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Enhancing Immunotherapeutic Interventions for Treatment of Chronic Lymphocytic Leukemia

Kamira K. Maharaj

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Enhancing Immunomodulatory Therapeutic Interventions for Chronic Lymphocytic Leukemia

by

Kamira K. Maharaj

A dissertation submitted in partial fulfillment
Of the requirements for the degree of
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DEDICATION

I would like to dedicate this dissertation first and foremost to my family, who have tirelessly supported me in the pursuit of education. To both of my grandmothers, who believed in the power of education for their daughters and granddaughters. This work has been possible because I stand upon the shoulders of those who came before me. To my parents, Sataish and Kamla Maharaj, who have always believed in me while I followed my dreams. To my sister, Satira, who is my role model personally and professionally. To my extended family, especially Ravi, Alicia, Maya and Milan, who have been with me every step of the way. On another note, I dedicate this work to all the biomedical researchers who have come before me and those who will come after. Last but not least, I dedicate this work to all people who have experienced cancer. May you find strength to persevere.
Firstly, I would like to acknowledge my extraordinary mentor, Dr. Javier Pinilla-Ibarz, for being the best mentor I could ask for during this process. I have learned so much from you. I am truly thankful to have had the incredible opportunity to train in your laboratory. Your influence has been the most instrumental in grooming my career. Thank you for your steady guidance, unwavering support on all fronts and for sharing your passion and expertise with me. Your patience, can-do attitude and tremendously exuberant personality have left a lasting imprint.

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# TABLE OF CONTENTS

List of Tables ........................................................................................................................................ iii

List of Figures ........................................................................................................................................ iv

Abstract ................................................................................................................................................ vi

Chapter 1 - Introduction
  Introduction to cancer....................................................................................................................... 1
  Introduction to chronic lymphocytic leukemia (CLL)......................................................................... 2
  Immunobiology of CLL B cells........................................................................................................ 2
  Immune dysfunction in CLL................................................................................................................ 4
  Therapeutic interventions................................................................................................................... 5
  Introduction to epigenetics................................................................................................................. 9
  Histone deacetylases........................................................................................................................ 10
  HDAC inhibitors................................................................................................................................ 11

Chapter 2 - Silencing HDAC6 as a therapeutic strategy in chronic lymphocytic leukemia
  Introduction........................................................................................................................................ 14
  Background and rationale................................................................................................................... 15
  Results
    Analysis of HDAC6 expression in CLL patient samples .................................................................. 16
    Genetic silencing of HDAC6 in the euTCL1 murine model.............................................................. 20
    RNA sequencing of HDAC6-deficient murine CLL B cells............................................................. 24
    Antitumor efficacy of ACY738 in murine CLL.............................................................................. 27
    ACY738 alters proliferative capacity and sensitivity to apoptosis in euTCL1 B cells ................. 30
    HDAC6 inhibition also alters proliferation and apoptosis
      in human CLL via downregulation of BCR signaling................................................................... 32
    Synergistic activity of ACY738 and ibrutinib in vitro................................................................... 35
    Combinatorial efficacy of ACY738 and ibrutinib in murine CLL
      in vivo........................................................................................................................................... 36
  Discussion......................................................................................................................................... 37
  Materials and methods.................................................................................................................... 40

Chapter 3 - HDAC6 inhibition relieves CLL T-cell dysfunction and augments antitumor efficacy of anti-PD-1
  Introduction....................................................................................................................................... 46
  Background and rationale.................................................................................................................. 47
  Results
    Reduction of PD-L1 on CLL B cells by HDAC6i............................................................................ 48
    Reversal of CLL T-cell phenotype by HDAC6i.............................................................................. 50
    HDAC6i counters CLL-induced T-cell suppression........................................................................ 53
LIST OF TABLES

Table 1. In-clinic CLL therapeutics (2018) .......................................................................................... 8
Table 2. HDAC inhibitors in clinical trial as anticancer agents (2018) .................................................. 12
Table 3. CLL patient characteristics .................................................................................................. 17
Table 4. Criteria used to diagnose immune-mediated toxicity ........................................................... 93
LIST OF FIGURES

Figure 1. CLL B cell .................................................................................................................. 4
Figure 2. Flow cytometry analysis of CLL patient and normal PBMCS ........................................... 18
Figure 3. Analysis of HDAC6 expression in CLL patient samples .................................................. 19
Figure 4. Analysis of HDAC6-deficient CLL mouse model .......................................................... 21
Figure 5. Genetic silencing of HDAC6 in euTCL1 mice .............................................................. 22
Figure 6. Quantification of tumor burden in CLL mice ............................................................... 23
Figure 7. Hierarchical clustering of top 50 differentially expressed genes ..................................... 25
Figure 8. RNA-sequencing analysis of young and old mice .......................................................... 26
Figure 9. SYK expression ........................................................................................................... 27
Figure 10. In vivo activity of selective HDAC6 inhibitor ACY738 in wildtype mice ....................... 28
Figure 11. In vivo activity of selective HDAC6 inhibitor ACY738 in CLL mice .............................. 29
Figure 12. Effect of in vivo ACY738 treatment on euTCL1 B cells ............................................... 31
Figure 13. HDAC6-selective range of ACY738 in CLL cell lines .................................................. 33
Figure 14. Effect of ACY738 treatment in human CLL cell lines .................................................. 34
Figure 15. Combinatorial efficacy of ACY738 and ibrutinib in CLL cells in vitro ......................... 35
Figure 16. Functional activity of ibrutinib drinking water .............................................................. 36
Figure 17. Combinatorial antitumor efficacy of ACY738 and ibrutinib treatment in CLL .............. 38
Figure 18. Immunoregulation of CLL B cells by HDAC6 ............................................................ 50
Figure 19. EuTCL1 T-helper and cytotoxic-T phenotype ............................................................. 51
Figure 20. EuTCL1 Memory-T and Treg phenotype ..................................................................... 52
Figure 21. Analysis of euTCL1 T-helper subsets ...................................................................... 53
Figure 22. T-cell activation in co-culture with CLL B cells .......................................................... 54
Figure 23. Combinatorial efficacy of ACY738 and anti-PD-1 in euTCL1 model ........................56
Figure 24. STAT3 signaling in murine B cells........................................................................57
Figure 25. Proposed model for immunomodulatory effects of HDAC6 in CLL ..................58
Figure 26. The CLL microenvironment.................................................................................78
Figure 27. PI3K inhibitors impair normal human T-cell survival and function...............85
Figure 28. Normal and CLL Tregs are differentially impaired by PI3K inhibitors............86
Figure 29. Gating strategy to identify Tregs in human or mouse samples .......................87
Figure 30. Effect of PI3K inhibitors on Tregs............................................................88
Figure 31. Treg suppressive capacity is only modestly impaired by umbralisib treatment ....89
Figure 32. Treatment with PI3K inhibitors differentially impairs Tregs in euTCL1 mice....91
Figure 33. Histology of immune-mediated toxicity in CLL murine model .....................93
Figure 34. Analysis of immune-mediated toxicity in CLL murine model .......................94
Figure 35. Effects of SR-4471 on normal human T cells .................................................96
Figure 36. Effects of SR-4471 on Treg suppressive capacity ...........................................97
Figure 37. CK1ε inhibition promotes CLL Treg survival ...............................................98
Figure 38. Surrogate markers of Treg functional capacity ..............................................99
Figure 39. Proposed model for action of CK1ε inhibition in T cells on canonical
Wnt signaling..................................................................................................................99
ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most common leukemia in developed countries. It is characterized by the accumulation of CD5+ B lymphocytes in the peripheral blood, bone marrow, and lymphoid tissues of affected patients. Patients experience lymphadenopathy, fatigue, and are severely immunocompromised. Management of disease and symptoms is possible with chemo-immunotherapy for higher risk patients; however, CLL remains incurable unless bone marrow transplant is performed. Identification of novel targets and optimization of current therapeutics is therefore still necessary. The new targeted therapies are at the forefront of development for CLL treatment, but it remains challenging to maximize their efficacies while avoiding toxicities.

In this body of work, experimental results demonstrated a role for epigenetic modifier histone deacetylase 6 (HDAC6) in CLL B-cell biology and suggest that targeting of HDAC6 is an effective, novel strategy against chronic lymphocytic leukemia. Antitumor effects occurred through blockade of intrinsic survival signaling and through beneficial immunomodulation. Briefly, this data showed that genetic silencing or pharmacological inhibition of HDAC6 in preclinical CLL mouse models reduced tumor burden and increased survival through inhibition of pro-survival, anti-apoptotic B-cell receptor signaling in CLL B cells. Further interrogation of B-cell receptor signaling was performed ex vivo, showing transcriptional downregulation of spleen tyrosine kinase (SYK) and reduced protein expression of anti-apoptotic transcription factors myeloid cell leukemia (MCL)-1 and B cell lymphoma (BCL)-2.

Based on the ability of HDAC6 to alter intrinsic survival signaling, a rational combinatorial treatment with ibrutinib, a frontline CLL therapeutic, was tested. The combination of HDAC6
inhibitor plus ibrutinib showed synergistic cell kill in CLL cell lines and patient-derived B cells, reduced tumor burden, and increased survival in the preclinical CLL mouse model. HDAC6 was also found to play a role in immunomodulation of CLL B cells and CLL T cells in the microenvironment. HDAC6 silencing or inhibition modified the expression of immunoreactive molecules on CLL B and T cells, including programmed cell death (PD)-1, programmed cell death ligand (PD-L)-1, major histocompatibility complex (MHC)II and lymphocyte-activation gene (LAG)-3. Interestingly, alteration of STAT3 signaling was involved in the immunomodulatory activity of HDAC6. As a result, HDAC6 inhibition resulted in a beneficial reversal of T-cell dysfunction. Moreover, HDAC6 inhibition augmented the antitumor efficacy of immune checkpoint inhibitor, anti-PD-1.

B-cell receptor (BCR) and phosphoinositol-3-kinase (PI3K) signaling have emerged as therapeutic avenues that have changed the paradigm of CLL treatment. Inhibitors targeting these pathways abrogate survival signaling in B cells, but also can affect other immune compartments. Secondary immune targets result in a variety of physiological effects that are not thoroughly understood. On this note, we investigated the role of CLL T-cell subsets in the incidence of autoimmune-related toxicities occurring in patients treated with PI3K inhibitors idelalisib, duvelisib and umbralisib. The data established the regulatory T-cell subset (Tregs) as a definitive regulator of the autoimmune-like toxicities using preclinical CLL and graft-versus-host models. Finally, side-by-side comparison of clinically available PI3K inhibitors demonstrated that umbralisib exhibits differential and beneficial effects on Tregs through inhibition of casein kinase (CK)-1 epsilon and canonical Wnt signaling.

The work reported here contributes knowledge and furthers understanding of immunomodulatory approaches using small-molecule inhibitors for treatment of CLL. While chemo-immunotherapy is still used and appropriate for selected younger patients, alternative strategies are sought after for the unfit, elderly, resistant, relapsed and refractory patients. Novel therapeutic strategies or rational combinations will be most beneficial to those patients who
progress or develop resistance on established regimes. Ultimately, identification of novel targets and optimization of existing therapies is necessary to increase responses, eradicate minimal residual disease and mediate toxicity. These processes will advance the treatment regimen for chronic lymphocytic leukemia patients and impact quality and length of life.
CHAPTER ONE:
INTRODUCTION

Introduction to cancer

Cancer refers to a collection of related diseases arising from the unchecked proliferation of a tumorigenic cell. According to the National Cancer Institute (NCI), approximately 38.4% of people will be diagnosed with cancer at some point in their lifetimes. Malignant tumors may begin almost anywhere in the human body and can spread through the vascular system to invade a variety of host tissues, disrupting their normal functions. This can lead to a host of life-threatening conditions, altogether causing a huge socioeconomic burden in the United States and other countries around the world. The most common cancers according to 2018 NCI statistics are breast, lung, prostate, colon, skin, bladder, non-Hodgkin lymphoma, kidney, endometrial, leukemia, pancreatic, thyroid and liver.

A cancer cell exhibits numerous differences compared to a normal cell. Researchers have studied and defined these differences over the years, attempting to identify and exploit cancer cell weaknesses as therapeutic strategies. The “hallmarks” of cancer cells comprise several universal biological traits acquired during tumorigenesis. Hallmark traits include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Further, it is now widely recognized that tumor beds recruit other types of cells to their physical niches that support acquisition of hallmarks and ultimately form a tumor microenvironment.
Introduction to chronic lymphocytic leukemia (CLL)

Blood cancers are classified as leukemias, lymphomas, or myelomas according to their cell of origin. Chronic lymphocytic leukemia (CLL), a B-cell malignancy, is the most common leukemia in the western countries, accounting for approximately one-quarter of new leukemia cases. It is typical of the aging population and rarely diagnosed in children (average age of diagnosis was 71 years in 2018). The American Cancer Society estimated 20,940 new cases of CLL and 4,510 deaths due to CLL in 2018. Although it can be manageable, the disease has a highly variable clinical course and most patients eventually require therapeutic intervention. Symptoms can include enlarged lymph nodes, spleen and liver, fatigue, fever, weight loss, night sweats and frequent infections. In addition, cytopenia and hypogammaglobulinemia are typical. Risk groups are defined by prognostic score (CLL-IPI) and the Rai and Binet staging systems

Immunobiology of CLL B cells

CLL diagnosis is established with blood smear, lymphocyte count and immunophenotyping of the circulating clonal B cells. The clonal B cells are arrested at the pro B-cell differentiation stage, typically expressing cluster of differentiation (CD)19, dim CD20, CD5 and CD23, while morphologically resembling mature B lymphocytes. CLL B cells also express dim immunoglobulin (Ig)M and or IgD, with a predominance of either kappa or lambda light chain. Deletion of chromosome 11q or 17p, p53 mutation, immunoglobulin heavy-chain variable-region (IgHV) unmutated expression in B cells of CLL patients are standard prognostic markers that can predict clinical course and resistance to chemotherapy.

CLL B cells exhibit features that facilitate responses to intrinsic and extrinsic survival signals. Intrinsic survival signals derive primarily from altered B-cell receptor (BCR) signaling, resulting in expression of genes that promote a proliferative and anti-apoptotic phenotype. The mutational status of surface IgVH on the BCR of CLL B cells is a strong predictor of disease outcome, where the unmutated subtype is a surrogate of high BCR signaling and indicates
shorter time to progression as cells grow more rapidly, whereas the mutated subtype suggests a more latent disease\textsuperscript{6}. Some evidence suggests a role for autoantigen or microbial antigen recognition by the BCR in selection and accumulation of the clonal B cells\textsuperscript{5}.

Engagement of the BCR on a CLL B cell induces phosphorylation of its cytoplasmic regions by src-family kinases, followed by activation of spleen tyrosine kinase (SYK). This triggers a cascade resulting in activity of 1) Bruton’s tyrosine kinase (BTK), 2) phospholipase-C gamma (PLC-\textgamma), 3) phosphoinositide 3-kinases (PI3Ks) and downstream signaling, 4) mitogen-activated protein (MAP) kinase and RAS signaling, and 5) transcription factors nuclear factor (NF)-kappaB, nuclear factor of activated T cells (NF-AT) and AP-1\textsuperscript{5,7}. Therapeutic targeting of BCR-associated kinases has recently exploded into a major field of interest due to CLL cells’ dependence on tonic BCR signaling. Notwithstanding, other pathways besides BCR signaling have been intricately studied and linked to survival of CLL B cells. Notably, therapeutics are being developed to target receptor tyrosine kinase-like orphan receptor 1 (ROR-1), which activates downstream Wnt signaling\textsuperscript{8,9}.

On the other hand, the tumor microenvironment provides extrinsic survival, anti-apoptosis, and trafficking signals, via secretion of soluble factors and direct interaction with cell surface ligands. CLL B cells’ ability to respond to interleukin (IL)-4, IL-6, IL-10, tumor necrosis factor (TNF)\textalpha, CD40 ligand (L), BAFF, APRIL, CXCL12 and CXCL13 secreted by the microenvironment establish that other immune cells play significant roles in pathogenesis of the disease\textsuperscript{10}. This has led to widespread interest in targeting the CLL immune-microenvironment. Finally, CLL B cells display the ability to evade tumor immune surveillance. Evidence has shown that CLL B cells can accomplish this task by modulating immunoreactive surface ligands and cytokines. For example, CLL B cells upregulate coinhibitory immune checkpoints programmed cell death protein (PD)-1 and PD-L1, downregulate major histocompatibility complex (MHC)II expression and secrete higher quantities of suppressive cytokines compared to normal B cells, especially IL-10\textsuperscript{11}. These molecular changes CLL B cells undergo serve to dampen antitumor T-cell responses. Preclinical
studies and clinical trials are underway to investigate the utility of checkpoint blockade and immunomodulatory inhibitors for re-invigorating the antitumor T-cell responses in CLL.\textsuperscript{12}

\textbf{Figure 1. CLL B cell.} Constitutive BCR stimulation and phosphorylation of the intracellular domain leads to signal transduction through PI3K and BTK signaling. BCR signaling elicits upregulation of intrinsic survival and anti-apoptosis signals. Altered expression of immunoreactive surface molecules (e.g. PD-L1 and CD40) and secretion of suppressive soluble factors (e.g. IL-10) impact tumor immune evasion abilities and T-cell dysfunction in CLL patients.

\textbf{Immune Dysfunction in CLL}

Due to accumulation of clonal B cells in bone marrow, blood and lymphoid tissue, CLL patients harbor skewed proportions of other immune cell populations. Immune cells in CLL patients have been found to exhibit characteristic phenotypes that contribute toward malignant progression and CLL B-cell survival. Resulting immune dysfunction increases susceptibility to infections and CLL patients often die from related complications. In addition to the classically
known bone marrow stromal cells and nurse-like cells that provide extrinsic survival signals for CLL B cells, abnormal T cells act in collaboration with the microenvironment to support proliferation of malignant B cells\textsuperscript{10}.

Trends in CLL T-cell changes have emerged: imbalance of T-cell subsets, exhausted phenotype, dysregulation of coinhibitory molecules, increase in suppressive numbers and phenotype, abnormal cytokine secretion, immune synapse and toxicity defects. Numerous studies support the idea of targeting the CLL microenvironment specifically through the T-cell defects to achieve antitumor effects, and therapies thought to primarily target the CLL B cells have also been shown to beneficially impact microenvironment populations\textsuperscript{13,14,15}. Besides T cells, other immune populations that are expanded in CLL patients and correlated with disease, such as myeloid-derived suppressor cells, are being studied and could be potential targets to augment antitumor immunity\textsuperscript{16}.

**Therapeutic interventions**

A variety of approaches has been used to control CLL disease in the past few decades. In this section, the current major options for CLL treatment will be discussed. Most patients typically are monitored and do not require treatment. For the symptomatic, patients with progressive lymphadenopathy or splenomegaly and for patients who develop anemia and thrombocytopenia, treatment is indicated. Frontline therapy with chemo-immunotherapy (fludarabine, cyclophosphamide, and rituximab)\textsuperscript{17} is still used and appropriate for younger patients that carry a IGHV mutated status, however, less-toxic alternatives are sought after for high risk younger patients, elderly, unfit or relapsed and refractory (R/R) patients. Elucidation of underlying molecular pathways driving CLL progression and resistance to therapy has been crucial for development of revolutionary therapeutics in recent years to address this niche of patients. In addition, allogeneic stem cell transplant can be a viable option for CLL patients, although it is very limited by age\textsuperscript{3}.
CLL therapies can be divided into several broad categories: chemotherapy, immunotherapy, and targeted therapies. Chemotherapies typically utilized for CLL treatment are the DNA-damaging agents chlorambucil, fludarabine, and bendamustine. These work by preferentially preventing the replication of tumor cells. Due to frequent relapse and resistance, it has been advantageous to combine immunotherapy with chemotherapy. Several monoclonal antibodies (mAb) against CD20 antigen are clinically approved, including rituximab, ofatumumab, and obinutuzumab. These work to increase cell-dependent and antibody-dependent cellular cytotoxicity. In addition to the CD20 antigen, mAbs that target a slew of CLL B cell surface proteins are in development (Table 1).

Other immunotherapeutic agents are being investigated with the intention of augmenting host immunity. Pembrolizumab, an anti-PD1 immune checkpoint-blocking antibody, is in clinical trial for R/R CLL patients alone or in combination with ibrutinib. Notably, pembrolizumab elicited response in 4 out of 9 CLL patients who progressed after ibrutinib treatment and developed Richter’s transformation, a phenomenon where B-CLL becomes fast-growing diffuse large B-cell lymphoma (DLBCL). Atezolizumab (anti-PD-L1 antibody) was found to block progression in a preclinical CLL mouse model and is currently in clinical trial in combination with obinutuzumab and ibrutinib for relapsed, refractory, or high-risk untreated CLL. Numerous other immune checkpoints have been shown to be upregulated in CLL that function to dampen immune responses and evade immune surveillance. Preclinical development of therapeutics capable of checkpoint inhibition is therefore burgeoning. Last but not least, chimeric antigen receptor (CAR) T cells bearing domains recognizing CD19 and additional signaling through 4-1BB or CD28 have demonstrated efficacy in clinical trials with refractory CLL patients. Several types of CAR T therapies are in clinical trial for CLL, utilizing autologous or allogeneic T cells recognizing various domains. Recently, interest has been generated in utilizing immunomodulatory small-molecule inhibitors to reconfigure the phenotype of CLL patient T cells, so that they are more conducive to ex vivo expansion and transduction with CAR constructs. Preclinical studies surrounding this
concept are underway and will provide rationale to combine CAR therapy with small-molecule inhibitors.

The third group of CLL therapeutics encompasses the small-molecule inhibitors. The BCR kinase inhibitors have gained most attention in this group. Approval of ibrutinib (BTK inhibitor), a first-in-class, once-daily treatment by the United States Food and Drug Administration (FDA) as frontline therapy for CLL patients shifted the paradigm of CLL treatment\textsuperscript{23}. Idelalisib (PI3K\(\delta\) inhibitor) in combination with rituximab has also been approved for R/R patients\textsuperscript{24}. Most recently, duvelisib (PI3K\(\delta/\gamma\) inhibitor) was approved for R/R CLL patients who failed at least two prior therapies. Alternative BCR inhibitors are being tested in clinical trial but are not yet approved for CLL, such as acalabrutinib\textsuperscript{25} (BTK), entospletinib (SYK) and umbralisib\textsuperscript{26} (PI3K\(\delta/casein kinase (CK)1\epsilon\)). Another small-molecule inhibitor at the forefront is venetoclax, targeting the B-cell lymphoma (BCL)-2 protein, a key regulator of apoptosis. Venetoclax\textsuperscript{27} has been approved for CLL patients alone or in combination with rituximab after one prior therapy and has shown promising initial results in combination with ibrutinib as well as obinutuzumab for frontline therapy\textsuperscript{28}. Although venetoclax combination offers a time limited therapy, there are not enough data to know whether these patients will progress. Several other small-molecule inhibitors targeting various CLL survival proteins are in clinical development, including HDAC inhibitors (Table 1).

BCR inhibitors have been shown to display immunomodulatory effects, and can robustly target secondary immune populations like T cells and myeloid cells\textsuperscript{12}. The effects of BCR inhibitors on secondary target populations are under investigation. These studies will elucidate novel mechanisms of action, unveil potential repurposing and provide rationale for combinations. Lenalidomide (targeting cereblon), although not a BCR inhibitor, is a small-molecule immunomodulatory drug that exhibits antitumor effects in CLL while changing the immune microenvironment to increase immune surveillance. Studies demonstrating immunomodulation of
CLL immune subsets by lenalidomide have paved the way for elucidating the immunomodulatory effects of BCR inhibitors\textsuperscript{29}.

**Table 1: In-clinic CLL therapeutics.** Notable chemotherapy, immunotherapy, and small-molecule inhibitors for treatment of CLL that are either approved or in Phase I-III trial (2018).

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Class</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>alkylating agent</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>purine analog</td>
<td>FDA approved</td>
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<tr>
<td>Bendamustine</td>
<td>purine analog</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>purine analog</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Rituximab</td>
<td>anti-CD20 mAb</td>
<td>FDA approved</td>
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<td>Ofatumumab</td>
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<td>FDA approved</td>
</tr>
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<td>Obinutuzumab</td>
<td>anti-CD20 mAb</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Ublituximab</td>
<td>anti-CD20 mAb</td>
<td>Phase III</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>anti-PD-1 mAb</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Nivolumab</td>
<td>anti-PD-1 mAb</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Atezolizumab</td>
<td>anti-CD52 mAb</td>
<td>FDA approved</td>
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<td>Cirmtuzumab</td>
<td>anti-ROR1 mAb</td>
<td>Phase I</td>
</tr>
<tr>
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<td>anti-NKG2A mAb</td>
<td>Phase I/II</td>
</tr>
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<td>Daratumumab</td>
<td>anti-CD38 mAb</td>
<td>Phase II</td>
</tr>
<tr>
<td>Lirilumab</td>
<td>anti-IgG1 mAb</td>
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<td>Duvelisib</td>
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<td>Buparlisib</td>
<td>pan-PI3K inhibitor</td>
<td>Phase II</td>
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<td>Umbralisib</td>
<td>PI3K&amp;/CK1&amp; inhibitor</td>
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<tr>
<td>Fenebrutinib</td>
<td>BTK inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>Selinexor</td>
<td>XPO1 inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>Entospletinib</td>
<td>SYK inhibitor</td>
<td>Phase II</td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>JAK inhibitor</td>
<td>Phase II</td>
</tr>
<tr>
<td>AZD6738</td>
<td>ATM/ATR inhibitor</td>
<td>Phase I/II</td>
</tr>
</tbody>
</table>
Table 1 Cont’d: In-clinic CLL therapeutics. Notable chemotherapy, immunotherapy, and small-molecule inhibitors for treatment of CLL that are either approved or in Phase I-III trial (2018).

<table>
<thead>
<tr>
<th>Drug/Inhibitor</th>
<th>Category</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pevonedistat</td>
<td>NEDD8-activating enzyme inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>CYC065</td>
<td>CDK2/9 inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>Venetoclax</td>
<td>BCL-2 inhibitor</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Navitoclax</td>
<td>BCL-2/BCL-xL/BCL-w inhibitor</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Lenalidomide</td>
<td>thalidomide analog</td>
<td>FDA approved</td>
</tr>
<tr>
<td>ACY-1215</td>
<td>HDAC6 inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>Vorinostat</td>
<td>pan-HDAC inhibitor</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Romidepsin</td>
<td>pan-HDAC inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>MS-553</td>
<td>PKC-β inhibitor</td>
<td>Phase I</td>
</tr>
<tr>
<td>Axicabtagebi ciloleucel</td>
<td>anti CD19 CAR T</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Tisagenlecleucel-T</td>
<td>anti CD19 CAR T</td>
<td>Phase I</td>
</tr>
<tr>
<td>Lisocabtagene maraleucel</td>
<td>anti CD19/4-1BB CAR T</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>CD19/CD22 CAR T</td>
<td>anti CD19/CD22 CAR T</td>
<td>Phase I</td>
</tr>
<tr>
<td>CD19.CAR-aNKT cells</td>
<td>CAR T/ NKT</td>
<td>Phase I</td>
</tr>
<tr>
<td>INVAC-1</td>
<td>DNA vaccine</td>
<td>Phase II</td>
</tr>
<tr>
<td>NeoVax</td>
<td>personal neoantigen vaccine</td>
<td>Phase I</td>
</tr>
<tr>
<td>CD3/28 activated T cells</td>
<td>adoptive cell</td>
<td>Phase I/II</td>
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Introduction to epigenetics

Epigenetics is the study of heritable changes in gene expression occurring without alteration to primary DNA sequence. Chromatin refers to the macromolecular complex of DNA and histone proteins which comprise the genome. The nucleosome, the unit of chromatin, contains 147 base pairs of DNA wrapped around a histone octamer. The histone octamer contains two histones H2A, H2B, H3 and H4. The components of the nucleosome can be covalently modified, altering the structural organization and expression of genes. Noncovalent modifications also occur. Epigenetic dysregulation of biological processes such as transcription, DNA repair, and replication, have been found to occur frequently in cancer. In addition, therapeutics targeting epigenetic regulators have demonstrated anticancer activity.
In total, there are numerous types of DNA and histone modifications that are regulated by chromatin-modifying enzymes. DNA methylation refers to the addition of methyl groups to CpG islands in the genome. This is catalyzed by DNA methyltransferases (DNMTs)\textsuperscript{31}. This modification generally represses transcription and ensures stability by silencing transposable elements. A link between cancer and epigenetics was first observed from DNA methylation studies, mostly characterizing silencing of tumor suppressors by promoter methylation. Aside from DNA methylation, post-translational histone modifications have been characterized, including methylation, acetylation, phosphorylation, ubiquitination and others\textsuperscript{32}. Modifications to histone tails regulate gene expression by changing chromatin to a condensed or relaxed conformation.

Acetylation of histone tails is regulated by enzymes called histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs and HDACs catalyze the addition or removal of an acetyl group derived from acetyl-CoA at the N-terminal amino group of lysine residues, respectively. Acetylation neutralizes the positively charged lysine and relaxes chromatin structure to encourage transcription, while deacetylation condenses chromatin structure to block transcription\textsuperscript{33}. HATs and HDACs, along with numerous binding partners, have been found to be dysregulated in some types of cancer\textsuperscript{34}. Alteration of cancer cell processes through targeting of HATs and HDACs has therefore been a major field of interest.

**Histone deacetylases**

Eighteen mammalian histone deacetylases have been identified, which can be divided into classes according to structural homology\textsuperscript{33}. Class I includes HDACs 1, 2, 3 and 8. Class IIa includes HDACs 4, 7 and 9, while class IIb includes HDACs 6 and 10. Class II HDACs show tissue-specific expression and both nuclear and cytoplasmic localization- suggesting that this class may regularly acetylate non-histone proteins. The newest HDAC member, HDAC11, is the only member of class IV. Finally, class III comprises sirtuins 1-7. The expression levels of various HDACs have been found to be dysregulated in numerous cancer types\textsuperscript{35}. These findings suggest
that transcriptional regulation by aberrant HDACs could be a common phenomenon in tumor initiation. The role of HDACs in cancer may also depend on their regulation of non-histone proteins. For example, tumor suppressor p53 activity has been shown to be affected by acetylation status which can be modulated by pharmacological inhibition of HDACs\(^3\)\(^6\).

**HDAC inhibitors**

The potential for epigenetic manipulation of cellular processes makes HDACs compelling targets for therapeutics. In addition, HDAC inhibitors have been used as tools to elucidate the cellular activities of the HDAC family of proteins. Some HDAC inhibitors block the entire family of proteins and are referred to as “pan-HDAC inhibitors”. Others specifically silence one HDAC or a class of HDACs, referred to as “specific” or “selective” HDAC inhibitors. Four pan-HDAC inhibitors are currently FDA-approved for treatment of specific malignancies: vorinostat (SAHA) and romidepsin for cutaneous T-cell lymphoma, belinostat for peripheral T-cell lymphoma, and panobinostat for multiple myeloma. Several others are in clinical trials for various cancer types, including hematological malignancies and solid tumors\(^3\)\(^7\). The HDAC inhibitors in clinical trial as anticancer agents current for 2018 is summarized in Table 2. Many more are in preclinical development\(^3\)\(^7\).

It is now recognized that mechanisms of action responsible for the antitumor effects of the HDAC inhibitors differ. For example, HDAC inhibitors have been shown to cause cell cycle arrest, induce apoptosis, reduce angiogenesis, and downregulate oncogenic signaling in cancer cells\(^3\)\(^8\). In preclinical studies, the HDACs have been shown to regulate various processes depending on cell type and disease context. Notably, several HDACs have been implicated in control of immune responses and as a result, HDAC inhibitors are being explored as immunomodulatory agents\(^3\)\(^9\). Some pan-HDAC inhibitors tested in patients have not progressed past clinical trials due to toxicity. Toxicity with pan-HDAC inhibitors is not altogether surprising, given the wide range of
HDAC expression and function. For this reason, growth of isoform-selective HDAC inhibitors is currently at the forefront^{40}. Since the aberrant expression and function of individual HDACs in cancers may differ, isoform-selective inhibitors can be utilized for potent and specific targeting. The use of selective HDAC inhibitors promises more deliberate, rationale-based trials leading to less toxicity, greater efficacy, mechanistic understanding, novel utility, and combination approaches with other therapeutics.

Table 2: HDAC inhibitors in clinical trial as anticancer agents (2018).

<table>
<thead>
<tr>
<th>HDAC inhibitor</th>
<th>Class</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorinostat</td>
<td>pan-HDAC</td>
<td>melanoma, solid tumors, uterine sarcoma, NSCLC, CLL</td>
</tr>
<tr>
<td>Panobinostat</td>
<td>pan-HDAC</td>
<td>melanoma, NHL, multiple myeloma, AML, MDS</td>
</tr>
<tr>
<td>Abexinostat</td>
<td>pan-HDAC</td>
<td>follicular lymphoma</td>
</tr>
<tr>
<td>Romidepsin</td>
<td>pan-HDAC</td>
<td>lymphoid malignancies</td>
</tr>
<tr>
<td>Givinostat</td>
<td>pan-HDAC</td>
<td>chronic myeloproliferative neoplasms</td>
</tr>
<tr>
<td>Tefinostat</td>
<td>pan-HDAC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>Mocetinostat</td>
<td>Class I HDAC</td>
<td>melanoma, solid tumors, NSCLC</td>
</tr>
<tr>
<td>Entinostat</td>
<td>Class I HDAC</td>
<td>solid tumors, MDS, CML, AML, breast cancer</td>
</tr>
<tr>
<td>CUDC-907</td>
<td>Class I/II HDAC / PI3Ka, B, d</td>
<td>solid tumors</td>
</tr>
<tr>
<td>Martinostat</td>
<td>Class I/IIb HDAC</td>
<td>breast cancer</td>
</tr>
<tr>
<td>KA2507</td>
<td>HDAC6</td>
<td>solid tumors</td>
</tr>
<tr>
<td>ACY-241</td>
<td>HDAC6</td>
<td>NSCLC, multiple myeloma</td>
</tr>
<tr>
<td>ACY-1215</td>
<td>HDAC6</td>
<td>lymphoid malignancies, breast cancer, multiple myeloma</td>
</tr>
</tbody>
</table>
NOTE TO THE READER

Portions of the following chapter have been previously published: Maharaj, K et al. Silencing of HDAC6 as a Therapeutic Target in Chronic Lymphocytic Leukemia. Blood Advances 2, 3012-3024 (2018) and have been included here with the journal's permission (see Appendix A for copyright policy).
CHAPTER TWO: SILENCING OF HDAC6 AS A THERAPEUTIC STRATEGY IN CHRONIC LYMPHOCYTIC LEUKEMIA

Introduction

Although the treatment paradigm for chronic lymphocytic leukemia (CLL) is rapidly changing, the disease remains incurable, and resistance, relapsed disease, and partial responses persist as significant challenges. Recent studies have uncovered roles for epigenetic modification in the regulation of mechanisms contributing to malignant progression of CLL B cells. However, the extent to which epigenetic modifiers can be targeted for therapeutic benefit in CLL patients remains poorly explored. We report for the first time that expression of epigenetic modifier histone deacetylase 6 (HDAC6) is upregulated in CLL patient samples, cell lines, and euTCL1 transgenic mouse models compared to HDAC 6 in normal controls. Genetic silencing of HDAC6 conferred survival benefit in euTCL1 mice. Administration of isoform-specific HDAC6 inhibitor ACY738 in the euTCL1 aging and adoptive transfer models deterred proliferation of CLL B cells, delayed disease onset via disruption of B-cell receptor signaling, and sensitized CLL B cells to apoptosis. Further, coadministration of ACY738 and ibrutinib displayed synergistic cell kill against CLL cell lines and improved overall survival compared to either single agent in vivo. These results demonstrate for the first time the therapeutic efficacy of selective HDAC6 inhibition in preclinical CLL models and suggest a rationale for the clinical development of HDAC6 inhibitors for CLL treatment, either alone or in combination with Bruton’s tyrosine kinase inhibition.
Background and rationale

Epigenetic reprogramming in CLL B cells has previously been described in several studies\textsuperscript{42, 43, 44}. These studies documenting DNA hypomethylation, dysregulated microRNAs, and histone modifications in CLL B cells have proven beneficial in identifying biomarkers and have offered a fresh perspective on therapeutic strategies. Our group has also reported findings establishing a role for epigenetic modifiers in altering CLL biology\textsuperscript{15, 45}. Epigenetic modification with demethylating agent 5-aza-2‘deoxycytidine and HDAC inhibitor LAQ824 restored the immunogenicity of CLL cells through the induction of antigenic markers to facilitate interaction of CLL B cells and T cells. Demethylation of T helper 1 promoters by 5-aza-2‘deoxycytidine repolarized T helper 2-skewed CLL T cells to a more beneficial inflammatory phenotype. Interestingly, various groups have reported elevated HDAC expression in CLL B cells compared to HDAC expression in normal B cells and correlations of HDAC expression levels with prognostic factors\textsuperscript{46, 47, 48, 49}. Several studies have also begun to explore the therapeutic potential of HDAC inhibitors for CLL treatment\textsuperscript{60, 51}. However, the roles of individual HDACs in CLL biology have not been fully elucidated.

Class IIb HDAC6 can form a complex with nuclear proteins such as p300,\textsuperscript{52} HDAC11,\textsuperscript{53} Runx2,\textsuperscript{54} and LCoR\textsuperscript{55} to regulate transcriptional repression. On the other hand, HDAC6 can deacetylate cytoplasmic proteins alpha-tubulin,\textsuperscript{56} cortactin,\textsuperscript{57, 58} HSP90\alpha,\textsuperscript{59} and GRP78,\textsuperscript{60} regulating cell motility, migration, division, and proteosomal degradation\textsuperscript{61}. Further, HDAC6 has been explored in the context of various cancers. It was found to be upregulated in oral squamous cell carcinoma,\textsuperscript{62} ovarian carcinoma,\textsuperscript{63, 64} and primary acute myeloid leukemia,\textsuperscript{65} relative to nonmalignant controls. Preclinical studies have demonstrated that HDAC6 inhibitors display antitumor activity when used either alone or in combination with other agents to treat multiple myeloma,\textsuperscript{66, 67} B-cell lymphoma,\textsuperscript{60, 68} breast cancer,\textsuperscript{69, 70} lung cancer,\textsuperscript{71, 72} and melanoma\textsuperscript{73, 74}. At present, HDAC6-selective inhibitors are in clinical trials to treat several solid tumor types, multiple myeloma, and lymphoma (NCT03008018, NCT02935790, NCT20635061, NCT01323751,
Finally, HDAC6 has been explored in the context of immune regulation. In the current study, we asked whether there is a role for HDAC6 in CLL B-cell pathobiology and explored the potential therapeutic value of targeting HDAC6 in preclinical CLL models.

Results

Analysis of HDAC6 expression in CLL patient samples

We examined the expression of HDAC6 in peripheral blood samples from a cohort of 38 CLL donors and 13 healthy donors. Table 3 displays clinical features of all CLL patients. PBMCs were gated on normal B cells (CD19+ CD5-) in healthy donors and CLL B cells (CD19+ CD5+) in CLL donors (Figure 2). HDAC6 protein expression was determined by flow cytometry and quantified by median fluorescence intensity. HDAC6 protein expression was found to be upregulated in CLL B cells compared to normal healthy donor B cells (Figure 3A). Correlation analyses demonstrated a weak positive association between HDAC6 protein expression and CD19+ CD5+ CLL B-cell percentages in CLL patient samples (Figure 3B). In addition, HDAC6 protein expression was higher among CD38+ patients than CD38- patients (Figure 3C). However, HDAC6 protein expression did not correlate with gender (p = .2), age at diagnosis (p = .6), del 11q/ATM gene (p = .2), del 17p/p53 gene (p = .7), del 13q (p = .5), trisomy 12 (p = .7), ZAP70 status (p = .4), immunoglobulin heavy chain variable region-mutated/unmutated status, or relapsed status (p = .9; data not shown).
Table 3. CLL patient characteristics

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender</th>
<th>Age at Diagnosis</th>
<th>Cytogenetics (FISH)</th>
<th>CD38 Expression &gt;7%</th>
<th>ZAP70 Expression &gt;10%</th>
<th>IgVH Status</th>
<th>Relapsed</th>
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</thead>
<tbody>
<tr>
<td>CLL1</td>
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<td>47</td>
<td>trisomy 12</td>
<td>-</td>
<td>-</td>
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<td>Yes</td>
</tr>
<tr>
<td>CLL2</td>
<td>Female</td>
<td>50</td>
<td>normal</td>
<td>-</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL3</td>
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<td>71</td>
<td>del 13q, del 17p</td>
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<td>+</td>
<td>Mutated</td>
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</tr>
<tr>
<td>CLL4</td>
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<td>51</td>
<td>trisomy 12</td>
<td>-</td>
<td>-</td>
<td>Mutated</td>
<td>Yes</td>
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<tr>
<td>CLL5</td>
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<td>62</td>
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<td>-</td>
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<td>No</td>
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<tr>
<td>CLL6</td>
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<td>59</td>
<td>trisomy 12</td>
<td>Unknown</td>
<td>+</td>
<td>Unknown</td>
<td>Yes</td>
</tr>
<tr>
<td>CLL7</td>
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<td>58</td>
<td>del 11q, del 13q</td>
<td>+</td>
<td>+</td>
<td>Unmutated</td>
<td>Yes</td>
</tr>
<tr>
<td>CLL8</td>
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<td>39</td>
<td>del 11q</td>
<td>+</td>
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<td>61</td>
<td>del 13q</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CLL10</td>
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<td>62</td>
<td>normal</td>
<td>-</td>
<td>-</td>
<td>Mutated</td>
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<tr>
<td>CLL11</td>
<td>Female</td>
<td>55</td>
<td>trisomy 12</td>
<td>-</td>
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<td>Unmutated</td>
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<tr>
<td>CLL12</td>
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<td>57</td>
<td>del 13q</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>CLL13</td>
<td>Male</td>
<td>46</td>
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<td>-</td>
<td>-</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL14</td>
<td>Male</td>
<td>56</td>
<td>del 13q</td>
<td>+</td>
<td>+</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL15</td>
<td>Male</td>
<td>69</td>
<td>del 11q, del 13q</td>
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<td>Unknown</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL16</td>
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<td>58</td>
<td>del 13q</td>
<td>+</td>
<td>-</td>
<td>Unknown</td>
<td>No</td>
</tr>
<tr>
<td>CLL17</td>
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<td>61</td>
<td>del 13q</td>
<td>-</td>
<td>-</td>
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<td>No</td>
</tr>
<tr>
<td>CLL18</td>
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<td>49</td>
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<td>-</td>
<td>-</td>
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<td>Yes</td>
</tr>
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<td>Male</td>
<td>65</td>
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</tr>
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<td>-</td>
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</tr>
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<td>-</td>
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<td>No</td>
</tr>
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<td>CLL22</td>
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<td>trisomy 12</td>
<td>+</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL23</td>
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<td>64</td>
<td>trisomy 12</td>
<td>+</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL24</td>
<td>Male</td>
<td>53</td>
<td>normal</td>
<td>+</td>
<td>+</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL25</td>
<td>Male</td>
<td>77</td>
<td>del 13q, del 17p</td>
<td>-</td>
<td>-</td>
<td>Mutated</td>
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</tr>
<tr>
<td>CLL26</td>
<td>Female</td>
<td>74</td>
<td>trisomy 12</td>
<td>-</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
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<tr>
<td>CLL27</td>
<td>Male</td>
<td>49</td>
<td>IgH rearrangement</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
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</tr>
<tr>
<td>CLL28</td>
<td>Female</td>
<td>49</td>
<td>del 11q, del 13q</td>
<td>+</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL29</td>
<td>Female</td>
<td>46</td>
<td>del 11q</td>
<td>Unknown</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL30</td>
<td>Male</td>
<td>61</td>
<td>del 11q, del 13q</td>
<td>-</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL31</td>
<td>Female</td>
<td>60</td>
<td>del 13q</td>
<td>-</td>
<td>-</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL32</td>
<td>Male</td>
<td>65</td>
<td>normal</td>
<td>+</td>
<td>-</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL33</td>
<td>Female</td>
<td>76</td>
<td>normal</td>
<td>-</td>
<td>+</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL34</td>
<td>Female</td>
<td>49</td>
<td>del 11q, IgH rearrangement</td>
<td>+</td>
<td>Unknown</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL35</td>
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<td>52</td>
<td>normal</td>
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<td>Unknown</td>
<td>Unmutated</td>
<td>Yes</td>
</tr>
<tr>
<td>CLL36</td>
<td>Female</td>
<td>55</td>
<td>del 13q</td>
<td>+</td>
<td>-</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL37</td>
<td>Male</td>
<td>56</td>
<td>del 13q</td>
<td>-</td>
<td>Unknown</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>CLL38</td>
<td>Female</td>
<td>57</td>
<td>del 11q, del 13q</td>
<td>+</td>
<td>-</td>
<td>Unmutated</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 2. Flow cytometry analysis of CLL patient and normal peripheral blood mononuclear cells (PBMCs). Cells of interest were gated on singlets using forward scatter height vs. forward scatter area parameters and nonviable cells (Live Dead Yellow-positive) were excluded. Median fluorescence intensity of HDAC6 was determined for the CD19+ CD5+ (CLL B cells) population. All markers were gated according to fluorescence-minus-one or isotype controls. HDAC6 fluorescence-minus-one control is indicated as grey histogram. Red arrows denote the order of gating strategy. (A) Representative of CLL patient cells analysis. (B) Representative of normal donor cells analysis. Abbreviations: CLL, chronic lymphocytic leukemia; PBMC, peripheral blood mononuclear cells.
Figure 3. Analysis of HDAC6 expression in CLL patient samples. (A) Intracellular HDAC6 protein expression in CD19+ CD5- B cells from normal donors (n = 13) and CD19+ CD5+ B cells from CLL patient peripheral blood samples (n = 38), as determined by flow cytometry. (B) Correlation analyses showing relationship between HDAC6 protein expression and CLL B-cell percentages in CLL patient samples. (C) HDAC6 protein expression in CD38+ or CD38- CLL patient samples. Error bars correspond to standard deviations and *, **, *** represent p < .01, p < .001, p < .001, respectively. Abbreviations: CLL, chronic lymphocytic leukemia; HDAC6, histone deacetylase 6; MFIR, median fluorescence intensity ratio (value normalized to isotype control).
Genetic silencing of HDAC6 in the euTCL1 murine model

To study the role of HDAC6 in CLL B cells, we employed the euTCL1 transgenic murine model. This mouse model is genetically engineered to express the T-cell leukemia 1 (TCL1) gene in B cells, leading to development of a CLL-like phenotype at 6 to 9 months of age. At 6 months of age, euTCL1 mice demonstrated higher expression of HDAC6 protein in peripheral CD19+ B cells compared to age-matched wild-type (WT) mice (Figure 4B, 5A). Here, we generated an HDAC6-deficient euTCL1 mouse model by crossing euTCL1 mice with HDAC6KO mice. In euTCL1/HDAC6KO mice, HDAC6 protein levels were significantly decreased and TCL1 protein expression in B cells remained unchanged compared to euTCL1 mice. We also detected increased acetylation of α-tubulin in HDAC6-deficient mice, suggesting that HDAC6 was functionally impaired (Figure 4D). Age-matched cohorts of euTCL1 and euTCL1/HDAC6KO mice were observed for survival alongside WT and HDAC6KO controls. Interestingly, the euTCL1/HDAC6KO cohort demonstrated increased overall survival (median survival, 375 days) compared to euTCL1 controls (median survival, 318.5 days; Figure 5B). However, euTCL1/HDAC6KO mice eventually succumbed to leukemia.

To determine the effect of HDAC6 genetic deletion on CLL burden, 9-month-old mice were sacrificed for the collection of spleens and peripheral blood. We quantified tumor burden by gating on CD3- CD19+ B220Lo-Hi CD5+ B cells (Figure 6). The total B-cell number (CD19+ B220Lo-Hi) was lower in the euTCL1/HDAC6KO group than the euTCL1 group, in both peripheral blood and spleen compartments. In addition, the ratio of CD5+ B cells (defined as “malignant”) to CD5- B cells (defined as “normal”) was reduced in the euTCL1/HDAC6KO group compared to the euTCL1 group. The reduction in malignant/normal B-cell ratios was evident in both peripheral blood and spleen (Figure 5C). Finally, splenomegaly, was reduced in euTCL1/HDAC6KO mice compared to euTCL1 controls (Figure 5D-E). Genetic deletion of HDAC6 therefore slowed down the accumulation of malignant B cells in vivo, resulting in lower CLL tumor burden over time and improved overall survival rates.
Figure 4. Analysis of HDAC6-deficient CLL mouse model. (A) Western blot showing expression of HDAC6 protein in WT, euTCL1, HDAC6KO or euTCL1/HDAC6KO murine B cells. (B) Flow cytometry showing expression of HDAC6 protein in WT, euTCL1, HDAC6KO or euTCL1/HDAC6KO murine B cells. (C) Flow cytometry analysis showing TCL1 protein expression is similar in euTCL1 B cells and euTCL1/HDAC6KO B cells. (D) Flow cytometry analysis showing functional inhibition of HDAC6 in euTCL1/HDAC6KO B cells demonstrated by increased acetylation of alpha-tubulin, a cytoplasmic substrate of HDAC6, compared to the WT cohort. Abbreviations: CLL, chronic lymphocytic leukemia; WT, wild-type mouse.
Figure 5. Genetic silencing of HDAC6 in euTCL1 mice. (A) Intracellular HDAC6 protein expression in CD19+ B220+ B cells from WT or euTCL1 peripheral blood, as determined by flow cytometry (n = 9 per group). (B) Overall survival of indicated groups of mice (n = 5 per group), representing results of 3 independent experiments. (C) CLL burden quantified in peripheral blood or spleen by flow cytometry (n = 5 per group), representing results of at least 3 independent experiments. (D) Compilation of spleen-weight data at the end of the study (n = 3 per group). (E) Representative spleens from each group indicated. Abbreviations: CLL, chronic lymphocytic leukemia; HDAC6, histone deacetylase 6; MFIR, median fluorescence intensity ratio; PB, peripheral blood; WT, wild-type mice.
Figure 6. Quantification of tumor burden in CLL mice. Flow cytometry analysis of tumor burden in splenocytes of (A) euTCL1 and (B) euTCL1/HDAC6KO mice. Cells of interest were gated on singlets using forward scatter height vs. forward scatter area parameters, and nonviable cells (4’,6-Diamidino-2-Phenylindole, Dihydrochloride -positive) were excluded. The CD3+ CD19+ B220+ CD5- were gated as normal B cells and CD3- CD19+ B220+ CD5+ were gated as malignant CLL B cells. CD5 fluorescence-minus-one control is displayed as grey dots. Red arrows denote the order of gating strategy.
RNA sequencing of HDAC6-deficient murine CLL B cells

Next, we investigated transcriptional changes related to disease progression in HDAC6-deficient CLL B cells. RNA-sequencing was performed on B cells isolated from spleens of young (3-month-old) and aged (9-month-old) cohorts of euTCL1, euTCL1/HDAC6KO, HDAC6KO, and WT mice. Gene expression patterns in young mice differed from aged mice. Hierarchal clustering demonstrated that gene expression was most dissimilar between euTCL1 and euTCL1/HDAC6KO in both young and aged groups, suggesting that silencing of HDAC6 initiated widespread transcriptional changes in the CLL setting (Figure 7). In euTCL1 mice, CLL progresses as the mice age. Therefore, gene expression in aged euTCL1 may depend heavily on both the aging process and disease progression. Consequently, young and aged WT and HDAC6KO mice were compared in order to identify and eliminate genes that were differentially expressed due to the aging process.

Figure 8A-B describes the analyses of the changed genes uniquely regulated in euTCL1/HDAC6KO mice compared to euTCL1 mice as a consequence of slower disease progression. The Venn diagram in Figure 8C depicts the group of 334 disease-related genes that were uniquely regulated in euTCL1/HDAC6KO mice compared to euTCL1 mice. Gene ontology analysis was performed on these 334 genes. The majority of the 50 pathways corresponded to immune-related signaling. Spleen tyrosine kinase (SYK) was common among many of these pathways and was one of the most significantly changed genes within the focus group. It was confirmed by quantitative real-time PCR that SYK is transcriptionally downregulated in euTCL1/HDAC6KO and HDAC6KO mice compared to euTCL1 and WT mice, respectively (Figure 9A). Interestingly, SYK is involved in transduction of BCR signaling, which is required for the survival of CLL B cells. Given the importance of SYK to BCR signaling, this result suggested to us that transcriptional regulation of SYK might potentially result in changes to downstream BCR
signaling in CLL B cells lacking HDAC6. Immunoblotting confirmed that SYK protein was downregulated in euTCL1/HDAC6KO mice compared to euTCL1 mice (Figure 9B).

Figure 7. Hierarchical clustering of top 50 differentially expressed genes. Heat maps show similarities in gene expression patterns among mice used in RNA-sequencing analyses. (A) Young group. (B) Aged (old) group.
Figure 8. RNA-sequencing analysis of young and old mice. (A) Table showing cohorts of mice used for analysis and types of genes identified by each. (B) Flow chart describing step-by-step analysis process used to identify genes of interest. (C) Venn diagram displaying the number of differentially expressed genes either exclusive to or common to each cohort. The Venn diagram was used in the identification of genes of interest.
Antitumor efficacy of ACY738 in murine CLL

Considering the results described, we asked whether selective HDAC6 inhibition could be harnessed for therapeutic benefit in CLL. First, orally bioavailable selective HDAC6 inhibitor ACY738 was incorporated into chow and fed to WT mice at levels equivalent to a final concentration of 25 mg/kg per day. At this concentration, ACY738 was detected in plasma (Figure 10A), and acetylation of α-tubulin was increased in the ACY738-treated group compared to a group fed chow (vehicle) alone (Figure 10B), confirming reduction of HDAC6 enzymatic activity with ACY738 oral treatment. ACY738-treated WT mice did not lose weight or show other signs of drug-related toxicities (data not shown). Percentage of normal CD19+ B cells, CD3+ T cells, CD4/CD8 ratio, and lymphocyte activation in spleen were not significantly affected after 1 month of continuous ACY738 treatment (Figure 10C).

Next, groups of aging euTCL1 mice were treated with ACY738 or were fed vehicle alone, beginning at 3 months of age until either death or euthanasia due to disease symptoms. Similar to HDAC6-deficient euTCL1 mice, ACY738-treated euTCL1 mice displayed significantly longer overall survival compared to mice fed vehicle only (Figure 11A). Figure 11B displays the gross changes in percentage of B and T cells observed in the periphery of these mice. Specifically, tumor burden and malignant-to-normal B-cell ratios were decreased in peripheral blood and
spleen samples following 3 months of treatment (Figure 11C). To extend these results, this experiment was replicated in the adoptive transfer euTCL1 model. In this model, leukemic euTCL1 splenocytes were injected into 6- to 8-week-old WT mice to establish CLL disease within 3 weeks of engraftment. Mice were then randomized to ACY738 or vehicle groups. ACY738 treatment significantly prolonged overall survival in this model as well (Figure 11D). Finally, in mice sacrificed at the end of the experimental period (12 weeks after adoptive transfer), onset of CLL was delayed, and splenomegaly was reduced in the ACY738 group compared to the vehicle-only group (Figure 11E-G). Taken together, these data suggest that pharmacological inhibition of HDAC6 with ACY738 had resulted in a therapeutic benefit in our CLL models.

Figure 10. In vivo activity of selective HDAC6 inhibitor ACY738 in wildtype mice. Peripheral blood and spleens were collected from wild-type mice after 1 month of being fed vehicle (n = 4) or treated with ACY738 orally at 25 mg/kg (n = 5). (A) Plasma was separated by centrifugation from whole blood, and the presence of ACY738 was detected by high-performance liquid chromatography. (B) Acetylation level of alpha-tubulin was quantified via flow cytometry in peripheral blood CD19+ B cells from indicated mice (n = 4 per group). (C) Characterization of immune subsets and activation status from splenocytes (n = 4 per group).
Figure 11. *In vivo* activity of selective HDAC6 inhibitor ACY738 in CLL mice. (A) Overall survival for euTCL1 aging (n = 7 per group) mice fed vehicle only or treated with ACY738. Data is representative of 3 independent experiments. (B) Characterization of immune subsets from splenocytes derived from aging euTCL1 mice treated with vehicle or ACY738 orally (n = 7 per group). (C) CLL burden was quantified in aging euTCL1 mice fed vehicle only or treated with ACY738 for a duration of 3 months (n = 6 per group). Data were compiled from 2 independent experiments. (D) Overall survival for adoptive transfer euTCL1 mice (n = 6 per group) fed vehicle only or treated with ACY738. Data is representative of 3 independent experiments. (E) CLL burden was also quantified in adoptive transfer CLL mice (n = 8 per group) fed vehicle only or treated with ACY738 for a 12-week duration, and results are representative of 5 independent experiments. (F) Compilation of adoptive transfer CLL mice spleen weights after 12 weeks of indicated treatments (n = 6 per group). (G) Representative spleens from indicated groups. Error bars correspond to standard errors of the means and *, **, *** represent p < .01, p < .001, p < .001, respectively, compared to vehicle-only controls. Abbreviations: CLL, chronic lymphocytic leukemia; conc, concentration; HDAC6, histone deacetylase 6; MFI, median fluorescence intensity; PB, peripheral blood; WT, wild-type mice.
ACY738 treatment alters proliferative capacity and sensitivity to apoptosis in euTCL1 B cells

To investigate the direct effects of pharmacological HDAC6 inhibition on CLL B cells, euTCL1 splenocytes were injected into SCID mice, which intrinsically lack B and T cells. Mice were randomized into 2 groups treated with ACY738 or fed vehicle only. Rapid expansion of adoptively transferred euTCL1 B cells was detected in peripheral blood 7 days after transfer. However, oral ACY738 treatment significantly attenuated the expansion of malignant B cells over time (Figure 12A). This led us to hypothesize that ACY738 may directly affect intrinsic survival signals of malignant B cells, creating a cytostatic effect and, therefore, a more indolent less aggressive disease. To test this hypothesis, all animals were sacrificed after 21 days of treatment and B cells were isolated from splenocytes by negative selection. First, the proliferative status of B cells was measured by Ki67 staining, and as expected, it was significantly reduced in the ACY738 treatment group compared to the vehicle-only group (Figure 12B). In addition, isolated B cells from these mice were cultured ex vivo with lipopolysaccharide for 72 hours. B cells from ACY738-treated mice were found to be more sensitive to lipopolysaccharide-induced apoptosis than B cells in untreated mice, as measured by annexin V and viability staining (Figure 12C-D).

The malignant phenotype of CLL B cells depends on constitutive activation of molecular pathways that promote proliferation, resistance to apoptosis, and survival signals. BCR signaling is one of the most crucial pathways that CLL B cells depend on for intrinsic survival signals. Within this pathway, Bruton’s tyrosine kinase (BTK) is constitutively activated in CLL and is a critical kinase for CLL development and expansion. To examine whether activation status of this pathway was impaired, phosphorylation of BTK was measured following ex vivo stimulation with antimouse immunoglobulin M in the isolated B cells. Phosphorylation of BTK was found to be decreased in the ACY738 group compared to the vehicle-only group (Figure 12E). These results
suggested that ACY738 treatment reduced BCR signaling in the malignant B cells, possibly disrupting downstream proliferation and antiapoptotic signals.

Figure 12. Effect of in vivo ACY738 treatment on euTCL1 B cells. (A) euTCL1 B cells were injected into SCID mice, which were then fed vehicle only or treated with oral ACY738 (n = 6 per group). Tumor burden was quantified in peripheral blood at the time points indicated. Data are representative of 2 independent experiments. (B) Total B cells were isolated from spleens of immunocompromised adoptive transfer CLL mice. Proliferative capacity was measured by Ki67 staining and flow cytometric analysis (n = 5 per group). (C) Isolated B cells were cultured with 1 μg/mL of lipopolysaccharide for 72 hours and the percentage of apoptotic B cells was quantified by Annexin V staining (n = 3 mice per group). (D) B cells were stimulated ex vivo with 10 μg/mL of antimouse immunoglobulin M, and phosphorylated Bruton's tyrosine kinase (Y223) was analyzed by flow cytometry (vehicle-only group, n = 6 and ACY738-treated group, n = 5). Error bars correspond to standard errors of means and *, **, *** represent p < .01, p < .001, p < .001, respectively. Abbreviations: p-BTK, phosphorylated Bruton's tyrosine kinase; FI, median fluorescence intensity; PB, peripheral blood; SCID, severe combined immunodeficient; WT, wild-type mice.
HDAC6 inhibition also alters proliferation and apoptosis in human CLL via downregulation of BCR signaling

To validate the relevance of these results in the human setting, 2 aggressive patient-derived CLL cell lines were treated with ACY738 in vitro to selectively inhibit HDAC6 (Figure 13A). Figure 12B displays a heat map showing the selectivity of ACY738 for HDAC6 compared to other HDACs. The CLL cell lines OSU-CLL and Mec2 were found to express HDAC6 protein at higher levels than normal healthy human donor B-cell controls (Figure 14A). First, these cell lines were cultured with ACY738 or dimethyl sulfoxide (DMSO) control.

Growth kinetics were significantly delayed in ACY738-treated cells (Figures 14B and 13C) with reduced cell-cycle progression and arrest in G1 phase (Figures 14C and 13D). Additionally, CLL cells experienced increased, dose-dependent apoptosis following treatment with ACY738 relative to DMSO (Figures 14D-E and 13E). To further examine the role of BCR signaling in altering CLL proliferation after HDAC6 inhibition, we measured the phosphorylation status of several BCR-signaling molecules after ACY738 treatment.

Phosphorylation of several BCR-signaling molecules was dose-dependently abrogated in CLL cell lines after ACY738 treatment compared to phosphorylation in DMSO-treated controls (Figures 14F and 13F). Considering these results, we looked at MCL1 and BCL-2, transcription factors which mediate resistance to apoptosis in CLL cells. Both MCL1 and BCL-2 levels were reduced following 24 hours of culture with ACY738, in a dose-dependent manner (Figures 14G and 13G).
Figure 13. HDAC6-selective range of ACY738 in CLL cell lines. (A) OSU-CLL and Mec2 were treated with ACY738 in increasing concentrations for 24 hours, and flow cytometry analysis was performed to detect acetylation status of alpha-tubulin and histone 3 in viable cells. Increased acetylation of alpha-tubulin compared to histone 3 indicated selectivity for HDAC6 inhibition (B) Heat map showing biochemical potency of ACY738 for HDAC1-9 compared to pan-HDAC inhibitor panobinostat and another HDAC6 selective inhibitor, ACY1215. (C) Growth kinetics of Mec2 in culture with ACY738 at 1 μM or vehicle for indicated period. (D) Cell cycle analysis in Mec2 determined by Ki67/7AAD staining after 24 hours of indicated treatments. (E) Apoptosis was measured by Annexin V/viable staining following treatment of Mec2 with ACY738 for 24 hours. (F) Phosphorylation of indicated molecules was measured following stimulation with 10 μg/mL antihuman immunoglobulin M in Mec2. (G) Expression of intracellular MCL1 and BCL-2 protein analyzed by flow cytometry, after incubating Mec2 with indicated concentrations of ACY738.
Figure 14. Effect of ACY738 treatment in human CLL cell lines. (A) HDAC6 protein expression quantified by flow cytometry in normal donor B cells (n = 5) and CLL cell lines; data were compiled from 3 independent experiments. (B) Growth kinetics of OSU-CLL in culture with ACY738 at 1 μM or vehicle for indicated period. (C) Cell cycle analysis in OSU-CLL determined by Ki67/7AAD staining after 24 hours of indicated treatments. (D–E) Apoptosis was measured by Annexin V/viable staining following treatment of OSU-CLL with ACY738 for 24 hours. (F) Phosphorylation of indicated molecules was measured following stimulation with 10 μg/mL antihuman immunoglobulin M in CLL cell lines. (G) Expression of intracellular MCL1 and BCL-2 protein analyzed by flow cytometry, after incubating OSU-CLL with indicated concentrations of ACY738. All error bars correspond to standard deviations and *, **, *** represent p < .01, p < .001, p < .001, respectively. Abbreviations: CLL, chronic lymphocytic leukemia; HDAC6, histone deacetylase 6.
Synergistic activity of ACY738 and ibrutinib in vitro

Due to the antiproliferative and proapoptotic effects of HDAC6 inhibition, we hypothesized that combining HDAC6 and BTK (ibrutinib) inhibitors would be beneficial for CLL therapy. Indeed, combining ACY738 and ibrutinib demonstrated synergistic cell kill in both OSU-CLL and Mec2 cells in vitro (Figure 15A-B). To determine whether this was relevant in primary cells, we cultured CLL patient samples with ACY738, ibrutinib, or both. Single treatments decreased viability dose-dependently, however the combination further decreased viability compared to single treatments. Decreased viability was more noticeable in the unmutated patients, suggesting that patients with a more proliferative disease may particularly benefit from this combination (Figure 15C).

![Figure 15. Combinatorial efficacy of ACY738 and ibrutinib in CLL cells in vitro](image)

(A) CellTiter-Blue assay was performed to detect cell kill synergy in CLL cell lines treated with ACY738 and ibrutinib. (B) CellTiter-Blue assay was performed to detect cell kill in CLL patient peripheral B cells with unmutated or mutated IGVH status after 48 hours of indicated treatments (n = 8 patients per group). All error bars correspond to standard deviations and *, **, *** represent p < .01, p < .001, p < .001, respectively. Abbreviations: CLL, chronic lymphocytic leukemia; IgVH, immunoglobulin variable heavy chain region.
Combinatorial efficacy of ACY738 and ibrutinib in murine CLL in vivo

To assess the in vivo relevance of this drug combination, groups of adoptively transferred euTCL1 mice were treated with ACY738 alone, ibrutinib alone, or ACY738 in combination with ibrutinib, or fed vehicle only from disease engraftment until death or euthanasia. Prior to these experiments, we confirmed that WT mice treated with ibrutinib via the drinking water had exhibited reduced phosphorylation of BTK in isolated splenic B cells after only 3 days of ibrutinib treatment (Figure 16). First, longer overall survival was confirmed in both the ACY738-only and ibrutinib-only groups, as compared to the vehicle group. However, the combined treatment further increased overall survival compared to either single-agent treatment group (Figure 17A). Median survival of the dual treatment group was 194 days compared to 112.5 days for the ACY738-only group and 138.5 days for the ibrutinib-only group. When sacrificed after 12 weeks, tumor burden and splenomegaly were observed to be lower in the dual-treatment group (Figure 17B-C) than in either single-agent group (Figure 17D-E). Mice on dual treatment did not overtly exhibit any symptoms associated with drug-related toxicities, such as weight loss or diarrhea (data not shown). These results suggested that dual inhibition of HDAC6 and BTK showed combinatorial antitumor efficacy against CLL in this murine model.

![Western blot showing activity of ibrutinib delivered via drinking water.](image)

**Figure 16. Functional activity of ibrutinib drinking water.** Western blot showing activity of ibrutinib delivered via drinking water. WT mice (n = 3 per group) were supplied with ibrutinib drinking water or regular drinking water (vehicle) for 3 days. Mice were sacrificed, and total B cells were isolated by magnetic separation from homogenized splenocytes. Phosphorylation of BTK at Y223 was measured following 30 minutes of stimulation with plate-bound antimouse IgM (10 μg/mL).
Figure 17. Combinatorial antitumor efficacy of ACY738 and ibrutinib treatment in CLL mice. (A) Overall survival data for adoptive transfer CLL mice treated as indicated until death or euthanasia (n = 8 per group), representative of 2 independent experiments. (B-C) CLL burden was quantified in peripheral blood or spleen after 12 weeks of indicated treatments; data are representative of 3 independent experiments. (D) Spleen weight at conclusion of experiment. (E) Representative spleens from indicated treatment groups are shown. Error bars correspond to standard errors of means and * represent p < .01, ** represent p < .001, *** represent p < .001, respectively.

Discussion

In this study, we found that protein expression of HDAC6 was elevated in B cells of CLL patients compared to normal B-cell controls (Figure 3). Previously, Van Damme et al demonstrated that HDAC6 messenger RNA was found to be higher in CLL patient B cells compared to normal B-cell controls. In addition, HDAC6 mRNA expression was found to correlate
with treatment-free survival but not with CD38, ZAP70, or IgVH status. In our study, HDAC6 protein expression was higher in CD38+ patients compared to CD38- patients. CD38+ CLL patients typically represent a population of intermediate- to high-risk patients. It is interesting to note that, in separate studies, both HDAC6 mRNA and protein were found to be elevated in CLL B cells. Our data also showed elevation of HDAC6 protein in CLL cell lines (Figure 14) and euTCL1 B cells (Figure 5) compared to normal controls. The discrepancies between these studies may be due to differences in methods. Protein level was measured via flow cytometry in the current study, whereas mRNA level was measured via qRT-PCR in Van Damme et al. In addition, cohort size and average CLL B-cell percentage present in the patient samples were different between the 2 studies. Altogether, our current observations and the prior literature suggested that HDAC6 expression may be a clinically relevant factor for CLL patients and warrants further investigation to determine whether HDAC6 may be able to regulate processes supporting the malignant progression of CLL B cells.

Our experiments showed that HDAC6 inhibition downregulated BCR signaling in murine and human CLL B cells, leading to defects in proliferation and sensitivity to apoptosis. This translated to delayed disease progression, lower tumor burden, and increased overall survival when HDAC6 was genetically silenced or pharmacologically inhibited in the euTCL1 model. Molecules such as BTK and AKT that are constitutively activated downstream of the BCR are particularly crucial to transduction of oncogenic signaling in CLL B cells. HDAC6 has been shown to regulate oncogenic mitogen-activated protein kinase- and phosphoinositide 3 kinase-signaling pathways in multiple cell types via deacetylation of cytoplasmic targets. In our experiments, we found no evidence of hyperacetylation of SYK, BTK, or AKT following HDAC6 inhibition in CLL cell lines (data not shown). However, RNA-seq analyses demonstrated transcriptional regulation of several genes that may be involved in CLL progression. Of note, SYK was included in this list. Given the importance of SYK to BCR signaling transduction, this result indicated to us that HDAC6 might be acting in the nucleus to regulate the expression of this
kinase, thereby affecting downstream BCR signaling. Indeed, SYK protein was downregulated in euTCL1/HDAC6KO mice compared to euTCL1 mice (Figure 9). Although the exact mechanism is not yet clear, we have further studies that are fully exploring this possibility.

Single-agent therapy with BCR inhibitors has met with clinical success; however, relapsed patients are emerging as a population with unmet needs. Characterization of mutations in BCR-signaling components, such as BTK (C481S) and/or PLC-2γ, has shown a role for these components in resistance to ibrutinib. Further characterization also found genetic alterations of TRAIL-R, EP300, MLL2, and EIF2A in ibrutinib-resistant patients. Mutations could be detected for up to 15 months before manifestation of clinical progression, and these patients were transitioned to alternative targeted agents with moderate success. A study of clonal evolution in 8 patients who were resistant to venetoclax (BCL-2 inhibitor) reported mutations in BTG1 and BRAF deletions affecting CDKN2A and amplification of CD274 (PD-L1). A recently reported study classified acquisition of Gly101Val mutation in BCL2 as a resistance mechanism to venetoclax. Other researchers have predicted that resistance to venetoclax may occur via upregulation of alternative antiapoptotic BCL-family proteins. Resistance to idelalisib mechanisms (PI3Kδ inhibitors) have been predicted to occur, for example, via upregulation of class 1A PI3K. Recently reported data showed mutations in mitogen activated pathway kinase (MAPK) genes occurring in CLL patients treated with PI3K inhibitors. In these patients, idelalisib treatment did not downregulate IgM-induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. Combination approaches with other inhibitors were recommended to be tested in these patients. Combination approaches using ibrutinib are being tested in previously treated high-risk patients and in treatment-naïve patients (e.g. NCT02301156, NCT02420912, NCT02756897). Results reported after six months of a clinical trial testing ibrutinib plus venetoclax in relapsed and refractory (R/R) CLL showed high overall response rates, eradication of minimal residual disease, and manageable adverse events. Although results are promising, long-term follow-up has not been reported, and it is not known whether some of these patients may ultimately progress.
In conclusion, this study establishes for the first time the potential therapeutic value of pharmacological HDAC6 inhibition for CLL treatment. ACY738 and ibrutinib demonstrated greater cell kill than either single treatment in CLL B cells, particularly in patients with unmutated IgVH, and significant combinatorial antitumor efficacy in our preclinical CLL models. Despite improvements in care, CLL currently remains incurable and demonstrates a variable course with many R/R patients. In anticipation of the drawbacks of the currently available therapies, it is imperative to develop alternative inhibitors and rational combination approaches to cater for the niche that does not respond or experiences progression on available regimens. One strategy being explored to combat ibrutinib-resistance is combination with immunotherapy or inhibitors whose antitumor efficacy results from a differentiated mechanism of action. Given our preclinical data, we believe selective HDAC6 inhibitors are promising candidates for this niche and are worthy of further testing either alone, in combination with BTK inhibitors, or as an alternative therapeutic agent for R/R patients.

Materials and methods

CLL patient samples

Peripheral blood mononuclear cells (PBMCs) were obtained from patients with chronic lymphocytic leukemia (CLL). All participants gave written institutional review board-approved informed consent for their blood to be used for research (#CR6_Pro00000316). Blood was collected at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL). CLL patients were diagnosed according to International Workshop on CLL 2018 guidelines. Patients indicated as “relapsed” in Table 1 had been previously treated with chemotherapy at the time of sample collection. All other patients were treatment-naïve at the time of sample collection.
In vivo studies

An HDAC6-deficient CLL murine model (referred to as euTCL1/HDAC6KO) was generated by crossing HDAC6KO and Eu-TCL1 (C57BL/6 background) mice\textsuperscript{78, 93}. For the accelerated CLL model, several aged leukemic euTCL1 mice ("aged leukemic" is defined as > 9 months of age and showing > 70% CD5+ B cells out of total viable lymphocytes by flow analysis) were sacrificed and their splenocytes were pooled. Freshly obtained splenocytes were then resuspended in phosphate-buffered saline and adoptively transferred via tail vein into 6- to 8-week old C57BL/6 wild-type (WT) mice at 25 x 10^6 per mouse. In severe combined immunodeficient (SCID) mice, leukemic splenocytes were injected at 5 x 10^6 per mouse (WT- or SCID-recipient animals were purchased from Charles River, Wilmington, MA). CLL induction was confirmed at 3 weeks after adoptive transfer by high complete blood count, and a significantly greater CD19+ B220+ CD5+ B lymphocyte population in peripheral blood than in peripheral blood from a healthy age-matched WT cohort. Groups were randomized prior to treatment. For survival analyses, mice were monitored until death or euthanasia due to disease symptoms such as lethargy, difficulty moving, lack of grooming, and enlarged spleen and/or lymph nodes. Mice were kept in pathogen-free conditions and handled in accordance with Guidelines for Animal Experiments requirements.

Cell culture

Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% nonessential amino acids, and MycoZap (Lonza, Basel, Switzerland). Cell lines were authenticated prior to use in these assays. Cells were incubated in 5% CO\textsubscript{2} at 37°C for the duration of assays.

Cell viability assay

Cell-kill assays for viability and synergy studies were conducted using CellTiter-Blue Cell Viability Assay (Promega, Madison, WI). Cells were plated at a density of 1 x 10^6/mL in a 96-well flat-bottom plate and treated with inhibitors in a final volume of 100uL for 24 hours prior to the
addition of CellTiter-Blue reagent. Synergy was determined by combination index. The combination index method is based on that previously described, in which a combination index value of 0.85 to 0.9 indicated slight synergism, 0.7 to 0.85 indicated moderate synergism, 0.3 to 0.7 indicated synergism, 0.1 and 0.3 indicated strong synergism, and < 0.1 indicated very strong synergism.

**Ex vivo murine CLL-B cell studies**

Murine B cells were isolated from homogenized splenocytes by negative selection, using magnetic separation (B-cell Isolation Kit; StemCell Technologies, Vancouver, Canada). To detect proliferation or B-cell receptor signaling activation, B cells were stimulated by plate-bound goat antimouse immunoglobulin M F(ab')2 fragment (Southern Biotech, Birmingham, AL) for 50 minutes, prior to fixing for flow cytometry assessment.

**B-cell receptor activation in CLL cell lines**

Mec2 or OSU-CLL were cultured with ACY738 for 24 hours then stimulated with plate-bound goat antihuman immunoglobulin M F(ab')2 fragment (Southern Biotech, Birmingham, AL) for 50 minutes prior to fixing for flow cytometry assessment.

**Immunoblotting**

Samples were analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), followed by transfer to nitrocellulose membrane blocked with 5% nonfat milk and incubation with indicated antibodies. Blots were developed using LI-COR system. Cell lysis was performed with radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol) supplemented with protease inhibitors (Roche, Basel, Switzerland) and phospho-protease inhibitors (Santa Cruz, Dallas, TX). Quantities of proteins were determined by bicinchoninic acid assay (Pierce, Waltham, MA). Antimouse antibodies were used against pBTK-Y223, BTK (Cell Signaling, Danvers, MA), and GAPDH (ThermoFisher Scientific, Waltham, MA).
Flow cytometry

For murine CLL immunophenotyping, 100 µL of peripheral blood was obtained from submandibular bleeds and fresh spleens were homogenized and resuspended in an equal volume of flow cytometry (FACS) buffer prior to analysis. Cells were stained with indicated antibodies according to manufacturers’ dilutions for 1 hour at room temperature. To complete phospho-flow, cells were stimulated then fixed/permeabilized in 1% BD CytoFix for 15 minutes followed by 90% ice-cold methanol for 1 hour. Cells were stained with the phospho-specific antibody or isotype control for 1 hour prior to analysis in FACS buffer in the presence of Fc block. For intracellular staining, cells were fixed/permeabilized using transcription factor fixation/permeabilization buffer set (BD Biosciences, San Jose, CA), in accordance with manufacturer’s protocol. Cell cycling was achieved by fixing cells in ice-cold 80% ethanol dropwise and incubating them at -20°C overnight followed by Ki67 staining (BD Biosciences, San Jose, CA). Acquisition was performed on an LSRII Cytometer (BD Biosciences, San Jose, CA), and data were analyzed with FlowJo software v10.1 (Tree Star Inc., Ashland, OR).

RNA Sequencing

Isolated splenic B cells from 3 young (3-month-old) and 3 aged (9-month-old) mice per group (euTCL1, euTCL1/HDAC6KO, HDAC6KO, and WT) were lysed in Trizol, and messenger-RNA extraction was performed. Normalized expression data from DESeq2 were log transformed. Samples were divided into 2 groups by age (young and old). For each group, top-expressed genes were used to create hierarchical cluster analyses.

Reverse transcription and polymerase chain reaction

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from RNA using iScript Reverse Transcriptase (BioRad, Hercules, CA). Primers against mouse SYK and mouse 18s (Qiagen, Venlo, Netherlands) were used together with iScript Reaction Mix (BioRad, Hercules, CA).
Reagents and Antibodies

ACY738 was supplied by Acetylon Pharmaceuticals, Boston, MA. All other inhibitors used in experiments were commercially obtained. All inhibitors used for in vitro assays were dissolved in dimethyl sulfoxide and stored as 20 mM stock at -20°C.

For murine studies, ACY738 was incorporated into chow at 25 mg/kg and administered to mice orally. Ibrutinib was dissolved in drinking water with 10% cyclodextrin at a final concentration of 15 mg/kg per day per mouse and administered orally, in accordance with a previously established protocol.\(^8\)

For immunophenotyping analysis of murine CLL, DAPI (4',6-diamino-2-phenylindole, dihydrochloride) exclusion was used for viability staining. Antimouse antibodies were used against CD3 BV786, CD19 Alexa Fluor 700, CD5 PerCP, immunoglobulin M PE, CD4 Pacific Blue, CD8 FITC, CD69 BUV737 (BD Biosciences, San Jose, CA), and B220 Alexa Fluor 488 (Biolegend, San Diego, CA). Absolute cell counts were determined using Accucheck counting beads (ThermoFisher Scientific, Waltham, MA), following the manufacturer's protocol.

For phospho-flow analysis, antibodies were used against pBTK-Y223, pAKT-S473, pERK-T202/Y204 (BD Biosciences, San Jose, CA), and pSyk-Y525/526 (Cell Signaling, Danvers, MA), conjugated to Alexa Fluor 647. Phospho-IKK α/β-S176/180 (Cell Signaling, Danvers, MA) was used with antirabbit secondary antibodies conjugated to Alexa Fluor 647.

For assessment of HDAC6 expression in CLL patient samples, antibodies were used against HDAC6 Alexa Fluor 488 (Novus Biologicals, Centennial, CO), acetylated alpha-tubulin PE (Cell Signaling, Danvers, MA), CD19 Alexa Fluor 647, and CD5 PerCP (BD Biosciences, San Jose, CA). Frozen aliquots of CLL peripheral blood mononuclear cells were thawed and allowed to recover at 37°C in RPMI media with 50% fetal bovine serum for 1 hour. Samples were fixed using nuclear transcription factor staining buffer set (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s protocol. Cells were stained in the presence of human Fc block (BD Biosciences, San Jose, CA).
For apoptosis assays, cells were stained with Annexin V BV605 (BD Biosciences, San Jose, CA) and Live Dead Near Infrared (ThermoFisher Scientific, Waltham, MA) in the presence of annexin V staining buffer, in accordance with the manufacturer’s protocol.

**Statistical analyses**

For normally distributed data, statistical significance of comparisons between data sets was determined by unpaired 2-tailed Student’s t test or 1-way ANOVA followed by Tukey’s multiple comparisons test. For data that did not demonstrate normal distribution, Mann-Whitney U unpaired test or 1-way ANOVA followed by Kruskal-Wallis test were used. Kaplan-Meier survival curves were compared using the log-rank test. Overall survival was defined as time from birth until death or euthanasia in the aged euTCL1 model or time from leukemic adoptive transfer until death or euthanasia in the accelerated model. For correlation studies, linear regression was performed on data sets. A p value < .05 was considered significant. All analyses were conducted using GraphPad Prism software v7.
CHAPTER THREE:
HDAC6 INHIBITION RELIEVES CLL T-CELL DYSFUNCTION AND AUGMENTS ANTITUMOR EFFICACY OF ANTI-PD-1

Introduction

Development of chronic lymphocytic leukemia (CLL) is associated with severe immune dysfunction characterized by exhausted T cells. As a result, CLL patients are severely susceptible to infectious complications that increase morbidity and mortality. T cell exhaustion, immune checkpoint upregulation and increase of suppressive T cells contribute to an immunosuppressive microenvironment. It has been postulated that reversal of T-cell dysfunction in CLL may be beneficial to reduce tumor burden. Here, we report for the first time that selective HDAC6 inhibition exerts immunomodulatory effects on CLL B cells and relieves immunosuppression of CLL T cells. In the euTCL1 murine model genetically lacking HDAC6 or treated with ACY738 (HDAC6 inhibitor), PD-L1 protein expression and IL-10 production was decreased on CLL B cells. This occurred alongside a reversal of the exhausted T-cell phenotype, demonstrated by alteration of coinhibitory molecules and activation status. Our experiments suggest these effects may be mediated through downregulation of the STAT3 pathway in the CLL B cells. Based on the beneficial immunomodulatory activity of HDAC6, we rationalized that HDAC6 could be combined with immune checkpoint blockade in CLL. Indeed, combination treatment with ACY738 augmented the antitumor efficacy of anti-PD-1 in the euTCL1 model. Taken together, these data highlight a role for HDAC inhibitors in combination with immunotherapy. Specifically, HDAC6 inhibition together with checkpoint inhibition should be investigated for use in CLL patients to reduce tumor burden while relieving T-cell dysfunction.
Background and rationale

CLL T-cell dysfunction has been rigorously documented both in patients and the euTCL1 murine model\(^\text{95, 96}\). Dysfunctional CLL T cells serve two main purposes: 1) they support tumor immune evasion and 2) provide survival and expansion signals to the malignant clone. In particular, T-cell exhaustion and upregulation of immune checkpoint inhibitors has been implicated in evasion of tumor immune surveillance\(^\text{97}\). Additionally, increased regulatory T cells (Tregs) suppress T effector function. Based on these observations, it has been postulated that reversal of T-cell dysfunction may reinvigorate T-cell driven, anti-CLL cytolytic activity. T-cell changes occurring alongside CLL progression have been reviewed in detail in Chapter 2\(^\text{12}\).

Several studies have explored the idea of reversing T-cell exhaustion in CLL. Brusa et al initially identified that the PD-1/PD-L1 axis drives T-cell exhaustion in CLL\(^\text{98}\). This study reported that PD-L\(^1\)\(^\text{hi}\) CLL cells and associated closely with PD-1\(^+\) T cells in lymphoid tissue. Gassner et al found that pretreatment of euTCL1 splenocytes with recombinant PD-1 blocking fragments reduced engraftment of disease when these cells were adoptively transferred to wildtype recipients. This study concluded that the antitumor effects were due to T-cell based tumor cell lysis\(^\text{99}\). In another study, McClanahan et al characterized CLL-specific PD-1 induction on euTCL1 T cells. Interestingly, the PD-1\(^+\) T cells exhibited variable effector function\(^\text{100}\). This group also reported antitumor efficacy of PD-L1 blocking antibody in the euTCL1 model\(^\text{19}\).

Anti-PD-1 antibodies pembrolizumab and nivolumab have demonstrated clinical activity in solid tumors and are being tested in CLL. In a Phase II trial, pembrolizumab elicited responses in 4 out of 9 CLL patients who had underwent Richter transformation (RT) but 0 out of 16 patients who had relapsed after prior therapy with ibrutinib without RT\(^\text{18}\). Increased PD-L1 on B cells and a trend of increased PD-1 on T cells were detected in responders versus non-responders before therapy. However, levels of other checkpoints were not determined. Nivolumab in combination with ibrutinib also elicited responses in CLL patients that underwent RT\(^\text{101}\). These results suggest that PD-1 blockade may be useful for RT patients. For the non-RT, relapsed CLL patients, further
optimization will be required. We speculated that in relapsed CLL patients, multiple mechanisms of immune suppression and possible upregulation of numerous immune checkpoints may occur. We therefore hypothesized that novel combination approaches with other agents that block immune checkpoints could maximize efficacy of anti-PD-1.

CLL cells in circulation contain constitutively phosphorylated signal transducer and activator of transcription 3 (STAT3) at serine 727 residues. STAT3 can also be phosphorylated at tyrosine 705 residues when CLL cells are stimulated with extracellular factors. IL-6, IL-10, and IgM stimulation have been shown to initiate transient STAT3 phosphorylation at the tyrosine 705 residue in CLL cells. Phosphorylated STAT3 undergoes nuclear localization and activates expression of anti-apoptosis genes.

Roles for histone deacetylases in cancer-related immune biology have been reported by our group and others. HDAC6 inhibition has been found to augment immunogenicity of melanoma cells through modulation of STAT3/PD-L1. HDAC6 was also found to regulate the activity of the STAT3/IL-10 pathway in antigen-presenting cells. More recently, we have described the antitumor activity of histone deacetylase 6 (HDAC6) inhibition in CLL. However, the therapeutic potential for combination of HDAC inhibition with immunotherapy remains unexplored to this date. In the current study, we investigated the immunomodulatory effects of HDAC6 in CLL and tested the rational combination of ACY738 (HDAC6 inhibitor) with anti-PD-1 antibody in vivo.

Results

Reduction of PD-L1 on CLL B cells by HDAC6i

To examine the role of HDAC6 in CLL immunomodulation, we utilized the euTCL1 adoptive transfer model. Aged euTCL1 or euTCL1/HDAC6KO splenocytes were injected into syngeneic wildtype recipients. After engraftment was confirmed, a group of euTCL1 recipients was treated orally with selective HDAC6 inhibitor, ACY738, or vehicle. Both ACY738-treated and
euTCL1/HDAC6KO recipient mice exhibited less CLL progression after six weeks compared to controls (Figure 18A).

Previous studies by other groups have demonstrated increased expression of PD-L1 on CLL B cells compared to normal controls. In our euTCL1 adoptive transfer experiment, PD-L1 was found to be upregulated on the malignant B cell subset (CD5+) compared to the normal B cell subset (CD5-, Figure 18B). PD-L1 expression also correlated with tumor burden, confirming a role for the PD-1/PD-L1 axis in driving CLL progression in this model (Figure 18C). Notably, we observed that PD-L1 expression was significantly decreased on ACY738-treated and euTCL1/HDAC6KO total and malignant B cells, and trended down in normal B cells (Figure 18B).

To determine if modulation of PD-L1 was a general antitumor aftereffect, or a direct effect of HDAC6 inhibition, we treated wildtype mice orally with ACY738 or ibrutinib as a positive control. Ibrutinib has previously been shown to downregulate PD-L1 in B cells\(^1\). All treated mice downregulated PD-L1 on B cells (Figure 18D).
Figure 18. Immunoregulation of CLL B cells by HDAC6. Peripheral blood mononuclear cells (PBMCs) were collected and stained with appropriate antibodies for 1 hour at room temperature. (A) Tumor burden (malignant B cells) was defined as CD19^{+} B220^{LO} IgM^{+} CD5^{+} cells. Total B cells were gated as CD3^{-} CD19^{+} cells. Normal B cells were gated as CD19^{+} B220^{HI} CD5^{-} cells. (B) PD-L1 expression on total, malignant and normal B cells. (C) Correlation analysis of PD-L1 and total B cells. (D) WT mice were treated with ACY738 or ibrutinib orally for 3 weeks. Expression of PD-L1 on CD19^{+} B220^{HI} B cells from periphery. n = 8 mice per group. Results are representative of 3 independent experiments. Data are expressed as average and error bars represent standard error of mean. gMFI: geometric mean fluorescence intensity; PB: peripheral blood. *p<0.05, **p<0.005, ***p<0.0005.

Reversal of CLL T-cell phenotype by HDAC6i

Expression of immune checkpoints PD-1 and LAG-3 was downregulated on both CD4+ and CD8+ subsets in the periphery (Figure 19A-D). Although HDAC6KO mice demonstrated a reduction of absolute T cells, CD4-to-CD8 ratios were not changed by HDAC6 silencing or ACY738 (Figure 19E). Ratio of memory subsets was also not changed in the periphery (Figure 20A). Interestingly, PD-1 and LAG-3 was also downregulated on CD8+ effector memory T cells
Finally, the absolute number of regulatory T cells (Tregs) was reduced along with checkpoint expression (Figure 20C-F).

Next, T cells were isolated from splenocytes and differentiation factors were assessed by q-RT-PCR. Compared to euTCL1 mice, euTCL1/HDAC6KO and ACY738-treated mice favored the Th1 phenotype. Expression of Tbet, a transcription factor involved in T helper (Th) 1 lineage differentiation, as well as IL-2, was substantially increased on euTCL1/HDAC6KO T cells, and on ACY738-treated euTCL1 T cells to a lesser extent (Figure 21A-B). Expression of Eomes (Th2 transcription factor) was decreased on euTCL1/HDAC6KO T cells and ACY738-treated euTCL1 T cells (Figure 21C).

![Figure 19. EuTCL1 T-helper and cytotoxic-T phenotype.](image)

PBMCs were stained with appropriate antibodies for 1 hour at room temperature. Cells were gated on singlets, CD3+ viable cells, CD4+, and CD8+. (A-B) Immune checkpoint expression on CD4+ T cells. (C-D) Immune checkpoint expression on CD8+ T cells. (E) Absolute counts of CD4+ and CD8+ T cells. Significant p values in bold type or *p<0.05, **p<0.005, ***p<0.0005.
**Figure 20. EuTCL1 Memory-T and Treg phenotype.** PBMCs were stained with appropriate antibodies for 1 hour at room temperature. Cells were gated on singlets, CD3+ viable cells, CD4+, and CD8+. (A) CD8+ memory subsets were defined as naïve (CD25- CD44-), terminal (CD25-, CD44+) and effector (CD25+ CD44+) in accordance with a previously published strategy for euTCL1 mice. (B-C) Immune checkpoint expression on CD8+ effector memory T cells. (D) Absolute Treg count. Tregs were defined as CD4+ CD25HI CD127- cells. (E-F) Immune checkpoint expression on Tregs. n = 8 per group. Results are representative of 3 independent experiments. Data are expressed as average and error bars represent standard error of mean. Significant p values are in bold type or *p<0.05, **p<0.005, ***p<0.0005.
Figure 21. Analysis of euTCL1 T-helper subsets. CD3+ T cells were isolated from spleens using magnetic separation (negative selection) and lysed in Trizol. RNA was extracted and qRT-PCR was performed using indicated primers. (A) Expression of Tbet (B) Expression of IL-2 (C) Expression of Eomes. n = 3 per group. Data are expressed as average and error bars represent standard deviation. *p<0.05, **p<0.005, ***p<0.0005.

HDAC6i counters CLL-induced T-cell suppression

It has been previously established that CLL B cells suppress T cell responses. At this point, we asked whether inhibition of HDAC6 in CLL B cells could reverse suppression of T-cell responses. To answer this question, a mixed lymphocyte assay was conducted. EuTCL1 B cells were isolated from spleens and pretreated with HDAC6 inhibitor ACY1215, washed and cocultured with OTII CD4+ T cells in the presence of OVA peptide. Interferon gamma (IFNγ) was measured as a readout of T-cell activation. ACY1215 pretreatment of B cells dose-dependently increased T cell-activation (Figure 21A). This data suggested that HDAC6 inhibition was able to counter the immunosuppressive effects of CLL B cells. These effects were further confirmed in human CLL cell lines co-cultured with activated normal, human T cells (Figure 21B-C).
Figure 21. T-cell activation in co-culture with CLL B cells. (A) EuTCL1 B cells were isolated from spleens by magnetic separation (negative selection) and pretreated overnight with ACY1215. Then B cells were washed and plated together with OTII CD4+ T cells in a 1:5 ratio. Stimulation was achieved by addition of OVA peptide. Concentration of IFNγ secreted into supernatant was measured by cytokine bead array after 24 hours. (B) Mec2 or OSU-CLL cells were pretreated with ACY1215 overnight, washed, and co-cultured with normal human T cells in a 1:5 ratio. Stimulation was achieved by addition of anti-CD3/28 dynabeads. Concentration of IFNγ secreted into supernatant was measured by cytokine bead array after 24 hours. Results are typical of three independent experiments.
Combinatorial efficacy of ACY738 and anti-PD-1

Given that HDAC6 silencing or inhibition relieved CLL T-cell dysfunction in vivo, we rationalized that it may be beneficial to combine HDAC6 inhibition with immune checkpoint inhibition in CLL. For this experiment, euTCL1 adoptive transfer mice were treated with either ACY738, PD-1, or both. CLL burden was sampled in the periphery of mice. Both ACY738 and PD-1 treatment singlehandedly reduced CLL progression. Compared to either single agent, combination of ACY738 and anti-PD1 treatment further decreased CLL progression (Figure 2A).

Previously, anti-PD-1 treatment was shown to demonstrate antitumor activity through increasing tumor-specific cytolysis⁹⁹. Here, we hypothesized that relief of T-cell exhaustion by ACY738 treatment may augment the antitumor activity of T cells in PD-1 treated mice. To examine this hypothesis, mice were sacrificed and T cells were assessed from spleens. The combination of PD-1 and ACY738 induced a trend of increased percentage of antigen-experienced cytotoxic T cells (CD8+ CD44+, Figure 2B) In addition, the ratio of CD107a and perforin-expressing CD8+ CD44+ T cells were increased in the combination treatment group (Figure 2C-D). These results suggest greater effector T function in the combination group leading to increased antitumor activity.
Alteration of STAT3 signaling in CLL B cells by HDAC6i

HDAC6 has been found to alter STAT3/IL-10 pathway in antigen-presenting cells. STAT3 signaling is also active in CLL B cells and has previously been determined to control PD-L1 expression after ibrutinib treatment. We therefore examined whether STAT3 signaling was affected after HDAC6 inhibition in CLL B cells. Ex vivo, we observed that ACY-treated or euTCL1/HDAC6KO B cells secreted less IL-10 compared to euTCL1 B cells (Figure 23A). Next,
activation of STAT3 pathway was assessed by detection of phosphorylated STAT3 protein at serine 727 and tyrosine 705. In euTCL1 B cells, HDAC6 inhibition downregulated pSTAT3 after stimulation (Figure 23B-C). Ruxolitinib, a JAK2 inhibitor, was utilized as a positive control. Based on these results, we predict a model where regulation of STAT3 signaling by HDAC6 can counter the immunoregulatory phenotype of CLL B cells through reduction of PD-L1 and IL-10. In turn, CLL T-cell phenotype is generally less suppressive and less exhausted due to less regulatory signals from the CLL B cells (Figure 24). The CLL T cells can then carry out effector functions to support antitumor activity.

**Figure 23. STAT3 signaling in murine B cells** (A) IL-10 secretion from wildtype, euTCL1 or euTCL1/HDAC6KO B cells was measured after ex vivo lipopolysaccharide stimulation for 24 hours. Cells were plated at 5 x 10^6 per mL. (B) Phosphorylation of STAT3 was detected by flow cytometry for intracellular proteins. Cells were isolated from splenocytes, fixed permed and stained with antibodies for 1 hour at 4 degrees. Data is expressed as average and *p<0.05, **p<0.005, ***p<0.0005.
Figure 24. Proposed model for immunomodulatory effects of HDAC6 in CLL. We predict a model where regulation of STAT3 signaling by HDAC6 can counter the immunoregulatory phenotype of CLL B cells through reduction of PD-L1 and IL-10. In turn, CLL T-cell phenotype is generally less suppressive and less exhausted due to less regulatory signals from the CLL B cells. As a result, increased activation and cytolytic activity translate to antitumor effects.

Discussion

Based on this series of studies, we report for the first time that HDAC6 exhibits immunomodulatory effects in CLL and can be combined with anti-PD1 antibody to augment therapeutic effects. These effects occurred through increased activation and cytolytic activity of CLL T cells. We propose a model where downregulation of STAT3 signaling by HDAC6 inhibition counters the immunoregulatory phenotype of the CLL B cells.

To date, HDAC inhibitors are clinically utilized for various diseases, including cancers. Besides epigenetic regulation of gene expression, HDACs may regulate targets that are non-histone proteins. In some cases, these properties relate to HDAC regulation of immune signaling and therefore, immune function. The ability of HDACs to regulate the immune system suggest
promising potential therapeutic strategies, however, therapeutic potential for combination of HDAC inhibition with immunotherapies has not been fully explored.

HDAC6 is a class IIb member with both histone and non-histone targets. Previously, it was shown that HDAC6 physically interacts with STAT3 protein in antigen presenting cells. This study showed that HDAC6 inhibition led to decrease of STAT3 phosphorylation, decreased recruitment of STAT3 to the IL-10 promoter, and abrogation of IL-10 production. Communication between antigen presenting cells and T cells is critical for T-cell responses. During this process, T cells activate, expand and are primed to exert cytolytic effects. Normal B cells can present antigens to T cells, although they are not professional antigen presenting cells. However, CLL B cells exhibit an immunosuppressive phenotype and immune synapse with T cells is defective. This phenotype serves to avoid tumor immune surveillance and ultimately, escape clearance from cytotoxic T cells.

Immune cells tend to harbor homogenous or related signaling pathways due to their common lineage. Similar to antigen presenting cells, B cells also utilize STAT3 signaling. STAT3 signaling can be initiated via several surface receptors, including the B-cell receptor, IL-6 receptor, and IL-10 receptor. CLL B cells are known for active STAT3 signaling, although the reason for this activity is not clear. It may be a combination of several reasons, including tonic BCR signaling, and increased concentrations of IL-6 and IL-10 in the CLL microenvironment. Interestingly, a recent study demonstrated that ibrutinib treatment downregulated STAT3 signaling, and consequently, PD-L1 expression on CLL B cells and PD-1 expression on CLL T cells. This effect was cited as a reason why ibrutinib exerts immunomodulatory effects. In this study, we focused on whether HDAC6 could downregulate STAT3 activation in the context of IgM (BCR) stimulation or IL-6 (IL-6R) stimulation. In both cases, activation was downregulated. We predict this may be due to the physical interaction of HDAC6 with STAT3 notwithstanding which receptor initiated signaling.
In this study, we did not explore whether HDAC6 inhibition in T cells directly inhibited STAT3 signaling. It is possible that HDAC6 could directly impact STAT3 signaling in T cells. Downregulation of multiple immune checkpoints on T cells and reduction of Tregs in vivo suggested non-specific reversal of CLL T cell dysfunction, prompting us to focus on CLL B cells. Further, our data from ex vivo mixed lymphocyte reactions implied that direct HDAC6 inhibition of CLL B cells robustly countered suppression of T-cell activation. It must be acknowledged that the mechanisms investigated here may not be the only mechanism of immune suppression that is affected by HDAC6 inhibition, as CLL B cells employ multiple immunoregulatory strategies. In the future, it will be interesting to continue to explore how HDAC6 interacts with signaling impacting other immunoregulatory strategies in CLL B cells, T cells and other supportive immune fractions.

In conclusion, this data provides rationale that HDAC6 inhibition should be tested in combination with anti-PD1 or other immune checkpoint inhibitors in CLL patients. This could provide benefit for the subset of patients who relapse or progress after prior therapy.

Materials and methods

Cell culture

Cells were cultured in RPMI supplemented with 10% fetal bovine serum, 5% penicillin-streptomycin, 5% non-essential amino acids and 1% Mycozap, incubated at 37°C with 5% CO2. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque density gradient separation medium. Cell isolations were performed using magnetic separation kits (StemCell Tech, Cambridge, MA) and viability assays were conducted using Cell Titer-Blue® Cell Viability Assay (Promega, Madison, WI) according to manufacturers’ protocols.

Reverse transcriptase quantitative polymerase chain reaction

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from RNA using iScript Reverse Transcriptase (BioRad, Hercules, CA).
Primers against human Tbet, GATA-3, FoxP3, Axin2, 18s (Qiagen, Venlo, Netherlands) were used together with iScript Reaction Mix (BioRad, Hercules, CA).

**euTCL1 mouse model**

25 x 10⁶ leukemic splenocytes from euTCL1 or euTCL1/HDAC6KO transgenic mice were adoptively transferred via tail vein into wild type (6-8 weeks old) syngeneic C57BL/6 mice to induce CLL disease. Leukemic euTCL1 mice were defined as >6 months of age and >70% B cells out of total lymphocytes in peripheral blood by flow analysis. Disease induction was confirmed by significantly elevated white blood cell count within 3 weeks of adoptive transfer.

**Flow cytometry**

Cells were stained with indicated antibodies for 1 hour at room temperature. For intracellular proteins, cells were fixed and permed in 90% methanol prior to staining for 1 hour at 4 degrees Celcius. Antibodies utilized were against mouse or human CD19, B220, IgM, CD5, PD-L1, CD3, CD4, CD8, CD25, CD127, CD44, PD-1, LAG-3, STAT3 (s727), STAT3 (y705). Antibodies were obtained from BD Biosciences, Franklin Lakes, NJ. Anti-mouse or human Th1/Th2 cytokine bead array kit was obtained from BD Biosciences, Franklin Lakes, NJ and protocol was conducted according to manufacturer’s instructions. Assays were run on LSRII (BD Biosciences, Franklin Lakes, NJ. or iQue Screener (Intellicyt, Albuquerque, NM) and analyzed with FlowJo software (TreeStar Inc., Ashland, OR).

**Inhibitors**

ACY738 and ACY1215 was supplied by Acetylon Pharmaceuticals. All other inhibitors were obtained commercially. ACY738 was administered orally in chow at 25mpk per day. Anti-PD-1 antibody was administered intraperitoneally at 3mpk, 3 times per week, for 6 weeks total. It was diluted in sterile phosphate buffered saline. For in vitro assays, inhibitors were dissolved in DMSO and stored in -20 degrees until use.
**Statistical analysis**

Statistical significance between data sets was determined by unpaired, 2-tailed, or Student’s t test if data were normally distributed, or using a Mann-Whitney U unpaired test if the data were not normally distributed. For groups of 3 or more, 1-way ANOVA followed by Tukey’s multiple comparisons test was used if the data were normally distributed, or a Kruskal-Wallis test was used if the data were not normally distributed. For correlation studies, linear regression was performed on data sets. A p value < .05 was considered significant. All analyses were conducted using GraphPad Prism software v7 or Microsoft Excel v16.
NOTE TO THE READER

Portions of the following chapter have been previously published: Maharaj, K et al. Emerging Role of BCR Inhibitors in Immunomodulation of Chronic Lymphocytic Leukemia. Blood Advances 1, 1867-1875 (2017) and have been included here with the journal's permission (see Appendix A for copyright policy).
CHAPTER FOUR:
EMERGING ROLE OF BCR INHIBITORS IN IMMUNOMODULATION OF CLL

Introduction

Approved therapies that target the B-cell receptor (BCR) signaling pathway, such as ibrutinib and idelalisib, are known to show activity in chronic lymphocytic leukemia (CLL) via their direct impacts on crucial survival pathways in malignant B cells. However, these therapies also have effects on T cells in CLL by mediating toxicity and possibly controlling disease. By focusing on the effects of BCR signaling inhibitors on the T-cell compartment, we may gain new insights into the comprehensive biological outcomes of systemic treatment to 1) further understand mechanisms of drug efficacy, 2) predict toxicity or adverse events, and 3) identify novel combinatorial therapies as well as diseases where the drugs can be repurposed. Here, we review the occurrence of T-cell abnormalities in CLL disease in preclinical models and patient samples, finding that, as well as supporting B cells directly, CLL T cells orchestrate immune dysfunction and immune-related serious adverse events. We then continue to address the effects of clinically available small molecule BCR signaling inhibitors on the immune cells, especially T cells, in the context of concomitant immune-mediated adverse events and implications for future treatment strategies. Our review suggests potentially novel mechanisms of action related to BCR inhibitors, providing a rationale to extend their use to other cancers and autoimmune disorders.

Background

Components of the BCR signaling pathway are attractive therapeutic targets in CLL and other B-cell malignancies. Selective inhibitors of BTK and PI3Kδ (such as ibrutinib and idelalisib,
respectively) have gained attention for significant clinical activity in CLL patients with relapsed or refractory (R/R) disease.\textsuperscript{105} Because of pathway homology, BCR inhibitors also inhibit T-cell signaling and activation to some degree.\textsuperscript{106} The effects of clinically available BCR inhibitors on T cells need to be considered to fully understand mechanisms of efficacy and the occurrence of adverse events. In this review, we will highlight the importance of T-cell biology in relation to CLL development and discuss its possible role in treatment efficacy and the occurrence of adverse events after current treatments.

T-cell abnormalities in CLL patients

Investigators have widely reported immune defects, including T-cell dysfunction, occurring alongside CLL development in patients. Abnormal T cells act in collaboration with the CLL microenvironment to support the growth of malignant B cells. In addition, T-cell abnormalities are evidence of mechanisms of tumor immune-surveillance escape. Effects of T-cell changes on CLL have emerged, including imbalance in T-cell subsets, exhausted phenotypes, dysregulation of co-inhibitory molecules, increase in suppressive numbers and phenotypes, abnormal cytokine secretion, and immune synapse and cytotoxicity defects.\textsuperscript{107} Below, we review studies that support the possibility of targeting the tumor microenvironment (TME) by exploiting CLL T-cell defects.\textsuperscript{108}

Imbalance of T-cell subsets

Overall changes in T-cell ratios have consistently been described in human CLL.\textsuperscript{109} One such description is inverted CD4-to-CD8 ratio being attributed to the expansion of CD8+ T cells in circulation, accompanied by Th2 preponderance and preferential expression of Th2-type chemokine receptors on T cells.\textsuperscript{110, 111} CD4+ T cells accumulate in lymphoid tissue and associate with CLL B cells to provide survival signals\textsuperscript{112} and to drive malignant progression. Interestingly, T-cell ratios may differ between niches in, for example, circulation versus lymphoid tissue.\textsuperscript{113} Evidence suggests that CD8+ expansion in CLL may be related to a CLL-specific adaptive
immune response. Next-generation sequencing of CLL T cells have documented clonal architecture and provided evidence that antigen drive could underlie expansion in a CLL-specific context.\textsuperscript{114} Another theory postulated that chronic viral infection is a likely culprit for inducing T-cell changes in CLL patients.\textsuperscript{115} However, T-cell defects have not been shown to correlate with cytomegalovirus-positive or cytomegalovirus-negative status in CLL patients. Subsequent studies did not find functional impairment in cytomegalovirus-specific CD8+ T cells.\textsuperscript{116, 117} Further studies on how cytomegalovirus-specific T cells in CLL maintain functionality may offer insight into repairing dysfunctional CLL T cells.

Another description of T-cell imbalance is the observation that expansion of CD4+FoxP3+ regulatory T cells (Tregs) is characteristic of changes in T-cell ratios in CLL. These changes have been correlated with progression and prognostic markers like IGVH mutation status and CD38 expression. Increases in CD8+CD25^{hi}FOXP3+ cells have also been correlated with progression.\textsuperscript{118} After fludarabine or thalidomide treatment, Treg decreases indicate a mutualistic relation between Tregs and malignant B cells necessary for immune homeostasis in CLL.\textsuperscript{119, 120} Interestingly, Tregs are not the only suppressive population involved, as myeloid-derived suppressor cells (MDSCs) are also implicated in CLL progression.\textsuperscript{16} Evidence indicated that low T-helper 17 (Th17) numbers, interleukin (IL)-17+ cytotoxic T-cell numbers, and decreased IL-17 expression levels are correlated with poor prognoses.\textsuperscript{121} Induction of Th17 population with lenalidomide implies a protective role for the Th17 subset against CLL progression.\textsuperscript{122} On the other hand, induction of Th17 cells and associated cytokines may increase the possibility of complications like autoimmune cytopenia. Accordingly, increased Th17 cells have been detected in patients experiencing autoimmune cytopenia, with decreased Treg-to-Th17 ratio.\textsuperscript{123} The mechanism responsible for this skewing has not been described, although it is suspected that IL-10 secretion by malignant B cells may modulate Treg/Th17 differentiation.\textsuperscript{121, 122, 124}
**Terminally differentiated and exhausted T cells**

CLL patients have been shown to have decreased naive T cells and a shift toward the CD8+ effector memory phenotype.\textsuperscript{97, 126, 126} The accumulation of terminal memory in CLL may be due to chronic antigen stimulation. Peptide-specific effector memory T cells recognizing the cytoskeletal proteins vimentin and coflin-1 have been detected in patient samples, establishing possible autoantigens recognized by CLL B cells.\textsuperscript{127} In CLL patients, exhausted T cells classically exhibit defective effector function, express inhibitory receptors, and proliferate poorly in response to chronic infection.\textsuperscript{128} CLL T cells extensively upregulate exhaustion markers PD-1, CD244, CD160, and intracellular CTLA-4, translating to defective proliferation and cytotoxicity.\textsuperscript{129, 130} Furthermore, an increase of T-effector cells in CLL patients correlated with lowered PD-1 expression and better prognoses.\textsuperscript{131} These data suggest a role for T-cell dysfunction as a therapeutic target to improve tumor-induced immune suppression.

**T-cell function**

Differentially expressed genes in CD4+ CLL T cells occur in cell growth, differentiation, proliferation, survival, cytoskeleton formation, and vesicle trafficking pathways.\textsuperscript{95} These changes predict Th2 differentiation consistent with the data mentioned above. In CD8+ CLL T cells, differentially expressed genes are involved in cytoskeleton formation, intracellular transport, vesicle trafficking, and cytotoxicity. CLL B cells have been found to induce similar changes in normal CD4+ and CD8+ T cells after \textit{ex vivo} co-culture in a contact-dependent manner, suggesting a reciprocal interaction.\textsuperscript{95}

Subsequent functional analyses confirmed predicted defects.\textsuperscript{132} When CLL T cells form impaired immune synapses with antigen-presenting cells (APCs), activation function is reduced. It has been proposed that degranulation of cytotoxic T lymphocytes (CTLs) is a regulatory mechanism of evasion utilized by malignant B cells to interfere with immune synapse formation.\textsuperscript{133} Further, abnormal cytoskeleton formation and vesicle packaging contributed toward defective
cytotoxic response. Immunomodulation with lenalidomide improved immune synapse formation between CLL T cells and B cells, validating the concept of targeting T-cell dysfunction in the CLL TME.\textsuperscript{132} Cytokine profiles have also illustrated CLL-induced T-cell dysfunction. Increased IL-10 and IL-6 secretion by CLL B cells affords protection from CTLs,\textsuperscript{134} and increased IL-4 production by circulating CD4+ T cells reiterates Th2 differentiation.\textsuperscript{113} IFN-γ and TNF-α secretion by CLL T cells also provides extrinsic survival signals as part of the TME. When IL-21 and lenalidomide treatment are combined, Bid-dependent CLL B-cell apoptosis was shown to be upregulated and BCR signaling was inhibited.\textsuperscript{135}

With improved understanding of the TME, novel targets continue to emerge. Recently, CD4+ CLL T cells were shown to internalize vesicles containing miR-363 secreted by CLL B cells and silencing of miR-363 prevented T-cell alteration.\textsuperscript{136} Genome-wide analyses comparing CD8+ CLL T cells to normal CD8+ T cells identified differentially methylated immune-regulatory genes, including PD-1 promoter, KLRG1, and CCR6, confirming epigenetic reprogramming in CLL.\textsuperscript{111} We previously demonstrated that treatment of CTLs from CLL patients with hypomethylating agent 5-aza-2-deoxycytidine caused a potentially beneficial Th1 polarization through demethylation of Th1-specific promoters.\textsuperscript{15} In a later study, the epigenetic modifiers 5-aza-2-deoxycytidine and LAQ824 effectively restored immunogenicity in CLL cell lines and primary CLL patient cells.\textsuperscript{15} The combination treatment simultaneously improved T-cell activation and APC function of CLL B cells. These works support the use of immunomodulating agents and dual targeting to improve normative function of both T and B cells in CLL.

Lessons from preclinical CLL models

Preclinical mouse models are particularly advantageous for investigating CLL immunobiology. Examining the CLL T-cell compartment has resulted in novel mechanisms for CLL progression and interest in immunotherapeutic strategies.\textsuperscript{96, 100, 137} To study the dependence of CLL B cells on immune subsets, carboxyfluorescein succinimidyl ester-labeled human CLL
cells were injected into NSG mice alongside various immune components, such as CD34+ progenitor cells, mesenchymal stromal cells, or mature APCs. Data demonstrated that T cells activated by allogeneic APCs were required for leukemic cell survival and proliferation. Administration of anti-CD3 or anti-CD4 antibodies consistently reduced leukemic growth. Another study proved that blockade of B-cell maturation is imposed by TME signals in CLL. These findings relate to the efficacy of CLL therapies that function to 1) eliminate autologous T-cell support for leukemic cells or 2) improve T-cell immune surveillance capacity to increase response to CLL antigens.

Immunological studies in euTCL1 transgenic mice (which spontaneously develop CLL-like CD5+ B-cell leukemia) confirmed that leukemic B cells impaired T-cell function, which could be reversed by lenalidomide treatment. Another study confirmed that a shift from naive to memory T-cell phenotype in lymphoid tissue correlated with inverted CD4-to-CD8 ratio. In an accelerated euTCL1 model, T-cell subset alterations induced by disease progression were found to be antigen-driven and clonally skewed. McClanahan et al investigated CLL T-cell function in aging and accelerated euTCL1 models in various niches. CD8+ T-cell proliferation was increased in the spleen, in contrast to previous data reported from peripheral blood of late-stage CLL patients. EuTCL1 B and T cells upregulated expression of PD-L1/PD-L2 and PD-1, respectively, to control T-cell dysfunction. Other inhibitory receptors, KLRG-1, 2B4, and LAG-3, also showed CLL-induced upregulation. Interestingly, PD-1+ euTCL1 T cells exhibited heterogeneous functionality. Investigations of T-cell function in the spleen indicated that immune synapse formation was increased at 6 months but impaired at 12 months, indicating overcompensation during early-stage disease. Considering these data, the authors speculated that T-cell exhaustion is not irreversible in CLL.

Gassner et al also reported that euTCL1-derived T cells exhibited exhaustion. CD4+ T cells upregulated PD-1 and LAG-3 expression in periphery and lymphoid tissues, and CD8+ T cells upregulated LAG-3 expression. PD-L1 expression was modestly increased in CLL B cells.
from periphery tissue but was significantly increased in CLL cells from lymph nodes, implying a TME effect. To investigate whether blocking the PD-1/PD-L1 axis in CLL has therapeutic potential, euTCL1 mice were engrafted with a mixture of fluorescently labeled syngeneic leukemic cells, some of which had been treated ex vivo with PD-L1 blocking antibody. These experiments implied that PD-1/PD-L1 blockade could reactivate the CTL response within the euTCL1 transgenic recipients to target malignant cells. Overall survival was not reported.99

Together, these exciting reports reiterate previously established trends in human CLL and provide evidence that CLL T cells can be targeted with the intention of improving prognoses.98

Effect of BCR signaling inhibitors on T cells and other immune subsets and future therapeutic implications

Although frontline therapy with chemo-immunotherapy is still appropriate for a subgroup of younger CLL patients, it is often not well-tolerated in the older population.140, 141 Alternative drugs ibrutinib and idelalisib have been developed to meet the needs of these patients. To date, ibrutinib is approved as both a frontline and R/R CLL patient therapy, while idelalisib as well as duvelisib is approved for R/R patients.142

Although consequences of the BCR signaling blockade are well-characterized in the targeted malignant B cell, BCR inhibitors, including ibrutinib, idelalisib, and others, can interact with multiple immune cell types, especially T cells. Dysfunctional T cells are related to the occurrence of autoimmune hemolytic anemia complications in CLL patients. Recently, the control of autoimmune hemolytic anemia with ibrutinib has been described in case reports, but available data do not describe its incidence or outcomes in patients receiving idelalisib.143 Although how these drugs interact with immune deficiency occurring in CLL is currently unknown, the complex interaction of immune cells in this environment, as illustrated in Figure 25, makes the effects of BCR inhibitors in immune subsets especially interesting.
**PI3K inhibitors and toxicity**

PI3Kδ shows higher intrinsic activity in leukemic B cells compared to normal B cells and can be therapeutically targeted in CLL. Idelalisib (targeting PI3Kδ) and duvelisib (also known as IPI-145, targeting PI3Ky/δ) have both demonstrated clinical responses attributed to their direct effects on malignant B cells’ dependence on intrinsic and extrinsic survival signals. Nevertheless, their reported toxicities are a cause for concern. While data from early idelalisib CLL trials in R/R patients reported only a 3% occurrence of serious adverse event (SAE) diarrhea, a subsequent trial in previously untreated patients reported a 42.2% occurrence of diarrhea or colitis SAEs, followed by high discontinuation rates. Other SAEs reported included hepatotoxicity and pneumonitis. Grade 3/4 diarrhea and hepatotoxicity were reported in clinical trials with duvelisib and the pan-PI3K inhibitor pilaralisib. These data indicate that this toxicity pattern may be a class effect of PI3K inhibitors. In contrast, TGR-1202, a structurally different next-generation PI3Kδ inhibitor with CK1ε activity, has been associated with substantially fewer adverse events. However, despite TGR-1202 showing promising clinical efficacy in various B-cell malignancies, incidences of hepatotoxicity and colitis in comparison with previous PI3Kδ inhibitors remain lower, even with long-term follow up.

T cells represent a secondary target cell population for PI3K inhibitors due to their dependence on PI3Kδ and PI3Ky isoforms for signaling. Early in vitro studies reported no cytotoxic properties of idelalisib against normal CD3+ T cells but described a reduction of inflammatory and anti-apoptotic cytokines. Recently, preliminary studies have reinvestigated PI3K inhibitors in specific T-cell subsets to determine potential culprits with a role in autoimmune toxicity. Matos et al reported that patients who received idelalisib showed decreased Treg number and function. Tregs in patients experiencing autoimmune toxicities showed decreased expression of the functional markers GITR, T-bet, CXCR3, granzyme-B, and TIM-3. Tregs also downregulated pro-survival BCL-2 and increased pro-apoptotic CD95. Our group subsequently described the differential effect of TGR-1202 in normal human T cells and murine CLL T cells.
compared with idelalisib and duvelisib.\textsuperscript{156, 157} Treg numbers and function were depleted by all 3 inhibitors but were maintained closer to normal levels after TGR-1202 treatment. These data are of relevance to determining why this inhibitor causes fewer incidences of SAEs in patients. Further, Deng et al recently reported a previously unknown off-target effect of TGR-1202. TGR-1202 silenced c-myc translation in leukemia and lymphoma cells through inhibition of casein kinase 1 epsilon (CK1\(\epsilon\)).\textsuperscript{68} The impact of this inhibition on T cells is currently unknown.

**PI3K inhibition in immune subsets**

Class I PI3-kinases are composed of a regulatory (p85 or p55) and a catalytic (class IA p110 alpha, beta, delta, or class IB gamma) subunit. Upon recruitment of subunits to the membrane at phosphorylated YXXM motifs, PI3K signaling is initiated, which acts downstream to control varied cellular functions, such as growth, proliferation, and apoptosis. While alpha and beta subunits are ubiquitously expressed, delta and gamma are mainly expressed by leukocytes.\textsuperscript{158} Currently, relative expression of the 4 class I PI3K catalytic subunit isoforms in immune cell types has not been comprehensively characterized.

Data previously reported from genetically silenced mouse models might be useful in predicting the modulation of immune subsets by PI3K inhibitors. The PI3K\(\delta\)-inactive mouse model showed normal thymic development and T-subset ratios, but T cells exhibited reduced CD44 expression, indicating a role for the \(\delta\) subunit in the differentiation of effector and/or memory T cells. PI3K\(\delta\)-inactive T cells exhibited diminished proliferation and IL-2 production post-stimulation. PI3K\(\delta\)-inactive mice developed mild inflammatory bowel disease characterized by intestinal leukocyte infiltration, which was thought to result from dysfunctional Tregs.\textsuperscript{159} Impairment of the T-cell-mediated immune response has been partly explained by the recent discovery that PI3K\(\delta\) promotes CD4+ T-cell interactions with APCs by increasing LFA-1 binding to ICAM-1.\textsuperscript{160} In addition, PI3K\(\delta\)-inactive Tregs produced less IL-10 and expressed lower levels of CD38+, correlating to defective suppressive function.\textsuperscript{161} PI3K\(\delta\)-inactive Tregs were incapable
of protecting against a model of induced colitis. RAG-knockout (KO)/PI3Kδ inactive mice receiving CD4+ T cells developed severe colitis, showing increased percentages of IFN-γ- and IL-17A-producing lamina propria CD4+ T cells compared with RAG-KO mice. Despite overall reduced inflammatory T-cell response, PI3Kδ-inactive mice exhibited improved resistance to bacterial infections, possibly due to reduced Treg expansion and tissue homing.

Investigations with PI3Kγ-inactive or PI3Kγ-KO mice have also highlighted roles for this catalytic isoform in T-cell development, trafficking, activation, and Th1 and Th17 responses. Unlike PI3Kδ, PI3Kγ has been linked to chemokine-receptor signaling through G-protein-coupled receptors but not T-cell receptors (TCRs). The tumor-reactive CD8+ effector T-cell population is of interest because of its involvement in anti-tumor immunity. Murine PI3Kγ-KO CD8+ effector T cells displayed impaired migration following viral challenge, suggesting that PI3Kγ could regulate tumor-reactive CD8+ effector T cells. PI3Kγ is also involved in the regulation of dendritic cell (DC), neutrophil, and monocyte migration. Further, studies of PI3Kγ/δ double KO mice showed dramatic reductions in T cells in peripheral blood, lymph nodes, and spleen alongside symptoms of lymphopenia. Functionally, Tregs of γ-KO/δ-inactive mice were deficient in suppressive assays and expressed low levels of GITR and FoxP3. It was noted that lymphopenia is associated with autoimmunity; however, a secondary factor is necessary to induce autoimmune disease, such as dysfunctional Tregs or local tissue inflammation.

Although there is now evidence that peripheral Treg function is compromised after PI3Kδ or γ/δ genetic inactivation, experiments utilizing PI3K inhibitors to interrogate Treg function in vitro have reported variable results, likely due to the context of dosing and stimulation. Tregs derived from normal human peripheral blood mononuclear cells were selectively spared by PI3Kα, PI3Kδ, or MEK inhibitors when compared with CD4+ CD25- (Tcon) and CD8+ (Teff) subsets. Tregs retained closer to normal levels of proliferation, activation, and suppressive capacity following anti-CD3/CD28 stimulation in the presence of these inhibitors. Most interestingly, PI3Kδ and PI3Kα protein expression levels were lower in Tregs than in Tcons. This is consistent with
observations that PI3K signaling inhibition can differentially affect Tregs versus Tcon or Teff and suggests that protein expression of the PI3K catalytic isoforms should be quantified and compared among immune subsets. Thus far, the clinical application of PI3K inhibitors resembles the PI3Kδ-inactive mouse model, since peripheral Treg numbers and functions are compromised in patients following PI3K inhibitor treatments.

Abu-Eid et al showed that PI3K-Akt pathway inhibition decreased Treg infiltration of the TC-1 tumor and enhanced the anti-tumor effects of tumor-specific vaccinations. In another study, Ali et al found that both genetic and pharmacological inactivation of PI3Kδ in Tregs conferred resistance to solid tumor growth (4T1, EL4, and LLC) in mice. Although PI3Kδ blockade weakened the cytotoxic T-cell response, Treg-mediated immune suppression was overridden and an anti-tumor effect was achieved. PI3Kδ may be more essential for Treg response than effector T-cell response, and PI3Kδ blockade may reinvigorate adaptive anti-tumor responses, implying additional mechanisms for PI3K inhibitor efficacy against hematological and solid malignancies.

Alongside Tregs, other immune subsets have been implicated in PI3K-mediated anti-tumor immunity. It was found that PI3K inhibition could relieve immunosuppression to augment the use of Toll-like receptor (TLR) agonist for improving anti-tumor immunity in combination with DC vaccines. In a comprehensive analysis utilizing inhibitors of all class I PI3K catalytic isoforms, class I PI3K inhibition in DCs suppressed IL-10 and TGF-β secretion. However, PI3K inhibition did not hinder pro-inflammatory induction of IL-12 and IL-1B after TLR5 ligand (flagellin) treatment. The combination of pathogen-derived flagellin with pan-PI3K inhibition suppressed tumor growth in subcutaneous B16, CT26, and LLC solid tumor models, with and without DC vaccines. Here, it was demonstrated that both DC and tumor-infiltrating-lymphocyte populations were functionally modulated by PI3Ki treatment and that both were involved in the anti-tumor response.
**Ibrutinib in T cells and other immune cells**

Although ibrutinib is known for its clinical success as an irreversible BTK inhibitor, it is also the first clinically available inhibitor of inducible T-cell kinase (ITK). ITK is expressed in T cells and belongs to the Tec kinase family. It is a major player in TCR signaling, activating PLC-γ and downstream NFAT, nuclear factor-κB, MAPK pathway, calcium mobilization, cytoskeleton reorganization, and synapse formation and adhesion. Gomez-Rodriguez et al demonstrated the importance of ITK in regulating Th17/Treg lineage differentiation. Considering the immunomodulatory effects of ITK inhibition, it is possible for ibrutinib to be repurposed for use in other contexts.

Dubovsky et al explored the effects of ibrutinib in T cells using healthy human T cells, CLL patient T cells, Jurkat T cells, and mouse models. This study established that ibrutinib treatment irreversibly inhibited ITK to decrease downstream activation in Th2 cells but not Th1 or CD8+ T cells, due to compensation by redundant resting lymphocyte kinase. Ibrutinib-treated CLL T cells exhibited Th1 skewing via T-bet upregulation and JunB downregulation. Systemic treatment confirmed these effects in euTCL1 mice. These data imply that ibrutinib may function through a 2-pronged approach to target malignant B cells directly, while reinvigorating a beneficial inflammatory T-cell response in CLL. Using mouse models of leishmaniasis, leukemia, and listeriosis, this study highlighted the therapeutic potential of ibrutinib in other diseases involving disproportionate polarization of Th2 immunity.

Other studies have explored the effects of ibrutinib on the CLL immune microenvironment. Niemann et al reported decreased T-cell activation, proliferation, and PD-1 expression in CLL patients following ibrutinib therapy. Consistent with prior findings in ITK knockout mice, the authors demonstrated reduced proliferation of circulating Th17 cells in these patients and inhibition of Th17 differentiation in ex vivo assays. Additionally, ibrutinib treatment disrupted CLL-macrophage interactions in bone marrow specimens. Yin et al described an increase in CLL patient TCR diversity following 1 year of ibrutinib therapy. Using next-generation sequencing,
this paper demonstrated that pre-therapy TCRβ clones decreased while the number of productive, unique clones increased during treatment. TCR diversity was positively correlated with clinical efficacy and lower infection rates. In CLL mouse models, Chen et al determined that ibrutinib treatment deregulated B cell surface membrane CXCR4 expression and signaling, disrupted the homing of B-CLL cells to lymphoid tissue, and ultimately contributed to improved survival via this novel mechanism.\textsuperscript{179} The effect of ibrutinib on CLL T cell migration and homing, however, is yet to be studied.

In a pioneering work, Barfi et al harnessed the immunomodulatory capacity of ibrutinib in combination with an immune checkpoint blockade (anti-PD-L1 antibody) to treat hematological malignancies without intrinsic sensitivity to ibrutinib and solid tumors without BTK expression.\textsuperscript{180} In ibrutinib-insensitive PD-L1+ A20 lymphoma established in mice, combining ibrutinib with anti-PD-L1 synergistically delayed tumor growth and prolonged survival. Tumor-specific T cells were detected in mice treated with the combination. This result was recapitulated in ibrutinib-insensitive J558 myeloma and 4T1 (breast cancer) with appreciably fewer metastatic lesions. Similar results were seen with CT26 (colon cancer) tumor growth, with detection of T cells specific to the CT26 tumor antigen. The cured mice displayed long-term memory to CT26 antigens. Ibrutinib may therefore enhance response to T-cell therapies.

In a simultaneous study, Barfi et al explored the concept of repurposing ibrutinib by combining intratumoral vaccine with ibrutinib treatment in a mouse model of subcutaneous lymphoma.\textsuperscript{181} Intratumoral administration of CpG activated local natural killer cells, macrophages, and DCs through TLR9 agonist activity, resulting in local tumor regression but not systemic anti-tumor response. Systemic administration of ibrutinib alone caused mild tumor growth delay at all sites. In contrast, combining CpG and ibrutinib resulted in complete and permanent tumor regression at all sites. This effect was found to be CD4+ and CD8+ T-cell dependent. Infused T cells that had been pre-treated with the combination prevented the outgrowth of tumors in new recipient mice.
The full extent of the immunomodulatory properties of ibrutinib remains unclear. Although natural killer T cells and mast cells also express ITK, their responses to ibrutinib treatment are not yet known. Natarajan et al identified that ibrutinib treatment decreased expression of MHCII and CD86 but increased expression of CD80 on DCs. Ibrutinib-treated DCs also promoted T-cell proliferation and enhanced IL-17 cytokine production in co-culture via TLR4-modulated activation.\textsuperscript{182} Additionally, in a report from Stiff et al on the effects of ibrutinib treatment on MDSCs, which express BTK, ibrutinib inhibited BTK phosphorylation.\textsuperscript{183} Together with reduced cell migration and inhibition of \textit{in vitro} MDSC generation, ibrutinib treatment also reduced MDSCs in spleen and EMT6 murine mammary tumors. Further, ibrutinib treatment reduced MDSCs in a melanoma model in a BTK-dependent fashion. These data potentiate alternative modes of action for ibrutinib in previously unexplored immune cell types.

Acalabrutinib is a more selective BTK inhibitor than ibrutinib, demonstrating on-par antitumor efficacy in CLL, both in preclinical models and patient trials.\textsuperscript{25, 184} Preliminary comparison of patients treated with ibrutinib or acalabrutinib suggests that the immunomodulatory capacity of acalabrutinib is differentiated from that of ibrutinib, likely due to less off-target effects on Tec-family kinases, including ITK. Ibrutinib but not acalabrutinib increased absolute numbers of CD4+ and CD8+ T cells enriched for effector memory subsets over naïve and central memory subsets. Acalabrutinib did not change Treg-to-CD4 ratio or activation-induced cell death. Both treatment groups did however, exhibit reduced PD-1 and CTLA-4 expression in CD4+ and CD8+ T cells.
Figure 25: The CLL microenvironment. Immune cells in the CLL microenvironment show typical phenotypic changes that support survival of the malignant B cell including alterations to T-cell subsets and MDSCs (numbered 1-15). BCR inhibitors target intrinsic survival signals of CLL B cells but can also affect signaling in other immune cell types.

Conclusions

CLL T-cell dysregulation trends have been well described, showing that altered subset ratios and gene expression and function are necessary for and supportive of malignant progression. In addition to supporting malignant B cells directly, CLL T cells orchestrate immune dysfunction and immune-related SAEs. Immunomodulatory agents, monoclonal antibodies, and epigenetic modifiers targeting CLL T-cell dysfunction have yielded promising preclinical results. Clinically administered BCR inhibitors also display immunomodulatory properties, affecting a wide
range of immune cell categories. Emerging data describing the immunomodulatory capacity of BCR inhibitors predicts feasible combination strategies for ibrutinib with other immunomodulatory agents in CLL, such as lenalidomide or histone deacetylase inhibitors. In addition, the modulation of T cells by ibrutinib or PI3K inhibitors could augment anti-tumor responses from checkpoint blockade in CLL, such as with anti-PD-L1 antibody. Our review of the impact of BCR inhibitors on T cells and other immune compartments suggests potentially novel mechanisms of action, providing a rationale to extend their use to other cancers and autoimmune disorders.
CHAPTER FIVE:
THE DUAL PI3KD/CK1ε INHIBITOR UMBRALISIB HAS DIFFERENTIAL AND
BENEFICIAL IMMUNOMODULATORY EFFECTS ON CLL T CELLS

Introduction

The in-clinic PI3K inhibitors idelalisib (CAL-101) and duvelisib (IPI-145) have demonstrated high rates of response and progression-free survival in clinical trials of B cell malignancies such as chronic lymphocytic leukemia (CLL). However, a high incidence of adverse events has led to frequent discontinuations, limiting the clinical development of these inhibitors. By contrast, the dual PI3Kδ/casein kinase-1-epsilon (CK1ε) inhibitor umbralisib (TGR-1202) also shows high rates of response in clinical trials but has an improved safety profile with fewer severe adverse events. The toxicities typical of this class of PI3K inhibitors are largely thought to be immune-mediated, but these are poorly characterized. Here we report the effects of idelalisib, duvelisib and umbralisib on regulatory T cells (Tregs) on normal human T cells, T cells from CLL patients and T cells in an EuTCL1 adoptive transfer mouse CLL model. Ex vivo studies revealed differential effects of these PI3K inhibitors, where only umbralisib treatment sustained normal and CLL associated FoxP3+ human Tregs. Further, while all three inhibitors exhibit anti-tumor efficacy in the EuTCL1 CLL model, idelalisib or duvelisib-treated mice displayed immune-mediated toxicities and impaired function and reduced numbers of Tregs, whereas Treg number and function was preserved in umbralisib-treated CLL-bearing mice. Finally, studies using an inhibitor that selectively impairs CK1ε suggests that the improved safety profiles of umbralisib are due to its roles as a dual PI3Kδ/CK1ε inhibitor that preserves Treg numbers and functions.
Background and rationale

In mammalian cells PI3K signaling occurs upon the binding of a regulatory subunit (p85 or p55) with a catalytic subunit (p110α, β, δ, or γ) and this leads to activation of AKT and other downstream effectors that control cell growth, survival and metabolism. In cells of hematopoietic origin, the expression of the p110 PI3Kδ catalytic subunit predominates and plays essential roles in B-cell development, survival and function. For example, in mature B cells, PI3K signaling is initiated downstream of B-cell receptor (BCR) signaling, where it controls activation and differentiation responses, and mice lacking a catalytically active p110δ are viable but have impaired B cell responses. Notably, p110δ-deficient mice also display impaired T-cell functions, and develop an inflammatory bowel syndrome that has been attributed to defective regulatory T cells (Tregs) and that is manifest as marked lymphocyte infiltrates into intestinal tissues. Given of these observations, the role of PI3K signaling in T cell differentiation, function and immune tolerance has also been studied. The p110γ catalytic subunit is also known to play important roles in PI3K signaling downstream of G-protein–coupled receptors in T cells and myeloid cells. PI3K inhibitors targeting p110γ isoform have been investigated in the context of cancers and autoimmunity. However, it is unclear how p110δ and p110γ subunits interact to enable PI3K signaling in T cells, what specific functions they each control, and if they can act in a compensatory manner in cells lacking or inhibited in one isoform.

Dependence on PI3K signaling is conserved in malignant B cells, including chronic lymphocytic leukemia (CLL) B cells, and this has led to the recent clinical development and FDA approval of PI3K inhibitors for the treatment of B-cell malignancies. However, there is a lack of robust data characterizing the effects of these inhibitors in secondary target cell populations. Due to the importance of PI3K signaling in multiple immune cell types, PI3K inhibitors targeting one or more catalytic isoforms can have unexpected immunomodulatory effects, which may vary depending on each inhibitor’s selectivity, potency and off-target profile.
The immunomodulatory properties of PI3K inhibitors are significant in the context of hematological malignancies and other cancers. For example, while the anti-tumor effects of the PI3K inhibitors idelalisib and duvelisib are clear, the high incidence of immune-mediated severe adverse events (AEs) in clinical trials among patients with B-cell malignancies, including colitis, pneumonitis and hepatotoxicity, has caused high rates of discontinuation that have limited the development of a potentially long-term beneficial treatment. In contrast, treatment with umbralisib, a next-generation dual inhibitor of PI3Kδ and casein kinase 1-epsilon (CK1ε), has shown an improved safety profile characterized by less-severe AEs, even with long-term follow-up. IC$_{50}$ values of idelalisib, duvelisib, and umbralisib against p110 isoforms in a cell-free enzymatic assay have been previously published.

It has been postulated that T cells may modulate PI3K-inhibitor-related AEs. Increased inflammatory cytokine levels and decreased Treg numbers have been associated with the development of toxicities in patients on idelalisib, and younger treatment-naïve patients, who likely have more robust immune systems, were at higher risk of developing toxicities. There is a clear need to understand the effects of pharmacological PI3K inhibition in T cells and other immune populations.

In the context of hematological malignancies, T cells may develop a phenotype supporting malignant progression, differing substantially from that of healthy T cells. For example, CLL patients often harbor low numbers of T cells that display markers of exhaustion, are less proliferative, and have inverted Th1/Th2 and CD4/CD8 ratios, and can also have increased Treg numbers. Here we characterized the immunomodulatory effects of clinically available PI3Kδ inhibitors, and report marked and differential effects of these drugs on Tregs in the setting of CLL. Importantly, these analyses revealed superior toxicity profiles of the dual PI3K/CK1ε inhibitor umbralisib versus idelalisib and duvelisib, and that these are associated with improved function and sustained numbers of Tregs, supporting the clinical utility of this agent in the treatment of CLL.
Results

Suppressive T-cell phenotypes are differentially modulated by PI3K inhibitors

To initially assess potential immunomodulatory effects of PI3K inhibitors, normal human T cells were isolated from healthy donor PBMCs. Isolated total T cells were cultured with PI3K inhibitors idelalisib, duvelisib, umbralisib or vehicle (DMSO) for 24 hours at various doses, and viability was measured by CellTiter-Blue assay. Treatment with these inhibitors did not compromise the survival of normal T cells at doses of 0.1 to 10 μM (Figure 26A); hence, this dose range was selected for all in vitro experiments. To confirm if these agents were on target, the phosphorylation of AKT (pAKT) was used as a readout of PI3K pathway inhibition in normal human T cells. As predicted, reductions in the levels of pAKT were observed and were found to be similar among all inhibitors (Figure 26B).

To assess potential effects on T-cell functions, cytokine secretion was measured from the supernatant of total T cells incubated with the three PI3K inhibitors (Figure 26C-D). Overall, Th1 and Th2 cytokines were decreased following treatment with PI3K inhibitors, yet levels of IL-10 production were higher following umbralisib treatment compared to idelalisib or duvelisib treatment (Figure 26D). Finally, the low levels of IL-4 and IL-6 that were produced by normal T cells were comparably reduced by treatment with the three PI3K inhibitors.

The potential effects of these PI3K inhibitors on T-cell fate was assessed by examining the expression of T-helper (Th) lineage transcription factors by quantitative RT-PCR. The expression of Tbet (Th1) was markedly and comparably suppressed by all three PI3K inhibitors, whereas the expression of the Th2 determination factor GATA-3 was unaffected (Figure 26E and data not shown). Idelalisib or duvelisib treatment also led to a profound (>25-fold) suppression of FoxP3 (Treg) expression, whereas FoxP3 levels were more sustained in umbralisib-treated T cells (Figure 26E). Thus, PI3K inhibitors impair T-helper cell differentiation, and idelalisib or duvelisib treatment has more suppressive effects on FoxP3+ Tregs than treatment with umbralisib.
Given these findings, the effects of these PI3K inhibitors on anti-CD3/anti-CD28 activation of total T cells from normal or CLL donors were assessed. Although PI3K treatment with did not affect total T cell count or the CD4 to CD8 ratio (Figure 27A), total Treg numbers were reduced in a dose-dependent fashion following treatment with idelalisib or duvelisib (gating strategy shown in Figure 28). In contrast, Treg numbers were more sustained in umbralisib-treated T cells (Figure 27A and Figure 29A). Interestingly, the cell surface expression of the immunosuppressive checkpoint molecules PD-1 and CTLA-4 on Tregs was sustained on umbralisib-treated samples but was suppressed by idelalisib or duvelisib treatment (Figure 27B and Figure 29B). These findings were confirmed in a cohort of 6 normal donor samples and 5 CLL patient samples, where percentage of Tregs, and surface levels of PD-1 and CTLA-4 on Tregs, was relatively preserved in umbralisib-treated normal or CLL patient T cells (27C-F).

To assess the potential effects of PI3K inhibitors on Treg function, we performed a Treg suppression assay, where normal donor Tregs were pre-treated with inhibitors for 24 hours, washed, and then co-cultured with autologous responder CD4+ T cells. Treatment with duvelisib and idelalisib impaired the suppressive capacity of Tregs, whereas umbralisib-treated Tregs retained their suppressive capacity (Figure 30A, B).
Figure 26. PI3K inhibitors impair normal human T-cell survival and function. (A) CellTiter-Blue assay of T-cell viability after 24 hr treatment with the indicated concentrations of PI3K inhibitors. (B) Inhibition of PI3K signaling in CD3+ T cells was measured by assessing levels of pS473-AKT after 24 hr of incubation with inhibitors followed by stimulation with anti-CD3/anti-CD28. (C-D) Cytokine bead array analysis of Th1/Th2 cytokines secreted from CD3+ T cells incubated with the indicated inhibitors (1µM, for 24 hr) following anti-CD3/anti-CD28 stimulation (24 hr). (E) The expression of Th transcription factors was assessed by qRT-PCR in CD3+ T cells incubated with the indicated inhibitors (1µM, 24 hr) followed by anti-CD3/anti-CD28 stimulation (24 hr). Graph displays ΔΔCT for each transcription factor. All data are representative of 3 independent experiments using a unique donor for each experiment. Graphs display mean values and error bars represent SD of technical triplicates from a representative donor. *p<0.05, **p<0.005, ***p<0.0005.
Figure 27. Normal and CLL Tregs are differentially impaired by PI3K inhibitors. (A) CD3+ T cells from normal donor peripheral blood were cultured with the indicated concentrations of PI3K inhibitors in dose titration in the presence of anti-CD3/anti-CD28 for 72 hr. (B) PD-1 and CTLA-4 expression in Tregs (C) T cells (n=6) were isolated from peripheral blood and cultured with the indicated inhibitors (1µM, 72 hr) followed by immunophenotyping. (D) CD3+ T cells were isolated from previously frozen CLL patient PBMCs (n=5) and cultured with PI3K inhibitors and anti-CD3/anti-CD28 for 72 hr followed by immunophenotyping. (E) PD-1 and CTLA-4 expression (MFI) on normal donor Tregs (n=6) treated with the indicated inhibitors (1µM, 24 hr). (F) PD-1 and CTLA-4 expression (MFI) on Tregs from CLL patients (n=5) treated with the indicated inhibitors (1µM, 24 hr). PD-1 and CTLA-4 MFI were normalized to FMO controls. Graphs display mean values and error bars represent standard deviation. *p<0.05, **p<0.005, ***p<0.0005.
Figure 28. Gating strategy to identify Tregs in human or mouse samples. PBMCs were stained with antibodies indicated for 1 hour at room temperature in the dark. Cells were fixed and permeabilized, then intracellular proteins were stained with antibodies indicated for 1 hour at 4 degrees. Cells were gated on single, viable lymphocytes. Accuchek beads were used to determine absolute counts.
Figure 29. Effect of PI3K inhibitors on Tregs (A) Flow cytometry to detect Treg population in normal human T cells treated ex vivo with PI3K inhibitors at various concentrations. Treg is identified as CD3+ CD4+ CD25^{HI} CD127^{LO} FoxP3+. (B) PD-1 and CTLA-4 expression on Treg population. Median fluorescence intensity is displayed corresponding to each histogram.
Figure 30. Treg suppressive capacity is only modestly impaired by umbralisib treatment. (A) Naïve CD4⁺ T cells were isolated from normal donor peripheral blood and cultured in Treg-polarizing conditions for 5 days. Tregs were washed and pre-treated as indicated for 24 hours, and were then co-cultured with autologous, cell trace violet-labeled, responder T cells. Cell trace violet-labeled cells were assessed by flow cytometry 4 days after beginning co-culture. (B) Histograms depicting division of responder T cells measured by flow cytometry from 1 representative donor. Graph shows quantification of suppression assay (n=3). Graphs display mean values and error bars represent standard deviation. Data are representative of 3 independent experiments. *p<0.05, **p<0.005, ***p<0.0005.
Differential effects of PI3K inhibitor treatment on CLL Tregs

To assess if differential regulation of Tregs by PI3K inhibitors was also manifest in the setting of CLL, we utilized the EuTCL1 adoptive transfer mouse model of CLL. As predicted, oral administration of idelalisib, duvelisib or umbralisib led to marked reductions in pAKT in EuTCL1 T cells (Figure 31A). Pharmacokinetic analyses established that all three inhibitors were detectable in plasma at 1 hour post-dosing (Figure 31B) and remained detectable ≤ 16 hours (data not shown). Following oral administration of idelalisib, duvelisib or umbralisib (21 days, once daily, at 100 mg/kg) CLL burden (CD19⁺ CD5⁺ B cells) in peripheral blood was significantly decreased compared to control (Figure 31C). Reduction in tumor burden was confirmed in splenocytes (data not shown). Total CD3⁺ T-cell numbers were also lower in inhibitor- versus vehicle-treated mice (Figure 31D), yet the CD4/CD8 ratio remained unchanged (Figure 31E). Notably, total Treg numbers were higher in the umbralisib than the idelalisib or duvelisib groups (Figure 31F). The expression of several surface markers involved in Treg suppressive function (TGFB-1, GITR, CD73) was also higher in the umbralisib-treated cohort compared to idelalisib- and duvelisib-treated mice, suggesting umbralisib-treated Tregs also retain functional capacity in the CLL setting (31G). Finally, to assess functional capacity, the four cohorts of EuTCL1 mice were sacrificed and splenic CD3⁺ T cells were activated ex vivo. Notably, IL-10 production was more sustained in T cells isolated from umbralisib-treated EuTCL1 mice (Figure 31H).
Figure 31. Treatment with PI3K inhibitors differentially impairs Tregs in EuTCL1 mice. (A) In vivo activity of PI3K inhibitors was assessed by monitoring levels of pS473-AKT in splenic T cells after treatment for 3 days (100 mg/kg, once daily, oral gavage) in wild type mice (n=4 per group). (B) EuTCL1 CLL-bearing recipient mice were randomized and treated for 21 days with the indicated inhibitors (100 mg/kg, once daily, n=5 mice per group). The plasma concentration of each PI3K inhibitor was measured by HPLC after collection of peripheral blood 1 hr after oral gavage. (C) Anti-tumor efficacy of inhibitors was determined by quantifying CLL B cells in peripheral blood following 21 days of treatment (100 mg/kg, n=5 mice per group). (D) Total CD3+ T-cell count in peripheral blood in the animals treated in (C). (E) CD4/CD8 ratio in peripheral blood in the animals treated in (C). (F) Treg count in peripheral blood of animals treated in (C). (G-H) Total CD3+ splenic T cells from the mice treated in (C) were stimulated ex vivo for 24 hr with anti-CD3/CD28. Expression of functional markers on surface of Tregs was then determined by flow cytometry and IL-10 secretion was measured from supernatant by CBA. MFI of functional markers were normalized to FMO controls. Graphs display mean and error bars represent standard deviation. Data are representative of 3 independent in vivo experiments. *p<0.05, **p<0.005, ***p<0.0005.
Treg number is associated with incidence of immune-mediated toxicities in CLL

B-cell cancer patients treated with idelalisib, duvelisib and umbralisib can suffer from immune-mediated toxicities.\(^{197}\) To investigate this phenomenon, we collected tissues susceptible to AEs from EuTCL1 mice treated with the PI3K inhibitors. In a blinded histological analysis, each mouse was assigned a toxicity grade based on histological features of the small intestine, colon and liver (Table 4 and Figure 32). Idelalisib- and duvelisib-treated EuTCL1 mice accrued the greatest toxicity grades, while umbralisib-treated EuTCL1 mice displayed a much lower incidence of AEs (Figure 33A). This result was reminiscent of available toxicity data derived from clinical use of these inhibitors.\(^{196,198}\)

It has been postulated that deleterious effects on Tregs may be involved in the regulation of immune-mediated toxicities characteristic of PI3K inhibitors.\(^{197}\) Given the differences in peripheral Treg number and function following treatment of EuTCL1 mice with these three PI3K inhibitors, a correlation analysis was performed to determine if Treg number was related to incidence of toxicities. Interestingly, a reduced number of peripheral Tregs positively correlated with overall toxicity grade in EuTCL1 treated mice (Figure 33B).

To assess the effects of these PI3K inhibitors on Treg suppressive capacity in vivo, a MHC-mismatched graft (C57Bl/6 bone marrow donor)-versus-host (Balb/c recipient) disease (GVHD) mouse model was used where functional Tregs are known to dampen the course of GVHD.\(^{200}\) Following engraftment, recipient mice were treated orally with umbralisib, idelalisib or vehicle, and were followed for their course of disease. Notably, GVHD was exacerbated in mice treated with idelalisib, while mice treated with umbralisib did not show any difference from the vehicle cohort (Figure 33C). Finally, umbralisib-treated mice had 2-fold greater numbers of Tregs than idelalisib-treated mice (Figure 33D). Thus, umbralisib treatment does not compromise Treg suppressive functions in vivo.
Table 4. Criteria for histological analysis of immune-mediated toxicity

<table>
<thead>
<tr>
<th>Organ Site</th>
<th>Criteria for immune-mediated toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>inflammatory foci, hepatocyte injury</td>
</tr>
<tr>
<td>Small intestine</td>
<td>immune infiltrate, shortened villi, denuded mucosa</td>
</tr>
<tr>
<td>Colon</td>
<td>immune infiltrate, shortened villi, denuded mucosa</td>
</tr>
</tbody>
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Figure 32. Histology of immune-mediated toxicity in CLL murine model. Representative histology findings in liver, small intestine and colon of EuTCL1 CLL-bearing recipient mice treated as described in Figure 4. Circles and arrows depict sites showing signs of toxicity by alterations in histology. All images were captured at 10x magnification.
Figure 33. Analysis of immune-mediated toxicity in CLL murine model. (A) Average toxicity grade per group (n=5 mice per group, from Figure 4). (B) Linear regression of peripheral Treg count versus incidence of toxicity establishes an inverse correlation. (C) Percent weight loss in the GVHD murine model normalized to weight prior to sub-lethal irradiation (100mg/kg, once daily, oral gavage, n=6 mice per group). (D) Submandibular bleeds were collected from each mouse on Day 8 and immunophenotyping was performed by flow cytometry to detect peripheral CD4+ CD25Hi FoxP3+ Tregs. Graphs display mean and error bars represent standard deviation. Data are representative of 3 independent in vivo experiments. *p<0.05, **p<0.005, ***p<0.0005.
**CK1ε inhibition protects Tregs from the detrimental effects of PI3K inhibitors**

Umbralisib is the only PI3K inhibitor known to inhibit CK1ε.195 Anti-CK1ε activity has been implicated in the inhibitor’s antitumor activity toward B-cell lymphomas due to downregulation of c-MYC (MYC).195 CK1ε drives canonical Wnt signaling in mammalian cells,201 where it suppresses the activity the β-catenin destruction complex and promotes β-catenin nuclear translocation and the transcription of WNT/β-catenin target genes.202 Accordingly, CK1ε silencing suppresses the expression and activity of β-catenin and its target genes.203 Notably, WNT signaling has been implicated in the differentiation and function of T-cell subsets,204 yet roles of CK1ε in T-cell function have not been investigated.

To assess if CK1ε inhibition by umbralisib contributes to sparing normal and CLL Treg number and function, we utilized a newly developed and potent CK1ε-selective small-molecule inhibitor coined SR-4471, which does not inhibit the closely related isoform CK1δ or the more distantly related family member CK1α.205 SR-4471 treatment did not affect normal T-cell survival at doses of 0.5μM and below (Figure 34A), but did lead to marked reductions in the expression of the WNT effector gene *Axin2*. Levels of *Axin2* transcripts in normal T cells were also suppressed by umbralisib treatment, but not by treatment with idelalisib (Figure 34B), suggesting that inhibition of CK1ε by umbralisib was necessary for suppression of *Axin2* expression.

To test if CK1ε inhibition also affects Treg biology, a Treg suppression assay was conducted using human Tregs pre-treated with vehicle, SR-4471, the three PI3K inhibitors, or combinations of SR-4471 plus PI3K inhibitors. As expected, treatment with PI3K inhibitors impaired the suppressive functions of Tregs, and the repressive effects of umbralisib on Tregs were more modest than those of idelalisib or duvelisib (Figure 35A, B). Further, treatment with SR-4471 did not impair the suppressive activity of Tregs (Figure 35A, B). Notably, dual treatment with SR-4471 impaired the deleterious effects of PI3K inhibitors on the suppressive functions of Tregs (Figure 35A-B).
To test if these findings were also manifest in the context of CLL, we treated splenic EuTCL1 T-cells with SR-4471, umbralisib, idelalisib or duvelisib and assessed effects on the expression of β-catenin and TCF-1, which are easily detectable by flow cytometry. Treatment with SR-4471 or umbralisib led to a dose-dependent suppression of β-catenin and TCF-1 levels, indicating ablation of WNT signaling, whereas this was not evident in idelalisib or duvelisib treated cells (Figure 36A). Further, treatment of splenic EuTCL1 CD4+ T cells under Treg-polarizing conditions revealed that, surprisingly, treatment with SR-4471 alone was sufficient to provoke significant increases in Treg numbers compared to control, and that SR-4471 treatment improved Treg numbers when added in combination with PI3K inhibitors (Figure 36B-C). The expression of functional Treg markers TGFβ-1, GITR and CD73 were also generally increased by the combination versus single treatments (Figure 37). Collectively, these findings are consistent with the notion that CK1ε inhibition by umbralisib impairs reductions in Treg number and function that are provoked by the idelalisib or duvelisib PI3K inhibitors (Figure 38).

**Figure 34. Effects of SR-4471 on normal human T cells.** (A) CellTiter-Blue assay showing viability of normal human T cells after 24 hrs of culture with SR-4471. (B) Normal human T cells were treated for 24 hrs with the indicated inhibitors and then lysed in trizol. Axin2 mRNA levels were measured by qRT-PCR. Graph displays ΔΔCT. Error bars denote technical triplicates of a representative donor.
Figure 35. Effects of SR-4471 on Treg suppressive capacity (A) Treg suppression assay was performed as in Figure 3, following treatment with PI3K inhibitors (1 μM) +/- SR-4471 (500 nM). Flow diagrams depict division of responder T cells measured by flow cytometry from 1 representative donor. (B) Graph showing quantification of Treg suppression assay (n=3). Graph displays mean values and error bars represent standard deviation. Data are representative of 3 independent experiments. *p<0.05, **p<0.005, ***p<0.0005.
Figure 36. CK1ε Inhibition promotes CLL Treg survival (A) Effects of PI3K (1 µM) and CK1ε inhibitors (500 nM) on protein levels of β-catenin and TCF-1 in isolated EuTCL1 T cells as determined by flow cytometry (n=3). (B-C) EuTCL1 splenic CD4⁺ T cells from 6-month-old EuTCL1 mice were cultured with PI3K inhibitors +/- SR-4471 (500 nM) in Treg-polarizing conditions (n=3). Graphs display mean values and error bars represent standard deviation. Data are representative of 3 independent experiments. *p<0.05, **p<0.005, ***p<0.0005.
Figure 37. Surrogate markers of Treg functional capacity. Expression of functional markers on the surface of EuTCL1 Tregs following treatment with PI3K inhibitor (1 μM) +/- SR-4471 (500 nM) (n=3). Graph displays mean values and error bars represent standard deviation. Data are representative of 3 independent experiments. *p<0.05, **p<0.005, ***p<0.0005.

Figure 38. Proposed model for action of CK1ε in T cells in canonical Wnt signaling. We propose a model where downregulation of canonical Wnt signaling by CK1ε inhibition allows more binding of FoxP3 to IL-2 promoter and thus encourages Treg differentiation and function.
Discussion

PI3K signaling is dysregulated in many cancers, including solid tumors and lymphoid malignancies. Activating mutations occur in multiple genes that direct PI3K signaling, including P\text{I3KCA}, P\text{TEN}, A\text{KT}, and m\text{TOR}.\textsuperscript{206} Mutations also occur in downstream effectors of PI3K signaling, including antiapoptosis BCL-family proteins.\textsuperscript{207, 208} Furthermore, mutations and the resulting pathway activation are associated with acquired resistance to therapeutic agents.\textsuperscript{209, 210} Based on these data, PI3K inhibitors were developed for clinical application. Interestingly, several antitumor mechanisms of action have been described, including inhibition of pro-survival signaling, disruption of microenvironment signals and enhanced anti-tumor immunity.\textsuperscript{197} Accordingly, there has been a drive to develop isoform-selective inhibitors to enhance selectivity in targeted cell types and reduce toxicity. Here we assessed the immunomodulatory effects of PI3K inhibitors idelalisib, duvelisib and umbralisib, which differ in their clinical safety profiles.\textsuperscript{197}

Idelalisib is the pioneer PI3K inhibitor that was FDA-approved in 2014 for the treatment of patients with relapsed follicular B-cell non-Hodgkin lymphoma (NHL), for small lymphocytic leukemia (SLL) patients who had received at least two prior systemic therapies. It was also approved relapsed CLL patients in combination with rituximab.\textsuperscript{211} Although high response rates and increased overall survival were demonstrated in patients treated with idelalisib, severe AEs were well documented,\textsuperscript{194} and frontline trials for CLL patients were halted due to their high incidence. Notably, disruption of the immune system has been linked to these AEs.\textsuperscript{155} For example, idelalisib treatment was reported to impair production of IL-6, IL-10 and TNF-\textalpha by activated CD3\textsuperscript{+} T cells and IFN-\gamma levels in activated NK cells.\textsuperscript{154} However, \textit{in vitro} studies of idelalisib did not characterize effects on CLL T cells and, considering the differences manifest in T-cell subsets and function in CLL\textsuperscript{12}, we reasoned that the immunomodulatory effects of PI3K inhibitors could be more profound in this context. While our data agree with prior studies showing no cytotoxicity of idelalisib against normal human T cells and a general reduction of Th1/Th2
cytokine secretion, the data clearly indicate profound reductions in Treg number and function in both normal and CLL human T cells following in vitro treatment.

Clinical data indicate a correlation between inhibition of p110δ and autoimmune toxicities,¹⁵⁵, ¹⁹⁸ and a decreased percentage of Tregs has been associated with the development of toxicities in patients treated with idelalisib.¹⁹⁸ To thoroughly address the immunomodulatory effects of the currently clinically available PI3Kδ inhibitors we directly compared the effects of several PI3K inhibitors in a validated mouse model of CLL. Interestingly, peripheral Treg number negatively correlates with incidence of autoimmune toxicity, suggesting that functional Tregs are indispensable to protect against autoimmune toxicity. Thus, peripheral Treg count and/or IL-10 secretion from peripheral blood T cells could be explored as potential predictive biomarkers for autoimmune toxicities in patients that are treated with idelalisib or other PI3K inhibitors.

Duvelisib is an inhibitor of both p110δ and p110γ.²¹² It is, however, more potent against p110δ than idelalisib in cell-free enzymatic assays.¹⁹⁶ Duvelisib has been tested as a monotherapy and in combination with other agents to treat B-cell malignancies, and was recently approved for R/R CLL patients in 2018. Its toxicity profile was similar to idelalisib and included transaminitis, neutropenia, colitis and pneumonitis.¹⁹⁷ Further, in vitro studies have demonstrated cytotoxicity of duvelisib toward human CLL T cells and reductions in IL-2, TNF-α and IFN-γ production from normal healthy human T cells.¹⁴⁵ Concordant with these findings, duvelisib treatment of normal and CLL T cells provokes reductions in Th1/Th2 cytokines, and in Treg number and function. Further, despite duvelisib plasma levels being lower than idelalisib and umbralisib in the EuTCL1 CLL model, the reductions in p-AKT in T cells were similar, the decreases in Treg numbers were more profound and there was a higher incidence of toxicity compared to vehicle or umbralisib-treated mice.

Umbralisib has enhanced selectivity for inhibiting p110δ over other PI3K isoforms and is unique in that it also inhibits CK1ε.¹⁹⁵ Umbralisib has been tested in clinical trials encompassing
several B-cell malignancies with high response rates and progression-free survival similar to those previously demonstrated with idelalisib. Notably, however, umbralisib has an improved safety profile, with fewer incidences of immune-mediated AEs and less discontinuations due to toxicities. Concordant with these findings, in the EuTCL1 model, umbralisib-treated mice experienced the lowest level of immune-mediated toxicity among the three treatments, and this was associated with higher numbers of functional Tregs. Although data from patients treated with umbralisib that assesses lymphocyte subset number and distribution is not yet available, a prediction from the findings presented herein is that the improved safety profile of umbralisib will correlate with increased Tregs in treated patients.

Janovska and colleagues recently explored inhibition of CK1δ/CK1ε as a therapeutic target in CLL. Interestingly, the noncanonical WNT receptor ROR1 is upregulated in the malignant B-cells of CLL patients. CK1ε is essential for signaling downstream of ROR1, and treatment with the dual CK1δ/CK1ε inhibitor PF-670462 elicited an anti-tumor benefit in the EuTCL1 model. Since umbralisib is unique in its ability to selectively inhibit CK1ε, we reasoned that this might account for the therapeutic and toxicity profile of this PI3K inhibitor. To our surprise, treatment of EuTCL1 T cells with SR-4471, a newly developed and highly selective and potent CK1ε inhibitor, led to increased numbers of Tregs, and improved Treg numbers when combined with PI3K inhibitors. Further, the effects of SR-4471 were associated with suppression of β-catenin and TCF-1 in Tregs. Interestingly, others have recently shown that WNT pathway stimulation in Tregs provokes TCF-1-dependent induction of IL2 transcription and the suppression of FoxP3 transcriptional activity, resulting in decreased Treg function. Collectively, these findings support a model whereby PI3Kδ inhibitors compromise Treg survival and function. This effect can be disabled by inhibition of CK1ε, via a WNT-β-catenin pathway. The results suggest that this circuit would be operational for a broad spectrum of malignancies treated with PI3K inhibitors. They also suggest that once a CK1ε inhibitor safety candidate is developed that a CLL
clinical trial testing its utility as a single agent or as combination therapeutic with PI3K inhibitors should be considered.

**Materials and methods**

**Cell culture**

Cells were cultured in RPMI supplemented with 10% fetal bovine serum, 5% penicillin-streptomycin, 5% non-essential amino acids and 1% Mycozap, incubated at 37°C with 5% CO₂. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque density gradient separation medium. Cell isolations were performed using magnetic separation kits (StemCell Tech, Cambridge, MA) and viability assays were conducted using Cell Titer-Blue® Cell Viability Assay (Promega, Madison, WI) according to manufacturers’ protocols.

**CLL patient samples**

PBMCs were obtained from CLL patients. All participants gave written IRB-approved informed consent for their blood to be used for research (#CR6_Pro00000316). Blood was collected at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL). CLL patients were diagnosed according to International Workshop on CLL 2018 guidelines. Patient characteristics are detailed in Table 1 and patients were treatment-naïve at the time of sample collection.

**Treg polarization and suppression assay**

Naïve CD4⁺ T cells were isolated from human whole blood using a Human CD4⁺ T cell isolation kit and were cultured in Treg-polarizing conditions (50 IU/mL human IL-2, 5 ng/mL human TGF-β1, Immunocult CD3/CD28 stimulation) for 5 days. Tregs were washed and treated with inhibitors at 1 μM for 24 hr. Tregs were then cultured with autologous responder CD4⁺ T cells labeled with CellTrace Violet (ThermoFisher Scientific, Waltham, MA) in a 1 to 5 (Treg:CD4⁺) ratio for 5 days. CellTrace Violet dilution was measured via flow cytometry.
**PI3K and CK1ε Inhibitors**

Idelalisib (CAL-101) and duvelisib (IPI-145) were obtained commercially (SelleckChem, Houston, TX). Umbralisib (TGR-1202) was kindly provided by TG Therapeutics. The CK1ε-selective inhibitor SR-4471 was synthesized by using appropriate modifications of a published synthetic procedure for CK1δ/CK1ε inhibitors. Inhibitors were dissolved and stored in DMSO at -20°C.

**CLL mouse model**

25 x 10⁶ leukemic splenocytes from EuTCL1 mice were adoptively transferred via tail vein into wild type (6-8 weeks old) syngeneic C57BL/6 mice to induce CLL disease. Leukemic EuTCL1 mice were defined as >6 months of age and >70% B cells out of total lymphocytes in peripheral blood by flow analysis. Disease induction was confirmed by significantly elevated white blood cell count within 3 weeks of adoptive transfer. Inhibitors were administered as a suspension in Tween-20 and 90% methylcellulose/deionized water once daily by oral gavage at indicated final concentrations. At end point, animals were sacrificed for collection of organs. Peripheral blood and spleen were used to assess tumor burden, immunophenotyping analysis and other in vitro assays. Liver and gastrointestinal tract from tumor-bearing recipients were fixed in formaldehyde, paraffin-embedded and H&E stained prior to histology analysis.

**GVHD mouse model**

5 x 10⁶ bone marrow cells isolated from femurs of wild-type C57Bl/6 mice were adoptively transferred into wild-type Balb/c mice that were previously irradiated with 900 rads. Treatment was administered via oral gavage beginning one day post adoptive transfer. Incidence of GVHD was tracked by weight loss over time.

**Flow cytometry**

Immunophenotyping analysis was performed via flow cytometry. All markers were gated according to fluorescence minus one or isotype controls. Acquisition was performed on a LSR II.
Cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed with FlowJo software v10.1 (Tree Star, Ashland, OR). For staining and antibodies, see Supplemental Methods.

**Statistical analysis**

Statistical significance between data sets was determined by unpaired, 2-tailed, or Student’s $t$ test if data were normally distributed, or using a Mann-Whitney U unpaired test if the data were not normally distributed. For groups of 3 or more, 1-way ANOVA followed by Tukey’s multiple comparisons test was used if the data were normally distributed, or a Kruskal-Wallis test was used if the data were not normally distributed. For correlation studies, linear regression was performed on data sets. A $p$ value < .05 was considered significant. All analyses were conducted using GraphPad Prism software v7.
CHAPTER SIX:
CONCLUSIONS AND FUTURE PERSPECTIVES

Treatment of disease conditions in cancer patients has been a challenge for physicians and scientists for a long time. Despite vast improvements over the past few decades, we are still in the infancy of understanding the biology of cancer. Aside from the option of bone marrow transplant, CLL is a manageable but currently incurable disease. Studies over the past several decades have collectively pieced together a picture of the characteristic molecular signatures of CLL B cells. Researchers have identified signaling pathways, receptors, ligands and lineage differentiation markers that are crucial for survival and function of malignant B cells.

The B-cell receptor signaling pathway has emerged at the forefront as the major source of intrinsic survival, anti-apoptosis and proliferation signals, providing numerous opportunities for developing small-molecule kinase inhibitors. The recent FDA approvals of ibrutinib, idelalisib and duvelisib have certainly changed the paradigm for treatment of CLL patients, promising greater efficacy, long duration of responses, and more options for the niche of patients that are unfit, elderly, or have relapsed from conventional therapy. More options are also available for younger patients with high risk features. However, these inhibitors are in their early stages and far from perfect. The preclinical development of more selective, less toxic, and overall better optimized compounds has been ongoing.

Overall, this work has contributed knowledge of antitumor targets for CLL patients, and has developed understanding of how antitumor targeting affects signaling pathways to modulate efficacy and safety of inhibitors. The studies reported here describe the potential of an epigenetic modifier, histone deacetylase 6, as a therapeutic target in CLL. Histone deacetylase 6 silencing
or pharmacological inhibition was found to downregulate BCR signaling, causing less survival, proliferation and anti-apoptosis signals for CLL B cells. Further, the concept was tested using a selective HDAC6 inhibitor, ACY738, in a preclinical murine CLL model. The rational combination of ACY738 plus ibrutinib reduced tumor burden and increased survival of mice with CLL significantly longer than either single agent, suggesting that this combination should be tested in clinical trial.

Immune dysfunction of CLL patients is well described. Specific CLL immune phenotypes have been reported, especially characterizing T-cell subsets. However, the picture is vague at best. With development of technology and increasing knowledge of immune biology, the importance of CLL immune interactions, costimulatory and coinhibitory signals, and tumor microenvironment is becoming clear. But, there is still a general lack of understanding of how the immune components work in tandem to progress the disease. This is partly due to the fact that technical challenges are plentiful. For example, the isolation and activation of CLL patient T cells has proved very difficult given the “exhausted” phenotype they portray. Opportunities to conduct functional studies for CLL patient T cells remain limited.

Experiments with other immune compartments, for example, cells of the myeloid lineage, are also difficult given that the functional cells reside within specific physiological niches. When removed from their niches, function may be compromised. Considering these challenges, preclinical mouse models, such as the euTCL1 transgenic model, have been extremely useful in the study of the CLL microenvironment. It has long been postulated that targeting of the CLL immunomicroenvironment can disrupt malignant cell survival. Interestingly, BCR inhibitors have recently been found to modulate other CLL immune subsets in ways that may be contributing to their antitumor mechanisms of action. The studies reported here demonstrated that HDAC6 exhibited immunomodulatory activity and implies that these properties can harnessed to reverse T-cell dysfunction in CLL.
Our studies provided evidence that HDAC6 inhibition impaired mechanisms of B-cell derived immunosuppression, ultimately improving T-cell numbers and function in euTCL1 mice. HDAC6 silencing or pharmacological inhibition resulted in altered levels of immune checkpoints and other surface molecules important for T-cell antigen presentation on the CLL B cells and on the CLL T cells. Based on this, the rational combination of ACY738 augmented the antitumor activity of clinically available immune checkpoint inhibitor anti-PD1. Although not tested here, ACY738 may have the potential to also augment the activity of other immune checkpoint inhibitors in preclinical development, such as anti-PD-L1 and LAG-3. Future studies to investigate these possibilities will be of interest.

Concerning clinical development of therapeutic agents, it has always been important to strike a delicate balance between efficacy and toxicity. The BCR inhibitors and other immunomodulatory agents and immunotherapies are notorious for their efficacy, but also for sometimes unpredicted toxicities. As such, translational studies are more necessary than ever to manage toxicities that hamper the development of the effective inhibitors. The case of the PI3K inhibitors is particularly interesting. The primary research base focused on characterizing the effects of PI3K inhibitors in normal and malignant B cells. When unexpected autoimmune-like toxicities occurred, the culprit was suspected to be another immune population that harbored similar signaling pathways, like T cells. Prior literature has indicated that T-cell function is susceptible to PI3K inhibition, however, how these effects related to clinical applications was unknown.

The studies described here facilitated comparison of the incidence of autoimmune-like side effects of clinically available PI3K inhibitors in a CLL mouse model. Specifically, these results definitively elucidated the contribution of Tregs to the incidence of side effects. Finally, these studies described alternative pathway modulation by an off-target, CK1ε, responsible for the differential and beneficial effects of next-generation inhibitor, umbralisib, on Tregs. This study reinforces the idea that all inhibitors are not created equal and immunomodulatory differences
perceived as small can have huge clinical repercussions. Although not performed here, this study could be extended to investigate other T-cell subsets after PI3K inhibition. For example, stem-like memory CD8 and Th17 cells are also heavily regulated by PI3K signaling and have been found to impact autoimmune disease conditions in the literature. The impact of PI3K inhibitors on these T-cell subsets and other immune compartments in the context of efficacy and toxicity in CLL patients will be interesting topics for future study.

CAR T cells have been very popular subjects for research and development in cancer therapeutics. FDA approval of CAR therapies tiagenlecleucel and axicabtagene ciloleucel for non-Hodgkin lymphoma and diffuse large B-cell lymphoma prove that CAR T cells are useful for treatment of B-cell malignancies. However, CAR T therapy has been tested but not yet approved in CLL. Because the T cells of CLL patients are few and difficult to expand, there is a growing interest in the use of immunomodulatory agents to alter the phenotype of patient-derived T cells to make them more amenable to transduction, survive longer, and persist in vivo. Investigations surrounding CLL T-cell biology and the immunomodulatory drugs have become immensely important. BCR inhibitors are being tested for the purpose of altering CLL T-cells, however, epigenetic modifiers have not yet been tested. Epigenetic manipulation of T cells using immunomodulatory histone modifiers could be useful for CAR T therapy and possibly other adoptive cell therapies. Thus, interest in the role of epigenetic modifiers in the biology of normal and malignant immune function continues to grow.

Within the bigger picture, the findings presented here imply that during development of HDAC inhibitors, BCR inhibitors and other novel immunomodulatory therapeutics, basic and translational research elucidating impacts on tumor cells and various immune subsets is very useful to maximize efficacy and mediate toxicity. This includes modulation of complex tumor survival signaling, along with signaling that controls immune evasion tactics. For immune cells that home to the tumor microenvironment and or circulate the periphery, this includes modulation of signaling that may offer support to the malignant progression of the tumor cells; signaling that
controls disease symptoms and toxicities; and signaling that controls a suppressive versus inflammatory response. On the other hand, elucidation of biology at this level will also point to plausible biomarkers for development of clinical assays to gauge what is occurring in real time when a patient is treated with an immunotherapy.

Recognition of the need for a comprehensive picture about how tumor cells and immune cells interact is underway in the scientific community. To address this emerging need, the application of interdisciplinary, new technologies and methods to research clinical challenges will be paramount. New technologies, such as high-throughput genomics, proteomics, metabolomics and the use of bioinformatics analysis to predict immune pathway modulation, will be instrumental to advance cancer immunology and no doubt advance our ability to fully understand and harness the potentially life-saving antitumor effects of the human immune system.
REFERENCES


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APPENDIX B
IACUC APPROVAL

USF UNIVERSITY OF SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM
TO: Eduardo Sotomayor, M.D.

FROM: Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 2/12/2015

PROJECT TITLE: Targeting Negative Regulatory Pathways for Immunotherapy of B-cell Lymphomas

FUNDING SOURCE: NIH/NCI
IACUC PROTOCOL #: R 800000904
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 2/12/2015:
3/1/2018

Javier Pinilla, M.D., Ph.D.
H Lee Moffitt Cancer Center
12902 Magnolia Dr., MRC-3 3057J
Tampa, FL 33612

RE: Expedited Approval for Continuing Review
IRB#: CR8_Pro00000316
Title: Epigenetic Modifiers to Augment the Immunogenicity of Chronic Lymphocytic Leukemia

Study Approval Period: 3/16/2018 to 3/16/2019

Dear Dr. Pinilla:

On 2/27/2018, the Institutional Review Board (IRB) reviewed and APPROVED the above application and all documents contained within including those outlined below.

Approved Item(s):
Protocol Document(s):
16118.CLL.protocol.7-20-11.CLEAN.doc

Consent/Assent Document(s)*:
16118.ICF.v02.CLEAN.2011-07-25.docx.pdf