UNIVERSITY OF CALIFORNIA,

IRVINE

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
Professor Craig M. Walsh
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.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

Page

ACKNOWLEDGMENTS

This work could not have been completed without the support, advice, and guidance from incredible individuals who have taught me how persevere through the hardships, celebrate the little victories, and pursue my goals with both energy and rigor.

First and foremost, I'd like to thank Dr. David Fruman for giving me the opportunity to pursue my training under his mentorship. Through his support and belief in my capabilities, David has always encouraged my growth and challenged me to realize more of my potential. His patience with the stumbles and excitement in the accomplishments have made the journey both memorable and meaningful. David's enthusiasm for science and down-to-earth perspective have made him an exceptional mentor and role-model and are ideal qualities that I hope to one day integrate into my own research career.

The Fruman lab members have been outstanding role models, supporters, and friends. I'd especially like to thank Thanh-Trang Vo for all her help and support both inside the lab and out. She has pushed me to become a better person, lab mate, and scientist. My fellow Fruman lab grad students Jose, Solomon, Christine, Honyin, Lee-or, and Dennis, thank you for teaching me to be resilient, patient, humble, and confident. Their discussions and advice have helped so much in developing these projects and their friendship helped me persevere through the hardships and take pride and joy in the work that we do. I'd also like to thank the undergraduate students whom I mentored, Veronica Ortiz and Sarah Tang, for reminding me of why I pursued research, for putting

up with my lessons and lame jokes, and for putting their hearts into the work we did together.

I'd like to thank Drs. Aimee Edinger and Craig Walsh for their invaluable insights and suggestions during joint lab meetings. Their perspectives added a richness to my graduate training that has been essential to my growth as a scientist. Their collegiality and willingness to collaborate are perfect examples of the type of scientist strive to be. Drs. Christopher Hughes and Irene Pedersen also deserve thanks for serving on my advancement committee and emphasizing the big picture.

CURRICULUM VITAE

Jong-Hoon Scott Lee

Education	**Doctor of Philosophy in Biological Sciences**	November 2016

University of California Irvine, Irvine, CA
    Thesis Committee: David Fruman, Aimee Edinger, Craig Walsh

Bachelor of Arts in Biology, magna cum laude   May 2011
University of Pennsylvania, Philadelphia, PA

Bachelor of Arts in Biochemistry, magna cum laude   May 2011
University of Pennsylvania, Philadelphia, PA

Technical Skills

- Mouse handling
- BH3 Profiling
- MTS Assay
- Mutagenesis
- shRNA
- qRT-PCR

- Flow Cytometry
- CRISPR
- Intracellular Staining
- Gibson Assembly
- Viability Assays
- Subcellular Fractionation

- Immunoblotting
- Viral Transduction
- Plasmid cloning
- Immunoprecipitation
- Tissue culture
- Lymphocyte purification

Research Experience

Graduate Researcher   July 2012 – Present
Department of Molecular Biology and Biochemistry, University of California Irvine
Laboratory of David Fruman

- Identified and characterized novel synergy between statins and BCL-2 inhibitors in B cell lymphomas.
- Identified a non-canonical mechanism of synergy between PI3K and BCL-2 inhibitors that supports future clinical testing of this combination.
- Confirmed predictive capability of dynamic BH3 profiling.
- Confirmed little off-target toxicity of these combination therapies on normal immune cells.

Undergraduate Research Assistant   Mar 2009 – May 2011
Department of Neuroscience, University of Pennsylvania
Laboratory of Dr. Jon Lindstrom

- Developed system to express a single isoform of human acetylcholine receptor in *Xenopus laevis* oocytes.
- Characterized differential pharmacological response of this isoform using voltage clamp experiments.
- Work enables identification of isoform-selective drugs for treating nicotine addiction and Parkinson's disease.

Grants and Fellowships	TL-1 Pre-doctoral Training Fellowship in Translational Biomedical Research	2016
	Francisco J Ayala Graduate Fellowship	2011

Publications

Vo TT*, **Lee JS***, Gotesman M, Mallya S, Fruman DA. mTOR inhibitors sensitizes B-ALL cells to dasatinib through eIF4F inhibition. *In preparation*. [*co-first authors].

Lee JS, Vo TT, Bhatt S, Fleischman AG, O'Brien S, Letai A, Schatz JH, Fruman DA. Statins synergize with ABT-199 in blood cancers. *In preparation*.

Vo TT, **Lee JS**, Pandori W, Nguyen D, Lui B, Khaw A, Mallya S, Lu M, Fruman DA. mTOR inhibition induces resistance to methotrexate and 6-mercaptopurine in high-risk-B-ALL. *Submitted*.

Lee JS, Vo TT, Fruman DA. Targeting mTOR for the treatment of B cell malignancies. Br J Clin Pharm. 2016. *Epub Jan 25.* PMID: 26805380.

Lee JS, Tang SS, Ortiz V, Vo TT, Fruman DA. MCL-1-independent mechanisms of synergy between dual PI3K/mTOR and BCL-2 inhibition in diffuse large B cell lymphoma. Oncotarget. 2015;6(34):35202–17.

Mallya S, Fitch BA, **Lee JS**, So L, Janes MR, Fruman DA. Resistance to mTOR kinase inhibitors in lymphoma cells lacking 4EBP1. PLoS ONE. 2014;9(2):e88865.

Presentations

Poster Presentations

Lee JS, Vo TT, Fruman DA. (2016) "Statins synergize with BCL-2 inhibitors in blood cancers" UCI Campus-wise Symposium on Basic Cancer Research, University of California Irvine, Irvine, CA May 7

Lee JS, Vo TT, Fruman DA. (2016) "Statins synergize with BCL-2 inhibitors in blood cancers" AACR Annual Meeting, New Orleans, LA Apr 16-20.

Lee JS, Tang S, Ortiz V, Fruman DA (2015) "Vertical Inhibition of the PI3K Pathway Potently Sensitizes Diffuse Large B Cell Lymphoma to BCL-2 Antagonism" Keystone Symposia on PI3-Kinase Signaling Pathways in Disease, Vancouver, British Columbia, Canada Jan 13-18

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

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" AACR Special Conference:

Targeting the PI3K-mTOR Network in Cancer, Philadelphia, PA Sep 14-17

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" UCI Clinical Translation Research Day, University of California Irvine, Irvine, CA June 27

Lee JS, Tang S, Ortiz V, Frumand DA (2014) "Vertical Inhibition of the PI3K Pathway Potently Sensitizes Diffuse Large B Cell Lymphoma to BCL-2 Antagonism" UCI Campus-wise Symposium on Basic Cancer Research, University of California Irvine, Irvine, CA May 3

Lee JS, Beagle B, Lee C, Fruman DA (2012) "Overcoming Resistance to Active-Site TOR Inhibitors in Diffuse Large B Cell Lymphoma" 10th Annual UCI Immunology Fair, University of California Irvine, Irvine, CA Nov 15-16

Lee JS, Kuryatov A, Lindstrom J (2011) "Functional Expression of $\alpha6\beta2\beta3*$ Acetylcholine Receptors in *Xenopus laevis* oocytes" University of Pennsylvania Department of Biochemistry Annual Poster Session, University of Pennsylvania, Philadelphia, PA May 10

Awards and Honors	First place poster at UC Irvine Symposium on Basic Cancer Research	2016
	First place poster at UC Irvine Immunology Fair	2014
	Featured poster at UC Irvine Clinical and Translational Research Day	2014
	Vagelos Scholar in Molecular and Life Sciences	2011

Professional Development

SciPhD, The Business of Science for Scientists   2015
University of California Irvine
Focus: the core business competencies necessary for transitioning from academia to industry

Project Management Introductory Course   2015
University of California Irvine
Focus: the core principles in project management as covered in the PMBOK. Including topics in scope, time, quality, risk, and stakeholder management.

Teaching and Mentorship

Graduate Mentor   2012-2015
University of California, Irvine
- Mentored two undergraduate students in data collection and analysis
- Developed students' written and oral presentation skills
- Guided students through graduate school applications leading to acceptance at USC and Memorial Sloan-Kettering

Teaching Assistant, Molecular Biology Laboratory   2013, 2014
University of California, Irvine
- Prepared supplementary content for professor-led coursework to ensure students understood material
- Led undergraduate laboratory classes in basic molecular biology techniques
- Graded exams and laboratory reports for understanding of scientific communications and methodology

Professional Memberships
American Association for Cancer Research (AACR)
American Society for Cell Biology (ASCB)
UC Irvine Graduate Professional Success for PhD Students and Postdocs in the Biomedical Sciences (GPS-BIOMED)

References

David Fruman, Professor and Associate Director of Cancer Research Institute
Department of Molecular Biology and Biochemistry
University of California Irvine
(949) 824-1947, dfruman@uci.edu

Aimee Edinger, Associate Professor
Department of Developmental and Cell Biology
University of California Irvine
(949) 824-1921, aedinger@uci.edu

Abstract of the Dissertation

Enhancing the efficacy of BH3 mimetics in blood cancers

By

Jong-Hoon Scott Lee

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2016

Professor David A. Fruman, Chair

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 *in vitro* and *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

Parts of the following introduction 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".

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).
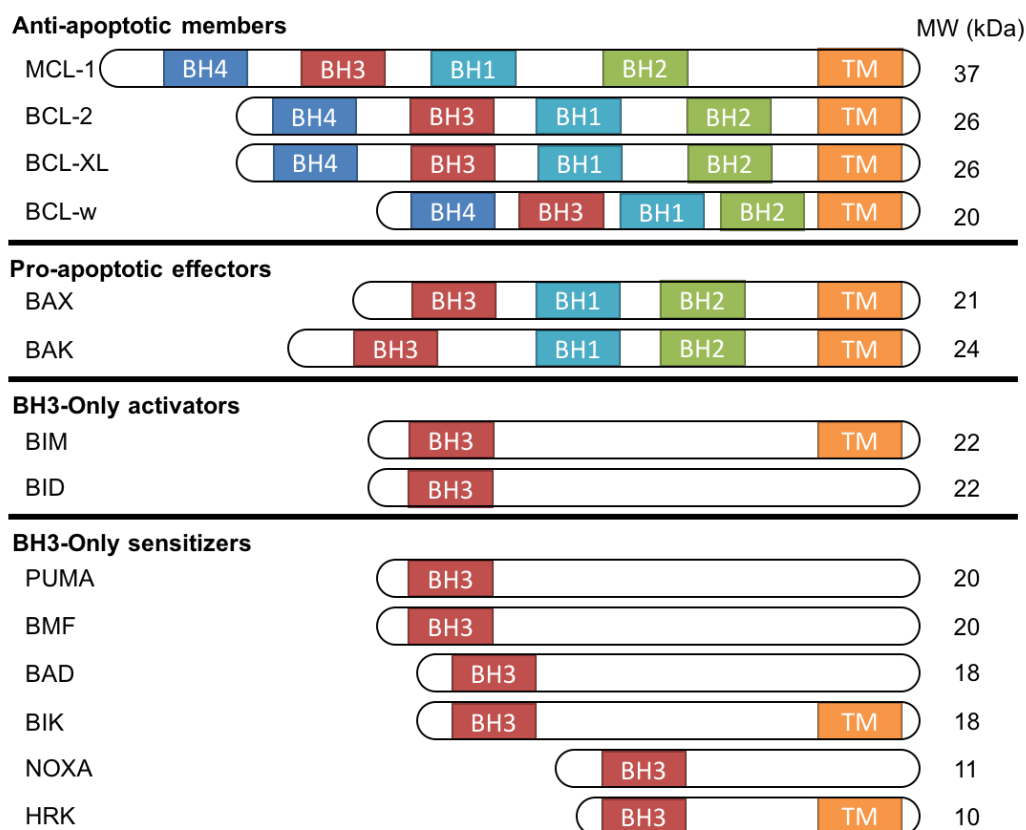
Figure 1.1. BCL-2 family proteins. BCL-2 family proteins can be broken into major groups based on their BH domains, which define their binding partners and function.

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.

A

Apoptotic Activators:
Bid | Bim

Apoptotic Effectors:
Bax | Bak

Death

Anti-Apoptotic Factors
BCL-2
BCL-XL
MCL-1

ABT-199
ABT-263

Apoptotic Sensitizers
Bad
Puma
Noxa

B

BH3-only proteins	Anti-apoptotic proteins			
	BCL-2	BCL-XL	BCL-w	MCL-1
BIM	■	■	■	■
PUMA	■	■	■	■
BID		■	■	■
BAD	■	■	■	
BMF	■	■	■	
NOXA				■
HRK		■		
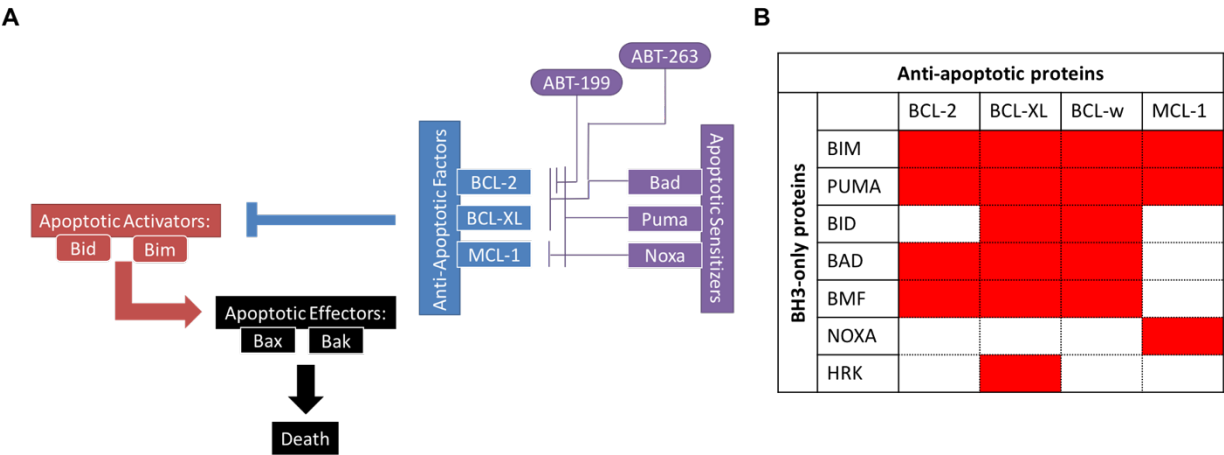
Figure 1.2. Overview of BCL-2 family protein interactions. (**A**) Overview of mitochondrial apoptotic pathway, with binding interactions highlighted. (**B**) Binding affinity of BH3 only peptides for anti-apoptotic BCL-2 family proteins, figure adapted from Deng et al (**50**).

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.
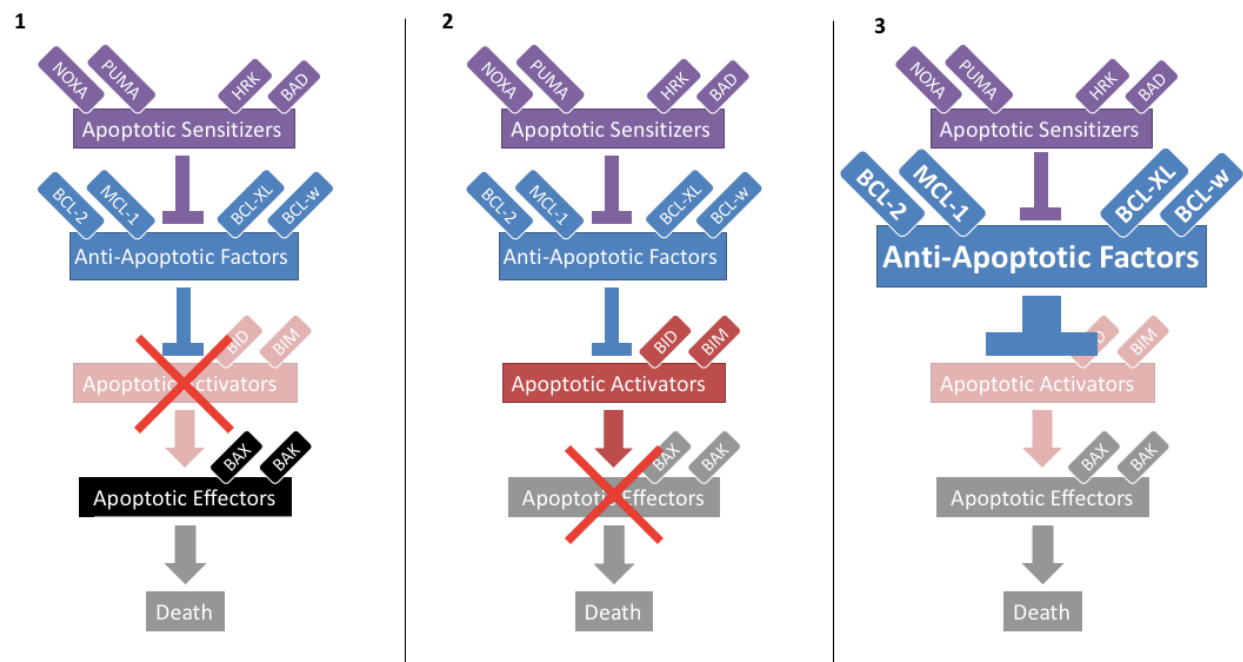
Figure 1.3. Strategies for evading apoptosis. Evading apoptosis is a hallmark of cancer, as such there are many strategies that cancer cells use to achieve this. For example, cancers can silence apoptotic activators (panel 1) or effectors (panel 2) to limit induction of MOMP. Alternatively, some cancers 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

10

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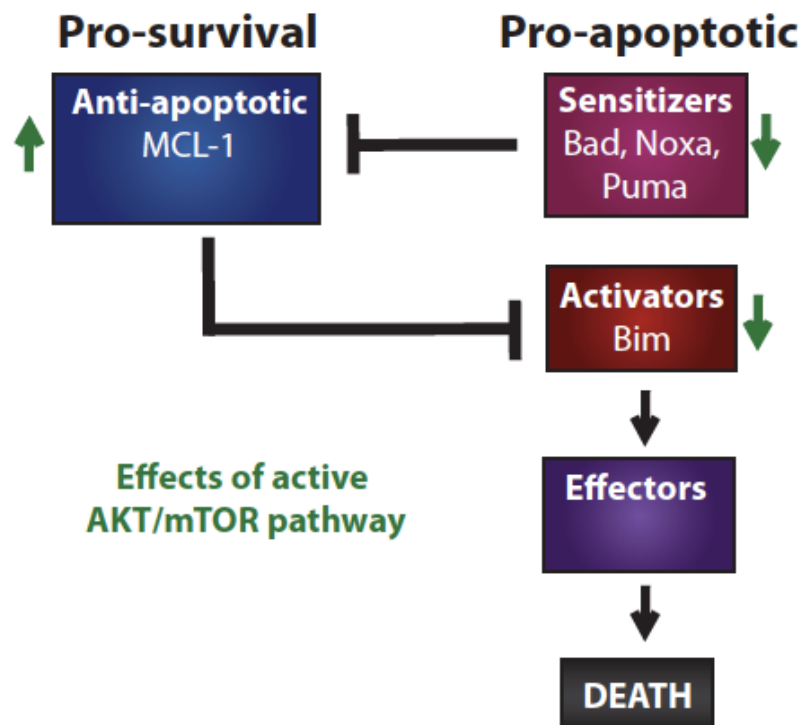
Figure 1.4. Known interactions between the PI3K pathway and BCL-2 family proteins. Aberrant PI3K activation affects the expression/activity of many BCL-2 family proteins, skewing cells towards survival by reducing pro-apoptotic proteins (BAD, NOXA, PUMA, Bim (120-122)) and increasing pro-survival proteins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

(114,124-127). 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).
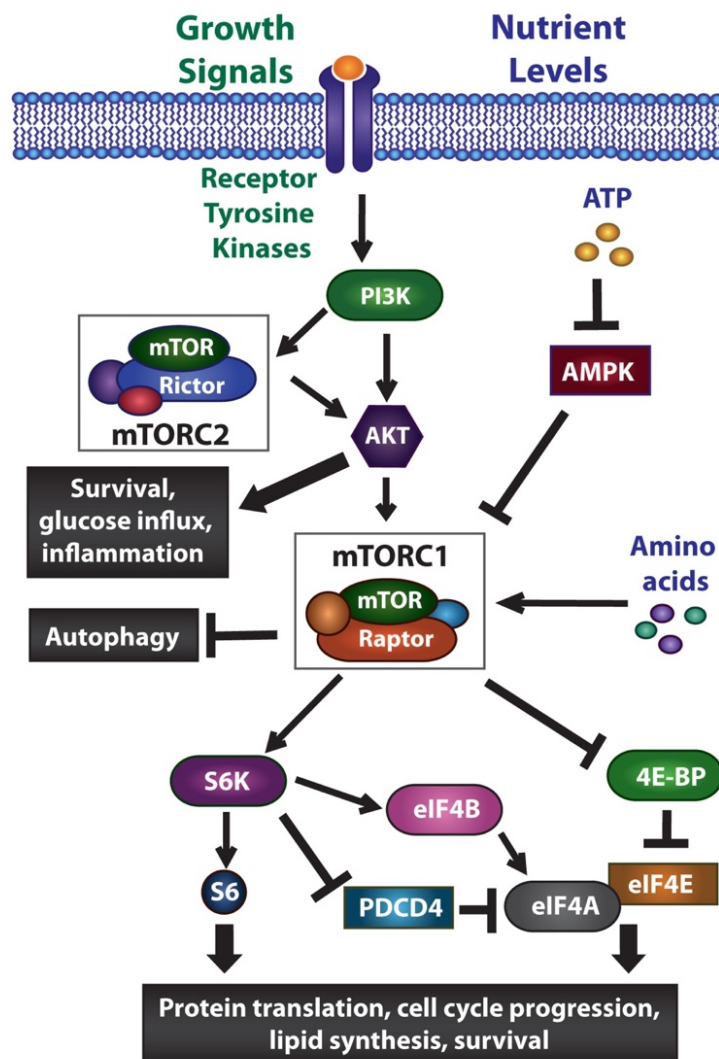
Figure 1.5. Overview of PI3K/AKT/mTOR signaling pathway. Activation of PI3K by growth factor receptor leads to generation of PIP$_3$. This recruits PDK1 and AKT to the plasma membrane where AKT is activated. This in turn activates Rheb-GTP and mTORC1 by suppressing the TSC1/2 complex.

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).

Target	Inhibitor	IC50 (nM)					
		p110α	p110β	p110δ	p110γ	AKT (1,2,3)	mTOR
PI3K	GDC-0941 (176,177)	3	33	3	75		580
	ZSTK474 (178)	16	44	4.6	49		
AKT	AKT Inhibitor VIII (179-181)					58, 210, 2120	
	MK-2206 (182)					8, 12, 65	
mTOR	MLN0128 (183)	219	5293	230	221		1
	AZD8055 (184)	3590		3200			0.8
PI3K/ mTOR	NVP-BEZ235 (185)	4		7	5		6
	GDC-0980 (186)	5	27	7	14		17

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.
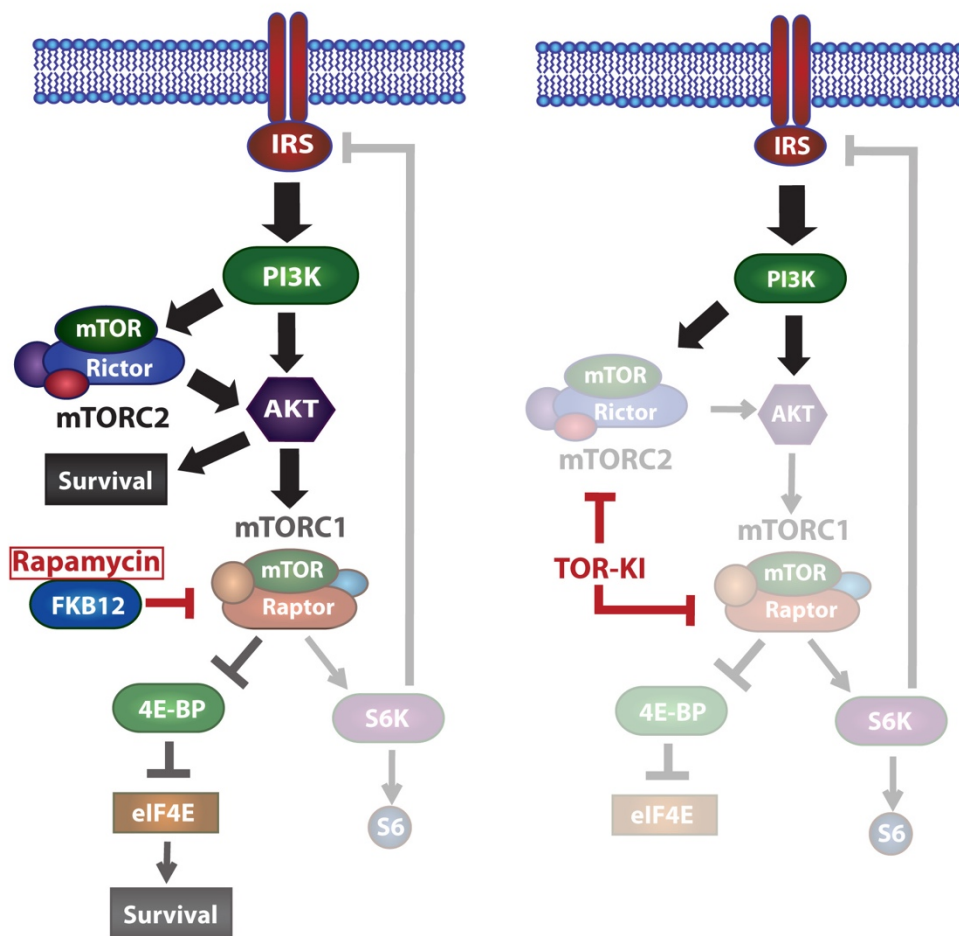
Figure 1.6. Rapamycin versus TOR kinase inhibitors. Rapamycin only partially inhibits mTORC1 activity, promoting activation of upstream PI3K, mTORC2, and AKT via loss of feedback inhibition through IRS1. Conversely, TOR-KIs suppress all mTOR outputs.

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 isozymes. 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

21

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.
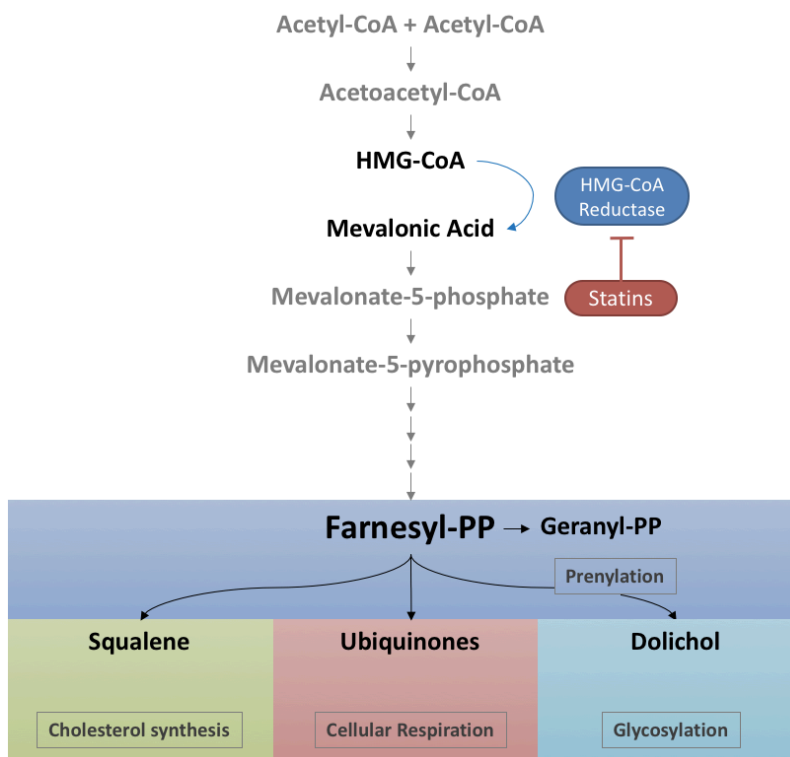
Figure 1.7. Overview of the mevalonate pathway. Mevalonate pathway is a multi-step metabolic pathway that converts acetyl-CoA to multiple bioactive molecules. The rate limiting step is catalyzed by HMG-CoA reductase, which can be inhibited using statins. Following production of farnesyl-PP, the pathway 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.
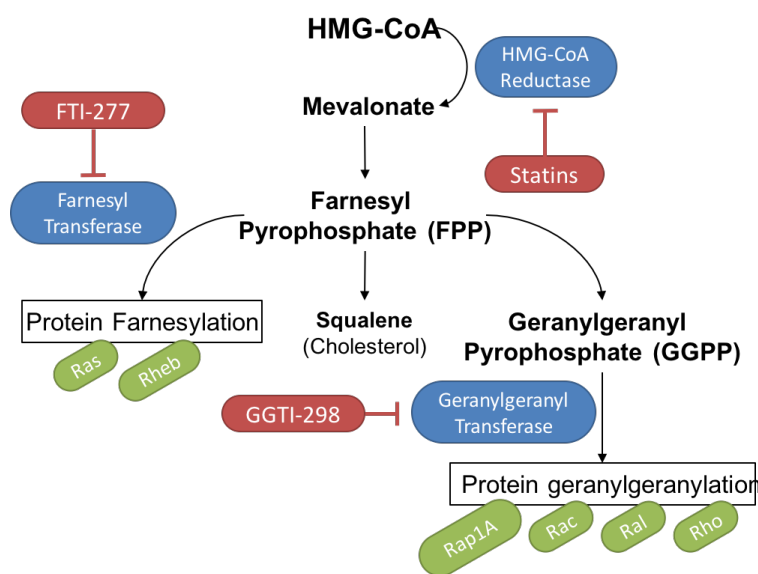
Figure 1.8. Targeting protein prenylation pathways. Two key isoprenoids, FPP and GGPP, are used to prenylate CAAX-containing proteins, many of which are small GTPases of the Ras, Rac, Rheb, and Rho sub-families (green). Distinct enzymes carry out these reactions (blue) that are targetable using small molecule inhibitors (red).

Farnesyltransferase 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-kB 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.
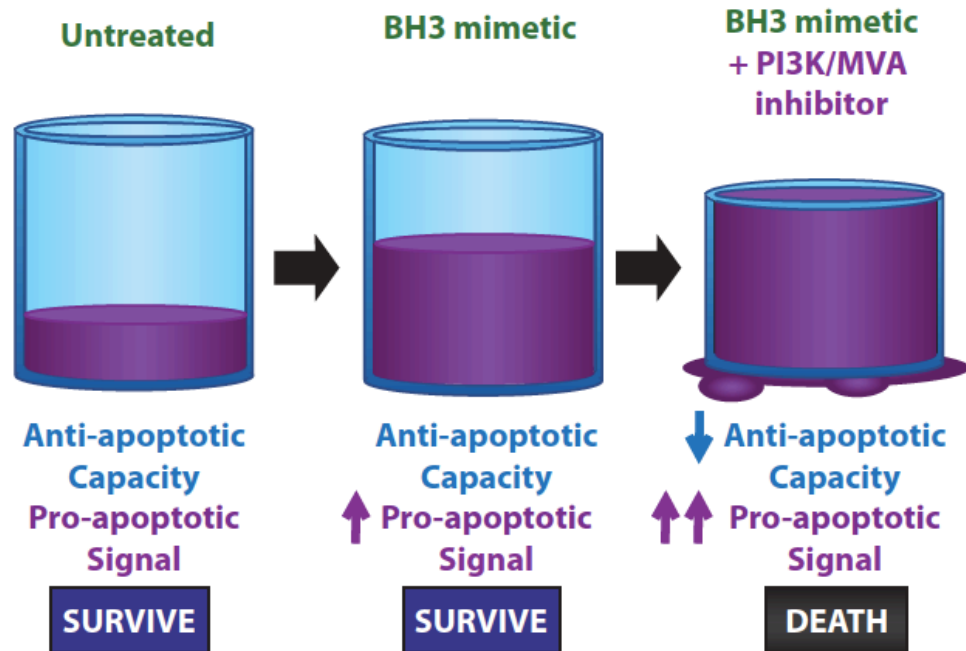
Figure 1.9. Central hypothesis: combination approaches to treating cancer using BH3 mimetics. In some contexts, treatment with BH3 mimetics may be insufficient to kill cells as single agents. The addition of other targeted agents may prime these cells for apoptosis, leading to an effective combination.

References

1.   Farber S, Diamond LK, Mercer RD, Sylvester RF Jr., Wolff JA. Temporary Remissions in Acute Leukemia in Children Produced by Folic Acid Antagonist, 4-Aminopteroyl-Glutamic Acid (Aminopterin). N Engl J Med. Massachusetts Medical Society; 1948;238:787–93.
2.   Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, et al. Activity of a Specific Inhibitor of the BCR-ABL Tyrosine Kinase in the Blast Crisis of Chronic Myeloid Leukemia and Acute Lymphoblastic Leukemia with the Philadelphia Chromosome. N Engl J Med. 2001;344:1038–42.
3.   Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000;100:57–70.
4.   Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011;144:646–74.
5.   Morton LM. Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. Blood. 2006;107:265–76.
6.   Smith A, Howell D, Patmore R, Jack A, Roman E. Incidence of haematological malignancy by sub-type: a report from the Haematological Malignancy Research Network. Br J Cancer. 2011;105:1684–92.
7.   van de Schans SAM, Wymenga ANM, van Spronsen DJ, Schouten HC, Coebergh JWW, Janssen-Heijnen MLG. Two sides of the medallion: poor

treatment tolerance but better survival by standard chemotherapy in elderly patients with advanced-stage diffuse large B-cell lymphoma. Annals of Oncology. 2012;23:1280–6.

8. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503–11.

9. Lenz G, Wright GW, Emre NCT, Kohlhammer H, Dave SS, Davis RE, et al. Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. Proceedings of the National Academy of Sciences. 2008;105:13520–5.

10. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, et al. The Use of Molecular Profiling to Predict Survival after Chemotherapy for Diffuse Large-B-Cell Lymphoma. N Engl J Med. 2002;346:1937–47.

11. Mathews Griner LA, Guha R, Shinn P, Young RM, Keller JM, Liu D, et al. High-throughput combinatorial screening identifies drugs that cooperate with ibrutinib to kill activated B-cell–like diffuse large B-cell lymphoma cells. Proceedings of the National Academy of Sciences. 2014;111:2349–54.

12. Ezell SA, Mayo M, Bihani T, Tepsuporn S, Wang S, Passino M, et al. Synergistic induction of apoptosis by combination of BTK and dual mTORC1/2 inhibitors in diffuse large B cell lymphoma. Oncotarget. 2014;5:4990–5001.

13. Basso K, Dalla-Favera R. Germinal centres and B cell lymphomagenesis. Nat Rev Immunol. 2015;15:172–84.

14. Iqbal J, Meyer PN, Smith LM, Johnson NA, Vose JM, Greiner TC, et al. BCL2 Predicts Survival in Germinal Center B-cell-like Diffuse Large B-cell Lymphoma Treated with CHOP-like Therapy and Rituximab. Clinical Cancer Research. 2011;17:7785–95.

15. Iqbal J, Neppalli VT, Wright G, Dave BJ, Horsman DE, Rosenwald A, et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. J Clin Oncol. 2006;24:961–8.

16. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicologic Path. 2007;35:495–516.

17. Lindsten T, Ross AJ, King A, Zong W-X, Rathmell JC, Shiels HA, et al. The Combined Functions of Proapoptotic Bcl-2 Family Members Bak and Bax Are Essential for Normal Development of Multiple Tissues. Molecular Cell. 2000;6:1389–99.

18. Cory S, Adams JM. The bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. 2002;2:647–56.

19. Hockenbery D, Nuñez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 1990;348:334–6.

20. Boise LH, González-García M, Postema CE, Ding L, Lindsten T, Turka LA, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 1993;74:597–608.

21. Gibson L, Holmgreen SP, Huang DC, Bernard O, Copeland NG, Jenkins NA, et al. bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene. 1996;13:665–75.

22. Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood. 1997;89:630–43.

23. Puthalakath H. Bmf: A Proapoptotic BH3-Only Protein Regulated by Interaction with the Myosin V Actin Motor Complex, Activated by Anoikis. Science. American Association for the Advancement of Science; 2001;293:1829–32.

24. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Molecular Cell. 2001;7:683–94.

25. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell. 1995;80:285–91.

26. Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, et al. Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene. 1995;11:1921–8.

27. Inohara N, Ding L, Chen S, Nuñez G. harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L). The EMBO Journal. 1997;16:1686–94.

28. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science. 2000;288:1053–8.

29. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, et al. Bim: a novel member of the Bcl-2 family that promotes apoptosis. The EMBO Journal. 1998;17:384–95.

30. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ. BID: a novel BH3 domain-only death agonist. Genes & Development. 1996;10:2859–69.

31. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993;74:609–19.

32. Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI, et al. Induction of apoptosis by the Bcl-2 homologue Bak. Nature. 1995;374:733–6.

33. Zong WX. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes & Development. 2001;15:1481–6.

34. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 2001;292:727–30.

35. Green DR. The Pathophysiology of Mitochondrial Cell Death. Science. 2004;305:626–9.

36. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ. 2000;7:1166–73.

37. Wang X. The expanding role of mitochondria in apoptosis. Genes & Development. 2001;15:2922–33.

38. Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem.

1999;274:11549–56.

39. Rodriguez J, Lazebnik Y. Caspase-9 and APAF-1 form an active holoenzyme. Genes & Development. 2000;13:3179–84.

40. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, et al. BH3 Domains of BH3-Only Proteins Differentially Regulate Bax-Mediated Mitochondrial Membrane Permeabilization Both Directly and Indirectly. Molecular Cell. 2005;17:525–35.

41. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell. 2002;2:183–92.

42. Certo M, Moore VDG, Nishino M, Wei G, Korsmeyer S, Armstrong SA, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell. 2006;9:351–65.

43. Petros AM, Olejniczak ET, Fesik SW. Structural biology of the Bcl-2 family of proteins. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2004;1644:83–94.

44. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol. 2013;15:49–63.

45. Cheng EHYA, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, et al. BCL-2, BCL-XL Sequester BH3 Domain-Only Molecules Preventing BAX- and BAK-Mediated Mitochondrial Apoptosis. Molecular Cell. 2001;8:705–11.

46. Lanave C, Santamaria M, Saccone C. Comparative genomics: the evolutionary history of the Bcl-2 family. Gene. 2004;333:71–9.

47. Day CL, Smits C, Fan FC, Lee EF, Fairlie WD, Hinds MG. Structure of the BH3 Domains from the p53-Inducible BH3-Only Proteins Noxa and Puma in Complex with Mcl-1. Journal of Molecular Biology. 2008;380:958–71.

48. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Molecular Cell. 2005.

49. O'Connor L. Bim: a novel member of the Bcl-2 family that promotes apoptosis. The EMBO Journal. 1998;17:384–95.

50. Deng J, Carlson N, Takeyama K, Cin PD, Shipp M, Letai A. BH3 Profiling Identifies Three Distinct Classes of Apoptotic Blocks to Predict Response to ABT-737 and Conventional Chemotherapeutic Agents. Cancer Cell. 2007;12:171–85.

51. Fanidi A, Harrington EA, Evan GI. Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature. 1992;359:554–6.

52. Bissonnette RP, Echeverri F, Mahboubi A, Green DR. Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature. 1992;359:552–4.

53. Richter-Larrea JA, Robles EF, Fresquet V, Beltran E, Rullan AJ, Agirre X, et al. Reversion of epigenetically mediated BIM silencing overcomes chemoresistance in Burkitt lymphoma. Blood. 2010;116:2531–42.

54. Reynolds C, Roderick JE, LaBelle JL, Bird G, Mathieu R, Bodaar K, et al. Repression of BIM mediates survival signaling by MYC and AKT in high-risk T-cell acute lymphoblastic leukemia. Leukemia. 2014;28:1819–27.

55. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, et al. E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell. 2008;13:272–86.

56. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, et al. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science. 1997;275:967–9.

57. Meijerink JP, Mensink EJ, Wang K, Sedlak TW, Slöetjes AW, de Witte T, et al. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. Blood. 1998;91:2991–7.

58. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. 1988;335:440–2.

59. Albinger-Hegyi A, Hochreutener B, Abdou M-T, Hegyi I, Dours-Zimmermann MT, Kurrer MO, et al. High Frequency of t(14;18)-Translocation Breakpoints Outside of Major Breakpoint and Minor Cluster Regions in Follicular Lymphomas. The American Journal of Pathology. 2002;160:823–32.

60. Tsujimoto Y, Finger LR, Yunis J, Nowell PC. Cloning of the chromosome breakpoint of neoplastic B cells with the t (14; 18) chromosome translocation. Science. 1984.

61. Hermine O, Haioun C, Lepage E, d'Agay MF, Briere J, Lavignac C, et al. Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. Groupe d'Etude des Lymphomes de l'Adulte (GELA). Blood. 1996;87:265–72.

62. Gascoyne RD, Adomat SA, Krajewski S, Krajewska M, Horsman DE, Tolcher AW, et al. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. Blood. 1997;90:244–51.

63. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest. 2007;117:112–21.

64. Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. Leukemia. 1996;10:456–9.

65. Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, et al. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 1993;81:3091–6.

66. Strasser A, Harris AW, Vaux DL, Webb E, Bath ML, Adams JM, et al. Abnormalities of the immune system induced by dysregulated bcl-2 expression in transgenic mice. Curr Top Microbiol Immunol. 1990;166:175–81.

67. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell. 1991;67:879–88.

68. Nuñez G, London L, Hockenbery D, Alexander M, McKearn JP, Korsmeyer SJ. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol. 1990;144:3602–10.

69. Nuñez G, Seto M, Seremetis S, Ferrero D, Grignani F, Korsmeyer SJ, et al. Growth- and tumor-promoting effects of deregulated BCL2 in human B-

lymphoblastoid cells. Proceedings of the National Academy of Sciences. National Acad Sciences; 1989;86:4589–93.

70. Tsujimoto Y. Stress-resistance conferred by high level of bcl-2 alpha protein in human B lymphoblastoid cell. Oncogene. 1989;4:1331–6.

71. McDonnell TJ, Deane N, Platt FM, Nuñez G, Jaeger U, McKearn JP, et al. bcl-2-Immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell. 1989;57:79–88.

72. Strasser A, Harris AW, Cory S. E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. Oncogene. 1993;8:1–9.

73. Strasser A, Harris AW, Bath ML, Cory S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature. 1990;348:331–3.

74. McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature. 1991;349:254–6.

75. Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene. 1991;6:1915–22.

76. Aukema SM, Siebert R, Schuuring E, van Imhoff GW, Kluin-Nelemans HC, Boerma EJ, et al. Double-hit B-cell lymphomas. Blood. 2011;117:2319–31.

77. Reed JC, Stein C, Subasinghe C, Haldar S, Croce CM, Yum S, et al. Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. Cancer Research. 1990;50:6565–70.

78. Varadarajan S, Vogler M, Butterworth M, Dinsdale D, Walensky LD, Cohen GM. Evaluation and critical assessment of putative MCL-1 inhibitors. Cell Death Differ. 2013;20:1475–84.

79. Lei X, Chen Y, Du G, Yu W, Wang X, Qu H, et al. Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome c release via a conformational change in Bcl-2. FASEB J. 2006;20:2147–9.

80. Konopleva M, Watt J, Contractor R, Tsao T, Harris D, Estrov Z, et al. Mechanisms of Antileukemic Activity of the Novel Bcl-2 Homology Domain-3 Mimetic GX15-070 (Obatoclax). Cancer Research. 2008;68:3413–20.

81. Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, et al. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science. 1997;275:983–6.

82. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature. 2005;435:677–81.

83. Vogler M, Weber K, Dinsdale D, Schmitz I, Schulze-Osthoff K, Dyer MJS, et al. Different forms of cell death induced by putative BCL2 inhibitors. Cell Death Differ. Nature Publishing Group; 2009;16:1030–9.

84. van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell.

2006;10:389–99.

85. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, et al. ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. Cancer Research. 2008;68:3421–8.

86. Davids MS, Deng J, Wiestner A, Lannutti BJ, Wang L, Wu CJ, et al. Decreased mitochondrial apoptotic priming underlies stroma-mediated treatment resistance in chronic lymphocytic leukemia. Blood. American Society of Hematology; 2012;120:3501–9.

87. Wilson WH, O'Connor OA, Czuczman MS, LaCasce AS, Gerecitano JF, Leonard JP, et al. Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. The Lancet Oncology. 2010;11:1149–59.

88. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL, et al. Substantial Susceptibility of Chronic Lymphocytic Leukemia to BCL2 Inhibition: Results of a Phase I Study of Navitoclax in Patients With Relapsed or Refractory Disease. Journal of Clinical Oncology. 2012;30:488–96.

89. Rudin CM, Hann CL, Garon EB, Ribeiro de Oliveira M, Bonomi PD, Camidge DR, et al. Phase II Study of Single-Agent Navitoclax (ABT-263) and Biomarker Correlates in Patients with Relapsed Small Cell Lung Cancer. Clinical Cancer Research. 2012;18:3163–9.

90. Zhang H, Nimmer PM, Tahir SK, Chen J, Fryer RM, Hahn KR, et al. Bcl-2 family proteins are essential for platelet survival. Cell Death Differ. 2007.

91. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, et al. Programmed Anuclear Cell Death Delimits Platelet Life Span. Cell. 2007;128:1173–86.

92. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013;19:202–8.

93. Vogler M, Dinsdale D, Dyer MJS, Cohen GM. ABT-199 selectively inhibits BCL2 but not BCL2L1 and efficiently induces apoptosis of chronic lymphocytic leukaemic cells but not platelets. British Journal of Haematology. 2013;163:139–42.

94. Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2016;374:311–22.

95. Seymour JF, Gerecitano JF, Kahl BS, Pagel JM. The single-agent Bcl-2 inhibitor ABT-199 (GDC-0199) in patients with relapsed/refractory (R/R) non-Hodgkin lymphoma (NHL): responses observed in all …. 2013.

96. Konopleva M, Pollyea DA, Potluri J, Chyla B, Hogdal L, Busman T, et al. Efficacy and Biological Correlates of Response in a Phase II Study of Venetoclax Monotherapy in Patients with Acute Myelogenous Leukemia. Cancer Discovery. 2016.

97. Hata AN, Engelman JA, Faber AC. The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. Cancer Discovery. 2015;5:475–87.

98. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis. 2009;49:1749–55.

99. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. 2002;9:505–12.

100. Kelekar A, Thompson CB. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. 1998;8:324–30.

101. Ryan JA, Brunelle JK, Letai A. Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. Proceedings of the National …. 2010.

102. Ryan J, Letai A. BH3 profiling in whole cells by fluorimeter or FACS. Methods. 2013;61:156–64.

103. Del Gaizo Moore V, Letai A. BH3 profiling--measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. Cancer Letters. 2013;332:202–5.

104. Vo T-T, Ryan J, Carrasco R, Neuberg D, Rossi DJ, Stone RM, et al. Relative Mitochondrial Priming of Myeloblasts and Normal HSCs Determines Chemotherapeutic Success in AML. Cell. 2012;151:344–55.

105. Colak S, Zimberlin CD, Fessler E, Hogdal L, Prasetyanti PR, Grandela CM, et al. Decreased mitochondrial priming determines chemoresistance of colon cancer stem cells. Cell Death Differ. 2014;21:1170–7.

106. Touzeau C, Ryan J, Guerriero J, Moreau P, Chonghaile TN, Le Gouill S, et al. BH3 profiling identifies heterogeneous dependency on Bcl-2 family members in multiple myeloma and predicts sensitivity to BH3 mimetics. Leukemia. 2015.

107. Montero J, Sarosiek KA, DeAngelo JD, Maertens O, Ryan J, Ercan D, et al. Drug-induced death signaling strategy rapidly predicts cancer response to chemotherapy. Cell. 2015;160:977–89.

108. Beagle BR, Nguyen DM, Mallya S, Tang SS, Lu M, Zeng Z, et al. mTOR kinase inhibitors synergize with histone deacetylase inhibitors to kill B-cell acute lymphoblastic leukemia cells. Oncotarget. 2015;6:2088–100.

109. Lee JS, Tang SS, Ortiz V, Vo T-T, Fruman DA. MCL-1-independent mechanisms of synergy between dual PI3K/mTOR and BCL-2 inhibition in diffuse large B cell lymphoma. Oncotarget. 2015;6:35202–17.

110. Chonghaile TN, Letai A. Mimicking the BH3 domain to kill cancer cells. Oncogene. 2008;27:S149–57.

111. Suryani S, Carol H, Chonghaile TN, Frismantas V, Sarmah C, High L, et al. Cell and Molecular Determinants of In Vivo Efficacy of the BH3 Mimetic ABT-263 against Pediatric Acute Lymphoblastic Leukemia Xenografts. Clinical Cancer Research. 2014;20:4520–31.

112. Del Gaizo Moore V, Schlis KD, Sallan SE, Armstrong SA, Letai A. BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. Blood. 2008;111:2300–9.

113. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. Blood. 2010;115:3304–13.

114. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and

opportunities. Nat Rev Drug Discov. 2014;13:140–56.

115. Yamasaki F, Johansen MJ, Zhang D, Krishnamurthy S, Felix E, Bartholomeusz C, et al. Acquired Resistance to Erlotinib in A-431 Epidermoid Cancer Cells Requires Down-regulation of MMAC1/PTEN and Up-regulation of Phosphorylated Akt. Cancer Research. 2007;67:5779–88.

116. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A Functional Genetic Approach Identifies the PI3K Pathway as a Major Determinant of Trastuzumab Resistance in Breast Cancer. Cancer Cell. 2007;12:395–402.

117. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nature Reviews Cancer. 2009;9:550–62.

118. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET Amplification Leads to Gefitinib Resistance in Lung Cancer by Activating ERBB3 Signaling. Science. 2007;316:1039–43.

119. Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borras AM, Gale CM, et al. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. 2006;116:2695–706.

120. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery. Cell. 1997;91:231–41.

121. Sunters A, Fernandez de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, et al. FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines. 2003;278:49795–805.

122. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011;1813:1978–86.

123. Mills JR, Hippo Y, Robert F, Chen SMH, Malina A, Lin CJ, et al. mTORC1 promotes survival through translational control of Mcl-1. Proceedings of the National Academy of Sciences. 2008;105:10853–8.

124. Cantley LC. The Phosphoinositide 3-Kinase Pathway. Science. 2002;296:1655–7.

125. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 2006;7:606–19.

126. Samuels Y. High Frequency of Mutations of the PIK3CA Gene in Human Cancers. Science. 2004;304:554–4.

127. Courtney KD, Corcoran RB, Engelman JA. The PI3K Pathway As Drug Target in Human Cancer. Journal of Clinical Oncology. 2010;28:1075–83.

128. Kang S, Denley A, Vanhaesebroeck B, Vogt PK. Oncogenic transformation induced by the p110beta, - , and - isoforms of class I phosphoinositide 3-kinase. Proceedings of the National Academy of Sciences. 2006;103:1289–94.

129. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. Nat Rev Cancer. 2002;2:489–501.

130. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, et al. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proceedings of the National Academy of Sciences. 1998;95:13513–8.

131. Bellacosa A, Testa, Staal S, Tsichlis P. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science. 1991;254:274–7.

132. Franke TF, Yang S-I, Chan TO, Datta K, Kazlauskas A, Morrison DK, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell. 1995;81:727–36.

133. Sarbassov DD. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science. 2005;307:1098–101.

134. Guertin DA, Sabatini DM. Defining the Role of mTOR in Cancer. Cancer Cell. 2007;12:9–22.

135. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in Mice of the mTORC Components raptor, rictor, or mLST8 Reveals that mTORC2 Is Required for Signaling to Akt-FOXO and PKCα, but Not S6K1. Developmental Cell. 2006;11:859–71.

136. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. The EMBO Journal. 1996;15:6541–51.

137. Manning BD, Cantley LC. AKT/PKB Signaling: Navigating Downstream. Cell. 2007;129:1261–74.

138. Marte BM, Downward J. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. Trends in Biochemical Sciences. 1997;22:355–8.

139. Avruch J, Long X, Lin Y, Ortiz-Vega S, Rapley J, Papageorgiou A, et al. Activation of mTORC1 in two steps: Rheb-GTP activation of catalytic function and increased binding of substrates to raptor 1. Biochem Soc Trans. 2009;37:223–6.

140. Manning B. Rheb fills a GAP between TSC and TOR. Trends in Biochemical Sciences. 2003;28:573–6.

141. Kim D-H, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al. mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery. Cell. 2002;110:163–75.

142. Sarbassov DD, Ali SM, Kim D-H, Guertin DA, Latek RR, Erdjument-Bromage H, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004;14:1296–302.

143. Liu P, Gan W, Chin YR, Ogura K, Guo J, Zhang J, et al. PtdIns(3,4,5)P3-Dependent Activation of the mTORC2 Kinase Complex. Cancer Discovery. 2015;5:1194–209.

144. Laplante M, Sabatini DM. mTOR Signaling in Growth Control and Disease. Cell. 2012;149:274–93.

145. Hay N. Upstream and downstream of mTOR. Genes & Development. 2004;18:1926–45.

146. Kamada Y, Yoshino K-I, Kondo C, Kawamata T, Oshiro N, Yonezawa K, et al. Tor directly controls the Atg1 kinase complex to regulate autophagy. 2010;30:1049–58.

147. Chang Y-Y, Neufeld TP. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Molecular Biology of the Cell. 2009;20:2004–14.

148. Grabiner BC, Nardi V, Birsoy K, Possemato R, Shen K, Sinha S, et al. A Diverse Array of Cancer-Associated MTOR Mutations Are Hyperactivating and Can Predict Rapamycin Sensitivity. Cancer Discovery. 2014;4:554–63.

149. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. Oncogene. 2006;25:6416–22.

150. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. Nature Reviews Cancer. 2005;5:921–9.

151. Dennis MD, Jefferson LS, Kimball SR. Role of p70S6K1-mediated Phosphorylation of eIF4B and PDCD4 Proteins in the Regulation of Protein Synthesis. 2012;287:42890–9.

152. Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proceedings of the National Academy of Sciences. 2001;98:9666–70.

153. Lin S-J, Chang C, Ng A-K, Wang S-H, Li J-J, Hu C-P. Prevention of TGF-β-induced apoptosis by interlukin-4 through Akt activation and p70S6K survival signaling pathways. Apoptosis. 2007;12:1659–70.

154. Wu X, Reiter CEN, Antonetti DA, Kimball SR, Jefferson LS, Gardner TW. Insulin Promotes Rat Retinal Neuronal Cell Survival in a p70S6K-dependent Manner. 2004;279:9167–75.

155. Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, et al. S6K1-/-/S6K2-/- Mice Exhibit Perinatal Lethality and Rapamycin-Sensitive 5'-Terminal Oligopyrimidine mRNA Translation and Reveal a Mitogen-Activated Protein Kinase-Dependent S6 Kinase Pathway. Molecular and Cellular Biology. 2004;24:3112–24.

156. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes & Development. 1999;13:1422–37.

157. Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature. 2005;433:477–80.

158. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol. 2009;10:307–18.

159. Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell. 2009;136:731–45.

160. Jackson RJ, Hellen CUT, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol. 2010;11:113–27.

161. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, Sabatini DM. A unifying model for mTORC1-mediated regulation of mRNA translation. Nature. 2012;485:109–13.

162. Ruggero D, Montanaro L, Ma L, Xu W, Londei P, Cordon-Cardo C, et al. The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. Nat Med. 2004;10:484–6.

163. Morita M, Gravel S-P, Chénard V, Sikström K, Zheng L, Alain T, et al. mTORC1 Controls Mitochondrial Activity and Biogenesis through 4E-BP-Dependent Translational Regulation. 2013;18:698–711.

164. Matsumoto M, Seike M, Noro R, Soeno C, Sugano T, Takeuchi S, et al. Control of the MYC-eIF4E axis plus mTOR inhibitor treatment in small cell lung cancer.

BMC Cancer. 2015;15:11.

165. Schatz JH, Oricchio E, Wolfe AL, Jiang M, Linkov I, Maragulia J, et al. Targeting cap-dependent translation blocks converging survival signals by AKT and PIM kinases in lymphoma. J Exp Med. 2011;208:1799–807.

166. Choi J, Chen J, Schreiber SL, Clardy J. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science. 1996;273:239–42.

167. Yang H, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, Pavletich NP. mTOR kinase structure, mechanism and regulation. Nature. 2013;497:217–23.

168. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6:1122–8.

169. Sarbassov DD, Ali SM, Sengupta S, Sheen J-H, Hsu PP, Bagley AF, et al. Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB. Molecular Cell. 2006;22:159–68.

170. Kang SA, Pacold ME, Cervantes CL, Lim D, Lou HJ, Ottina K, et al. mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin. Science. 2013;341:1236566–6.

171. Choo AY, Yoon S-O, Kim SG, Roux PP, Blenis J. Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. Proc Natl Acad Sci U S A. 2008;105:17414–9.

172. Dowling RJO, Topisirovic I, Alain T, Bidinosti M, Fonseca BD, Petroulakis E, et al. mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science. American Association for the Advancement of Science; 2010;328:1172–6.

173. Ono A, Oike R, Okuhashi Y, Takahashi Y, Itoh M, Nara N, et al. Comparative effects of PP242 and rapamycin on mTOR signalling and NOTCH signalling in leukemia cells. Anticancer Res. 2013;33:809–13.

174. Mallya S, Fitch BA, Lee JS, So L, Janes MR, Fruman DA. Resistance to mTOR kinase inhibitors in lymphoma cells lacking 4EBP1. Sobol RW, editor. PLoS ONE. 2014;9:e88865.

175. Janes MR, Limon JJ, So L, Chen J, Lim RJ, Chavez MA, et al. Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. Nat Med. 2010;16:205–13.

176. Folkes AJ, Ahmadi K, Alderton WK, Alix S, Baker SJ, Box G, et al. The Identification of 2-(1 H-Indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2- d]pyrimidine (GDC-0941) as a Potent, Selective, Orally Bioavailable Inhibitor of Class I PI3 Kinase for the Treatment of Cancer †. J Med Chem. 2008;51:5522–32.

177. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, et al. Ligand-Independent HER2/HER3/PI3K Complex Is Disrupted by Trastuzumab and Is Effectively Inhibited by the PI3K Inhibitor GDC-0941. Cancer Cell. 2009;15:429–40.

178. Kong D, Yamori T. ZSTK474 is an ATP-competitive inhibitor of class I phosphatidylinositol 3 kinase isoforms. Cancer Science. 2007;98:1638–42.

179. Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, et al.

Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. Biochem J. 2005;385:399–408.

180. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, et al. Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. Bioorg Med Chem Lett. 2005;15:761–4.

181. Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, et al. Discovery of 2,3,5-trisubstituted pyridine derivatives as potent Akt1 and Akt2 dual inhibitors. Bioorg Med Chem Lett. 2005;15:905–9.

182. Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, et al. MK-2206, an Allosteric Akt Inhibitor, Enhances Antitumor Efficacy by Standard Chemotherapeutic Agents or Molecular Targeted Drugs In vitro and In vivo. Molecular Cancer Therapeutics. 2010;9:1956–67.

183. Jessen K, Wang S, Kessler L, Guo X, Kucharski J, Staunton J, et al. Abstract B148: INK128 is a potent and selective TORC1/2 inhibitor with broad oral antitumor activity. Molecular Cancer Therapeutics. 2009;8:B148–8.

184. Chresta CM, Davies BR, Hickson I, Harding T, Cosulich S, Critchlow SE, et al. AZD8055 Is a Potent, Selective, and Orally Bioavailable ATP-Competitive Mammalian Target of Rapamycin Kinase Inhibitor with In vitro and In vivo Antitumor Activity. Cancer Research. 2010;70:288–98.

185. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Molecular Cancer Therapeutics. 2008;7:1851–63.

186. Sutherlin DP, Bao L, Berry M, Castanedo G, Chuckowree I, Dotson J, et al. Discovery of a Potent, Selective, and Orally Available Class I Phosphatidylinositol 3-Kinase (PI3K)/Mammalian Target of Rapamycin (mTOR) Kinase Inhibitor (GDC-0980) for the Treatment of Cancer. J Med Chem. 2011;54:7579–87.

187. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, Ruggero D, et al. Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2. Hunter T, editor. PLoS Biol. 2009;7:e1000038.

188. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, et al. An ATP-competitive Mammalian Target of Rapamycin Inhibitor Reveals Rapamycin-resistant Functions of mTORC1. 2009;284:8023–32.

189. Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR, et al. Genetic Dissection of the Oncogenic mTOR Pathway Reveals Druggable Addiction to Translational Control via 4EBP-eIF4E. Cancer Cell. 2010;17:249–61.

190. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell. 2006;127:125–37.

191. Neri LM, Cani A, Martelli AM, Simioni C, Junghanss C, Tabellini G, et al. Targeting the PI3K/Akt/mTOR signaling pathway in B-precursor acute lymphoblastic leukemia and its therapeutic potential. Leukemia. 2014;28:739–48.

192. Petrich AM, Leshchenko V, Kuo PY, Xia B, Thirukonda VK, Ulahannan N, et al.

Akt Inhibitors MK-2206 and Nelfinavir Overcome mTOR Inhibitor Resistance in Diffuse Large B-cell Lymphoma. Clinical Cancer Research. 2012;18:2534–44.

193. García-Martínez JM, Moran J, Clarke RG, Gray A, Cosulich SC, Chresta CM, et al. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem J. 2009;421:29–42.

194. Pike KG, Malagu K, Hummersone MG, Menear KA, Duggan HME, Gomez S, et al. Optimization of potent and selective dual mTORC1 and mTORC2 inhibitors: the discovery of AZD8055 and AZD2014. Bioorg Med Chem Lett. 2013;23:1212–6.

195. Mortensen DS, Fultz KE, Xu S, Xu W, Packard G, Khambatta G, et al. CC-223, a Potent and Selective Inhibitor of mTOR Kinase: In Vitro and In Vivo Characterization. Molecular Cancer Therapeutics. 2015;14:1295–305.

196. Jackson SP, Schoenwaelder SM, Goncalves I, Nesbitt WS, Yap CL, Wright CE, et al. PI 3-kinase p110beta: a new target for antithrombotic therapy. Nat Med. 2005;11:507–14.

197. Lannutti BJ, Meadows SA, Herman S. CAL-101, a p110δ selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood. 2011.

198. Furman RR, Sharman JP, Coutre SE, Cheson BD, Pagel JM, Hillmen P, et al. Idelalisib and Rituximab in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2014;370:997–1007.

199. Gopal AK, Kahl BS, de Vos S, Wagner-Johnston ND, Schuster SJ, Jurczak WJ, et al. PI3Kδ Inhibition by Idelalisib in Patients with Relapsed Indolent Lymphoma. N Engl J Med. 2014;370:1008–18.

200. Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene. 2005;24:7482–92.

201. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature. 1990;343:425–30.

202. Hirsch HA, Iliopoulos D, Joshi A, Zhang Y, Jaeger SA, Bulyk M, et al. A transcriptional signature and common gene networks link cancer with lipid metabolism and diverse human diseases. Cancer Cell. 2010;17:348–61.

203. Griffiths B, Lewis CA, Bensaad K, Ros S, Zhang Q, Ferber EC, et al. Sterol regulatory element binding protein-dependent regulation of lipid synthesis supports cell survival and tumor growth. Cancer Metab. 2013;1:3.

204. Nohturfft A, DeBose-Boyd RA, Scheek S, Goldstein JL, Brown MS. Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. Proceedings of the National Academy of Sciences. 1999;96:11235–40.

205. Horton JD. Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochem Soc Trans. 2002;30:1091–5.

206. Sakakura Y, Shimano H, Sone H, Takahashi A, Inoue N, Toyoshima H, et al. Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis. Biochemical and Biophysical Research Communications. 2001;286:176–83.

207. Buhaescu I, Izzedine H. Mevalonate pathway: a review of clinical and

therapeutical implications. Clin Biochem. 2007;40:575–84.

208. Botham KM, Mayes PA. Chapter 26. Cholesterol Synthesis, Transport, & Excretion. 2012.

209. Warburg O. On the Origin of Cancer Cells. Science. 1956;123:309.

210. Advani AS, McDonough S, Copelan E, Willman C, Mulford DA, List AF, et al. SWOG0919: A Phase 2 Study of Idarubicin and Cytarabine in Combination with Pravastatin for Relapsed Acute Myeloid Leukaemia. British Journal of Haematology. NIH Public Access; 2014;167:233–7.

211. Wong W, Wong WWL, Dimitroulakos J, Minden MD, Penn LZ. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia. 2002;16:508–19.

212. Clendening JW, Pandyra A, Boutros PC, Ghamrasni SE, Khosravi F, Trentin GA, et al. Dysregulation of the mevalonate pathway promotes transformation. Proceedings of the National Academy of Sciences. 2010;107:15051–6.

213. Li HY, Appelbaum FR, Willman CL, Zager RA, Banker DE. Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses. 2002;101:3628–34.

214. Banker DE, Mayer SJ, Li HY, Willman CL, Appelbaum FR, Zager RA. Cholesterol synthesis and import contribute to protective cholesterol increments in acute myeloid leukemia cells. 2004;104:1816–24.

215. Lucken-Ardjomande S, Montessuit S, Martinou JC. Bax activation and stress-induced apoptosis delayed by the accumulation of cholesterol in mitochondrial membranes. Cell Death and Differentiation. 2007;15:484–93.

216. García MJ, Reinoso RF, Sánchez Navarro A, Prous JR. Clinical pharmacokinetics of statins. Methods Find Exp Clin Pharmacol. 2003;25:455.

217. Istvan ES. Structural Mechanism for Statin Inhibition of HMG-CoA Reductase. Science. 2001;292:1160–4.

218. Farmer JA, Torre-Amione G. Comparative Tolerability of the HMG-CoA Reductase Inhibitors. 2000;23:197–213.

219. Davidson MH. Safety Profiles for the HMG-CoA Reductase Inhibitors. Drugs. 2001;61:197–206.

220. Ucar M, Mjörndal T, Dahlqvist R. HMG-CoA reductase inhibitors and myotoxicity. Drug Saf. 2000;22:441–57.

221. Keyomarsi K, Sandoval L, Band V, Pardee AB. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Research. 1991;51:3602–9.

222. Newman A, Clutterbuck RD, Powles RL, Millar JL. Selective inhibition of primary acute myeloid leukaemia cell growth by simvastatin. Leukemia. 1994;8:2023–9.

223. Dimitroulakos J, Nohynek D, Backway KL, Hedley DW, Yeger H, Freedman MH, et al. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. 1999;93:1308–18.

224. Newman A, Clutterbuck RD, Powles RL, Catovsky D, Millar JL. A Comparison of the Effect of the 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitors Simvastatin, Lovastatin and Pravastatin on Leukaemic and Normal Bone Marrow Progenitors. Leuk Lymphoma. 2015;24:533–7.

225. Minden MD, Dimitroulakos J, Nohynek D, Penn LZ. Lovastatin induced control of

blast cell growth in an elderly patient with acute myeloblastic leukemia. Leuk Lymphoma. 2001;40:659–62.

226. Nowakowski GS, Maurer MJ, Habermann TM, Ansell SM, Macon WR, Ristow KM, et al. Statin use and prognosis in patients with diffuse large B-cell lymphoma and follicular lymphoma in the rituximab era. J Clin Oncol. 2010;28:412–7.

227. Samaras P, Heider H, Haile SR, Petrausch U, Schaefer NG, Siciliano RD, et al. Concomitant statin use does not impair the clinical outcome of patients with diffuse large B cell lymphoma treated with rituximab-CHOP. Ann Hematol. 2010;89:783–7.

228. Ennishi D, Asai H, Maeda Y, Shinagawa K, Ikeda K, Yokoyama M, et al. Statin-independent prognosis of patients with diffuse large B-cell lymphoma receiving rituximab plus CHOP therapy. Ann Oncol. 2010;21:1217–21.

229. Luskey KL, Faust JR, Chin DJ, Brown MS, Goldstein JL. Amplification of the gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not for the 53-kDa protein, in UT-1 cells. J Biol Chem. 1983;258:8462–9.

230. Ravid T, Avner R, Polak-Charcon S, Faust JR, Roitelman J. Impaired Regulation of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Degradation in Lovastatin-resistant Cells. Journal of Biological Chemistry. 1999;274:29341–51.

231. Xia Z, Tan MM, Wei-Lynn Wong W, Dimitroulakos J, Minden MD, Penn LZ. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia. 2001;15:1398–407.

232. Wood WG, Igbavboa U, Muller WE, Eckert GP. Statins, Bcl-2, and apoptosis: cell death or cell protection? Mol Neurobiol. 2013;48:308–14.

233. Wang M, Casey PJ. Protein prenylation: unique fats make their mark on biology. Nat Rev Mol Cell Biol. 2016;17:110–22.

234. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Annual Review of Biochemistry. 1996;65:241–69.

235. Lane KT. Thematic review series: Lipid Posttranslational Modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. The Journal of Lipid Research. 2006;47:681.

236. Perez-Sala D. Protein isoprenylation in biology and disease: general overview and perspectives from studies with genetically engineered animals. Frontiers in Bioscience. 2007;12:4456.

237. Solski PA, Helms W, Keely PJ, Su L, Der CJ. RhoA biological activity is dependent on prenylation but independent of specific isoprenoid modification. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research. NIH Public Access; 2002;13:363–73.

238. Roskoski R, Ritchie P. Role of the carboxyterminal residue in peptide binding to protein farnesyltransferase and protein geranylgeranyltransferase. Archives of Biochemistry and Biophysics. 1998;356:167–76.

239. Reid TS, Terry KL, Casey PJ, Beese LS. Crystallographic analysis of CaaX prenyltransferases complexed with substrates defines rules of protein substrate selectivity. Journal of Molecular Biology. 2004;343:417–33.

240. Moores SL, Schaber MD, Mosser SD, Rands E, O'Hara MB, Garsky VM, et al.

Sequence dependence of protein isoprenylation. J Biol Chem. 1991;266:14603–10.

241. Ashby MN. CaaX converting enzymes. Curr Opin Lipidol. 1998;9:99–102.
242. Winter-Vann AM, Casey PJ. Post-prenylation-processing enzymes as new targets in oncogenesis. 2005;5:405–12.
243. Berndt N, Sebti SM. Measurement of protein farnesylation and geranylgeranylation in vitro, in cultured cells and in biopsies, and the effects of prenyl transferase inhibitors. Nat Protoc. 2011;6:1775–91.
244. Mijimolle N, Velasco J, Dubus P, Guerra C, Weinbaum CA, Casey PJ, et al. Protein farnesyltransferase in embryogenesis, adult homeostasis, and tumor development. Cancer Cell. 2005;7:313–24.
245. Anraku Y, Friesen JD, Ohya Y, Goebl M, Goodman LE, Petersen-Bjørn S, et al. Yeast CAL1 is a structural and functional homologue to the DPR1 (RAM) gene involved in ras processing. J Biol Chem. 1991;266:12356–60.
246. Therrien M, Chang HC, Solomon NM, Karim FD, Wassarman DA, Rubin GM. KSR, a novel protein kinase required for RAS signal transduction. Cell. 1995;83:879–88.
247. Jackson JH, Cochrane CG, Bourne JR, Solski PA, Buss JE, Der CJ. Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. Proceedings of the National Academy of Sciences. 1990;87:3042.
248. Stokoe D, Macdonald S, Cadwallader K, Symons M, Hancock J. Activation of Raf as a result of recruitment to the plasma membrane. Science. 1994;264:1463.
249. Ding H, McDonald JS, Yun S, Schneider PA, Peterson KL, Flatten KS, et al. Farnesyltransferase inhibitor tipifarnib inhibits Rheb prenylation and stabilizes Bax in acute myelogenous leukemia cells. Haematologica. 2013;99:60.
250. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, et al. Phosphatidylinositol-3-OH kinase direct target of Ras. Nature. 1994;370:527.
251. Berndt N, Hamilton AD, Sebti SM. Targeting protein prenylation for cancer therapy. Nature Reviews Cancer. 2011;11:775–91.
252. Prendergast GC. Farnesyltransferase inhibitors: antineoplastic mechanism and clinical prospects. Current Opinion in Cell Biology. 2000;12:166–73.
253. Sebti SM. Protein farnesylation: Implications for normal physiology, malignant transformation, and cancer therapy. Cancer Cell. 2005;7:297–300.
254. Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, et al. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. 1995;1:792–7.
255. Barrington RE, Subler MA, Rands E, Omer CA, Miller PJ, Hundley JE, et al. A Farnesyltransferase Inhibitor Induces Tumor Regression in Transgenic Mice Harboring Multiple Oncogenic Mutations by Mediating Alterations in Both Cell Cycle Control and Apoptosis. Molecular and Cellular Biology. 1998;18:85–92.
256. Kazi A, Carie A, Blaskovich MA, Bucher C, Thai V, Moulder S, et al. Blockade of Protein Geranylgeranylation Inhibits Cdk2-Dependent p27Kip1 Phosphorylation on Thr187 and Accumulates p27Kip1 in the Nucleus: Implications for Breast Cancer Therapy. Molecular and Cellular Biology. 2009;29:2254–63.

257. Sjogren A-KM, Andersson KME, Liu M, Cutts BA, Karlsson C, Wahlstrom AM, et al. GGTase-I deficiency reduces tumor formation and improves survival in mice with K-RAS–induced lung cancer. J Clin Invest. 2007;117:1294–304.

258. Dan HC, Jiang K, Coppola D, Hamilton A, Nicosia SV, Sebti SM, et al. Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis. Oncogene. 2004;23:706–15.

259. Vogt A, Sun J, Qian Y, Hamilton AD, Sebti SM. The Geranylgeranyltransferase-I Inhibitor GGTI-298 Arrests Human Tumor Cells in G0/G1 and Induces p21WAF1/CIP1/SDI1 in a p53-independent Manner. Journal of Biological Chemistry. 1997;272:27224–9.

260. Liu M, Bryant MS, Chen J, Lee S, Yaremko B, Lipari P, et al. Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. Cancer Research. 1998;58:4947–56.

261. Jiang K, Coppola D, Crespo NC, Nicosia SV, Hamilton AD, Sebti SM, et al. The Phosphoinositide 3-OH Kinase/AKT2 Pathway as a Critical Target for Farnesyltransferase Inhibitor-Induced Apoptosis. Molecular and Cellular Biology. 2000;20:139–48.

262. Chen S, Fu L, Raja SM, Yue P, Khuri FR, Sun S-Y. Dissecting the roles of DR4, DR5 and c-FLIP in the regulation of Geranylgeranyltransferase I inhibition-mediated augmentation of TRAIL-induced apoptosis. Mol Cancer. 2010;9:23.

263. Zhang B, Prendergast GC, Fenton RG. Farnesyltransferase inhibitors reverse Ras-mediated inhibition of Fas gene expression. Cancer Research. 2002.

264. Takada Y, Khuri FR, Aggarwal BB. Protein Farnesyltransferase Inhibitor (SCH 66336) Abolishes NF- B Activation Induced by Various Carcinogens and Inflammatory Stimuli Leading to Suppression of NF- B-regulated Gene Expression and Up-regulation of Apoptosis. Journal of Biological Chemistry. 2004;279:26287–99.

265. Van Cutsem E, van de Velde H, Karasek P, Oettle H, Vervenne WL, Szawlowski A, et al. Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. J Clin Oncol. 2004;22:1430–8.

266. Rao S, Cunningham D, de Gramont A, Scheithauer W, Smakal M, Humblet Y, et al. Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. J Clin Oncol. 2004;22:3950–7.

267. Harousseau JL, Martinelli G, Jedrzejczak WW, Brandwein JM, Bordessoule D, Masszi T, et al. A randomized phase 3 study of tipifarnib compared with best supportive care, including hydroxyurea, in the treatment of newly diagnosed acute myeloid leukemia in patients 70 years or older. 2009;114:1166.

268. Balint B, Scagliotti G, De Marinis F, Cihon F, Hanna N, Grossi F, et al. Phase III Study of Carboplatin and Paclitaxel Alone or With Sorafenib in Advanced Non-Small-Cell Lung Cancer. Journal of Clinical Oncology. 2010;28:1835.

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% CO_2.

Cells were routinely tested to ensure absence of mycoplasma, and were maintained at

or below 2×10^6 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-X_L, 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 10^4 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 IC_{50} 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

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'-GGGAGCCGAACGCGAGCGTCCT-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 MgCl$_2$, 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 10^6 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 10^6 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

Inhibitor	Inhibitor Class	Selective Concentration (nM)
GDC-0941	Pan-PI3K	100
ZSTK-474	Pan-PI3K	100
AKT Inhibitor VIII	Allosteric AKT	1000
MK2206	Allosteric AKT	300
MLN0128	mTOR kinase	50
AZD8055	mTOR kinase	50
Rapamycin	Allosteric mTOR	10
BEZ235	Dual PI3K/mTOR	50
GDC-0980	Dual PI3K/mTOR	300

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.
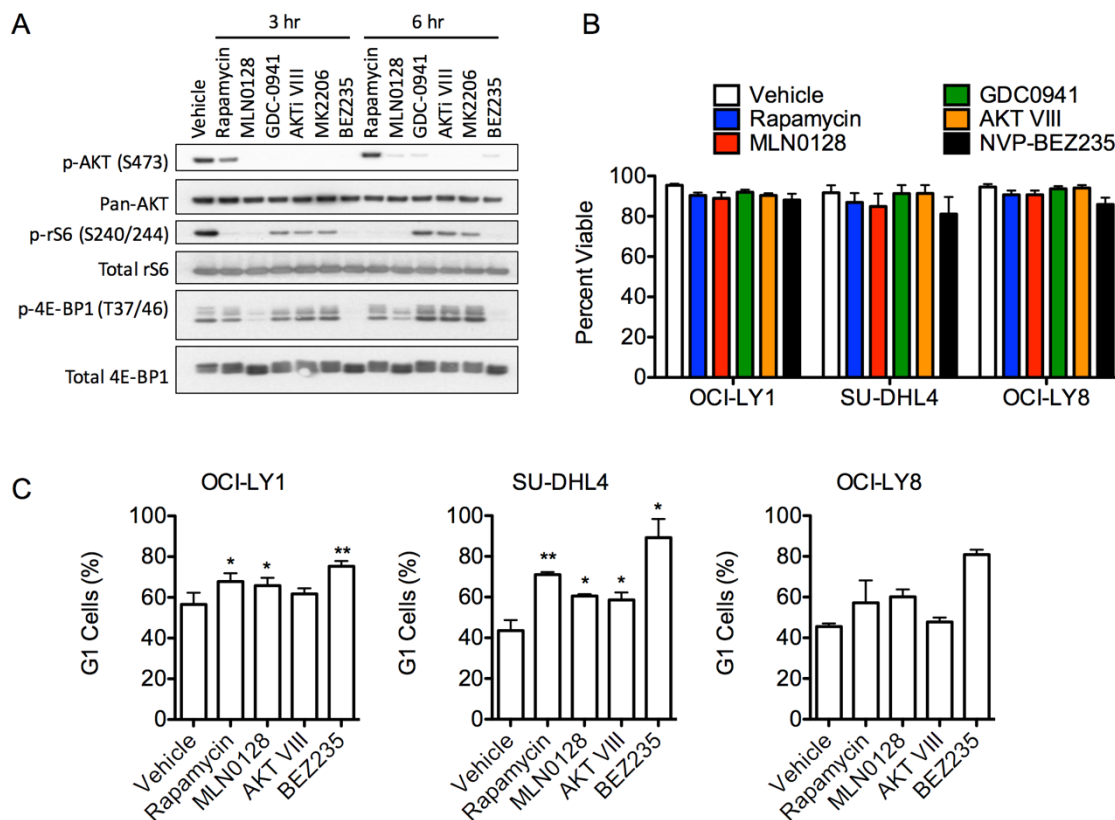
Figure 2.1. Suppression of the PI3K/AKT/mTOR pathway components is cytostatic in DLBCL. (**A**) western blot of OCI-LY1 cells treated with indicated inhibitors (concentrations indicated in Table 2.1) for 3 or 6 hours. (**B**) Viability of DLBCL cell lines (OCI-LY1, SU-DHL4, and OCI-LY8) treated with 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-X_L). 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-X_L antagonist, ABT-737 (23). Indeed, combined PI3K and BCL-2/BCL-X_L 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.
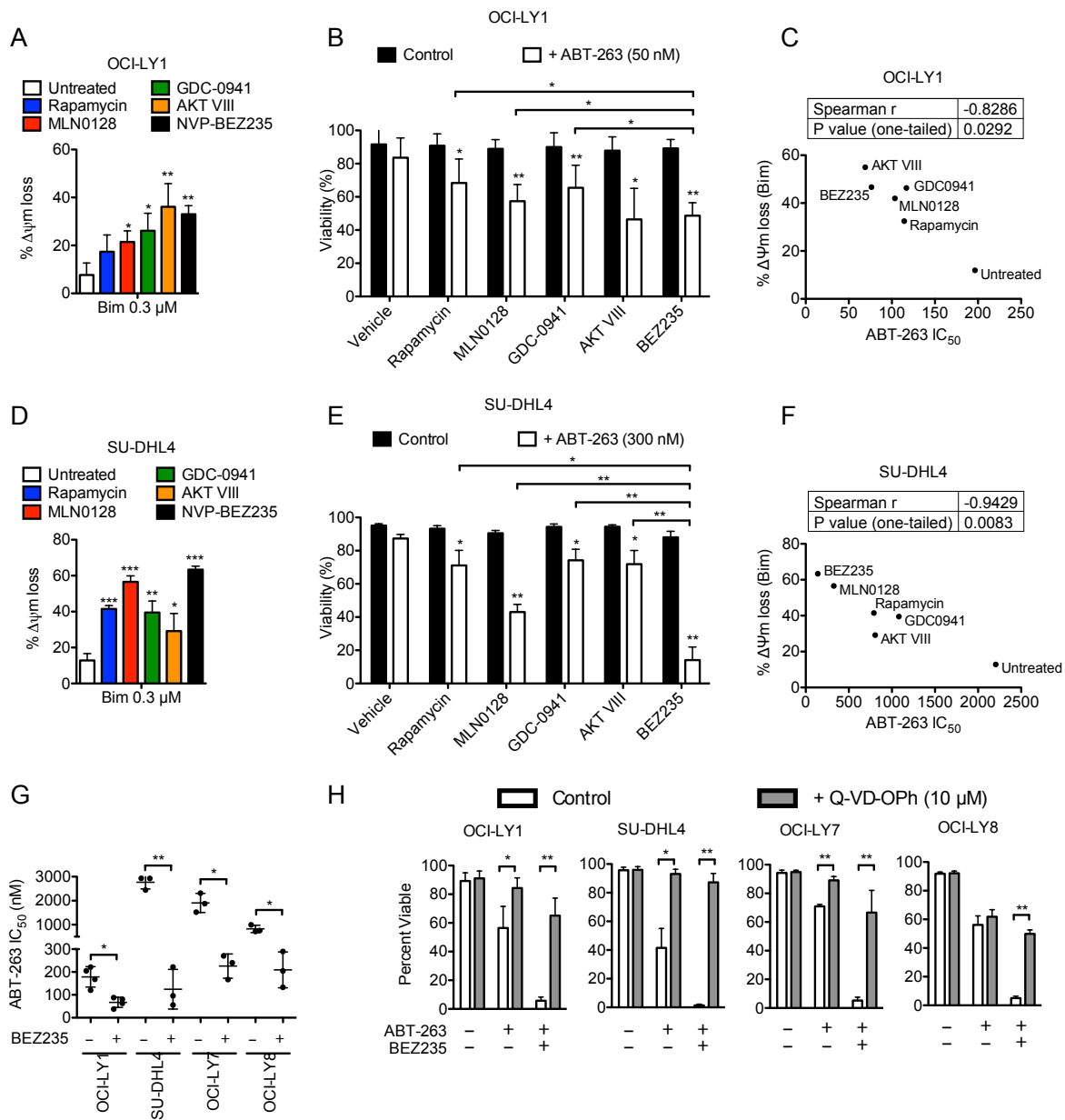
Figure 2.2. PI3K pathway inhibition increases mitochondrial priming and enhances efficacy of ABT-263 in DLBCL cell lines. (**A, D**) BH3 profile of OCI-LY1 and SU-DHL4 cells treated with inhibitors for 16 hours. (**B, E**) Viability of OCI-LY1 and SU-DHL4 cells treated with ABT-263 with or without PI3K 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).
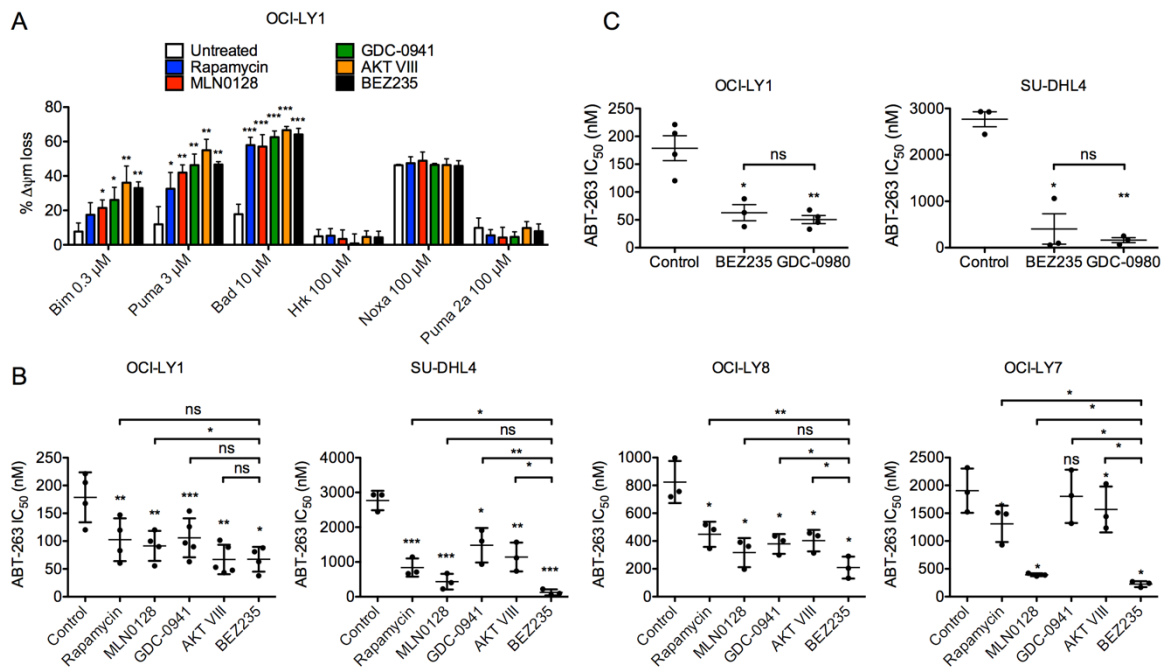
Among the classes of PI3K pathway inhibitors used, the dual PI3K/mTOR inhibitors, BEZ235 and GDC-0980, were consistently the most potent sensitizers to ABT-263 across several DLBCL cell lines tested (**Figures 2.2G** and **2.3B, C**). Thus, we focused further experiments on the effects of dual PI3K/mTOR 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-
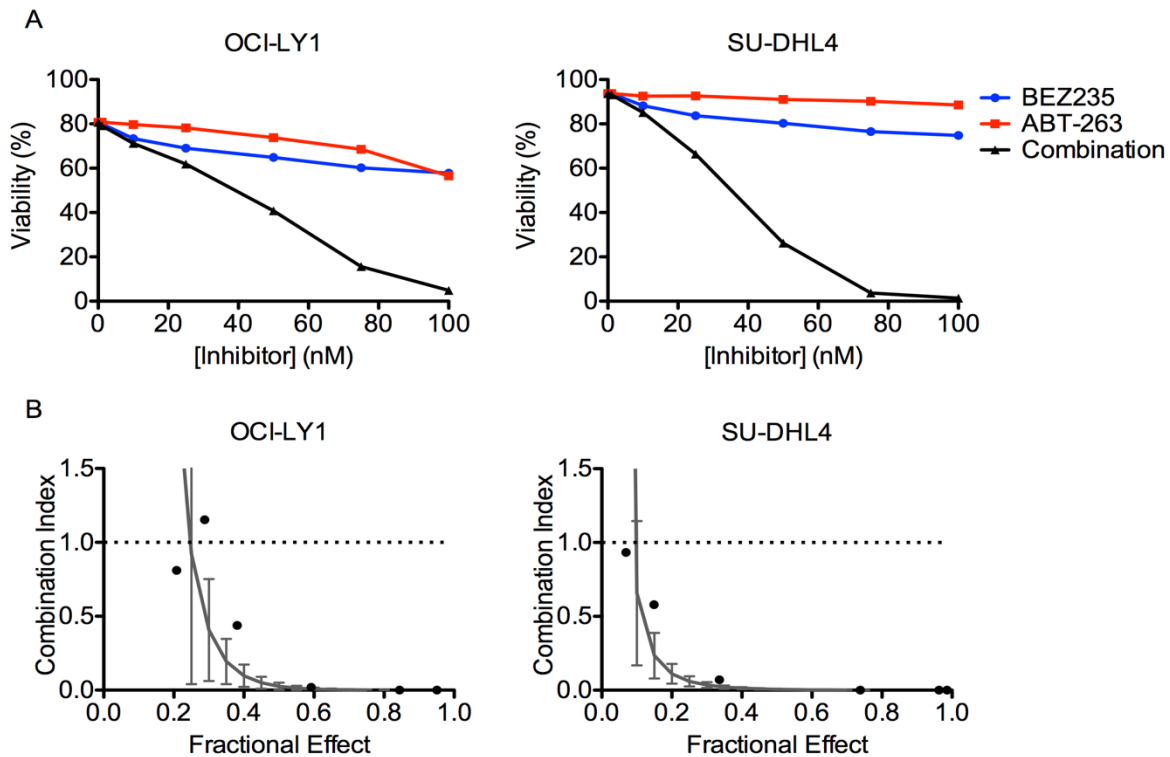
feedback loop (26).

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**).
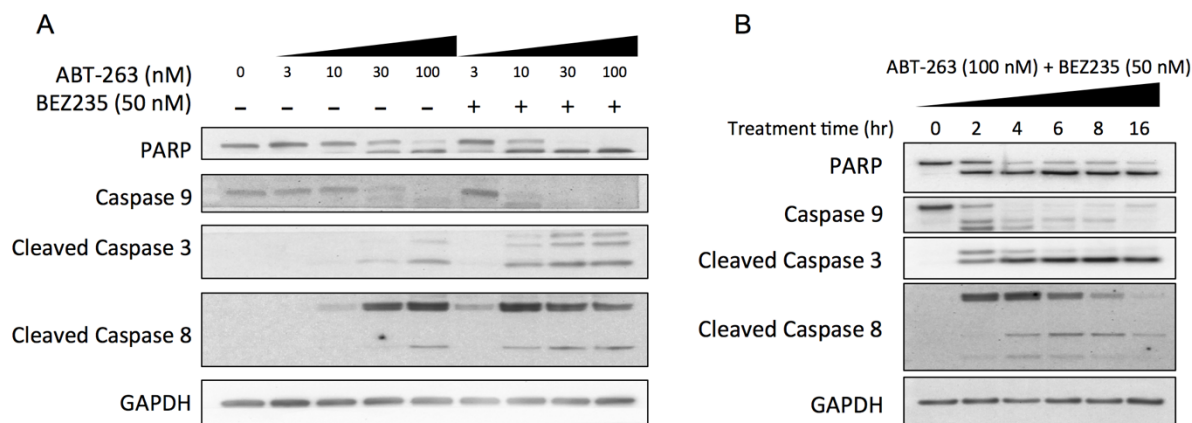
Figure 2.5. Combining BEZ235 and ABT-263 induces caspase and PARP cleavage. (**A**) Western blot of OCI-LY1 cells treated with increasing doses of ABT-263 with or without BEZ235 for 6 hours. (**B**) Western blot of OCI-LY1 cells treated with ABT-263 (100 nM) in combination with BEZ235 (50 nM) for indicated 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 mimetic, 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**).
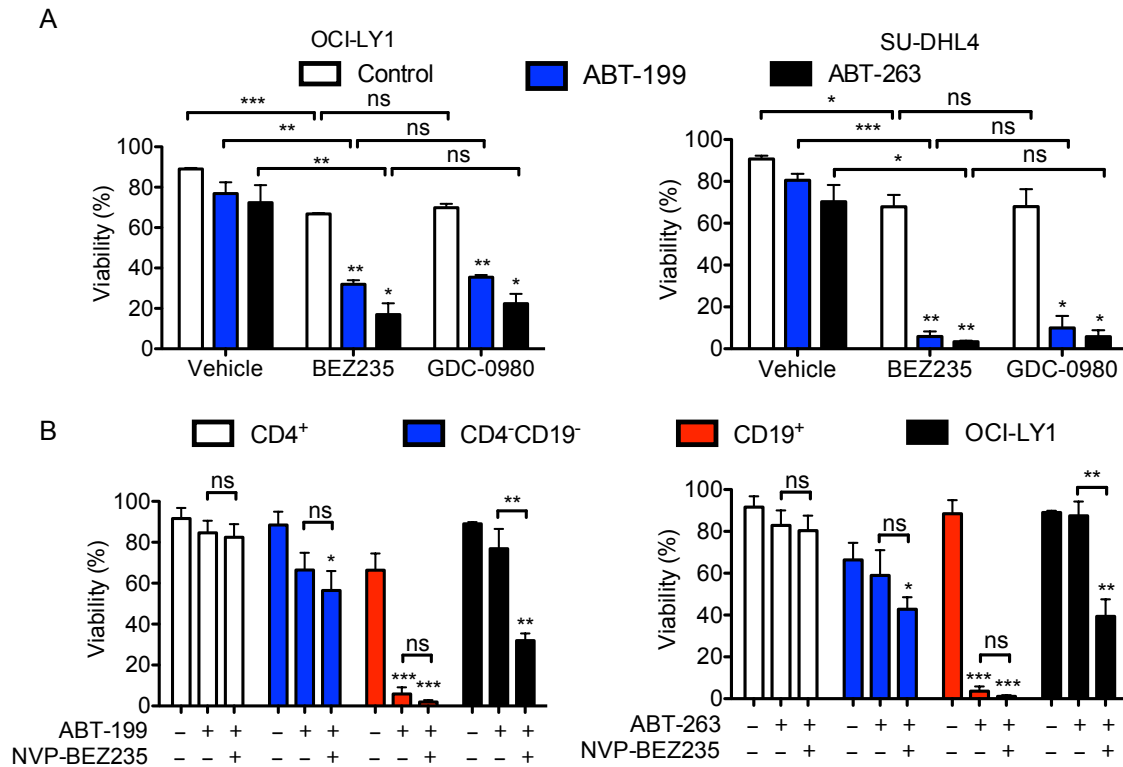
Figure 2.6. BEZ235 does not enhance the toxicity of BH3 mimetics in normal human T cells. (**A**) Viability of OCI-LY1 and SU-DHL4 cells treated with ABT-263 (50 or 300 nM, respectively) or ABT-199 (5 or 50 nM, respectively) with or without BEZ235 or GDC-0980. (**B**) Viability of PBMCs isolated from 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**).
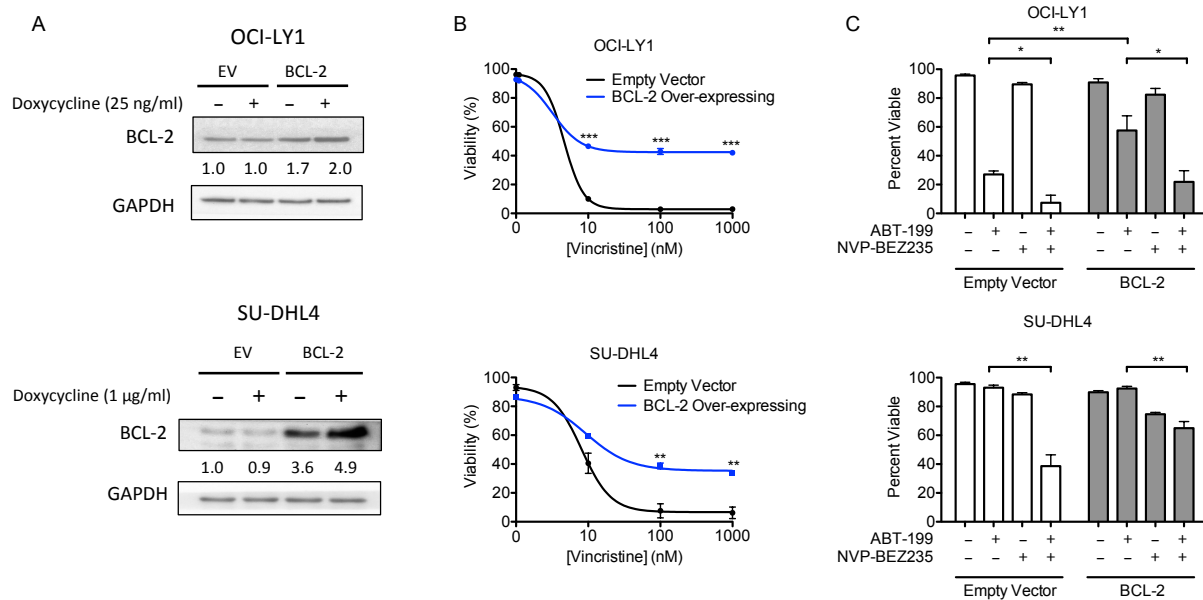
Figure 2.7. DLBCL cells over-expressing BCL-2 are resistant to a chemotherapeutic drug but remain sensitive to BEZ235 and ABT-199. (**A**) Western blot of cells treated with indicated doxycycline doses for 24 hours. (**B**) Viability of cells pre-treated with doxycycline (25 ng/ml or 1 μg/ml, respectively) 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).
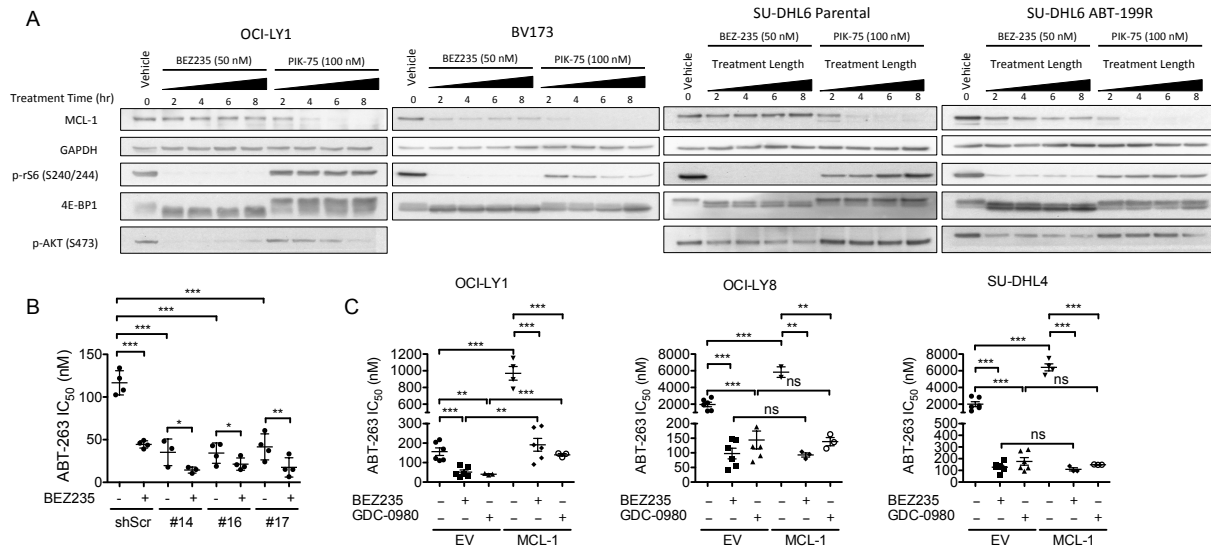
Figure 2.8. BEZ235 does not affect MCL-1 expression in OCI-LY1 cells. (**A**) Western blot of cells treated with BEZ235 or PIK-75 for increasing duration. (**B**) Sensitivity of cells stably transduced with shMCL-1 or shScramble to ABT-263 with or without BEZ235. (**C**) Sensitivity of cells stably transduced 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}.
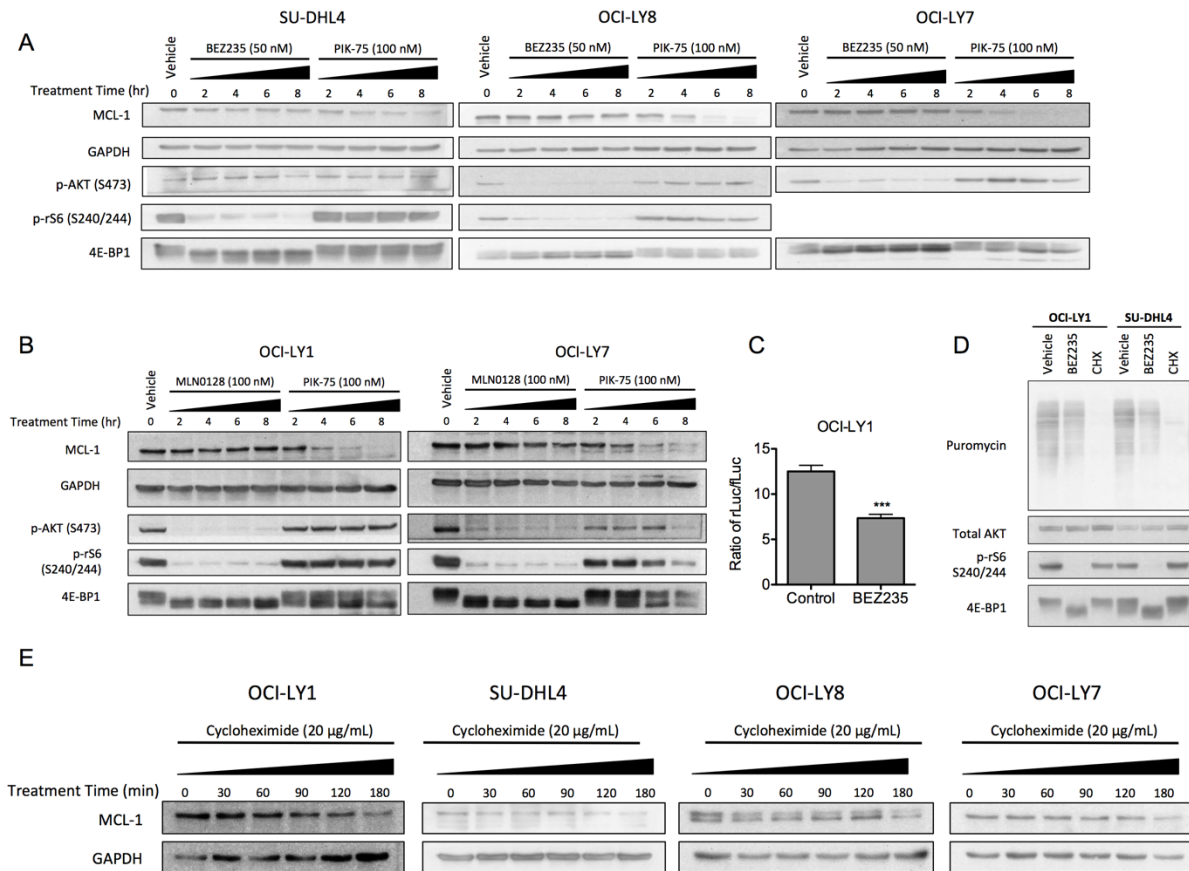
Figure 2.9. BEZ235 does not affect MCL-1 expression in DLBCL cell lines. (**A,B**) Western blot of cells treated with PIK-75 and either BEZ235 (**A**) or MLN0128 (**B**), concentrations indicated. (**C**) Dual luciferase cap-dependent reporter assay of cells treated with BEZ235 for 16 hours. (**D**) 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 pre-treated with doxycycline (1 μg/ml) for 24 hours before treatment with BEZ235 for 24 or 48 hours.
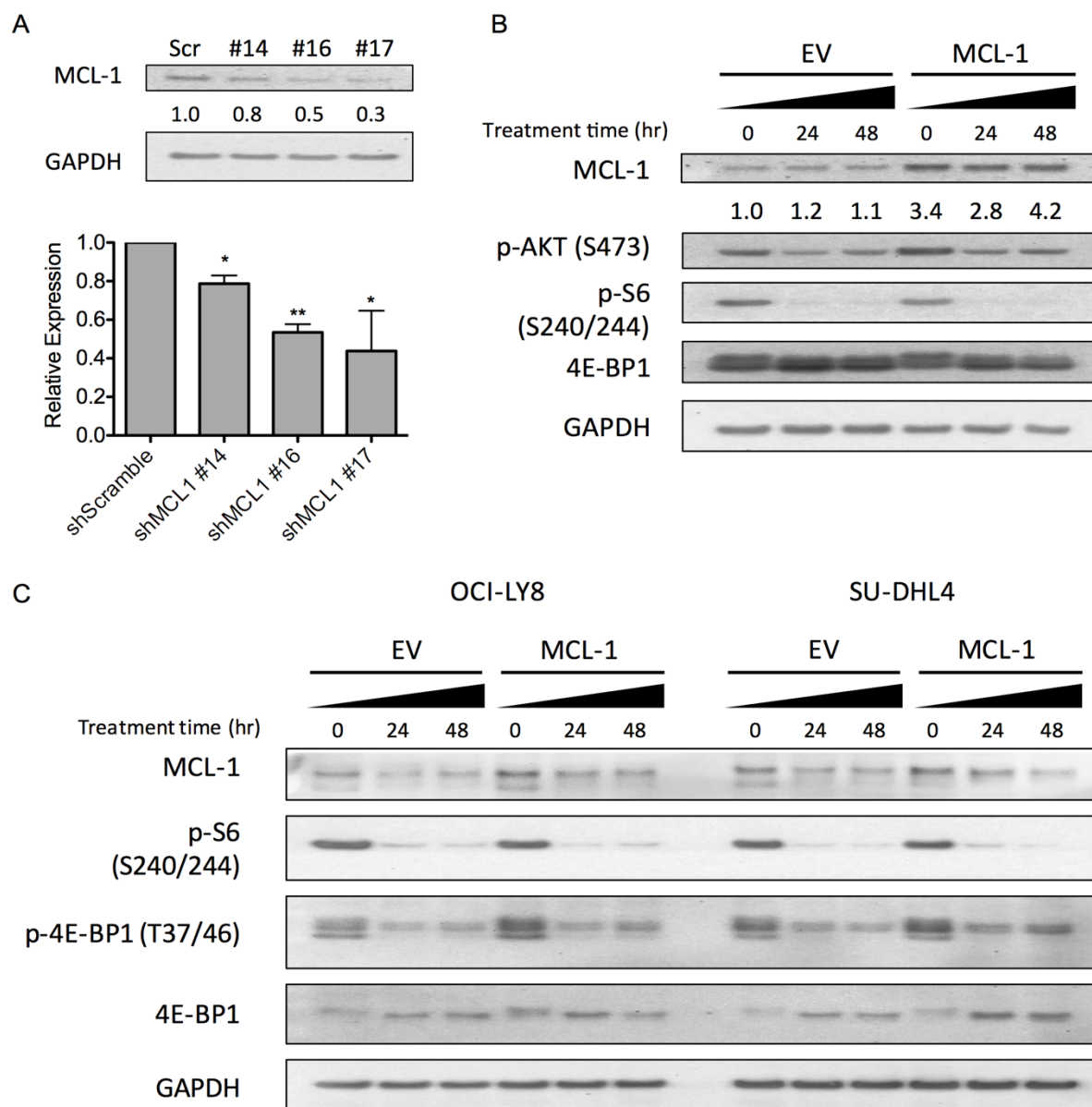
PI3K pathway inhibition increases mitochondrial localization of BAD and BIM

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.
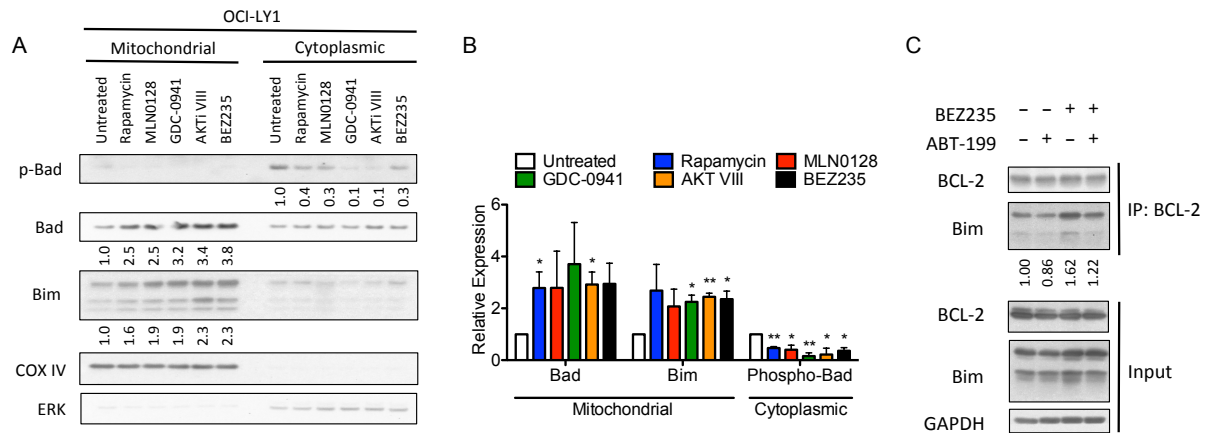
Figure 2.11. **PI3K pathway inhibitors increase mitochondrial abundance of BAD and BIM.** (**A**) Western blot of mitochondrial and cytoplasmic fractions of OCI-LY1 cells treated with indicated PI3K pathway inhibitors for 16 hours. (**B**) Average densitometry values of three replicates of panel (**A**). (**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.
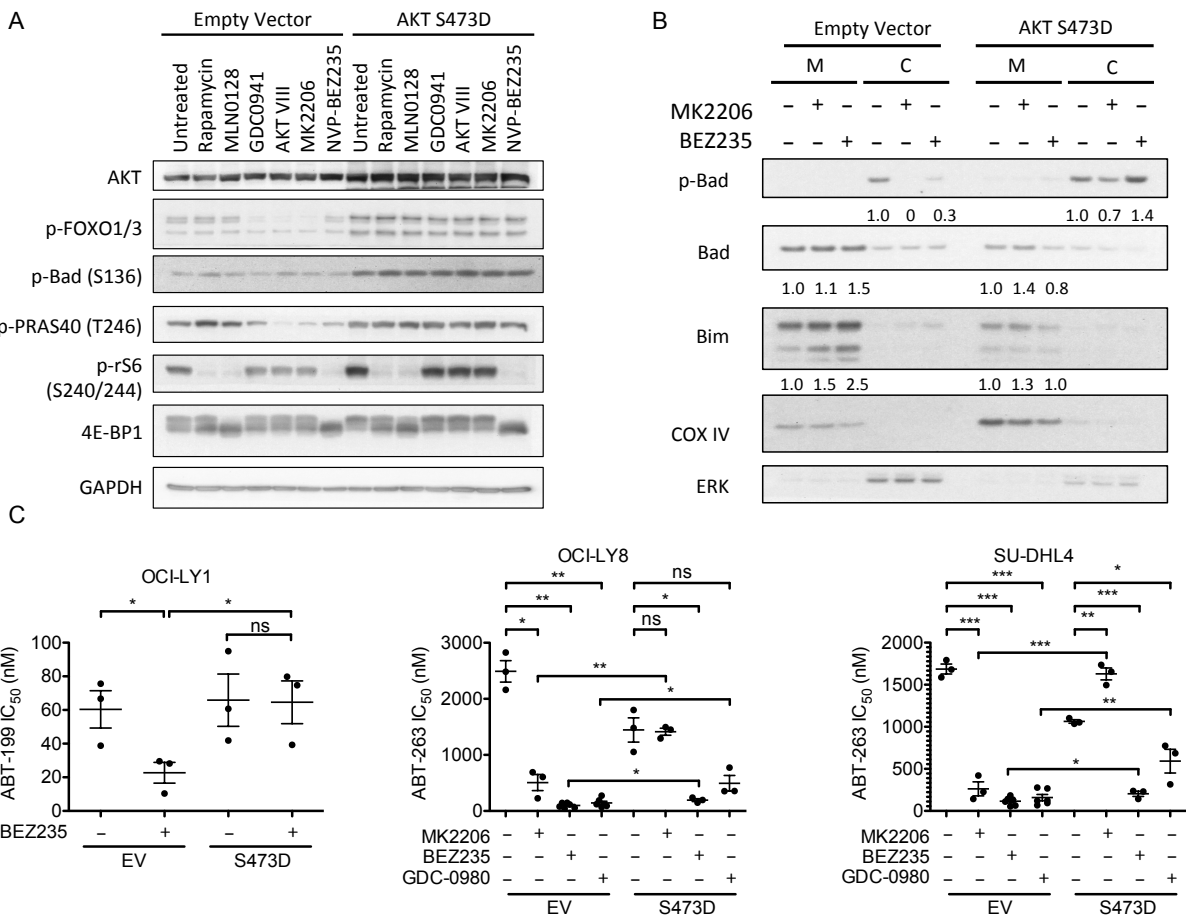
Figure 2.12. AKT suppression is a critical component of synergy between BEZ235 and ABT-199. (**A**) Western blot of OCI-LY1 cells expressing either empty vector or phospho-mimetic AKT (S473D) treated with indicated inhibitors for 3 hours. Cells were pre-treated with doxycycline (1 µg/ml) for 24 hours 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.
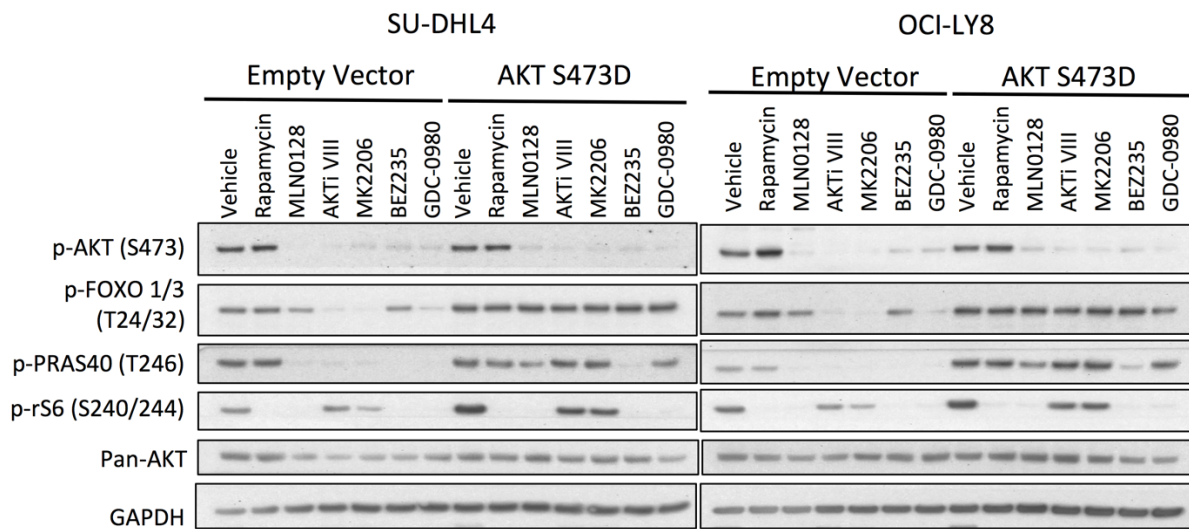
Figure 2.13. Confirmation of expression of AKT S473D in SU-DHL4 and OCI-LY8 cells. Western blot of SU-DHL4 and OCI-LY8 cells expressing either empty vector or phospho-mimetic AKT (S473D). Cells were pre-treated with doxycycline (1 μg/ml) for 24 hours prior to treatment with indicated PI3K pathway inhibitors for an additional 3 hours.
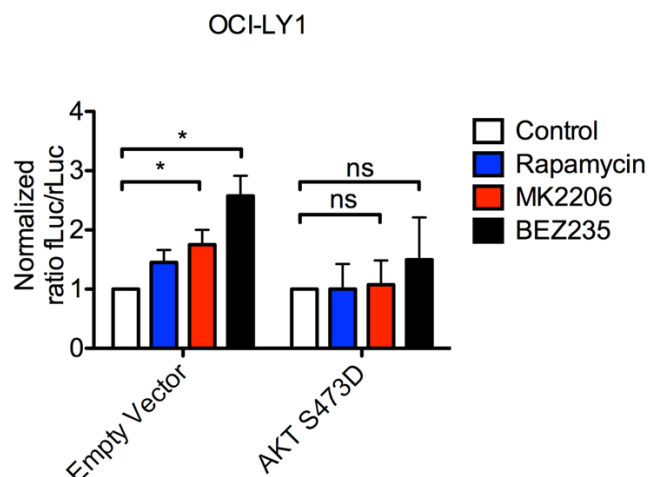
Figure 2.14. Expression of AKT S473D suppresses FOXO activation following inhibition of AKT. Relative FOXO transcriptional activity measured using a luciferase reporter assay system. Cells were co-transfected with pRL-TK (renilla) and firefly luciferase downstream of the putative FOXO3 binding site prior 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.
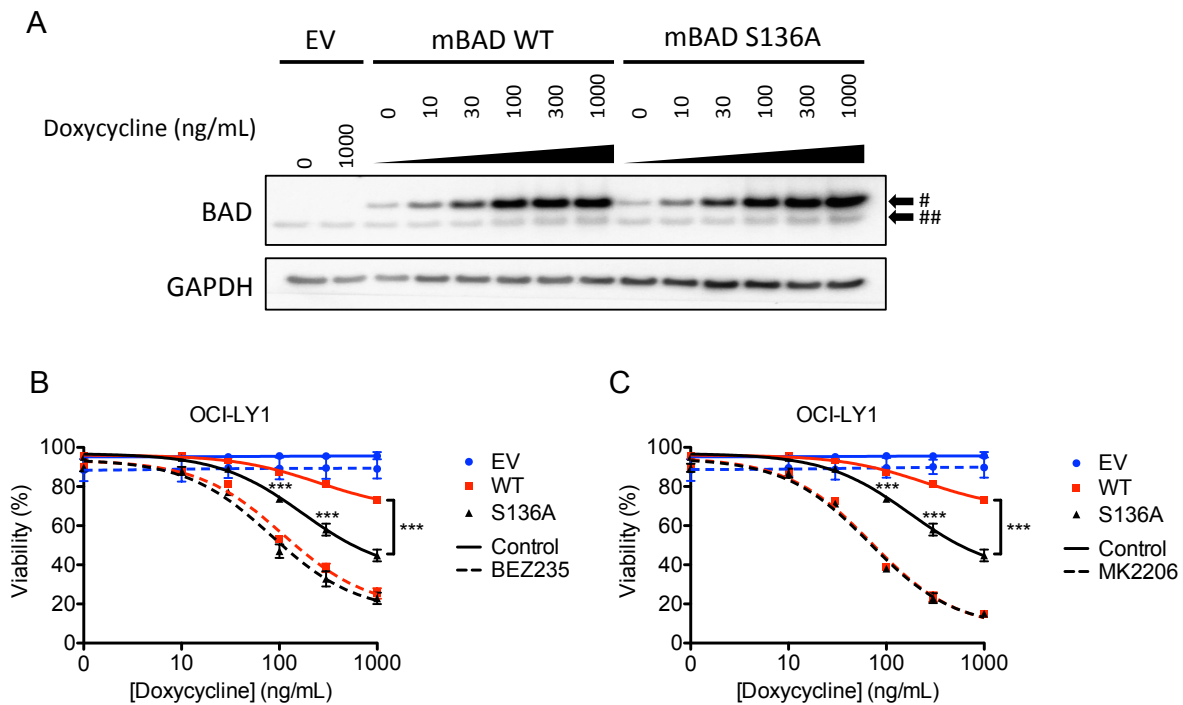
Figure 2.15. Expression of exogenous murine Bad sensitizes OCI-LY1 cells to AKT inhibition. (**A**) Western blot of murine BAD (mBAD) induction following 24 hour treatment with indicated doses of doxycycline. Cells were also treated with 10 µM Q-VD-OPh to prevent caspase cleavage of BAD. # Indicates 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.
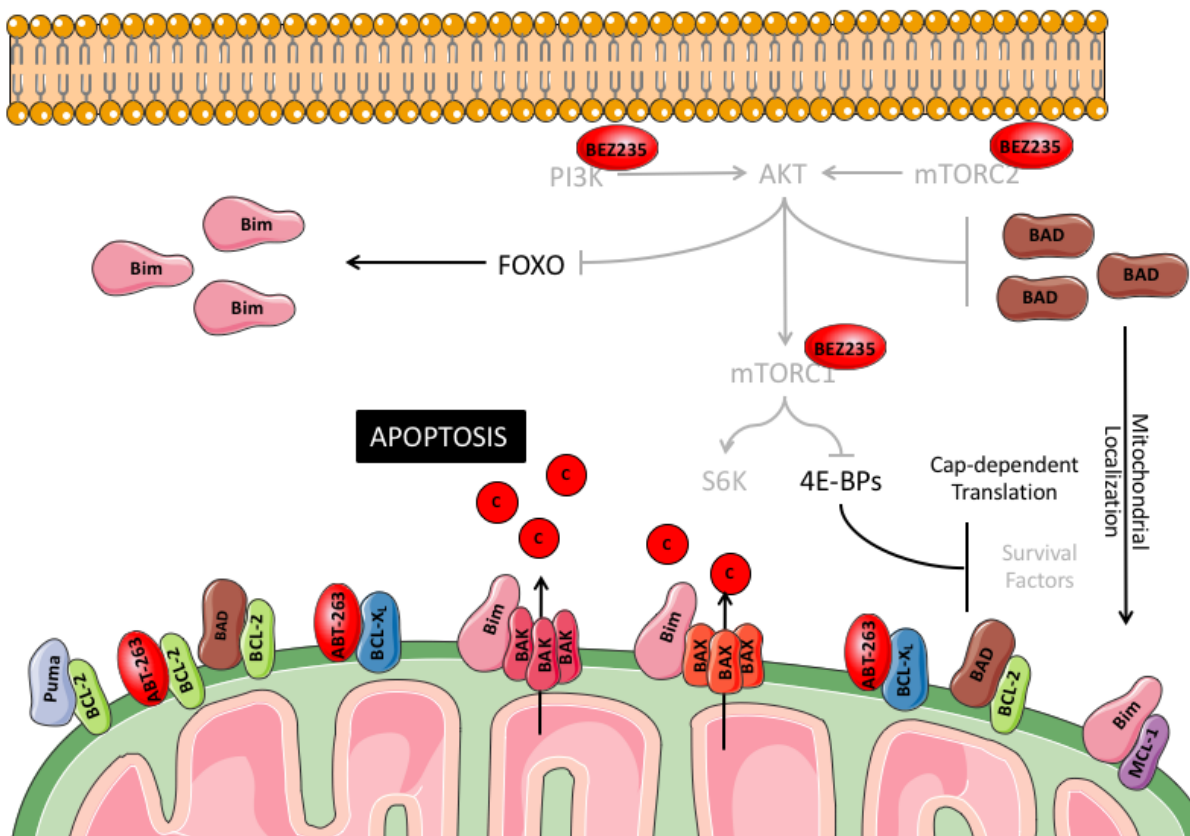

Figure 2.16. Model of synergy between BEZ235 and ABT-263 in DLBCL cell lines. Treatment with BEZ235 in DLBCL cell lines completely inhibits signaling through the PI3K and downstream effectors, AKT and mTORC1. Loss of AKT activity promotes FOXO-mediated transcription of BIM and facilitates mitochondrial 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

1. Fruman DA, Cantley LC. Idelalisib — A PI3Kδ Inhibitor for B-Cell Cancers. N Engl J Med. 2014;370:1061–2.
2. Witzig TE, Reeder CB, LaPlant BR, Gupta M, Johnston PB, Micallef IN, et al. A phase II trial of the oral mTOR inhibitor everolimus in relapsed aggressive lymphoma. Leukemia. 2011;25:341–7.
3. Mathews Griner LA, Guha R, Shinn P, Young RM, Keller JM, Liu D, et al. High-throughput combinatorial screening identifies drugs that cooperate with ibrutinib to kill activated B-cell–like diffuse large B-cell lymphoma cells. Proceedings of the National Academy of Sciences. 2014;111:2349–54.
4. Ezell SA, Mayo M, Bihani T, Tepsuporn S, Wang S, Passino M, et al. Synergistic induction of apoptosis by combination of BTK and dual mTORC1/2 inhibitors in diffuse large B cell lymphoma. Oncotarget. 2014;5:4990–5001.
5. Iqbal J, Meyer PN, Smith LM, Johnson NA, Vose JM, Greiner TC, et al. BCL2 Predicts Survival in Germinal Center B-cell-like Diffuse Large B-cell Lymphoma Treated with CHOP-like Therapy and Rituximab. Clinical Cancer Research. 2011;17:7785–95.
6. Basso K, Dalla-Favera R. Germinal centres and B cell lymphomagenesis. Nat Rev Immunol. 2015;15:172–84.
7. Hasselblom S, Hansson U, Olsson M, Torén L, Bergström A, Nilsson-Ehle H, et al. High immunohistochemical expression of p-AKT predicts inferior survival in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. British Journal of Haematology. 2010;149:560–8.
8. Pfeifer M, Grau M, Lenze D, Wenzel SS, Wolf A, Wollert-Wulf B, et al. PTEN loss defines a PI3K/AKT pathway-dependent germinal center subtype of diffuse large B-cell lymphoma. Proceedings of the National Academy of Sciences. 2013;110:12420–5.
9. Faber AC, Coffee EM, Costa C, Dastur A, Ebi H, Hata AN, et al. mTOR Inhibition Specifically Sensitizes Colorectal Cancers with KRAS or BRAF Mutations to BCL-2/BCL-XL Inhibition by Suppressing MCL-1. Cancer Discovery. 2014;4:42–52.
10. Pareja F, Macleod D, Shu C, Crary JF, Canoll PD, Ross AH, et al. PI3K and Bcl-2 Inhibition Primes Glioblastoma Cells to Apoptosis through Downregulation of Mcl-1 and Phospho-BAD. Molecular Cancer Research. 2014;12:987–1001.
11. Jebahi A, Villedieu M, Pétigny-Lechartier C, Brotin E, Louis M-H, Abeilard E, et al. PI3K/mTOR dual inhibitor NVP-BEZ235 decreases Mcl-1 expression and sensitizes ovarian carcinoma cells to Bcl-xL-targeting strategies, provided that Bim expression is induced. Cancer Letters. 2014;348:38–49.
12. Choudhary GS, Al-harbi S, Mazumder S, Hill BT, Smith MR, Bodo J, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. Cell Death Dis. 2015;6:e1593.
13. Coloff JL, Macintyre AN, Nichols AG, Liu T, Gallo CA, Plas DR, et al. Akt-Dependent Glucose Metabolism Promotes Mcl-1 Synthesis to Maintain Cell Survival and Resistance to Bcl-2 Inhibition. Cancer Research. 2011;71:5204–13.
14. Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR, et al. Genetic

Dissection of the Oncogenic mTOR Pathway Reveals Druggable Addiction to Translational Control via 4EBP-eIF4E. Cancer Cell. 2010;17:249–61.

15. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. Cell. 1999;96:857–68.

16. Dijkers PF, Medema RH, Lammers J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Current Biology. 2000.

17. Khoury MK, Parker I, Aswad DW. Acquisition of chemiluminescent signals from immunoblots with a digital single-lens reflex camera. Analytical Biochemistry. 2010;397:129–31.

18. Ryan J, Letai A. BH3 profiling in whole cells by fluorimeter or FACS. Methods. 2013;61:156–64.

19. Certo M, Moore VDG, Nishino M, Wei G, Korsmeyer S, Armstrong SA, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell. 2006;9:351–65.

20. Kutuk O, Letai A. Displacement of Bim by Bmf and Puma rather than increase in Bim level mediates paclitaxel-induced apoptosis in breast cancer cells. Cell Death Differ. 2010;17:1624–35.

21. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nature Methods. 2009;6:275–7.

22. Jang GM, Leong LEC, Hoang LT, Wang PH, Gutman GA, Semler BL. Structurally Distinct Elements Mediate Internal Ribosome Entry within the 5'-Noncoding Region of a Voltage-gated Potassium Channel mRNA. 2004;279:47419–30.

23. Del Gaizo Moore V, Schlis KD, Sallan SE, Armstrong SA, Letai A. BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. Blood. 2008;111:2300–9.

24. Chou TC. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. Cancer Research. 2010;70:440–6.

25. Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis. 2003;8:345–52.

26. Ferreira KS, Kreutz C, MacNelly S, Neubert K, Haber A, Bogyo M, et al. Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes. Apoptosis. 2012;17:503–15.

27. Rudin CM, Hann CL, Garon EB, Ribeiro de Oliveira M, Bonomi PD, Camidge DR, et al. Phase II Study of Single-Agent Navitoclax (ABT-263) and Biomarker Correlates in Patients with Relapsed Small Cell Lung Cancer. Clinical Cancer Research. 2012;18:3163–9.

28. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013;19:202–8.

29. Kennedy R, Celis E. Multiple roles for CD4 +T cells in anti-tumor immune responses. Immunol Rev. 2008;222:129–44.

30. Rakhra K, Bachireddy P, Zabuawala T, Zeiser R, Xu L, Kopelman A, et al. CD4+ T Cells Contribute to the Remodeling of the Microenvironment Required for

Sustained Tumor Regression upon Oncogene Inactivation. Cancer Cell. 2010;18:485–98.

31.   Aukema SM, Siebert R, Schuuring E, van Imhoff GW, Kluin-Nelemans HC, Boerma EJ, et al. Double-hit B-cell lymphomas. Blood. 2011;117:2319–31.

32.   Lheureux S, N'Diaye M, Blanc-Fournier C, Dugué AE, Clarisse B, Dutoit S, et al. Identification of predictive factors of response to the BH3-mimetic molecule ABT-737: An ex vivoexperiment in human serous ovarian carcinoma. Int J Cancer. 2014;136:E340–50.

33.   Konopleva M, Contractor R, Tsao T, Samudio I, Ruvolo PP, Kitada S, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell. 2006;10:375–88.

34.   Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. Blood. 2010;115:3304–13.

35.   van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell. 2006;10:389–99.

36.   Thomas D, Powell JA, Vergez F, Segal DH, Nguyen NYN, Baker A, et al. Targeting acute myeloid leukemia by dual inhibition of PI3K signaling and Cdk9-mediated Mcl-1 transcription. Blood. 2013;122:738–48.

37.   Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen Synthase Kinase-3 Regulates Mitochondrial Outer Membrane Permeabilization and Apoptosis by Destabilization of MCL-1. Molecular Cell. 2006;21:749–60.

38.   Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014;13:140–56.

39.   Masters SC, Yang H, Datta SR, Greenberg ME, Fu H. 14-3-3 inhibits Bad-induced cell death through interaction with serine-136. Molecular Pharmacology. 2001;60:1325–31.

40.   Ren D, Tu HC, Kim H, Wang GX, Bean GR, Takeuchi O, et al. BID, BIM, and PUMA Are Essential for Activation of the BAX- and BAK-Dependent Cell Death Program. Science. 2010;330:1390–3.

41.   Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010;11:621–32.

42.   Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K–Akt signaling pathway. Current Opinion in Neurobiology. 2001;11:297–305.

43.   Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proceedings of the National Academy of Sciences. 2001;98:9666–70.

44.   Spender LC, Inman GJ. Phosphoinositide 3-kinase/AKT/mTORC1/2 Signaling Determines Sensitivity of Burkitt's Lymphoma Cells to BH3 mimetics. Molecular Cancer Research. 2012;10:347–59.

45.   Mills JR, Hippo Y, Robert F, Chen SMH, Malina A, Lin CJ, et al. mTORC1 promotes survival through translational control of Mcl-1. Proceedings of the National Academy of Sciences. 2008;105:10853–8.

46.   Chen S, Dai Y, Pei XY, Myers J, Wang L, Kramer LB, et al. CDK Inhibitors Upregulate BH3-Only Proteins to Sensitize Human Myeloma Cells to BH3 Mimetic

Therapies. Cancer Research. 2012;72:4225–37.

47. Stewart DP, Koss B, Bathina M, Perciavalle RM, Bisanz K, Opferman JT. Ubiquitin-Independent Degradation of Antiapoptotic MCL-1. Molecular and Cellular Biology. 2010;30:3099–110.

48. Mazumder S, Choudhary GS, Al-harbi S, Almasan A. Mcl-1 Phosphorylation Defines ABT-737 Resistance That Can Be Overcome by Increased NOXA Expression in Leukemic B cells. Cancer Research. 2012;72:3069–79.

49. Inuzuka H, Fukushima H, Shaik S, Liu P, Lau AW, Wei W. Mcl-1 Ubiquitination and Destruction. Oncotarget. 2011;2:239–44.

50. Weber K, Harper N, Schwabe J, Cohen GM. BIM-Mediated Membrane Insertion of the BAK Pore Domain Is an Essential Requirement for Apoptosis. Cell Reports. 2013;5:409–20.

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

88

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

89

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% CO_2. Cells were routinely

90

tested to ensure absence of mycoplasma, and were maintained at or below 2×10^6 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) 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 µg/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, 6×10^4 cells were cultured in 200 µL 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 cytomety 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 x 104 cells/well. Confluence was confirmed by phase-contrast microscopy before seeding of CLL cells at 6 x 105 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

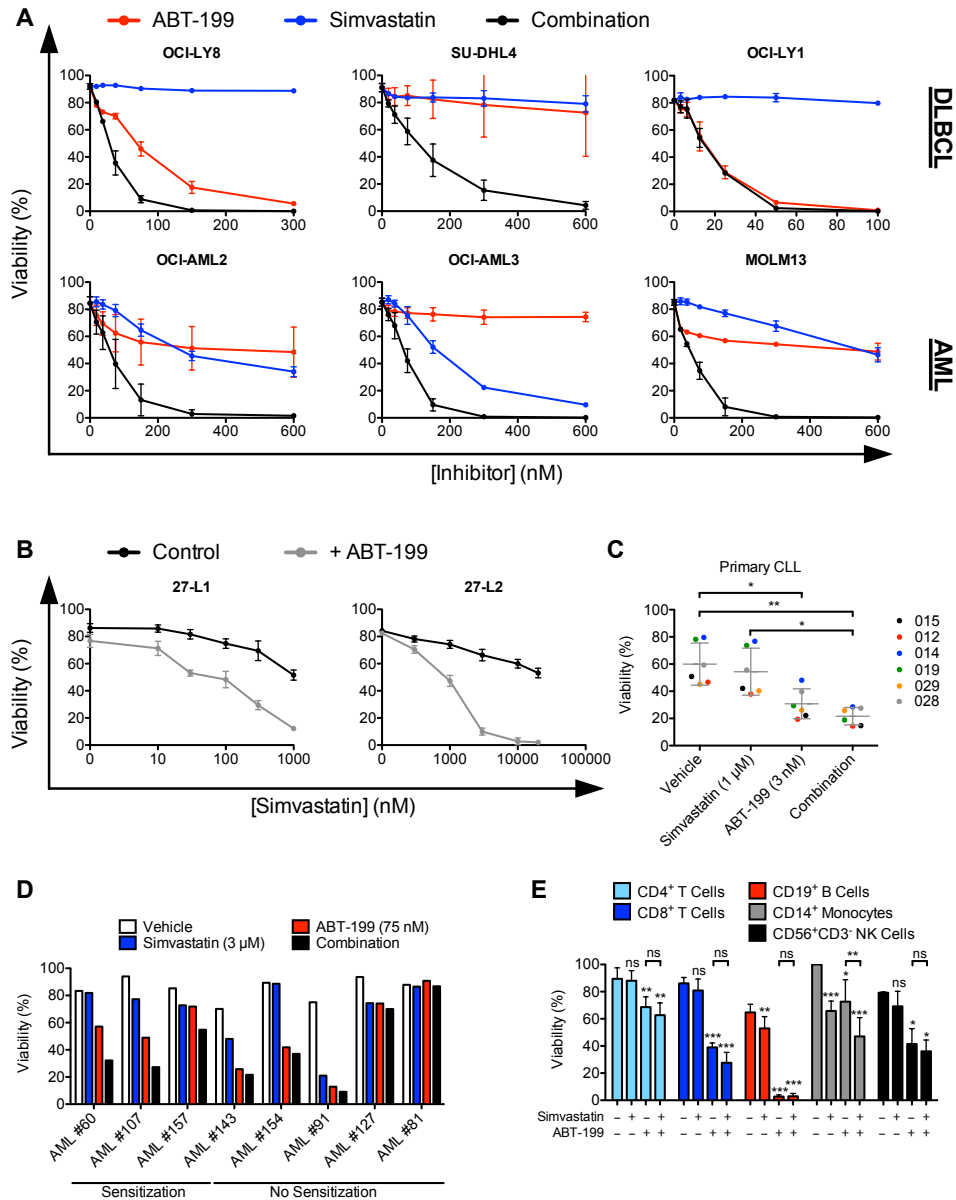poor prognosis (**Figures 3.1B** and **3.2C**).

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.

96

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.
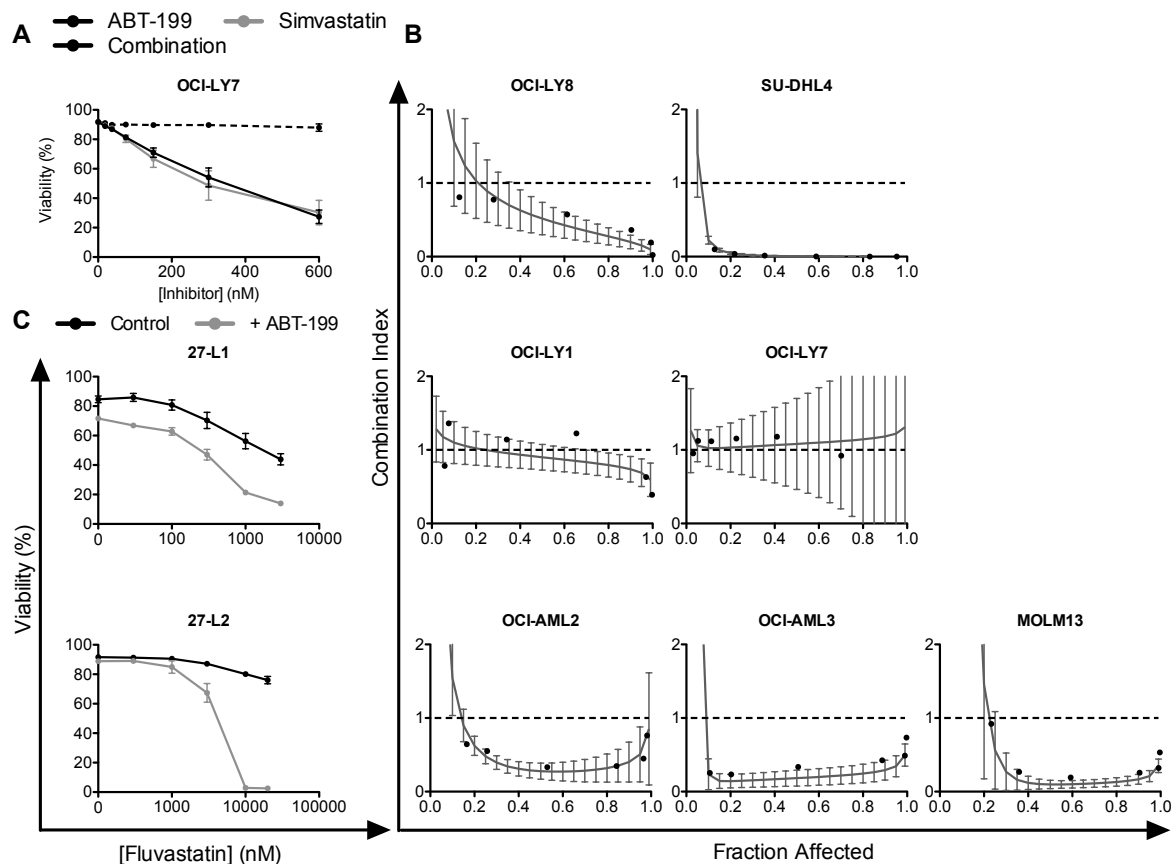
Figure 3.2. Statins synergize with ABT-199 in blood cancer cells. (**A**) viability of OCI-LY7 cells treated with increasing doses of ABT-199 (1X indicated dose), simvastatin (33.33X indicated dose), or the combination for 48 hours. (**B**) formal synergy analysis of DLBCL and AML cell lines using data 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.
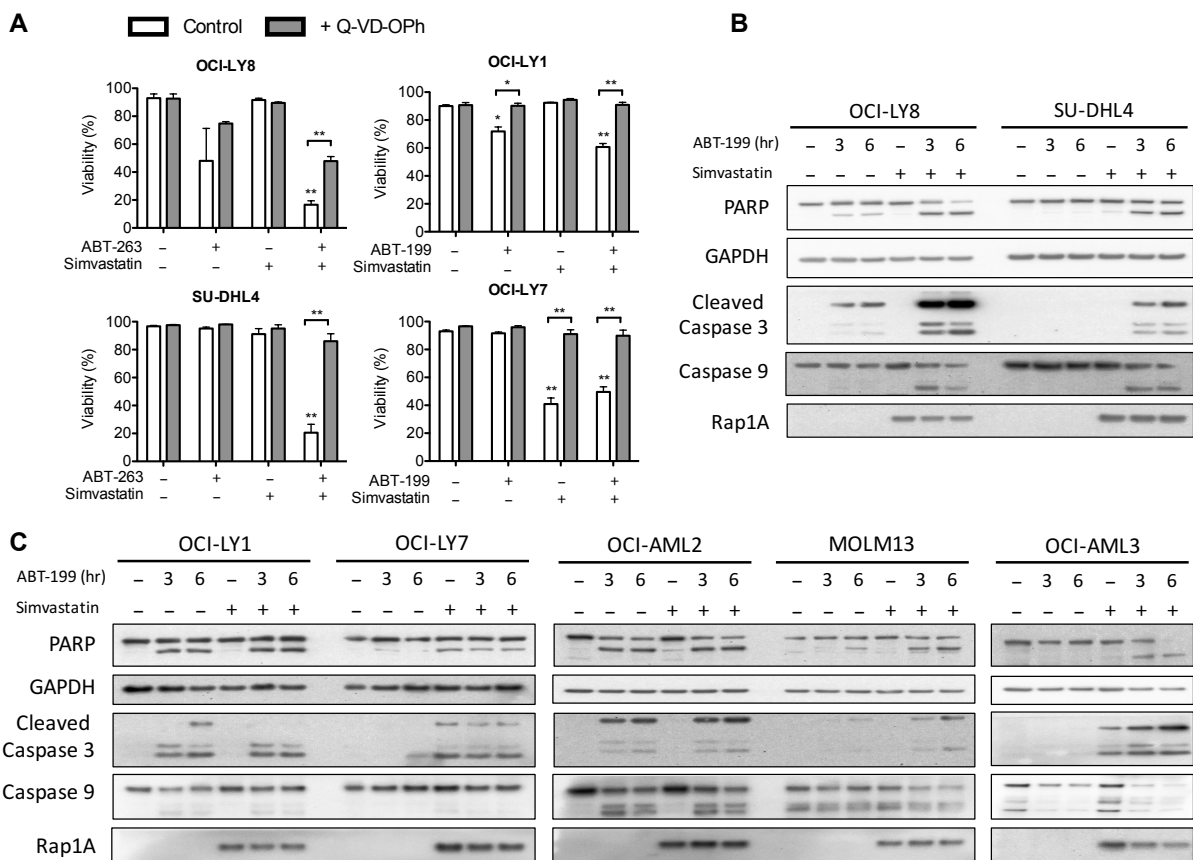
Figure 3.3. Simvastatin plus ABT-199 induce apoptosis in DLBCL and AML cell lines. (**A**) viability of cells treated with indicated drugs with (grey bars) or without (white bars) pan-caspase inhibitor Q-VD-OPh (10 µM). (**B-C**) western blot of cells pre-treated with vehicle or simvastatin for 16 hours 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.

Sample ID	Therapies	Cytogenetics
2016-012	NA	46XY +12, t(14;18)(q32;q21), del(17p)
2016-014	Simvastatin	NA
2016-015	Ibrutinib	NA
2016-019	Bendamustine, Rituximab Ibrutinib	NA
2016-028	Rosuvastatin	NA
2016-029	Bendamustine, Rituximab	del(17p), trisomy 12

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).
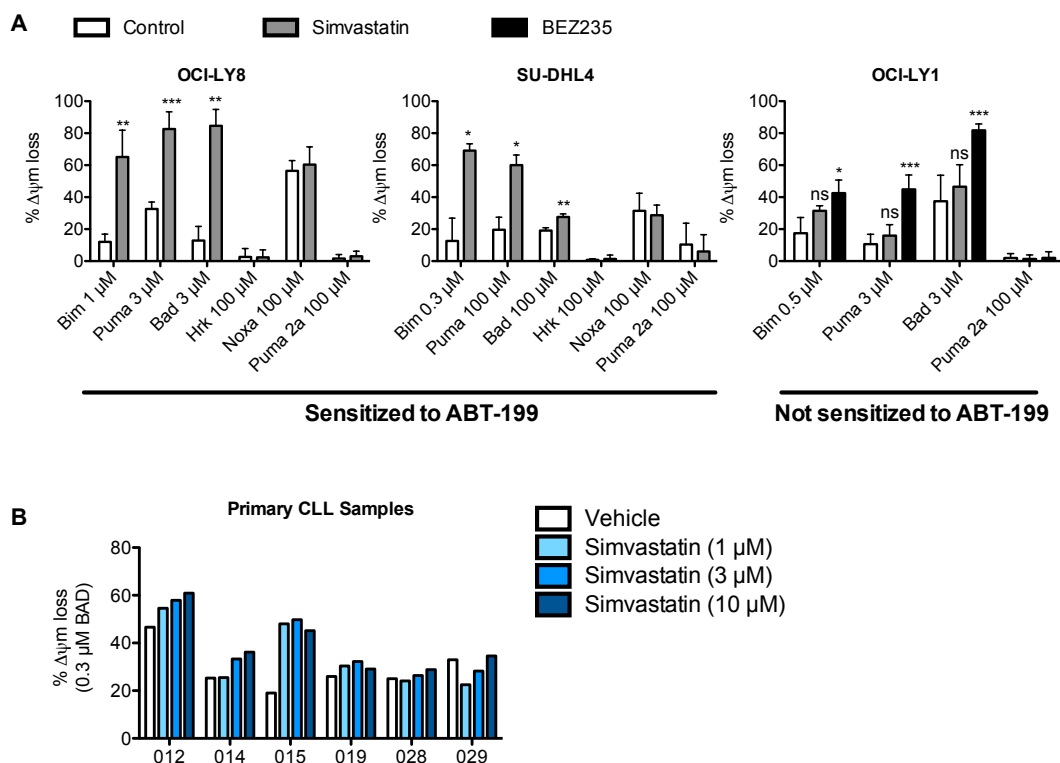
Figure 3.4. Dynamic BH3 profiling predicts sensitization to ABT-199 by simvastatin. (**A**) dynamic BH3 profiles for all cells treated with 10 µM simvastatin or 50 nM BEZ235 for 16 hours. (**B**) limited dynamic BH3 profile (BAD peptide) of primary CLL samples treated with indicated concentration of simvastatin for 16 hours.
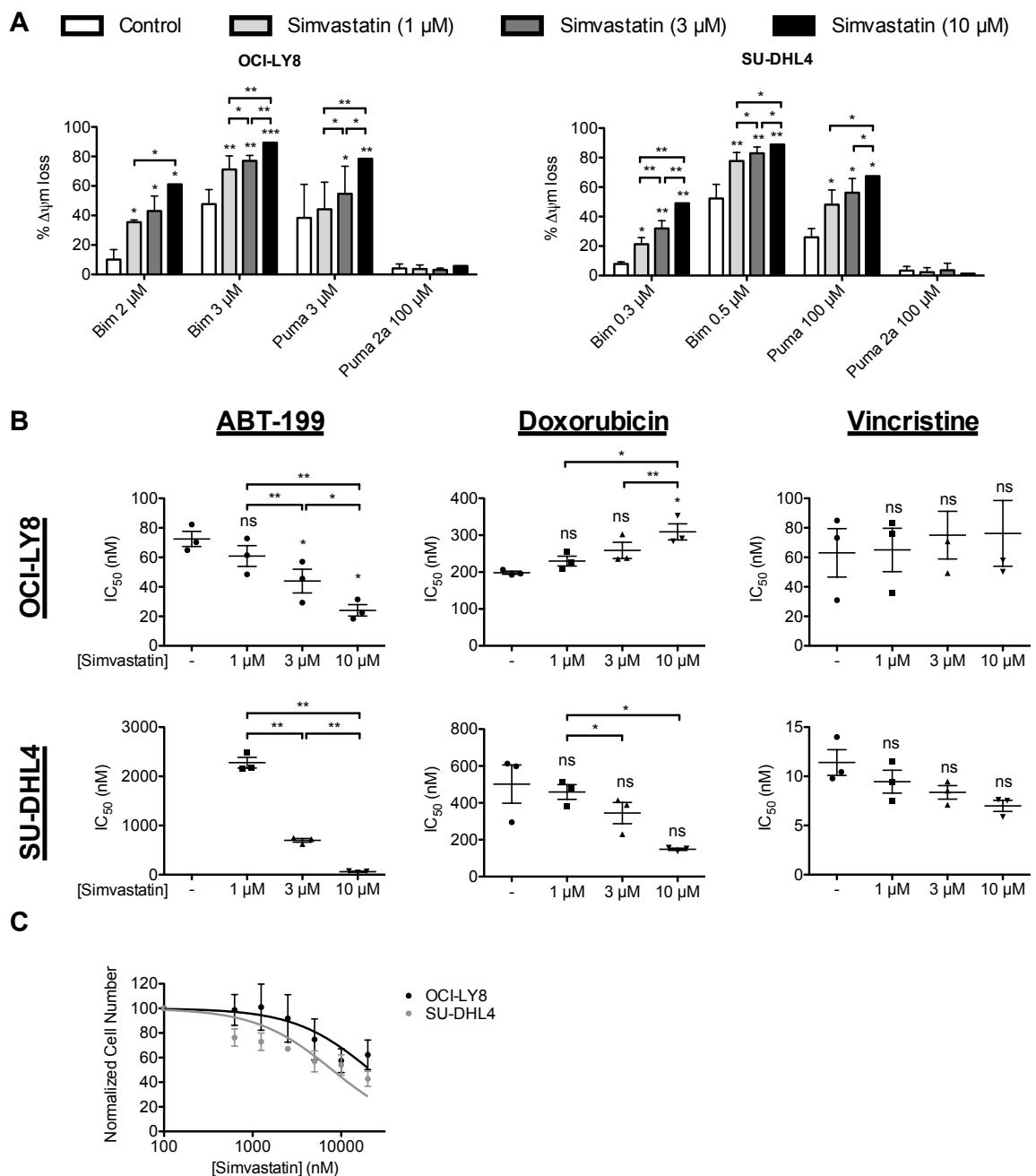
Figure 3.5. Statins induce a dose-dependent increase in mitochondrial priming, but do not sensitize to chemotherapy. (**A**) dynamic BH3 profiles for cells treated with indicated doses of simvastatin for 16 hours. (**B**) sensitivity (IC50) of two DLBCL cell lines to ABT-199 or two chemotherapies. Cells 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).
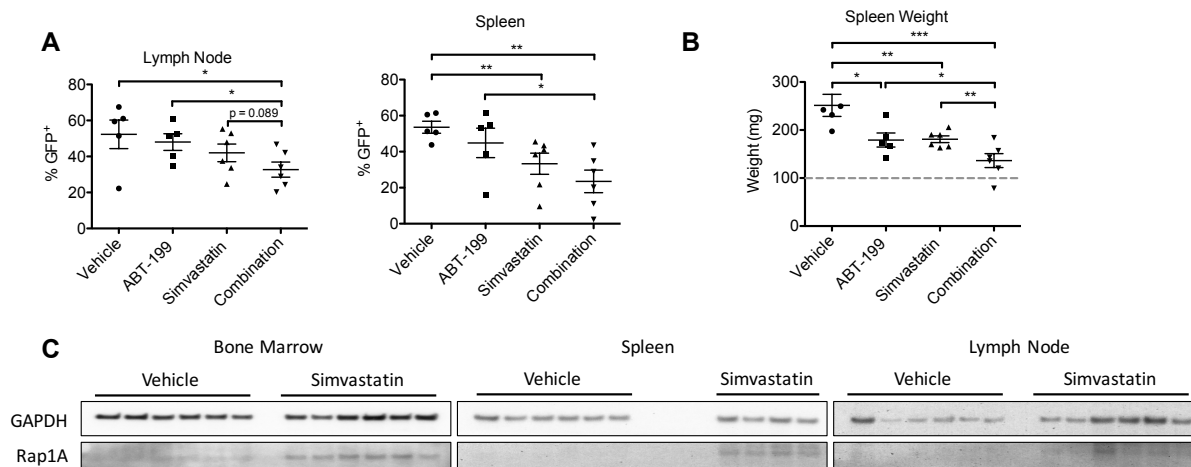
Figure 3.6. Combination of simvastatin and ABT-199 is effective in a syngeneic mouse model of lymphoma. (**A**) percent lymphoma burden in indicated organs of mice treated with ABT-199 (75 mg/kg/day), simvastatin (50 mg/kg/day), or the combination for 5 days. (**B**) spleen weights for mice treated as in **A**. (**C**) western blots for pharmacodynamic effect of simvastain (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.
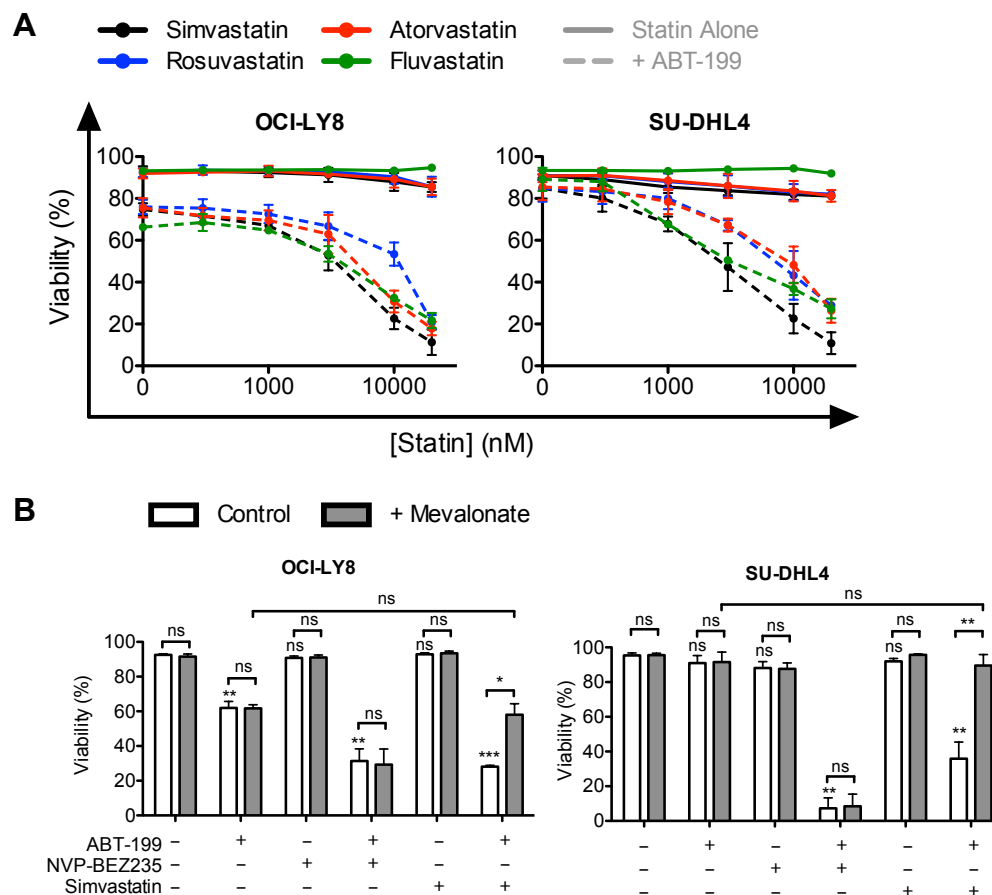
Figure 3.7. Effect of statins is due to on-target HMGCR inhibition. (**A**) viability of cells treated with increasing doses of different chemically distinct statins (colors) with (dashed lines) or without (solid lines) ABT-199 (30 nM for LY8, 300 nM for HL4). (**B**) viability of cells treated with 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.
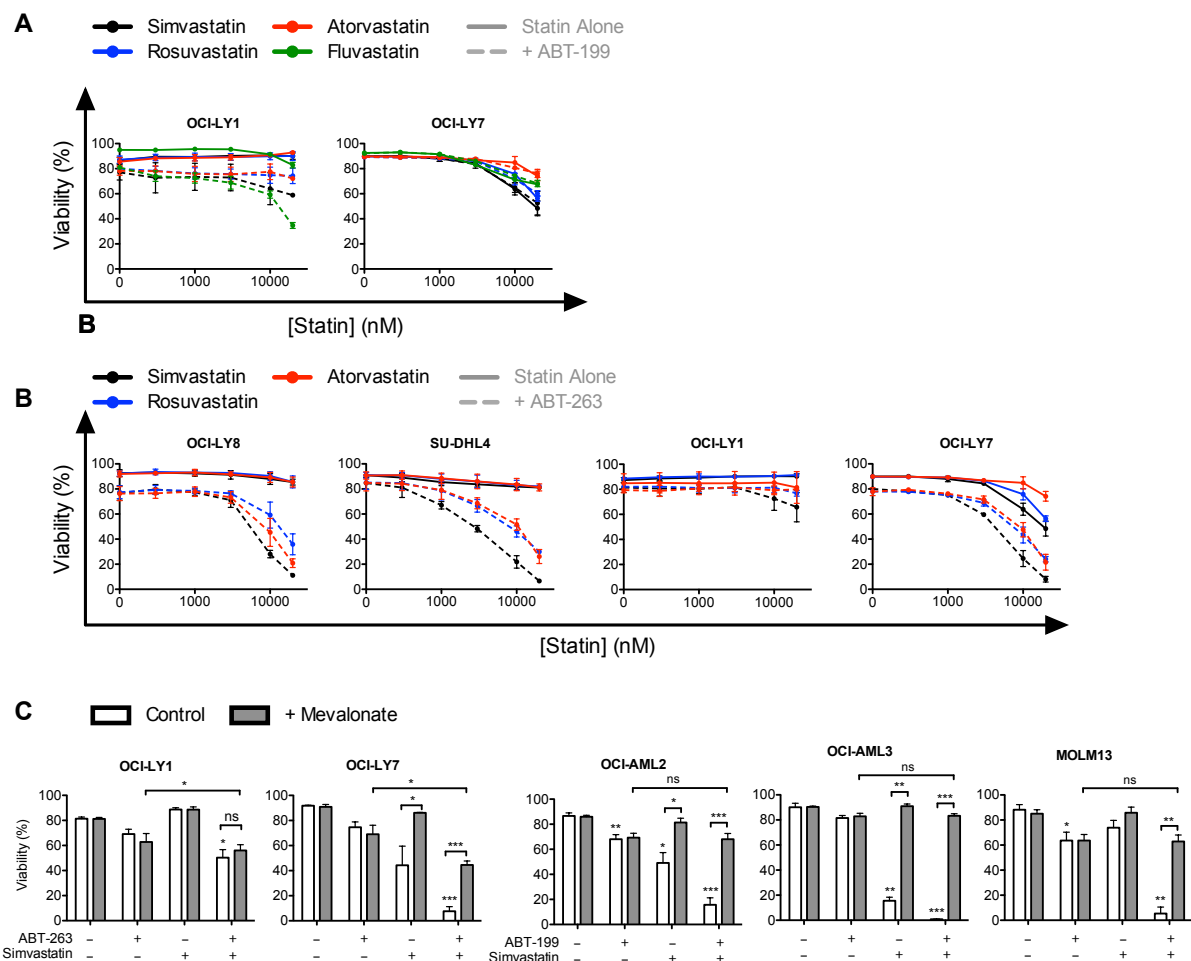
Figure 3.8. **Sensitization by statins is on-target.** (**A**) viability of cells treated with increasing doses of different chemically distinct statins (colors) with (dashed lines) or without (solid lines) ABT-199 (10 nM for LY1, 300 nM for LY7). (**B**) viability of cells treated with increasing doses 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**).
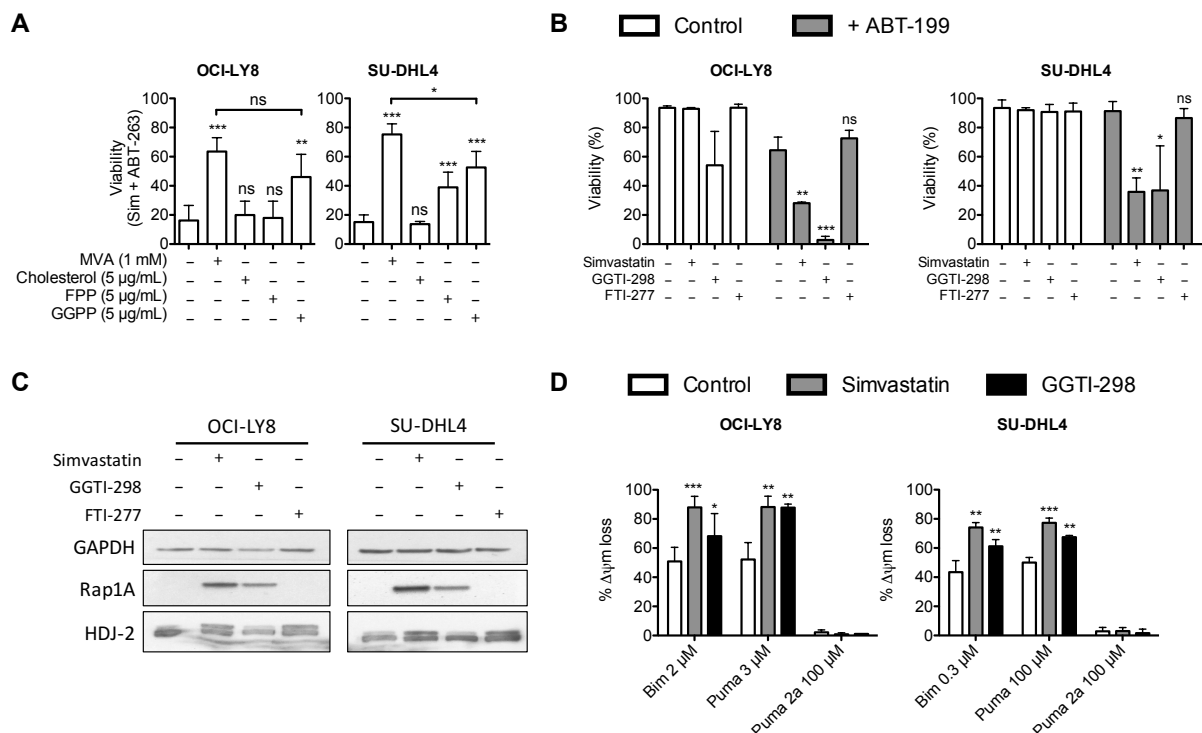
Figure 3.9. Sensitization to ABT-199 requires inhibition of protein geranylgeranylation. (**A**) viability of cells treated with the combination of 300 nM ABT-263 and 10 µM simvastatin supplemented with indicated metabolites for 48 hours. Abbreviations are as follows; MVA: mevalonate, FPP: farnesyl pyrophosphate, 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.
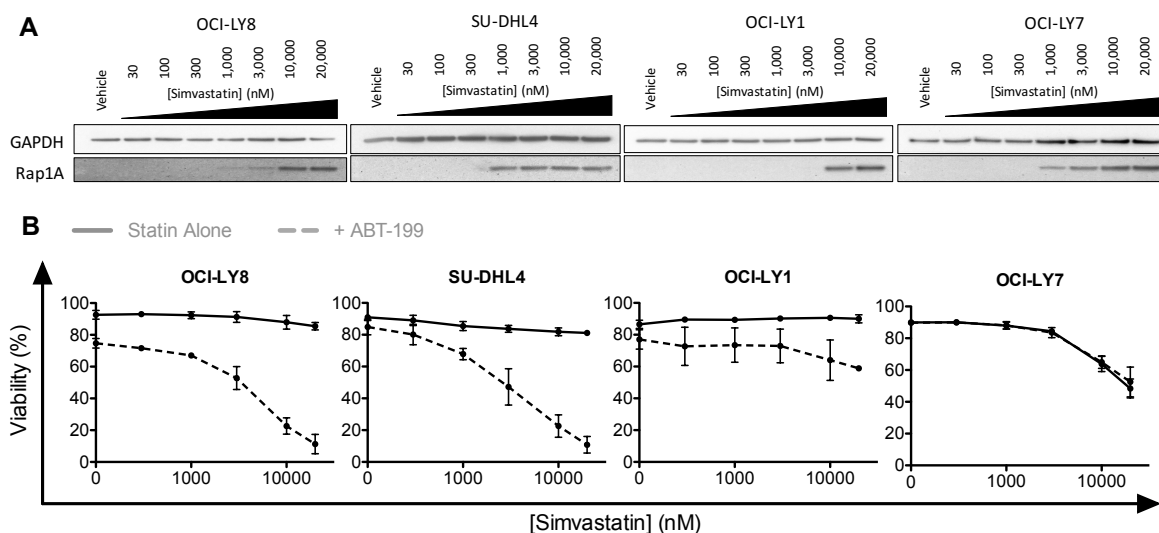
Figure 3.11. Simvastatin inhibits protein geranylgeranylation in a dose-dependent manner in DLBCL. (**A**) western blots of DLBCL cell lines treated with increasing doses of simvastatin for 16 hours. (**B**) viability of ce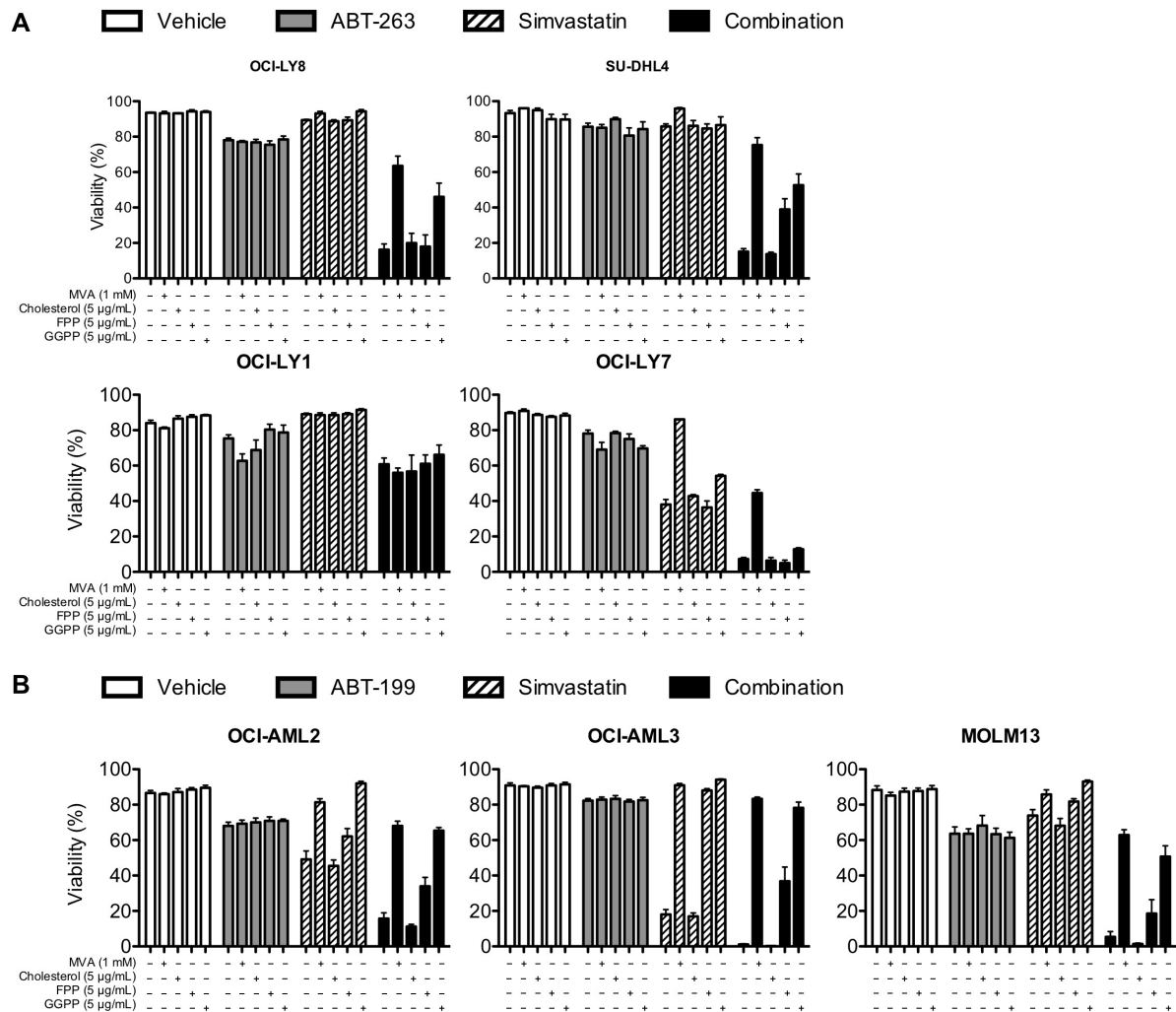lls treated with doses of simvastatin in **A** for 48 hours with (dashed lines) or without (solid lines) ABT-199. Doses of ABT-199 are as follows: 30 nM for OCI-LY8, 10 nM for OCI-LY1, 300 nM for OCI-LY7 and SU-DHL4.

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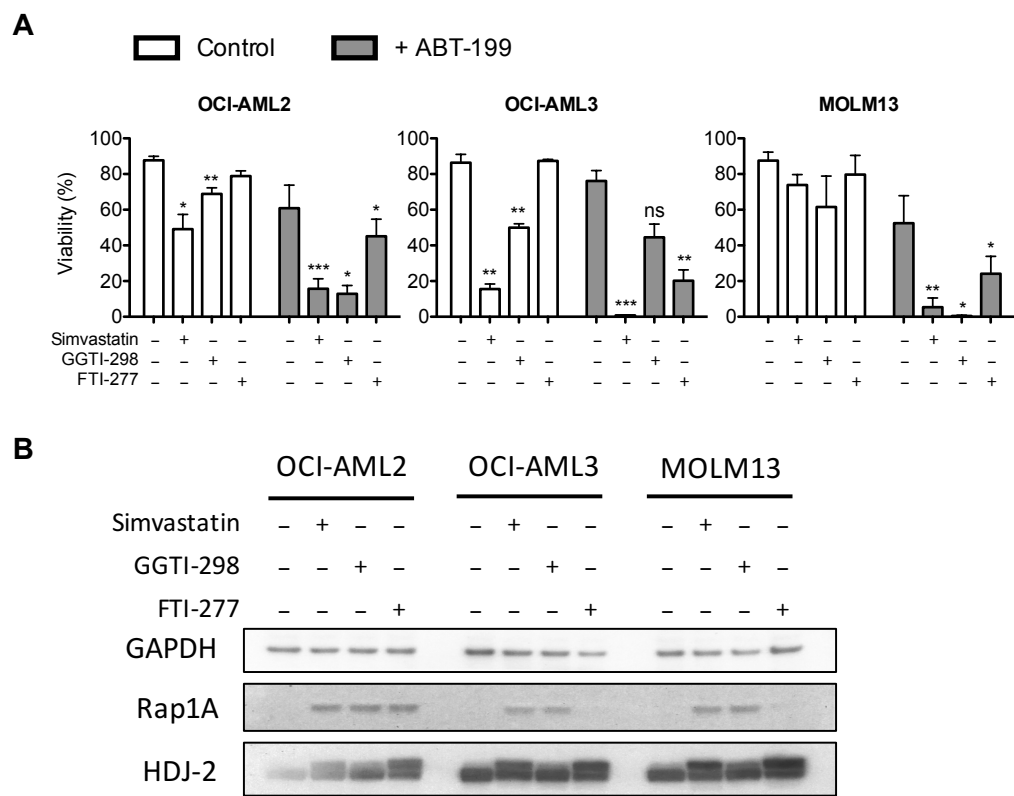
Figure 3.12. **Inhibition of GGT is sufficient to recapitulate the effects of simvastatin in AML cell lines.** (**A**) viability of AML cell lines treated with indicated inhibitors for 48 hours. (**B**) western blot of AML cell lines treated with 10 µM indicated inhibitors for 16 hours.

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**).
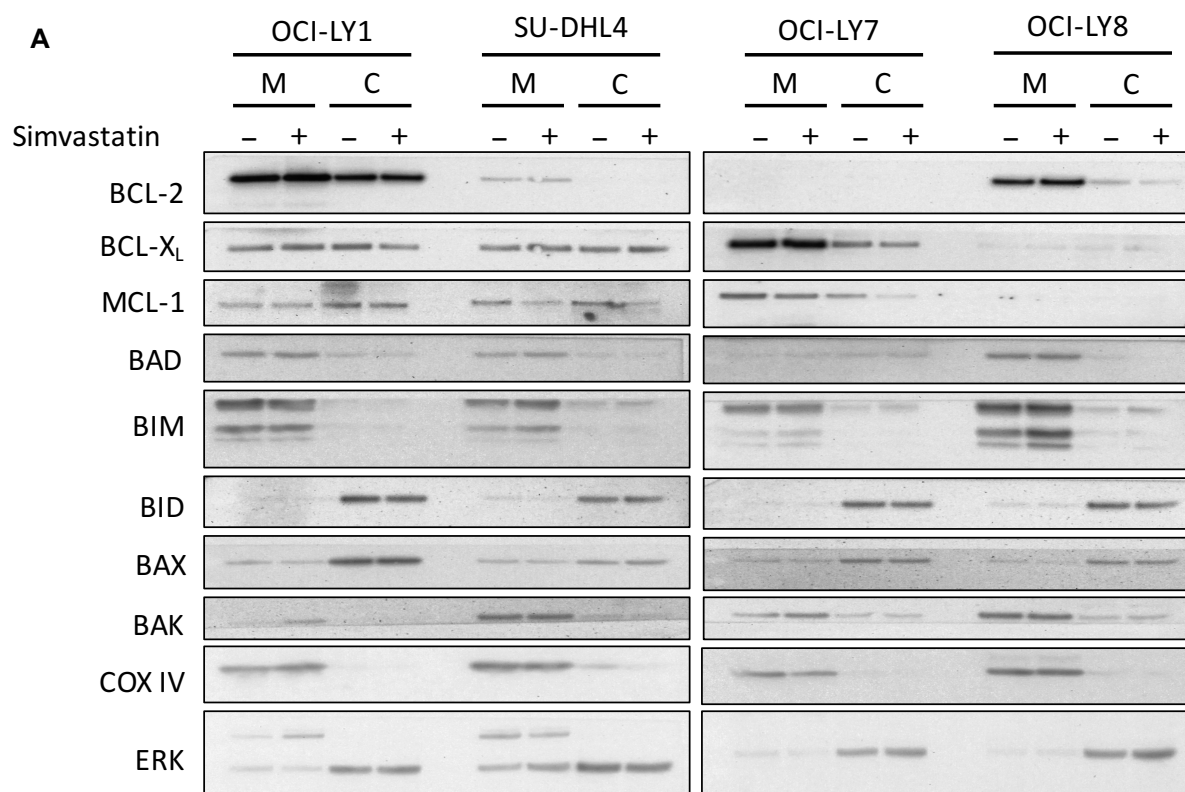
Figure 3.13. Simvastatin does not affect expression of many BCL-2 family proteins. Western blot of cell lysate fractions enriched for heavy membranes (M) or cytoplasmic (C) proteins following 16 hour treatment with 10 μM simvastatin.
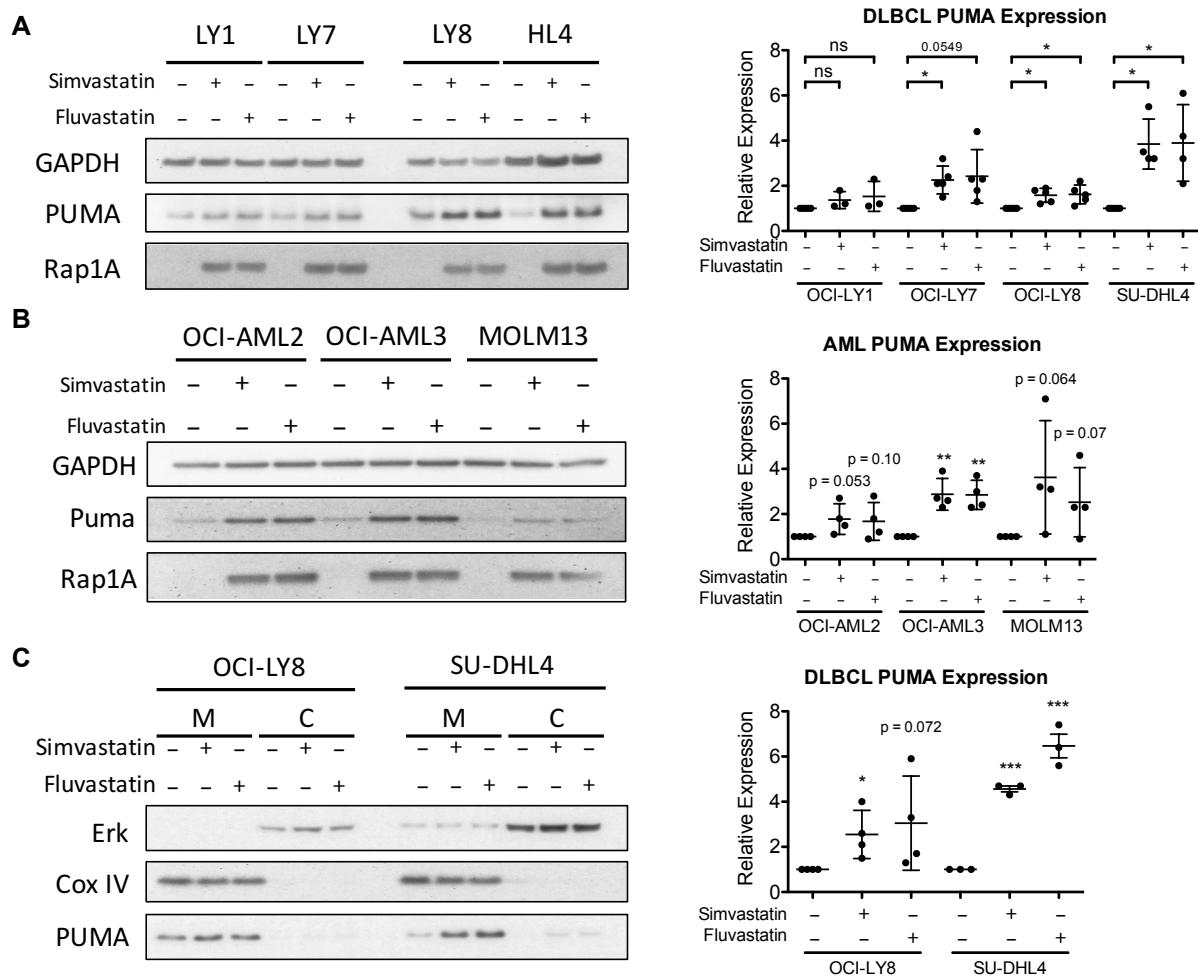
Figure 3.14. **Statins induce up-regulation of PUMA in DLBCL and AML cell lines.**
(**A-B**) representative western blots of cells treated with 10 µM indicated statin for 16 hours (left) and quantitation across replicates (right). (**C**) representative western blot of mitochondrial (M) and cytoplasmic (C) fractions isolated from cells treated with 10 µM indicated statin for 16 hours (left) and quantitation across replicates (right).
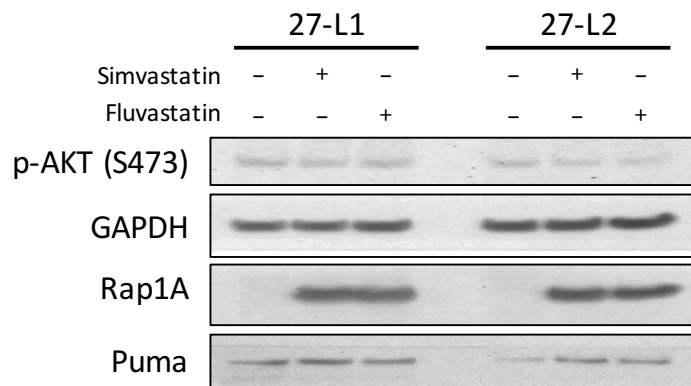
Figure 3.15. Statins induce PUMA in one primary mouse lymphoma cell line. Western blot of primary mouse lymphoma cells treated with 10 μM indicated statin for 16 hours.

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 over-expression 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 sensitizers 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

1. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest. 2007;117:112–21.
2. Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. Leukemia. 1996;10:456–9.
3. Iqbal J, Meyer PN, Smith LM, Johnson NA, Vose JM, Greiner TC, et al. BCL2 Predicts Survival in Germinal Center B-cell-like Diffuse Large B-cell Lymphoma Treated with CHOP-like Therapy and Rituximab. Clinical Cancer Research. 2011;17:7785–95.
4. Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, et al. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 1993;81:3091–6.
5. Basso K, Dalla-Favera R. Germinal centres and B cell lymphomagenesis. Nat Rev Immunol. 2015;15:172–84.
6. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature. 2005;435:677–81.
7. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, et al. ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. Cancer Research. 2008;68:3421–8.
8. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013;19:202–8.
9. van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell. 2006;10:389–99.
10. Rudin CM, Hann CL, Garon EB, Ribeiro de Oliveira M, Bonomi PD, Camidge DR, et al. Phase II Study of Single-Agent Navitoclax (ABT-263) and Biomarker Correlates in Patients with Relapsed Small Cell Lung Cancer. Clinical Cancer Research. 2012;18:3163–9.
11. Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2016;374:311–22.
12. Seymour JF, Gerecitano JF, Kahl BS, Pagel JM. The single-agent Bcl-2 inhibitor ABT-199 (GDC-0199) in patients with relapsed/refractory (R/R) non-Hodgkin lymphoma (NHL): responses observed in all …. 2013.
13. Choudhary GS, Al-harbi S, Mazumder S, Hill BT, Smith MR, Bodo J, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. Cell Death Dis. 2015;6:e1593.
14. Lee JS, Tang SS, Ortiz V, Vo T-T, Fruman DA. MCL-1-independent mechanisms of synergy between dual PI3K/mTOR and BCL-2 inhibition in diffuse large B cell lymphoma. Oncotarget. 2015;6:35202–17.

15. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014;13:140–56.
16. Janes MR, Limon JJ, So L, Chen J, Lim RJ, Chavez MA, et al. Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. Nat Med. 2010;16:205–13.
17. Gupta SC, Sung B, Prasad S, Webb LJ, Aggarwal BB. Cancer drug discovery by repurposing: teaching new tricks to old dogs. Trends in Pharmacological Sciences. 2013;34:508–17.
18. Karp I, Behlouli H, LeLorier J, Pilote L. Statins and Cancer Risk. The American Journal of Medicine. 2008;121:302–9.
19. Demierre M-F, Higgins PDR, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nature Reviews Cancer. 2005;5:930–42.
20. Wong W, Wong WWL, Dimitroulakos J, Minden MD, Penn LZ. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia. 2002;16:508–19.
21. Davidson MH. Safety Profiles for the HMG-CoA Reductase Inhibitors. Drugs. 2001;61:197–206.
22. Clendening JW, Pandyra A, Boutros PC, Ghamrasni SE, Khosravi F, Trentin GA, et al. Dysregulation of the mevalonate pathway promotes transformation. Proceedings of the National Academy of Sciences. 2010;107:15051–6.
23. Dimitroulakos J, Nohynek D, Backway KL, Hedley DW, Yeger H, Freedman MH, et al. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. 1999;93:1308–18.
24. Minden MD, Dimitroulakos J, Nohynek D, Penn LZ. Lovastatin induced control of blast cell growth in an elderly patient with acute myeloblastic leukemia. Leuk Lymphoma. 2001;40:659–62.
25. Dong W, Vuletic S, Albers JJ. Differential effects of simvastatin and pravastatin on expression of Alzheimer's disease-related genes in human astrocytes and neuronal cells. J Lipid Res. 2009;50:2095–102.
26. Certo M, Moore VDG, Nishino M, Wei G, Korsmeyer S, Armstrong SA, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell. 2006;9:351–65.
27. Khoury MK, Parker I, Aswad DW. Acquisition of chemiluminescent signals from immunoblots with a digital single-lens reflex camera. Analytical Biochemistry. 2010;397:129–31.
28. Chou TC. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. Cancer Research. 2010;70:440–6.
29. Li L, Pongtornpipat P, Tiutan T, Kendrick SL, Park S, Persky DO, et al. Synergistic induction of apoptosis in high-risk DLBCL by BCL2 inhibition with ABT-199 combined with pharmacologic loss of MCL1. Leukemia. 2015;29:1702–12.
30. Friedman AA, Letai A, Fisher DE, Flaherty KT. Precision medicine for cancer with next-generation functional diagnostics. Nat Rev Cancer. 2015;15:747–56.
31. Vo T-T, Ryan J, Carrasco R, Neuberg D, Rossi DJ, Stone RM, et al. Relative Mitochondrial Priming of Myeloblasts and Normal HSCs Determines Chemotherapeutic Success in AML. Cell. 2012;151:344–55.

32.   Del Gaizo Moore V, Letai A. BH3 profiling--measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. Cancer Letters. 2013;332:202–5.

33.   Montero J, Sarosiek KA, DeAngelo JD, Maertens O, Ryan J, Ercan D, et al. Drug-induced death signaling strategy rapidly predicts cancer response to chemotherapy. Cell. 2015;160:977–89.

34.   Samaras P, Heider H, Haile SR, Petrausch U, Schaefer NG, Siciliano RD, et al. Concomitant statin use does not impair the clinical outcome of patients with diffuse large B cell lymphoma treated with rituximab-CHOP. Ann Hematol. 2010;89:783–7.

35.   Ennishi D, Asai H, Maeda Y, Shinagawa K, Ikeda K, Yokoyama M, et al. Statin-independent prognosis of patients with diffuse large B-cell lymphoma receiving rituximab plus CHOP therapy. Ann Oncol. 2010;21:1217–21.

36.   Nowakowski GS, Maurer MJ, Habermann TM, Ansell SM, Macon WR, Ristow KM, et al. Statin use and prognosis in patients with diffuse large B-cell lymphoma and follicular lymphoma in the rituximab era. J Clin Oncol. 2010;28:412–7.

37.   Kohn KW, O'Connor PM. Cell cycle regulation and the chemosensitivity of cancer cells. Cancer Treatment An Update. Paris: Springer Paris; 1994. pages 85–9.

38.   Holdaway KM, Finlay GJ, Baguley BC. Relationship of cell cycle parameters to in vitro and in vivo chemosensitivity for a series of Lewis lung carcinoma lines. European Journal of Cancer. 1992;28:1427–31.

39.   Miwa S, Yano S, Kimura H, Yamamoto M, Toneri M, Matsumoto Y, et al. Cell-cycle fate-monitoring distinguishes individual chemosensitive and chemoresistant cancer cells in drug-treated heterogeneous populations demonstrated by real-time FUCCI imaging. Cell Cycle. 2015;14:621–9.

40.   Berndt N, Sebti SM. Measurement of protein farnesylation and geranylgeranylation in vitro, in cultured cells and in biopsies, and the effects of prenyl transferase inhibitors. Nat Protoc. 2011;6:1775–91.

41.   Wang M, Casey PJ. Protein prenylation: unique fats make their mark on biology. Nat Rev Mol Cell Biol. 2016;17:110–22.

42.   Iqbal J, Neppalli VT, Wright G, Dave BJ, Horsman DE, Rosenwald A, et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. J Clin Oncol. 2006;24:961–8.

43.   Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL, et al. Substantial Susceptibility of Chronic Lymphocytic Leukemia to BCL2 Inhibition: Results of a Phase I Study of Navitoclax in Patients With Relapsed or Refractory Disease. Journal of Clinical Oncology. 2012;30:488–96.

44.   Chan KKW, Oza AM, Siu LL. The statins as anticancer agents. Clinical Cancer Research. 2003;9:10–9.

45.   Pan HY, DeVault AR, Wang-Iverson D, Ivashkiv E, Swanson BN, Sugerman AA. Comparative pharmacokinetics and pharmacodynamics of pravastatin and lovastatin. J Clin Pharmacol. 1990;30:1128–35.

46.   Kornblau SM, Banker DE, Stirewalt D, Shen D, Lemker E, Verstovsek S, et al. Blockade of adaptive defensive changes in cholesterol uptake and synthesis in AML by the addition of pravastatin to idarubicin + high-dose Ara-C: a phase 1 study. Blood. American Society of Hematology; 2007;109:2999–3006.

47. Kornbrust DJ, MacDonald JS, Peter CP, Duchai DM, Stubbs RJ, Germershausen JI, et al. Toxicity of the HMG-coenzyme A reductase inhibitor, lovastatin, to rabbits. J Pharmacol Exp Ther. 1989;248:498–505.

48. Ucar M, Mjörndal T, Dahlqvist R. HMG-CoA reductase inhibitors and myotoxicity. Drug Saf. 2000;22:441–57.

49. Blanco-Colio LM, Villa A, Ortego M, Hernández-Presa MA, Pascual A, Plaza JJ, et al. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by downregulation of Bcl-2 expression and Rho A prenylation. Atherosclerosis. 2002;161:17–26.

50. Barrington RE, Subler MA, Rands E, Omer CA, Miller PJ, Hundley JE, et al. A Farnesyltransferase Inhibitor Induces Tumor Regression in Transgenic Mice Harboring Multiple Oncogenic Mutations by Mediating Alterations in Both Cell Cycle Control and Apoptosis. Molecular and Cellular Biology. 1998;18:85–92.

51. Vogt A, Sun J, Qian Y, Hamilton AD, Sebti SM. The Geranylgeranyltransferase-I Inhibitor GGTI-298 Arrests Human Tumor Cells in G0/G1 and Induces p21WAF1/CIP1/SDI1 in a p53-independent Manner. Journal of Biological Chemistry. 1997;272:27224–9.

52. Berndt N, Hamilton AD, Sebti SM. Targeting protein prenylation for cancer therapy. Nature Reviews Cancer. 2011;11:775–91.

53. O'Dwyer PJ, Gallagher M, Nguyen B, Waddell MJ, Chiorean EG. Phase I accelerated dose-escalating safety and pharmacokinetic (PK) study of GGTI-2418, a novel geranylgeranyltransferase I inhibitor in patients with refractory solid tumors. 8th International Symposium on Targeted Anticancer Therapies. Bethesda, MD: Oxford University Press; 2010.

54. Perez-Sala D. Protein isoprenylation in biology and disease: general overview and perspectives from studies with genetically engineered animals. Frontiers in Bioscience. 2007;12:4456.

55. Chan LN, Hart C, Guo L, Nyberg T, Davies BSJ, Fong LG, et al. A novel approach to tag and identify geranylgeranylated proteins. Electrophoresis. 2009;30:3598–606.

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-X$_L$, 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.
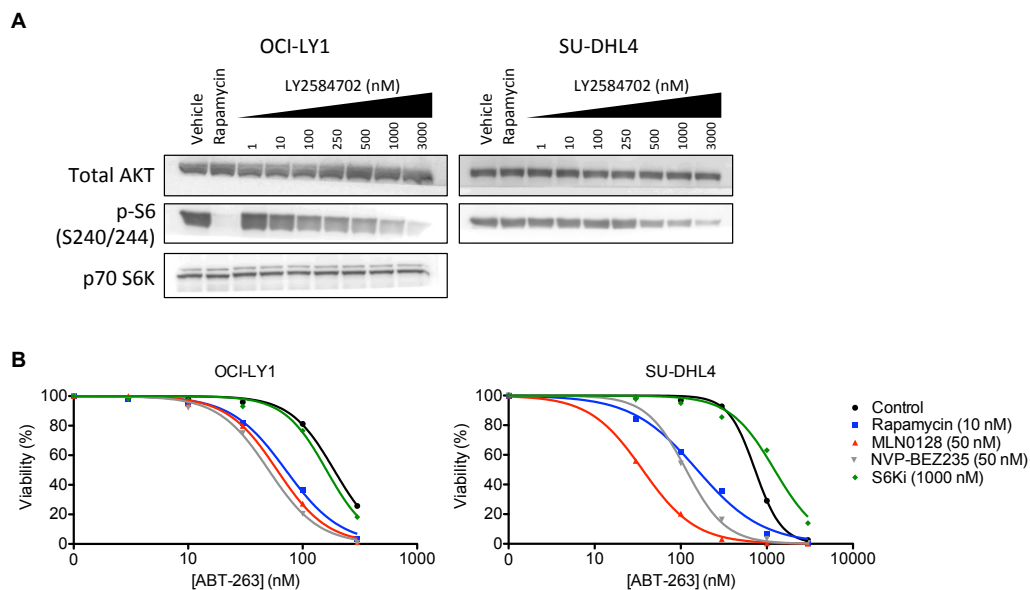

Figure 4.1. S6K inhibition does not sensitize DLBCL cells to ABT-263. (**A**) Western blot of cells treated with increasing doses of S6K1 inhibitor for 3 hours. (**B**) Viability of cells treated with indicated inhibitors for 48 hours. Viability was assessed using 7-AAD dye exclusion by FACS.

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.
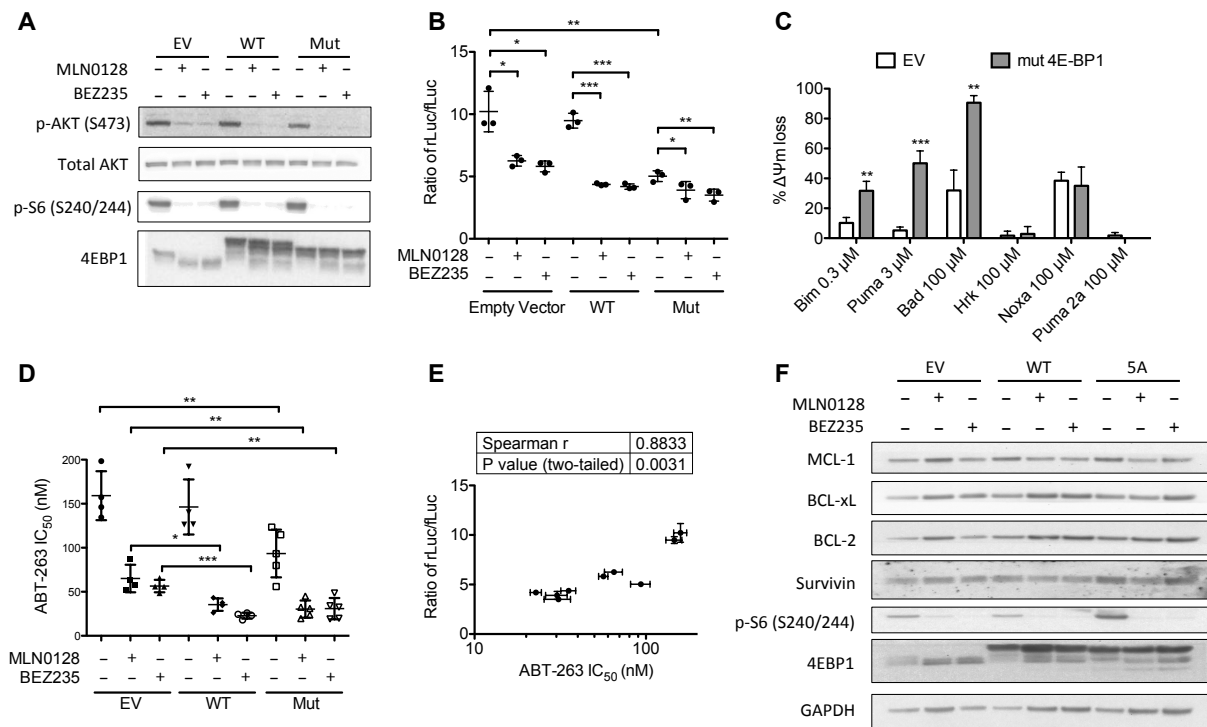
Figure 4.2. Expression of constitutively active 4E-BP1 sensitizes OCI-LY1 cells to ABT-263. (**A**) Western blot of OCI-LY1 cells expressing empty vector, 4E-BP1 wild type (WT), or 4E-BP1-5A mutant (Mut) treated with indicated inhibitors for 3 hours. (**B**) Dual luciferase assay measuring ratio of cap-dependent 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**).
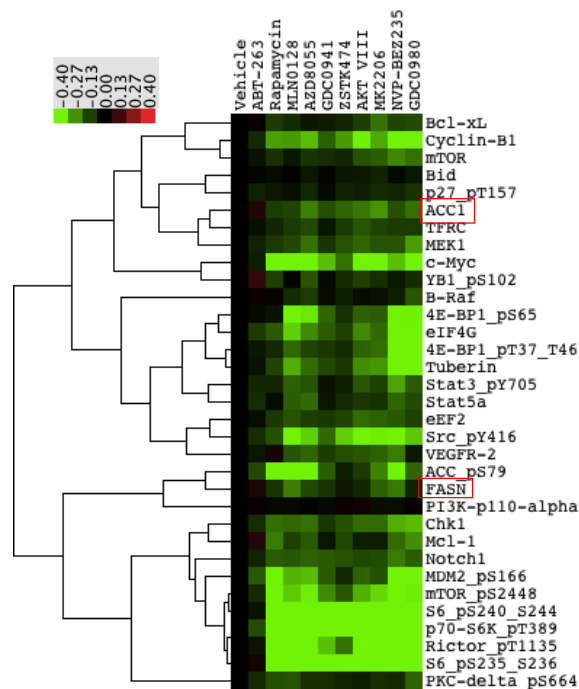
Figure 4.3. PI3K pathway inhibitors suppress expression of SREBP target genes.
Heat map of reverse phase protein array (RPPA) data on SU-DHL4 cells treated with
indicated inhibitors for 3 hours.

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
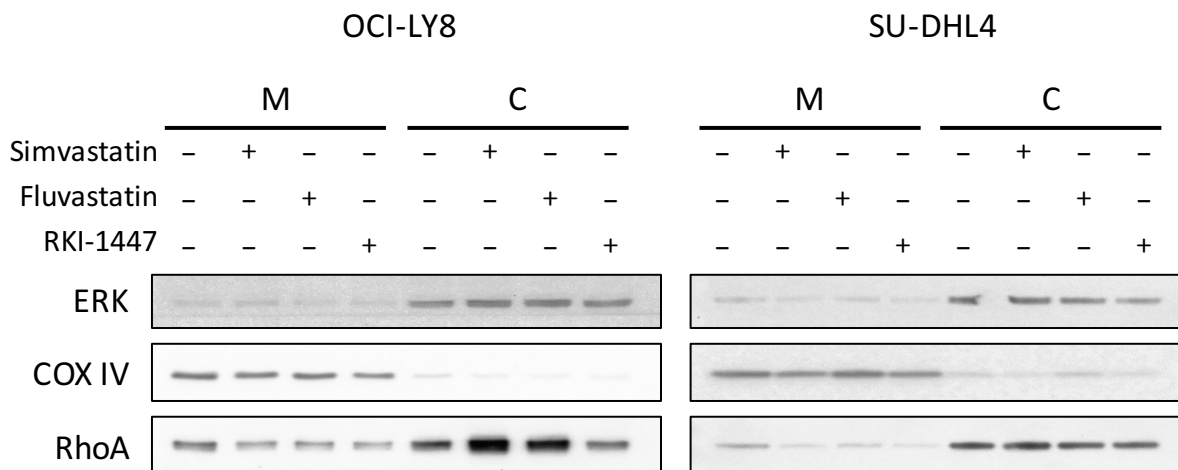
apoptosis and sensitize them to BCL-2 antagonism.

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.
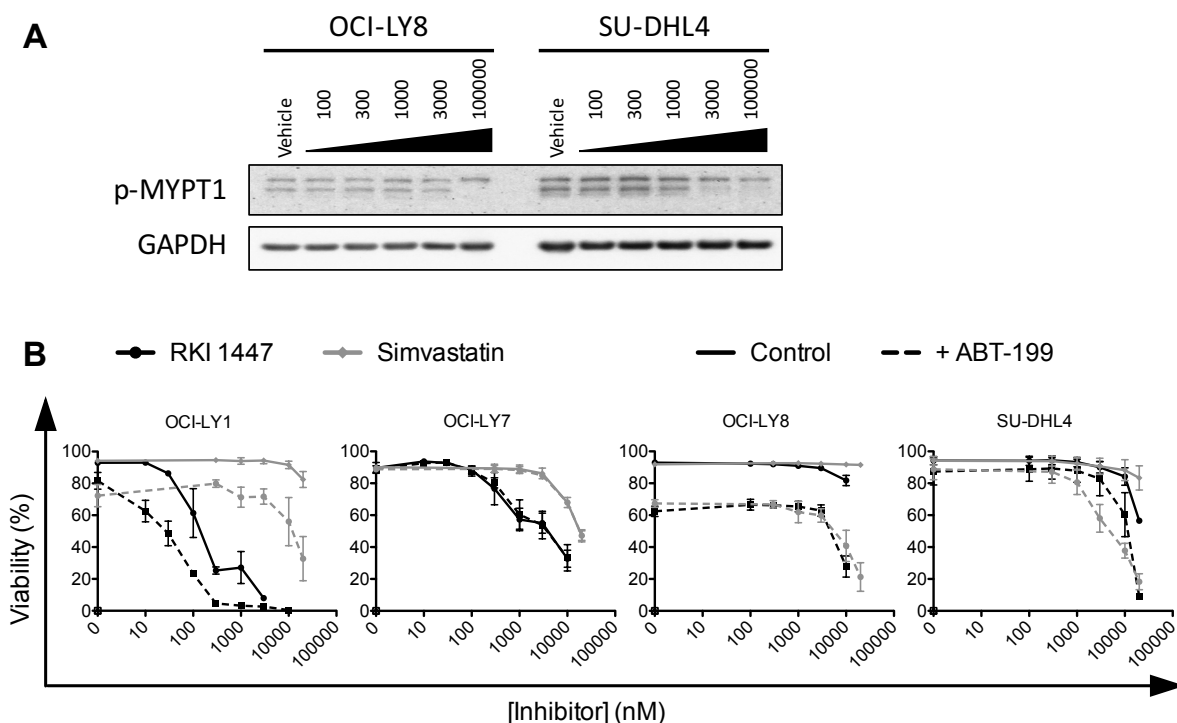
Figure 4.5. ROCK inhibitors recapitulate the sensitizing effect of statins in DLBCL cell lines. (**A**) Western blot of cells treated with indicated concentration (nM) of RKI-1447 for 3 hours. (**B**) Viability of DLBCL cells treated with increasing doses of RKI-1447 (black lines) or simvastatin (grey 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 de-polymerization, 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.

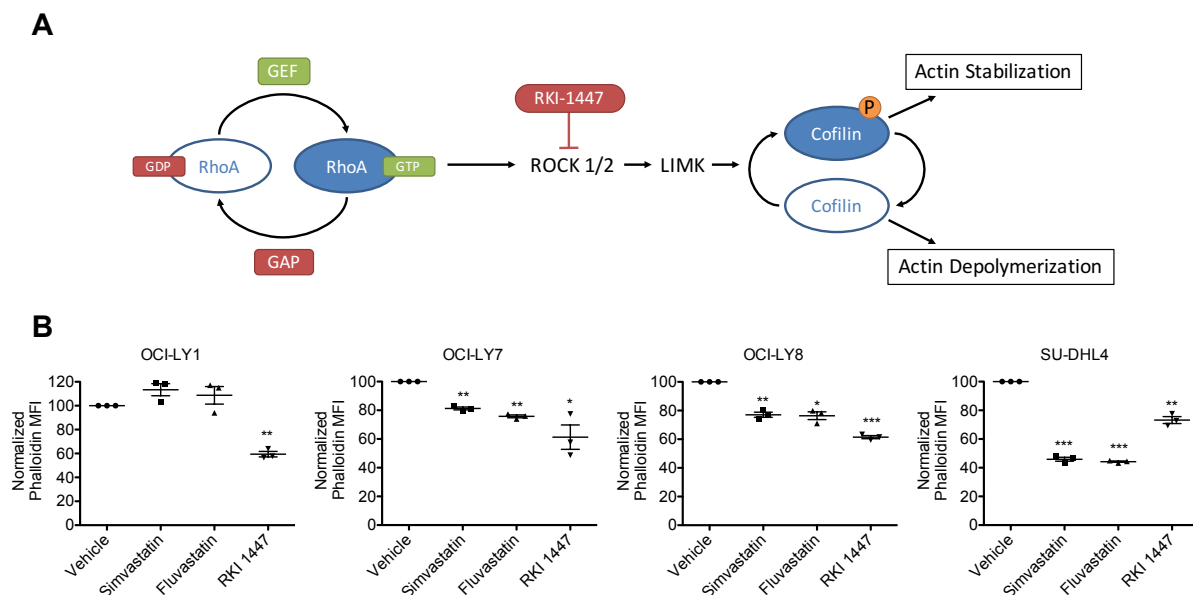**Figure 4.6. Rho and ROCK regulate actin dynamics.** (**A**) Schematic of Rho/ROCK/Actin pathway. Inhibition of RhoA/ROCK releases cofilin from inhibition by LIMK leading to actin de-polymerization. (**B**) MFI of phalloidin staining in DLBCL cells treated with 10 μM indicated inhibitor for 16 hours. Cells were permeabilized and fixed prior to staining with Phalloidin-AlexFluor 647 to measure total f-actin levels.
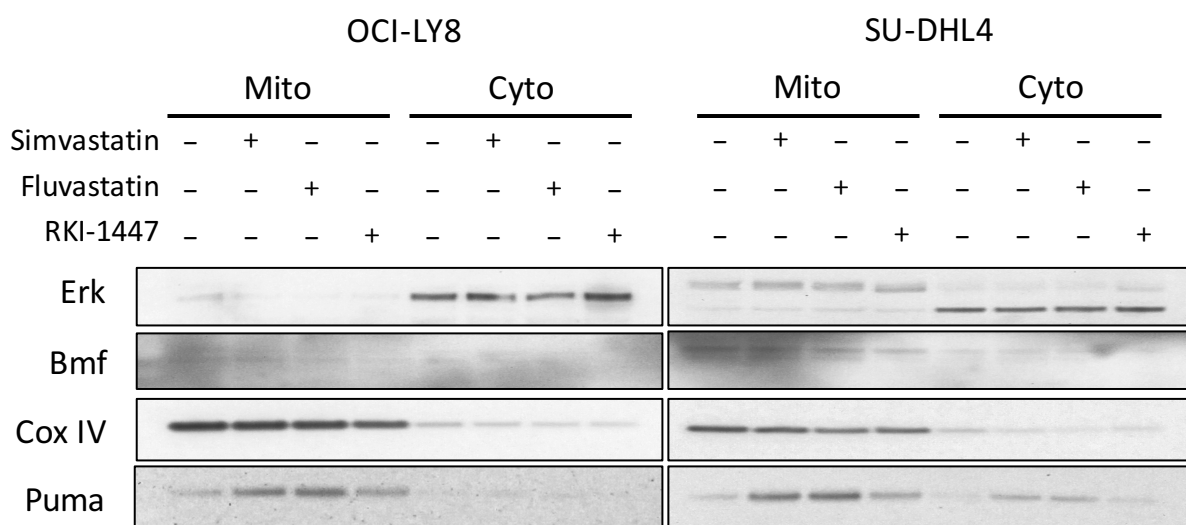
OCI-LY8 / SU-DHL4

	OCI-LY8								SU-DHL4							
	Mito				Cyto				Mito				Cyto			
Simvastatin	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–	–
Fluvastatin	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–
RKI-1447	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+
Erk																
Bmf																
Cox IV																
Puma																

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.

p53-independent mechanisms of PUMA up-regulation

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.
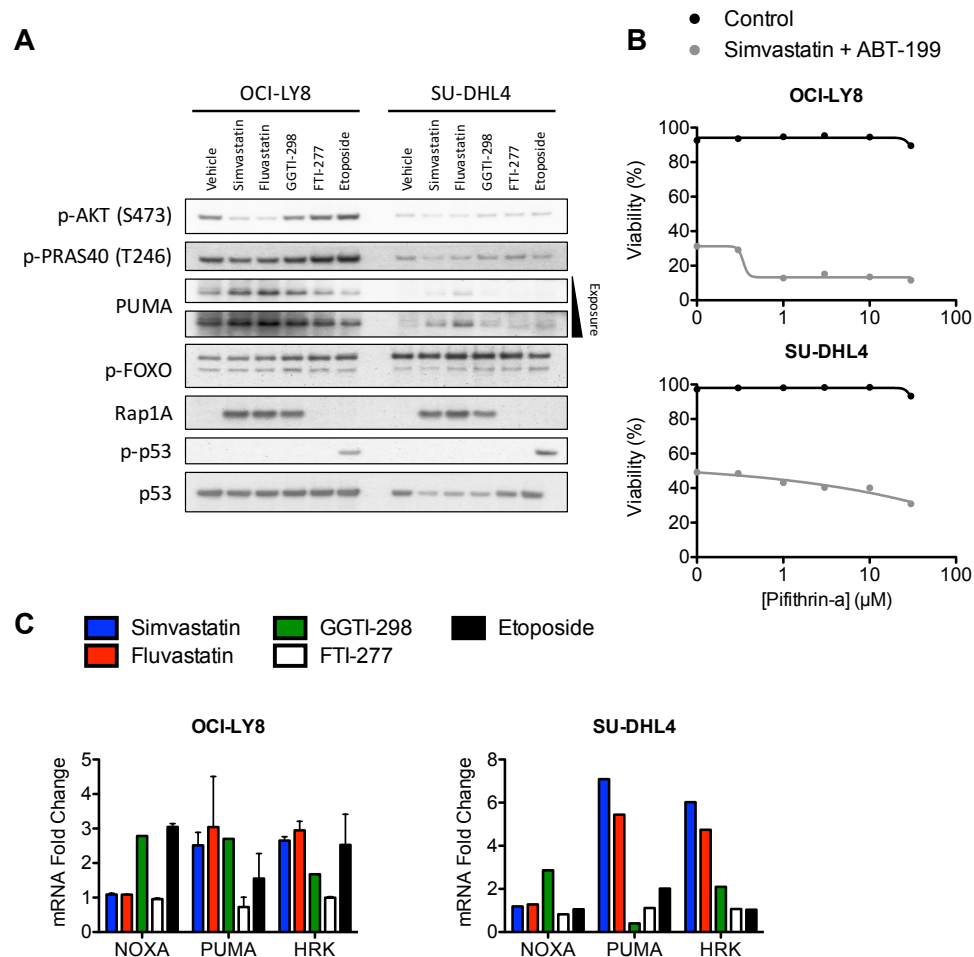
Figure 4.8. Statins increase PUMA transcription in a p53-independent manner. (**A**) Western blot of cells treated with 10 µM of indicated inhibitors for 16 hours prior. (**B**) Viability of cells treated with increasing doses of pifithrin-a. Cells were co-treated with vehicle (black lines) or simvastatin 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.
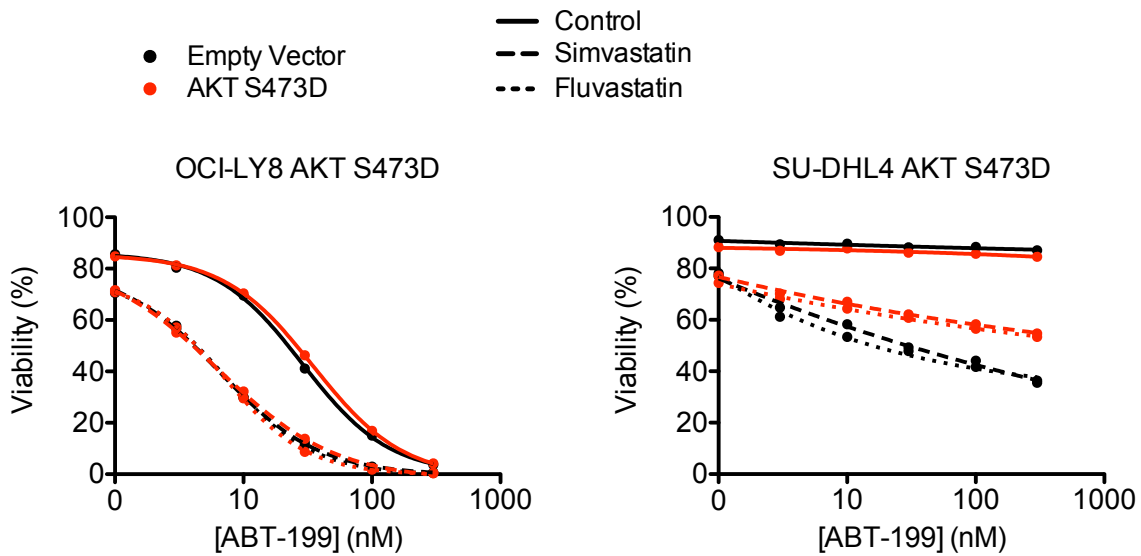
Figure 4.9. AKT S473D expression does not protect from statin sensitization.
Viability of cells expressing AKT S473D (red lines) or empty vector (black lines). Cells were pre-treated with doxycycline to induce expression 24 hours before treating cells with increasing concentrations of ABT-199 with vehicle (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.
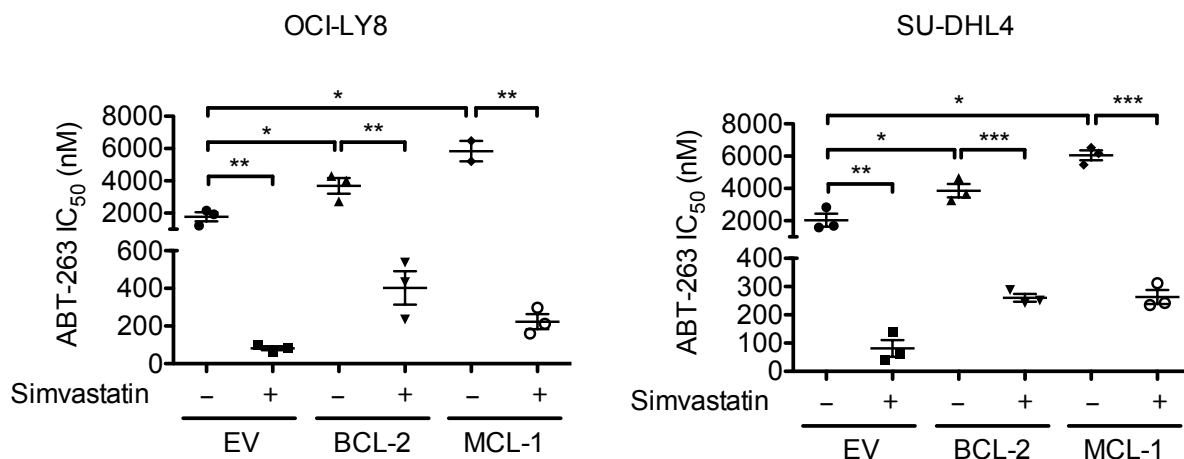
Figure 4.10. BCL-2 and MCL-1 over-expression do not rescue from statin sensitization. Viability of cells pre-treated with doxycycline for 24 hours prior to assessment of ABT-263 IC50 +/- simvastatin (10 µM) after 48 hour treatment.

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.

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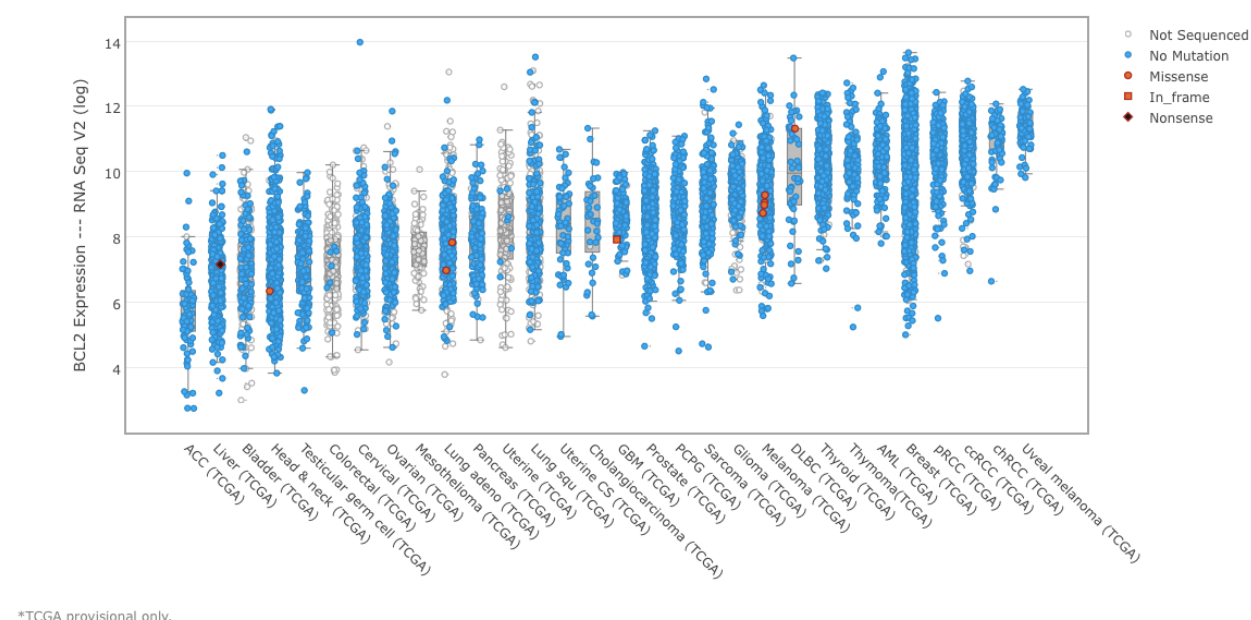
Figure 4.11. Relative expression of BCL-2 mRNA across cancers. Data are taken directly from cBioPortal.org and show relative BCL-2 mRNA expression across indicated cancers subtypes. Data are derived from The Cancer Genome Atlas (TCGA) database.

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,

148

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.
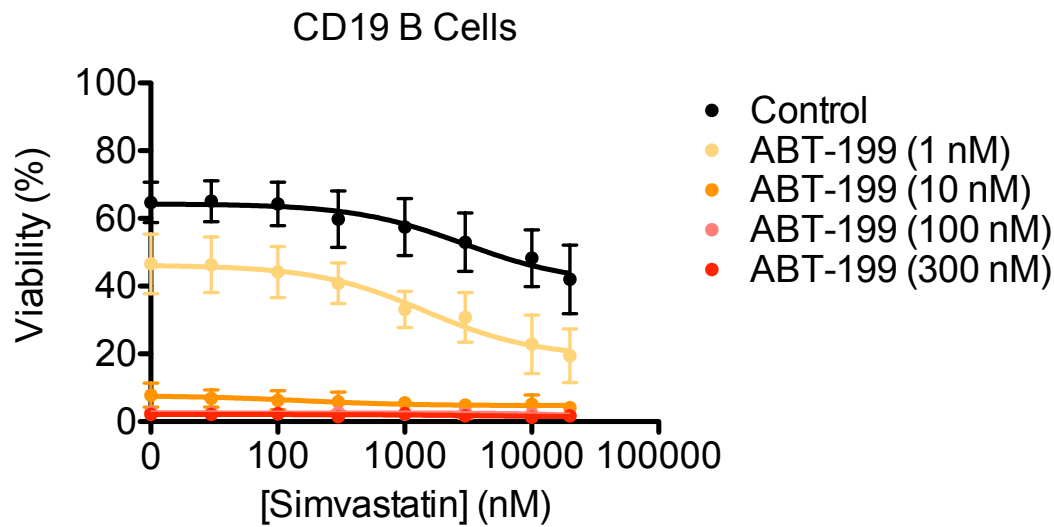
CD19 B Cells

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+).

Mice Weights

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.

151

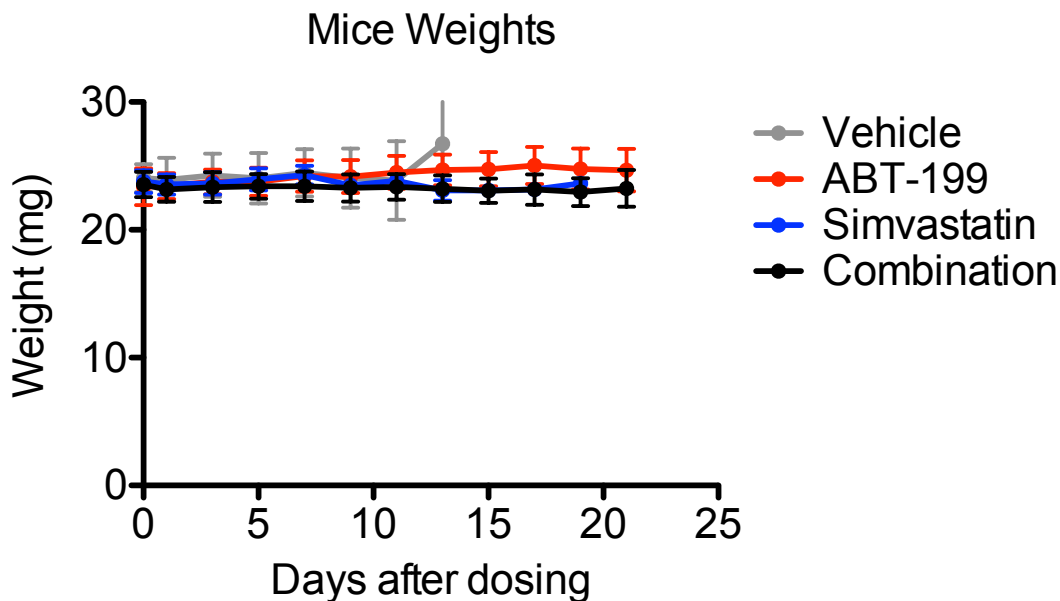Identification/prediction of combination toxicities must be fully investigated using preclinical approaches. Thus, when choosing which combinations to combine with ABT-199, it will be important to consider the toxicities of each individual drug. The safety profile of statins is well-described, with the 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.

References

1. Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. Leukemia. 1996;10:456–9.
2. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest. 2007;117:112–21.
3. Tolcher A, Goldman J, Patnaik A, Papadopoulos KP, Westwood P, Kelly CS, et al. A phase I trial of LY2584702 tosylate, a p70 S6 kinase inhibitor, in patients with advanced solid tumours. European Journal of Cancer. 2014;50:867–75.
4. Yang D, Brunn GJ, Lawrence JC Jr. Mutational analysis of sites in the translational regulator, PHAS-I, that are selectively phosphorylated by mTOR. FEBS Letters. 1999;453:387–90.
5. Alain T, Sonenberg N, Topisirovic I. mTOR inhibitor efficacy is determined by the eIF4E/4E-BP ratio. Oncotarget. 2012;3:1491–2.
6. Feng Y, Pinkerton AB, Hulea L, Zhang T, Davies MA, Grotegut S, et al. SBI-

0640756 Attenuates the Growth of Clinically Unresponsive Melanomas by Disrupting the eIF4F Translation Initiation Complex. Cancer Research. 2015;75:5211–8.

7.   Svitkin YV, Pause A, Haghighat A, Pyronnet S, Witherell G, Belsham GJ, et al. The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. RNA. 2001;7:382–94.

8.   Cencic R, Carrier M, Galicia-Vázquez G, Bordeleau M-E, Sukarieh R, Bourdeau A, et al. Antitumor Activity and Mechanism of Action of the Cyclopenta[b]benzofuran, Silvestrol. Preiss T, editor. PLoS ONE. Public Library of Science; 2009;4:e5223.

9.   Bordeleau M-E, Mori A, Oberer M, Lindqvist L, Chard LS, Higa T, et al. Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. Nat Chem Biol. 2006;2:213–20.

10.   Lucas DM, Edwards RB, Lozanski G, West DA, Shin JD, Vargo MA, et al. The novel plant-derived agent silvestrol has B-cell selective activity in chronic lymphocytic leukemia and acute lymphoblastic leukemia in vitro and in vivo. Blood. 2009;113:4656–66.

11.   Korf U, Löbke C, Sahin Ö, Haller F, Sültmann H, Arlt D, et al. Reverse-phase protein arrays for application-orientated cancer research. Prot Clin Appl. 2009;3:1140–50.

12.   Bengoechea-Alonso MT, Ericsson J. SREBP in signal transduction: cholesterol metabolism and beyond. Current Opinion in Cell Biology. 2007;19:215–22.

13.   Jeon T-I, Osborne TF. SREBPs: metabolic integrators in physiology and metabolism. Trends in Endocrinology & Metabolism. 2012;23:65–72.

14.   Porstmann T, Griffiths B, Chung Y-L, Delpuech O, Griffiths JR, Downward J, et al. PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. Oncogene. 2005;24:6465–81.

15.   Düvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a Metabolic Gene Regulatory Network Downstream of mTOR Complex 1. Molecular Cell. 2010;39:171–83.

16.   Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth. 2008;8:224–36.

17.   Peterson TR, Sengupta SS, Harris TE, Carmack AE, Kang SA, Balderas E, et al. mTOR Complex 1 Regulates Lipin 1 Localization to Control the SREBP Pathway. Cell. 2011;146:408–20.

18.   Puthalakath H. Bmf: A Proapoptotic BH3-Only Protein Regulated by Interaction with the Myosin V Actin Motor Complex, Activated by Anoikis. Science. American Association for the Advancement of Science; 2001;293:1829–32.

19.   Solski PA, Helms W, Keely PJ, Su L, Der CJ. RhoA biological activity is dependent on prenylation but independent of specific isoprenoid modification. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research. NIH Public Access; 2002;13:363–73.

20.   Ying H, Biroc SL, Li W-W, Alicke B, Xuan J-A, Pagila R, et al. The Rho kinase inhibitor fasudil inhibits tumor progression in human and rat tumor models. Molecular Cancer Therapeutics. American Association for Cancer Research;

2006;5:2158–64.

21. Morgan-Fisher M, Wewer UM, Yoneda A. Regulation of ROCK Activity in Cancer. Journal of Histochemistry and Cytochemistry. Histochemical Society; 2013;61:185–98.

22. Berndt N, Sebti SM. Measurement of protein farnesylation and geranylgeranylation in vitro, in cultured cells and in biopsies, and the effects of prenyl transferase inhibitors. Nat Protoc. 2011;6:1775–91.

23. Perez-Sala D. Protein isoprenylation in biology and disease: general overview and perspectives from studies with genetically engineered animals. Frontiers in Bioscience. 2007;12:4456.

24. Chan LN, Hart C, Guo L, Nyberg T, Davies BSJ, Fong LG, et al. A novel approach to tag and identify geranylgeranylated proteins. Electrophoresis. 2009;30:3598–606.

25. Diehl P, Tedesco D, Chenchik A. Use of RNAi screens to uncover resistance mechanisms in cancer cells and identify synthetic lethal interactions. Drug Discov Today Technol. 2014;11:11–8.

26. Silva JM, Marran K, Parker JS, Silva J, Golding M, Schlabach MR, et al. Profiling essential genes in human mammary cells by multiplex RNAi screening. Science. 2008;319:617–20.

27. Luo B, Cheung HW, Subramanian A, Sharifnia T, Okamoto M, Yang X, et al. Highly parallel identification of essential genes in cancer cells. Proc Natl Acad Sci U S A. 2008;105:20380–5.

28. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, et al. A Chemical Inhibitor of p53 That Protects Mice from the Side Effects of Cancer Therapy. Science. American Association for the Advancement of Science; 1999;285:1733–7.

29. Karpinich NO, Tafani M, Rothman RJ, Russo MA, Farber JL. The Course of Etoposide-induced Apoptosis from Damage to DNA and p53 Activation to Mitochondrial Release of Cytochromec. Journal of Biological Chemistry. American Society for Biochemistry and Molecular Biology; 2002;277:16547–52.

30. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011;1813:1978–86.

31. Choudhary GS, Al-harbi S, Mazumder S, Hill BT, Smith MR, Bodo J, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. Cell Death Dis. 2015;6:e1593.

32. Del Gaizo Moore V, Schlis KD, Sallan SE, Armstrong SA, Letai A. BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. Blood. 2008;111:2300–9.

33. Friedman AA, Letai A, Fisher DE, Flaherty KT. Precision medicine for cancer with next-generation functional diagnostics. Nat Rev Cancer. 2015;15:747–56.

34. Derijcke S, Vansteenkiste J. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. Breathe; 2009.

35. Kwak EL, Bang Y-J, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J

Med. 2010;363:1693–703.

36. Piccart-Gebhart MJ, Procter M. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. … England Journal of …. 2005.

37. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005;353:1673–84.

38. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010;363:809–19.

39. Chapman PB, Hauschild A, Robert C. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. … England Journal of …. 2011.

40. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med. 2009;361:123–34.

41. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. Nature. 2014;505:495–501.

42. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discovery. 2012;2:401–4.

43. Dienstmann R, Jang IS, Bot B, Friend S, Guinney J. Database of genomic biomarkers for cancer drugs and clinical targetability in solid tumors. Cancer Discovery. American Association for Cancer Research; 2015;5:118–23.

44. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nature Reviews …. 2013.

45. Wheler JJ, Yelensky R. Prospective study comparing outcomes in patients with advanced malignancies on molecular alteration-matched versus non-matched therapy. Science. 2015.

46. Vo T-T, Ryan J, Carrasco R, Neuberg D, Rossi DJ, Stone RM, et al. Relative Mitochondrial Priming of Myeloblasts and Normal HSCs Determines Chemotherapeutic Success in AML. Cell. 2012;151:344–55.

47. Davids MS, Deng J, Wiestner A, Lannutti BJ, Wang L, Wu CJ, et al. Decreased mitochondrial apoptotic priming underlies stroma-mediated treatment resistance in chronic lymphocytic leukemia. Blood. American Society of Hematology; 2012;120:3501–9.

48. Colak S, Zimberlin CD, Fessler E, Hogdal L, Prasetyanti PR, Grandela CM, et al. Decreased mitochondrial priming determines chemoresistance of colon cancer stem cells. Cell Death Differ. 2014;21:1170–7.

49. Negishi I, Nakayama K, Kuida K, Shinkai Y. Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science. 1993.

50. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell. 1993;75:229–40.

51. Touzeau C, Ryan J, Guerriero J, Moreau P, Chonghaile TN, Le Gouill S, et al. BH3 profiling identifies heterogeneous dependency on Bcl-2 family members in multiple myeloma and predicts sensitivity to BH3 mimetics. Leukemia. 2015.

52. Woo D. Apoptosis and loss of renal tissue in polycystic kidney diseases. N Engl J Med. 1995;333:18–25.
53. Gobé G, Rubin M, Williams G, Sawczuk I, Buttyan R. Apoptosis and expression of Bcl-2, Bcl-XL, and Bax in renal cell carcinomas. Cancer Investigation. 2002;20:324–32.
54. Huang A, Fone PD, Gandour-Edwards R, White RW, Low RK. Immunohistochemical analysis of BCL-2 protein expression in renal cell carcinoma. J Urol. 1999;162:610–3.
55. Vogler M. Targeting BCL2-Proteins for the Treatment of Solid Tumours. Adv Med. Hindawi Publishing Corporation; 2014;2014:943648.
56. Seymour JF, Gerecitano JF, Kahl BS, Pagel JM. The single-agent Bcl-2 inhibitor ABT-199 (GDC-0199) in patients with relapsed/refractory (R/R) non-Hodgkin lymphoma (NHL): responses observed in all …. 2013.
57. Zhang H, Nimmer PM, Tahir SK, Chen J, Fryer RM, Hahn KR, et al. Bcl-2 family proteins are essential for platelet survival. Cell Death Differ. 2007.
58. Wilson WH, O'Connor OA, Czuczman MS, LaCasce AS, Gerecitano JF, Leonard JP, et al. Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. The Lancet Oncology. 2010;11:1149–59.
59. Vlahovic G, Karantza V, Wang D, Cosgrove D, Rudersdorf N, Yang J, et al. A phase I safety and pharmacokinetic study of ABT-263 in combination with carboplatin/paclitaxel in the treatment of patients with solid tumors. Invest New Drugs. 2014;32:976–84.
60. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013;19:202–8.
61. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. Blood. 2010;115:3304–13.
62. Fesik SW. Abstract SY16-02: The discovery of Mcl-1 inhibitors using fragment-based methods and structure-based design. Cancer Research. 2016.
63. Lee T, Bian Z, Belmar J, Shaw S, Tarr JC, Zhao B. Discovery of orally bioavailable novel Mcl-1 inhibitors that exhibit selective anti-proliferative activity in Mcl-1 sensitive cancer cell lines. Cancer Research. 2016.
64. Sensintaffar JL, Arnold A, Goodwin C, Hogdal L, Shaw S, Tarr JC, et al. Small molecule Mcl-1 inhibitors induce apoptosis and death in multiple cancer subtypes in vitro. Cancer Research. 2016.
65. Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G, et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. Nature. Nature Research; 2016;538:477–82.
66. Placzek WJ, Wei J, Kitada S, Zhai D, Reed JC, Pellecchia M. A survey of the anti-apoptotic Bcl-2 subfamily expression in cancer types provides a platform to predict the efficacy of Bcl-2 antagonists in cancer therapy. Cell Death Dis. 2010;1:e40.
67. Opferman JT. Obligate Role of Anti-Apoptotic MCL-1 in the Survival of Hematopoietic Stem Cells. Science. 2005;307:1101–4.

68.   Ucar M, Mjörndal T, Dahlqvist R. HMG-CoA reductase inhibitors and myotoxicity. Drug Saf. 2000;22:441–57.

69.   Blanco-Colio LM, Villa A, Ortego M, Hernández-Presa MA, Pascual A, Plaza JJ, et al. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by downregulation of Bcl-2 expression and Rho A prenylation. Atherosclerosis. 2002;161:17–26.

70.   Kim WS, Kim MM, Choi HJ, Yoon S-S, Lee MH, Park K, et al. Phase II Study of High-Dose Lovastatin in Patients with Advanced Gastric Adenocarcinoma. Invest New Drugs. Kluwer Academic Publishers; 2001;19:81–3.

71.   Thibault A, Samid D, Tompkins AC, Figg WD, Cooper MR, Hohl RJ, et al. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clinical Cancer Research. 1996;2:483–91.

72.   Bargossi AM, Grossi G, Fiorella PL, Gaddi A, Di Giulio R, Battino M. Exogenous CoQ10 supplementation prevents plasma ubiquinone reduction induced by HMG-CoA reductase inhibitors. Mol Aspects Med. 1994;15 Suppl:s187–93.

73.   Effect of atorvastatin on left ventricular diastolic function and ability of coenzyme Q 10 to reverse that dysfunction. The American journal of …. 2004.

74.   Serra V, Markman B, Scaltriti M, Eichhorn PJA, Valero V, Guzmán M, et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Research. 2008;68:8022–30.

75.   Wallin JJ, Edgar KA, Guan J, Berry M, Prior WW, Lee L, et al. GDC-0980 is a novel class I PI3K/mTOR kinase inhibitor with robust activity in cancer models driven by the PI3K pathway. Molecular Cancer Therapeutics. 2011;10:2426–36.

76.   Dienstmann R, Rodón J, Serra V, Tabernero J. Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. Molecular Cancer Therapeutics. 2014;13:1021–31.

77.   Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014;13:140–56.

78.   Nitulescu GM, Margina D, Juzenas P, Peng Q, Olaru OT, Saloustros E, et al. Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use (Review). Int J Oncol. 2016;48:869–85.

79.   Ahn DH, Li J, Wei L, Doyle A, Marshall JL, Schaaf LJ, et al. Results of an abbreviated phase-II study with the Akt Inhibitor MK-2206 in Patients with Advanced Biliary Cancer. Sci Rep. 2015;5:12122.

80.   Hess G, Herbrecht R, Romaguera J, Verhoef G, Crump M, Gisselbrecht C, et al. Phase III Study to Evaluate Temsirolimus Compared With Investigator's Choice Therapy for the Treatment of Relapsed or Refractory Mantle Cell Lymphoma. Journal of Clinical Oncology. 2009;27:3822–9.

81.   Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. 2014;19:373–9.

82.   Santulli G, Totary-Jain H. Tailoring mTOR-based therapy: molecular evidence and clinical challenges. Pharmacogenomics. 2013;14:1517–26.

83.   Basu B, Dean E, Puglisi M, Greystoke A, Ong M, Burke W, et al. First-in-Human Pharmacokinetic and Pharmacodynamic Study of the Dual m-TORC 1/2 Inhibitor AZD2014. Clinical Cancer Research. 2015;21:3412–9.

84. Bendell JC, Kelley RK, Shih KC, Grabowsky JA, Bergsland E, Jones S, et al. A phase I dose-escalation study to assess safety, tolerability, pharmacokinetics, and preliminary efficacy of the dual mTORC1/mTORC2 kinase inhibitor CC-223 in patients with advanced solid tumors or multiple myeloma. Cancer. 2015;121:3481–90.

85. Infante JR, Tabernero J, Cervantes A, Jalal S. Abstract C252: A phase 1, dose-escalation study of MLN0128, an investigational oral mammalian target of rapamycin complex 1/2 (mTORC1/2) catalytic inhibitor, in …. Mol Cancer. 2013.