The third most common abnormality is trisomy 12 occurs in 13-16% of patients. The fourth most common abnormality is loss of 17p13 occurs in 7-10% of patients and results in deletion of the tumor suppressor p53 gene. Rare cytogenetic abnormalities include loss of 6q21. Patients with no cytogenetic abnormality or loss of 13q14 as the only abnormality have the best prognosis. Patients with loss of 11q23 or 17p12 have the most aggressive disease and worst survival. These cytogenetics can change over time as CLL cells are exposed to additional stresses, such as therapy.

Serum proteins levels can also serve as biomarkers in CLL. In particular, the level of β2 microglobulin (β2M) independently predicts progression-free survival. Levels above 3.5 mg/L are considered high. This protein is associated with the α chain of MHC class I molecules, but can be found in a soluble form in serum. In vitro stimulation of CLL cells induces β2M secretion. Elevated levels of any protein in the serum may indicate kidney malfunction, and
thus levels of β2M are often adjusted according to serum creatinine or glomerular filtration rate. When adjusted for the glomerular filtration rate, high levels of β2M predicted a shorter treatment-free survival. Levels can also be useful for predicting patient outcome when treated with FCR. Levels of β2M are higher in patients with bulky disease.

Aside from β2M levels, the levels of other proteins such as cytokines can also serve as biomarkers in CLL. One group showed that ratios of different cytokine groups predicted time to first treatment and overall survival. Prognosis can also be predicted by specific levels of cytokines such as IL-6, IL-8, B cell activating factor (BAFF), tumor necrosis factor α (TNFα), vascular endothelial growth factor (VEGF), and thymidine kinase.

Expression of certain plasma membrane proteins also has predictive value in CLL. The most studied is CD38. This protein is a cyclic ADP-ribose hydrolase which catalyzes the production of cyclic ADP ribose. CD38 is expressed on the surface of numerous different lymphocytes, but high CD38 expression on CLL cells is associated with aggressive disease and mortality particularly in low Rai stage patients. Various different cut-off values have been employed. A common cut-off is if >30% of cells express CD38 then that disease is CD38+, and if <30% of cells express CD38 then that disease is CD38-. However, one group has shown that irrespective of cut-off value, presence of CD38+ CLL cells indicated progressive disease. While some have shown that CD38 expression can change over the course of the disease, others have shown that they do not.

Kinase expression can also dictate important biological and clinical information about CLL. The most studied kinase in this regard is zeta chain-associated protein 70 (ZAP-70). The CLL clone is consider to be ZAP-70+ if at least 20% of the cells express ZAP-70 as determined
by intracellular staining and flow cytometry. However, methylation of the ZAP-70 gene, specifically at 223 nucleotides downstream of the transcription start site, is a better predictor than protein expression of time to first treatment and overall survival. ZAP-70 expression dictates shorter time to treatment, shorter progression-free survival, and shorter overall survival. Many have suggested that ZAP-70 expression is a surrogate maker for IgVH mutational status as the majority of IgVH unmutated CLL cases are ZAP-70+. The slight discordance can be explained by the presence other high-risk characteristics such as 11q or 17p deletion. ZAP-70 expression is 4.3-5.5 times higher in unmutated CLL cells than in mutated CLL cells and it is the most tightly discriminating gene between these two subsets.

Lastly, one of the most useful biomarkers in CLL is immunoglobulin status. As mentioned previously, B cells somatically hypermutate immunoglobulin genes to improve the affinity for the target antigen, particularly in the variable region of the immunoglobulin heavy chain (IgVH). However, some CLL clones can retain germline or unmutated IgVH, while others have mutated IgVH. This mutational status is determined by diagnostic polymerase chain reactions (PCR) and DNA sequencing; samples that have at least 98% sequence homolog with the nearest germline are considered un-mutated while samples with more than 2% discordance are considered mutated. IgVH Un-mutated CLL have more aggressive disease and a worse prognosis compared to mutated CLL. Interestingly, the gene expression profiles of unmutated CLL and mutated CLL cases are strikingly similar and intermingled. One study found only 175 genes differentially-expressed between unmutated and mutated cases. The most tightly discriminating gene between unmutated and mutated cases was zeta chain-associated protein 70 (ZAP-70).
While some biomarkers can change over the course of disease, the IgVH does not; CLL clones can acquire additional cytogenetic abnormalities and gain expression of ZAP-70 and CD38 over time but never change their mutational status. In contrast, MBL clones can show signs of continual ongoing mutation.

1.11. Chemotherapies and Immunotherapies in CLL

Fludarabine use was the first big advance in CLL treatment. Fludarabine is a nucleoside analog that mimics the purine nucleotide deoxyadenosine 5’ triphosphate. Upon DNA replication, ligation, or transcription, the active metabolite of fludarabine incorporates into the growing chain of nucleic acids and prevents DNA enzymes from completing their tasks. This prevents DNA replication, transcription, and repair thus inducing cell death.

The addition of cyclophosphamide to fludarabine (FC) greatly increased patient response and survival. Cyclophosphamide is a nitrogen mustard which crosslinks DNA by adding alkyl groups to guanine bases. This causes DNA damage, prevents DNA replication, and causes cell death.

The addition of rituximab to fludarabine and cyclophosphamide (FCR) further improved patient response and survival, and resulted in a lower level of minimal residual disease. These positive results occurred in both treatment-naïve cohorts and previously-treated cohorts. Although the achievement of minimal residual disease is an independent predictor of overall and progression-free survival, many argue that MRD is not enough, and instead a cure should be the aim. Rituximab is an anti-CD20 antibody which binds to CD20 on the surface of CLL cells. This facilitates complement fixation and phagocytosis.

Therefore, the first-line standard of care in CLL is FCR. Although FCR is the standard of care, drug resistance is common and toxicities limit its use in elderly and unfit
Regimens appropriate FCR-ineligible patients include chlorambucil, bendamustine, and other antibodies.

Chlorambucil is a nitrogen mustard and acts as an alkylating agent like cyclophosphamide. It is not often used as monotherapy, but instead is used in combination with anti-CD20 antibodies to treat elderly, unfit patients.\textsuperscript{285}

Bendamustine is a unique drug in CLL as it contains a chemical group of an alkylating agent and also a chemical group of a nucleoside analog.\textsuperscript{286} As a single agent, it is superior than chlorambucil for untreated patients ineligible for FCR.\textsuperscript{287,288} Bendamustine is also used in combination with rituximab,\textsuperscript{289} other antibodies,\textsuperscript{290} or novel agents\textsuperscript{291} for fit relapsed patients.

Like rituximab, ofatumumab is a type I human anti-CD20 antibody. However, this antibody has a completely unique binding site on CD20. Both of these type I antibodies redistribute CD20 molecules into lipid rafts and promote complexes with BCR and complement. Ofatumumab alone is effective in fludarabine-refractory CLL.\textsuperscript{292} This antibody induces the highest degree of complement fixation, and results in complement exhaustion in the patient.

Obinutuzumab is another anti-CD20 antibody. Unlike rituximab and ofatumumab, obinutuzumab is a glycoengineered type II antibody. Glycoengineered antibodies have an increased affinity for Fc receptors and thus promote greater antibody-dependent cellular cytotoxicity. They also promote homotypic adhesion and lysosome-mediated cell death. Obinutuzumab shows activity in previously treated and relapsed patients.\textsuperscript{293} Type II antibodies bind to half as many CD20 molecules as type I antibodies. This may be because type II antibodies bind to CD20 in a cyclic conformation, while type I antibodies bind to the naïve conformation.\textsuperscript{294}
Alemtuzumab is an anti-CD52 antibody. It promotes greater antibody-dependent phagocytosis compared with anti-CD20 antibodies. However, due to the widespread expression of CD52 on immune cells, this antibody presents greater toxicities than CD20 antibodies. This therapy is used in relapsed/refractory CLL and shows promise in combination with FC for upfront treatment of high risk CLL.

Other antibodies that have been tested in CLL but have yet to receive FDA approval include type II anti-CD20 tositumumab, radio-labeled anti-CD20 ibritumomab, anti-CD22 epratuzumab, anti-CD23 lumiliximab, and anti-CD44 RG7356. Complement deficiencies, present in 38% of CLL patients, can limit the use of antibodies in CLL.

Of all these therapies, none have been shown to cure CLL. The only chance of a cure is through bone marrow stem cell transplant. Autologous transplantation using the donor’s own cells has high relapse rates and is not curative; only allogeneic transplant using cells from a donor has the potential to cure thanks to the graft versus leukemia effect. The graft versus leukemia effect can effectively eliminate the CLL cells and provide long-term remission. However, rates of acute and chronic graft versus host disease (GVHD) are high in allogeneic transplant; chronic GVHD can occur in up to 75% of transplant patients and mortality rates can reach up to 40%. Therefore, allogeneic transplant is preceded with myeloablative conditioning. Several studies have shown that reduced intensity non-myeloablative conditioning can still maintain the graft-versus-leukemia effect. In all of these studies it is important to assess minimal residual disease in the most precise manner possible. Overall, stem cell transplant is a challenging therapeutic option due to the lack of a suitable donor, the poor fitness of the patient, and the high rates of GVHD and mortality.
1.12. Kinase Inhibitors

In addition to chemoimmunotherapy options in CLL, numerous targeted therapies have been investigated. Many targeted therapies are kinase inhibitors which prevent activation of the BCR pathway and other pathways including those in response to growth factors and cytokines. These inhibitors are small molecules that bind to the active pocket of kinases thus preventing the phosphorylation of key amino acid residues on the kinase itself or other targets. Inhibitors that have shown efficacy in CLL include ibrutinib, idelalisib, ruxolitinib, sorafenib, dasatinib, and fostamatinib (Figure 1.5). Until this study, gefitinib and erlotinib had yet to be tested in CLL.

**Figure 1.5. Tyrosine kinase inhibitors target the BCR and cytokine pathways.** Dasatinib can inhibit Src family tyrosine kinases such as Lck and Lyn. Fostamatinib can inhibit Syk. Ibrutinib can inhibit Btk. Sorafenib can inhibit multiple kinases, including Raf. Idelalisib can inhibit PI3K. Ruxolitinib can inhibit JAK.

Ibrutinib is a small molecule inhibitor of the tyrosine kinase Btk. It was recently approved by the FDA in 2014 for use in CLL patients that have received at least one prior
therapy. It is also approved for use in Mantle cell lymphoma and Waldenstrom macroglobulinemia. Within 24 hours of treatment there is an increase in lymphocytosis due to the release of CLL cells from the lymph nodes. The lymphocytosis is not associated with any adverse effects and is not associated with an inferior patient response. Combination treatment with rituximab can reduce this. Despite the efficacy of ibrutinib, CLL cells are developing resistance mechanisms. Mutation of Btk and mutation of the downstream enzyme PLCγ are two known mechanisms of ibrutinib resistance. Furthermore, due to the fact that ibrutinib is a long-term treatment, adverse side effects accumulate. A recent study found that hair and nail abnormalities occur with prolonged ibrutinib treatment.

Idelalisib is a small molecule inhibitor of the lipid kinase PI3K. It was recently approved by the FDA in 2014 for use along with rituximab in relapsed CLL. It is also approved for use in follicular lymphoma. This came after results of a phase 3 study showed that adding idelalisib to rituximab significantly improved patient response rate, progression-free survival, and overall survival. Like ibrutinib, there is an initial lymphocytosis that peaks within the first 8 weeks of treatment. Early phase I clinical trials results of idelalisib alone reported that 54% of patients discontinued treatment due to progressive disease or adverse effects. This number dropped to 19% when idelalisib was combined with rituximab. However, adverse effects are still common and can be serious in nature, particularly diarrhea and colitis which can be fatal.

Ruxolitinib is a small molecular inhibitor of the Jak tyrosine kinases. It is FDA approved for use in myelofibrosis and polycythemia vera. This drug inhibits BCR-induced and cytokine-induced Jak/STAT signaling which then leads to apoptosis. This drug is currently being tested in a phase I/II (NCT02015208) and a phase II (NCT02131584) clinical trials. Like other tyrosine kinase inhibitors, ruxolitinib treatment induces lymphocytosis accompanied by decreased
lymphadenopathy. Results of a small phase II study showed that anemia worsened with ruxolitinib treatment, and 10 of the 13 trial patients required transfusions.

Dasatinib is a small molecule inhibitor of the Abl and Src tyrosine kinase families. It was first developed as an inhibitor of the fusion protein BCR-Abl which is aberrantly expressed in CML cells. It received FDA approval for this purpose in 2006. Dasatinib is also approved for use in adults with BCR-Abl+ ALL. In CLL dasatinib was found to inhibit autophosphorylation of the Src kinases Lyn and Lck and inhibits phosphorylation of downstream proteins such as Erk, Akt, and p38. This treatment can sensitize CLL cells to fludarabine, chlorambucil, and dexamethasone, and is effective even in CLL samples with unmutated IgVH. Dasatinib efficacy can be predicted by the downstream kinases Syk and PLCγ. Resistance is mediated by p53 and autophagy. Dasatinib has progressed through Phase II clinical trials.

Sorafenib is a multi-kinase inhibitor that has been shown to inhibit Raf, VEGFR, and insulin-like growth factor-1 receptor. Sorafenib is FDA-approved for use in renal cell carcinoma and hepatocellular carcinoma, and has entered clinical trials for use in CLL. The exact targets for sorafenib in CLL are unknown, but the Raf kinase is necessary for cytokine-induced survival and it is mutated in 2.8% of CLL cases. In CLL sorafenib can inhibit cytokine-induced Raf signaling, reduce Mcl-1 levels, and initiate apoptosis. It can block CXCL12-induced migration and can overcome protection from stroma and from nurse-like cells. Sorafenib can also limit leukemia progression in mouse models.

Fostamatinib is a small molecule inhibitor of the tyrosine kinase Syk. This kinase has been shown to regulate the survival of CLL cells. Fostamatinib prodrug is termed R788 and the active metabolite is termed R406. In vitro fostamatinib inhibits BCR signaling, decreases Mcl-1, and prevents migration. It kills even in the presence of nurse-like cells and
stromal cells. In vivo it inhibits BCR signaling, prolongs survival in mice, and has efficacy in a Phase I/II clinical trial. Due to the off-target effects of fostamatinib, novel Syk inhibitors have been developed such as PRT318, P505-15, and GS-9973. P505-15 is effective in vitro and synergizes with fludarabine. GS-9973 is effective in vitro and has been tested in Phase II clinical trials. However, 29% of patients tested had serious adverse events.

Gefitinib and erlotinib are small molecule inhibitors of the epidermal growth factor receptor (EGFR). This kinase is often mutated and involved in the pathogenesis of numerous cancers, such as non-small cell lung cancer (NSCLC). In 2003 gefitinib was approved by the FDA for use as a monotherapy in NSCLC. A year later, erlotinib received FDA approval for use as a monotherapy in NSCLC. In addition to inhibiting EGFR, gefitinib and erlotinib can also inhibit other kinases, such as Syk in AML cells and Myelodysplastic Syndrome (MDS) cells. However, gefitinib and erlotinib have yet to be tested in CLL. It is possible that these tyrosine kinase inhibitors may inhibit both Syk and its homolog ZAP-70 in CLL cells.

1.13. CLL Microenvironments

Therapeutics are often tested on only CLL cells, thus ignoring the numerous other cell types in contact with CLL cells. Although CLL is defined by excess B cells in the peripheral blood, these cells are also abundant in other sites in the body. CLL cells commonly occupy lymph nodes, bone marrow, spleen, kidneys, and liver. Less common sites include adrenal glands, heart, pancreas, lungs, and skin. The lymph nodes and bone marrow are the most common sites of CLL infiltration; 100% of patients have infiltration to some degree.
Within the lymph node, there is an increased quantity of high endothelial venules which mediates the extravasation of CLL cells from the peripheral blood into the lymph node. This process is mediated by L-selectin\(^3^{54}\). The influx of CLL cells into lymph nodes can disturb the normal architecture and increase the physical size of the node causing lymphadenopathy\(^3^{55}\). Proliferation centers also may be increased\(^3^{56}\), however, the article reporting this finding was later retracted for an unknown reason. The lymph node is the site for CLL proliferation\(^3^{57}\). Within the lymph node and peripheral blood, T cells have been shown to respond to CLL antigens\(^3^{58–60}\). Patients with anti-CLL T cell responses have an improved survival\(^3^{61}\). However, numerous therapies eliminate T cells along with CLL cells\(^3^{62–64}\), thus hindering any beneficial anti-tumor immune response and instead promoting infections\(^3^{65}\).

Within the bone marrow, there is increased angiogenesis\(^3^{66}\) and disrupted architecture\(^3^{67}\). Infiltration of CLL cells into the bone marrow can take two different patterns: nodular or diffuse. Nodular bone marrow infiltration is associated with mutated IgVH and ZAP-70\(^-\)\(^3^{68}\), while the diffuse bone marrow infiltration is associated with unmutated IgVH and ZAP-70\(^+\)\(^3^{68,69}\). Furthermore, ZAP-70 expression promotes CLL infiltration into the bone marrow. The mechanism of this infiltration relies on the enhanced signaling and migration post CXCR4 stimulation\(^3^{70}\). In addition, ZAP-70\(^+\) CLL cells have stronger adhesion to bone marrow stromal cells than do ZAP-70\(^-\) CLL cells\(^1^{57}\). These bone marrow-derived stromal cells protect CLL cells from cell death and promote drug resistance\(^2^{22,371}\).

Overall, CLL is not just a disease of blood, but also of bone marrow and lymph nodes. CLL cells establish a bidirectional crosstalk with numerous different cells within these microenvironments resulting in immune dysregulation.

A recently recognized hallmark of cancer is the reprogramming of their surrounding environment to create a tumor microenvironment\(^\text{372}\). CLL cells have a large impact on their surrounding microenvironments. CLL cells can distort the structure of lymphoid environments\(^\text{367}\) and can adversely affect stromal cells, DCs, nurse-like cells, endothelial cells, B cells, T cells, and NK cells.

Stromal cells are a diverse family of different cell types that form the support in many different organs. CLL cells signal through the lymphotoxin-β-receptor to remodel the stromal environment for their benefit\(^\text{373}\). In addition, CLL cells can release exosomes to induce the transition of stromal cells into cancer-associated fibroblasts which promote a tumor-supportive environment\(^\text{374}\). Stromal cells can protect CLL cells from apoptosis\(^\text{371,375}\), and likewise CLL cells can also protect stromal cells from apoptosis. This requires integrins\(^\text{376}\), CD27\(^\text{377}\), cysteine\(^\text{378}\), growth factors such as VEGF\(^\text{379}\), and chemokines such as SDF-1\(^\text{380}\). CLL cell chemotaxis to stromal cells can result in pseudoemperipolesis: the migration of CLL cells beneath stromal cells\(^\text{380}\). The benefit of this form of migration is not known. Bone marrow stromal cells not only protect CLL cells from apoptosis, but they also promote CLL cell metabolism, particularly glycolysis\(^\text{381}\).

Dendritic cells (DCs) are also altered in CLL patients. Dendritic cells (DCs) have been shown to recruit CLL cells through the chemokine receptor CXCR5\(^\text{373}\) and protect them from apoptosis which was dependent on the adhesion molecule CD44\(^\text{382}\). CLL patients have reduced numbers of plasmacytoid DCs and the plasmacytoid DC-derived cytokine IFNα\(^\text{383}\). Maturation of DCs from peripheral blood monocytes of CLL patients is inhibited by the presence of CLL cells. Furthermore, these DCs have lower expression of CD80 and CD40 compared to DCs.
matured from healthy monocytes. This DC dysfunctions increases as disease progresses \(^{384}\). However, a group that used a similar protocol to generate monocyte-derived dendritic cells found similar antigen processing capabilities, surface molecules, and cytokine expression on healthy and CLL-derived dendritic cells \(^{385}\). When an alternative method was employed to negatively select mature DCs from peripheral blood, a deficiency of CD80 expression compared to healthy donors was identified. These CLL DCs also produce less IL-10 and IL-12 \(^{386}\). Therefore, DC dysfunction exists in CLL but it is not fully defined.

Nurse-like cells are blood-derived cells that have fibroblast-like appearance. They secrete SDF-1 which promotes the survival and drug-resistance of CLL cells \(^{387}\). This survival is contact-dependent and involves the up-regulation of anti-apoptotic genes in CLL cells \(^{388}\). These nurse-like cells are considered by some to be tumor-associated macrophages \(^{389}\). Increased numbers of nurse-like cells in lymph nodes correlates with increased proliferation of CLL cells \(^{390}\).

Endothelial cells are found lining blood vessels. These cells can interact with CLL cells during the process of extravasation when CLL cells exit the peripheral blood and enter the surrounding tissue \(^{354}\). They produce BAFF, APRIL, CD40 \(^{391}\), and IL-6 \(^{392}\) which promote the survival of CLL cells \(^{393,394}\).

B cells are outcompeted and inhibited by malignant CLL B cells. One proposed mechanism for this inhibition is through CD95 on plasma cells and CD95L on CLL cells \(^{395,396}\). CLL patients often present with hypogammaglobulinemia and thus there is a humoral defect in CLL \(^{397,398}\). This is present in up to 30-50\% of CLL patients, and can worsen as disease progresses \(^{399}\). It can predict early death \(^{400}\). This deficiency is the same for unmutated and mutated IgVH CLL \(^{401}\). This defect can be reversed by drugs such as lenalidomide \(^{397}\), ibrutinib
Healthy B cells can secrete anti-CLL antibodies such as anti-ROR1 and anti-immature laminin receptor (iLR). Quantities of anti-ROR1 antibodies and anti-iLR antibodies can correlate with patient disease progression. CLL patients have intact anti-CMV humoral immune response.

T cells are severely impaired in CLL patients. These cells have diminished helper activity, less proliferation, and reduced signaling. Diminished helper activity may be caused by impaired cell:cell contact due to defective immune synapse formation. Impaired immune synapse formation may be caused by the expression of the inhibitory ligands CTLA-4, CD200, CD270, CD274 (PD-L1), and CD276. Blockade of CD200 can restore T cell activity. Lack of proliferation may be caused by less IL-2 secretion, although the later study has cited this finding as data not shown, and an additional study has shown normal IL-2 production. Reduced signaling may be caused by decreased expression of the T cell receptor co-receptor CD3ζ and the costimulatory molecule CD28. Furthermore, T cells exhibit signs of exhaustion. Some T cell abnormalities are not present in MBL. T cells can be stimulated by the immune-stimulatory drug lenalidomide and by the bi-specific anti-CD3 and anti-CD19 antibody blinatumomab. CLL cells have more regulatory T cells. Aside from CLL-mediated immunosuppression, nurse-like cells can also contribute. Nurse-like cells inhibit T cell proliferation but support expansion of T regulatory cells.

NK cells are expanded in CLL patients; however, their activity is impaired. Some groups have shown that NK cells from CLL patients have reduced cytolytic activity against NK-sensitive cell types, but others disagree. The reason for this discrepancy cannot be the target cells used, as each study uses the NK-sensitive K-562 target cell line. Despite this disagreement on whether CLL NK cells are impaired in killing known NK-sensitive cell lines,
researchers agree that CLL NK cells cannot kill CLL cells\textsuperscript{425,426}. In addition, CLL cells cannot be killed by healthy donor NK cells\textsuperscript{425}. This demonstrates that CLL cells evade NK cell-mediated lysis. CLL cells may evade recognition by NK cells by producing soluble BAG3\textsuperscript{425,427} which engages the activating receptor NKp30. This soluble receptor can act as a decoy\textsuperscript{427}. CLL NK cells have reduced expression of the activating receptor NKG2D\textsuperscript{422}. However, another group found no difference in NKG2D, but a slight decrease in NKp30\textsuperscript{425}. In addition, CLL NK cells have fewer cytotoxic granules than healthy NK cells\textsuperscript{424}. NK cell activity can be restored by therapies such as anti-CD20 therapy\textsuperscript{425,428,429}, lenalidomide\textsuperscript{422}, and cytokines such as IL-2, IL-15, IL-21\textsuperscript{422}, and IFN\textsuperscript{430}.

Overall, CLL cells have shaped the microenvironments and the cells within (Figure 1.6) to promote their survival, proliferation, and drug resistance. The cells within these microenvironments should be considered when testing novel therapies.

\textbf{Figure 1.6. Immune dysregulation in CLL.} CLL cells secrete extracellular vesicles and numerous soluble factors, such as IL-10, which remodels its surrounding microenvironment. This environment includes T cells, NK cells, plasma cells, dendritic cells, stromal cells, endothelial cells, and nurse-like cells. These cells can all promote the recruitment, proliferation, and/or survival of CLL cells.
1.15. Study Rationale, Hypothesis, and Objectives

CLL is a common and rarely curable cancer. Disease relapse and drug resistance remain common occurrences. This underscores the importance of investigating novel therapies and therapeutic targets. Therefore, the aim of this thesis project is to investigate novel targeted therapies in CLL. First, this thesis will examine the novel use of Gefitinib targeting a known susceptibility: the BCR pathway. Secondly, this thesis will investigate the use of novel lysosomotropic agents to target an unexplored susceptibility of CLL cells: the lysosome. Lastly, this thesis will focus on the clinical applicability of these targeted therapies by assessing the immune impact and clinical correlations. Overall, these therapeutic strategies are focused on inhibiting the pro-survival BCR pathway, and promoting the pro-cell death pathway initiated by lysosome membrane permeabilization.

I hypothesize that the altered biology of CLL cells will render them sensitive to gefitinib and to lysosomotropic agents. Specifically, I hypothesize that gefitinib will inhibit the BCR pathway and kill ZAP-70+ CLL cells, and that lysosomotropic agents will induce lysosome permeabilization and kill CLL cells, regardless of poor prognostic indicators. These studies will confirm that the BCR pathway is a susceptibility of CLL cells, and will provide evidence that the lysosome is a novel susceptibility of CLL cells.

The specific objectives of my research are divided into the following three chapters and are outlined below.

Chapter 2

- Investigate gefitinib in CLL cells.
- Determine the impact of gefitinib on the BCR pathway.
- Explore the role of ZAP-70 in gefitinib mechanism of action.
Chapter 3

- Investigate lysosomotropic agents in CLL cells.
- Determine how lysosomotropic agents kill CLL cells.
- Explore the ways in which lysosomes and metabolism are altered in CLL.

Chapter 4

- Investigate the effect of targeted therapies on T cells.
- Investigate the effect of targeted therapies on CLL cells in the presence of stromal cells.
- Calculate clinical correlations of patient characteristics with drug response.

This thesis will conclude with Chapter 5 which presents final conclusions and future directions.
Chapter 2

2. Targeting the B Cell Receptor Pathway in Chronic Lymphocytic Leukemia.

Information presented in this chapter has been previously published in the article entitled “Gefitinib targets ZAP-70-expressing chronic lymphocytic leukemia cells and inhibits B-cell receptor signaling” (Dielschneider et al., Cell Death and Disease, 2014).

2.1. Abstract

Chronic Lymphocytic Leukemia (CLL) cells alter the pro-survival B cell receptor (BCR) pathway by changing the BCR itself and upregulating many downstream signaling components. CLL cells frequently have un-mutated immunoglobulin heavy chain (IgVH), over-express Syk, and ectopically express the Syk homolog ZAP-70. Both un-mutated IgVH and ZAP-70 enhance BCR signaling and are indicators of aggressive disease and poor patient prognosis. Although tyrosine kinase inhibitors have been shown to effectively inhibit the BCR pathway, none target ZAP-70 and none are without toxicities and limitations. The tyrosine kinase inhibitors gefitinib and erlotinib were previously shown to inhibit Syk in acute myeloid leukemia; however, their effects on ZAP-70 and their effects in CLL cells have yet to be investigated. Therefore, the purpose of this work is to investigate gefitinib and erlotinib in ZAP-70+ and ZAP-70- cell lines and CLL cells. MTT viability assays and Annexin V apoptosis assay both demonstrated an increased susceptibility of ZAP-70+ CLL cells to gefitinib, but not erlotinib. This increased sensitivity was not seen with fludarabine. This trend persisted when investigating cell lines; the ZAP-70+ Jurkat cell line responded significantly better as compared to the ZAP-70- cell lines BJAB, NALM6, and I83. Western blot analysis demonstrated that gefitinib inhibited both Syk
and ZAP-70 phosphorylation in ZAP-70+ CLL cells and ZAP-70 phosphorylation in the Jurkat cell line. Furthermore, gefitinib inhibited downstream BCR signaling by preventing Erk and Akt phosphorylation, and preventing the pro-survival response. Introduction of ZAP-70 into the B cell line Raji by lentiviral transduction significantly increased their sensitivity to gefitinib as measured by two independent viability assays. Therefore, gefitinib is an effective BCR pathway inhibitor in ZAP-70+ cells, and shows promise as an effective treatment for aggressive ZAP-70+ CLL patients.
2.2. Introduction

Chronic Lymphocytic Leukemia (CLL) is a cancer of B cells. These cells exhibit several immunobiological differences as compared to their healthy counterparts. CLL cells have altered immunoglobulins which are stereotyped and/or un-mutated, and have altered expression and activation of numerous proteins downstream of the BCR. One such alteration in the BCR pathway is the ectopic expression of ZAP-70 in CLL cells. This kinase is normally expressed in T cells, Natural Killer (NK) cells, and in pro/pre-B cells; however, its expression in mature B cells found within the peripheral blood is abnormal.

The role of ZAP-70 in CLL cells has been debated. ZAP-70 associates with CD79b after BCR stimulation and increases tyrosine phosphorylation and BCR signaling; however, one group showed that expression of ZAP-70 with or without its kinase domain both increased BCR signaling. Furthermore, ZAP-70 is inefficiently activated in CLL cells as compared to Syk, but its ability to recruit downstream mediators such as PI3K and Shc is intact. Moreover, ZAP-70 can prolong the activation of kinases such as ERK and Akt. Thus, the kinase function of ZAP-70 may not mediate the increased BCR signaling; instead it is possible that ZAP-70 could play the role of an adaptor protein.

In addition to promoting BCR signaling, ZAP-70 expression in CLL can promote migration. ZAP-70+ CLL cells are more responsive to chemokine signals, such as CXCL12. ZAP-70+ CLL cells migrate and adhere better to stromal cells as compared to ZAP-70- CLL cells. Furthermore, ZAP-70+ CLL cells transferred into mice preferentially migrate to the bone marrow.
Aside from complicating CLL biology, ZAP-70 expression has prognostic value in CLL. Indeed, CLL patients that express ZAP-70 in at least 20% of their cells have shorter time to treatment, shorter progression-free survival, and shorter overall survival.

Overall, abnormal ZAP-70 expression in CLL promotes BCR signaling and migration, leading to aggressive disease. This supports the notion of ZAP-70 as a therapeutic target in CLL. Several targeted therapies have been investigated in CLL; however, none have focused on ZAP-70. Based on evidence that gefitinib and erlotinib can inhibit Syk, the homolog of ZAP-70, in AML and MDS, we hypothesize that these drugs will be effective in CLL cells, particularly those expressing ZAP-70. Therefore, the purpose of this study was to investigate the efficacy of gefitinib and erlotinib in ZAP-70+ and ZAP-70- CLL cells, and to determine the impact of these drugs on BCR signaling.
2.3. Materials and Methods

2.3.1. Cell Processing and Culture

The malignant B cell lines BJAB (ATCC), NALM6 (DSMZ), I83 (kind gift from Dr. Panasci, McGill University), and Raji +/- ZAP-70 (kind gifts from Dr. Aaron Marshall, University of Manitoba) and the malignant T cell line Jurkat (ATCC) were all cultured in RPMI (Hyclone) with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific) and 1X penicillin and streptomycin (Gibco, Thermo Fisher Scientific). Raji- ZAP-70 and Raji-Vector cells were made by lentiviral transduction.

Primary CLL cells were processed from peripheral blood samples within 24 hours of blood draw. Patient samples were collected following their informed consent in accordance with the Research Ethics Board of the University of Manitoba. Blood was mixed with RosetteSep (Stemcell Technologies) for 30 minutes if lymphocyte count < 40 x 10^9 cells/L, then purified by centrifugation on a Ficoll-Paque gradient (GE Healthcare). Red Blood Cells (RBCs) were lysed using the RBC lysis buffer (eBiocience) for 10 minutes. Purified cells were cultured in Hybridoma Serum-Free Medium (SFM; Life Technologies) with no additives. All cells were kept in a humid 37°C incubator with 5% CO₂.

2.3.2. Drugs and Stimuli

The following drugs were dissolved in DMSO and stock solutions were kept at -80 °C: gefitinib (LC Laboratories), dasatinib (LC Laboratories), erlotinib (LC Laboratories), ibrutinib (SelleckBio), and fludarabine (Sigma).

B cells were stimulated with biotinylated Fab2 anti-IgM (Southern Biotech). For western blot experiments, 10 µg/ml anti-IgM was added and the cells were lysed within an hour. For cell viability analysis, 0.1 µg/ml anti-IgM in Hanks’ Buffered Salt Solution (HBSS; Life
Technologies) was immobilized in a 96-well Falcon plate (BD) for 24 hours, then washed prior to CLL cell addition. T cells were stimulated with 2 µg/ml LEAF-purified anti-CD3 (BioLegend).

2.3.3. **Flow Cytometry**

For intracellular staining of ZAP-70 in cell death experiments, RosetteSep and ficoll-purified CLL cells were first surface stained with Annexin V-APC and 7AAD, then fixed with solution A (Beckman Coulter) for 12 min at 37 °C, washed with PBS, permeabilized with solution B (Beckman Coulter) for 5 min at room temperature, and then stained with mouse anti-human ZAP-70-FITC (Beckman Coulter) for 15 min at room temperature. Mouse IgG1-FITC (BD) was used as an isotype control. Unstained and single-stained controls were always included in all flow cytometry experiments. All sample data were acquired on BD FACSCalibur and analyzed using CellQuest Pro software (BD). CLL cell samples were considered ZAP-70+ if ≥20% of the cells stained positive.

The ZAP-70 status of each patient sample was determined by diagnostic flow cytometry. Whole blood was stained with anti-CD19, anti-CD5, anti-CD38, and anti-ZAP-70. The negative control is a normal blood sample that should not have CD19+CD5+ leukemic cells. The positive control is the autologous CD19-CD5+ZAP-70+ T cells.

2.3.4. **Cell Viability Assays**

Cell viability was measured by two different methods: MTT assays and Annexin V/7AAD flow cytometry assay. The former assay was performed with 3 x 10⁷ CLL cells in 3 ml Hybridoma SFM in round bottom tubes (Sarstedt). Cells were treated with 1, 2, 5, 10, or 15 µM gefitinib, erlotinib, or fludarabine for 24 hours. Cells were then washed in HBSS, seeded into 96-well U-bottom plates, and cultured. Cells were seeded in 1, 2, 3, or 4 x 10⁶ cells per well in
quadruplicate. Three days later, MTT (Sigma) was added at a final concentration of 0.25 mg/ml and incubated for 5 hours. Absorbance was measured at a wavelength of 540 nm. The inhibitory concentration of at 50% (IC\textsubscript{50}) was calculated from the absorbance of drug or solvent-treated cells. The survival curves for each drug treatment seeded in the 4 different cell concentrations were plotted and the slope of the trend line was determined. The surviving cell fraction (SCF) was determined by dividing the slope of the trend line of the DMSO-treated sample to the slope of the trend line of each drug-treated sample. Therefore, the SCF of DMSO sample was always 1. The SCF values were plotted on a log scale versus the concentration of drug, and the concentration at 0.5 was determined from the equation of the trend line.

For flow cytometry assay, 5 x 10\textsuperscript{5} cells were collected and washed with 1X Annexin V binding buffer (BD Biosciences). Washed cells were stained with 7AAD (BD) and Annexin V-FITC (BD) or Annexin V-APC (BD). The decision to use FITC or APC was made based on the expression, or not, of a GFP vector. Samples were acquired on the BD FACSCalibur and analyzed using CellQuest Pro software (BD).

**2.3.5. Western Blot and Immunoprecipitation**

Cell lysates were collected at the indicated times in 1% NP-40 lysis buffer. This buffer contained 1% NP-40, 20 mM Tris base (Sigma), 150 mM NaCl, 10% glycerol, 2 mM EDTA, complete protease inhibitor tablet (Roche), 1 mM phenylmethanesulfonylfluoride (PMSF), and 2 mM sodium orthovanadate (New England BioLabs). The later three ingredients were always added immediately prior to use. Protein levels were quantified with a Pierce BCA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Prior to gel loading, samples were mixed with gel loading buffer containing a final concentration of 20 mM Tris, 2% SDS, 5% glycerol, 1% \(\beta\)-mercaptoethanol (Sigma), and 0.05% bromophenol blue (Sigma). Samples were
run on 8% or 10% polyacrylamide gels, transferred onto 0.45 µm nitrocellulose membranes (Bio-Rad), and then blocked in 5% BSA (Sigma) or milk in TBS-T for 1 hour at room temperature.

Primary antibodies included rabbit or mouse anti-ZAP-70 (Cell Signaling), rabbit anti-Syk/ZAP-70-P (Cell Signaling), rabbit anti-Lyn (Cell Signaling), rabbit anti-Lyn-P (Epitomics), mouse anti-Lck (Cell Signaling), rabbit anti-Btk (Cell Signaling), rabbit anti-Btk-P (Cell Signaling), rabbit anti-ERK1/2 (Cell Signaling), rabbit or mouse anti-ERK1/2-P (Cell Signaling), rabbit anti-Akt (Cell Signaling), rabbit anti-Akt Ser-P (Cell Signaling), mouse anti-tyrosine-P (Millipore), rabbit anti-Mcl-1 (Cell Signaling), rabbit anti-PARP (Cell Signaling), rabbit anti-cleaved caspase 3 (Cell Signaling), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; Sigma), rabbit anti-α-tubulin (Cell Signaling), and rabbit or mouse anti-β-actin (Sigma). Secondary antibodies were goat anti-rabbit-HRP or anti-mouse-HRP (Bio-Rad). Detection of protein on nitrocellulose membranes was done using Pierce ECL or Pierce Supersignal Pico (Thermo Fisher Scientific) reagents.

Co-immunoprecipitation was carried out with 500 µg of protein in 0.2% CHAPS lysis buffer containing 150 mM NaCl, 20 mM Tris, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2 mM sodium orthovanadate, and complete protease inhibitor tablet (Roche). Lysates were incubated at 4 °C with primary antibody mouse anti-Syk (Abcam) or mouse anti-ZAP-70 (Cell Signaling) on a rotator overnight. Pierce protein G plus agarose bead slurry (Thermo Fisher Scientific) was added in a final dilution of 1:10 for 2 hours and the procedure for western blot was followed. Entire immunoprecipitate supernatant was loaded on to a gel lane. Immunoprecipitations were done in the same way, with the exception of using 1% NP-40 lysis buffer.

2.3.6. Statistical Analysis
All graphs were created and statistics were performed using GraphPad Prism4 software (GraphPad Software Inc.). Unless otherwise noted, a paired or unpaired two-tailed t-test was performed according to the nature of data. Statistical significance was noted in the figures as *$p<0.05$, **$p<0.01$, or ***$p<0.001$. Densitometry was calculated using ImageJ (Wayne Rasband; National Institute of Mental Health, Bethesda, MD, USA).
2.4. Results

2.4.1. Gefitinib kills ZAP-70+ CLL cells and malignant lymphoid cell lines.

The EGFR inhibitors gefitinib and erlotinib have inhibited Syk and shown cytotoxicity in AML; however, their activity in CLL is unknown. Therefore, we investigated gefitinib and erlotinib in CLL cells that were positive or negative for the Syk homolog ZAP-70. CLL cells were treated with various concentrations of each drug or the standard of care fludarabine. Viability was assessed by MTT assay and the inhibitory concentration at 50% (IC\textsubscript{50}) was determined. The median IC\textsubscript{50} of gefitinib was 4.5 µM in ZAP-70+ CLL cells, and was > 15 µM in ZAP-70- CLL cells (Table 2.1). Overall, 30% of ZAP-70- and 77% of ZAP-70+ CLL samples responded to gefitinib treatment with an IC\textsubscript{50} value < 10 µM (Figure 2.1 A). Analysis of the IC\textsubscript{50} values of all patients, excluding non-responders, revealed a significant difference between ZAP-70+ CLL samples and ZAP-70- CLL samples (Figure 2.1 B). The number of non-responders to gefitinib decreased as ZAP-70 expression increased (Figure 2.1 C). This trend was not observed with another EGFR inhibitor erlotinib as the IC\textsubscript{50} was > 40.0 µM in both ZAP-70+ and ZAP-70- samples. Furthermore this trend was not observed with the standard of care fludarabine (Table 2.1). In addition, there was no cross-resistance to fludarabine in the ZAP-70+ samples; most fludarabine-resistant cases were sensitive to gefitinib.

**Table 2.1. Gefitinib is most effective in ZAP-70+ CLL.** Gefitinib, erlotinib, and fludarabine IC\textsubscript{50} values determined by MTT assays in ZAP-70+, ZAP-70-, mutated (M) Ig\textsubscript{VH}, and unmutated (U) Ig\textsubscript{VH} primary CLL cells treated for 24 hours.

<table>
<thead>
<tr>
<th>CLL Patients</th>
<th>Gefitinib Median IC\textsubscript{50}</th>
<th>Erlotinib Median IC\textsubscript{50}</th>
<th>Fludarabine Median IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP-70+</td>
<td>4.5 µM (n=22)*</td>
<td>&gt; 40.0 µM (n=8)</td>
<td>5.4 µM (n=14)</td>
</tr>
<tr>
<td>ZAP-70-</td>
<td>&gt; 15.0 µM (n=23)</td>
<td>&gt; 40.0 µM (n=10)</td>
<td>7.0 µM (n=16)</td>
</tr>
<tr>
<td>ZAP-70+ &amp; M- Ig\textsubscript{VH}</td>
<td>4.0 µM (n=9)</td>
<td>&gt; 40.0 µM (n=2)</td>
<td>13.2 µM (n=6)</td>
</tr>
<tr>
<td>ZAP-70+ &amp; U-Ig\textsubscript{VH}</td>
<td>6.0 µM (n=11)</td>
<td>&gt; 40.0 µM (n=5)</td>
<td>5.4 µM (n=8)</td>
</tr>
</tbody>
</table>

* represents statistical significance of gefitinib median IC\textsubscript{50} (p<0.05) between ZAP-70+ and ZAP-70- cells.
Figure 2.1. Gefitinib is most effective in ZAP-70+ CLL cells. CLL cells were treated with 1, 2, 5, 10, or 15 µM gefitinib for 24 hours and viability was assessed by MTT assay (A-C) or Annexin V and 7AAD staining (D-E). (A) Gefitinib IC$_{50}$ values as determined by MTT assay can be divided into >10 and <10 µM groups. (B) MTT IC$_{50}$ values in ZAP-70+ and ZAP-70- CLL samples, excluding non-responders. (C) Number of non-responders to gefitinib treatment versus the ZAP-70 expression. (D) Annexin V staining after gefitinib treatment in ZAP-70+ and ZAP-70- CLL samples. (E) After treatment, CLL cells were also stained with anti-ZAP-70. (F) CLL cells were treated with 1, 5, or 10 µM Gefitinib for 72 hours then stained with Annexin V and 7AAD.

MTT results were confirmed by another independent viability assay: Annexin V and 7AAD. CLL cells were treated with gefitinib in vitro and after 24 hours cell death was analyzed by flow cytometry. The trend observed in the MTT assay results held true: ZAP-70+ CLL cells were more sensitive to gefitinib than ZAP-70- CLL cells. After 24 hours, gefitinib at a dose of 10 µM killed between 25% and 85% of ZAP-70+ CLL cells (Figure 2.1 D). Despite classification of a sample as ZAP-70+, not all cells may express ZAP-70. To be considered ZAP-70+, at least
20% of the cell must express the protein as determined by intracellular staining and flow cytometry. Intracellular staining for ZAP-70 confirmed that viable ZAP-70-expressing CLL cells decreased after gefitinib treatment (Figure 2.1 E). Gefitinib-induced cell death was dose-dependent, starting with as little as 1 µM of drug (Figure 2.1 F).

The preference of gefitinib for ZAP-70+ CLL cells could not be explained by the expression of another target of gefitinib; CLL cells do not express EGFR and do not differentially express RIP2 or GAK1 (Figure 2.2).

![Figure 2.2. CLL cells differentially express ZAP-70 but not EGFR, RIP2, or GAK.](image)

To further investigate the trend of gefitinib efficacy in ZAP-70+ cells, we examined four different leukemia/lymphoma cell lines: BJAB, NAML6, I83, and Jurkat. The cell lines BJAB, NAML6, and I83 are B cell-derived and express Syk but not ZAP-70. The cell line Jurkat is T cell-derived and expresses ZAP-70 but not Syk. This expression was confirmed by western blot. The HEK293 cell line was used as a negative control for both Syk and ZAP-70 expression (Figure 2.3 A). Doses of 0.1 to 30.0 µM gefitinib were tested in all cell lines, and the Jurkat cell line was the most responsive. After 18 hours a dose of 30.0 µM gefitinib killed >80% Jurkat cells and <40% of the ZAP-70- B cell lines (Figure 2.3 B, C). Likewise, gefitinib treatment over an 18 hour time course revealed that only the Jurkat cell line showed significant cell death (Figure 2.3)
C). As a control experiment, the four cell lines were treated with the Src tyrosine kinase inhibitor dasatinib which showed no bias for the Jurkat cell line (Figure 2.3 D).

**Figure 2.3. Gefitinib is most effective in ZAP-70+ cell lines.** (A) Lymphoid cell lines Jurkat, NALM6, I83, and BJAB were lysed and analyzed by western blot for ZAP-70 and Syk expression. (B) Lymphoid cell lines were treated with various dosing of gefitinib for 24 hours, (C) or with 30 µM and collected at various times. (D) Lymphoid cell lines were treated with 30 µM dasatinib and collected at various times. Cell viability was assessed with Annexin V and 7AAD staining and analyzed by flow cytometry.

**2.4.2. Gefitinib inhibits BCR signaling.**

To determine the effect of gefitinib on signaling within cells, western blot experiments were performed. In cell lines, gefitinib treatment did not reduce Syk phosphorylation in BCR-stimulated BJAB cells (Figure 2.4 A, C), but did reduce tyrosine phosphorylation of ZAP-70 in a dose-dependent manner in CD3-stimulated Jurkat T cells (Figure 2.4 B, C). There was no decrease in Lyn or Lck phosphorylation in BJAB or Jurkat cells, respectively.
Figure 2.4. Gefitinib inhibits ZAP-70 phosphorylation in Jurkat cells, but not Syk in BJAB cells. BJAB cells (A) and Jurkat cells (B) were stimulated with anti-IgM or anti-CD3 respectively for 10 minutes and were then untreated (Control or C as shown in figure), or treated with vehicle control DMSO, or treated with 5, 10, or 20 µM Gefitinib for 1 hour then lysed. Phosphorylations of Syk, ZAP-70, Lyn, and Lck were determined by western blot analysis. (C) Densitometry was done using ImageJ and represents 3 independent biological experiments.

In primary CLL cells, we found gefitinib treatment reduced BCR-stimulated total cellular tyrosine phosphorylation in ZAP-70+ CLL cells over a range of gefitinib concentrations and incubation times (Figure 2.5 A, B). However, this was not observed in ZAP-70− CLL cells. The decrease in overall cellular tyrosine phosphorylation was evident after 1 h, and decreased further after 24 hours (Figure 2.5 B). Unlike cell lines, there were decreases in both Syk/ZAP-70 and Lyn/Lck phosphorylation in ZAP-70+ CLL cells after 1 hour of gefitinib treatment (Figure 2.5 C). Quantification of these decreases showed that Syk/ZAP-70 phosphorylation decreased more than Lyn/Lck phosphorylation (Figure 2.5 D).

To compare the impact of gefitinib on downstream BCR signaling to that of other tyrosine kinase inhibitors, CLL cells were treated with gefitinib, the Src inhibitor dasatinib, or the Btk inhibitor ibrutinib. The downstream effect of gefitinib was the same as these other two drugs; all three drugs prevented Akt and Erk phosphorylation after BCR stimulation. This inhibition was not observed with fludarabine that was used as a negative control. BCR stimulation alone served as a positive control (Figure 2.5 E, F).
Figure 2.5. Gefitinib inhibits BCR signaling in ZAP-70+ CLL cells. (A) A ZAP-70+ CLL cell sample was left unstimulated and untreated or stimulated with anti-IgM and treated with 10, 20, or 30 µM gefitinib for 1 hour. Representative blot is shown from n=3 different CLL samples. (B) Lysates of a ZAP-70+ and a ZAP-70- CLL patient sample treated with 10 µM gefitinib for 3, 6, 16 and 24 hours analyzed by slot blot for tyrosine phosphorylation. (C) ZAP-70+ CLL cells were left unstimulated and untreated, or stimulated with anti-IgM and treated with 10, 20, or 30 µM gefitinib for 1 hour. (D) Densitometry of Syk/ZAP-70 and Lyn/Lck phosphorylation in n=4. (E) ZAP-70+ CLL cells were left unstimulated and untreated or stimulated with anti-IgM and treated with DMSO, 30 µM gefitinib (lane G), 10 µM Dasatinib (lane D), 10 µM ibrutinib (lane I), or 10 µM fludarabine (lane F) for 1 hour. (F) Densitometry of Erk and Akt phosphorylation in n=3. All protein expression was analyzed by western blot and densitometry was calculated using ImageJ software.
The phospho-antibodies used detect both Syk and ZAP-70 phosphorylation due to protein homology. Therefore, immunoprecipitation was used to discern the effect on Syk and on ZAP-70 separately. Gefitinib treatment decreased the phosphorylation of both Syk and ZAP-70 equally in CLL cells (Figure 2.6).

**Figure 2.6. Gefitinib inhibits both Syk and ZAP-70 phosphorylation.** (A) ZAP-70+ CLL cells were stimulated with anti-IgM and treated with DMSO or 30 µM gefitinib for 1 hour then lysed for immunoprecipitation of ZAP-70 or Syk and western blot analysis of phosphorylations. (B) Densitometry was performed on n=3 using Image J software.

As signaling through the BCR can promote cell survival, we determined whether inhibition of this signaling pathway by gefitinib decreased survival. Primary CLL cells were treated with 10 µM gefitinib, cultured on immobilized anti-IgM for 30 minutes to stimulate the BCR, and then cultured alone for 24 hours. We found that anti-IgM protected CLL cells from spontaneous apoptosis, a common occurrence in CLL cells *ex vivo*, but failed to protect CLL cells from gefitinib treatment (Figure 2.7).
Figure 2.7. Gefitinib inhibits BCR-induced survival. CLL cells were pretreated with 10 μM gefitinib for 30 minutes, stimulated with plate-immobilized anti-IgM for 30 minutes, and cultured for 24 hours. Samples were stained with Annexin V-FITC and 7AAD and analyzed by flow cytometry (unpaired t test). Single representative experiment done in triplicate with standard deviations is shown. Experiment repeated with 3 different patient samples.

2.4.3. ZAP-70 overexpression increases sensitivity to gefitinib.

To further elucidate the role of ZAP-70 in the susceptibility to gefitinib, we tested the response of the ZAP-70-negative lymphoma-derived B-cell line Raji transduced with GFP-expressing vector or ZAP-70-expressing vector. Expression of ZAP-70 was confirmed by both western blot (Figure 2.8 A) and intracellular staining and expression of GFP was confirmed by flow cytometry. Gefitinib treatment lowered Syk/ZAP-70 phosphorylation but not Lyn phosphorylation only in ZAP-70-expressing Raji (Figure 2.8 B). Western blot analysis showed constitutive phosphorylation of Syk/ZAP-70 in Raji cells overexpressing ZAP-70 (Figure 2.8 B), which agrees with previously published literature.

Upon BCR activation, Syk/ZAP-70 binds to the tyrosine phosphorylated co-receptor CD79a. Therefore, we performed co-immunoprecipitation experiments to investigate the binding of Syk and ZAP-70 to CD79a after gefitinib treatment. There was no difference in Syk or ZAP-70 binding to CD79a after gefitinib treatment (Figure 2.8 C). This suggests that gefitinib does not interfere with Syk or ZAP-70 binding to CD79a, but rather interferes with downstream signaling and cell survival.
We next investigated the sensitivity of gefitinib in ZAP-70-overexpressing Raji cells. Raji cells overexpressing ZAP-70 had increased sensitivity to gefitinib compared with cells with vector alone as measured by greater DNA fragmentation indicated by sub-G1 peak analysis (Figure 2.8 D) and greater Annexin V and 7AAD staining (Figure 2.8 E). In addition, gefitinib treatment was compared with dasatinib and ibrutinib. Gefitinib had the greatest effect of all the drugs on Raji cells overexpressing ZAP-70, as compared with Raji cells with vector alone. Dasatinib had little effect on Raji cells, whether they overexpressed ZAP-70 or not, only increasing cell death by 3–10%. Ibrutinib had a greater effect on ZAP-70-overexpressing Raji cells, as compared with the non-ZAP-70-expressing cells, but there was less cell death than seen with gefitinib using this same drug dosage (20 μM). This increased sensitivity of the ZAP-70-expressing Raji cells was not seen with fludarabine treatment, which was used as a negative control (Figure 2.8 F).
Figure 2.8. ZAP-70 expression sensitizes B cells to gefitinib treatment. (A) Raji cells were transduced to express GFP vector or ZAP-70 vector. Expression was confirmed by western blot. (B) Raji-Vector and Raji-ZAP-70 were treated with DMSO, 20, or 30 µM gefitinib for 20 minutes and lysed for western blot analysis of Syk/ZAP-70 and Lyn phosphorylation. (C) ZAP-70 and Syk were immunoprecipitated from Raji-ZAP-70 cells treated with 30 µM gefitinib for 1 hour. Mouse isotype serves as a negative control and lysate serves as positive control for western blot. Raji vector and Raji-ZAP-70 cells were treated with DMSO or 20 µM gefitinib for 24 hours and cell viability was assessed by propidium iodide (D) and Annexin V and 7AAD staining (E). (F) These cells were also treated with 30 µM dasatinib, 20 µM ibrutinib, or 20 µM fludarabine for 24 hours and then stained with Annexin V-APC and 7-AAD and analyzed by flow cytometry. Cell death of Raji-ZAP-70 cells normalized to Raji-vector cells. Results from 3 independent experiments are shown. The Raji-Vector and Raji-ZAP-70 cell lines were transduced by collaborators Hongzhao Li and Dr. Aaron Marshall (University of Manitoba).
2.5. Discussion

In this study the tyrosine kinase inhibitor gefitinib, originally used to inhibit EGFR kinase activation in lung cancer, is also cytotoxic to primary CLL cells that overexpress ZAP-70. When these cells undergo BCR activation, gefitinib can inhibit phosphorylation of Lyn/Lck, Syk/ZAP-70, Erk1/2, and Akt within 1 hour. These results are similar to those seen with 5–10 μM gefitinib in AML and MDS cells\textsuperscript{432,433}, where gefitinib functions through an EGFR-independent mechanism targeting Syk activation. Using the MTT assay, which we and others have shown to be predictive of clinical response to fludarabine and chlorambucil\textsuperscript{434}, the median IC\textsubscript{50} of gefitinib was 4.5 μM in ZAP-70+ CLL cells but >15 μM in ZAP-70− cells.

The therapeutic approach of targeting the BCR pathway in CLL has been well explored. This pathway is implicated in the pathogenesis of CLL\textsuperscript{85,435,436}. Due to the numerous alterations to the BCR itself, and to downstream kinases, there has been considerable interest in the evaluation of tyrosine kinase inhibitors for the treatment of CLL. However, each kinase inhibitor comes with challenges. Whether it be drug resistance, toxicity, cost, or lack of activity in aggressive cells, no agent is without disadvantages. The long-term toxicity and efficacy of some of these tyrosine kinase inhibitors in CLL is unknown, as many of these agents such as Idelalisib and Ibrutinib have only received FDA approval in the last 2 years. In contrast, gefitinib is already approved by the FDA for the treatment of NSCLC and has been used for over a decade in the clinic. It has the advantage in CLL of having more cytotoxicity towards ZAP-70+ CLL cells, and it is not myelo- or immuno-suppressive. This is the first instance of a targeted therapy toward ZAP-70 (Figure 2.9).
Figure 2.9. Tyrosine kinase inhibitors in CLL. Dasatinib can inhibit the Src kinases such as Lck and Lyn. Gefitinib can inhibit ZAP-70 and Syk, and potentially other kinases. Fostamatinib can inhibit Syk. Ibrutinib can inhibit Btk. Idelalisib can inhibit PI3K.

Gefitinib has recently been shown to accumulate in solid tumors. Haura et al. found 22 μM in lung tumor 437, and McKillop et al. found 16.7 μM in breast tumor 438. These doses were 40 and 42 times higher than the concentration observed in the plasma, respectively. We predict that this accumulation of gefitinib at the cancer site would also occur in leukemia patients. Therefore, low plasma concentrations from patients with solid tumors may not be appropriate values to consider when testing doses of gefitinib on leukemic cells that reside in the blood and lymphoid tissues. In addition, in vitro experiments cannot recapitulate the dosing scheme that would be used in vivo. Gefitinib cytotoxic concentrations in ZAP-70+ CLL cells were similar to its concentrations that induce apoptosis in AML cells 432,433.

The exact target of gefitinib and the precise mechanism by which it inhibits the BCR pathway remains unknown. This work implicates ZAP-70; however, further investigation into the binding of gefitinib to Syk and ZAP-70 is needed. Furthermore, the role of other kinase targets cannot be ignored. Although gefitinib decreased ZAP-70 phosphorylation but not Syk, Lyn, or Lck phosphorylation in cell lines, this was not the case in CLL cells: the
phosphorylations of Lyn/Lck and Syk/ZAP-70 all decreased after gefitinib treatment. It is known that Syk and ZAP-70 have different abilities and dependencies on Src family kinases: Syk can phosphorylate ITAMs independent of Src kinases and still transmit the BCR signal, albeit delayed, but ZAP-70 does not have this ability. This creates great complexity within the BCR pathway in CLL cells. The complex interplay between these kinases, and the exact target and binding of gefitinib warrants future investigation.

Despite inefficient tyrosine kinase activity in CLL, ZAP-70 still plays an important role in the over-activation of the BCR pathway. Although the kinase domain is not required for enhanced signaling, inhibition of its kinase activity may cause steric hindrance or prevent conformational changes of signaling complexes preventing downstream signaling events. Indeed, binding of small molecule or of a non-hydrolyzable form of ATP can stabilize the active conformation of the kinase. Furthermore, small molecules can prevent protein-protein interactions.

Although kinases present alluring targets for therapy, adaptor proteins have also been recognized as therapeutic targets. Adaptor proteins can play pivotal roles in the malignant transformation and actions of cancer cells. Examples of critical adaptor proteins include NEDD9 in melanoma, Crk in epithelial cancers, and insulin receptor substrate proteins in endometrial carcinoma. Thus, targeting ZAP-70 as an adaptor in CLL cells is not an unfounded notion.

Cells expressing ZAP-70 used in this study, namely ZAP-70+ CLL cells and ZAP-70+ Raji cells, tended to have higher basal cell death compared to ZAP-70- CLL cells and ZAP-70- Raji cells, respectively. This may be caused by the enhanced ability of ZAP-70, and not Syk, in inducing caspase activation in the process of activation-induced cell death.
Overall, gefitinib selectively kills CLL cells expressing ZAP-70. This indicates that tyrosine kinase inhibitors could be used to selectively treat patients with high ZAP-70-expressing CLL cells. As gefitinib is already in clinical use in lung cancer patients, and lacks suppression of the bone marrow or immune system, further studies are warranted to investigate the clinical activity of gefitinib in ZAP-70+ CLL patients.
Chapter 3

3. Targeting the Lysosome in Chronic Lymphocytic Leukemia.

Information presented in this chapter has been previously published in the article entitled “Lysosomotropic agents selectively target chronic lymphocytic leukemia cells due to altered sphingolipid metabolism” (Dielschneider et al., Leukemia, 2016).

3.1. Abstract

Chronic Lymphocytic Leukemia (CLL) cells alter many different cell death pathways to accomplish their survival goals. These pathways include apoptosis, necroptosis, and autophagic cell death. An unexplored cell death pathway in CLL is lysosome-mediated cell death. Thus, the purpose of this work was to investigate lysosome-mediated cell death in CLL cells with known drugs such as the anti-depressants siramesine, nortriptyline, and desipramine; the antibiotic ciprofloxacin; and the anti-malarial drugs mefloquine, tafenoquine, primaquine, and atovaquone. All of these drugs except ciprofloxacin, primaquine, and atovaquone permeabilized lysosomes and killed CLL cells. Siramesine was one of the most effective at the lowest doses, and was investigated further. Lysosome permeabilization was confirmed with flow cytometry and confocal microscopy. Furthermore, loss of lysosome integrity was shown by the translocation of TFEB into the nucleus. Lysosome permeabilization was accompanied with lipid peroxidation within minutes of drug treatment. Downstream events include loss of mitochondrial membrane potential and release of soluble reactive oxygen species. Only inhibition of lipid peroxidation, but not cathepsins nor soluble reactive oxygen species, prevented cell death. CLL cells were more sensitive to lysosome disruption compared to healthy B cells, and this may be due to the over-expression of SPP1 and its product sphingosine in CLL cells. The addition of this sphingolipid induced rapid lysosome permeabilization and cell death, but only in CLL cells and
not healthy B cells. This work demonstrates for the first time that CLL cells are sensitive to lysosome disruption induced by well characterized anti-depressants and anti-malarial drugs. Furthermore, this work has found that CLL cells are more sensitive than their healthy counterparts to lysosome-mediated cell death perhaps due at least in part to their altered sphingolipid metabolism.
3.2. Introduction

Drug resistance and drug toxicity in CLL highlight the need for different therapeutic approaches. CLL cells evade cell death by upregulating the pro-survival BCR pathway and altering the expression of numerous cell death proteins. A cell death pathway that has yet to be investigated in CLL cells is the pathway initiated by lysosome membrane permeabilization.

Lysosome membrane permeabilization (LMP) has been shown to be an effective therapeutic strategy in many cancer models. Despite the ubiquitous nature of lysosomes in all mammalian cell types, cancer cells have been shown to increase lysosome biogenesis and alter cellular biology thus affecting lysosomes. One such biological process that impacts lysosomes is sphingolipid metabolism.

Sphingolipids are lipids based from sphingosine. They are found in cellular membranes and play important functions in cell signaling and recognition. Sphingolipid metabolism comprises numerous dynamic pathways that form and destroy sphingolipids. Altered sphingolipid metabolism has been found in many cancers. Different cancer cell types over-express sphingosine kinase (SK) and down-regulate acidic sphingomyelinase (ASM). Alterations of ASM in transformed cells, including breast cancer cells, was shown to sensitize cells to lysosome disruption. Changes in lipid metabolism have been investigated in IgVH mutated versus unmutated CLL cases, but has never been compared in CLL cells versus their healthy B cell counterparts.

Sphingosine is the most simple of all sphingolipids. Sphingosine is the backbone onto which many other chemical groups can be added to generate more complex sphingolipids. Sphingosine can be phosphorylated to form the signaling molecule sphingosine 1-phosphate, or a fatty acid can be added to form ceramide. Recently, sphingosine has been shown to induce LMP.
Like many lysosomotropic agents, sphingosine is protonated and trapped within lysosomes where it acts like a detergent. It can permeabilize lysosomes within minutes and leads to loss of mitochondrial membrane potential. Sphingosine can rigidify membranes and form transient channels of < 2 nm in diameter. These channels are weakly anion selective and are not large enough to allow passage of large proteins. The role of sphingosine in LMP has been investigated in T cell lines, macrophage cell lines, prostate cancer cell lines, and liver cancer cell lines. The role of sphingosine has yet to be tested in B cells.

Thus, little is known about lysosomes and sphingolipid metabolism in CLL. Furthermore, it is not known if these cells are selectively susceptible to lysosome disruption. Therefore, in the present study we have investigated lysosomotropic agents as potential therapeutics in CLL cells, and examined the role of sphingolipid metabolism in this process.
3.3. Materials and Methods

3.3.1. Drugs and Stimuli

Siramesine (kindly provided by Lundbeck) was dissolved in DMSO and kept at room temperature. Nortriptyline (Sigma) was dissolved in ethanol and stored at 4 °C. Desipramine (Sigma) was dissolved in water and stored at 4 °C. Fludarabine (Sigma) was dissolved in DMSO and frozen stocks were stored at -80 °C. Ciprofloxacin (Sigma) was dissolved in 1 mM acetic acid and stored at 4 °C. Mefloquine (Sigma) was dissolved in DMSO and stored at -20 °C. Tafenoquine (Sigma) was dissolved in DMSO and stored at -20 °C. Primaquine (Sigma) was dissolved in DMSO and stored at -20 °C. D-Sphingosine (Sigma) was dissolved in DMSO and stored in single-use aliquots at -20 °C.

Various inhibitors were added 1 hour prior to drug treatment: α-tocopherol (Sigma) was dissolved in ethanol and prepared fresh for each experiment; lycopene (Sigma) was dissolved in ethanol; N-acetyl cysteine (NAC, Sigma) was dissolved in 1X phosphate-buffered saline (PBS) at pH 7.4 and made fresh for each experiment; Glutathione (Sigma) was dissolved in 1X PBS and made fresh for each experiment; Ca-074-Me (Enzo Life Sciences) was dissolved in DMSO; Chymostatin (Sigma) was dissolved in DMSO; E64 (Sigma) was dissolved in water; zVAD-fmk (Caspase Inhibitor VI, Millipore) was dissolved in DMSO; and SKI II (Sigma) was dissolved in DMSO. Unless preparations were made fresh, drug stocks were frozen at -20 °C.

3.3.2. Western Blot

Cell lysates were collected at the indicated times in 1% NP-40 lysis buffer. This buffer contained 1% NP-40, 20 mM Tris base, 150 mM NaCl, 10% glycerol, 2 mM EDTA, complete protease inhibitor tablet (Roche), 1 mM PMSF, and 2 mM sodium orthovanadate (New England
BioLabs). The later three ingredients were always added immediately prior to use. Protein levels were quantified in triplicate with Pierce BCA kit (Thermo Scientific) according to manufacturer’s instructions. Samples were run on 10% polyacrylamide gels and transferred onto 0.45 µm nitrocellulose membranes (BioRad) blocked in 5% BSA (Sigma) in tris-buffered saline with 0.1% tween-20 (TBS-T, Sigma). Primary antibodies included anti-rabbit SPP1 (#108435, Abcam), anti-TFEB (#4240, Cell Signaling), anti-Sp1 (#07-645, Millipore), anti-hexanoyl lysine (HEL, #93056, Abcam), anti-GADPH (#G8795, Sigma), and anti-actin (#A2066 or A3853, Sigma). Secondary antibodies were goat anti-rabbit-HRP or goat anti-mouse-HRP (BioRad). Detection of protein was with Pierce ECL or Pierce Supersignal Pico (Thermo Scientific) reagents.

3.3.3. Flow Cytometry

For cell viability analysis, cells were stained with Annexin V-FITC (BD) and 7AAD (BD) for 15 minutes at room temperature. For lysosome staining, cells were stained with 50 nM Lysotracker Red DND-99 (Invitrogen) for 30 minutes at 37 °C. For mitochondrial membrane potential and soluble reactive oxygen species analysis, cells were stained with 25 nM DiOC6 (Sigma) and 3.2 µM dihydroethidium (DHE, Sigma) together for 30 minutes at 37 °C. For lipid peroxidation analysis, cells were stained with 1 µM BODIPY 581/591 (Invitrogen) for 30 minutes at 37 °C. Prior to analysis, all stained cells were diluted in 1X PBS or 1X Annexin V Binding Buffer (Invitrogen). Flow cytometry experiments were done alongside unstained and single stained controls using a BD FACSCalibur machine and CellQuestPro software.

3.3.4. Confocal Microscopy

CLL cells were isolated from patient peripheral blood and cultured in Nunc Lab-Tek II chambered coverglass (Thermo Scientific) overnight. The following morning, after adhering to
the coverglass, cells were stained with 50 nM Lysotracker for 30 minutes in a humid incubator at 37 °C. Staining solution was removed and Live Cell Imaging Solution (Life Technologies) was added prior to viewing under the Olympus IX82 confocal microscope and viewed using FLUOVIEW 4.3 software.

3.3.5. Lipid Analysis

Cell pellets were re-suspended in LC/MS-grade water, vortexed for 15 seconds and sonicated for 20 minutes. Then 50 µl of a 1 µM solution containing each of the internal standards (IS) C17:1-Sphingosine, C17:0-Sphinganine, C17:1-Sphingosine-1-phosphate, C17:0-Sphinganine-1-phosphate, C17:0-Ceramide, C17:0-Glucosylceramide was added to 80 µl of the cell solution. To this solution was added 300 µL of extraction solvent (chloroform/methanol, 1:2). The mixture was vortexed, centrifuged at 2500 rpm for 15 minutes and the resultant supernatant was transferred into a separate glass vial. The extraction procedure was repeated and the two supernatants were combined. The final extract was dried under a nitrogen stream, re-dissolved into 500 µL of methanol and then filtered through a 0.22 µm membrane for subsequent analysis.

The analysis of sphingolipids was performed on an Agilent 1200 series HPLC (Agilent Technologies) coupled to a 3200 QTRAP mass spectrometer (AB SCIEX) with Analyst 1.4.2 software. The identification of individual sphingolipid molecules in the cell extracts was achieved by comparison of their retention times to the known standards, accurate mass measurements, and fragmentation patterns. Their quantification was achieved via calibration curves of analyte to IS peak area ratio vs. the concentration for each sphingolipid molecule.

Sphingosine-1-phosphate (S1P) was measured by ELISA (Echelon Biosciences). Experiment was done exactly according to kit manual. Cell were lysed in lysis buffer at pH 7.0
containing 1% Triton X-100 (Sigma), 20 mM PIPES (Sigma), 150 mM NaCl (Sigma), 1mM EGTA (Sigma), 1.5 mM MgCl2 (Sigma), 0.1% SDS (Sigma), and 1mM sodium orthovanadate. Lysates were quantified using the Pierce BCA kit (Thermo Scientific) according to manufacturer’s instructions and 30 µg was used for the S1P ELISA.

3.3.6. Statistical Analysis

All graphs were created and statistics were performed using GraphPad Prism4 software (GraphPad Software Inc.). Unless otherwise noted, a paired or unpaired, non-parametric two-tailed t-test was performed according to the nature of data. Statistical significance was noted in the figures as *p<0.05, **p<0.01, or ***p<0.001. Densitometry was calculated using ImageJ (Wayne Rasband; National Institute of Mental Health, Bethesda, MD, USA).
3.4. Results

3.4.1. CLL cells are sensitive to lysosome disruption.

Lysosome membrane permeabilization (LMP) has been recognized as a novel therapeutic strategy in cancer \(^{459}\), but little is known regarding its efficacy in CLL cells. In the present study we treated primary CLL cells with a variety of known lysosomotropic agents: siramesine \(^{207,225}\), ciprofloxacin \(^{214,232}\), nortriptyline \(^{212}\), desipramine \(^{212}\), mefloquine \(^{210}\), primaquine \(^{230,231}\), and atovaquone \(^{230}\). Tafenoquine was also chosen but its lysosomotropism was unknown. All agents except ciprofloxacin, primaquine, and atovaquone induced cell death (Figure 3.1) at doses reported in the literature (Table 1.1) as measured by the Annexin V apoptotic assay. The doses of drugs required to kill 50% of CLL cells were 5 µM siramesine, 100 µM desipramine, 100 µM nortriptyline, 20 µM mefloquine, and 5 µM tafenoquine (Figure 3.1). Since siramesine had the greatest activity at the lowest doses, it was chosen for follow-up study. Siramesine induced cell death was an early event as measured after 1 hour and remained the same after 24 hours. MTT assays were done on 4 different CLL samples to confirm the loss of cell viability. The IC\(_{50}\) values for siramesine within these four samples was 2.4, 2.8, 5.4, and 6.0 µM. Thus, the dose of 5 µM siramesine was effective at killing CLL cells as measured by Annexin V staining and MTT assay and was used in subsequent experiments.
Figure 3.1. **CLL cells are sensitive to lysosomotropic agents.** CLL cells were treated with siramesine (A, n=8), nortriptyline (B, n=6), desipramine (C, n=6), ciprofloxacin (D, n=4), tafenoquine (E, n=6), mefloquine (F, n=8), primaquine (G, n=4), or atovaquone (H, n=5) for one hour. Cell viability was measured by Annexin V and 7AAD staining and analyzed by flow cytometry.

To confirm that these agents were acting through LMP, primary CLL cells were stained with lysotracker and the staining intensity was measured using flow cytometry both before and after drug treatment in 5 minute time intervals. All drugs which caused cell death induced permeabilization of lysosomes (Figure 3.2). Primaquine was unable to be investigated in this way as it emitted fluorescence which directly overlapped with the emission of Lysotracker. Atovaquone induced a slight permeabilization of lysosomes immediately upon addition; however, lysosomes recovered by 5 minutes post addition (Figure 3.2 G). LMP was further confirmed using confocal microscopy (Figure 3.3 A) and western blot for the nuclear translocation of transcription factor EB (TFEB), a master regulator of lysosome biogenesis. Approximately 50% of the cytosolic TFEB moved into the nucleus at 30 minutes post-drug treatment.
treatment (Figure 3.3 B, C). This confirms that lysosomotropic agents rapidly induce LMP in CLL cells which is followed by cell death.

**Figure 3.2. Anti-depressants and anti-malarials permeabilize lysosomes.** CLL cells were stained with 50 nM Lysotracker for 30 minutes and fluorescence was acquired on the flow cytometer in 5 minute intervals before, during, and after treatment with 5 µM siramesine (A, representative of n=5), 100 µM desipramine (B, representative of n=3), 100 µM nortriptyline (C, representative of n=3), 20 µg/ml ciprofloxacin (D, representative of n=2), 1 µM tafenoquine (E, representative of n=5), 15 µM mefloquine (F, representative of n=4), or 60 µM atovaquone (G, representative of n=3). In each case, CLL cells were stained and treated in triplicate. Standard deviations are shown.
Among these drugs, siramesine is the most well-studied lysosomotropic agent and was the most effective at the lowest doses. Thus, this drug was used for subsequent mechanistic studies. A total of 123 different CLL patient samples were tested in various experiments.

3.4.2. Siramesine-induced LMP causes mitochondrial dysfunction and subsequent cell death.

As the mechanism of cell death with lysosome disruption appears to depend on the type of cell studied \textsuperscript{202, 214, 232, 460}, we evaluated the biological changes leading to cell death following siramesine treatment of CLL cells. Primary CLL cells were stained with lysotracker, BODIPY 581/591, DiOC6, or DHE which identify intact lysosomes, lipid peroxidation, mitochondrial membrane potential, and soluble reactive oxygen species (ROS), respectively. Staining intensity...
was measured by flow cytometry in 5 minute intervals before and after drug treatment. Within 5 minutes of drug treatment, lysotracker (Figure 3.4 A) and BODIPY red fluorescence decreased (Figure 3.4 B) while BODIPY green fluorescence increased (Figure 3.4 C), indicating lysosome permeability and lipid peroxidation. At the same time, the lipid peroxidation product hexanoyl lysine (HEL) adduct on proteins was slightly increased by western blot. This slight increase in HEL adduct formation was observed after 5 minutes of siramesine treatment (Figure 3.4 D, E). Over a 60 minute time course, there was a gradual decrease in DiOC6 (Figure 3.4 F) and increase in DHE fluorescence (Figure 3.4 E), indicating a loss of mitochondrial membrane potential and increased levels of soluble ROS, respectively. These results indicate that LMP with siramesine in CLL cells occurs prior to mitochondrial dysfunction.

Figure 3.4. Siramesine permeabilizes lysosomes, and induces lipid peroxidation and loss of mitochondrial membrane potential. Primary CLL cells were stained with lysotracker (A) or BODIPY 581/591 (B and C) and fluorescence was measured in 5 minute intervals before, at the time of, and after treatment with drug or control. Both red (B) and green (C) fluorescence was measured in BODIPY stained cells. Representative experiments of n=3 are shown. D) CLL cells were treated with DMSO or 5 µM siramesine for 30 minutes at which time cells were lysed and lysates were probed by western blot with anti-hexanoyl lysine (HEL) and anti-actin (E, n=4). CLL cells were stained with DIOC6 (F) or DHE (G) and fluorescence was measured in 5 minute intervals before, at the time of, and after treatment with drug or control. Representative experiment of n=3 is shown.
3.4.3. *Siramesine-induced cell death requires lipid peroxidation.*

It has previously been shown that siramesine-induced cell death is mediated by proteases and ROS \(^{207,460}\). Furthermore, the protease cathepsin B is required by valproic acid and fludarabine to kill CLL cells \(^{461}\). To investigate the role of proteases and ROS in siramesine-induced CLL cell death, CLL cells were pretreated with various inhibitors and scavengers for 1 hour. Scavengers of lipid ROS, such as α-tocopherol and to a lesser extent lycopene, blocked siramesine-induced cell death, whereas soluble ROS scavengers N-acetyl cysteine (NAC) and glutathione failed to prevent cell death (Figure 3.5 A-D). In contrast, protease inhibitors CA-074-Me, Chymostatin, E64, and z-VAD-fmk did not prevent siramesine-induced cell death (Figure 3.5 E-I). To determine if lipid peroxidation involved oxidases, inhibitors of xanthine oxidase and NADPH oxidase were tested. These failed to prevent siramesine-induced cell death (Figure 3.5 J, K).
Figure 3.5. Siramesine-induced cell death requires lipid ROS. CLL cells were pretreated with α-tocopherol (A), lycopene (B), glutathione (C), N-acetyl cysteine (NAC, D), Ca-074-Me (E and F), E64 (G), Chymostatin (H), zVADfmk (I), allopurinol (J), diphenyleneiodoium (DPI, K), or neopterin (K) for 1 hour, then treated with 5 µM siramesine for 1 hour. Cell viability was assessed by Annexin V and 7AAD staining measured by flow cytometry (A–D, F–K). Cathepsin B activity was assessed using fluorescent cathepsin B substrate (E).

To further investigate the role of lipid ROS in the process of siramesine-induced cell death, we evaluated the effect of α-tocopherol on LMP and mitochondrial membrane potential in CLL cells treated with siramesine. α-Tocopherol failed to prevent LMP (Figure 3.6 A, B) but blocked lipid peroxidation (Figure 3.6 C, D), changes in mitochondrial membrane potential (Figure 3.6 E, F), and the increase in soluble ROS (Figure 3.6 G, H). These results indicate that lipid ROS are not required for LMP but are a consequence of LMP, leading to mitochondria dysfunction and cell death.
3.6. Early lipid peroxidation is required to affect mitochondria. CLL cells were either not pretreated (A,C,E,G) or pretreated with α-tocopherol (B,D,F,H) for one hour, then stained with lysotracker (A,B), BODIPY (C,D), DIOC6 (E,F), or DHE (G,H) for 30 minutes then analyzed in 5 minute intervals before, at the time of, and after treatment with drug or control. Representative experiment of n=3 is shown.

3.4.4. CLL cells are more sensitive than healthy B cells to LMP.

To determine whether CLL cells are more sensitive than normal B cells to siramesine treatment, peripheral blood mononuclear cells (PBMCs) from CLL patients and age-matched normal donors were isolated and treated with 5 μM siramesine. Results showed that CLL cells were more sensitive to siramesine treatment than normal B cells. A median of 15% of normal B cells or 40% of CLL cells were killed by 5 μM siramesine (Figure 3.7 A). To confirm these results with another drug, PBMCs from CLL patients and age-matched normal donors were
isolated and treated with various doses of tafenoquine. Like with siramesine, CLL cells were more sensitive than healthy B cells to tafenoquine (Figure 3.7 B). A median of 32% of normal B cells or 80% of CLL cells were killed by 3 μM tafenoquine. Thus, CLL cells are more sensitive than normal B cells to lysosome-mediated cell death mediated by siramesine and by tafenoquine.

![Figure 3.7. Lysosomotropic agents target CLL cells over healthy B cells.](image)

3.4.5. Altered sphingolipid metabolism in CLL cells primes the lysosomes for disruption.

To investigate the possible mechanisms for the enhanced sensitivity of CLL cells to LMP, we first determined the number of lysosomes in cells. Lysosomes from 100 CLL cells or normal B cells were counted from images acquired using confocal microscopy. Lysosomes were counted from a single focal plane. CLL cells were found to have a slight, but statistically-significant, higher average number of lysosomes per cell than normal B cells, with means of 6 lysosomes/cell and 4 lysosomes/cell, respectively (Figure 3.8 A, C, D). Furthermore, there were more lysosomes in cells from male donors compared to female donors (Figure 3.8 B). This difference lost its significance when males and females were divided into CLL and healthy groups, possibly due to low sample size.
Figure 3.8. CLL cells and male B cells have more lysosomes per cell than healthy/normal B cells and female B cells, respectively. Lysosomes were counted from a single plane view under the confocal microscope using 40X objective and 2X zoom from 100 lysotracker-stained cells from normal age-matched donors (dark grey squares or bars) or CLL donors (light grey triangles or bars). Average counts (A) of healthy B cells and CLL cells, and of male cells and female cells (B) stained with Lysotracker (C), and individual counts per donor (D) are shown.

Sphingolipid metabolism was compared in CLL and healthy/normal B cells as the lysosome is a major storage site for lipids in the cell and alterations in lipid metabolism have profound effects on lysosome function. To determine differences between normal B and CLL cells, the GEO and ONCOMINE databases were mined for differences in lysosome or lipid metabolism genes. One alteration that was found consistently was an over-expression of sphingosine 1-phosphate phosphatase 1 (SGPP1 or SPP1) in CLL cells compared to normal B cells (Figure 3.9 A, B). Western blot analysis confirmed this over-expression at the protein level (Figure 3.9 C). In addition, the product of this enzyme, C18:1 sphingosine, was also increased in CLL cells, as compared to healthy/normal B cells (Figure 3.9 D). Levels of other sphingolipids such as sphingosine-1-phosphate (Table 3.1), C18:0 ceramide, C16:0
glucosylceramide, and C20:0 glucosylceramide (Table 3.2) did not differ between CLL and normal B cells.

**Figure 3.9. CLL cells have more SPP1 and its product sphingosine.** Compared with normal/healthy B cells, CLL cells have more *SGPP1* mRNA (A and B) as measured by microarray, SPP1 protein (C) as measured by western blot, and its product sphingosine (D) as measured by HPLC-MS. Normal sample sizes are n=11 (B), n=10 (C), and n=4 (D). CLL sample sizes are n=44 (B), n=14 (C), and n=5 (D). HPLC-MS experiments were performed by collaborators Lisa Mi and Dr. Jonathan Curtis (University of Alberta). Analysis performed by myself.

**Table 3.1. CLL cells and B cells have similar sphingosine-1-phosphate levels.** Sphingosine-1-phosphate levels were determined in lysates from CLL cells (n=8) and healthy/normal B cells (n=8) by ELISA. Both mean and median values are shown. Statistical Mann Whitney test (unpaired, non-parametric t test) showed that differences between the medians were non-significant (ns).

<table>
<thead>
<tr>
<th></th>
<th>CLL Cells</th>
<th>B Cells</th>
<th>Statistical significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Mean</td>
</tr>
<tr>
<td>Sphingosine-1P</td>
<td>0.943 µM</td>
<td>0.479 µM</td>
<td>0.558 µM</td>
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Table 3.2. CLL cells have more C18:1 sphingosine and more C24:1 glucosylceramide than B cells. Sphingolipid levels were measured in lipid extracts from CLL cells (n=5) and healthy/normal B cells (n=4) by HPLC-MS. Both mean and median values are shown. Mann Whitney test (unpaired, non-parametric t test) determined if differences in the medians were significant or non-significant (ns). HPLC-MS experiments were performed by collaborators Lisa Mi and Dr. Jonathan Curtis (University of Alberta). Analysis performed by myself.

<table>
<thead>
<tr>
<th>Sphingolipid</th>
<th>CLL Cells</th>
<th></th>
<th>B Cells</th>
<th></th>
<th>Statistical significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Mean</td>
<td>Median</td>
<td></td>
</tr>
<tr>
<td>C18:1 Sphingosine</td>
<td>22.7 nM</td>
<td>19.8 nM</td>
<td>5.6 nM</td>
<td>4.0 nM</td>
<td>* (0.0317)</td>
</tr>
<tr>
<td>C16:0 Glucosylceramide</td>
<td>31.5 nM</td>
<td>31.9 nM</td>
<td>22.7 nM</td>
<td>19.0 nM</td>
<td>ns (0.2857)</td>
</tr>
<tr>
<td>C18:0 Glucosylceramide</td>
<td>4.6 nM</td>
<td>4.5 nM</td>
<td>3.9 nM</td>
<td>2.0 nM</td>
<td>ns (0.5714)</td>
</tr>
<tr>
<td>C20:0 Glucosylceramide</td>
<td>6.8 nM</td>
<td>5.0 nM</td>
<td>8.7 nM</td>
<td>7.3 nM</td>
<td>ns (0.5556)</td>
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<tr>
<td>C22:0 Glucosylceramide</td>
<td>17.5 nM</td>
<td>17.6 nM</td>
<td>12.1 nM</td>
<td>12.3 nM</td>
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<tr>
<td>C24:1 Glucosylceramide</td>
<td>89.7 nM</td>
<td>80.8 nM</td>
<td>34.3 nM</td>
<td>29.8 nM</td>
<td>* (0.0317)</td>
</tr>
<tr>
<td>C24:0 Glucosylceramide</td>
<td>5.9 nM</td>
<td>4.8 nM</td>
<td>3.9 nM</td>
<td>3.4 nM</td>
<td>ns (0.1905)</td>
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<tr>
<td>C16:0 Ceramide</td>
<td>143.9 nM</td>
<td>135.6 nM</td>
<td>98.5 nM</td>
<td>49.3 nM</td>
<td>ns (0.2857)</td>
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<tr>
<td>C18:0 Ceramide</td>
<td>14.9 nM</td>
<td>13.1 nM</td>
<td>12.5 nM</td>
<td>5.1 nM</td>
<td>ns (0.2857)</td>
</tr>
<tr>
<td>C20:0 Ceramide</td>
<td>10.0 nM</td>
<td>8.6 nM</td>
<td>6.6 nM</td>
<td>3.0 nM</td>
<td>ns (0.2857)</td>
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<tr>
<td>C22:1 Ceramide</td>
<td>16.0 nM</td>
<td>10.5 nM</td>
<td>3.8 nM</td>
<td>2.1 nM</td>
<td>ns (0.1111)</td>
</tr>
<tr>
<td>C22:0 Ceramide</td>
<td>28.8 nM</td>
<td>23.2 nM</td>
<td>12.2 nM</td>
<td>9.0 nM</td>
<td>ns (0.1111)</td>
</tr>
<tr>
<td>C24:1 Ceramide</td>
<td>261.1 nM</td>
<td>227.3 nM</td>
<td>80.2 nM</td>
<td>48.5 nM</td>
<td>ns (0.0635)</td>
</tr>
<tr>
<td>C24:0 Ceramide</td>
<td>26.7 nM</td>
<td>19.8 nM</td>
<td>13.0 nM</td>
<td>9.6 nM</td>
<td>ns (0.1905)</td>
</tr>
</tbody>
</table>

To confirm the role of sphingosine in sensitizing lysosomes to disruption, we treated CLL cells directly with sphingosine. The addition of sphingosine significantly increased siramesine-induced cell death (Figure 3.10 A). Likewise, increasing sphingosine levels by inhibiting sphingosine kinase (SK) with sphingosine kinase inhibitor (SKI) II also increased siramesine-induced cell death (Figure 3.10 B). A lower dose of 3 µM siramesine was used for these experiments. Similar to siramesine (Figure 3.10 C, D), sphingosine was found to disrupt lysosomes and cause permeabilization within minutes which led to a later effect on mitochondria (Figure 3.10 E, F). In addition, 5 and 10 µM sphingosine caused significant cell death in CLL cells within 1 hour but was not toxic to healthy/normal B cells (Figure 3.10 G, H). This effect
was presumably because CLL cells already have high levels of sphingosine. These results show that sphingolipid metabolism, particularly sphingosine metabolism, is altered in CLL cells and the high levels of sphingosine increase lysosomal disruption.
Figure 3.10. Excess sphingosine causes lysosome disruption. CLL cells were pretreated with 0.5 μM sphingosine (A) or 20 μM Sphingosine Kinase Inhibitor II (SKI II) (B) for one hour, followed by 3 μM siramesine for one hour. The cells were then stained with Annexin V-FITC and 7AAD and analyzed by flow cytometry. CLL cells were stained with lysotracker (C, E) or DiOC6 (D, F) then analyzed in 5 minute intervals before, at the time of, and after treatment with siramesine or sphingosine or control. Results shown are representative of n=3. Primary CLL cells (G, n=4) or healthy/normal PBMCs (H, n=4) were treated with 1.0, 5.0, or 10.0 μM sphingosine for one hour then stained with Annexin V-FITC, 7AAD, and anti-CD19-APC then analyzed by flow cytometry.
3.5. Discussion

Since their discovery as the suicide bags of the cell, lysosomes have been explored as therapeutic targets in cancer. However, little is known about lysosomes and lysosome-mediated cell death in CLL. The results of the present study demonstrate that CLL cells are susceptible to lysosome-mediated cell death, particularly to siramesine treatment. In addition, we demonstrated that following siramesine induced LMP, lipid peroxidation occurs which leads to mitochondrial permeabilization and cell death. Moreover, siramesine had little effect on normal B cells and the selective killing of CLL cells may be related to the increased number of lysosomes in these cells and increased lysosome fragility due to altered sphingolipid metabolism.

Clinically-tested agents were chosen for evaluation in CLL cells. Siramesine was originally developed as a sigma receptor antagonist for the treatment of depression \(^466\). Nortriptyline and desipramine are FDA-approved anti-depressants \(^467\). Ciprofloxacin is a FDA-approved antibiotic \(^468\). Mefloquine, primaquine, and atovaquone are all FDA-approved anti-malarial drugs \(^469\); tafenoquine has received FDA breakthrough therapy status \(^470\). All these agents have been shown to induce LMP in cancer cells (Table 3.1) except for tafenoquine whose lysosomotropism was unknown. Thus, this work is the first to report that tafenoquine can permeabilize lysosomes. Furthermore, this report was the first to test tafenoquine in a cancer model. These agents induced a rapid permeabilization of lysosomes followed by relatively early signs of cell death. This rapid response may lead to tumor lysis syndrome \textit{in vivo}; however, this has not been noted in any of the published \textit{in vivo} animal studies in other cancer models.

Lysosomotropic agents induce cell death through increased ROS and activation of cathepsins. We found that siramesine caused lipid peroxidation leading to cell death. The pivotal role of lipid peroxidation in siramesine-induced cell death has been demonstrated in other
CLL cells may be particularly sensitive to lipid peroxidation, as one report found that they lack endogenous inhibitors of lipid peroxidation: tocopherols; while healthy B cells were found to have 6.0 µg of tocopherols/10^9 cells, CLL cells were found to have 2.6 µg/10^9 cells. Thus, CLL cells have less than half of the tocopherols found in healthy counterparts. Furthermore, B cells have previously been shown to be more sensitive to oxidative stress than other cells, including T cells.

Lysosomotropic agents, such as siramesine, activate cathepsins leading to degradation of anti-apoptotic proteins in a variety of different cancer cells. In CLL cells, it was previously demonstrated that valproic acid and fludarabine in combination increases cathepsin B expression and cathepsin B-mediated cell death in CLL cells. Despite the role of cathepsin B in siramesine treatment in other cancer cells, we found that cathepsin B was not required for siramesine-induced cell death in CLL cells. This suggests the main mechanism of action for siramesine in CLL cells is through lipid peroxidation and not cathepsin B. Although, it is possible that other cathepsins could contribute.

The direct mechanism by which lysosome permeabilization leads to loss of mitochondrial membrane potential is unknown in CLL cells. We speculate that lipid peroxidation generated at the lysosomal membrane could directly oxidize and open the mitochondrial permeability transition pore, as this has been shown in other models. The exact mechanism of lysosome-mitochondria crosstalk is unknown.

Aside from characterizing the efficacy and the mechanism of action of siramesine, we investigated alterations in lysosomes that could explain the differential sensitivity of CLL cells to normal B cells. To this end, we showed that CLL cells tend to have more lysosomes compared with normal B cells. An increase in lysosome size or biogenesis has been noted in other cancers.
such as AML which contributes to their sensitization to lysosomotropic agent treatment. CLL cells may have more lysosomes as a result of activation; lysosomes are required to extract and present antigens but their biogenesis in activated B cells and cancerous B cells remains to be studied. The role of CLL lysosome numbers in response to siramesine is unknown, and would require future investigation.

Similar to the slight increase in lysosome number, a slight increase in mitochondria has been noted in CLL cells compared to normal B cells. Mitochondrial functions are also altered in cancer cells, including CLL cells, contributing to increased ROS levels. However, the role of mitochondria in siramesine-induced cell death is controversial. Indeed, one study has suggested that siramesine functions through mitochondria rather than through lysosomes to induce cell death. In CLL cells, siramesine permeabilized lysosome membranes before changes in mitochondrial function occur, and blocking caspases or mitochondrial soluble ROS production did not prevent siramesine-induced cell death. This suggested that although mitochondria are involved in cell death, lysosome permeabilization is the initiating event.

Aside from alterations in lysosome numbers, lysosome function could be affected by altered sphingolipid metabolism. Altered sphingolipid metabolism has been described in cancer cells, however this is the first study that has found an increased expression of sphingosine-1-phosphate phosphatase (SPP1). Studies have found alterations in sphingosine kinase (SK) and acidic sphingomyelinase (ASM) in cancer cells; however, according to cancer expression databases these proteins are not altered in CLL cells. Although siramesine inhibits ASM, it is unlikely that this plays a role in the enhanced sensitivity of CLL cells to lysosome disruption compared to normal B cells. Instead, the elevated sphingosine content appears to make CLL cells more sensitive to sphingosine or siramesine, as compared to
normal B cells. This agrees with published studies that have shown that addition of sphingosine causes cell death \(^{453,454,458}\).

Sphingolipid metabolism is a complex process which centers on the reversible metabolism of sphingosine-1-phosphate into sphingosine, then into ceramide, and onto more complex glucosylceramides. Many of these components, particularly sphingosine-1-phosphate and ceramide, act as second messengers to promote signaling and affect the balance between cell survival and cell death \(^{464}\). Sphingosine, but not sphingosine-1-phosphate or ceramide, was elevated in CLL cells compared to normal B cells. Excess sphingosine could affect all membranes within the cell, but the lysosome membrane can be specifically permeabilized with a pH-activated drug like siramesine. It is unclear at this point if excess sphingosine confers any benefit to the CLL cells, and this will be the focus of future investigation.

Sphingolipid analysis revealed that aside from sphingosine, the only other sphingolipid altered in CLL cells was C24:1 glucosylerceramide. Furthermore, GEO and ONCOMINE databases reveal that a glucosylceramide-generating enzyme UGCG is over-expressed in CLL cells compared to healthy B cells. This enzyme was shown to be controlled by BCR signaling \(^{480}\). Excess glucosylceramides have been linked to drug resistance and cancer progression \(^{445,481,482}\). Interestingly, the accumulation of glucosylceramides in Gaucher’s disease, a lysosomal storage disease, has been linked to the development of B cell cancers \(^{483}\). The biological implications of excess glucosylceramide in CLL cells requires confirmation and future investigation.

Overall, this work identifies yet another cell death pathway that is altered in CLL (Figure 3.11). Taken together, our findings provide evidence that lysosomotropic agents such as siramesine selectively induce cell death in primary CLL cells compared to normal B cells. This selectivity is at least partially due to alterations in sphingolipid metabolism and provides
rationale to develop lysosomotropic agents for treatment of CLL. Using lysosomotropic agents in combination with chemotherapeutic agents is the focus of future investigation.

**Figure 3.11. CLL cells alter many cell death mechanisms.** CLL cells alter apoptosis by overexpressing Bcl-2 and IAPs while downregulating Smac. CLL cells alter necroptosis by overexpressing IAPs and downregulating CYLD and RIP3. CLL cells alter autophagy by overexpressing ATG4, UVRAG, and ULK1. CLL cells alter the pathway of lysosome-mediated cell death by an overabundance of sphingosine, and a decreased quantity of tocopherols. For illustrative purposes, upregulations are shown in green and downregulations are shown in red.
Chapter 4

4. Immune Impact and Clinical Correlation of Targeted Therapies.

Information presented in this chapter has been previously published in the articles entitled “Gefitinib targets ZAP-70-expressing chronic lymphocytic leukemia cells and inhibits B-cell receptor signaling” (Dielschneider et al., Cell Death and Disease, 2014) and “Lysosomotropic agents selectively target chronic lymphocytic leukemia cells due to altered sphingolipid metabolism” (Dielschneider et al., Leukemia, 2016).

4.1. Abstract

Chronic Lymphocytic Leukemia (CLL) is a cancer plagued with drug resistance and disease relapse. Thus, novel therapies are needed. These novel therapies must target susceptibilities of the leukemic cells while sparing healthy cells. Therefore, the purpose of this work is to assess impact on immune cells and to perform clinical correlations of the targeted therapies gefitinib and lysosomotropic agents. Firstly, the toxicity of these therapies towards T cells and towards CLL cells in the protective presence of the bone-marrow stromal cell line HS-5 was assessed. Gefitinib and siramesine were both more effective in CLL cells compared to T cells isolated from CLL patients. Both of these treatments were also effective in the presence of stromal cells, unlike fludarabine. Secondly, these therapies were investigated in various CLL samples with different prognostic indicators, treatment histories, ages, and genders. Gefitinib was equally effective in CLL cells of different Rai stages and IgVH mutational status, but was more effective in ZAP-70+ CLL cells and its effectiveness increased as ZAP-70 expression increased. Siramesine was effective in CLL cells of all Rai stages, IgVH mutated and unmutated, and ZAP-70- and ZAP-70+. Gefitinib was effective in both treatment naïve and previously-treated CLL cells; in contrast, siramesine was more effective in previously-treated CLL cells. Gefitinib was
least effective in CLL cells from elderly patients, while siramesine was equally effective in CLL cells regardless of patient age. Gefitinib was effective in CLL cells from both male and female patients, but siramesine was more effective in CLL cells from male patients. Therefore, this work demonstrates that the targeted therapies gefitinib and siramesine have favourable preclinical characteristics and show promise as CLL therapeutic agents.
4.2. Introduction

Chronic Lymphocytic Leukemia (CLL) is a heterogeneous cancer of B cells; patients can present with various different prognostic markers and some patients require aggressive treatment while others do not. Furthermore, this cancer is not uniform in its distribution around the body; CLL cells accumulate not only in the blood but also in the bone marrow and lymph nodes.

CLL cells interact with numerous cell types within the body, and these interactions should be considered during therapeutic investigation. T cells are the most abundant lymphocyte population in the human body, and these cells are impaired in CLL patients. In spite of these functional impairments, T cells from some CLL patients can mount an effective anti-CLL immune response improving patient survival. However, treatment can reduce T cell numbers causing toxicity and reducing the potential anti-tumor effect. In addition, stromal cells can reduce the effect of therapy by protecting CLL cells from cell death. Therefore, the cytotoxicity toward T cells and the efficacy amid protective stromal cells should be investigated.

CLL can be indolent or aggressive, and these different clinical courses can be defined by poor prognostic markers. Patients vary in disease characteristics such as Rai stage, IgVH mutational status, ZAP-70 expression, and more. Patients can also vary in age, gender, and treatment status. All these variables can impact patient management and treatment decisions. Male patients and those with advanced Rai stage, unmutated IgVH, and ZAP-70 expression are likely to have aggressive disease and will have a shorter time to first treatment. In addition, those who were previously-treated may be eligible for treatments that are unavailable to treatment-naïve patients. Similarly, elderly patients are not eligible for as many treatments as more young and fit patients. Therefore, biomarkers of poor prognosis,
treatment status, age, and gender are all variables that should be considered when evaluating drug efficacy.

The targeted therapies gefitinib and siramesine have now been tested in CLL cells, but little is known about their efficacy on other cell types in CLL patients, and in CLL cells from patients with different clinical characteristics. Therefore, the purpose of this study was to focus on the targeted agents gefitinib and siramesine and assess their T cell toxicity, investigate efficacy amid stromal cells, and correlate drug toxicity with clinical characteristics.
4.3. Materials and Methods

4.3.1. Cell Cultures and Co-cultures

Primary CLL cells were processed from peripheral blood samples within 24 hours of blood draw. Patient samples were collected following their informed consent in accordance with the Research Ethics Board of the University of Manitoba. Blood was mixed with RosetteSep (Stemcell Technologies) for 30 minutes if lymphocyte count < 40 x 10^9 cells/L, then purified by centrifugation on a Ficoll-Paque gradient (GE Healthcare). If T cells were required, the RosetteSep was omitted. Red Blood Cells (RBCs) were lysed using the RBC lysis buffer (eBioscience) for 10 minutes. Purified cells were cultured in Hybridoma Serum-Free Medium (SFM; Life Technologies) with no additives. All cells were kept in a humid 37°C incubator with 5% CO₂.

The human bone marrow-derived stromal cell line HS-5 (kind gift from Dr. Peng Huang, MD Anderson Cancer Center, USA) was cultured in DMEM (Hyclone) with 10% FBS (Thermo Fisher Scientific) and 1X penicillin and streptomycin (Gibco, Thermo Fisher Scientific). Cells were seeded into 48-well plates at a concentration of 5 x 10^5 cells/well 1 day prior to CLL addition. Primary CLL cells were added onto HS-5 cells at a co-culture ratio of 100 CLL:1 HS-5 cell; therefore, 5 x 10^6 CLL cells were added to 5 x 10^4 HS-5 cells. All cells were kept in a humid 37°C incubator with 5% CO₂.

4.3.2. Drugs and Stimuli

The following drugs were dissolved in DMSO: Gefitinib (LC Laboratories), Dasatinib (LC Laboratories), Fludarabine (Sigma), Siramesine (kindly provided by Lundbeck), Mefloquine (Sigma), and Tafenoquine (Sigma). All drugs stocks were stored at -20 °C except for siramesine which was stored at room temperature.
4.3.3. Flow Cytometry

For extracellular staining of T and B cells, peripheral mononuclear cells were stained with Annexin V-FITC, anti-CD3-PE, 7AAD, and anti-CD19-APC (BD Biosciences). Cells gated on either CD19+ or CD3+ were then analyzed for expression of Annexin V and 7AAD. Likewise, CLL cells from co-cultures were collected and stained for Annexin V-FITC, 7AAD, and anti-CD19-APC. Cells were gated on CD19 and then analyzed for Annexin V and 7AAD. All flow cytometry experiments were done using the BD FACSCalibur and analyzed using CellQuest Pro software.

4.3.4. Clinical Correlations

Drug efficacy was measured by MTT assay for gefitinib and Annexin V staining for siramesine. These experiments were done blinded to clinical characteristics of samples, except in the case of ZAP-70 expression. Drug efficacy was compared with the age, sex, treatment status, Rai stage, and presence of poor prognostic factors such as ZAP-70 expression and IgVH mutational status. Treatment status was denoted as treatment naïve or previously treated. Previously-treated samples were donated from patients that had received treatment, but was at least 6 months prior to the blood sample donation; this previous treatment was given to the patient in vivo, not given to the cells in vitro.

4.3.5. Statistical Analysis

All graphs were created and statistics were performed using GraphPad Prism4 software (GraphPad Software Inc.). Unless otherwise noted, a paired or unpaired two-tailed non-parametric t-test was performed according to the nature of data. Statistical significance was noted in the figures as *p<0.05, **p<0.01, or ***p<0.001. Densitometry was calculated using ImageJ (Wayne Rasband; National Institute of Mental Health, Bethesda, MD, USA).
4.4. Results

4.4.1. Targeted therapies spare T cells.

Targeted therapies should not only be able to kill malignant cells, but should do so without harming healthy cells. To test if the targeted therapies investigated in this study affect healthy cells, PBMCs from CLL patients were treated with drug then cell death of T cells and CLL cells was analyzed. Both gefitinib (Figure 4.1 A) and siramesine (Figure 4.1 B) are significantly more effective in CD19+ CLL cells than they were in CD3+ T cells from CLL patients. Gefitinib and siramesine were both 6X more effective in CLL cells compared to autologous and non-malignant T cells.

![Figure 4.1. Targeted therapies spare autologous T cells.](image)

Peripheral blood mononuclear cells from CLL patients were treated with 10 µM gefitinib (n=4, A) or 5 µM siramesine (n=4, B) and stained with Annexin V-FITC, anti-CD3-PE, 7AAD, and anti-CD19-APC. Cell viability of CD3+ T cells and CD19+ CLL cells was measured by flow cytometry.

4.4.2. Stromal cells protect from drug toxicity.

ZAP-70+ CLL cells migrate into the bone marrow more efficiently than ZAP-70- CLL cells. Furthermore, bone marrow-derived stromal cells promote drug resistance in CLL cells. Thus, we decided to investigate the effect of the ZAP-70-targeted therapy gefitinib, as well as other targeted therapies, in a co-culture model of CLL cells with the human bone marrow-
derived stromal cell line HS-5 (Figure 4.2 A). The presence of HS-5 cells protected from spontaneous cell death in DMSO-treated cells and fludarabine-induced cell death (Figure 4.2 B, E). However, HS-5 cells did not protect from gefitinib or dasatinib-induced cell death (Figure 4.2 C, D). There is a trend for HS-5 protection from siramesine-induced cell death; however, this did not reach statistical significance as it did with tafenoquine and mefloquine (Figure 4.2 F-H). Results shown are normalized to DMSO treatment, meaning that cell death from CLL + DMSO was subtracted from CLL + Gefitinib, and cell death in HS-5 + CLL + DMSO was subtracted from HS-5 + CLL + Gefitinib, and so on.

Figure 4.2. Kinase inhibitors are just as effective in the presence of stromal cells. CLL cells were co-cultured with the HS-5 cell line (A) and treated with DMSO (B), gefitinib (C), Dasatinib (D), fludarabine (E), siramesine (F), tafenoquine (G), or mefloquine (H). Cell viability was assessed by flow cytometric analysis of cells stained with Annexin V-FITC and 7AAD. All results for drug treatments are normalized to DMSO vehicle control.

4.4.3. Efficacy of targeted therapies in CLL subsets.

It is advantageous to know the efficacy of therapies in subsets of CLL. Treatment decisions depend on variables such as poor prognostic indicators like Rai stage, IgVH mutational
status, and ZAP-70 expression; previously treatments; patient age; and patient gender.

Knowledge of drug response in these patients can guide drug use and application.

Advanced Rai stage indicates aggressive and metastatic disease. To investigate the response of low Rai stage and high Rai stage CLL patients to gefitinib and siramesine, the patients of various Rai stages were treated with these therapies. Response to gefitinib was measured by MTT assay. Response to siramesine was measured by flow cytometric analysis of Annexin V and 7AAD staining. There was no significant difference between the responses of patients with different Rai stages to gefitinib or siramesine; patients with advanced Rai stage 3 or 4 responded equally as well as patients with Rai stage 0 or 1 (Figure 4.3 A, B).

As previously discussed, one of the most robust poor prognostic indicators is IgVH mutational status; CLL patients with unmutated IgVH have a significantly worse outcome than those with mutated IgVH. The efficacy of gefitinib and siramesine were tested in CLL cases of differing mutational status. Data from gefitinib MTT assays and from siramesine viability flow cytometry assays demonstrate that both targeted therapies are equally effective in unmutated and mutated CLL cases (Figure 4.3 C, D).

Aside from Rai stage and IgVH mutational status, the expression of ZAP-70 is an indicator of aggressive CLL disease with increased BCR signaling and a high migratory potential. The response to the targeted therapies gefitinib and siramesine was compared in ZAP-70+ and ZAP-70- CLL samples. As previously shown, gefitinib was more effective in ZAP-70+ CLL cells as the IC_{50} was lowest in these samples. In contrast, siramesine was equally effective in both ZAP-70+ and ZAP-70- CLL cells (Figure 4.3 E, F). In addition, there is a trending correlation between high ZAP-70 expression and low gefitinib IC_{50}. This demonstrates that
gefitinib efficacy increased with increasing percentage of ZAP-70 expression CLL cells.

Siramesine was effective regardless of the percentage of ZAP-70 expression (Figure 4.3 G, H).

Figure 4.3. Targeted therapies are effective in good and poor prognostic CLL cases. CLL cells of various Rai stages (A and B), of unmutated and mutated IgVH (C and D), and with or without ZAP-70 expression (E - H) were treated with gefitinib or siramesine. Gefitinib IC₅₀ was measured by MTT assay. Cell death from 5 µM siramesine was measured by flow cytometric analysis of Annexin V-FITC and 7AAD staining. (G) n=20. (H) n=68.
CLL patients that have been previously treated may be difficult to treat again due to drug resistance. It is important that new therapies are effective in these patients. Thus, the efficacy of the targeted therapies gefitinib and siramesine were investigated in treatment naïve and in previously-treated CLL cells. Both treatments were effective in each subgroup; however, siramesine was more effective in cells from previously-treated CLL patients (Figure 4.4).

Figure 4.4. Targeted therapies are effective in treatment naïve and previously-treated CLL cases. Both previously treated and untreated, or treatment naïve, CLL cells were treated with gefitinib (A) or siramesine (B). Gefitinib IC\textsubscript{50} was measured by MTT assay. Cell death from 5 \( \mu \text{M} \) siramesine was measured by flow cytometric analysis of Annexin V-FITC and 7AAD staining.

Elderly CLL patients are difficult to treat due to comorbidities and lack of fitness. The standard of care FCR has been deemed too toxic for these patients \textsuperscript{284}. Thus, new agents should be effective in elderly patients to provide more therapeutic options in this population. The targeted therapy siramesine was effective in CLL samples, regardless of age. In contrast, the targeted therapy gefitinib was effective in CLL cells from patients of all ages, but less effective in cells from elderly patients as indicated by a higher IC\textsubscript{50} value (Figure 4.5).
Figure 4.5. Targeted therapies are effective in CLL samples regardless of age. CLL cells of various ages were treated with gefitinib (A, n=29) or siramesine (B, n=70). Gefitinib IC$_{50}$ was measured by MTT assay. Cell death from 5 µM siramesine was measured by flow cytometric analysis of Annexin V-FITC and 7AAD staining.

Males have a higher incidence of CLL and respond poorly to treatments as compared to females $^{490,491}$. Therefore, it is necessary to determine the effectiveness of targeted treatments in both female and male cells. Results show that gefitinib was equally effective in CLL cells regardless of gender; however, siramesine was more effective in CLL cells from male patients those CLL cells from female patients (Figure 4.6).

Figure 4.6. Targeted therapies are effective in CLL samples regardless of gender. Both male and female CLL cells were treated with gefitinib (A) or siramesine (B). Gefitinib IC$_{50}$ was measured by MTT assay. Cell death from 5 µM siramesine was measured by flow cytometric analysis of Annexin V-FITC and 7AAD staining.
4.5. Discussion

This work investigates the impact on immune cells and clinical correlations of targeted therapies against ZAP-70 and the lysosome. These were measured by the response of these drugs in CLL T cells; in stromal cell co-cultures; in CLL cells with poor prognostic indicators such as advanced Rai stage, unmutated IgVH, and ZAP-70 expression; in CLL cells that were previously treated; in CLL cells from elderly patients; and in CLL cells from male and female patients. These are all important parameters to consider before translation of a therapy in a clinical setting.

Many treatments, such as fludarabine and anti-CD52 therapy, that are effective in CLL cells unfortunately also target T cells. This toxicity toward T cells results in exaggerated cytopenia and infections. It is imperative that T cells remain viable and functional, as they are necessary for numerous immune responses, such as those against tumors and viruses. Infections are a common cause of death in CLL. Furthermore, it is important to ensure that T cells remain intact and viable because they have the capacity to mount an anti-CLL immune response. T cells can be provoked, activated, and will respond to live and apoptotic CLL cells. One study found 5 out of 8, while another study found 6 out of 11 CLL patients had T cells that could mount an anti-CLL response. In fact, when T cells from CLL patients respond to at least 2 different CLL-associated antigens, patient survival is profoundly improved. Therefore, it is advantageous for therapies, such as gefitinib and siramesine, to selectively kill CLL cells and spare T cells. Gefitinib may be more effective in CLL cells compared to healthy T cells due to the overexpression of gefitinib targets, such as Syk and ZAP-70. It is not known if autologous T cells from CLL donors share the same abnormal lysosome numbers and sphingolipid metabolism as CLL cells, but these investigations could explain the higher efficacy of siramesine in CLL cells compared to T cells.
The co-culture system can recapitulate the direct and indirect contact between CLL cells and stromal cells. Various different co-culture stroma:CLL ratios of 1:20, 1:50, and 1:100 have all shown similar levels of protection to the CLL cells from fludarabine-induced apoptosis. Similar to the tyrosine kinase inhibitor ibrutinib, the effects gefitinib and dasatinib were not abrogated by the presence of stromal cells.

IgVH mutational status, ZAP-70 expression, and high Rai stage are all indicators of a poor prognosis in CLL. The targeted therapies investigated in this study are all effective, if not more effective, in CLL cases that have high Rai stage, unmutated IgVH, and ZAP-70 expression. In the case of gefitinib, as shown previously, it was more effective in aggressive ZAP-70+ CLL cells.

Previously-treated CLL cases are often drug resistant and require different therapeutic options. Although only one third of CLL cases will require treatment, many of these will receive more than one treatment regimen in the course of their disease. The targeted therapies in this study, gefitinib and siramesine, are effective in previously-treated CLL cells, and siramesine is more effective in previously-treated CLL cells. Previously-treated cells may have increased levels of autophagy and thus may have increased lysosome number, which has been demonstrated in many cancer models of drug resistance. This could possibly account for their increased sensitivity to the lysosomotropic agent siramesine. Siramesine has been shown to have efficacy in models of drug resistance.

With a median age of diagnosis of 71 years, CLL is a disease that can affect elderly adults. Elderly CLL patients are frequently frail and difficult to treat due to low fitness. These patients cannot tolerate the standard of care of FCR, and thus other treatment options are necessary. Gefitinib and siramesine were effective in CLL cells of all different ages, but
gefitinib was less effective in CLL samples from elderly CLL patients. This may not be a universal finding of tyrosine kinase inhibitors as ibrutinib is effective and improves survival in elderly frail CLL patients. Like gefitinib, rituximab is most effective in young patients.

Not only are more men diagnosed with CLL than women, but men also have more aggressive disease and respond poorly to treatment. Therefore, it is necessary to determine the effectiveness of targeted treatments in both female and male cells. While gefitinib was equally effective in cells from men and women, siramesine was more effective in cells from men than from women. This may be due to the fact that male cells had more lysosomes - the targets of siramesine. Thus, male patients may benefit more from siramesine treatment than female patients. This finding should be explored further, particularly with a larger sample size and different lysosomotropic agents. The male:female ratio of approximately 1.5-2:1 that is observed in large CLL cohorts is confirmed in this study.

In conclusion, this work demonstrates that the tyrosine kinase inhibitor gefitinib and the lysosomotropic agent siramesine show promising preclinical activity. Both gefitinib and siramesine spared patient T cells and retained efficacy in the presence of stromal cells. Furthermore, these drugs were effective regardless of poor prognostic factors. Lastly, siramesine was more effective in previously-treated CLL cells and in male CLL cells. This work supports further development of gefitinib and siramesine as therapeutics in CLL, and can guide future clinical investigation.
Chapter 5

5. Conclusions

5.1. Synthesis

The overarching theme of this thesis is drug repurposing for novel uses in CLL. There are great advantages in testing known therapies for novel purposes, compared to de novo novel drug testing. Drugs that have already been tested clinically and those that have received regulatory approval have known safety and pharmacological profiles. Knowledge of these features reduces cost and saves time because dosing schedules, drug formulations, routes of administration, and the maximum tolerated dose are already optimized and established. De novo drug development is a 10-12 year process from idea to a marketable drug; however, drug repurposing can take as few as 3 years. Thus, the application and translation of already-approved drugs into the clinic can be expedited. This repurposing and repositioning of drugs is a common strategy in numerous research fields, and has led to the optimal use of drugs such as Viagra (originally developed to treat angina) for erectile dysfunction, and Thalidomide (originally developed to treat morning sickness) for multiple myeloma and for an inflammatory leprosy condition termed erythema nodosum laprosum.

The repurposed therapies investigated in this thesis were used to inhibit susceptible signaling pathways in CLL (Figure 5.1). Firstly, this thesis identifies the novel use of gefitinib to target a known susceptibility in CLL cells: the BCR pathway. Secondly, this thesis identifies novel agents that target a novel susceptibility in CLL cells: the lysosome. Lastly, this thesis explores the effect of targeted therapies in regards to the immune system and different patient clinical characteristics.
Figure 5.1. CLL cells are uniquely sensitive to gefitinib and lysosomotropic agents. CLL cells ectopically express ZAP-70, which renders them sensitive to gefitinib treatment. Likewise, CLL cells overexpress SPP1 which produces more sphingosine (blue line) and renders the lysosome more easily permeabilized by lysosomotropic drugs. Gefitinib inhibits the pro-survival BCR pathway while lysosomotropic drugs permeabilize lysosomes, leading to cell death.

It is established that the BCR pathway is overactive in CLL cells. Not only do CLL cells have an altered BCR, often un-mutated$^{254,265}$ and stereotyped$^{79,80,84}$, but CLL cells also overexpress many of the downstream signaling proteins$^{136,139,191,336}$ and ectopically express additional kinases such as ZAP-70$^{137}$. This protein identifies a subset of CLL cases that have aggressive disease due to increased BCR signaling$^{151,154,155}$ and increased migratory potential$^{370,505}$. Until this study, there was no targeted therapy for CLL that over-expressed ZAP-70. This thesis identifies that the tyrosine kinase inhibitor gefitinib is more effective in ZAP-70+ CLL cells and cell lines as compared to ZAP-70- CLL cells and cell lines. Gefitinib inhibited Syk and ZAP-70 phosphorylation and downstream BCR signaling, ultimately preventing the pro-survival
BCR response. Furthermore, ectopic expression of ZAP-70 into a malignant B cell line increased their sensitivity to gefitinib.

It is established that permeabilization of lysosomes leads to cell death \(^{202,204}\). Moreover, the lysosome has been shown to be altered in many cancer cell types; increased lysosome biogenesis has been noted in AML \(^{210}\), bladder cancer \(^{506}\), melanoma \(^{198}\), and pancreatic cancer \(^{211}\). Until this study, it was not known if lysosomes were altered in CLL cells. Furthermore, it was not known if CLL cells were susceptible to lysosome-mediated cell death. This thesis has demonstrated that CLL cells are susceptible to lysosome-mediated cell death induced by several different anti-depressants and anti-malarial drugs. This study confirmed the lysosomotropism of siramesine, nortriptyline, desipramine, and mefloquine, and showed for the first time that tafenoquine is also a lysosomotropic agent. CLL cells are more sensitive to this form of cell death as compared to their healthy B cell counterparts. This sensitivity is due, at least in part, to the excess sphingosine produced by the over-expressed SPP1. Altered sphingolipid metabolism has been identified in several cancers \(^{212,444,449}\); however, the overexpression of SPP1 and overproduction of sphingosine may be unique to CLL.

The overall readout in several experiments performed in this thesis was cell death. This was often measured by Annexin V/7AAD staining, and results were shown as Annexin V+ as it was felt that Annexin V+7AAD+ underestimated the loss of cell viability. Therefore, although Annexin V+ cells may not be dead yet, they are not viable either. However, because Annexin V staining alone is not conclusive, complementary MTT assays and trypan blue staining were performed with gefitinib and siramesine to confirm Annexin V results.

This work goes beyond the identification of novel susceptibilities in CLL by identifying novel targeted therapies. All of the therapies used in this thesis have been previously tested in
human patients, and some like gefitinib and mefloquine have been used for decades. Gefitinib is an FDA-approved drug as an EGFR inhibitor for NSCLC\textsuperscript{345}. Siramesine was developed as a sigma-2 receptor antagonist and entered clinical trials as an anti-depressant\textsuperscript{466}. Nortriptyline and desipramine are FDA-approved tricyclic anti-depressants that inhibit the re-uptake of the neurotransmitters serotonin and norepinephrine, respectively\textsuperscript{467,507}. Ciprofloxacin is an FDA-approved antibiotic that inhibits bacterial DNA enzymes\textsuperscript{468}. Mefloquine, primaquine, and atovaquone are all FDA-approved anti-malarial drugs that inhibit metabolism and the mitochondrial transport chain in malaria parasites\textsuperscript{469,508}. Tafenoquine is an emerging anti-malarial drug which inhibits metabolism in malarial parasites. It has received FDA breakthrough therapy status\textsuperscript{470,509}. In order to repurpose these drugs in CLL, additional investigation is needed.

\textbf{5.2. Future Directions}

The future directions of this work are to investigate the benefit of excess sphingosine, role of excess glucosylceramides, lysosome biology in different sexes, drug efficacy in combination, drug efficacy in an \textit{in vivo} mouse model, and lastly drug efficacy in a clinical trial.

This thesis exploits two susceptibilities of CLL cells: ZAP-70 expression and sphingosine-destabilized lysosomes. The benefit of ZAP-70 to the CLL cell is the boost in pro-survival BCR signaling and migration into protective microenvironments\textsuperscript{151,370}. Unlike ZAP-70, the benefit of excess sphingosine is unknown. I hypothesize that excess sphingosine may form more lipid rafts and thus support the excessive signaling in CLL cells. I expect that the addition of non-toxic concentrations of sphingosine will promote lipid raft formation and enhance BCR signaling in CLL cells. In the event that this is not the case, it may be necessary to study forms of sphingosine that cannot be readily metabolized, or whose metabolism can be monitored. Thus, the role of sphingosine can be separated from that of sphingosine-1-phosphate and ceramide.
Like sphingosine, the role of excess glucosylceramide is unknown in CLL. Excess glucosylceramides may uniquely promote cancers, because the accumulation of glucosylceramides in Gaucher’s disease is specifically linked to increased rate of cancers \(^{510-512}\). Among the cancers in this population, those of B cell origin are the most common. When the synthesis of glucosylceramides is inhibited in a mouse model of the disease, the onset of B cell cancers is prevented \(^{483}\). Thus, glucosylceramides may play a role in promoting the onset of the B cell cancer CLL. Inhibition of a glucosylceramide-producing enzyme has been shown to kill CLL cells \(^{480}\), but this has not been tested \textit{in vivo} and its effect on preventing leukemia development is unknown. I expect that the addition of glucosylceramides will promote cell cycle progression and proliferation in CLL cells. A possible pitfall with this experiment is the complexity of glucosylceramides. To address this, different glucosylceramides, in particular C24:1 glucosylceramide which is in excess in CLL cells, could be tested and compared.

Further work is needed to determine the differences in lysosome numbers in healthy donors and CLL donors. In addition, lysosome counts should be done in more male and female samples, as the preliminary counts in this study found that male B cells had more lysosomes compared with female B cells. Interestingly, siramesine was marginally more effective in male CLL cells compared to female CLL cells. As this was the only lysosomotropic drug tested in a sufficient number of samples, it is unknown if the other lysosomotropic agents have this efficacy in male cells. Sophisticated multivariate analysis with a larger sample cohort is needed to determine if gender and/or lysosome number is correlated with response to lysosomotropic drugs.

One therapy alone may not cure CLL, and instead may lead to drug resistance. These malignant cells have proven that they have the ability to mutate kinase targets, such as Btk, to
resist ibrutinib treatment. Thus, gefitinib may not be suitable as a monotherapy. Mutations in ZAP-70 and Syk have not been identified in CLL; however, mutations in Btk were only found in ibrutinib-treated patients. Thus, gefitinib treatment could push CLL cells to adopt novel mechanisms of resistance, such as mutation of ZAP-70 or Syk. As half of the patients that developed Btk mutations were treated with ibrutinib as a single agent, the use of combination therapies may help prevent the development of resistance mutations. I propose testing the combination of gefitinib and lysosomotropic agents with each other, and also with treatments already approved for use in CLL such as fludarabine, rituximab, obinutuzumab, and ibrutinib. The combination of tyrosine kinase inhibitors and lysosomotropic agents has shown synergism in other cancer models (unpublished data), and therefore this combination warrants investigation in CLL. Furthermore, the combination of the targeted therapies in this study with chemoimmunotherapies already used in CLL is justified because these are standard treatments used in CLL and this data could guide in vivo testing and clinical trial design.

Following in vitro characterization, the targeted therapies should be evaluated in vivo first in mouse models, and then in human clinical trials. The most commonly-used animal models of CLL involve mice. Although the disease is found in other animals such as dogs, mice are often the most easily-accessible and cost-effective models to use. Mouse models include the xenograft model, New Zealand Black (NZB) model, Tcl-1 oncogene model, and p53−/− Tcl-1 model. The xenograft model is beneficial in that it uses human cells in vivo and has a fast disease establishment, but the effect of the immune system is absent due to the immunocompromised mouse. The NZB model has the benefit of natural CLL development, but is complicated by the simultaneous development of autoimmune diseases. The Tcl-1 model has the benefit of consistent phenotype with 100% penetrance, but only represents aggressive disease. This
model, whether with wildtype p53 or deleted p53, is the most studied model of CLL \(^{523}\). I propose testing gefitinib in the NZB model or Tcl-1 model as the CLL cells in both models express ZAP-70 \(^{523,524}\). I propose testing lysosomotropic agents in the p53\(^{-/}\) Tcl-1 model, as this model recapitulates aggressive CLL disease that is often drug resistant. I expect that each targeted therapy will decrease CLL disease burden in these mouse models. The possible pitfall of lack of efficacy can be resolved with optimization of dosing and routes of administration.

Clinical trials are required to determine if a treatment is safe in humans and effective in the desired patients. I propose Phase I/II dose escalation and efficacy study in a CLL cohort to determine the safety, maximum tolerated dose, and efficacy of a given targeted therapy: gefitinib or mefloquine. The lysosomotropic agent of choice for this proposed study is mefloquine due to the promising \textit{in vitro} results and its FDA and Health Canada approvals. For the investigation of gefitinib, ZAP-70 expression of CLL cells (>20% of cells expressing ZAP-70) will be an enrollment criterion because work presented in this thesis indicated that gefitinib is most effective in ZAP-70+ CLL cells. Patients will be stratified based on percentage of ZAP-70 expression (20-100%) and the study will be powered to investigate whether gefitinib is more effective in ZAP-70 high expressers as compared to ZAP-70 low expressers, as suggested by preliminary data presented in this thesis. For the investigation of mefloquine, relapsed/refractory CLL patients will be enrolled because these patients are most in need of novel therapies. This study will be powered to investigate whether mefloquine has more benefit in male patients, as suggested by preliminary data presented in this thesis. Blood will be collected at various time points to ensure that the drug is decreasing the CLL burden and acting on target, whether that target be ZAP-70 in the case of gefitinib or the lysosome in the case of lysosomotropic agents. This type of trial will provide valuable information needed for further clinical study. I expect that
clinically-achievable doses of gefitinib and mefloquine can decrease the quantity of CLL cells in the peripheral blood. In addition, I expect that gefitinib and mefloquine will act on target by either decreasing ZAP-70 phosphorylation or inducing lysosome permeabilization, respectively.

5.3. Conclusion

In conclusion, the work presented in this thesis identifies novel susceptible signaling pathways of CLL cells and exploits these using targeted therapies. The findings of this work show that the expression of ZAP-70 and enhanced BCR signaling in aggressive CLL cells can be exploited with gefitinib treatment. In addition, the fragile lysosomes due to excess sphingosine of CLL cells can be exploited with lysosomotropic agents. Ultimately, this work enhances the biological understanding of ZAP-70, lysosomes, and sphingolipid metabolism in CLL cells. Furthermore, this work provides promising evidence for the use of gefitinib and lysosomotropic agents as therapeutics in CLL. This work can guide future investigation of gefitinib and lysosomotropic agents in CLL, and in other cancers.
Chapter 6

6. References


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