ANTIHISTAMINES INDUCE SYNERGISTIC CELL DEATH WHEN COMBINED WITH IBRUTINIB IN MALIGNANT B CELL LINES AND PRIMARY CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

by

Aaron P. Chanas-LaRue

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Department of Immunology
University of Manitoba
Winnipeg, Manitoba

ABSTRACT

In chronic lymphocytic leukemia (CLL), malignant monoclonal B cells display aberrant levels of survival related to defects in apoptosis. These cells often develop resistance to therapies targeting susceptible components of the disease, and therefore additional druggable targets are necessary to circumvent this resistance. One such therapeutic target may be the lysosome; CLL cells have previously been shown to be selectively susceptible to cell death induced by lysosomal membrane permeabilization. Furthermore, lysosome targeting drugs have been demonstrated to induce synergistic cell death when combined with tyrosine kinase inhibitors. In this study, H1 antihistamines clemastine, desloratadine, and loratadine have been demonstrated to target lysosomes in malignant B cell lines and primary CLL cells through an oxidative apoptotic mechanism. These drugs were also able to induce synergistic cell death when combined with the tyrosine kinase inhibitor ibrutinib. This synergistic cell death was found to occur through an increase in intracellular reactive oxygen species, mitochondrial dysfunction, and degradation of the anti-apoptotic protein Mcl-1. Taken together, this study proposes to repurpose over-the-counter allergy medications in combination with drugs targeting kinase signalling networks for use as a therapeutic strategy in B cell cancers including CLL.

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AIF(1) – Apoptosis Inducing Factor (1)
Akt – Protein Kinase B
AML – Acute Myeloid Leukemia
APAF-1 – Apoptotic Protease Activating Factor 1
APRIL – A Proliferation-Inducing Ligand
ASM- Acid Sphingomyelinase
ATP- Adenosine Triphosphate
BAFF – B Cell Activating Factor
Bcl-2 – B Cell Lymphoma 2
Bcl-xL – B Cell Lymphoma-Extra Large
BCR – B Cell Antigen Receptor
BH3 – Bcl-2 Homology Domain 3
BLK – B Lymphocyte Kinase
BLNK – B Cell Linker Protein
BMSC – Bone Marrow Stromal Cell
BTK – Bruton's Tyrosine Kinase
CAD – Cationic Amphiphilic Drug
CD – Cluster of Differentiation
CI – Combination Index
CLL – Chronic Lymphocytic Leukemia
CXCR – CXC Chemokine Receptor
ddH_20 – Double-Distilled Water
DC – Dendritic Cell
DHE – Dihydroethidium
DNA – Deoxyribonucleic Acid
DMSO – Dimethyl Sulfoxide
EC50 – Effective Concentration to Kill 50% of Cells
ECL – Enhanced Chemiluminescence
ERK1/2 – Extracellular Signal-Related Kinase 1/2
FBS – Fetal Bovine Serum
FDA – United States Food and Drug Administration
FISH – Fluorescent In-Situ Hybridization
GPx4 – Glutathione Peroxidase 4
GSH – Glutathione
HBSS – Hank's Balanced Salt Solution
HRP – Horseradish Peroxidase
(X)IAP – (X-Linked) Inhibitor of Apoptosis Protein
IL – Interleukin
ITAM – Immunoreceptor Tyrosine-Based Action Motifs
IGVH – Immunoglobulin Variable Heavy Chain
LAMP- Lysosomal-Associated Membrane Protein
LEF1 – Lymphoid Enhancer Binding Factor 1
LMP – Lysosomal Membrane Permeabilization
MAPK – Mitogen Activated Protein Kinase

MBL – Monoclonal B Cell Lymphocytosis
Mcl-1 – Myeloid Cell Leukemia 1
MMP – Mitochondrial Membrane Potential
NAC – N-Acetylcysteine
NF-κB – Nuclear Factor Kappa-Light Chain Enhancer of Activated B Cells
NLC – Nurse-Like Cell
NSCLC – Non-Small Cell Lung Cancer
NP-40 – Nonidet P-40
OMM – Outer Mitochondrial Membrane
PBMC – Peripheral Blood Mononuclear Cell
PBS – Phosphate-Buffered Saline
PI3Kδ – Phosphatidylinositol-4,5-bisphosphate 3-kinase Delta
PLCγ2 – Phospholipase C-gamma-2
RBC – Red Blood Cell
RIPK – Receptor-Interacting Protein Kinase
RIPA – Radioimmunoprecipitation Assay
ROS – Reactive Oxygen Species
S1PP- Sphingosine-1 Phosphate Phosphatase
SFM – Serum-Free Media
SK(I)- Sphingosine Kinase (Inhibitor)
SLL – Small Lymphocytic Lymphoma
Smac – Second Mitochondrial Activator of Caspases
Syk – Splenic Tyrosine Kinase
TBS(T) – Tris-Buffered Saline (Tween)
TKI – Tyrosine Kinase Inhibitor
TLR – Toll-like Receptor
TNF(R) – Tumor Necrosis Factor Receptor
TMRM – Tetramethylrhodamine
TRADD – Tumor Necrosis Factor Receptor Type 1-associated Death Domain Protein
TRAIL – TNF-related Apoptosis Inducing Ligand
VEGF – Vascular Endothelial Growth Factor
WBC – White Blood Cell
ZAP-70 – Zeta Chain Associated Protein Kinase 70

CHAPTER 1: INTRODUCTION

1.1 HALLMARKS OF CANCER AND CHRONIC LYMPHOCYTIC LEUKEMIA

In all multicellular organisms, a balance between cell survival and cell death is necessary to maintain life. When this balance goes awry through alterations in cellular machinery and/or their surrounding tissues, cancer may arise. The term "cancer" cannot be simplified to just one disease. The word spans a huge variety of maladies, each with different cells of origin and affected organs. Cancers can be indolent or aggressive, require immediate treatment or no treatment at all, and develop from a range of tissue types from epithelial cells to immune cells. Even within the same diagnosis, cancers can present heterogeneously based on biomarker expression. It has long been debated what exactly defines a cancer, but it is generally agreed upon that there are 6 major alterations that occur in cancer development. These hallmarks are increased proliferation, avoiding growth suppression, resistance to apoptosis, indefinite replication, promoting abnormal angiogenesis, and invading/metastasizing to surrounding tissues [1]. Recently, research has identified two more additions to this list: altered metabolism and evasion of the immune system [2].

In Canada alone, it is estimated that there were over 200,000 new diagnoses and 80,000 death from cancer in the year 2017 [3]. Nearly a quarter of Canadians are expected to die from some form of cancer, and these numbers will only increase with the increasing aging population. Among these diseases is chronic lymphocytic leukemia (CLL), a cancer of the B cell and the most common leukemia. CLL can exemplify many of the hallmarks of cancer described above, including apoptotic resistance, proliferation, altered metabolism, and immune evasion. This disease is incurable, with long-term treatments imposing a strong burden on the quality of life of patients at significant cost to the Canadian health care system. It is estimated that the average

yearly cost of treating each case of CLL is over $150,000 and will increase 4-fold by 2025 with an increasing shift to first-line targeted therapies [4]. Therefore, the work in this thesis proposes to investigate susceptible drug targets in CLL using over-the-counter drugs to improve the current paradigm of CLL treatment.

1.2 CHRONIC LYMPHOCYTIC LEUKEMIA

1.2.1 DIAGNOSIS AND STAGING

CLL is the most common leukemia in North America, with over 15,000 new diagnoses per year in the United States and over 2500 in Canada [5, 6]. The disease occurs predominantly in males, with 30% more diagnoses compared to females [7, 8]. CLL is characterized by the accumulation of monoclonal B lymphocytes in the peripheral blood (lymphocytosis) and lymphoid organs (lymphadenopathy and splenomegaly). It is diagnosed by detection of pronounced lymphocytosis through blood tests, with greater than 5×10^9 monoclonal B cells liter of blood required for diagnosis [9]. CLL is preceded by monoclonal B cell lymphocytosis (MBL). This event occurs when a clone of B cells is present at concentrations less than 5×10^9 /L, however, most of these cases never develop into CLL [10]. A disease with the same cell biology is small lymphocytic lymphoma (SLL), but this disease primarily presents in the lymph nodes and spleen [5] in contrast to the majority of CLL cells being in peripheral blood.

The Rai and Binet staging systems are most commonly used criteria to describe CLL progression [11]. The Rai staging system is mostly used in North America while Binet is applied more commonly in Europe, but both systems are based upon clinical examination of patients. Rai staging is major staging system used Manitoba (Consensus Recommendations for Management of CLL at Cancercare Manitoba) and consists of stages 0-4. Low-risk stages include 0 and 1:

stage 0 presents with lymphocytosis, while stage 1 also includes lymphadenopathy. Rai stage 2 includes splenomegaly with or without lymphadenopathy. Rai stages 3-4 are typically advanced high-risk disease, with median survival time as low as 1.5 years. These stages involve iron deficiency (anemia) or reduced platelets (thrombocytopenia), respectively, in addition to the above criteria [11].

1.2.2 CLL CELL BIOLOGY

CLL B cells bear a unique set of surface markers. These cells are CD5+, CD19+, CD23+, CD20+ (low), CD38 (variable), and have low expression of surface antibody [9]. Clonality is assessed using flow cytometry, a singular clone of CLL will contain either κ or λ light chains in its B cell antigen receptor (BCR) [12]. Unlike non-malignant B cells, CLL cells provide poor antigen presentation and antibody production [13]. Some CLL cells display methylation patterns and gene expression consistent with a mature, memory B cell phenotype [14]. In normal B cell selection, autoreactive B cells undergo somatic hypermutation to improve antigen specificity and prevent autoreactivity. Up to 75% of CLL cells can react to self-antigen, indicating that these cells may be generated before or after germinal center selection processes depending on the individual [15]. Studies have found that patients whose cells had not undergone somatic hypermutation have nearly identical (stereotyped) BCR structure [16]. Therefore, CLL cell biology can vary between individual cases and one hypothesis believes that the cell of origin is variable.

1.2.3 PROGNOSTIC INDICATORS

Though individual cases of CLL share the same diagnostic criteria, the disease may present in a heterogenous way between different patients. The presence and/or expression level of several different biomarkers can help predict the clinical course of CLL. These include, immunoglobulin variable heavy chain mutational status (IGVH), zeta chain-associated protein kinase 70 expression (ZAP-70), cytogenetic abnormalities, CD38 expression and β2 microglobulin plasma concentration. These markers, along with age, sex, and Rai staging criteria can predict time to first treatment and overall survival, and can even dictate if or which treatment is needed (Table 1.1) [17].

Immunoglobulin variable heavy chain (IGVH) mutational status refers to the conformation of genes of the BCR. If the receptor has undergone somatic hypermutation, the clone is considered mutated. A mutated IGVH status confers a better prognosis with a statistically significant difference in median survival compared to unmutated status. Approximately 40% of CLL patients have unmutated IGVH, and this is associated with increased adverse cytogenetic abnormalities [18]. Women are statistically more likely to have a mutated IGVH status and respond better to drugs than males [8].

ZAP-70 is a protein normally found in T cells and plays an important role in T cell receptor signalling. It is aberrantly expressed in CLL, with greater expression of the protein being associated with poorer overall survival and event free survival. The level of ZAP-70 expression in a patient may change over time [19, 20]. ZAP-70 positivity also has a strong positive correlation with unmutated IGVH. The advantage that ZAP-70 confers to CLL cells is not fully understood, but it has been shown to enhance cell activation by increasing intracellular calcium and phosphorylation of other kinases [21].

One of the strongest predictors of the course of CLL is cytogenetics. Cytogenetic abnormalities are detected by fluorescent in-situ hybridization (FISH) or karyotyping and can change over time as cells are subjected to selectional bias through treatment [22]. Mutations can occur in both the p or q arms of chromosomes. Deletion of 17p13 is the most serious adverse prognostic marker and is associated with loss of *TP53*, a vital tumor suppressor gene. 11q23 deletion results in loss of the *ATM* gene involved in DNA repair. Loss these genes can confer resistance to chemotherapeutics that target DNA; these cells often require other treatment options [23]. Trisomy 12q is associated with slightly shortened survival, integrin expression, and *NOTCH1* mutations and confers an intermediate prognosis. A unmutated IGVH status is associated with greater risk of developing the above abnormalities. Deletions in 13q14 are the most common in CLL, can be monoallelic or biallelic, and are associated with a better prognosis and mutated IGVH [24]. Patients can possess one or more of these alterations simultaneously, with 80% of patients having at least one abnormality [25].

Other biomarkers, such as greater expression of the surface protein CD38 and higher concentrations of β2 microglobulin in plasma confer a negative prognosis. The predictive value of these markers is debated. In one study, ZAP-70+ patients who were CD38- had better prognosis than those with the reverse expression [21]. A list of clinically-relevant prognostic biomarkers is provided below.

Biomarker	Inferior Prognosis	Intermediate Prognosis	Superior Prognosis
Rai Stage	3-4	2	0-1
Age	>75 years		<75 years
Sex	Male		Female
IGVH	Unmutated		Mutated
ZAP-70	>20%	20%	<20%
FISH Status	17p13 deletion 11q23 deletion	Trisomy 12	13q14 deletion
CD38	>20%	20%	<20%
β2 Microglobulin	>4.0	2.0-4.0	<2.0

Table 1.1: Prognostic Indicators in CLL. (Chart adapted from recent literature and CLL Global Research Foundation Criteria).

1.3 APOPTOTIC PATHWAYS IN CLL

Apoptosis is the major form of programmed cell death necessary for homeostasis and turnover of tissues in living organisms. The dysregulation of apoptosis is one of the key features responsible for the accumulation of cells seen in CLL. There is evidence that CLL is also a disease of proliferation – one study found that a maximum of 3.3% of the CLL clone is recycled within the lymph nodes per day [26]. However, ~95% of CLL cells remain arrested in G_0/G_1 phase of the cell cycle and do not divide (dependent on disease aggressiveness) [27]. Apoptosis has been canonically divided into two major pathways: extrinsic and intrinsic apoptosis. These pathways are not mutually exclusive, and cross-talk or sharing of initiator or executioner caspases can occur between the two. Microenvironmental signals and intrinsic cellular alterations are major contributors to an imbalance between cell survival and cell death in CLL[28].

1.3.1 EXTRINSIC APOPTOSIS

The "extrinsic" pathway of apoptosis is triggered by the binding of death receptors to their ligands. In CLL, these include tumor necrosis factor receptor (TNFR), Fas, and death receptors (DR) 3 through 6. Ligands of these receptors are TNF, Fas-ligand, and TNF-related apoptosis inducing ligand (TRAIL), respectively [29]. It was found that altered TRAIL expression can contribute to survival of CLL cells [30]. Moreover, chemotherapy and TP53 transcriptional activity can overexpress Fas or DR4/5 in CLL cells to sensitize these cells to death through extrinsic apoptosis [31, 32]. DR ligation recruits adaptor domain molecules such as tumor necrosis factor receptor type 1-associated death domain protein (TRADD) which convert procaspase 8 into the initiator caspase 8 [29]. Fas-induced apoptosis can also feed into the mitochondrial pathway by caspase 8-mediated cleavage of the protein Bid into its truncated pro-apoptotic conformation. Initiator caspase 8 cleaves substrates such as the executioner caspases 3 and 7 to induce cytoskeletal and nuclear degradation [33].

1.3.2 INTRINSIC APOPTOSIS

The "intrinsic" or mitochondrial pathway of apoptosis involves the mitochondria. Triggers of mitochondrial apoptosis in CLL typically include intracellular insults such as DNA damage through radiation, chemotherapy, or through a loss of pro-survival signals [28]. Oxidative stress can also contribute to cell death through this pathway [34]. This pathway involves several major events: recruitment of pro-apoptotic Bcl-2 proteins such as Bax and Bak to the mitochondria, mitochondrial permeabilization, and release of cytochrome C/second mitochondria-derived activator of caspases (Smac) from the mitochondrial intermembrane space. Pro-apoptotic Bcl-2 family proteins inhibit the oligomerization of pro-apoptotic Bcl-2 family

members and formation of mitochondrial pores [35]. Upon permeabilization, cytochrome C is released from mitochondria and forms a multimeric complex with apoptotic protease activating factor 1 (APAF-1), procaspase 9 and adenosine triphosphate (ATP) known as the apoptosome [34]. Inhibitors of apoptosis (IAP) proteins prevent apoptosome activity; X-linked IAP (XIAP) is upregulated in CLL and contributes to defects in apoptosis [36]. Smac released from mitochondria functions to inhibit IAP activity, allowing caspase 9 to cleave procaspases 3 and 7 into their active forms. Smac has been similarly been found to be downregulated in CLL [37].

Other molecules retained within mitochondria can induce apoptosis independent of caspase activity. Among these are apoptosis inducing factors (AIF) and reactive oxygen species (ROS). AIF1 is released upon mitochondrial permeabilization to induce chromatin condensation and fragmentation of DNA. ROS such as hydrogen peroxide/superoxides are common byproducts of mitochondrial redox reactions involved in ATP metabolism. ROS produced by the mitochondria contribute to apoptosis and can irreversibly damage structural components such as nucleic acids and membrane lipids [38]. Antioxidants such as glutathione (GSH), tocopherols and thioredoxin are present in all cells, to prevent excessive oxidative damage [39]. Interestingly, GSH levels are reduced and easily depleted in CLL cells due to low expression of the system X_c transporter responsible for its synthesis [40]. ROS can also modulate kinase phosphorylation to a significant degree; in B cells, Akt signalling has been shown to be affected by ROS [41]. While many cancers tend to rely on anaerobic glycolysis to meet their energy needs (also known as the Warburg Effect) [42], CLL is unique in that cells display increased mitochondrial biogenesis and oxidative phosphorylation rates contributing to overall ROS levels [43]. A summary of relevant apoptotic effectors discussing in th

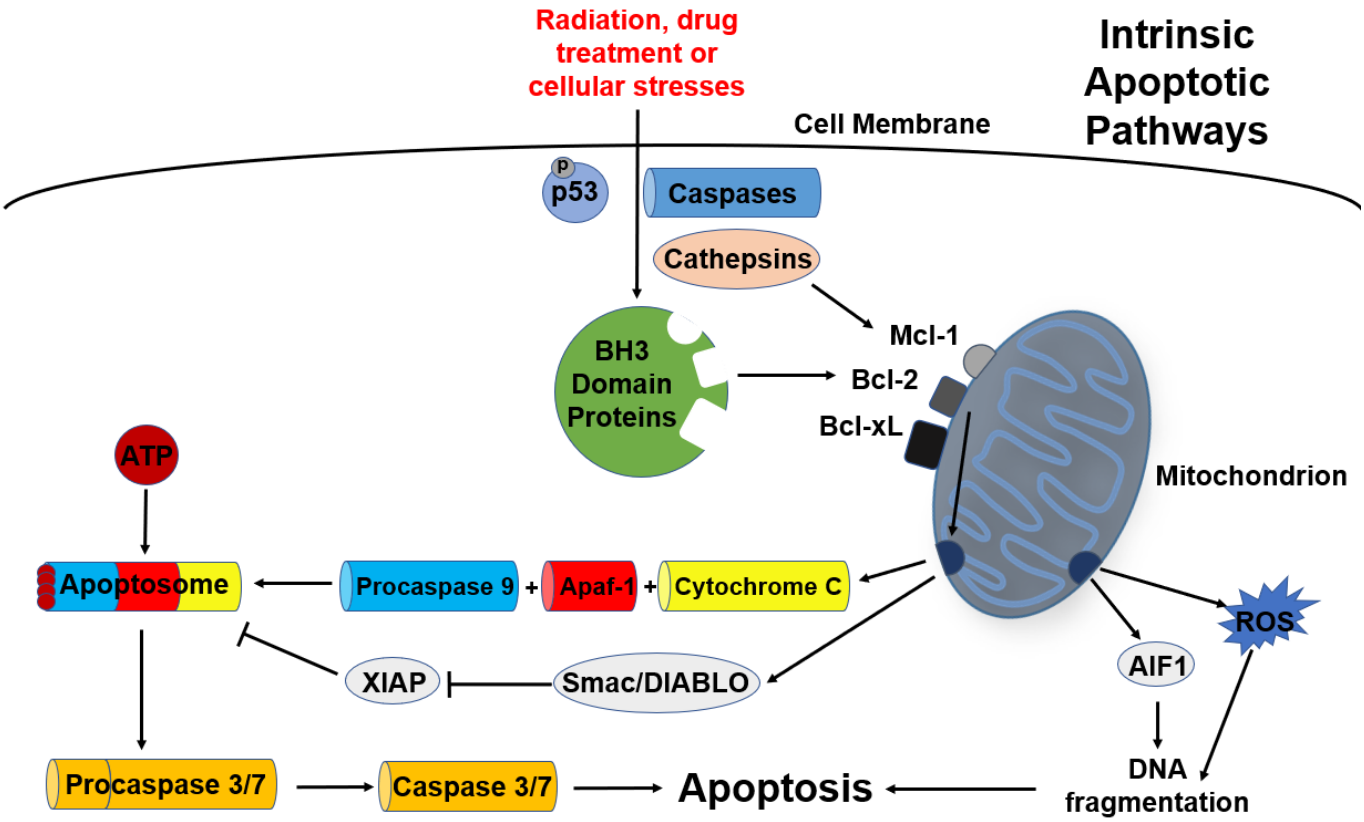


Figure 1.1: Effectors and regulators of intrinsic apoptosis in CLL cells. Summary of mitochondrial pathway of apoptosis. Bcl-2 family anti-apoptotic proteins are upregulated in CLL, and are inhibited or cleaved by a variety of proteins and proteases. Recruitment of BH3-only proteins such as Bax or Bak to the mitochondria induces release of mitochondrial contents. Cytochrome C release from mitochondria promotes caspase-dependent apoptosis, while AIF1 and ROS can promote caspase-independent apoptosis. XIAP is upregulated in CLL, and is inhibited by Smac which is downregulated in CLL [36, 37].

1.3.3 BCL-2 FAMILY PROTEINS

The Bcl-2 family consists of 20 members, and have been referred to as both "assassins" and "bodyguards". These connotations refer to their function; pro-apoptotic Bcl-2 proteins permeabilize mitochondria, while anti-apoptotic proteins function to inhibit this permeabilization. The ratio of pro-apoptotic proteins to anti-apoptotic proteins can determine cell fate, with lower ratios seen in CLL contributing to apoptosis resistance [35]. Most pro-apoptotic proteins, such as Bid and Bad, contain a Bcl-2 homology 3 (BH3) domain and directly

bind to anti-apoptotic proteins to inhibit their function and allow Bax and Bak recruitment to the mitochondria [44]. This has led to the development of novel drugs such as venetoclax which mimic the structure of these BH3 proteins to directly induce mitochondrial dysfunction [45]. Anti-apoptotic Bcl-2 proteins are equally important regulators of CLL cell death. These include Bcl-2, Bcl-xL and Mcl-1, which are overexpressed in CLL. Mcl-1 is a major player in CLL cell death, as it easily cleaved by caspases and has quick turnover [35]. Anti-apoptotic Bcl-2 proteins display clinical significance in CLL as well, as overexpression of these proteins correlates with drug resistance [46]. Mcl-1 expression has been found to be variable between CLL patients and its expression correlates with other biomarkers including ZAP-70 and IGVH status predicting drug resistance and progression [47–50]. Greater mRNA expression of Mcl-1 is correlated with reduced survival, and base insertions in the Mcl-1 promoter are associated with more aggressive disease [51, 52]. Novel BH3 mimetics targeting Mcl-1 such as S63845 are in development and show promise in the future of CLL treatment [53]. Anti-apoptotic Bcl-2 protein expression has been demonstrated to be strongly modulated by the presence of microenvironmental signals *in vivo*, which promote CLL cell activation through the BCR [54, 55].

1.4 THE CLL MICROENVIRONMENT

1.4.1 MICROENVIRONMENTAL SURVIVAL FACTORS

In normal hematopoiesis, immune cells and their precursors depend on bone marrow stromal cells (BMSCs) and lymphoid tissues as both a physical docking site and source of soluble survival signals. In healthy organisms, these signals support survival, proliferation, and differentiation of the immune system. However, in CLL the immune microenvironment is a primary provider of survival signals supporting apoptosis resistance of malignant B cells,

contributing to disease pathogenesis [56]. CLL cells can interact with BMSCs, nurse-like cells (NLCs), T cells and dendritic cells (DCs) among others.

In addition to peripheral blood, CLL cells can be found in the lymph nodes, spleen, and bone marrow with less common infiltration of other organs such as the skin and lungs [56–58]. The microenvironment is a complex regulatory network of supporting signals and cells. CLL cells can easily adhere to stromal cells through β integrin-mediated adhesion, which is amplified by the CXCR4/CXCR5 based homing mechanisms [59]. Stromal cells protect CLL cells from chemotherapy-induced apoptosis, while other novel agents inhibit these interactions. CD38 on CLL cells interacts with CD31 expressed on BMSCs and NLCs to confer a survival advantage through kinase signalling [60]. NLCs also express B cell activating factor (BAFF) and A proliferation inducing ligand (APRIL) on their surface which interact with receptors on CLL cells [56]. CLL cells express CD40 on their surface which can be ligated by CD40L on T cells or NLC's to upregulate anti-apoptotic proteins such as survivin [61]. Additionally, IL-4 produced by BMSCs or T cells can stimulate many pathways including BCR signalling [62]. Together, IL-4 and CD40L stimulation is one of the strongest rescuers of CLL cell viability *in vitro* [63].

CLL cells are not just recipients of microenvironmental signals; they are also modulators of their environment. Results of one study indicated that CLL cells could release exosomes to transform stromal cells into pro-inflammatory fibroblasts producing IL-6 and IL-8 [64]. CLL cells are both recipients and producers of vascular endothelial growth factor (VEGF) which can stimulate JAK/STAT signalling pathways in various cells to prevent cell death [65]. They can also secrete chemokines CCL3 and CCL4 to attract T cells or monocytes to further interactions

[66]. Therefore, cross-talk between CLL cells and their supporting cells is responsible for characteristics seen in CLL.

1.4.2: B CELL RECEPTOR SIGNALLING

Stimulation by self-antigen is an important process in the selection of functioning B cells. Constitutively-active BCR signalling is induced by interaction of CLL cells with their environment leading to uncontrolled cell survival. Increased BCR signalling has been demonstrated to decrease apoptosis and increase expression of Bcl-2 family proteins such as Mcl-1 [67].

Stimulation of BCR signalling through autoantigen or cellular interactions promotes pro-survival pathways through phosphatidylinositol-4,5-bisphosphate 3-kinase delta (PI3Kδ), extracellular related protein kinase (ERK) 1/2, protein kinase B (Akt), and the nuclear factor kappa B (NF-κB) transcriptional pathway [68]. Ligation of the BCR results in its recruitment to lipid rafts where CD19, CD79a and CD79b are phosphorylated by Lyn kinase at immunoreceptor tyrosine-based action motifs (ITAM) [69]. This provides a binding site for spleen tyrosine kinase (Syk), which phosphorylates targets including the B cell linker protein (BLNK) serving as a docking site for Bruton's tyrosine kinase (BTK) and phospholipase C gamma 2 (PLCγ2) which modulate intracellular calcium [70]. CD19 recruits PI3Kδ which phosphorylates the phospholipid PIP2 to PIP3. Interactions between these kinases ultimately trigger activation of the Raf/ERK and Akt/mTOR signalling axes to activate survival gene transcription [71].

Alterations in BCR signalling in CLL such as expression of Lck and ZAP-70 assist in signal transduction to improve survival [72, 73]. Additionally, Syk overexpression has been

demonstrated in CLL [74]. Moreover, Akt and ERK1/2 have specifically been demonstrated as important regulators of Mcl-1 expression in CLL [75]. Inhibition of BCR signalling has been shown to impair interactions of CLL cells with stromal cells and is a highly-relevant clinical target of novel kinase inhibitor therapies [63]. These therapies will be discussed in the next chapter.

1.5 CURRENT LANDSCAPE OF CLL THERAPY

While CLL is currently incurable in most cases, recent advancements in the understanding of disease biology have drastically improved the outlook for patients with both favourable and unfavourable prognoses. The only current curative option for CLL is an allogeneic bone marrow transplant, a procedure only available for high-risk younger patients, with mortality rates upwards of 20% [76]. The decision between available therapies is made on a patient-by-patient basis, with variables including age, renal function, prognostic biomarkers, and other comorbidities factoring into the choice of treatment strategy. Diverse families of drugs such as nucleoside analogues, alkylating agents, targeted therapies and monoclonal antibodies are available as monotherapies, or in combination with each other based on International Workshop on CLL (IWCLL) recommendations [77]. Cells can often develop resistance mechanisms to these drugs, emphasizing the need for additional therapies targeting novel pathways. The most common mechanisms of resistance to therapies are outlined in this chapter.

1.5.1 CHEMOTHERAPY AND CHEMOIMMUNOTHERAPY

Agents which induce cell death through DNA damage are included in a larger class of drugs referred to as chemotherapeutics. In CLL, these drugs include nucleoside analogues and alkylating agents. Purine analogues such as fludarabine, pentostatin, and cladribine are prodrugs which are metabolized by cells prior to insertion into the DNA sequence upon replication. These molecules induce cell death via inhibition of DNA polymerases and adenosine deaminase, and can terminate the growing strand [78]. Fludarabine in particular was a major breakthrough in CLL treatment, with greater than 70% response rate in untreated patients. One prominent side effect of nucleoside analogues is depletion of blood cell counts; patients often experience neutropenia or thrombocytopenia [79].

Alkylating agents were the first agents used to treat lymphoid malignancies, and still remain a necessary component of CLL treatment to this day. These drugs cross-link DNA to inhibit separation during synthesis and transcription, and add alkyl groups to bases causing DNA fragmentation during enzymatic repair attempts [80]. Alkylating agents displaying significant clinical use in CLL include cyclophosphamide, chlorambucil, and bendamustine. Cyclophosphamide was added to the regimen with fludarabine to target multiple pathways, improving response rates in combination [81]. Chlorambucil is often given to patients deemed unfit for a harsher treatment schedule with fludarabine, while bendamustine has recently shown comparable or greater effectiveness than chlorambucil in elderly patients with high-risk disease [82]. Resistance mechanisms to chemotherapy can occur as a result of mutations in DNA repair genes TP53 and ATM, transposon mutagenesis, or increased ceramide metabolism to glucosylceramides [83–85].

Monoclonal anti-CD20 antibodies are a form of immunotherapy often given in combination with the aforementioned chemotherapeutics, and are being investigated in combination with targeted therapies. The two most commonly used antibodies in CLL are rituximab and obinutuzumab, but ofatumumab can also be considered. Rituximab is a type I human antibody, which acts through antibody-dependent cellular cytotoxicity (ADCC), massive complement activation, and direct induction of apoptosis [86]. It has been combined with fludarabine and cyclophosphamide to further improve the treatment regimen (FCR), considered standard first-line treatment for fitter patients. FCR can be "curative" in some cases, producing durable minimal residual disease-negative responses in up to 60% of patients for greater than 6 years after treatment [87]. Obinutuzumab performs the above functions, but is unique in that it is glycoengineered to improve Fc receptor specificity. One standout characteristic of obinutuzumab is its Actin-based internalization into the lysosome of CLL cells to induce cell death through cathepsin B release. This finding corresponds to rapid reduction of lymphocyte counts within minutes of antibody infusion, but can induce higher rates of toxicity as a result which can be unsuitable for less-fit patients [88]. Resistance mechanisms to anti-CD20 antibodies are not completely defined, but immune suppression and increased NF-κB signalling and corresponding Bcl-2 upregulation can antagonize cell death caused by these drugs [89].

1.5.2 TARGETED THERAPIES

Targeted therapies are a broader class of drugs referring to small molecule inhibitors which specifically target various mechanisms of CLL survival. These include tyrosine kinase inhibitors (TKI's) and BH3 mimetics.

BTK plays a crucial role in the BCR signalling pathway for CLL cell activation, survival, and interaction with supporting cells. Ibrutinib, a small molecule inhibitor of BTK, has been approved by the FDA as a treatment for CLL, Mantle cell lymphoma, and Waldestrom macroglobulinemia. The drug is approved by IWCLL for treatment of patients with relapsed refractory CLL, and those possessing TP53 dysfunction [90]. In clinical trials, it has shown effectiveness as a frontline therapy and when combined with rituximab [91]. Ibrutinib covalently binds to the CS481 binding site of the protein irreversibly and prevents phosphorylation of this site [92]. Inhibition of BCR signal transduction prevents the ability of CLL cells to adhere to supporting cells in the lymphoid tissues. This results in lymphocytosis for 4 to 6 weeks after treatment corresponding to a reduction of splenomegaly and lymphadenopathy [93]. The cells in peripheral circulation undergo apoptosis through loss of interaction with surrounding stroma, corresponding with a reduction in expression of antiapoptotic proteins Mcl-1 and Bcl-xL, with a resolution of lymphocytosis occurring in 80% of patients within 1 year of therapy [94, 95]. Though ibrutinib is generally well-tolerated, toxicities such as bleeding, diarrhea, and skin rashes, as well abnormalities in hair and nail growth may occur after acute or prolonged treatment. Additionally, infections are a serious side effect of ibrutinib treatment. Ibrutinib has been characterized to have other targets outside of BTK in B cells. It is known to inhibit BTK in platelets which may be responsible for excessive bleeding, as well as targeting other kinases such as IL-2 inducible T cell kinase (ITK), which is theorized to impair T cell function and increase infection rates [92, 96]. Mechanisms of resistance to ibrutinib may arise in some cases, the most common of which being the CS481 BTK mutation, and mutations in PLCγ2 which override the need for BTK signalling [97, 98]. Acalabrutinib, an inhibitor with greater specificity for BTK

and potentially fewer side effects, was approved for treatment of Mantle Cell Lymphoma in 2017 and is presently being investigated for use in CLL [94].

Similarly, idelalisib is a small molecule inhibitor of PI3Kδ which blocks BCR signalling and is approved for use in relapsed refractory CLL and follicular lymphoma. Use of the drug also achieves lymphocytosis, with peak response occurring up to 8 weeks after treatment. Despite effectiveness in controlling CLL progression in chemo-resistant and TP53 dysfunctional disease, idelalisib use was shown to cause short-term and long-term adverse effects in 89% of patients [99]. Colitis and hepatitis are among long-term complications of idelalisib treatment. These events are theorized to be autoimmune in nature due to increased lymphocytic infiltration in colon biopsies and impaired regulatory T cell function in idelalisib treated patients [100]. Thus, idelalisib is less likely to be used as a treatment for CLL than ibrutinib. Addition of rituximab to the idelalisib treatment regimen reduced adverse effects and increased treatment efficacy [99]. Other kinase inhibitors including duvelisib, a dual inhibitor of PI3K isoforms δ and γ, fostamatinib, a Syk inhibitor, and dasatinib, a BCR-Abl and Src family kinase inhibitor, have shown significant clinical activity in CLL patients [101].

In addition to TKI's, the BH3 mimetic venetoclax was more recently approved in 2018 for use as a second-line therapy in CLL. Venetoclax fills a previously lacking therapeutic niche: a directly cytotoxic agent specifically targeting CLL survival mechanisms. The drug acts as a BH3 analog, directly binding to and inhibiting the Bcl-2 protein to initiate apoptosis [102]. The most common toxicity was tumor lysis syndrome, which was ameliorated by dose escalation. Response rates were favourable and were further improved to up to 89% through combinations with rituximab or obinutuzumab, with complete remission occurring in 8% of cases of high-risk disease [103]. Resistance to venetoclax can be developed by upregulation of Bcl-xL and Mcl-1

by CLL cells [104]. However, venetoclax has shown synergy when combined with ibrutinib both *in vitro* and in recent clinical trials, overcoming Bcl-2 dependence induced by ibrutinib and directly killing CLL cells in the peripheral blood outside of their protective niches [105]. This highlights how cell survival pathways in CLL display redundancy, while also illustrating how combining therapies with differing mechanisms can be an effective strategy to overcome resistance.

1.6 LYSOSOMES AND CELL DEATH

1.6.1 LYSOSOMAL BIOLOGY AND FUNCTION

Lysosomes are acidic organelles that are crucial for maintenance of cellular homeostasis. They consist of a double-layered membrane with many embedded proteins including lysosomal-associated membrane proteins (LAMP) 2 and 3, proton pumps to maintain lysosomal pH, and sphingolipid-converting enzymes [106]. They have several diverse functions in mammalian cells, although their primary role, metabolism, is exemplified by diverse enzymatic content within their compartment. Lysosomes contain over 60 different hydrolytic enzymes, and are a major center of protein, nucleic acid, sugar, iron and lipid metabolism in most functioning cells [107].

Macromolecules are degraded within the lysosome by several families of enzymes, including cathepsins cleaving different amino acid residues. Cysteine cathepsins are the largest family, and include 11 members such as cathepsins B, K and L . These are the most active cathepsins at a lysosomal pH of approximately 4.8 [108]. Aspartic cathepsins include D and E, while cathepsins A and G cleave serine residues. Alpha acid glucosidase is an enzyme responsible for breaking down glycogen into glucose within the lysosome [109]. Ribosomes,

mitochondria, and nuclei can also be delivered to the lysosome in a process called autophagy, where their RNA and DNA are degraded for re-use or release upon cell death [110].

Lysosomes are also a center of iron metabolism. Lysosomes degrading iron-rich macromolecules can become iron-rich themselves, becoming sensitive to oxidative stress. This iron can react with peroxides to produce hydroxyl radicals, which can oxidize lipids, proteins, and DNA [111]. The molecule apo-ferritin can help maintain lysosomal integrity by protecting lysosomes from oxidative stress. Iron found in lysosomes can be a major source of lipid ROS implicated in many cell death pathways [112].

Lastly, lysosomal membranes are implicated in lipid metabolism and storage. Lysosomal membranes contain enzymes involved in the processing and interconversion of sphingolipids. Sphingosine 1-phosphate is converted to sphingosine by sphingosine 1-phosphate phosphatase (S1PP) and the reverse reaction is catalyzed by sphingosine kinase (SK). Sphingosine is further converted to ceramide, which can be interconverted to sphingomyelin by sphingomyelin synthase (SMS) and reversed by acid sphingomyelinase (ASM) [113]. Sphingolipids are important signalling molecules in cellular homeostasis, with sphingosine and ceramide being proapoptotic while their phosphorylated counterparts are associated with proliferation [114]. Sphingosine and sphingomyelin can also make lysosomal membranes rigid when present in greater concentrations. Aberrant activity of sphingolipid metabolic enzymes can result in lysosomal storage diseases such as Niemann-pick type C disease (ASM deficiency) and Wolman disease (acid lipase deficiency), and dysregulation of these pathways is a common feature in cancer cells [115].

1.6.2 LYSOSOMAL MEMBRANE PERMEABILIZATION

In normal cells, the contents of lysosomes are used to degrade and/or modify macromolecules for re-use and storage. In many cancer cells, lysosomal biogenesis and lipid metabolism is dysregulated, possibly due to the immense metabolic demand of these cells. This property was found to be exploitable to induce cell death through lysosomal membrane permeabilization (LMP). Many cancer types, including acute myeloid leukemia (AML), brain cancer, breast cancer, CLL, colon cancer, and lung cancer, are susceptible to cell death through LMP [116–122]. Several of these cancers have reduced ASM and increased SK activity, promoting increased sphingomyelin and sphingosine synthesis [123–126]. In CLL, no major alterations in ASM or SK activity were found. However, S1PP activity was found to be overexpressed in the disease, as was an increase in lysosomal sphingosine content [119]. Direct addition of sphingosine to cells, as well as inhibition of SK, can increase membrane fragility and sensitize cancer cells to or directly cause LMP [127, 128].

Release of lysosomal cathepsins and ROS into the cytoplasm are common events associated with LMP. Lysosomal cell death bears apoptosis-like cell morphology and typically hijacks apoptotic effectors, but it can occur in the absence of cleaved caspases 3 and 9 [129, 130]. If LMP occurs at extremely high drug doses or stresses, the cell death can present with necrotic morphology. Following lysosomal membrane permeabilization, cathepsins are implicated in the degradation of cellular machinery to initiate apoptosis. Cathepsins B and D are commonly involved in lysosomal cell death, relocating to the cytosol and inducing cleavage of Bid, caspase 8, and antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1 and XIAP [131, 132]. Cathepsins can also contribute to free radical formation and oxidative stress [133]. Additionally, lysosomal iron can generate lipid ROS and initiate a cell death pathway known as ferroptosis

[134]. Oxidation of the lysosomal membrane can induce an influx of calcium and amplify cell death responses. Lipid peroxidation by ROS is another hallmark of LMP, with cell membranes including the outer mitochondrial membrane being oxidized and permeabilized [135, 136]. Thus, mitochondrial dysfunction through these pathways is a secondary mechanism often following LMP.

1.6.3 LYSOSOMOTROPIC AGENTS

Drugs that induce LMP are collectively known as lysosomotropic agents. These drugs belong to several different pharmaceutical classes including antibiotics, antidepressants, antihistamines, antimalarials, and antiepileptics. The best-studied of these drugs, siramesine, is a sigma-receptor agonist developed for treatment of depression. The drug can permeabilize lysosomes and induce release of cathepsins and ROS, although oxidative activity occurred exclusively in CLL[118, 119]. Siramesine has demonstrated strong anti-tumor activity in chemotherapy-resistant tumor cells including lung cancer, glioblastoma and breast cancer [117, 134, 137]. It has also successfully prevented tumor formation in an MCF-7 xenograft mouse model of breast cancer, along with other lysosomotropic antidepressants [118, 123].

Drugs with cationic amphiphilic (CAD) structure such as antimalarials and tricyclic antidepressants are among compounds that display lysosomotropism. CADs typically contain a weakly basic nitrogen with a free electron pair and large, sterically hindered hydrophobic ring structures. This property allows these drugs to freely diffuse across cell membranes, becoming protonated and trapped within lysosomes [138–140]. Accumulation of these drugs is marked by a volumetric expansion and breakage of lysosomes through formation of puncta in the membrane immediately following drug treatment [138, 141]. This process, from the diffusion of CAD

drugs into the cell to initiation of LMP, is described in Figure 1.2. Many lysosomotropic drugs can act as sphingosine metabolism inhibitors or act directly as a detergent on the lysosomal membrane to induce breakage (Figure 1.2). Drugs used to induce lysosomotropism in CLL cells include fludarabine and valproic acid, desipramine, nortriptyline, siramesine, mefloquine, tafenoquine, and obinuzutumab [88, 119, 120, 142]. In particular, fludarabine and obinutuzumab are already used to treat CLL clinically, although lysosomotropism has only been shown to contribute to a major therapeutic effect in the case of obinutuzumab. Many of these drugs require higher concentrations than are clinically achievable by standard dosing to kill CLL cells (Table 1.2). Indeed, additional investigation into FDA-approved lysosomotropic drugs that can kill CLL cells is needed.

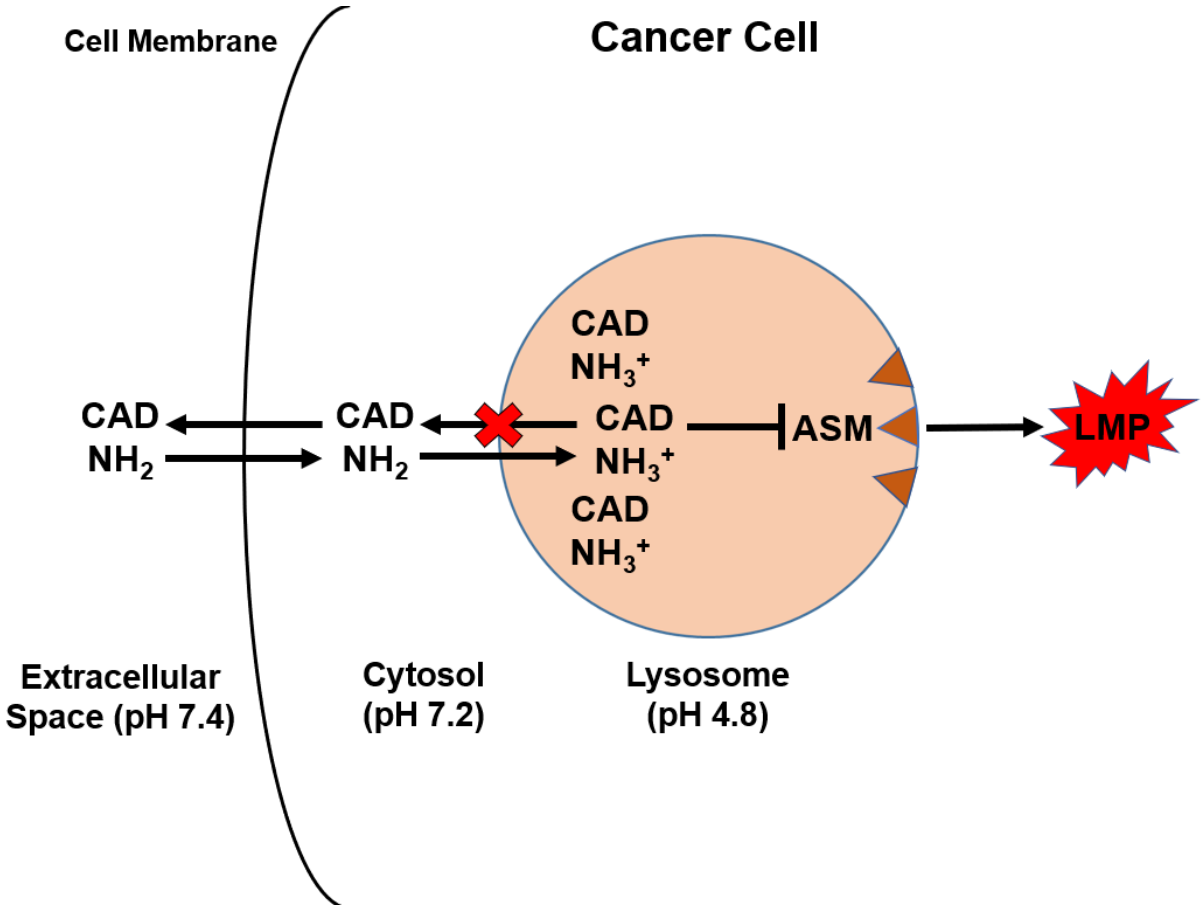


Figure 1.2: Lysosomal trapping of CAD compounds. Drugs with hydrophobic ring structures and a nitrogen with a free electron pair can freely diffuse across the plasma membrane and lysosomal membranes. These compounds can gain a partial positive charge within acidic vesicles, making exiting these vesicles an energetically unfavourable process. Accumulation of CAD compounds within lysosomes can solubilize lysosomal membranes to detach enzymes including ASM, leading to accumulation of the pro-apoptotic sphingolipids: sphingomyelin and sphingosine, and subsequent LMP. Puncta formation (represented by triangles) allows for release of lysosomal contents.

Drug Name	Drug Class	Lysosomotropic Concentrations *In Vitro*	Clinically Relevant Plasma Concentrations	FDA Approved?
Fludarabine/ Valproic Acid	Nucleoside Analogue (Flu)/ Antiepileptic (VPA)	5μM (Flu)/ 1mM (VPA) [142]	3.0μM (Flu) [143]/ 1mM (VPA) [142]	Yes/Yes
Desipramine	Tricyclic Antidepressant	50-100μM [119]	0.47μM [144]	Yes
Nortriptyline	Tricyclic Antidepressant	50-100μM [119]	0.20μM-1.0μM [145]	Yes
Siramesine	σ-Receptor Agonist (Antidepressant)	1-5μM [119]	0.50μM [123]	No
Mefloquine	Antimalarial	10-20μM [120]	0.48μM [146]	Yes
Tafenoquine	Antimalarial	1-10μM [147]	0.97μM [148]	Yes
Obinutuzumab	Anti-CD20 Monoclonal Antibody	10-40μg/mL [149]	100-1500μg/mL [150]	Yes

Table 1.2: Summary of Lysosomotropic Drugs Investigated in CLL Cells

1.6.4 H1 ANTIHISTAMINES AS LYSOSOMOTROPIC AGENTS

The first use of H1 antihistamines as cytotoxic agents in cancer was explored in cutaneous T cell lymphoma cell lines. Though their lysosomotropism was not demonstrated in this study, clemastine and desloratadine were found to directly induce cell death in these cells through inhibition of transcription factors c-Myc and STAT3 [151]. These antihistamines, in addition to loratadine, were later screened as part of a CAD drug library, and could induce cell death in non-small cell lung cancer (NSCLC) cell lines. The cell death was determined to be acting via an LMP pathway through galectin-1 puncta formation, a sign of lysosomal leakage in solid tumor cells [152]. More recently, a study in brain cancer used clemastine to kill chemotherapy-resistant invasive glioblastoma cells through LMP. In this study, a clinically-achievable dosing schedule was able to eliminate invasive glioblastoma cells in a mouse

xenograft model with minimal side effects in the animals. This significantly prolonged mouse survival with low rates of tumor recurrence [153]. Therefore, the efficacy of antihistamines as lysosomotropic agents in the above cancer models has been demonstrated both *in vitro* and *in vivo*. Despite the effectiveness of these drugs in killing solid tumor cells, the lysomotropism of H1 antihistamines has yet to be investigated in B cell cancer models.

1.7 ALTERNATIVE CELL DEATH MECHANISMS

1.7.1 FERROPTOSIS

Abnormal iron homeostasis is implicated in ageing and disease. Ferroptosis is a recently discovered iron-dependent form of cell death with a mechanism distinct from that of apoptosis. Though a comprehensive summary of all ferroptotic effectors is beyond the scope of this thesis, hallmarks include iron-dependent increases in intracellular ROS and lipid peroxidation. The small molecule erastin, initially designed to specifically kill tumor cells with oncogenic *Ras* mutations, was determined to induce cell death despite apoptotic, necroptotic and autophagic inhibition [134]. This mechanism was later demonstrated to be ferroptotic, acting through an excess of iron from the lysosome contributing to ROS generation through an oxidative catalytic process called the Fenton reaction. Iron chelation and radical-trapping antioxidants both showed potent inhibition of erastin-induced cell death, demonstrating the necessity of iron and ROS in this cell death pathway [154].

Ferroptosis was found to be induced synergistically by the lysosomotropic agent siramesine in combination with the TKI lapatinib in many cancer models including lung, breast and glioblastoma cell lines [155–157]. Deletion of the antioxidant enzyme glutathione peroxidase 4 (GPx4) could similarly induce ferroptosis through mitochondrial lipid peroxidation

[158]. Additionally, knockdown of GPx4 was shown to increase ferroptosis induced by either erastin or chemotherapy [159]. Thus, simultaneous induction of ROS through LMP and inhibition of antioxidant mechanisms has the potential for synergistic anti-tumor activity. While ferroptotic cell death is inducible in solid tumor and AML cells, its mechanism has not yet been demonstrated in CLL [160].

1.7.2 AUTOPHAGY

Autophagy directly translates to "self-eating", referring to a regulated process of self-digestion which occurs in all mammalian cells. Though several autophagic processes have been described, the best-understood of these is macroautophagy. Through macroautophagy, cellular components are isolated within a double-layered vesicle called the autophagosome. This vesicle fuses with the lysosome to form the autophagolysosome, degrading components for future use [161]. This process is crucial for cellular homeostasis, recycling damaged proteins and organelles under periods of starvation and hypoxia, as well as processing intracellular pathogens [162].

While autophagy can promote cell survival, it has been commonly referred to as a "double-edged sword", inducing cell death upon exhaustion of resources under sustained stress. The mTOR pathway prevents autophagy in mammalian cells, however, cellular stresses inhibit mTOR signalling to promote autophagic flux [163]. Additionally, the CAD antimalarial compounds chloroquine and hydroxychloroquine act to inhibit autophagy by inhibiting lysosome acidity and blocking lysosome-autophagosome fusion. These drugs have been used in clinical trials to inhibit cancer cell autophagy and enhance chemotherapy effectiveness in many different

malignancies including melanoma and brain cancer [164, 165]. The potential of autophagy as a therapeutic target in oncology is the subject of ongoing study.

1.7.3 NECROSIS

Necrosis is a more deregulated or "unprogrammed" form of cell death when compared to apoptosis. Cells exposed to excessive adverse stimuli such as extreme temperatures and mechanical stress often undergo necrosis [166]. In contrast to apoptosis, necrosis does not require significant ATP or additional protein synthesis and is described as a more passive process [167]. While characteristics of apoptosis include chromatin condensation and plasma membrane blebbing, necrosis features uncontrolled DNA degradation and cell membrane swelling or endocytosis. Direct release of ROS and damaged organelles into the extracellular space are common in necrotic cell death [168]. Components released are highly immunogenic and immune responses to necrosis can result in significant collateral damage to tissues [169]. Thus, cellular necrosis is generally an unfavourable therapeutic outcome.

1.7.4 NECROPTOSIS

Necroptosis is regulated form of cell death displaying characteristics of both necrosis and apoptosis, yet is distinct from either pathway. Physical characteristics of necroptosis include breakage of the plasma membrane and swelling of the cell. Necroptosis is dependent on receptor-interacting protein kinase 1 (RIPK1) and RIPK3, which interact to produce a complex called the necrosome through their receptor homology domain [170]. This dependence on RIPK1 was illustrated by blockage of necroptosis by Necrostatin-1, a RIPK1 inhibitor [171]. The necrosome can then phosphorylate the mixed lineage kinase domain like protein (MLKL),

which can act as a Na+ or Ca2+ ion channel or directly rupture the plasma membrane [172]. The necroptotic pathway can only be induced in the absence of caspase 8 activity; active caspase 8 inhibits necrosome formation and cells will preferentially undergo apoptosis [173].

RIPK recruitment can be initiated by ROS or ligation of toll-like receptors (TLR), tumor TNFR, or other death receptors [170]. In CLL cells, necroptosis has been demonstrated to occur through treatment with a combination of TNF and the pan-caspase inhibitor z-VAD, in the absence of lymphoid enhancer-binding factor 1 (LEF1) [174].

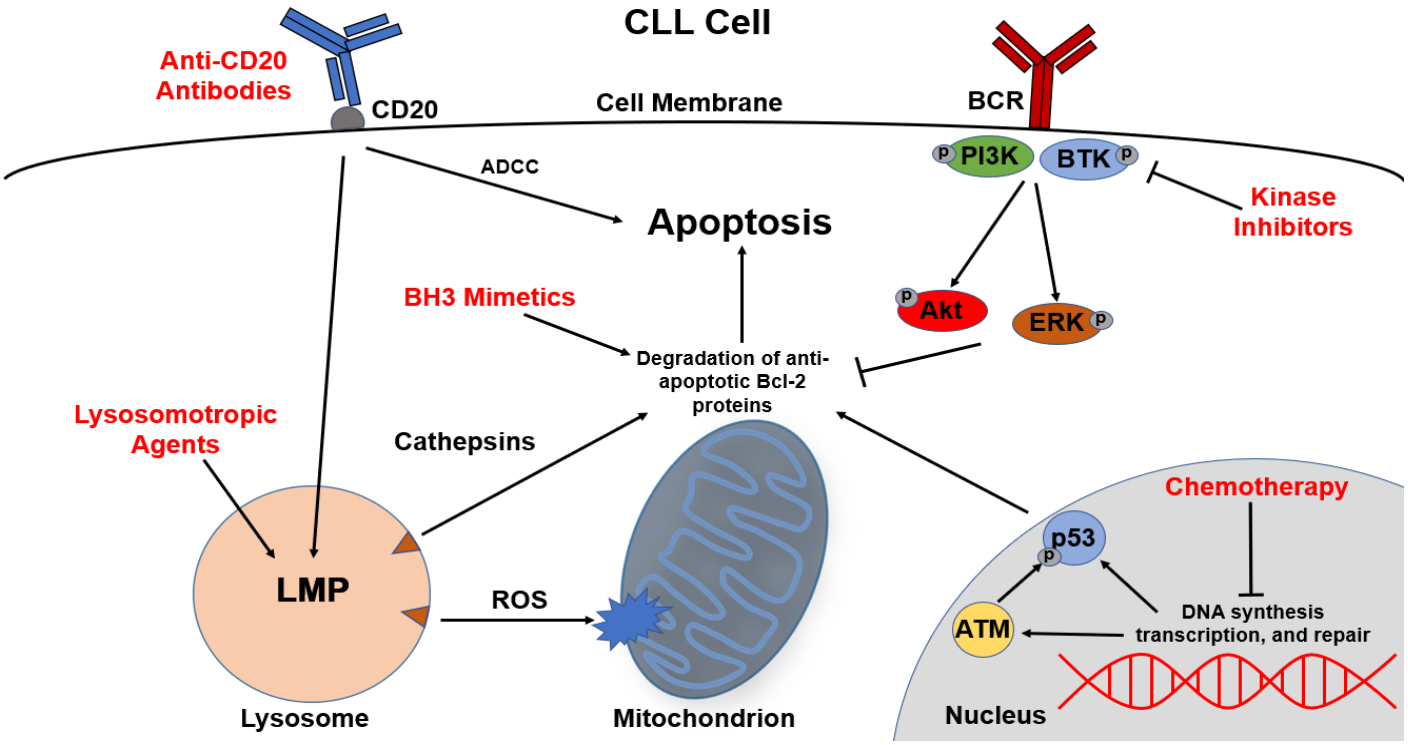


Figure 1.3: Summary of druggable targets in CLL cells described in this thesis. Chemotherapy includes nucleoside analogues and alkylating agents, targeting DNA synthesis, repair and transcriptional mechanisms. Proteins such as ATM kinase and p53 primarily keep the genome intact by initiating DNA repair, but are also involved in initiating apoptosis. Kinase inhibitors include ibrutinib and idelalisib, which target BTK and PI3Kδ respectively, as well as the Akt and ERK1/2 axes downstream of these targets. BH3 mimetics, which currently include venetoclax, bind to the BH3 region on Bcl-2 to inhibit its anti-apoptotic function. Anti-CD20 antibodies include rituximab and obinutuzumab, tag CD20 on the surface of CLL cells for immune cell or compliment-mediated killing. Obinutuzumab specifically can lyse lysosomes, along with other lysosomotropic agents, leading to mitochondrial dysfunction through cathepsins and ROS.

CHAPTER 2: RATIONALE, HYPOTHESIS, AND RESEARCH AIMS

2.1 RATIONALE

CLL cells often develop mechanisms of resistance to cell death induced by drugs targeting susceptible pathways of the disease. Therefore, drugs targeting alternative cell death pathways may serve to circumvent this resistance and provide other therapeutic options. One proposed pathway is the lysosome; CLL cells have previously been shown to be selectively susceptible to cell death by LMP. While many drugs including antidepressants and antimalarials have demonstrated lysosomotropism in CLL, they have experienced barriers to clinical implementation such as lack of FDA approval and severe toxic side effects. H1 antihistamines are a promising alternative to these drugs in that they are FDA approved and display minimal toxicities. Since H1 antihistamines are known to induce LMP in solid tumor cells, CLL cells may be sensitive to antihistamine-induced LMP as well.

Combinatorial approaches are another commonly used way to improve treatment options. These strategies can reduce the necessary doses of each individual drug to lower toxicities and improve the overall effect of treatment. Previous research done in our laboratory has shown that lysosomotropic agents can be combined with many forms of cancer therapy to produce increased or even synergistic cell death. In CLL cells, the lysosomotropic agent valproic acid was first combined with fludarabine to increase cell death induced by the agent. Moreover, many lysosomotropic agents were combined with TKIs to induce consistently synergistic cell death in breast, lung, and brain cancer models. Therefore, as ibrutinib is a TKI approved for clinical use in CLL, it may synergize with H1 antihistamines such as clemastine, desloratadine and

loratadine in CLL models. Synergistic drug combinations may lead to novel therapeutic strategies in specific subsets of patients.

2.2 HYPOTHESIS

We hypothesized that malignant B cell lines and CLL cells would be sensitive to H1 antihistamine-induced cell death, and that this cell death would occur through LMP. We also predicted that clemastine, desloratadine and loratadine would induce synergistic cell death when combined with the TKI ibrutinib, but not other CLL therapies.

2.3 RESEARCH AIMS

The aims of this study were to:

1. Determine if clemastine, desloratadine, and loratadine could kill malignant B cell lines and CLL cells, and identify the mechanism of cell death.
2. Determine if clemastine, desloratadine, and loratadine could induce synergistic cell death when combined with drugs relevant to CLL, and identify the mechanism of cell death caused by a synergistic combination.

CHAPTER 3: MATERIALS AND METHODS

3.1 CELL CULTURE

3.1.1 CELL LINES

The malignant B cell lines BJAB (Thermo Fisher) and I83 (obtained as a kind gift from Dr. Panasci, McGill University) were used as models for many experiments described in this thesis. BJAB is an immortalized EBV-negative Burkitt-like lymphoma cell line possessing a TP53 mutation, derived from a 5 year old female Burkitt's lymphoma patient. The I83 cell line was derived from 75-year old CLL patient and is EBV positive [186, 187]. These cell lines were cultured in RPMI-1640 medium (HyClone, Life Technologies) with 5% fetal bovine serum (FBS; Life Technologies) and 1% penicillin/streptomycin (Gibco). Both cell lines were grown in T-75 suspension cell culture flasks (Sarstedt) positioned horizontally to allow greater access to O_2, and maintained in a humid incubator with 5% CO_2 at 37°C. Cells were passaged at approximately 80% confluency and split at ratios of 1:2, 1:3, 1:5 or 1:10. Cell lines were maintained up to a maximum of passage number 15, or the approximate equivalent of one month since thawing. For experiments, cell lines were seeded at 5 x 10^5 cells/mL in 12, 24, or 96-well suspension cell culture plates (Sarstedt).

3.1.2 PASSAGING OF CELL LINES

BJAB and I83 cells were grown in suspension to a concentration of 1.5x10^6 cells/mL in T-75 flasks containing 25mL of media. All cells were removed from the culture flask, and the appropriate ratio of media containing cells was removed from the cell suspension. These cells

were then pipetted into a new flask containing the appropriate volume of fresh media to bring the total volume up to 25mL.

3.1.3 FREEZING OF CELL LINES

BJAB or I83 cells were harvested at a concentration of $1.5x10^6$ cells/mL (or approximately 80% of maximal healthy concentration as described in the provider datasheets). Cells were pelleted via centrifugation at 290 x g for 5 minutes, and growth media was aspirated off of the pellet. Cells were resuspended in 10mL of freezing media consisting of RPMI-1640 supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and 10% FBS. This suspension was then aliquoted into cryovials (Sarstedt) at a final volume of 1mL. Cryovials were then slotted into the Mr. Frosty Freezing Container (Nalgene), which surrounds cells with 100% isopropyl alcohol to slow freezing rates and minimize cellular stress. Cells were stored in this container at -80°C for immediate future use, or transferred to liquid nitrogen containers for longer-term storage.

3.1.4 THAWING OF CELL LINES

Cryovials containing BJAB or I83 cells were removed from freezing and placed in a 37°C water bath for 1 minute. Thawed cells were resuspended via pipetting, and quickly transferred from cryovials into 9mL of pre-warmed RPMI-1640 media containing 10% FBS and 1% penicillin/streptomycin to reduce DMSO concentration and improve cell viability. This suspension was then centrifuged at 290 x g and all media was aspirated off of the pellet. Finally, the pellet was resuspended in 10mL of fresh RPMI-1640 media with 10% FBS and 1% penicillin/streptomycin, and transferred into a T-25 flask (Corning) for incubation. Upon

reaching greater than 90% viability (measured via trypan blue exclusion), cell lines were transferred to a T-75 flask and transitioned to media containing 5% FBS for use in experiments.

3.1.5 PRIMARY SAMPLES

Primary lymphocytes were isolated for use in this project, which included both CLL cells and non-leukemic peripheral blood mononuclear cells (PBMC). To obtain these samples, blood was donated with informed consent by CLL patients and age-matched healthy donors in compliance with University of Manitoba Research Ethics Board policy. CLL cells were isolated from whole blood collected in anti-coagulant tubes within the Manitoba Tumor Bank using a Ficoll-Paque gradient (GE Healthcare). If lymphocyte counts were less than 40×10^9 cells/litre of blood (counted by the Manitoba Tumor Bank), RosetteSep (StemCell Technologies) was mixed into blood for 30 minutes to cross-link unwanted cells to red blood cells (RBC). Finally, RBC lysis buffer (eBioscience) was added for 10 minutes to eliminate RBC's from the final purification. PBMC's were also isolated from whole blood as previously described, without the use of RosetteSep to maximize lymphocyte yield. Cells were centrifuged at 290 x g for 10 minutes, and resuspended in Hybridoma Serum Free Media (SFM; Gibco). Primary cells were seeded for experiments at a final concentration of 5×10^6 cells/mL in 12, 24, or 96-well suspension cell cultures plates (Sarstedt), and stored in a humid incubator with 5% CO_2 at 37°C.

3.1.6 CELL COUNTING

Cell lines or CLL cells suspended in media were mixed in a 1:1 ratio with 0.4% Trypan Blue solution (Gibco), and 10µL of the mixture was pipetted into cell counting slides (Bio-Rad). The slides were then inserted into the TC-20 Automated Cell Counter (Bio-Rad), and debris was

gated out based on size to provide a more accurate cell count and viability reading. Cell suspensions were diluted based on final cell counts to achieve the desired seeding concentrations for models described above.

3.2 DRUGS, STIMULI, AND INHIBITORS

3.2.1 DRUGS

All relevant drugs used for main experiments in this study are summarized in Table 3.1. Drugs were stored in single-use aliquots and thawed once for each experiment. Cell lines or CLL cells were treated with a range of increasing doses of drugs to generate dose-response curves. Cell death responses were measured at 24h post-treatment unless otherwise specified. Concentrations for combination experiments were determined from the lowest doses to achieve 5-20% additional cell death compared to vehicle controls, as these doses are where synergy is most evident. Specific concentrations used for individual experiments are stated in figure legends (See Chapter 4: Results). Additionally, siramesine was included as a positive control for lysosomotropic agents in mechanistic studies.

Table 3.1: Summary of Drugs Used in this Study

Drug Name	Drug Class	Concentration Range Tested	Solvent/Storage Temperature	Provider
Clemastine	H1 Antihistamine	3.25-25μM (CLL cells) 20-80μM (Cell Lines)	DMSO/-20°C	Sigma
Desloratadine	H1 Antihistamine	6.25-50μM (CLL cells) 40-120μM (Cell Lines)	DMSO/-20°C	Sigma
Loratadine	H1 Antihistamine	3-25μM (CLL cells) 20-80μM (Cell Lines)	DMSO/-20°C	Sigma
Siramesine	Antidepressant	2-5μM (CLL cells) 10μM (Cell Lines)	DMSO/Room Temperature	Sigma
Bendamustine	Alkylating Agent	6-50μM (Cell Lines)	DMSO/-80°C	Sigma
Chlorambucil	Alkylating Agent	6-50μM (Cell Lines)	DMSO/-80°C	Sigma
Fludarabine	Nucleoside Analogue	3-25μM (Cell Lines)	DMSO/-80°C	Sigma
Ibrutinib	BTK Inhibitor	3.25-25μM (CLL cells) 5-50μM (Cell Lines)	DMSO/-80°C	Selleckchem
Idelalisib	PI3Kδ Inhibitor	6.25-50μM (Cell Lines)	DMSO/-80°C	Selleckchem
Venetoclax	BH3 Mimetic	3-25μM (Cell Lines)	DMSO/-80°C	Selleckchem

3.2.2 STIMULI AND INHIBITORS

All relevant inhibitors of cell death mechanisms to this project are summarized in Table 3.2. Inhibitors were used at a concentration that could inhibit their target without additionally reducing cell viability, and were optimized prior to experiments using concentrations from previous literature. All inhibitors were added directly to cell media at desired concentrations 1 hour prior to drug addition. All inhibitors were pre-dissolved and stored in single-used aliquots, while antioxidants α-Tocopherol and N-acetylcysteine were made fresh for each experiment in sterile solvents. In a separate experiment to simulate microenvironmental effects on drug treatment, CLL cells were stimulated with 100ng/mL IL-4 and 100ng/mL CD40L concurrently for 1 hour prior to drug addition (not shown in Table 3.2).

Table 3.2: Summary of Inhibitors Used in this Study

Inhibitor Name	Function	Concentration Used	Solvent/Storage Temperature	Provider
SKI II	Sphingosine Kinase Inhibitor	10μM	DMSO/-20°C	Sigma
CA074-ME	Cathepsin B Inhibitor	10μM	DMSO/-20°C	Sigma
E64	Cathepsin B Inhibitor	10μM	ddH_20/-20°C	Sigma
Chymostatin	Cathepsin D Inhibitor	25μM	DMSO/-20°C	Sigma
α-Tocopherol	Lipid ROS Scavenger	200μg/mL	Ethanol/4°C	Sigma
N-acetylcysteine	Antioxidant/ GSH Precursor	5mM	PBS/4°C	Calbiochem
Ferrostatin-1	Ferroptosis Inhibitor	10μM	DMSO/-20°C	Sigma
Diferoxamine	Iron Chelator	200μM	DMSO/-20°C	Sigma

3.3 FLOW CYTOMETRY

3.3.1 CELL DEATH ANALYSIS

Cell death for the majority of experiments in this thesis was measured using Trypan Blue exclusion. Trypan Blue is a protein dye that normally excluded from entering viable cells by the plasma membrane. When a cell loses its membrane integrity during cell death, Trypan Blue can enter the membrane through newly formed pores and stains cytoplasmic proteins, emitting a red fluorescence with a wavelength of approximately 650 nm. A 0.4% Trypan Blue solution was mixed with drug-treated cells in media at a ratio of 1:10 v/v and immediately analyzed with the Novocyte flow cytometer (Acea Biosciences) to prevent nonspecific staining. Positive and negative events were gated in the PerCP channel of the flow cytometer based on drug-treated controls. Events positive for Trypan Blue fluorescence were considered dead.

For assessment of apoptosis, Annexin V and 7-Aminoactinomycin D (7AAD) staining was used. Annexin V is a fluorescent probe which binds phosphatidylserine on the surface of cells undergoing apoptosis, while 7AAD is a DNA stain which undergoes a spectral shift similar to Trypan Blue when it enters dead cells and binds its target. Together, these stains simultaneously detect apoptosis and total cell death. A staining solution consisting of Annexin V Binding Buffer, Annexin V-FITC conjugated antibody, and 7AAD was added to cells in media. After 15 minutes at room temperature, PBS was added to quench the reaction and cells were analyzed with the Novocyte flow cytometer. Events were gated in the FITC and PerCP channels with appropriate gating and compensation controls. Those events negative for Annexin V or 7AAD fluorescence were considered viable, while all events positive for either stain were considered dead.

3.3.2 LYSOSOME STAINING

To examine the integrity of the lysosomal membrane in cells treated with lysosomotropic agents, acidophilic dyes Lysotracker Green DND-26 (Invitrogen) and Acridine Orange (AO; Sigma-Aldrich) were used. A significant decrease in fluorescence of either stain indicates loss of acidity and permeabilization of acidic organelles. For Lysotracker staining, cells were suspended in a solution of 50nM Lysotracker Green in media for 30 minutes at 37°C. For AO staining, cells were suspended in a 5µg/mL solution of AO in PBS for 15 minutes at room temperature. After staining, baseline median fluorescent intensity (MFI) was measured using the Novocyte flow cytometer prior to drug addition. DMSO or lysosomotropic agents were added at doses indicated in figure legends. MFI readings were determined at 1 minute and in 5-minute intervals after drug treatment over a 15 minute time course.

3.3.3 DIHYDROETHIDIUM (DHE) STAINING FOR DETECTION OF ROS

DHE is a probe commonly used to detect intracellular ROS. DHE binds superoxide to become oxyethidium, which intercalates with DNA and emits a red fluorescent signal with a wavelength of approximately 590nm. To detect ROS, drug-treated cells were centrifuged at 290 x g and resuspended in PBS containing 3.2µM DHE for 30 minutes at 37°C. Cells were washed once with PBS and fluorescence was analyzed in the PerCP channel of the Novocyte flow cytometer. Positive and negative events were based on the fluorescence of vehicle and unstained controls, and events positive for DHE fluorescence were determined to be positive for ROS.

3.3.4 TETRAMETHYLRHODAMINE (TMRM) STAINING OF MITOCHONDRIA

TMRM is a cationic dye that can cross the mitochondrial outer membrane and is sequestered by active mitochondria. The dye accumulates within intact mitochondria; a loss of TMRM fluorescence indicates that mitochondrial membrane potential has been lost. For assessment of mitochondrial membrane potential, drug-treated cells were centrifuged at 290 x g and resuspended in PBS containing 25nM TMRM for 30 minutes at room temperature. Cells were washed once with PBS and fluorescence was analyzed in the PE channel of the Novocyte flow cytometer. Positive and negative events were gated based on the fluorescence of vehicle and unstained controls, and events negative for TMRM fluorescence were determined to have permeabilized mitochondria.

3.4 CONFOCAL MICROSCOPY

To examine if fluorescence was localized to the lysosome in Lysotracker experiments, the Zeiss Cell Observer SD was used to visualize primary CLL cells. 4-chamber coverslip-bottom slides (Lab-Tek) were coated with Cell-Tak (Corning) mixed with binding buffer containing $NaHCO_3$ and NaOH for 20 minutes. Cell-Tak was aspirated and the chambers were washed with ddH_2O and allowed to dry. Primary CLL cells were seeded in chambers at a concentration of 5×10^5 cells/mL and allowed to bind to the chambers for 1 hour at 37°C. Fresh media was then added containing 50nM Lysotracker Deep Red (Invitrogen) for 30 minutes at 37°C to stain lysosomes. Finally, stained cells were treated with either DMSO or clemastine for 5 minutes and analyzed via spinning disk confocal microscope at 100x magnification.

3.5 GEL ELECTROPHORESIS AND WESTERN BLOTTING

3.5.1 SAMPLE PREPARATION

Control or drug treated cells were harvested and washed once with PBS prior to lysis. Cells were resuspended in radioimmunoprecipitation assay (RIPA) lysis buffer containing Complete Mini Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich). Lysis was done on ice and cells were vortexed for 30 seconds at 5-minute intervals over 20 minutes. Lysates were centrifuged at 16,000 x g for 15 minutes and debris was removed. Protein was quantified using the Pierce BCA (bicinchoninic acid; Thermo Fisher) kit and samples were diluted in 6 x Laemmli loading buffer and RIPA buffer so that each final sample contained 50µg of total protein in 1x loading buffer. Samples were boiled at 95°C for 10 minutes, cooled on ice, vortexed, and centrifuged prior to loading.

3.5.2 GEL ELECROPHORESIS AND TRANSFER

Samples were loaded into wells in 10% acrylamide TGX FastCast Gels (Bio-Rad) alongside a well containing PageRuler Plus Prestained Protein Ladder (Thermo-Fisher). Protein samples were electrophoresed at 80V for 30 minutes to clear the stacking gel before being switched to 120V for the remaining 30 minutes. Upon appropriate ladder separation, gels were placed in the Trans-Blot Turbo transfer apparatus (Bio-Rad) and transferred to 0.22mm or 0.45mm nitrocellulose membranes at constant 2.5A/up to 25V for 12 minutes. Membranes were then blocked for 1 hour in tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% non-fat milk. For short-term storage, membranes were sealed in airtight packages with TBS-T and stored at 4°C. For long-term storage, membranes were kept dry in airtight packages at 4°C.

3.5.3 BLOTTING OF MEMBRANES

Membranes containing protein samples were sealed in airtight packages with the appropriate primary antibody dilution in blocking buffer. Membranes with antibody were placed on an agitator overnight at 4°C to allow uniform antibody binding. They were then washed 3 times for 10 minutes with TBS-T to remove excess primary antibody. Membranes were placed in blocking buffer containing horseradish peroxidase (HRP) conjugated secondary antibodies at room temperate for 1 hour. Again, membranes were washed 3 times for 10 minutes with TBS-T to remove excess secondary antibody. Pierce ECL (enhanced chemiluminescence; Thermo-Fisher) reagent was used to blot membranes for HRP antibody binding, and images were exposed using a film developer. For re-use, membranes were stripped of antibodies using a 1 x Western Re-Probe Reagent (Calbiochem) dilution for 30 minutes at room temperature. Images were scanned and analyzed via densitometry using ImageJ software. Density of bands was normalized to loading controls. A summary of all primary and secondary antibodies used for this study is provided in Table 3.3.

Table 3.3: Summary of Antibodies Used in this Study

Target Antigen	Primary or Secondary	Species	Dilution	Provider	Product Number
Phospho-Akt (Ser473)	Primary	Rabbit	1:1000	CST	9271S
Akt	Primary	Rabbit	1:1000	CST	9272S
Phospho-ERK1/2 (Thr202/Tyr204)	Primary	Rabbit	1:1000	CST	4370S
ERK1/2	Primary	Rabbit	1:1000	CST	4695S
Bcl-2	Primary	Rabbit	1:1000	CST	2876S
Bcl-xL	Primary	Rabbit	1:1000	CST	2762S
Mcl-1	Primary	Mouse	1:200	SCBT	sc-12756
Actin	Primary	Mouse	1:10000	Sigma	A3853
Actin	Primary	Rabbit	1:4000	CST	4970S
Mouse IgG	Secondary	Goat	1:1000 (Mcl-1) 1:20000 (actin) 1:4000 (others)	Bio-Rad	170-6515
Rabbit IgG	Secondary	Goat	1:4000	Bio-Rad	170-6516

CST=Cell Signalling Technology; SCBT=Santa Cruz Biotechnology

3.6 DATA ANALYSIS

All graphs relevant to this thesis were generated using Graphpad Prism 7 software. Bars for each graph represent mean and standard error to compare variation between individual means. Statistical significance was determined by two-tailed unpaired t-test comparing two different conditions with an N of 3 or greater. A p value of <0.05 indicates statistically significant difference between two populations. In the case of combinations comparing 4 different conditions, ANOVA was instead used to determine statistical differences with post-hoc analysis comparing controls to combination treatments. Degree of significance was noted in figures. * represents $p<0.05$; ** represents $p<0.01$; *** represents $p<0.001$; **** represents $p<0.0001$.

Drug interactions were assessed through the generation of a combination index (CI) for a single combination of drug doses. First, sigmoidal dose-response curves for each individual

drug were generated using Graphpad Prism 7. The effective concentration required to kill 50% of cells (EC50) was determined for each drug using this method. Additionally, combinations were assigned a CI value from these dose response-curves by calculating the ratio of doses required to achieve an effect in combination, when compared to doses required to achieve the same effect as a single agent. A CI less than 1 indicates synergy (more desirable), a CI equal to 1 indicates additivity, and a CI greater than 1 indicates antagonism (less desirable). CI values were represented graphically using isobolograms [175, 176].

CHAPTER 4: RESULTS

4.1: AIM 1: To determine if H1 antihistamines could kill malignant B cell lines and CLL cells, and to identify the mechanism of this cell death.

4.1.1: H1 antihistamines induce dose-dependent cell death in the malignant B cell lines BJAB and I83, and preferentially kill primary CLL cells compared to PBMC's.

To determine if H1 antihistamines could kill malignant B cells, BJAB and I83 cells were treated with a range of doses of clemastine (20-80μM), desloratadine (40-120μM), or loratadine (20-80μM). Dose ranges were chosen based off of initial results in the lung cancer study and our solid tumor models [152, 175]. Cell death was measured using Trypan Blue exclusion 24 hours after drug treatment. All three antihistamines induced dose-dependent cell death in both cell lines (Figure 4.1). Clemastine had the lowest EC50 value in both cell lines with approximately 50μM of the drug killing 50% of cells (Table 4.1). This concentration was chosen for future experiments to study the mechanism of death induced by clemastine as an individual agent. These results demonstrate that the H1 antihistamines clemastine, desloratadine, and loratadine could display cytotoxicity in malignant B cell lines.

To translate this result to CLL, primary CLL cells or PBMC's were treated with a range of doses of clemastine (3.25-50μM), desloratadine (6.25-100μM), or loratadine (3.25-50)μM. In CLL cells, maximal cell death was achieved at a concentration of 25μM clemastine, 50μM desloratadine or 25μM loratadine. Clemastine was again the most cytotoxic of the 3 agents, with a mean EC50 concentration of 12.3μM (Table 4.1). Therefore, a concentration of 12.5μM clemastine was chosen to study the mechanism of death induced by clemastine as an individual agent. Additionally, CLL cells required approximately half as much drug to kill 50% of cells

compared to PBMC's (Figure 4.2 d.e.f.), indicating that CLL cells are more sensitive to antihistamine-induced death than normal mononuclear cells.

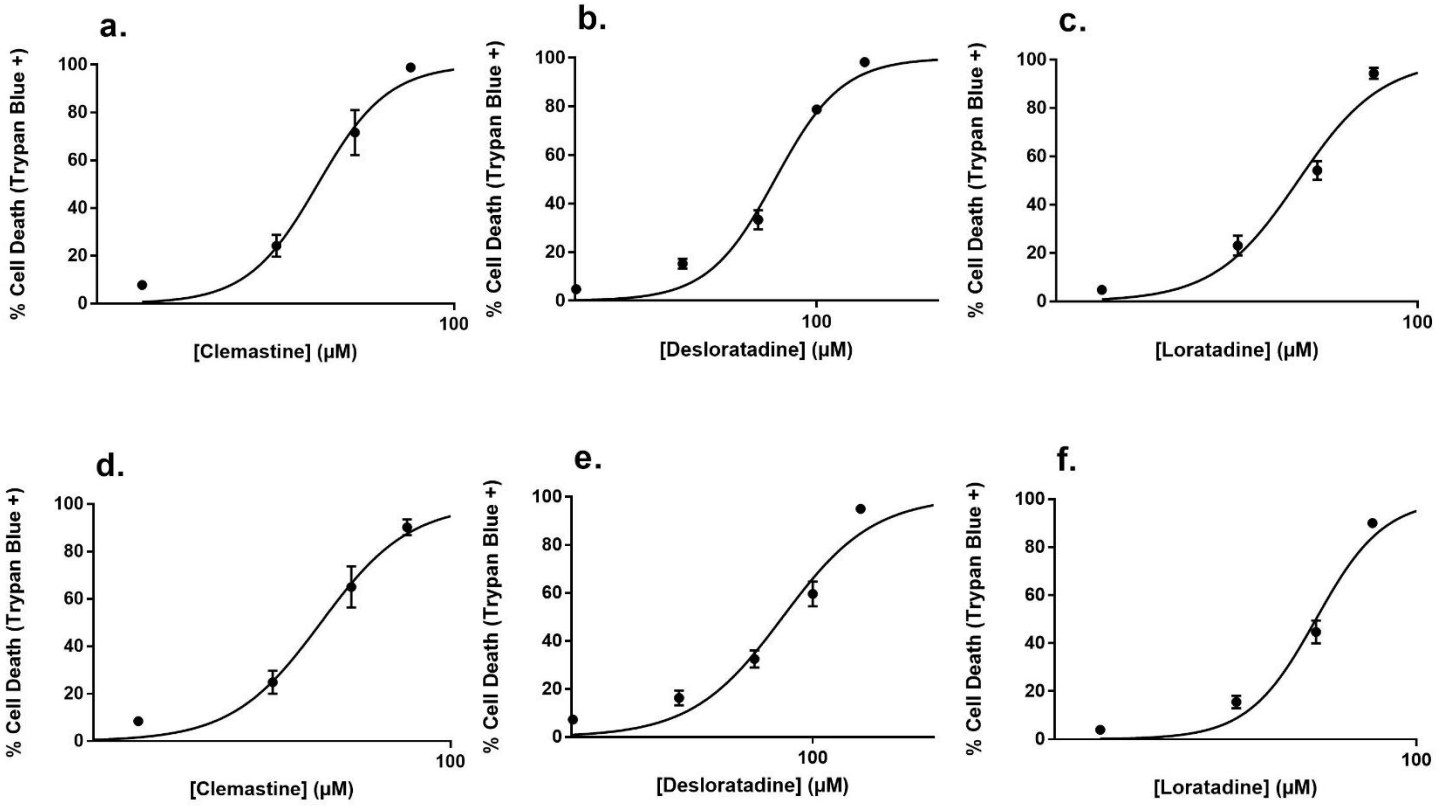


Figure 4.1: Antihistamines induce dose-dependent cell death in BJAB and I83 cells. BJAB (a.)(b.)(c.) or I83 cells (d.)(e.)(f.) were cultured with an increasing range of doses of clemastine, desloratadine, or loratadine (c.) for 24 hours. X-axes represent the antilog micromolar dose of each drug. Cells were stained with Trypan Blue and subjected to further analysis via flow cytometry. Cells displaying positive Trypan Blue fluorescence were considered dead. Concentrations required to kill 50% of cells (EC50) for each sample were determined from dose-response curves. Error bars represent mean and standard error. N=3.

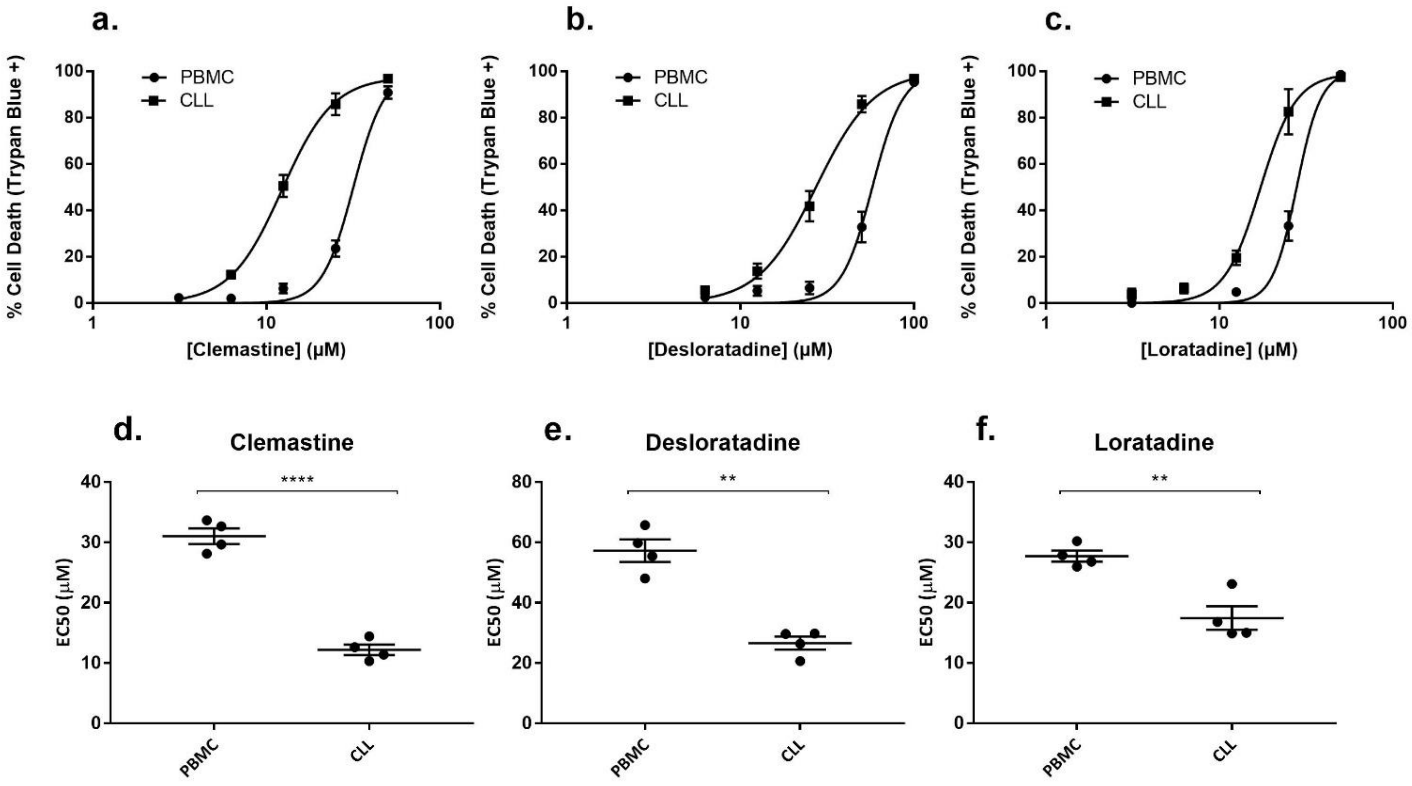


Figure 4.2: CLL cells are more sensitive to antihistamine-induced cell death compared to PBMC's from donors without CLL. CLL cells or PBMC's from age-matched healthy donors were treated with an increasing range of doses of clemastine (a.) desloratadine (b.) or loratadine (c.) for 24 hours. Cells were stained with Trypan Blue and subjected to further analysis via flow cytometry. Cells displaying positive Trypan Blue fluorescence were considered dead. Concentrations required to kill 50% of cells (EC50) for each sample were determined from dose-response curves for individual donors (d.)(e.)(f.). Error bars represent mean and standard error. N=4 independent patients or donors. ** $p<0.01$; **** $p<0.0001$.

Drug Name	BJAB	I83	CLL	PBMC
Clemastine	49.5μM	51.3μM	12.3μM	31.7μM
Desloratadine	85.3μM	89.1μM	27.2μM	57.5μM
Loratadine	53.5μM	60.0μM	17.2μM	27.9μM

Table 4.1: Absolute EC50 Concentrations Calculated from Dose-Response Curves for B Cell Models.

4.1.2: H1 antihistamines induce lysosomal membrane permeabilization in BJAB and primary CLL cells

Clemastine, desloratadine and loratadine are CAD drugs that have been demonstrated to permeabilize lysosomes in cancer models. One characteristic of CAD-induced LMP is a reduction of acidity in the lysosome due to lysosomal membrane breakage [141, 152]. LMP can also be induced or enhanced by addition of excess sphingosine, which can be achieved through inhibition of sphingosine kinase with SKI II [126].

To assess whether LMP was occurring in BJAB or CLL cells, I stained these cells with the acidophilic dyes Lysotracker or AO and treated them with EC50 concentrations of clemastine and desloratadine or a DMSO control. MFI was measured by flow cytometry over a 15-minute time course and normalized to DMSO control. In BJAB cells, fluorescence of clemastine-treated cells was decreased by a maximum of 57% in Lysotracker-stained cells or 37% in AO-stained cells compared to the control (Figure 4.3 a. b.). In CLL cells, clemastine treatment reduced fluorescence by a maximum of 48% and desloratadine reduced fluorescence by a maximum of 24% compared to the control (Figure 4.3 d. e.). Lysotracker fluorescence was shown to be localized to the lysosome through confocal imaging, and was reduced in clemastine-treated cells compared to control cells (Figure 4.4).

CLL cells were also pre-treated with SKI II at a dose that could inhibit sphingosine kinase and subsequently treated with clemastine. Results indicate that SKI II significantly enhanced clemastine-induced cell death (Figure 4.5). Additionally, SKI II induced approximately 20% cell death by itself, potentially due to its ability to induce LMP through increases in sphingosine. This may be evidence that antihistamines affect sphingosine

metabolism. Together, these findings indicate that H1 antihistamines clemastine and desloratadine induce LMP in BJAB and CLL cells.

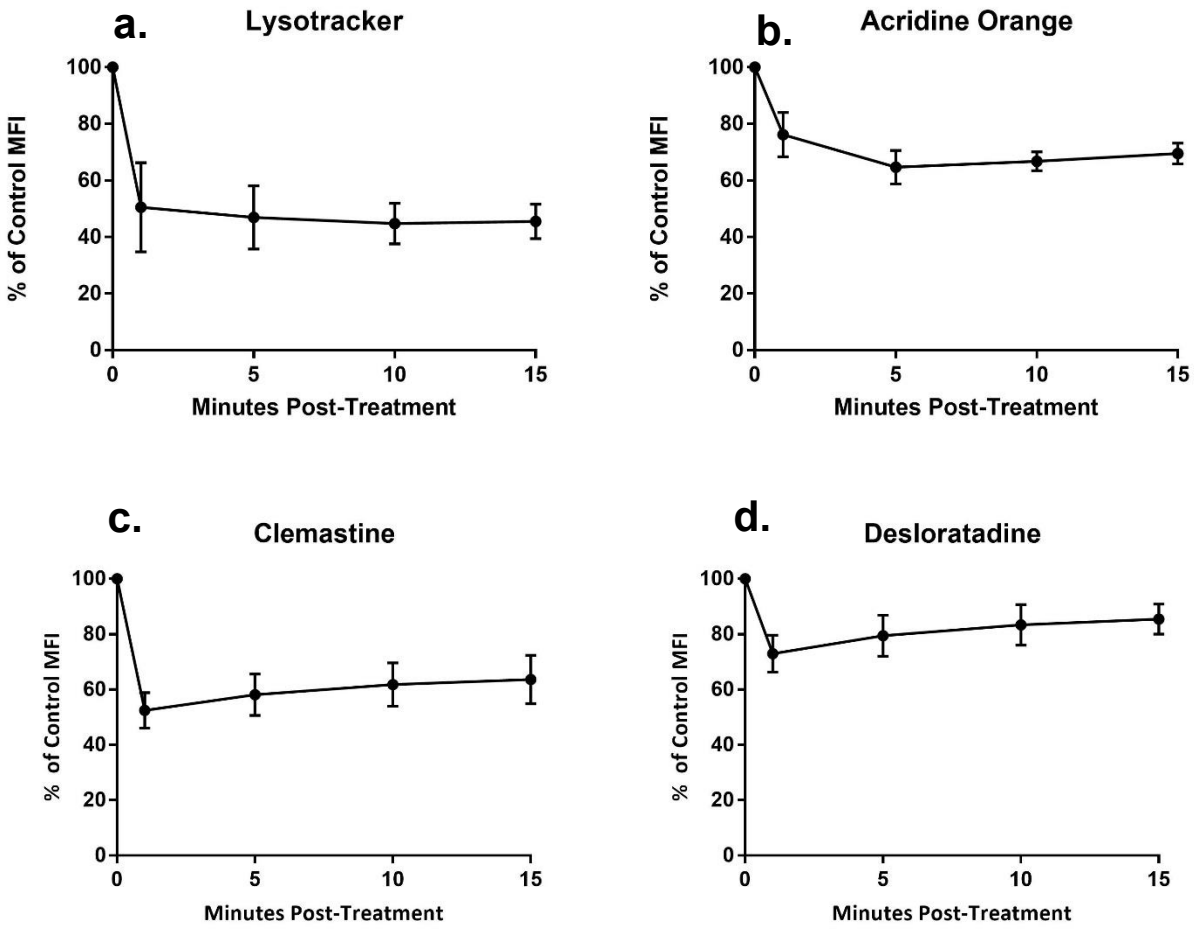


Figure 4.3: H1 antihistamine treatment reduces fluorescence of acidophilic dyes in BJAB and CLL cells. (a.)(b.) BJAB cells were stained with Lysotracker Green DND-26 or AO and treated with 50μM clemastine (N=3) (d.)(e.) CLL cells were stained with Lysotracker Green DND-26 and treated with 12.5μM clemastine or 25μM desloratadine. (N=4) Fluorescence was measured via flow cytometry prior to treatment, at 1 minute post-treatment, and in 5 minute intervals post-treatment. Values are represented as a percentage of vehicle control median fluorescent intensity (MFI) measured via flow cytometry.

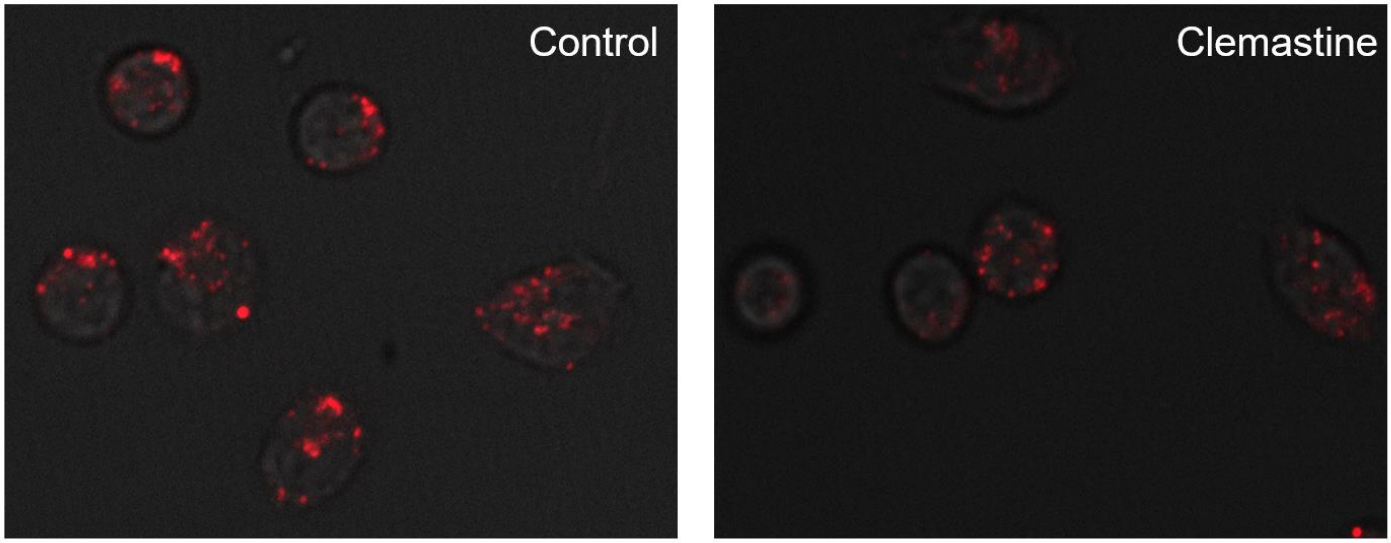


Figure 4.4: Reduction of Lysotracker fluorescence by clemastine is localized to the lysosome in CLL cells. CLL cells were stained with Lysotracker Deep Red and treated with either a DMSO control or 12.5µM clemastine. Images were taken 5 minutes post-treatment under 100x magnification using the Zeiss Cell Observer. One representative view of several images for each condition is shown.

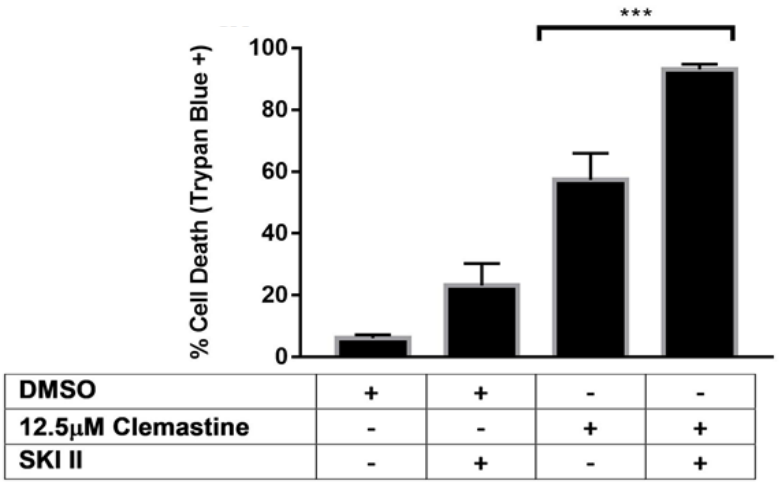


Figure 4.5: Clemastine-induced cell death is enhanced by inhibition of sphingosine kinase in CLL cells. CLL cells were pre-treated with 20µM SKI II for 1h and subsequently treated with 12.5µM clemastine for 24h. Cell death was quantified using Trypan Blue exclusion via flow cytometry. N=3 independent patient samples. Error bars represent mean and standard error. *** $p<0.001$.

4.1.3: Clemastine induces apoptotic cell death through an increase in ROS and decrease in mitochondrial membrane potential

Cell death induced through LMP can bear characteristics of apoptotic or necrotic cell death [177]. LMP induced by drugs often involves cross-talk between lysosomal ROS and mitochondrial lipids through lipid peroxidation [118]. A physical event that occurs in apoptosis is the translocation of phosphatidylserine from the inner layer of the cell membrane to the outer layer, which can be detected by the Annexin V probe [29].

To determine if increases in ROS or mitochondrial dysfunction were occurring, CLL cells treated with 12.5 μM clemastine were stained with DHE (for ROS detection) or TMRM (to assess MMP). I found that there was approximately a 50% increase in ROS at 4h post-treatment compared to the vehicle control, and a corresponding reduction of 40% in cells with intact MMP at 24h post-treatment (Figure 4.6 a. b.). To determine if this cell death was apoptotic, CLL cells were stained with Annexin V/7AAD. CLL cells treated with a range of doses of clemastine produced Annexin V/7AAD positive events in a dose-dependent manner similar to our results using Trypan Blue staining (Figure 4.6 c.). Together, this data indicates that CLL cells treated with 12.5μM clemastine undergo changes consistent with the intrinsic pathway of apoptosis and involve an early increase in ROS and subsequent mitochondrial dysfunction.

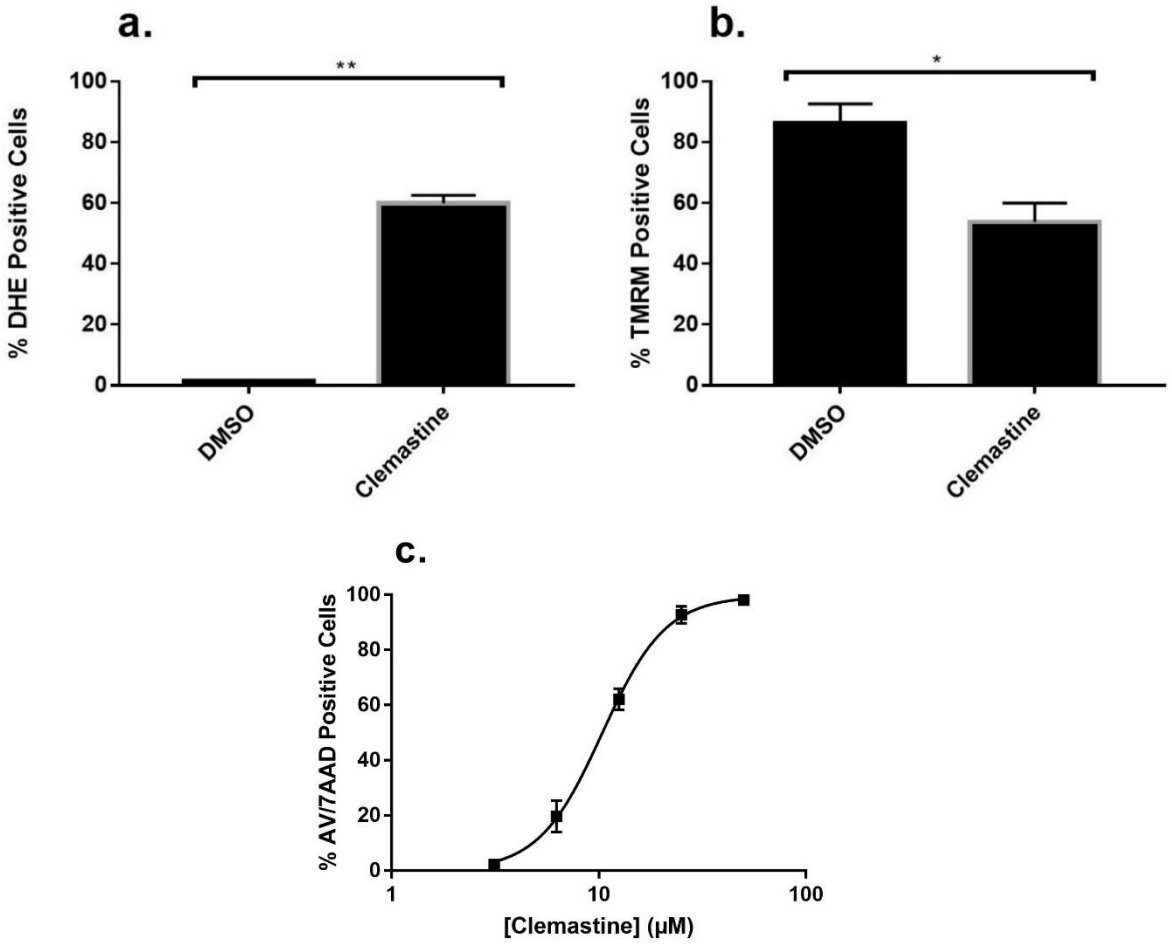


Figure 4.6: Clemastine-induced cell death increases ROS, decreases MMP, and induces apoptosis in CLL cells. (a.) CLL cells were treated with a vehicle control, or 12.5μM clemastine for 4h. Cells were stained with DHE and fluorescence was measured via flow cytometry based on vehicle control. (b.) CLL cells were treated for 24h with 12.5μM clemastine. Cells were stained with TMRM and analyzed via flow cytometry. Cells negative for TMRM fluorescence were considered to have lost mitochondrial membrane potential. (c.) CLL cells were treated with 3.25-25μM clemastine and stained with Annexin V/7AAD and analyzed via flow cytometry after 24h treatment. Cells positive for AV/7AAD were considered apoptotic. Error bars represent mean and standard error. N=3. ** $p<0.01$. * $p<0.05$.

4.1.4: Clemastine-induced LMP kills CLL cells through an oxidative mechanism but not cathepsins

LMP is associated with the release of cathepsins B and D, as well as ROS, from the lysosomal into the cytosol. Cathepsins can cleave anti-apoptotic mitochondrial proteins, while ROS can oxidize the mitochondrial membrane directly [118, 131, 132, 178] . Cathepsin B can be inhibited by a variety of compounds including the specific inhibitor CA074-ME, as well as the inhibitor of cysteine peptidases E64. Cathepsin D can be inhibited nonspecifically by a variety of aspartic protease inhibitors including chymostatin [179]. Alpha-tocopherol is an endogenous scavenger of lipid ROS, also known as vitamin E, while NAC is a GSH precursor and a ROS scavenger.

To determine the mechanism of mitochondrial dysfunction induced by clemastine, I pre-treated CLL cells with the inhibitors of cathepsins or lipid peroxidation previously discussed. Cells were then treated with 12.5μM clemastine and cell death was measured. I found that the cathepsin inhibitors CA074-ME, E64 and chymostatin were not able to protect CLL cells from clemastine-induced death. Interestingly, CA074-ME actually significantly increased cell death (Figure 4.7 b.). In contrast, both ROS scavengers were able to partially protect CLL cells from clemastine-induced death. Alpha tocopherol conferred a greater degree of protection to CLL cells than did NAC (Figure 4.7 a.). Siramesine was used as a positive control for these experiments and cell death induced by the drug was completely prevented by alpha-tocopherol (data not shown). Together, this data implies that lipid peroxidation by lysosomal ROS, but not cathepsins, may be playing a role in the mitochondrial dysfunction induced by clemastine. However, the partial protection from clemastine-induced cell death by antioxidants may indicate that additional mechanisms of cell death are occurring in tandem with lipid peroxidation

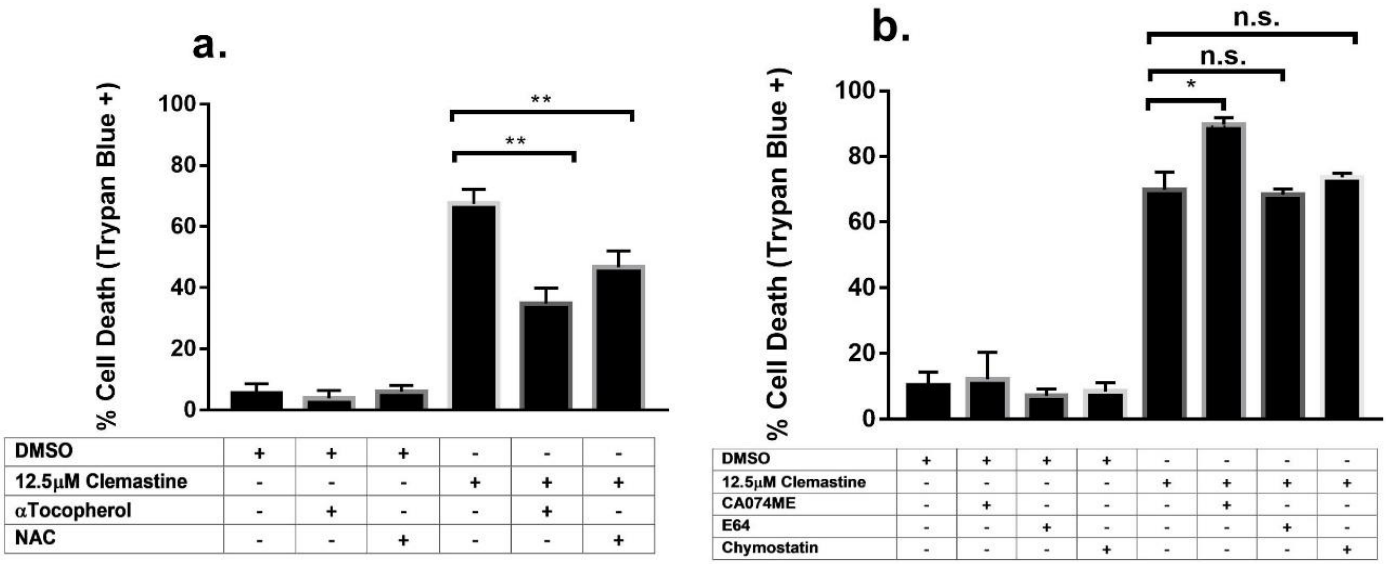


Figure 4.7: Cell death induced by clemastine is reduced by antioxidants but not cathepsin inhibitors in CLL cells. (a.) CLL cells were pretreated for 1h in the presence or absence of antioxidants alpha-tocopherol (200µg/mL) or NAC (5mM) and subsequently with 12.5µM clemastine for 24h. (b.) CLL cells were pretreated for 1h in the presence or absence of inhibitors of cathepsin B (E64; 10µM)(CA-074-ME; 10µM) or cathepsin D (chymostatin; 25µM) and subsequently treated with 12.5µM clemastine for 24h. Cell death was quantified using Trypan Blue exclusion via flow cytometry. N=3 independent patient samples. * $p<0.05$; ** $p<0.01$.

4.2: AIM 2: To determine if H1 antihistamines could induce synergistic cell death when combined with drugs relevant to CLL, and to identify the mechanism of cell death caused by a synergistic combination

4.2.1: The combination of clemastine with targeted therapies, but not chemotherapy, significantly increases total cell death in BJAB cells

Combination treatments are commonly used to target multiple pathways in CLL to reduce doses and toxicities and increase treatment specificity. Lysosomotropic agents have shown synergy with both fludarabine and TKI's in cancer models [142, 157, 175]. Therefore, I wanted to determine if the lysosomotropic agent clemastine could increase total cell death when combined with therapies relevant to CLL. The BJAB cell line was used to screen combinations of drugs for synergy. In BJAB cells, doses of drugs producing less than 20% cell death were chosen for combinations, as these doses have indicated where synergy is most evident in our previous studies [175]. A dose of 25μM clemastine was chosen from its dose-response curve in BJAB cells to be combined with chlorambucil, bendamustine, fludarabine, ibrutinib, idelalisib, and venetoclax for 24h. Results show that combinations of clemastine with chemotherapy did not produce significantly increased cell death compared to vehicle or single-agent controls (Figure 4.8 a.-c.). In contrast, combinations of clemastine with targeted therapies did produce significantly increased cell death (Figure 4.8 d.-f.). The combination of 25μM clemastine and 5μM ibrutinib induced the greatest amount of cell death (45%) compared to controls, and was thus chosen to be investigated further. This combination was investigated for synergy in the next section.

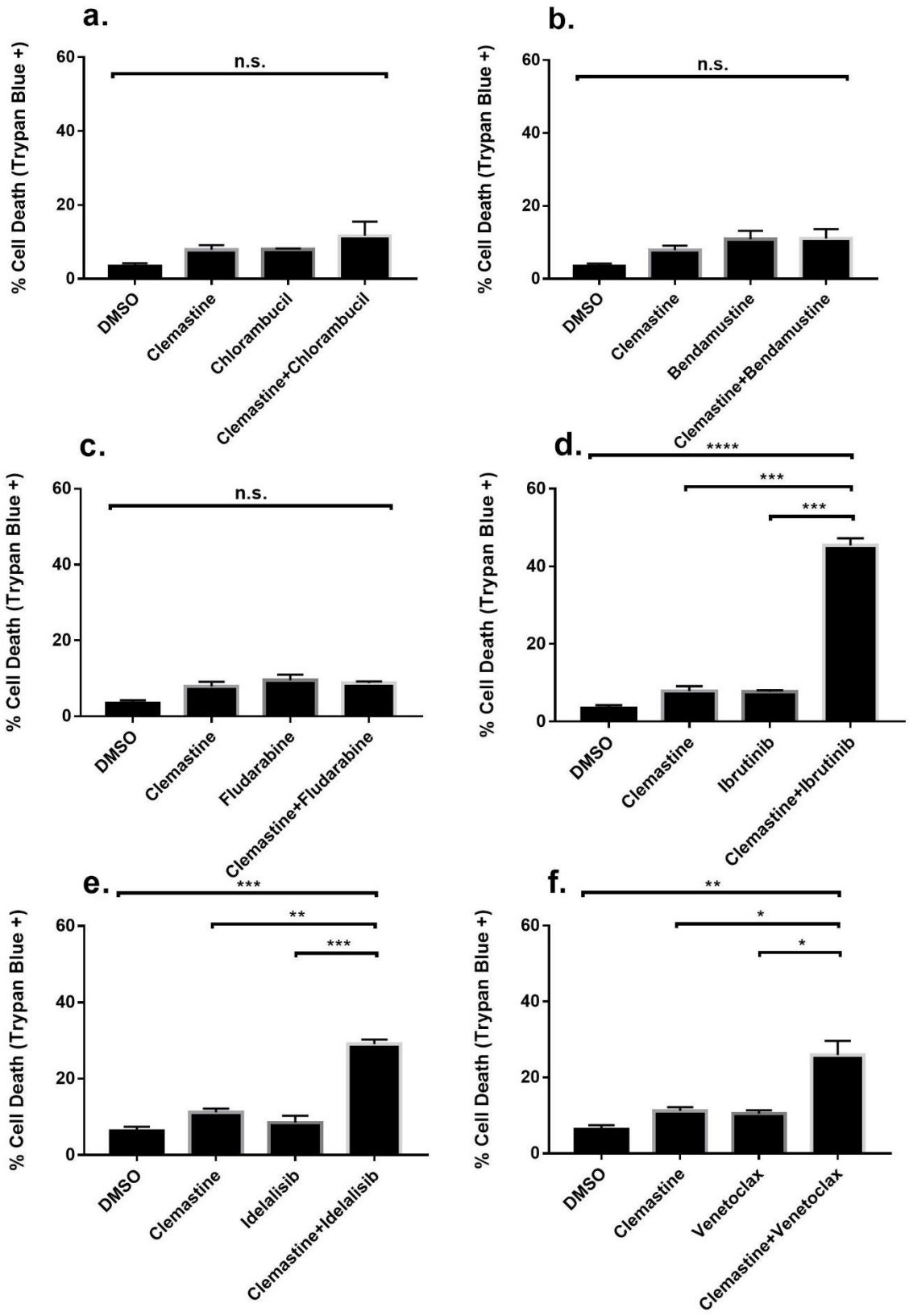
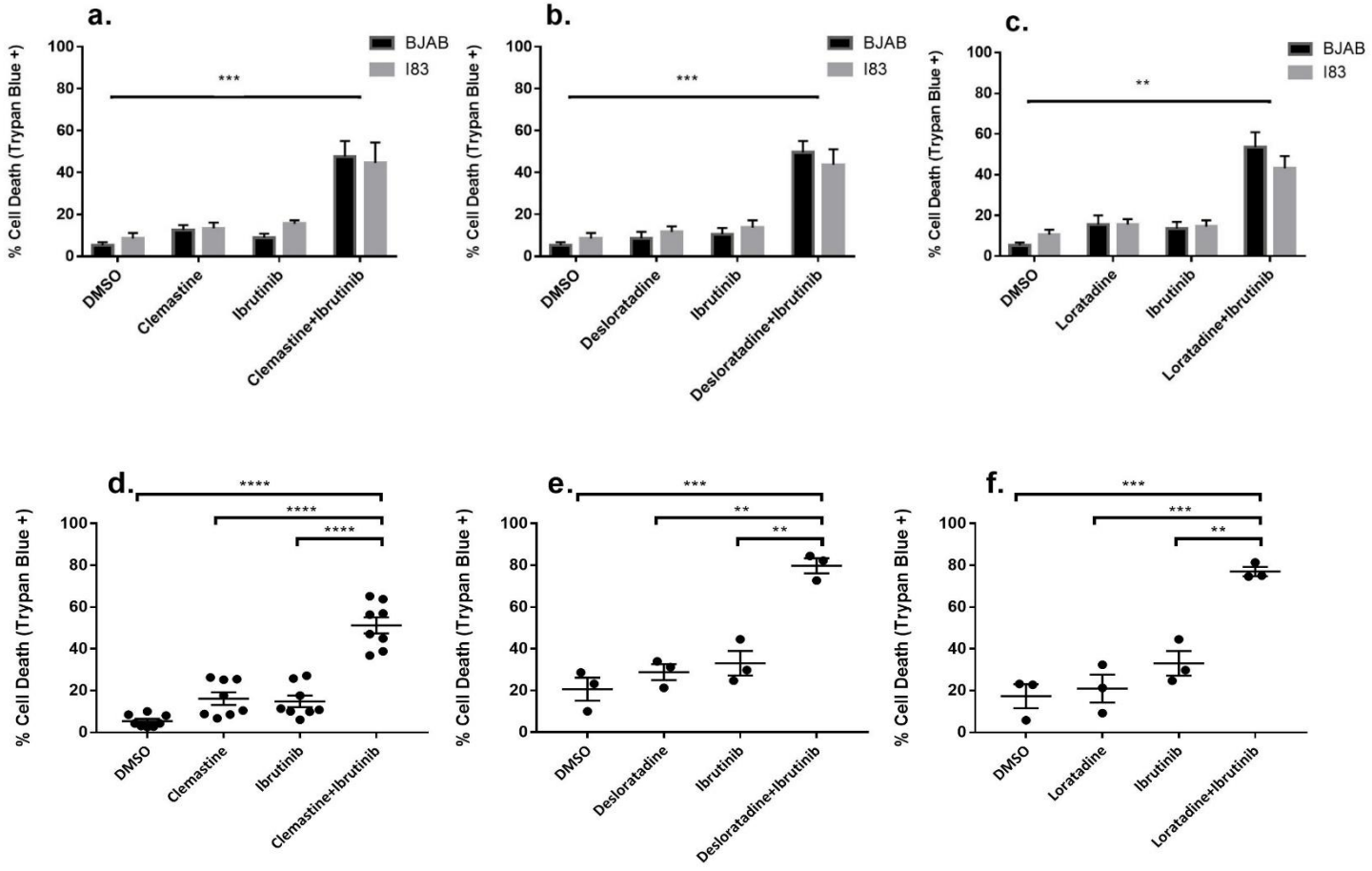

a.
% Cell Death (Trypan Blue +)
n.s.
DMSO
Clemastine
Chlorambucil
Clemastine+Chlorambucil
b.
% Cell Death (Trypan Blue +)
n.s.
DMSO
Clemastine
Bendamustine
Clemastine+Bendamustine
c.
% Cell Death (Trypan Blue +)
n.s.
DMSO
Clemastine
Fludarabine
Clemastine+Fludarabine
d.
% Cell Death (Trypan Blue +)
****
***
***
DMSO
Clemastine
Ibrutinib
Clemastine+Ibrutinib
e.
% Cell Death (Trypan Blue +)
***
**
***
DMSO
Clemastine
Idelalisib
Clemastine+Idelalisib
f.
% Cell Death (Trypan Blue +)
**
*
*
DMSO
Clemastine
Venetoclax
Clemastine+Venetoclax

Figure 4.8: Clemastine increases total cell death in BJAB cells when combined with targeted therapies but not chemotherapy. (a.-f.) BJAB cells were treated with a either a vehicle control, individual doses or combination of clemastine and a targeted therapy for 24h. Doses causing less than 20% cell death individually were chosen: 25μM clemastine, 12.5μM chlorambucil, 25μM bendamustine, 5μM fludarabine, 5μM ibrutinib, 20μM idelalisib or 10μM venetoclax. Cell death was quantified using Trypan Blue exclusion via flow cytometry. N=3. Statistical analysis portrays degree of significant difference between combination and controls. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, $p<0.05$.

4.2.2: The combination of antihistamines and ibrutinib is synergistic in malignant B cell models

To determine whether the combination of H1 anthistamines and ibrutinib could induce cell death that was truly synergistic, dose-response curves were generated for each individual drug in the BJAB and I83 cell lines and in CLL cells. Doses were chosen that could produce less than 20% additional cell death as individual agents, and combined in the above models. Combinations of clemastine, desloratadine, and loratadine were identified that produced significantly increased cell death compared to controls when combined with ibrutinib in BJAB, I83, and CLL cells (Figure 4.9 a.-f). These combinations produced approximately 50% cell death or greater in each case. Desloratadine and loratadine produced even higher amounts of cell death than clemastine when combined ibrutinib in CLL cells, although is most likely due to the lower (~80%) baseline viability of the cells. Isobolograms were generated from these combinations and a CI value was assigned to each combination of each drug as previously described (Figure 4.9 g.-i.). In the literature, a CI of 0.6 or lower represents strong synergy, a CI of approximately 0.75 represents moderate synergy and a CI of approximately 0.9 or higher represents weak synergy [175, 176]. All combinations of clemastine, desloratadine, and loratadine with ibrutinib; however, this synergy varied between drugs, models and individual patients (Table 4.2). Clemastine displayed the most consistent synergy at doses chosen with values ranging from

0.65-0.79, indicating moderate synergy. Combinations of desloratadine or loratadine varied greatly, from 0.51-0.93, indicating strong to weak synergy dependent on dose and patient. Indeed, synergy can be a spectrum, and one combination in 3 models is not representative of all interactions of drugs at all doses, especially when patient responses are typically heterogeneous. However, for the purposes of studying combinatorial mechanisms at one selected dose, a single synergistic combination will suffice. As clemastine was the most cytotoxic of the 3 antihistamines at the lowest doses, as well as displaying the most consistent synergy at doses chosen, a combination of clemastine and ibrutinib was chosen for further mechanistic study in our models.



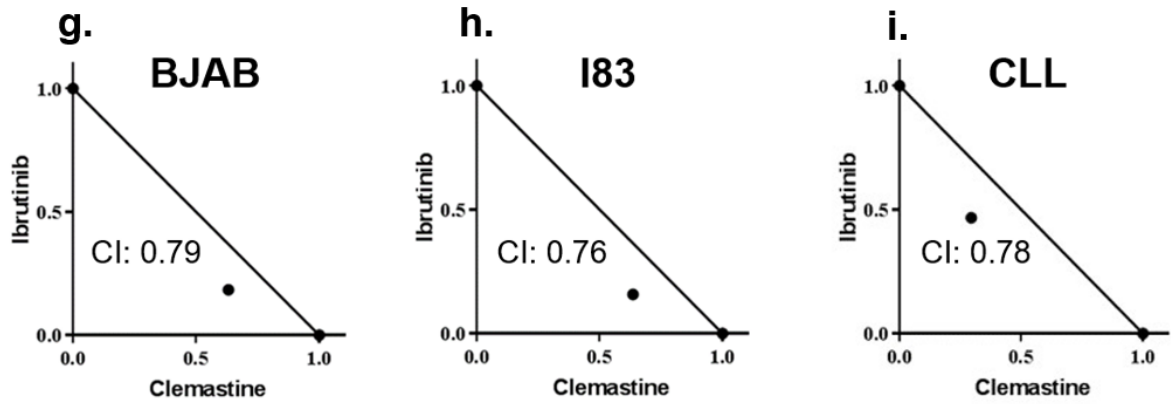


Figure 4.9: The combination of clemastine and ibrutinib induces synergistic cell death in malignant B cell models. Combinations of clemastine, desloratadine, and loratadine with ibrutinib in BJAB, I83 (a.)-(c.) (N=3). and CLL cells (d.)-(f.) (N=8;3;3). Doses of drugs were as follows: clemastine: 25µM (cell lines), 6.25µM (CLL); desloratadine: 45µM (cell lines), 12.5 µM (CLL); loratadine: 25µM (cell lines), 6.25µM (CLL); ibrutinib: 5µM (cell lines), 6.25µM (CLL). Cell death was measured at 24h post-treatment with Trypan Blue exclusion and analyzed via flow cytometry. *** $p<0.0001$, ***$p<0.001$, ** $p<0.01$ (g.)-(i.) Representative isobolograms of combinations of clemastine with ibrutinib in cell lines and 1 CLL patient. CI<1 represents antagonism; CI>1 represents synergy

Model	Clemastine+ Ibrutinib	Desloratadine+ Ibrutinib	Loratadine+ Ibrutinib
BJAB	0.79	0.89	0.85
I83	0.76	0.92	0.88
CLL 1	0.65	0.52	0.51
CLL 2	0.78	0.79	0.83
CLL 3	0.79	0.93	0.88

Table 4.2: Combination Indices for Combinations of Antihistamines and Ibrutinib

4.2.3: The combination of clemastine and ibrutinib kills CLL cells through an oxidative mechanism but not ferroptosis

Previous research in our lab has shown that combinations of lysosomotropic drugs and TKI's can produce synergistic cell death through a ferroptotic mechanism . This mechanism involves increases in ROS and lipid peroxidation pathways which can often induce mitochondrial dysfunction [134]. Ferrostatin-1 is a radical-trapping antioxidant while DFO is an iron chelator; both of these drugs can inhibit ferroptotic pathways [157]. Alpha tocopherol and NAC are ROS scavengers and inhibit lipid peroxidation [119].

To screen for the presence of a ferroptotic mechanism, I pre-treated CLL cells with ferrostatin-1 or DFO. To assess for lipid peroxidation, I pre-treated CLL cells with alpha-tocopherol or NAC. CLL cells were subsequently treated with a combination of clemastine and ibrutinib or a vehicle control. I found that neither inhibitor of ferroptosis could protect CLL cells from clemastine and ibrutinib-induced cell death (Figure 4.10 a.). However, both antioxidants exerted a significant protective effect on CLL cells from the drug combination (Figure 4.10 b.). This protection was only partial; the antioxidants only reduced cell death induced by the drug combination by approximately 50%. Together, these results show that the combination of clemastine and ibrutinib can kill CLL cells through an oxidative mechanism independently of ferroptosis. Additionally, protection by lipid ROS scavengers may indicate that this oxidative mechanism is through lipid peroxidation.

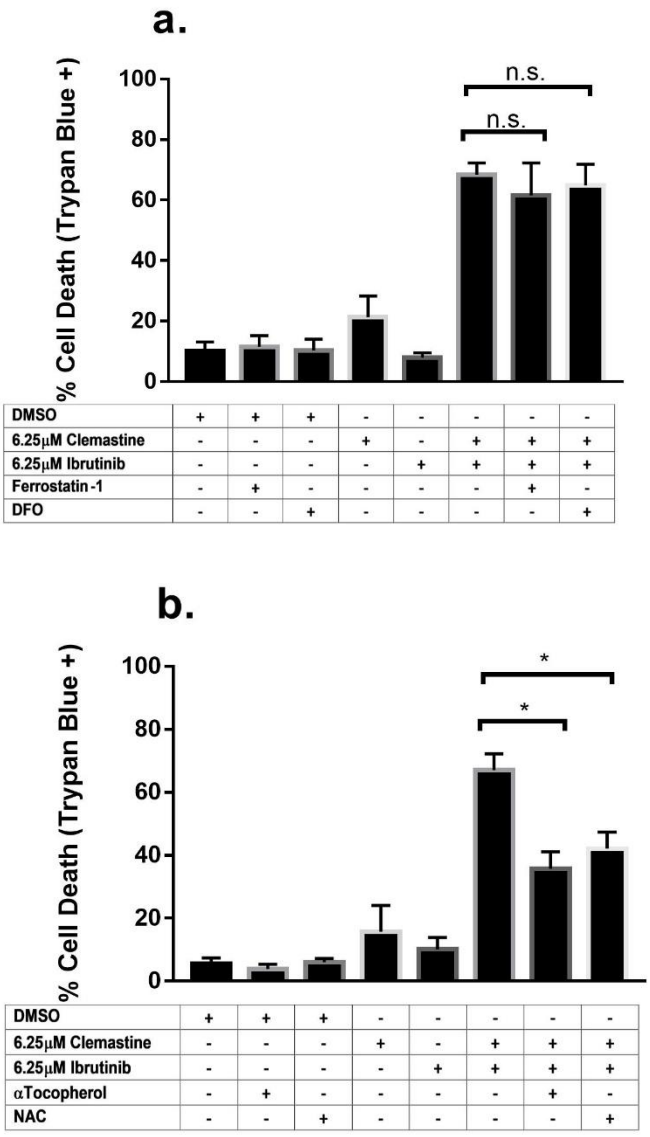


Figure 4.10: Clemastine and ibrutinib-induced cell death is reduced by antioxidants but not ferroptosis inhibitors in CLL cells. (a.) CLL cells were pretreated for 1h in the presence or absence of inhibitors of ferroptosis Ferrostatin-1 (10μM) or DFO (200μM) and subsequently treated with 6.25μM clemastine and 6.25μM ibrutinib for 24h. (b.) CLL cells were pretreated for 1h in the presence or absence of antioxidants alpha-tocopherol (200μg/mL) or NAC (5mM) and subsequently with 6.25μM clemastine and 6.25μM ibrutinib for 24h. Cell death was quantified using Trypan Blue exclusion via flow cytometry. N=3 independent patient samples. * $p<0.05$; ** $p<0.01$.

4.2.4: The combination of clemastine and ibrutinib decreases phosphorylation of ERK1/2 and Akt in BJAB and CLL cells

Ibrutinib's inhibition of BTK is well-characterized. This inhibition can also have effects on kinase signalling involving proteins that interact with BTK in B cells such as PI3Kδ. Survival pathways downstream of these pathways, such as the Raf/ERK axis and the Akt/mTOR axis can thus be inhibited as well by treatment of the drug . I wanted to assess the effect of ibrutinib on these pathways and determine if they play a potential role in the synergy.

BJAB or CLL cells were treated with a vehicle control, clemastine, ibrutinib, or a combination of the drugs and lysed 1h post-treatment. Phosphorylation of ERK1/2 or Akt was determined by western blot. In BJAB cells, ibrutinib and its combination with clemastine decreased phosphorylation of ERK1/2, but this decrease was nearly identical between treatment groups (Figure 4.11 a.). Ibrutinib and the combination also reduced Akt phosphorylation to an undetectable level in BJAB cells, so it could not be determined whether this decrease was enhanced by addition of clemastine (Figure 4.11 b.). In CLL cells, the combination of drugs decreased ERK1/2 phosphorylation in a similar manner to BJAB cells (Figure 4.12 a.). The combination also decreased Akt phosphorylation in CLL cells, but the bands were too faint at maximum exposure to determine whether this is a substantial effect (Figure 4.12 b.). Additional samples must be assessed before these results can be directly translated to CLL. Nevertheless, this preliminary data shows that ibrutinib is affecting signalling downstream of BTK in BJAB and CLL cells and the drug combination with clemastine does not reduce this effect.

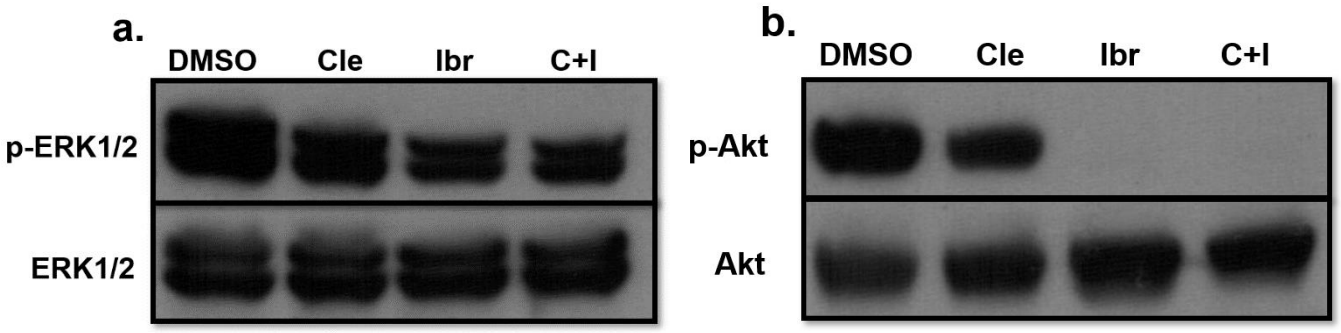


Figure 4.11: The combination of clemastine and ibrutinib decreases phosphorylation of ERK1/2 and Akt in BJAB cells (a.)(b.) Representative western blots of BJAB cells treated with a combination of 25µM clemastine and 5µM ibrutinib for 1h prior to lysis. Phosphorylation of ERK1/2 or Akt is shown in relation to pan-expression of protein. (N=3).

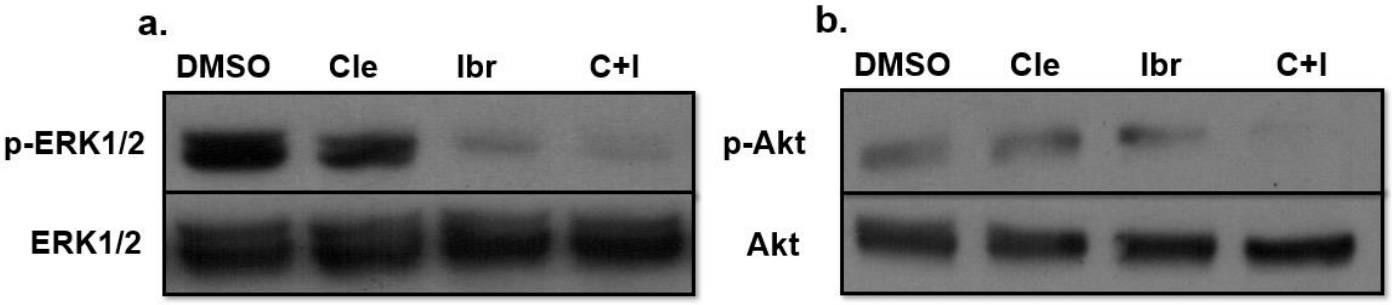


Figure 4.12: The combination of clemastine and ibrutinib decreases phosphorylation of ERK1/2 and Akt in CLL cells (a.)(b.) Western blots of BJAB cells treated with a combination of 6.25µM clemastine and 6.25µM ibrutinib for 1h prior to lysis. Phosphorylation of ERK1/2 or Akt is shown in relation to pan-expression of protein N=1.

4.2.5: The combination of clemastine and ibrutinib induces cell death through an increase in ROS and decrease in mitochondrial membrane potential

Ibrutinib has been demonstrated to reduce expression of Mcl-1 and Bcl-xL through its inhibition of the BCR [94]. This corresponds with activation of the mitochondrial pathway of apoptosis. Clemastine and other lysosomotropic drugs have also been shown to damage mitochondria through induction of ROS [119]. Furthermore, TKI's can enhance lipid peroxidation through ROS when combined with lysosomotropic agents [157]. Therefore, a combination of these drugs may act through similar mechanisms to their individual agents.

To assess this, I treated BJAB cells with a combination of clemastine and ibrutinib and measured ROS and TMRM via flow cytometry. I found that clemastine was a major generator of ROS at 4h post-treatment, producing 20% DHE positive cells. Addition of ibrutinib could enhance the production of ROS to 63% DHE positive cells (Figure 4.13 a.). This corresponded to a synergistic reduction in TMRM-positive cells indicating that the drug combination enhances reduction in MMP to approximately 50% (Figure 4.13 b.). To determine the time point at which this mitochondrial dysfunction occurs, I stained BJAB cells at various intervals post-treatment over a 24h time course. The greatest degree of difference between controls and combination treatment occurred at 24h post-treatment, indicating that mitochondrial dysfunction is a late event after drug treatment (Figure 4.13 c.). Together, these results may suggest that induction of ROS could precede mitochondrial dysfunction induced by a combination of clemastine and ibrutinib. However, experiments may have to be performed at identical time points to determine a causative relationship between them.

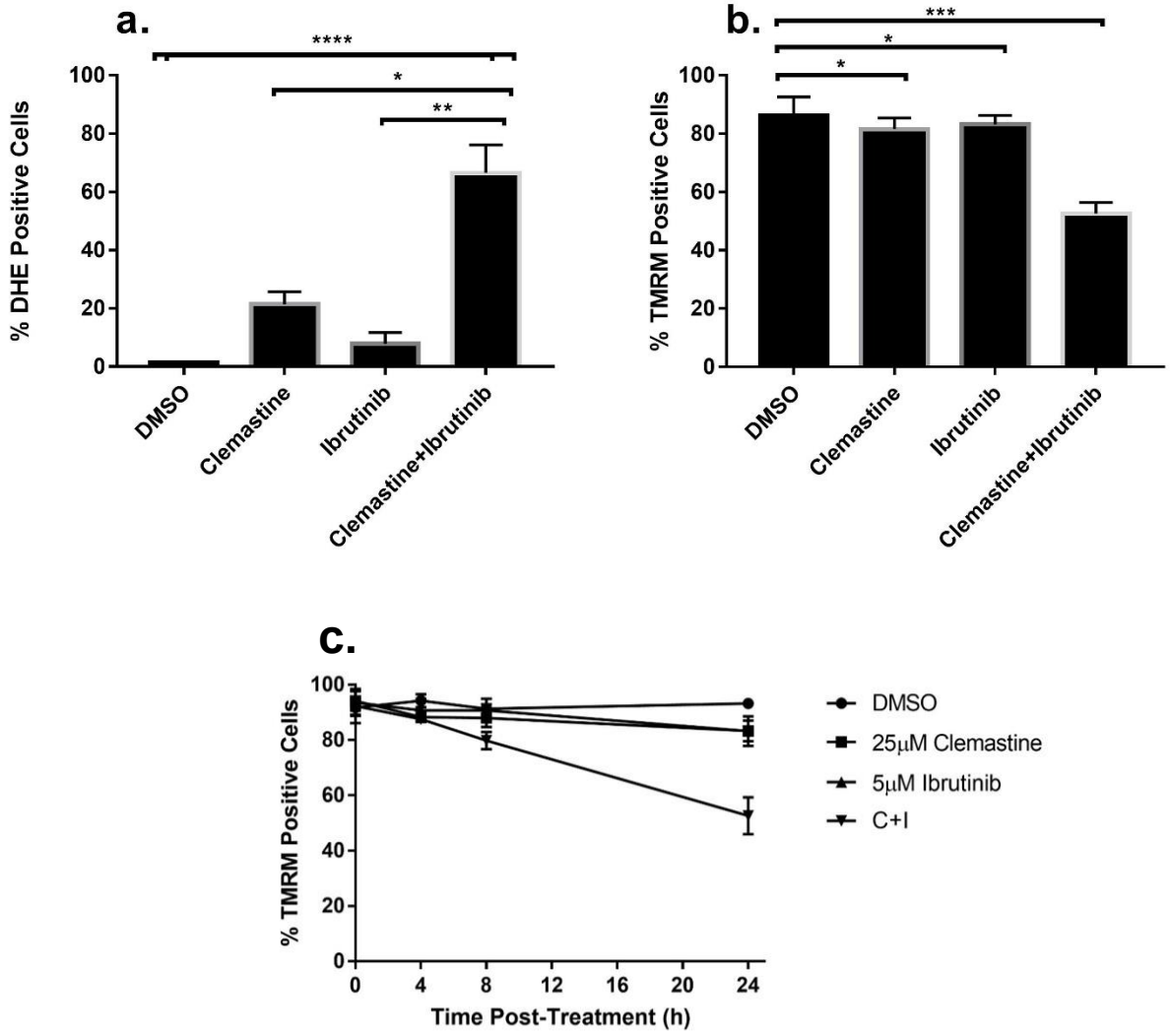


Figure 4.13: The combination of clemastine and ibrutinib increases total ROS and decreases MMP in BJAB cells. BJAB cells treated with a combination of clemastine and ibrutinib were stained with (a.) DHE after 4h treatment. (b.) TMRM after 24h treatment or (c.) TMRM at 0h, 4h, 8h or 24h post-treatment. Events were detected via flow cytometry. Error bars represent mean and standard error. Statistics represent degree of significant difference between combination and controls. N=3. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, $p<0.05$

4.2.6: The combination of clemastine and ibrutinib decreases expression of Mcl-1 but not other antiapoptotic Bcl-2 family proteins in BJAB and CLL cells

The mitochondrial dysfunction induced by a combination of clemastine and ibrutinib is indicative of engagement of the intrinsic apoptosis pathway. One of the hallmarks of intrinsic apoptosis induced by drugs is the inhibition and degradation of anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. Therapeutics inducing cell death in CLL cells generally reduce expression of one or more, but not all, of these proteins [35]. Thus, I wanted to determine if any Bcl-2 family anti-apoptotic proteins were targeted by a combination of clemastine and ibrutinib.

BJAB cells were treated with a combination of 25μM clemastine and 5μM ibrutinib for 24h and expression of Bcl-2, Bcl-xL and Mcl-1 were measured by western blot. Additionally, viability of each sample was measured via Trypan Blue exclusion at 24h post-treatment to ensure synergistic cell death occurred. Results indicate that Bcl-2 and Bcl-xL expression remained unchanged in BJAB cells. In contrast, Mcl-1 levels decreased significantly in combination compared to single-agent or vehicle controls. This indicates that degradation of Mcl-1, but not Bcl-2 or Bcl-xL, is a component of the combination-induced cell death pathway. To determine if the same was true in CLL, primary CLL cells were treated with 6.25μM clemastine and 6.25μM ibrutinib for 24h and expression of Mcl-1 was measured by western blot. A similar decrease in Mcl-1 expression was seen when data was compared to results in BJAB cells. These results show that degradation of Mcl-1 may play a role in the mitochondrial dysfunction induced by a combination of clemastine and ibrutinib in CLL cells. However, data in primary CLL cells is limited and additional samples must be analyzed before a direct conclusion about the role of Mcl-1 in this cell death can be drawn.

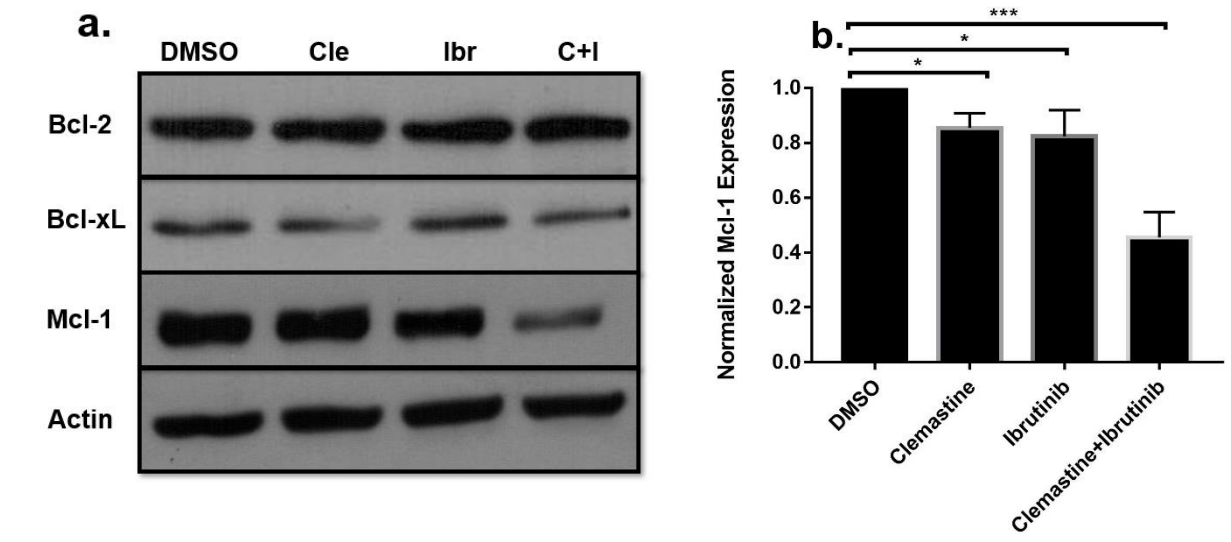


Figure 4.14: The combination of clemastine and ibrutinib decreases expression of Mcl-1 but not Bcl-2 or Bcl-xL BJAB cells. (a.) Representative western blots of BJAB cells treated with a combination of 25μM clemastine and 5μM ibrutinib for 24h prior to lysis. Expression of Bcl-2, Bcl-xL or Mcl-1 is shown. **(b.)** Levels of Mcl-1 in relation to loading control were quantified via densitometry. Values represent relative expression of Mcl-1 in relation to actin, normalized to DMSO control. N=3. Error bars represent mean and standard error. *** $p<0.01$, * represents statistical significance $p<0.05$.

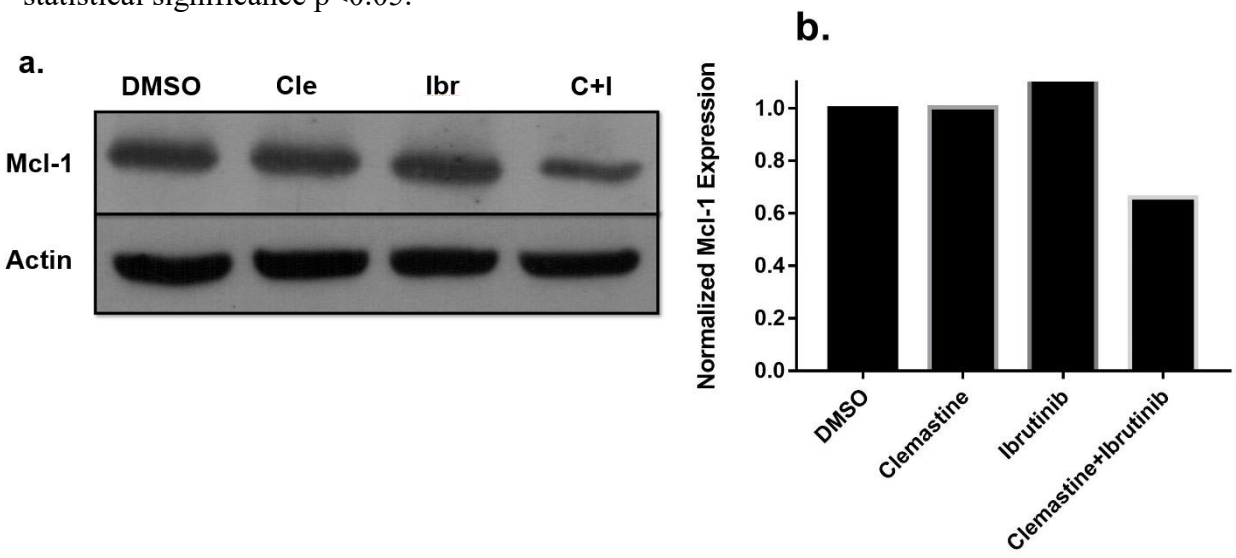


Figure 4.15: The combination of clemastine and ibrutinib decreases expression of Mcl-1 p in CLL cells. (a.) Western blots of CLL cells treated with a combination of 6.25μM clemastine and 6.25μM ibrutinib for 24h prior to lysis. Expression of Mcl-1 is shown. **(b.)** Levels of Mcl-1 in relation to loading control were quantified via densitometry. Values represent relative expression of Mcl-1 in relation to actin, normalized to DMSO control. N=1

4.2.7: Microenvironmental factors protect CLL cells from the combination of clemastine and ibrutinib

Microenvironmental factors such as anti-IgM stimulation, cytokines and interactions with BMSC's can protect CLL cells from apoptosis when cultured with CLL cells *in vitro*. These represent a barrier to clinical effectiveness of therapies and may mirror effects of these signals on drug response *in vivo* [180]. To determine if this was true with the combination of clemastine and ibrutinib, I stimulated CLL cells with CD40/IL-4 or cocultured them at a 100:1 ratio with HS-5 stromal cells. Stromal cells were seeded in RPMI-1640 media with 10% FBS for 24h prior to this experiment. CD40/IL-40 stimulation was chosen because studies have demonstrated that it is one of the strongest rescuers of CLL cell viability *in vitro* [180]. The HS-5 stromal cell line does not produce IL-4 and thus may provide data independent of results seen with CD40/IL-4 stimulation. [181]

Results indicate that stimulation with CD40/IL-4 reduced cell death induced by clemastine and ibrutinib 3-fold. (Figure 4.16 a.) Despite this, the combination of drugs was still able to induce cell death in CLL cells. Similarly, co-culture with HS-5 stromal cells was able to partially rescue CLL cells from combination-induced death, albeit with reduced effectiveness compared to CD40/IL-4 (Figure 4.16 b.). Interestingly, the combination produced only 24% increased cell death in the co-culture experiment compared to the DMSO control, most likely due to the presence of FBS required to support HS-5 growth. Together, these results provide preliminary evidence that doses of clemastine and ibrutinib required to directly induce cell death *in vivo* will be higher than concentrations used in this study.

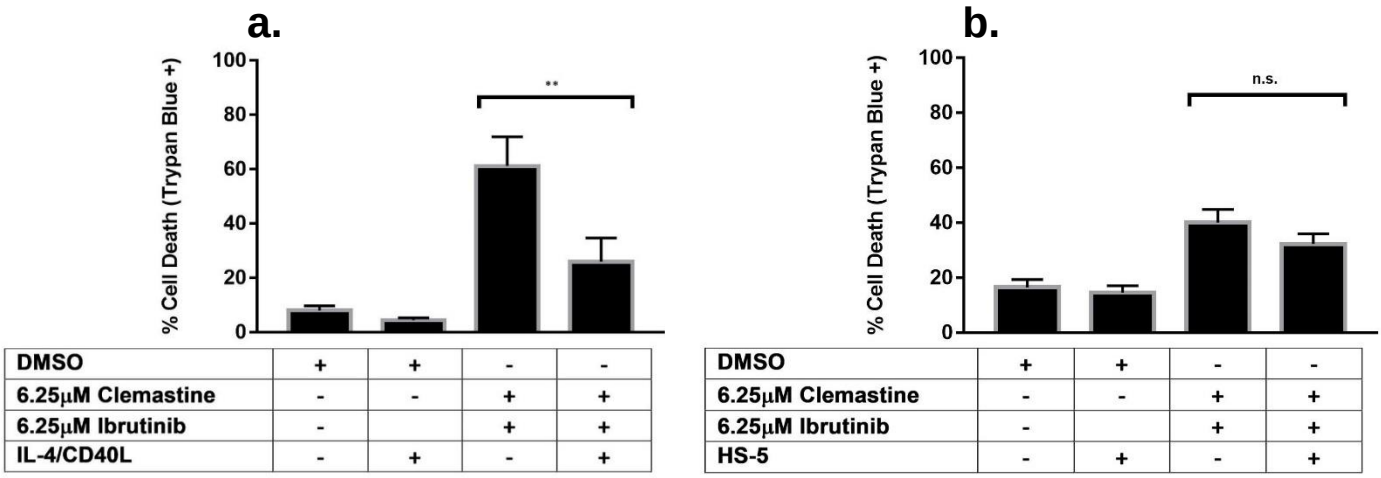


Figure 4.16: Microenvironmental factors protect CLL cells from clemastine and ibrutinib-induced cell death. (a.) CLL cells were stimulated with 50ng/mL IL-4 and 50ng/mL CD40L for 1h and treated with 6.25μM clemastine and 6.25μM ibrutinib or a DMSO control for 24h. (N=4) (b.) CLL cells were cultured with HS-5 stromal cells at a 100:1 ratio CLL:HS-5 for 1h, then treated with 6.25μM clemastine and 6.25μM ibrutinib for 24h (N=2). Cells were stained with Trypan Blue and analyzed via flow cytometry. Bars represent mean and standard error. ** $p<0.01$.

4.2.8: Other molecular targets aside from BTK may be responsible for the synergy between antihistamines and ibrutinib

Drugs including TKI's can often target other signalling pathways, despite being more specific for their primary target. A more specific inhibitor of BTK, acalabrutinib, was recently approved for clinical use in CLL. Therefore, I combined it with clemastine at concentrations inducing less than 20% individual cell death to determine if the drugs could induce synergistic cell death in combination. I found that at the specific dose of 10μM acalabrutinib chosen, in contrast to ibrutinib, did not induce synergistic cell death.

In the case of ibrutinib, the drug can target molecules aside from BTK including ITK in T cells and B lymphocyte kinase (BLK) in B cells. Bosutinib and Dasatinib are pan-SRC kinase inhibitors that include c-Abl as a primary target. Masitinib is a pan-SRC kinase inhibitor that targets BLK. I combined each of these agents with clemastine in the BJAB cell line to determine if these combinations could induce synergistic cell death. Results indicated that combinations of clemastine with both inhibitors of c-Abl were synergistic at selected doses, but masitinib demonstrated additivity. Therefore, combinations of clemastine and other TKI's may provide preliminary evidence that other targets aside from BTK may contribute to the synergy between these agents.

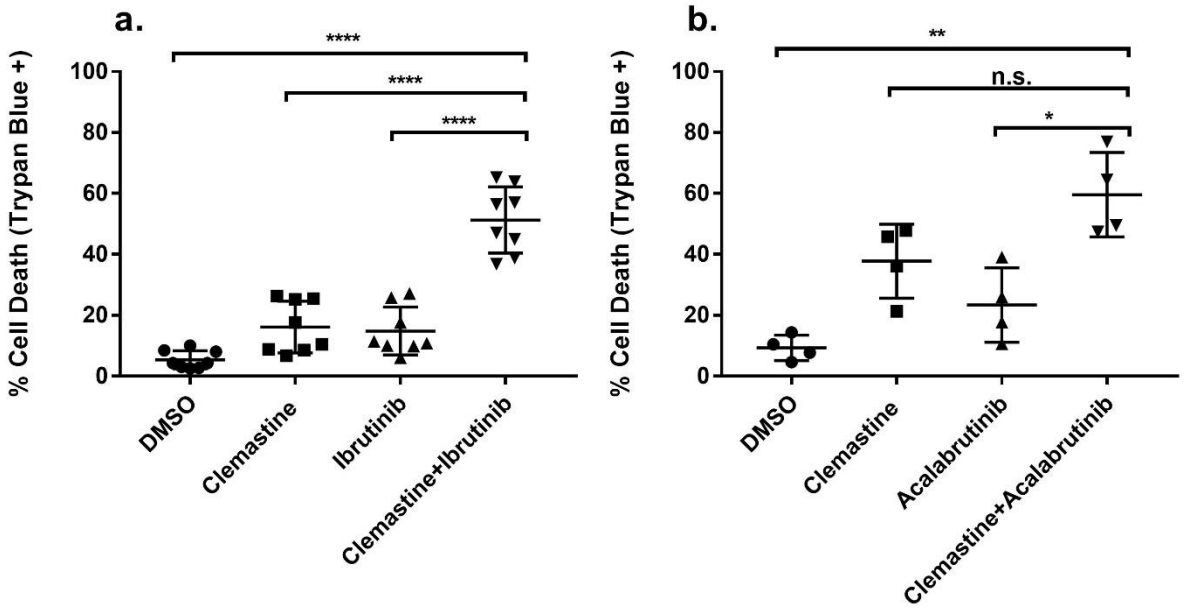


Figure 4.17: Clemastine does not synergistically increase total cell death when combined with acalabrutinib in CLL cells. 6.25μM clemastine was combined with (a.) 6.25μM ibrutinib, **(b.)** 10μM acalabrutinib, for 24h in CLL cells and cell death was measured with Trypan Blue. Bars represent mean and standard error. Statistics represent degree of significant difference between combination and single-agent controls. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$

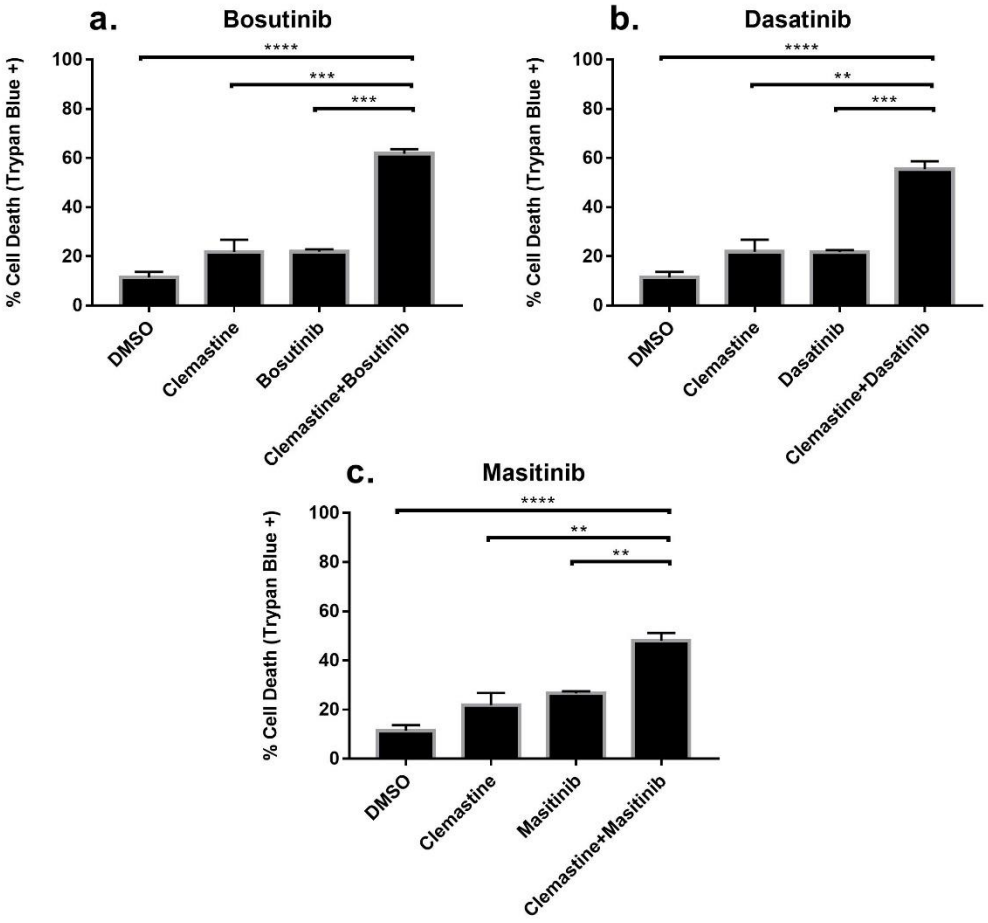


Figure 4.18: Treatment of BJAB cells with combinations of clemastine and inhibitors of c-Abl or BLK produced significantly increased cell death. 25µM clemastine was combined with (a.) 5µM bosutinib, (b.) 20µM dasatinib, or (c.) 20µM masitinib for 24h and cell death was measured with Trypan Blue. CI values for combinations of the three kinase inhibitors with clemastine were as follows: bosutinib=0.86; dasatinib=0.88; masitinib=1.03. Bars represent mean and standard error. Statistics represent degree of significant difference between combination and single-agent controls. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, * $p<0.05$

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

Since the discovery of the lysosomal cell death pathway and its alterations in cancer cells, it has been explored as a promising target for novel cancer therapies. CLL cells in particular have been demonstrated to be highly susceptible to death through this pathway, due to alterations in sphingosine content and processing. Many classes of drugs including antidepressants and antimalarials have been successfully repurposed to induce LMP in CLL cells. These drugs showed no significant reduction of cytotoxic activity in 17p or TP53 deleted patients, indicating that lysosome-targeting drugs may show promise in chemotherapy-resistant CLL .[119, 120] Siramesine is highly potent and is the best-studied lysosomotropic agent, however it lacked effectiveness as an antidepressant and was denied approval by the FDA. While the antimalarial drugs mefloquine and tafenoquine are FDA-approved, these drugs can induce serious toxicities such as long-term neuropsychiatric damage and hemolysis [182]. Such toxicities may be undesirable in elderly CLL patients, and therefore alternatives for these drugs are needed. H1 antihistamines may be one answer to the search for better-tolerated and FDA-approved lysosomotropic drugs.

H1 antihistamines are the active ingredients found in many over-the-counter allergy medications. Collectively, these drugs act as inverse agonists of the H1 histamine receptor, which is expressed in many cells of the body including smooth muscle cells, epithelial cells and lymphocytes. Their competitive activity in blocking histaminergic responses ameliorates symptoms of allergic rhinitis and normalizes immune responses [183]. Despite similar levels of clinical efficacy, these drugs have different molecular structures, metabolic routes, and side

effects [184]. Clemastine (brand name Tavist) is a first-generation antihistamine with the ability to cross the blood-brain barrier and can act as a sedative. While this sedative property is considered an adverse effect, it proved beneficial in the glioblastoma study by allowing the drug to cross the blood-brain barrier [153]. Loratadine (brand-name Claritin) and its metabolite desloratadine (brand name Aerius) are non-sedative second-generation antihistamines. Loratadine and desloratadine are tricyclic antihistamines possessing molecular structures similar to the lysosomotropic antidepressants desipramine and nortriptyline [185]. These three antihistamines are approved for use by both Health Canada and the FDA. As a group, H1 antihistamines are well-tolerated with very low rates of severe toxicities when taken at standard doses [184]. Because of this, they have recently been revisited for their promising lysosomotropic anti-cancer activity.

The malignant B cell lines BJAB and I83 were chosen as models to study many of the antihistamine-induced cell death mechanisms presented in this thesis. Results in I83 cells may be more representative of true CLL compared to BJAB cells, due to the age, sex, and disease of the cell of origin. Both cell lines were previously shown to be susceptible to LMP through a combination of fludarabine and valproic acid [142]. BJAB and I83 are commonly used as models for BCR signalling studies, with constitutively active phosphorylation of BCR-associated kinases independent of stimulation. This constitutive activation of cells and proliferation may better mimic responses to drugs seen *in vivo*. These cell lines also express Bcl-2 family proteins chosen for investigation in this study [142]. However, primary CLL cells remain as the gold standard model for CLL *in vitro* and were used to supplement cell line data based on sample availability. Cell line data followed similar trends to results seen in primary CLL cells.

In the present study, I tested an increasing range of concentrations of H1 antihistamines to determine if they could kill the BJAB and I83 cell lines, as well as CLL cells. We found that clemastine, desloratadine, and loratadine were all cytotoxic at micromolar concentrations, and that these drugs could kill these models in a dose-dependent manner. Concentrations inducing cytotoxicity in the BJAB and I83 cell lines were consistent with doses of 20-120μM used in solid tumor cell lines in previous studies [152, 175]. CLL cells showed greater sensitivity than cell lines to antihistamine-induced death, and this may be in part due to lack of proliferative activity, and the lack of serum in culture media of primary cells. CLL cells were also demonstrated to be more sensitive to antihistamine-induced cell death than non-malignant PBMC's from healthy donors. This finding is in agreeance with previous data in CLL cells and normal B cells using similar concentrations of the lysosomotropic agents siramesine, tafenoquine and mefloquine [119, 120]. No trend between response to clemastine treatment and patient prognostic markers was seen in this study; all CLL cells treated with same dose of clemastine experienced between 40% and 75% cell death.

In a study by Kornhuber et. al. in 2011, the H1 antihistamines clemastine, desloratadine and loratadine were found to inhibit the enzyme acid sphingomyelinase (ASM). CAD compounds such as these H1 antihistamines are theorized to solubilize and detach ASM and other enzymes involved in sphingolipid metabolism from the lysosomal membrane. These enzymes are then degraded within the lysosomal compartment. While CLL cells do not display altered activity of ASM or sensitivity to ceramide, their S1PP activity is increased resulting in an increase in membrane sphingosine content [119]. The present study has not demonstrated a precise mechanism by which clemastine inhibits sphingosine metabolism, but hallmarks of LMP are present such as loss of lysotracker staining and increased cell death when SK is inhibited.

Another characteristic of drugs classified as ASM inhibitors is their synergy with TKI's. CAD antimalarial drugs, in contrast to antihistamines and antidepressants, do not act to inhibit ASM. This has been demonstrated in our solid tumor models, and in CLL. In CLL cells, antimalarial drugs interacted antagonistically with ibrutinib, while antihistamines could induce synergistic cell death in combination with the drug [120]. Combinations of ibrutinib with other classes of ASM inhibitors such as antidepressants and antiepileptics were not explored in our B cell models.

Previous research has shown that mitochondrial dysfunction can be a component of the lysosomal cell death pathway. This loss of MMP can be induced by degradation of mitochondrial antiapoptotic proteins by cathepsin proteases, or by direct oxidation of the mitochondrial membrane by lipid ROS. While some lysosome targeting drugs such as siramesine and tafenoquine act through lipid peroxidation, others such as mefloquine can kill cells without this event occurring [118, 120, 147, 188]. Our research shows that clemastine-induced cell death was partially blocked by inhibitors of lipid peroxidation and ROS accumulation. This is in contrast to inhibitors of cathepsins B or D, which had no protective effect on CLL cells. E64 and chymostatin are less-specific inhibitors of cathepsins B and D, respectively, and can inhibit a broader range of lysosomal cysteine proteases including cathepsins K and L [179]. This supports our conclusion that H1 antihistamines kill CLL cells through LMP and a subsequent oxidative mitochondrial mechanism independent of cathepsin activation. Since CLL cells do not produce tocopherols, this may explain their susceptibility lipid peroxidation or other mechanisms of oxidative damage [189]. Moreover, mitochondria in CLL cells have higher levels of oxidative phosphorylation and contribute to ROS generation and oxidative stress when disrupted. One important endogenous antioxidant pathway CLL cells used

to reduce oxidative stress from mitochondria is expression of heme-oxygenase-1 (HO-1) [43]. I would encourage future research to investigate the potential roles of this molecule in resistance to clemastine-induced cell death.

In solid tumor models, synergistic combinations of lysosomotropic agents and TKI's were found to induce cell death through ferroptosis. Common effectors in the ferroptotic cell death pathway include labile iron release from the lysosome, and subsequent mitochondrial lipid peroxidation. Ferroptosis holds promise in cancer cells as a method to kill apoptosis-resistant cells as it occurs through a distinct pathway [190]. The combination of clemastine and ibrutinib was found to kill CLL cells despite inhibition of ferroptosis or chelation of reactive iron. However, lipid ROS scavengers were able to partially protect CLL cells from this combination. The combinatorial cell death also induced a synergistic loss of MMP following an increase in ROS. Therefore, I believe that this mitochondrial dysfunction may be occurring through lipid peroxidation in tandem with another cell death mechanism. This mechanism has been supported by other research, which has identified ibrutinib as an inhibitor of cellular GSH, targeting antioxidant pathways in CLL cells [191]. Additionally, combinations of lysosome targeting drugs and TKI's in breast cancer models were found to kill cells through autophagy in independently of ferroptosis [156]. One of the characteristics of autophagy is the dysfunction and degradation of mitochondria, which occurred in our B cell models. Thus, the role of autophagy in the combination of clemastine and ibrutinib may be worth investigation in CLL cells.

The finding that this drug combination acts through degradation of Mcl-1 but not other antiapoptotic Bcl-2 family members is a subject of importance. As the large splice variant of Mcl-1 functions to sequester proapoptotic factors within the mitochondria, the loss of Mcl-1

provides a preliminary mechanism through which MMP is lost [35]. Other factors, such as the phosphorylation of Akt and ERK1/2 regulate expression of Mcl-1 [75]. Our data shows that these kinases are dephosphorylated by the combination of clemastine and ibrutinib, and these proteins may be responsible in part for the decrease in Mcl-1. The role of Mcl-1 in response to this drug combination also has clinical implications, with variability in expression between patients. Increased Mcl-1 expression has been correlated with a poor prognosis in CLL, with events such as chemotherapy resistance and short progression-free survival being associated with higher levels of the protein [47, 48]. Therefore, agents which can reduce Mcl-1 expression and kill cells via this pathway are required. As the combination of clemastine with the Bcl-2 inhibitor venetoclax also increased total cell death, together these agents may another useful combination to simultaneously target multiple mechanisms of survival and escape in CLL cells.

Stimulation with peptides found in the CLL microenvironment can have varied effects on CLL cells, due to the highly regulated and complex nature of cell signalling. Treatment with Il-4 and CD40L can activate transcription factor pathways such as STAT6 and NF-κB, while also upregulating expression and signalling of cell surface IgM [62, 180]. Furthermore, these peptides have demonstrated the ability to increase activity of DNA repair pathways in CLL such as ATM [192]. These alterations can help protect CLL cells from spontaneous or drug-induced apoptosis. In the present study, stimulation with these factors antagonized cell death induced by antihistamines and their combination with ibrutinib. This represents a potential barrier to application of these drugs *in vivo*, but it also may provide hints toward alternative mechanisms of cellular damage induced by these drugs. The role of the drugs used in this project on DNA transcriptional activity and repair was not explored in this study, but many of these factors relate to apoptotic effectors discovered within this work. For example, ROS and oxidative stress can

induce single-stranded and double-stranded DNA breaks, can activate DNA repair pathways. It is unlikely that previous lysosomotropic agents induced DNA damage as a major component of the pathway, however, as these agents were still effective in p53-deficient patient cells.

Though the apoptotic effectors involved in the combinatorial cell death pathway have been identified, the specific mechanism of the synergy between clemastine and ibrutinib has yet to be uncovered. While antagonism often occurs between drugs targeting the same mechanism, drugs targeting different but overlapping mechanisms can have synergistic interactions [193]. From results of previous research, I believe sphingosine metabolism inhibition and the generation of ROS are the mechanisms of clemastine treatment responsible for the synergy. However, the target(s) of ibrutinib responsible for this synergy have not yet been identified. Ibrutinib was used at a concentration of 5-6.25μM for this study to induce direct cytotoxicity. This is higher than the maximal plasma concentrations of 0.2μM seen in patients receiving standard ibrutinib therapy [143]. In addition, while ibrutinib reduced phosphorylation of kinases downstream of BTK including Akt and ERK1/2, this reduction was not synergistic when combined with clemastine. Therefore, ibrutinib may be inhibiting one or more alternative targets in addition to BTK. While some alternative kinase targets of ibrutinib such as ITK are not expressed in CLL cells, others such as B lymphocyte kinase (BLK) and c-Abl display relevance to CLL cell viability[194]. c-Abl kinase in particular has shown importance in regulating Mcl-1 expression, which was synergistically degraded by the drug combination used in this study [195]. There may also be the possibility that ibrutinib is targeting an antioxidant or transcriptional pathway. To fully explore this mechanism of synergy, additional studies of the effects of clemastine and ibrutinib on CLL cell signalling networks are necessary.

Drug repurposing, also known as repositioning, in the field of oncology remains an encouraging prospect. This strategy is the most expeditious way to implement drugs into a new therapeutic role. It can take upwards of 10 years for a drug to be developed and assessed *de novo*, while drugs can be successfully repurposed in a minimum of 3 years. There are additional benefits to repurposing drugs other than efficiency. FDA approved drugs have already been examined for safety and their pharmacological data and dosing information have been characterized [196]. Furthermore, repurposing readily available drugs such as over-the-counter medications can be economically beneficial to patients requiring expensive cancer therapies and to the systems providing these therapies.

The question remains as to whether concentrations of H1 antihistamines used to kill cells in this study are clinically achievable. Many factors such as plasma protein binding and specificity for the H1 receptor can reduce bioavailability of these drugs. Plasma concentrations of antihistamines *in vivo* are not readily available in the literature, and peak H1 receptor occupancy occurs within hours at nanomolar concentrations[184]. These drugs have high affinity for plasma proteins due to their hydrophobic nature, with up to 85% binding [184]. Standard doses of clemastine include 1mg, 2mg, and 4mg tablets. Despite this, a 50mg/kg daily dosing schedule of clemastine in the brain cancer study was used to kill glioblastoma cells and significantly improve overall survival in mice [153]. Doses of this magnitude may induce adverse affects *in vivo* which are not seen in pharmacological characterization of the drugs. We also may need to consider the implications of antagonizing histaminergic responses in patients: will this lead to immune suppression and increased infections? Lysosomal drug delivery systems such as gelatin-conjugated drugs may be one way to make lysosome-targeting more specific and

minimize adverse effects at high doses [197]. These doses must be determined using an animal model.

Taken together, the present study has identified repurposing H1 antihistamines as a potential therapeutic strategy in CLL and other B cell cancers. Antihistamines are often given pre-emptively in CLL to prevent adverse immune reactions to rituximab and obinuzutumab infusion [198]. Aside from this, the biological relevance of current antihistamine use to the outcomes of CLL patients is unknown. In a retrospective epidemiological study, a statistically significant correlation between allergies in CLL patients and an increased time to first treatment, or better prognosis was demonstrated. This correlation was theorized to be due to the presence of increased IgE antibodies and improved immune function [199]. Our data implies that antihistamine use may be an additional metric to track in clinic, especially pertaining to those requiring ibrutinib treatment, and may correlate to improved patient outcomes. Despite this correlation, there is currently limited data on the over-the-counter antihistamine use of CLL patients.

There may be broader implications to the results of this study. The majority of patients receiving ibrutinib therapy have high risk disease. These patients often must continue to receive the therapy for the rest of their lives, as the drug does not completely eradicate CLL cells. As previously discussed, ibrutinib induces peripheral blood lymphocytosis by flushing CLL cells outside of their protective niches [93]. These cells then die slowly over time due to lack of interaction with lymphoid tissues, with more directly cytotoxic agents such as venetoclax being successfully combined with ibrutinib to kill the cells. Our data may suggest that H1 antihistamines, due to their selectivity to CLL cells and synergy with ibrutinib, may be able to fill this niche. These drugs may enhance the efficacy and reduce the duration of ibrutinib

treatment to improve patient care. Therefore, a potential therapeutic niche for the combination of antihistamines and ibrutinib could be chemotherapy-resistant patients requiring other treatment options. To explore this potential, additional pre-clinical studies are needed.

5.2 CONCLUSIONS

The first aim of this project was to determine if H1 antihistamines could kill malignant B cell lines and CLL cells and identify the mechanism of this cell death. The results in this thesis indicate that CLL cells are selectively sensitive to cell death induced by the H1 antihistamines clemastine, desloratadine and loratadine. This cell death was determined to be mediated by the lysosome through reduction of acidophilic stains, and was enhanced by the modulator of sphingosine metabolism SKI II. Clemastine was the most cytotoxic of these agents at the lowest concentrations. The drug could kill CLL cells despite cathepsin inhibition, but this cell death was partially blocked by antioxidants.

The second aim of this project was to determine if antihistamines could induce synergistic cell death when combined with drugs relevant to CLL and identify the mechanism of cell death caused by a synergistic combination. Our data shows that the antihistamine clemastine demonstrated increased cell death when combined with targeted therapies but not chemotherapy in the BJAB cell line. When combined with the TKI ibrutinib in B cell models, this cell death was found to be synergistic. The synergistic cell death was not blocked by inhibitors of ferroptosis, but was again partially reduced by antioxidants. In the BJAB cell line, this cell death was accompanied by a synergistic increase in ROS, decrease in MMP, and reduction in Mcl-1 expression, all of which are hallmarks of apoptosis. This cell death could target kinases associated with BTK and could still induce cell death when cells were stimulated with IL-4 and

CD40L. Together, these findings demonstrate that clemastine acts as a lysosomotropic agent and specifically synergizes with the TKI ibrutinib via an apoptotic mechanism in malignant B cell models (Figure 5.1).

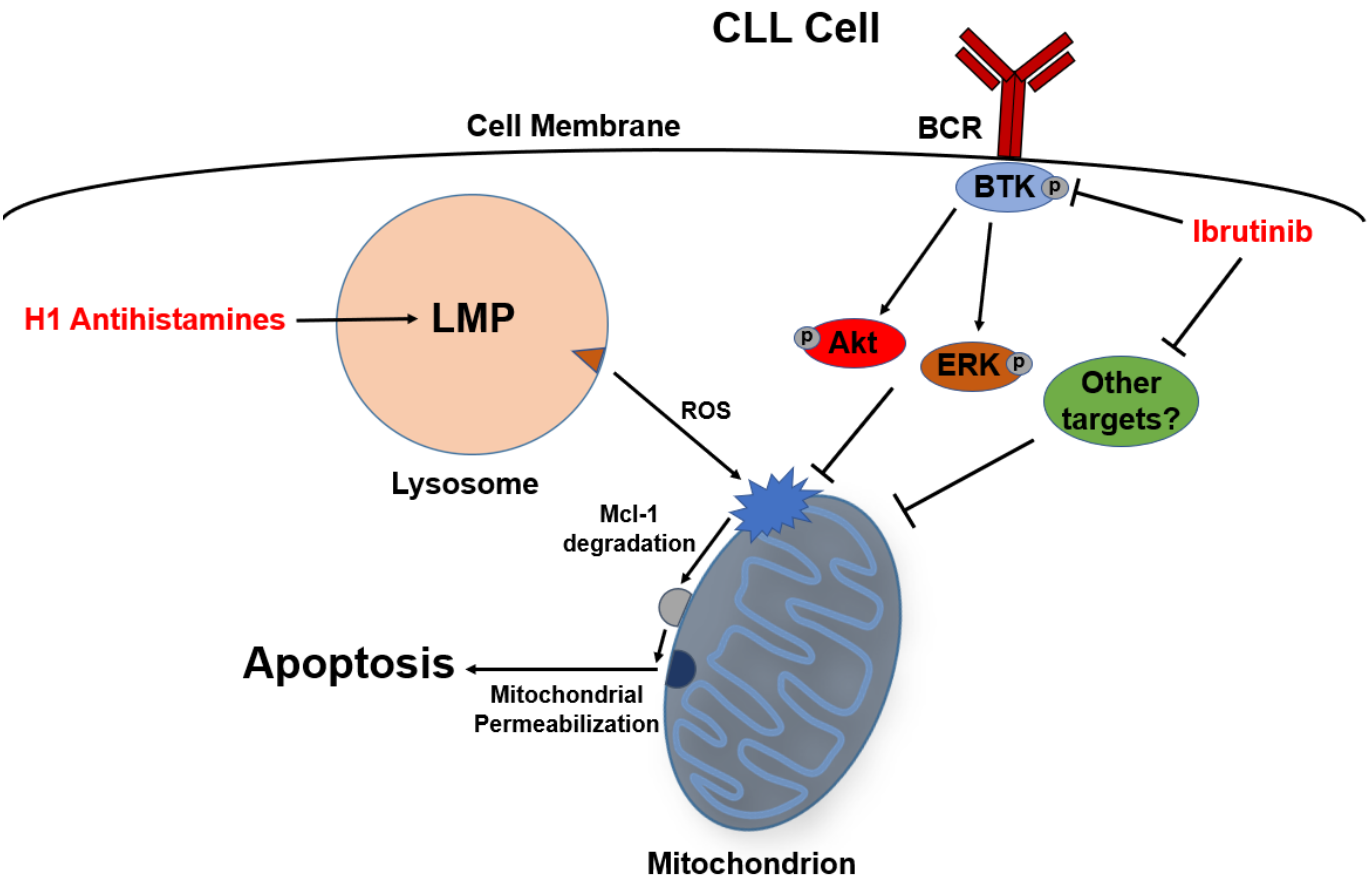


Figure 5.1: Summary of the preliminary cell death mechanism induced by a combination of clemastine and ibrutinib in malignant B cells. Targeting lysosomes with clemastine induces an increase in intracellular cellular ROS and mitochondrial dysfunction. Ibrutinib treatment reduces phosphorylation of kinases downstream of BTK such as Akt and ERK1/2. These two drugs together synergistically increase ROS levels and reduce Mcl-1 expression, leading to mitochondrial permeabilization and cell death through apoptosis.

CHAPTER 6: FUTURE DIRECTIONS

One of the primary goals of this study was to identify a clinically-relevant synergistic combination of drugs specifically targeting CLL cells, and to understand its preliminary mechanism. Therefore, experimental steps to continue investigation of the cytotoxicity of antihistamines as single agents and in combination with ibrutinib can be divided into two parts: further assessment of preclinical activity of these drugs and additional exploration of their inducible cell death mechanisms.

6.1 PRECLINICAL ASSESSMENT

Microenvironmental factors are a significant contributor to drug resistance in CLL and represent a barrier to clinical implementation of many drugs. Current models of the microenvironment using co-culture with stromal cells and stimulation with IgM ligation or cytokines are often done in 2-dimensional *in vitro* cell culture. 3-dimensional cell culture environments may be a more biologically-relevant model to simulate the protective effects of actual lymphoid tissues on the viability of CLL cells. This warrants the use of a 3-dimensional culture system such as the tissue roll for analysis of cellular environment and response (TRACER) system [200]. The system can be used to investigate the role of clemastine and ibrutinib on the interactions of CLL cells with stromal cells in an artificial lymph node model.

Animal studies are also crucial for understanding the efficacy and safety of compounds *in vivo*. TCL-1 transgenic mice are commonly used as a tool for preclinical drug development in CLL, with strains representing both indolent and aggressive disease [201]. Antihistamines are well-tolerated drugs when taken at FDA-approved doses, but concentrations of the drugs used in

this project may be higher than clinically-relevant plasma concentrations. Therefore, to determine if antihistamines are effective at selectively killing CLL cells *in vivo* at safe doses, I propose using dose-escalation in the TCL-1 mouse model and tracking lymphocyte counts from blood samples over time. To determine if toxicity is an issue, I would also use flow cytometry to measure counts of other blood cells including erythrocytes, thrombocytes and normal leukocytes. Additionally, I would monitor renal and liver function to assess health. Using the maximal dose displaying anti-cancer activity without adverse effects, I would then combine antihistamines with ibrutinib at a clinically-relevant dosing schedule to identify if this combination is synergistic in an animal model. However, I acknowledge that doses used to achieve an effect in mice may not be equivalent to those used in humans, so a clinical trial will eventually be necessary to directly translate results to a human model.

Finally, to examine if clinically relevant doses of ibrutinib sensitize CLL cells to antihistamine-induced death, I would use an *ex-vivo* human model. This would involve isolating cells from CLL patients prior to starting ibrutinib therapy and performing a dose-response cell death analysis using increasing doses of antihistamines. I would then request sequential samples from the same patients at intervals following initiation of treatment, again tracking the responses of their cells to antihistamines using dose-response curves over the course of ibrutinib therapy. EC50 concentrations would then be compared between pre-treatment samples and post-treatment samples. I hypothesize that CLL cells from patients receiving ibrutinib therapy would be more sensitive to antihistamine-induced cell death than they were prior to ibrutinib therapy.

6.2 MECHANISTIC STUDIES

Though the preliminary mechanism of the synergy between clemastine and ibrutinib was explored in this study, additional mechanisms of cell death may be occurring in tandem with apoptosis caused by LMP and Mcl-1 degradation.

Most notably, autophagy was not one of the mechanisms of cell death analyzed in response to this drug combination. There are several pieces of evidence suggesting that autophagy may be worth investigation. CAD compounds such as chloroquine inhibit autophagy in many cell types by raising lysosomal pH, and can also be lysosomotropic at higher concentrations [202]. Lysosomotropism has been shown to kill cells through an autophagic mechanism at later time points (24h) after drug treatment as it exhausts cellular resources. As clemastine, desloratadine, and loratadine also possess CAD structure and enter lysosomes, they may play a role in inhibiting autophagy to induce cell death. Moreover, the combination of siramesine and lapatinib induced cell death via autophagy in breast cancer cells at later time points. This mechanism was found to occur independently of ferroptosis [156]. Therefore, I would explore the role of autophagy in the cell death induced by a combination of clemastine and ibrutinib in CLL cells. This could be done by using the autophagy inhibitor 3-methyladenine (3-MA) in concert with this drug combination, and subsequently measuring cell death. I predict that 3-MA would partially protect cells from clemastine and ibrutinib induced cell death since these drugs may be inducing cell death through autophagy.

Additionally, many mononuclear blood cells including B lymphocytes express the H1 receptor at varying levels. There is evidence to suggest that H1 receptor signalling influences levels of lymphocyte activation and immune responses [203]. Therefore, treating CLL cells with an H1 receptor agonist such as H1 antihistamines may affect cell signalling associated with this

receptor. To further investigate this in the context of this project, I propose knocking down or overexpressing the H1 receptor in a malignant B cell line and determining if this influences sensitivity to antihistamine-induced death.

Lastly, an overarching pattern in this field of study is the synergy between lysosomotropic agents and TKI's. While a combination of the lysosomotropic agent clemastine and TKI ibrutinib induced synergistic cell death in BJAB cells, it did not synergistically inhibit kinases downstream of BTK. I believe that this synergy may be due to an off-target effect of ibrutinib, supported by experiments demonstrated in Chapter 4.2.8. Clemastine was combined with acalabrutinib, a more specific inhibitor of BTK, and did not exhibit a significant increase cell death in CLL cells. Furthermore, clemastine was combined in BJAB cells with inhibitors of off-target tyrosine kinases with the greatest degree of therapeutic index between ibrutinib and acalabrutinib. These included inhibitors of c-Abl (dasatinib and bosutinib) or BLK (masitinib). Only the inhibitors of c-Abl were synergistic with clemastine at selected concentrations in BJAB cells, indicating that c-Abl may play a role in this synergy. I suggest a kinome activity screen identifying kinase pathways synergistically dephosphorylated by this drug combination to help identify future targets. A complete understanding of this cell death pathway could identify novel targets in CLL for further drug repurposing to improve patient treatment and quality of life. However, it may also be relevant to study the downstream effectors of these pathways, as synergy also occurs at the level of ROS generation and mitochondrial effectors.

CHAPTER 7: REFERENCES

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APPENDIX A: CLINICAL CHARACTERISTICS OF CLL SAMPLES

Sex	RAI Stage	Zap 70	IGVH	FISH Status	Prior Treatment
M	RAI 0	Negative	Mu	13q14 del	Flu/R, Ibr
F	RAI 1	Negative	Mu	13q14 del	
M	RAI 1	Negative	Mu	13q14 del	Clb /Ob
M	Unk	Negative	Mu	Normal	Clb, Clb/Ob
M	RAI 0	Negative	Mu	Unk	
M	RAI 0	Negative	Mu	Unk	
F	RAI 0	Negative	U	Unk	
M	RAI 0	Unk	Mu	Unk	
M	RAI 1	Unk	Mu	13q14 del	Ibr
M	Unk	Unk	Unk	13q14 del, 17p13 del	Ibr
M	RAI 0	Negative	Mu	Unk	
F	RAI 0	Positive	Mu	Trisomy 12, 17p13 del	Clb, RCD, Ibr
M	RAI 0	Positive	U	Unk	
M	RAI 2	Positive	Unk	Normal	Ibr
M	Unk	Positive	Mu	13q14 del	
F	RAI 0	Positive	Unk	11q22 del, 13q14 del	Flu, Ibr, Ven
M	RAI 0	Negative	Mu	Normal	
M	RAI 0	Negative	Mu	Unk	
M	RAI 1	Positive	Unk	13q14 del	
M	RAI 0	Negative	Unk	Unk	
M	RAI 0	Negative	U	11q22 del, 13q14 del, 17p13 del	Ibr
F	RAI 1	Negative	Mu	13q14 del	FCR

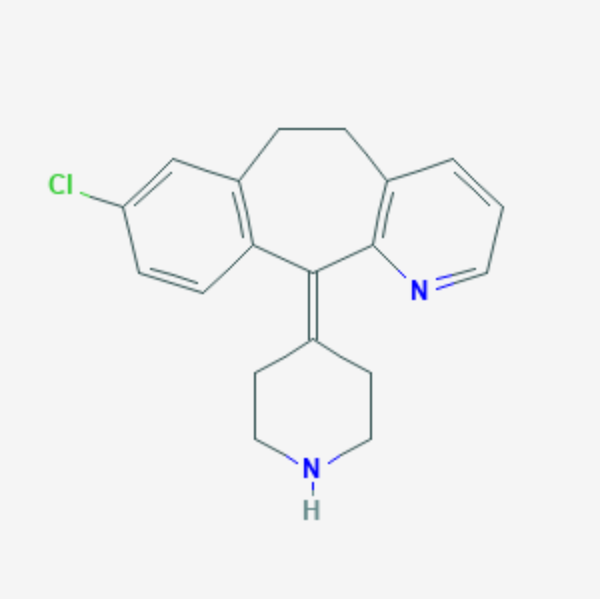
M=Male; F=Female

Mu=Mutated; U=Unmutated

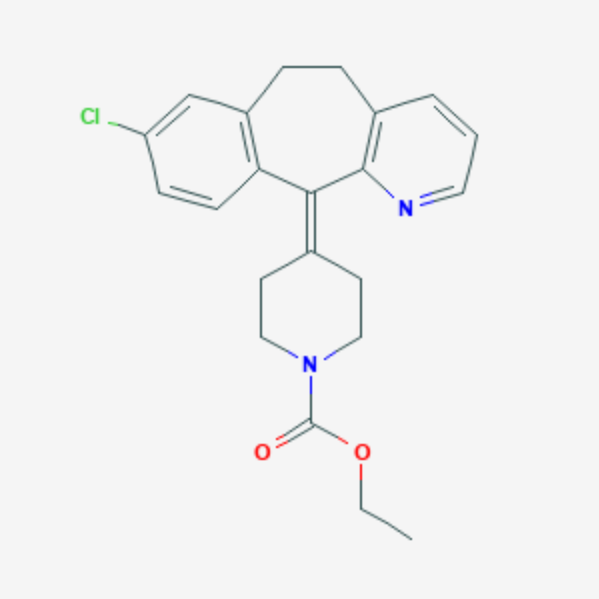
Flu=Fludarabine; R=Rituximab; Ibr=Ibrutinib; Clb=Chlorambucil; Ob=Obinutuzumab; C=Cyclophosphamide; D=Dexamethasone; Ven=Venetoclax

Unk=Unknown

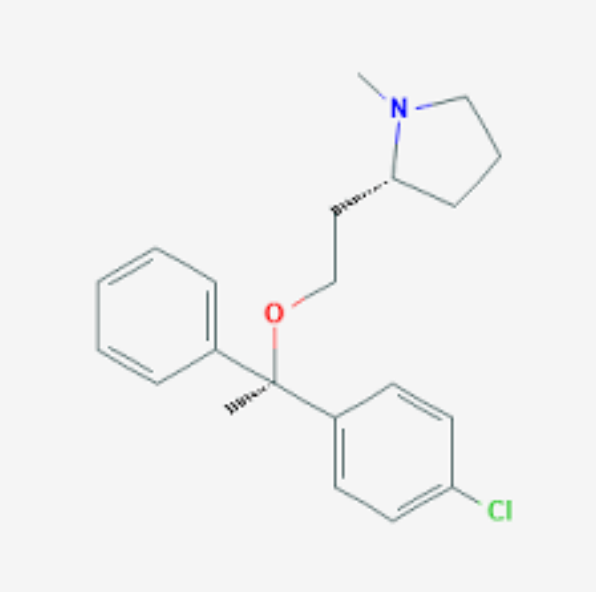
APPENDIX B: MOLECULAR STRUCTURES OF H1 ANTIHISTAMINES



Desloratadine



Loratadine



Clemastine

APPENDIX C: ADDITIONAL KINASE INHIBITORS

Drug Name	Drug Class	Concentration Used In Combination	Solvent/Storage Temperature	Provider
Acalabrutinib	BTK Inhibitor	10µM (CLL cells)	DMSO/-80°c	Selleckchem
Bosutinib	SRC/c-Abl Inhibitor	5µM (Cell Lines)	DMSO/-80°c	Sigma
Dasatinib	c-Abl Inhibitor	20µM (Cell Lines)	DMSO/-80°c	Sigma
Masitinib	BLK Inhibitor	20µM (Cell Lines)	DMSO/-80°c	Sigma

Table 8.1: Additional Kinase Inhibitors Used in this Study

APPENDIX D: LIST OF SOLUTIONS

Cell Culture

Full Media (+5% FBS/1%Pen/Strep)

RPMI-1640 Media	500mL
FBS	25mL
Pen/Strep	5mL
Total Volume	**530mL**

Freezing Media (+10% FBS/10% DMSO)

RPMI-1640 Media	10mL
FBS	1mL
DMSO	1mL
Total Volume	**12mL**

PBS (pH 7.4)

KCl	6.0g
NaCl	240.0g
KH_2PO_4	7.2g
Na_2HPO_4	80.4g
1M HCl	Adjust pH to 7.4
ddH_20	2.4L, Adjust volume to 3.0L
Total Volume	**3.0L**

Flow Cytometry

Lysotracker Staining Solution

50µM Lysotracker Stock Solution	1µL
RPMI-1640 Media	1000µL
Final Concentration	**50nM**

AO Staining Solution

5mg/mL AO Stock Solution	1µL
PBS	1000µL
Final Concentration	**5µg/mL**

DHE Staining Solution

1.6mM DHE Stock Solution	2µL
PBS	1000µL
Final Concentration	**3.2µM**

TMRM Staining Solution

1mM TMRM Stock Solution	1µL
PBS	39µL
25µM TMRM Dilution	1µL
PBS	1000µL
Final Concentration	**25nM**

Confocal Microscopy

Cell-Tak Binding Solution

100mM $NaHCO_3$ (Sterile)	962.6µL
1M NaOH	18.7µL
1.2mg/mL Cell-Tak Stock Solution	18.7µL
Total Volume	**1.0mL**

Western Blotting

RIPA Lysis Buffer Stock (1% NP-40)

1M Tris-Cl pH 7.4	5mL
5M NaCl	30mL
20% NP-40	5mL
10% Sodium Deoxycholate	5mL
20% SDS	500µL
ddH_2O	55mL
Total Volume	**100mL**

6x Laemmli Buffer Stock

Sodium Dodecyl Sulfate	1.2g
Bromophenol Blue	6.0mg
Glycerol	4.7mL
0.5M Tris-Cl pH 6.8	1.2mL
ddH_2O	2.1mL
DTT	0.93g
Total Volume (Stock Solution)	**10mL**
β-Mercaptoethanol (BME)	12.5µL
6x Laemmli Buffer Stock	100µL
Total Volume (BME added fresh 1:8 v/v)	**112.5µL**

Running Buffer

Tris Base	3.03g
Glycine	14.1g
Sodium Dodecyl Sulfate	1.0g
ddH_2O	800mL, adjust volume to 1.0L
Total Volume	**1.0L**

Transfer Buffer

5x Trans-Blot Turbo Buffer Stock Solution	200mL
100% Ethanol	200mL
ddH_2O	600mL
Total Volume	**1.0L**

TBS (pH 7.4)

Tris Base	6.05g
NaCl	8.76g
ddH_2O	800mL, adjust volume to 1.0L
1M HCl	Adjust pH to 7.4
Total Volume	**1.0L**

TBS-T (0.1% Tween-20)

TBS	1.0L
Tween-20	1mL
Total Volume	**1.0L**

5% Nonfat Milk

TBS-T	100mL
Nonfat Milk	5.0g
Total Volume	**100mL**