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## ACTIONS OF PI3K-DELTA INHIBITOR, IDELALISIB, AND ITS COMBINATION WITH BENDAMUSTINE IN CHRONIC LYMPHOCYTIC LEUKEMIA

Prexy Modi

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**ACTIONS OF PI3K-DELTA INHIBITOR, IDELALISIB, AND ITS COMBINATION  
WITH BENDAMUSTINE IN CHRONIC LYMPHOCYTIC LEUKEMIA**

by

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**ACTIONS OF PI3K-DELTA INHIBITOR, IDELALISIB, AND ITS COMBINATION  
WITH BENDAMUSTINE IN CHRONIC LYMPHOCYTIC LEUKEMIA**

**A**

**THESIS**

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

**MASTER OF SCIENCE**

by

Prexy Modi, B.S.

Houston, Texas

May 2015

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## **Dedication**

To my loving and supportive husband, parents and parents-in-law, grandparents and my  
siblings.

## **Acknowledgement**

I wish to express my sincere gratitude towards my advisor, Dr. Varsha Gandhi. Your passion, support, teachings and mentorship on my academic and professional development has been the most vital experience of my graduate training. Your genuine dedication to science and education is truly inspirational and without your generous support, I would not have reached my goal.

I would like to extend my deepest thank you to Dr. Kumudha Balakrishnan. You have taught me more than laboratory techniques and experimental design. I could not have done it without your constant support in the laboratory, committee meetings and throughout my research project.

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Many thanks and support I received from my lab members especially Carly Yang, Shadia Zaman, Fab Gomez, Viral Patel, Alope Sarkar, Rong Chen, Tina Stellrecht, Betty Lamothe and Hima Vangapandu for all the help and suggestions throughout my graduate training.

Finally, big thanks to the Experimental Therapeutics Academic Program (ETAP) manager, Lidia Vogelsang, for constant assistance. Thank you to ETAP faculty and students for advice and support.

# **ACTIONS OF PI3K-DELTA INHIBITOR, IDELALISIB, AND ITS COMBINATION WITH BENDAMUSTINE IN CHRONIC LYMPHOCYTIC LEUKEMIA**

Prexy Modi, B.S.

Advisor: Varsha Gandhi, Ph.D.

Class I phosphatidylinositol 3-kinase isoforms ( $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ) play a major role in cancer cell growth and survival. PI3K  $\alpha$  and  $\beta$  are most studied. PI3K pathway is highly dysregulated in many cancers and aberrant PI3K signaling is associated with oncogene mutations and disease progression in solid tumors and in hematologic malignancies.

Chronic lymphocytic leukemia (CLL) is driven by B-cell receptor (BCR) signaling that promotes B-cell proliferation and survival. PI3K is a critical node in BCR pathway and PI3K $\delta$  has a pivotal role in B-cell development and maintenance and this isoform is over-expressed in many B-cell malignancies, including CLL.

Idelalisib is a FDA approved small molecule PI3K $\delta$  inhibitor. Idelalisib promotes apoptosis in CLL by disrupting molecular pathways related to BCR signaling, leukemic migration and signals from the microenvironment. Importantly, idelalisib inhibits BCR-derived PI3K signaling, dampening survival signals. We hypothesized that similar to inhibition of  $\alpha$  and  $\beta$  isoforms, attenuation of PI3K $\delta$  will repress transcription, reduce short-lived anti-apoptotic proteins, induce DNA damage and repair responses, leading to enhanced apoptosis of malignant CLL cells.

Idelalisib treatment induced moderate levels of apoptosis in CLL lymphocytes. Idelalisib treatment with IgM stimulation decreased phosphorylation of AKT, a downstream signaling

molecule. We observed a significant decrease in global RNA synthesis and a decline in Mcl-1 transcript and protein levels, with no changes to Bcl-2 protein and mRNA expression. Interestingly, we observed that idelalisib induced  $\gamma$ H2AX, a hallmark for DNA damage and repair response.

Bendamustine is a FDA approved alkylating agent for CLL therapy. We hypothesized that idelalisib-mediated decline in Mcl-1 and bendamustine-induced DNA damage will sensitize B-CLL cells and this mechanism-based combination will lead to a synergistic interaction.

At clinically relevant concentrations, bendamustine and idelalisib as single agents induced moderate level of apoptosis; however, combination treatment resulted in enhanced CLL cell death. Combination index assessment demonstrated that idelalisib and bendamustine couplet resulted in synergistic cytotoxicity. Mechanistic investigations suggest that the synergy maybe due to modulation of Mcl-1 protein levels and DNA damage and repair responses in CLL. Collectively, the emerging role of PI3K inhibitor in combination with bendamustine provides a unique modality for CLL therapy.

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## CHAPTER 1: Introduction

### **PI3K: Function and expression**

The phosphoinositide 3-kinase (PI3K) family plays a major role in nearly all aspects of cellular biology and it is a key node in human cancers, metabolism and aging. Signaling through the PI3K families of enzymes play a multifunctional role in plethora of cellular activities mediating functions such as cellular growth, proliferation, differentiation, motility, intracellular trafficking, and metabolism. PI3K pathway is highly dysregulated in many cancers and aberrant PI3K signaling is associated with oncogene mutations and disease progression in solid tumors and in hematologic malignancies (1). PI3Ks are activated and recruited by broad range of growth factors; these enzymes convert phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to yield phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>) (2). This lipid product acts as second messenger by binding and activating downstream cellular targets.

There are three distinct classes of PI3Ks; I, II and III grouped according to their structure and function. Class I PI3K is most investigated and has two subclasses, IA and IB. Class IA PI3K is a heterodimer consisting of a regulatory subunit and a catalytic subunit and it is found to be highly implicated in human cancers (3-5). The regulatory subunit consists of p85 and the three mammalian genes, *PIK3CA*, *PIK3CB*, and *PIK3CD*, encode for the catalytic isoforms, p110 $\alpha$  (alpha), p110 $\beta$  (beta) and p110 $\delta$  (delta) (6). Class IB PI3Ks are composed of two regulatory subunits (p101 or p87) and has one catalytic subunit p110 $\gamma$  (gamma), encoded by gene *PIK3CG*. Class IA PI3Ks are activated by direct interaction with receptor tyrosine kinases (RTKs), non-receptor tyrosine kinases (non-RTKs), G-protein coupled receptors (GPCRs) and Ras, whereas class IB PI3Ks are activated by only GPCRs and Ras (6,7). The main function of

the regulatory subunit is to facilitate membrane localization, receptor binding and activation, while the catalytic subunit mediates phosphorylation of (PIP2) to yield (PIP3) (8). Class I PI3K signaling is activated in human cancers through various mechanisms. Such activity is due to mutational activation or amplification of genes encoding the distinct isoforms and their subunits of the PI3K pathway.

### **PI3K Class I: Isoform expression and function**

There are four class I catalytic isoforms that share overlapping but distinct functions and differential expression. The p110 $\delta$  and p110 $\gamma$  isoforms function and expression are restricted in immune cells, whereas p110 $\alpha$  and p110 $\beta$  are ubiquitously expressed in mammalian tissues (3). Genetic ablation of p110 $\alpha$  or p110 $\beta$  isoforms results in embryonic lethality, indicating its essential and non-redundant roles during development (9). P110 $\alpha$  isoform has a role in insulin-dependent signaling and p110 $\beta$  has been shown to have a role in platelet aggregation, insulin signaling and thrombosis (10). P110 $\delta$  and p110 $\gamma$  play a key role in lymphocyte activation and signaling, chemotaxis and mast cell degranulation. In contrast to mice lacking PI3K  $\alpha$  and  $\beta$ , mice deprived or with mutant phenotypes of p110 $\delta$  and p110 $\gamma$  have severely impaired immune response (11,12). P110 $\delta$  knockout and kinase inactive knock-in mice show very specific impairment in B-cell signaling and response leading to defects in B-cell development, T-cell dependent and –independent antigen stimulated antibody generation (3,4). Studies in humans have been reported that gain-of-function mutations in *PIK3CD*, which encodes p110 $\delta$ , severely impaired development and function of memory B-cells (12).

### **PI3K $\delta$ in B-cell development and hematologic malignancies**

PI3K signaling is activated or altered in many solid tumors as well as in hematologic malignancies and is found to display varying levels of mechanisms to achieve the malignancy

status. Numerous reports have identified the pivotal role of class I PI3Ks in leukemias and lymphomas. In chronic lymphocytic leukemia, PI3K is not often mutated, but it signals downstream of the B-cell receptor pathway, leading to increased expression of many anti-apoptotic proteins (13,14). The constitutive activation of the PI3K in CLL is dependent on the p110 $\delta$  isoform (13). Similarly, in Hodgkin's lymphoma (HL) cell lines, compared to other isoforms, PI3K $\delta$  expression is found at higher levels (15). PI3K $\delta$  is widely expressed in mantle cell lymphoma (MCL) and is shown to have modest activity (16,17). There is also constitutive activation of the PI3K pathway through the B-cell receptor which leads to dysregulation of cell-cycle progression in MCL cell lines and in primary samples (17). Even in non-B-cell malignancies, such as acute myelogenous leukemia, p110 $\delta$  plays a critical role in PI3K activity. From the recent studies in Ali et al, inhibition of the PI3K $\delta$  isoform in mice disrupts the function of the regulatory T cells and shifts the response towards effective anti-tumor activity (18). This mechanism further provides a strong rationale for p110 $\delta$  inhibition both in a broad range of solid tumors and in hematologic malignancies

### **PI3K $\delta$ in BCR signaling**

The regulatory subunits of PI3Ks facilitate membrane localization, receptor binding and activation, whereas the catalytic subunit phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to yield phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>). The PIP<sub>3</sub> initiates downstream signaling by recruiting 3-phospho-inositide-dependent kinase 1 (PDK1) to the membrane, activating AKT by phosphorylating the threonine 308 residue (19) (Figure 1). Activation of the AKT also occurs by phosphorylation at serine 473 by mammalian target of rapamycin (mTOR). The B-cell receptor pathway (BCR) signaling pathway is critical for development and maturation of normal B-cells. The class 1 PI3K isoform recruits downstream proteins of the

BCR pathway such as the activation of the PI3K, Bruton's tyrosine kinase (BTK) and AKT promoting cell survival by activating NF- $\kappa$ B and inhibiting apoptotic signals (20,21). The BCR signaling is initiated with an antigen specific immunoglobulin (Ig) that forms a non-covalent complex with CD79a and CD79b which have cytoplasmic domains that contain the Immunoreceptor Tyrosine-based Activation motif (ITAMs). Upon BCR ligation, this complex can recruit multiple kinases and adaptor molecules stimulating BCR signaling cascade for the growth, survival and expansion of the B cells. Activated ITAMs can phosphorylate Lyn, recruit SRC family kinases, and further downstream kinases such as BTK. Signaling through BCR also activates the PI3K/AKT/mTOR pathway (22). Neoplastic B cell lymphomas maintain the expression of surface immunoglobulin and have functional BCR signaling. The complex BCR pathway interacts with multiple factors from the microenvironment and other stimulating factors (chemokines and cytokines) leading to constitutively active state, further contributing to the growth, survival and expansion of the malignant B-cells (23).

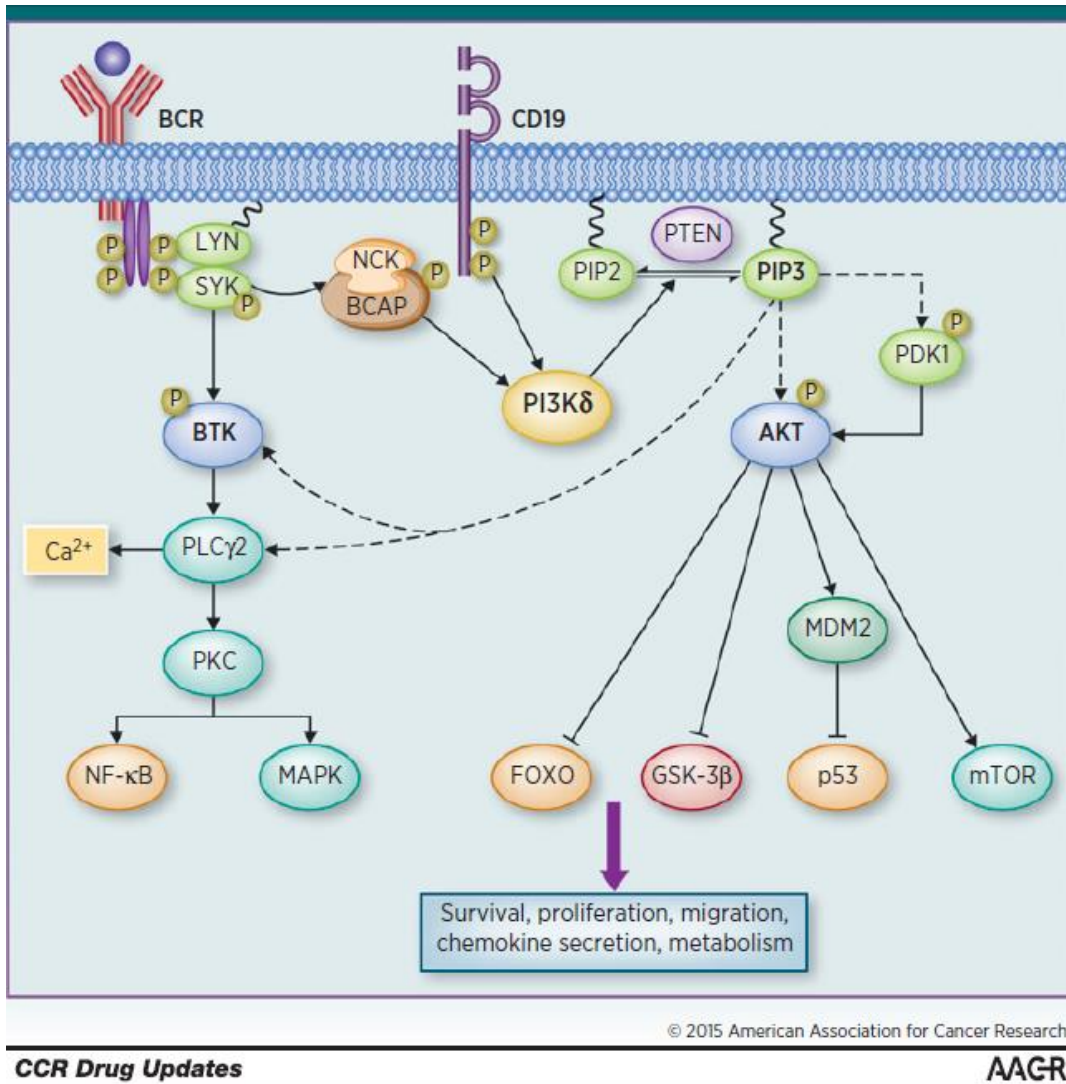
### **PI3K $\delta$ regulation and function**

PI3K $\delta$  critically regulates a number of signaling pathways in normal and malignant B cells. These pathways are driven by multitude of receptors including BCR, Fc-gamma receptor (Fc $\gamma$ R), TLR, C-X-C chemokine receptor type 4/5 (CXCR4/5), and the tumor necrosis factor (TNF) receptor family (24,25). PI3K $\delta$  also regulates B-cell responses to CD40-ligand, B-cell activating factor (BAFF), IL-4, and to the homing chemokines CXCL12/13 (23,25-27). All of these interactions feed into the overall B-cell signaling. PI3K $\delta$  is a key player downstream of BCR activation from all the microenvironmental factors and thereby promoting malignant B-cell proliferation, growth, survival, adhesion, and homing and metabolism. BCR activation recruits and phosphorylates tyrosine kinase Lyn and spleen tyrosine kinase (Syk) at the plasma

membrane, and it also recruits an adaptor protein non-catalytic region of tyrosine kinase adaptor (Nck) (28). PI3K $\delta$  is recruited to the cell membrane, mediated by the association of p85 regulatory subunit to the phosphorylated tyrosine motifs in the B-cell antigen CD19 and the B-cell PI3K adaptor protein (BCAP) (29). CD19 and BCAP get phosphorylated and act as docking sites for the p85 regulatory subunits, a critical step for recruitment and activation of p110 $\delta$  catalytic subunit (30). Refer to figure 1 for the role of PI3K $\delta$  in BCR pathway.

Importantly, key pathways coordinate downstream PI3K $\delta$ ; AKT/mammalian target of rapamycin (mTOR), mitogen-activated protein kinases (MAPK), and nuclear factor kappa light-chain enhancer of activated B cells (NF- $\kappa$ B), are turned on in B-cell malignancies upon BCR activation (31,32). AKT is the most characterized downstream effector of PI3K $\delta$ . Further oncogenic effectors downstream of AKT play critical roles in regulating cell cycle and cell survival (mouse double minute 2 homolog (MDM2), p53, forkhead box O (FOXO), cell trafficking (glycogen synthase kinase 3 beta (GSK-3 $\beta$ ), DNA repair (MDM2, p53), chemoresistance (NF- $\kappa$ B), and energy metabolism (GSK-3 $\beta$ , mTOR) (33,34).

**Figure 1.**



Yang Q, Modi P, Newcomb T, Quéva C, Gandhi V. Idelalisib: first-in-class PI3K delta inhibitor for the treatment of chronic lymphocytic leukemia, small lymphocytic leukemia, and follicular lymphoma. Clin Cancer Res;21:1537-42.

**Figure 1: BCR pathway and the role of PI3Kδ**

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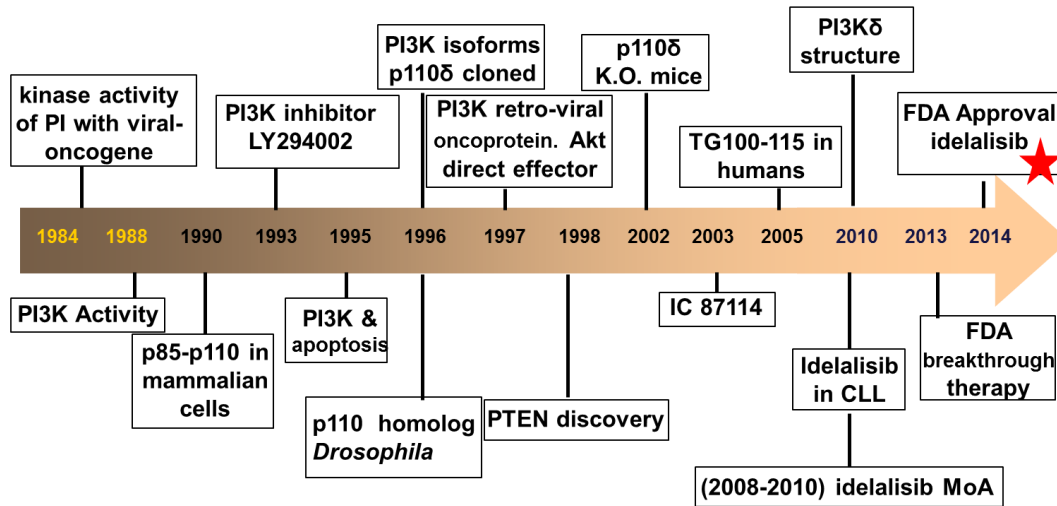
## **Development of PI3K pan or isoform specific inhibitors**

For more than two decades, the PI3Ks field has evolved from their initial discovery, the understanding their physiologic activities and function as lipid kinase with PIP3 as a product, and their oncoprotein properties to specifically targeting these kinases to treat cancer and inflammation. The timeline figure 2; highlights the key events of PI3K discovery and the progressive findings that have led to the growth of PI3K $\delta$  specific therapy. Initially, scientists focused on the development of pan-PI3K inhibitors such as LY294002 (35). However, with the discovery of PI3K $\delta$  selective expression in hematopoietic cells, isoform-specific inhibitors were designed (31,36). IC87114 was the first isoform-selective inhibitor of PI3K $\delta$  subunit (37). Several new isoform-specific inhibitors have been developed by different pharmaceutical companies; their potency and specificity are reviewed in detail by Brana and Siu (38).

## **Idelalisib, PI3K delta inhibitor**

Idelalisib (Zydelig<sup>TM</sup>), (5-fluoro-3-phenyl-2-[(*S*)-1-(9*H*-purin-6-ylamino)-propyl]-3*H*-quinazolin-4-one) formally called CAL-101 or GS-1101, is a small molecule antagonist ATP-competitor of p110 $\delta$ , the catalytic subunit of PI3K $\delta$  (Figure 3). Idelalisib was identified as a selective p110 $\delta$  inhibitor through high throughput screening and a kinome-wide screen using purified enzymes and in cell-based PI3K isoform specific assays. The IC<sub>50</sub> of idelalisib for PI3K $\delta$  was 19 nM, whereas the IC<sub>50</sub> values for PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\gamma$  were 8,600; 4,000; and 2,100 nM, respectively (34). Idelalisib at a concentration of 10  $\mu$ M did not significantly interfere with ligand-receptor binding in a panel of 61 receptors including G-protein coupled receptors (GPCRs), ion channels, receptor tyrosine kinases, steroid receptors, and transporters. Idelalisib at 10  $\mu$ M did not interact or inhibit any other kinases except for PI3K isoforms (34).

**Figure 2.**



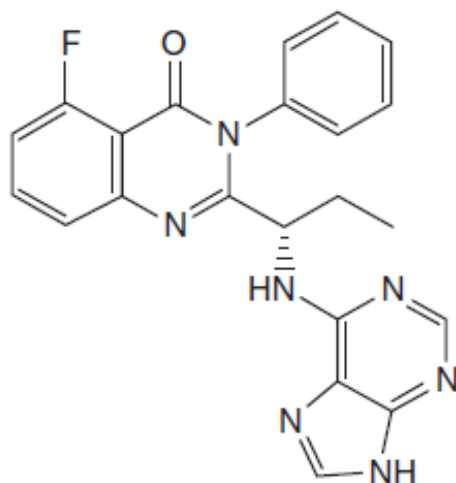
Yang, Q and Modi, P, Ramanathan, S, Quéva, C, Gandhi, V  
Idelalisib for the treatment of B-cell malignancies.

*Expert Opinion on Orphan Drugs* 2015 3:1 , 109-123 doi=10.1517%2F21678707.2014.978858

**Figure 2: Timeline of PI3 Kinase and Idelalisib**

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**Figure 3.**



Yang, Q and Modi, P, Ramanathan, S, Quéva, C, Gandhi, V

Idelalisib for the treatment of B-cell malignancies

*Expert Opinion on Orphan Drugs* 2015 3:1, 109-123 doi=10.1517%2F21678707.2014.978858

**Figure 3. Chemical structure of idelalisib**

**(5-fluoro-3-phenyl-2-[(1S)-1-(9H-purin-6-ylamino)propyl]quinazolin-4(3H)-one)**

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### **Other PI3K delta inhibitors**

To date, besides idelalisib, several other PI3K $\delta$ -specific inhibitors are currently being investigated in clinical trials. ACP-319 (Amgen Inc. – Acerta Pharma) is currently in phase I trials for patients with relapsed or refractory CLL in combination with Acerta's Bruton's tyrosine kinase (BTK) inhibitor (39). INCB40093 (Incyte Pharmaceuticals) entered phase I evaluation for refractory B-cell malignancies and may be further tested in combination with Incyte Pharmaceuticals' Janus kinase 1 (JAK1) inhibitor (clinicaltrials.gov NCT01905813). TGR-1202 (TG Therapeutics) is in phase I clinical trial with the most notable combinations being with brenduximab vedotin in Hodgkin's lymphoma and with chlorambucil and obinutuzumab for CLL (clinicaltrials.gov). Finally, IPI-145, also known as duvelisib (Infinity Pharmaceutical), is a dual PI3K $\delta$  and  $\gamma$  inhibitor, and is currently being evaluated as a single agent in Phase 3 trials for patients with advanced CLL and other hematologic malignancies (40).

### **Chronic lymphocytic leukemia (CLL) and its molecular pathogenesis**

Chronic lymphocytic leukemia (CLL), a B-cell malignancy is the most common adult leukemia in the western world; with predominant cases in North America and Europe regions. The rate of incidence is double in men and the median age of diagnosis is 72 years. CLL is characterized by the clonal expansion of CD5<sup>+</sup> CD23<sup>+</sup> B cells in the peripheral blood, bone marrow and secondary lymphoid tissues. Generally, CLL is asymptomatic in most patients and symptoms in advanced stages include anemia, enlargement of lymph node and bone marrow failure (41-43).

The malignant B CLL cells are mature lymphocytes that are found to be replicationally quiescent and many of these CLL B cells exist in G0 and G1 phase of the cell cycle. The CLL lymphocytes get accumulated in the lymph nodes, bone marrow, spleen and peripheral blood.

Circulating malignant CLL cells have been associated with dysfunctional apoptotic machinery thereby causing prolonged survival (44). Overall, genetic defects and the extrinsic signals from the tumor microenvironment both contribute to survival, proliferation and abnormal accumulation of CLL cells (45).

There are genetic lesions such as deletions, translocations and polysomy in CLL which contribute to CLL pathogenesis, disease progression, poor prognosis and treatment outcome. First studies from Gahrton et al reported Trisomy 12 as a genetic abnormality which is associated with poor treatment outcome (46). Genetic aberrations in CLL include deletion of 13q14, 6q21, 11q22.3, 11q23.1 and 17p13. Deletion of genetic region 17p13.1 is associated with loss of TP53 gene function and deletion of 11q22-23 is associated with loss of ATM gene function. These specific genetic deletions are strongly correlated with treatment outcome and poor patient survival. Furthermore, the most common genetic deletion at 13q14.3 is associated with loss of mir-15a and mir-16-1 (47). Loss of mir-15a and mir-16-1 regulate expression of anti-apoptotic proteins Bcl-2 and Mcl-1, thereby causing an overexpression of these two proteins.

### **PI3K $\delta$ in CLL and its relevance**

The PI3K pathway is a critical component of cell survival in CLL. Initial studies determined the importance targeting the delta isoform of PI3K in CLL. Studies from Herman et al, first reported overexpression of PI3K $\delta$  isoform in CLL patient lymphocytes compared to normal B cell lymphocytes (27). When compared to other PI3K isoforms, expression of PI3K $\gamma$  isoform was also evident, however essentially no PI3K  $\alpha$  and  $\beta$  isoforms observed in CLL patient lymphocytes. Additionally, CLL cells had higher PI3K activity when compared to normal B cell, and this higher PI3K activity has also been reported previously (27).

Furthermore, Lannutti and other colleagues observed increased resting PI3K activity in CLL compared to normal B cells. These studies also extended downstream constitutive phosphorylation of AKT, suggesting the role of PI3K $\delta$  in CLL and survival signals (13). These key findings provided a rationale for specifically targeting PI3K $\delta$  in CLL.

### **Bcl-2 anti-apoptotic family proteins**

Apoptosis is a cell death program that is extensively coordinated for normal cell biology however; defective apoptotic machinery is a hallmark feature in CLL (48). B-cell lymphoma 2 (Bcl-2) family anti-apoptotic proteins are primarily responsible for abnormal survival and proliferation of malignant CLL cells. Bcl-2 family proteins play central roles in cell death regulation modulating diverse cell death mechanisms and alterations in their expression and function leads to pathogenesis (44,49). Of the 30 mammalian Bcl-2 family proteins, 14 are of human origin and are categorized into 3 different subclasses based on the structural and functional Bcl-2 homology BH domains (50).

The first subclass of Bcl-2 family proteins is the anti-apoptotic proteins which include Bcl-2, Bcl-b, Bcl-xL, Bfl-1, Bcl-w, and Mcl-1 proteins. These six proteins mainly function as the inhibitors of the apoptosis pathway. There has been critical evidence for the vital functional role of these anti-apoptotic proteins. Bcl-2 protein maintains homeostasis of the adult B cell population in the peripheral blood (51). Bcl-xL protein is critically required for survival of the immature thymocytes (52). Studies also document the role of Bcl-w to be linked with sperm cells rather than lymphocytes (53). Mcl-1 has a unique role in cell physiology; it is critically essential for early embryonic development, survival of hematopoietic stem cells and lymphocytes as well as development of mature lymphocytes (54,55). In transgenic mice studies, over-expression of Mcl-1 induced B-cell lymphomas (56).

The second subclass of Bcl-2 family proteins is the multi-domain pro-apoptotic proteins Bax, Bak, and Bok. These proteins are involved in facilitating apoptosis pathway thereby inducing apoptosis. Bax and Bak activate apoptosis through mitochondrial outer membrane permeabilization (MOMP) process leading to release of the cytochrome c, activating the caspases cascade and cell death (57,58). Cells lacking Bax and Bak fail to undergo MOMP and apoptosis in response to many death signals (57). Expression of Bax protein in lymphocytes plays a key role in programmed cell death (59). *In vivo* studies have also demonstrated the role of Bak protein in B-cell homeostasis.

The third subclass of Bcl-2 family consists of the BH3 only pro-apoptotic proteins. Among the 9 proteins in this group; Bim and Bid functions as the activators of the apoptosis and Bad, Bmf, Bik, Hrk, Bcl-rambo, Puma and Noxa function as sensitizers of apoptosis process. BH3 only pro-apoptotic protein, Bid, functions in cell maintenance of myeloid cells (60); while Bim protein plays a role in B-cells program cell death, both in memory and plasma B cells. In addition to BH3 only pro-apoptotic proteins having a prominent role in apoptosis, it's been documented that several BH3-only proteins are involved in cell-cycle regulation, metabolism and DNA repair mechanisms (61,62).

Overall, differential expressions of Bcl-2 family proteins orchestrate cellular death and survival signals. The new role of Mcl-1 has been explored and it is found to be associated with DNA damage and repair pathway.

### **Role of Mcl-1 in DNA repair**

Mcl-1 has an extensive and a critical role in apoptosis and in CLL, however; several studies have demonstrated a new and unique role of Mcl-1 in DNA damage response and repair

mechanisms. Cells have an elaborate response to DNA damage and elicit DNA repair responses in order to protect genetic changes. To this date, mammalian cells have evolved to variety of mechanisms to reduce DNA damage. One of the mechanisms that is critical before the initiation of DNA repair process is growth-arrest of the cells following DNA damage. Another critical mechanism is cell death by apoptotic program in response to DNA damage. Studies have reported cytotoxic DNA damaging agents leading with an early apoptosis response leads to an enhanced expression of MCL1 gene in a p53-independent manner (63-66). More studies showed role of Mcl-1 linked to regulating cell cycle progression, partially mediated through proliferating cell nuclear antigen (PCNA), interactions with cyclin dependent kinase 1 (CDK-1), and ATR-dependent activation of checkpoint 1 protein (CHK-1) following DNA damage (67-69). Overall, Mcl-1 is highly overexpressed in many human cancers, manipulated by malignant cells to escape apoptosis regulation and have further implications for its unique role in DNA damage response.

### **Alkylating and DNA damaging agents in CLL**

For past several decades, alkylating agents are being used as front line therapy in CLL. Initially, chlorambucil, a nitrogen mustard compound, was considered gold standard for CLL therapy. Even today, chlorambucil therapy is used for elderly, unfit patient population. Cyclophosphamide is another alkylating agent that has been used in CLL for many years. Fludarabine, pentostatin, and cladribine have been used as purine analogues for CLL therapy. In the 1990's, fludarabine was compared to chlorambucil in previously untreated CLL patients. In the front line treatment study, fludarabine resulted in higher response rates and longer duration of remission and progression-free survival compared to chlorambucil (70). Also, when assessed for better alkylating agent in combination, fludarabine and cyclophosphamide regimen was

preferred compared to fludarabine or chlorambucil (71). Overall, combination regimen of alkylating agents with purine analogues have enhanced anti-leukemic activity; resulting in better overall response rate and progression-free survival when compared as single agent treatment. In recent years, rituximab, a chimeric anti-CD-20 monoclonal antibody had shown some efficacy in CLL however, combination with chemotherapy have proven to be very efficacious in CLL therapy. After rituximab, many other new generation antibodies have been tested as single agent or in combination. Several studies in the literature suggested that chemo-immunotherapy provided patients with improved overall and progression-free survival in CLL.

Together, fludarabine, cyclophosphamide, and rituximab (FCR) combination makes the most effective regimen in CLL therapy. From the clinical studies with FCR regimen, the response rate achieved was 95%, with complete response rate of 72% and the median time to disease progression was 80 months (72). Since then, long-term study results extended up to 142 months were just published (73).

When bendamustine was introduced, it was compared to chlorambucil for toxicity and efficacy, and the overall response rate to bendamustine resulted in 68%, more than double the observed response rate to chlorambucil (74).

### **Bendamustine**

Bendamustine is FDA approved for the treatment of CLL and indolent non-Hodgkin's lymphoma (NHL). Bendamustine hydrochloride (TREANDA®, Cephalon, Inc) is an alkylating agent and for the treatment of CLL, it is administered intravenously for 30 minutes on days 1 and 2 of a 28-day cycle, up to six cycles (National Cancer Institute).

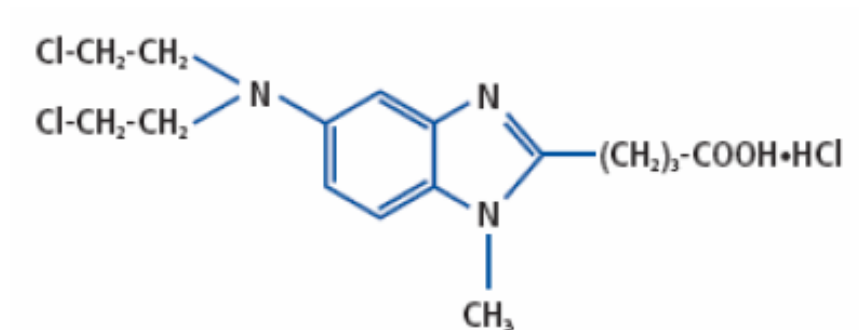
Bendamustine was first synthesized in the 1960's and was widely used in Europe, but it did not gain its FDA approval until 2008 (75). Bendamustine is an alkylating agent is efficacious in CLL therapy. Chemically, bendamustine is 4-(5-[bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl) butyric acid hydrochloride, as shown in the figure 4. Structurally, bendamustine contains an alkylating group (2-chloroethylamine), a benzimidazole ring and finally to enhance water solubility, a side chain of butyric acid was added (75). Functionally, the 2-chloroethylamine represents a nitrogen mustard group responsible for the alkylating mechanism of the molecule. The nitrogen mustard group in bendamustine resembles previously used alkylating agents such as, chlorambucil and cyclophosphamide.

Bendamustine primarily acts as an alkylating agent causing formation of intrastrand and interstrand cross-links within and between DNA strands. This feature of bendamustine generates DNA damage by inhibiting DNA replication, repair and transcription (76-78). DNA damage response initiates repair response and the cells repair the damage with minimal response. Notably, the CLL lymphocytes are characterized with an increased DNA repair ability (79,80).

### **Alkylating agents and induction of CLL cell death**

Among different damages elicited by alkylating agents, interstrand crosslinks are considered to be mainly responsible for apoptosis including death of quiescent CLL lymphocytes. Extent of DNA damage is primary lesion, followed by its maintenance and induction of DNA repair processes. Lesions are repaired by DNA repair assemblies and hence inhibition of these repair processes facilitates induction of cell death. Agents that inhibit repair processes such as purine nucleoside analog (fludarabine) result in increased damage with corollary increased cell death (81).

**Figure 4.**



**Figure 4. Chemical structure of alkylating agent, bendamustine hydrochloride.**

Chemical structure of bendamustine obtained from the FDA website,

<http://www.accessdata.fda.gov>

## **DNA alkylation and repair process**

Alkylating agents are one of the oldest classes of anticancer agents for treatment of cancer. Alkylating agents interact with nitrogen (N) and oxygen (O) atoms of DNA bases to generate a variety of covalent adducts; resulting in addition of simple methyl groups to complex alkyl groups (82). Bifunctional alkylating agents generally consist of two reactive groups that can chemically bond to separate DNA bases to form interstrand crosslinks. There are two major classes of bifunctional alkylating compounds for anti-cancer treatment; the nitrogen mustards and aziridine compounds, both of which are capable of crosslinking DNA through formation of aziridinium-ring intermediate (83). Bendamustine, chlorambucil and cyclophosphamide are all considered as nitrogen mustard compounds. These groups of alkylating compounds react with N7-guanine to form bulky N-monoadducts (84). Formation of these adducts interact with DNA bases to form guanine–guanine (G–G) and guanine–adenine (G–A) interstrand crosslinks (85). Following DNA alkylation damage response, complex repair responses are initiated with multiple enzymes and repair pathways. Major DNA damage repair mechanisms include; direct DNA repair with family of  $\alpha$ -ketoglutarate dependent dioxygenous enzymes and DNA methyl transferase (MGMT) repair enzymes (86), as well as intricate pathways of base excision repair (BER) and nucleotide excision repair (NER) (87,88).

Similar to DNA repair pathways, DNA damage recognition response is required to initiate downstream checkpoints to correct DNA damage. The DNA damage response is mediated by phosphoinositide 3-kinase-like kinase (PIKK) family members, Ataxia-telangiectasia mutated (ATM), ATM and Rad 3-Related (ATR), and DNA-PK and by the proteins poly(ADP-ribose) polymerase (PARP) family (89,90). Once the DNA lesions are recognized, ATM and ATR phosphorylate multiple mediator proteins and substrates amplifying

the DNA damage response. These mediator proteins are either directly phosphorylated by ATM/ATR or by check-point kinase 1 (CHK1) and check-point kinase 2 (CHK2) kinases (89). In response to DNA damage, ATM and CHK2 are both regulated by p53. P53 induces apoptosis, cell-cycle arrest or senescence in response to DNA damage (91,92). Overall, there are multiple controlled pathways, mediators and sensors that respond to DNA damage signals and then coordinate DNA repair pathways or lead to cell-cycle arrest and apoptosis.

## **Idelalisib and bendamustine in combination: Rationale**

PI3K pathway plays a pivotal role in many aspects of normal cellular biology and malignant tumor cells. This pathway is dysregulated in many solid tumors and hematologic malignancies. In CLL; oncogenic mutations, microenvironmental factors from BCR signaling, and constitutive activation of PI3K due to over-expressed PI3K $\delta$  isoform drive malignant B-cell proliferation and survival. Given the importance of BCR and PI3K pathway, delta-specific isoform inhibition by idelalisib was tested and FDA approved for CLL. It inhibits BCR-derived survival signals and promotes apoptosis. In solid tumors, studies have demonstrated that inhibition PI3K $\alpha/\beta$  isoforms leads to decrease in transcription in many cancer cells. Additionally,  $\alpha/\beta$  isoform-specific inhibition promoted DNA damage response. CLL is also driven by many survival factors specifically, the anti-apoptotic proteins, Bcl-2 and Mcl-1. Studies have also elucidated the role of Mcl-1 in DNA damage and repair response. Bendamustine is a DNA-damaging alkylating agent FDA approved for CLL therapy.

## **Specific Aims**

### **1. Analyze biological consequences and biomarkers impacted by idelalisib**

Investigate the biological consequences and biomarkers that are impacted by idelalisib treatment in primary CLL lymphocytes. Test the dose- and time- dependent effect on apoptosis and global RNA synthesis. Based on the RNA synthesis, evaluate the effect of idelalisib on transcript and protein expression of anti-apoptotic proteins, Mcl-1 and Bcl-2. Investigate the downstream signaling protein expression impacted by idelalisib treatment. Finally, evaluate the effect of idelalisib treatment on DNA damage and repair response.

### **2. Combination of idelalisib with bendamustine in CLL lymphocytes**

Based on the rationale, investigate the biological consequences impacted by idelalisib and bendamustine in combination treatment. Furthermore, evaluate whether the combination treatment results in synergistic, additive or antagonistic interaction for cytotoxicity. Test the effect of combination treatment on Mcl-1 and Bcl-2 transcript and protein expression and whether there are any molecular changes that affect the DNA damage and repair response pathway in combination.

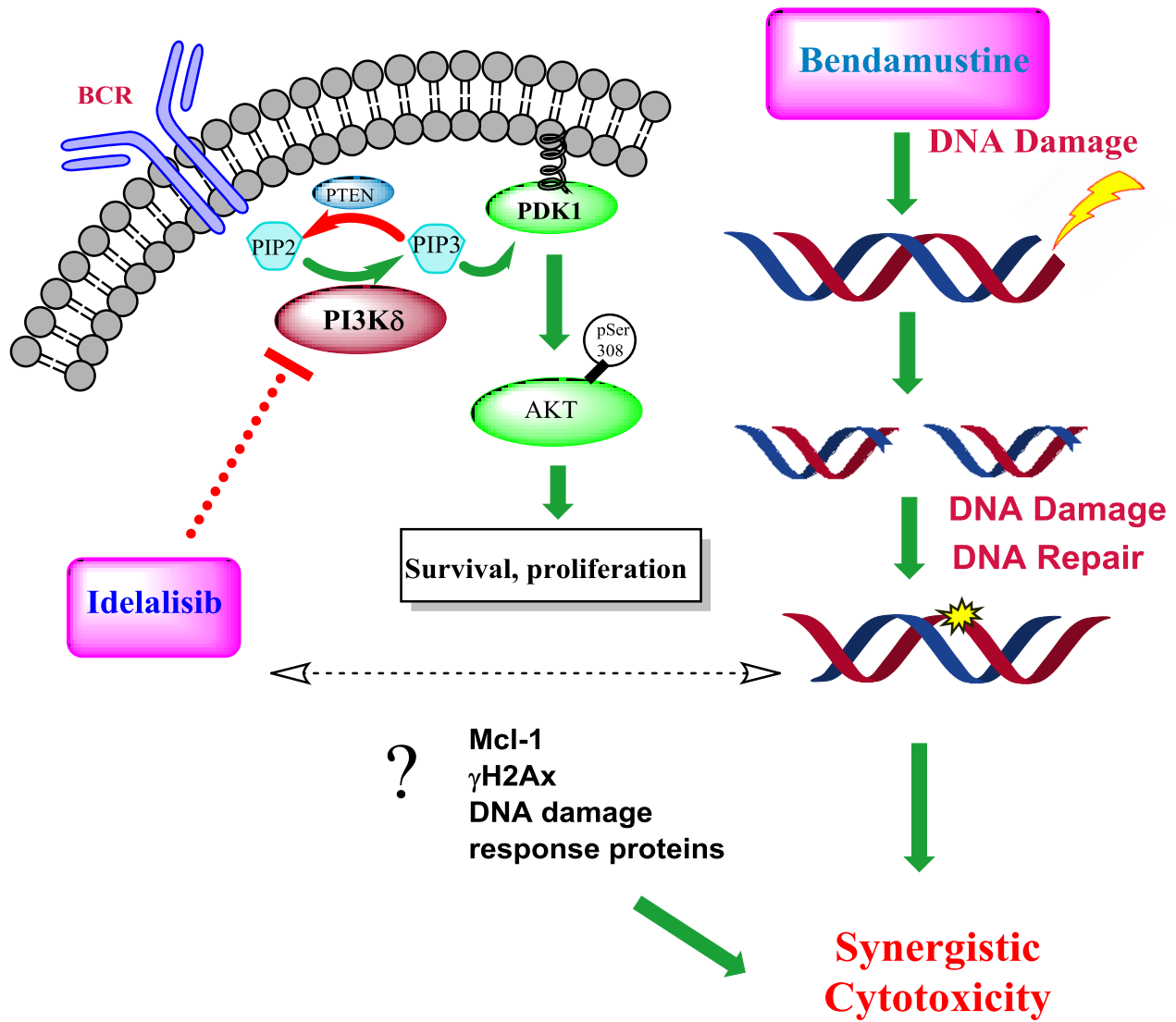
### **3. Mechanism for combination effect of idelalisib and bendamustine**

To further elucidate the mechanism of the synergy with combination treatment, evaluate Mcl-1 deletion mouse embryonic fibroblasts (Mefs) with the WT Mefs in response to bendamustine and if bendamustine treatment in Mcl-1 deleted cells sensitizes cells to apoptosis.

### **Hypothesis**

We hypothesized that idelalisib treatment will impact transcriptional and translational changes; specifically RNA synthesis, short-lived anti-apoptotic proteins, and DNA damage and repair responses, leading to enhanced apoptosis of malignant CLL cells. Combination of idelalisib with bendamustine will lead to a synergistic cytotoxicity due to decrease in survival proteins and bendamustine-induced DNA damage that will sensitize CLL cells to apoptosis (Figure 5).

**Figure 5.**



**Figure 5. Hypothesis model for combination of idelalisib and bendamustine.**

## CHAPTER 2: Materials and methods

### **Primary cells from CLL patients**

Peripheral blood samples were obtained from CLL patients at MD Anderson Cancer Center after informed consents through institutional review board-approved protocols that were in agreement with Dr. William Wierda in the Leukemia Department. All patient characteristics are listed in Table 1. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using the standard Ficoll-Hypaque (Invitrogen) density gradient centrifugation. A Coulter channelyzer counter (Beckman Coulter, Inc.) was used to determine the cell count and size for each experiment. Primary cells were cultured in RPMI-1640 medium with 10% autologous patient serum at the density of  $10^7$  cells/mL during the experiments. Endogenous cell death levels were measured for experiments using flow cytometry, and only samples with less than 50% inherent cell death were used for experiments and drug treatments.

### **Cell lines**

Mouse embryonic fibroblasts (Mefs), wild-type and Mcl-1 deficient cell lines were generously provided by Dr. Joseph T. Opferman at St. Jude Children's Research Hospital. Both cell lines are Simian virus (SV40)-transformed and the cells were maintained in Dulbecco modified Eagle medium with L-glutamine (DMEM; Invitrogen) media supplemented with 10% fetal bovine serum (FBS; Invitrogen), Pen/Strep, L-Glut, and non-essential amino acids (NEAA; GIBCO). These cells are extensively confirmed by Dr. Opferman's group as well as with Western blots. Cell lines were tested for *Mycoplasma* contamination using a MycoTect kit.

**Table 1.**

Patient	Sex	Age	Prior Rx	IGVH Mutation Status	ZAP Status (FLOW)	ZAP Status (IHC)	RAI Stage	WBC (x10 <sup>3</sup> /μL)	β2M	Genetic Aberrations
741	F	69	0	UNMUTATED	ND	POS	1	122.8	3	p53
89	M	67	0	NR	ND	NEG	0	34.8	3.2	NEG
778	F	54	0	MUTATED	ND	NEG	0	37.9	2.1	NEG
637	M	69	7	UNMUTATED	2.5	POS	0	96.2	8.6	ATM
527	M	78	0	NOT DONE	POS	ND	3	50.3	7.1	ATM
10	F	76	1	MUTATED	ND	NEG	4	58.4	4.6	T12
514	F	70	0	MUTATED	3.1	NEG	1	107.6	2.1	ND
57	M	68	1	UNMUTATED	ND	POS	1	122.2	3.2	NEG
785	M	55	6	UNMUTATED	ND	POS	1	40.3	3.2	ND
455	M	57	1	UNMUTATED	NEG	POS	1	183.5	3.1	NEG
327	F	73	0	MUTATED	ND	NEG	0	178.5	4.4	D13
607	F	72	0	MUTATED	ND	NEG	1	92.8	2.8	NEG
56	M	62	1	ND	ND	ND	2	42.7	2.1	ND
81	F	68	0	MUTATED	ND	NEG	0	214.6	2.6	D13
514	F	70	0	MUTATED	3.1	NEG	1	104.2	1.7	D13
87	F	76	0	NR	1.76	ND	1	58.1	2.4	NEG
592	F	60	0	MUTATED	2.65	ND	0	42.2	2	ND
103	M	53	0	UNMUTATED	ND	POS	1	132	2.6	ATM
419	M	73	5	NR	ND	POS	4	122.9	10.8	ATM
189	M	64	0	UNMUTATED	ND	POS	1	92.7	2.7	ATM
661	M	62	0	MUTATED	ND	NEG	0	22	2.2	D13
525	F	64	0	MUTATED	NEG	NEG & POS (2 CLONES)	0	160.3	2.2	NEG
345	F	52	0	MUTATED	36.1	POS	1	21.8	21.8	D13
424	M	74	1	MUTATED	ND	POS	4	61.9	3.9	D13
516	F	75	1	MUTATED	1.8	ND	2	120.5	2.1	D13
944	M	55	0	NR	ND	NEG	4	29.3	2.3	T12
267	F	49	0	MUTATED	ND	NEG	0	37.2	1.2	D13
247	F	67	0	MUTATED	2.02	ND	0	118.4	2.1	D13
109	M	61	1	UNMUTATED	ND	POS	1	131.4	3.1	NEG

**Table 1 continued.**

Patient	Sex	Age	Prior Rx	IGVH Mutation Status	ZAP-70 Status (Flow)	ZAP-70 Status (IHC)	RAI Stage	WBC (x10 <sup>3</sup> /μL)	β2M	Genetic Aberrations
973	M	78	3	MUTATED	1.5	ND	0	49.5	2.4	ND
68	M	93	2	UNMUTATED	30.9	POS	3	224.9	20.8	D13
773	M	48	0	MUTATED	ND	POS	2	77.7	4.7	D13
112	M	59	0	UNMUTATED	POS	POS	0	27.6	1.3	T12
781	M	72	3	UNMUTATED	ND	NEG	1	96.1	3	ATM
969	M	69	0	UNMUTATED	ND	POS	1	241.5	4.1	ATM
599	M	84	0	MUTATED	NEG	ND	0	20.7	2	D13
607	F	72	0	MUTATED	ND	NEG	0	95.9	2.8	NEG
85	F	65	6	NR	ND	NEG	4	121.8	9.3	D13
654	M	57	1	UNMUTATED	ND	POS	0	67.3	2.8	p53
820	F	59	0	UNMUTATED	ND	POS	1	72.3	2	T12
417	M	42	0	MUTATED	1.09	ND	2	157.5	2.5	ND
841	F	62	1	UNMUTATED	ND	POS	3	286.8	4.8	ND
79	F	59	0	UNMUTATED	NEG	ND	1	77.7	2.1	ATM
252	M	60	0	UNMUTATED	ND	NEG	1	29.2	3.3	D13
354	M	70	2	UNMUTATED	ND	ND	1	101	6	ND
516	F	75	1	UNMUTATED	1.8	ND	2	125	3	D13
79	F	59	0	UNMUTATED	NEG	ND	1	73.6	2.5	ATM
606	M	61	1	UNMUTATED	10.8	ND	0	19	2	ND
203	M	66	1	NR	ND	POS	4	123.8	3.7	ATM
14	M	72	0	NR	POS	ND	1	30.3	2.6	ND
90	M	68	0	MUTATED	ND	POS	4	117.7	3.3	D13
785	M	66	0	NR	ND	NEG	3	134.1	7	p53
653	M	66	0	UNMUTATED	POS	ND	2	114.9	5.4	D13
33	M	68	0	UNMUTATED	ND	POS	0	111.9	3.8	T12
758	M	49	0	MUTATED	ND	ND	0	46.1	1.4	D13
827	F	72	0	MUTATED	ND	NEG	1	41.5	2.6	D13
294	M	68	0	UNMUTATED	ND	NEG	0	21.8	2	p53
599	M	84	0	MUTATED	NEG	NEG	3	20.5	3.2	D13

**Table 1. CLL patient characteristics.**

**Key:** Prior Rx – Prior treatment; IgVH – Immunoglobulin Variable Heavy Cluster; ZAP70 – Zeta-Chain (TCR) Associated Protein Kinase 70kda; WBC – White Blood Cells; ND – Not Determined; ATM – Ataxia Telangiectasia Mutated; T12 – Trisomy 12; D13 – Deletion 13q14.3

(Invitrogen). All experiments were conducted in cell passages less than 15 and were maintained at a logarithmic growth concentration between  $10^5$  cells/mL and  $10^6$  cells/mL with 80% confluency as determined by a Coulter channelyzer with less than 10% endogenous cell death confirmed by flow cytometry.

## **Drugs**

Idelalisib (formerly known as GS-1101 or CAL-101) was provided by Calistoga Pharmaceuticals (now Gilead Sciences, Inc.). Bendamustine hydrochloride was originally obtained from Cephalon (now Teva Pharmaceuticals Industries, Ltd.) and was later purchased from Selleckchem, USA. Idelalisib was shipped in a powder form and was dissolved in dimethyl sulfoxide (DMSO); stock solutions were made at 100 mM concentrations and stored in  $-80^{\circ}\text{C}$ . Bendamustine hydrochloride was dissolved in DMSO and stock solutions were made at 30 mM concentration and stored in  $-80^{\circ}\text{C}$ .

In our study, both drugs were used in micromolar concentrations and the highest concentration for idelalisib was 10 $\mu\text{M}$  and 30 $\mu\text{M}$  for bendamustine hydrochloride. These concentrations were chosen based on reported plasma concentrations (27,34,75,77,81). Idelalisib has greater than 84% of the drug bound to human plasma proteins(34).

## **Apoptosis assays**

Primary cells were treated with DMSO alone or with drugs, then  $10^5$  cells per experiment were washed with PBS and then incubated with Annexin V (BD Pharmingen) followed by propidium iodide (PI) along with the Annexin binding buffer. After 15 minutes incubation, Annexin/PI positivity for cell death was measured using FACS Caliber flow cytometer (BD Biosciences). For WT and Mcl-1 $^{-/-}$  Mefs, cells in the supernatant and in the attached cells

(removed by Accutase; Sigma) were incubated with Annexin V followed by PI and the Annexin binding buffer were then used to measure cell death on flow cytometer.

### **Macromolecule synthesis assays**

Primary cells from CLL patients were incubated with [5,6-  $^3\text{H}$ ]-uridine (1.0mCi/mL stock; Moravek Biochemicals) for 30-45minutes for each experiment.  $10^6$  cells from primary samples were plated in 12-welled plate and each treatment was done in triplicate. 4mCi/mL of radioactive nucleoside was used for primary samples. The incorporated radioactivity was used to measure RNA synthesis using a Packard Tri-Carb liquid scintillation analyzer (GMI, Inc Perkin Elmers).

### **Gene expression assays**

Total RNA from primary cells was extracted using the RNAeasy mini kit (Qiagen N.V.). NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific) was used to quantify RNA. RNA extract was diluted to a desirable concentration of 50ng/mL for target genes and for *18S* housekeeping control gene, and then assayed with a 1-step TaqMan reverse transcription polymerase chain reaction (RT-PCR) master mix (Applied Biosystems). Gene expression levels were measured with an ABI prism 7900 sequence detection systems (Applied Biosystems). Primers and probes for *MCL1* (Hs0172036\_m1), *BCL2* and *18S* (4333760) (Applied Biosystems) were used to detect the expression levels of the corresponding genes. Each treatment and experiment was performed in triplicate and the relative gene expression levels were normalized to *18S*, a housekeeping control.

### **Immunostaining for $\gamma$ H2AX**

Post-treated primary CLL cells ( $5 \times 10^6$  cells) were washed twice with PBS and then fixed with ice-cold 70% ethanol overnight at 4°C or for longer storage in -20°C. Ethanol was removed by centrifugation and then cells were washed twice with PBS and twice with PBS with 1% bovine serum albumin (BSA) and blocked with 1% BSA. After blocking, cells were incubated with primary phospho-Histone 2AX (Ser139) or  $\gamma$ H2AX antibody (EMD Millipore) for 2hr at room temperature with gentle shaking. After incubation, cells were washed three times with PBS with 0.5% BSA and cells were incubated with FITC-labeled IgG secondary antibody for 1hr at room temperature protected from light followed by three washes with PBS with 0.5% BSA. Cells were then co-incubated with 10  $\mu$ g/mL PI and 2.5  $\mu$ g/mL DNase-free RNase for 30 mins at room temperature. The samples were analyzed by flow cytometry and compared to the baseline, an upward shift of the florescent signal was gated as percent positive for DNA damage.

### **Protein expression assays with immunoblots**

Control (untreated) and drug-treated primary CLL cells were lysed using a radioimmunoprecipitation assay buffer (RIPA; Upstate Biotechnology) and sonication to collect lysates. The protein concentration was measured using Bio-Rad DC protein assay (Bio-Rad Laboratories). Total prepared protein lysates (30-50 $\mu$ g) were loaded into 10% or 4-12% gradient bis-tris polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Bio-Rad Laboratories), followed by overnight (4°C) probe with target-specific primary antibodies. Membranes were washed three times with PBS-tween (0.1%) from the primary antibody and then incubated with infrared-labeled secondary antibodies for 1 h at room temperature, and then the protein bands were visualized and quantitated using an Odyssey

infrared imager (LI-COR Biosciences). Table 2 lists antibodies name and source that were used in the project.

### **Data analyses**

All plotted graphs were prepared and analyzed using Graphpad Prism software version 5 (San Diego, California). Primary cells and cell lines data were performed at least in triplicates (more n's for patient samples) and cell line data was presented as mean value  $\pm$  standard error of the mean (SEM). For each drug treatments, DMSO was used as a vehicle control. Single drug treatment experiments compared to the DMSO control, were analyzed by the Student paired, 2-tailed *t*-test. For combination drug treatment study, fractional analysis was used to determine if the combination methods lead to less than, equal to or more than the additive effect on induction of apoptosis (Calculusyn Software, Chou-Chou Talalay method) (93). Phosphorylated and total protein expression levels detected by immunoblots were quantified using Odyssey software for the Odyssey Infrared Imaging System (LI-COR Biosciences), and then normalized to the DMSO control. For immunoblot analyses, a ratio of phosphorylated protein to the total protein level was calculated for the phosphorylated proteins, and the ratio of protein to GAPDH level was done for total protein targets.

**Table 2.**

	<b>Primary Antibodies</b>	<b>Company Source</b>
<b>1</b>	Mcl-1	Santa Cruz Biotechnology, Santa Cruz, CA
<b>2</b>	Bcl-2	Dako, Carpinteria, CA
<b>3</b>	Phospho-H2AX (Ser139)	EMD Millipore, Billerica, MA
<b>4</b>	Total H2AX	EMD Millipore, Billerica, MA
<b>5</b>	GAPDH	Cell Signaling Technology, Beverly, MA
<b>6</b>	Phospho-ATM (Ser1981)	EMD Millipore, Billerica, MA
<b>7</b>	Total ATM	Abcam, Cambridge, MA
<b>8</b>	Phospho-p53 (Ser15) CS9284	Cell Signaling, Danvers, MA
<b>9</b>	Total p53	EMD Chemicals, Gibbstown, NJ
<b>10</b>	Phospho-GSK3 $\beta$ (Ser9) CS9336	Cell Signaling, Danvers, MA
<b>11</b>	Total GSK3 $\beta$ CS9315	Cell Signaling, Danvers, MA
<b>12</b>	Mcl-1 CS5453	Cell Signaling, Danvers, MA
<b>13</b>	Phospho-AKT (Ser473) CS9271	Cell Signaling, Danvers, MA
<b>14</b>	Total AKT	Cell Signaling, Danvers, MA
<b>15</b>	Phospho-Chk2 (Thr68) CS2661	Cell Signaling, Danvers, MA
<b>16</b>	Total Chk2 SC5278	Santa Cruz, Biotechnology, Santa Cruz, CA

**Table 2. Antibodies Source**

## **CHAPTER 3: Results**

### **Aim 1: Analyze biological consequences and biomarkers impacted by idelalisib**

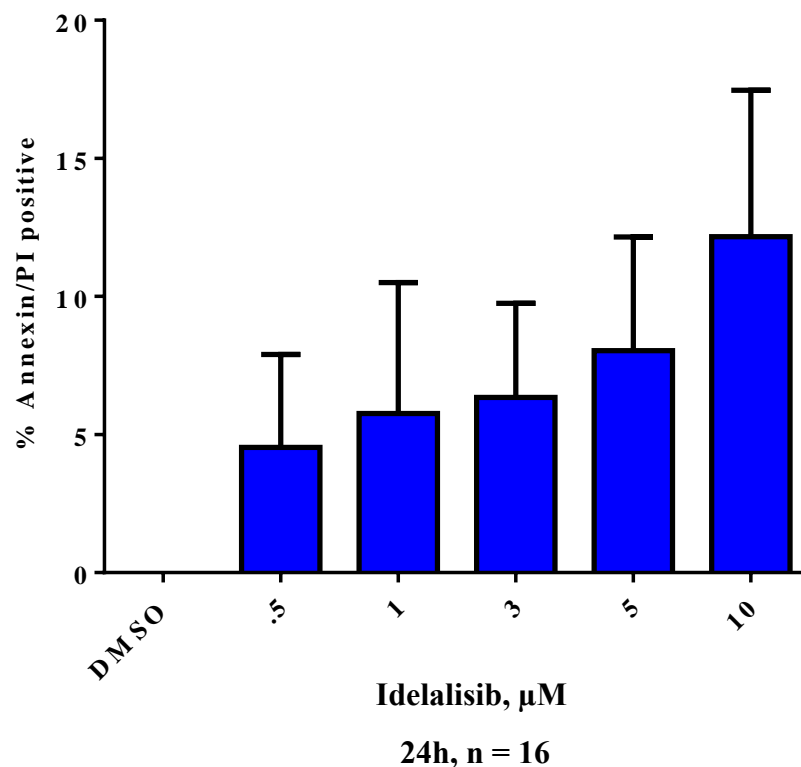
#### **1.1 Idelalisib treatment induced dose-dependent apoptosis in CLL lymphocytes**

To investigate the biological effect of idelalisib in primary CLL cells, we isolated malignant PBMCs from peripheral blood of patients with CLL. These cells were used fresh for all investigations. Primary cells were treated with DMSO (served as a control) or varying doses of idelalisib (0.5, 1, 3, 5, 10  $\mu$ M) for 24 h. These doses were selected based on literature review and company recommendations. Extent of apoptosis was measured using flow cytometry and was compared with the control. Generally in control (DMSO treated), there was 1 and 3% cell death. This percent increased in a dose-dependent manner after 24 h treatment with idelalisib, as seen in figure 6. Each graph represents percent Annexin/PI positive cell death observed in these CLL patient lymphocytes (n=16 for 24 h).

#### **1.2 Idelalisib treatment induced time-dependent apoptosis in CLL lymphocytes**

To determine if the cell death was further enhanced after long-term treatment with idelalisib, CLL PBMCs from 3 different patients were treated with 5  $\mu$ M of idelalisib for 24, 48, and 72 h and cell death was measured using flow cytometry (Figure 7). % Annexin/PI positive cells, normalized to DMSO control for each patient samples were 23, 24, and 22 at 24 h, 35, 30, and 46 at 48 h, and 48, 55 and 52 at 72 h. We observed time-dependent increase in apoptosis ranging from 10-30% from 24 h to 72 h. These data suggest that overall with idelalisib there was only modest cell death of CLL lymphocytes.

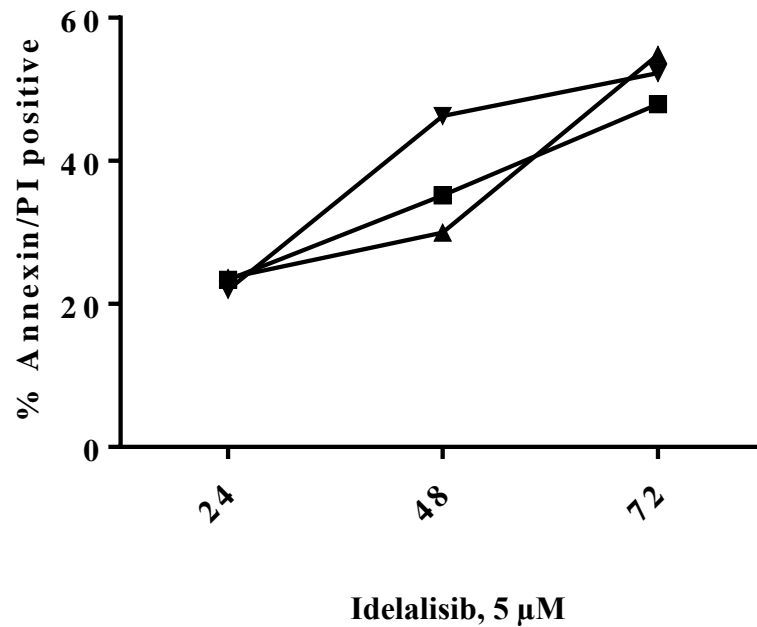
**Figure 6.**



**Figure 6. Induction of dose-dependent apoptosis by Idelalisib treatment in primary CLL cells measured by Annexin V/PI assay.**

CLL PBMCs isolated from blood samples of patients were either treated with DMSO vehicle control or different doses of idelalisib (0.5, 1, 3, 5, 10  $\mu\text{M}$ ) for 24 h. The cells were then harvested and then stained with Annexin V for 15 mins followed by 5 mins of PI. Apoptosis for each treatment was measured using flow cytometry; the apoptotic cells population was indicated in the upper right and lower right quadrants. Dose response experiments were performed in 16 CLL patients (CLL516, 944, 267, 109, 781, 973, 056, 741, 089, 778, 637, 527, 785, 455, 327, and 419). The graph represents percent Annexin/PI positive cells.

**Figure 7.**



**Figure 7. Induction of time-dependent apoptosis by idelalisib treatment in primary CLL cells measured by Annexin V/PI assay.**

Time-dependent Annexin-V/PI positivity was measured by idelalisib in primary CLL cells treated with DMSO control or 5  $\mu$ M idelalisib for different time points (24, 48, 72 h), stained with Annexin V/PI followed by measuring the apoptosis levels on flow cytometry. Time-dependent experiments were performed in 3 patients (CLL514, 087, 592).

### **1.3 Effect of idelalisib treatment on global RNA synthesis**

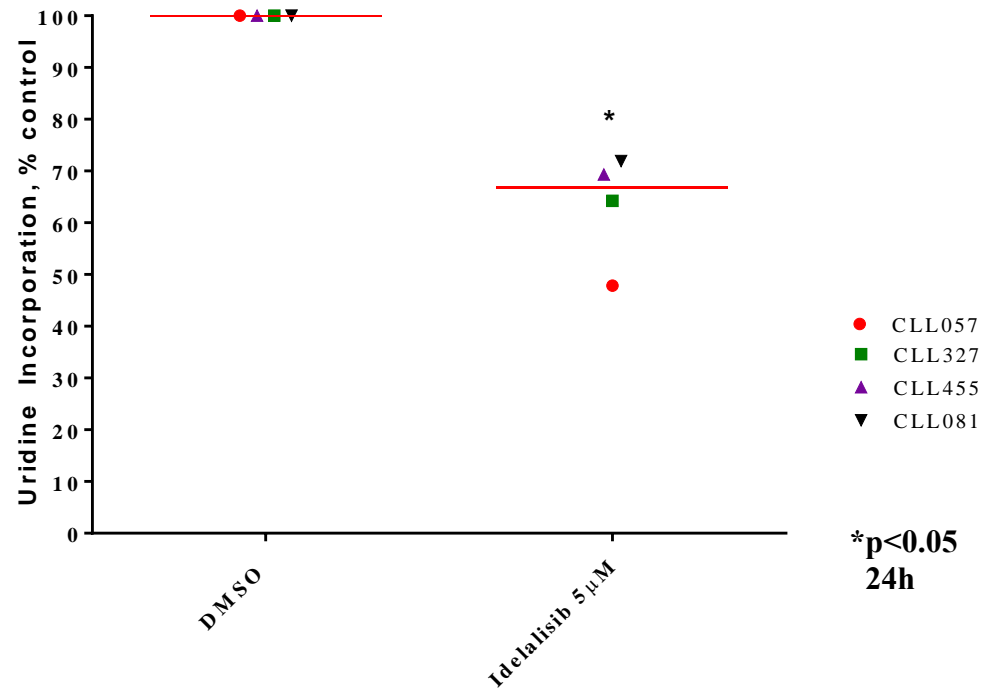
Previous studies have demonstrated that inhibition of PI3K  $\alpha$  and  $\beta$  isoforms result in a decrease in transcription. To assess if idelalisib, a PI3K $\delta$  inhibitor, will have a similar response, primary CLL cells were treated with DMSO control or 5  $\mu$ M of idelalisib for 24 h. During the last 30 mins of incubation (prior to harvesting cells), radioactively-labeled uridine was added for incorporation into the newly synthesized RNA. The incorporated radioactivity was measured using scintillation counter and the results were measured as DPM/cell, and then normalized to the DMSO control.

The graph in figure 8 represents four patient sample data points at 24 h after treatment with 5  $\mu$ M idelalisib. In control (DMSO treated only) samples, the RNA synthetic capacity varied and was between 0.002 and 0.005 dpm/cell. Treatment with idelalisib significantly reduced RNA synthesis capability in these CLL patient lymphocytes, ranging between 0.001 and 0.002 dpm/cell. The observed decrease with idelalisib treatment in RNA synthesis, when normalized to DMSO control, ranges between 47% and 71%. Results from these 4 patient samples were analyzed using paired 2-tailed student's t-test.

### **1.4 Effect of idelalisib on anti-apoptotic gene expression levels**

Given that idelalisib treatment decreases RNA synthesis in CLL lymphocytes, short-lived mRNA expression levels may also be affected. CLL cells have a malfunctioning apoptotic response and the Bcl-2 family anti-apoptotic proteins mainly contribute for abnormal CLL cell proliferation and survival.

**Figure 8.**



**Figure 8. Inhibition of global RNA synthesis by idelalisib in primary CLL cells.**

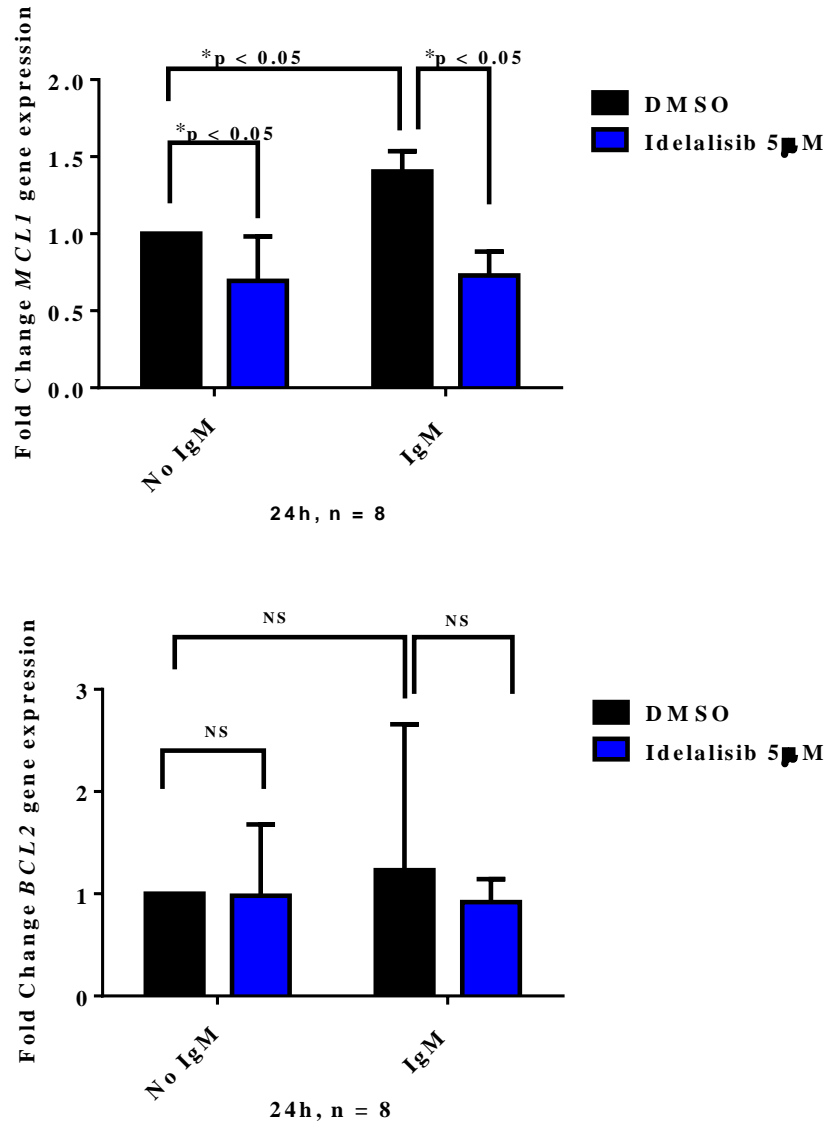
CLL PBMCs isolated from patient samples were treated with DMSO or with 5  $\mu$ M idelalisib for 24 h. The cells were co-incubated with [5,6- $^3$ H]-uridine for 30 mins before harvesting, then the amount of radioactivity incorporated into cells was measured using a scintillation counter. DPM/cell was calculated and normalized to DMSO control. Experiments from 4 patient samples (CLL081, 057, 327, 455) were done in triplicates. A Student's paired 2-tailed t-test was performed for these patient samples.

Activation of the BCR pathway also contributes to cell maintenance, proliferation and survival. Previous studies have shown that Mcl-1, a critical component in the survival pathway, is up-regulated in CLL cells and plays a role in patient prognosis and treatment outcomes. Therefore, we assessed the effect of idelalisib on mRNA expression levels of *MCL1* and *BCL2*. CLL PBMCs were either left unstimulated or stimulated with IgM for BCR pathway activation, then treated with DMSO control or 5 $\mu$ M of idelalisib for 24 h. Post 24 h treatment, cells were harvested and total RNA was extracted for real time RT-PCR experiment.

Our data in figure 9 show that without any IgM stimulation, idelalisib-treatment results in a significant 30% decrease in *MCL1* gene expression compared to DMSO control. CLL PBMCs with IgM stimulation alone increased *MCL1* gene expression by 40% when compared to unstimulated DMSO control. Interestingly, combination of IgM stimulation with idelalisib treatment resulted in 28% decrease in *MCL1* gene expression.

Bcl-2 is another anti-apoptotic protein known to be up-regulated in CLL and plays a major role in CLL proliferation and survival. Our results in figure 9 show no changes in the *BCL2* gene expression without IgM stimulation and idelalisib treatment in the CLL cells. There was a modest increase in *BCL2* mRNA levels when the CLL cells were stimulated with IgM alone. Combination of IgM and idelalisib did not have any significant changes in the *BCL2* gene expression levels. The results in the figure represent 8 different patient samples and were analyzed by 2-tailed paired student's t-test.

**Figure 9.**



**Figure 9. Decline of *MCL1* mRNA gene expression levels with no changes in *BCL2* gene expression levels by idelalisib treatment in primary CLL cells.**

Primary cells were either untreated or stimulated with 10  $\mu$ g/ml IgM for 30 mins. Following the stimulation, cells were treated with DMSO or 5  $\mu$ M idelalisib for 24 h. Cells were harvested and total RNA was extracted and quantified. Isolated RNA was analyzed by real-time RT-PCR with primers and probes for *MCL1* and *BCL2* mRNA transcripts. *MCL1* and *BCL2* gene expression levels were measured and normalized to the *18S* endogenous control and each experiment was normalized to the DMSO control in the experiment. The results are shown for 8 patient samples (CLL075, 483, 454, 293, 068, 354, 653, and 516).

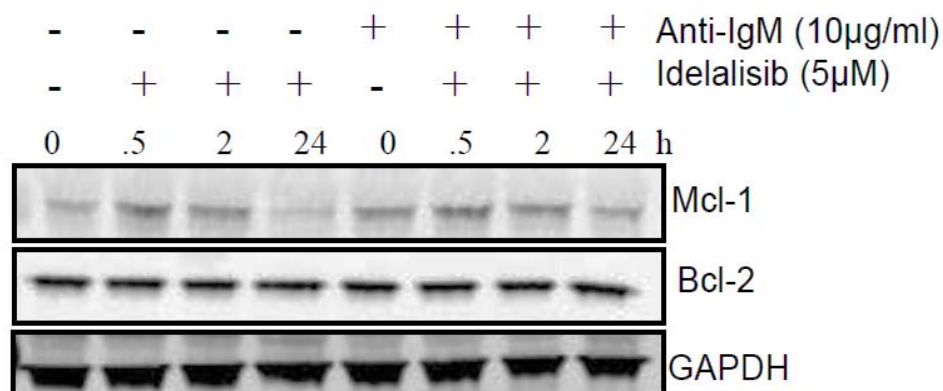
### **1.5 Effect of idelalisib on anti-apoptotic protein expression levels**

Our observation includes decrease in global RNA synthesis, decrease in Mcl-1 mRNA transcript levels and an overall modest cell death with idelalisib treatment. We further investigate the changes of anti-apoptotic protein levels following idelalisib treatment. To evaluate changes of the Mcl-1 and Bcl-2 protein expression levels, CLL cells were treated with 5  $\mu$ M of idelalisib or DMSO for 24 h and 48 h. Mcl-1 and Bcl-2 protein levels were measured by immunoblots and normalized to GAPDH compared to DMSO. Immunoblots in figure 10 show a dramatic decrease in Mcl-1 protein levels with idelalisib treatment at 24 and 48 h. Compared to GAPDH, Bcl-2 protein levels showed no changes with idelalisib treatment at 24 h and 48 h. Overall, Mcl-1, a survival factor in CLL, is significantly decreased by idelalisib both at transcript and protein levels and no significant changes in the Bcl-2 transcript and protein levels were observed.

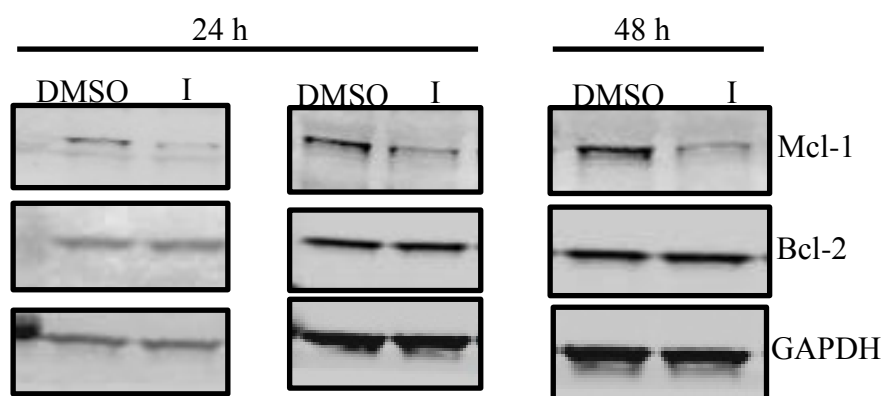
### **1.6 Effect of idelalisib on downstream signaling protein expression levels**

BCR signaling pathway plays an important role in CLL. BCR signaling kinases such as PI3K delta amplify signals and induce survival and proliferation in CLL cells through the activation of downstream kinases such as MAPK, PI3K/AKT and NF- $\kappa$ B signaling pathways. These signaling events are either amplified due to over-expression or have constitutive activation of these kinases. To investigate the effect of idelalisib on signaling proteins downstream of PI3K $\delta$ , we analyzed the phosphorylation of AKT, GSK3 $\beta$  and Mcl-1 levels. CLL PBMCs were either left untreated or stimulated with IgM for 30 mins followed by treatment with DMSO or 5  $\mu$ M of idelalisib. We measured phosphorylation and total protein levels at 0, 0.5, 1, 2, and 24 h.

**Figure 10.**



**CLL599**



**CLL525**

**CLL103**

**CLL103**

**Figure 10. Decrease of Mcl-1 protein expression level, with no changes to the Bcl-2 protein expression level by idelalisib treatment in primary CLL cells.**

Primary cells were either untreated or stimulated with 10 µg/ml IgM for 30 mins and then treated with 5 µM of idelalisib (I) for different times (0, 0.5, 2, 24 h). Cells were harvested and protein lysates were analyzed using immunoblots to detect the protein expression levels for Mcl-1 and Bcl-2. GAPDH was used as control for equal protein loading. Quantitation for the immunoblots for each patient sample was performed by measuring the ratios of Mcl-1 or Bcl-2 to GAPDH for idelalisib treated samples, and then normalized to DMSO.

Notably, inhibition of PI3K delta by idelalisib decreases phospho-AKT (Ser473) protein expression levels in a time-dependent manner, compared to the total AKT levels in these CLL patient cells in figure 11. Although, given patient heterogeneity, there were variable levels of phospho-protein expression levels observed.

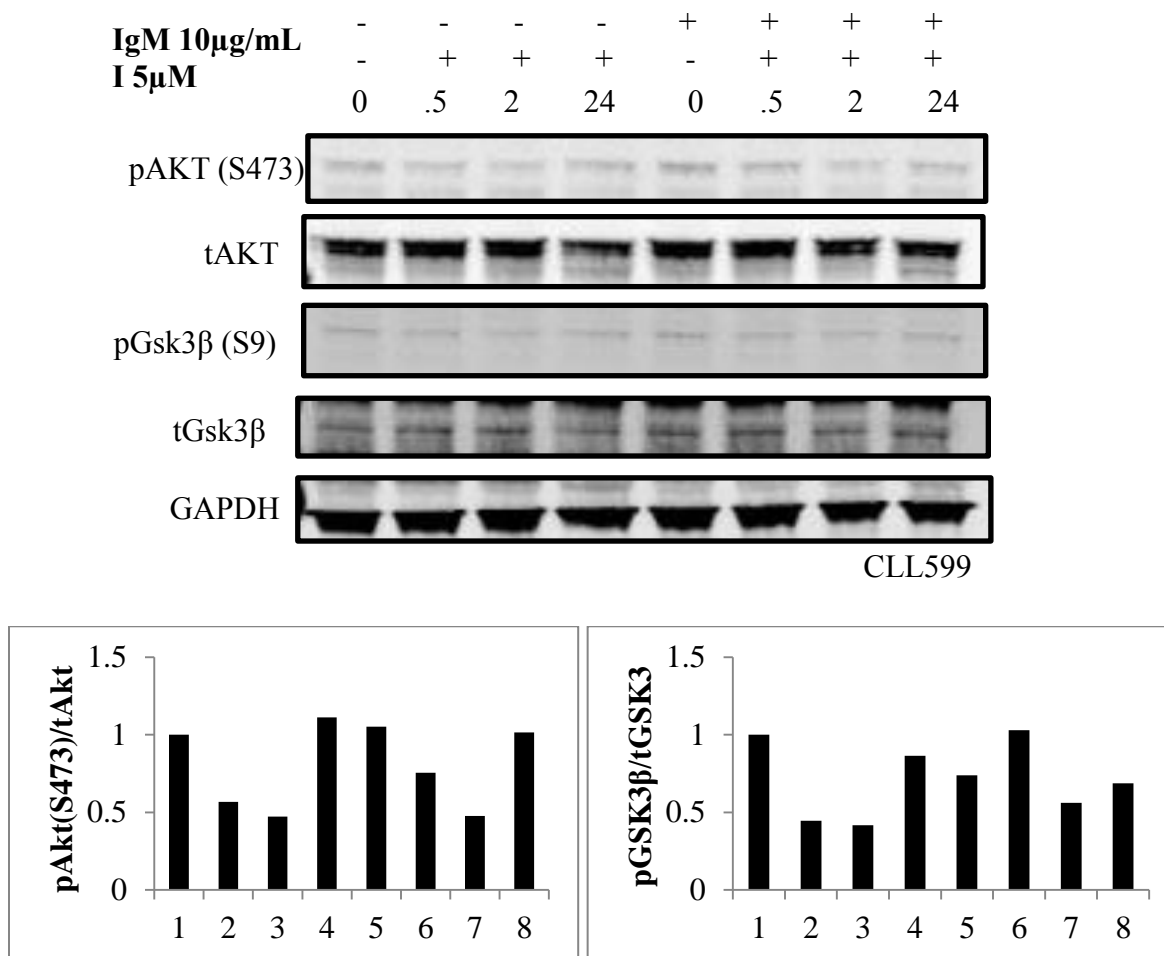
Downstream of PI3K/AKT activation, GSK3 phosphorylation is reported to regulate Mcl-1 stability directly. Mcl-1 protein has a short half-life, with rapid induction and destruction of Mcl-1 and this is proposed by several mechanisms. Therefore, we wanted to investigate if there was a direct relationship of GSK3 $\beta$  and phospho-Mcl-1 activity that would lead to decrease in total Mcl-1 protein levels. Interestingly, we detected variable expression levels of phospho-GSK3 $\beta$  and total GSK3 $\beta$  protein with or without idelalisib treatment and IgM stimulation (figure 11). Similarly, compared to the total protein, phospho-Mcl-1 (Ser159/Thr163) revealed variable protein expression with or without idelalisib treatment and no significant changes in these CLL patients were observed, data not shown (n = 3).

### **1.7 Effect on DNA damage marker $\gamma$ H2AX by idelalisib in CLL lymphocytes**

There have been brief reports in the literature that suggests inhibition of PI3K  $\alpha$  and  $\beta$  isoforms result in changes in the DNA damage responses. DNA damage response can be initiated with variety of stress signals in physiological processes. To evaluate if PI3K $\delta$ isoform inhibition by idelalisib would impact in any damage response, CLL PBMCs from 6 different patient samples were either treated with DMSO or 5  $\mu$ M idelalisib for 24 h. Post treatment, cells were harvested and we performed immuno-staining of CLL cells to analyze changes in the levels of phospho-Histone 2A variant X (H2AX) at Ser139, also known as  $\gamma$ H2AX. As shown in Figure 12, in the DMSO control, there was 1-3 % H2AX phosphorylation observed by flow cytometry. Interestingly, after idelalisib-treatment, in each patient sample there was an increase

between 2-8% in  $\gamma$ H2AX. Our results demonstrate that inhibition of PI3K delta isoform by idelalisib induces modest increase in the DNA damage response marker. These 6 patient samples concluded in a significant increase in DNA damage response with idelalisib treatment when compared to vehicle control and the samples were analyzed by paired 2-tailed student's t-test.

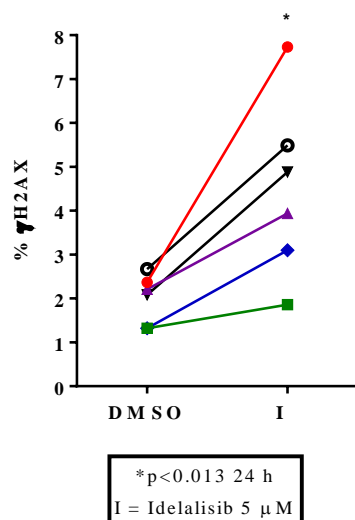
**Figure 11.**



**Figure 11. Effect on downstream signaling proteins by idelalisib treatment in primary CLL cells.**

Primary cells were either untreated or stimulated with 10 µg/mL IgM for 30 mins and then treated with 5 µM of idelalisib for different times (0h, 0.5h, 1h, 2h, 24h). Cells were harvested and protein lysates were analyzed using immunoblots to detect the protein expression levels for phospho-AKT (Ser473), phospho-GSK3β (Ser9) and phospho-Mcl-1. GAPDH was used as control for equal protein loading. Quantitation for the immunoblots for each patient sample was performed by measuring the ratios of phospho- to total proteins for idelalisib treated samples, and then normalized to DMSO.

**Figure 12.**



**Figure 12. Idelalisib treatment induces  $\gamma$ H2AX expression in primary CLL cells.**

Primary cells were either treated with DMSO control or with 5  $\mu$ M of idelalisib for 24 h. The cells were then harvested and fixed with 70% ethanol overnight. Cells were then washed with PBS and then incubated with 0.5% goat serum for 1 h, then probed with the primary antibody phospho-histone H2AX (Ser139) or  $\gamma$ H2AX (1:500) for 2 h. Cells were washed and then co-incubated with 10 $\mu$ g/mL propidium iodide and 2.5  $\mu$ g/mL DNase-free RNase for 15 mins. Cells were analyzed for fluorescence signal on flow cytometry for DNA damage response. Fluorescence-positive cells were marked as  $\gamma$ H2AX positive treated cells. The figure represents 6 CLL patients (CLL081, 514, 455, 327, 137, and 103).

## **Aim 2: Explore combination strategy of idelalisib with bendamustine in CLL**

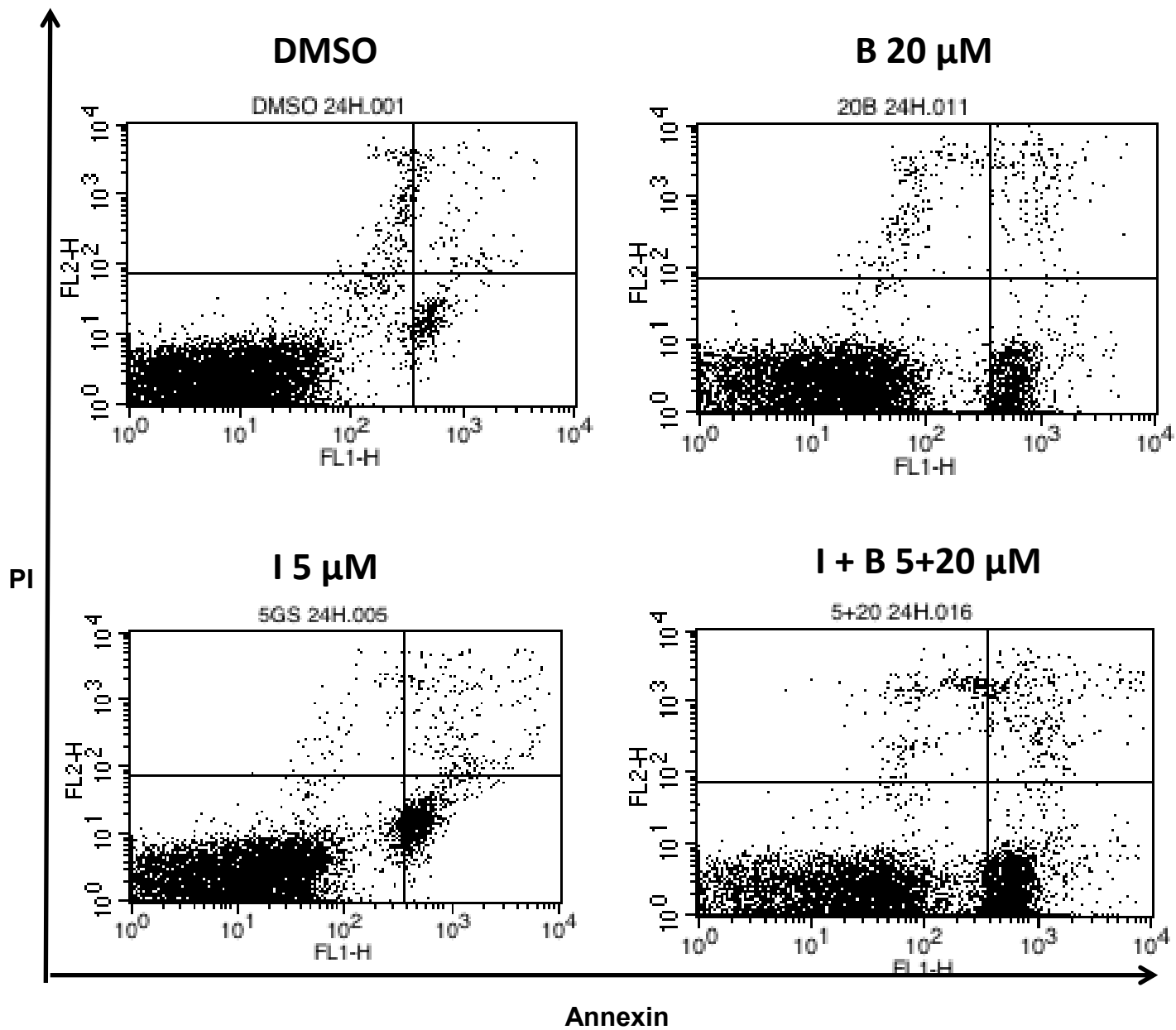
Bendamustine, an alkylating agent, is a FDA-approved drug for NHL and CLL. Bendamustine induces DNA damage response by disruption of DNA replication and transcription processes evident in both replicating and in quiescent cells. Our studies in CLL have demonstrated that idelalisib-induced cytotoxicity is modest in CLL and it targets transcriptional and translational changes as well as it leads to DNA damage response, leading to cell death of the malignant CLL cells. Based on bendamustine-induced DNA damage along with idelalisib-mediated cytotoxicity, we hypothesize that the combination of the treatment would enhance DNA damage in CLL cells and will further result in a synergistic cytotoxicity in malignant CLL cells.

### **2.1 Effect on apoptosis induction by idelalisib treatment in combination with bendamustine in primary CLL samples**

To test this hypothesis, we used 5  $\mu$ M of idelalisib and 20  $\mu$ M bendamustine. Idelalisib concentration was selected based on the single agent data in CLL cells as previously described. Bendamustine concentration of 20  $\mu$ M was selected because it is a clinically relevant dose, easily achieved in plasma at tolerated dose (74,75,77).

CLL PBMCs in figure 13 were treated with DMSO, 5  $\mu$ M idelalisib, 20  $\mu$ M bendamustine or combination of both (5 + 20  $\mu$ M) for 24 h. Cells were harvested, washed with PBS and then stained with Annexin V/PI, and the levels of apoptosis were measured by flow cytometry. The histogram in figure 13 represents induction of apoptosis indicated by the increasing number of cells in the lower and upper right quadrants paired with decreasing number of cells in the lower and upper left quadrants. Percentages of apoptotic cells resulting from each drug at its concentration and the combination are shown. When normalized to DMSO

Figure 13.



**Figure 13. Increase of apoptosis with idelalisib treatment in combination with bendamustine in primary CLL cells measured by Annexin V/PI assay.**

Primary cells were treated with DMSO, 5  $\mu$ M of idelalisib, 20  $\mu$ M of bendamustine, and then combination of idelalisib and bendamustine (5  $\mu$ M + 20  $\mu$ M, respectively). Cells were harvested and then stained with Annexin V for 15 mins followed by 5 mins of PI. The levels of apoptosis for each treatment was detected using flow cytometry and the apoptotic cells were indicated by the cell population present in the lower right, upper right, and upper left quadrants.

control, single agent treatment with idelalisib alone induced 5% cell death, bendamustine alone induced 11% and the combination of both drugs induced 28% cell death. At this dose concentration, there was enhanced apoptosis observed in combination when compared to single agent treatment.

## **2.2 Dose-dependent induction of apoptosis by combination treatment of idelalisib with bendamustine**

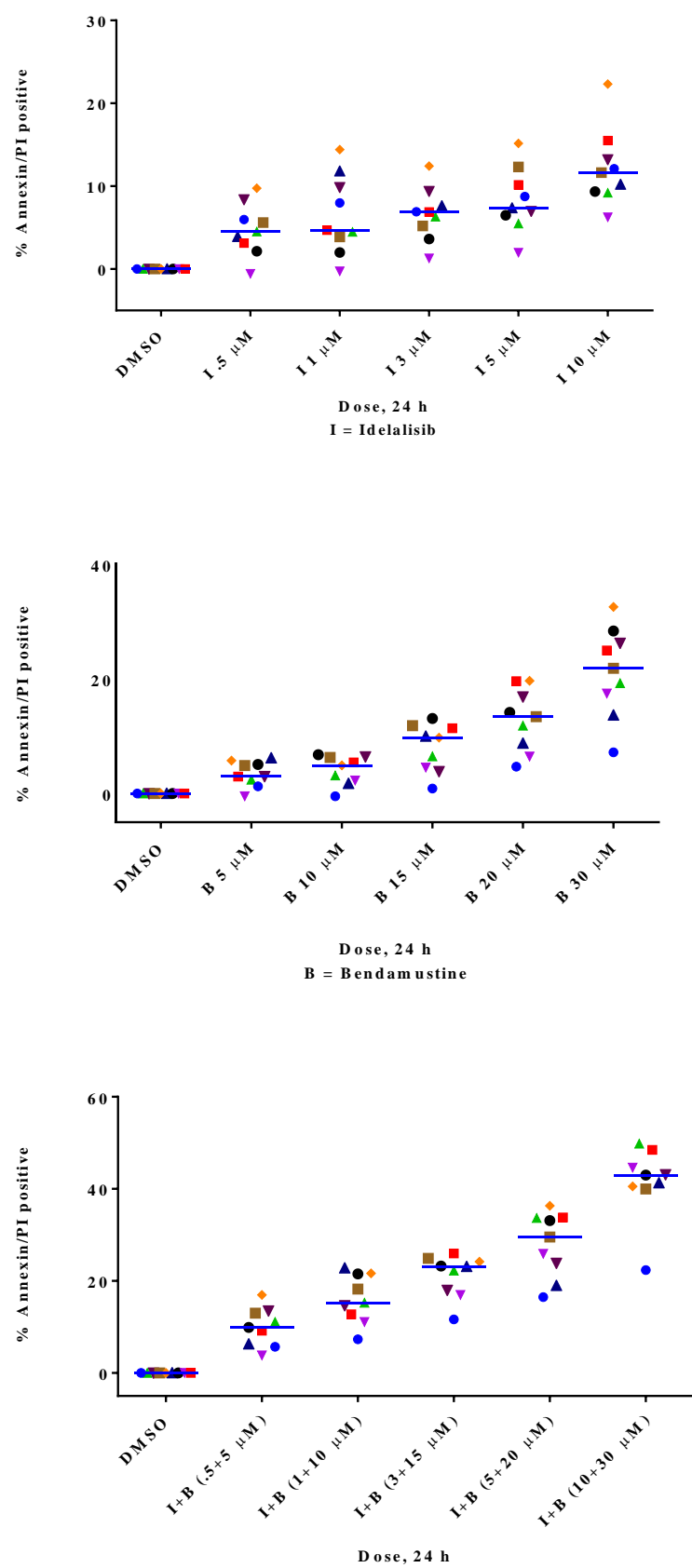
To further evaluate apoptosis in combination, we performed a dose response experiment with varying dose as single agent and in combination. CLL PBMCs were treated with DMSO, 0.5, 1, 3, 5, and 10  $\mu\text{M}$  of idelalisib, 5, 10, 15, 20 and 30  $\mu\text{M}$  of bendamustine and then in combination 0.5+5, 1+10, 3+15, 5+20, and 10+30  $\mu\text{M}$  of idelalisib and bendamustine, respectively. Cells were harvested, washed with PBS and then stained with Annexin V/PI, and the levels of apoptosis were measured by flow cytometry. Results in Figure 14 are separated into 3 graphs and represents 9 different CLL patients at 24 h treatments. Each colored symbol represents a different patient at a given dose concentration.

Idelalisib treatment as a single agent induced dose-dependent apoptosis; at 0.5  $\mu\text{M}$ , cell death ranged 1-9%, at 1  $\mu\text{M}$  cell death ranged 1-14%, at 3  $\mu\text{M}$  cell death ranged 2-13%, at 5  $\mu\text{M}$  cell death ranged 2-16%, and finally at 10  $\mu\text{M}$  cell death ranged 6-16%.

Similarly, bendamustine treatment as a single agent induces dose-dependent apoptosis; at 5  $\mu\text{M}$  cell death ranged 1-6%, at 10  $\mu\text{M}$  cell death ranged 1-7%, at 15  $\mu\text{M}$  cell death ranged 1-13%, at 20  $\mu\text{M}$  cell death ranged 5-20%, and finally at 30  $\mu\text{M}$  cell death ranged 7-33%.

Combination of idelalisib and bendamustine in non-constant ratios of concentrations showed an enhanced apoptosis in a dose-dependent manner in these malignant CLL cells. %

Figure 14.



**Figure 14. Increase of apoptosis with idelalisib treatment in combination with bendamustine in primary CLL cells measured by Annexin V/PI assay.**

Primary cells were treated with DMSO, idelalisib (0.5, 1, 3, 5, or 10  $\mu$ M), bendamustine (5, 10, 15, 20 or 30  $\mu$ M) and then combination of idelalisib and bendamustine (0.5+5, 1+10, 3+15, 5+20, 10+30  $\mu$ M respectively) for 24 h. Cells were harvested and then stained with Annexin V for 15 mins followed by 5 mins of PI. The levels of apoptosis for each treatment was detected using flow cytometry and the apoptotic cells were indicated by the cell population present in the lower right, upper right, and upper left quadrants. Each color shape represents a CLL patient sample, showing 9 CLL patient samples (516, 944, 267, 109, 973, 781, 247, 661, and 112).

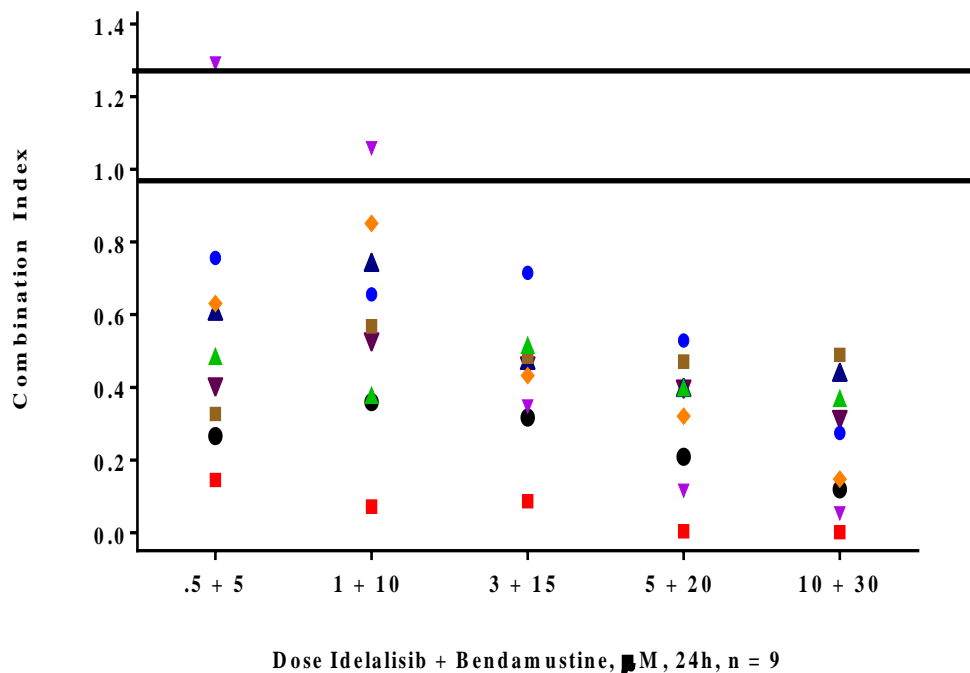
Annexin/PI positivity with 0.5+5  $\mu$ M I+B ranged 4-13%, 1+10  $\mu$ M ranges 7-23%, 3+15  $\mu$ M ranged 11-26%, 5+20  $\mu$ M ranged 16-36%, and finally 10+30  $\mu$ M of the combination treatment ranged between 23-49%. Overall, given patient heterogeneity, idelalisib and bendamustine induced dose-dependent apoptosis and this effect was enhanced in combination treatment.

### **2.3 To evaluate the combination index of idelalisib treatment in combination with bendamustine**

From figure 14, extensive dose responses of the two drugs are given and we wanted to further investigate whether this combination study results in synergistic interaction between idelalisib and bendamustine. Combination of idelalisib with bendamustine resulted in enhanced apoptosis when compared to single agents alone. Drug combination studies and their synergy quantifications are widely used to achieve therapeutic effect, reduce dose and toxicity, and minimize drug resistance. The apoptotic population from the combination treatment was used for fractional analysis. Calcsyn software was used for an output of the combination index given by the fraction affected with the non-constant ratios of the drugs. A combination index (CI) of less than 0.8 is indicative of strong synergistic interaction of the drugs; CI between the range of 0.8 and 1.2 is additive, and CI greater than 1.2 is considered as an antagonistic drug interaction (93).

Combination index of five dose ratios are shown in figure 15 (0.5+5, 1+10, 3+15, 5+20, and 10+30  $\mu$ M) and except for one CLL patient, all the combination ratios indicate a strong synergistic interaction of idelalisib and bendamustine. At lower combination concentrations of the drugs, one CLL patient showed an antagonistic (0.5+5  $\mu$ M) and two CLL patients showed an additive interaction (1+10  $\mu$ M). Overall, CI values from 9 different patients suggest the

**Figure 15.**



*Combination Index  $> 1.2$  is Antagonism*

*Combination Index  $0.8 < x < 1.2$  is Additive*

*Combination Index  $< 0.8$  is Synergism*

**Figure 15. Evaluation of the combination index of idelalisib and bendamustine in combination treatment in primary CLL cells.**

The apoptotic population from the combination treatment was used for fractional analysis. The CalcuSyn software was used for an output of the combination index calculated by the fraction affected and the non-constant ratios of the drugs. A combination index (CI) of less than 0.8 is indicative of synergistic interaction of the drugs, CI between 0.8 and 1.2 is additive, and CI above 1.2 is antagonistic drug interaction.

combination of idelalisib with bendamustine resulted in enhanced toxicities and synergistic interaction.

#### **2.4 Effect on anti-apoptotic gene expression levels by idelalisib and bendamustine combination treatment**

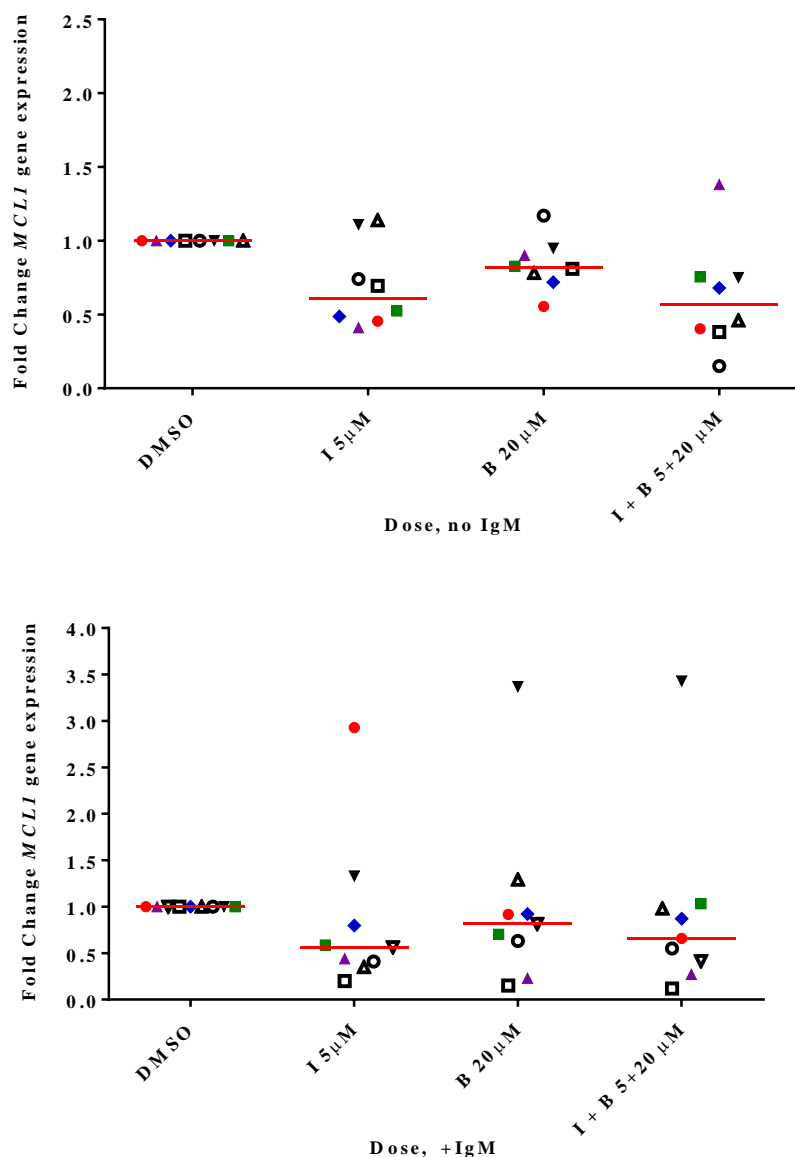
Our data strongly suggests that combination of idelalisib with bendamustine results in synergy. We next investigated the mechanism of synergy resulting from idelalisib and bendamustine treatments. Mcl-1 is a survival factor in CLL and it is known to be highly up-regulated in CLL. Findings from literature suggest a decrease in Mcl-1 radio-sensitizes cells and promotes cell cytotoxicity. Further evidence shows Mcl-1 involved in DNA damage and repair response. Taken together, we hypothesize that decrease in Mcl-1 by idelalisib may sensitize CLL cells to bendamustine and promote toxicity of the malignant cells.

To test the effect of idelalisib and bendamustine on short-lived mRNA expression levels, we carried out experiments to analyze the change of *MCL1* gene levels with combination effect. CLL PBMCs were either left untreated or stimulated with IgM for 30 mins, followed by treatments with DMSO control, 5  $\mu$ M idelalisib, 20  $\mu$ M of bendamustine, and combination of both at these concentrations. Post 24 h treatment, cells were harvested and total RNA was extracted for real time RT-PCR experiment.

Figure 16 shows CLL cells without IgM stimulation, idelalisib treatment resulted in a decrease in 30% *MCL1* gene expression compared to DMSO. Combination of idelalisib with bendamustine also resulted in decrease in *MCL1* gene expression.

Similarly, when CLL cells were stimulated with IgM and then treated with DMSO, idelalisib, and bendamustine, and combination of both with same concentrations, the results

**Figure 16.**



**Figure 16. Effect of idelalisib and bendamustine combination treatment on *MCL1* gene expression in primary CLL cells.**

Primary cells were either untreated or stimulated with 10 µg/mL IgM for 30 mins. The cells were then treated with DMSO, 5 µM of idelalisib (I), 20 µM of bendamustine (B), and then combination of idelalisib and bendamustine (5 µM + 20 µM, respectively). Cells were harvested and total RNA was extracted and quantified. Isolated RNA was analyzed by real-time RT-PCR with primers and probe for *MCL1* mRNA transcript. *MCL1* gene expression levels were measured and normalized to the *18S* endogenous control and each experiment was normalized to the DMSO control in the experiment. The figures above represent 8 CLL patients (CLL 516, 068, 454, 483, 354, 203, 653, and 075).

indicated in 28% decrease in *MCL1* gene expression levels as single agent and remained at lower levels in combination effect. Our results for both figures represent 8 different patient samples.

## **2.5 Effect on anti-apoptotic gene expression levels by idelalisib and bendamustine combination treatment**

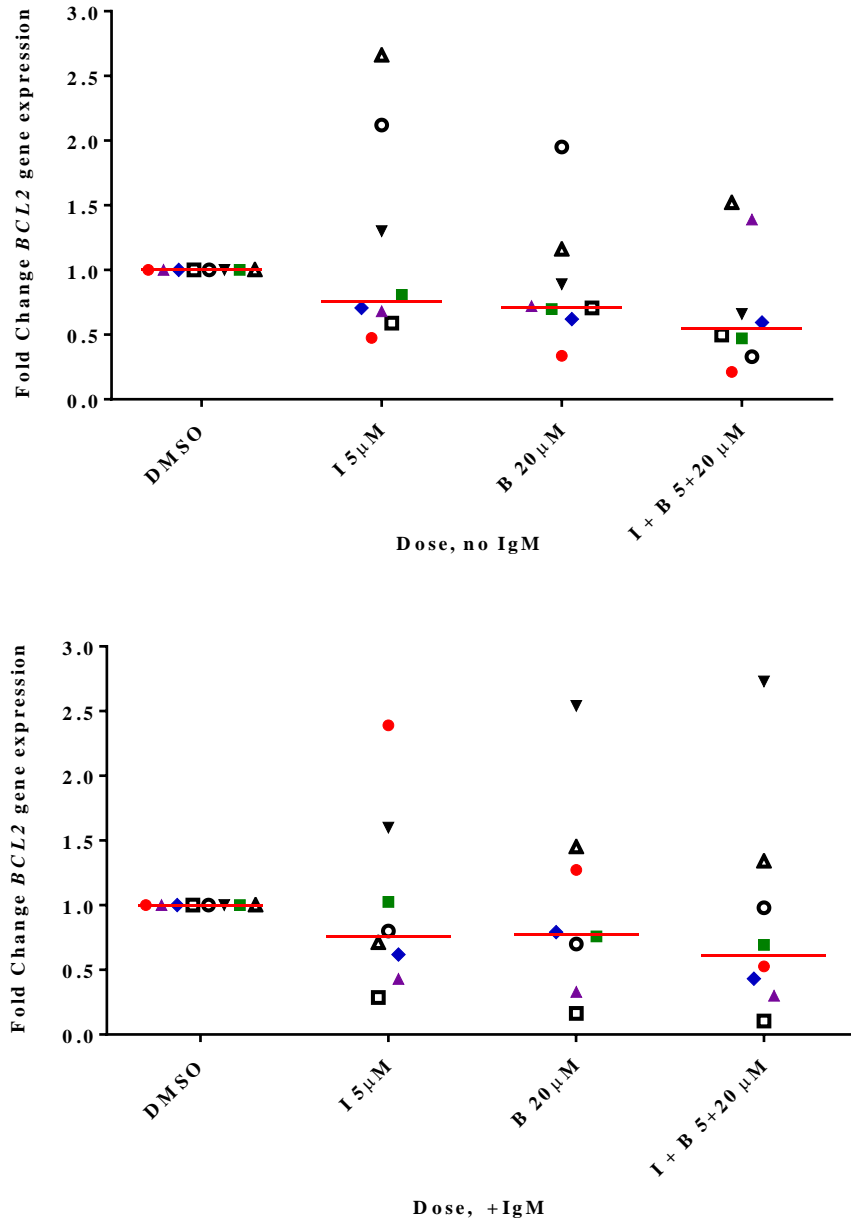
We further evaluated the effect of idelalisib on the *BCL2* mRNA expression with combination of idelalisib and bendamustine. Post 24 h, DMSO, idelalisib (5  $\mu$ M), bendamustine (20  $\mu$ M), and combination of both (5+20  $\mu$ M) treated cells were harvested and total RNA was extracted for real time RT-PCR experiment. Figure 17 shows that without IgM stimulation, there was a decrease in *BCL2* gene expression in some patients, while some patients had elevated *BCL2* mRNA levels. Similarly, combination of idelalisib with bendamustine also resulted in decrease in *BCL2* mRNA for majority of the patients, except for two patient samples with elevated mRNA levels.

Consistent with no IgM effect on *BCL2* expression levels, when CLL cells were stimulated with IgM and then treated with DMSO, idelalisib, and bendamustine, and combination with same concentrations, the results indicated a decrease in *BCL2* gene expression levels as single agents and in combination in some patients with variable elevated levels for other patients. Our results for both figures represent 8 different patient samples.

## **2.6 Effect on anti-apoptotic protein expression levels by idelalisib and bendamustine combination treatment**

We have observed even in combination treatment, changes in the *MCL1* and *BCL2* gene expression levels. Accordingly, we evaluated the changes of Mcl-1 and Bcl-2

**Figure 17.**

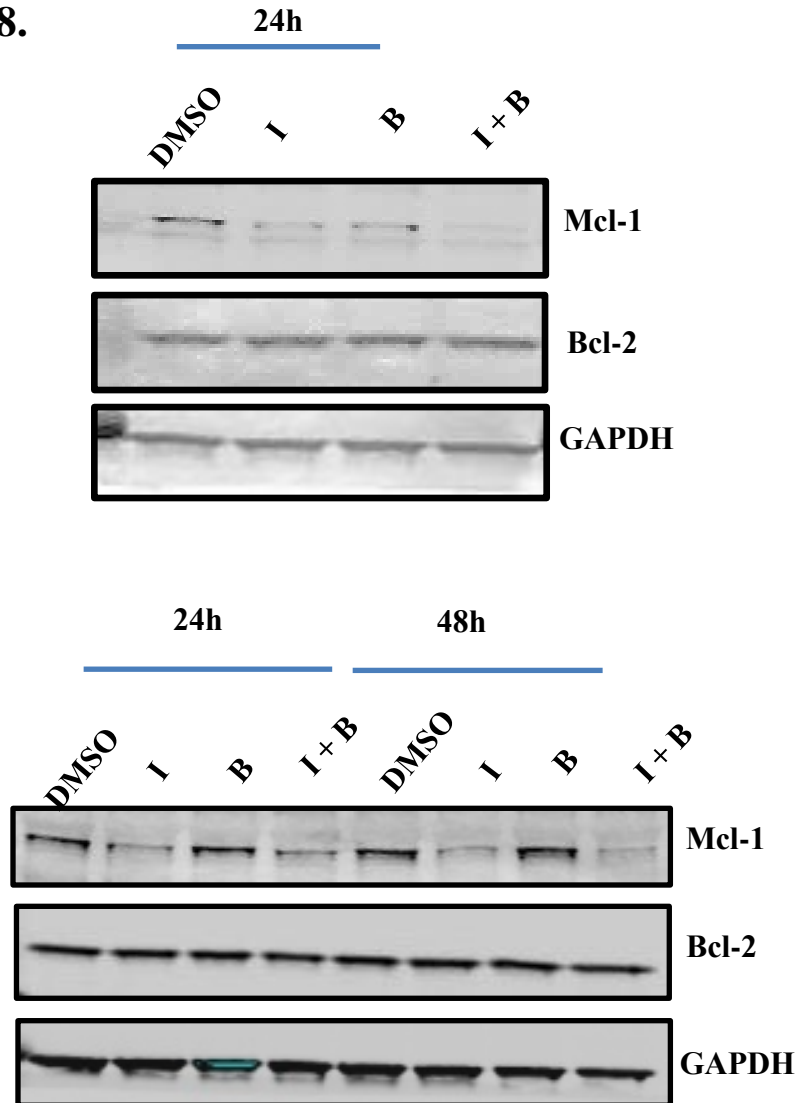


**Figure 17. Effect of idelalisib and bendamustine combination treatment on *BCL2* gene expression in primary CLL cells.**

Primary cells were either untreated or stimulated with 10  $\mu$ g/mL IgM for 30 mins. The cells were then treated with DMSO, 5  $\mu$ M of idelalisib (I), 20  $\mu$ M of bendamustine (B), and then combination of idelalisib and bendamustine (5 $\mu$ M + 20 $\mu$ M, respectively) for 24 h. Cells were harvested and total RNA was extracted and quantified. Isolated RNA was analyzed by real-time RT-PCR with primers and probes for *BCL2* mRNA transcript. *BCL2* gene expression levels were measured and normalized to the *18S* endogenous control and each experiment was normalized to the DMSO control in the experiment. The figures above represent 8 CLL patients (CLL 516, 068, 454, 483, 354, 203, 653, and 075).

protein expression. CLL PBMCs were treated with DMSO, 5  $\mu$ M of idelalisib, 20  $\mu$ M bendamustine and then the combination for 24 h and 48 h. Mcl-1 and Bcl-2 protein levels were measured by immunoblots and normalized to GAPDH compared to DMSO. Results in figure 18 indicated decrease in Mcl-1 protein levels at 24 h and 48 h with idelalisib alone and in combination treatment. There was variable expression of Mcl-1 protein with bendamustine treatment. Bcl-2/GAPDH protein expression levels did not change with treatments of idelalisib, bendamustine or their combination.

**Figure 18.**



**Figure 18. Effect of apoptotic protein levels by idelalisib and bendamustine combination treatment in primary CLL cells.**

Primary cells were either treated with DMSO, 5  $\mu$ M of idelalisib (I), 20  $\mu$ M of bendamustine (B), and then combination of idelalisib and bendamustine (5  $\mu$ M + 20  $\mu$ M, respectively) for 24 h and 48 h. Cells were harvested and protein lysates were analyzed using immunoblots to detect the protein expression levels for Mcl-1 and Bcl-2. GAPDH was used as control for equal protein loading. Quantitation for the immunoblots for each patient sample was performed by measuring the ratios of Mcl-1 and Bcl-2 to GAPDH for idelalisib and bendamustine treated samples, and then normalized to DMSO.

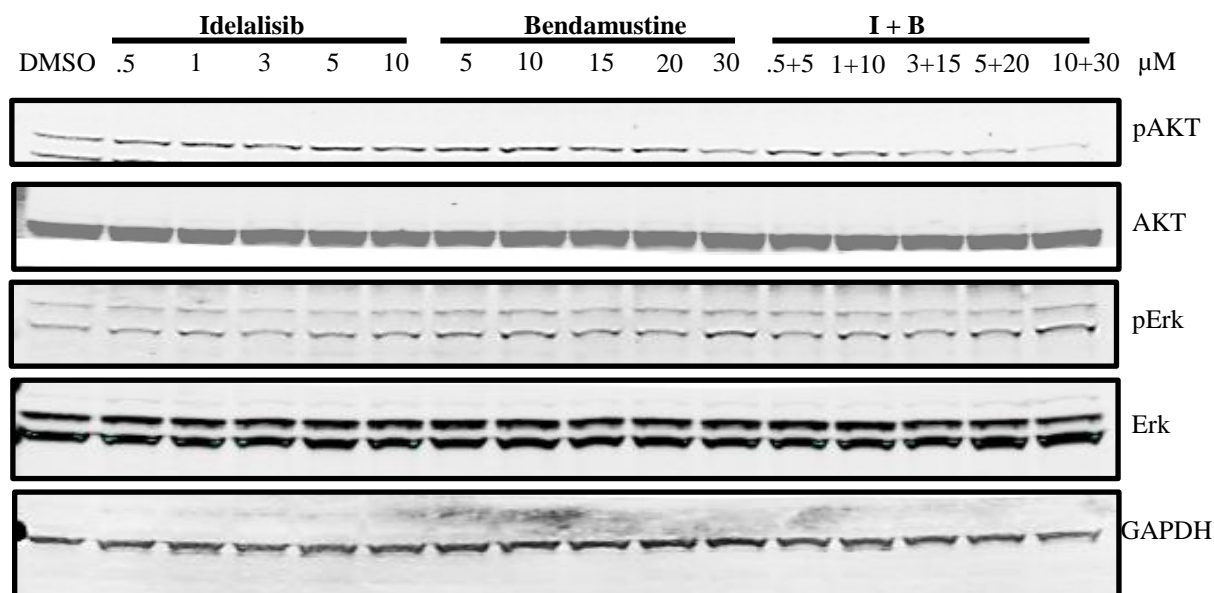
## **2.7 Effect on downstream signaling protein expression by idelalisib and bendamustine combination treatment**

We further assessed downstream signaling protein expression of PI3K and MAPK pathways with combination of the two drugs and performed a dose response experiment with varying dose as single agent and in combination. CLL PBMCs were treated with DMSO, 0.5, 1, 3, 5, and 10  $\mu$ M of idelalisib, 5, 10, 15, 20 and 30  $\mu$ M of bendamustine and then in combination 0.5+5, 1+10, 3+15, 5+20, and 10+30  $\mu$ M of idelalisib and bendamustine, accordingly. Post 24 h treatment, cell lysates were harvested and immunoblot assay was performed for phospho-AKT and phospho-Erk expression. In figure 19, when compared to the total protein levels of AKT, there was modest decrease in phospho-AKT (Ser473) in combination effect. Phospho-Erk protein levels showed no changes in the protein levels with single agent treatment or with combination treatments.

## **2.8 Effect on DNA damage marker $\gamma$ H2AX by idelalisib and bendamustine combination treatment**

From our study, idelalisib alone induced DNA damage response. We evaluated if bendamustine as a single agent and in combination with idelalisib further enhanced DNA damage response. CLL PBMCs were either left untreated or stimulated with IgM for 30 mins, then treated with DMSO control, 5  $\mu$ M idelalisib, 20  $\mu$ M bendamustine or combination for 24 h.

**Figure 19.**



**Figure 19. Effect on downstream signaling proteins by idelalisib and bendamustine combination treatment in primary CLL cells.**

Primary cells were treated with DMSO, idelalisib (0.5, 1, 3, 5, or 10 μM), bendamustine (5, 10, 15, 20 or 30 μM) and then combination of idelalisib and bendamustine (0.5+5, 1+10, 3+15, 5+20, 10+30 μM respectively) for 24 h. Cells were harvested and protein lysates were analyzed using immunoblots to detect the protein expression levels for phospho-AKT (Ser473) and phospho-Erk. GAPDH was used as control for equal protein loading. Quantitation for the immunoblots for each patient sample was performed by measuring the ratios of phospho- to total proteins for idelalisib treated samples, and then normalized to DMSO.

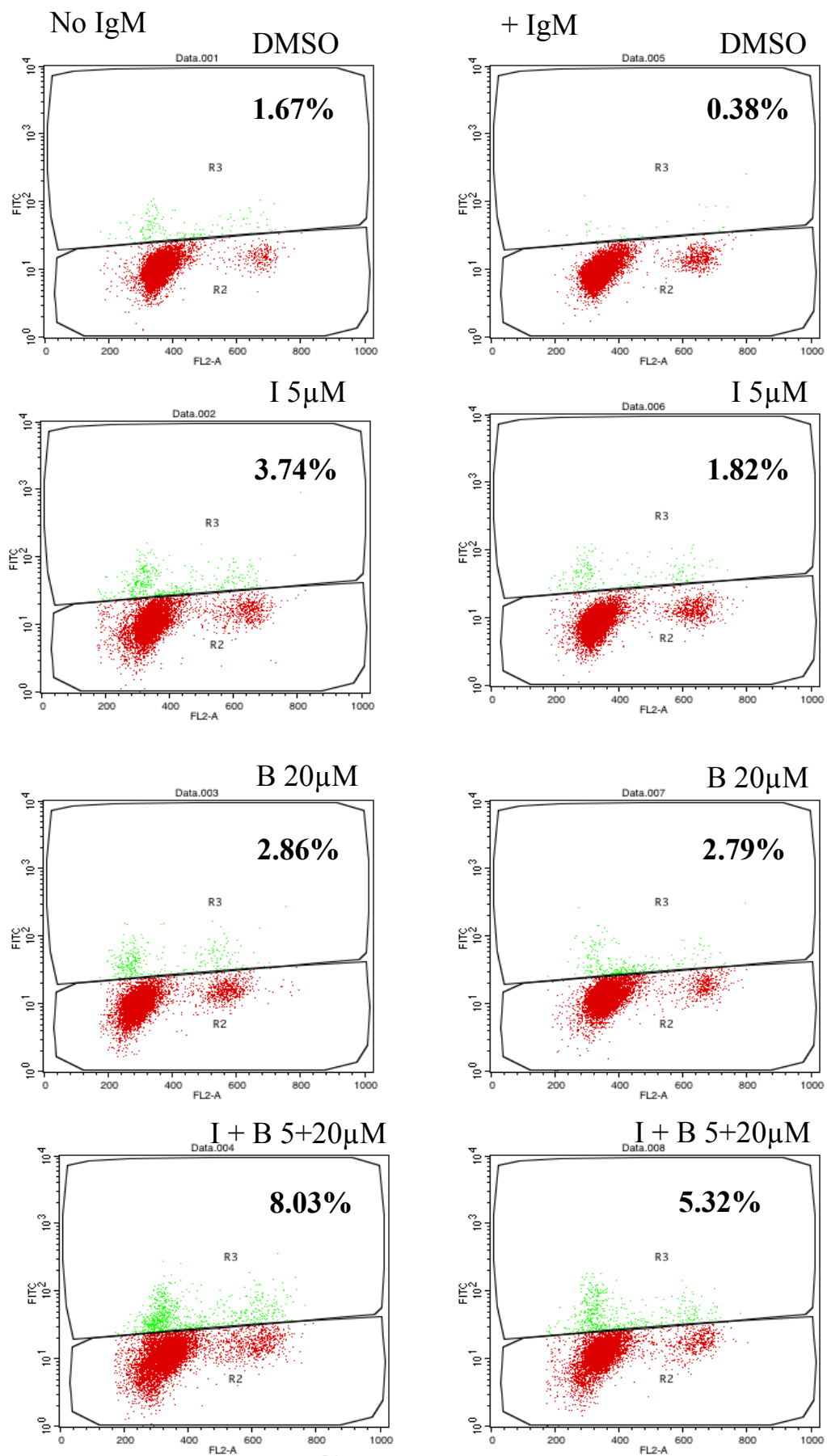
As shown in Figure 20, with no IgM stimulation, there was %  $\gamma$ H2AX increase in idelalisib and bendamustine treated samples. However, combination of drugs had a significant increase in DNA damage response as shown in the increased FITC-labeled  $\gamma$ H2AX signal from flow cytometry. When the same CLL PBMCs, with IgM stimulation alone, there was a decrease in DNA damage response. Interestingly, even with single agents or combination, there was a reduced DNA damage effect observed with IgM stimulation. Overall, there was a significant decrease in %  $\gamma$ H2AX phosphorylation in 5 separate CLL patients with IgM stimulation alone.

### **2.9 Effect on DNA damage marker $\gamma$ H2AX by idelalisib and bendamustine combination treatment**

To determine the extent of DNA damage with idelalisib, bendamustine and in combination, we evaluated 5 different patient samples for DNA damage response to the treatments. CLL PBMCs were treated with DMSO, 5  $\mu$ M idelalisib, 20  $\mu$ M bendamustine or combination for 24 h. Cells were harvested and we performed immunostaining of CLL cells to analyze changes in the levels of phospho-Histone 2A variant X (H2AX) at Ser139,  $\gamma$ H2AX by flow cytometry. In general, DMSO treated cells induced 1-3%  $\gamma$ H2AX. In figure 20, idelalisib treated PBMCs induced 2-5%  $\gamma$ H2AX while bendamustine alone induced 2-4%  $\gamma$ H2AX. Finally, the combination of idelalisib with bendamustine resulted in enhanced DNA damage response, ranging from 4-7%  $\gamma$ H2AX. Our study show enhanced apoptosis, decrease in Mcl-1 protein and gene expression levels, and increased DNA damage response with combination of idelalisib and bendamustine, when compared to single agents alone.

Similar experiment was performed in five additional patient samples (Figure 21). In each case idelalisib-induced H2AX phosphorylation above base-line level and this molecular event was highest when bendamustine and idelalisib were combined.

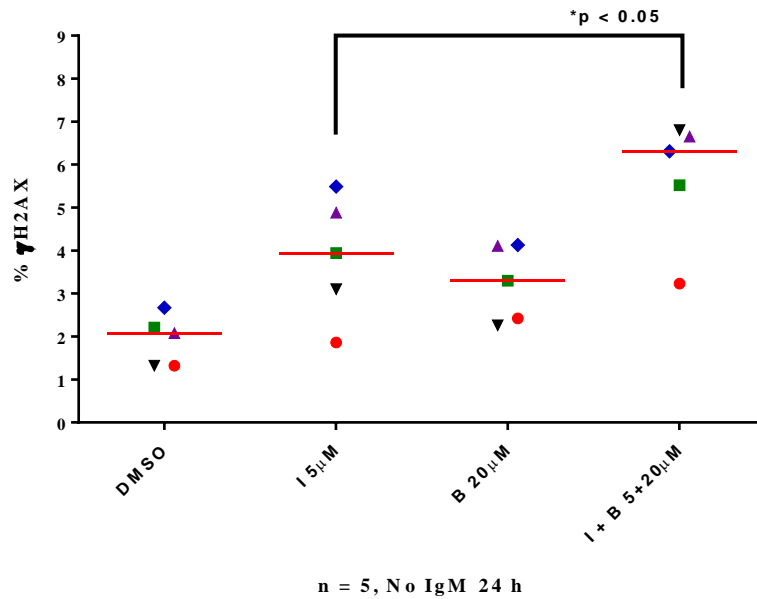
**Figure 20.**



**Figure 20. Idelalisib treatment in combination with bendamustine induces  $\gamma$ H2AX expression in primary CLL cells.**

Primary cells were either untreated or stimulated with 10 $\mu$ g/mL IgM for 30 mins. The cells were then either treated with DMSO, 5  $\mu$ M of idelalisib (I), 20  $\mu$ M of bendamustine (B), or combination of idelalisib and bendamustine (5  $\mu$ M + 20  $\mu$ M, respectively) for 24 h. The cells were then harvested and fixed with 70% ethanol overnight. Cells were then washed with PBS and then incubated with 0.5% goat serum for 1 h, then probed with the primary antibody phospho-histone H2AX (1:500) for 2 h. Cells were washed and then co-incubated with 10  $\mu$ g/mL propidium iodide and 2.5  $\mu$ g/mL DNase-free RNase for 15 mins. Cells were analyzed for fluorescence signal on flow cytometry for DNA damage response. Fluorescence-positive cells, with an upward shift in green, were marked as  $\gamma$ H2AX positive treated cells.

**Figure 21.**



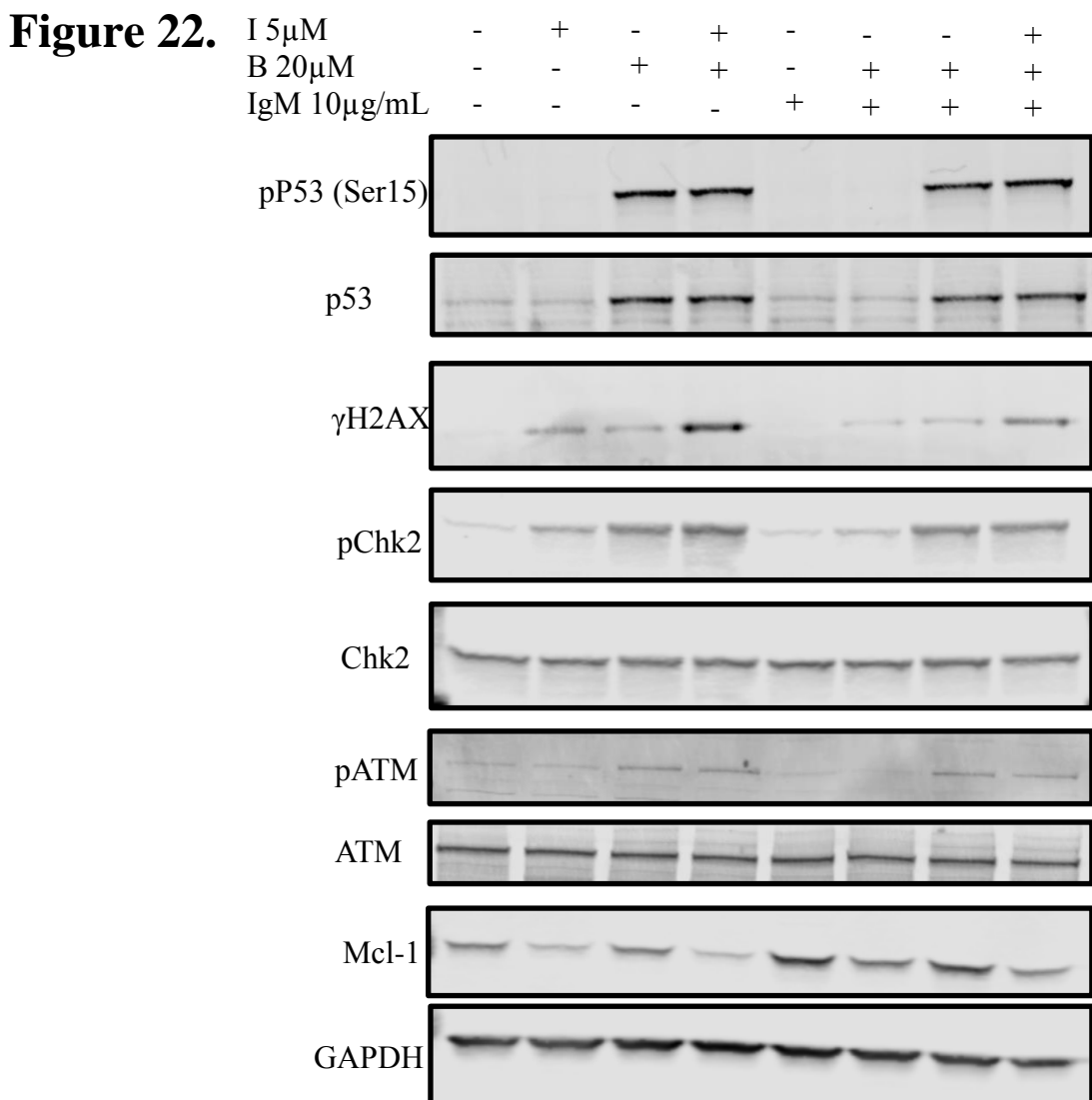
**Figure 21. Idelalisib treatment in combination with bendamustine induces  $\gamma$ H2AX expression in primary CLL cells.**

Primary cells were either treated with DMSO, 5  $\mu$ M of idelalisib (I), 20  $\mu$ M of bendamustine (B), or combination of idelalisib and bendamustine (5  $\mu$ M + 20  $\mu$ M, respectively) for 24 h. The cells were then harvested and fixed with 70% ethanol overnight. Cells were then washed with PBS and then incubated with 0.5% goat serum for 1 h, then probed with the primary antibody phospho-histone H2AX (1:500) for 2 h. Cells were washed and then co-incubated with 10 $\mu$ g/mL propidium iodide and 2.5  $\mu$ g/mL DNase-free RNase for 15 mins. Cells were analyzed for fluorescence signal on flow cytometry for DNA damage response. Fluorescence-positive cells, with an upward shift in green, were marked as  $\gamma$ H2AX positive treated cells. The figure represents 5 different CLL patients (CLL514, 455, 327, 137, and 103). A Student's t-test, paired 2-tailed was performed for these patient samples.

## **2.10 Effect on DNA damage protein levels by idelalisib and bendamustine combination treatment**

Collectively, both idelalisib and bendamustine induced DNA damage response and this response was enhanced with combination. Therefore, we investigated changes in kinases and mediators involved in DNA damage and repair signaling pathway.

CLL PBMCs were either untreated or stimulated with IgM for 30 mins and then treated with DMSO, 5  $\mu$ M idelalisib, 20  $\mu$ M bendamustine or combination for 24 h. We measured immunoblots for expression of total and phospho-protein levels. In parallel to the flow cytometry data that showed phosphorylation of H2AX with the drug treatments, Western blot analysis in figure 22 shows an increase in phospho-H2AX expression with idelalisib and bendamustine. In combination effect, there is a greater induction of phospho-H2AX, indicating more DNA damage response. This effect was slightly decreased when drug treatments were done with IgM stimulation. The total ATM remain unchanged across all treatments, however there was an increase in phospho-ATM with bendamustine alone and in combination. Idelalisib treatment did not induce phosphorylation of ATM with or without IgM stimulation. Phosphorylation of p53 is a response to DNA damage and repair. With bendamustine alone and in combination with idelalisib, there was stabilization of p53 protein, marked by phosphorylation of p53 at Ser15. Phosphorylation of p53 at Ser20 was also measured; however we observed variable expression with the treatments in different patient samples. Chk1 and Chk2 have also been recognized as the mediators of DNA damage response which phosphorylate p53 and are activated by ATR and ATM kinases. Idelalisib and bendamustine



**Figure 22. Effect on DNA damage protein levels by idelalisib and bendamustine combination treatment in primary CLL cells.**

Primary cells were either untreated or stimulated with 10  $\mu$ g/mL IgM for 30 mins. The cells were then either treated with DMSO, 5 $\mu$ M of idelalisib (I), 20 $\mu$ M of bendamustine (B), or combination of idelalisib and bendamustine (5  $\mu$ M + 20  $\mu$ M, respectively) for 24 h. Cells were harvested and protein lysates were analyzed using immunoblots to detect the protein expression levels for phospho-p53,  $\gamma$ H2AX, phospho-Chk2, and phospho-ATM. GAPDH was used as control for equal protein loading. Quantitation for the immunoblots for each patient sample was performed by measuring the ratios of phospho to total proteins for idelalisib and bendamustine treated samples, and then normalized to DMSO.

alone induced phosphorylation of Chk2 at Thr68; combination of both enhanced the expression of phospho-Chk2. There were no changes in the expression of total Chk2 protein with all treatments. We did not detect any phospho-Chk1 levels in our samples (data not shown).

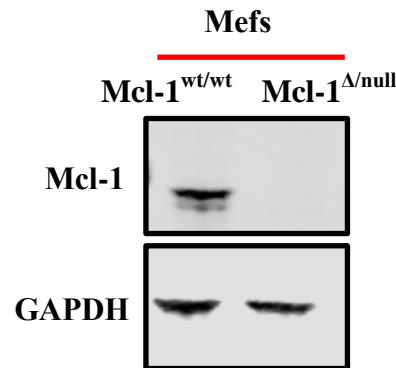
### **Aim 3: Effect of Mcl-1 deleted mouse embryonic fibroblasts (Mefs) in response to bendamustine**

#### **3.1 Dose- and time-dependent apoptosis in Mcl-1 $\Delta$ /null Mefs in response to bendamustine**

First, the cell lines generously provided by Dr. Opferman were validated to ensure Mcl-1 expression is not evident in the Mcl-1 $\Delta$ /null Mefs when compared to Mcl-1<sup>wt/wt</sup>, and indeed in figure 23, we observed no expression of Mcl-1 protein in the Mcl-1 deleted Mefs. To further validate the role of Mcl-1 in bendamustine-induced sensitivity and the enhanced combination cytotoxicity, we used Mcl-1<sup>wt/wt</sup> and Mcl-1 $\Delta$ /null murine embryonic fibroblasts (Mefs) cell lines. Mcl-1<sup>wt/wt</sup> and Mcl-1 $\Delta$ /null Mefs were treated with DMSO, idelalisib (0.5, 1, 3, 5, or 10  $\mu$ M), bendamustine (5, 10, 15, 20 or 30  $\mu$ M) and then combination of idelalisib and bendamustine (0.5+5, 1+10, 3+15, 5+20, 10+30  $\mu$ M respectively) for 24, 48, and 72 h. Cells were harvested, washed with PBS and then stained with Annexin V/PI, and the levels of apoptosis were measured by flow cytometry. Results in figure 24 show modest apoptosis with idelalisib treatment in Mcl-1 $\Delta$ /null Mefs; cell death from 2% - 3% at 24 h, 2% - 4% at 48 h, and 3% - 6% at 48 h, when compared to Mcl-1<sup>wt/wt</sup> and DMSO control. Interestingly, bendamustine treated Mcl-1 $\Delta$ /null Mefs were sensitive to cell death when compared to Mcl-1<sup>wt/wt</sup> Mefs. Apoptotic cell population ranged from 5% - 8% at 24 h, 8% - 13% at 48 h, and 10% - 20% at 72 h. Inclusive, combination of idelalisib with bendamustine in Mcl-1 $\Delta$ /null Mefs resulted in a dose- and time-dependent cytotoxicity when compared to the Mcl-1<sup>wt/wt</sup> Mefs. %Apoptosis observed for combination ranges from 5% - 10% at 24 h, 12% - 23% at 48 h, and finally 20% - 36% at 72 h.

The Mcl-1<sup>wt/wt</sup> and Mcl-1<sup>Δ/null</sup> Mefs experiments were done in triplicates and graphs represent three individual experiments with SEM.

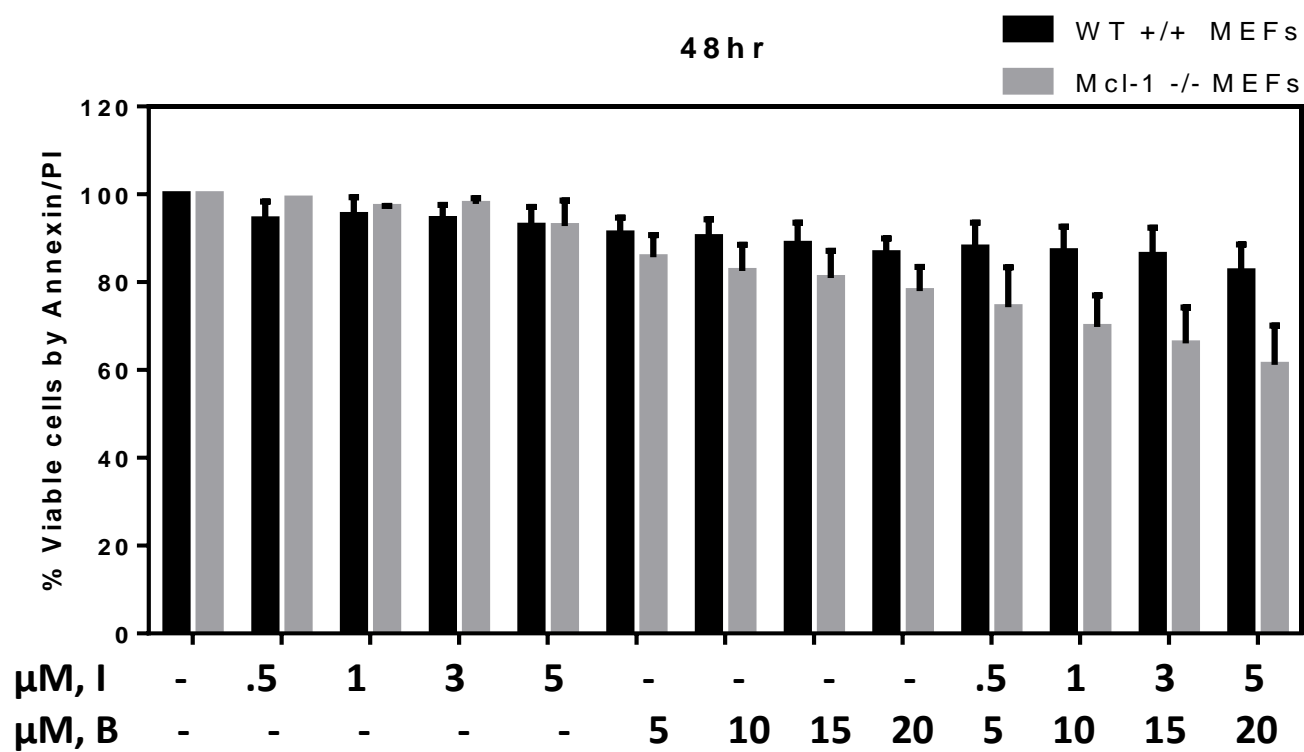
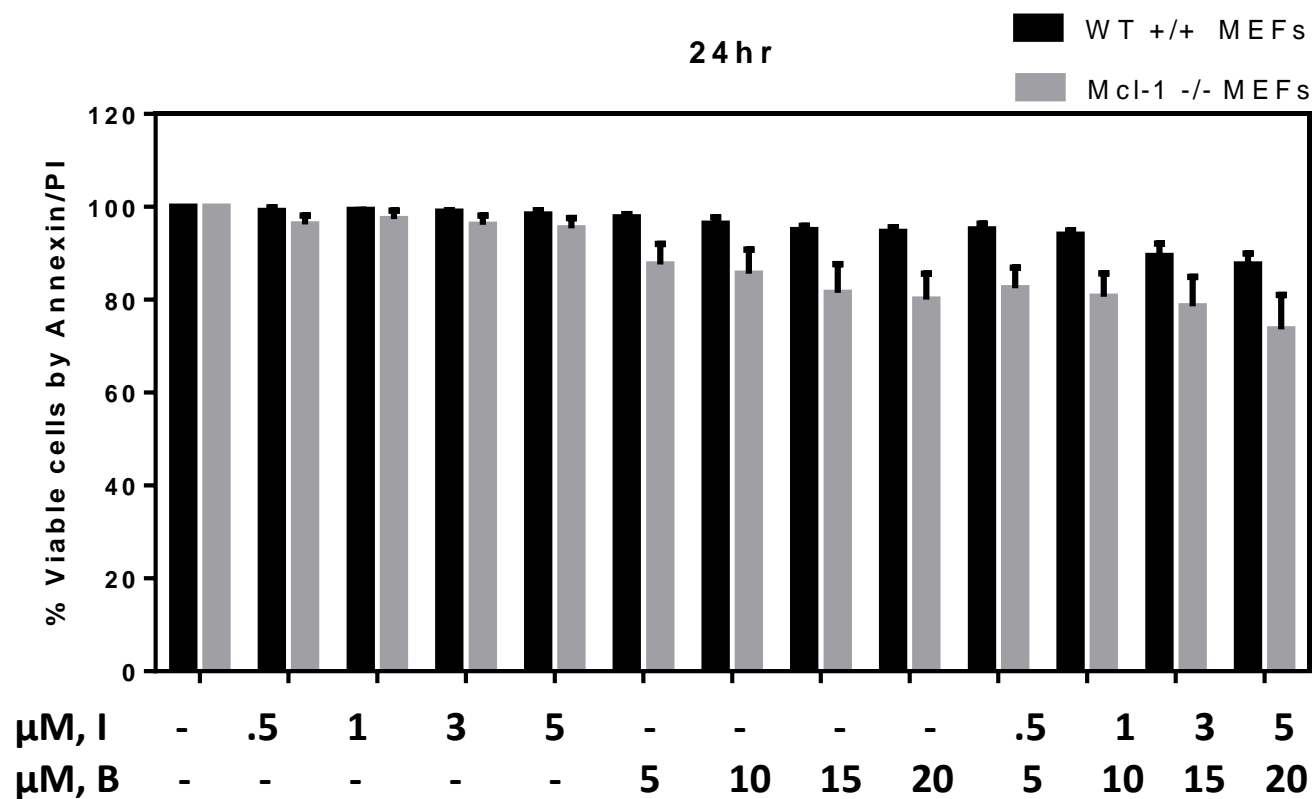
**Figure 23.**



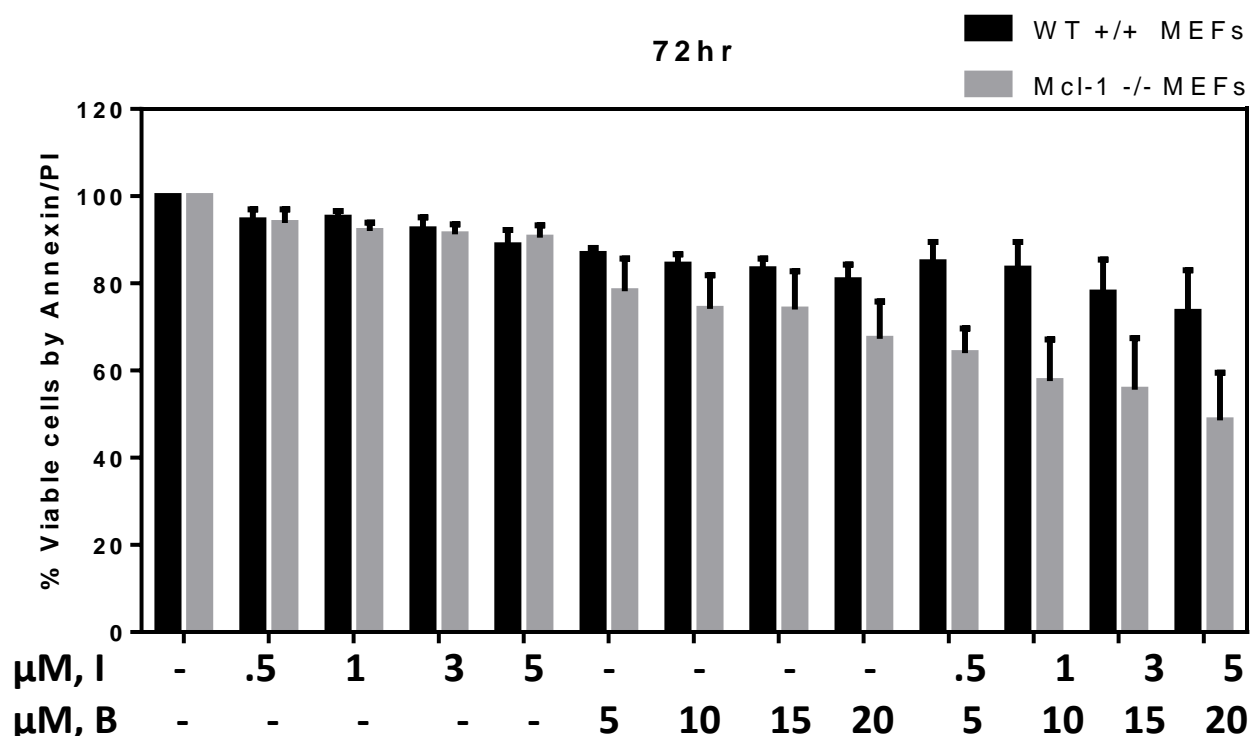
**Figure 23. Mcl-1 protein expression in Mcl-1<sup>Δ/null</sup> Mefs.**

Mcl<sup>wt/wt</sup> and Mcl-1<sup>Δ/null</sup> Mefs were cultured according to the method presented in materials and methods. Cells were harvested and protein lysates were analyzed using immunoblots to detect the protein expression levels Mcl-1 to ensure the cell lines with Mcl-1 deletion does not express Mcl-1 protein. GAPDH was used as control for equal protein loading. A mouse specific antibody detected the expression of mouse Mcl-1 in wild-type and Mcl-1 deleted isogenic cell lines.

**Figure 24.**



**Figure 24.**



**Figure 24. Dose and time-dependent decrease in cell viability in Mcl<sup>wt/wt</sup> and Mcl-1<sup>Δ/null</sup> Mefs cell lines with combination of idelalisib and bendamustine treatments.**

Mcl<sup>wt/wt</sup> and Mcl-1<sup>Δ/null</sup> Mefs were treated with DMSO, idelalisib (0.5, 1, 3, 5, or 10 μM), bendamustine (5, 10, 15, 20 or 30 μM) and then combination of idelalisib and bendamustine (0.5+5, 1+10, 3+15, 5+20, 10+30 μM respectively) for 24, 48, and 72 h. Cells were harvested and then stained with Annexin V for 15mins followed by 5mins of PI. The levels of apoptosis for each treatment was detected using flow cytometry and the apoptotic cells were indicated by the cell population present in the lower right, upper right, and upper left quadrants. Experiments were done in triplicates and the results shown are average  $\pm$  SEM.

## CHAPTER 4: Discussion and Conclusions

Our findings indicate moderate level of cytotoxicity observed with idelalisib treatment in CLL lymphocytes. We observed a dose- and time-dependent apoptosis in primary CLL lymphocytes. Idelalisib treatment with IgM stimulation decreased phosphorylation of AKT, a critical downstream survival signal. We observed a significant decrease in global RNA synthesis in idelalisib treated CLL lymphocytes. Idelalisib treatment resulted in a decline in Mcl-1 transcript and protein expression, with no changes to Bcl-2 protein and transcript levels. Interestingly, we observed that idelalisib treatment induced  $\gamma$ H2AX, a hallmark of DNA damage response, evident with protein expression.

Idelalisib and bendamustine as single agents induced moderate level of apoptosis in CLL lymphocytes; however, combination treatment resulted in enhanced apoptosis. We assessed combination index, and at all non-fixed ratios doses, we observed idelalisib and bendamustine couplet resulted in synergistic cytotoxicity. Furthermore, we observed both idelalisib and bendamustine as single agents and in combination induced changes in protein expression related to DNA damage response pathway, particularly,  $\gamma$ H2AX. We also evaluated the role of Mcl-1 in the mouse embryonic fibroblasts deleted with Mcl-1, and whether bendamustine-induced cytotoxicity related with depletion of Mcl-1 and sensitizing cells to apoptosis. To elucidate the mechanism of synergy, our data suggest that modulating Mcl-1 protein and transcript levels, as well as changes in DNA damage response and repair in CLL may contribute to the observed cytotoxicity. Collectively, there are multiple pathways involved in the combination interaction with PI3K/AKT, members of the Bcl-2 family, as well as the DNA damage and repair responses.

### **Apoptosis and Bcl-2 family of anti-apoptotic proteins**

Apoptosis is a controlled physiological process critical for normal cell biology and remains a key target in cancer. The Bcl-2 family proteins are extensively involved in the regulation of the mitochondrial outer membrane permeabilization process and apoptosis. The Bcl-2 family of proteins are subdivided into three functionally and structurally distinct subgroups based on BH domain homology as mentioned in the introduction. The 6 members of the anti-apoptotic proteins consist of Bcl-2, Bcl-b, Bcl-xL, Bfl-1, Bcl-w, and Mcl-1 proteins. Among these six proteins, three of them play a critical role in the pathophysiology of CLL. These anti-apoptotic proteins sequester the pro-apoptotic members Bax and Bak. Upon stimulation, the BH3-only protein members bind to antagonize the anti-apoptotic proteins. Ultimately, these six proteins function as the inhibitory effectors of the apoptotic machinery.

### **Bcl-2 family of proteins and CLL**

CLL is a hematologic malignancy characterized with a clonal expansion of CD5+ B lymphocytes; found to be replicationally quiescent, accumulating in the peripheral blood, lymph nodes, and bone marrow. These malignant quiescent cell populations in the blood compartments fail to start apoptosis program due to defects in the apoptotic pathway and the survival signals secreted from the microenvironment. Factors from the microenvironment include bone marrow stromal cells, nurse-like cells, and T-cells which produce cytokines and chemokines feeding into the NF- $\kappa$ B survival pathway as well as constitutively activating PI3K/AKT pathway. Overall activation of these pathways in CLL leads to overexpression of important anti-apoptotic proteins. Three key Bcl-2 anti-apoptotic proteins involved in CLL include Bcl-xL, Bcl-2 and Mcl-1. In CLL, there are high levels of *BCL2* gene expression maybe caused due to hypo-methylation of the gene through epigenetic regulation (94) and it is post transcriptionally

regulated by the lack of microRNA -15 and -16 (95). It is now well established that Mcl-1 is a critical player in the defective apoptotic program in CLL cells, with up-regulation of Mcl-1 resulting in poor clinical outcome and resistance to chemotherapy (96-99). Furthermore, patients with rituximab resistance, an antiCD-20 antibody used for treating B-cell malignancies, revealed high expression of Mcl-1. Knocking down Mcl-1 with small interfering RNA (siRNA) was sufficient to induce apoptosis of the malignant CLL cells (100). It has also been established in BCR signaling pathway, particularly through PI3K/AKT axis, Mcl-1 expression is highly up-regulated. Mcl-1 is found to be induced from the factors in the bone marrow stromal interactions in the CLL microenvironment (101). Therefore, Mcl-1 protein in CLL is pivotal in survival pathways and its complex regulation, expression and function play an important role in CLL cell survival.

### **Mcl-1 and hematopoiesis and hematologic malignancies**

Mcl-1 protein remains to be distinctive and has a fundamental role in normal cell physiology and in cancer. Mcl-1 has wide tissue and cell-specific expression and plays an essential role for survival and development. One prominent example includes lack of Mcl-1, but not Bcl-2, resulting in peri-implantation lethality during mouse embryo development (51). It has been demonstrated that the Mcl-1 is essential for the development and maintenance for T- and B-lymphocytes, and vital for neural development (55,102). Furthermore, Mcl-1 is important for hematopoietic stem cell survival (54). Mcl-1 was originally identified as early expression gene induced from a differentiation process in human myeloid leukemia cell line (66). Given Mcl-1's prominent role in differentiation, development and apoptosis, Mcl-1 has also been implicated in cell cycle progression and now distinctly in DNA damage response and repair.

### **Regulation of Mcl-1 expression**

Mcl-1 is essential for survival of several cell lineages and overexpression of Mcl-1 contributes to tumor malignancies. Therefore, its expression and function are tightly controlled, with intricate level of regulation at transcriptional, post-transcriptional and post-translational processes. Transcriptionally, cell-type specific regulation of Mcl-1 is evident. In hematopoietic cells, the PI3K/AKT and signal transducers and activators of transcription (STAT) and MAPK pathways are necessary for Mcl-1 expression (103,104). Additionally, conditional factors such as inflammatory cytokines, such as TNF $\alpha$  and IL-6 as well as growth factors may also rapidly induce Mcl-1 expression. The Mcl-1 promoter region contains multiple transcription factor binding sites including STAT response elements and nuclear factor kappa B binding sites (105). Importantly, Mcl-1 is down-regulated transcriptionally through number of conditional factors such as growth factor deprivation or induction of apoptosis through cytotoxic treatments (106,107). In general, Mcl-1 is down-regulated by inactivating the transcriptional factors which promote Mcl-1 transcription. However, binding of the E2F-1 transcription factor to the Mcl-1 promoter region directly shuts down Mcl-1 transcription (108).

### **Mcl-1 transcript**

Mcl-1 transcript is subject to alternative splicing, producing two protein isoforms; short form of Mcl-1 (Mcl-1s) and the long form of this protein (Mcl-1l) (109). Interestingly, the short isoform of Mcl-1 is unable to sequester the pro-apoptotic Bcl-2 family members, and therefore induces apoptosis (110). Similar to short half-life of Mcl-1 protein, Mcl-1 mRNA is also short-lived and therefore Mcl-1 mRNA translation is regulated and targeted by microRNA 29b and the RNA binding protein CUGBP2 (111,112). Both of these inhibitors directly bind to the 3' UTR of the Mcl-1 mRNA transcript. Finally, studies have demonstrated that eIF2a and

mTORC1 are also involved in overall Mcl-1 translation and apoptosis regulation (113,114).

Similar to the PEST regions that are responsible for rapid protein turnover rate, Mcl-1 contains ARE sequences that signal for rapid mRNA transcript turnover (96). These specific sequences consist of the adenylate/uridylate-rich elements (AREs) found in the 3' untranslated regions of the Mcl-1 mRNA transcript. The A-U rich elements are responsible for the rapid degradation of the short-lived (~1 hour) Mcl-1 mRNA transcript (96).

### **Mcl-1 protein structure**

Mcl-1 has a unique structure which allows for complex molecular interactions and differential functional activities. Structurally, Mcl-1 anti-apoptotic protein contains 3 BH3 domains, unlike the other anti-apoptotic members which contain 4 BH3 domains. Mcl-1 is a relatively large protein, compared to other members, containing 350 amino acid residues and it contains an N terminus which affects the proteins function and localization (115-117). Similar to Bcl-2, Mcl-1 contains a carboxy terminus with a hydrophobic transmembrane domain and BH2, BH1 and BH3 domains within the cytosolic area. Studies from Kozopas et al., identified Mcl-1 containing regions with proline (P), glutamic acid (E), serine (S), and threonine (T) residues, known as the PEST domains, a common feature in short-lived proteins (66). Mcl-1 protein has rapid turnover, with a short half-life of 30 minutes to 3 hours, depending on the cellular conditions and stimuli; hence another unique feature of this protein is PEST domains and faster turn-over (118).

### **Mcl-1 protein post-translation modifications/regulation**

A complex regulation of Mcl-1 is also evident at translational and post-translational levels. Several studies have elucidated phosphorylation-dependent degradation of Mcl-1 or phosphorylation-dependent increase in stability of Mcl-1 protein. This feature is dictated by the

multiple phosphorylation sites within the PEST region of Mcl-1 protein. Several studies have demonstrated that extracellular regulated protein kinase-1(ERK) targets Thr163 phosphorylation site to stabilize Mcl-1, increasing the proteins half-life (119). In contrary, phosphorylation at Thr163 by c-Jun N-terminal kinase (JNK) coordinated with Glycogen Synthase Kinase 3 (GSK) leads to Mcl-1 degradation (120-124) GSK-3-mediated phosphorylation at Ser159 leads to ubiquitination-dependent degradation of Mcl-1 (122) Additionally, Maurer group also demonstrated that phosphorylation at Ser159 by GSK-3 inhibits Mcl-1 interaction with a pro-apoptotic member, Bim, further inhibiting Mcl-1's anti-apoptotic function (122). Phosphorylation at Ser155 and Thr163 by GSK-3 in combination results in destabilization of Mcl-1 and impairs the anti-apoptotic function of Mcl-1 (120). Additional phosphorylation sites include ERK target at Thr92 phosphorylation with Thr163, stabilizing Mcl-1 and it is required for Mcl-1's anti-apoptotic function. Similarly, the phospho-residue sites at Ser64 and Ser121 targeted by other kinases also results in stability and inactivation of Mcl-1 protein function (125-127). Overall, Mcl-1 contains rich phosphorylation sites on the PEST regions and these phospho-residue interactions have a significant outcome on Mcl-1 function.

### **Mcl-1 and caspase-dependent cleavage**

Mcl-1 has unique structural components further giving a depth of regulation at post-translational level. Unlike other Bcl-2 family proteins, Mcl-1 consists of long N-terminus region involved with multiple modification sites. These modulations include Mcl-1 degradation, localization and interaction with other Bcl-2 family members, and phosphorylation, ultimately providing protein stability and cell survival and apoptotic responses. The PEST region within the N-terminus of Mcl-1 contains two caspases cleavage sites of Mcl-1 protein and several phosphorylation sites. In order to dampen the pro-survival signals, Mcl-1 protein can be cleaved

by caspases and granzyme B at two specific aspartate sites within Mcl-1, Asp127 and Asp157, both of these sites are targets for cleavage by caspase 3. Caspase-dependent cleavage of Mcl-1 not only inhibits the anti-apoptotic function of Mcl-1; however, some studies argue that cleaved Mcl-1 may promote pro-apoptotic feature of Mcl-1 (128,129).

### **Mcl-1 and proteosomal degradation**

Mcl-1 protein degradation is also attributed to the ubiquitin-proteasome system. Mcl-1 undergoes rapid turnover through ubiquitin-dependent degradation by the 26S proteasome and Mcl-1 ubiquitin ligase E3 (MULE) (130). Studies have identified MULE as it contains a conserved BH3 domain and it was demonstrated that the BH3 domain of MULE only associates with Mcl-1 and not any other members such as Bax, Bcl-2 and Bcl-xl (130,131). Studies from Zhong et al discovered that it was the lysine residues that were targeted for ubiquitination and accordingly; *in vitro* studies demonstrated MULE is essential for constitutive degradation of Mcl-1 (130,131). Importantly, knockdown of MULE by shRNA resulted in an accumulation of Mcl-1 and inhibited apoptosis of cells caused by DNA damaging agents. Contrary to the ubiquitination process, deubiquitination of Mcl-1 is processed by ubiquitin specific peptidase 9 X-linked (USP9X) enzyme. USP9X was identified as one of the proteins associated with Mcl-1 and its directing binding to the protein resulted in Mcl-1 stabilization; leading to cell survival (132). The well-regulated Mcl-1 at multiple levels is very significant in normal cellular biology as well as in malignant processes.

### **Interaction of Mcl-1 with pro-apoptotic Bcl-2 family proteins**

Mcl-1 has very distinctive characteristics relating to its structure and complex regulation. Mcl-1 has the ability to interact with diverse anti-apoptotic and pro-apoptotic proteins in the group. Mcl-1 belongs to a family of anti-apoptotic proteins with their main function as the

inhibitors of the apoptosis pathway. Mcl-1 can inhibit apoptotic signals by binding and sequestering the members of the pro-apoptotic proteins Bax and Bak. Bax and Bak facilitate apoptotic activation through outer mitochondrial membrane permeabilization causing release of cytochrome c and activating the caspases, leading to cell death. Studies have demonstrated that Mcl-1 can bind with BH3-only pro-apoptotic Bcl-2 family members and block the polymerization of Bax and Bak (96,133-135). It is well established that Mcl-1 also selectively interacts with BH3-only proteins such as Puma, Noxa, Bim and Bik (133,135,136). When Mcl-1 is not bound to Bax or Bak, activator BH3-only proteins such as Bim and Puma directly bind and activate the pro-survival functions of Bax and Bak (136-138). Alternatively, Noxa binding to Mcl-1 leads to proteasomal degradation of Mcl-1 (135). Studies have also elucidated that over-expression of BH3-only proteins such as Puma can stabilize Mcl-1 and can no longer interact with MULE for its degradation. In general, Mcl-1 has many interactions with the members of the apoptotic family however; Mcl-1 may have even more unique role in DNA damage and repair pathways.

### **Mcl-1 and DNA repair**

Mcl-1 plays a very critical role in apoptosis program and few studies have further attempted to characterize the role of Mcl-1 in DNA damage response. Decrease in Mcl-1 enhances apoptosis through DNA damage response (113). Additionally, stressed cells undergoing DNA damage can either go through apoptosis or cell cycle arrest, which can go through p53 dependent or independent pathways. Another study explored the role of Mcl-1 in regulating both apoptosis as well as regulating cell cycle; where Mcl-1 interacts with proliferating cell nuclear antigen, PCNA (67). Key findings from adeno virus infected cells resulted in down-regulation of Mcl-1 due to DNA damage, increase in phosphorylated H2AX

and ATM; caused from double strand breaks in DNA (139). Studies have demonstrated the interaction of Mcl-1 with cyclin dependent kinase-1 (Cdk-1) and expression of Mcl-1 leads to suppression of cell proliferation. Jamil S et al revealed Mcl-1 coordinates DNA damage mediated checkpoint response by binding at the sites of double strand breaks in DNA, associating with key DNA damage response proteins (68,140). Interestingly, this study also concluded that ATR signaling pathway and not ATM pathway was mediating Mcl-1 interaction with  $\gamma$ H2AX (140). Taken together, all the previous studies have explored the interaction of Mcl-1 with DNA damaging agents and its outcome in DNA damage and repair response.

### **Idelalisib-induced DNA damage response**

PI3K $\delta$  is an important PI3K family class I isoform expressed exclusively in B-cells and is known to promote malignant cell growth and survival. BCR survival pathway in CLL contributes to the activation of the PI3K/AKT survival pathway and is therefore a therapeutic target. PI3K $\delta$  plays a critical role in BCR signaling and the delta isoform is over-expressed in many B-cell malignancies including CLL. It is well established in solid tumors that the PI3K/AKT axis regulates multitude of translation and transcription processes and further targets downstream transcription factors and proteins (33,141). In addition, studies from Cantley et al and others demonstrate PI3K $\alpha$  and PI3K $\beta$  have a very important role in DNA damage and repair mechanisms (142,143). Interestingly, our investigation suggests inhibition of the delta isoform with idelalisib may have a potential role in DNA damage and repair responses.

Consistent with these prior observations with PI3K  $\alpha$  and  $\beta$  isoforms, our data demonstrated that CLL lymphocytes treated with idelalisib induced phosphorylation of  $\gamma$ H2AX both with immunostaining as well as protein immunoblot assays. Interestingly, when idelalisib was treated with IgM stimulation, the signal and the protein expression of  $\gamma$ H2Ax decreased.

Furthermore, our data suggest an important role of PI3K $\delta$  inhibition in other DNA damage and repair proteins. As shown by Western blot analysis, idelalisib treatment also led to phosphorylation of Chk2 and variable expression of phospho-ATM. This unique observation needs to be explored more in details regarding the role and the potential cross-talk with PI3K/AKT pathway and DNA damage and repair pathways.

### **Bendamustine-induced DNA damage response**

DNA damage signaling transduction pathway is initiated with a DNA damage recognized by the upstream kinases ATM and ATR (144). Downstream of these proteins are the Chk1 and Chk2 kinases. Several contrasting studies show the regulation of p53 by Chk2, however; these studies remain to be unclear. Although it is established that Chk2 regulates ionizing radiation (IR)-induced p53-dependent apoptosis, there seems to be ambiguity about the role of Chk2 in apoptosis induced by chemotherapeutic agents, especially alkylating agents (145-148). Furthermore, various kinases upstream such as ATM, Chk1 and Chk2 are responsible for activation of p53 in response to DNA damage. Activation of p53 can lead to both cell cycle arrest and DNA repair or apoptosis processes. DNA damage induces p53 at Ser15 and Ser20, furthermore; p53 can be phosphorylated by ATM at Ser15 (147,149).

Our study shows that DNA damage induced by bendamustine in CLL lymphocytes induced phosphorylation of ATM as well as p53 at Ser15. In addition, our data show phosphorylation of Chk2 protein with bendamustine treatment in CLL lymphocytes regardless of IgM stimulation of the BCR pathway. Phosphorylation of H2AX ( $\gamma$ H2AX) is a well-known marker for DNA double-stranded breaks, which is associated with interstrand crosslinks in the DNA (77). Phosphorylation of H2AX is a hallmark of recognizing DNA damage and further elicits and recruits DNA repair and cell-cycle checkpoint proteins to the site of DNA damage

(150,151). Our results from Western blot analysis show an increase in  $\gamma$ H2Ax protein expression in bendamustine treated CLL lymphocytes; however, this effect was decreased under combination treatment of bendamustine and IgM stimulation of the BCR pathway. These results may suggest a possible cross-talk between the DNA damage pathway and the BCR pathway. Overall, in line with DNA damage response, our study shows that bendamustine treatment in CLL lymphocytes elicited DNA damage response through activation of p53, ATM, Chk2 and  $\gamma$ H2AX.

### **Idelalisib and bendamustine in combination: mechanism of synergy**

As explained in the rationale for combination of idelalisib and bendamustine and the observed synergy in CLL, our data elucidated a potential role of members of the anti-apoptotic protein family and the DNA damage and repair response. Studies from Juvekar et al demonstrated that inhibition of PI3K  $\alpha$  and  $\beta$  isoforms result in modulating DNA damage and repair responses (142). Our data suggest that the mechanism of synergy may be due to decline in Mcl-1 and modulating DNA damage responses such as induction of  $\gamma$ H2AX and other downstream signaling proteins, further validation with mechanistic studies would elucidate the potential cross-talk with the two pathways. Bendamustine-induced DNA damage in Mcl-1 deleted Mefs sensitized cells to apoptosis in dose- and time-dependent manner. While some studies suggest the role of Mcl-1 in DNA damage and once exposed to DNA damage response, Mcl-1 is not only essential but it is translocated to nucleus and facilitates DNA damage and repair responses (140). Our study described provided a strong rationale for the feasibility of PI3K $\delta$  inhibitor, idelalisib in combination with bendamustine and further mechanistic studies would improve our understanding of the combination synergy and efficacy translating to applicable therapy with minimal toxicity in patients.

## **Conclusion and future directions**

The investigation described in this thesis focuses on the emerging role of PI3K $\delta$  inhibitors as therapeutic targets and evaluating the effect of PI3K $\delta$  inhibitor, idelalisib, and a combination strategy with a chemotherapeutic agent, bendamustine in chronic lymphocytic leukemia. Our findings elucidate idelalisib and bendamustine, as single agent and in combination, are cytotoxic in CLL. These agents target PI3K and DNA damage pathways, and furthermore target transcription and translation of anti-apoptotic and DNA damage to disrupt survival and proliferation signals in CLL.

Our study with idelalisib and bendamustine provides an initial mechanism of action that may guide further use of this combination and support the use of PI3K $\delta$  inhibitors and in combination with chemotherapeutics agents. Given the complexity of the two extensive pathways involved with treating idelalisib and bendamustine, questions remain unclear. One prominent question is whether there are other mechanisms of cell death that maybe involved with idelalisib treatment. It is well established that multiple survival pathways may be activated during the survival signals and therefore, there may be redundant pathways activated. Besides the phosphorylation targets, direct modulation of apoptosis and DNA damage response pathways may elucidate the exact mechanism of idelalisib and bendamustine as single agents or in combination. Evaluating other Bcl-2 family members and pharmacological inhibitors of Mcl-1 and Bcl-2, such as ABT-737 and ABT-199 may strengthen the mechanism of action with idelalisib. Additionally, it would be interesting to also explore the cross-talk of the pathways on a global scale through reverse-phase protein analysis and to detail in the up-regulation or down-regulation of important proteins.

In addition, effect of idelalisib under microenvironment conditions is a critical part of the study that needs to be further explored. Our study showed the IgM stimulation aspect of idelalisib treatment analysis but more detailed models of the CLL microenvironment would characterize the mechanism of action of idelalisib. Moreover, based on the recent finds from Ali et al 2014, it would be interesting to speculate how idelalisib and bendamustine modulate responses in T cell immunity (18). Idelalisib, when used as a single agent results in an increase in lymphocytes in the peripheral blood, presumably due to egress of CLL cells from lymph nodes (152). If bendamustine is added, it will result in apoptotic cell death of those lymphocytes resulting in enhanced clinical response. As a final point, idelalisib and bendamustine are already approved in the clinic for CLL therapy, and currently, idelalisib is in clinical trials with bendamustine and rituximab in previously treated, relapsed and untreated patient populations (NCT01569295, NCT01980888 and NCT01088048). Further understanding the mechanism and the biomarkers targeted with these drug treatments will constitute optimal treatment regimen options for CLL patient therapy in clinic.

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## **Vita**

Prexy Tapan Modi was born in Gujarat, India on May 10<sup>th</sup>, 1987 to parents Atul Shah and Priti Shah as the oldest daughter. Prexy moved to the United States of America in 1998 with her family and resided in Worthington, Ohio. Prexy attended Thomas Worthington High School from 2001-2005. She was admitted to The Ohio State University, Columbus, Ohio for her undergraduate studies. Prexy majored in Microbiology and graduated in 2009. Prexy continued her post-graduate studies and research at The Ohio State University and in 2012; Prexy was enrolled in The University of Texas, Graduate School of Biomedical Sciences. Prexy joined the Experimental Therapeutics Academic Program at MD Anderson Cancer Center. Prexy conducted her Master of Science thesis work under Dr. Varsha Gandhi's supervision and she will complete all the degree and program requirements by May, 2015.

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