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Enhancing the efficacy of BH3 mimetics in blood cancers

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Enhancing the efficacy of BH3 mimetics in blood cancers

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Jong-Hoon Scott Lee

Dissertation Committee:
Professor David A. Fruman, Chair
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Professor Aimee L. Edinger

2016
Dedication

To my parents, Robert and Mihae

For their love and support
For encouraging me to open my mind and heart,
and to view the world from every perspective.
For giving me the freedom to pursue my own goals, make my own mistakes.
For their selfless sacrifices that have paved my growth.
For teaching me the value of perseverance and independence.
For instilling in me a strong sense of character and morality.
For raising me from that little sparkplug,
I would be not be the man I am today without you

To my brother and sister, Bobby and Janet

For being exemplary role models and teaching me the little things.
For lending a sympathetic ear or a helpful hand,
I have always felt supported through my endeavors.

To all my family and friends

For all the birthday surprises, food excursions, game nights, happy hours, distractions from the bad days, celebrations of the good days, and for all the memories.
Grad school would not have been joyful without you.
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Lee JS, Tang S, Ortiz V, Fruman DA (2014) “Vertical Inhibition of the PI3K Pathway Potently Sensitizes Diffuse Large B Cell Lymphoma to BCL-2 Antagonism” 12th Annual UCI Immunology Fair, University of California Irvine, Irvine, CA Nov 20-21

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Evasion of cell death is one of the hallmarks of cancer that has only recently become targetable using small molecule inhibitors. By antagonizing the pro-survival function of BCL-2 family proteins, these so-called BH3 mimetics skew cells towards undergoing apoptosis. Though single-agent approaches have yielded promising clinical efficacy in some contexts, it is often insufficient to induce substantial patient responses. This dissertation investigates the potential of using rational targeted therapies to enhance the efficacy of BH3 mimetics.

In Chapter 2, we pursue the simple hypothesis that targeting a pathway whose aberrant activation is associated with cancer (the PI3K/AKT/mTOR pathway) can sensitize cells to BCL-2 inhibition. We demonstrate that inhibitors of this pathway synergize strongly with two BH3 mimetics, ABT-263 and ABT-199, and that the extent of this synergy can be predicted using dynamic BH3 profiling. By suppressing AKT activity, PI3K pathway inhibitors induced mitochondrial accumulation of pro-apoptotic
proteins. This work provided insight into markers of pharmacodynamic response, tools for predicting efficacy, and evidence warranting further investigation.

In Chapter 3 we pursue an alternative approach: that targeting a key metabolic pathway for which inhibitors are already FDA-approved, can also sensitize cells to BH3 mimetics. We show that targeting HMG-CoA reductase using statins selectively primes cancer cells for apoptosis and enhances the efficacy of ABT-199 \textit{in vitro} and \textit{in vivo}. We build on the work from Chapter 2 to demonstrate the predictive capabilities of dynamic BH3 profiling in cell lines and primary patient samples. Additionally, we identify downstream targets contributing to the sensitization effect, supporting further investigation.

Lastly, in Chapter 4, I present incomplete or unpublished data that follows up on the work presented in Chapters 2 and 3. I also discuss potential future directions derived from those experiments as well as the implications of this work.
Chapter One

Introduction
Resistance to apoptosis: a hallmark of cancer

The goal of modern cancer therapies is to deliver treatments that can selectively kill cancer cells and spare normal cells. The first effective chemotherapies exploited the tendency of transformed cells to rapidly proliferate (1), but were associated with significant toxicity against normal dividing cells. While advances in modern chemotherapies have improved patient responses and managed toxicities, many patients still do not respond and require alternative therapies. As a result, a large effort has been to identify effective therapies that target cancer-specific mutations. By targeting pathways or processes on which tumor cells are more dependent, these so-called targeted therapies promised efficacy with minimal toxicity. Indeed, the prototypical example of targeted therapy, imatinib, yielded impressive responses in chronic myelogenous leukemias driven by the BCR-ABL translocation with few toxicities (2). Coincident with this early success, decades of research that expanded our understanding of the defining characteristics of cancer cells were aggregated in the seminal paper: “The Hallmarks of Cancer” (3). By summarizing the features required for cancer, Drs. Hanahan and Weinberg highlighted multiple potential vulnerabilities that could be exploited using targeted therapies. After only a decade, a follow-up report revealed that nearly every hallmark of cancer could be targeted using an existing or newly discovered compound (4). Among these hallmarks, resisting cell death is a
promising target for therapies to induce tumor-selective apoptosis. This dissertation extends upon this idea and focuses on identifying rational combinations that directly target cell death machinery to achieve selective killing of tumor cells, particularly B cell lymphoma.

**Diffuse large B cell lymphoma (DLBCL)**

Diffuse large B cell lymphoma is the most common form of adult non-Hodgkin’s lymphoma representing over a quarter of all new B cell neoplasm diagnoses each year (5). Given that DLBCL is predominantly diagnosed in elderly patients (median age ~70 (6)), tolerability of therapies is a growing concern. Indeed, a recent retrospective study of ~400 DLBCL patients age 75 or older revealed that fewer than a quarter of patients completed the full regimen of chemotherapy (7). Major reasons for failure to receive therapy included personal/family decisions, comorbidities, and overall lower resilience. Five year survival rates for those patients that receive combination chemotherapy (R-CHOP: rituximab, cyclophosphamide, hydroxydaunorubicin, oncovine, and prednisone) are quite good (approaching 60%). However, a subset of patients still respond poorly to the standard of care. Thus, while there has been substantial progress in treating the disease, there is still a significant need for effective alternative therapies.

While early diagnosis of DLBCL hinged on histological identification, whole exome sequencing of DLBCL patient samples revealed three distinct subtypes with unique expression profiles. These were named activated B cell like (ABC), germinal center-like (GCB), and primary mediastinal B cell lymphoma (PMBL) based on similarities to normal gene expression programs (8,9). Importantly, each subtype is
separable from another in terms of oncogenic driver mutations (9) as well as chemo-
sensitivity (10). As a result, targeted therapy approaches to treating each disease varies
greatly between subtypes. For example, while therapies that target activating mutations
in B cell receptor signaling are effective in the ABC subtype, they have little efficacy in
the GCB subtype (11,12). Instead, poor patient responses in GCB-DLBCL are often
associated with over-expression of BCL-2 (13-15). Consequently, for the majority of this
dissertation we focus on approaches to treating GCB-DLBCL using small molecule
inhibitors of BCL-2.

**BCL-2 family proteins**

Apoptosis is a critical process whereby cells undergo a controlled form of cell
death that is crucially required for diverse biological processes ranging from mounting
immune responses to maintaining tissue homeostasis (16,17). However, aberrant
suppression of this pathway has been linked to tumorigenesis, and evasion of cell death
is widely acknowledged as a hallmark of cancer (4). Apoptosis is primarily regulated by
the B cell lymphoma 2 (BCL-2) family proteins, which interact in a dynamic balance to
control whether a cell lives or dies (18). Currently, there are over 25 known proteins in
the BCL-2 family, each of which exerts either a pro- or anti-apoptotic effect. Based on
their function, these proteins can be divided into one of four major groups: the anti-
apoptotic proteins (BCL-2, BCL-XL, BCL-w, and MCL-1 (19-22)), the apoptotic
sensitizers (PUMA, BMF, BAD, BIK, NOXA, and HRK (23-28)), apoptotic activators
(BIM and BID (29,30)), or the apoptotic effectors (BAX and BAK (31,32)).
The function of BCL-2 family proteins is largely determined by their BCL-2 homology (BH) domains (Figure 1.1). For example, the multi-domain “effectors” BAX and BAK serve as the final arbiters of apoptosis (33,34). Upon activation, these proteins oligomerize to form pores that promote mitochondrial outer membrane permeabilization (MOMP) and allow the release of cytochrome c and other apoptotic factors into the cytoplasm (35-37). Cytochrome c then interacts with pro-caspase-9 and apoptosis protease-activator factor-1 (APAF-1), forming a holoenzyme that promotes the auto-proteolytic cleavage and activation of caspase-9 (38,39). Upon activation, caspase-9 then initiates a cascade of proteolytic activity which promote effector caspase (3, 6, and 7)-mediated proteolysis and DNA fragmentation (16).

In order for BAX and BAK to oligomerize, they must first interact with the apoptotic “activators” BIM or BID. Upon binding with the BH3 domains of BIM/BID, BAX/BAK undergo a conformational change that allows for oligomerization (34,36,40,41) (Figure 1.2A). Importantly, these BIM/BID BH3 domains are both necessary and sufficient for activator function (26,29,30,41,42). As such, suppression of apoptosis requires the prevention of BIM/BID interactions with BAX/BAK (43). Indeed, all anti-apoptotic proteins possess a hydrophobic pocket formed at the interface of BH1, BH2, and BH3 domains that establishes a binding site for BH3 domains (43-45). By directly binding to pro-apoptotic BH3 domains, anti-apoptotic proteins not only prevent BIM/BID from activating BAX/BAK (Figure 1.2A), but can also directly suppress the effector functions of BAX/BAK (43).
**Figure 1.1. BCL-2 family proteins.** BCL-2 family proteins can be broken into major groups based on their BH domains, which define their binding partners and function.

Unlike the activators, which also only contain a single BH3 domain, the sensitizers proteins cannot directly activate BAX and BAK (41), likely due to the absence of strictly conserved amino acid motifs across BH3 domains (46,47). Instead, sensitizer proteins neutralize the anti-apoptotic proteins by competing for access to the BH3 domain binding pocket. However, due to the lack of conservation among the BH3 domain, different BH3-only proteins exhibit preferential binding affinities for different anti-apoptotic proteins (48). Among all BH3-only proteins, only BIM and PUMA demonstrate comparable binding affinities to all anti-apoptotic factors. On the other hand, BAD and BMF preferentially bind BCL-2, BCL-XL, and BCL-w, NOXA binds to...
MCL-1, and BID, BIK, and HRK bind to BCL-XL and BCL-w (24-26,28,30,49) (Figure 1.2A, B). Thus, sensitizers promote apoptosis by displacing activators from anti-apoptotic proteins, allowing activation of BAX and BAK and subsequent initiation of apoptosis.

**Figure 1.2. Overview of BCL-2 family protein interactions.** (A) Overview of mitochondrial apoptotic pathway, with binding interactions highlighted. (B) Binding affinity of BH3 only peptides for anti-apoptotic BCL-2 family proteins, figure adapted from Deng et al (50).

The nature of cancer requires an enhanced survival phenotype. Several hallmarks of cancer like genomic instability, oncogene activation, and loss of survival signaling promote induction of apoptosis (4,51,52). Thus, evading apoptosis is essential in the development and progression of cancer. Based on the interaction of BCL-2 family proteins, it is feasible to imagine several potential strategies whereby cancer may resist death (Figure 1.3). First, silencing of BH3-only proteins (especially BIM) may skew cells towards survival and hinder effective activation of BAX/BAK (53-55). Second, loss of apoptotic effectors suppresses induction of MOMP (56,57). Lastly, over-expression of
anti-apoptotic factors, via translocation, gene amplification, or copy number alteration can similarly buffer cancer cells against induction of apoptosis (58,59). Thus, identifying and targeting these cancer-specific evasion tactics may lead to effective therapies.

**Figure 1.3. Strategies for evading apoptosis.** Evading apoptosis is a hallmark of cancer, as such there are many strategies that cancer cells use to achieve this. For example, cancers can silence apoptotic activators (panel 1) or effectors (panel 2) to limit induction of MOMP. Alternatively, some cancers over-express anti-apoptotic factors to prevent BAX/BAK activation (panel 3).

**Targeting BCL-2 in blood cancers**

Since its identification as the proto-oncogene involved in the t(14;18) translocations defining human follicular lymphoma (FL) (58,60), BCL-2 has been inextricably linked to lymphoid malignancies. Indeed, over-expression of this protein is widely associated with poorer patient outcomes across blood cancers (13-15,61-65), where it enables aberrant cell survival (19,58,66-70). While insufficient to initiate
tumorigenesis itself, extended survival conferred by BCL-2 over-expression enhances the acquisition of additional oncogenic mutations that can drive transformation (71-74). In particular, BCL-2 cooperates exceedingly well with mutations promoting cell proliferation, like MYC, which often promotes forms of death that BCL-2 can suppress (51,52,75). Indeed, lymphomas that over-express both MYC and BCL-2 (“double-hit” lymphomas) represent the most difficult to treat subset of lymphomas in human patients (76).

Given their clear role in cancer cell survival, the development of therapeutics targeting this family of proteins has progressed rapidly over the past two decades. While early approaches included antisense oligonucleotides (77) and drugs identified through compound screens, the ability of these compounds to kill BAX/BAK-deficient cells suggested non-specific off-target toxicity (78-80). Fortunately, following the elucidation of the structure of BCL-XL in complex with the BH3 domain of BAK (81), a team of scientists working in conjunction with Abbott Laboratories (now AbbVie) succeeded in developing compounds that could mimic the function of BH3-only proteins (82). Termed BH3-mimetics, the first-in-class molecule, ABT-737, could effectively bind BCL-2, BCL-XL, and BCL-w and demonstrated potent activity against multiple cancer lines (82), but not BAX/BAK-null cells (83,84). However, due to poor oral bioavailability, ABT-737 was succeeded by its sister compound, ABT-263 (navitoclax) (85). In clinical trials, ABT-263 demonstrated tremendous single-agent activity in patients with chronic lymphocytic leukemia (CLL), a disease in which the leukemia cells are known to depend on BCL-2 for survival (86-88). Unfortunately, due to on-target BCL-XL inhibition, ABT-263
presented with dose-limiting thrombocytopenia that hindered its clinical advancement (87-91).

In response to this on-target toxicity, second-generation BH3 mimetics were reverse engineered to reduce activity against BCL-XL. The result was ABT-199 (venetoclax or Venclexta®), a compound that exhibits several orders of magnitude increased selectivity for BCL-2 over BCL-XL (92,93). Importantly, subsequent clinical trials with ABT-199 revealed profound single-agent efficacy and minimal platelet toxicity, leading to FDA approval as a second line therapy in p53-deleted (17p(del)) CLL (94). However, despite BCL-2 being associated with poor prognosis in acute myeloid leukemia (AML), DLBCL, and FL (14,65), ABT-199 monotherapy demonstrated limited efficacy in these malignancies (95,96). Thus, the main premise of this dissertation is to investigate whether rational combinations may augment the efficacy of BH3 mimetics in these cancers.

BH3 profiling

Since most effective cytotoxic therapies are dependent on induction of apoptosis (97), understanding how a cell decides to die may be the key to predicting effective therapies. Unlike in other fields where approaches like ex vivo drug screening routinely yield clinically relevant predictions (98), predictive diagnostics have yet to mature in a cancer setting. Indeed, most studies tying BCL-2 family proteins to clinical cancer outcomes have been prognostic rather than predictive (e.g. correlative associations between BCL-2 expression and poorer outcomes). While logistical challenges in acquiring viable specimens is a key consideration, predicting the induction of apoptosis
has its own biological challenges as well. For example, different stimuli can result in vastly different BH3-only responses between different cells (99,100). Additionally, many BCL-2 proteins can be sequestered away from the mitochondria where they do not affect mitochondrial apoptosis, complicating the relationship between expression levels and functional activity.

Despite these challenges, emerging functional diagnostic technology is beginning to yield clinically relevant predictive biomarkers. In particular, dynamic BH3 profiling (DBP), which is based off a previously described assay (static BH3 profiling), directly measures a key cellular attribute known as “mitochondrial priming” to predict chemosensitivity (50,101,102). Conceptually, this attribute represents how close a cell is to the threshold for undergoing apoptosis; the higher the priming, the closer the cell is to dying. Both static and dynamic BH3 profiling detect the loss of mitochondrial charge as a surrogate readout for MOMP. By exposing cells to synthetic peptides that mimic the function of BH3-only proteins (102,103), both assays essentially ask how much apoptotic input is required to induce apoptosis. If a higher degree of MOMP can be induced by relatively lower concentrations of BH3 peptide, it can be inferred that this cell is more primed for apoptosis. Importantly, studies have shown that static BH3 profiling can accurately predict differential chemo-sensitivity within cell lines and primary samples (50,104-106). More recently, by pre-treating cells with candidate compounds for a few hours before profiling the cells, DBP has been shown to accurately predict whether the cells are likely to respond to prolonged exposure (107).

While both static and dynamic BH3 profiling have yielded compelling evidence that mitochondrial priming may correlate with clinical responses, their predictive power
is still limited in some cases. For example, static BH3 profiling may not accurately predict whether cells will die in response to some chemotherapies (104). Furthermore, recent DBP data demonstrates that while some inhibitors can significantly increase mitochondrial priming, they may not induce apoptosis after prolonged treatment (108,109). Fortunately, some of these challenges can be circumvented when considering BH3 mimetics. Because these compounds act directly at the mitochondria, static BH3 profiling has consistently predicted single-agent sensitivity to this class of compounds (82,85,92) across several cancer cell types (42,110-113). In Chapters 2 and 3, we demonstrate that while DBP of cells pretreated with targeted inhibitors did not predict apoptosis, it did predict enhanced sensitivity to BH3 mimetics (109). Together, these studies highlight the potential of DBP to identify inhibitors that can synergize with BH3 mimetics.

**PI3K/AKT/mTOR pathway**

*Phosphoinositide 3-kinase (PI3K)*

The PI3K pathway is one of the most commonly activated pathways in human cancer where aberrant upregulation promotes cell growth, proliferation, metabolism, and survival. Activation of this network has been associated with virtually every hallmark of cancer (4,114), and elevated activity is associated with poor prognosis and drug resistance. In support, many models of resistance to tyrosine kinase inhibitors (TKIs, e.g. imatinib, erlotinib, gefitinib, trastuzumab), show sustained PI3K activation (115-118). Additionally, PI3K-activating mutations are sufficient to confer resistance in many cases (119), and affect expression or localization of many BCL-2 family proteins to
promote survival (Figure 1.4 (114)). Given this interplay with the apoptotic pathway, in Chapter 2 we explore the potential of using PI3K pathway inhibitors to enhance the efficacy of BH3 mimetics.

**Figure 1.4. Known interactions between the PI3K pathway and BCL-2 family proteins.** Aberrant PI3K activation affects the expression/activity of many BCL-2 family proteins, skewing cells towards survival by reducing pro-apoptotic proteins (BAD, NOXA, PUMA, Bim (120-122)) and increasing pro-survival proteins ((123)).

There are three major classes of PI3Ks (class I, II, and III), which differ in structure and substrate specificity. Class I PI3K will be the focus of this dissertation as they are primarily responsible for survival signaling. Class I PI3Ks function as heterodimers and preferentially phosphorylate the 3 position of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) to generate the crucial second messenger, PI(3,4,5)P3.
All class I PI3Ks consist of a catalytic subunit (p110) bound to a regulatory subunit, the exact composition of which defines the class IA and IB PI3K subtypes. Class IA PI3Ks contain one of three catalytic subunits: p110α (PIK3CA), p110β (PIK3CB), or p110δ (PIK3CD) bound to one of five regulatory subunits: p85α, p55α, or p50α (alternative products from PIK3R1), p85β (PIK3R2), or p85γ (PIK3R3). On the other hand, class IB PI3Ks consist of p110γ (PI3K4G) bound to either p84 (PIK3R6) or p101 (PIK3R5).

In addition to these structural differences, class IA and IB PI3K also have distinct mechanisms of activation. Facilitated by Src homology (SH) domains that selectively bind phospho-tyrosine residues, class IA PI3Ks are activated downstream of receptor tyrosine kinases (RTKs). On the other hand, class IB PI3Ks are activated by G protein-coupled receptors (GPCRs). Ultimately, both interactions localize PI3K to the plasma membrane where it can efficiently generate PIP3. Importantly, each of the genes encoding class I PI3K have demonstrated potential for oncogenic transformation (128), and the negative regulators of PI3K activity, the lipid phosphatases PTEN and SHIP, are frequently lost or silenced in many types of cancer (129,130).

The generation of the PIP3 induces rapid co-localization of enzymes containing pleckstrin-homology (PH) domains. Following recruitment to the membrane, AKT, a serine/threonine kinase (131,132), is phosphorylated at Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and Ser473 by mechanistic target of rapamycin complex 2 (mTORC2) (133-136). Upon activation via these phosphorylation events, AKT then transduces a significant portion of the PI3K signal through interactions with a diverse substrate pool. Some key AKT targets include Forkhead box group O (FOXO)
transcription factors, tuberous sclerosis complex (TSC)-2, proline-rich AKT substrate of 40 kDa (PRAS40), and the BH3-only protein BAD. In addition to these substrates directly contributing to cell survival and proliferation (Figure 1.4 and 1.5) (129,137,138), they also connect PI3K signaling to mTOR complex 1 (mTORC1), a master regulator of cell growth and proliferation (139,140).

**Figure 1.5. Overview of PI3K/AKT/mTOR signaling pathway.** Activation of PI3K by growth factor receptor leads to generation of PIP₃. This recruits PDK1 and AKT to the plasma membrane where AKT is activated. This in turn activates Rheb-GTP and mTORC1 by suppressing the TSC1/2 complex.
Mechanistic target of rapamycin (mTOR)

mTOR is a serine/threonine kinase that is active in two distinct multi-protein complexes (mTORC1 and mTORC2) characterized by the defining subunits RAPTOR and RICTOR, respectively (141,142). Each complex is differentially regulated and has a distinct set of substrates (Figure 1.5). mTORC2 activation is directly regulated by the levels of PIP3 (143), and is required for full activity of AKT (133). On the other hand, mTORC1 functions by integrating growth factor and nutrient signals (i.e. from the PI3K/AKT pathway) to ensure that the cell is at an appropriate bioenergetic state to support cell growth and division (144,145). Upon activation, mTORC1 promotes key biosynthetic pathways including translation, transcription, and lipogenesis, while suppressing apoptotic and autophagic processes (146,147). Importantly, hyper-activating mutations in mTOR have been identified in many cancers, providing a clear link between mTOR and tumorigenesis (148).

The most well-characterized downstream targets of mTORC1 include the p70 ribosomal-S6 kinases (S6Ks) and eukaryotic initiation factor 4E (eIF4E, Figure 1.5), which coordinately regulate mRNA translation (149,150). In addition to promoting translation via interactions with PDCD4, eIF4B, and ribosomal protein S6 (rS6) (151), S6Ks also regulate ribosome biogenesis, lipid and nucleotide synthesis, and metabolic reprogramming (152-154). There are two known isoforms of S6K (S6K1 and S6K2) that have partially redundant functions. While single knockout mice exhibit different phenotypes, double knockout mice lacking both S6K1 and S6K2 exhibit perinatal lethality, emphasizing the importance of S6Ks in growth, development, or survival (155).
Whereas mTORC1 activates S6Ks directly, it activates eIF4E indirectly by suppressing the eIF4E binding proteins (4E-BPs) (156). When mTORC1 activity is low, 4E-BPs compete with eIF4G for binding to eIF4E, preventing the assembly of the eIF4F translation initiation complex (157). However, upon phosphorylation by activated mTORC1, 4E-BPs lose this capacity to bind eIF4E. As a result, eIF4E can facilitate assembly of eIF4F at the 5’ mRNA cap to initiate translation (158-160). Importantly, this regulation is specific for a subset of oncogenic mRNAs (161,162), including the proliferative proteins, MYC and cyclin D1, as well as the anti-apoptotic protein, MCL-1 (123,163-165).

Targeting the PI3K/AKT/mTOR pathway

Given that each major kinase in the PI3K pathway has its own distinct set of substrates, a plethora of chemical inhibitors specific for each node in the pathway have been developed. In Chapter 2, we take advantage of these tool compounds to isolate and study the contribution of PI3K, AKT, or mTOR inhibition to apoptotic sensitization. These inhibitors, their targets, and in vitro kinase activities are summarized in Table 1.1.

Rapamycin and its analogs (“rapalogs”) are highly potent allosteric inhibitors of mTORC1. Upon entry into a cell, rapalogs bind to FKBP12, forming a complex that selectively suppresses mTORC1 kinase activity by limiting substrate access to the active site (166,167). Importantly, the rapamycin-FKBP12 complex cannot bind to mTORC2 (142,168), though prolonged exposure may limit the assembly of mTORC2 (169). Due to their unique mechanism of action, rapalogs demonstrate unique biological effects. For example, rapalogs are known to incompletely inhibit the phosphorylation of a subset of mTORC1 substrates (170). Additionally, mTORC1 inhibition induces robust
feedback activation of upstream PI3K/AKT and MAPK pathways (Figure 1.6). Despite restricting access to the active-site, mTORC1-mediated phosphorylation of 4E-BP1 is refractory to long-term treatments (171). Consequently, many cells maintain 4E-BP phosphorylation even in the presence of rapamycin (172-175).

<table>
<thead>
<tr>
<th>Target</th>
<th>Inhibitor</th>
<th>p110α</th>
<th>p110β</th>
<th>p110 δ</th>
<th>p110γ</th>
<th>AKT (1,2,3)</th>
<th>mTOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K</td>
<td>GDC-0941 (176,177)</td>
<td>3</td>
<td>33</td>
<td>3</td>
<td>75</td>
<td></td>
<td>580</td>
</tr>
<tr>
<td></td>
<td>ZSTK474 (178)</td>
<td>16</td>
<td>44</td>
<td>4.6</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AKT Inhibitor VIII (179-181)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>58, 210, 2120</td>
<td>8,12,65</td>
</tr>
<tr>
<td></td>
<td>MK-2206 (182)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTOR</td>
<td>MLN0128 (183)</td>
<td>219</td>
<td>5293</td>
<td>230</td>
<td>221</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AZD8055 (184)</td>
<td>3590</td>
<td></td>
<td>3200</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>PI3K/mTOR</td>
<td>NVP-BEZ235 (185)</td>
<td>4</td>
<td></td>
<td>7</td>
<td>5</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>GDC-0980 (186)</td>
<td>5</td>
<td>27</td>
<td>7</td>
<td>14</td>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>

Table 1.1 *in vitro* kinase activity of PI3K pathway inhibitors.

The timely development of mTOR kinase inhibitors (TOR-KIs) directly addressed the biochemical disadvantages of rapalogs. By competing with ATP for binding to the mTOR active site, not only do TOR-KIs more completely block mTORC1 substrate phosphorylation (namely 4E-BPs), but they also inhibit mTORC2 activity (173-175,187-189). This results in reduced phosphorylation of AKT at Ser473 (Figure 1.6), dampening the feedback activation of PI3K/AKT (190-192). Several structurally distinct
mTOR-selective inhibitors have been developed. Most notable among them are PP242 (188), Torin1 (187), Ku-0063794 (193), AZD8055 (194), AZD2014 (194), MLN0128 (previously INK128 (183)), and CC-223 (195). However, it is important to note that by competing with ATP, TOR-KIs inhibit several other related kinases at higher doses, including PI3K. Conversely, several compounds that are often used pre-clinically as PI3K inhibitors (wortmannin, LY294002) also directly inhibit mTORC1 and mTORC2 at similar concentrations. Interestingly, some companies have capitalized on the similarities between PI3K and mTOR active sites, leading to the development of optimized compounds with dual specificity for both kinases (e.g. NVP-BEZ235 and GDC-0980) (185,186).

Through additional optimizations, novel PI3K-selective inhibitors have also been developed. In particular, GDC-0941 (176,177) is an ATP-competitive inhibitor with comparable activity against all class I PI3K isoforms (p110α, p110β, p110γ, and p110δ) but has limited efficacy against mTOR (Table 1.1). Similarly, ZSTK474 is another PI3K inhibitor with even less activity against mTOR (178). In addition to these pan-isoform selective PI3K inhibitors, isoform selective inhibitors also exist (e.g. TGX-221 for p110β (196) and CAL-101 for p110δ (197)). While these inhibitors have demonstrated unique anti-cancer potential in certain contexts (198,199), for the purposes of this dissertation, we chose to focus on pan-PI3K inhibitors in Chapter 2.
Figure 1.6. Rapamycin versus TOR kinase inhibitors. Rapamycin only partially inhibits mTORC1 activity, promoting activation of upstream PI3K, mTORC2, and AKT via loss of feedback inhibition through IRS1. Conversely, TOR-KIs suppress all mTOR outputs.

In addition to PI3K and mTOR inhibitors, compounds selectively targeting AKT are also under development. However, neither ATP-competitive inhibitors nor phosphatidylinositol analogs have yielded compounds selective for the AKT isozymes (200). As a result, in Chapter 2 we primarily utilize allosteric inhibitors. Despite being PH domain dependent, allosteric AKT inhibitors retain selectivity for AKT and commonly display preferential inhibition of single isoforms. For example, AKT Inhibitor VIII,
preferentially inhibits the AKT1 (179-181). Nevertheless, second generation allosteric
AKT inhibitors such as MK-2206, demonstrate more widespread activity against all
three (182). These inhibitors suppress activation of AKT downstream of PI3K by
stabilizing inactive conformations of the protein, suppressing phosphorylation at both
the PDK1 (Thr308) and mTORC2 (Ser473) sites.

**Mevalonate (MVA) pathway**

The mevalonate pathway is a metabolic network that produces several key
bioactive molecules that are necessary for diverse cellular processes (Figure 1.7). The
most widely known function of this lipid metabolism pathway is the de novo production
of sterol isoprenoids (i.e. cholesterol) (201). However, mevalonate is also used to
produce non-sterol isoprenoids that have essential roles in regulating cell growth,
differentiation, and survival. Collectively, these mevalonate-derived products are crucial
in maintaining lipid homeostasis and sustaining tumor growth and survival (202,203).

Entry into the mevalonate pathway begins with the two-step condensation of
acetyl-CoA into acetoacetyl-CoA then 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)
(Figure 1.7). This is followed by the rate-limiting step in the mevalonate pathway,
reduction of HMG-CoA to mevalonic acid. Importantly, transcription of the enzyme
catalyzing this reaction (HMG-CoA reductase, HMGCR) is tightly regulated by sterol
regulatory element-binding proteins (SREBPs) (204,205) in a classic negative feedback
loop. In the absence of sterol isoprenoids, SREBP cleavage activating protein (SCAP)
facilitates the cleavage of SREBP allowing translocation to the nucleus and activation of
downstream target genes (204). In addition to HMGCR, SREBPs are also thought to
regulate nearly every enzyme in the cholesterol biosynthesis pathway (206), making them critical regulators of flux through the mevalonate pathway.

Upon production of mevalonic acid, two phosphorylation steps catalyzed by mevalonate kinase and phosphomevalonate kinase yield mevalonate pyrophosphate. Followed by a series of additional reactions (207), the generation of farnesyl pyrophosphate (FPP) represents a critical branch point in the pathway (Figure 1.7). At this junction, FPP can be metabolized into one of several intermediates that yield distinct products. For example, squalene synthase (SS) catalyzes the first committed step of the cholesterol biosynthesis pathway yielding squalene from FPP. After an additional 21 steps, squalene is then converted to cholesterol for use in membrane integrity or as a precursor for bile acids and steroid hormones (208).

**HMG-CoA reductase inhibitors (statins)**

Beginning with the observation that cancer cells consume a disproportionately large amount of glucose (209), altered metabolism is now widely acknowledged as a hallmark of cancer (4). In essence, it is thought that the transformed state requires increased demand for key metabolic products including those products of the mevalonate pathway (210,211). In support, dysregulation of the mevalonate pathway (via over-expression of HMGCR) enhances tumorigenesis and cooperates with oncogenic RAS to promote cellular transformation (212). Additionally, many cancers up-regulate lipid and cholesterol synthesis genes, with high cholesterol levels correlating with reduced chemo-sensitivity (202,213,214) and resistance to apoptosis (215).
Collectively, these studies further suggest that suppressing the mevalonate pathway may yield therapeutic benefit in cancer.

**Figure 1.7. Overview of the mevalonate pathway.** Mevalonate pathway is a multi-step metabolic pathway that converts acetyl-CoA to multiple bioactive molecules. The rate limiting step is catalyzed by HMG-CoA reductase, which can be inhibited using statins. Following production of farnesyl-PP, the pathway branches to produce molecules that are used in distinct cellular processes (colored boxes).

Initially developed and heralded as a blockbuster drug for the treatment of hypercholesterolemia (201,211), the statin family of drugs may also have selective anti-cancer potential. By inhibiting the HMGCR, the rate-limiting enzyme in the mevalonate pathway (Figure 1.7), statins effectively block production of mevalonate and suppress all downstream metabolic pathways. In this manner, statins induce a remarkable homeostatic feedback loop which activates SREBPs to compensate for the loss of
cholesterol biosynthesis. As a result, SREBP-mediated up-regulation of the low-density lipoprotein (LDL) receptor induces rapid internalization of LDL-cholesterol thereby lowering circulating cholesterol levels. While different statins may have widely divergent pharmacokinetic properties (211,216), they all share common structural features that allow them to bind to catalytically active HMGCR (217). Importantly, statins are among the most widely prescribed drugs, and their tolerability has been well documented (218,219), with the major adverse effect being muscle toxicity (220). These characteristics, coupled with promising preclinical work using cancer cell lines, suggest statins may be an attractive cancer therapy.

Early evidence of statin efficacy revealed a potent anti-proliferative effect cancer cells that stemmed from a block in the G1/S phase transition. In fact, lovastatin was routinely used to synchronize cancer cells in vitro by reversing its potent cell cycle arrest with exogenous mevalonate (221). Later studies revealed that some cancers, particularly acute myeloid leukemias (AML) were exceptionally sensitive to statins as single agents (222,223), and that statins seemed selective for transformed rather than normal cells (223,224). However, despite these promising preclinical studies, clinical trials, retrospective analyses, and epidemiological studies have yielded mixed results regarding the anti-cancer efficacy of statins. While there are some cases of response (particularly in AML (225)), several retrospective analyses of DLBCL suggested statins had no effect on chemotherapy (211,226-228). While the lack of response may in part be due to known resistance mechanisms that up-regulate HMGCR (229,230), a better understanding of statin efficacy may also yield more informative biomarkers. For example, while mechanistic studies implicate downstream protein prenylation pathways,
particularly protein geranylgeranylation (231), as well as BCL-2 modulation (211,232), the exact mechanisms are still largely unknown. Nevertheless, both these findings provide a strong rationale to 1) test statins in combination with BH3 mimetics, and 2) investigate the potential of targeting downstream prenylation pathways.

**Protein prenylation**

Apart from cholesterol biosynthesis, FPP can also be converted to geranylgeranyl pyrophosphate (GGPP) via GGPP synthase. Both FPP and GGPP are substrates for protein prenylation (Figure 1.8), a post-translational modification whereby hydrophobic lipid groups are covalently attached to proteins. The three enzymes that carry out this modification in eukaryotes are farnesyltransferase (FT), geranylgeranyl transferase 1 (GGT-1), and geranylgeranyl transferase 2 (GGT-2) (Figure 1.8). While all proteins undergoing this modification share a common carboxy-terminal cysteine-containing motif, the exact composition of the motif dictates whether it receives an isoprene farnesyl (C-15) or isoprene geranylgeranyl group (C-20). GGT-2 exclusively prenylates Rab GTPases and recognizes CXC or CC motifs, where X is any amino acid (233). On the other hand, both FT and GGT-1 recognize CAAX motifs where C is cysteine and A is any aliphatic amino acid. While FT prefers X to be methionine, serine, glutamine, or cysteine, GGT-1 prefers leucine or isoleucine at this residue (234-236). While these preferences can be exploited by introducing mutations that alter or switch dependence on farnesylation or geranylgeranylation (237), it is important to note that the preferences are not absolute, as some proteins may be subject to both farnesylation and geranygeranylation (238-240). Following prenylation, proteins are then further
processed to augment the functionality of the lipid group. These processing steps are executed by RAS-converting CAAX endopeptidase 1 (RCE1) and isoprenylcysteine carboxylmethyltransferase (ICMT), and yield a carboxy-terminal Cys residue that is both prenylated and methylated (241,242).

Functionally, both farnesylation and geranylgeranylation enhance protein interactions with cellular membranes, ultimately affecting subcellular localization, protein-protein interactions, and/or protein stability. While the exact number of proteins that undergo prenylation is unknown, contemporary reviews estimate that there are several hundred that may be subject to this modification (236,243). Importantly, both farnesylation and geranylgeranylation are essential processes for normal development. In support, knockout of FT is embryonic lethal in mice (244), though it is dispensable for postnatal development and adult tissue homeostasis. Parallel studies have not yet been done with GGT-1 in mice, though results from model organisms support the notion that GGT-1 is a similarly essential protein (245,246).

Several proteins that are confirmed substrates for protein prenylation have key roles in regulating cell growth, proliferation, and survival. Perhaps the most famous example is RAS, one of the most frequently activated oncogenes across human cancers, which undergoes farnesylation. Subsequent work later elucidated that this modification of RAS was required for its transforming activity (247), suggesting that targeting of prenyltransferases may be effective at combating cancer. In support, several other prenylation substrates with relevance to known oncogenic signaling pathways have also been identified (Figure 1.8). In particular, small GTPases like those of the Rac, Rho, and Rheb subfamilies, make up a large majority of the known
“prenylome.” These proteins play major roles in oncogenesis, contributing to critical pathways including the Raf-MEK-ERK (248), PI3K/AKT/mTOR (249,250), and Rho-ROCK pathways.

**Figure 1.8. Targeting protein prenylation pathways.** Two key isoprenoids, FPP and GGPP, are used to prenylate CAAX-containing proteins, many of which are small GTPases of the Ras, Rac, Rheb, and Rho sub-families (green). Distinct enzymes carry out these reactions (blue) that are targetable using small molecule inhibitors (red).

**Farnesytransferase and geranylgeranyl transferase inhibitors**

The finding that inhibiting farnesylation also suppresses oncogenic RAS, a protein that is notoriously difficult to target by direct small molecule inhibitors, launched a global effort to develop FT and GGT-1 inhibitors (FTIs and GGTIs) as cancer therapeutics (251). The result of these efforts were CAAX peptidomimetics that mimic substrate binding and compete for access to the active site. Consequently, structural differences between prenyltransferase inhibitors (PTIs) dictate both the selectivity and potency of these drugs. Pre-clinically, both classes of PTIs demonstrate potent
apoptotic and anti-proliferative effects in a variety of cancer settings. For example, FTIs consistently induce mitotic arrest and apoptosis in RAS-driven cancer cells (251-253) and can induce tumor regression in vivo (254,255). Similarly, conditional GGT-1 deficiency and GGTI treatment also suppresses tumor outgrowth in transgenic mouse models of lung and breast cancer (256,257).

Mechanistically, the anti-cancer effects of both FTIs and GGTIs involves cell cycle inhibition and p53-independent apoptosis (255,258-260). Depending on the context, PTIs have been shown to suppress AKT-mediated inhibition of BAD (258,261), enhance death receptor signaling (262,263), or suppress NF-κB survival outputs (264). However, despite the clear link between prenylation and RAS activity, it is clear that other farnesylated proteins may contribute to the efficacy of FTIs (252). Consequently, incomplete understanding of which substrates are required for sensitivity to FTIs represents a substantial hurdle towards improving clinical responses (265-268).

Collectively, these studies suggest potential synergism between PTIs and agents targeting the apoptotic machinery and indicate the need for developing improved companion diagnostic and/or predictive tools.

**Organization of chapters**

Evasion of apoptosis is a hallmark of cancer that is now directly targetable using small molecule BCL-2 inhibitors. While clearly effective in some settings (namely CLL), it becoming increasingly apparent that these so-called BH3 mimetics induce incomplete responses in other contexts. A promising approach to improving their efficacy is through rational combinations. Several oncogenic pathways feed into regulation of the BCL-2
family proteins and may contribute to poor sensitivity to BCL-2-targeting therapies. Thus, this dissertation addresses whether targeting two different pathways important to cancer (PI3K and MVA) can sensitize cells to BCL-2 inhibition by priming cells for undergoing apoptosis (Figure 1.9). Understanding how this sensitization occurs, and whether it can be predicted using techniques like BH3 profiling, is critical for efforts to improve the use of BCL-2 antagonists in a clinical setting.

In Chapter 2, we present a mechanistic investigation into the efficacy of combining PI3K pathway inhibitors with BH3 mimetics. Using pharmacological and genetic approaches, we identify a mechanism of action that complements previously published studies and strongly supports further clinical investigation. In addition, we demonstrate the merits of DBP in predicting the most potent sensitizers to BCL-2 inhibition. These findings led to a first-author publication in Oncotarget.

Chapter 3 investigates the potential repurposing an FDA-approved drug (statins) to enhance the efficacy of BH3 mimetics in blood cancer. Using several preclinical models, we demonstrate the efficacy and selectivity of this combination across different blood cancers. We also reaffirm the merits of DBP as a predictive tool, this time in identifying which cells are likely to respond to combinations. Lastly, we characterize a mechanism involving potentially-targetable downstream effectors.

Lastly, Chapter 4 summarizes the major findings of this dissertation, discusses unpublished or incomplete data, and provides insight into unanswered mechanistic questions regarding the efficacy of the two combinations presented. We also present some future directions for enhancing the efficacy of BH3 mimetics in cancer.
Figure 1.9. Central hypothesis: combination approaches to treating cancer using BH3 mimetics. In some contexts, treatment with BH3 mimetics may be insufficient to kill cells as single agents. The addition of other targeted agents may prime these cells for apoptosis, leading to an effective combination.

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Chapter Two

PI3K/AKT/mTOR inhibitors enhance the efficacy of BCL-2 inhibitors in diffuse large B cell lymphoma
This chapter is largely derived from a manuscript published in *Oncotarget* 6(34) pages 35202-35217 (2015), entitled “MCL-1-independent mechanisms of synergy between dual PI3K/mTOR and BCL-2 inhibition in diffuse large B cell lymphoma”. Supplementary materials originally published from this manuscript have been incorporated into the main text.

**Abstract**

Aberrant activation of the PI3K/AKT/mTOR pathway is associated with poor prognosis in patients with diffuse large B cell lymphoma (DLBCL). However, inhibitors targeting this pathway are often insufficient to evoke strong clinical responses as single agents, suggesting that combinations may hold potential. In support, previous studies have demonstrated marked synergy with BCL-2 inhibitors in other cancer subsets (e.g. lung cancer) where PI3K/mTOR inhibitors suppress expression of MCL-1. Here, we demonstrate that PI3K/AKT/mTOR inhibitors also synergize with BCL-2 antagonists in DLBCL cell lines, a phenomenon that could be predicted using dynamic BH3 profiling (DBP). Unlike in solid tumors, mTORC1 inhibition did not suppress MCL-1 expression. Instead, inhibition of AKT induced mitochondrial accumulation of pro-apoptotic proteins BAD and BIM. Thus, our work identifies an additional mechanism of synergy between PI3K pathway inhibitors and BCL-2 antagonists that strengthens the rationale for testing this combination in DLBCL.
**Introduction**

PI3K inhibitors have recently received increased attention in blood cancers, where an inhibitor of the p110δ catalytic isoform (idelalisib) elicits significant patient responses in both chronic lymphocytic leukemia (CLL) and indolent non-Hodgkin’s lymphoma (1). However, more aggressive blood cancers such as diffuse large B cell lymphoma (DLBCL) do not respond to monotherapy with inhibitors targeting this network (2). Nevertheless, inhibition of the PI3K pathway may still be effective in rational combinations with other therapies. Indeed, in the activated B cell (ABC) subtype, the combination of PI3K pathway inhibitors with the BTK inhibitor, ibrutinib, has shown promise (3,4). Despite this efficacy in ABC-DLBCL, combined BTK and PI3K inhibition fails to kill the germinal center subtype (GCB) cells (4). In this context, elevated expression of BCL-2 (a hallmark of the GCB subtype (5,6)) may limit the cytotoxic potential of PI3K inhibitors (7,8). These observations suggest that PI3K pathway inhibitors may still sensitize GCB-DLBCL to BH3 mimetics.

Previously, others have demonstrated synergism between PI3K inhibitors and BCL-2 antagonists in several contexts (9-11). For example, in DLBCL sublines that are selected for resistance to ABT-199/737, mTOR inhibition can revert the resistance (12,13). In other cancer subtypes, similar efficacy is observed where mTORC1 inhibition decreases MCL-1 expression (14). However, the PI3K pathway has several other survival outputs, particularly from AKT (15,16), that may also enhance BCL-2 inhibitor sensitivity. Additionally, the effect of these drug combinations on normal lymphocytes has not yet been explored.
Here, we show that the combination of PI3K pathway and BCL-2 inhibitors synergistically induces apoptosis in a panel of GCB-DLBCL cell lines, with dual PI3K/mTOR inhibition providing the greatest effect. Importantly, we also demonstrate that dynamic BH3 profiling of cells treated with PI3K pathway inhibitors correlates strongly with the degree of synergy, suggesting this assay may be used to predict treatment responsiveness. Furthermore, the combination is also effective in chemo-resistant DLBCL lines, but lack toxicity in normal T lymphocytes. Contrary to other tumor cell contexts, MCL-1 expression in GCB-DLBCL cells does not decrease following PI3K/mTOR inhibition. Instead, treatment with dual PI3K/mTOR inhibitors increases mitochondrial accumulation of BAD and BIM. These effects are dependent on suppression of AKT activity, as a constitutively active mutant of AKT abolishes both the synergy and up-regulation effects. These findings identify a promising combination approach to achieve selective GCB-DLBCL death, and highlight a previously unpredicted mechanism of synergy.

Materials and Methods

Chemicals

We obtained rapamycin, MLN0128, GDC-0941, and NVP-BEZ235 from LC Laboratories (Woburn, MA, USA); ABT-263, ABT-199, MK2206 and GDC-0980 from Active Biochem (Wan Chai, Hong Kong), and AKT inhibitor VIII from Chemdea (Ridgewood, NJ, USA). InSolution Q-VD-OPh was obtained from EMD Millipore (Billerica, MA, USA), dimethyl sulfoxide (DMSO) from Fisher Scientific (Waltham, MA, USA) and doxycycline from Sigma-Aldrich (St. Louis, MO).
Cell Culture

OCI-LY1, OCI-LY7, OCI-LY8, and SU-DHL4 cell lines (a gift from Dr. Laura Pasqualucci, Columbia University) were cultured in IMDM (GE Healthcare Hyclone, Little Chalfont, UK) supplemented with 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM L-Glutamine, 100 I.U. penicillin, and 100 µg/ml streptomycin. Cells were grown in a humidified 37°C incubator with 5% CO2. Cells were routinely tested to ensure absence of mycoplasma, and were maintained at or below 2 x 10^6 cells/mL. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% calf serum, 100 I.U. penicillin, and 100 µg/mL streptomycin. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by centrifugation through Ficoll-Paque™ (GE Healthcare, Piscataway, NJ, USA) and were grown in RPMI (Corning, NY, USA) with identical additives as IMDM.

Western blotting

Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, 2 mM EDTA, 50 mM NaF) supplemented with protease inhibitor cocktail (Calbiochem, USA) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). Protein concentrations were normalized using a Bradford protein assay (Bio-Rad). Lysates were prepared at 1 µg/µl concentration in 1X XT Sample Buffer (Bio-Rad) and 5% 2-mercaptoethanol (Sigma-Aldrich). Lysates were run on 4-12% Bolt® Bis-Tris Plus gels.
(Life Technologies), and transferred onto nitrocellulose membranes. The following antibodies were used: phospho-AKT (S473), phospho-PRAS40 (T246), phospho-rS6 (S240/244), phospho-BAD (S136), 4E-BP1, GAPDH, PARP, caspase 9, cleaved caspase 3, cleaved caspase 8, MCL-1, BIM, COX IV, ERK, phospho-FOXO1 (T24)/FOXO3 (T32), HA-Tag, BCL-Xₐ, Bad (Cell Signaling Technology, Beverly, MA, USA), BCL-2 (BD Pharmingen, San Diego, CA, USA), and Bad (Santa Cruz Biotechnology, Dallas, TX, USA). The following secondary HRP-conjugated antibodies were used: anti-mouse IgG, anti-rabbit IgG (Promega, Madison, WI, USA), and Protein A (BD Pharmingen). Blots were developed using Pierce ECL Western Blotting Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Life Technologies) and detected using a Nikon D700 SLR camera as described previously (17). Images were processed using Adobe Photoshop software and densitometry was quantified using ImageJ software.

**Cell Viability**

Cell viability assays were performed in 96-well plates, with 6 x 10⁴ cells in 200 µl. Cells were harvested by centrifuging the 96-well plate in a plate spinner centrifuge at 500 g for 5 minutes. Cells were incubated in 1 µg/ml 7-aminoactinomycin D (Life Technologies) in Hank’s Balanced Salt Solution (HBSS; Life Technologies) supplemented with 2.5% bovine serum albumin for 10 min at room temperature. Cell fluorescence was assessed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Analysis of the data was completed using FlowJo Software v10.0.7 (TreeStar, Ashland, OR). All IC₅₀ values were calculated using GraphPad Prism.
version 5c software (GraphPad Software, La Jolla, CA, USA) from the average of three independent experiments using half-log dilutions of the indicated inhibitor.

**BH3 profile**

OCI-LY1 and SU-DHL4 cell lines were profiled as previously described,(18) with modifications. Cells were plated at 8 x 10^6 cells per 10 ml of media and treated with inhibitors for 16 hours. 4 x 10^5 cells were incubated in T-EB buffer (300 mM trehalose, 10 mM HEPES, 80 mM potassium chloride, 1 mM EGTA, 1 mM EDTA, 0.1% BSA, and 5 mM succinic acid) with 200 nM JC-1 (Life Technologies), 0.001% digitonin (Sigma-Aldrich), and 10 µg/ml oligomycin (Sigma-Aldrich) with either DMSO or BH3-only peptides for 60 minutes prior to analysis using a FACScalibur (Becton-Dickinson). The sequences and method of synthesis of BH3-only peptides were described previously (19). Percent depolarization caused by each BH3-only peptide was calculated as the percent difference in the JC-1 red fluorescence (590 nm) relative to DMSO-treated control cells.

**Retro/lentiviral Transductions**

For all viral productions, 293T HEK cells were transfected using X-tremeGene HP DNA Transfection Reagent (Roche, Switzerland). 293T cells were incubated for 24 hours prior to replacing medium with IMDM. These virus-containing media were then harvested after an additional 24 hours and used to transduce DLBCL cell lines. For retroviral production, 293T cells were co-transfected with pCL-ampho viral packaging vector (Novus Biologicals, Littleton, CO, USA) whereas pCMV-VSVG (Addgene plasmid 54
8454) and psPAX2 (Addgene plasmid 12260) were co-transfected for lentivirus production. To transduce DLBCL cell lines, we incubated cells in viral supernatants for 72 hours (changing supernatant every 24 hours) with 10 µg/ml 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene, Sigma-Aldrich). Cells were treated with either blasticidin (8 µg/ml) or puromycin (2 µg/ml) for 5 days after transduction to select for stably transduced cells. Plasmid-positive cells were maintained with blasticidin (4 µg/ml) or puromycin (1 µg/ml).

Expression plasmids

To generate DLBCL cells with doxycycline-inducible expression of a gene of interest, cells were first transduced with pMA2640 (Addgene plasmid #25434) and selected for blasticidin resistance. Expression of the improved tetracycline-controlled transactivator (rtTA-Advanced) allowed for doxycycline-inducible expression of genes downstream of the modified Tet-responsive element provided in the pLVX-tight-puro vector (Clontech). To generate MCL-1 expression plasmid, the human MCL-1 cDNA was cloned from pCMV-Flag-hMCL-1 (Addgene plasmid #25392) into pUC118 using BamHI and EcoRV. MCL-1 was then cloned into plvx-tight-puro using BamHI and NotI. To generate the AKT(S473D)-pLVX-tight-puro plasmid, we cloned AKT (S473D) from a plasmid received from Dr. Bing Su (Yale University) into pLVX-tight-puro using NotI and EcoRI. To generate BAD expression plasmids, murine Bad (S136A) in pcDNA3 (Addgene plasmid #8798) was cloned into plvx-tight-puro using EcoRI. WT murine Bad was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) to introduce a point mutation to restore
expression of a serine rather than alanine at position 136. The following primers were used for this purpose 5’-AGGACGCTCGCGTTCGGCTCCCC-3’ and 5’-GGGAGCCGAACGCGAGCGTCCTCCT-3’. To generate BCL-2 expression plasmid, the human BCL-2 cDNA was cloned from pMIG-BCL-2 (Addgene plasmid #8793) into pLVX-tight-puro using EcoRI. pLKO.1 shRNA expression plasmids containing MCL-1 hairpins (TRCN0000005514, TRCN0000005516, TRCN0000005517) were a gift from Dr. Anand Ganesan (UC Irvine). All pLVX-tight-puro plasmids were sequenced using the following primer, 5’-AGCTCGTTTAGTGAACCGTCAGATC-3’.

Subcellular Fractionation

Subcellular fractionation was performed as described previously (20). In brief, cells were harvested and resuspended in isotonic buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Calbiochem, USA), and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)). Cells were lysed by passing through 28 gauge insulin syringes and resulting lysates were spun at 800 g for 10 minutes at 4˚C four times to remove intact cells and nuclear fractions. Supernatants were then spun at 10,000 g for 30 minutes at 4˚C to separate the mitochondria-enriched heavy membrane pellet from the supernatant containing cytoplasmic fractions. Pellets were then lysed using RIPA buffer and run for immunoblotting as described above.
Co-immunoprecipitation

Co-immunoprecipitations were performed as described previously (20). Briefly, total cell lysates were prepared using 1% CHAPS buffer (5 mM MgCl₂, 137 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 20 mM Tris-HCL (pH 7.5), and protease inhibitor cocktail (Calbiochem, USA)). BCL-2 was immunoprecipitated from 500 µg protein using anti-BCL-2 (BD Pharmingen) and a slurry of protein G-Sepharose beads (GE Healthcare) at 4˚C for 16 hours. Immunoprecipitates were washed three times in 1% CHAPS buffer and eluted from beads by boiling in 1X XT Sample Buffer (Bio-Rad) in 1% CHAPS buffer with 5% 2-mercaptoethanol (Sigma-Aldrich) for 10 minutes.

Cell Cycle

Cells were plated at 1 x 10⁶ cells/ml and treated with inhibitors for 48 hours. Cells were then harvested and permeabilized in 90% ethanol. RNA was degraded using RNAse A (100 µg/ml) before staining DNA with propidium iodide (50 µg/ml) in 5 mM EDTA in 1X PBS. Fluorescence was measured using FACScalibur (Becton-Dickinson) and cell cycle populations were analyzed using FlowJo Software v10.0.7 (TreeStar).

SUnSET puromycin incorporation

We performed SUnSET puromycin incorporation in OCI-LY1 and SU-DHL4 cells as previously reported (21), with modifications. Cells were plated at 3 x 10⁶ cells in 3 ml and treated with inhibitors for 24 hours. Nascent peptide chains were labeled by incubating cells in 1 µg/ml puromycin (Sigma-Aldrich) for 30 minutes. Control cells were co-treated with 20 µg/ml cycloheximide (Sigma-Aldrich) during puromycin labeling.
Lysates from these cells were run for immunoblotting as described above. An anti-puromycin primary antibody (EMD Millipore, Billerica, MA, USA) was used and visualized using chemiluminescence after incubation with an anti-mouse HRP-conjugated secondary (Promega).

**Luciferase Assays**

For measurement of FOXO transcriptional activity, cells were co-transfected with equal amounts of the pRL-TK plasmid (Promega) and the FOXO3 Firefly luciferase reporter plasmid (gift from Dr. Anne Brunet, Stanford University). For cap-dependent translation studies, the pRSTF-CVB3 dual-luciferase reporter plasmid was used as described previously (22). For all experiments, cells were transfected using a GenePulser Xcell™ (Bio-Rad) at 280 V and 0.975 F. All cells were treated with indicated inhibitors for 16 hours, prior to harvesting. Luciferase activity was measured using substrates from the Dual-Luciferase Reporter Assay System kit (Promega) and a Sirius single tube luminometer (Titertek-Berthold, Pforzheim, Germany).

**Statistical Analysis**

Statistical analyses were performed in the GraphPad Prism software version 5c (GraphPad Software). Unless otherwise indicated, results indicate mean±S.D. of three independent experiments. Unless otherwise indicated, all western blots are representative of three independent experiments. P < 0.05 was considered statistically significant and was annotated throughout as: * P < 0.05, ** P < 0.005, *** P < 0.001. For
drug synergy calculations, combination index versus fraction affected curves were generated using CalcuSyn software (Biosoft, Cambridge, UK).

Results

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Class</th>
<th>Selective Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0941</td>
<td>Pan-PI3K</td>
<td>100</td>
</tr>
<tr>
<td>ZSTK-474</td>
<td>Pan-PI3K</td>
<td>100</td>
</tr>
<tr>
<td>AKT Inhibitor VIII</td>
<td>Allosteric AKT</td>
<td>1000</td>
</tr>
<tr>
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<tr>
<td>Rapamycin</td>
<td>Allosteric mTOR</td>
<td>10</td>
</tr>
<tr>
<td>BEZ235</td>
<td>Dual PI3K/mTOR</td>
<td>50</td>
</tr>
<tr>
<td>GDC-0980</td>
<td>Dual PI3K/mTOR</td>
<td>300</td>
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</table>

Table 2.1. Minimum required dose of PI3K pathway inhibitors required for full inhibition of targets in DLBCL. The above listed concentrations were determined by treating cells with half-log dilutions of each inhibitor for 3 hours. Doses reflect the minimum dose required to fully block phosphorylation of substrate proteins.

PI3K pathway inhibition increases mitochondrial priming and enhances cytotoxicity of ABT-263 in DLBCL

To evaluate the impact of PI3K pathway inhibitors as single agents, we used several classes of chemical inhibitors targeting distinct nodes in the PI3K/AKT/mTOR axis. For each class of inhibitor, we compared the effects of two chemically distinct
compounds to limit the contribution of off-target effects (Table 2.1). Using the minimum dose of the inhibitors required to completely inhibit their intended nodes (Figure 2.1A), we demonstrate that PI3K inhibitors alone do not significantly kill GCB-DLBCL cell lines (Figure 2.1B). However, all inhibitors caused an accumulation of cells in the G1 phase (Figure 2.1C), indicative of a cytostatic response.

Figure 2.1. Suppression of the PI3K/AKT/mTOR pathway components is cytostatic in DLBCL. (A) western blot of OCI-LY1 cells treated with indicated inhibitors (concentrations indicated in Table 2.1) for 3 or 6 hours. (B) Viability of DLBCL cell lines (OCI-LY1, SU-DHL4, and OCI-LY8) treated with inhibitors for 48 hours. (C) Cell cycle analysis of cells treated for 48 hours. Percentage of cells in the G1 stage of cell cycle was obtained using FlowJo (v10) software.
Since PI3K pathway inhibitors can synergize with BCL-2 antagonists in other contexts (9-12), we used BH3 profiling to investigate whether these inhibitors were priming cells for apoptosis without killing them outright (18). Interestingly, all inhibitors significantly increased mitochondrial priming, indicated by elevated MOMP by the BIM, PUMA, and BAD peptides (Figures 2.2A, D and 2.3A). Importantly, sensitivity to the NOXA peptide, a readout for MCL-1 modulation, remained unchanged (Figure 2.3A).

Heightened sensitivity to the BAD peptide (Figure 2.3A) suggested that PI3K inhibitors were increasing dependence on anti-apoptotic factors that could neutralize BAD (e.g. BCL2 and BCL-XL). Consistent with this interpretation, previous studies have shown that increased sensitivity to the BAD peptide correlates with higher efficacy of the dual BCL-2/BCL-XL antagonist, ABT-737 (23). Indeed, combined PI3K and BCL-2/BCL-XL inhibition killed significantly more DLBCL cells compared to single-agent treatments (Figures 2.2B, E). In addition, the degree of enhanced apoptosis correlated strongly with the extent of BIM-induced MOMP (Figures 2.2C, F). In other words, DBP seemed to predict which PI3K inhibitor would best enhance ABT-263 cytotoxicity.
Figure 2.2. PI3K pathway inhibition increases mitochondrial priming and enhances efficacy of ABT-263 in DLBCL cell lines. (A, D) BH3 profile of OCI-LY1 and SU-DHL4 cells treated with inhibitors for 16 hours. (B, E) Viability of OCI-LY1 and SU-DHL4 cells treated with ABT-263 with or without PI3K pathway inhibitors for 48 hours. (C, F) Spearman correlation between ABT-263 sensitivity (IC_{50}) and MOMP induced by BIM peptide. (G) ABT-263 sensitivity of four DLBCL cell lines with or without BEZ235. (H) Viability of cells treated with combinations of ABT-263 and BEZ235 with or without Q-VD-OPh (pan-caspase inhibitor).
Figure 2.3. Dual PI3K/mTOR inhibition consistently demonstrates greatest enhancement of ABT-263 efficacy. (A) BH3 profile of OCI-LY1 cells treated with various PI3K pathway inhibitors for 16 hours prior. (B) Sensitivity of four DLBCL cell lines to ABT-263 in the presence or absence of various PI3K pathway inhibitors. (C) Sensitivity of two DLBCL cell lines to ABT-263 in combination with two chemically distinct dual PI3K/mTOR inhibitors (BEZ235 and GDC-0980).

Among the classes of PI3K pathway inhibitors used, the dual PI3K/mTOR inhibitors, BEZ235 and GDC-0980, were consistently the most potent sensitizers to ABT-263 across several DLBCL cell lines tested (Figures 2.2G and 2.3B, C). Thus, we focused further experiments on the effects of dual PI3K/mTOR inhibitors. Using the median-effect method (24), we confirmed that combining BEZ235 and ABT-263 demonstrated formal synergy in both OCI-LY1 and SU-DHL4 cell lines (Figure 2.4). To confirm the induction of apoptosis, we co-treated DLBCL cells with the pan-caspase inhibitor, Q-VD-OPh (25), which rescued the death effects of BEZ235 and ABT-263 (Figure 2.2H). We further confirmed that the combination induced dose- and time-
dependent cleavage of caspase 3, caspase 9, and poly ADP ribose polymerase (PARP, Figure 2.5), indicative of an activated apoptosis pathway. Cleavage of caspase 8 also occurred concurrently with caspase 3 cleavage, and may be the result of a positive-feedback loop (26).

**Figure 2.4.** BEZ235 and ABT-263 synergistically kill DLBCL cell lines. (A) Viability of OCI-LY1 and SU-DHL4 were treated with indicated inhibitors (note that concentration for ABT-263 in the SU-DHL4 cells is 6-fold higher than the indicated dose). (B) Formal synergy analysis of (A).
**Figure 2.5. Combining BEZ235 and ABT-263 induces caspase and PARP cleavage.** (A) Western blot of OCI-LY1 cells treated with increasing doses of ABT-263 with or without BEZ235 for 6 hours. (B) Western blot of OCI-LY1 cells treated with ABT-263 (100 nM) in combination with BEZ235 (50 nM) for indicated duration.

**Combined PI3K/mTOR and BCL-2 inhibition spares normal T cells**

By inhibiting BCL-XL, ABT-263 results in the on-target toxicity of thrombocytopenia (27), which is not observed with the selective BCL-2 inhibitor, ABT-199 (28). Thus, to test whether PI3K/mTOR inhibitors could also enhance the efficacy of the more clinically relevant BH3 mimic, we treated DLBCL cells with both ABT-263 and ABT-199 in combination with BEZ235. The lack of change in sensitivity to the HRK peptide (Figure 2.3A) suggested that BCL-XL inhibition was dispensable for the observed synergy between ABT-263 and BEZ235. Indeed, while ABT-199 was effective at 10-fold lower concentrations than ABT-263, the efficacies of both BCL-2 inhibitors were significantly enhanced by the addition of either BEZ235 or GDC-0980 (Figure 2.6A).
Figure 2.6. BEZ235 does not enhance the toxicity of BH3 mimetics in normal human T cells. (A) Viability of OCI-LY1 and SU-DHL4 cells treated with ABT-263 (50 or 300 nM, respectively) or ABT-199 (5 or 50 nM, respectively) with or without BEZ235 or GDC-0980. (B) Viability of PBMCs isolated from normal human blood donors treated with ABT-263 (30 nM) or ABT-199 (3 nM) with or without BEZ235 for 48 hours.

To evaluate the tolerability of this combination, we tested whether BEZ235 plus ABT-199 is toxic to normal human lymphocytes, particularly the CD4⁺ T cells that contribute to anti-tumor immune responses (29,30). We isolated PBMCs from healthy human donors and treated the cells with BEZ235 and either ABT-199 or ABT-263. In agreement with previous work (27), treatment with either BCL-2 antagonist alone for 48 hours was sufficient to eradicate normal B cells (Figure 2.6B). However, at doses of ABT-263 and ABT-199 that synergized with BEZ235 in OCI-LY1 cells, there was no significant effect on the viability of CD4⁺ T cells (Figure 2.6B). Importantly, the addition
of BEZ235 did not further enhance the cytotoxicity of ABT-199 in CD4 T cells, suggesting that the combination may be selective for cancer cells.

*Chemo-resistant DLBCL cells over-expressing BCL-2 remain sensitive to BEZ235 with ABT-199*

Overexpression of BCL-2 is associated with chemo-resistance, particularly in GCB-DLBCL (5,31). Thus, to determine whether the combination of ABT-199 and BEZ235 could efficiently eliminate cells that over-express BCL-2, we established stable cell lines that could inducibly over-express BCL-2 (**Figure 2.7A**). While excessive over-expression of BCL-2 was toxic (via cleavage to a pro-apoptotic isoform, data not shown), modest increases in BCL-2 expression were sufficient to induce resistance to the chemotherapeutic agent, vincristine (**Figure 2.7B**). As expected, increased BCL-2 expression also reduced sensitivity to ABT-199 as a single agent (**Figure 2.7C**). Nevertheless, the addition of BEZ235 enhanced the killing effect of ABT-263 in BCL-2-over-expressing cells (**Figure 2.7C**).
Figure 2.7. DLBCL cells over-expressing BCL-2 are resistant to a chemotherapeutic drug but remain sensitive to BEZ235 and ABT-199. (A) Western blot of cells treated with indicated doxycycline doses for 24 hours. (B) Viability of cells pre-treated with doxycycline (25 ng/ml or 1 µg/ml, respectively) for 24 hours, prior to treatment with vincristine for an additional 48 hours. (C) Viability of cells pre-treated with doxycycline (25 ng/ml or 1 µg/ml, respectively) for 24 hours prior to treatment with ABT-199 (100 nM, respectively) with or without BEZ235 for 48 hours.

**Dual PI3K/mTOR inhibition does not affect MCL-1 expression in DLBCL cell lines**

Previous studies have revealed a critical role for MCL-1 in modulating sensitivity to BCL-2 antagonists (32-35). In particular, suppression of mTORC1-mediated translation of MCL-1 sensitized various cancer cells, including ABT-199-resistant DLBCL, to BH3 mimetics (9-12). Thus, to test whether mTORC1 inhibition could also suppress MCL-1 expression in our DLBCL cell lines, we treated cells with either BEZ235, MLN0128, or PIK-75 (a compound that suppresses MCL-1 transcription (36)) and monitored MCL-1 expression by western blot. Surprisingly, while PIK-75 fully suppressed MCL-1 expression within 8 hours, neither BEZ235 nor MLN0128 reduced
expression of MCL-1 (Figures 2.8A and 2.9A, B). By contrast, BEZ235 strongly suppressed expression of MCL-1 in a human leukemia cell line (BV173). Interestingly, comparing parental and ABT-199-resistant SU-DHL6 cells, BEZ235 only reduced MCL-1 expression in resistant cells (Figure 2.8A), consistent with previous findings (12).

Figure 2.8. BEZ235 does not affect MCL-1 expression in OCI-LY1 cells. (A) Western blot of cells treated with BEZ235 or PIK-75 for increasing duration. (B) Sensitivity of cells stably transduced with shMCL-1 or shScramble to ABT-263 with or without BEZ235. (C) Sensitivity of cells stably transduced with empty vector or a doxycycline-inducible MCL-1 expression vector to ABT-263. Cells were pre-treated with doxycycline (1 µg/ml) for 24 hours prior to determining IC_{50}.
Figure 2.9. BEZ235 does not affect MCL-1 expression in DLBCL cell lines. (A,B) Western blot of cells treated with PIK-75 and either BEZ235 (A) or MLN0128 (B), concentrations indicated. (C) Dual luciferase cap-dependent reporter assay of cells treated with BEZ235 for 16 hours. (D) Western blot of cells pre-treated with vehicle (DMSO) or BEZ235 for 6 hours prior to pulse treatment of puromycin (1 µg/ml) for 30 minutes, or co-treatment with cycloheximide (CHX, 20 µg/ml) and puromycin for 30 minutes. Representative of two independent experiments. (E) Western blot of cells treated with cycloheximide (20 µg/ml) for indicated duration.

We considered two possibilities to explain why dual PI3K/mTOR inhibition did not reduce MCL-1 expression. First, we tested the possibility that BEZ235 does not sufficiently suppress cap-dependent translation in DLBCL cells. However, a dual-luciferase reporter assay confirmed that BEZ235 strongly inhibits cap-dependent translation (Figure 2.9C). In addition, BEZ235 reduced the rate of puromycin
incorporation into newly synthesized peptides (SUnSET (21), Figure 2.9D). Second, we considered whether MCL-1 was aberrantly stabilized. In particular, because mutations in MCL-1 may confer increased protein stability (37), we assessed whether cycloheximide could abolish MCL-1 expression in a time frame consistent with normal stability. In agreement with previous studies (37), we confirmed MCL-1 has a half-life of roughly 90 minutes in DLBCL (Figure 2.9E). Thus, our data suggest that MCL-1 levels are not regulated by mTORC1-dependent translation in these DLBCL cell lines.

To further assess whether MCL-1 contributes to the enhancement effect, we modulated MCL-1 expression and monitored the degree of sensitization. Using shRNA, we knocked down expression of MCL-1 (Figure 2.10A). Knocked down cells were expectedly more sensitive to ABT-263 (Figure 2.8B). However, the addition of BEZ235 further enhanced ABT-263 killing (Figure 2.8B), consistent with a model in which BEZ235 likely primes for apoptosis through an alternative mechanism. To determine whether uncoupling MCL-1 translation from regulation by mTORC1 could rescue from the synergy, we expressed of a form of MCL-1 lacking its endogenous 5' UTR (Figure 2.10B) (14). Despite conferring resistance to ABT-263, ectopic expression of MCL-1 was insufficient to abolish the synergy between BEZ235 and ABT-263 (Figure 2.8C). Thus, despite the clear influence of MCL-1 expression levels on ABT-263 sensitivity, suppression of PI3K/mTOR likely synergizes with BCL-2 antagonists through a non-MCL-1-dependent mechanism.
Figure 2.10. Confirmation of MCL-1 knockdown and overexpression. (A) Representative western blot of MCL-1 knockdown using three distinct shRNAs (upper) and quantification (lower). (B, C) Western blot of MCL-1 expression in cells transduced with empty vector or a doxycycline-inducible MCL-1 expression vector. Cells were pretreated with doxycycline (1 µg/ml) for 24 hours before treatment with BEZ235 for 24 or 48 hours.
PI3K pathway inhibition increases mitochondrial localization of BAD and BIM

Other than suppressing mTORC1-dependent translation of pro-survival factors (e.g. MCL-1 and BCL-XL), inhibition of the PI3K pathway also affects the expression of several other BCL-2 family proteins (38). Since some BCL-2 family proteins are regulated by subcellular localization (39), we examined the abundance of BCL-2 family proteins in mitochondria-enriched lysate fractions. After 16 hours, PI3K pathway inhibition induced mitochondrial accumulation of BAD and BIM (Figure 2.11A, B). To confirm that these BH3-only proteins were functionally contributing to priming, we used immunoprecipitation to assess whether there was increased loading onto BCL-2. BEZ235 increased both the total abundance of BIM as well as its direct binding to BCL-2 (Figure 2.11C), suggesting that BCL-2 mitigates the induction of BH3-only proteins to support survival. These results are consistent with increased sensitivity to the BAD peptide (Figure 2.3A), which similarly suggested that DLBCL cells rely on BCL-2 for survival in the absence of PI3K activity. However, the addition of ABT-199 displaced BIM from BCL-2 (Figure 2.11C) allowing BIM-mediated induction apoptosis (40). In addition to changes in BIM expression, the reduction of cytoplasmic phospho-BAD (S136), and concomitant increase in the mitochondrial abundance of BAD, also support a model of increased dependence on BCL-2 following PI3K pathway inhibition (Figure 2.11A). Mitochondrial accumulation of BAD is likely a result of its binding to BCL-2 (41), suggesting that BAD may amplify the effect of BIM up-regulation by limiting the amount of BCL-2 that can counteract BIM. Together, these data suggest that inhibition of PI3K/mTOR by BEZ235 enhances the effect of BCL-2 antagonists by increasing the abundance of BIM and BAD at the mitochondria.
**Figure 2.11.** PI3K pathway inhibitors increase mitochondrial abundance of BAD and BIM. (A) Western blot of mitochondrial and cytoplasmic fractions of OCI-LY1 cells treated with indicated PI3K pathway inhibitors for 16 hours. (B) Average densitometry values of three replicates of panel (A). (C) Western blot of immunoprecipitation of BCL-2 (upper) or whole cell lysates (lower) following 16 hour treatment with BEZ235, ABT-199 (100 nM), or the combination. All cells were also treated with 10 µM Q-VD-OPh to prevent cleavage of BCL-2 family proteins by caspases.

*Inhibition of AKT is required for apoptotic sensitization in DLBCL cell lines*

Previous work has established that both BAD and BIM can be regulated in part by AKT (42). Thus, to test whether sustained activation of AKT could abolish the synergy enhancement effect of PI3K inhibitors, we used a doxycycline-inducible system to express a phospho-mimetic mutant of AKT (S473D). Expression of this mutant not only elevated basal AKT activity, but also rendered AKT activity insensitive to direct PI3K or allosteric AKT inhibitors (Figures 2.12A and 2.13). Importantly, AKT S473D also completely blocked the ability of MK2206 and BEZ235 to increase the mitochondrial abundance of BIM and BAD (Figure 2.12B). Lastly, AKT S473D expression completely abrogated the synergy between AKT inhibitors and BCL-2 inhibitors in DLBCL cells, and partially reversed the sensitization by dual PI3K/mTOR inhibitors (Figure 2.12C).
Together, these data support the importance of AKT in modulating sensitivity to BCL-2 antagonists by regulating the mitochondrial abundance of BAD and BIM.

Figure 2.12. AKT suppression is a critical component of synergy between BEZ235 and ABT-199. (A) Western blot of OCI-LY1 cells expressing either empty vector or phospho-mimetic AKT (S473D) treated with indicated inhibitors for 3 hours. Cells were pre-treated with doxycycline (1 µg/ml) for 24 hours prior to treatment. (B) Western blot of mitochondrial (M) and cytoplasmic (C) fractions of cells in panel (A). (C) Sensitivity of three DLBCL cell lines expressing AKT S473D to ABT-199 in the presence or absence of MK2206, BEZ235, or GDC-0980. Cells were pre-treated with doxycycline (1 µg/ml) for 24 hours prior to treatment.

The capacity for AKT to modulate BIM expression has been attributed to direct regulation of FOXO transcription factors (15). Thus, to confirm that AKT inhibition can activate FOXOs in DLBCL, we used a luciferase reporter assay to measure the
transcriptional activity of FOXOs (15). Treatment with either MK2206 or BEZ235 significantly increased FOXO activity in control cells, but neither inhibitor affected FOXO activity in cells expressing AKT S473D (Figure 2.14) where phospho-FOXO levels are maintained (Figure 2.12A). These results support a model where activation of FOXOs downstream of AKT inhibition contributes to BEZ235-mediated apoptotic sensitization.

Figure 2.13. Confirmation of expression of AKT S473D in SU-DHL4 and OCI-LY8 cells. Western blot of SU-DHL4 and OCI-LY8 cells expressing either empty vector or phospho-mimetic AKT (S473D). Cells were pre-treated with doxycycline (1 µg/ml) for 24 hours prior to treatment with indicated PI3K pathway inhibitors for an additional 3 hours.
Figure 2.14. Expression of AKT S473D suppresses FOXO activation following inhibition of AKT. Relative FOXO transcriptional activity measured using a luciferase reporter assay system. Cells were co-transfected with pRL-TK (renilla) and firefly luciferase downstream of the putative FOXO3 binding site prior to treatment with indicated inhibitor for 16 hours.

To investigate the contribution of AKT-mediated phosphorylation of BAD to the sensitization effect, we over-expressed either wild-type or phospho-null (S136A) mutant of murine Bad (Figure 2.15A) (43). While expression of either form of Bad was sufficient to induce apoptosis (Figure 2.15B, C), the phospho-null mutant induced significantly more death than wild-type Bad, despite being expressed at comparable levels (Figure 2.15). These data suggest that basal AKT activity can limit the cytotoxic potential of wild-type Bad. In support, when cells were treated with BEZ235 and MK2206, both forms of Bad induced comparable levels of cell death (Figures 2.15B, C). Together these data support a model where the amount of de-phosphorylated (active) Bad determines the degree of apoptosis. Overall, these data indicate that inhibition of AKT, and subsequent
accumulation of BAD and BIM, is a key component of the synergy between BEZ235 and ABT-199.

**Figure 2.15. Expression of exogenous murine Bad sensitizes OCI-LY1 cells to AKT inhibition.** (A) Western blot of murine BAD (mBAD) induction following 24 hour treatment with indicated doses of doxycycline. Cells were also treated with 10 µM Q-VD-OPh to prevent caspase cleavage of BAD. # Indicates murine isoform, ## indicates human isoform. (B, C) Viability of OCI-LY1 cells transduced with empty vector, mBAD wild-type (WT), or phospho-null mBAD (S136A) treated with increasing concentrations of doxycycline ± BEZ235 (B) or MK2206 (C) for 48 hours.

**Discussion**

Despite showing promising clinical efficacy in some blood cancers (1), PI3K/mTOR inhibitors lack single-agent cytotoxicity in aggressive diseases like DLBCL (38). However, there is an increasing body of evidence suggesting that these inhibitors may be effective in combination therapies. In this study, we show combined inhibition of
PI3K/mTOR and BCL-2 synergistically induced cell death, even in cells over-expressing BCL-2 or MCL-1, which are predictors of poor therapeutic response (5,31,34).

Using DBP, we demonstrate that degree of priming correlates with the degree of enhanced killing by ABT-263, suggesting the assay may have predictive capabilities. Importantly, we also show that this combination lacks toxicity in normal human T lymphocytes, which important for mediating durable anti-tumor responses (29,30).

However, unlike in other cancers where suppression of mTORC1 has been shown to reduce MCL-1 expression (9-11), our analysis reveals an unpredicted mechanism. DBP identified that BCL-2 and BCL-XL maintain survival following PI3K pathway inhibition. Consistent with these findings, induction of BAD and BIM (which are both BCL-2/BCL-XL antagonists) was the primary mechanism of sensitization (Figure 2.16). Together, these data provide an alternative rationale for combining PI3K/mTOR and BCL-2 inhibitors as a promising therapy for GCB-DLBCL.

It is surprising that unlike in other contexts (9-11), inhibiting mTORC1-dependent translation did not reduce MCL-1 levels. A simple explanation is that MCL-1 regulation may be cell-type-specific. In support, PI3K/mTOR inhibition significantly reduced MCL-1 expression in cells derived from a different B cell malignancy (BV173, B-ALL cells). However, work from others demonstrating the sensitivity of MCL-1 expression to PI3K/mTOR inhibitors in ABT-199/737-resistant GCB-DLBCL cells (12,13), requires an alternative explanation. In this situation, it is plausible that the selection of ABT-199-resistant cells enriches for those cells that up-regulate MCL-1 in an mTORC1-dependent manner (34,44,45). Indeed, when compared to the parental SU-DHL6 line, PI3K/mTOR inhibition down-regulated MCL-1 only in ABT-199-resistant cells. It is
important to note that dysregulated MCL-1 expression can be conferred by defects in any of the multiple layers of regulation (transcriptional (36,46), translational (45), and post-translational levels (47-49)). Nevertheless, our data that knockdown or over-expression of MCL-1 can modulate sensitivity to BCL-2 antagonists strongly supports the work of others that describe the potential of targeting MCL-1 in rational combinations involving BH3 mimetics.

**Figure 2.16. Model of synergy between BEZ235 and ABT-263 in DLBCL cell lines.** Treatment with BEZ235 in DLBCL cell lines completely inhibits signaling through the PI3K and downstream effectors, AKT and mTORC1. Loss of AKT activity promotes FOXO-mediated transcription of BIM and facilitates mitochondrial accumulation of de-phosphorylated BAD.
Complementary to previous reports, we demonstrate that PI3K/mTOR inhibitors induce the mitochondrial accumulation of BAD and BIM, which sensitizes cells to BH3 mimetics. Both effects require inhibition of AKT, as constitutively active AKT nullified the induction. The requirement for BIM in the initiation of apoptosis has been well-characterized (50). Indeed, BIM up-regulation is required for synergy of BEZ235 and ABT-737 in ovarian cancer cells (11). Similarly, the consequences of over-activating BAD are straightforward. By activating BAD, PI3K pathway inhibitors increase the concentration of endogenous BCL-2 antagonists (BAD and BIM protein), which enhances the effects of pharmacological BCL-2 antagonists (ABT-199/263) (10).

Despite sensitizing cells to BCL-2 inhibitors, PI3K pathway inhibitors are insufficient to induce apoptosis as single agents. As such, it is also unlikely that suppression of AKT would confer sensitivity to BH3 mimetics in cells that are fundamentally resistant to apoptosis (e.g. BAX/BAK null or MCL-1 over-expressing cells). Regardless, these data identify an alternative mechanism of synergy between PI3K/mTOR and BCL-2 inhibitors in which suppression of AKT enhances the activity/expression of pro-apoptotic factors.

In conclusion, while the mechanism may differ depending on the context, the combination of PI3K/AKT/mTOR inhibitors and BCL-2 antagonists strongly synergizes to kill DLBCL cells. The existence of multiple mechanisms of synergy may prove beneficial in combating tumor heterogeneity and preventing acquired resistance in a clinical setting. In addition, our results define alternative markers of response and identify situations in which tracking MCL-1 expression may not be predictive of patient responses. Thus, the results of this and other studies provide a strengthening rationale for testing dual PI3K/mTOR inhibitors with BCL-2 inhibitors in GCB-DLBCL patients.
References

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Chapter Three

Statins enhance the efficacy of BCL-2 inhibitors in blood cancers
Abstract

BCL-2 is a key pro-survival protein that is highly expressed in many leukemias and lymphomas. ABT-199 (venetoclax) is a small molecule inhibitor of BCL-2 that has demonstrated promising clinical potential in chronic lymphocytic leukemia (CLL). However, other hematologic malignancies are less responsive to ABT-199 as a single agent, suggesting that combinations of targeted therapies may be required to elicit more promising responses. We have investigated the potential of combining ABT-199 with HMG-CoA reductase (HMGCR) inhibitors (statins), which have known anti-cancer potential in hematologic malignancies. Using multiple chemically distinct statin compounds, we observed profound synergistic induction of apoptosis when combined with ABT-199 in both human diffuse large B cell lymphoma (DLBCL) as well as acute myeloid leukemia (AML) cell lines. This synergy was also seen in primary murine B lymphoma cells over-expressing MYC and BCL-2. Importantly, addition of exogenous mevalonate completely rescued cells from the combination, confirming on-target efficacy of HMGCR inhibition. Using BH3 profiling, we found that simvastatin significantly primed lymphoma cells for undergoing apoptosis (termed mitochondrial priming). Notably, the degree of priming correlated with its ability to synergize with ABT-199, suggesting that this method may be used to predict patient responses. Strikingly, the combination did not synergize to kill normal human peripheral blood mononuclear cells from healthy donors, suggesting that statins may selectively prime cancer cells for apoptosis. Mechanistic studies support the hypothesis that statins synergize with ABT-199 by suppressing protein prenylation, particularly protein geranylgeranylation. In support, the addition of exogenous geranylgeranyl pyrophosphate (GGPP) completely
rescued cells from the effects of simvastatin. Furthermore, selective inhibition of protein geranylgeranyl transferase (GGT) was sufficient to recapitulate the effects of simvastatin in combination with ABT-199. Lastly, we have identified Rap1A de-prenylation and PUMA upregulation as markers of pharmacodynamic response to statins in vivo. Thus, this project highlights a novel combination for use in aggressive lymphomas, establishes its efficacy and tolerability using preclinical models, and provides proof-of-concept to warrant investigation of its clinical potential.

**Introduction**

BCL-2 is the founding member of a family of proteins that interact in a dynamic balance to regulate cell fate. Initially cloned from the t(14;18) translocation that characterizes follicular lymphoma (FL), BCL-2 has since been shown to promote survival and chemo-resistance in multiple lymphoid cancers. Indeed, over-expression of this protein is frequently associated with poorer patient outcomes in CLL, AML, and DLBCL (1-5). BH3 mimetic drugs are an exciting class of anticancer drugs that mimic the activity of BCL-2 antagonists to promote apoptosis (6-9). While the first potent inhibitor of BCL-2, navitoclax, demonstrated marked in vitro efficacy in a variety of cancers, dose-limiting thrombocytopenia stemming from its activity against BCL-XL precluded its clinical potential (10). A second generation inhibitor, ABT-199, which lacks this on-target toxicity (8), has recently been granted FDA approval as a second line therapy in 17p(del) CLL (11). However, in phase I trials, ABT-199 monotherapy demonstrated limited efficacy in other lymphoid malignancies (12), warranting further
investigation into whether rational combinations may augment its efficacy in these contexts.

Previous studies have identified potent synergy between BH3 mimetics and inhibitors of the PI3K/AKT/mTOR pathway in DLBCL (13,14). However, the clinical use of this combination is hindered by a lack of regulatory approval (15), and immune cell toxicity (16). As part of a growing effort to “repurpose” FDA-approved drugs to treat cancer (17), several groups have reported HMGCR inhibitors (statins) may have an anti-cancer potential related to BCL-2 family modulation (18-20). While statins are commonly used to safely control plasma cholesterol levels (21), by inhibiting the rate-limiting enzyme of the mevalonate pathway, statins also suppress production of isoprenoids that are required for the normal function of key oncogenic proteins like the Ras superfamily (22). Consequently, statins also have single-agent anti-cancer activity in certain contexts like AML cell lines (23,24). Therefore, we hypothesized that statins would enhance the efficacy of ABT-199 in a broad selection of blood cancers.

Here, we report that multiple chemically distinct statin compounds profoundly enhance the ability of ABT-199 to kill DLBCL, CLL, and AML cells. In addition, the combination was effective at reducing lymphoma burden in a syngeneic mouse model of BCL-2/MYC-driven “double-hit” lymphoma. We also show that statins significantly prime lymphoma cells for undergoing apoptosis (termed mitochondrial priming) and that dynamic BH3 profiling (DBP) may be used to predict which samples are likely to respond to this combination. Consistent with an increase in mitochondrial priming, we show that statins induce up-regulation of BH3-only protein PUMA. Mechanistically, inhibition of protein geranylgeranylation is both necessary and sufficient to recapitulate
the sensitizing effect of simvastatin. Thus, this project establishes the efficacy and selectivity of a novel combination for use in aggressive lymphomas.

**Materials and Methods**

*Chemicals*

Simvastatin, atorvastatin calcium salt, rosuvastatin calcium salt, and fluvastatin sodium salt were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Simvastatin was activated as reported elsewhere (25). ABT-199 and ABT-263 were obtained from Active Biochem (Wan Chai, Hong Kong). InSolution Q-VD-OPh was obtained from EMD Millipore (Billerica, MA, USA). NVP-BEZ235 was obtained from LC laboratories (Woburn, MA, USA). Doxorubicin, vincristine, mevalonate, squalene, cholesterol, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

*Cell culture*

OCI-LY1, OCI-LY7, OCI-LY8, and SU-DHL4 cell lines (a gift from Dr. Laura Pasqualucci, Columbia University) were cultured in IMDM (GE Healthcare Hyclone, Little Chalfont, UK) supplemented with 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM L-Glutamine, 100 I.U. penicillin, and 100 μg/ml streptomycin. OCI-AML2, OCI-AML3, and MOLM13 cell lines (a gift from Dr. Anthony Letai) were cultured in RPMI (Corning, NY, USA) supplemented with 10% FBS, 10 mM HEPES, 10 mM L-Glutamine, 100 I.U. Penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified 37°C incubator with 5% CO₂. Cells were routinely
tested to ensure absence of mycoplasma, and were maintained at or below $2 \times 10^6$ cells/mL. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% calf serum, 100 I.U. penicillin, and 100 $\mu$g/mL streptomycin. Human peripheral blood mononuclear cells (PBMCs) and primary CLL samples were isolated from blood samples by centrifugation through Ficoll-PaqueTM (GE Healthcare, Piscataway, NJ, USA) and were grown in RPMI with 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM L-Glutamine, 100 I.U. penicillin, 100 $\mu$g/ml streptomycin. In the case of primary CLL samples, cells were grown on NK.Tert immortalized bone marrow stromal cell line.

Cell viability

Cell viability assays were performed in 96-well format as described previously (14). Briefly, $6 \times 10^4$ cells were cultured in 200 $\mu$L growth medium with inhibitors for 48 hours. Cells were harvested and stained with Annexin V, Alexa Fluor 647 conjugate and propidium iodide (Life Technologies). Fluorescence was measured by flow cytometry using FACScalibur (Becton-Dickinson, San Jose, CA, USA) and viability of cells was quantified using FlowJo software v10.1r7 (FlowJo LLC, Ashland, OR, USA). In the case of primary CLL samples, stromal cells were seeded onto 96-well plates 24 hours before each experiment at $6 \times 10^4$ cells/well. Confluence was confirmed by phase-contrast microscopy before seeding of CLL cells at $6 \times 10^5$ cells/well. Cells were then treated with simvastatin for 16 hours prior to addition of ABT-199 for an additional 8 hours. Cells were then harvested and stained for CD19, and viability was assessed using Annexin V.
BH3 profiling

BH3 profiling of DLBCL cells was performed as described previously (14). Briefly, cells were incubated in T-EB buffer (300 mM trehalose, 10 mM HEPES, 80 mM potassium chloride, 1 mM EGTA, 1 mM EDTA, 0.1% BSA, and 5 mM succinic acid) with 200 nM JC-1 (Life Technologies), 0.001-0.005% digitonin (Sigma-Aldrich), and 10 μg/ml oligomycin (Sigma-Aldrich) with either DMSO or BH3-only peptides for 60 minutes prior to analysis using a FACScalibur (Becton-Dickinson). The sequences and method of synthesis of BH3-only peptides were described previously (26). Percent depolarization caused by each BH3-only peptide was calculated as the percent difference in the JC-1 red fluorescence (590 nm) relative to DMSO-treated control cells.

Western blotting

Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, 2 mM EDTA, 50 mM NaF) supplemented with protease inhibitor cocktail (Calbiochem, USA) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). Protein concentrations were normalized using a Bradford protein assay (Bio-Rad). Lysates were prepared at 1 μg/μl concentration in 1X XT Sample Buffer (Bio-Rad) and 5% 2-mercaptoethanol (Sigma-Aldrich). Lysates were run on 4-12% Bolt® Bis-Tris Plus gels (Life Technologies), and transferred onto nitrocellulose membranes. The following antibodies were used: GAPDH, PARP, caspase 9, cleaved caspase 3, MCL-1, BCL-XL, BIM, BID, BAX, BAK, PUMA, COX IV, ERK (Cell Signaling Technology, Beverly, MA,
USA), BCL-2 (BD Pharmingen, San Diego, CA, USA), Bad, Rap1A, HRK (Santa Cruz Biotechnology, Dallas, TX, USA), and HDJ-2 (Thermo Scientific). The following secondary HRP-conjugated antibodies were used: anti-mouse IgG, anti-rabbit IgG (Promega, Madison, WI, USA), anti-goat IgG (Santa Cruz Biotechnology), and Protein A (BD Pharmingen). Blots were developed using Pierce ECL Western Blotting Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Life Technologies) and detected using a Nikon D700 SLR camera as described previously (27). Images were processed using Adobe Photoshop software and densitometry was quantified using ImageJ software.

Animal studies

All animal studies were conducted in accordance with guidelines of the University of California Institutional Animal Care and Use Committee. 10 week old female C57BL/6N mice were purchased from Charles River and sub-lethally irradiated (4G) 24 hours prior to injection of lymphoma cells by tail vein. Lymphoma burden was measured by FACS using peripheral blood collected via Goldenrod animal lancets (Braintree Scientific, Inc, Braintree, MA, USA). Drug administration was performed by oral gavage with vehicle formulations as follows: ABT-199 in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG and simvastatin in 0.5% methylcellulose and 0.1% Tween-80.
Subcellular fractionation

Cells were harvested and re-suspended in isotonic buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Calbiochem, USA), and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)). Cells were lysed by passing through 28 gauge insulin syringes and resulting lysates were spun at 800 g for 10 minutes at 4˚C six times to remove intact cells and nuclear fractions. Supernatants were then spun at 10,000 g for 30 minutes at 4˚C to separate the mitochondria-enriched heavy membrane pellet from the supernatant containing cytoplasmic fractions. Pellets were then lysed using RIPA buffer and run for western blotting as described above.

Statistical Analysis

Statistical analyses were performed in the GraphPad Prism software version 5c (GraphPad Software, La Jolla, CA, USA). Unless otherwise indicated, results indicate mean±S.D. of three independent experiments. P < 0.05 was considered statistically significant and was annotated throughout as: * P < 0.05, ** P < 0.005, *** P < 0.001. For drug synergy calculations, combination index versus fraction affected curves were generated using CalcuSyn software (Biosoft, Cambridge, UK).

Results

Statins selectively enhance the efficacy of ABT-199 against cancer cells in vitro

We first tested several human germinal center B cell-like (GCB) DLBCL (where BCL-2 plays a clear role in disease progression and outcomes (3,5)) and AML cell lines
for sensitivity to ABT-199, simvastatin, or the combination. Strikingly, in all three AML cell lines and in two out of four DLBCL cell lines, the combination of simvastatin and ABT-199 induced significantly more death than either treatment alone (Figures 3.1A and 3.2A). We confirmed that this interaction was synergistic using the median-effect method (Figure 3.2B (28)), and also confirmed the combination kills cells via the intrinsic apoptosis pathway. In particular, the pan-caspase inhibitor, Q-VD-OPh, completely rescued the viability of cells treated with the combination (Figure 3.3A). In addition, we also observed rapid cleavage of caspase 3, caspase 9, and PARP following treatment with simvastatin and ABT-199 (Figures 3.3B, C). Having confirmed efficacy using cell lines, we next tested whether the combination was equally effective in both primary murine lymphoma cells and primary human patient cells. Both simvastatin and fluvastatin were effective at sensitizing primary murine lymphoma cells expressing MYC and BCL-2 oncogenes (29), representing human “double-hit” lymphoma that has poor prognosis (Figures 3.1B and 3.2C).
Figure 3.1. Statins selectively enhance the efficacy of ABT-199 against blood cancer cells. (A) viability of DLBCL and AML cell lines treated with increasing doses of ABT-199, simvastatin, or the combination for 48 hours. Concentrations for each drug are as follows; ABT-199: 1X indicated dose for all cell lines, Simvastatin: 66.67X for LY8, 200X for LY1, 33.33X for all other cell lines. (B) viability of primary murine lymphoma cells co-cultured on irradiated 3T3 stroma and treated for 48 hours with indicated inhibitors. (C) viability of primary CLL cells grown on NK.tert stroma and treated with simvastatin for 16 hours prior to addition of ABT-199 for an additional 8 hours. (D) viability of primary AML cells treated with simvastatin for 16 hours prior to addition of ABT-199 for an additional 8 hours. (E) viability of PBMC subsets treated with simvastatin (3 µM), ABT-199 (100 nM), or the combination for 48 hours before staining. Significance testing was performed by two-tailed paired Student’s t-test relative to vehicle-treated control samples unless otherwise indicated.
CLL cells are very sensitive to ABT-199 alone (1,2). At low concentrations of ABT-199, simvastatin augmented killing of primary CLL samples including some with 17p(del) (Figure 3.1C and Table 3.1). The combination had mixed efficacy in primary AML samples where three of eight samples tested showed an increased effect of the combination relative to single-agent treatments (Figure 3.1D). Given this broad efficacy across blood cancers, we next investigated whether the combination would exacerbate any toxicities in normal human peripheral blood mononuclear cell (PBMC) subsets. Importantly, we found that while ABT-199 alone had some toxicity in most subsets (particularly CD19+ cells), the addition of simvastatin did not enhance this toxicity in any subset except CD14+ monocytes where simvastatin also exhibited single-agent toxicity (Figure 3.1E). Collectively, these data identify a promising combination effect between statins and ABT-199 in a broad spectrum of blood cancers. However, the variability of response among some sample sets emphasizes the importance of identifying biomarkers that are predictive of response.
Figure 3.2. Statins synergize with ABT-199 in blood cancer cells. (A) viability of OCI-LY7 cells treated with increasing doses of ABT-199 (1X indicated dose), simvastatin (33.33X indicated dose), or the combination for 48 hours. (B) formal synergy analysis of DLBCL and AML cell lines using data from Figure 3.1A and 3.2A. (C) viability of primary murine lymphoma cells co-cultured on irradiated 3T3 stroma and treated for 48 hours with indicated inhibitors.
Figure 3.3. Simvastatin plus ABT-199 induce apoptosis in DLBCL and AML cell lines. (A) viability of cells treated with indicated drugs with (grey bars) or without (white bars) pan-caspase inhibitor Q-VD-OPh (10 µM). (B-C) western blot of cells pre-treated with vehicle or simvastatin for 16 hours before addition of ABT-199 for indicated times. Doses of ABT-199 are as follows: 10 nM for OCI-LY1, 30 nM for OCI-LY8, 300 nM for all other cell lines.

Table 3.1. Characteristics of human samples used in Chapter 3.
Dynamic BH3 profiling predicts sensitization to ABT-199 by simvastatin

The development and utilization of functional diagnostics for patient selection criteria has been an area of growing emphasis in precision medicine (30). A promising approach in these efforts has been dynamic BH3 profiling (DBP), which seeks to measure the effect of treatments on how readily cells undergo mitochondrial outer membrane permeabilization (MOMP) (31,32). In so doing, DBP can rapidly predict whether a treatment is likely to elicit an apoptotic response and a characteristic that can be used to match patients with drugs to which their cancers are likely to be sensitive (33). We previously reported that DBP could be used to predict enhanced sensitivity to combinations involving BCL-2 antagonists (14). Therefore, we sought to investigate whether DBP could also be used as a diagnostic tool to predict which cells were sensitive to the combination of statins with ABT-199. Strikingly, in DLBCL cell lines where the combination of simvastatin with ABT-199 was synergistic (Figure 3.1A), DBP identified increased mitochondrial priming (Figure 3.4A). Notably, in OCI-LY1 cells where simvastatin did not synergize with ABT-199 (Figure 3.1A), there was no measurable increase in priming (Figure 3.4A). Both the priming effect and the sensitization to ABT-199 were dose-dependent, with significant priming observed using 1 µM simvastatin (Figures 3.5A, B). In addition, simvastatin primed most primary CLL samples tested except sample 029 (Figure 3.4B). Consistent with our hypothesis that DBP may be used to predict responses, the addition of simvastatin did not enhance killing by ABT-199 in this sample (Figure 3.1C).
Previous studies using BH3 profiling have demonstrated that in untreated cells, basal priming levels can predict chemo-sensitivity (31). Therefore, we next tested whether the increase in priming by simvastatin could also enhance the efficacy of various chemotherapies. Surprisingly, simvastatin did not significantly increase sensitivity to either doxorubicin or vincristine, as measured by the change in IC50 for chemotherapy (Figure 3.5B). While these data are in agreement with numerous retrospective studies in DLBCL indicating no effect of statin-use on chemotherapy outcomes (34-36), they are at odds with the notion that basal priming can predict chemo-sensitivity. However, it is possible that simvastatin may antagonize the effects of chemotherapy. In particular, simvastatin significantly reduces cell proliferation (Figure 3.5C), an attribute which also determines chemo-sensitivity (37-39).
Figure 3.4. Dynamic BH3 profiling predicts sensitization to ABT-199 by simvastatin. (A) dynamic BH3 profiles for all cells treated with 10 µM simvastatin or 50 nM BEZ235 for 16 hours. (B) limited dynamic BH3 profile (BAD peptide) of primary CLL samples treated with indicated concentration of simvastatin for 16 hours.
Figure 3.5. Statins induce a dose-dependent increase in mitochondrial priming, but do not sensitize to chemotherapy. (A) dynamic BH3 profiles for cells treated with indicated doses of simvastatin for 16 hours. (B) sensitivity (IC50) of two DLBCL cell lines to ABT-199 or two chemotherapies. Cells were treated with inhibitors for 48 hours.
**Combination of statins and ABT-199 is effective in a syngeneic mouse model of lymphoma**

Given the efficacy of the combination *in vitro*, we next sought to determine whether statins could synergize with ABT-199 in an *in vivo* model of mouse lymphoma. To this end, we injected murine lymphoma cell line 27-L1, which were sensitive to the combination *in vitro* (**Figure 3.1B**), into C57BL6/N mice as a syngeneic mouse model of lymphoma (29). After only five days of dosing, we observed efficacy of simultaneous targeting of both HMGCR and BCL-2. In particular, the combination markedly reduced lymphoma burden (%GFP+) in both lymph nodes and spleens compared to ABT-199 treatment alone (**Figure 3.6A**). Additionally, the degree of splenomegaly was significantly reduced in mice receiving both simvastatin and ABT-199 relative to control groups (**Figure 3.6B**). We next confirmed that the dose of simvastatin was sufficient to inhibit HMGCR by western blot for un-prenylated Rap1A (**Figure 3.6C**), a marker of suppressed mevalonate production (40).
Figure 3.6. Combination of simvastatin and ABT-199 is effective in a syngeneic mouse model of lymphoma. (A) percent lymphoma burden in indicated organs of mice treated with ABT-199 (75 mg/kg/day), simvastatin (50 mg/kg/day), or the combination for 5 days. (B) spleen weights for mice treated as in A. (C) western blots for pharmacodynamic effect of simvastatin (Rap1A de-prenylation) in indicated organs of mice treated as in B. Note two spleens from simvastatin-treated groups were lost due to errors in processing. (D) Kaplan-Meier survival curve of mice injected with 27-L2 cells and treated with ABT-199 (100 mg/kg/day), simvastatin (50 mg/kg/day), or the combination. (E) spleen weights of mice from D, measured at sacrifice. (F) percent lymphoma burden in indicated organs of mice from D, measured at sacrifice.

Effect of statins is due to on-target inhibition of HMGCR

In order to exclude the possibility of drug artifacts derived from simvastatin use, we tested several chemically distinct statins for their ability to sensitize DLBCL cell lines to ABT-199 or ABT-263. Fluvastatin, atorvastatin, and rosuvastatin all similarly enhanced the efficacy of ABT-199 across all DLBCL cell lines tested (Figure 3.2C, 3.7A and 3.8A). Furthermore, in cells where statins sensitized to ABT-199, all statins also similarly sensitized to ABT-263 (Figure 3.8B). While the concordance of these results suggested the effects of statins were due to a shared on-target effect, in order to directly test this we next investigated whether the addition of mevalonate was sufficient to rescue from simvastatin. Supplementing cells with the product of HMGCR activity
completely negated the enhanced killing effect conferred by simvastatin in DLBCL and AML cells (Figures 3.7B and 3.8C). Importantly, mevalonate specifically counteracted the effects of simvastatin, but not the dual PI3K/mTOR inhibitor BEZ235, which also synergizes with ABT-199 in DLBCL (14). Collectively, these data show that the ability of statins to enhance the killing of BCL-2 antagonism in DLBCL and AML cell lines stems from on-target inhibition of HMGCR.

Figure 3.7. Effect of statins is due to on-target HMGCR inhibition. (A) viability of cells treated with increasing doses of different chemically distinct statins (colors) with (dashed lines) or without (solid lines) ABT-199 (30 nM for LY8, 300 nM for HL4). (B) viability of cells treated with indicated drugs with (grey bars) or without (white bars) mevalonate (1 mM). Doses of drugs are 10 µM simvastatin, 30 nM (LY8) or 300 nM (HL4) ABT-199, and 50 nM NVP-BEZ235.
Figure 3.8. Sensitization by statins is on-target. (A) viability of cells treated with increasing doses of different chemically distinct statins (colors) with (dashed lines) or without (solid lines) ABT-199 (10 nM for LY1, 300 nM for LY7). (B) viability of cells treated with increasing doses of different chemically distinct statins (colors) with (dashed lines) or without (solid lines) ABT-263 (50 nM for LY1, 300 nM for all other cell lines). (C) viability of cells treated with indicated inhibitors with (grey bars) or without (white bars) 1 mM mevalonate.

Sensitization to ABT-199 requires inhibition of protein geranylgeranylation.

Mevalonate is utilized in several key cellular processes including cholesterol biosynthesis, protein synthesis, and signal transduction, any of which could mediate enhanced sensitivity to BCL-2 antagonism (20). In order to determine which pathways downstream of HMGCR were critical for the sensitizing effect of statins, we investigated
whether the addition of mevalonate metabolites required for these processes could also rescue cells from simvastatin. While mevalonate reliably rescued DLBCL and AML cell lines from simvastatin, we found that only addition of geranylgeranyl pyrophosphate (GGPP) could also consistently rescue viability (Figures 3.9A and 3.10). A related metabolite, farnesyl pyrophosphate (FPP), also moderately rescued some cells, but addition of exogenous cholesterol or squalene had no effect on viability of cells treated with a combination of simvastatin and ABT-199 or ABT-263. Both FPP and GGPP are required for protein prenylation, a post-translational modification that mediates membrane localization (41). Thus, to confirm whether protein prenylation was suppressed by HMGCR inhibition, we performed western blotting for un-prenylated Rap1A (Figure 3.11A), a marker of reduced geranylgeranylation (40). In all DLBCL cell lines tested, treatment with simvastatin inhibited prenylation of Rap1A in a dose-dependent manner that correlated with the degree of sensitization to ABT-199 (Figure 3.11B).
Figure 3.9. Sensitization to ABT-199 requires inhibition of protein geranylgeranylation. (A) viability of cells treated with the combination of 300 nM ABT-263 and 10 µM simvastatin supplemented with indicated metabolites for 48 hours. Abbreviations are as follows; MVA: mevalonate, FPP: farnesyl pyrophosphate, GGPP: geranylgeranyl pyrophosphate. (B) viability of cells treated with indicated 10 µM simvastatin, GGTI-298, or FTI-277 with (grey bars) or without (white bars) ABT-199 (30 nM for LY8, 300 nM for HL4) for 48 hours. Viability was assessed by flow cytometry using Annexin-V and PI double-negativity. (C) western blot of cells treated with 10 µM of indicated inhibitors for 16 hours. (D) dynamic BH3 profile of cells treated with 10 µM simvastatin (grey bars) or GGTI-298 (black bars) for 16 hours. Significance testing was performed by two-tailed paired Student’s t-test relative to vehicle-treated control samples unless otherwise indicated.
Figure 3.10. Mevalonate and geranylgeranyl pyrophosphate are sufficient to rescue from the effects of simvastatin. (A-B) viability of DLBCL cell lines treated with indicated inhibitors for 48 hours with or without addition of indicated metabolites.
Figure 3.11. Simvastatin inhibits protein geranylgeranylation in a dose-dependent manner in DLBCL. (A) western blots of DLBCL cell lines treated with increasing doses of simvastatin for 16 hours. (B) viability of cells treated with doses of simvastatin in A for 48 hours with (dashed lines) or without (solid lines) ABT-199. Doses of ABT-199 are as follows: 30 nM for OCI-LY8, 10 nM for OCI-LY1, 300 nM for OCI-LY7 and SU-DHL4.

Given that both FPP and GGPP rescued cells from the effects of simvastatin, we next sought to determine which pathway was more critical for the enhancement effect of simvastatin. Because FPP can be shunted to GGPP (41), we tested whether selective inhibition of either geranylgeranyl transferase (GGT) or farnesyl transferase (FT) was sufficient to recapitulate the effects of simvastatin. In most cell lines tested, the FT inhibitor (FTI-277) did not enhance the killing by ABT-199 whereas the GGT inhibitor (GGTI-298) consistently sensitized cells to ABT-199 (Figures 3.9B and 3.12A). We also confirmed each inhibitor adequately suppressed their intended process by western blotting for band shifting of HDJ-2 (a marker of FT inhibition) or the appearance of an un-prenylated Rap1A band (Figures 3.9C and 3.12B). Collectively, these data suggest...
that inhibition of protein geranylgeranylation is both required and sufficient to sensitize cells to BCL-2 antagonism in both DLBCL and AML cells.

Because we had previously observed that DBP could accurately predict enhanced sensitivity to ABT-199 by simvastatin, we next tested whether GGTI-298 could also prime DLBCL cells for apoptosis. In both OCI-LY8 and SU-DHL4 cell lines, where GGTI-298 sensitized cells to ABT-199, DBP detected significant increases in mitochondrial priming (Figure 3.9D), supporting the notion that DBP may be used as a functional diagnostic to predict responses to combinations involving ABT-199.

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**Figure 3.12.** Inhibition of GGT is sufficient to recapitulate the effects of simvastatin in AML cell lines. (A) viability of AML cell lines treated with indicated inhibitors for 48 hours. (B) western blot of AML cell lines treated with 10 µM indicated inhibitors for 16 hours.
Statins induce expression of PUMA

The anti-cancer effect of statins as a single agent has been previously studied, particularly in AML, where efficacy was associated with a reduction in BCL-2 expression (20). Thus, to investigate whether statins could affect expression of BCL-2 family members similarly, we enriched for mitochondria-containing lysate fractions and probed for expression of the major BCL-2 family proteins by western blot. Using this approach, we observed that none of the major pro-survival factors (BCL-2, MCL-1, BCL-XL), apoptotic effectors (BAX and BAK), or activators (BID and BIM) were substantially affected by treatment with simvastatin (Figure 3.13). Instead, we observed consistent induction of PUMA expression in both DLBCL and AML cell lines (Figures 3.14A, B). Furthermore, we confirmed that this increase in PUMA expression also correlated with an accumulation of PUMA in the mitochondria-enriched fractions where it exerts its pro-apoptotic function (Figure 3.14C). This increase was also seen in at one 27-L2 primary mouse lymphoma cells, but not 27-L1 (Figure 3.15).
Figure 3.13. Simvastatin does not affect expression of many BCL-2 family proteins. Western blot of cell lysate fractions enriched for heavy membranes (M) or cytoplasmic (C) proteins following 16 hour treatment with 10 µM simvastatin.
**Figure 3.14. Statins induce up-regulation of PUMA in DLBCL and AML cell lines.** (A-B) representative western blots of cells treated with 10 µM indicated statin for 16 hours (left) and quantitation across replicates (right). (C) representative western blot of mitochondrial (M) and cytoplasmic (C) fractions isolated from cells treated with 10 µM indicated statin for 16 hours (left) and quantitation across replicates (right).
Figure 3.15. Statins induce PUMA in one primary mouse lymphoma cell line. Western blot of primary mouse lymphoma cells treated with 10 µM indicated statin for 16 hours.

Discussion

Despite promising single-agent efficacy in patients with CLL (11), ABT-199 has yielded limited responses in patients with AML and DLBCL (12). Though BCL-2 overexpression correlates with poor prognosis in these settings (3,4,42), dependence on these proteins for survival may be dampened relative to CLL (1,2,43). Consequently, combination therapies might be required to enhance the efficacy of BCL-2 inhibition. In the past, we and others have demonstrated the potential of combining PI3K/AKT/mTOR and BCL-2 inhibitors (13,14). However, regulatory approval for PI3K/AKT/mTOR inhibitors has not been achieved in GCB-DLBCL or AML. Here, we report that statins and ABT-199, two drugs that are currently FDA approved for other indications, synergize strongly to selectively kill blood cancer cells both in vitro and in vivo.

Since both drugs are currently FDA approved, this combination has clear potential for rapid clinical application. Nevertheless, a major concern is whether the approved doses of statins will be sufficient for the efficacy observed in vitro (44,45).
Whether doses exceeding the anti-hypercholesterolemia doses may further augment statin efficacy is also currently under investigation. In humans, a phase I clinical trial using doses of 1680 mg/day yielded serum concentrations nearing 1 µM with few toxicities (46). Additionally, preclinical studies in rabbits estimate that doses of 200 mg/kg/day can achieve circulating concentrations reaching 20 µM, though with substantial toxicity (47). Thus, it will be important to carefully test statin dosing levels and schedules and to balance the efficacy of the combination against high-dose toxicity.

Despite few toxicities being reported in the high-dose statin trial (46), a major secondary concern is whether the combination will exacerbate any single-agent toxicities. While the dose-limiting toxicity of ABT-199 is tumor lysis syndrome, statins are associated with myotoxicity (particularly myositis (48)). Previous work on smooth muscle cells suggest that statins can induce apoptosis via downregulation of BCL-2 (49), cautioning that ABT-199 may exacerbate this toxicity through shared targeting of BCL-2. However, significant myotoxicity from ABT-199 has not yet been reported in any clinical trials and statin use did not appear to exacerbate any adverse events. In addition, simvastatin did not enhance ABT-199 toxicity among most PBMC subpopulations. Collectively, these results suggest that the combination may retain an acceptable safety profile and achieve reasonable cancer cell selectivity.

Accompanying the growing emphasis on precision medicine, a key area of research is to develop biomarkers and/or diagnostics that can match patients with the efficacious drugs. Here, we provide compelling evidence that illustrates the predictive capacity of dynamic BH3 profiling as a functional diagnostic. Across DLBCL cell lines, DBP accurately predicted which lines would respond best to combined statin and ABT-
199 treatment. Further, statin-induced sensitivity to the BAD peptide correlated with additivity between statins and ABT-199 in primary CLL samples. In addition to DBP, we also identify protein markers of pharmacodynamic response and enhanced sensitivity to ABT-199. While Rap1A de-prenylation can determine whether statins suppress protein prenylation *in vivo*, PUMA up-regulation may be used to predict cells that respond to the combination. Indeed, across DLBCL and AML cell lines, only those cell lines that significantly up-regulated PUMA expression after statin treatment were more sensitive to the combination.

Up-regulation of PUMA suggested that p53 may be activated by statin treatment. However, the combination of statins and ABT-199 was equally effective across primary CLL samples irrespective of p53 status. In support, early work using prenyltransferase inhibitors identified p53-independent mechanisms of apoptosis (50,51). These data broaden the potential for statins as apoptotic sensitzers and provide a strong rationale for testing statins prospectively.

With any targeted therapy, identifying alternative targets in the pathway may be key to circumventing potential bypass, resistance, or feedback mechanisms that can limit efficacy. Our approach uncovered a key downstream target whose inhibition is both necessary and sufficient for sensitization to ABT-199. By inhibiting protein geranylgeranylation directly, GGT-1 inhibitors obviate statin effects on cholesterol biosynthesis, ubiquinone production, and glycosylation. Whether isolating this effect will amplify or dampen the efficacy and/or tolerability of statins remains to be determined. Clinically, only GGTI-2418 has progressed to clinical trials where it had favorable tolerability and plasma concentrations in patients with refractory solid tumors (52,53).
Combinations of GGTIs and BH3 mimetics have not yet been studied in preclinical or clinical studies.

A goal for future research is to identify the proteins whose de-prenylation is required for statin and GGTI efficacy. There is an unknown number of proteins in the prenylome, though contemporary reviews place this number at approximately 500 (40,54). Identifying which proteins mediate the effect of statins and GGTIs will be key to identifying novel targets that may elevate responses and mitigate concerns regarding toxicity or clinical feasibility. Advances in standard methods to identify and study prenylation using novel tagging methods may facilitate the annotation of a complete prenylome (55). Coupled with recent advances in CRISPR/Cas9 gene editing, it is reasonable to anticipate prenylome-wide screens will identify genomic mediators or predictors of response to statins and/or prenyltransferase inhibitors.

The identification of rational combinations of targeted therapies has yielded several treatments with promising preclinical efficacy. We report a novel combination involving two FDA-approved treatments that induces selective cancer cell death across several blood cancers both in vitro and in vivo. Our results strengthen an existing rationale for the development of DBP as a predictive diagnostic tool and support a growing body of evidence regarding the importance of the mevalonate pathway in cancer. More generally, this work provides a solid preclinical foundation warranting clinical evaluation of combining statins and BH3 mimetics to treat blood cancers.
References


Chapter Four

Conclusions and future directions
Parts of the following conclusion contain excerpts taken from our review article published in *The British Journal of Clinical Pharmacology* (2016), entitled “Targeting mTOR for the treatment of B cell malignancies”.

In this Chapter, we provide a summary of the main conclusions from Chapters 2 and 3, then present unpublished data derived from these studies. Subsequently, we discuss potential future directions as well as the implication of this dissertation work as a whole.

The discovery of BH3 mimetics opened a new avenue by which a key hallmark of cancer (resisting apoptosis) could be directly targeted to induce cancer cell death. With initial success in generating BCL-2 antagonists, early work revealed potent activity of these molecules in many contexts, especially where BCL-2 dependence could be established (1,2). Due to their selectivity for BCL-2, second generation BH3 mimetics maintained this efficacy while also being well tolerated with minimal toxicities against normal cells. However, given the diversity of methods to evade apoptosis (Figure 1.3), it is not surprising that BH3 mimetics have not been universal cancer killers. A straightforward approach to improving efficacy is to simultaneously target survival pathways that are important for cancer cells. This dissertation investigates two targeted therapies with which to combine BCL-2 inhibitors in order to augment their anti-cancer efficacy while maintaining their tolerability.
Targeted therapies that enhance the efficacy of BH3 mimetics

**PI3K/AKT/mTOR pathway**

Pursuing the simple hypothesis that targeting two pathways whose over-expression/activation are correlated with poor prognosis, in Chapter 2 we uncovered synergy between PI3K/AKT/mTOR pathway inhibitors and BH3 mimetics in DLBCL. By using DBP to measure mitochondrial priming, we provide evidence that this assay has potential in predicting efficacious combinations. We also show the addition of PI3K/mTOR inhibitors does not exacerbate toxicity of BH3 mimetics among PBMC subsets, providing preclinical evidence for tolerability of this combination. Unlike previous reports implicating suppression of MCL-1 expression, we demonstrate that PI3K/AKT/mTOR inhibitors increase mitochondrial accumulation of BAD and BIM. Using pharmacological and genetic approaches, we identify that AKT inhibition is required for this effect. This work supports the notion that as combinations of PI3K and BCL-2 inhibitors mature, it will be critical to fully understand the breadth of interactions with BCL-2 family proteins to identify predictive markers of response.

**Mevalonate pathway**

In Chapter 3, we investigated the potential of targeting the mevalonate pathway with HMGCR inhibitors (statins) to enhance the efficacy of BH3 mimetics. We uncovered striking synergy between these two drug classes, and expanded these findings beyond DLBCL to other blood cancers where ABT-199 is under clinical investigation (i.e. AML and CLL). By again applying the DBP approach, we reaffirm that therapy-induced priming is a strong predictor of enhanced responses to BH3 mimetics.
across cell lines and primary patient samples. Mechanistically, we implicate a crucial role for suppressing protein geranylgeranylation, which leads to upregulation of PUMA. These findings hint at alternative downstream targets, though the relative efficacy and tolerability of these strategies remains to be seen. Collectively, our data provide compelling evidence for the tolerability and efficacy of the statin/ABT-199 combination and strongly support further clinical investigation. As this clinical data matures, it will be important to monitor the tolerability and efficacy of these mevalonate-targeting strategies in combination with BH3 mimetics, and verify the utility of DBP as a predictive companion diagnostic.

**Targeting downstream of PI3K/AKT/mTOR**

In Chapter 2, we observed that both rapamycin and TOR-KIs also enhanced the efficacy of BH3 mimetics in DLBCL, similar to dual PI3K/mTOR inhibitors. This suggested a role for mTORC1 in mitochondrial priming. To test this hypothesis we used chemical and genetic approaches to selectively inhibit two major downstream mTORC1 effectors, 4E-BP and S6K. Using an S6K1-specific small molecule inhibitor (LY2584702 (3)) that suppresses phosphorylation of S6 protein in DLBCL (Figure 4.1A), we observed no significant enhancement of ABT-263 (Figure 4.1B), suggesting that the kinase activity of S6K1 is not important to the sensitization effect.

To mimic selective inhibition of the mTORC1-4E-BP-eIF4E axis, we expressed a constitutively active form of 4E-BP1, which has all five phosphorylation sites mutated to alanine residues and cannot be inhibited by mTOR kinase activity (4). Treatment of cells with doxycycline strongly induced expression of 4E-BP1, suppressed cap-dependent
translation and increased the extent of MOMP induced by BIM, BAD, and PUMA (Figure 4.2A-C), all patterns that phenocopied pharmacological PI3K pathway inhibition. In contrast to S6K1 inhibition, genetic suppression of eIF4E was also sufficient to enhance the efficacy of ABT-263 (Figure 4.2D). Interestingly, the sensitivity to ABT-263 exhibited an inverse correlation with the amount of cap-dependent translation, implicating protein synthesis in survival signaling (Figure 4.2E). Induction of mutant 4EBP1 did not significantly affect expression of survivin, BCL-XL, or MCL-1 (Figure 4.2F), candidate pro-survival proteins reported to be regulated by cap-dependent translation downstream of mTORC1 in other systems. Together, our data suggest a key role for the mTORC1-4EBP1-eIF4E axis in modulating sensitivity to BCL-2 antagonists, via translational control of gene products yet to be determined.

![Figure 4.1](image)

**Figure 4.1.** S6K inhibition does not sensitize DLBCL cells to ABT-263. (A) Western blot of cells treated with increasing doses of S6K1 inhibitor for 3 hours. (B) Viability of cells treated with indicated inhibitors for 48 hours. Viability was assessed using 7-AAD dye exclusion by FACS.
Given this data, it is likely that there is a complementary mTORC1-dependent mechanism that augments the priming effects of AKT inhibition that we described in Chapter 2. This hypothesis is supported by the observation that dual PI3K/mTOR inhibitors consistently produced the strongest effect, likely due to their ability to suppress both AKT and mTORC1 activity. Indeed, the addition of MLN0128 or BEZ235 to cells expressing mutant 4E-BP1 further sensitized cells to BH3 mimetics, likely due to inhibition of AKT (Figure 4.2D). Interestingly, expression of wild type 4E-BP1 also enhanced the sensitizing effects of TOR-KIs. This is likely a consequence of altering the ratio of eIF4E to 4E-BP, which augments the severity of cap-dependent translation inhibition by mTORC1 inhibition (5). Thus, an alternative means with which to sensitize cells to BH3 mimetics may be to target eIF4E downstream of mTORC1. A promising approach is to disrupt the assembly of the cap-dependent translation initiation complex (eIF4F). The compound SBI-0640756 achieves this by preventing eIF4G from binding to eIF4E, thereby disrupting complex formation and suppressing cap-dependent translation (6). Thus, an interesting question is whether this inhibitor may also enhance the efficacy of BH3 mimetics in blood cancers and whether this approach may be more effective/tolerable than TOR-KIs. Distinct from disruption of complex assembly, another approach is to target the enzymatic activity of other proteins in the eIF4F complex. For example, the helicase eIF4A represents an attractive target due to its demonstrated importance in the translation of certain mRNAs (7), and the availability of compounds that directly inhibit its activity (silvestrol and hippuristanol (8,9)). Interestingly, silvestrol has already demonstrated efficacy in B cell leukemias, and is also associated with B cell toxicity (10). Moving forward, key questions will be to compare these inhibitors with
TOR-KIs and note whether these therapies are 1) more effective at priming cells and 2) whether they are more tolerable.

Figure 4.2. Expression of constitutively active 4E-BP1 sensitizes OCI-LY1 cells to ABT-263. (A) Western blot of OCI-LY1 cells expressing empty vector, 4E-BP1 wild type (WT), or 4E-BP1-5A mutant (Mut) treated with indicated inhibitors for 3 hours. (B) Dual luciferase assay measuring ratio of cap-dependent translation after treatment for 16 hours. (C) BH3 profile of cells expressing mutant 4E-BP1. (D) Sensitivity of indicated cells to ABT-263 with or without MLN0128 or BEZ235. (E) Spearman correlation between data in (B) and (D). (F) Western blot of cells treated with inhibitors for 16 hours. In all cases, cells were pre-treated with doxycycline (1 µg/µL) for 24 hours prior to indicated treatments.

SREBPs connect PI3K and Mevalonate

The pathways discussed in Chapters 2 and 3 are distinct and seemingly unrelated. In this section we discuss how we stumbled upon the priming effects of targeting the mevalonate pathway based off the findings of Chapter 2.
In an effort to expand the findings of Chapter 2, we used a proteomics approach to identify additional downstream effects of PI3K pathway inhibition in DLBCL cells. In collaboration with the MD Anderson Cancer Center (Houston, TX) we performed reverse phase protein array (11) on DLBCL cells treated with several PI3K pathway inhibitors for 3 hours. Importantly, two sterol-regulatory element binding protein (SREBP) target genes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) were significantly down-regulated by PI3K pathway inhibitors (Figure 4.3). SREBPs are transcription factors that promote expression of genes required for cholesterol and fatty acid biosynthesis (12). Recently, it has become appreciated that the PI3K/AKT/mTOR pathway plays a critical role in both the activation, expression, and stability of SREBPs (13). In support, activation of PI3K and AKT is required for both the expression of SREBPs (14) and its nuclear accumulation (15,16). In addition, both mTORC1 and p70S6K1 inhibit the function of Lipin 1, which suppresses SREBP1 transcriptional activity (17). Collectively, these data suggested that inhibition of PI3K may also affect cholesterol or fatty acid synthesis, warranting an investigation of direct inhibitors of these pathways (i.e. statins). While these experiments ultimately led us to the discovery that statins prime blood cancers for apoptosis, it has become clear that sensitization by PI3K pathway inhibitors is independent of effects on mevalonate (Figure 3.7B).
Figure 4.3. PI3K pathway inhibitors suppress expression of SREBP target genes. Heat map of reverse phase protein array (RPPA) data on SU-DHL4 cells treated with indicated inhibitors for 3 hours.

The missing link between isoprenylation and mitochondrial priming

Rho/ROCK/actin pathway

In Chapter 3, we established a solid link between protein geranylgeranylation and mitochondrial priming, but were not able to identify the proteins whose de-prenylation are required for this effect. While it is likely that the effects of statins and GGTIs rely on de-prenylating multiple proteins, we used a candidate approach to investigate the contribution of Rho GTPases. After 16 hour treatment with simvastatin or fluvastatin we observed a marked decrease in membrane-associated RhoA with a concomitant increase in cytoplasmic accumulation (Figure 4.4). Having confirmed that statins affect Rho prenylation in DLBCL cell lines, we next investigated whether pharmacological
inhibition of Rho-associated protein kinase (ROCK, a major effector of Rho activity) could similarly sensitize DLBCL cells to ABT-199. Impressively, treatment with RKI-1447 inhibited phosphorylation of the ROCK substrate, MYPT1 (Figure 4.5A), and almost completely reproduced the effects of statin treatment (Figure 4.5B). Collectively, these data suggested that ROCK inhibition is sufficient to prime DLBCL cells for apoptosis and sensitize them to BCL-2 antagonism.

**Figure 4.4. Statins suppress prenylation of RhoA.** Heavy membrane preparation of cells treated with 10 µM of indicated inhibitors for 16 hours. Heavy membrane fractions (M) were separated from cytoplasmic (C) fractions by lysis in isotonic buffer and differential centrifugation.
Figure 4.5. ROCK inhibitors recapitulate the sensitizing effect of statins in DLBCL cell lines. (A) Western blot of cells treated with indicated concentration (nM) of RKI-1447 for 3 hours. (B) Viability of DLBCL cells treated with increasing doses of RKI-1447 (black lines) or simvastatin (grey lines) with (dashed) or without (solid) ABT-199 for 48 hours. Viability was assessed using Annexin V and propidium iodide.

Since ROCK plays a key role in regulating actin dynamics (Figure 4.6A), we next tested whether statins or RKI-1447 could induce actin de-polymerization. Using phalloidin to stain for total filamentous (f)-actin, we observed significant actin de-polymerization following statin or RKI-1447 treatment (Figure 4.6B). Interestingly, the occurrence of de-polymerization correlated with sensitivity to single-agent HMGCR/ROCK inhibition and/or enhanced sensitivity to ABT-199. In particular, in OCI-LY1 cells where statins did not synergize with ABT-199, statins did not reduce phalloidin staining (Figure 4.6B). Conversely, RKI-1447 both reduced actin staining and was sufficient to kill OCI-LY1 cells as a single agent (Figure 4.5B). We next investigated
which BCL-2 family proteins may be regulated by this phenotype. Interestingly, the BH3-only protein BMF can be sequestered away from the mitochondria through an interaction with myosin and actin (18). Thus, we hypothesized that by inducing actin depolymerization, ROCK inhibition may free BMF to prime cells for apoptosis. However, when we examined the mitochondrial abundance of BMF following statin or RKI-1447 treatment, we observed no significant increase (Figure 4.7). Furthermore, RKI-1447 did not induce PUMA accumulation (Figure 4.7), suggesting that the effects of ROCK inhibition may be distinct from the effects of statins.

Figure 4.6. Rho and ROCK regulate actin dynamics. (A) Schematic of Rho/ROCK/Actin pathway. Inhibition of RhoA/ROCK releases cofilin from inhibition by LIMK leading to actin de-polymerization. (B) MFI of phalloidin staining in DLBCL cells treated with 10 µM indicated inhibitor for 16 hours. Cells were permeabilized and fixed prior to staining with Phalloidin-AlexFluor 647 to measure total f-actin levels.
Figure 4.7. Statins and RKI-1447 do not induce mitochondrial accumulation of BMF. Western blot of cells treated with 10 µM of indicated inhibitors for 16 hours. Mitochondrial (mito) and cytoplasmic (cyto) fractions were separated by centrifugation.

While these results suggested that the effects of Rho and ROCK inhibition may not be directly connected to the mevalonate pathway, an approach to directly test this connection is to generate a CAAX-mutant form of constitutively active RhoA. By mutating the CAAX domain, we can effectively switch the RhoA post-translational modification from geranylgeranylation to farnesylation (19). Subsequently, if RhoA is the primary GTPase whose de-prenylation is required for the priming effect of statins and GGTIs, then this switch should render cells insensitive to GGTIs (but not statins). Similarly, if a constitutively active ROCK can rescue cells from the effects of both statins and ROCK inhibitors, then these data would also support a role for the Rho/ROCK pathway in mediating the efficacy of statins. Nevertheless, irrespective of whether inhibition of Rho is the primary mechanism by which statins prime DLBCL cells for apoptosis, the promising synergy between RKI-1447 and ABT-199 suggest that the
Rho/ROCK pathway may be another target for combination therapies. Clinically, ROCK inhibitors (fasudil and K-115) have found success in treatment of glaucoma with favorable tolerability, and recent data demonstrating preclinical efficacy of ROCK inhibitors support further investigation into their anti-cancer potential (20,21).

**Identification of geranylgeranylated proteins in DLBCL**

Identification of the exact mechanisms that induce mitochondrial priming downstream of protein geranylgeranylation may lead to alternative targets or biomarkers that would supplement strategies for how best to deploy MVA/BCL-2 targeting combinations. However, given the vast number of proteins that are potentially subject to this post-translational modification (22,23), this task is nontrivial. Emerging techniques to study prenylation may yield useful tools with which to identify and validate substrates of GGT-1 that may be important for modulating apoptotic sensitivity. In particular, novel tagging strategies utilizing GGPP labeled with an azide group enables easy pulldown when coupled with a alkyne-labeled beads (24). Commercially available (Life Technologies) azido-GGPP has already been confirmed to be cell permeant and can be incorporated directly into newly prenylated proteins. Following an azide-alkyne cycloaddition, these proteins can be covalently linked to beads, purified from whole cell lysates, and utilized in mass spectrometry analysis to yield a comprehensive list of geranylgeranylated proteins. Performing these experiments in DLBCL cell lines will circumvent any context-dependent differences in the prenylome. Standard protocols for confirming geranylgeranylation are also readily available to validate the targets from such an experiment (22).
Given this abbreviated list of geranylgeranylated proteins, a straightforward approach to identify those proteins whose loss of function contributes to mitochondrial priming is by knockout screen. Using pooled RNAi or CRISPR/Cas9 approaches, one could selectively knockout expression of geranylgeranylated proteins. Once cells are placed under ABT-199 selection (at doses that do not significantly kill parental cells), only those short-hairpin RNAs (shRNAs) or guide RNAs (gRNAs) targeting relevant proteins would “dropout” of the pool (i.e. a synthetic lethality screen (25)). The relative abundance of each shRNA/gRNA can then be identified using high-throughput sequencing (26,27) to yield a list of those geranylgeranylated proteins whose loss of expression sensitizes cells to ABT-199. With any screen, the most important factors are approaches to follow-up and validate those hits in a biologically meaningful way. For these experiments, the most pertinent experiment is to specifically alter the geranylgeranylation of these target proteins and measure changes in sensitivity to combined HMGCR/BCL-2 inhibition. For example, introducing CAAX-mutant variants of these candidate proteins will render proteins dependent on FTs rather than GGTs. Thus, only those proteins whose CAAX-mutant variants can rescue cells from GGTI would be important for mediating the effects of statins and GGTIs. Overall, much is still unknown regarding how geranylgeranylation affects mitochondrial priming and which proteins mediate this effect. Nonetheless, as candidates begin to be revealed from large scale screens, it will be important to test whether targeting these proteins directly will be more effective/tolerable relative to statins.
In Chapter 3 we describe a statin-induced increase in PUMA expression, but do not identify the mechanism by which this occurs. Typically, PUMA up-regulation is linked to p53 activation (hence, p53-upregulated modulator of apoptosis). However, since simvastatin plus ABT-199 demonstrated efficacy in 17p(del) primary CLL patient samples, we hypothesized a mechanism of priming that was independent of p53 activation. In support, neither statins nor GGTI-298 increased p53 phosphorylation or stabilization in DLBCL after 16 hour treatment (Figure 4.8A). While this data supports the notion that p53 is not activated by statins, in order to test this directly we used pifithrin-α (PFT), a chemical inhibitor of p53 activity (28). If p53 activity is required for the synergism between statins and ABT-199, then inhibition of p53 should protect from the combination. However, we observed no rescue from the combination using up to 30 µM PFT (Figure 4.8B). While p53 suppression was not confirmed (either by qPCR or western blot of p53 and its target genes), these data strongly suggest that statin-induced PUMA expression is p53-independent.

To investigate whether increased PUMA expression occurs via up-regulation of transcription, we used qPCR to measure the abundance of PUMA mRNAs following 16 hour treatment with statins or PTIs. In both DLBCL cells tested, both simvastatin and fluvastatin increased mRNA levels of PUMA (Figure 4.8C). In addition, statins also induced up-regulation of HRK, a BH3-only protein with high affinity for BCL-XL. Unexpectedly, treatment with etoposide (which causes DNA-damage-induced p53 activation (29)) did not induce PUMA transcription, suggesting that these cells express non-functional p53. Consistent with this interpretation, etoposide also failed to promote
p53 accumulation despite inducing its phosphorylation (Figure 4.8A). Collectively, the data support the hypothesis that statins do not induce p53 activation.

**Figure 4.8.** Statins increase PUMA transcription in a p53-independent manner. (A) Western blot of cells treated with 10 µM of indicated inhibitors for 16 hours prior. (B) Viability of cells treated with increasing doses of pifithrin-a. Cells were co-treated with vehicle (black lines) or simvastatin plus ABT-199 (grey lines). Doses for cells are 10 µM simvastatin, and 30 nM (OCI-LY8) or 300 nM (SU-DHL4) ABT-199. (C) qPCR of cells treated with 10 µM indicated inhibitor for 16 hour. N = 2 for OCI-LY8, N = 1 for SU-DHL4.
Our data suggest that statins induce transcription of both PUMA and HRK to promote mitochondrial priming in a mechanism that is independent from p53 activation. An alternative explanation is through activation of FOXO transcription factors, which also regulate PUMA transcription (30). Supporting this hypothesis, we observed that statins consistently suppressed phosphorylation of AKT at the Ser473 site (Figure 4.8A). However, GGTI-298 did not inhibit this phosphorylation. Thus, either 1) statins and GGTI-298 have alternative mechanisms of priming, or 2) inhibition of AKT is dispensable for priming. Given that GGPP completely rescues cells from the effects of simvastatin, it is highly unlikely that their mechanisms are not shared. Furthermore, AKT-dependent up-regulation of PUMA and/or HRK in these cell lines would be inconsistent with the phenotypes we presented in Chapter 2 (BAD and BIM accumulation). In addition, preliminary experiments using a phospho-mimetic form of AKT (S473D) suggested that this mutant was insufficient to dampen the synergy (Figure 4.9). Overall, the exact mechanism whereby PUMA and HRK transcription is induced remains to be identified. Moving forward, identifying the precise mechanism may give clues to which pathways downstream of geranylgeranylation are important for the effects of statins and GGTI. In addition, this mechanistic insight may also yield novel targets whose efficacy and tolerability might have advantages relative to statins or GGTIs.
Figure 4.9. AKT S473D expression does not protect from statin sensitization. Viability of cells expressing AKT S473D (red lines) or empty vector (black lines). Cells were pre-treated with doxycycline to induce expression 24 hours before treating cells with increasing concentrations of ABT-199 with vehicle (solid lines), simvastatin (10 µM, dashed lines), or fluvastatin (10 µM, dotted lines).

**BCL-2 and MCL-1 over-expression do not rescue from effects of statins**

Since statins up-regulate PUMA, which has the capacity to neutralize all anti-apoptotic BCL-2 family proteins, we also investigated whether the combination of statins could work in cells over-expressing either MCL-1 or BCL-2. Over-expression of either of these proteins is associated with resistance to BCL-2 inhibitors (31,32). In support, expression of BCL-2 or MCL-1 significantly reduced sensitivity to ABT-263 (Figure 4.10). However, simvastatin retained its ability to enhance the killing effects of BCL-2 inhibition (Figure 4.10), suggesting the combination may be effective in contexts of known resistance to BCL-2-inhibitors.
Figure 4.10. BCL-2 and MCL-1 over-expression do not rescue from statin sensitization. Viability of cells pre-treated with doxycycline for 24 hours prior to assessment of ABT-263 IC50 +/- simvastatin (10 µM) after 48 hour treatment.

BH3 profiling as a functional diagnostic with predictive power

In both Chapters 2 and 3 we present compelling data regarding the power of dynamic BH3 profiling to predict not only effective drug combinations, but also which samples are likely to respond. This addresses a growing concern in the development of cancer therapeutics, which is to develop methods to match patients with those drugs from which they will most likely benefit (33). Indeed, many patients receive therapies to which their cancers remain unresponsive highlighting two major clinical issues: (1) patients that receive ineffective therapies are not receiving potentially effective treatments and (2) they experience unnecessary toxicities and paying unnecessary costs for drugs that they do not need. As a result, there is great interest in being able to improve patient outcomes by improving treatment/patient selection criteria.

In general, successful approaches have been dependent on molecular, often genomic, indicators and are rooted in the notion that targeting driver genetic alterations
can yield lasting clinical responses. These efforts have been strongly supported over the years, with the most poignant example being the dramatic efficacy of imatinib in targeting the BCR-ABL translocation in chronic myelogenous leukemia (CML).

Subsequent discoveries of epidermal growth factor receptor 1 (EGFR) or anaplastic lymphoma kinase (ALK) mutations in non-small cell lung cancer (NSCLC) (34,35), HER2/neu mutations or amplifications in breast cancer (36,37), and BRAF mutations in melanoma (38,39) have all yielded promising targeted therapies in their respective settings.

Further success in biomarker identification has also stemmed from an understanding of synthetic lethality, which supports utilizing non-druggable alterations as predictive biomarkers of therapeutic responses. For example, in ovarian cancer, the loss BRCA or BRCA-like genes is strongly predictive of clinical responses to PARP inhibition (40). Given these successes, it is unsurprising that there has been a recent surge in studies seeking to exploit cancer genomics for use as predictive biomarkers (41,42). However, only a small percentage of patients have diseases where an FDA-approved biomarker-treatment combination exists (43). In addition, none of the subsequently identified biomarker-therapy combinations have come close to producing the magnitude of response that was observed in CML (44). In support, a recent prospective trial comparing biomarker paired genomics-to-therapy approaches to control groups showed only modest gains in progression free survival (PFS) and overall survival (OS) across cancer subtypes (45). Given the lack of substantial responses, it is likely that genomics based approaches to biomarker discovery do not fully capture the complexity of translating genotypes to phenotypes. Thus, our work demonstrating the
clear capacity of DBP to predict effective combinations provides compelling evidence that DBP might be an exciting opportunity to develop a functional diagnostic with predictive power.

**Further development of DBP**

While our profiling data with primary CLL patient samples corresponded nicely with the efficacy of the combination *in vitro*, a key concern is whether this *ex vivo* testing accurately reflects/predicts patient responses *in vivo*. Correlative evidence from the Letai lab demonstrates clear potential for BH3 profiling to predict chemo-sensitivity (46-48), but the clinical utility of DBP to specifically predict sensitivity to combinations with BH3 mimetics has not yet been tested prospectively. Our data support that DBP may be used in two distinct methods: 1) screen for compounds that prime cells or 2) screen for patients that respond to a given compound. While the two approaches are not mutually exclusive, it will be important to prioritize which clinical use would be most effectively implemented. Given the skill- and time-intensive nature of DBP, it is likely that these restrictions would favor the latter method. Nonetheless, as DBP matures and more therapies receive regulatory approval, the utility of DBP might expand such that *ex vivo* screens would be clinically feasible.

Apart from these clinical considerations, it is important to note that our work suggests that changes in mitochondrial priming do not necessarily provide insight into the molecular mechanisms driving those changes. While it is tempting to interpret enhanced sensitivity to specific peptides as indicative of potential mechanisms, the complexity of the binding interactions among BCL-2 family proteins makes this analysis
non-trivial. Indeed, treatments that increase priming may do so in any number of ways. For example, any increase in priming may be reflective of 1) decreases in anti-apoptotic expression, 2) increases in pro-apoptotic proteins, 3) changes in post-translational modifications, or 4) changes in subcellular localization. Thus, a thorough mechanistic investigation is required. Nevertheless, the capacity for DBP to predict enhanced sensitivity to BH3 mimetics has consistently been captured by increased sensitivity to the BAD peptide, suggesting that limited profiling based on this peptide may be sufficient for predicting ABT-199 sensitivity.

Lastly, a common issue that limits the efficacy of targeted therapies is the existence of intra-tumoral heterogeneity and the emergence of resistance. Clinically, this may lead to changes in DBP responses as non-sensitive cell populations are selected for, or as resistance emerges. Whether sequential profiling can be used to identify alternative effective therapies is another aspect that must be tested. Ongoing developments in DBP are also likely to yield advantages over the FACS-based assays presented in this dissertation. For example, the kinetics of depolarization may be entirely circumvented by modifications to the protocol. By permeabilizing and fixing cells, depolarization can be accurately measured by cytochrome C intracellular staining (depolarized cells lose intracellular cytochrome C). Alternatively, cells can be mounted for microscopy to detect single-cell differences in responses to BH3 only peptides (using cytochrome C). While accompanying computational methods and validation will be needed to test whether this accurately reflects patient responses, these advancements are likely to directly address current shortcomings of DBP. Thus, DBP represents an attractive strategy to predict which combinations of drugs can be used in combination
with BH3 mimetics to elicit patient responses and warrants further preclinical and clinical testing.

**Targeting BCL-2 family proteins**

*Future directions for BCL-2 antagonism*

Moving forward, a key question is whether combinations with BCL-2 inhibitors will have broader efficacy in other cancer settings. The rationale for targeting BCL-2 in blood cancers is derived from their dependence on BCL-2 for survival (1,2). Indeed, the earliest evidence of this dependence came from knockout studies where loss of BCL-2 caused massive apoptosis in lymphoid organs following maturation (49,50). Later studies using BH3 profiling confirmed that blood cancer cells are both inherently primed for apoptosis, and reliant on BCL-2 for survival (46,51), making them prime targets for BCL-2 inhibitors. Following this logic, it plausible that since mice lacking BCL-2 develop polycystic kidney disease (PKD) as a result of kidney cell apoptosis (52), renal cell cancers may also be sensitive to BH3 mimetics (50). RNA-seq data compiled by Memorial-Sloan Kettering (cBioPortal) clearly illustrates the tendency of renal cell carcinomas (RCC) cells to express high levels of BCL-2 (**Figure 4.11**) and, elevated BCL-2 expression is correlated with poor prognosis in patients with RCC (53,54). These studies suggest that BCL-2 antagonists may have efficacy in this setting, but to date, there have been no publications examining this. In addition to RCC, uveal melanomas and breast cancers also express elevated levels of BCL-2 (**Figure 4.11**). Preclinical data from these settings suggest that single agent BH3 mimetics are insufficient to induce apoptosis (55). However, combinations have shown promising efficacy (55).
Ongoing clinical trials in breast cancer cell are also reporting encouraging responses in patients treated with ABT-199 combined with chemotherapy (ISRCTN98335443). Lastly, follicular lymphoma, a disease characterized by BCL-2 over-expression, may also benefit from BCL-2 antagonists. However, early clinical trial data suggest limited single-agent efficacy (56). Overall, BCL-2 inhibitors may find success in contexts beyond blood cancers, though combinations may be required to unleash its full potential.

**Figure 4.11. Relative expression of BCL-2 mRNA across cancers.** Data are taken directly from cBioPortal.org and show relative BCL-2 mRNA expression across indicated cancers subtypes. Data are derived from The Cancer Genome Atlas (TCGA) database.

*TCGA provisional only.

**Alternative targets of BH3 mimetics**

The development of current BCL-2-selective inhibitors directly demonstrates the importance of choosing which BCL-2 family protein to target. First generation inhibitors (ABT-263 and ABT-737) directly mimicked the molecular function of the BAD. However,
because they inhibited BCL-XL these drugs were inseparable from on-target, dose-limiting thrombocytopenia (57-59). The second generation compound, ABT-199, lacks this toxicity due to reduced affinity for BCL-XL (60), emphasizing the striking difference between BCL-2 and BCL-XL inhibition. While it is tempting to believe that potent inhibition of multiple BCL-2 family proteins will be more effective at eliminating cancer cells, this approach will likely have massive toxicities and limited tolerability. Instead, selective inhibition of single BCL-2 family members and rational combinations may be the key to uncovering effective and tolerable anti-cancer therapies.

Whether targeting other pro-survival BCL-2 family members may yield broader efficacy without added toxicity is still unknown. A promising target for new approaches is MCL-1. Indeed, cancers that become resistant to BCL-2-targeting therapies often up-regulate MCL-1 (31,61). Thus, it stands to reason that those cancers that are resistant to single agent BCL-2 inhibition, may instead rely on MCL-1 for survival. Spearheaded by work from Steven Fesik’s laboratory, development of small molecule MCL-1 inhibitors is under way (62-64). Recent preclinical data using a novel molecule, S63845, demonstrated promising anti-cancer effects in vitro and in vivo in a variety of blood cancer models (65). However, it will be important to test pre-clinically which normal and cancer cells are most likely to be affected. Given elevated levels of MCL-1 mRNA in lung, prostate, breast, ovarian, renal, and glial cancers (66), MCL-1 inhibitors may also find success in these settings. Additionally, whether MCL-1 inhibitors can be given to patients whose tumors become resistant to ABT-199 may be another promising approach. However, knockout studies in mice caution of potentially devastating toxicities. In particular, inducible loss of MCL-1 caused depletion of hematopoietic stem
cells (HSCs (67)). Thus, it will be important to test these inhibitors extensively in mouse models to investigate potential complications from acute MCL-1 inhibition.

**Balancing combination toxicities**

A major goal of targeted therapies is to maximize efficacy against cancer cells and minimize the toxicity against normal cells. Based off previous studies, we examined lymphocyte subpopulations due to the role of BCL-2 in lymphocyte survival (49,50). With both combinations, we observed little enhancement of ABT-199 toxicity among PBMC subsets, but did not extend these findings to other contexts. Thus, a key outstanding question is whether these priming agents will also sensitize normal cells and reveal new toxicities. In an expanded test of CD19 B cell toxicity, we saw no effect between ABT-199 and simvastatin until very high concentrations (Figure 4.12), suggesting that statins selectively prime malignant but not normal B cells. Furthermore, in a prolonged survival assay, we observed no signs of toxicity (i.e. weight loss) in mice dosed with ABT-199 and simvastatin for up to 3 weeks (Figure 4.13). While we did not directly test whether statins prime normal cells, it is possible that statins selectively affect cancer cells due to a lower requirement for mevalonate products in normal cells compared to cancer cells. However, further mechanistic insight into how statins prime cancer cells will be required to shed light on the likelihood of this explanation. Nonetheless, these studies may provide invaluable insight into potential predictive biomarkers (i.e. cancers that are likely to be primed by statins) or markers of response.
Figure 4.12. Simvastatin does not enhance sensitivity of normal CD19+ B cells to ABT-199. PBMCs from healthy human donors treated with indicated inhibitors for 48 hours prior to measuring viability by 7-AAD dye exclusion. Cells were stained with subpopulation markers to distinguish B cells (CD19+).

Figure 4.13. Mice treated with ABT-199 and/or simvastatin do not show excessive weight loss after 3 weeks. Tumor bearing C57BL6/N were treated with simvastatin (50 mg/kg/day), ABT-199 (100 mg/kg/day), or the combination for up to 3 weeks. Dosing followed a 6-day-ON, 1-day-OFF weekly schedule.
Identification/prediction of combination toxicities must be fully investigated using preclinical approaches. Thus, when choosing which combinations to combine with ABT-199, it will be important to consider the toxicities of each individual drug. The safety profile of statins is well-described, with the major adverse effect (AE) being myotoxicity (68). While there is little evidence of overlapping toxicity between statins and ABT-199, the combination might augment myotoxicity through shared targeting of BCL-2 (69). A direct approach to assess myotoxicity in a preclinical setting might be to measure serum creatinine kinase (a measure of myotoxicity, CK) levels in mice treated with single agent statin or the combination. Complete blood counts and weight loss/muscle function may also be monitored in these mice to determine whether unforeseen toxicities may arise. Nonetheless, preliminary analysis of retrospective data of patients on ABT-199 suggested that there were no additional toxicities associated with concurrent statin use. Furthermore, supplementation by coenzyme Q10 may be sufficient to counteract statin toxicities (70-73). Thus, despite the potential for some toxicities, the combination of statins and ABT-199 seems like an appealing combination with potential for rapid clinical application.

Contrary to statins where literature regarding AEs is abundantly available, toxicities from PI3K pathway inhibitors are still incompletely understood. In Chapter 2, we focused on dual PI3K/mTOR inhibitors due to the consistency of their sensitization to BH3 mimetics across DLBCL cell lines. Indeed, clinical data support that PI3K/mTOR inhibitors have the broadest activity profile across cancer types (74,75). However, this approach tends to be less tolerable relative to other approaches (76). While pan-PI3K did not exacerbate toxicity in PBMCs, it is unclear what effect immune modulation may
have on patient responses (76,77). Thus, it may be necessary to test whether isoform selective inhibitors also synergize with BH3 mimetics. Another approach is to directly target AKT. While few AKT inhibitors have progressed to clinical trials, MK-2206 was recently shown to have mild and tolerable side effects (78,79). Indeed, our mechanistic studies support that AKT inhibition is required for enhanced sensitivity to BH3 mimetics, and thus warrant further clinical investigation.

An alternative to targeting PI3K/AKT is to target downstream mTORC1. The most well-tolerated mTOR inhibitors are rapalogs, which have gained European approval in mantle cell lymphoma (80). Nonetheless, clinical evidence of several toxicities including thrombocytopenia, mucositis, and hyperlipidemia suggests that prolonged treatment will be difficult to manage (81). Rapamycin was also the least potent at priming DLBCL cells for apoptosis. On the other hand, TOR-KIs were more effective than rapamycin, yet are generally less tolerable (82). A single agent tolerability test of AZD2014 showed dose-limiting toxicities that were similar to rapalogs (83), but both CC-223 and MLN0128, also induced hyperglycemia (84,85). While it is possible that lowering the dose of TOR-KIs may improve their tolerability, it will also impinge on their ability to fully suppress mTOR kinase activity. Moving forward, it will be important to determine whether these potentially suboptimal doses, which only partially inhibit mTOR, will be more effective than clinically tolerable doses of rapalogs, which potently inhibits phosphorylation of some, but not all, mTORC1 substrates. Overall, despite advances in PI3K pathway inhibitors, much is still unknown regarding how best to position them in a clinical setting. Thus, as the clinical data matures, it will be important to revisit which strategies may
best combine with BH3 mimetics, in what contexts they will be effective, and how to manage or minimize the toxicities.

Concluding remarks

The development of agents that directly target apoptotic machinery has yielded new strategies by which to selectively induce cancer cell death. While BCL-2-targeting strategies have proven effective in some contexts, responses have been limited in others. In this dissertation we demonstrate that two combinations can enhance the efficacy of BH3 mimetics in blood cancers without increasing toxicities. Whether new combinations or targeting of other BCL-2 family proteins may extend the efficacy of BH3 mimetics to other contexts remains to be seen. Nonetheless, the work presented here offers insight into exploitable mechanisms of sensitization, reveals the promising predictive capacity of DBP, and provides a strong rationale for prospectively testing combinations of BH3 mimetics in blood cancer.

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