Elderly patients with Chronic Lymphocytic Leukaemia (CLL): Predicting their Survival and Managing their Disease with Valproic Acid and Fludarabine

by

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ABSTRACT

Chronic Lymphocytic Leukaemia (CLL) is a disease of B-lymphocytes that account for significant morbidity and mortality in mostly elderly patients (aged ≥ 70 years). The relative survival of patients with CLL has been shown to decrease with patient age, and this age-related reduction in survival was found to correlate with the levels of two inflammatory cytokine levels in the patients' plasma. The levels of two inflammatory cytokines, interleukin-6 and -8 (IL-6, IL-8) were found to correlate positively with patient age, and increased levels were associated with lower overall survival. Addition of IL-6 or IL-8 to a co-culture system of CLL cells with bone marrow stromal cells increased the CLL-stromal cell adhesion, and co-culturing increased IL-8 secretion. In a search of a treatment regimen that may be effective and readily tolerated by elderly patients, we examined the combination of fludarabine with valproic acid (VPA), an epileptic that was found to inhibit histone deacetylases (HDACs). The combination was synergistic against human leukaemic cells, including primary CLL cells. In a phase II clinical trial where six elderly patients with relapsed, previously treated CLL were enrolled (half of whom were clinically refractory to fludarabine), the VPA-fludarabine combination induced reduction in the peripheral and lymph node tumour loads. Mechanistically, the fludarabine treatment induced disruption of the lysosomes, while VPA induced increase in the level and activity of cathepsin B, a lysosomal protease. The VPA-induced increase in cathepsin B levels was observed in in cell lines (*in vitro*), primary CLL cells (*ex vivo*) and in patients treated with VPA (*in vivo*). Chemical inhibition of cathepsin B was sufficient to dampen the VPA-fludarabine cytotoxicity, and the addition activated cathepsin B to leukaemic cell lysates was sufficient to induce

caspase cleavage and reduction in anti-apoptotic protein levels. The VPA-fludarabine combination also lowered phospho-Akt levels and ATM activation, which also contributed to the VPA-fludarabine synergy, and VPA treatment lowered ATM levels and phospho-Akt levels *in vivo*.

In summary, there lies a biological explanation for the poor survival observed with elderly patients, and the VPA-fludarabine may be a useful regimen for these patients.

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부모님께. 오랫동안 건강하시길.

(To my parents. Wishing them good health.)

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AIF (Apoptosis Inducing Factor)

ALC (Absolute Lymphocyte Count)

APAF-1 (Apoptotic Protease Activating Factor 1)

APE1 (Apurinic-apyrimidinic Endonuclease 1)

ATR (Ataxia Telangiectasia and Rad3-related protein)

ATRIP (ATR-Interacting Protein)

ATRX (α-Thalassaemia/mental Retardation syndrome X-linked)

BCL-2 (B-cell CLL/lymphoma 2)

BCR (B-Cell Receptor)

BER (Base Excision Repair)

BH (Bcl-2 homology)

BLNK (B-cell linker)

BMI (Body Mass Index)

BTK (Bruton's Tyrosine Kinase)

CaMKII (Ca^{2+}/Calmodulin-Dependent Protein Kinase)

c-FLIP (FLICE/Caspase-8-inhibitory proteins)

CIRS (Cumulative Illness Rating Scale)

CK2 (Casein Kinase 2)

CLL (Chronic Lymphocytic Leukaemia)

CREB (cAMP Response Element-Binding)

CRP (C-Reactive Protein)

CVD (Cardiovascular Disease)

DAXX (Death-Domain Associated protein)

DISC (Death-Inducing Signalling Complex)

DLBCL (Diffuse Large B-Cell Lymphoma)

DNA (Deoxyribose Nucleic Acid)

DSB (Double-Stranded Break)

DSBR (Double-Stranded Break Repair)

ER (Endoplasmic Reticulum)

FADD (Fas-Associated protein with Death Domain)

FCR (Fludarabine/Cyclophosphamide/Rituximab)

G6P (Glucose-6-Phosphate)

GBM (Glioblastoma Multiforme)

GSK-3β (Glycogen Synthase Kinase 3β)

HAT (Histone Acetyltransferase or Lysine acetyl-transferases)

HBXIP (Hepatitis B virus X-Interacting Protein)

HDAC (Histone Deacetylase)

HDI (Histone Deacetylase Inhibitor)

HNPCC (Hereditary Non-Polyposis Colon Cancer)

HR (Homologous Recombination)

HSP70 (Heat Shock Protein 70)

IAP (Inhibitors of Apoptosis Protein)

ICAD (Inhibitor of Caspase-Activated DNase)

ICL (Inter-strand Cross-Links)

IL (Interleukin)

KAT (Lysine Acetyltransferase)

LAMP (Lysosome-Associated Membrane glycoprotein)

LC3 (Microtubule-Associated Protein1 Light Chain 3)

LC-PUFA (Long-Chain Polyunsaturated Fatty Acid)

LDT (Lymphocyte Doubling Time)

LMP (Lysosomal Membrane Permeabilization)

LSD (Lysosomal Storage Disorders)

LYN (Lck/Yes-related Novel protein tyrosine kinase)

MBL (Monoclonal B-cell Lymphocytosis)

MCL-1 (Myeloid Cell Leukemia 1)

MDS (Myelodysplastic Syndrome)

MIP (Myo-Inositol-1-Phosphate)

MMR (Mismatch Repair)

MOMP (Mitochondrial Outer Membrane Permeabilization)

MRD (Minimal Residual Disease)

MRN (MRE11-RAD50-NBS1)

MSC (Mesenchymal Stromal Cell)

MSDH (O-Methyl-Serine Dodecylamide Hydrochloride)

MSNs (Mesoporous Silica Nanoparticles)

NAIP (NLR family Apoptosis Inhibitory Protein)

NER (Nucleotide Excision Repair)

NF-κB (Nuclear Factor-κB)

NHEJ (Non-Homologous End-Joining)

NSCLC (Non-Small Cell Lung Cancer)

PARP (poly (ADP-ribose) polymerase)

PIDD (p53-Induced protein with a Death Domain)

PIKK (Phosphatidylinositol 3-Kinase-related Kinases)

PIP (Phosphatidylinositol-4-Phosphate)

PIP2 (Phosphatidylinositol 4,5-Bisphosphate)

PIP3 (Phosphatidylinositol-(3,4,5)-Trisphosphate)

PLA2 (Phospholipase A2)

PNKP (Polynucleotide Kinase 3'-Phosphatase)

PP1 (Protein Phosphatase 1)

PTEN (Phosphatase and Tensin Homolog Deleted On Chromosome Ten)

RAIDD (RIP-Associated ICH-1/CAD-3 homologous protein with a Death Domain)

RIP-1 (Receptor-Interacting Protein 1)

RNA (Ribonucleic Acid)

ROS (Reactive Oxygen Species)

SAHA (suberoylanilide hydroxamic acid)

SPi2A (Serpin 2A)

SLL (Small Lymphocytic Lymphoma)

SMAC (Second Mitochondria-derived Activator of Caspase)

SMC (Structural Maintenance of Chromatin)

SSB (Single-Stranded Break)

SSBR (Single-Stranded Break Repair)

SYK (Spleen Tyrosine Kinase)

TFIIH (Transcription Factor II H)

TLR3 (Toll-Like Receptor 3)

TRADD (Tumor necrosis factor Receptor type 1-Associated Death Domain)

TRAIL (TNF-Related Apoptosis-Inducing Ligand

TSA (Trichostatin A)

TTFT (Time-To-First-Treatment)

TWEAK (TNF-related weak inducer of apoptosis, APO3 ligand)

VEGF (Vascular Endothelial Growth Factor)

VPA (Valproic Acid)

XIAP (X-linked Inhibitor of Apoptosis Protein)

XP (Xeroderma Pigmentosum)

XRCC1 (X-ray Repair Cross-Complementing protein 1)

ZAP-70 (Zeta-Associated Protein 70)

1 INTRODUCTION

1.1 Chronic Lymphocytic Leukaemia (CLL) – Diagnosis, Staging and Investigations

Chronic Lymphocytic Leukaemia (CLL) is a chronic haematological malignancy, where the term generally refers to a disease of the B lymphocytes (1). It is a monoclonal disease, with light chain restriction to either lambda or kappa, and the latest diagnostic criteria now requires the presence of $\geq 5 \times 10^9$ B lymphocytes/L, with clonality confirmed by flow cytometry (1). Persons displaying monoclonal expansion of the B lymphocytes where the count does not reach 5×10^9/L is categorized as Monoclonal B-cell Lymphocytosis (MBL), and CLL can be distinguished from Small Lymphocytic Lymphoma (SLL), where the patient lacks the leukaemic presentation but presents with enlarged lymph nodes and/or spleen (1).

CLL is further distinguished from other leukaemias by the surface markers that are examined via flow cytometry (Reviewed in 1). While CLL cells express the typical B-cell surface antigens CD19, CD20 and CD23, they also express CD5, a surface marker typically restricted to T lymphocytes. The expression level of surface immunoglobulin, CD20 and CD79b are typically of diminished intensity on CLL cells. There are rare cases, however, where the immunophenotype may be atypical (Reviewed in 2). These cases of atypical CLL can show strong surface immunoglobulin staining, weak or negative CD23 staining or CD5 negativity.

When viewed by their gene expression profile, the CLL cells are most closely related to memory B-cells when compared against naïve B-cells, $CD5^+$ B-cells, germinal centre centroblasts and centrocytes (3). Approximately 50-70% CLL cases have somatic

hypermutation in IgV_H gene, as expected of mature B-cells that have undergone the maturation process. In the rest of the cases, however, IgV_H gene is unmutated, and unmutated CLL cases, defined by < 2% differences from most similar germline gene in both the expressed V_H and V_L genes, have profoundly worse prognosis (4, 5). While it is thought that unmutated and mutated CLL may have very different sources of origin, as being pre- and post-germinal centre B-cells (6), the gene expression profiles between unmutated and mutated CLL were found to be very similar (3, 7).

Clinical course of CLL is heterogeneous, ranging from never requiring therapy to aggressive disease that leads to early death (Reviewed in 8). Consequently, there is great interest in finding biological markers to predict progression, response to therapy and survival. While a large number of investigations have been shown to predict overall survival and time-to-first-treatment (TTFT), a relatively small number of investigations are performed as part of the standard test in CLL clinics around the world. Rai staging combines physical exam for lymphadenopathy and splenomegaly with crude markers for a patient's bone marrow function, i.e. haemoglobin and platelet counts (9). Rai stage 0 describes a patient where lymphocytosis (increased lymphocyte count) is the only phenotype detectable by physical exam and blood count. Rai stages I and II represent intermediate risk patients, where lymphadenopathy (enlarged lymph nodes, stage I) and splenomegaly (enlarged spleen, stage II) also accompanies the lymphocytosis. "Bulky" lymphadenopathy has been demonstrated to be a strong prognostic marker, and presence of lymph nodes that measures 10 cm in longest diameter is an indication of active disease (1). High stage patients (Rai stages III/IV) also present with anaemia (haemoglobin < 110 g/dL, stage III) and/or thrombocytopaenia (platelet < 100 x 10^9/L, stage IV).

Although generally considered to be the prognostic gold standard, the predictive value of Rai stage, especially in comparison to other laboratory investigations, is rather argued to be poor, at least in some studies (10). The Binet staging system is another clinical staging system that is similar to the Rai staging system (11), but the Rai staging system is routinely used in North America. Lymphocyte doubling time (LDT) is another routinely performed older prognostic marker, and LDT is defined as the time period during which the absolute lymphocyte count (ALC) is doubled (12).

While bone marrow histology is not required for diagnosis or staging of CLL, diffuse bone marrow infiltration is predictive of greater likelihood of progression and shorter survival (13-15). Autoimmune haemolysis and reduced bone marrow function are the two prominent causes of anaemia in CLL. While patients with autoimmune anaemia have comparable or sometime better overall survival, reduced bone marrow function, as seen in patients with true high-stage CLL, assessed by bone marrow biopsy, is associated with poor prognosis (15).

A number of laboratory investigations now routinely complement physical exam and blood counts. Many of the prognostic markers assess changes in the CLL cells themselves, and certain specific genetic lesions assessed by Fluorescent *In Situ* Hybridization (FISH) have been shown to be some of the strongest prognostic markers. In a study by Döhner *et al*., a number of genetic lesions, detected by FISH, were found to be recurrent, and these lesions were del13q14, del11q22-q23, del17p13 and trisomy 12q (16). Among them, del11q22-q23, del17p13 have been associated with a particularly poor prognosis, representing a sub-cohort with some of the worst outcomes observed among patients with CLL (16), and these lesions are discussed elsewhere in the

introduction. While these defects have been associated with genetic complexity and inferior survival, their role in the pathogenesis of the disease and their contribution to the acquisition of genetic lesions are unknown. Telomere attrition and telomeric dysfunction have been shown in CLL cells (17-20), and these defects may be contributing to the increased genomic complexity of CLL cells.

There are also proteins markers that have been linked to prognosis, both intracellular and surface markers. Approximately 1/3 of CLL patients are $CD38^+$ at diagnosis, and CD38 positivity has been associated with poor prognosis (4, 21). CD38 ligation induces *in vitro* proliferation of the CLL cells (22), and high CD38 positivity is closely associated with Ki-67 (23), an index of proliferation. Another important prognostic marker is Zeta-Associated Protein 70 (Zap-70), a cytoplasmic protein that normally functions downstream of the T-cell receptor. While *ZAP-70* expression level is not detected in majority of CLL cases, in a large proportion of unmutated CLL cases, CLL cells express high levels of ZAP-70 (7). Zap-70 level measurement by flow cytometry is now a standard test in many centres around the world, as high levels are associated with poor prognosis (24, 25).

Finally, certain plasma markers are also assessed routinely in CLL patients. β_2-microglobulin has been shown to be a strong prognostic marker by a number of studies (26, 27). The β_2-microglobulin is a component of the class I major histocompatibility complex (MHC) that is present on all cells (excluding the red blood cells), and β_2-microglobulin can be readily shed into the plasma and is subsequently degraded by the kidney. The level of this protein is thus believed to reflect the tumour burden in CLL, although the level may also increase with age, which is generally associated with a

decrease in renal function (28). In order to compensate for this decline, an algorithm has been proposed for adjusting for renal function when considering the level of β_2-microglobulin in the context of CLL patients (29). Other plasma markers that have been shown to be independent prognostic markers include soluble CD23 (30, 31), serum thymidine kinase (32, 33) and serum vitamin D (34). Other tests that may be performed on CLL patients include flow cytometric or PCR-based tests for minimal residual disease (MRD) following treatment (35) and imaging tests, including abdominal computed tomography scan for assessing lymphadenopathy and other nodal involvement (36).

1.1.1 Management of Patients with CLL

Not all CLL cases require treatment. The standard practice for early stage, non-progressing disease is a conservative watch-and-wait approach, as there is no evidence suggesting benefits of early chemotherapy (37). Indications for treatment include active Rai stage I & II disease, high Rai stage (III, IV) disease, progressive disease (demonstrated by increasing lymphocytosis with a LDT < 6 months and/or rapidly enlarging lymph nodes, spleen, and liver) and systemic symptoms (i.e. weakness, night sweats, weight loss, painful lymphadenopathy and/or fever) (1). Allogeneic haematopoietic cell transplantation using non-myeloablative, reduced intensity regimens can offer long-term disease control, and it does seem to offer long-term survival benefit for patients with del17p (38), a cohort of patients that have often demonstrated the worst survival in numerous studies. The conditioning regimen, however, is associated with treatment-related mortality and cannot be tolerated by many patients with CLL (39), and thus chemo- or chemoimmunotherapy are the mainstay management strategies.

The appropriate treatment regimen depends on the biological fitness of patients. Cumulative Illness Rating Scale (CIRS) distinguishes patients into three groups: 1) "Go-Go" (i.e. fit) patients for whom intensive chemo- or chemoimmunotherapy is appropriate, 2) "Slow-Go" (i.e. intermediate) patients for whom dose-reduced regimens may be more appropriate, and 3) "No-Go" (i.e. frail) patients for whom chemo- or chemoimmunotherapy is not appropriate (40, 41). For the "Go-Go" patients, the consensus on the frontline therapy is the Fludarabine/Cyclophosphamide/Rituximab (FCR) combined chemoimmunotherapy, based on a phase III clinical trial that demonstrated the survival benefit provided the addition of rituximab, an anti-CD20 monoclonal antibody (42). While FCR does appear to be effective in relapsed CLL as well (43), there is currently no consensus regarding treatment of relapsed cases of CLL. Tolerability of standard FCR in elderly patients is debatable, and the tolerance of even the dose-reduced FCR-Lite regimen appears to be reduced compared to that in younger patients (44). While there are some regimens that appear to be promising, such as the chlorambucil and rituximab combination, the elderly and slow-go patients, two cohorts that are often overlapping, remain greatly under-studied (41).

A number of other agents are also used by medical oncologists on off-label basis, and a number of clinical trials are taking place to examine their effects. Broadly categorized, the different agents in use to manage CLL include: 1) nucleoside analogues (ex. fludarabine, cytarabine), 2) alkylating agent (ex. cyclophosphamide, bendamustine), 3) steroids (ex. prednisone), 4) immunotherapy (ex. rituximab, GA101, ofatumumab), 5) immunomodulatory drugs (ex. lenalidomide), 6) kinase inhibitors (ex. dasatinib,

flavopiridol), 7) anti-metabolites (excluding nucleoside analogues, ex. methotrexate), and 8) other targeted therapies (ex. pan-Bcl-2 antagonist GX15-070).

1.2 DNA Damage Response Pathways

For many chemotherapeutic drugs used in CLL, the induction of DNA damage is an essential action of the drug. Yet, DNA damage is a common occurrence in a cell's day, and a normal cell in our body is estimated to encounter 10 double-stranded breaks (DSBs) per day (45). DNA damaging agents include exogenous factors, such as radiation, various environmental agents and genotoxic agents in a cancer patient undergoing treatment, as well as endogenous factors, such as reactive oxygen species (ROS) being generated in the cell (46, 47). Other endogenous DNA damages include depurination, cytosine deamination and methylation (induced by S-adenosyl methionine) (46, 47). These factors vary in the type of DNA damage induced, as well as in the severity of the damage. DSBs are generally thought to be the most severe form of DNA damage, as a single DSB is sufficient to induce cell cycle arrest, and, if not repaired, eventual death of the cell (48, 49). Cells are able to respond to different types of damage due to the variety of sensor proteins, which also represent the first steps in the DNA damage response. The importance of this first step has been demonstrated by experiments where sensor proteins were tethered to chromatin, and the tethering was shown to be sufficient to elicit the DNA damage response signalling cascade in the absence of DNA damage (50, 51). By recruiting different factors, a cell is able to dynamically repair different types of DNA damages.

At least four partially independent sensors have been described to be able to sense

DSBs: Poly (ADP-ribose) polymerase (PARP), Ku70/Ku80, the MRE11-RAD50-NBS1 (MRN) complex and Replication protein A (RPA) (with DSB processing) (Reviewed in 47). PARP1/2 proteins can directly sense DSBs, as well as single-stranded breaks, and PARP1/2 is capable of recruiting both the MRN complex and ATM to the damaged sites. The MRN (Mre11/NBS1/Rad50) complex can directly interact with the DNA ends of the DSBs, process the ends (via its endo/exonuclease activity) and recruit the ATM proteins to promote the repair. Following the initial detection of the DSB, four independent pathways can repair the lesions, namely homologous recombination (HR), non-homologous end-joining (NHEJ), alternative-NHEJ and single-strand annealing (SSA). The main mechanism by which a cell chooses the type of the DSB repair pathway is thought to be dependent on the initial DNA end resection; HR, alternative-NHEJ and SSA are mechanisms of DSB repair that are dependent on DSB resection, while (classical) NHEJ is not dependent on DSB resection. One of the factors that determine the extent of DSB end resection is the cell cycle phase, and DSB resection is primarily induced in the S and G2 phases of the cell cycle (52). This choice is a sensible one, considering that the homologous copy required for homologous repair is unavailable during the G1 phase. Limited DSB resection can be carried out by CtIP in the G1 phase, but this resection promotes alternative-NHEJ, where the required resection is much less smaller (5-25 nucleotides).

During the G2/S phase, extensive resection of DSB ends can take place, which is necessary for the homologous recombination to follow (Reviewed in 47). Nucleases and helicase work in conjunction to create long 3'-ssDNA ends, which is rapidly bound by the ssDNA-binding protein RPA, allowing for the assembly of Rad51 filaments. The

Rad51 filament will perform homology search, after which the filament will invade the appropriate DNA. Repair continues following the processing of the synapsis and gap filling, a process that can occur via three sub-pathways of HR. SSA is another homology-directed DNA repair pathway, which can take place when DSBs occur in an area with repeated sequences. Unlike homologous recombination, SSA does not involve DNA-strand invasion and leads to the deletion of the DNA sequences that lie between the repeated sequences.

The type of repair also depends on the sensor proteins involved (Reviewed in 47). The Ku70/80 heterodimer can directly bind DSB ends and serve as a docking site for the subsequent NHEJ factors to bind. DNA-PKcs, another member of the Phosphatidylinositol 3-Kinase-related Kinases (PIKK) family (which includes ATM), is recruited, activated (via auto-phosphorylation) and phosphorylates a number of downstream target, including H2A.X (resulting in γH2A.X, which is H2A.X phosphorylated at serine 139). Following processing of the DSB ends and gap-filling by specialized polymerases (members of the Pol X family), the DSB ends are ligated by XRCC4/LIG4.

Single-stranded breaks (SSBs) are also detected by PARP1/2, where PARP1, the main sensor of the two PARPs, sense SSBs arising directly from disintegration of oxidized deoxyribose (Reviewed in 53). As endogenous ROS is one of the most prominent causes of SSBs, the SSB repair (SSBR) pathway represents an important mechanism for the maintenance of genomic integrity. Upon the recognition of the SSBs and subsequent binding of RPA, a ssDNA-binding protein, to the sites, another member of the PIKK family, Ataxia Telangiectasia and Rad3-related protein (ATR), is recruited

along with ATR-Interacting Protein (ATRIP). SSBs are generally damaged such that their ends require processing, and thus the recruitment of processing enzymes, including X-ray Repair Cross-Complementing protein 1 (XRCC1), Polynucleotide Kinase 3'-Phosphatase (PNKP) and Apurinic-apyrimidinic Endonuclease 1 (APE1), are facilitated by the DNA-bound PARP1/2. Following the processing of the SSB ends, gaps are filled by DNA polymerases and ends are ligated. SSBs can also arise during the process of base excision repair (BER), which will recognize oxidized, alkylated, deaminated bases, as well as inappropriately inserted uracil, excise the base of interest, then engage the SSBR mechanism by activating PARP1/2.

Nucleotide excision repair (NER) recognizes damaged bases, including thymine dimers (Reviewed in 54). The mechanism of the recognition involves the detection of the distortion in the DNA double helix by the XPC-hR23B-Cen2 complex. The DNA is separated at the site of the lesion by the action of the helicase Transcription Factor II H (TFIIH), which recruits the proteins Xeroderma Pigmentosum group B (XPB), XPD, XPA and RPA. The DNA is incised at both sides of the lesion, and the created gap is filled and ligated. NER can also be coupled to transcription via the transcription-coupled repair, a sub-pathway of NER. As in the aforementioned mechanism, TFIIH, as part of the RNA II polymerase holoenzyme, recognizes the DNA lesion.

Other repair pathways are also in play, dynamically maintaining the genomic integrity within a cell. These pathways include the mismatch repair (MMR) and the Inter-strand Cross-Links (ICL) repair pathway.

1.3 Histones and Histone Deacetylases (HDACs)

The dynamic interaction between the histones and DNA modulates the accessibility of the DNA by the DNA damaging agents and the DNA damage repair machineries. A nucleosome consists of the octameric histone complex, composed of two of each of the core histones, H2A, H2B, H3, H4, with 146 bp of DNA wrapped around the complex (Reviewed in 55, 56). H1, the linker histone, also serves an important component for higher order structure of chromatin. In a cell, the histones undergo various post-translational modifications (PTMs) including acetylation, phosphorylation, methylation, ubiquitination and sumoylation (56). Depending on the residue and the PTM in question, these different PTMs influence the DNA-histone interaction in different ways to modulate gene expression. Generally speaking, however, increased acetylation is generally associated with open chromatin, while the vice versa is also true.

Histone acetylation is a dynamically regulated by two class of enzymes, histone acetyl-transferases (HATs, also known as lysine acetyl-transferases or KATs) and histone deacetylases (HDACs or KDACs) (56). HDACs are categorized into four different classes based on their homology to the founding members from yeast (Reviewed in 57). Class I HDACs, homologous to Rpd3p in yeast, are composed of HDACs 1, 2, 3 and 8. Class II HDACs share homology to Hda1p in yeast and can be divided into two sub-classes, Class IIA and IIB. Class IIA HDACs are composed of HDACs 4, 5, 7 and 9, where the C-terminal catalytic domain is conserved among the members. HDACs 6 and 10 form the class IIB HDACs and are distinguished from the IIA HDACs by having their catalytic domain in the N-terminus (*vs.* C-terminus). HDAC6 is also unique in that in contains two tandem deacetylase domains. Class III HDACs are composed of the Sir2p

homologs, SIRT1-7, and HDAC11 is the sole member of the class IV. Class I, II and IV HDACs share a common Zn^{2+}-dependent catalytic site, while class III HDACs-mediated deacetylation is dependent on NAD^{+} (58).

1.3.1 Histone Metabolism and Cancers

HDACs have been found to be overexpressed in a number of cancer cell lines and tissues (57, 59). With regards to which HDAC is overexpressed, there appears to be heterogeneity among the different cancers, although this may be associated with a certain bias in the field that has focused on class I HDACs, more specifically HDAC1-3 (57), perhaps due to the availability of drugs that have been shown to target class HDAC1-3. There appears to be significant heterogeneity in the clinical significance of HDAC levels as well. In hepatocellular carcinoma, increased HDAC1 levels also correlated with high tumour stage and poorer overall survival after resection (60). High levels of HDAC1-3 was an independent prognostic factor in gastric cancer (61). HDAC1-3 were found to be strongly expressed in majority of prostate cancer tissues, but only HDAC2 levels were found to be a significant prognostic marker (62). In colorectal cancer, levels of HDAC1-3 were all significant predictors of overall survival, though HDAC2 levels were the only independent prognostic marker (63). It is important to note that high HDAC levels are not always associated with inferior survival. In lung cancer, while *HDAC1* expression levels do not appear to be different from that of non-cancerous counterparts, higher levels were observed in high-grade lung cancer tissues, but this difference did not correlate with inferior overall survival (64). In renal cancer, the levels of class I HDACs were found to be not associated with inferior survival (65). In breast cancer, higher levels *HDAC1* or

HDAC6 mRNA levels or HDAC1 protein levels actually correlated with better prognosis (66-68). Significance of HDAC levels has also been examined in haematological malignancies. While HDAC1 and HDAC2 levels were not predictive of patient survival, high HDAC6 levels correlated with worse survival in PTCL, while low levels correlated with worse survival in Diffuse Large B-Cell Lymphoma (DLBCL) (69). HDAC1 and HDAC2 levels were also not predictive of survival in cutaneous T-cell lymphoma (70), an interesting observation in the consideration of cutaneous T-cell lymphoma being one of the few cancers where a HDAC inhibitor (HDI) is approved for therapy by the U.S. Food and Drug Administration. In fact, while many studies report the anti-tumour effects of HDIs in almost all types of cancers, actual studies that report the level of histone acetylations is relatively scarce. In CLL, all class I HDACs are expressed at higher levels compared to normal lymphocytes in a small cohort of samples (71), providing the first mechanistic rationale for using HDAC inhibitors in CLL (*vs.* effect-driven rationale, such as the demonstration that HDIs are cytotoxic against CLL cells). The high level of HDACs was associated with suppression of certain miRNAs, namely *miR-15a*, *miR-16*, and *miR-29b*. Among those HDAC targets, low expression of *miR-15a* and *miR-16* had been linked to the deletion of the 13q14 locus, making them some of the first miRNAs to be linked to cancer pathogenesis (72). Low expression of *miR-15a* and *miR-16* were also observed in some of CLL patient samples with no evidence of 13q14 deletion, and this transcriptional repression was found to be reversible with HDIs LBH589 or MS275 (73).

In some cancers, differences in histone PTMs have also been linked to survival differences. Fraga *et al.* observed loss of monoacetylated and trimethylated forms of histone 4 in various leukaemia cell lines (ex. HL60, Jurkat), primary lymphoma and

primary colon tumours, specifically the loss of acetyl-K16 (H4K16ac) and trimethyl-K20 (H4K20me3) (74). H3K4me2 & H3K18ac levels predicted clinical outcome of lung and kidney cancer patients. H3K4-methyl (mono-, di and tri-methyl combined) levels predicted progression-free survival in renal cell carcinoma (75). High H3K4me2 or low H3K9ac levels were associated with better survival in non-small cell lung cancer (76). In gastric adenocarcinoma, high levels of H3K9me3 were associated with poor prognosis (77). In breast cancer, clustering of 408 cases for seven histone PTMs (H3K18ac, H3K9ac, H3K4me2, H4K12ac, H4K16ac, and H4K20me3 and H4R3me2) resulted in three groups, where the "hypomodified" group showed low levels of six of the seven PTMs (excluding H4K16ac), and this group showed lower patient survival (78). Differences in histones between cancerous and non-cancerous tissues are not limited to PTMs. In paediatric glioblastoma multiforme (GBM), somatic mutations were found in the *H3F3A* gene, which encodes for the histone variant H3.3 (79). The mutations in *H3F3A* were associated with two genes associated with H3.3 function, namely α-thalassaemia/mental retardation syndrome X-linked (ATRX) and death-domain associated protein (DAXX). The mutations, however, appeared to be of no clinical significance, as the presence of mutations were not associated with inferior overall survival or progression-free survival, despite the observation that mutations in *H3F3A* and/or *ATRX* were associated with higher proportion of samples being positive for *TP53* mutations (79).

1.3.2 HDAC inhibitors (HDIs) and CLL

Recognizing the high level of HDAC expression in a number of cancers (57), inhibition of HDACs using HDAC inhibitors (HDIs) has been examined as a chemotherapeutic strategy . A number of criteria have been used in reviews to categorize HDIs (Reviewed in 59). HDIs can be categorized based on their chemical structures into six classes: 1) hydroxamate derivatives, 2) cyclic tetrapeptides, 3) short-chain fatty acids, 4) synthetic pyridyl carbamate derivatives, 5) synthetic benzamide derivatives, and 6) ketones. HDIs have also been categorized based on their targets, to group them into 1) pan-HDAC inhibitors (ex. Trichostatin A), 2) class I inhibitors (ex. MS-275), 3) class III inhibitors (ex. sirtinol), and 4) HDAC-specific inhibitors (such as HDAC6 inhibitors). While the HDIs vary in their potency and selectivity, the different HDIs converge on their ability to target HDACs to enhance the level of acetylated proteins in variety of *in vitro* and *in vivo* models.

Inhibition of HDACs using pharmacological agents, such as depsipeptide, suberoylanilide hydroxamic acid (SAHA) is a novel strategy in treating CLL. *In vitro* experiments with HDIs using depsipeptide (80, 81), LBH589 (82) and MS-275 (83) were promising, resulting in significant levels of cell death. In the clinic, however, studies examining HDI monotherapy in CLL were disappointing. Phase I trial with depsipeptide observed no response, despite obvious increase in the level of acetylated histones and p21 levels (84). Phase II trial using MGCD0103 in previously treated CLL also observed no response, despite some patients receiving concomitant rituximab (85). In other haematological malignancies, results with belinostat (PXD101) in patients with advanced hematological neoplasms were disappointing with no complete or partial response

observed (86). Disappointing response with HDI therapy has been reported in other malignancies as well. Mocetinostat in relapsed or refractory Hodgkin's lymphoma resulted in disease control rate of only 35% (87), whereas overall response rate using belinostat in myelodysplastic syndrome (MDS) was 5% (88). Response rate using Belinostat in thymic epithelial tumours was also low, with response rate of 8% (89).

Two important issues are highlighted by the clinical trials with HDI monotherapy. One important issue is poor tolerance of some of the HDIs at the doses administered. In a number of trials, haematological grades 3-4 toxicities were observed, and such high-grade toxicities were observed in some cases high as 50% of patients enrolled, including neutropaenia, thrombocytopaenia and anaemia (88). In some trials, belinostat in thymic epithelial tumours for example, HDI monotherapy was well tolerated, but the overall response rates from such trials were disappointing (89). Considering the poor tolerance observed some trials, high-dose HDI monotherapy is not appropriate in CLL patients, where the median age of patients is high and biological fitness is often lower than most patients enrolled in clinical trials. Thus, rather than HDI monotherapy, HDI combination therapy would be a more appropriate strategy in CLL management.

1.3.3 Valproic Acid (VPA)

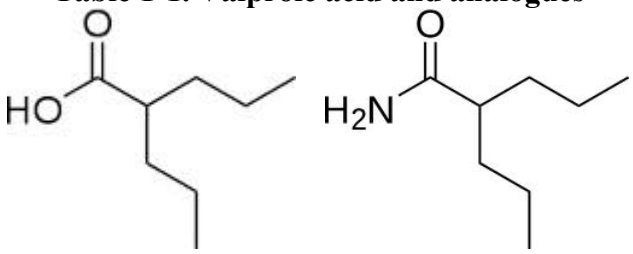
Valproic Acid (2-propylpentanoic acid) is a branched fatty acid that was serendipitously found to have anti-epileptic activity. Its anti-epileptic activity has been contributed to its ability to elevate GABA levels and inhibit voltage-gated sodium channels (90). The physical structure of VPA is important for its activity, as its activity is altered upon changing the location of the propyl chain or upon the substitution of its

carboxyl head group (91-94). While not all substitutions enhanced the anti-epileptic activity, substitution of the carboxyl head group to amide (i.e. valpromide) (92), hydroxamic acid (93) or urea (94) enhanced the anti-epileptic activity.

VPA is a first-generation anti-epileptic that has been shown to have HDAC inhibition activity (95, 96), with IC_{50} values for HDAC1-3 being $\leq$ 1 mM (96). In a more recent study using tagged VPA, its HDAC inhibition appears to be limited to class I HDACs, more specifically HDAC1-3 (97). Treatment of a number of different cells *in vitro* induced hyper-acetylation of histones 3 and 4 (95, 98), and valproate also induced hyper-acetylation on H2A, with little effects on H2B (97). VPA treatment of breast cancer cells resulted in the depletion of a number of chromatin-associated proteins, including structural maintenance of chromatin (SMC) proteins, SMC-associated proteins, DNA methyltransferase, and heterochromatin proteins (99). The hyper-acetylated histones, at least H3K9ac in embryonic stem cells, were found to be enriched at gene promoters, which, in turn, correlated with gene expression levels (100). VPA has also been shown to influence DNA methylation status, being able to revert chemically induced hyper-methylation of the *reelin* promoter in mice (101), and VPA has been shown to increase acetylated H4 in CpG island and shores in astrocytes (102). Associated with these various effects on the chromatin, VPA has been shown to alter the expression of many genes. Hundreds to thousands of genes have been reported to be modulated in response to VPA in larger scale studies (103-105). Interestingly, valpromide, where the head carboxyl group is substitute with amide, did not demonstrate HDAC inhibition activity, despite being the stronger anti-epileptic agent (106), suggesting that HDAC inhibition may not be necessary for its anti-epileptic activity. On

the other hand, the HDAC inhibition does appear to be important for its anti-tumour activity. Comparing AML cell lines, two cell lines that expressed higher levels of HDAC1-3, had much higher IC_{50} value for valproic acid (107).

Table 1-1. Valproic acid and analogues



Valproic Acid **Valpromide**

VPA has been shown to have anti-tumour effects against a number of tumour models, including prostate (108), breast (109), gliomas (110), neuroblastoma (111) and numerous haematological malignancies (112-114). VPA monotherapy of primary CLL cells *in vitro* was sufficient to induce apoptosis of CLL cells, as judged by caspase activation and annexin V staining (115, 116), and VPA inhibited proliferation of CLL cells induced by oligonucleotide and IL-2 co-stimulation (103). Single-agent VPA was sufficient to induce alterations in the gene expression level of a large number of genes in CLL cells (103) and was sufficient to change the Bcl-2/Bax ratio at the protein level (103, 117). Furthermore, VPA enhanced the effects of DNA-damaging agents against CLL cells, including fludarabine, bortezomib, flavopiridol, thalidomide and lenalidomide (103, 115).

1.4 Cell Death – Apoptosis and Caspases

In general, the desired effects of chemotherapeutic drugs are to induce sufficient amount of DNA damage, overwhelm the DNA damage repair systems and induce cell death. According to the Nomenclature Committee on Cell Death, dead cells are defined by one of the three features: 1) loss of plasma membrane integrity, 2) cell fragmentation (and formation of apoptotic bodies), or 3) engulfment by adjacent cells (118-120). Bona fide *dead* cells are distinguished from *dying* cells, which have not completely crossed the points of no return. Necrosis and apoptosis are the two better understood pathways leading to bona fide cell death. Necrosis is an energy-independent process that results in karyolysis (nuclear lysis), oncosis (cell swelling) and eventual rupture of the cell (118, 119). Other morphological features include cytoplasmic vacuolization, distended ER, cytoplasmic blebs, condensed, swollen or ruptured mitochondria and detachment of ribosomes (118, 121). As cells undergo lysis, there is an efflux of cell constituents, for example the nuclear protein HMGB1, which can trigger inflammatory processes (122, 123).

Apoptosis is an active, energy-dependent process that requires ATP. Morphologically, apoptosis is characterized by membrane blebbing, pyknosis (cell volume shrinkage), karyorrhexis (nuclear condensation) and phosphatidyl-serine externalization in the plasma membrane (118, 121). Modifications that occur on cytoplasmic organelles are generally minor in apoptosis. The externalized phosphatidyl-serine acts as a tag recognized by macrophages that engulf the apoptotic cells (124, 125). *In vitro*, where macrophages are absent, apoptotic cells can undergo secondary necrosis, eventually undergoing oncosis and rupture (126, 127). While these morphological and

molecular differences between necrosis and apoptosis are noted, it is important to note that the two terms, together, describe a spectrum of cell death events. Apoptosis is also distinguished from necrosis by their dependence on caspases. Caspases are cysteine proteases that are produced as zymogens, in their pro-enzyme forms, and, like many other proteases, cleavage of the pro-enzyme form is required for their activation (128, 129). Depending on their place in the caspase cascade, they may be categorized as upstream/apical or downstream/executioner caspases (130). Caspase-2, caspase-8 and caspase-9 are examples of apical caspases, while caspases-3, -6 and -7 are executioner caspase. While the cleavage of executioner caspases is sufficient for their activation, a number of initiator caspases require formation of larger complexes that often act to bring the initiator caspases as multimers (129). Once the cascade succeeds in activating the executioner caspases, a number of proteins are targeted, bringing the host cell to its ultimate demise. Targets of caspases include the components of the DNA repair pathway (ex. poly (ADP-ribose) polymerase (PARP)), structural nuclear proteins (ex. lamins), DNA metabolism (ex. Inhibitor of Caspase-Activated DNase (ICAD), the degradation of which contributes to the eventual DNA fragmentation observed in apoptosis), cytoskeletal dynamics (ex. gelsolin, the cleavage of which contributes to the membrane blebbing observed) and many others (131).

Two pathways in apoptosis can lead to the eventual activation of the executioner caspases, the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways. The intrinsic pathway is initiated by the disruption of the mitochondrial integrity that leads to the loss of the mitochondrial membrane potential ($\Delta\psi m$) (132). While transient loss can be physiological, prolonged loss leads to the eventual mitochondrial outer membrane

permeabilization (MOMP). MOMP is accomplished by actions of the Bax sub-family members of the Bcl-2 family, i.e. Bax, Bak and Bok, as well as the pro-apoptotic, activator BH3 members, i.e. Bid and Bim (133). Other pro-apoptotic members of the Bcl-2 family promote apoptosis in a less direct manner. Bad, Bmf, Bik, Hrk, Noxa and Puma cannot induce the activation of Bax or Bak directly, but rather these sensitizers act by binding to the anti-apoptotic members of the Bcl-2 family, promoting the release of the activator Bcl-2 family members (134, 135). The anti-apoptotic members of the Bcl-2 family and their relationships with CLL cells are described in another section.

Upon receiving the apoptotic signals, the activator Bcl-2 proteins migrate and bind to the mitochondrial membrane, forming a ring that functions as "permeability transition pores" (133). Formation of the pore allows for the egress of a number of mitochondrial proteins, including cytochrome *c* and Apoptosis Inducing factor (AIF) (132). The released Aif enters the nucleus to induce chromatin condensation and DNA fragmentation, while cytochrome *c* binds to Apoptotic Protease Activating Factor 1 (APAF-1), allowing for APAF-1 to undergo conformational change. Following (d)ATP-(d)ADP exchange, the seven-membered oligomer of activated Apaf-1, i.e. the apoptosome, is allowed form, and the apoptosome activates pro-caspase-9 (136).

The extrinsic pathway involves the ligation of a death receptor (DR) complex, downstream of which caspase-8 or caspase-10 is activated, leading to apoptosis (130). The ligands trigger that extrinsic pathway and their respective receptors are summarized in Table 1-2. Not all death receptors play a role in promoting apoptosis; there are a number of decoy receptors that act by sequestering TRAIL (TRAIL-R3, TRAIL-R4, OPG) or FasL (DcR-3/TR6) (137, 138). In a typical extrinsic apoptotic pathway, ligation

of a DR leads to their aggregation, resulting in trimerization of the DRs (130). Ligation also results in the recruitment of adaptor proteins, either Fas-Associated protein with Death Domain (FADD) or Tumor necrosis factor Receptor type 1-Associated Death Domain protein (TRADD), which then binds the initiator caspases caspase-8 or caspase-10 (Reviewed in 130). Juxtaposition of caspase-8 or -10 allows for cross-cleavage of the caspases and their activation. Downstream of caspase-8 or -10, the executioner caspases may be directly cleaved, ultimately leading to the apoptotic processes. In some cell death scenarios, for example downstream of Fas ligation, caspase-2 has been described to be activated downstream of caspase-8, thereby presumably working to enhance caspase-3 activation (139). However, as discussed below, caspase-2 does not always function downstream of caspase-8.

Table 1-2. Death receptors and their ligands

Ligand	**Receptor**
FasL	Fas (CD95)
TNF-α	TNFR1
TNF-Related Apoptosis-Inducing Ligand (TRAIL)	TRAIL-R1/DR4 TRAIL-R2/DR5
TNF-related weak inducer of apoptosis, APO3 ligand (TWEAK)	FN14

While caspase-8 or -10 can directly cleave and activate the executioner caspase caspase-3, the extrinsic pathway can also mediate the activation of the intrinsic pathway, thereby amplifying the initial apoptotic signals in certain cells (140, 141). The necessity of the amplification of the extrinsic triggers distinguishes the type I cells in which amplification is unnecessary *vs.* type II cells in which extrinsic triggers rely heavily on the amplification in order to elicit cell death (140, 141). In type II cells, an important target of caspase-8/10 is Bid, where, upon the caspase-mediated truncation of Bid, truncated Bid (tBid) will translocate to the mitochondria, inducing MOMP and,

subsequently, the activation of the intrinsic apoptotic pathway (140, 141). Bid can also be targeted by caspase-2, albeit in a manner less efficient than caspase-8, but nonetheless engaging the intrinsic apoptotic pathway downstream of caspase-2 (142, 143).

Caspase-2, -8 and -10 can also be activated independent of the typical death receptor complexes. Mitochondrial activation of caspase-8 has been described, involving the integration of caspase-8 into cardiolipin-rich domains of the outer mitochondrial membrane in response to Fas ligation (144). More recently, formation of a high molecular weight complex containing Receptor-Interacting Protein 1 (RIP1), caspase-8 and Fadd has recently been described downstream of genotoxic damage and Toll-Like Receptor 3 (TLR3) ligation (145, 146), forming a complex named the RIPoptosome. Another recently described caspase-8 activating platform has been described to form on the cytosolic surface of autophago-lysosomes (147, 148). The complex involved was composed of caspase-8, p62 and LC3 that formed in response to MG132, a proteasome inhibitor, and the oligomerization of caspase-8 involved its poly-ubiquitination of caspase-8 (147, 148).

While caspase-8/10 and caspase-9, in general, appear to be dedicated to the extrinsic and intrinsic apoptotic pathways, respectively, the platforms required for the activation and the place in the caspase cascade of other apical caspases are less clear. Caspase-4 activation has been observed downstream of endoplasmic reticulum (ER) stress, and caspase-4 has been seen to be localized at the ER and activated by ER stress (149). Interestingly, spontaneous apoptosis of CLL cells has been associated with the activation of caspase-4 and is thought to be associated with ER stress (150).

Caspase-2 is another caspase whose role with respect to the extrinsic and intrinsic

apoptotic pathways remains unclear (151, 152). In some studies, caspase-2 has been shown to cleave Bid and has been placed upstream of caspase-9 in response to a number of different stimuli, including Trichostatin A (153), saikosaponin A (154), ceramide (155) and ER stress (156). Yet, in a study examining thymocytes lacking either Apaf-1 or caspase-9, caspase-2 activation was not observed after γ-irradiation (157), suggesting caspase-2 is acting downstream of caspase-9. Caspase-2 has also been placed upstream of caspase-8 in some cases. For example, inhibiting caspase-2 reduced activation of caspase-8 and -3 in response to tunicamycin (158), saikosaponin A (154), ceramide (155) and docetaxel (159). Yet, caspase-2 activation has also been reported downstream of Fas and TNFR ligation and caspase-8 activation (139, 160, 161). However, most studies do agree that caspase-2 acts as an initiator caspase, upstream of caspase-3. In cases where caspase-2 acts upstream of other initiator caspases, one mechanism for its activation is the formation of a high molecular weight complex called the PIDDosome (162, 163). PIDDosome is a signalling platform for caspase-2 activation that contains the proteins p53-Induced protein with a Death Domain (PIDD) and RIP-Associated ICH-1/CAD-3 homologous protein with a Death Domain (RAIDD) (162, 163). Since the initial characterization of the PIDDosome, other PIDDosome-related complexes have been described that play roles in NF-κB activation and the DNA damage response pathway (163). Thus, the initially described PIDDosome complex is now referred to as the "Caspase-2 PIDDosome", and is distinguished from "NEMO-PIDDosome", "PCNA-PIDDosome" and "DNA-PKc-PIDDosome" (163). The formation of the Caspase-2-PIDDosome is not the only mechanism of caspase-2 activation, as its activation is observed in the absence of detectable PIDDosome formation (164).

1.4.1 Inhibitors of Apoptosis and CLL cells

CLL cells are able to undergo different modes of deaths, and both the intrinsic and extrinsic apoptotic pathways can be activated in CLL cells. CLL cells, however, are highly resistant to apoptosis, and two families of proteins, Bcl-2 and IAP (inhibitors of apoptosis) proteins, are of particular importance in suppressing apoptosis. B-cell CLL/lymphoma 2 (Bcl-2), the founding member of the Bcl-2 family, is the first proto-oncogene to be described with anti-apoptotic function (165). The Bcl-2 family is characterized by the presence of at least one Bcl-2 homology (BH) domain, where the different classes differ in which BH domain(s) are contained in the protein; while the members of the "BH3-only" class contain BH3 domain only, the other "multi-domain" members may contain domains BH1-4 (166, 167). The multi-domain Bcl-2 family members are further divided into two classes, pro- and anti-apoptotic classes. The multi-domain pro-apoptotic class is composed of Bax, Bak and Bok, and the best described mechanism of their action involves inducing permeabilization of the outer mitochondrial membrane as described in the previous section. Anti-apoptotic members of the Bcl-2 family members, including Bcl-2, Bcl-2A1, Bcl-W, Bcl-xL and Mcl-1, regulate the mitochondrial permeability by terminating the oligomerization of pro-apoptotic proteins (166, 167). BH3-only proteins, including Bad, Bik, Bid, Hrk, Bim, Bmf, Noxa and Puma, interact with core Bcl-2 family proteins and can promote or inhibit apoptosis.

A number of the anti-apoptotic Bcl-2 proteins are overexpressed in a large number of cancers. Bcl-2 was first identified in human follicular lymphoma, where t(14;18) translocation is directly responsible for the aberrantly high expression of *BCL-2* (165). Most B-CLL cells express high levels of Bcl-2 proteins but in the absence of any

detectable t(14;18) translocation (168-170). The mechanism of *BCL-2* overexpression involves the genetic deletion of the 13q14 locus, leading to the downregulation of *miR-15* and *miR-16*, two miRNAs that negatively regulate Bcl-2 at the post-transcriptional level (72). Myeloid Cell Leukemia sequence 1 (MCL-1) is another anti-apoptotic Bcl-2 protein that plays a crucial role in promoting cell survival in CLL cells, and targeting Mcl-1 has been shown to be sufficient to induce apoptosis in a number of B-cell tumour models, including CLL (171-174). It is unclear, however, whether Bcl-2 or Mcl-1 is the important therapeutic target in CLL. Using BH3 domain sequence peptides, cancer cells have been categorized as being "Bcl-2-dependent" *vs.* "Mcl-1-dependent" by Letai *et al*. (134). Using this BH3-profiling approach, Letai *et al*. described CLL cells as being Bcl-2 dependent (i.e. *vs*. other Bcl-2 proteins), and they reported that ABT-737, a Bcl-2 antagonist, is active against CLL cells (175). Bcl-2 is also expressed at a much higher level compared to Mcl-1 *ex vivo* in CLL cells (175), suggesting that, at least in un-stimulated CLL cells, Bcl-2 is of particular importance to cell survival. As well, CLL cells expressed Mcl-1 at lower levels even compared to tonsillar B cells from healthy donors (173). However, there is an abundance of data now suggesting that Mcl-1 is a therapeutically relevant target. siRNA-mediated knockdown of *MCL-1* in CLL cells was sufficient to reduce viability, albeit at a modest decrease of approximately 40% after 48 hours (174). Importantly, low Mcl-1 levels were associated with longer progression-free survival (in a cohort of patients treated with pentostatin, cyclophosphamide and rituximab) and overall survival (176, 177). Thus, taken together, both Bcl-2 and Mcl-1 are likely to be therapeutically relevant targets.

The Inhibitor of Apoptosis Protein (IAP) family includes cIAP-1/2 (cellular IAPs),

XIAP (X-linked), NLR family Apoptosis Inhibitory Protein (NAIP) and Survivin (178). While initially described as a direct, biochemical inhibitor of caspases, XIAP is now thought to be the only member that acts by directly inhibiting caspases (178). Genetic modulation of *XIAP* has been reported to be sufficient for modulating the level of apoptosis in a number of different cells (179-182), and Xiap has been shown to directly bind and inhibit the caspases-3, -7 and -9 via its E3 ubiquitin ligase activity (183, 184). Inhibition of apoptosis by Xiap is not limited to the intrinsic apoptotic pathway, as Xiap also suppresses apoptosis downstream of FasL-induced caspase-8 activation (185, 186). While other members of the IAP family also act to inhibit apoptosis, their ability to inhibit caspases is limited and their mechanism of action is thought to be more indirect. While survivin alone is a poor inhibitor of caspases, survivin complexes with another protein, Hepatitis B virus X-Interacting Protein (HBXIP), and the complex functions as a potent inhibitor of caspase-9 by inhibiting its interaction with Apaf-1 (187). c-Iap1/2 are considered to inhibit caspases by targeting them for proteasomal degradation (188).

Interestingly, cells have a built-in mechanism to inhibit the inhibitors of apoptosis, at least when the intrinsic apoptotic pathway has been engaged. Second Mitochondria-derived Activator of Caspase (SMAC, Diablo) is yet another mitochondrial protein that is released into the cytoplasm during apoptosis, where Smac disrupts the interaction between XIAP and caspases and induce the proteasomal degradation of Xiap (189). FLICE/Caspase-8-inhibitory proteins (c-FLIP) is another endogenous caspase inhibitor that has been associated with cancer cell survival. c-Flip shares extensive homology with procaspase-8, thereby binding as a decoy form of caspase-8 and can compete with caspase-8 for the binding to Fadd (190). CLL cells express c-Iap-1/2, Naip, Xiap,

survivin and c-Flip, and they have been found express c-Iap-2 and Xiap at higher levels compared to healthy donor counterparts (173, 191-193). The Notch signalling pathway has been reported to be constitutively active in CLL cells, leading to increased NF-κB activity and resultant increased levels of c-Iap2 and Xiap (194).

The role of different anti-apoptotic proteins become enhanced in the tumour microenvironment in which CLL cells interact with a number of different supporting cells and various soluble factors. In the lymph nodes, CLL cells up-regulate Mcl-1, Bcl-xL and Bcl-2A1, a phenomenon that can be mimicked by CD40 ligation (195, 196). Mcl-1 up-regulation is also observed in response to co-culturing of CLL cells with Mesenchymal Stromal Cells (MSCs) (197), treatment with cytokines (especially Vascular Endothelial Growth Factor (VEGF), Interleukin-2 (IL-2)) (198), adhesion of CLL cells to extracellular matrix proteins (including pro-Matrix MetalloProteinase-9 (MMP-9) and Vascular Cell Adhesion Molecule (VCAM)) (199) and, importantly, B-Cell Receptor (BCR) engagement (173, 200, 201). Xiap is up-regulated when co-cultured with human stromal HS5 cells, which also resulted in Mcl-1, but not Bcl-2, up-regulation (202). Xiap up-regulation has also been reported in response to VEGF treatment (203). While the basal *Survivin* expression level has been described to be low compared to B-cells from healthy donors (191), Survivin is up-regulated upon CD40 stimulation or co-stimulation with DSP30 and IL-2 (204, 205). Interestingly, a number of stimuli that have been reported to up-regulate Bcl-2 is rather scarce, perhaps owing to the high basal level of its expression; by the author's literature search, IL-4 appears to be the only reported inducer of Bcl-2 in CLL cells (206). The survival signalling pathways responsible for up-regulation of various anti-apoptotic proteins and the tumour microenvironment are

discussed elsewhere.

1.4.2 Cell Death – Lysosomes and Cell Death

While the role of the mitochondria in cell death has been recognized and extensively studied, lysosome is an organelle whose role in cell death, while generally recognized, remains greatly under-studied. Lysosomes were discovered in the 1950s and were discovered to play an important role in protein metabolism (Reviewed in 207, 208). Lysosomes can be discriminated from the endosomes by being more acidic (pH 4.6-5.0) and by the absence of the mannose-6-phosphate receptors. The low pH, maintained by proton-pumping vacuolar ATPases, is crucial for the activity of the lysosomal acid hydrolases. Lysosomes contain many different types of hydrolytic enzymes including proteases, lipases, nucleases, glycosidases, phospholipases, phosphatases and sulfatases that usually exert their maximal enzymatic activity at low pH. All newly synthesized acid hydrolases use a common pathway for delivery via the Golgi network, utilizing the mannose-6-phosphate tag to target the luminal proteins to the lysosome. The acidic nature of the lysosomal lumen allows for targeting of lysosomal function by various chemical agents, including chloroquine, NH_4Cl and Bafilomycin A1 (209, 210).

Another important attribute of the lysosomes is their fusogenic nature (Reviewed in 207, 208). Lysosomes are dynamic organelles that receive and degrade macromolecules from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways. Lysosomal membrane is protected from the acidic hydrolases by lysosome-specific expression of membrane proteins such as Lysosome-Associated Membrane glycoprotein 1 and 2 (LAMP-1, 2), which are heavily glycosylated and hence

resist digestion.

The importance of lysosomal activity is highlighted by lysosomal storage disorders (LSDs) (Reviewed in 211). There are over 40 LSDs that involve soluble hydrolases, which all result in the accumulation of unmetabolized substrates. Deficiency of different lysosomal enzymes leads to various clinical signs, including peripheral neuropathy, neurodegeneration, ataxia, and cardiovascular diseases. While a systemic examination of lysosomal enzymes in CLL has not been done, a few studies do report lower levels of lysosomal enzymes in CLL; levels and/or activities of lysosomal acid hydrolases and acid phosphatases were reported to be lower compared to healthy donors or other haematological malignancies (212-214).

Cathepsins comprise one family of acid hydrolases that function inside endolysosomal/lysosomal vesicles (Reviewed in 215). Cathepsins, term derived from *kata*- "down" (Greek) and *hepsein* "boil" (Greek), is a family of proteases that include aspartic proteases (Cathepsins D & E) and cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, W and X). Among them, the two aspartic proteases and four of the cysteine proteases (cathepsins B, C, H and L) are known to be ubiquitously expressed. While most cathepsins are endo-peptidases, cathepsin B has been reported to have both endo- and exo-peptidase activity (216). Like many proteases, cathepsin B is synthesized as a zymogen and must undergo post-translational processing in order to become active (216). Processing of cathepsin B involves directing of pre-pro-cathepsin B to the Endoplasmic Reticulum (ER), removal of the signal peptide, glycosylation and limited proteolysis to result in the mature single-chain form of ~27 kDa (216, 217).

While the role of lysosomal proteases in cell death is a rather less known role

compared to their better known cousins, caspases, lysosomal enzymes were among the first proteases to be associated with programmed cell death, tracing their roots to the study of tadpole tail during the 1960s (218). A number of different lysosomal enzymes have been described to mediate cell death, including cathepsin B, cathepsin L, cathepsin D, acid sphingomyelinase and acid ceramidase (219). Like the role of the mitochondria in the intrinsic apoptotic pathway which involves its permeabilization, the role of the lysosome also appears to involve some level of their permeabilization during the cell death process (218). Studies appear to deviate, however, in the degree of lysosomal permeabilization and the type of cell death that takes place in response to their permeabilization, and it is thought that the type of cell death depends on the extent of LMP and the amount and types of lysosomal proteases released (220). In a number of studies, a moderate level of lysosomal membrane permeabilization (LMP) was sufficient to induce programmed cell death, though the morphological features were non-apoptotic in a number of cases. For example, GA101 treatment of primary CLL cells induced non-apoptotic cell death that is dependent on Cathepsin B (221). Partial LMP can also result in cell death, but such events largely appear to be apoptotic and are usually associated with caspase activation. Lysosomal protease-dependent caspase-3 activation has been reported in cell death induced by various chemotherapeutic drugs, including bortezomib, camptothecin, doxorubicin and etoposide (222-226). Other agents that have been described to induce LMP include lysosomal detergents (ex. N-dodecylimidazole (227, 228)), free fatty acids and bile salts (229-231), cytokines (ex. Tumour Necrosis Factor (TNF) (232), Interferon-γ (233)) and kinase inhibitors (ex. imatinib (234), staurosporine (235)).

The exact mechanism through which the lysosomes rupture is currently unclear, though some models have been presented. Sphingosine or lysophosphatidylcholine accumulation in the lysosomal membrane is thought to directly impact the lysosomal membrane integrity by insertion into the membrane (236-238). Such a mechanism may be induced in a less direct manner, and the activation of Phospholipase A2 (PLA2) is thought to be responsible for lysophosphatidylcholine generation in some cases (238). Lysosomal accumulation of redox-active iron and lipid peroxidation chain reaction can destabilize the lysosomal membrane (239). Yet another mechanism of LMP implicates a pro-apoptotic member of the Bcl-2 family, Bax. Studies using *bax* knockout mice and a model using increased *BCL-XL* expression (thereby sequestering Bax) demonstrated that Bax is necessary for palmitate-induced LMP and death in hepatocytes (229). It is unclear, however, whether this mechanism involves direct insertion of Bax into the lysosomal membrane, as in the case of mitochondrial permeabilization. Cathepsins can also directly target Bid *in vitro* (240, 241), thereby activating the intrinsic apoptotic pathway by inducing mitochondrial permeabilization.

In short, the roles of lysosomal proteases in cell death are unclear, intriguing and complex. As a number of chemotherapeutic agents appear to be dependent on this pathway for the cell death process, further study on the lysosomal pathway is clearly warranted.

1.5 Nucleoside Analogues and CLL

Despite the CLL cells' appreciable resistance to apoptosis, F-ara-AMP (Fludarabine), a nucleoside analogue, was found to be effective *in vitro* for inducing CLL

cell death, and the introduction of fludarabine to CLL therapy regimen has resulted in an appreciable improvement in patient survival (242). Fludarabine is a nucleoside analogue, where arabinose sugar replaces the ribose sugar backbone of adenosine (242, 243). Compared to its non-fluorinated counterpart, Ara-A (Vidarabine), the fludarabine exhibits increased water solubility and increased resistance to deamination by adenosine deaminase. (242, 244). Fludarabine is a pro-drug that becomes rapidly de-phosphorylated to F-ara-A in the body by serum phosphatase, and it enters the cells via the nucleoside transport mechanism. After entering the cell, F-ara-A undergoes phosphorylation to form F-ara-ATP, the active metabolite, and is incorporated into DNA via replication and the DNA damage repair mechanisms (242, 245). Irradiation of quiescent lymphocytes with UV light in the presence of radiolabelled fludarabine resulted in detectable incorporation of fludarabine into the DNA within 2 hours of treatment (245). However fludarabine becomes incorporated, the resulting assault on the genome is the induction of DNA damage, at least part of which appears to be double stranded breaks, assessed by H2A.X phosphorylation (i.e. γH2AX induction) and comet tail formation (246, 247). The induction of DSBs is thought to depend on direct termination of DNA synthesis, be it in the process of repairing DNA or replicating DNA (248). It is imaginable, however, that actively replicating cells may incorporate a greater level of fludarabine, compared to repair-mediated incorporation observed in quiescent cells. In support of this idea, increased level of DNA degradation was observed when human T lymphoblastoid cells were synchronized in S phase before treatment with fludarabine (248). In patients, unlike unstimulated CLL cells *in vitro*, CLL cells have been shown to undergo active cell division using heavy water-mediated imaging (249), and these events

represent opportunities for enhanced fludarabine incorporation. Upon treatment with fludarabine, CLL cells respond by turning on the expression of a large number of genes as seen in a microarray study (250). Among them, a number of genes, such as *BAX*, TRAIL-receptor 2, were pro-apoptotic genes, and this response was found to be p53 dependent. Among the p53-up-regulated genes, *CASP2* (encoding Caspase-2) and its inducer *ITGB3BP*, are both up-regulated in response to fludarabine *in vivo* (251), and caspase-2 has been shown to be important for p53-mediated apoptosis (252). In CLL, the role of caspase-2 in fludarabine-induced apoptosis is unknown.

Interestingly, there is mounting evidence that Fludarabine can modulate the DNA damage repair activity. NER activity in lymphocytes has been shown to be suppressed by fludarabine in the context of cisplatin-DNA adducts and UV irradiation (245, 253). This ability to suppress DNA repair has been reported with other nucleoside analogues as well, as clofarabine (254) and gemcitabine (255) have also been reported to inhibit NER. Fludarabine is also thought to inhibit DNA damage repair in a less direct fashion as well by inhibiting Ribonucleotide Reductase, thereby reducing the pool of deoxynucleotides necessary for DNA repair (256). Fludarabine treatment also results in production of reactive oxygen species (ROS) (257), which in turn can also impact the genome integrity and produce DNA breaks. The impact of fludarabine is not restricted to DNA metabolism, as it has been shown to impact both RNA and proteins. Fludarabine has been shown to be incorporated into RNA (258), and treatment of CLL cells reduced RNA transcription, as judged by measuring [3H]-uridine incorporation (259). Fludarabine treatment also reduced protein synthesis, as judged by [3H]-leucine incorporation (259).

1.5.1 DNA Damage Repair, Drug Resistance and CLL

In cancers, defects in DNA damage repair and ensuing genomic instability have been associated with the pathogenesis of the disease (Reviewed in 260). Acquisition of mutations in the various DNA damage repair pathways has been demonstrated to be step stones toward the genesis of different cancers. Acquisition of defects in mismatch repair (MMR) process has been associated with the acquisition of microsatellite instability (MIN), while mutations in NER are associated with nucleotide instability (NIN). Hereditary non-polyposis colon cancer (HNPCC) is an example of germline mutations in a DNA damage repair pathway that predisposes patients to the eventual carcinogenesis. In CLL, there is no clear link between the acquisition of defects in DNA repair process and its genesis. Approximately ½ of the CLL cases carry the deletion of the 13q14 locus, which contains two micro RNAs, namely *miR-15a* & *miR-16-1* (16, 261). The deletion of the two miRNAs has been shown to be sufficient to induce CLL-like phenotype in mouse models (262), thus directly linking the del13q14 karyotype with CLL pathogenesis. However, the mechanism by which the 13q14 locus becomes deleted is unknown, though thought to be a product of genomic instability.

At diagnosis, some patients also may harbour deletions in two loci, 11q22-q23 locus, containing the *ATM* gene, and the 17p13 locus, containing the *TP53* gene, and del11q23 and del17p13 karyotypes are described as "high-risk genomic CLL" (263-265). The lower overall survival and treatment-free interval observed in patients with those deletions are associated the role of Atm and p53 in DNA damage repair and its appropriate response to excessive DNA damage, i.e. apoptosis (263, 264). As with other cancers, *TP53* mutations in CLL are associated with increased resistance to various

genotoxic agents, including γ-irradiation, chlorambucil and fludarabine (264, 266). The loss of ATM function results in reduced p53 phosphorylation (which occurs downstream of ATM activation) in response to ionizing radiation, chlorambucil, cyclophosphamide and fludarabine *in vitro* (264, 267). del11q karyotype in CLL was associated with worse progression-free survival in response to fludarabine plus cyclophosphamide (268). Response was greatly enhanced by the addition of Rituximab (i.e. FCR) in del11q patients, but not in del17p patients (42). Unfortunately, CLL will generally relapse after treatment, and patients can acquire mutations in *TP53*, especially following DNA-directed therapy (266). In the clinic, refractoriness to fludarabine therapy is defined as CLL that fails to respond or relapses within 6 months of fludarabine therapy (269).

While the loss of p53 function has been linked to drug resistance in CLL and other cancers, with regard to other components of the DNA damage response pathways, their activity and sometimes hyper-activity has been linked to drug resistance. Dampening the DNA damage response pathway combined with DNA-directed therapy is thus a potential strategy in cancer management. For example, a number of poly(ADP-ribose)polymerase (PARP) inhibitors are in clinical trial, either as monotherapy (exploiting the inherent DNA damage repair in cancer cells as a part of synthetic lethality strategy) or in combination with genotoxic drugs (270). CLL cells are sensitive to PARP inhibition, and PARP inhibition sensitized CLL cells to various cytotoxic agents, including fludarabine and VPA (271). It is important to note, however, that not all repair pathways are of the same importance; different pathways play the dominant role in response to different DNA-damaging agents, and different pathways are hyper-active in different cancers.

Considering that fludarabine induces DSBs (246), the DSB repair pathway is of great importance in cancer cells' tolerance of DNA damage induced by fludarabine, as well as other genotoxic agents. An interesting attribute of CLL cells is that, at least *in vitro*, CLL cells are mostly arrested in the G0/G1 phase of the cell cycle (272). Thus, the sister chromatid required for homologous recombination (HR) is lacking in most of the CLL cells, and HR cannot serve as the main mechanism of repair *in vitro*. When it comes to DSBs, rather, Non-Homologous End-Joining (NHEJ) appears to be the main mechanism of repair. Indeed, only 15 minutes after the induction of double-stranded breaks (DSBs) using γ-irradiation, NHEJ activity, including DNA-PK activity, is observed to increase in CLL cells (273). In fact, the high NHEJ activity contributes to drug resistance in CLL cells, and chemical inhibition of DNA-PK using NU7026 restored sensitivity to DNA damage-induced apoptosis (273). NHEJ has shown to be the main mechanism of repair in response to fludarabine-induced DSBs. In line with this idea, genetic manipulation of immortalized cells, either the DNA-PKcs mutant or the Ku80 mutant, resulted in increased sensitivity to fludarabine, and such enhancements were not observed in cells deficient for HR activity (274).

DNA damage induced by fludarabine is not restricted to DSBs, as Base Excision Repair (BER) has also been shown to be another important mechanism for DNA repair in fludarabine-treated cells, and F-ara-A:Thymidine mismatched base pair can be excised by uracil-DNA glycosylase *in vitro* (246). The expression level of uracil-DNA glycosylase, the initial sensor in the BER pathway, was found to be higher in CLL cells compared to healthy donor counterparts, thereby discovering a mechanism by which CLL cells can acquire increased resistance to fludarabine treatment (246). Fludarabine treatment of

CLL cell by comet assay was shown to also induce single-strand breaks (SSBs) (247), and thus both DSBR and SSBR should be of importance in determining fludarabine cytotoxicity. SSBR is active in CLL cells, but no differences in SSBR kinetics was found between normal and CLL cells after radiation-induced SSBs (275). However, another study comparing alkylator-sensitive and alkylator-resistant CLL showed more robust SSBR activity in resistant CLL (276), suggesting that SSBR may also be contributing to chemo-resistance.

Chlorambucil is a DNA-alkylating agent that is also commonly used in the CLL clinic. While chlorambucil can also cause DSBs, its initial action is to alkylate the DNA, which causes bulky DNA lesions. Such lesions would be first recognized and, potentially, repaired by the nucleotide excision repair (NER) pathway. In CLL, however, the NER activity appears to be low. NER activity was observed to be undetectable in 14/18 CLL samples examined, seven of which were from previously treated CLL patients, while the remaining 4/18 CLL samples exhibited lower levels of NER activity compared to control samples (277). As stated previously, the NHEJ pathway appears to be the more important pathway of repair in cells treated with chlorambucil, suggesting that the CLL cells may wait until the induction of DSB by chlorambucil before acting upon the lesion.

Once inside the cell and metabolized, induction of apoptosis is the main mechanism of fludarabine-induced cell death (278). It is important to note that resistance to spontaneous apoptosis is often not synonymous with resistance against cancer therapeutic agents, and the mechanism of spontaneous apoptosis and drug-induced apoptosis for many drugs differ in cancer cells. In spontaneous apoptosis of CLL cells, ER stress and the resultant caspase-4 activation have been reported to be a core driver of

spontaneous apoptosis, and caspase-9 plays only a minor role, despite the mitochondria clearly being disrupted (150). In contrast, p53-response and the subsequent activation of caspase-9 is important for fludarabine sensitivity (250, 279, 280). As caspase-9 activation is dependent on the disruption of the mitochondria and the formation of the apoptosome complex, the Bcl-2 family anti-apoptotic proteins are likely to be important for modulating the intrinsic apoptotic pathway in response to fludarabine. In a study that examined the protein levels of Bcl-2 and Mcl-1, low Mcl-1 levels, but not Bcl-2 levels, were associated with better clinical response to fludarabine monotherapy (170). BCR signalling up-regulates Mcl-1 but not Bcl-2, and this up-regulation was associated with *in vitro* resistance to fludarabine, and the up-regulation of Mcl-1 was dependent on the Phosphoinositide 3-kinase (PI3K)/Akt pathway (201). Activation of the PI3K/Akt pathway by a mitogen phorbol 12-myristate 13-acetat (PMA) also protected CLL cells from fludarabine (281). Thus, while CLL cells may depend heavily on Bcl-2 for the suppression of spontaneous apoptosis (134), targeting anti-apoptotic proteins other than Bcl-2, especially Mcl-1, is a suitable strategy in sensitizing CLL cells to drug-induced cell death.

The levels of Xiap and c-Flip are thought to be deterministic factors that modulate a cell's response to caspase-8 activation and the activation of the extrinsic apoptotic pathway. Loss of Xiap by genetic ablation or Smac-mimetic re-sensitizes *bid*-/- cells to Fas ligation (282), and Xiap also inhibits caspase-8-dependent apoptosis associated with proteo-toxicity-induced cell death and RIPoptosome-mediated cell death (146, 283). c-Flip is also thought to enhance cell's dependence on the intrinsic pathway, and combined inhibition of c-Flip and Xiap has been shown to render cells less dependent on the

intrinsic pathway (284). c-Flip also inhibits caspase-8 activation downstream of RIPoptosome formation that forms in response to genotoxic damage (145), which had been previously thought to only involve the intrinsic apoptotic pathway. While CLL cells express both c-Flip and Xiap (173, 191-193), Xiap inhibition was sufficient to enhance Fas-mediated cell death (285), whereas c-FLIP inhibition did not sensitize CLL cells to Fas ligation (286). As well, CLL cells are highly resistant to TRAIL-induced apoptosis (287), and Xiap inhibition also sensitized CLL cells to TRAIL-induced cell death (192, 288). Therefore, with regards to drugs inducing apoptosis in a caspase-8 dependent manner, Xiap inhibition is a rational adjunct strategy.

Other mechanisms of fludarabine resistance involve the processes upstream of DNA damage induction, and one such mechanism involves the modulation of intracellular fludarabine levels. CLL cells express concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) type of transporters, specifically hCNT2, hCNT3, hENT1 and hENT2, which are involved in transport of nucleosides, including fludarabine into cells (289, 290). While the expression level of each nucleoside transporter did not correlate with *ex vivo* fludarabine sensitivity, there was a correlation between the total level of fludarabine uptake and fludarabine sensitivity (289). When nucleotide transporter levels were examined at the protein levels, ex vivo sensitivity to fludarabine correlated with hENT2 protein proteins (290). After the uptake of fludarabine, fludarabine may exit the cell in an export-pump-dependent manner. CLL cells do express Multidrug Resistance genes 1, 2 and 3 (*MDR1-3*) and Multidrug Resistance-associated Proteins 1, 4, 5 and 7 (*MRP1, 4, 5, 7*), and the expression levels are comparable to that in normal B-cells (291-293). High *MDR3* expression level was

associated with high Rai stage disease (291), and MDR3 positivity was associated with lower patient survival (294). However, another study observed no correlation between *MDR1* or *MDR3* expression levels with Rai stage or clinical response to therapy, including fludarabine (295), and thus the role of fludarabine efflux in fludarabine resistance is unclear.

1.6 Survival Signals and Tumour Microenvironment in CLL

CLL cells examined *ex vivo* have been shown to have a number of signalling pathways to be constitutively active when examined *ex vivo*, by which studies generally refer to CLL samples obtained from the peripheral circulation. A much smaller number of studies have examined lymph node and bone marrow biopsies to examine CLL cells in their tumour microenvironment, and there appears to be a number of differences in the activation state of pro-survival signalling pathways, supporting the idea that the tumour microenvironment provides a certain sanctuary for CLL cells, especially in the context of chemotherapeutic therapy.

Among the pathways, the B-cell receptor (BCR) signalling has been the target of intense scrutiny in CLL, as a number of components are druggable, and preliminary results using such drugs have thus been very promising (296). CLL cells, being closely related to mature B-cells, do express BCR (6, 296). Depending on the mutational status of IgV_H, different response to IgM ligation has been reported; whereas mutated CLL show comparatively weak signalling and thought to be in anergic, unmutated CLL respond with stronger signalling, leading to antigen-dependent proliferation of CLL cells (297). Much like signalling in mature B-cells, IgM ligation in CLL cells lead to the

activation of the MAPK (ERK1/2), PI3K/Akt and NF-κB pathways (296), all of which are discussed further in this section. Formation of the BCR signalosome complex, composed of Lck/Yes-related novel protein tyrosine kinase (LYN), Spleen Tyrosine Kinase (SYK), Bruton's Tyrosine Kinase (BTK), Vav, along with adaptor proteins, Grb2 and B-cell linker (BLNK), is an important event that lies upstream of the three aforementioned signalling pathways, and many of the components of the BCR Signalosome are thought to be potential therapeutic targets (296). Syk activity and level are both elevated in CLL cells (298), and Syk inhibition using small chemical inhibitors or genetic knockdown was sufficient to reduce downstream signalling events and induce apoptosis (298, 299). Similarly, Lyn is constitutively active in CLL, and Lyn inhibitors induced apoptosis (300). Unlike Lyn and Syk, the expression level of *BTK* was found to be slightly lower in CLL compared to normal B-cells in a small study (301). Nonetheless, Btk inhibition using PCI-32765 induced apoptosis (301), and preliminary clinical trial data, thus far, is very promising, with excellent tolerability (302).

An interesting difference in BCR signalling between normal and CLL cells is the involvement of 70-kD zeta-associated protein (Zap-70). Normally a component of the T-cell receptor (TCR) signalling, *ZAP-70* is expressed in a subset of CLL patients, where more unmutated CLL cases express *ZAP-70* (24, 25). Zap-70 is thought to enhance BCR signalling independent of its kinase activity, acting as an adaptor protein (303, 304). In line with the association between Zap-70 expression and BCR signalling, Zap-70 expression is associated with poor prognosis in CLL (25, 305). Though widely used as a prognostic marker, it is unknown whether Zap-70 is a therapeutic target.

The PhosphoInositide 3-Kinase (PI3K)/Akt signalling pathway is another pathway of great clinical interest; administration of CAL-101 (GS-1101), an orally available PI3K (p110δ isoform) inhibitor has thus far resulted in promising results with excellent tolerability (306). The PI3K/Akt pathway is constitutively activated in CLL, as judged by detectable Akt phosphorylation status at both Ser473 and Thr308 (307-309), both residues that are important for the activation of Akt. In a study examining 44 CLL patient samples, Thr308 phosphorylation levels were clearly higher in CLL samples than compared to healthy donor samples (309). Ser473 phosphorylation could be readily detected in both CLL and healthy donor samples, though authors claimed the levels to be higher in CLL (309). While another study examining Akt phosphorylation status reported Akt phosphorylation (Ser473 or Thr308) to be undetectable by immunoblotting (310), this appears to be a mere technical issue, as cryopreservation was demonstrated to greatly diminish phospho-Akt detection levels (307). Akt phosphorylation has been shown to increase in response to a number of different stimulants, and these stimulants are summarized in the table below.

Table 1-3. Stimulants of the PI3K/Akt pathway in CLL.

Stimulants		References
Cytokines	CCL21	(311)
	CXCL12	(311, 312)
	CXCL13	(311)
	SDF-1α	(307),
	VEGF	(203)
Integrins	α4β1	(199)
	VCAM-1	(199, 312)
Co-Cultures	HS5	(281)
	M2-10B4	(312, 313)
	primary bone marrow-derived stromal cells	(312)
Others	Albumin	(314)
	BCR stimulation	(173, 200, 201)
	CD180 ligation	(315)
	CpG oligonucleotide	(316)
	insulin	(317)
	Lenalidomide	(318)
	LysoPhosphatidic Acid (LPA)	(319)
	Microvesicles	(320)
	Phorbol Myristate Acetate (PMA)	(307)
	TCL1 overexpression	(281)

The importance of Akt signalling has been demonstrated in a number of studies. Genetic modulation of *AKT* has been shown to be sufficient to modulate cell survival in CLL cells. *AKT* over-expression was sufficient to enhance survival by upregulating Mcl-1 and Bcl-xL, albeit the increase was modest (173). Downregulation of *AKT* using siRNA was also sufficient to reduce Mcl-1 levels and induce apoptosis (308). Chemical inhibition of Akt using small-molecule inhibitors, including A-443654, AiX, Akti-1/2 or LY294002 also reduced viability in CLL cells (281, 308, 311, 321). Interestingly, while a number of mechanisms are possible, the pro-apoptotic effects of Akt inhibition is thought to involve Glycogen Synthase Kinase 3 (GSK-3), which can phosphorylate Mcl-1, leading to its degradation via the ubiquitin-proteasome pathway (322). Inhibition of Akt is thought to result in liberation of GSK-3, allowing GSK-3 to phosphorylate Mcl-1,

resulting in its degradation. Inhibition of the PI3K/Akt pathway has also been reported to sensitize CLL cells to fludarabine treatment (323, 324).

The Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway has also been shown to be important for CLL cell survival, where among the STATs, Stat3 has been most clearly demonstrated to play a pro-survival role in CLL. In two studies examining CLL patient samples, STAT3 was found to be constitutively phosphorylated at Ser727 in CLL, while Tyr705 phosphorylation could not be detected in most patient samples (325, 326). Serine-phosphorylated STAT3 was found to be DNA-bound in the nucleus, and downregulation of STAT3 resulted in reduced expression of Stat3-regulated genes, including *BCL2* and *BCL-xL* (325). This downregulation of *STAT3* was sufficient to induce apoptosis in CLL cells, albeit at a modest level (325). Chemical reduction of Stat3 phosphorylation using JSI-124 resulted in Xiap downregulation and apoptosis (327). Increase in Stat3 phosphorylation has been reported in response to various cytokines, including IL-6 (325), IL-10 (328, 329), IL-15 (330), IL-21 (330), Interferons (331) and VEGF (332), as well as being up-regulated when co-cultured with stromal cells (202).

Nuclear Factor κB (NF-κB) pathway has been described to be constitutively active in CLL cells, where the DNA binding has been reported to be higher than healthy donor samples (333). This activity could be further enhanced by CD40 ligation (333), a stimulant that is thought to be pivotal for CLL cell survival in the lymph node. Basal DNA binding activity of a NF-κB subunit RelA was heterogeneous, and the level of binding showed a moderate, negative correlation with the level of spontaneous apoptosis (334), i.e. higher binding correlated with lower spontaneous apoptosis. Chemical

inhibition of NF-κB was sufficient to induce cell death in CLL cells (334-336). Beside CD40 ligation, activators of NF-κB include cytokines (APRIL, BAFF and VEGF)(107, 198, 337), CD180 ligation (315), and BCR activation (201).

Mitogen-Activated Protein Kinase (MAPK) pathway is yet another pathway that is constitutively active in a subset of patients. In a study of 51 patient samples, phospho-Extracellular signal-Regulated Kinase 1/2 (ERK-1/2) was detectable by immunoblotting in 49% of patients (310). As discussed above, ERK1/2 undergoes strong phosphorylation in response to BCR stimulation, and thus ERK1/2 phosphorylation used in a number of studies as a reporter for BCR stimulation. Increase in ERK1/2 phosphorylation has also been reported in response to IL-15 (330), CXCL-12 (199, 311), CXCL-13 (311), CCL-21 (338), PMA (311) and co-culturing of CLL cells with stromal cells (339). Overexpression of MAPK/ERK Kinase 2 (MEK2), the kinase that lies upstream of ERK1/2, was sufficient to enhance Erk phosphorylation and increase Xiap levels in primary CLL cells, but this was not sufficient to suppress the level of spontaneous cell death (173). Inhibition of Erk1/2 using UO126 was also not sufficient to induce a significant decrease in CLL cell viability (311). While the role of Erk1/2 on cell survival may be limited, Erk1/2 is thought to play an important role in CLL cell proliferation. IgM stimulation of CLL cells resulted in up-regulation of Myc in an Erk1/2-dependent manner, and both Myc and phospho-Erk1/2 were detectable in lymph node biopsy samples (340). Chemical inhibition of Erk1/2 using UO126 also inhibited CLL proliferation in response to CpG stimulation (340). Roles of other MAP kinases, i.e. c-Jun N-terminal kinases (JNK) and p38, in CLL cell survival are also thought to be limited. Neither chemical inhibition nor siRNA knockdown of JNK were sufficient to induce cell

death (341). Prolonged incubation of CLL cells with higher doses of p38, SB203580, did induce apoptosis, but the level of apoptosis was modest (342).

1.7 Cytokines and CLL

As glanced from the previous sections, cytokines play an important role in modulating CLL cell survival. CLL is a disease of the elderly, with the median age at diagnosis being 71.5 years, and advanced age is associated with reduced patient survival (343, 344). Data from a number of studies examining hundreds of patients suggest that such an age-related trend may be exacerbated in patients with CLL, while a number of *in vitro* studies suggest possible mechanisms by which the cytokines can contribute to disease progression (345, 346). The role of cytokines in cancer biology has been and is being extensively studied, and the field of cytokine biology is in its renaissance in cancer research, as the cancer researchers continue to gain greater insight into the role of the tumour microenvironment. In patients with CLL, cytokine-CLL cell interaction cannot be limited to the CLL cells alone but must be expanded to other cells with which CLL cells interact. CLL cells constitutively produce and secrete a number of cytokines, while some cytokines require stimulation of CLL cells for production. While decades of research cannot be summarized in a single chapter, perhaps a catalogue of information could be compiled.

1.7.1 Source of Cytokines in CLL patients

From a review of the literature, heterogeneity with regards to the method used to determine cytokine levels was noted. While most studies were performed *in vitro*, a few

in vivo studies do exist; for example, Ad-CD154-transduced cells were infused into patients with CLL and measured the cytokines in the plasma (347). Among the *in vitro* studies, some studies resorted to measuring the mRNA levels, while most others measured the secreted (i.e. plasma/serum) protein levels by Enzyme-Linked ImmunoSorbent Assays (ELISA). With the greater understanding of the tumour microenvironment, more studies are being done where CLL cells are co-cultured with other cell types, where the exact source of cytokine secretion cannot be discerned between the cells in the culture system. Unless specifically stated as being a co-culture study, the referenced study was done *in vitro* using CLL cell mono-cultures. Also, a number of cytokines are also reported to be up-regulated in response to different stimuli, as noted in the table below.

There is great heterogeneity also in terms of amount of research done on each cytokine. While certain inflammatory cytokines such as TNF-α and IL-6 have been extensively examined, the literature was limited with regards to certain cytokines, where, for example, research on effects of IL-9 was limited to a review citing unpublished data (348). There were a number of cytokines for which no references could be found by the author, and the table below is restricted to cytokines where references could be found. Finally, conflicting findings are also noted between different studies.

Table 1-4. Cytokines produced by CLL cells and known stimulants

Cytokine	Cells of Origin/Stimulant	References
IL-1α	CLL cells	(349, 350)
	not expressed by CLL cells (n = 10)	(351)
	Staphylococcus aureus Cowan strain I (SAC), phorbol ester	(349)
IL-1β	CLL cells	(350-352)
	up-regulated in CD38+ CLL cells	(353)
	TLR7 agonist	(354)
IL-2	T-cells in CLL PBMCs	(355, 356)
IL-3	not expressed by CLL cells (n = 10)	(351)
IL-4	not expressed by CLL cells (n = 10)	(351)
IL-5	not expressed by CLL cells (n = 9)	(351)
IL-6	CLL cells	(351, 357, 358)
	class B CpG ODN	(359)
	TLR7 agonist	(354)
	CD160 activation	(360)
	co-culture with HS-5	(361)
	endothelial cells	(362)
	co-culture with nurse-like cells	(363)
	adherent marrow stromal layer	(364)
	Ad-ISF35 injection*	(365)
IL-7	CLL cells	(366)
	not expressed by CLL cells (n = 9)	(351)
	endothelial hybrid cells	(366)
IL-8	CLL cells	(351, 367)
	Lymph node CLL cells	(367)
	TLR7 agonist	(354)
	co-culture with HS-5	(361)
	co-culture with S17	(346)
IL-9	T-cells	(368)
IL-10	CLL cells	(369-371)
	TLR7 agonist	(354)
	class B CpG ODN	(359, 371)
	infusion of Ad-CD154-CLL cells	(347)
IL-11	CLL cells	(372)
IL-12	CLL cells	(369)
	infusion of Ad-CD154-CLL cells*	(347)
IL-13	CLL cells	(370)
IL-16	CLL cells	(373)
IL-18	expressed but not produced by CLL cells	(374)
IL-24	CLL cells express & produce intracellular IL-24	(342)

Table 1--4 (continued).

Cytokine	Cells of Origin/Stimulant	References
CCL1	Not expressed by CLL cells (n = 12)	(367)
CCL2 (MCP-1)	CLL cells	(375)
	up-regulated in CD38+ CLL cells	(353)
	Monocytic production induced by CLL cells	(361)
	co-culture with HUVEC	(376)
	co-culture with nurse-like cells	(363)
CCL3 (MIP-1α)	CLL cells	(367)
	anti-IGM	(363, 377)
	co-culture with HS-5	(378)
	co-culture with nurse-like cells	(363)
CCL4 (MIP-1β)	anti-IGM	(363, 377)
	co-culture with HS-5	(361, 378)
	T-cells express higher levels in CLL	(379)
	co-culture with nurse-like cells	(363)
CCL5 (RANTES)	CLL cells	(367)
	T-cells express higher levels in CLL	(379)
CCL7 (MCP-3)	CLL cells	(363)
	co-culture with nurse-like cells	(363)
CCL8 (MCP-2)	co-culture with HS-5	(378)
CCL17 (TARC)	Lymph node CLL cells	(367)
CCL18 (DC-CK1)	Not expressed by CLL cells (n = 12)	(367)
CCL19 (MIP-3β)	Not expressed by CLL cells (n = 12)	(367)
CCL20 (MIP-3α)	Not expressed by CLL cells (n = 12)	(367)
	co-culture with HS-5	(378)
CCL21 (6Ckine/SLC/exodus-2)	Not expressed by CLL cells (n = 12)	(367)
CCL22 (MDC)	CLL cells	(363, 367)
	CD40 ligation	(367)
	co-culture with nurse-like cells	(363)
CCL24 (MPIF-2)	co-culture with HS-5	(378)
CCL25 (TECK)	Not expressed by CLL cells (n = 12)	(367)
CXCL1	co-culture with HS-5	(361)
CXCL2	CLL cells	(375)
CXCL3	up-regulated in CD38+ CLL cells	(353)
CXCL7	co-culture with HS-5	(378)
CXCL8	CLL cells	(367)
CXCL9	Not expressed by CLL cells (n = 12)	(367)
CXCL10	Not expressed by CLL cells (n = 12)	(367)
	TLR7 agonist	(354)
CXCL12/SDF-1	nurse-like cells	(380)
	Not expressed by CLL cells (n = 12)	(367)

Table 1--4 (continued).

Cytokine	Cells of Origin/Stimulant	References
CXCL13	nurse-like cells	(380)
	Not expressed by CLL cells (n = 12)	(367)
XCL1	T-cells express higher levels	(379)
XCL2	T-cells express higher levels	(379)
CX3CL1	detectable in lymph node CLL cells	(381)
	Not expressed by CLL cells (n = 12)	(367)
TNF-α	CLL cells	(279, 351, 358, 363)
	TLR7 agonist	(354)
	CD40 ligation	(279)
	class B CpG ODN	(359)
	co-culture with nurse-like cells	(363)
VEGF	CLL cells	(320, 382, 383)
	up-regulated in CD38+ CLL cells	(353)
	hypoxia	(383)
	class B CpG ODN	(359)
	co-culture with HUVEC (VEGF-C)	(376)
TGF-β	CLL cells	(358)
APRIL	CLL cells	(384)
	nurse-like cells	(337)
BAFF	CLL cells	(384)
	nurse-like cells	(337)
IFN-α	CLL cells	(383)
IFN-γ	CLL cells	(385)
	T cells	(356)
	TLR7 agonist	(354)
	infusion of Ad-CD154-CLL cells	(347)
	Ad-ISF35 injection*	(365)
IGF-1	CLL cells	(386)
GM-CSF	not expressed by CLL cells (n = 10)	(351)
G-CSF	CLL cells	(386)

*performed *in vivo*; **inferred from physiological phenomena

1.7.2 Expression of Cytokine Receptors in CLL

For a given cytokine to impact the CLL cells directly, the cells of interest must harbour the receptors for those cytokines. Cross-reactivity can occur, and this may be an issue in the *in vitro* studies where the doses of cytokines provided are generally in supra-

physiological ranges. In the body of patients with CLL, even in cases where the cytokine levels are elevated, cytokines are present at lower levels, and cross-reactivity is much less likely to be an issue. A number of studies have examined the expression of receptors for various cytokines in CLL, and a comprehensive compilation of these studies was attempted, as shown in the table below. Once again, because much of the research has been done on certain cytokines decades before the present time, certain technical limitations are noted, and the interpretation becomes a challenge in compiling the data. For example, a number of the cytokine receptors have multiple subunits, a fact that was not dissected out in older studies. It was thus noted that some older studies simply describe the receptor for a cytokine, while the more recent studies report the expression pattern of the specific subunits. Also, it is important to note that only a small number of studies have reported the expression of many cytokine receptors.

Table 1-5. Cytokine Receptor Expression on CLL cells

Cytokine	Receptor	Expression on CLL cells	References
IL-2	IL-2R	Some	(352, 387)
	IL-2Rα	Yes (3/3)	(388)
	IL-2Rα (CD25)	Yes – high in subset (46/281)	(389)
	soluble IL-2R	Yes – high in CLL	(390)
	γc/IL-2Rγ/CD132	Yes	(330)
IL-3	IL-3R (IL-3Rα/CD123 + CSF2RB/CD131)	Yes	(391)
IL-4	IL-4Rα	Yes	(392)
	γc/IL-2Rγ/CD132	Yes	(330)
IL-6	IL-6R	Yes – lower in CLL	(393)
	soluble IL-6R	increased in CLL	(393, 394)
	gp130	Yes	(393)
IL-7	IL-7Rα/CD127	Yes – lower in CLL	(395)
	γc/IL-2Rγ/CD132	Yes	(330)
IL-8	CXCR1/IL-8Rα	CXCR1 (3/13)	(396)
	CXCR2/IL-8Rβ	Not expressed (n = 13)	(396)
IL-9	IL-9R	No (n = ?)	(348)
IL-10	IL-10R (α, β)	Yes	(328)
IL-11	IL-11Rα	Yes	(372)
IL-12	IL-12β1	Yes (15/15)	(397)
	IL-12β2	No	(397, 398)
IL-13	IL-13Rα1	(inferred to express)*	
	IL-4Rα	Yes	(392)
IL-15	IL-15Rα	Low expression (6/6), up-regulated by CD40	(330)
	IL-2R (β)	Yes (3/3)	(388)
	γc/IL-2Rγ/CD132	Yes	(330)
IL-16	CD4	No	
IL-18	IL-18Rα, IL-18Rβ	Yes (12/12)	(374)
IL-21	IL-21R	expressed in majority	(330)
		up-regulated by CD40	(330)
	γc/IL-2Rγ/CD132	Yes	(330)
IL-24	IL22R, IL20R1, IL20R2	Yes	(342)
CCL2 (MCP-1)	CCR2	CCR2 (6/13)	(396)
CCL3 (MIP-1α)	CCR1, CCR5	CCR1 (9/13), CCR5 (1/13)	(396)
CCL4 (MIP-1β)	CCR5	CCR5 (1/13)	(396)
CCL5 (RANTES)	CCR1, CCR3, CCR5	CCR1 (9/13), CCR3 (13/13), CCR5 (1/13)	(396)

Table 1--5 (continued)

Cytokine	Receptor	Expression on CLL cells	References
CCL7 (MCP-3)	CCR1, CCR2, CCR3	CCR1 (9/13), CCR2 (6/13), CCR3 (13/13)	(396)
CCL8 (MCP-2)	CCR3	CCR3 (13/13)	(396)
CCL11 (Eotaxin)	CCR3	CCR3 (13/13)	(396)
CCL13 (MCP-4)	CCR2, CCR3	CCR2 (6/13), CCR3 (13/13)	(396)
CCL14 (HCC-1)	CCR1	CCR1 (9/13)	(396)
CCL15 (HCC-2)	CCR1, CCR3	CCR1 (9/13), CCR3 (13/13)	(396)
CCL16 (HCC-4)	CCR1	CCR1 (9/13)	(396)
CCL17 (TARC)	CCR4	Yes – lower in CLL	(399)
CCL19 (MIP-3β)	CCR7	Yes	(400)
CCL20 (MIP-3α)	CCR6	Yes (13/13)	(396)
CCL21 (6Ckine/SLC/exodus-2)	CCR7	Yes	(400)
CCL22 (MDC)	CCR4	Yes – lower in CLL	(399)
CCL23 (MPIF-1)	CCR1	CCR1 (9/13)	(396)
CCL24 (MPIF-2)	CCR3	CCR3 (13/13)	(396)
CCL25 (TECK)	CCR9	Yes – low expression	(401)
CCL26 (Eotaxin-3)	CCR3	CCR3 (13/13)	(396)
CCL20/MIP-1-α MIP-1-β RANTES	CCR5	No (0/23)	(402)
		Yes (1/13)	(396)
CCL20	CCR6	Yes (13/18)	(402)
		Yes (13/13)	(396)

Table 1--5 (continued)

Cytokine	Receptor	Expression on CLL cells	References
CXCL1	CXCR2 > CXCR1	CXCR1 (3/13)	(396)
		No CXCR2 (0/13)	(396)
CXCL2	CXCR2/IL-8Rβ	No (0/13)	(396)
CXCL3	CXCR2/IL-8Rβ	No (0/13)	(396)
CXCL4	CXCR3	Yes (13/13 (396))	(396, 403, 404)
CXCL5	CXCR2/IL-8Rβ	No (0/13)	(396)
CXCL6	CXCR1/IL-8Rα	CXCR1 (3/13)	(396)
	CXCR2/IL-8Rβ	No (0/13)	(396)
CXCL7	CXCR2	No (0/13)	(396)
CXCL8	CXCR1, CXCR2	CXCR1 (3/13)	(396)
CXCL9	CXCR3	Yes (13/13 (396))	(396, 403, 404)
CXCL10	CXCR3	Yes (13/13 (396))	(396, 403, 404)
CXCL11	CXCR3	Yes (13/13 (396))	(396, 403, 404)
CXCL12/SDF1	CXCR4	Yes (23/23 (402), 13/13 (396))	(396, 402, 405-407)
CXCL13/BCA-1	CXCR5	Yes (23/23 (402), 13/13)	(380, 396, 402, 404)
CXCL16	CXCR6	Yes	(401)
CX3CL1	CX3CR1	Yes	(381)
BAFF	BR3	Yes	(107)
Interferon-α	IFNAR1	Yes	(408)
	IFNAR2		
TNF	TNF-R1, TNF-R2	Yes	(279, 391, 409)
VEGF	VEGFR1	Yes (4/5(383))	(332, 383)
	VEGFR2	Yes (5/5(383))	(332, 383)
GM-CSF	(IL-3Rα/CD123 + CSF2RB/CD131)	Yes. Up-regulated by IL-2.	(391)
G-CSF	G-CSF-R/ CD114	Yes. Up-regulated by IL-2.	(391)
M-CSF	M-CSF-R/ CD115	Yes (6/10)**	(410)
Insulin	INSR	Yes (app. 60%)	(317)
IGF-1	IGF-1R	Yes (12/27)	(386)

*(inferred to express) indicates no known reports specifically examining receptor expression, while *in vitro* effects with isolated CLL cells have been reported

** Japanese patients examined

1.7.3 *In Vitro* Effects of Cytokines on CLL

For a given cytokine, the downstream events are often pleiotropic, activating a number of signalling pathways. For example, the Interleukin-6 receptor (IL-6R) is present in the body as both membrane-bound and soluble forms, and ,upon their ligation with IL-6, IL-6R will bind gp130, leading to activation of the receptor-associated kinases, Jak1, Jak2 and Tyk2 (411). The activated kinases phosphorylate transcription factors STATs, including Stat3. Phosphorylated Stat3 can translocate to the nucleus, whereupon the its binding to DNA will facilitate transcription of numerous oncogenic and pro-inflammatory genes (412). Beside JAK/STAT signalling, other signalling pathways downstream of IL-6 include the Ras/MAPK and the PI3K/Akt signalling pathways (411). IL-6 can initiate its own negative feedback pathway by activating transcription of Suppressors Of Cytokine Signalling (SOCS) genes (413). Upon their expression, SOCS proteins bind to gp130 or phosphorylated JAKs, switching off the IL-6 signalling. An important focus in translational research, however, is the net effect of the cytokine stimulation and their impact on disease progression by impacting cell survival, proliferation and invasive behaviour. The table below is a compilation of the reported effects of the different cytokines on CLL cells as examined *in vitro*.

Table 1-6. *In vitro* effects of cytokines on CLL cells

Cytokine	*in vitro* effects	References
IL-2	↑proliferation	(414)
	↑colony formation in PHA/TPA-co-stimulated CLL cells	(387)
	↓spontaneous apoptosis	(415, 416)
IL-3	no induction of proliferation	(391)
IL-4	↓spontaneous apoptosis	(379)
	↓IL-2-induced proliferation	(417)
	no induction of proliferation	(418)
IL-5	↑apoptosis	(419)
IL-6	↓spontaneous apoptosis	(362, 375)
	no change in spontaneous apoptosis	(415)
IL-8	↓spontaneous apoptosis	(375, 420)
	↓hydrocortisone-induced apoptosis	(420)
IL-10	↓spontaneous apoptosis	(328)
	↓TPA-induced proliferation	(328)
	↓hydrocortisone-induced apoptosis	(328)
	↓proliferation in response to PMA and PMA/anti-Ig	(421)
	↑proliferation, differentiation (↑IL-2R)	(422)
IL-11	morphologic alterations, ↑cell size, ↑cell number	(372)
IL-13	↓spontaneous apoptosis	(418, 423)
	no change in spontaneous apoptosis	(415)
	↑CD40 expression, ↑apoptosis in response to CD40 ligation & anti-CD95 mAb	(424)
	↑proliferation	(388)
	no induction of proliferation	(418)
	↓IL-2-induced proliferation	(418)
IL-24	no change in spontaneous apoptosis	(342)
	↓apoptosis in response to p38 inhibition	(342)
	↑apoptosis combined with IL-2	(425)
	reduced S/G2/M in response to IL-2	(425)
IFN-α	↓spontaneous apoptosis	(427, 428)
	variable effects on proliferation	(428)
IFN-γ	↓spontaneous apoptosis	(328, 385)
Insulin	↓spontaneous apoptosis	(317)
VEGF	↓spontaneous apoptosis	(203, 332)
G-CSF	no induction of proliferation	(391)
GM-CSF	↑proliferation	(391)
TNF-α	no change in spontaneous apoptosis	(415)

Table 1--6 (continued)

Cytokine	*in vitro* effects	References
CCL2	↑cell survival (PBMCs, but not purified CLL cells)	(375)
CCL3	↑migration	(396)
CCL5	↑migration	(396)
CCL19	↓cell death in response to TNF when combined with CXCL13	(426)
	↑migration	(400)
CCL20	↑migration	(396)
CCL21	↓spontaneous apoptosis	(311)
	↑migration	(400)
CCL23	↑migration	(396)
CCL24	↑migration	(396)
CX3CL1	↑migration	(381)
CXCL12	↓spontaneous apoptosis	(311, 375)
	↑migration	(363, 377, 400)
CXCL13	↓spontaneous apoptosis	(311)
	↓cell death in response to TNF when combined with CCL19	(426)
	↑migration	(363, 377, 380)
CX3CL1	↑migration (select patients)	(381)

1.7.4 Clinical Significance of Cytokines in CLL

Among all the different soluble factors measured in the plasma of patients with CLL, β_2-microglobulin remains the most widely examined laboratory investigation for CLL (26). However, a substantial number of other soluble factors, especially cytokines, have been shown to have significant prognostic value, even by multi-variate analyses. Because the laboratory investigations for soluble factors can be easily automated and standardized, there is a potential value in searching for a soluble factor or a combination of soluble factors that can be used in CLL. In line with this idea, a recent study by Yan *et al*. examined the value of multi-cytokine panels (429), while this study examined the prognostic value of specific cytokines in sub-cohorts of patients (i.e. patients with advanced age). Some of the cytokines listed are performed as part of the standard

investigation for other diseases, and thus re-examination of the value of at least some of the cytokines is warranted. Finally, discrepancies between studies were noted. For example, high IL-6 levels had been shown to be associated with lower overall survival using mono-cytokine ELISA methods (345, 346, 430), an observation that was not seen in the study by Yan *et al*. using a multiplex sandwich immunoassay-based protein array system (429). While the reason for discrepancies is unclear, differences in sensitivity of the assays and small cohort sizes may be a factor. Even the larger studies are limited to 100-200 patient samples, and thus it may be of interest for the CLL research community to coordinate their efforts to examine a multi-centre cohort to identify cytokines, either alone or in combination, that can better predict patient survival and response to therapy. Finally, for the purpose of simplicity, "serum" and "plasma" were used as interchangeable terms in the table.

Table 1-7. Clinical significance of cytokines in CLL patients

Cytokine	Clinical Correlates	References
IL-1α	serum levels elevated in CLL	(431)
IL-1β	serum levels elevated in CLL patients	(429)
	serum levels lower in CLL patients IL-1β levels were closer to normal in those patients who had non-progressive disease	(432)
	IL-1 receptor antagonist levels lower in CLL	(432)
	high serum levels (> 6.2 pg/mL) associated with better OS	(429)
	no significant difference in TTFT	
IL-2	serum levels elevated in CLL patients	(433)
	no significant difference in OS, TTFT	(429)
IL-3	serum levels elevated in CLL patients	(433)
IL-4	elevated PHA-stimulated IL-4 production in MS-DAT positive patient samples	(434)
	no significant difference in OS, TTFT	(429)
	IL4 polymorphism associated with ↓mortality	(435)
IL-5	serum levels elevated in CLL patients	(429)
	no significant difference in OS, TTFT	(429)
IL-6	serum levels elevated in CLL patients	(345, 375, 429, 433)
	high serum levels (> 3 pg/mL) associated with shorter OS	(346, 430)
	high serum levels (> 4.3 pg/mL) associated with shorter OS	(345)
	no significant difference in OS, TTFT	(429)
IL-7	serum levels elevated in CLL patients	(433)
	no difference in serum levels between CLL & HD	(407)
IL-8	serum levels elevated in CLL patients	(346, 375)
	high serum levels (> 7.4 pg/mL) associated with shorter OS	(346)
	high serum levels (> 26.2 pg/mL) associated with shorter OS	(436)
	no significant difference in OS, TTFT	(429)
	IL8RB polymorphism associated with ↑mortality	(435)
IL-10	serum levels elevated in CLL patients	(345, 429, 437)
	high serum levels (> 10 pg/mL) associated with shorter OS	(345)
	high serum levels (> 3.6 pg/mL) associated with shorter TTFT	(429)
	no significant difference in OS	
	high serum TNF-α + IL-10 associated with shorter OS, TFS	(437)
	IL10 polymorphism associated with ↓mortality	(435)

Table 1--7 (continued)

Cytokine	Clinical Correlates	References
IL-12	serum levels elevated in CLL patients	(429)
	low serum levels (< 145 pg/mL) associated with shorter TTFT	
	no significant difference in OS	
IL-13	serum levels elevated in CLL patients	(433)
IL-15	serum levels elevated in CLL patients	(433)
	no difference in serum levels between CLL & HD	(429)
	no significant difference in OS, TTFT	
IL-16	carrier status for *IL16* P434S SNP associated with better PFS	(438)
IL-17	serum levels elevated in CLL patients	(429)
	no significant difference in OS, TTFT	
IL-19	homozygosity for *IL19* S213F SNP associated with a worse PFS	(438)
IL-24	serum levels lower in CLL patients	(342)
TNF-α	serum levels elevated in CLL patients	(429, 433, 437, 439)
	high serum levels (> 14 pg/mL) associated with shorter OS	(439)
	no significant difference in OS, TTFT	(429)
	high serum TNF-α + IL-10 associated with shorter OS, TFS	(437)
IGF-1	serum levels elevated in CLL patients	(433)
	high serum levels (> 93 ng/mL) associated with shorter PFS in Binet A patients	(440)
APRIL	serum levels elevated in CLL	(441, 442)
	high serum levels (>1.7851 ng/mL) associated with shorter TTFT	(442)
VEGF	no difference in serum levels between CLL & HD	(443)
	high serum levels (>194.8 pg/mL) associated with shorter PFS	
G-CSF	serum levels elevated in CLL patients	(433)
GM-CSF	no significant difference in OS, TTFT	(429)
IFN-α	serum levels elevated in CLL patients	(429)
	no significant difference in OS, TTFT	(429)
	recombinant α2-interferon (IFN-α2a) reduced transient ALC reduction	(444)
IFN-γ	serum levels elevated in CLL patients	(433)
	no significant difference in OS, TTFT	(429)
	IFNGR1 polymorphism associated with ↓mortality	(435)
	IFNGR2 polymorphism associated with ↑mortality	(435)

Table 1--7 (continued)

Cytokine	Clinical Correlates	References
Insulin	high receptor expression levels associated with shorter OS & TTFT	(317)
CCL2	serum levels elevated in CLL patients	(429, 433)
	no difference in serum levels between CLL & HD	(375)
	no significant difference in OS, TTFT	(429)
CCL3	serum levels elevated in CLL patients	(429)
	high serum levels (> 35.5 pg/mL) associated with shorter TTFT	(429)
	no significant difference in OS	(429)
CCL4	serum levels elevated in CLL patients	(429)
	low serum levels (< 171 pg/mL) associated with shorter TTFT	(429)
	no significant difference in OS	(429)
CCL5	serum levels lower in CLL patients	(407)
CCL11	no difference in serum levels between CLL & HD	(429)
	no significant difference in OS, TTFT	(429)
CCL17	serum levels elevated in CLL patients	(429)
	no significant difference in OS, TTFT	(429)
CCL19	high serum levels (> 108 pg/mL) associated with lower OS	(429)
	no significant difference in TTFT	(429)
CXCL2	serum levels elevated in CLL patients	(375)
CXCL9	serum levels elevated in CLL patients	(429, 433)
	no significant difference in OS, TTFT	(429)
CXCL10	no difference in serum levels between CLL & HD	(429)
	high serum levels (> 83 pg/mL) associated with lower OS	(429)
	no significant difference in TTFT	(429)
CXCL11	serum levels elevated in CLL patients	(429)
	high serum levels (> 107 pg/mL) associated with lower OS	(429)
	no significant difference in TTFT	(429)
CXCL12	serum levels lower in CLL patients	(407)
	serum levels elevated in CLL patients	(433)
CXCL13	serum levels elevated in CLL patients	(380)

OS = Overall Survival; TTFT = Time-To-First Treatment; PFS = Progression-Free Survival

SNP = Single Nucleotide Polymorphism

1.8 Thesis Rationale, Hypothesis and Objectives

As patients progress through the CLL disease course, patients develop resistance to fludarabine, and, as the disease is chronic, patients continue to age. Old and frail patients that develop resistance to therapy are arguably the most difficult patients to manage in the clinic, and new, better-tolerated therapies are needed. In a population study, advanced age was an important factor related to patient survival (344), and the impact of patient age on CLL survival was further examined. Considering the age-related increase in inflammatory cytokines in the general population (445), it was reasoned that cytokines may also be contributing to decreased survival in older patients with CLL.

To manage these patients, it was reasoned that Valproic Acid (VPA), a widely used anti-epileptic with HDAC-inhibiting activity, would be useful in elderly patients. Per Oral formulation, with high bio-availability, makes VPA easy to administer to patients, and doses of 20-25 mg/kg/day are commonly used in adolescents and adults (446, 447). Valproate is well absorbed after an oral dose, with bioavailability greater than 80%. As an anti-epileptic, VPA has a long history in the clinics (447), and it was reasoned that side effects would be predictable and manageable and reasoned that VPA may be useful as either mono- or combined therapy with standard agents, including fludarabine.

Hypothesis: Inferior survival seen in elderly patients with CLL is associated with increased levels of inflammatory cytokines, and the addition of Valproic Acid, an HDAC inhibitor, would be a helpful adjunct to fludarabine therapy in elderly patients that would be well tolerated.

Objectives:

1. Examine the association of between patient survival and inflammatory cytokines, and examine the mechanism of their effects on CLL.
2. Determine the pharmacological interaction between VPA and Fludarabine, and examine the mechanism by which the pharmacological interaction is achieved.

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2. ASSOCIATION OF INTERLEUKIN-6 AND INTERLEUKIN-8 WITH POOR PROGNOSIS IN ELDERLY CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Running short title: Cytokines in Chronic Lymphocytic Leukemia

Key words: Chronic Lymphocytic Leukemia, IL-6, IL-8, TNF-alpha, Beta 2-Microglobulin

Condensed abstract:
The study demonstrates that the clinical course of CLL differs in the elderly vs. younger patients, with elderly patients having poorer prognosis, while dying of similar CLL-related causes. This difference may be related to increased levels of the age-related cytokines in the elderly, which may influence the CLL microenvironment.

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JY, SL, DD and SL performed the research.
CM performed pathological tests.
SBG and JBJ designed the research study.
RK and JJ contributed patient recruitment.
AM and SG contributed to data analysis.
JY wrote the paper which was adapted by SB and JBJ.
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2.1 ABSTRACT

In population studies, the relative survival in chronic lymphocytic leukemia (CLL) decreases with age. In this study, we have demonstrated in a cohort of 189 patients from a CLL clinic that overall survival was lower in the sub-cohort of patients aged ≥70 years, but causes of deaths were similar for all age groups, being progressive CLL, secondary malignancies and infections. As normal individuals age, the plasma levels of inflammatory cytokines, such as IL-6 and IL-8, can increase. In our CLL patients, IL-6, IL-8 and TNF-α levels increased with age to a greater degree than in normal individuals, and the levels correlated closely with the plasma β_2-Microglobulin and with one another. In addition, in patients ≥70 years, IL-6 was found to be a better prognostic marker than IgV_H status. *In vitro* studies demonstrated that IL-6 and IL-8 could enhance the binding of CLL cells to stromal cells suggesting that their clinical activity may be mediated through their effects on the microenvironment. Thus, plasma IL-6 is an important prognostic marker for the elderly with CLL, and this study highlights that the utility of prognostic markers may depend on patient age.

2.2 INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a disease of the elderly, with the median age at diagnosis being 71.5 years.(1, 2) Clinical course is heterogeneous, ranging from never requiring therapy to aggressive disease that leads to early death. Consequently, there is great interest in developing biological markers to predict progression, response to therapy and survival. Many of these markers assess cellular changes, with markers of poor prognosis being the absence of mutations in the variable portion of the heavy-chain immunoglobulin gene (IgV_H), zeta-associated protein (ZAP)-70 positivity, CD38 positivity, and the presence of del 11q22-23 or del 17p13.(3-6) Other prognostic markers measured in the plasma include CD23, β_2-microglobulin or inflammatory cytokines.(7-9) The inflammatory cytokines, interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) may be elevated in some patients with CLL, and increased levels of these cytokines have been associated with poor prognosis,(10-13) which may related to their abilities to suppress apoptosis.(10-15)

Despite the fact that the majority of CLL patients are elderly, few studies have focused on this age group. The relative survival of the CLL population, i.e., survival as compared to an age- and sex-matched control population, progressively worsens as patients age beyond 70 years,(1, 2) suggesting that elderly CLL patients in the population are dying more rapidly than younger patients from CLL or related causes. However, whether relative survival decreases with age in CLL clinics is unclear. At the MD Anderson Cancer Center (Houston, Texas), age over 65 years was a poor prognostic feature with the causes of death in two-thirds of patients (regardless of age) being progressive CLL, second malignancies and infections.(16, 17) A multicenter study

showed that for patients ≥80 years at diagnosis, survival also decreased with age, although 40% of patients in this age group died from cardiovascular causes (CVD).(18) A recent study by Shanafelt *et al.* (19) evaluated the cellular prognostic markers and age in CLL which suggested that these markers were less useful as markers for survival in patients older than 75 years than in younger patients. These data would suggest that factors extrinsic to the leukemic cells may be influencing survival in these older patients. In the general population, the plasma levels of the cytokines (including IL-6 and TNF-α) are increased in some individuals as they age beyond 65 years, and increased levels are associated with frailty and decreased survival related to cardiovascular disease.(20-22) As CLL cells may also produce these cytokines,(23, 24) we have evaluated these age-related cytokines in CLL patients of different age groups and determined whether elevated levels are predictive of survival or of the cause of death in CLL.

2.3 MATERIALS AND METHODS

2.3.1 Patient Selection and Clinical Data

The diagnosis of CLL was made by peripheral blood morphology and the presence of monoclonal B cells in the peripheral blood with typical immunophenotype ($CD19^+$, $CD5^+$ and $CD23^+$). As the median age of CLL patients at diagnosis in the clinic was found to be 64 years, samples were obtained in a sequential order from the Manitoba CLL Tumor Bank to reflect the age and sex-distribution observed in Manitoban population.(2) All patients were off treatment for at least six months prior to study. Thirty seven age- and sex-matched healthy donors were used as controls for IL-6 and IL-8 levels. Plasma samples isolated from peripheral blood samples were banked in -80°C. All

clinical data presented are values at or close to the date of study, and all survival data are reported from the date of study to censor dates. Death from progressive CLL was defined as death that occurred in a patient with increasing lymphocytes and or lymphadenopathy/splenomegaly, despite therapy. Frequently these patients had required red cell and/or platelet transfusions.

2.3.2 Enzyme-Linked Immunosorbent Assays For Measuring IL-6, IL-8 and TNF-α

Patient plasma samples and supernatants from *in vitro* experiments were analyzed by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Quantikine®). The values were measured in triplicates and mean concentrations were determined from standards provided.

2.3.3 Measurement of β_2-microglobulin, CD38 and ZAP-70 Status

Serum β_2-microglobulin level was measured by a micro-particle enzyme immunoassay (Abbott Diagnostics). ZAP-70 and CD38 positivity was measured by staining with anti-CD5-PE, anti-CD19-PE-Cy7 and anti-CD38-PE-Cy5 (all from Beckman Coulter) for 5 min at room temperature. Cells were subsequently fixed using IntraPrep™ Reagent 1 (Beckman Coulter) for 15 min at room temperature. After washing, cells were permeabilized with IntraPrep™ Reagent 2 (Beckman Coulter) for 5 min at room temperature. Anti-ZAP-70-FITC (Beckman Coulter) was added to the cells for 15 min at room temperature. After washing, cells were analyzed by flow cytometry. Patients were considered CD38 and/or ZAP-70 positive if ≥20% cells stained positively.

2.3.4 Mutational Status of the Immunoglobulin Variable Heavy Chain Gene

The IgV_H mutational status was analyzed as previously described.(25, 26) Briefly, RNA was isolated using commercially available reagents (Invitrogen and Qiagen) and cDNA synthesized using FirstStrand reverse transcriptase (GE). PCRs were performed using published primers, amplifying VH1-7 and β-actin.(25) The obtained bands were isolated and sequenced. ≥2% mutation was identified as mutated.

2.3.5 Statistical Analysis

Associations between patient characteristics for pairs of numerical, continuous variables were assessed by Spearman rank correlation. Associations between categorical and continuous variables were assessed by either the Mann-Whitney or the Kruskal-Wallis test. Associations between two categorical variables were assessed by Fisher's exact test. Overall survival was used to assess the clinical outcome, analyzed using the Kaplan-Meier method, and log-rank tests were performed to calculate the difference between two curves. In all analyses, *p*-value of 0.05 was used as the cut-off for significance.

2.3.6 Cell Culture and Treatment Conditions

Peripheral blood samples were obtained from normal individuals and CLL patients following informed consent, in agreement with the Research Ethics Board at the University of Manitoba. Mononuclear cells were isolated from the buffy coat using a Ficoll-Paque density gradient as previously described.(27) Freshly isolated lymphocytes

were cultured at 37°C in a humidified, 5% CO_2 incubator at an initial density of 5 x 10^6 cells/mL in RPMI-1640 culture medium supplemented with 100 U of penicillin, 100 mg of streptomycin and 10% fetal bovine serum (FBS). Recombinant IL-6 or IL-8 (PeproTech Inc.) were reconstituted in sterile phosphate-buffered saline (PBS; pH, 7.4) solution and used at 50 ng/mL (final concentration).

2.3.7 Stromal Cells and Adhesion Assay

In order to assess the impact of the cytokines in the *in vitro* model of the microenvironment, we examined adhesion of CLL cells to murine (S17) stromal cells using the system previously developed by Kurtova *et al* **(28)**. 48-well plates were seeded with S17 cells at 5 x 10^4/well in Opti-MEMI, supplemented with 5% FBS, 1% penicillin/streptomycin and 2-mercaptoethanol. Stromal cells were cultured over-night at 37^{o}C in a humidified, 5% CO_2 incubator. Culture medium was then aspirated from wells and CLL cells at 5 x 10^6/mL in complete RPMI-1640 culture medium were added.

After one day of co-culture, CLL cells not adhered to the stromal cells were harvested by pipetting three times. S17-bound cells were trypsinized for 7 min in the presence of 0.25% EDTA (Sigma). Trypsinized cells were harvested and wells were washed once with complete RPMI-1640. Cells were next stained for 15 min at 37°C with anti-CD19-V450 (B cell marker), anti-CD5-APC (B-CLL and T cell marker), anti-CD3-APC-Cy7 (T cell marker), 7-AAD (viability dye) and Annexin V-FITC (apoptotic marker – all antibodies from BD BioSciences). The absolute numbers of live B cells (CD19/CD5 positive, CD3/7-AAD/Annexin V negative) in the S17-bound or unbound fractions were determined by flow cytometry analysis on a FACS Canto-II instrument (BD Biosciences).

The percentage of adhesion to S17 was calculated by dividing the number of S17-bound B-CLL by the number of total cells (bound + unbound fractions).

2.4 RESULTS

2.4.1 Patient Characteristics, Survival and Causes of Death

We studied a cohort of 189 patients in the clinic sequentially chosen to reflect the age and sex distribution of CLL patients observed in the Manitoba population. Examining overall survival in our cohort (from date of study to censor date), we confirm that the survival of the older CLL patients was shorter than for the younger patients (Figure 1). Patient characteristics between the young and elderly cohorts are shown in **Table 2-1**. The median patient age at study was 68.5 years (range = 37-94). The male:female ratio was 1.3:1. The median age of the control population was 63 years (range = 22-84) with a male:female ratio of 1.1:1. Distribution of the cohort in terms of Rai stage at the time of study was 40% low (stage 0), 34% intermediate (I, II) and 26% high (III, IV).

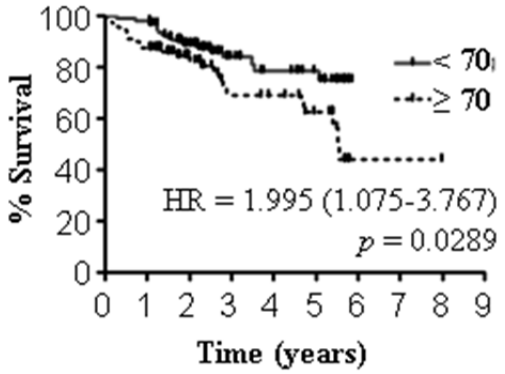


Figure 2-1. Overall survival of CLL patients aged <70 and ≥70 at the time of study.

Because CancerCare Manitoba is a referral center, the duration of time between the date of diagnosis and date of study varied between patients. The median time between

diagnosis and sample collection was 4.0 and 3.1 years for the young and elderly cohorts, respectively, and the difference was non-significant (p = 0.8219). The difference in the number of patients who had prior treatment was also non-significant (46.5% and 43.2%, p = 0.8831). There was a significant difference in the proportion of patients with unmutated IgV_H (p = 0.0151), but with the ≥70 age group being more likely to be mutated.

Table 2-1. Patient Characteristics.

Characteristic	Age < 70	Age ≥ 70	*p*-value
Number of Patients Examined	100	89	
Median Age (Years)*	58.5 (37-69)	75 (70-94)	
% Male***	57.6%	55.1%	0.7694
Disease duration in Years before study*	4.0 (0.1-27.9)	3.1 (0.01-39.1)	0.8219
Rai Stage**			0.8826
High	24 (25.5%)	23 (28.4%)	
Intermediate	33 (35.1%)	26 (32.1%)	
Low	37 (39.4%)	32 (39.5%)	
Mutational Status*			0.0151
Unmutated	47 (49%)	26 (30.6%)	
Mutated	49 (51%)	59 (69.4%)	
Previously Treated*			0.8831
Untreated	53 (53.5%)	49 (55.7%)	
Treated	46 (46.5%)	39 (44.3%)	

* Median value presented with range in the parentheses
** Chi-squared test
*** Fisher's exact test

To address the possibility that older patients were dying from co-morbidities unrelated to CLL, we examined the causes of death. In total, 40 patients died during the nine-year census period. The major causes of death were secondary malignancies (37.5%), CLL progression (35%) and infection (15%) (**Table 2-2**). This distribution of causes was similar in the younger or the older sub-cohorts. In the older sub-cohort, cardiovascular diseases (CVD) accounted for only 12.5% of all deaths. These numbers can be compared with the general population, where CVD normally accounts for over

one-third and all cancers account for about one fifth of all deaths in populations aged over 65 years.(29) The increased incidence of infections and second malignancies in CLL has been related to a compromised immune system.(30, 31) Thus, the older CLL patients died more rapidly than younger patients but the majority of deaths in both age groups could be attributed to CLL progression or to a complication of the leukemia.

Table 2-2. The Causes of Death in Patients < 70 and ≥ 70 years of age at study.

Cause of Death	Age at Study		Total Cohort
	< 70 Years	≥ 70 Years	
CLL Progression	6 (37.5%)	8 (33.3%)	14 (35%)
2nd Malignancy	6 (37.5%)	9 (37.5%)	15 (37.5%)
Infection	4 (25.0%)	2 (8.3%)	6 (15%)
CVD	0.0%	3 (12.5%)	3 (7.5%)
Frailty	0.0%	1 (4.2%)	1 (2.5%)
Unknown	0.0%	1 (4.2%)	1 (2.5%)
Total Deaths	16	24	40

2.4.2 Measurement of Cytokine Levels in CLL patients and Healthy Donors

Certain inflammatory cytokines, especially IL-6, have been demonstrated to increase in the general population with increasing age.(20, 22) In CLL, higher levels of IL-6 and IL-8 had been demonstrated to be associated with poorer survival,(10-12) and we thus focused on the two cytokines, with TNF-α also measured in a smaller subset of patients.

Cut-off values to define high levels of IL-6 or IL-8 were established as previously described for IL-6,(11) and the same method was used for IL-8, using the sums of the average value and one standard deviation for healthy donors (3 pg/mL for IL-6 and 7.4 pg/mL for IL-8). The median plasma IL-8 level was higher in CLL patients compared to healthy donors (p = 0.0056), while there was no statistically significant difference in median IL-6 levels. However, the proportion of CLL patients with a high IL-6 level (i.e.

≥ 3 pg/mL) was significantly higher compared to healthy donors (Fisher exact *p*-value = 0.0133).

To address the possibility of day-to-day variation in cytokines levels, we examined the IL-6 values in four CLL patients obtained at different time points. While small deviations were observed, plasma samples were consistently above or below the cut-off value (data not shown). In addition, as banked plasma proteins can degrade over time, we assessed the effect of storage duration on plasma IL-6 level. Spearman rank correlation between the duration of the sample storage and the measured IL-6 levels revealed a non-significant relationship ($p = 0.0871$) and a positive R (0.1294). Therefore, the analysis suggests a lack of bias related to the length of sample storage.

2.4.3 Relationship of the Cytokine Levels and other Prognostic Markers with Age

As our data demonstrated a significant difference in overall survival between younger (<70) and older (≥70) patients (Figure 1), we determined whether any of the standard prognostic markers or the cytokine levels correlated with patient age. The established predictors of survival examined were Rai stage, lymphocyte count, β_2-microglobulin, IgV_H mutational status, CD38 and ZAP-70 (**Table 2-3**).

For most of the listed predictors, no statistically significant relationship was observed with age. The exception was mutation status, but the difference does not account for the difference in survival. On the other hand, a significant relationship with age was observed with plasma β_2-microglobulin and the inflammatory cytokines, IL-6, IL-8 and TNF-α. Spearman R values ranged from 0.1586 (TNF-α) to 0.4081 (IL-6). There were also significant correlations between the levels of IL-6, IL-8, TNF-α and β_2-

microglobulin with each other (**Table 2-4**), though with a single exception (IL-8 *vs.* TNF-α). These results suggest a common, aging-related mechanism of induction.

Table 2-3. Correlation of Standard Prognostic Markers with Age.

	N	Spearman R	p-value
Markers of Tumor Burden			
Rai stage*	175	N/A	0.5224
Lymphocyte count	188	-0.1270	0.0823
Beta-2-microglobulin	159	0.3179	<0.0001
Biomarkers			
IgV_H mutational status**	175	N/A	0.0093
CD38	146	0.0041	0.9609
ZAP-70	114	-0.0369	0.6966
Telomere Length	146	0.1524	0.0664
Inflammatory Cytokines			
Interleukin-6	189	0.4081	<0.0001
Interleukin-8	175	0.1586	0.0361
TNF-α	30	0.3918	0.0323

*Rai stage comparison performed using Kruskal-Wallis test. Patients were grouped into either low (Rai 0), intermediate (Rai I or II) or high (Rai III or IV) groups.
**Comparison of age values using Mann-Whitney test.

Table 2-4. Correlation of IL-6 and IL-8 with β2-Microglobulin.

	IL-8	β2-microglobulin
IL-6	n = 158 R = 0.2850 p = 0.0003	n = 162 R = 0.3273 p <0.0001
IL-8		n = 135 R = 0.2786 p = 0.0011

2.4.4 Age, Cytokines and Patient Outcome

As the plasma levels of IL-6, IL-8 and TNF-α increased with patient age, we examined whether plasma levels of IL-6 and IL-8 correlated with poor outcome. The patient cohort was dichotomized into high or low plasma levels of IL-6 or IL-8 with respect to set cut-off values (described above). For our entire cohort, we confirmed previous observations that IL-6 and IL-8 were predictors of survival by univariate

analyses (Figure 2A, 2C).**(10-12)** IL-6 and IL-8 had similar hazard ratio values (2.366 and 2.266, respectively). When we performed sub-cohort analysis on patients ≥70 years at study, the hazard ratio values for IL-8 remained similar to that for the whole cohort, although significance was lost (n = 73, p = 0.1001). In contrast, IL-6 was considerably more predictive for survival in the elderly with the hazard ratio value being higher as compared to the whole cohort (4.909 *vs.* 2.366). The median survival for patients aged ≥70 with high IL-6 was half that of patients with low IL-6 (5.4 *vs.* 2.7 years). These results indicate that IL-6 is an important predictor of survival in CLL patients aged ≥ 70 years.

As the plasma levels of β_2-microglobulin correlated closely with the levels of the cytokines and age, we also determined the predictive value of β_2-microglobulin. The β_2-microglobulin levels was predictive of survival for the entire cohort but became non-significant when confined to the older cohort (hazard ratio = 2.93, *vs.* 4.32 for the entire cohort) and significance was lost (p = 0.0697). Thus, among IL-6, IL-8 and β_2-microglobulin, the IL-6 level was the only statistically significant prognostic factor among patients aged ≥ 70 years.

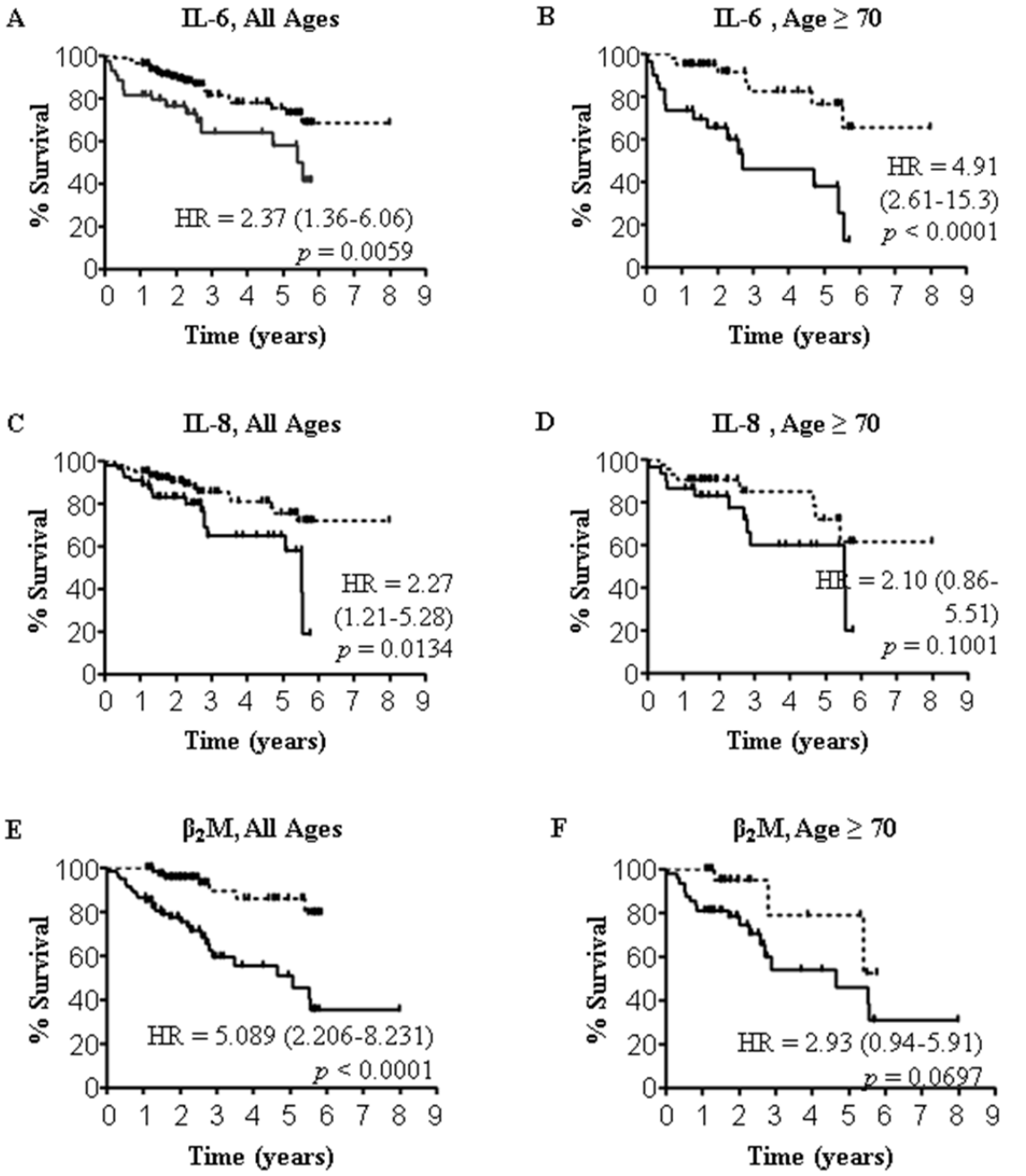
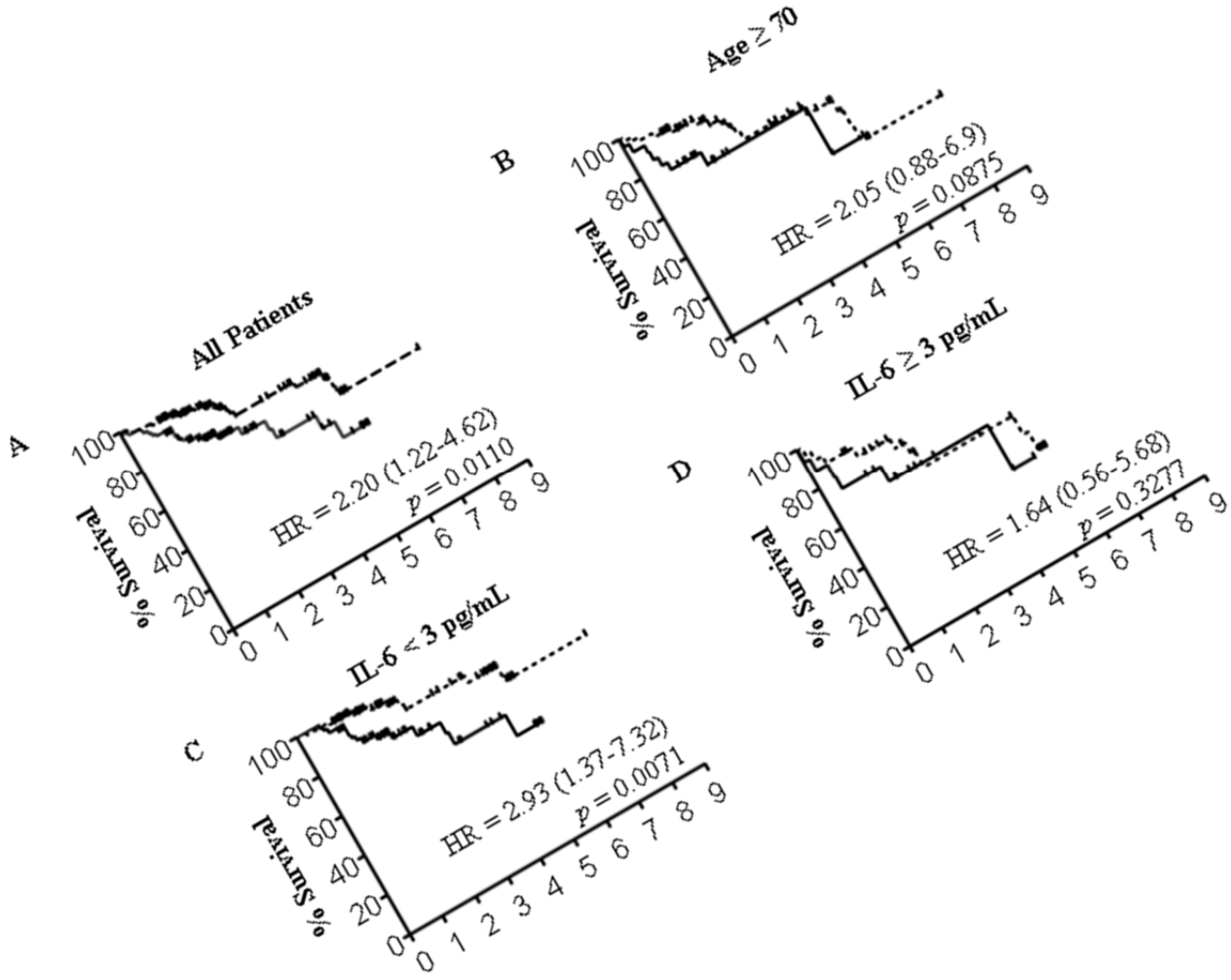


Figure 2-2. Overall survival of CLL patients dichotomized based on IL-6, IL-8 or β_2-microglobulin levels.

Low serum levels = dashed line, high serum levels = solid line. A) Survival of all patients stratified by their IL-6 levels (low < 3, high ≥3 pg/mL). B) Survival of patients whose age at study ≥ 70 years only, stratified by IL-6 levels. C) Survival of all patients stratified by IL-8 levels (low <7.4, high ≥7.4 pg/mL). D) Survival of patients whose age at study ≥ 70 years only, stratified by IL-8. E) Survival of all patients stratified by β_2-microglobulin (low < median value, high ≥ median value). F) Survival of patients whose age at study ≥ 70 years only, stratified by β_2-microglobulin.

2.4.5 Prognostic Value of Cytokine and β_2-Microglobulin Levels According to *Ig*V_H Mutational Status

Considering the difference in mutational status (Table I, III), we next examined the value of the cytokines as compared to mutational status. In examining the entire cohort, the mutational status was predictive of survival (HR = 2.2, $p = 0.011$) (Figure 3A). The hazard ratio value remained similar when the analysis focused on older patients (HR = 2.0), but significance was lost ($p = 0.0875$) (Figure 3B). The importance of *Ig*V_H mutational status for prognosis was maintained for patients with low levels IL-6 or IL-8. However, for patients with high levels of IL-6, the mutational status was no longer a predictor of survival (HR = 1.64, $p = 0.328$), though remained significant in cohort with high IL-8 (HR = 2.61, $p = 0.0373$). Strikingly, the mutational status did not predict survival in sub-cohort analysis for groups with either low or high β_2-microglobulin.


A
All Patients
HR = 2.20 (1.22-4.62)
p = 0.0110
% Survival
B
Age ≥ 70
HR = 2.05 (0.88-6.9)
p = 0.0875
% Survival
C
IL-6 < 3 pg/mL
HR = 2.93 (1.37-7.32)
p = 0.0071
% Survival
D
IL-6 ≥ 3 pg/mL
HR = 1.64 (0.56-5.68)
p = 0.3277
% Survival

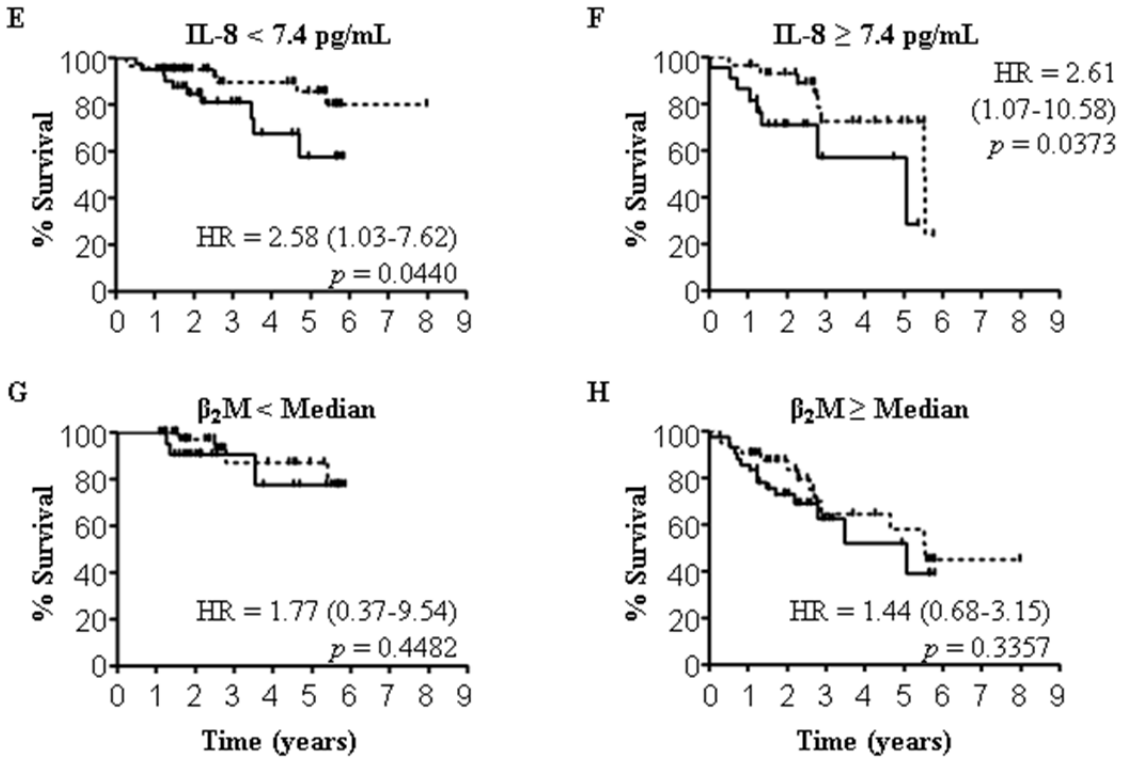


Figure 2-3. Overall survival of CLL patients dichotomized based on their IgVH mutational status.
Mutated = dashed line, unmutated = solid line. A) Survival of all patients stratified by their IgVH mutational status. B) Survival of patients whose age at study ≥ 70 years only. C) Survival of patients with low plasma IL-6 levels (< 3 pg/mL). D) Survival of patients with high plasma IL-6 levels (≥3 pg/mL). E) Survival of patients with low IL-8 levels (< 7.4 pg/mL). F) Survival of patients with high plasma IL-8 levels (≥ 7.4 pg/mL). G) Survival of patients with low β2-microglobulin (< median). F) Survival of patients with high β2-microglobulin (≥ median).

2.4.6 IL-6/IL-8 and the CLL microenvironment

A previous study demonstrated that the supernatant of co-cultured CLL and stromal cells has been shown to have high levels of IL-6 and IL-8,(32) suggesting that the microenvironment might also be a source of these cytokines. Studies have also demonstrated that the microenvironment is critical in CLL, with stromal cells inhibiting apoptosis of the leukemia cells.(33, 34) We thus used the CLL-stromal cell co-culture as a model for the microenvironment and examined the impacts of IL-6 and IL-8. When the

CLL cell/stromal cell co-cultures were supplemented with IL-6 or IL-8, the adherence of the CLL to stromal cells was increased (Figure 4A). Furthermore, while we observed that isolated CLL cells from some patients produced IL-8, co-culturing CLL cells with murine (S17) stromal cells markedly increased IL-8 production in all cases (Figure 4B). In contrast, we did not observe IL-6 secretion by CLL cells, either when incubated alone or with S17 cells (data not shown). Thus, both IL-6 and IL-8 can increase the adherence of CLL cells to stromal cells, and the adherence, in turn, induces an increase in IL-8 levels. This potential positive feedback loop may take place in the microenvironment, contributing to disease progression.

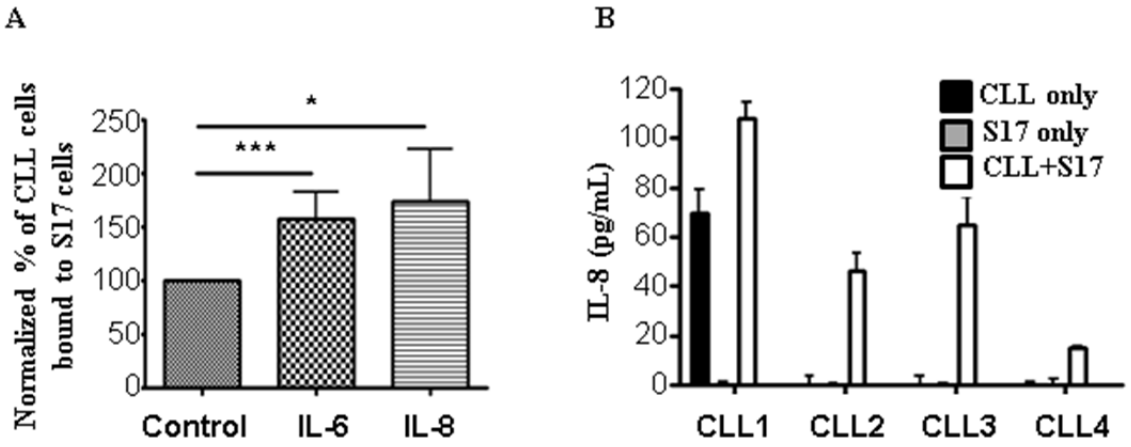


Figure 2-4. Impact of IL-6 or IL-8 on the microenvironment.
A) CLL cells were co-cultured with murine stromal (S17) cells and the number of CLL cells bound to stromal cells were quantified. Co-cultures were incubated in presence of vehicle, IL-6 or IL-8 (50 ng/mL). The data represents normalized results from seven different patient samples. Bar = mean value, error bar = one standard error of means. * $p < 0.05$, *** $p < 0.001$. B) The supernatant samples were obtained from cultures containing either CLL cells alone, stromal cells alone, CLL and S17 co-culture or CLL and S17 co-culture. Levels of IL-8 in the supernatant were measured using an ELISA assay. Each sample was measured three times. Bar = mean, error bar = 1SD.

2.5 DISCUSSION

Our results demonstrated that patients aged ≥ 70 years in the clinic had a poorer survival than younger patients, but the causes of death were the similar in both groups, i.e. progressive CLL, infections or second malignancies. This is in contrast to the normal population, where CVD is responsible for a one-third of death in the elderly.(29) This may be partly related to the age spectrum examined, as a study on CLL patients aged ≥80 years at presentation showed that 41% died from cardiac causes, while 50% died from progressive CLL, infections or second malignancies.(18) Although our results would suggest that CLL is more aggressive in the elderly, we found no difference in the cellular prognostic markers between the different age groups that explained poorer survival. Collectively, these results indicate that extrinsic factors might be influencing CLL growth in elderly CLL patients. Our results suggest that cytokines may be such an extrinsic factor. Among them, in this study, IL-6 was found to be the most powerful predictor of survival in the elderly and statistically more important than the IgV_H mutational status in patients ≥ 70 years. Further studies will be required to determine whether this is a consistent finding in elderly CLL patients.

A weakness of this study is that CLL clinics are likely not reflective of the CLL population, as the median age at diagnosis in our CLL clinic is 64 years (*vs.* 71.5 in the province). This difference occurs despite CancerCare Manitoba's CLL clinic being the primary referral center for the province. A similar finding has been observed at the Mayo Clinic, where the median age at diagnosis is also 64 years and relative survival of patients ≥75 years is not reduced,(19) the opposite finding to population studies.(1, 2) These findings suggest that a proportion of elderly patients with poor prognosis is not being

referred to the clinic, and may explain why the relative survival of CLL patients aged ≥80 years has not improved in the past 20 years.(1)

An important finding in this study was the close correlation observed between the levels of the IL-6, IL-8, TNF-α and β_2-microglobulin, which was previously observed when individual cytokines were studied alone.(10-13) Moreover, the cytokines and β_2-microglobulin increased with patient age. The plasma level of β_2-microglobulin is thought to reflect both tumor burden and renal function and has been shown to be a very important predictor of response to chemotherapy, time to treatment, duration of response and overall survival.(3, 6-9) However, one-third of Binet A patients have elevated levels of β_2-microglobulin levels, and these patients have a shorter treatment-free survival than those with normal levels, suggesting that other factors apart from tumor load can increase β_2-microglobulin levels.(3) CLL cells secrete β_2-microglobulin *in vitro*, especially upon being activated by TPA (12-O-tetradecanoylphorbol 13-acetate) or the B cell mitogen *Staphylococcus aureus* Cowan A.(35, 36) TNF-α did not influence secretion,(36) but whether IL-6 or IL-8 effects secretion is not yet known.

With normal aging, the two main sources of these cytokines are the presence of senescent cells and chronic inflammatory diseases.(22, 37) The number of senescent cells markedly increases with aging, and these cells secrete a wide variety of cytokines, particularly IL-6 and IL-8.(38, 39) The endothelial cells(40) or the CLL microenvironment(32-34) could also provide these cytokines. It has been demonstrated CLL-HS-5 stromal cell co-cultures produce high levels of IL-6 and IL-8,(32) and we demonstrate here that CLL-S17 murine stromal cell co-cultures also produced IL-8. We found that CLL cells did not secrete IL-6, and it is not known whether this is due to the

type of stromal cells used. Secreted IL-6 and IL-8 are expected to impact the biology of the microenvironment, and we demonstrated that IL-6 and IL-8 can increase adhesion of CLL cells to stromal cells. A number of downstream signalling events are likely to occur upon IL-6 or IL-8 stimulation of CLL cells. IL-6 can induce the MAPK, PI3K/Akt and STAT3 signalling pathways in various systems,(41) while IL-8 can induce both MAPK and PI3K/Akt pathways.(42) These pathways may thus be playing a role in facilitating the interaction between CLL and stromal cells.

In summary, we have demonstrated that the plasma levels of the inflammatory cytokines, IL-6, IL-8 and TNF-α, increase in parallel with aging in CLL and that high levels of IL-6 or IL-8 in the elderly are associated with poor survival. Indeed, in the elderly, the plasma IL-6 level is one of the most useful predictors of survival.

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3. SYNERGISTIC APOPTOTIC RESPONSE BETWEEN VALPROIC ACID AND FLUDARABINE IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS INVOLVES THE LYSOSOMAL PROTEASE CATHEPSIN B

This chapter was accepted for publication in *Blood Cancer Journal* as an Original Research Article.

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JY wrote the paper, which was edited by SBG and JBJ.

3.1. ABSTRACT

Fludarabine, a nucleoside analog, is commonly used in combination with other agents for the treatment of chronic lymphocytic leukemia (CLL). In previous studies, valproic acid (VPA), an inhibitor of histone deacetylases (HDACs), combined with fludarabine to synergistically increase apoptotic cell death in CLL cells. In the present study, we found that the combination of fludarabine and VPA decreases the level of the anti-apoptotic proteins Mcl-1 and XIAP in primary CLL cells. Treatment with fludarabine alone, or in combination with VPA, led to the loss of lysosome integrity, and chemical inhibition of the lysosomal protease cathepsin B, using CA074-Me, was sufficient to reduce apoptosis. VPA treatment increased cathepsin B levels and activities in primary CLL cells, thereby priming CLL cells for lysosome-mediated cell death. Six previously treated patients with relapsed CLL were treated with VPA, followed by VPA/fludarabine combination. The combined therapy resulted in reduced lymphocyte count in five out of six and reduced lymph node sizes in four out of six patients. In vivo VPA treatment increased histone-3 acetylation and cathepsin B expression levels. Thus, the synergistic apoptotic response with VPA and fludarabine in CLL is mediated by cathepsin B activation leading to a decrease in the anti-apoptotic proteins.

3.2. INTRODUCTION

Fludarabine-containing regimens are typically used in the front-line treatment of chronic lymphocytic leukemia (CLL), but relapse is common and response rate with re-treatment is generally inferior to response as first-line treatment.(1-4) There is thus a need to develop new and non-cross-resistant drug combinations for relapsing patients. Inhibition of Histone Deacetylases (HDAC) using pharmacological agents, such as depsipeptide, suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) is a novel strategy to treat such patients. *In vitro* results with HDAC inhibitors (HDIs) using depsipeptide, LBH589 and MS-275 were promising, implicating a number of different mechanisms associated with the inhibition of HDACs in CLL cells.(5-8) However, in the clinic, HDI monotherapy trials in CLL have been disappointing. A Phase I trial with depsipeptide observed no responses, despite obvious increases in the level of acetylated histones and p21 *in vivo*.(9) A Phase II trial using MGCD0103 in previously treated CLL patients also produced no responses, despite some patients receiving concomitant rituximab.(10) While both depsipeptide and MGCD0103 were tolerable, haematological grade 4 toxicity was observed with both drugs.(9, 10)

Since HDIs as monotherapy are not effective in CLL, we rationalized that VPA might be more useful if combined with chemotherapy. VPA is a first-generation anti-epileptic that has class I HDAC inhibition activity with IC_{50} values for HDAC1-3 of ≤ 1 mM.(11-13) Treatment of CLL cells *in vitro* with VPA has been shown to induce apoptosis, as judged by caspase activation and Annexin V staining, while VPA inhibited proliferation of CLL cells induced by oligonucleotide and IL-2 co-stimulation.(14-16) Single-agent VPA was sufficient to induce alterations in the gene expression level of a

large number of genes and to change the Bcl-2/Bax ratio at the protein level.(16, 17) Furthermore, VPA enhanced the effects of various chemotherapeutic agents on CLL cells, including fludarabine, bortezomib, flavopiridol, thalidomide and lenalidomide.(14, 16) Oral formulation, with high bio-availability, makes VPA easy to administer to patients, and doses of 20-25 mg/kg/day are commonly used in adolescents and adults.(18, 19) As an anti-epileptic, VPA has a long history in the clinic,(19) and we reasoned that side effects would be predictable and manageable.

Lysosomes are involved in the cell death produced by a number of different anti-tumour drugs, including doxorubicin, camptothecin, cisplatin and etoposide.(20-22) The mechanism of lysosome involvement in mediating cell death generally involves its disruption, a phenomenon known as Lysosome Membrane Permeabilization (LMP).(23, 24) LMP-associated cell death can be necrotic or apoptotic, and the mechanism of death is thought to be dependent on the degree of LMP, where partial LMP induces apoptosis.(23, 24) Once disrupted, various lysosomal enzymes are spilled into the cytoplasm, some of which maintain their activity at physiological pH to mediate cell death. Among them, cathepsins B, D and L have been implicated in LMP-associated cell death.(25) Cathepsin B is one of the most stable proteases at physiological pH and has been shown to mediate LMP-associated cell death in response to doxorubicin, bortezomib, tumor necrosis factor and during mammary involution in mice.(26-29) Released cathepsin B is active in the cytosol, where it can cleave many caspase targets and anti-apoptotic proteins, including Mcl-1 and XIAP.(30-32) However, the role of lysosome-mediated cell death for the activity of anti-tumour agents in primary CLL cells is largely

unstudied. In this study, we describe the role of cathepsin B in mediating VPA- and fludarabine-induced apoptosis in primary CLL cells.

3.3. MATERIALS AND METHODS

3.3.1 Cell culture and treatment conditions

The diagnosis of CLL was made by peripheral blood morphology and the presence of monoclonal B cells in the peripheral blood with typical immunophenotype ($CD19^+$, $CD5^+$ and $CD23^+$). Peripheral blood samples were obtained from CLL patients following informed consent, in agreement with the Research Ethics Board at the University of Manitoba. Peripheral blood mononuclear cells were isolated from the buffy coat using a Ficoll-Paque density gradient as previously described.(33) Freshly isolated CLL cells were cultured in RPMI-1640 culture medium supplemented with 100 U of penicillin, 100 mg of streptomycin and 0.5% Bovine Serum Albumin (BSA). BSA supplementation was chosen over FBS supplementation for culturing of primary CLL cells, as BSA supplementation was associated with lower levels of spontaneous apoptosis (Figure 3-1A). Three human B-cell leukemia/lymphoma cell lines, BJAB, I-83 and NALM-6, were cultured in RPMI-1640 culture medium supplemented with 100 U of penicillin, 100 mg of streptomycin and 10% fetal bovine serum (FBS).

3.3.2 Reagents used

Chloroquine, fludarabine, NH_4Cl, valproic acid, propidium iodide and trichostatin A were purchased from Sigma-Aldrich. Suberoylanilide hydroxamic acid (SAHA) was a gift from Dr. Jim Davie (University of Manitoba). Antibodies against Mcl-1, XIAP, and

Caspases-3, -8, -9 were from Cell Signalling. Antibodies against caspase-2 and cathepsin B were from AbCam. Antibodies against GAPDH and tubulin were from Sigma. Purified cathepsin B was purchased from AbCam. Chemical inhibitors of cathepsins, CA074-Me (Enzo) and Pepstatin A (Sigma), were also obtained commercially.

3.3.3 Cell death assays

Nuclear fragmentation was assessed by propidium iodide (PI) staining in hypotonic conditions and was performed with minor modifications using the direct DNA staining method.(34) Briefly, cell pellets were resuspended in the PI staining buffer (0.1% sodium citrate (w/v), 0.1% Triton X-100 (v/v), 50 mg/L PI, supplemented with RNase (100 μg/mL). Cells were stained for 30 minutes at room temperature in the dark, after which samples were kept on ice and analyzed on the flow cytometer within an hour.

Annexin V stains were performed as described previously (35).

3.3.4 Whole cell lysates and immunoblotting

Whole cell lysates were prepared from cell pellets which were subjected to a single cycle of freeze-thaw at -80°C, then resuspended in the radioimmunoprecipitation assay (RIPA) buffer supplemented with the Complete Mini protease inhibitor cocktail (Roche), phosphatase inhibitor cocktails (Sigma Aldrich) and sodium orthovanadate (1 mM, Sigma Aldrich). Protein samples were quantified using either the Bradford (BioRad) or the bicinchoninic acid (Pierce) protein quantification assay.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using standard protocols. Resultant blots were transferred onto nitrocellulose membranes and

blocked using 5% milk or 5% BSA in Tris-Buffered Saline-Tween-20 (TBST) (0.1% Tween-20). Blocked membranes were incubated overnight in the primary antibody mix, followed by incubation in the secondary antibody mix (Horse Radish Peroxidase (HRP)-conjugated anti-mouse/rabbit antibody (BioRad). Enhanced chemi-luminescence was performed using commercial reagents (Amersham or Pierce).

3.3.5 Measure of Synergy

Synergistic induction of apoptosis was examined by measuring %SubG1 events in response to increasing doses of fludarabine, VPA and the combination of 1 mM VPA with increasing dose of fludarabine. IC_{50} values were determined for three curves generated, and the following equation was used: Combination Index (CI) = $D_1/(D_m)_1$ + $D_2+(D_m)_2$, where $(D_m)_1$, $(D_m)_2$ are median doses (i.e. where 50% SubG1 events are observed) for drugs 1 and 2, and D_1, D_2 are doses are the doses in combination that induced 50% SubG1 events.

3.3.6 RNA isolation and Quantitative PCR (qPCR)

Total RNA was isolated using commercial RNA isolation kits (Qiagen) and quantified using ultraviolet. Complementary DNA (cDNA) was synthesized using FirstStrand II (Invitrogen) supplemented with RNase OUT (Invitrogen) as instructed by the manufacturer using 1-3 μg of total RNA. Quantitative PCR (qPCR) for XIAP mRNA levels were performed using previously described forward (5'-GCACGGATCTTTACTTTTGGG-3') and reverse (5'-TGCACCCTGGATACCATTTAG-3') primers,(36) and MCL1 mRNA levels were

performed using previously described forward (5'-GCCAAGGACACAAAGCCAAT-3') and reverse (5'-AACTCCACAAACCCATCCCA-3') primers.(37) GAPDH mRNA levels (forward = 5' TCCATGACAACTTTGGTATCGTGG-3', reverse = 5'-GACGCCTGCTTCACCACCTTCT-3') were used to normalize results. qPCRs were performed using the BioRad SYBR II qPCR master mix, with reactions run on BioRad C1000TM Thermal Cycler. Relative levels of expression were calculated using the ΔΔCq method, using the control expression level as the baseline.(38)

3.3.7 Lysosome visualization

Lysosomes were stained using either Acridine Orange or LysoTracker Red DND-99 (Molecular Probe). Briefly, cells were stained in Acridine Orange at 100 μg/mL for 10 minutes at 37°C, and then washed in fresh RPMI-1640 media before visualization. Cells were stained with LysoTracker at 62.5 nM for 30 minutes at 37°C and washed in fresh media before visualization or flow cytometry. In the author's hands, neither Acridine Orange nor LysoTracker staining were compatible with formaldehyde fixation, and thus visualization and flow cytometry were performed immediately.

3.3.8 *In vitro* cathepsin B experiments

Whole cell lysates were prepared in a lysis buffer containing NP40 supplemented with a protease inhibitor cocktail (Roche). Cell lysate protein was combined with ≥ 0.2 U of cathepsin B, which was activated for 15 minutes at room temperature in the presence of dithiothreitol (DTT) and phosphate-buffered saline (PBS) (pH 7.4). The

reaction mixtures were incubated at 37°C, and the reaction was terminated by boiling the samples after the addition of SDS-PAGE loading dye and DTT.

Benzyloxycarbonyl-Arg-Arg-7-amido-4-methylcoumarin (zRR-AMC) is a fluorogenic substrate of cathepsin B and was purchased from Enzo Life Sciences. Fresh lysates of treated cells were prepared using the NP40 buffer described above. Lysates were combined with PBS containing zRR-AMC (100 μM) and incubated for one hour at 37°C. After an hour of incubation at 37°C, zRR-AMC cleavage was measured using SpectraMax M5 plate reader (Molecular Devices). To assess the level of background fluorescence, a control without lysate was used.

3.3.9 Phase II clinical Trial

CLL patients aged 18 years or older who required therapy, had an ECOG performance score of two or less, and had been previously treated with a nucleoside analog were recruited into the trial. Patients were treated with oral VPA at a starting dose of 15 mg/kg/day orally in divided doses, with the goal of reaching a serum level of >1 mM through weekly therapeutic drug monitoring and dose escalation. For those with stable or progressive disease following 28 days of therapy, oral fludarabine at a dose of 40 mg/m^2/day on days 1-3 of each 28 day cycle was administered in conjunction with VPA. Treatment was continued to a maximum of six cycles. Therapy was discontinued if there was evidence of progressive disease after 2 cycles of fludarabine and VPA or if the patient experienced unacceptable toxicity attributable to the study drugs, e.g., ≥ 3 non-haematological toxicity or prolonged grade 4 haematological toxicity (NCI criteria).

The primary outcome of interest was best clinical response as defined by NCIWG criteria for CLL 6 months after commencing therapy.(39) Secondary outcomes of interest were the effect of treatment on histone acetylation status and toxicity, both haematological and non-haematological. This trial conformed to Good Clinical Practice Guidelines and is registered at NCT00524667.

3.3.10 Statistical Analyses

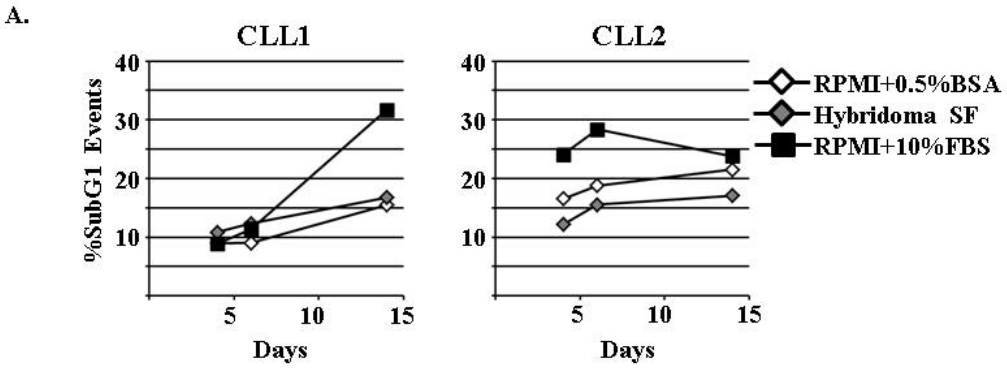
Analysis of variance (ANOVA) was used to compare multiple columns, and t-tests were used for two-column comparisons (Mann-Whitney). ANOVA was followed by post-test column comparison (Tukey's test).

3.4 RESULTS

3.4.1 A synergistic apoptotic response is observed when HDIs and fludarabine are combined.

VPA has been shown to interact with fludarabine in a synergistic manner against primary CLL cells,(14, 16) and this was confirmed when we examined primary CLL cells either co-treated or pre-treated for 24 hours with VPA and then treated with fludarabine (**Figure 3-1**B), and similar results were obtained using either Annexin V staining or the sub-G1 method (**Figure 3-1**D). There were no statistically significant differences in response to either fludarabine or the VPA/fludarabine combination in IgV_H mutated or unmutated CLL samples (**Figure 3-1**C). We next examined three B-cell neoplasm-derived cell lines, BJAB, I-83 and NALM-6, which were chosen to represent the spectrum of different B-cell neoplasms, representing immature (NALM-6) to mature

(CLL-like I-83) malignancies. Using sub-lethal dose of VPA (1 mM), the addition of VPA increased fludarabine cytotoxicity in all three cell lines (**Figure 3-2**A). This increased apoptosis was not observed between fludarabine and valpromide, the amide analogue of VPA that lacks the HDAC inhibition activity,(40) suggesting that HDAC inhibition is important for synergy (**Figure 3-2**A). Fludarabine cytotoxicity was also increased by the addition of three different HDAC inhibitors at sub-lethal doses, namely sodium butyrate, trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (**Figure 3-2**B). When the status of histone acetylation of histone 3 and histone 4 were examined, the VPA and fludarabine combination induced higher levels of histone acetylation than either agent alone (**Figure 3-1**E). This was associated with an increase in the levels of histone 3 and histone 4 by fludarabine treatment, albeit to a greater extent in BJAB and NALM-6 cells compared to I-83 cells, as well as downregulation of HDAC1 by the VPA/fludarabine combination (**Figure 3-2**E). The combination also induced activation of initiator caspases-2, -8, and -9 and the executioner caspase-3 in cell lines and primary CLL cells (**Figure 3-2**C), suggesting that both the intrinsic and extrinsic apoptotic pathways are activated.



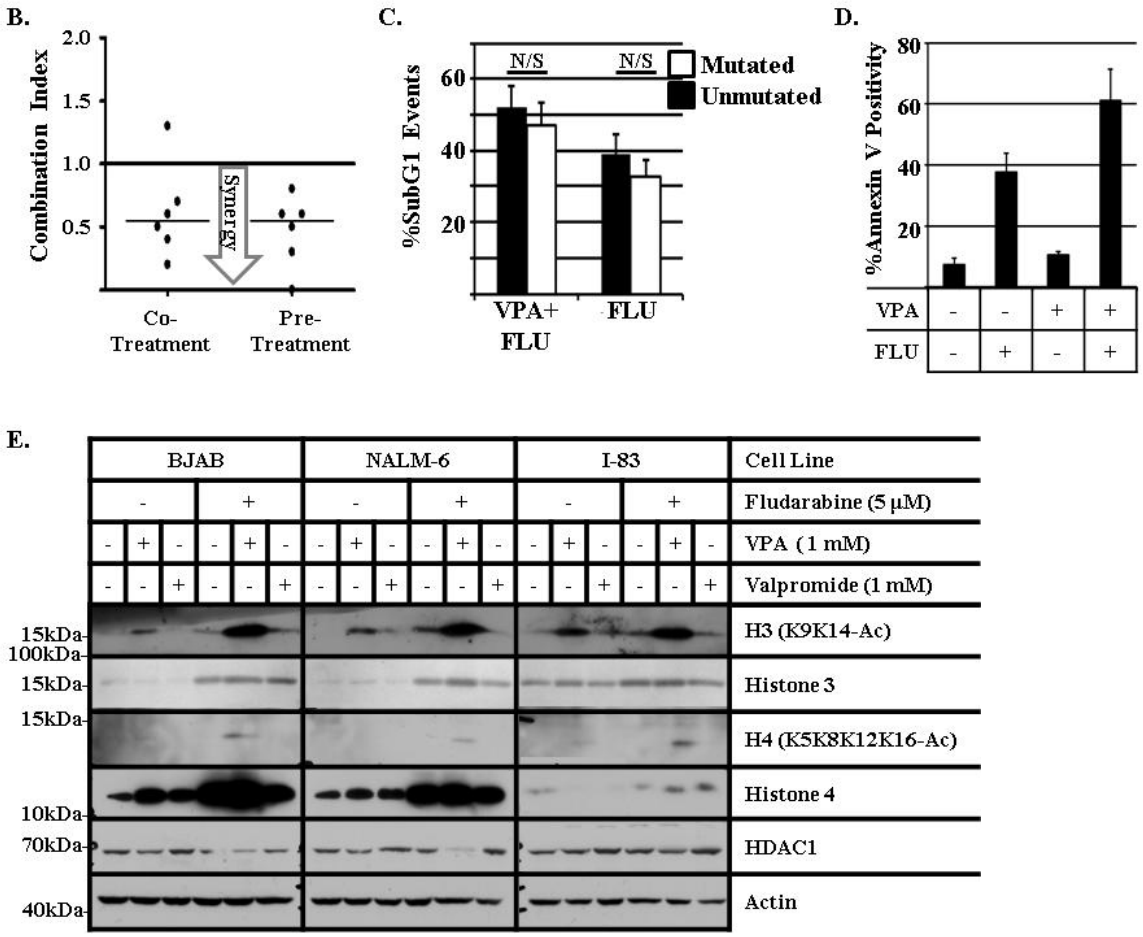


Figure 3-1. VPA-Fludarabine synergy in primary CLL.

A) Primary CLL cells from two different patients were incubated in the indicated media for up to 14 days. Spontaneous apoptosis in the absence of any cytotoxic drugs was measured using SubG1 quantification. B) Primary CLL cells were treated using combinations of fludarabine and VPA for two days, and combination index values, a measure of pharmacological synergy, were calculated as described in the supplementary methods. CLL cells were either co-treated on the same day, or pre-treated for one day with VPA before the addition of fludarabine. C) Comparison of unmutated and mutated primary CLL samples in response to fludarabine and the VPA/fludarabine combination. Cells were treated for two days with fludarabine (10 μM) ± VPA (1 mM), and SubG1 quantification was performed. Shown is data from 26 primary CLL samples (10 unmutated, 16 mutated). D) Cells were treated as described in C), with Annexin V staining performed to quantify cell death. Shown is data from 3 primary CLL samples. E) Three human leukaemic cell lines, BJAB, NALM-6 and I-83, were treated for one day using combinations of fludarabine (5 μM) with Valproic Acid (VPA, 1 mM) or valpromide (VPA analogue that does not target HDACs, 1 mM). Cell were harvested and whole cell lysates were examined via immunoblotting.

Bar = mean, error bar = one SEM. N/S indicates *p*-value > 0.05

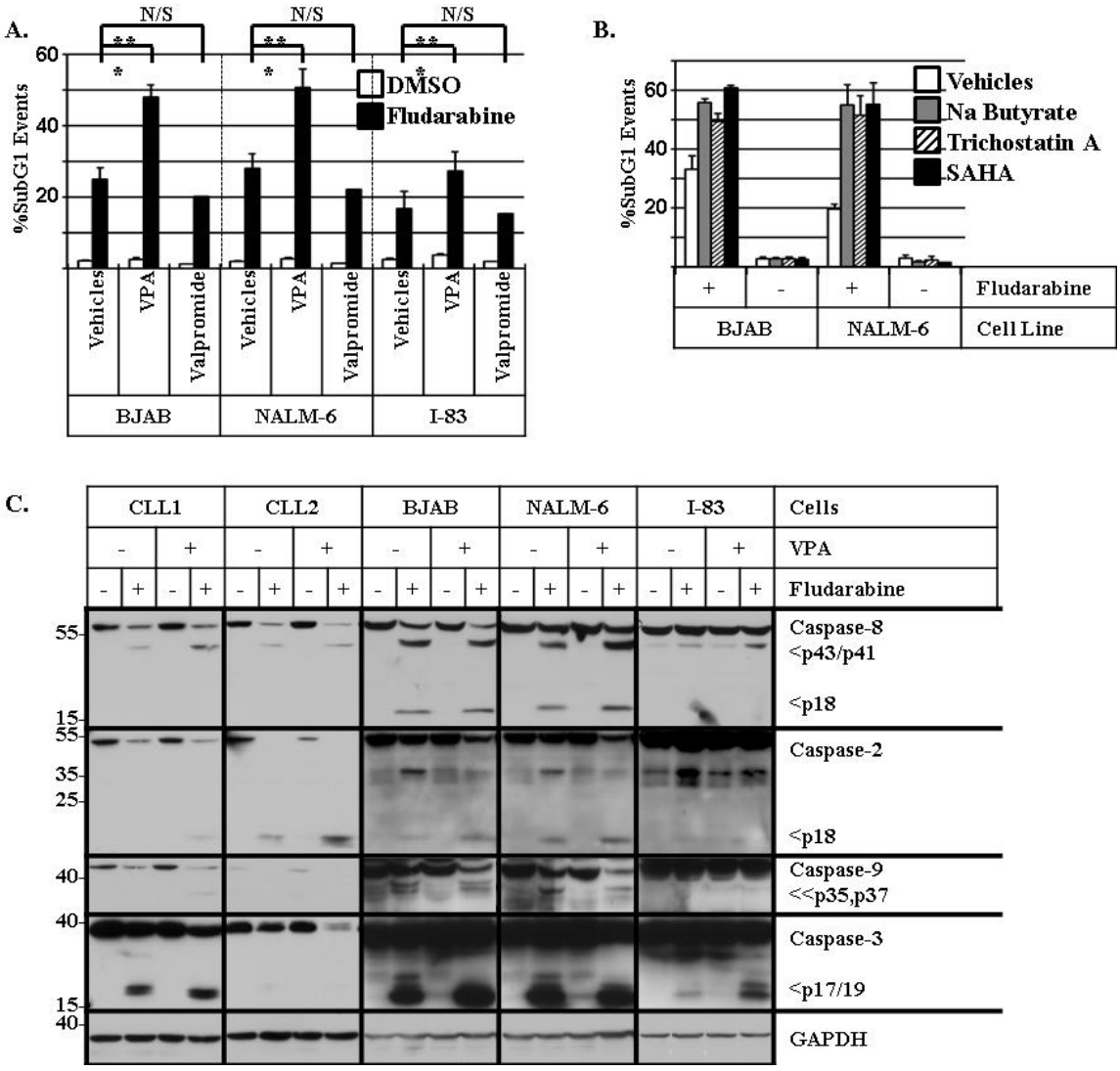


Figure 3-2. Cytotoxicity of fludarabine is enhanced by HDAC inhibitors.
A) Three human leukemic cell lines, BJAB, NALM-6 and I-83, were treated for one day using combinations of fludarabine (5 μM) with Valproic Acid (VPA, 1 mM) or valpromide (VPA analogue that does not target HDACs, 1 mM). DNA fragmentation was quantified by flow cytometry, examining for hypodiploid DNA content. B) Fludarabine (5 μM) was combined with three other HDAC inhibitors, sodium butyrate (1 mM), trichostatin A (5 nM) or SAHA (5 μM) in BJAB or NALM-6 cells for one-day treatment. C) Immunoblots using whole cell lysates to examine caspase activation. Cell lines for treated one day, and primary CLL cells were treated for two days using combinations of fludarabine (5 μM for cell lines, 10 μM for primary CLL cells) and VPA (1 mM). Bar = mean, error bar = one SEM. Primary CLL data shown is representative data, and all experiments were repeated at least three independent times.

3.4.2 The combination of VPA and fludarabine induces Mcl-1 and XIAP degradation in a lysosome-dependent manner

In CLL cells, various HDIs have previously been shown to down-regulate the anti-apoptotic proteins, Bcl-2, Mcl-1 and XIAP.(16, 17, 41) In the present study, we found that the levels of XIAP and Mcl-1 were reduced in both primary CLL and cell lines in response to fludarabine, and this reduction was further enhanced when fludarabine was combined with VPA (**Figure 3-3**A). In contrast, the Bcl-2 levels remained unchanged in primary CLL cells, while Bcl-2 was not expressed at detectable levels in BJAB or NALM-6 cells. In BJAB cells, the reduction in Mcl-1 and XIAP occurred with 16 hours of treatment, and the same reduction was observed with 24-hour treatment in primary CLL cells, and these observations were evident before significant apoptosis had occurred (**Figure 3-4**A, B). This suggests that the downregulation of the proteins was contributing to the apoptosis. Interestingly, when the mRNA levels for these proteins were examined, there was no reduction in transcription, and the *XIAP* mRNA levels increased after treatment (**Figure 3-4**C, D), suggesting that Mcl-1 and XIAP downregulation occurs post-transcriptionally.

Protein degradation can occur via a number of different pathways, including autophagy, lysosomal degradation and the ubiquitin-proteasome pathway.(42, 43) Under apoptosis, a large number of proteins also become targeted by caspases.(44) In order to investigate which pathways were involved in the degradation of Mcl-1 and XIAP, BJAB cells were pre-treated with chloroquine (inhibitor of autophagy and lysosomal pathways), zVAD-fmk (pan-caspase inhibitor) or bortezomib (26S proteasome inhibitor). We determined whether these inhibitors could block protein degradation and caspase

activation. Pre-treatment with either chloroquine or zVAD-fmk reduced activation of caspases-2 and -8 and partially stabilized Mcl-1 and XIAP in response to fludarabine, in the presence or absence of VPA (**Figure 3-3**B). In contrast, bortezomib increased caspase activation in cells treated with either VPA or fludarabine and did not stabilize XIAP in response to the VPA/fludarabine combination (**Figure 3-3**B). Bortezomib did, however, increase the levels of Mcl-1, as a result of blockage of the ubiquitin-proteasome pathways (**Figure 3-3**B). Taken together, both the lysosome and caspases appear to be involved in the degradation of XIAP and Mcl-1 by fludarabine, with or without VPA, and inhibition of the lysosomal protein degradation pathway suppressed caspase activation.

We next investigated the effects of lysosome alkalinization on the level of cell death. Pre-treatment with chloroquine or NH_4Cl reduced cell death in response to the combination of VPA and fludarabine in both BJAB and NALM-6 cells (**Figure 3-5**A). This protection by chloroquine or NH_4Cl is not associated with inhibition of autophagy, as fludarabine, in the presence or absence of VPA, did not induce the formation of LC3-II (an indicator of autophagy), as determined by immunoblotting and was not associated with increase in the number of acidic vacuoles assessed by acridine orange staining (**Figure 3-6**, **Figure 3-7**A,B). Rather, the addition of chloroquine resulted in the increase in steady state levels of LC3-II, due to blockage of its degradation by lysosomes (**Figure 3-6**). In primary CLL cells, pre-treatment with chloroquine or NH_4Cl reduced VPA/fludarabine- and fludarabine-induced apoptosis, although the magnitude of suppression was much lower, due to the toxicity associated with chloroquine or NH_4Cl pre-treatment (**Figure 3-5**B). In contrast to chloroquine or NH_4Cl, treatment of BJAB or NALM-6 cells with MG-132 or bortezomib increased VPA and fludarabine cytotoxicity

(**Figure 3-5**C). These observations suggest the lysosomes are playing a crucial, autophagy-independent role in VPA/fludarabine-induced cell death.

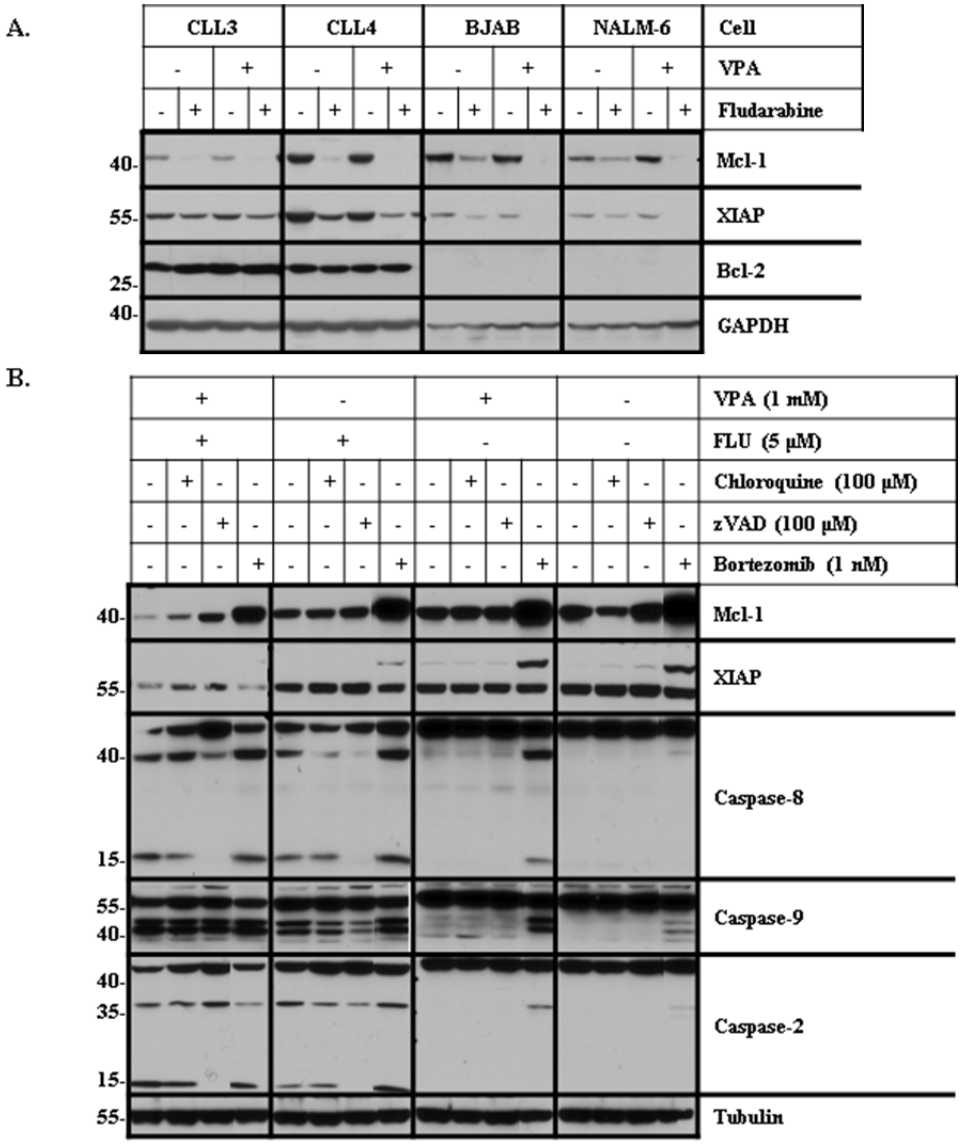


Figure 3-3. The role of different protein degradation pathways in Mcl-1 and XIAP degradation.

A) Cell lines for treated one day, and primary CLL cells were treated for two days using combinations of fludarabine (5 μM for cell lines, 10 μM for primary CLL cells) and VPA (1 mM). Whole cell lysates were examined for the levels of Bcl-2, Mcl-1 and XIAP in response to the indicated treatment combinations. Primary CLL data shown is representative data. B) BJAB cells were pre-treated with vehicles, chloroquine (an inhibitor of the lysosomal protein degradation), zVAD.fmk (a pan-caspase inhibitor) or bortezomib (a 26S proteasome inhibitor) for one hour, and then treated with the indicated combinations of fludarabine and VPA for 16 hours. Whole cell lysates were examined by immunoblotting.

The data shown for different treatments are obtained from a single representative experiment from the same membrane. All experiments were repeated three independent times.

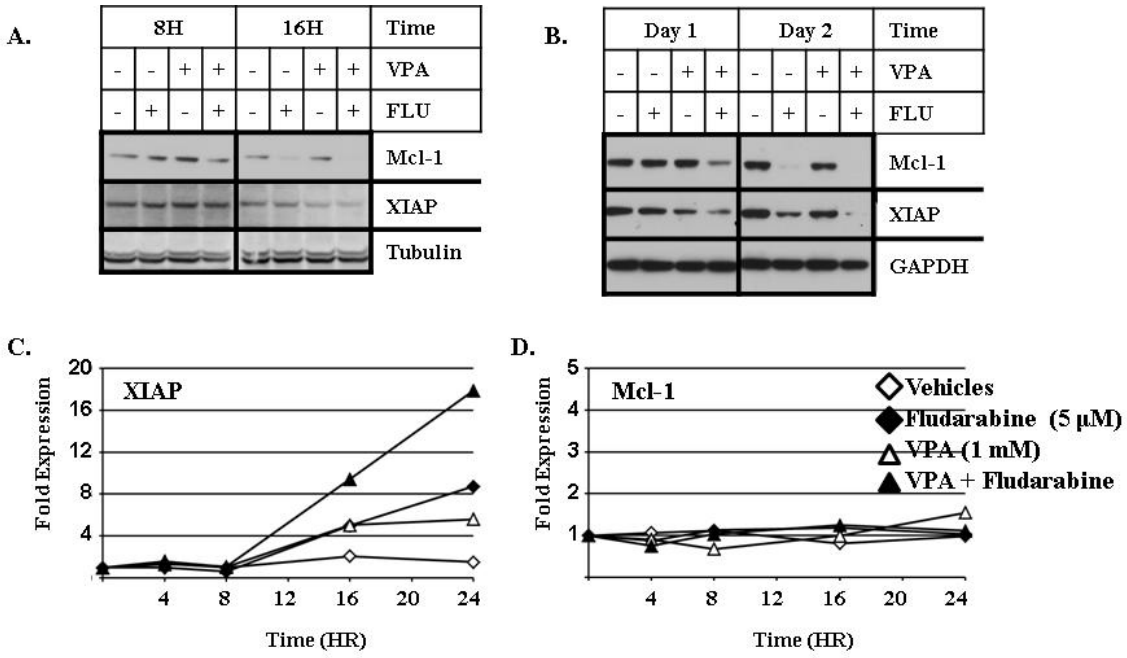
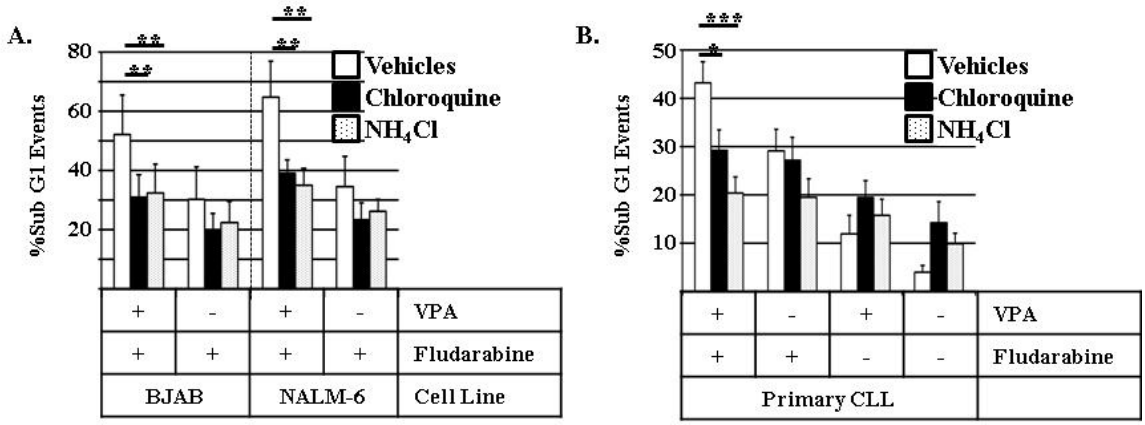


Figure 3-4. Mcl-1 and XIAP levels in response to VPA-fludarabine.
A) BJAB cells were treated for the indicated time durations using the indicated combinations of fludarabine (5 μM) and VPA (1 mM), and whole cell lysates were examined via immunoblotting. B) Primary CLL cells were treated for the indicated time durations using the indicated combinations of fludarabine (10 μM) and VPA (1 mM). C, D) NALM-6 cells were treated for the indicated time durations using the indicated combinations of fludarabine (5 μM) and VPA (1 mM). RNA was isolated, followed by cDNA synthesis and quantitative PCRs for mRNA levels for XIAP (D) and MCL1 (E).



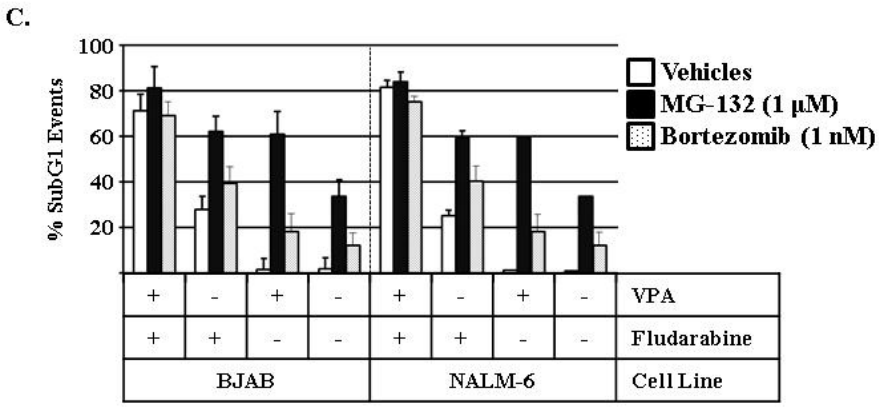


Figure 3-5. Inhibition of the endo-lysosomal pathway protects cells from VPA-fludarabine cytotoxicity.
A, B) BJAB, NALM-6 or primary CLL cells were pre-treated for one hour with two different inhibitors of the lysosomal protein degradation pathway, chloroquine (100 μM) or NH_4Cl (50 mM), then treated for one (cell lines) or two (primary CLL cells) days using the indicated combinations of fludarabine (5 μM for cell lines, 10 μM for primary CLL cells) and VPA (1 mM). Data is gathered from 11 CLL patient samples. C) BJAB or NALM-6 cells were pre-treated with two inhibitors of the 26S proteasomal protein degradation pathway, MG-132 (1 μM) or bortezomib (1 nM), then treated for one day using the indicated combinations of fludarabine (5 μM) and VPA (1 mM). Bar = mean, error bar = one SEM. All experiments in cell lines were repeated at least three independent times.

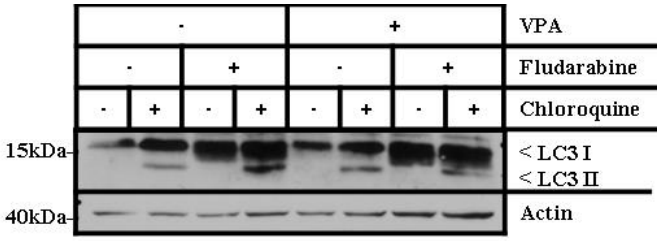


Figure 3-6. LC3 modifications in response to VPA-fludarabine.
BJAB cells were pre-treated for one hour using chloroquine (100 μM), followed by treatment with the indicated combinations of fludarabine (5 μM) and VPA (1 mM).

3.4.3 Apoptosis induced by the combination of VPA and fludarabine is cathepsin B-dependent

The lysosome has been implicated in cell death pathways that involve its disruption, known as LMP (lysosome membrane permeabilization), leading to the activation of caspases-2 and -8.(23) Since the VPA/fludarabine combination activated

caspase-2 and lysosome alkalinization protected the cells from the combination, we next determined whether LMP is involved in cell death induced by VPA and fludarabine. In cell lines, staining of acidic compartments using acridine orange (AO) showed a loss of acidic compartments in response to fludarabine and VPA (**Figure 3-7**A). When quantified, the percent of cells with loss of acidic compartments (i.e. no detectable acridine orange staining) corresponded with the level of apoptosis (**Figure 3-7**B). We next used LysoTracker, a commercial dye for acidic compartments, to examine primary CLL cells. LysoTracker staining showed a similar loss of acidic compartments in primary CLL cells following treatment, and the loss was visibly obvious by microscopy (**Figure 3-7**C) and by flow cytometry (**Figure 3-8**A). When signals were quantified, LysoTracker signals were significantly reduced in either fludarabine or VPA/fludarabine-treated cells (**Figure 3-7**D).

Disruption of lysosomes, however, is a phenomenon that is observed in many different modes of cell death, where lysosomes may or may not be playing a driving role in the induction of cell death.(45) When the disruption of lysosomes do actually drive cell death, such cell death scenarios involve the release of the lysosomal proteases into the cytosol, and, among the different proteases, cathepsins B and D have been implicated in such cell deaths.(23) When BJAB or NALM-6 cells were pre-treated with CA074-ME, an inhibitor of cathepsin B, there was a robust protection of cells from fludarabine- or VPA/fludarabine-induced apoptosis, while much higher concentrations of Pepstatin A, an inhibitor of cathepsin D, failed to show any protection (**Figure 3-8**B). Pre-treatment of NALM-6 cells with CA-074ME partially stabilized Mcl-1 and XIAP and also reduced the activation of caspases, including caspase-2 (**Figure 3-8**C). Unlike cell lines, treatment of

primary CLL cells with CA074-Me (20 μM) was toxic, but nonetheless resulted in mild suppression of the cytotoxicity seen with the VPA and fludarabine combination, where the difference approached statistical significance (**Figure 3-7**E).

While CA074-Me pre-treatment partially stabilized Mcl-1 and XIAP, the pre-treatment also inhibited the activation of caspases, which may be cleaving Mcl-1 and XIAP on their own. We thus determined whether cathepsin B could directly target Mcl-1 and XIAP, as cathepsin B has been shown to target purified Bcl-2 proteins, including Mcl-1 and XIAP in a cell-free system using purified proteins.(32) We thus simulated LMP by combining whole cell lysates from BJAB or NALM-6 cells with purified, activated cathepsin B, to determine whether cathepsin B could directly degrade Mcl-1 and XIAP *in vitro*. When BJAB or NALM-6 lysates were mixed with purified cathepsin B, there were reductions in Mcl-1 and XIAP levels (**Figure 3-8**D), thus demonstrating cathepsin B can target Mcl-1 and XIAP. Cathepsin B addition also resulted in the appearance of faster migrating bands with caspase-2 immunoblotting and a reduction in the pro-caspase-2 levels (**Figure 3-8**D). Taken together, treatment of leukemic cells with fludarabine and VPA induces LMP, and the resulting apoptosis is dependent on the lysosomal protease cathepsin B, which can directly target Mcl-1, XIAP and caspase-2.

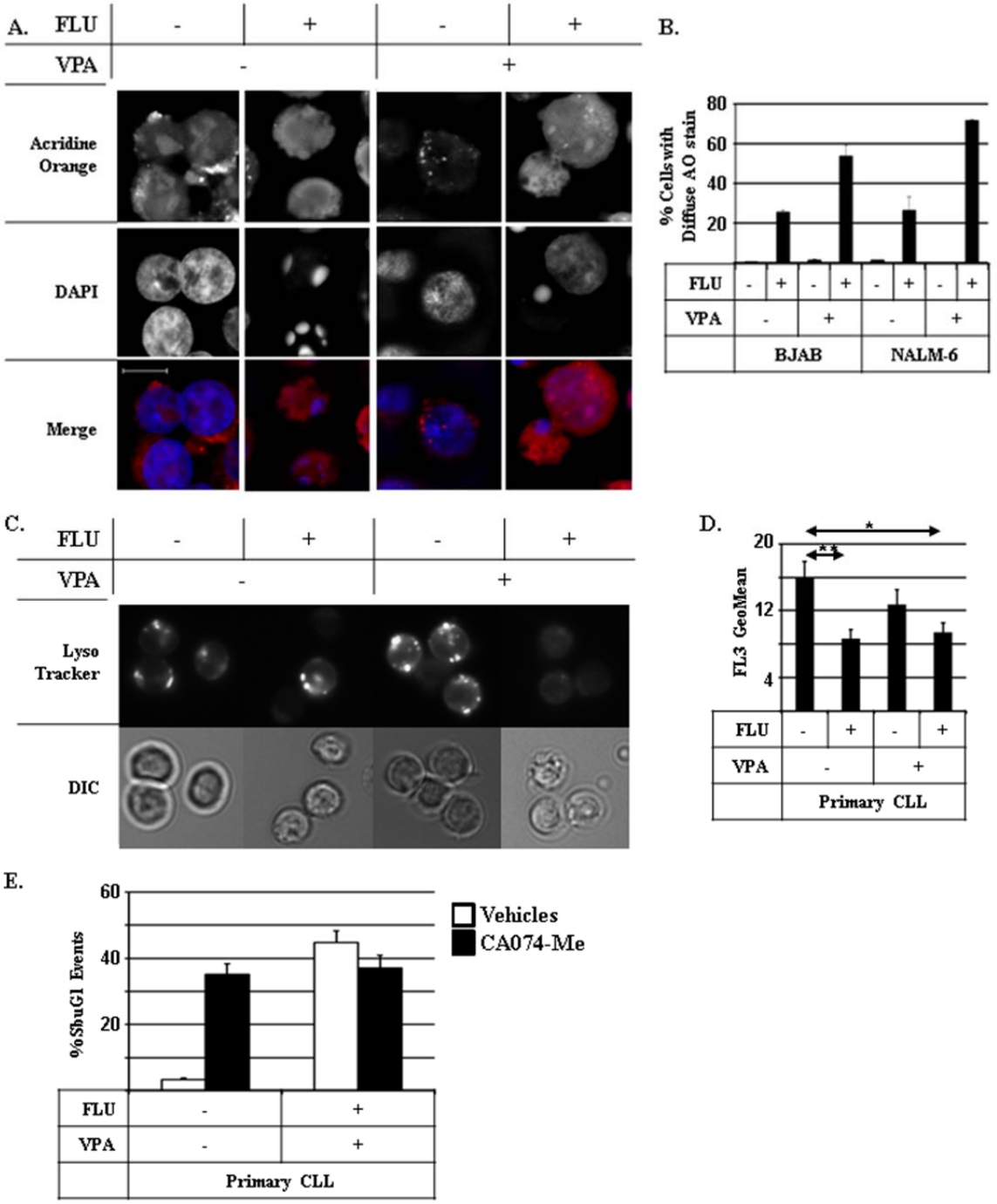


Figure 3-7. Lysosome morphology in response to VPA-fludarabine.
A) BJAB cells were treated for one day using the indicated combinations of fludarabine (5 μM) and VPA (1 mM), and acidic vesicles were stained using acridine orange. B) Manual counts of lysosome morphology in BJAB or NALM-6 cells in response to one day treatment using the indicated combinations of fludarabine (5 μM) and VPA (1 mM). C) Primary CLL cells were treated for two days using the indicated combinations of fludarabine (10 μM) and VPA (1 mM), after which they were stained using LysoTracker. D) Flow cytometric analysis of primary CLL cells stained as described in C). E) Primary CLL cells were pre-treated for one hour using CA074-Me (20 μM) and treated with either

vehicles (DMSO, PBS) or the combination of VPA (1 mM) and fludarabine (10 μM) for two days. Data shown is obtained from 16 different CLL samples.
Bar = mean, error bar = one SEM. * p-value < 0.05. ** p-value < 0.01. Scale: bar = 10 μm

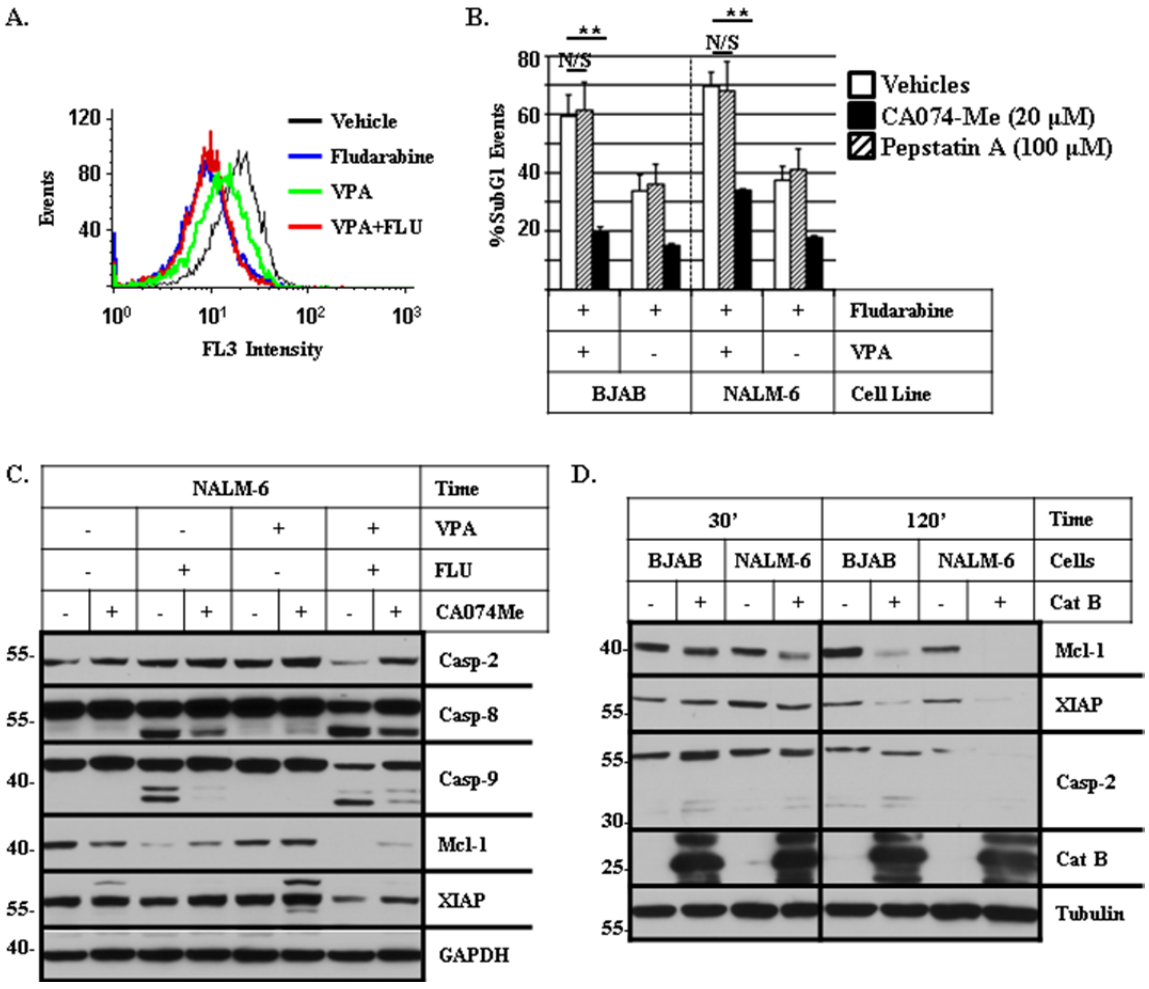


Figure 3-8. The role of cathepsin B in VPA-fludarabine cytotoxicity.
A) Flow cytometric analysis of a representative CLL patient sample using LysoTracker staining of the lysosomes. Primary CLL cells were treated for two days using the indicated combinations of fludarabine (10 μM) and VPA (1 mM). Loss of lysosomal integrity is indicated by decrease in LysoTracker staining intensity. B) BJAB or NALM-6 cells were pre-treated for one hour using CA074-Me, a permeable cathepsin B inhibitor, or pepstatin A, a cathepsin D inhibitor, then treated for one day using the indicated combinations of fludarabine (5 μM) and VPA (1 mM). C) Whole cell lysates from B) examined via immunoblotting. D) BJAB or NALM-6 cell lysates (10 μg each) was combined with activated, purified cathepsin B and incubated for the indicated durations. Reactions were stopped by adding SDS-PAGE loading dye supplemented with dithiothreitol (50 mM) and boiling the samples for five minutes before analysis via SDS-PAGE and immunoblotting. Bar = mean, error bar = one SEM. Primary CLL data shown

is representative data, and all experiments in cell lines were repeated at least three independent times

3.4.4 VPA up-regulates Cathepsin B levels and activity

A microarray study of VPA-treated CLL cells had reported cathepsin B to be up-regulated.(16) Treatment of BJAB cells with VPA resulted in increased levels of cathepsin B, which was not observed with valpromide (**Figure 3-9**A). We next asked whether this increase in cathepsin B level was also associated with increase in the cathepsin B activity. Fresh lysates from BJAB or NALM-6 cells that had been treated with either vehicle or 1 mM VPA was mixed with zRR-AMC, a fluorogenic substrate for cathepsin B whose cleavage product (7-amino-4-methylcoumarin) is fluorescent. Cleavage of zRR-AMC was detectable when combined with either vehicle-treated BJAB or NALM-6 lysates, and this activity was increased in lysates obtained from cells that had been treated for 24 hours with 1 mM VPA (**Figure 3-9**B). Co-treatment of cells with CA074-Me (20 μM) reduced the fluorescence to the background level, demonstrating that zRR-AMC cleavage is cathepsin B-dependent. In primary CLL cells, similar increase in cathepsin B levels in response to VPA was observable (**Figure 3-9**C), and VPA treatment was sufficient to increase zRR-AMC cleavage with lysates from primary CLL cells (**Figure 3-9**D). Thus, taken together, VPA increases cathepsin B protein levels, and this up-regulation is associated with increased cathepsin B activity.

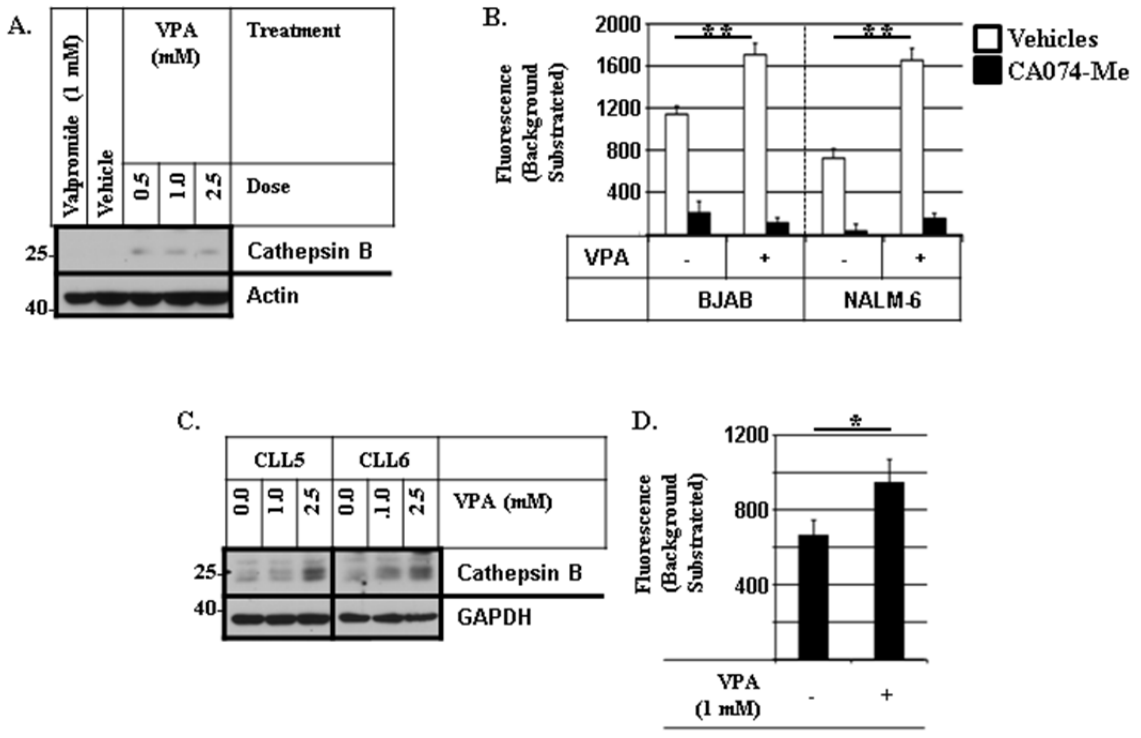


Figure 3-9. The effect of VPA on Cathepsin B level and activity.
A) BJAB cells were treated using either VPA at indicated doses or valpromide (1 mM, VPA analogue that does not inhibit HDACs) for one day, and whole cell lysates were examined. B) BJAB or NALM-6 cells were treated for one day using the indicated combinations of VPA (1 mM) and CA074-Me (20 μM, a cathepsin B inhibitor), then 40 μg of whole cell lysates were combined with zRR-AMC, a fluorogenic substrate for cathepsin B in PBS (pH 7.4). C) Two primary CLL samples were treated using the indicated doses of VPA for two days, and whole cell lysates were examined via immunoblotting. D) Primary CLL cells were treated for two days using VPA (1 mM). Cells were harvested, and 15 μg of whole cell lysates were combined with zRR-AMC as in B). The data is obtained from experiments with six different CLL samples.
Bar = mean, error bar = one SEM. Primary CLL data shown is representative data, and all experiments were repeated at least three independent times.

3.4.5 Phase I/II clinical trial data using a VPA and fludarabine combination

To determine whether VPA and fludarabine give increased clinical efficacy for CLL patients we conducted a phase I/II clinical trial. Of six patients registered in the trial, three patients were fludarabine-resistant and all had received a variety of chemotherapeutic regimens before trial entry (**Table 3-1**). Fludarabine resistance was

clinically defined as having failed treatment or relapsed within six months of completing prior fludarabine-based therapy as per the iwCLL guidelines.(46) Four patients had Rai stage IV disease and two patients Rai stage III disease. Response with a 28-day cycle of VPA monotherapy was modest at best, with no response in half of the patients, and VPA was then combined with fludarabine for the remainder of the treatment course. Patients had VPA concentrations in the serum between 0.54 to 1.1 mM (**Table 3-2**). Two patients had progressive disease following therapy, three patients had stable disease with greater than 50% decrease in lymphocyte count following five cycles of therapy, and one patient had a partial remission at the completion of six cycles of therapy (**Figure 3-10**A, **Table 3-3**). Two patients completed all planned cycles of therapy. Two patients developed significant anaemia (NCI CTC grade 4), and one patient developed significant fatigue (NCIC CTC grade 3) during the fifth cycle of therapy necessitating early treatment discontinuation. Nevertheless, out of the six patients, five showed a decrease in lymphocyte count and lymph node sizes (**Figure 3-10**A,B).

To evaluate whether VPA is targeting histone acetylation and cathepsin B expression *in vivo*, peripheral blood samples were obtained during the first 30 days of the treatment course on VPA monotherapy, and mononuclear cells were isolated for analysis by immunoblotting. Levels of both histone 3-acetyl initially increased and then fluctuated during the course of treatment (**Figure 3-10**C, S5). More importantly, cathepsin B expression was increased on average 2.3-fold in four patients' CLL cells over a 30 day time course following VPA treatment (**Figure 3-10**D). The increase in cathepsin B levels were similar to those seen in VPA-treated primary CLL cells *in vitro*

and corresponded to the subsequent decrease in lymphocyte count when fludarabine was added (**Figure 3-10**A).

Table 3-1. Patient Characteristics before study

Patient	Age at Study	Sex	Rai Stage	Previous Treatments	IgV_H	ZAP70	FLU Sensitivity*
1	78	M	IV	CLB → FLU+Dex →FRD → CR	U	N/A	Resistant
2	72	M	IV	FLU → FC	U	Positive	Sensitive
3	72	M	IV	CLB+Pred → FC	U	N/A	Resistant
4	70	M	IV	FLU → FC	M	Negative	Sensitive
5	79	F	III	CLB → FR → FLU → FCR → FR	N/A	Negative	Resistant
6	62	M	IV	CLB→Flu→CLB→ RCVP→RCHOP→ CLB→/RCVP	N/A	N/A	Sensitive

*FLU resistant = No response with FLU or relapse < 6M after the completion of treatment with FLU.

Table Legend:
FLU = Fludarabine; CLB = Chlorambucil; Dex = Dexamethasone; Pred = Prednisolone
CR = Cyclophosphamide/Rituximab, FC = Fludarabine/Cyclophosphamide
FCR = Fludarabine/Cyclophosphamide/Rituximab
FRD = Fludarabine/Rituximab/Dexamethasone
U = Unmutated; M = Mutated; N/A = Not Available

Table 3-2. Valproic Acid levels in the serum of treated CLL patients

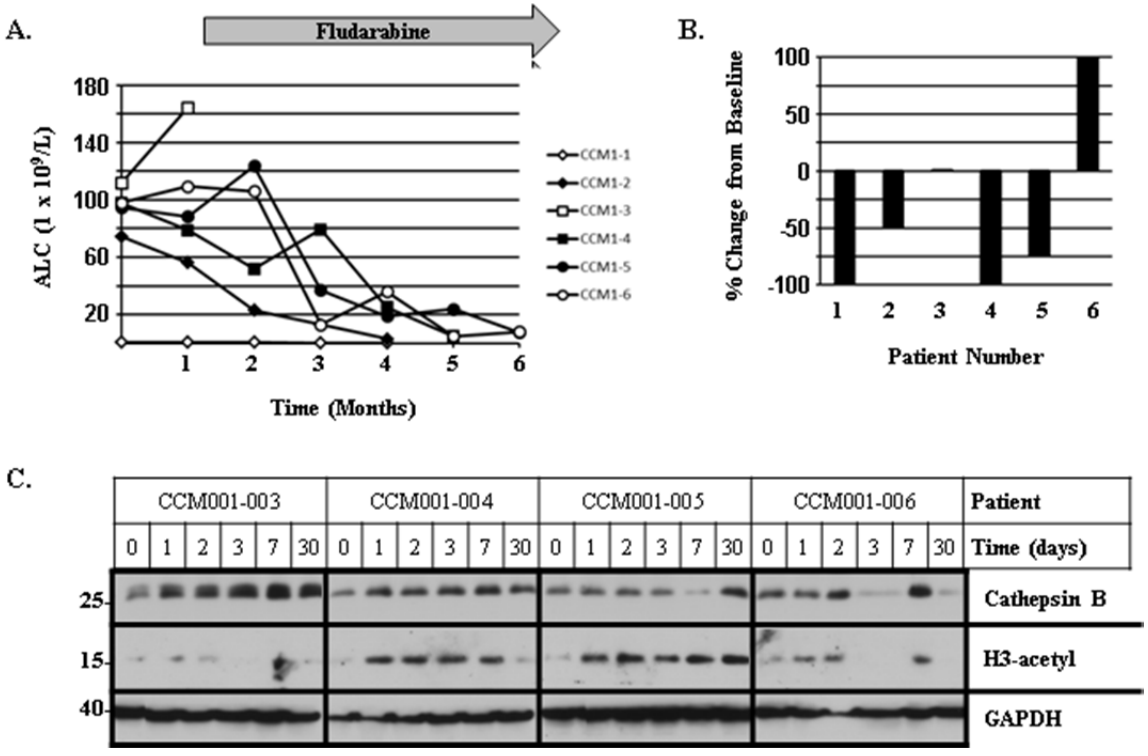
Patient	VPA final dose (mg/kg/day)	VPA final serum level	
		(mM)	(mg/L)
1	50	0.629	90.7
2	45	1.081	156.0
3	20	0.573	82.6
4	70	0.765	110.4
5	20	0.617	89.0
6	30	0.542	78

Table 3-3. Absolute Lymphocyte values in treated CLL patients

Patient	VPA alone		VPA+FLU						Overall Response
	pre-	post-	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	
CCM1-1	0.8	0.9	0.7	0.4	0.2	0.4	0.2	O/S[1]	SD
CCM1-2	84.5	74.4	59.3	56.2	80.4	12.2	3.2	O/S[3]	SD
CCM1-3	112.1	164.4	O/S[2]						PD
CCM1-4	88.8	97.7	78.9	51.7	79.3	25.3	5.0	O/S[3]	SD
CCM1-5	94.5	88.4	92.8	88.1	124.3	37.3	19.0	23.9	PR
CCM1-6	98.1	109.4	106.0	36.9	23.4	11.5	4.9	8.1	PD

O/S = Off Study, SD = Stable Disease, PR = Partial Response, PD = Progressive Disease

1. No further treatment required
2. Discontinued due to fatigue
3. Discontinued due to anemia



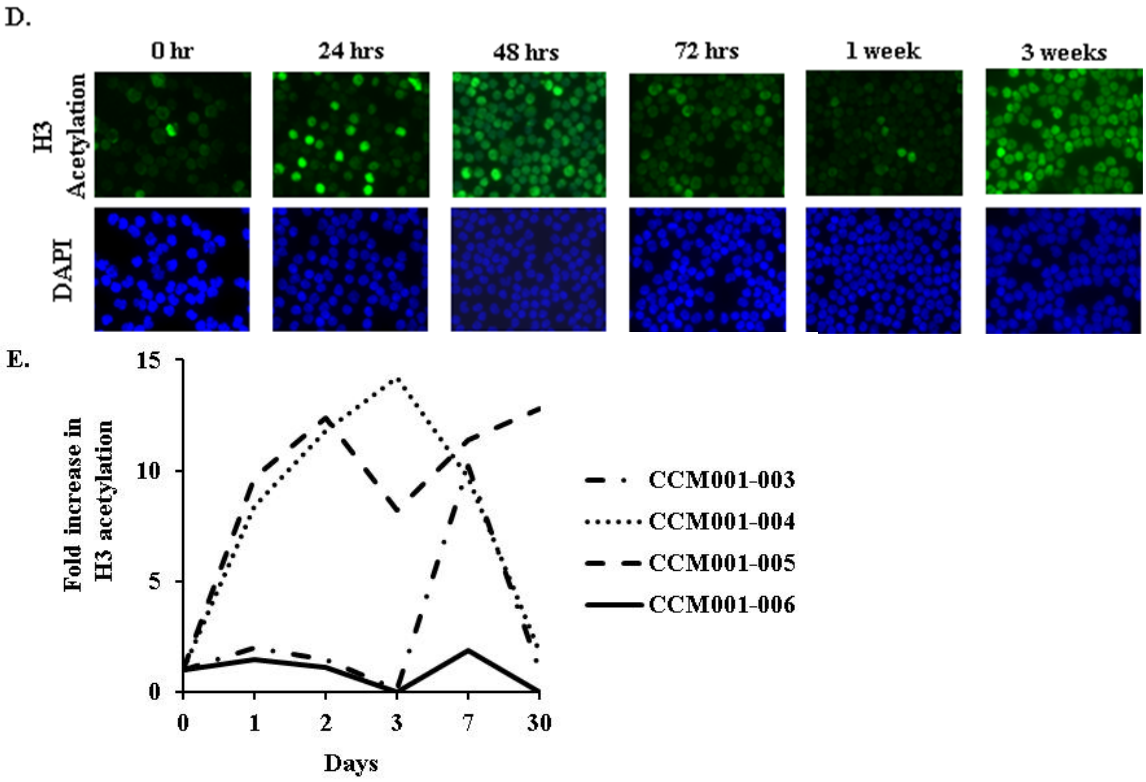


Figure 3-10. Clinical and translational data.
A) Absolute Lymphocyte Count values of the six patients enrolled in the clinical trial. B) Waterfall plot of best decrease in lymph node sizes. C) Whole cell lysates obtained from four patients while undergoing VPA monotherapy at indicated times. D) Densitometry was performed on cathepsin B cells in CLL cells and normalized to GAPDH. E) CLL cells from patients treated with VPA were collected over 30 days. The cells were lysed and western blotted for histone acetylation and densitometry performed. Fold increase was normalized to GAPDH.

3.5 DISCUSSION

Although it has recently become apparent that the lysosome can contribute to cell death, this activity has been largely unstudied in CLL. The lysosome is the ultimate target for the TOSO:IgM complex in CLL cells, thereby contributing to the regulation of the BCR signalling pathway.(47) In the context of rituximab, a type I anti-CD20 mAb, the lysosome is the ultimate destination for FcγRIIB-promoted internalization of CD20:anti-CD20 complexes leading to reduced expression of CD20 on the cell surface, thereby contributing to rituximab resistance.(48) On the other hand, GA101, a type II anti-CD20

mAb, induces lysosome-dependent cell death, which was increased with CD40 ligation.(49) Thus, lysosome mediated cell death may be a novel mechanism to kill CLL cells.

In this study, we describe the involvement of the lysosome in fludarabine- and VPA/fludarabine-induced death of CLL cells. While the GA101-induced cell death was described as being non-apoptotic,(49) VPA with fludarabine treatment induced DNA fragmentation, phosphatidylserine externalization, nuclear condensation (data not shown) and the activation of multiple caspases, thus demonstrating that the cell death is apoptotic in nature. The difference between the treatments with GA-101 *versus* VPA and fludarabine could be due to the extent of LMP. It is known that LMP can lead to both apoptosis and necrosis, depending on the extent of LMP; necrosis appears to be the dominant pathway of death in the setting of massive LMP, while partial LMP drives apoptotic cell death.(23) GA101-induced LMP was rapid and obvious after hours of treatment,(49) whereas fludarabine-induced LMP was much slower in comparison. By simulating LMP, we show that activated cathepsin B can target anti-apoptotic proteins, Mcl-1 and XIAP, as well as caspase-2. Considering the importance of the anti-apoptotic proteins in CLL cells survival, especially in the tumour microenvironment,(50) targeting their degradation through cathepsin B could be a novel strategy to reduce drug resistance in the CLL microenvironment. In line with this hypothesis, we observed shrinkage of the lymph nodes in four of the six patients treated with the VPA/fludarabine combination, and we believe cathepsin B activation and release contributed to this clinical observation. The role of cathepsin B in the tumour microenvironment will be a focus of future investigations.

Some HDIs have also been described to cause LMP itself. Sulforaphane monotherapy has been observed to induce LMP,(51) and the release of cathepsin B was found to contribute to the toxicity of doxorubicin when combined with HDIs.(52) In this study, VPA up-regulated cathepsin B levels in cell lines, primary CLL cells *in vitro* and in the leukemic cells of CLL patients treated in the clinic, resulting in increased protease activity in cell lysates. As cathepsin B itself has been found to be important for the disruption of lysosomes,(53) the increase in cathepsin B activity is likely to be sensitizing the lysosomes to LMP, as well as the subsequent cathepsin B-dependent protein degradation. In early studies that examined the biochemical activity of lysosomal enzymes in CLL, such as acid phosphatases and β-glucuronidases, peripheral blood CLL cells showed lower activity compared to normal lymphocytes,(54) while another study showed CLL samples to have lower lysosomal acid phosphatase activity compared to other lymphomas.(55) While VPA had little activity when used alone in CLL patients, it increased cathepsin B levels in the cells of all four patients examined. In addition, there was a synergistic response between VPA and fludarabine in primary CLL cells *in vitro*, and VPA produced a similar effect on the cathepsin B levels in CLL cells in treated patients, it is likely that VPA contributed to the clinical response. Thus, up-regulation of cathepsin B levels would be a sensible and rational adjunct strategy for lysosome-targeted therapy.

3.6 CHAPTER REFERENCES

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4. VALPROIC ACID ENHANCES FLUDARABINE INDUCED APOPTOSIS MEDIATED BY ROS AND INVOLVING DECREASED AKT AND AMT ACTIVATION IN B DERIVED LYMPHOID CELLS

Ju-Yoon Yoon[1,3], Ganchimeg Ishdorj[1,3], Bonnie A. Graham[1,3], James B. Johnston[2,3], Spencer B. Gibson[1,3]. Valproic acid enhances fludarabine-induced apoptosis mediated by ROS and involving decreased AKT and ATM activation in B-cell-lymphoid neoplastic cells. Apoptosis. 2013 Sep 22. [Epub ahead of print]

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Short Title: VPA modulates ROS, ATM, and AKT activation in CLL

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JY, SBG and JBJ designed the research study.
JY wrote the paper.

4.1. ABSTRACT

Histone deacetylase (HDAC) inhibitors sensitize leukemia cells to induce apoptosis. Unfortunately, T-cell derived leukemia is the only leukemia that HDAC inhibitors have shown clinical efficacy. In chronic lymphocytic leukemia (CLL), the first line therapy is based on the combination of fludarabine, a nucleoside analogue, and rituximab, an anti-CD20 monoclonal antibody, and there are presently no HDAC inhibitors are used to manage CLL. In the present study, we found that the addition of valproic acid (VPA), a HDAC inhibitor, increases cell death in B-cell-neoplasm-derived cell lines, BJAB, NALM-6 and I-83. This increased apoptosis caused release of mitochondrial cytochrome c, activation of caspases, and increased reactive oxygen species (ROS). The addition of a ROS scavenger inhibited cell death induced by the VPA-fludarabine combination. In contrast, blocking the death receptor pathway failed to inhibit VPA increased fludarabine induced apoptosis. Combination of VPA and fludarabine treatment decreased both total and phosphorylated levels of AKT, an important anti-apoptotic protein, and ATM, a pivotal protein in DNA damage response. Chemical inhibition of AKT or ATM was sufficient to enhance fludarabine-induced apoptosis. We next examined patient samples from a local clinical trial where relapsed CLL patients were treated with VPA and examined the effects of VPA on AKT and ATM *in vivo*. After 30 days, there was a reduction in ATM levels in three out of the four patients treated, while AKT phosphorylation was reduced only in one patient. Taken together, VPA reduces ATM levels, thereby increasing ROS-dependent cell death via the mitochondrial apoptotic pathway when combined with fludarabine, and the effect of VPA on ATM spans *in vitro* to *in vivo*.

4.2. INTRODUCTION

Fludarabine is a nucleoside analogue that forms the cornerstone of therapy in the management of Chronic Lymphocytic Leukemia (CLL) (1). The disease is characterized by the accumulation of slow-proliferating monoclonal CD19+/CD5+/CD23+ lymphocytes with progressive immune deficiency as the disease advances until the patient succumbs to the disease. Fludarabine combinational treatment involving rituximab has shown superior clinical responses compared to fludarabine and cyclophosphamide combined chemotherapy,(2), but unfortunately these patients also relapse. Besides CLL, fludarabine in combinational therapies is used to treat non-Hodgkin's lymphoma and other leukemias, but fludarabine resistance remains a major issue (3, 4). Thus, improved fludarabine-based combinations are needed.

Inhibition of histone deacetylases (HDAC) using pharmacological agents, such as depsipeptide, suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) is a novel strategy to treat leukemias (5, 6). *In vitro* results with HDAC inhibitors (HDIs) using depsipeptide, LBH589 and MS-275 were promising, implicating a number of different mechanisms associated with the inhibition of HDACs in CLL cells (7-10). However, in the clinic, monotherapy trials using HDIs in leukemia patients have been disappointing. A Phase I trial with depsipeptide observed no responses, despite obvious increases in the level of acetylated histones and p21 *in vivo* (11). A Phase II trial using MGCD0103 in previously treated CLL patients also produced no responses, despite some patients receiving concomitant rituximab (12). While both depsipeptide and MGCD0103 were tolerable, hematological grade 4 toxicities were observed with both drugs.(11, 12).

Since HDIs as monotherapy are not effective, we rationalized that VPA might be more useful if combined with chemotherapy. VPA is a first-generation anti-epileptic that has class I HDAC inhibition activity with IC_{50} values for HDAC1-3 of $\leq$ 1 mM (13-15). Treatment of CLL cells *in vitro* with VPA has been shown to induce apoptosis, as judged by caspase activation and Annexin V staining, while VPA inhibited proliferation of CLL cells induced by oligonucleotide and IL-2 co-stimulation (16-18). Single-agent VPA was sufficient to induce alterations in the gene expression level of a large number of genes and to change the Bcl-2/Bax ratio at the protein level (18, 19). Furthermore, VPA enhanced the effects of various chemotherapeutic agents on CLL cells, including fludarabine, bortezomib, flavopiridol, thalidomide and lenalidomide (5).

In this study, we found that VPA enhanced fludarabine-induced apoptosis through the mitochondrial apoptotic pathway, but independent of the classic death receptor pathway in B-cell neoplasm-derived cell lines. A part of the VPA-fludarabine *in vitro* was associated with reduction in ATM levels and AKT phosphorylation, but independent of their effects on NF-κB. When examined *in vivo*, however, VPA was insufficient to lower AKT phosphorylation, but VPA treatment was sufficient to lower ATM levels *in vivo*.

4.3. MATERIALS AND METHODS

4.3.1 Cell culture and treatment conditions

The diagnosis of CLL was made by peripheral blood morphology and the presence of monoclonal B cells in the peripheral blood with typical immunophenotype ($CD19^+$, $CD5^+$ and $CD23^+$). Peripheral blood samples were obtained from CLL patients

following informed consent, in agreement with the Research Ethics Board at the University of Manitoba. Peripheral blood mononuclear cells were isolated from the buffy coat using a Ficoll-Paque density gradient as previously described (20). Freshly isolated CLL cells were cultured in RPMI-1640 culture medium supplemented with 100 U of penicillin, 100 mg of streptomycin and 0.5% Bovine Serum Albumin (BSA). Three human B-cell leukemia/lymphoma cell lines, BJAB, I-83 and NALM-6, were cultured in RPMI-1640 culture medium supplemented with 100 U of penicillin, 100 mg of streptomycin and 10% fetal bovine serum (FBS).

4.3.2 Reagents used

Fludarabine, valproic acid, propidium iodide and trichostatin A were purchased from Sigma-Aldrich. Suberoylanilide hydroxamic acid (SAHA) was a gift from Dr. Jim Davie (University of Manitoba). Antibodies against AKT, phospho-AKT, acetyl-H3, RelA, BID and Caspases-3, -8, -9 were from Cell Signalling. Antibodies against γH2A.X, DR4, DR5 and TNFR1 were purchased from AbCam, and antibodies against β-actin and GAPDH were from Sigma. Antibody against TRAIL was purchased from BD Biosciences, and anti-TNF antibody was from R&D Systems. Anti-total ATM was from Novus Biologicals, and anti-phospho-ATM (ser1981) was from Epitomics. Antibodies against Fas and I-κB were purchased from Santa Cruz. Soluble super-TRAIL, FASL, and TNFα was purchased from ALEXIS, as were the fusion proteins DR4-Fc, TNFR-Fc, and FAS-Fc.

4.3.3 Cell death assays

Nuclear fragmentation was assessed by propidium iodide (PI) staining in hypotonic conditions and was performed with minor modifications using the direct DNA staining method (21). Briefly, cell pellets were resuspended in the PI staining buffer, supplemented with RNase (100 μg/mL). Cells were stained for 30 minutes at room temperature in the dark, after which samples were kept on ice and analyzed on the flow cytometer within an hour.

4.3.4 Measure of Synergy

Synergistic induction of apoptosis was examined by measuring %SubG1 events in response to increasing doses of fludarabine, VPA and the combination of 1 mM VPA with increasing dose of fludarabine. IC_{30} (for fraction affected (Fa) = 0.3) or IC_{50} (Fa = 0.5) values were determined for three curves generated, and the following equation was used: Combination Index (CI) = $D_1/(D_m)_1 + D_2+(D_m)_2$, where $(D_m)_1$, $(D_m)_2$ are median doses (i.e. where 30% or 50% SubG1 events are observed) for drugs 1 and 2, and D_1, D_2 are doses are the doses in combination that induced 30% or 50% SubG1 events.

4.3.5 Whole cell lysates and immunoblotting

Whole cell lysates were prepared from cell pellets which were subjected to a single cycle of freeze-thaw at -80°C, then resuspended in the radioimmunoprecipitation assay (RIPA) buffer supplemented with the Complete Mini protease inhibitor cocktail (Roche), phosphatase inhibitor cocktails (Sigma Aldrich) and sodium orthovanadate (1

mM, Sigma Aldrich). Protein samples were quantified using either the Bradford (BioRad) or the bicinchoninic acid (Pierce) protein quantification assay.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using standard protocols. Resultant blots were transferred onto nitrocellulose membranes and blocked using 5% milk or 5% BSA in Tris-Buffered Saline-Tween-20 (TBST) (0.1% Tween-20). Blocked membranes were incubated overnight in the primary antibody mix, followed by incubation in the secondary antibody mix (Horse Radish Peroxidase (HRP)-conjugated anti-mouse/rabbit antibody (BioRad). Enhanced chemiluminescence was performed using commercial reagents (Amersham or Pierce).

4.3.6 Fluorescence microscopy

Cells were first stained with MitoTracker as suggested by the commercial protocol. Cells were cyto-spun onto microscope slide and fixed in 3.7% formaldehyde in PBS overnight at 4°C. Cells were washed three times for 5 min in PBS, then cells were permeabilized in 0.5% Triton X-100 for 10 minutes at room temperature. Slides were washed again in PBS, followed by incubation in the appropriate primary antibody for at least 1 hour at room temperature. The slides were then re-washed, followed by incubation in the FITC-conjugated secondary antibody solution for 1 hour at room temperature. Slides were washed again, and DNA was counterstained with 4'6-diamidino-2-phenylindole (DAPI).

4.3.7 Patients treated with VPA

CLL patients 18 years of age or older who required therapy, with an ECOG performance score of two or less, and had been previously treated with a nucleoside analog were recruited into the trial. Patients were treated with oral VPA at a starting dose of 15 mg/kg/day orally in divided doses, with the goal of reaching a serum level of > 1 mM through weekly therapeutic drug monitoring and dose escalation.

4.4 RESULTS

4.4.1 VPA increases fludarabine cytotoxicity against B-cell leukemoid cells

Burkitt lymphoma BJAB, acute lymphocytic leukemia NALM-6, and CLL-like I-83 cell lines were treated with the combination of fludarabine (5μM) and VPA (1mM), and we found that VPA increased fludarabine-induced apoptosis (**Figure 4-1**A). The interaction between VPA and fludarabine was confirmed to be synergistic when we determined the combination index (CI) value for the interaction in NALM-6 cells, which showed that the CI values were less than one for VPA, as well as two other HDAC inhibitors, SAHA and Trichostatin A (TSA), indicating a synergistic interaction (**Figure 4-1**B). In primary CLL cells, 1 mM VPA, a clinically attainable plasma concentration, was minimally toxic, as observed in cell lines, and higher doses were required to cause more considerable toxicity (**Figure 4-2**). However, combining 1 mM VPA with fludarabine resulted in enhancement of fludarabine toxicity over a range of concentrations, as previously reported (**Figure 4-2**B). Thus, VPA, in combination with fludarabine, increases apoptosis in B cell derived cell lines and primary CLL cells.

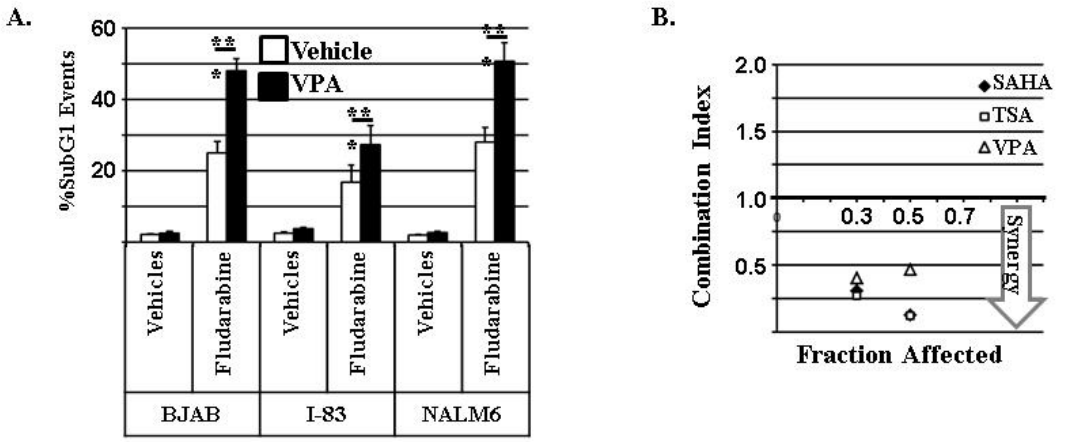

Figure 4-1. Valproic Acid (VPA) and Fludarabine increases apoptosis in B lymphoid cells.

A) BJAB, NALM-6 and I-83 cells were treated with VPA (1 mM), fludarabine (5μM) and in combination for one day, and the level of apoptosis was determined using the subG1 method, as described in the methods. The bars represent average values, error bars represent standard error of at least three independent experiments. *** indicates p-value < 0.01. B) Combination index (CI) values were determined for the drug interaction between fludarabine and three HDAC inhibitors, VPA, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), as described in the methods in NALM-6 cells. CI values less than one indicates a synergistic drug interaction.

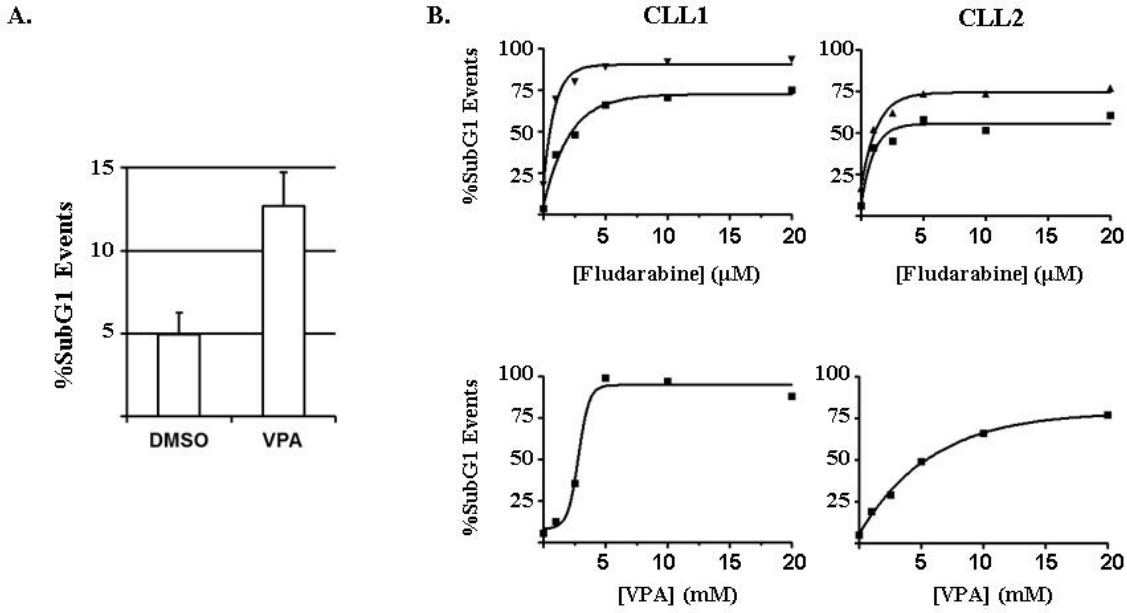

Figure 4-2. VPA and fludarabine combination in primary CLL cells.

A) Primary CLL cells were incubated with VPA (1 mM), and the level of apoptosis was determined by sub-G1 peak analysis. B) CLL cells were treated with increasing doses of fludarabine (top) in the presence or absence of 1mM VPA or increasing doses of VPA

(bottom) for two days. The amount of cell death was determined by subG1 peak analysis. Representative dose-response curves from two CLL samples are shown.
C) The level of reactive oxygen species was determined by Dihydroethidium staining in primary CLL cells. The percentage of stained cells was detected by flow cytometry after VPA treatment. Error bars represent standard error of three experiments. e) Primary CLL cells from six different patients were treated with fludarabine, VPA and in combination in the presence or absence of NAC. The amount of cell death was determined by subG1 peak analysis.

4.4.2 VPA and fludarabine combination increased apoptosis through the mitochondrial apoptotic pathway

There are two major apoptotic pathways, the mitochondrial and the death receptor pathways (22). The mitochondrial apoptotic pathway is characterized by the release of mitochondrial proteins, such as cytochrome *c* (22). We stained the mitochondria with MitoTracker and immunostained for cytochrome *c* in BJAB cells. In untreated and VPA-treated CLL cells, cytochrome *c* was localized in the mitochondria. Following fludarabine treatment, cytochrome *c* staining was diffuse and failed to co-localize to the mitochondria, and this pattern was more pronounced in cells treated with the VPA and fludarabine combination (**Figure 4-3**A). As expected in cell death scenarios involving disruption of the mitochondria, cell death in response to fludarabine alone or in combination with VPA resulted in elevated levels of superoxide, as measured by flow cytometry (**Figure 4-3**B). In order to determine whether reactive oxygen species (ROS) also contributed to the initiation of cell death, BJAB cells were pre-treated with the ROS scavenger *N*-acetylcysteine (NAC). NAC reduced activation of caspases-8, -9, -3 and BID cleavage and reduced γH2A.X levels, while increasing histone 3 acetylation (**Figure 4-4**). The addition of NAC reduced the cytotoxicity of fludarabine and the VPA/fludarabine combination in the three cell lines, as well as primary CLL cells

(**Figure 4-4**B,C). In summary, the above data suggest the mitochondrial pathway is involved in the synergistic apoptotic response, mediated at least partly by ROS.

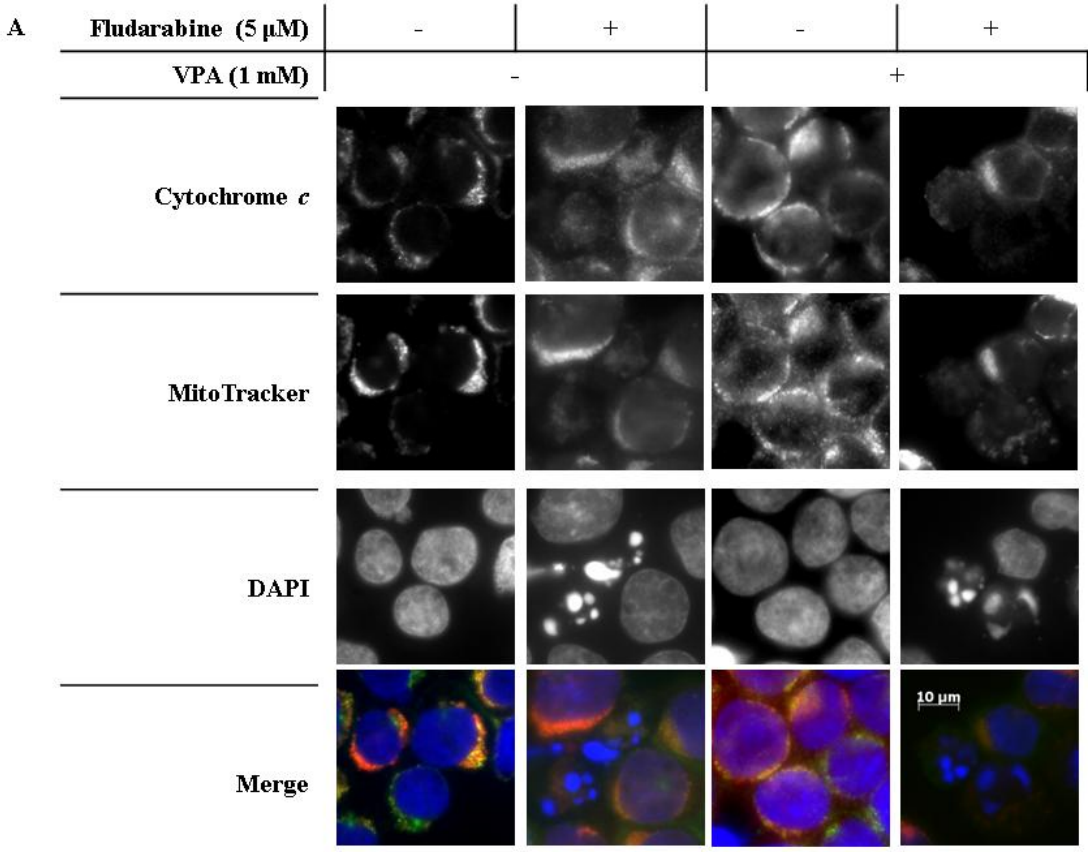

Figure 4-3. Effects of VPA and Fludarabine treatment on the mitochondria apoptotic pathway.
a) BJAB cells were treated with the indicated treatments for 24 hours, and the mitochondria were stained using MitoTracker before fixation in 3.7% formaldehyde. Cells were also then stained for cytochrome c and DNA was stained with DAPI. Scale bar = 10 µm. b) Quantification of ROS levels using dihydroethidium staining after one-day treatment of BJAB cells as indicated.

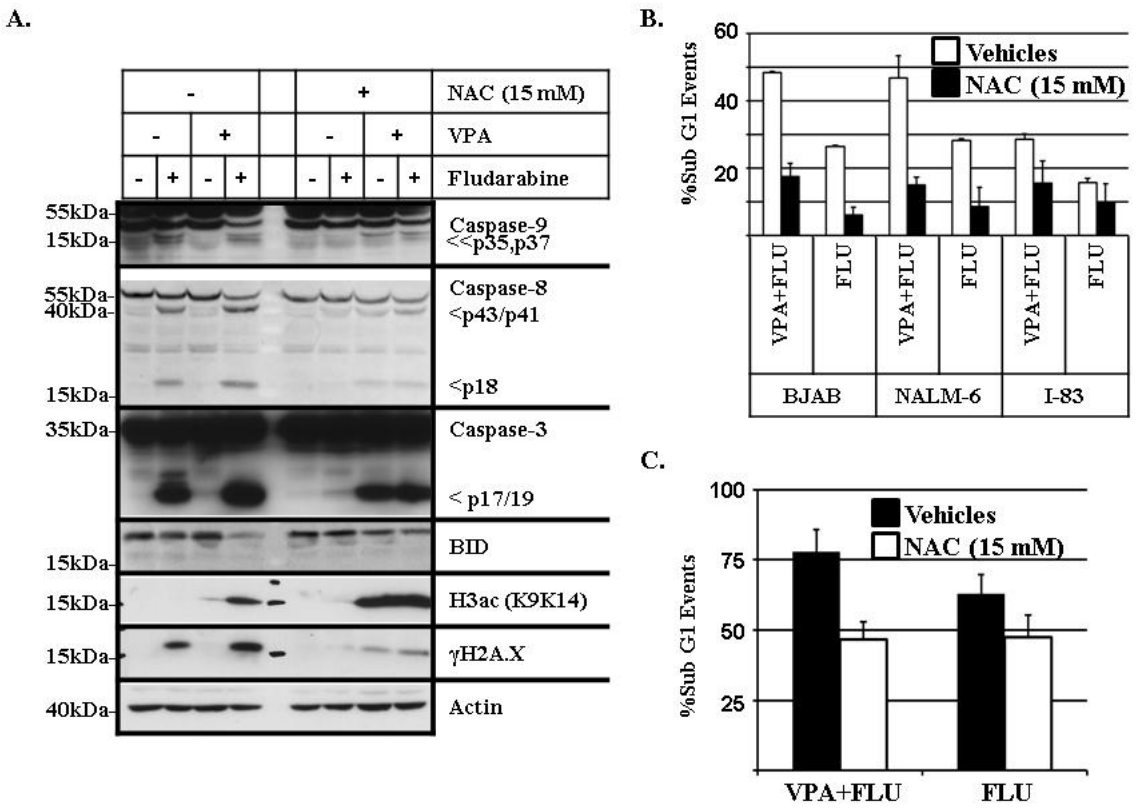

Figure 4-4. Effects of the ROS scavenger N-acetylcysteine (NAC) on VPA-fludarabine drug interaction.
A) BJAB cells were pre-treated with NAC (15 mM) or the vehicle control for 1 hour prior to treatment of the cells with different combinations of VPA and fludarabine as indicated for one day. Whole cell lysates were obtained and proteins were analyzed via immunoblotting. B) Three cell lines were pre-treated with either vehicle or NAC, prior to their treatment with fludarabine ± VPA for one day. Error bars represent standard errors of means from at least three independent experiments. C) Primary CLL cells were pre-treated with either vehicle or NAC, prior to their treatment with fludarabine ± VPA for two days.

The classic death receptor pathway involves the ligation of the death receptors, ex. death receptors 4 (DR4) and DR5 by TNF-related apoptosis-inducing ligand (TRAIL), followed by the caspase-8 activation (23), and we observed that the VPA and fludarabine combination leads to caspase 8 cleavage. Furthermore, HDI therapy has been shown to sensitize CLL cells to TRAIL-induced cell death (8, 24), leading to our hypothesis that part of the VPA-fludarabine synergy may be associated with the death receptor apoptotic

pathway. The combination of TRAIL, TNF-α or Fas ligand (FasL) with fludarabine resulted in significant increases in fludarabine-induced cell death, demonstrating that ligation of death receptor combined with fludarabine therapy enhances cell death in leukemic cells (**Figure 4-5**A). We then pre-treated cells with DR4:Fc, FAS:Fc or TNFR:Fc, which sequesters death receptor ligands away from their respective receptors, and they were pre-treated at doses that were determined to be sufficient to inhibit the recombinant ligands at doses of 100 ng/mL. In contrast to our expectation, this treatment failed to reduce VPA/fludarabine-induced apoptosis (**Figure 4-5**B). Furthermore, the expression levels of death receptors and ligands failed to be increased by VPA (with the exception of TRAIL), and their expressions were actually reduced by combining VPA with fludarabine in BJAB cells (**Figure 4-5**C). In summary, the above data suggest the death receptor pathway does not contribute to the VPA-fludarabine synergy.

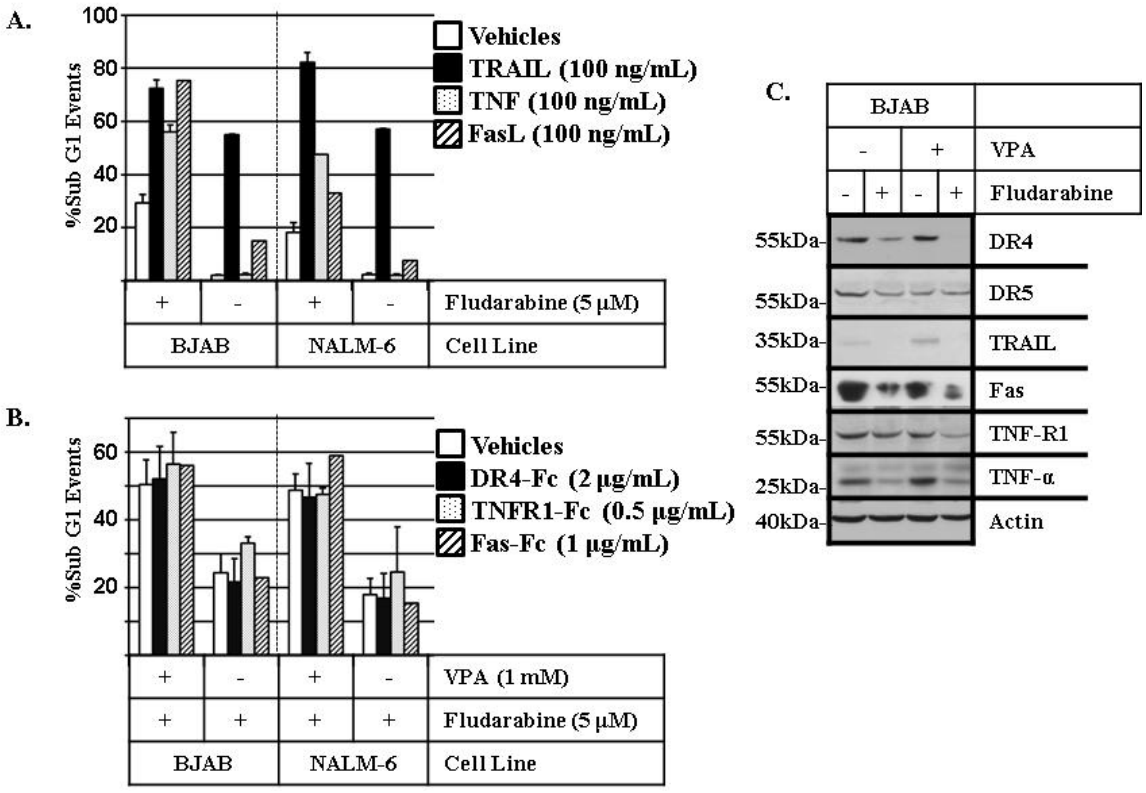

Figure 4-5. The role of death receptor ligands in VPA-Fludarabine induced apoptosis.
A) BJAB and NALM-6 cells were treated with fludarabine (5 µM) or the vehicle (DMSO) in combination with vehicle (PBS), recombinant TRAIL, TNF-α or Fas ligand at the indicated doses for one day. DNA fragmentation was quantified by flow cytometry, examining for hypodiploid DNA content. B) BJAB and NALM-6 cells were pre-treated for one hour using chimeric receptors at the indicated doses to sequester TRAIL, TNF-α or FasL, then treated with fludarabine ± VPA for one day. C) Whole cell lysates from BJAB cells were examined one-day post-treatment to examine the different death receptors and their ligands. Error bars represent standard error of three independent experiments.

4.4.3 VPA treatment decreases AKT activation and expression

In CLL cells, pro-survival signals through AKT activation are thought to be some of the main pro-survival pathways in CLL cells. A number of HDAC inhibitors, including VPA, trichostatin A and SAHA, have been shown to reduce AKT phosphorylation (25, 26). We thus hypothesized that VPA may be increasing apoptotic cell death by dampening the AKT pathway and examined whether AKT may be being

affected in response to the VPA-fludarabine combinatory treatment. We first confirmed that the inhibition of AKT is sufficient to enhance fludarabine-induced apoptosis. Using doses of the PI3K/AKT inhibitor LY-294002 at minimally toxic levels (< 10% Sub-G1 events), the combination of LY-294002 with fludarabine induced over two-fold enhancement in fludarabine cytotoxicity in BJAB and NALM-6 cells (**Figure 4-6**A). We next examined the impact of VPA treatment on human B lymphoid cell lines and primary CLL cells. In BJAB cells, 1 mM VPA (a sub-lethal dose) decreased AKT phosphorylation at Ser-473, while phosphorylation at Thr-450 remained unchanged. As a control, valpromide (a VPA analogue with no HDAC inhibitory properties (27)) had no effect at the 1 mM concentration (**Figure 4-6**B). Fludarabine reduced the total AKT levels in a dose-dependent manner after 24 hours treatment in BJAB cells (**Figure 4-6**B). Treatment of primary CLL cells with VPA also resulted in reduction in AKT phosphorylation on Ser-473 after 24 hour treatment, while higher doses also reduced phospho-Thr-450 and total AKT levels after 48 hours treatment (**Figure 4-6**C). Because both VPA and fludarabine targeted AKT as single agents, we next examined the impact of VPA and fludarabine as a combination. In both cell lines and primary CLL cells, the VPA-fludarabine combination resulted in greater reduction in AKT phosphorylation on both Ser-473 and Thr-450 and in total AKT expression (**Figure 4-6**D,E). Taken together, the VPA-fludarabine combination therapy resulted in greater reduction of phospho-AKT levels, and targeting AKT pathway was sufficient to enhance the activity of fludarabine.

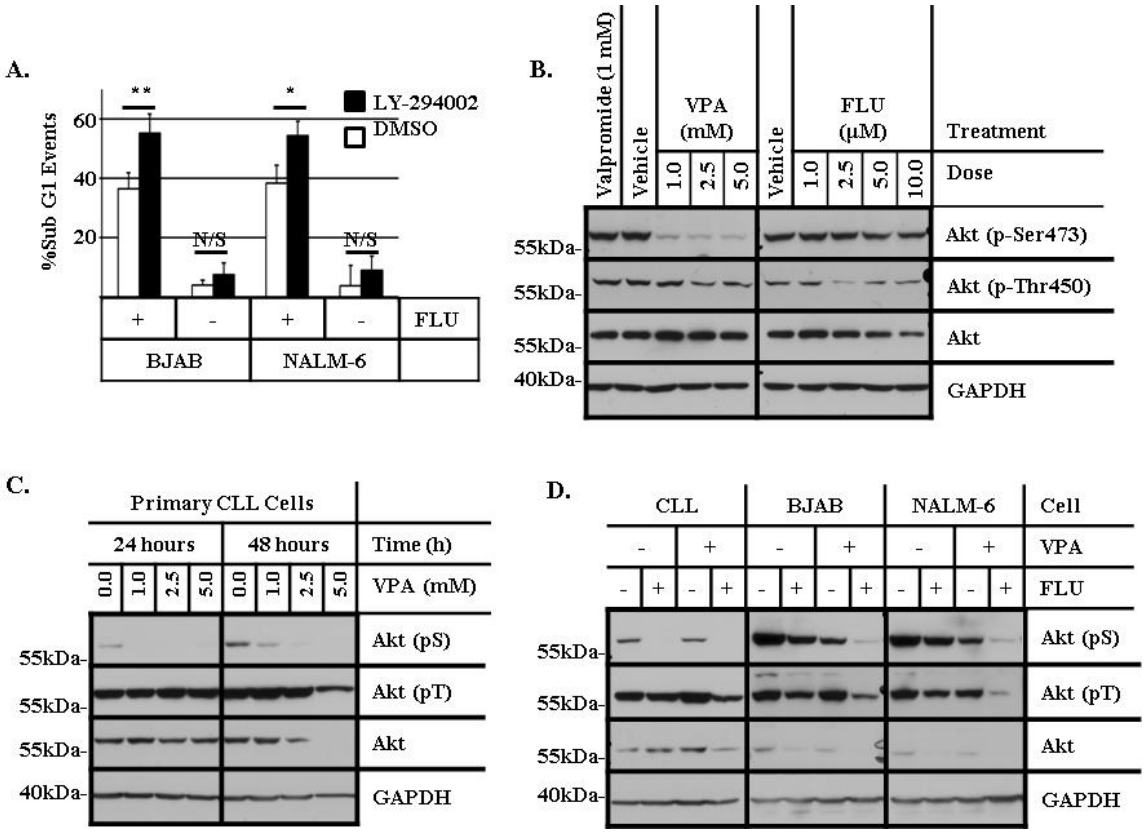

Figure 4-6. AKT and AMT activation and expression is decreased following VPA treatment.

A) BJAB and NALM-6 cells were pre-treated with for 1-hour with the AKT inhibitor LY-294002 and treated as indicated. The level of apoptosis was determined by sub-G1 peak analysis. Error bars represent standard error of three independent experiments. B) BJAB cells were treated with increasing doses of VPA (1-5 mM) or Fludarabine (1-10 μM) for 24 hours and whole cell lysates were analyzed by immunoblotting for phospho-AKT, AKT, phospho-AMT and AMT. GAPDH was used a loading control. Valpromide is a VPA analogue that lacks the HDAC inhibition activity. C) Effects of VPA on AKT and ATM levels in primary CLL cells. Primary CLL cells were treated with increasing dose of VPA for 24 and 48 hours, and whole cell lysates were analyzed via immunoblotting as above. D) Primary CLL (representative sample), BJAB or NALM-6 cells were treated with VPA (1 mM) and Fludarabine (5 μM for BJAB, NALM-6, 10 μM for CLL) and whole cell lysates were analyzed by immunoblotting for phospho-AKT, and AKT. GAPDH was used as a loading control. Blots are representative from at least three independent experiments.

4.4.4 VPA and Fludarabine increased apoptotic response is independent of NF-κB activation

Nuclear Factor κB (NF-κB) pathway is another important pro-survival signalling pathway in CLL cells, and it has been described to be constitutively active in CLL cells (28). While certain HDAC inhibitors, such as PCI-24781, have been shown to dampen NF-κB signalling (29), others, such as MS-275 and SAHA, have been shown to increase NF-κB activation (30). NF-κB inhibition has been shown to enhance fludarabine cytotoxicity in CLL cells (31), and we thus examined whether the NF-κB pathway may be involved in the VPA-fludarabine synergy. Treatment of BJAB cells with fludarabine reduced I-κB levels, which was enhanced by the addition of VPA (**Figure 4-7**A). However, knockdown of the NF-κB subunit RelA failed to protect BJAB cells from fludarabine or the VPA/fludarabine combination (**Figure 4-7**B**)**. In addition, suppression of the NF-κB signalling pathway by exogenous addition of either dominant-negative IKK (IKK-NBD) or cell-permeable inhibitory peptide (SN50) failed to change VPA and fludarabine induced apoptosis in BJAB cells (**Figure 4-7**C**)**. Taken together, the above data suggests that the VPA-fludarabine synergism is independent of NF-κB activation.

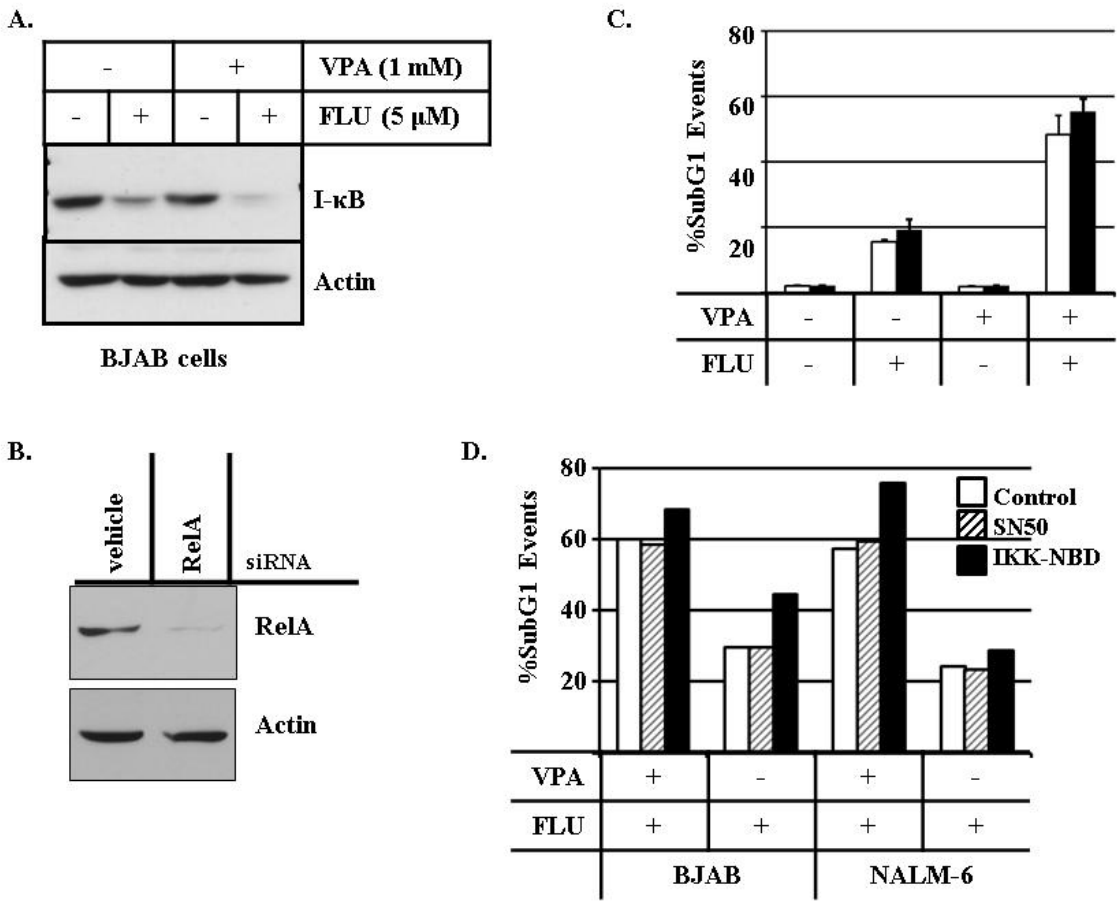

Figure 4-7. Activation of NF-κB fails to change the amount of fludarabine and VPA induced apoptosis.
A) BJAB cells were treated with VPA and/or fludarabine for 24 hours. Whole cell lysates were immunoblotted for I-κB and actin. B) BJAB cells were transfected with control or siRNA against RelA. The cells were lysed and immunoblotted for Rel A and actin. C) The transfected cells from B) were treated with VPA, Fludarabine or in combination and the level of apoptosis determined using the subG1 method. Error bars represent standard errors of means obtained from three independent experiments. D) BJAB or NALM-6 cells were also pre-treated with SN50 (NF-κB inhibitor) or transfected with IKK dominant negative domain peptide (IKK-NBD) for one hour, then the cells were treated with fludarabine ± VPA for 24hours. The amount of cell death was determined by subG1 peak analysis.

4.4.5 VPA decreases ATM activation and expression

In response to DNA damage, cells respond by activating the ATM kinase that is a member of the phosphatidyl inositol 3-kinase-related protein kinase (PIKK) family and plays a crucial role in the DNA damage repair pathways (32). VPA has been shown to

also induce DNA damage (33, 34), and VPA has been shown to reduce *ATM* expression levels in CLL (18), an observation unconfirmed at the protein level. We thus examined whether VPA may be down-regulating ATM to sensitize leukemic cells to fludarabine-induced cell death. We first determined whether targeting ATM is sufficient to enhance fludarabine cytotoxicity. Using minimally toxic doses of KU-55933, a known inhibitor of ATM (35), we observed dramatic enhancements in fludarabine cytotoxicity in BJAB and NALM-6 cells that was not observed in CLL like cell line I-83 (**Figure 4-8**A). When the three cell lines were compared for the level of ATM, I-83 cells were observed to have barely detectable level of ATM in contrast with higher expression in the other two cell lines (**Figure 4-7**B). As single agents, the effects of VPA and fludarabine on ATM in BJAB cells were modest, with reduction of total ATM levels only observed with higher doses of each agent, while fludarabine strongly induced ATM phosphorylation **(Figure 4-7**C**)**. Fludarabine treatment also induced cleavage of ATM, as seen by the appearance of faster migrating bands (**Figure 4-7**C). When primary CLL cells were examined, there was no change in ATM levels following 1 mM VPA treatment, but ATM level was reduced following treatment with higher VPA concentrations (2.5-5.0 mM), suggesting that this is a weak effect (**Figure 4-7**D). When VPA was combined with fludarabine, combined treatment resulted in more pronounced reduction in the levels of total ATM levels, and there were lower levels of phosphorylated ATM (**Figure 4-7**D). Such effects were not observed with the valpromide and fludarabine combination. Taken together, targeting ATM, at least those cells that express ATM, can enhance fludarabine cytotoxicity, and the VPA-fludarabine combination reduces total and phosphorylated ATM levels *in vitro.*

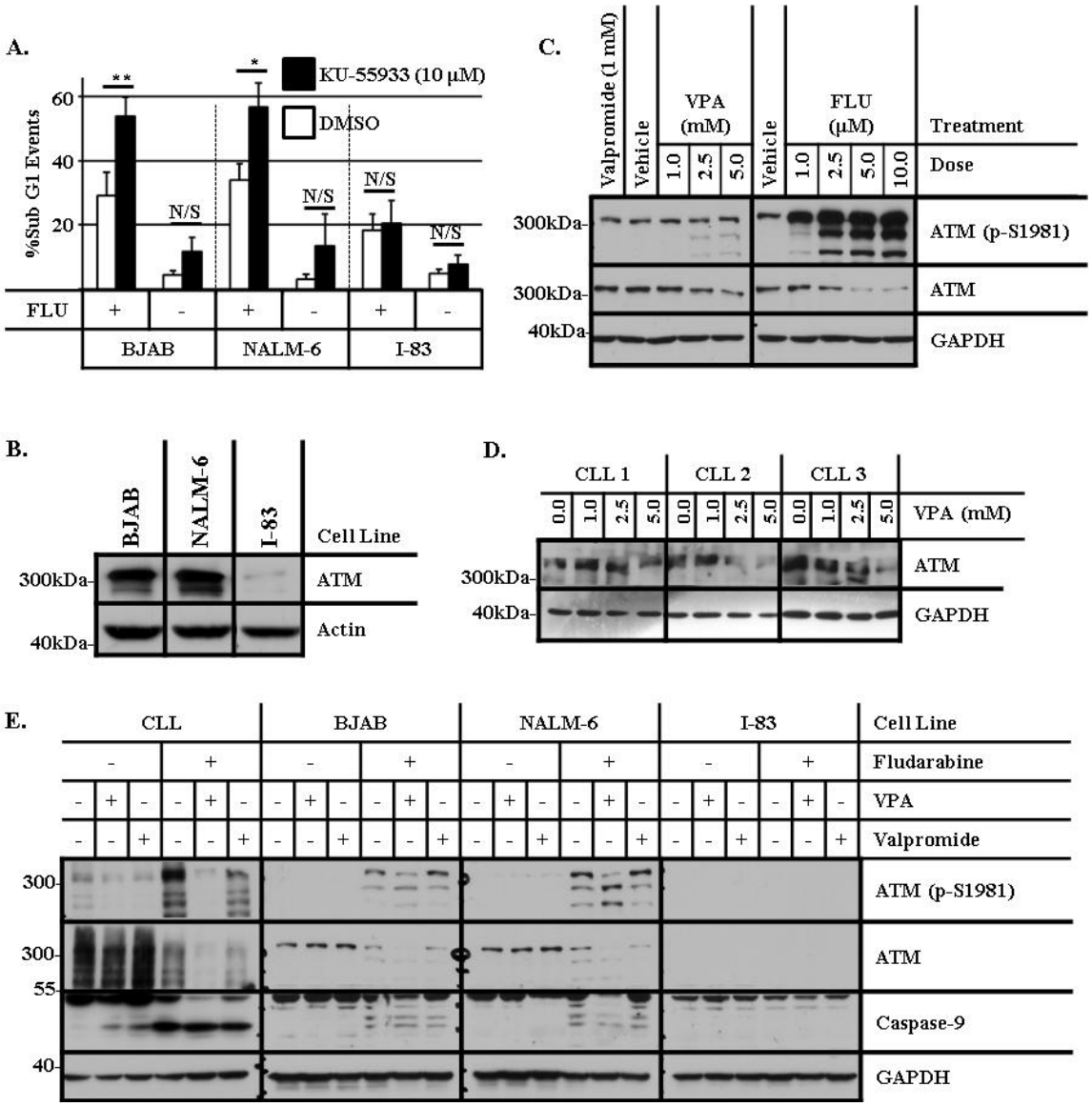

Figure 4-8. ATM activation and expression is decreased by VPA-fludarabine combination.

A) BJAB, NALM-6 and I-83 cells were treated with indicated treatments, and apoptosis was detected by subG1 peak analysis. B) To compare total levels of ATM in the three cell lines, whole cell lysate was immunoblotted for ATM, with actin used as a loading control. C) CLL cells were treated with the indicated dose of VPA, for 24 hours, and whole cell lysates were analyzed by immunoblotting for ATM. D) Primary CLL (representative), BJAB, NALM-6 and I-83 cells were treated (one day for cell lines, two days for primary CLL) with indicated combinations of VPA (1 mM), valpromide (1 mM) and fludarabine (5 μM for cell lines, 10 μM for primary CLL). Whole cell lysate were analyzed via immunoblotting.

4.4.6 CLL patients treated with VPA showed decreased ATM expression

Summarizing the above data, we have shown that VPA affects ATM and AKT *in vitro*, two mechanisms that contribute to the VPA-fludarabine interaction. In order to examine these findings *in vivo*, we examined CLL cells isolated from patients who were locally treated with VPA. CLL cells were obtained from relapsed, previously treated patients and samples were isolated on days 0, 1, 2, 3, 7 and 30 days post-VPA treatment. Results from this clinical trial are published elsewhere (This Study). We first examined the levels of histone-3 acetylation in CLL cells isolated from CLL patients by immunostaining. We found that levels of histone-3 acetylation initially increased after 24 and 48 hours of treatment, and the levels fluctuated over time (**Figure 4-9**A). Next, we examined these patient samples to determine the level of AKT and ATM activation. In contrast to the *in vitro* results, phospho-AKT (Ser-473) levels decreased in only one of the four patients analyzed (CCM001-003), and the total AKT levels remained unchanged (**Figure 4-9**B). In contrast, ATM levels were decreased in three of the four patients in response to VPA by day 30, while it remained unchanged in the remaining one (**Figure 4-9**B). Thus, our data indicates that VPA monotherapy in CLL patients is sufficient to reduce total ATM levels *in vivo*.

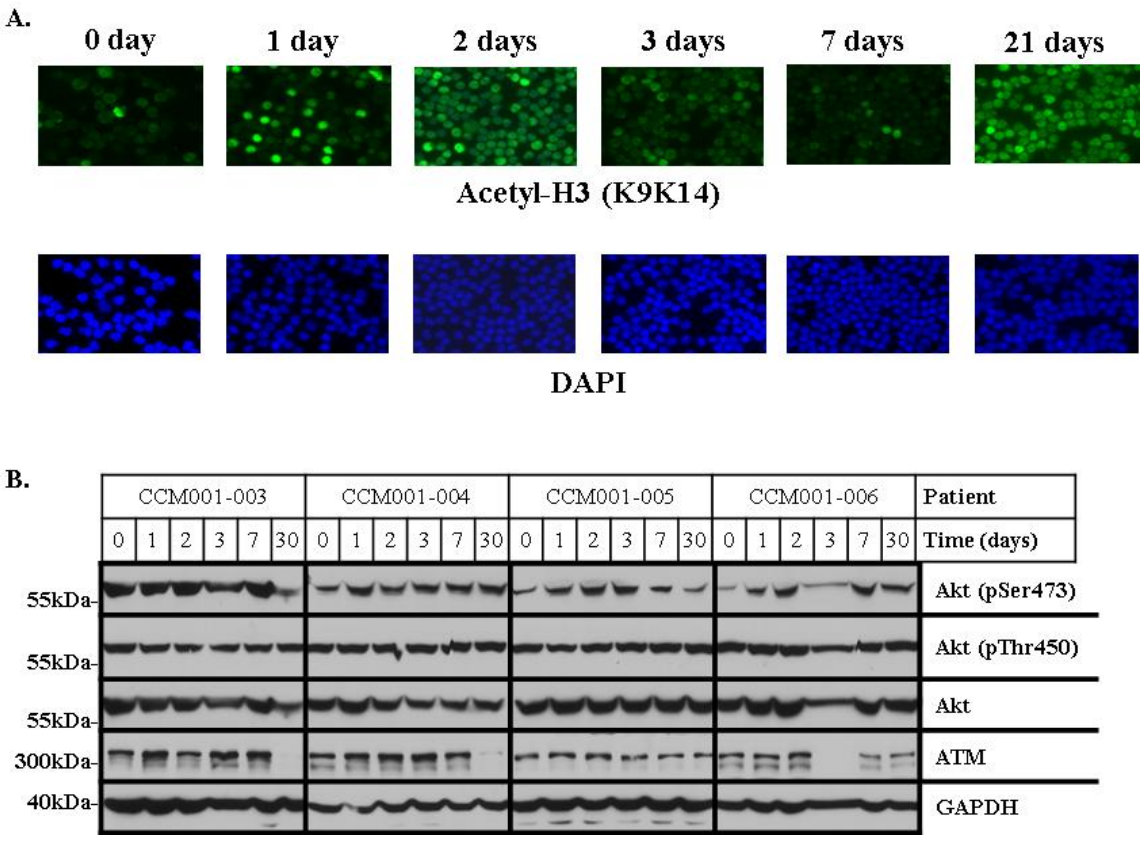

Figure 4-9. In vivo effects of VPA treatment on histone acetylation, AKT and ATM expression in CLL cells.
A) Primary CLL cells were isolated for a patient treated with VPA and immunostained for acetylated histone 3 (lysine 9 and lysine 14). DNA was stained by DAPI. B) Whole cell lysates from CLL cells obtained from four patients while undergoing VPA monotherapy at the indicated times. Whole cell lysates were immunoblotted for AKT (phosphorylated and total), ATM and GAPDH.

4.5 DISCUSSION

In this study, we described a synergistic interaction between VPA and fludarabine, and this interaction was shown to be dependent on ROS production and independent of the death receptors. A part of this interaction involves favorable combinations of effects of the drugs on both the AKT and ATM, while being independent of the NF-κB pathway. AKT activation leads to cell survival through phosphorylation of BAD, mTOR activation, activation of NF-κB, increased Bcl-2 family members and inhibiting caspase 9 (36).

HDAC inhibitors, at least in certain cancer cells, have been demonstrated to inhibit AKT activation (37), and VPA has been shown to down-modulate AKT in ovarian cancer and colon cancer models (38, 39). This could be cell type specific, since VPA has been shown to increase AKT phosphorylation in neuroblastoma cells (40). We showed in this study that VPA in combination with fludarabine reduced AKT phosphorylation and expression *in vitro*. Thus, VPA may be priming the leukemic cells for apoptosis by reducing AKT phosphorylation, thereby presumably down-regulating the pro-survival pathway. Despite the clear *in vitro* results, however, such an effect of VPA on AKT was not observed *in vivo* despite prolonged course of VPA treatment. This discordance between the *in vitro* and *in vivo* results may be due to a number of factors. One hypothesis is that the effect of VPA may be being negated by various pro-survival factors in the human circulation, as well as the tumor microenvironment. BCR activation in CLL cells is associated with AKT activation (41), and the effect of VPA on AKT may be too weak to overcome the other factors *in vivo*.

Another facet of the VPA-fludarabine drug interaction described in this study is the reduction of ATM by VPA. Incorporation of fludarabine into the DNA and subsequent induction of DNA damage are thought to be important aspect of fludarabine mechanism. In line with this model, CLL cells respond by upregulating a number of p53-downstream target genes, such as *bax*, *in vivo* (42). In this study, we found that ATM levels were reduced with administration of VPA *in vitro* and *in vivo*. As we have shown the importance of the mitochondrial pathway and ROS in the VPA-fludarabine interaction, a reduction in ATM levels would dampen the cells' response to the genotoxic assault from fludarabine, leading to enhanced activation of the mitochondrial apoptotic

pathway, as we observed. Similarly, dampening of the DNA damage response using the Poly (ADP-ribose) polymerase (PARP) inhibitor, olaparib, has been reported to enhance the cytotoxicity of fludarabine in CLL cells (43). Interestingly, among the reagents examined, the greatest synergism with olaparib was observed with high-dose VPA (5 mM) (43), a dose which should be sufficient to lower ATM levels, as well as causing DNA damage (likely via ROS generation). While we were unable to examine AKT and ATM levels in patients treated with the VPA-fludarabine combination in this study, this study predicts that ATM reduction would lead to increased DNA damage and enhanced cytotoxicity. Indeed, we previously reported that the VPA-fludarabine combined therapy leads to decreased peripheral and lymph node tumor burden in relapsed CLL patients (this study). Considering the excellent tolerability of VPA and the poor response of elderly patients to fludarabine and rituximab treatment due to toxicities (44), addition of VPA is an attractive and feasible strategy. Finally, as new more potent HDAC inhibitors are clinically investigated, their ability to be combined with standard chemotherapies such as fludarabine and rituximab could lead to better clinical responses and reduced toxicities in patients with leukemia and lymphoma.

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5. DISCUSSION

5.1 Targeting the Cytokines

In this study, a relationship between patient age, cytokine levels and patient survival was investigated. While a role for the cytokines IL-6 and IL-8 in promoting CLL cell survival in mono-cultures had been described (1-3), IL-6 and IL-8 were found to promote the physical interaction between CLL and stromal cells, describing a novel mechanism by which the cytokines can contribute to disease progression. In the settings of low-grade CLL not requiring immediate therapy, life-style modifications, rather than pharmacological intervention, may be beneficial and sufficient to target the increased cytokines. Higher body mass index (BMI) has also been associated with higher levels of IL-6 and C-reactive protein (CRP), and more active individuals tended to have lower levels of IL-6 and CRP (4, 5). Physical activity has been associated with reduced risk for various cancers, including breast, colon, pancreatic and prostate cancers (6-8), and exercise as an intervention has been examined in a number of different settings as anti-inflammatory therapy. Physical exercise is associated with reduced risk of mortality in colon cancer patients after diagnosis (9), and exercise interventions have been shown to improve the quality of life in cancer patients, associated with decreased fatigue and increased physical functioning (10). While the effects of physical activity in patients with CLL are unknown and no risk reduction is associated with exercise in the development of CLL (11), exercise may help to slow down the disease progression and increase the quality of life in CLL patients.

Supplements may also be of benefit in CLL patients with high cytokine levels. At least partly due to the advanced general age of patients with CLL, there exists an

interesting relationship between CLL, cardiovascular diseases (CVDs) and inflammatory cytokines. In the CLL cohort examined in this study, increased levels of IL-6 or IL-8 were associated with positivity for lifetime CVD history (data not shown). While CVDs were not a major cause of death in this study, a multicenter study examining patients ≥80 years at diagnosis reported 40% of patients dying from CVDs (12). As described in the introduction, CLL cells can secrete a variety of cytokines, and a number of cytokines, in turn, have been implicated in mediating vascular dysfunction (13). Long-chain polyunsaturated fatty acid (LC-PUFA) supplements have been shown to reduce incidence of CVDs and to reduce mortality in patients with CVDs (14), and this benefit may be associated with the anti-inflammatory effects of the LC-PUFAs. With such known benefits in CVDs and ease of administration, omega-3 fatty acid supplements are an attractive adjunct therapy. However, there is no clear consensus on the benefits of fish oil administration in cancer patients (15). In a double-blind placebo-controlled study examining 60 patients with advanced cancer (ranging from genitourinary to sarcomas), fish oil failed to show significant improvement in weight loss and other nutrition-related attributes (16). On the other hand, in another study examining patients with non-small cell lung cancer (NSCLC), fish oil administration was associated with maintained muscle mass, compared to the control group that lost muscle mass (17), and it was associated with increased response rate to platinum therapy (18). No clear consensus also has been reached on the beneficial role of LC-PUFAs on their ability to reduce inflammatory cytokines in various disease settings (19), and the effect on fish oil supplements in CLL is unknown. However, considering the protective benefits of LC-PUFAs regarding the

CVDs, patients with CLL are likely to benefit from LC-PUFA supplements, and minor CLL disease control may be at least indirectly attained.

In patients with extremely high levels of inflammatory cytokines, pharmacological intervention may be necessary, and the cytokines and signalling pathways downstream of the cytokines are druggable targets. There are a number of therapeutic means by which the IL-6 can be targeted, with a number of drugs in clinical trial for various inflammatory diseases, including rheumatoid arthritis and cancers (20). These options include the conventional monoclonal antibodies (chimeric or fully humanized), nanobodies (antibody-like proteins that contain single variable domain) and avimers (single-chain, multi-domain antigen-binding protein) (21). Such proteins that target IL-6 or the IL-6 receptor include Tocilizumab (humanized mAb against IL-6R), Sirukumab (human mAb against IL-6), ALX-0061 (nanobody against IL-6R) and C326 (anti-IL-6 avimer) (20). Tocilizumab has been reported to induce partial response for the control of graft-versus-host disease in two CLL patients post-stem cell transplant (22), suggesting an interesting role for anti-IL-6 therapy in CLL management. Outside of transplant, the effects of anti-IL-6 therapy on CLL disease progression are unknown. Antibodies against IL-8 also exist; ABX-IL8 was described to reduce the tumourigenicity and metastatic potential of human melanoma and bladder cancer cells (23, 24), but such therapies have not been examined in CLL. Interestingly, chronic administration of low-dose VPA may also be of benefit for chronic, anti-inflammatory therapy in CLL, especially in older patients. In a preliminary study examining a panel of cytokines in cell lines (BJAB, NALM-6) and one primary CLL sample, VPA treatment was associated with the reduction in the levels of a number of cytokines in the supernatant, and IL-6 was

reduced in all three cases (data not shown). While the significance and mechanism of this downregulation is unclear, this is an interesting observation, considering the clinical observation of CLL patients on VPA therapy reporting increased energy. Considering the reported role of inflammatory cytokines on cancer-related fatigue (25), the reduction in cytokine levels may be contributing to the increased quality of life that was evident in the charts of patients enrolled in the VPA trial.

5.2 Improving on Fludarabine-Based Therapy

In the clinic, refractoriness to fludarabine therapy is defined as CLL that fails to respond or relapses within 6 months of fludarabine therapy, and this sub-cohort represent patients with a very poor prognosis (26). In the elderly, the tolerability of standard FCR in elderly patients is debatable (27), and these patients become more difficult to treat as they continue to age and relapse through the CLL disease course. Furthermore, the elderly and the fludarabine refractory patients are often overlapping sub-cohorts, thus highlighting a dire need for a therapy that is better tolerated and more specific. Despite these needs to improve on fludarabine therapy, rather than abandoning current therapies, it would be prudent to better examine the mechanisms of current therapies and find ways to improve the quality of current therapies. Such improved therapies could function, then, as a *bridge* therapy that bridges the gap between the current and future therapies proven to be effective through phase III clinical trials, and this would be an important strategy considering the relatively low success rate (25-50%) of phase III trials in cancer therapy (28). In addition, using old drugs with new found effects in addition with current therapy (i.e. “drug recycling” adjunct) will facilitate the translation to the clinics and provide

rapidly improved therapy, as clinical trial using old drugs should be facilitated. Combining an old drug like VPA with a current therapy, i.e. fludarabine, is thus a rational bridging strategy, as long as efficacy and tolerability can be demonstrated.

In CLL therapy, a greatly desired effect of therapy is to dampen the protective effects of the tumour microenvironment. Mcl-1 and XIAP are key mediators of the protective effects of the CLL tumour microenvironment, with Mcl-1 being a key downstream target of Akt, and XIAP being a key downstream target of Mek/Erk pathway (29), both pathways that lie downstream of the B-Cell Receptor (BCR) signalling pathway. The overt downregulation of both Mcl-1 and XIAP in response to VPA-fludarabine *in vitro* is in accord with the robust reduction of lymph nodes observed in CLL patients *in vivo*. A number of other strategies have been demonstrated to sensitize cancer cells to genotoxin-based chemotherapy, including 1) inhibition of DNA damage repair pathways, 2) activation of DISC to enhance the caspase-8 activation, 3) inhibition of pro-survival pathways (such as MAPK, p38, Akt or JNK), and 4) inhibition of anti-apoptotic proteins, such as Bcl-2 and IAP family proteins (30, 31). A number of strategies have been examined *in vitro*, and a number of these studies are listed in **Table 5-1**.

Table 5-1. Drugs reported to enhance fludarabine cytotoxicity.

Type	Compound	References
Bcl-2 Inhibitors	GX15-070	(32)
	ODN 2009	(33)
Death Receptor Ligands	FasL, TNF	(This study)
	TRAIL	(This study)
DNA Damage Repair Pathway Inhibitor	KU55933 (ATM inhibitor)	(This study)
	Caffeine	(34)
	NU7026 (DNA-PK inhibitor)	(34)
	Olaparib (PARP inhibitor)	(35)
	vanillin (DNA-PK inhibitor)	(34)
Genotoxic Agents	4-Hydroperoxy-cyclophosphamide	(36)
	Cisplatin	(37)
	Gemcitabine	(38)
	Mitoxantrone	(39)
	Oxaliplatin	(40)
Kinase Inhibitors	Adaphostin	(41)
	CK2 inhibitor	(42)
	PI3K/Akt inhibitors	(43-45)
HDAC Inhibitors	SAHA, TSA, Sodium Butyrate	(This study)
	MS-275	(46)
	VPA	(This Study, 47)
Hsp90 Inhibitors	SNX-7081	(48)
	NVP-AUY922-AG	(49)
Monoclonal Antibody	Rituximab	(50, 51)
NF-κB Inhibitors	DHMEQ	(52)
	LC1	(53)
Proteasome Inhibitors	Bortezomib	(This study, 54)
	MG-132	(This study)
Others	Leflunomide	(55)
	PEITC	(56)
	Oncolytic virus	(57)

The common downstream target for all the combinations listed in **Table 5-1** is the caspases, the effectors in apoptosis. Caspases are clearly activated in response to fludarabine, as demonstrated in this study and other studies (58-60). While caspase activation should not always be equated with cells undergoing apoptosis, fludarabine induces apoptosis in leukaemic cells, judging by the nuclear morphology (DNA fragmentation and condensation), membrane morphology (membrane blebbing) and

inhibition of cell death by caspase inhibition (This study, 61, 62). In regard to caspase-8 activation, this study described enhancement of fludarabine cytotoxicity by the addition of recombinant FasL, TNF or TRAIL, the three ligands that organize the death receptors to form the death-inducing signalling complex (DISC). While the combination of fludarabine and recombinant TNF-related weak inducer of apoptosis (TWEAK) was not tested in this study, TWEAK has been shown to enhance RIPoptosome formation when combined with genotoxic damage (63), and thus TWEAK is likely to be another ligand that can enhance fludarabine cytotoxicity.

In addition to other caspases, this study described caspase-2 activation in response to fludarabine. Compared to other caspases, caspase-2 remains greatly understudied in many cancers, including CLL. Considering the prominent role of caspase-2 in lysosome-mediated cell death scenarios (64), caspase-2 is likely to be playing an essential role in fludarabine-mediated cell death. Addition of purified cathepsin B to leukaemic cell lysates was sufficient to reduce the levels of pro-caspase-2. Caspase-2 activation has been described to occur in independently of PIDDosome formation, as downregulation of *Casein Kinase 2* (*CK2*) allows for dephosphorylation serine-157, allowing for PIDDosome-independent activation of caspase-2, thereby priming caspase-8 for activation downstream of TRAIL activation in human cancer cells (65). CK2 inhibitors induced cell death in primary CLL cells, and the combination with fludarabine induced synergistic cell death (66, 67). It is unknown whether CK2 inhibitors enhance caspase-2 activation in response to fludarabine. Caspase-2 is also phosphorylated in a Ca^{2+}/calmodulin-dependent protein kinase (CaMKII)-dependent manner, suppressing caspase-2 activation (42), but the role of CaMKII in CLL is unknown.

In short, there are many ways by which fludarabine cytotoxicity may be enhanced. This study demonstrated that targets of the VPA-fludarabine combination are numerous, and the key targets described are discussed in detail in the following sections.

5.3 Targeting HDACs

Like TSA and SAHA, VPA is thought to directly target HDACs by inserting into the enzyme active site, where the carboxylic group may chelate the zinc ion (68-70). In line with this idea, substitution of the carboxylic group with amide (i.e. valpromide) results in the loss of HDAC inhibition activity (71). In this study, low-dose VPA (≤ 1 mM) was shown to modulate histone acetylation *in vitro* and *in vivo*, corroborating the number of *in vitro* studies reporting HDAC inhibition activity of VPA (47, 72-74). The inhibition of HDACs is thought to play a crucial role in the synergistic interactions observed between different HDIs and genotoxic drugs. In this study, in line with the idea, valpromide was observed to be unable to enhance fludarabine cytotoxicity at 1 mM, while other HDIs (sodium butyrate, SAHA and TSA) also enhanced fludarabine cytotoxicity at minimally lethal doses. Knockdown of HDACs has been shown to mimic the actions of HDIs in at least some aspects, and, for example, knockdown of *HDAC1* enhanced bortezomib-induced cell death in multiple myeloma cell lines, while its overexpression reduced bortezomib-induced cell death (75). Knockdown of *HDAC1* or *HDAC2* sensitized CLL cells to TRAIL (76), whereas *HDAC3, 6, 8* knockdown sensitized prostate cancer cells to TRAIL (77), mimicking the synergy observed between HDIs and TRAIL. Perhaps more convincing data has come from examining the class IIB HDAC inhibitors, such as tubacin, where the modulation of a single HDAC, i.e. HDAC6,

is sufficient to mimic the effects of such inhibitors (78-80). Furthermore, high level of Hdac1-3 expression was associated with higher IC_{50} values for VPA in pediatric AML cell lines (81), thus adding to the argument that HDACs are important targets of HDIs. As CLL cells express high levels of Hdac1-3 (82), the association between Hdac1-3 levels and VPA sensitivity also may explain why VPA monotherapy was largely ineffective in CLL. The lack of strong response with HDI monotherapy also may suggest that HDAC inhibition alone is, at least in CLL, likely to be insufficient for strong anti-tumour response, and thus this study focused on combination therapy with HDI and fludarabine.

What targets, then, are important for synergy with HDIs downstream of the HDACs? While HDAC inhibition does appear to be important, the different reports largely disagree on the targets downstream of HDACs. This heterogeneity is likely to be associated with a number of factors: 1) heterogeneity between the different cancer models, 2) heterogeneity between the actions of different agents (both of HDIs and genotoxic agents in the combination), and 3) the spectrum of different actions caused by a single combination in question. The number of genes modulated by VPA is large, reportedly ranging from hundreds to thousands (47, 83), and a microarray study on the effects of VPA in primary CLL cells reported 3,830 probe sets (14%) to be altered, some of which were up-regulated and others were down-regulated (47). Considering the pleiotropy of the effect of VPA on the transcriptome, the targets of VPA in the context of VPA-fludarabine synergy may be numerous. In CLL alone, VPA has been proposed to act through a number of different mechanisms, including Fas induction, c-FLIP downregulation and altered Bcl-2/Bax ratio (72, 73, 84). In this study, three targets were

implicated in the VPA-fludarabine synergy, and the following sections discuss each target.

5.4 Targeting the Lysosomes

In many cancers, increase in expression and activity of cathepsin B are observed, and cathepsin B secretion is also increased in some cancers (85). Interaction of cancer cells with the tumour microenvironment, especially in the bone, can up-regulate cathepsin B levels, where, upon its secretion, cathepsin B contributes to cancer cell-associated proteolysis of the basement collagen (85, 86). As this can contribute to the metastatic potential of the cancer cells, cathepsin B inhibition has been proposed as a therapeutic strategy (87, 88). Considering that LMP has been shown to drive cell death, often in cathepsin B-dependent manner (89, 90), cathepsin B, especially considering its high levels in certain cancers, is a double-edged sword that may be contributing to cancer progression and cancer therapy. As a therapeutic target, the lysosomes may be disrupted directly by a number of detergents that accumulate in the lysosomes, known as lysosomotropic detergents, and a number of these agents, including N-dodecylimidazole and O-methyl-serine dodecylamide hydrochloride, L-leucyl-L-leucine-methyl ester, O-methyl-serine dodecylamide hydrochloride, N-dodecylimidazole and siramesine, have been demonstrated to induce cell death (91-94).

Likely due to the toxicity of LMP, lysosomal membrane is not labile, and its permeabilization is protected by a number of means involving Heat shock protein 70 (HSP70), lysosome-associated membrane glycoprotein 1 and 2 (LAMP-1, 2) and endogenous glycoaminoglycans (89), among others. Hsp70 levels have been found to be

up-regulated in a number of cancers, and high levels have been correlated with poor prognosis in breast, endometrial, uterine cervical, and bladder carcinomas (95). *HSP70* overexpression protected cancer cells from a number of different chemotherapeutic drugs, including gemcitabine, imatinib and topotecan (96, 97), whereas downregulation sensitized to various drugs (98). A number of different mechanisms have been proposed for the role of Hsp70 in chemo-resistance, and blocking LMP is one of the mechanisms proposed. Hsp70 is localized on lysosomal membranes (99), and Hsp70 is important for stabilization of the lysosomal membrane (100). Hsp70 depletion in breast cancer cells was sufficient to induce LMP and cathepsin B-dependent programmed cell death (99). Hsp70 is a druggable target, and a Hsp70 inhibitor, pifithrin-μ, exhibited cytotoxicity against human leukaemic cells and enhanced the cytotoxicity of a number of different drugs, including cytarabine, a nucleoside analogue (101). Treatment of solid tumour cell lines with another Hsp70 inhibitor, 2-phenylethyenesulfonamide, was also toxic, with IC_{50} values ranging from 4-10 μM in 50 solid tumour cell lines (102). In CLL, a study of 40 CLL samples examined for intracellular Hsp70 levels showed a clear dichotomy within the cohort, where approximately half of the cohort had no detectable levels of Hsp70, and the rest of the cohort expressed higher levels of Hsp70 compared to non-leukaemic cells from CLL patients or healthy donors (103). Hsp70 levels were higher, however, in patients with stable disease (i.e. not requiring treatment) *vs.* progressive disease (103), and thus the contribution of Hsp70 levels to the clinical course is unclear. Whether Hsp70 is a druggable target in CLL or whether Hsp70 inhibition enhances fludarabine cytotoxicity in CLL is unknown.

Once the lysosomal contents are released into the cytoplasm, the activity of the lysosomal proteases are inhibited by intracellular cysteine protease inhibitors, including serpins and cystatins (89). In mice, serpin 2A (SPi2A) has been shown to block LMP- and cathepsin B-dependent cell death in response to TNF-α (104, 105). *SPi2A* expression has been shown to be modulated by Stat3- and NF-κB-dependent (106-108). NF-κB activation has been shown to up-regulate SPi2A levels to provide protection from lysosome-mediated cell death (106, 107), and this is in line with the protective role of NF-κB in fludarabine-induced death of CLL cells. Stat3, in contrast, has been described to promote lysosome-mediated cell death (108). Activation of Stat3 is associated with reduction in *spi2A* expression levels, leading to enhanced levels of cathepsin B and cathepsin L (108). In many cancers, however, activation of Stat3 has been reported to be protective (109), and CLL is not an exception (110), at least when it comes to spontaneous apoptosis. However, pre-treatment of BJAB or NALM-6 cells using AG490, a Jak inhibitor, which should be reducing Stat3 phosphorylation, protected cells from fludarabine ± VPA (data not shown), suggesting that Stat3 may be playing a pro-apoptotic role in response to fludarabine. No clear orthologue of SPi2A has been identified in humans, and thus the role of Stat3 in fludarabine-mediated cell death remains an open, interesting question.

Another strategy to sensitize cells to LMP in CLL is CD40 ligation. GA101, a type II anti-CD20 monoclonal antibody, induced rapid cell death in primary CLL cells (111). Although cleavage of the PARP protein was observed (an observation typical in apoptotic cell death), GA101-induced cell death could not be suppressed by caspase inhibition, suggesting the cell death is non-apoptotic (111). Increase in lysosome size is

associated with increased lysosomal fragility (112-114), and CD40 ligation induced lysosomal bloating and enhanced GA101-induced cell death (111). This is in contrast with fludarabine therapy, where CD40 ligation is associated with NF-κB activation and suppression of cell death (52, 115, 116). Interestingly, gefitinib, a tyrosine kinase inhibitor, induced overt lysosome bloating in primary CLL cells, and the gefitinib-fludarabine combination resulted in mild reduction in the level of apoptosis (data not shown). As GA101-mediated cell death appears to be non-apoptotic, the role of the lysosome in cell death induced by the two drugs may be quite different, and thus the effect of inducing lysosomal "bloating" on the cytotoxicity of the two drugs may be different. Gefitinib, thus, may interact with GA101 in a synergistic manner, while antagonizing fludarabine-induced cell death. Considering the cytotoxicity of gefitinib in CLL (Gibson lab, unpublished), the combination of gefitinib and GA101 is an interesting therapeutic combination worth examining.

5.5 Targets of VPA-Fludarabine – Cathepsin B and the Lysosome

While robust activation of caspases-2 and -8 were observed in response to fludarabine ± VPA in human leukaemic cells, no evidence for involvement of the typical platforms for caspase-8 activation, i.e. Fas, TNF receptor and Death Receptor 4/5, could be observed. While the addition of recombinant TNF, FasL or TRAIL was sufficient to enhance fludarabine cytotoxicity, the addition of antibodies to those ligands could not suppress fludarabine cytotoxicity. Caspase-8 activation has also been described to occur after the formation of the RIPoptosome complex, involving interaction between caspase-8, Rip1 and Fadd (63, 117), but no interaction between caspase-8 with Rip1 or Fadd

could be observed in BJAB or NALM-6 cells 8 hours post-treatment with fludarabine ± VPA (data not shown). Formation of PIDDosome, containing caspase-2, P53-Induced protein with a Death Domain (PIDD) and RIP-Associated ICH-1/CAD-3 homologous protein with a Death Domain (RAIDD) (118, 119), also could not be observed (data not shown). Caspase-8 has also been described to be activated by the protein complex forms on the surface of lysosomes in response to proteasome inhibitors and ER stress (120, 121). This complex formation involved ubiquitination and aggregation of caspase-8, forming a complex that contains LC3/p62/caspase-8, where LC3 (microtubule-associated protein1 light chain 3) on the intracellular membranes of autophago-lysosomes (120, 121). While the formation of the LC3/p62/caspase-8 complex involved conversion LC3 into the lipidated form, LC3-II, such conversion was not observed in response to fludarabine ± VPA. Rather, LC3-II formation was observed when cells were co-treated with chloroquine, addition of which suppressed apoptosis in response to VPA-fludarabine.

Thus, the involvement of other death-promoting platforms could not be observed, and this study implicated the lysosomal protease cathepsin B in the cytotoxicity of fludarabine ± VPA. In cell death scenarios involving the lysosome, a phenomenon known as Lysosome Membrane Permeabilization (LMP) is the driving event for cell death (89). Using acridine orange staining in cell lines and LysoTracker staining in primary CLL cells, a clear loss of lysosome staining was observed in response fludarabine ± VPA. However, whereas mitochondrial disruption can directly lead to caspase-9 activation by the formation of the apoptosome complex, the relationship between caspases and lysosomal proteases is less clear. A number of kinetic studies have placed LMP preceding caspase activation, suggesting that LMP can lie upstream of

caspase activation. For example, in apoptosis induced by bortezomib, a proteasome inhibitor, LMP precedes caspase-2 activation, and caspase-2 activation could be blocked by a cathepsin B inhibitor, while the caspase-2 inhibitor did not block LMP (122). However, in TNF-induced cell death, caspase-8 activation was detectable before LMP, suggesting that caspase activation precedes LMP (123). Unfortunately, interpretation of these experiments suffer from a number of issues: 1) full disruption of lysosomes is not necessary to initiate cell death; 2) the degree of LMP or caspase activation required for detection can vary greatly depending on the technique; and 3) the relationship between LMP and caspase activation may not be a simple linear relationship. To complicate the matter further, some studies suggest lysosomal proteases themselves are also involved in mediating LMP, and, for example, in mouse hepatocytes treated with either TNF or sphingosine, LMP induced in cathepsin B wildtype was not observable in hepatocytes from cathepsin B double knockout mice (124). In this study, alkalinization of lysosomes using chloroquine or NH_4Cl protected cells from fludarabine ± VPA, and this protection is likely to be at least partly related to the inhibition LMP by the inhibition of lysosomal protease activity within the lysosome. Comparison of wildtype *vs. caspase-9*$^{-/-}$ mouse embryonic fibroblasts showed that cathepsin B activity in staurosporine-treated cells can also be caspase-9 dependent (125). Using cell-free system with isolated lysosomes, caspase 8 was observed to induce the release of active cathepsin B (126). At the same time, however, knockdown of cathepsin B reduced and/or delayed the activation of caspase-9 and caspase-3, suggesting that cathepsin B contributes to caspase activation (125). It has been suggested that proteases form work as a dynamic network, rather than

simple linear sequences (127), and certainly the relationship between lysosomal proteases and caspases appears to be a complex one.

Once LMP is achieved, however, the different studies do agree that LMP can drive cell death. This study demonstrated that LMP is sufficient to drive caspase activation in leukaemic cells; in an experiment where whole cell lysates were combined with purified cathepsin B, thereby simulating LMP *in vitro*, the addition was sufficient to induce caspase-2 cleavage. The experiment does not prove, however, that cathepsin B can directly target caspase-2. Caspases, including caspase-2, have been shown to be poor substrates for various cathepsins *in vitro*, including cathepsin B (128, 129). This is in contrast with cathepsin D, which has been shown to be able to activate caspase-8 directly (130). Yet, in this study, based on the experiment using cathepsin B and cathepsin D inhibitors (CA074-Me and Pepstatin A, respectively), cathepsin B was the clear culprit, and the appearance of faster migrating bands with 30-minute incubation suggest that the cleavage may be direct. Once cleaved, caspase-2 has been described to be upstream of caspase-8 in some scenarios (131-134), and thus activation of caspase-2 should be sufficient to drive caspase-8, BID and caspase-3 cleavage. Once in the cytosol, cathepsin B has been shown to retain activity in the neutral pH, and examination of cathepsin B activity via activity-based probe assays showed that cathepsin B is active in the cytosolic pH and is capable of cleaving caspase substrates at both pH 4 and pH 7 (125), and this study demonstrated clear activity of purified cathepsin B at pH 7.4. In fact, a number of lysosomal proteases have been shown to be active at neutral pH for a time that ranges from a few minutes to an hour or more (135), duration that is argued to be sufficient to initiate the cell death process. Among the lysosomal proteases, cathepsin B has been

shown to be one of the most stable protease at neutral pH (135). The actions of lysosomal proteases may not be restricted to the cytoplasm, as cathepsin B has been shown to translocate to the nucleus in bile salt and amino-propanal-induced cell death (136, 137).

An interesting question that remains open is regarding the mechanism of LMP in fludarabine-induced cell death. Ceramide and ROS are thought to be two major inducers of LMP (89), and fludarabine has been shown to generate both ceramide and ROS (138, 139). LMP induction has also been reported to occur in a Bax-dependent manner, and *BAX* knockout was shown to suppress LMP in hepatocytes (140, 141). As these cell death scenarios involved formation of punctate Bax staining pattern (140, 141), Bax-dependent LMP is thought to also occur via membrane deposition of Bax in the lysosomal membrane, similar to mitochondrial disruption. Fludarabine treatment of CLL up-regulates *BAX* expression *in vivo* (142), but the effect of Bax levels on fludarabine sensitivity is unclear. Reports in the literature are in conflict on whether the levels of Bax, Bcl-2 or the Bcl-2/Bax ratio correlate with *in vitro* sensitivity to fludarabine or whether there is a difference in the levels of those proteins between fludarabine-sensitive and fludarabine-resistant CLL (143-145). However, *in vitro* fludarabine sensitivity correlated with the degree of conformational change in Bax in response to fludarabine treatment, resulting in translocation of Bax to the mitochondria and subsequent release of cytochrome *c* into the cytosol (146). Elucidating the exact mechanism by which LMP is achieved in response to fludarabine should facilitate the design of future lysosome-targeting therapies.

VPA may be acting at three different cellular locations to enhance cathepsin B-dependent apoptosis: 1) in the various lipid bilayers as it partitions into lipid bilayers; 2) in the cytoplasm; and 3) in the nucleus as an inhibitor of HDACs. One possibility is that VPA may be modifying the lysosomal membrane to facilitate the disruption of lysosomes. As an anti-epileptic, at least part of its actions is thought to be due to its interaction with the neuronal membrane, and VPA has been shown to increase potassium conductance and hyper-polarize the resting membrane potential (147). VPA has been shown to also interact with organellar membrane; VPA interacted with the mitochondrial membrane, modifying the conformation of some of the membrane proteins and modifying trans-membrane protein movement (148), a phenomenon that is thought to occur by partition of VPA into the lipid bilayer (149). Thus, VPA may be sensitizing cells to lysosome disrupting mechanisms by interacting with the lysosomal membrane.

As a fatty acid, VPA also may be impacting the fatty acid metabolism, including sphingolipid metabolism. VPA itself undergoes β-oxidation, and β-oxidation accounts for nearly 70% of phase I reactions to form 2-en-VPA, 3-OH-VPA and 3-keto-VPA (150, 151). VPA is thought to impact fatty acid metabolism by CoA sequestration (thus inhibiting acyl-CoA formation), carnitine depletion (thus impairing fatty acid transport) and by modulating β-oxidation enzyme level/activity (151). Considering the role of lysosomes described in VPA-fludarabine cytotoxicity, sphingolipids are of particular interest. While sphingolipids have been described to have a variety of effects on cancer cells, ceramide is arguably the best studied sphingolipids with pro-apoptotic effects and has been described to induce LMP (152, 153), and ceramide production has been reported

in response to two HDIs, butyric acid and LAQ824 (154, 155). Thus, increasing ceramide generation may be a mechanism by which VPA contributes to cell death.

Various HDIs have also been reported to induce ROS generation, and VPA does induce ROS formation in CLL and other cells (72, 156, 157). ROS generation has been described to be a crucial event for the synergy between HDIs and proteasome inhibitors (ex. Bortezomib) (158-160), kinase inhibitors (161) and fludarabine (46, 154), among others. The role of ROS as a causative agent for cell death is usually tested by combining the drugs with a ROS scavenger, ex. *N*-acetylcysteine (NAC), and NAC decreased cell death and caspase activation in response to fludarabine ± VPA. This was also associated with elevated levels of ROS when treated with fludarabine ± VPA. However, mitochondrial disruption, which is a driving event in apoptotic cell death (162), results in release of mitochondrial ROS (163), and thus it is difficult to discern between ROS release as a result of cells undergoing apoptosis or as a driving factor in cell death. NAC also impacts a number of different proteins, an effect that is thought to be due to its ability to scavenge ROS (164). As well, NAC is a general reducing agent, and the addition of NAC (15 mM) resulted in a significant change in the pH of the media (approximately 1-log scale decrease) (data not shown). Thus, the effects of NAC may not be limited to ROS scavenging. Furthermore, kinetic studies demonstrating ROS generation preceding caspase activation is also limited by the sensitivity of the assay for both ROS and caspase activation. Taken together, while ROS may be playing an important role in the synergy between HDIs and other drugs, a number of limitations in the study makes it challenging to discern the exact role of ROS.

While the roles of ROS, ceramide or VPA as a membrane-partitioning agent are unclear, this study described the role of VPA in its ability to enhance cathepsin B levels *in vitro* and *in vivo*. VPA administration to CLL patients resulted in increased cathepsin B protein levels, and this increase correlated roughly with changes in histone-3 acetylation. In a microarray study, *CTSB* (the gene encoding for cathepsin B) was shown to be up-regulated in CLL in response to VPA (47), and thus the increase in cathepsin B is, at least in part, is likely due to transcriptional increase in mRNA levels. It is unknown whether other HDAC inhibitors or knock down of specific HDACs would also result in increased cathepsin B. While the level of cathepsin B in CLL cells compared to other cell types is unknown, the level/activity of a number of lysosomal proteins has been reported to be low (165-167). Thus, despite LMP occurring, the activity of cathepsin B in the cytosol may be insufficient to drive apoptosis with fludarabine monotherapy, necessitating increased cathepsin B levels to further drive apoptosis.

5.6 Targets of VPA-Fludarabine – Akt

Akt is one of the targets of the VPA-fludarabine combination described in this study. VPA monotherapy was sufficient to strongly reduce Akt Ser473 phosphorylation in BJAB and NALM-6 cell lines at 1 mM. As 1 mM VPA did not induce any appreciable level of apoptosis in BJAB or NALM-6 cells, the decrease in Ser473 phosphorylation is an effect that is independent of apoptosis induction. While the reduction in Ser473 was much weaker in primary CLL cells compared to cell lines, the VPA-fludarabine combination resulted in enhanced reduction of both Ser473 and Thr450 phosphorylation.

Mechanism by which Akt contributes to cell survival is pleiotropic. Akt has been shown to phosphorylate XIAP, thereby preventing the ubiquitination of XIAP that leads to its degradation in a proteasome-dependent manner (168). While inhibition of the lysosome pathway, and not the proteasome pathway, was found to stabilize XIAP in response to the VPA-fludarabine combination, the appearance of higher molecular weight form of XIAP in bortezomib-treated cells suggest that at least a fraction of XIAP undergoes basal proteasome-dependent cleavage. It is also possible that the ubiquitinated XIAP may be targeted for lysosome-dependent degradation. *XIAP* overexpression has been shown to stabilize Akt and enhance Ser473 phosphorylation, while XIAP downregulation lead to Akt cleavage and caspase-9/3 cleavage (169). XIAP physically interacts with phosphatase and tensin homolog deleted on chromosome ten (PTEN), which negatively regulates Akt phosphorylation, and *XIAP* knockdown reduced mono- and poly-ubiquitinated PTEN (170). Akt also undergoes caspase-dependent cleavage (171), and caspase inhibition lead to Akt stabilization in response to the VPA-fludarabine combination (data not shown). XIAP also ubiquitinates the caspases (172), thereby also indirectly contributing to Akt stability, while Akt phosphorylates caspase-9 to suppress its activation (173). Thus, there may be a complex set of interactions between Akt, caspases and XIAP that function to promote cell survival, and this small network is disrupted in response to fludarabine and the VPA-fludarabine combination in leukaemic cells.

In CLL cells, *AKT* overexpression has been shown to up-regulate Mcl-1 levels and suppress spontaneous apoptosis (29). Mcl-1 up-regulation by Akt has been described to be transcriptional, involving a transcriptional complex containing cAMP response

element-binding (CREB) (174). Another interaction reported is post-transcriptional, where Akt inhibits glycogen synthase kinase 3β (GSK-3β)-dependent phosphorylation of Mcl-1, thereby stabilizing Mcl-1 levels (175, 176). Subsequent ubiquitination and degradation of Mcl-1 downstream of GSK-3β has been reported to be dependent on the E3 ligase, β-TrCP (177). In this study, in response to fludarabine or the VPA-fludarabine combination, Mcl-1 levels were clearly decreased, and the reduction is obvious 16H post-treatment. No obvious reduction in *MCL-1* mRNA levels was observed, however, suggesting that the Mcl-1 reduction is post-transcriptional. Inhibition of caspases, lysosome or proteasomes all stabilized Mcl-1 in response to VPA-fludarabine, where bortezomib, the proteasome inhibitor, resulted in the most obvious increase in basal and post-treatment Mcl-1 levels. However, despite the overt stabilization/enhancement of Mcl-1 in response to the proteasome inhibitor, the level of apoptosis was actually increased when combined with fludarabine ± VPA, suggesting that high Mcl-1 level is not sufficient to protect cells. Thus, the combined stabilization of Mcl-1 and XIAP may be required, as observed in the case of chloroquine or NH_4Cl pre-treated cells, in order for protection to take place.

How, then, does VPA modulate Akt phosphorylation? As a fatty acid, it is possible that VPA may impact the PI3K activity upstream of Akt by disrupting phospholipid metabolism. VPA has been shown to inhibit phospholipid turnover, reducing the production of phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) and its precursors, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) (178). This reduction in PIP species may in turn be related to the observation that VPA also reduced general inositol biosynthesis *in vivo* by inhibiting

myo-inositol-1-phosphate (MIP) synthase activity by blocking the conversion of glucose-6-phosphate (G6P) to inositol monophosphate (179, 180). Other HDAC inhibitors, including TSA and SAHA, have been shown to reduce Akt Ser473 phosphorylation via a mechanism where HDAC1 and protein phosphatase 1 (PP1) interaction is disrupted (181), and it has not been examined whether VPA is also capable of disrupting the HDAC1-PP1 interaction. It is also unknown whether TSA and SAHA can also inhibit inositol synthesis. Whatever the mechanism of Akt modulation by VPA, the observed action of VPA on Akt begs for the examination of the pharmacodynamics between VPA and PI3K inhibitors. PI3K inhibitors, including CAL-101 (GS-1101), are of particular interest for CLL management due to its efficacy and high tolerability (182). Low dose VPA was also found to be readily tolerable by elderly patients with CLL, and thus the VPA-and GA1101 combination is a combination worthy of further studies.

5.7 Targets of VPA-Fludarabine – Ataxia Telangiectasia, Mutated (ATM)

Another target of the VPA-fludarabine combination described in this study is ATM. A large number of chemotherapeutic drugs in the clinic are thought to work by inducing DNA damage; genotoxic drugs include platinum-derivatives (ex. cisplatin), nucleoside analogues (ex. fludarabine), nitrogen mustards (ex. chlorambucil) and alkylating agents (ex. cyclophosphamide), are such drugs (183). When the level of genotoxicity overwhelms the repair pathways, cell cycle arrest and cell death ensues (183), and thus, ATM is a desirable target as a strategy to lower the cancer cells' tolerance for genotoxic assaults. While the effect of low-dose VPA in acute, *in vitro* setting (i.e. one, two-day treatment) on ATM levels was small, the VPA-Fludarabine

combination induced stronger reduction in total ATM levels in cell lines and primary CLL cells. This downregulation of ATM was also observed *in vivo*, especially after prolonged, chronic treatment with low-dose VPA. The ATM downregulation is likely to be contributing to the VPA-fludarabine synergy based on: 1) chemical inhibition using (KU-55933) of ATM was sufficient to greatly enhance fludarabine cytotoxicity; 2) KU-55933-mediated enhancement of fludarabine cytotoxicity was not observed in I-83 cells, where ATM was nearly undetectable by immunoblotting; and 3) the level of apoptosis one-day post-treatment with VPA-fludarabine was lowest in I-83 cells compared to BJAB and NALM-6. In I-83 cells, the DNA damage response (assessed by γH2A.X) and subsequent induction of the intrinsic apoptotic pathway (assessed by caspase-9 activation) were also much lower compared to BJAB and NALM-6 cells. Enhancement of caspase-9 activation by VPA was also not observed in I-83 cells with one-day treatment, suggesting that VPA-mediated enhancement in caspase-9 activation requires ATM. In contrast, VPA-mediated enhanced activation of two other apical caspases, caspase-2 and caspase-8, and the executioner caspase-3 were observed in I-83 cells (data not shown), demonstrating that different "pathways" are engaged to induce apoptosis in response to the VPA-fludarabine combination.

In response to ATM downregulation and concomitant induction of DNA damage, the DNA damage repair pathways are presumably overwhelmed, driving cells toward apoptosis, which, in general terms, would requires intact p53 function. While the role of p53 in VPA-fludarabine was not directly examined in this study, all three cell lines were found to express p53 at detectable levels by immunoblotting (data not shown). Among them, BJAB cells have been described to be carry a mutation in *TP53* (His193Arg) (184),

a mutation that has been described in the Li-Fraumeni syndrome and other cancers (185, 186). His193 lies within the core DNA-binding domain of p53 and is thought to be one of the zinc-binding residues (187), suggesting at least the DNA-binding function of p53 may not be required for apoptosis induced by fludarabine ± VPA. It is unknown whether the VPA-fludarabine combination may be effective in patients with the 17p deletion karyotype, as karyotypes of the patients enrolled in the trial were not available. However, the relatively strong sensitivity of BJAB cells suggest that del17p patients, at least those with intact ATM function, may benefit from the VPA-fludarabine combination.

How, then, does VPA downregulate ATM? VPA treatment had been reported to reduce *ATM* expression by a microarray study (47), suggesting that the ATM downregulation is at least partially transcriptional. Considering that HDIs generally work by increasing histone acetylation and increasing transcription, reduced *ATM* expression in response to VPA is likely to be an indirect effect, and one possible mechanism may be through up-regulation of certain miRNAs. miRNA-mediated downregulation of ATM has been demonstrated, and *miR-18a*, *miR-421* and *miR-101* have been implicated (188-190). In turn, a number of miRNAs become up-regulated in ATM-dependent manner upon cells encountering DNA damage (191). VPA has been shown to modulate expression of a number of miRNAs (192, 193), and treatment of CLL cells with HDIs have been reported to alter miRNA expression (82). miRNA-mediated downregulation of ATM downstream of HDI treatment has yet to be reported.

5.8 CONCLUSIONS

Elderly patients with CLL fare poorly, and part of the difference (compared to younger patients) is clearly biological and at least partly associated with higher levels of elevated inflammatory cytokines. In a search of a treatment strategy that is appropriate and effective in the elderly, the VPA-fludarabine combination was examined with regard to its mechanism of action and the *in vivo* efficacy. Albeit a small study, this study did demonstrate efficacy of VPA-fludarabine in six elderly patients with relapsed CLL, half of which were clinically refractory to fludarabine therapy. Downregulation of ATM and Akt are contributing to the combined cytotoxicity. Closer examination of the fludarabine cytotoxicity revealed the involvement of the lysosomes, and VPA enhanced the cytotoxic potential of fludarabine by increasing the level of active cathepsin B in human leukaemic cells. Considering the high tolerability of the VPA-fludarabine, the efficacy of the combination should be further investigation in elderly patients with CLL, where the number of treatment options is limited.

5.9 FUTURE DIRECTIONS

Reducing therapy-associated toxicity for cancer therapy is desperately needed to reduce treatment-associated morbidity and improve the quality of cancer patients' lives. Targeting lysosomes is a novel approach that is expected to allow for specific and potent therapy. However, better understanding of the lysosome biology in leukaemic cells and elucidating other means of targeting lysosomes are needed for such advancements to take place.

HYPOTHESIS: We hypothesize that lysosome-mediated cell death is an effective targeted therapeutic strategy for CLL.

AIM 1. Understanding the Role of Lysosomes in Other Chemotherapeutic CLL Therapies: With the involvement of the lysosomes identified in fludarabine-mediated therapy, future studies will aim to examine the role of lysosomes in response to other chemotherapeutic drugs.

AIM 2. Lysosomal Attributes of CLL cells and the Effects of Other HDIs: With the goal of maximizing the efficiency of lysosome-targeting therapies and identifying patients that will benefit the most from such therapies, future studies will aim to determine the levels and activities of lysosomal enzymes in CLL patients, focusing on cathepsin B, and examine the effects of other HDIs on cathepsin B levels.

AIM 3. TLD (Targeted Lysosomal Disruption), a Proposed Strategy to improve Targeted Cancer Therapy: Using currently available techniques, it is hypothesized that targeted disruption of lysosomes is a possible therapeutic strategy. Future studies will aim to form a novel strategy to specifically disrupt the lysosomes within cancer cells.

5.9.1 Future Directions – Aim 1: Understanding the Role of Lysosomes in Other Chemotherapeutic CLL Therapies

RATIONALE: An important question for improving our understanding of chemotherapy mechanisms is whether other chemotherapeutic drugs are also dependent on the lysosomes and lysosomal proteases for their cytotoxicity. While the pro-death role of

lysosome has been examined in this study in the context of VPA-fludarabine therapy, as well as GA101 (111), lysosomes play a protective role in response to rituximab (194), and for most drugs, the role of lysosomes in CLL therapy is unknown. By identifying other chemotherapeutic drugs for which lysosomes play a pro-death role, those newly identified drugs then become rational adjuncts to fludarabine, GA101 and/or VPA.

APPROACH: While a number of chemotherapeutic drugs have been shown to induce LMP (89), observation of LMP is insufficient to conclude that LMP is driving cell death, as LMP can be observed late in cell death events as part of secondary necrosis (i.e. secondary to the initial apoptosis) as part of general cellular degradation *in vitro* (195). Thus, one must demonstrate that the inhibition of a lysosomal component results in suppression of cell death, be it apoptosis, necrosis or both. Examining dependence on cathepsin B in CLL is complicated by the toxicity of CA074-Me in CLL cells, but antagonism can still be demonstrated by examining combination index values, which is a measure of pharmacological synergy and antagonism (196). CA074-Me, at least at doses up to 20 μM, was minimally toxic in BJAB and NALM-6 cells, and thus the CA074-Me-mediated inhibition of cell death can easily be examined in those cell lines.

Future studies will focus on the drugs that are currently used in CLL therapy either as monotherapy or as a part of a combinatory treatment regimen. These drugs will include chlorambucil, cyclophosphamide, bendamustine and lenalidomide. Using propidium iodide staining to examine for the level apoptosis, a range of doses will be tested each drug in BJAB and NALM-6 cells in order to elucidate the doses required to induce approximately 50% apoptosis after one-day treatment (i.e. IC_{50} values). Alkalinization of lysosomes will be performed by pre-treating BJAB and NALM-6 cells

for one-hour using chloroquine (0-100 μM) or NH_4Cl (0-50 mM), and then the cells will be treated with chlorambucil, cyclophosphamide, bendamustine or lenalidomide at the IC_{50} doses. For drugs where lysosomes play a pro-death role, pre-treatment with chloroquine or NH_4Cl is expected to reduce cytotoxicity of such drugs in a manner dependent on the chloroquine/NH_4Cl dose. Using 96-well plate-based flow cytometry (Millipore Guava), this pharmacological study is greatly facilitated, allowing for a rapid pharmacological screen of lysosome-targeting drugs.

Next, focusing on the drugs where chloroquine/NH_4Cl clearly reduced the cytotoxicity, future studies will aim to identify the lysosomal protease driving cell death. BJAB and NALM-6 cells will be pre-treated using CA074-Me (0-20 μM) or Pepstatin A (0-100 μM) for one-hour, then treated with the drugs identified from above for one-day. This study will thus aim to group the drugs into three categories: 1) cathepsin B-dependent (i.e. cytotoxicity reduced by CA074-Me), 2) cathepsin D-dependent (i.e. reduced by pepstatin A), and 3) drugs independent of cathepsin B or D. This grouping would allow for the formation of rational combination treatment regimens, where the different drugs may be combined at low doses for maximal efficacy and tolerability. Using the combinations of drugs predicted to be synergistic, pharmacological synergy will be examined by combining different doses of the different drugs. While this study and most of other studies are generally limited to two-drug synergism, three-drug synergism can be examined using three-dimensional models (197), and such a strategy can be used as a basis for a facilitated clinical trial of a combinatory treatment regimen based on the observed *in vitro* synergism.

EXPECTED RESULTS: A number of known chemotherapeutic drugs are expected to induce lysosome disruption contributing to cell death. The drugs identified to work through the same target (whether that is cathepsin B or D) are expected to synergize with one another.

5.9.2 Future Directions – Aim 2: Lysosomal Attributes of CLL cells and the Effects of Other HDIs

RATIONALE: Cytotoxicity of drugs that drive LMP-mediated cell death would depend on a number of factors, two of which are the levels of the culprit lysosomal protein and the fragility of the lysosomes themselves. Since the early studies of lysosomal enzymes in CLL cells in the 1970s and 1980s, the lysosomes have been largely unstudied in CLL. Considering the newfound role of cathepsin B in both GA101 and fludarabine-induced cell death, a basic study of lysosomes and lysosomal enzymes is warranted. CLL cells have been shown to have more mitochondria than normal lymphocytes, and higher mitochondrial mass correlated with decreased sensitivity to fludarabine (198). This suggests that other organellar differences may be found, and the future studies will aim to examine the size and the number of lysosomes in CLL cells.

While cathepsin B is overexpressed in a number of cancers (85), it is unknown whether cathepsin B levels are high in CLL cells compared to other tissues. Some differences in cathepsin B levels are expected, based on the clinical data from this study. Fludarabine therapy is associated with a degree of bone marrow suppression (199), and bone marrow suppression was observed with the VPA-fludarabine therapy. As the Absolute lymphocyte count, a measure of peripheral tumour load, decreased in response

to the VPA-fludarabine therapy, however, other haematological cells increased in count, suggesting that CLL cells may be more sensitive to the VPA-fludarabine combination. If one were to assume that cell death induced by fludarabine in other cells is also cathepsin B-dependent, one can then infer that cathepsin B levels may be higher in CLL compared to other haematological cells, or that VPA-induced cathepsin B up-regulation is stronger in CLL. Alternatively, based on other studies that examined the activity of other lysosomal enzymes (200, 201), the levels of cathepsin B may be low in CLL cells, and thus up-regulation of cathepsin B levels may be a useful adjunct therapeutic strategy in combination with a number of other chemotherapeutic drugs in CLL.

This study examined the role of VPA in cathepsin B up-regulation in both *in vitro* and *in vivo*. This enhancement was not observed with valpromide, suggesting that the mechanism of enhancement may be transcriptional and dependent on HDAC inhibition. In line with this hypothesis, a microarray study had reported cathepsin B mRNA levels to be up-regulated in CLL in response to VPA (47). An interesting question that remains is whether a similar enhancement may be observed with other HDAC inhibitors, such as sodium butyrate, SAHA and TSA, the three HDAC inhibitors that were also synergistic with fludarabine. Considering the relatively good tolerability of the VPA-fludarabine combination in elderly patients, VPA may be the ideal cathepsin B-upregulating adjunct in elderly patients. However, should other HDAC inhibitors be also shown to up-regulate cathepsin B, a more potent HDAC inhibitor, such as SAHA, may be more appropriate in the setting of more aggressive disease and younger patients.

APPROACH: Using electron microscopy (Philips EM420 transmission electron microscope), the Gibson laboratory has successfully examined the lysosomes in breast

cancer cells, using para-formaldehyde/glutaraldehyde (2%/0.1%) fixation (202). Freshly isolated CLL cells, other white cells from CLL patients and B-cells from age-matched healthy donors will be fixed as before and transmission electron microscopy will be employed to compare the size and the number of lysosomes. Any differences in the gross lysosome morphology and numbers may suggest that CLL cells may respond differently to lysosome-targeting therapy. Another interesting question is the effect of the tumour microenvironment on the lysosomes. CD40 ligation had been reported to induce lysosomal bloating, sensitizing CLL cells to GA101-induced cell death (111). Future studies will thus aim to examine the effect of co-culturing of CLL cells with stromal cells and examine the effect of the lysosome size and number in both CLL and stromal cells. Should differences be observed in the *in vitro* recapitulation of the tumour microenvironment, examination of patient samples would be warranted. CLL cells in the peripheral circulation are known to exhibit biological differences when compared those in the lymph node and the bone marrow (203, 204). Aspirates can be obtained from the patients' lymph nodes and the bone marrow. While aspiration does not preserve the architecture of the tumour microenvironment, the technique is sufficient to isolate the CLL cells from the different microenvironments, and the isolated cells can be readily fixed as described above for electron microscopy.

Future studies will also examine the level and activity of cathepsin B in CLL cells and compare them to those in other white blood cells from CLL patients, as well as B-cells from age-matched healthy donors. Future studies will first examine the protein level of cathepsin B by immunoblotting. Banked samples will be used to prepare whole cell lysates, and equal amounts of protein samples will be loaded and analyzed for cathepsin

B levels. Multiple gels can be concatenated and transferred onto a single nitrocellulose membrane for larger scale immunoblotting, allowing for greater control of immunoblotting intensity. Samples will then be divided into three groups based on the level of cathepsin B, and the clinical attributes, including Rai stage and mutation status, will be compared between the groups. Most importantly, the patients will be divided into fludarabine-sensitive and fludarabine-refractory groups, and the level of cathepsin B, as well as lysosomal size and numbers, will be compared. Cathepsin B activity will also be examined in freshly isolated CLL cells, using the technique used in this study. BJAB and NALM-6 cell lysates will be used as internal controls to control for experimental differences.

In order to examine this, future studies will examine the effects of other HDIs on cathepsin B levels. BJAB, NALM-6 and primary CLL cells will be treated with increasing doses of sodium butyrate, SAHA and TSA, and the level of the active cathepsin B (approximately 25 kDa) will be examined. Based on the data with VPA and valpromide, other HDIs are expected to also up-regulate cathepsin B levels, and the importance of HDACs will be examined by knocking down *HDAC1-3* individually using siRNA to identify the important target of the HDIs. Should other HDIs be found to not up-regulate cathepsin B, such a finding would suggest that the VPA-induced upregulation of cathepsin B may not depend on HDAC inhibition and may not be transcriptional in mechanism. Cathepsin B undergoes post-transcriptional maturation to form the 25 kDa active form (205), and VPA may be facilitating this maturation process. In addition, we will examine the effect of HDIs on the cathepsin B levels in other white cells from CLL patients and B-cells from age-matched healthy donors.

EXPECTED RESULTS: The higher number of mitochondria in CLL cells may be reflecting whole organellar increase in CLL cells that match their higher level of metabolism, and CLL cells are expected to have matching increased number of lysosomes. In the microenvironment, one of the key signalling events that take place, at least in the lymph nodes, is thought to be CD40 ligation (204), and thus the lysosomes are also expected to be larger in the lymph nodes. Despite the expected differences in the lysosomes, cathepsin B levels are expected to be low based on the levels of others lysosome enzymes, and the levels are expected to rise in response to other HDIs.

5.9.3 Future Directions – Aim 3: TLD (Targeted Lysosomal Disruption), a Proposed Strategy to improve Targeted Cancer Therapy

RATIONALE: Immunotherapy using monoclonal antibodies has opened the door for targeted cancer therapy and has already made significant improvement in cancer therapy. The addition of rituximab to therapy for CLL, low-grade or follicular lymphoma and diffuse large B-cell lymphoma has made a significant difference in response rates to conventional chemotherapy, and a number of new antibodies are in clinical trials (206-208). An issue with the current targeted therapy using most targeted monoclonal antibodies is their reliance of the patients' immune system, and many monoclonal antibodies are thought to function via antibody-dependent and complement-dependent cytotoxicity (208). A number of defects in the immune system have been described in patients with CLL (209), however, thus resulting in less than desired effects with immunotherapy. T-cells from CLL patients show impaired immunological synapse formation (210, 211), and defects in the complement system have also been reported,

with defects being more prevalent among high Rai-stage disease patients (212). Furthermore, neutropaenia and infections are relatively commonly observed toxicities associated with fludarabine, and thus co-administration of fludarabine and rituximab may reduce complement- and antibody-dependent cytotoxicity of rituximab (212), thereby causing rituximab to rely on its ability to directly induce cell death when combined with fludarabine. This may explain why CLL patients with del17p failed to show improved response when rituximab was added to the treatment regimen (206).

Despite the weaknesses, immunotherapy offers a fantastic means of targeting cancer cells. CLL cells, for example, may be targeted by exploiting surface proteins that are enriched in B-lymphocytes, such as CD19 and CD20, as well as ROR1, a surface protein whose expression has been found to be largely limited to CLL cells and adipocytes (213). Targeting of these surface proteins may be achieved using whole antibodies or antibody fragments (214), and other antibody-like proteins, such as nanobodies or avimers, may also be a possible strategy. With the advances in bioengineering, antibodies can now be made conjugated to various molecules, thereby using targeted delivery of the said molecule to cancer cells and enhancing the drug potency. Currently available conjugated antibody therapy includes radioimmunotherapy, where radioisotopes are conjugated to antibodies. Examples are ibritumomab tiuxetan (Zevalin®, anti-CD20 conjugated to Yttrium-90) and tositumomab (Bexxar®, anti-CD20 conjugated with radioactive Iodine-131) (214). Immunotoxins are another example of such a drug, which include whole antibodies or antibody fragments linked to protein toxins, such as *Pseudomonas* exotoxin A and ricin (215). BT-1 is composed of truncated *Pseudomonas* exotoxin A conjugated to antigen-binding portion of antibodies against

ROR1, and BT-1 has been shown to induce apoptosis in ROR1-positive, but not ROR1-negative, cell lines (216).

Using either immunoconjugates or immunotoxins, it is also possible to target the lysosome. Rituximab has been demonstrated to be internalized and undergo lysosomal degradation, thereby contributing to rituximab resistance (194). At the same time, the study also demonstrates that CD20 can be exploited to target the lysosome. By delivering toxins or drugs that mediate LMP, internalization of the immunotoxin or the immunoconjugate would be sufficient to induce cell death in the targeted cancer cells. Lysosome targeting may also represent a strategy to reduce drug resistance. A number of toxins that have been tested, including *Pseudomonas* exotoxin and ricin, function in the cytosol of the target cells, and thus the immunotoxin must survive lysosomal degradation (215). In the case of LMB-2, the immunotoxin in the cytosol must also bind to the intracellular Lys-Asp-Glu-Leu (KDEL) receptor, which allows for transport to the ER, followed by translocation of the toxin to the cytoplasm, the site of its cytotoxic actions (215). In contrast, by targeting the lysosomes, a number of steps required for drug delivery can be greatly minimized; lysosomes are fusogenic in nature, being able to fuse with autophagosomes and endosomes (217), and thus targeting lysosomes can be attained via endocytosis-mediated internalization of surface proteins.

The acidic pH of lysosomes can also be exploited to ensure safer delivery of drugs or toxins to the target cells, and one possible means is to coat or conjugate the drugs to nanocarriers. Nanocarriers are nano-sized materials (diameter 1-100 nm), and the size of nanocarriers allow for avoidance of passive diffusion through tissues and renal clearance (218). Family of nanocarriers include polymer conjugates, polymeric nanoparticles,

lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, and gold nanoparticles, including nanoshells and nanocages (214, 218). Mesoporous silica nanoparticles (MSNs) are nanocarriers characterized by intrinsically large surface areas, large accessible pore volumes, highly ordered pore structures, and adjustable pore size (219-221). While MSNs are generally not accessible to enzymes, protons within acidic compartments, i.e. lysosomes, can access the MSNs, which can act to cleave bonds that link drug drugs to nano-channel walls (220, 221). MSNs can also be equipped with supramolecular nano-valves that are tightly closed at physiological pH (7.4) but capable of opening and delivering drugs in acidifying cellular compartments. β-cyclodextrin nano-valves that are responsive to the endosomal acidification conditions in human differentiated myeloid (THP-1) and squamous carcinoma (KB-31) cell lines have also been reported in 2010 (219). Targeted delivery of MSNs can be achieved using MSNs conjugated to antibodies; MSNs conjugated to anti-HER2 mAb has been described to target breast cancer cells (222), and the construction of cyclodextrin polycation nanoparticles conjugated to anti-CD20 (Rituximab) fragment has been previously achieved (223).

Summarizing the ideas discussed above, there are many potential paths by which the lysosomes may be targeted specifically in cancer cells, including CLL cells. The future studies will aim to construct CLL cell-targeting immunoconjugates composed of mAb conjugated to MSNs containing lysosome-rupturing agent.

APPROACH: mAb targeting ROR1, CD19 and CD20 will first be examined as the candidates for targeting CLL cells. The efficacy of lysosome targeting will depend on the extent of uptake of the immunoconjugate-target complex, as well as the copy number

of the target on the cell surface. In order to first examine the efficacy of lysosome targeting, immunoconjugates with empty MSNs conjugated to mAb against ROR1, CD19 or CD20. Cellular uptake of MSNs can be tracked using FITC-labelled MSNs immunoconjugates (222). Briefly, the construction of FITC-labelled immunoconjugate described in the literature involved the following steps: 1) Construction of FITC-conjugated MSNs, 2) Modification of the MSNs to allow for conjugation using 3-mercaptopropyltrimethoxysilane (MPTMS), 3) Removal of the template (the hexadecyl-trimethylammonium bromide template) by heating in acidic ethanol (to form the template-free sample), 4) Linking of mAbs to a polyethylene glycol spacer, and 5) Conjugation of the linked mAbs to the surface of the modified MSNs (222). The final product is mAb-conjugated, FITC-labelled MSNs with hollow inner structures. BJAB or NALM-6 cells will be exposed a range of the labelled immunoconjugates, incubated for 30 minutes at 37°C, then the lysosomes will be labelled using LysoTracker (Red). After washing, cells will be examined under confocal microscopy to assess the level of immunoconjugates in the lysosomes, and the immunoconjugate with the highest level of uptake will be used for subsequent loading of the MSNs.

Synthesis of MSN immunoconjugates result in highly porous structures with uniform (1.5-10 nm) pores, allowing for loading of the MSNs with various drugs. To induce disruption of the lysosomes upon their uptake, MSNs will be loaded with different lysosomotropic detergents that are capable of inducing LMP. A number of lysosomotropic detergents have been described, with varying degree of potency in various cancer cells (**Table 5-2**). Focusing on the detergents with higher reported toxicity, MSNs will be loaded with MSDH, N-dodecylimidazole or siramesine. Loading can be

performed by soaking the immunoconjugated MSNs in concentrated solutions of the drug, and the pores are subsequently plugged with β-cyclodextrin. The loaded MSNs are isolated via centrifugation, washed, vacuum-dried, sonicated and washed again in order to remove the drugs adsorbed (and not loaded) onto the surface of MSNs as described in the literature (224). BJAB, NALM-6 and primary CLL cells will be treated with the three different immunoconjugates, and their potency will be examined by measuring the levels of apoptosis (propidium iodide staining) and total cell death (trypan blue staining). LysoTracker staining will be used to examine the extent of LMP in response to the different immunoconjugates, and the role of lysosomes will be confirmed by pre-treating the cells with chloroquine, NH_4Cl or CA074-Me. The immunoconjugate identified to be the most potent will next be combined with different HDIs, including VPA, and the effect of the combination will be examined in cell lines and primary CLL cells.

EXPECTED RESULTS: Using the CLL cell-targeting immunoconjugated MSNs, CLL cells are expected to undergo targeted cell death through lysosome disruption, and the β-cyclodextrin nanovalves are expected to increase the potency and specificity of the drugs.

Table 5-2. Lysosomotropic detergents.

Lysosomotropic Detergent	Cells	Doses Examined	Reference
ciprofloxacin, norfloxacin	HeLa	10 μg/ml	(225)
LeuLeuOMe	MCF-7, SH-SY5Y	1 mM	(226)
O-methyl-serine dodecylamide hydrochloride (MSDH)	J-774	25 μM	(93)
N-dodecylimidazole	CHO fibroblasts	25-75 μg/ml	(92)
Siramesine	MCF-7	8 μM	(91)

5.9.4 Future Directions – Significance

Combining strategies to specifically target cancer cells with strategies to induce lysosomal disruption is expected to lead to a novel therapeutic strategy with improved

specificity and potency. The long-term goal of the future studies will be to form an anti-cancer strategy, where the high tolerability and high specificity would allow for easy administration of drugs, even by general physicians, as in the case of widely used antibiotics like amoxicillin. High specificity of such therapies should greatly lessen the morbidity associated with current cancer therapies and should greatly improve the quality of lives of cancer patients.

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