

DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD
DASH.HARVARD.EDU

HARVARD LIBRARY


Mitochondrial Priming and Anti-Apoptotic Dependencies in Aging and Diseased Bone Marrow

Citation

Hogdal, Leah Justine. 2015. Mitochondrial Priming and Anti-Apoptotic Dependencies in Aging and Diseased Bone Marrow. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

Link

http://nrs.harvard.edu/urn-3:HUL.InstRepos:17467490

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFlOWI2M2EwYTg

Accessibility

https://accessibility.huit.harvard.edu/digital-accessibility-policy

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. Submit a story

Mitochondrial Priming and Anti-apoptotic Dependencies in Aging and Diseased Bone Marrow

A dissertation presented

by

Leah Justine Hogdal

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological Chemistry and Molecular Pharmacology

Harvard University

Cambridge, Massachusetts

April 2015

© 2015 Leah Justine Hogdal

All rights reserved.

Dr. Anthony Letai

Leah Justine Hogdal

Mitochondrial Priming and Anti-apoptotic Dependencies in Aging and Diseased Bone Marrow

Abstract

This thesis explores two questions; how aging of the hematopoietic stem cell (HSC) may contribute to the increase in hematological malignancies with age and secondly, how hematological malignancies can be better treated when they arise. At the intersection of both of these questions is the mitochondrial apoptotic pathway and the tool, BH3 profiling, which measures the mitochondrial priming and anti-apoptotic dependencies within normal and malignant cells. Mitochondrial priming and anti-apoptotic dependencies are measured by assessing the sensitivity of cellular mitochondria to standardized amounts of BH3 peptides derived from the BH3 domains of pro-apoptotic proteins.

In our first study, we use BH3 profiling to identify anti-apoptotic dependencies to direct treatment of the anti-apoptotic BCL-2 inhibitor ABT-199 in acute myeloid leukemia (AML). We found that AML blasts are often dependent on the pro-survival protein BCL-2 and the mitochondrial dependence on BCL-2 measured by BH3 profiling correlated with cellular sensitivity to ABT-199. These pre-clinical results showed that ABT-199 was functioning on-target at the mitochondria and that BH3 profiling could be used to identify patients who would be most sensitive to BCL-2 inhibition. Importantly, these results directly led to a Phase II clinical trial of ABT-199 in relapsed/refractory AML patients and BH3 profiling was integrated into the study to test its efficacy as a predictive biomarker. In the clinical trial, we showed that BH3 profiling correlated well with clinical response.

Secondly, in our studies of aging in the HSC compartment, we used BH3 profiling to explore how mitochondrial priming is altered during normal hematopoietic differentiation and during differentiation of HSCs isolated from young and old mice. We found that HSCs are less primed than more differentiated progenitor cells and that old HSCs are even less primed than young HSCs which correlates with decreased sensitivity to apoptotic stimuli of old HSCs. These studies expand upon the biological understanding of functional defects of aged HSCs, and showed for the first time in a clinical setting that BH3 profiling may be used successfully to direct treatment of BH3 mimetics in the clinic.

CONTENTS

Chapter 1: Introduction 1
I. Overview 1
II. Mitochondrial apoptosis in normal physiology 3
BCL-2 family interactions control mitochondrial apoptosis 4
BH3 profiling identifies cells primed for death and Anti-apoptotic Addictions 9
III. Acute Myeloid Leukemia 13
Current standard of care for AML 14
Molecular characterization of AML 16
Novel therapies in AML 18
IV. Physiological hematopoiesis 24
Hematopoiesis during aging 29
Intrinsic and extrinsic factors effecting aging HSCs 33

Chapter 2: Selective BCL-2 inhibition by ABT-199 causes on-target cell death in pre-clinical models of acute myeloid leukemia 38
I. Abstract 38
II. Introduction 38
III. Methods 40
III. Results 46
IV. Discussion 58

Chapter 3: BH3 Profiling Predicts clinical response in a Phase II clinical trial of ABT-199 in Acute Myeloid Leukemia 61
I. Abstract 61
II. Introduction 61
III. Methods 63
IV. Results 65
V. Discussion 75

Chapter 4: Mitochondrial priming Increases during differentiation and Decreases with aging in Hematopoietic Stem Cells 79
I. Abstract 79
II. Introduction 79
III. Methods 82
IV. Results 85
V. Discussion 95

Chapter 5: Conclusions and Future Directions 99

Appendix: Supplemental figures and tables 115
References 120

Acknowledgements

This thesis could not have been accomplished without the support, encouragement, advice and guidance of many people.

To **Dr. Anthony Letai** who has been an excellent mentor throughout my graduate school career. He allowed me to take on the projects that most interested me, provided support and encouragement when I was struggling, and perhaps most importantly, taught me the importance of maintaining a balance between life and research. To all the members of the **Letai Lab** for their willingness to share their knowledge and expertise, and for being wonderful friends inside and outside of the lab. I'd like to especially thank my fellow office mates. **Jeremy Ryan** for his incredible technical expertise, and **Richard Oakley**, for his constant positive attitude and willingness to help me with any task no matter how big or how small. And to my **DAC committee**, Dr. David Frank, Dr. Joan Brugge and Dr. Tim Mitchison for their helpful guidance and perspectives in our meetings.

To **my parents** who taught me the value of hard work, perseverance, a positive attitude and reminding me to keep things in perspective. To **my sisters** Cassie, Meghan and Alicia who set the bar extremely high for me academics and athletics, and I am only where I am today because I was trying to meet the expectations they set. And to **Andrew Vesper** for making my life so much easier and happier.

CHAPTER 1: INTRODUCTION

I. OVERVIEW

Organismal aging is accompanied with a host of unwanted phenotypes, and is the major risk factor for a variety of human pathologies including diabetes, cardiovascular and neurological disorders as well as cancer, including hematological malignancies (**Figure 1.1**) (*1*). Therefore, it is necessary to understand how organismal cell maintenance is altered with age to improve quality of life for the aging and elderly. This thesis is composed of two projects involving the aging hematopoietic compartment. At the intersection of these two projects is the cell suicide pathway, apoptosis, and a tool called BH3 profiling a functional assay of mitochondrial readiness for apoptosis.

In the first project we investigate a novel method to treat acute myeloid leukemia (AML), a frequently diagnosed hematological malignancy in the elderly. Previous work in our lab identified that a portion of AML blasts are dependent on the anti-apoptotic protein BCL-2 for survival. The recent development of ABT-199, a potent and selective inhibitor of BCL-2, provided the opportunity to test if the small molecule could be effective in the treatment of AML patients. As previous work suggested that some, but not all AML blasts are dependent on BCL-2, we use BH3 profiling to identify anti-apoptotic dependencies of AML blasts. In this first project we test the hypothesis that BCL-2 dependent mitochondria, measured by BH3 profiling, will be more sensitive to inhibition of BCL-2. We test this hypothesis in pre-clinical *in vitro* and *ex vivo* experiments (Chapter 2), as well as *in vivo* in a Phase II clinical trial of ABT-199 in AML (Chapter 3).

In the second project we examine how mitochondrial apoptosis is controlled during normal hematopoietic stem cell (HSC) differentiation and stem cell aging. Preservation of HSC cell fate, whether the HCS undergoes differentiation, self-renewal or apoptosis, throughout life is necessary for organismal maintenance. However, there is a dearth of information on how HSCs control the apoptotic cell fate at steady state and during aging. As two hallmarks of HSC aging are increases in HSC number and impaired HSC function, we hypothesize that deficiencies in apoptosis contribute to these phenotypes. Therefore, in the second project we test the hypothesis that aging of the HSC is associated with a functional decline in apoptosis. We test this hypothesis by measuring mitochondrial priming of cells during differentiation and aging, as well as assessing the response of young and old HSCs to DNA damage.

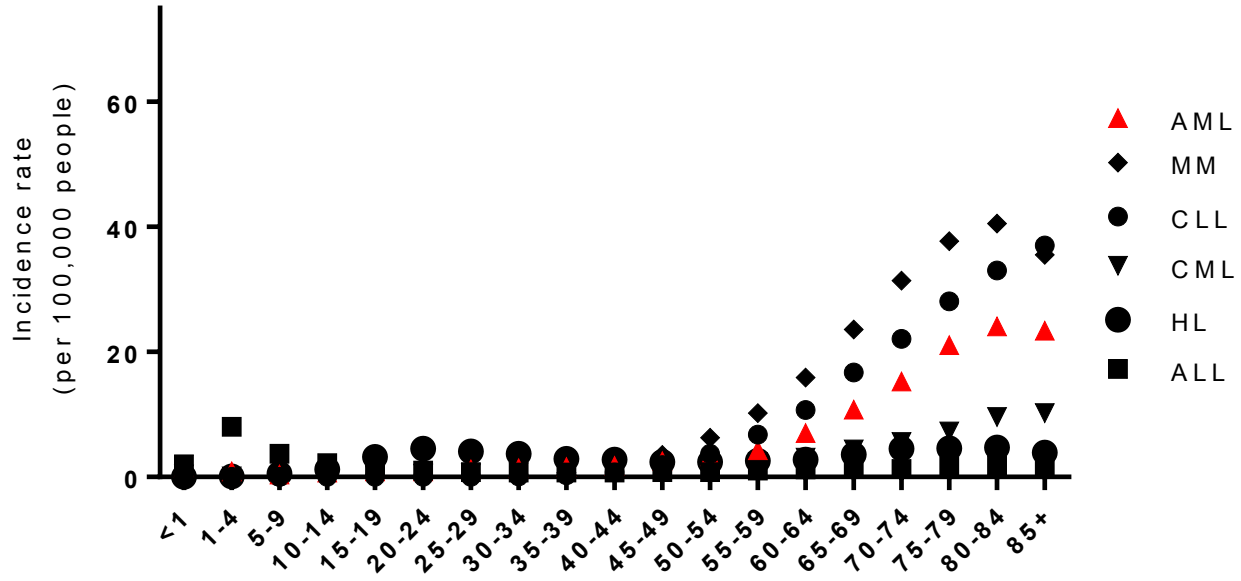


Figure 1.1 Incidence of hematological cancers increases with age.
There is an increase in the incidence of hematological malignancies with age (multiple myeloma (MM), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), Hodgkins lymphoma (HL) and acute lymphocytic leukemia (ALL)). Data generated from SEER.cancer.gov

The broader goal of both of these projects is to provide an enhanced understanding of how cancer develops and how to better treat cancer when it arises. The first project directly addresses this goal by testing a novel small molecule, ABT-199, in the treatment of AML. The second project provides evidence that aging of the HSC is accompanied with decreased sensitivity to apoptosis which may contribute the accumulation of DNA damaged HSCs with age. The purpose of this introduction is to put these projects in perspective by providing an overview of the current state of knowledge in mitochondrial apoptosis, AML and HSC aging.

II. Mitochondrial apoptosis in normal physiology

Programmed cell death, or apoptosis, is an important component of multi-cellular life. Early human development is dominated by continuous cell proliferation and apoptosis which is tightly regulated to support normal organismal maturation (*2*). Defects in apoptosis result in autoimmune and neurodegenerative disorders in the case of excessive apoptosis, and cancer in the case of insufficient apoptosis (*3*). Therefore, investigation of how apoptosis is controlled in the setting of normal organismal cell maintenance and in the development of disease is not only necessary to improve understanding of cell death biology, but is also essential for identifying potential novel therapeutic targets to stimulate or inhibit the apoptotic pathway.

Apoptosis is primarily controlled via two pathways which converge on the activation of executioner caspases, a class of cysteine proteases responsible for many of the phenotypes of apoptotic cells (*3*). Death receptor mediated cell killing, or the extrinsic apoptotic pathway, is initiated by the ligation of cell surface death receptors such as tumor necrosis factor receptor (TNFR) or Fas which contain a death domain in the cytoplasmic region of the receptor (*4, 5*). Activation of the death receptors by extracellular ligand binding results in recruitment of the initiator caspase, caspase 8, to the death domain. When activated, caspase 8 initiates a caspase cascade by directly or indirectly cleaving executioner caspases, caspase -3 and caspase -7, which are responsible for the execution of cell killing (*6*).

Commitment to death via the intrinsic mitochondrial apoptotic pathway is regulated by members of the B-cell leukemia/ lymphoma 2 (BCL-2) protein family. Responding to a of myriad upstream apoptotic

signals, members of this protein family control mitochondrial outer membrane permeabilization (MOMP), a critical point of no return in which cell death is inevitable when triggered (*7*). The BCL-2 family consists of three subfamilies which are categorized according to function and sequence homology within the four B-cell 2 homology (BH) domains (**Figure 1.2A**). The anti-apoptotic proteins inhibit apoptosis by binding to and sequestering pro-apoptotic proteins. These proteins include BCL-2, MCL-1, BCL-XL, BCL-w, and BFL1 and contain all four of the BH domains (BH1-4) (See box 1 for full names) (*8-11*). The pro-apoptotic effector proteins, BAX and BAK, contain BH domains 1-3, and are responsible for the execution of apoptosis by oligomerizing at the mitochondrial outer membrane to promote MOMP (*12, 13*) . The third class is the BH3-only proteins share only the 3rd BH3 domain. The BH3-only proteins can be further subdivided into two groups based on function – the "activators" (BID and BIM) and "sensitizers" (BAD, BIK, HRK, NOXA, PUMA, and BMF) (*14-19*). The activator BH3-only regulate apoptosis by directly binding to and activating BAX and BAK, whereas the sensitizer BH3-only proteins promote apoptosis by binding to and inhibiting the anti-apoptotic proteins. This allows the pro-apoptotic activator proteins to be displaced from that anti-apoptotics effectively sensitizing the cells to death (*14*).

BCL-2 FAMILY INTERACTIONS CONTROL MITOCHONDRIAL APOPTOSIS

In healthy cells, un-activated BAK is primarily tethered to the outer mitochondrial membrane by its C-terminal domain, whereas un-activated BAX is primarily localized to the cytosol (*20, 21*). When there is a death stimulus such as nutrient deprivation, radiation, or chemotherapy, upstream signals stimulate the activator BH3-only proteins BIM and BID either by post-translational modification or de novo translation (*22*). The activator BH3-only protein, BID, can also be directly activated by caspase-8 which cleaves full length BID to truncated BID (tBID), which targets it to the mitochondria to activate BAK (*23*). This is one way in which there is convergence between the extrinsic and intrinsic apoptotic pathways (*24*). When BIM and BID are activated they bind to and activate BAK and BAX which causes the effector proteins to

Box 1.1 Names of BCL-2 family members
BAK, BCL-2-antagonist/killer-1; BAX, BCL-2-associated X protein; BAD, BCL-2 antagonist of cell death; BCL-2, B cell lymphoma 2; MCL1,myeloid cell leukemia 1; BCL-XL, B-cell lymphoma extra-large; BCL-w BFL1, Bcl-2-related protein A1; BID, BH3-interacting domain death agonist; BIK, BCL-2-interacting killer; BIM, BCL-2-like-11; BMF, BCL-2 modifying factor; BOK, BCL-2-related ovarian killer; HRK, harakiri (also known as death protein-5); PUMA, p53 upregulator modulator of apoptosis

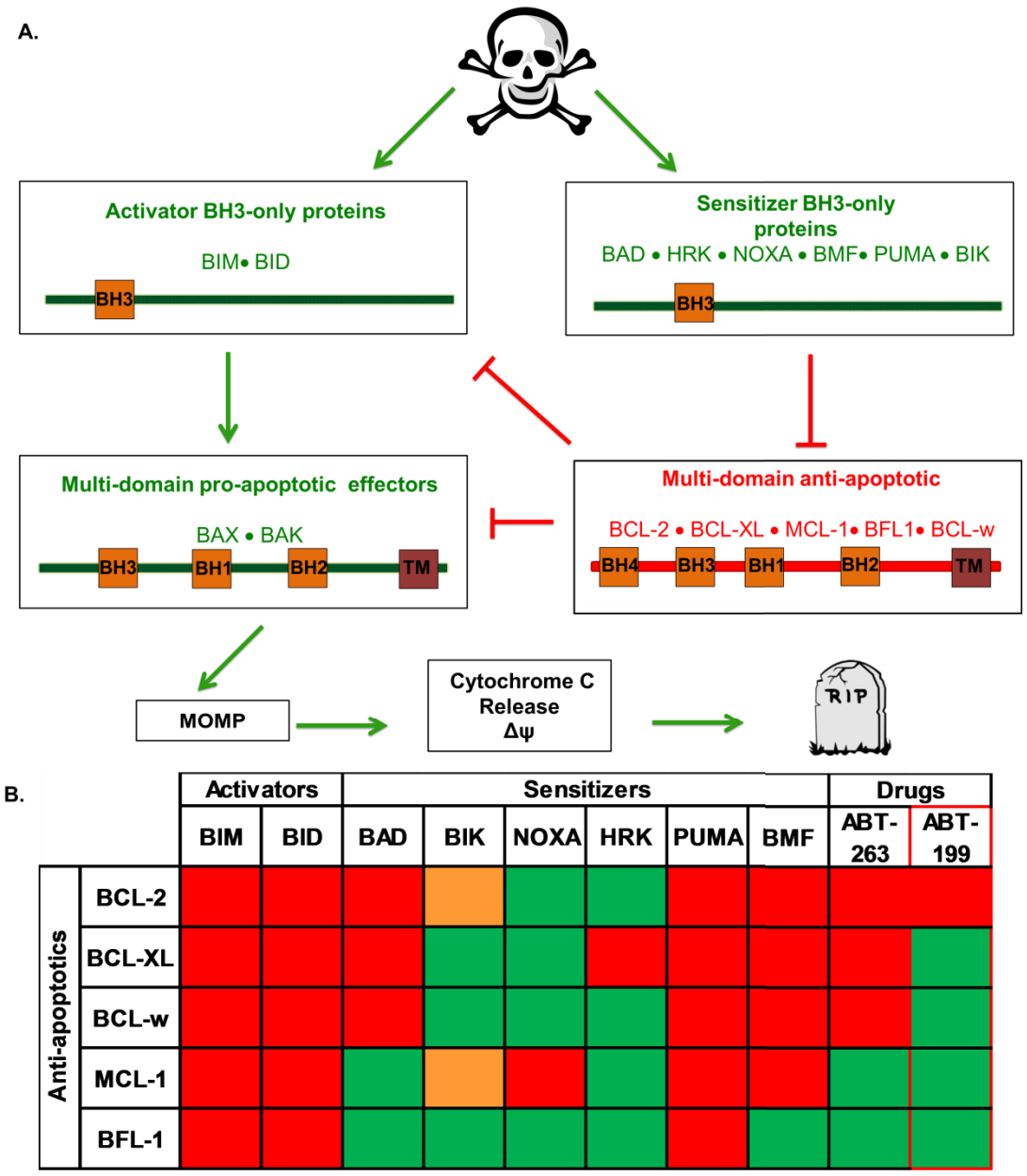


Figure 1.2. The mitochondrial apoptotic pathway.
A). Cell Death signals are relayed to the cytoplasm where activate activator proteins which bind to and activate the effector proteins to cause mitochondrial outer membrane permeabilization (MOMP), mitochondrial depolarization and the release of cytochrome c. Cytosolic cytochrome c binds to APAF to form the apoptosome which cleaves caspase 9 and initiates the caspase cascade. The anti-apoptotic proteins inhibit both the activator and effector pro-apoptotic proteins. The BH3-only sensitizer proteins bind to and inhibit the anti-apoptotic proteins. BH: Bcl-2 homology regions. TM: Transmembrane domains. **B.** Interactions of BH3-only proteins and BH3 mimetics with multi-domain anti-apoptotics. Red denotes high affinity binding, green- low affinity binding and orange - mild binding. ABT-199 is a BH3 mimetic and will be utilized in Chapter 2 and 3.

undergo extensive conformational changes allowing for BAX to be targeted to the mitochondria and both BAX and BAK to homo-oligomerize at the outer membrane of the mitochondria (*21, 25*). Oligomerization of BAX and BAK promotes MOMP by either directly forming pores themselves, or by inducing lipidic pore formation in the mitochondrial outer-membrane (*26, 27*).

Regardless of how pore formation occurs, MOMP results in mitochondria depolarization and the release of various proteins which are usually sequestered in the mitochondrial intermembrane space. The intermembrane space proteins include cytochrome c, second mitochondria-derived activator of caspase (SMAC), Omi and apoptosis inducing factor (AIF) which are responsible for the activation of caspases (*28, 29*). Once released into the cytoplasm, cytochrome c binds to the caspase adaptor molecule apoptotic-protease activating factor 1 (APAF-1) which promotes extensive conformational changes allowing for the exposure of caspase activation and recruitment domains (CARD) (*30*). The exposure of CARDs allows for the recruitment of pro-caspase 9 to form the apoptosome (*31*). The apoptosome is necessary for cleavage of the executioner caspases 3 and caspase 7 (*32*). The other mitochondrial intermembrane space proteins also have apoptotic functions. SMAC and Omi promote apoptosis by neutralizing x-linked inhibitor of apoptosis (XIAP) as XIAP is responsible for binding to and inhibiting the cleavage of caspases (*33, 34*).

Activation of the mitochondrial apoptotic pathway requires two events, activation of the effector proteins by BIM and BID, as described above, and the neutralization of anti-apoptotic proteins. Anti-apoptotic proteins inhibit the apoptotic pathway by binding to and preventing the activator and effector proteins from becoming activated. All of the anti-apoptotic proteins bind to BAX; whereas, BAK is preferentially bound by MCL-1 and BCL-XL (*35*). Upon apoptotic stimulation, the pro-apoptotic BH3-only proteins sequester the anti-apoptotic proteins by binding of the amphipathic BH3 α-helix of the BH3 only proteins to a hydrophobic groove within the anti-apoptotic proteins (*36*). The neutralization of the anti-apoptotic proteins allows for the release of the effector proteins to promote apoptosis.

For many years, there was a debate in the cell death field whether complete neutralization of the anti-apoptotic proteins was sufficient to activate apoptosis (indirect activation model of apoptosis), or activation of BAX and BAK was sufficient to promote apoptosis (direct activation model). However, a formative paper in the field showed that using mutated BIM, in which BIM's BH3 domain was replaced by

the BH3 domain of NOXA, PUMA or BAD (therefore, BIM could only bind to the anti-apoptotic proteins and not to BAX), that maximal apoptosis only occurred if the anti-apoptotics were inhibited and BAX was directly activated (*37*). Therefore, this data supported a consensus model in which apoptosis is promoted by the activation of the effectors BAX and BAK and the inhibition of the anti-apoptotic proteins.

The anti-apoptotic proteins are regulated by the BH3-only sensitizer proteins. The BH3-only sensitizer proteins indirectly promote apoptosis by binding to the anti-apoptotics effectively inhibiting the anti-apoptotic proteins from interacting with the activator and effector proteins (*14, 29*). Therefore, the BH3-only sensitizer proteins act by competitive displacement of BH3-only activators and effectors from the anti-apoptotic proteins. The sensitizer and activator BH3-only proteins exhibit specific interactions with the anti-apoptotic proteins due to subtle differences in the structure of the BH3-only domains (*38*). For instance, PUMA, BID and BIM interact promiscuously with all of the anti-apoptotic protein, whereas, other BH3-only sensitizers preferentially bind to particular anti-apoptotics with high affinity. For example, NOXA interacts with high affinity only with MCL-1, whereas HRK interacts with high affinity with only BCL-XL (**Figure 1.2B**) (*29, 39*).

Regulation of BCL-2 family members

As discussed above, the interactions of BCL-2 members control mitochondrial apoptosis. It is less well understood how the BCL-2 family members are regulated and activated upstream of the mitochondria. Some BCL-2 family members are transcriptionally regulated. For instance, following DNA damage, NOXA and PUMA are transcriptionally upregulated by p53 (*18, 40*). However, transcription of NOXA and PUMA can also be up-regulated by p-53 independent mechanisms (*41*). Post-translational modifications can affect the function of BCL-2 family members by enhancing or repressing the functions of apoptotic proteins. For example, phosphorylation of the anti-apoptotic protein BCL-2 on Ser70 enhances the affinity of the protein to BIM and BAK, rendering cells more resistant to certain chemotherapies (*42*). Whereas, phosphorylation of BCL-XL at Ser62 increases cell sensitivity of prostate cancer cells to apoptosis by allowing the release of BAX from BCL-XL (*43, 44*). The BCL-2 family can also be regulated by subcellular localization. Phosphorylation of BAD docks the BH3-only protein to 14-3-3 in the cytosol. Upon apoptotic stimulation such as growth factor withdrawal, BAD is dephosphorylated

and travels to the mitochondria where it can interact with the anti-apoptotic BCL-2 protein promoting apoptosis (*45*). Lastly, BCL-2 family proteins can be regulated be cleavage as was discussed above with the caspase 8 cleavage of Bid to tBid.

Events of apoptosis downstream of the mitochondrial

Once apoptosis is initiated by caspases, a series of downstream events is initiated to rid the organism of the dead cell in a tightly regulated, non-inflammatory fashion. Activation of caspases result in the distinct phenotype of apoptotic cells namely DNA degradation, cell blebbing and clearance by phagocytes. DNA degradation is stimulated by endogenous endonucleases which are responsible for the cleavage of chromosomal DNA into oligonucleosomal fragments. Caspase 3 degrades the inhibitor caspase DNase (ICAD) releasing caspase-activated DNase (CAD) (*46*). CAD activation results in the degradation of chromosomal DNA at internucleosomal A/T rich sites (*46, 47*). AIF, which is released from the mitochondria following MOMP, translocates to the nucleus where it promotes chromatin condensation and DNA degradation (*48*).

Executioner caspases contribute to loss of phospholipid asymmetry which results in phagocytosis of dead cells, the final step of apoptosis. In healthy cells, phospholipids are asymmetrically distributed on the outer and inner leaflet of cell membranes. Phosphatidylserine (PS) is an important phospholipid for recruiting phagocytes following apoptosis initiation which is usually restricted to the cytosol of the cell. The distribution of phospholipids is regulated by ATP dependent enzymes scramblases and flippases. The flippase ATP11C is responsible for regulating the exposure of PS to the inner leaflet. However, activated caspases cleave and inactivate ATP11C (*49*). The executioner caspases -3 and -7 also activate the scramblase XRP8 which scrambles the phospholipids resulting in loss of lipid asymmetry and the exposure of PS on the cell surface (*50*). When PS is flipped to the extracellular plasma membrane it acts as an “eat me” signal for patrolling macrophages allowing for engulfment of the apoptotic cells (*51*). All of these apoptotic events occur downstream of the mitochondria; therefore, the interactions of BCL-2 family members upstream of MOMP play a significant role in determining the cell’s fate.

BH3 PROFILING IDENTIFIES CELLS PRIMED FOR DEATH AND ANTI-APOPTOTIC ADDICTIONS

In order to simplify studying the apoptotic potential of individual cells and populations of cells, the Letai laboratory has developed a tool called BH3 profiling, which takes advantage of the interactions of the BCL-2 family members, to measure how close cells are to the apoptotic death threshold and anti-apoptotic dependencies (*29, 52*). BH3 profiling can be performed on clinical samples, and importantly, the assay does not require ex-vivo cell culture of primary tissue. For the BH3 profiling assay, cells are gently permeabilized with the detergent digitonin, exposed with standardized amounts of roughly 20-mer peptides derived from the alpha-helical BH3 domains of BH3-only proteins, and the mitochondrial response is measured (*53, 54*)

There are two methods used to measure the response of mitochondria to peptides in this thesis. The first method is measuring the release of cytochrome c following peptide incubation. In this method, cells are permeabilized, exposed to peptides, fixed with paraformaldehyde and stained with a cytochrome c antibody. As detailed above, cytochrome c is released from the mitochondria following MOMP and since cells are gently permeabilized, cytochrome c completely escapes from the cell. Therefore, antibodies directed against cytochrome c can be used to measure the amount of cytochrome c retained following peptide incubation by flow cytometry. This method is used for the BH3 profiling of AML primary patient cells.

The second method of measuring mitochondrial response to BH3 peptides is measurement of mitochondrial depolarization which occurs as a result of MOMP. This method utilizes the lipophilic cationic dye tetramethylrhodamine ethyl ester (TMRE). TMRE accumulates in charged mitochondria and fluoresces in the PE channel. When MOMP occurs, the membrane potential is lost and TMRE is no longer retained within the mitochondria, which results in decay of the PE signal (*55*). This method is used for BH3 profiling of hematopoietic stem cells during differentiation and aging as the HSCs. Both methods are highly correlative and provide similar results.

As mentioned above, for the BH3 profiling assays, cells are exposed to the BH3 domains of BH3-only peptides. It is important to note that information gathered from the BH3 profiling assay depends on

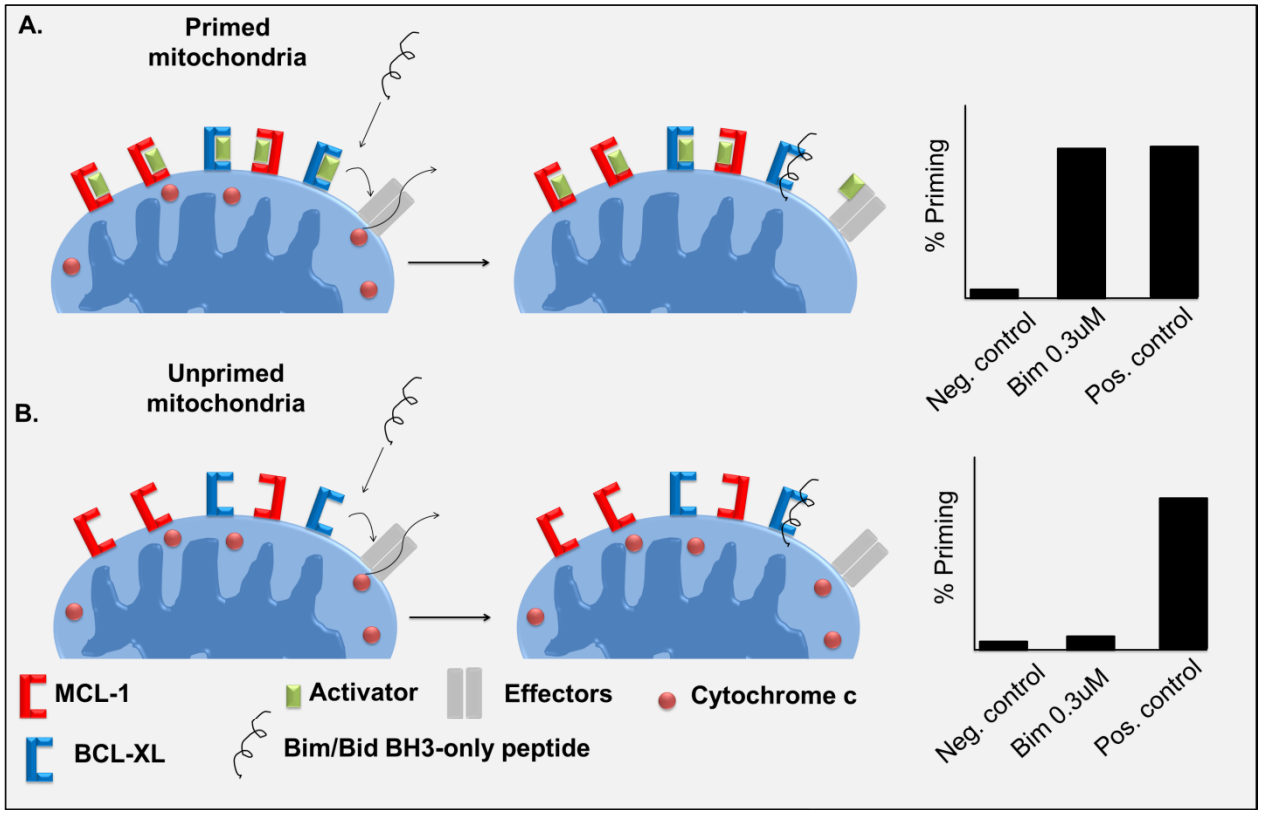


Figure 1.3 BH3 profiling measures the overall priming of a cell.
A). Primed mitochondria have activator BH3-only proteins pre-bound to the anti-apoptotics at the mitochondria outer membrane, whereas unprimed mitochondria do not (**B**). In the BH3 profiling assay, cells are gently permeabilized with digitonin to allow entry of BH3-only peptides. Primed cells require a lower concentration of BH3 peptides compared to unprimed mitochondria to cause cell depolarization or cytochrome c release. % Priming is calculated from the amount of cytochrome c released from the cell. Primed cells release more cytochrome c; therefore, they are more primed.

which peptides are used in the BH3 profiling assay. If promiscuous peptides which bind to all of the anti-apoptotic proteins are used, such as BIM, BID or PUMA, the level of overall priming of the cell is measured. As shown in **Figure 1.3A**, some cells are comprised of mitochondria that have pre-bound activator proteins to anti-apoptotic proteins at the mitochondria outer membrane. In contrast, some cells do not have pre-bound BIM and/or BID bound to the anti-apoptotics. When exposed to equivalent amounts of synthetic BH3 domain peptides, primed mitochondria depolarize and release cytochrome c more readily than unprimed cells (*29, 39, 52*). Mitochondria that depolarize and release cytochrome c readily are termed "primed for death", and are close to the threshold of apoptosis. Cells that do not release cytochrome c are labeled "unprimed" and are farther from the threshold **(Figure 1.3B)**. We have previously used this technique to show that chemosensitive tumors are closer to the death threshold compared to chemoresistant tumors (*52, 56*). Using cell surface markers to identify specific populations of cells, and then performing BH3 profiling, the priming of individual cells within a heterogeneous population can be assayed by flow cytometry (*57*). For this thesis, the BH3 profiling technique to measure overall priming is used in Chapter 4 to understand how mitochondrial priming changes during HSC differentiation and during HSC aging.

Non-promiscuous peptides can also be used in the assay and provides additional information about the anti-apoptotic dependencies of cells (*29, 39*). BH3 profiling can identify anti-apoptotic dependencies by taking advantage of the sensitizer BH3-only proteins specific interactions with certain anti-apoptotic proteins (described in **Figure 1.2B**). For instance, the NOXA BH3-only protein interacts with high affinity with only MCL-1 (*29*). Thus, treatment of MCL-1 dependent mitochondria, which have pre-bound activator peptides bound to MCL-1, with the NOXA peptide will result in MOMP, causing loss of mitochondrial potential and the release of cytochrome c (**Figure 1.4A**). In contrast mitochondria from cells that are dependent on a different anti-apoptotic, such as BCL-XL, will not release cytochrome c when exposed to the NOXA peptide (**Figure 1.4B**). Yet, BCL-XL dependent mitochondria will depolarize and release cytochrome c when incubated with the BCL-XL specific peptide HRK.

Different cell types are dependent on different anti-apoptotics. For instance, BCL-2 is highly expressed in chronic lymphocytic leukemia (CLL) cells and treating CLL cells with a specific BCL-2 inhibitor causes cell death (*58-60*). In contrast, human platelets are dependent on BCL-XL and selective

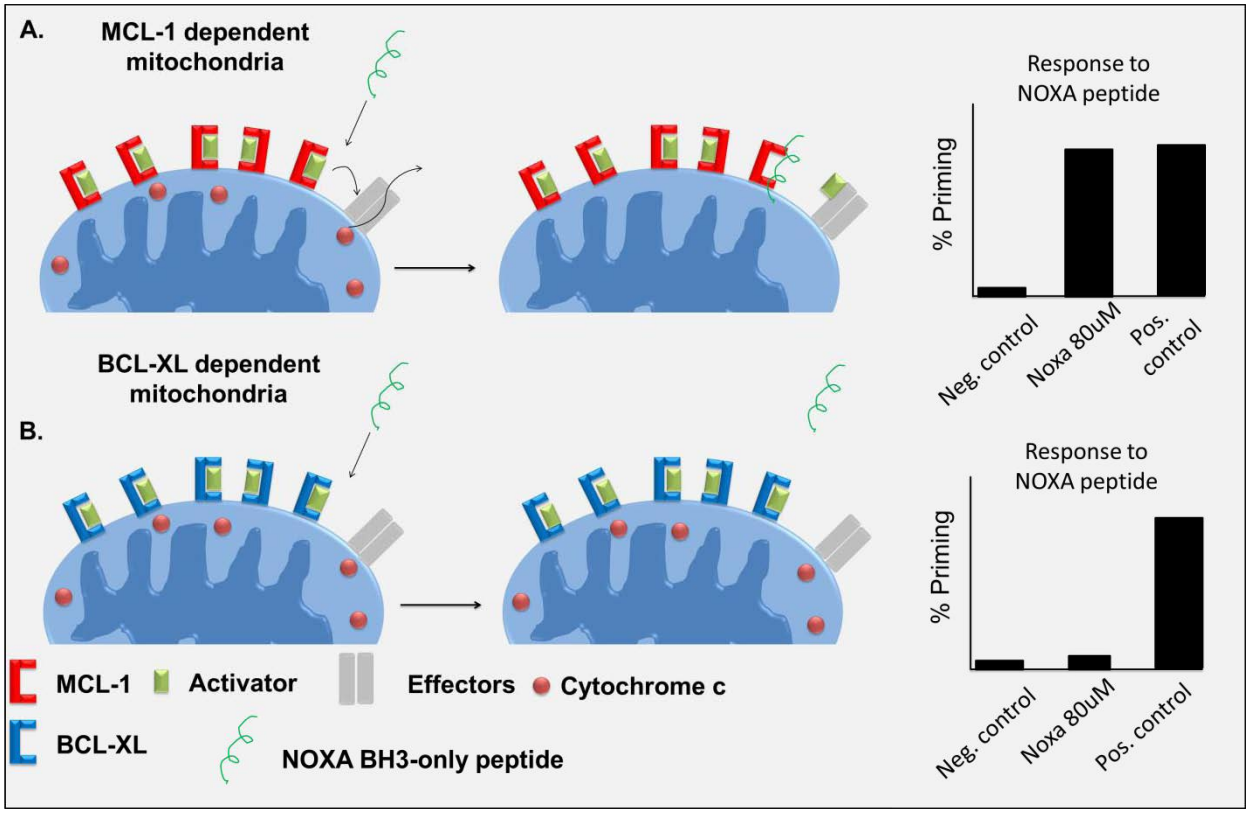


Figure 1.4. BH3 profiling measures anti-apoptotic dependencies of a cell.
A). MCL-1 dependent cells have a high amount of MCL-1 protein at the mitochondrial outer membrane pre-bound with activator BH3-only peptides. **B).** BCL-XL dependent mitochondria have a high amount of BCL-XL anti-apoptotic at the mitochondria outer membrane. When both cell types are incubated with the same concentration of the MCL-1 specific NOXA BH3-only peptide, the mitochondria of MCL-1 dependent cells depolarize and release cytochrome c, whereas BCL-XL dependent cells do not. % Priming is calculated from the amount of cytochrome c released from the cell.

inhibition of BCL-XL causes thrombocytopenia (*61*). Understanding and identifying anti-apoptotic addictions in different cell types is important for directing anti-apoptotic inhibitor treatment to patients whose cells are most dependent on those particular anti-apoptotics, as well as prospectively identifying potential dose limiting side effects on normal tissue. In this thesis, this technique is used to direct treatment of a novel BCL-2 inhibitor to cells that are BCL-2 dependent in the hematological cancer, acute myeloid leukemia (AML).

III. Acute Myeloid Leukemia

AML is a hematopoietic neoplasia characterized by the rapid, clonal growth of abnormal, immature myeloid cells. The overproduction of immature myeloid cells push normal blood cells from the bone marrow resulting in various blood disorders including bleeding, anemia and an increased risk of infection. AML is the most common acute leukemia in adults and approximately 250,000 adults are diagnosed worldwide each year (*62, 63*). AML is mainly a cancer of the elderly with the median age of AML diagnosis being 66 years of age (*64*). Although the prognosis for individual patients varies depending on mutation, cytogenetic status and age, the overall prognosis of AML patients is relatively poor with approximately 65-90% of patients diagnosed with AML dying of their disease within five years of diagnosis (*63, 65*). In Chapters 2 and 3 we examine the use of a novel BCL-2 inhibitor in AML, ABT-19 to improve treatment of AML patients. In this section, we discuss the current state of knowledge of AML pathogenies and biology as well as novel therapies in AML.

Classification of AML

AML is classified into various subtypes and prognostic classes based on different cytogenetic, and genetic criteria (*66, 67*). The goal of AML classification is to assist in guiding chemotherapy treatment and assign prognosis. One specific classification of AML is acute promyelocytic leukemia (APL). APL, which accounts for approximately 10-12% of all AML cases, is diagnosed by the presence of a translocation of the long arms of chromosomes 15 and 17 (t15;17) (*68*). APL is considered a distinct clinical entity with a different pathogenesis compared to all other AMLs; therefore, treatment of APL is distinct from all other AML treatment regimens (*69*). APL patients also experience much higher overall

survival compared to other AMLs (*68*). Due to these aspects of APL, while discussing AML, this thesis refers to all cases of AML with the exception of APL.

Beyond the diagnosis of APL, cytogenetic, molecular genetic data and clinical information are used to guide diagnoses and management of AML. The European Leukemia Network (ELN) published a set of guidelines which is widely used by American and European physicians to guide diagnoses and management of AML. ELN classifies AML prognostic factors by patient-related factors and leukemia related factors. Patient related factors include age, and comorbidities. Increasing age is a poor prognostic factor regardless of molecular genetic lesions and cytogenetic (*67*). Leukemia- related factors include cytogenetics and molecular genetics. Based off of these factors, prognosis is divided into four categories: favorable, intermediate I, Intermediate II and adverse (*67*). Patients classified with favorable cytogenetics have approximately 90% complete response rate to induction chemotherapy (discussed in the next section), whereas patients classified into the adverse cytogenetic group have a less than 50% CR rate following induction therapy (*70*). Furthermore, the 5-year survival rate for patients with favorable prognosis is 65%, whereas patients presenting with adverse cytogenetics have approximately 10-15% 5-year survival rate (*71*). The following section describes the current standard of care for AML patients and how prognosis and patient age may dictate treatment decisions.

CURRENT STANDARD OF CARE FOR AML

Despite tremendous advances in understanding the etiology and biology of the disease, the treatment of AML has not changed drastically over the past 40 years (*72*). Identifying the correct treatment for each individual patient is dependent on a multitude of factors including cytogenetic and molecular genetic risk, age, co-morbidities and performance status (*63, 73*). Most fit patients under the age of sixty receive at least two phases of treatment. The goal of the first phase, called induction chemotherapy, is to induce a complete remission (CR) which is defined by less than 5% blasts and recovery of normal elements in the bone marrow following treatment (*74*). Induction therapy consists of a combination of seven days of cytarabine with three days of an anthracycline such as daunorubicin or idarubicin (treatment called 7+3) (*67*). Cytarabine is a pyrimidine analog which inhibits DNA synthesis by incorporating into DNA as well as inhibiting DNA polymerase which effectively disrupts DNA synthesis

and DNA repair. Anthracyclines inhibit topoisomerase II which also inhibits DNA synthesis (*75*). With these drugs, a CR is achieved in approximately 60-80% of patients under the age of sixty (*67*).

Unfortunately, nearly all patients who achieve a CR following induction chemotherapy will eventually relapse if they receive no additional therapy due to the presence of residual blasts in the bone marrow following the first phase of therapy (*76, 77*). Therefore, the second phase of therapy called post-remission, or consolidation therapy, is critical for the cure of the patient. Consolidation therapy may consist of intensive conventional chemotherapy (high dose cytarabine), or high dose chemotherapy followed by allogeneic or autologous stem cell transplant (allo SCT or auto SCT) (*63, 67*). The choice of consolidation therapy depends on various factors including cytogenetic and molecular genetic risk, age, co-morbidities and availability of bone marrow donors (*67*). Allo SCT is the best option for cure for patients with patients who have adverse or intermediate prognosis because of the graft-versus-leukemia effect (GVL) in which donor cells eradicate residual host AML cells (*78*). Allo SCT has a relatively high treatment related mortality rate. Moreover, it can leave patients with lifelong chronic graft versus host disease. Therefore, it is most widely sought for patients who have adverse cytogenetic and molecular prognosis due to risk/benefit analysis (*79*).

Most patients diagnosed with AML are over the age of 60 and these older patients often present with lower fitness, additional co-morbidities and higher risk AML; therefore, their treatment may often differ from young AML patients (*73*). Older patients that do not present with prohibitive comorbidities receive induction chemotherapy; however, the CR rate following induction therapy for this age cohort is only around 30-50% (*67*). Since induction chemotherapy is a very intensive therapy regimen associated with treatment related mortality, older patients unfit for induction therapy may opt for other treatment options including hypomethylating agents, low dose cytarabine, enrollment in a clinical trial and/or supportive care (*80*).

With the aforementioned current standard of care for AML patients, the five year survival is far from ideal. For patients under the age of sixty, the five year survival rate is approximately 50% whereas for patients over the age of sixty it is only 10-15% (*65*). Therefore, there is an intense interest in understanding the recurrent genetic abnormalities found in AML and developing novel therapies to exploit these mutations to improve survival outcomes.

MOLECULAR CHARACTERIZATION OF AML

AML is a heterogeneous disease with considerable diversity in terms of its molecular and cytogenetic characterization among patients. Particular mutations and cytogenetic patterns dictate prognosis, but few of these mutations help direct treatment in a clinically useful manner.

Recurrent genetic mutations in AML

The most commonly mutated gene in AML is FMS-like tyrosine kinase 3 (FLT3). Mutations in FLT3 occur in two ways. The first is through an internal tandem duplication of the tyrosine kinase domain (FLT3-ITD), the second is by point mutations in the tyrosine kinase domain (FLT3-TKD) (*81*). FLT3-ITD and FLT3-TKD occur in approximately 20-35% and 5-10% of AML patients respectively (*82, 83*). FLT3-ITD mutations dictate an intermediate prognosis (*67*). Both aberrations result in constitutive activation of the pathway which promotes cell cycle progression, inhibition of apoptosis and differentiation (*84, 85*). Patients harboring FLT3-ITD have inferior treatment outcomes compared to FLT3 wild-type (WT) patients (*86*). Because of the inferior prognosis of this mutation, as well as the relatively high occurrence of FLT3-ITD mutations in AML blasts, therapies specifically targeting FLT3 have been developed and will be discussed in the following section.

In contrast to the intermediate prognosis marker of FLT3, mutations in nucleophosmin (NPM1), in the absence of FLT3-ITD mutation, are associated with favorable prognosis (*67, 87*). NPM1 is the second most commonly mutated gene in AML patients and occur in approximately 25% of AML patients with de-novo AML (*87*). NPM1 is a nuclear chaperone which is responsible for transporting proteins between the cytoplasm and nucleus, and also regulates the tumor suppressor p53 (*88-90*). Mutations in NPM1 result in the localization of NPM1 in the cytoplasm; however, it is not fully understood how this localization results in a better prognosis for NPM1 mutated AMLs (*87*).

The third most commonly mutated AML gene is DNA methyltransferase 3A (DNMT3A) which is mutated in approximately 22-26% of AML patients (*91*). DNMT3A is an enzyme responsible for catalyzing the addition of methyl groups to DNA cytosine sites at CpG residues (*92, 93*); however, mutations in DNMT3A do not appear to result in global hypomethylation of AML blast DNA, or cause global gene expression alterations (*91, 94*). DNMT3A mutation acquisition is thought to be an early event in the

evolution of AML as mutations in DNMT3A are observed in early multi-potent progenitors of individuals with AML, and is found in both the myeloid and lymphoid lineages of more differentiated cells (*95*). This finding, in combination with the observation that DNMT3A mutations are associated with decreased overall survival compared to patients who do not have mutation in DNMT3A in certain subgroups of AML (*91, 96*), have increased the efforts to identify specific DNMT3A inhibitors.

Mutations in isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) occur in approximately 15-20% of AML patients (*93, 97*). IDH1 and IDH2 are members of the citric acid cycle responsible for converting isocitrate to α-ketoglutarate (α-KG) (*98*). The neomorphic mutations found in IDH1 and IDH2 allow the enzymes to convert α-KG to the oncometabolite (R)-2-hydroxyglutarate (2-HG) which leads to downstream epigenetic dysregulation (*99-101*). Analysis of pre-treatment and relapse AML samples indicate IDH1 and IDH2 mutational clones survive initial treatment and contribute to the relapsed disease (*102*); therefore, there is interest in identifying inhibitors of these enzymes. A promising new series of drugs specifically targeting IDH1 and IDH2 mutations have recently entered clinical trials in AML and will be discussed in the next section.

Additional mutations occur in AML at lower frequencies. Mutations in the tumor suppressor gene TP53 occur in approximately 5-10% of all AML and is associated with particularly poor prognosis (*103*). Signal activating mutations in NRAS and KRAS occur in 12% of AML patients, mutations in the epigenetic regulator TET2 occur in 8% of AMLs and mutations in the transcription factor CEBPA occur in 6% (*93*). These lower frequency mutations are not routinely used as ELN associated prognostic factors, save for CEBPA and p53 which are a favorable and adverse prognostic factors respectively (*67*).

Recurrent cytogenetic abnormalities in AML

In contrast to many other cancer types, almost half of all AML cases have normal karyotypes and chromosomal instability is relatively rare (*104, 105*). Approximately 40-50% of AML patients present with normal cytogenetics which is a favorable prognostic factor (*67*). On the other side of the spectrum, patients presenting with complex karyotypes, defined as having three or more chromosomal abnormalities in the absence of certain translocations defined by the WHO, have an adverse prognosis (*67*). Complex karyotypes occur more commonly in older patients (*106*), and patients presenting with

complex karyotype are also more likely to have mutations in or loss of TP53 (between 60-70% of complex karyotype patients also have alterations in TP53 compared with 2% in non-complex karyotype patients), which is an adverse prognostic factor by itself (*107, 108*).

In between complex and normal karyotype, there are an array of monosomies, rearrangements and translocations that have been identified in association with AML. Several translocation indicate a favorable prognosis including t(8:21), inv(16) or t(16;16); whereas other translocation indicate adverse prognosis including inv(3), MLL rearranged, del(5q), t(6;9) (*67*).

Intra-patient heterogeneity

In addition to identifying mutational differences among different patients, recent molecular analysis has identified functional and genetic heterogeneity among individual clones within a single patient's AML (*109*). The evolution of AML is punctuated by the acquisition of different genetic and epigenetic lesions in normal HSCs and myeloid progenitor cells which give distinct subclones advantage in growth and differentiation (*110*). Analysis of AML subclones identified that some subclones exhibit superior ex-vivo cell growth, engraftment abilities compared to other clones from the same patient (*111*). Evidence suggests that chemotherapy selects for particularly chemoresistant subclones which eventually contribute to relapsed disease (*109*).

Leukemic stem cells (LSCs) have also been identified within the bulk AML. LSCs possess the capacity to self-renew as well as generate the heterogeneous cancer cells that comprise a tumor and most likely contribute to the relapse of AML (*112*). AML LSCs are often defined by their extracellular markers such as CD34+CD38-, and more recently CD123+ has been defined as a marker of leukemia stem cells (*113*). Patients who present with a high proportion of LSCs have worse progression free survival compared to patients who have less stem cells (*114*). The heterogeneity of AML subclones, as well as the presence of resistant LSCs underlies the difficulty in effectively treating the disease.

NOVEL THERAPIES IN AML

Despite great advances in understanding the pathogenesis and molecular genetics driving AML, no novel therapies have been approved for treatment in AML over the past 40 years (gemtuzumab

ozogamicin (GO), was approved in the early 2000's, but was later voluntarily withdrawn due to high toxicity and modest therapeutic benefit) (*115*). This is not for lack of effort. There are currently over 500 actively recruiting clinical trials for AML worldwide which are testing the efficacies of novel compounds as monotherapy or in combination with current treatment options (clinicaltrials.gov). **Table 1.1** summarizes some of the most encouraging novel therapies in pre-clinical and clinical trials. This section details novel therapies currently in clinical trials, concluding with the novel BCL-2 inhibitor ABT-199 which is the focus of Chapters 2 and 3.

Improving conventional chemotherapy

Since conventional chemotherapy has been most successful over the past 40 years in the treatment of AML, efforts to combine current standard of care with additional chemotherapies in the induction phase have been on going. Flavopiridol is an inhibitor of multiple cyclin dependent kinases which blocks progression of cells through the G1/S checkpoint (*116*). Early Phase II clinical trial results of a combination of flavopiridol followed by AraC and mitoxantrone (FLAM) showed higher response rates in patients treated with FLAM in comparison with patients treated with induction therapy alone in de novo AML and patients with secondary AML (*117*). The enhanced activity of FLAM vs conventional therapy is thought to be caused by flavopiridol arresting cells in S phase where the cells are more susceptible to the cell cycle dependent chemotherapies (*115*).

Instead of introducing additional therapies with the conventional induction therapy regimen, another new therapeutic modality is developing systems to optimally deliver current treatment therapeutics. CPX-351 is a bilamellar liposome which encapsulates AraC and daunorubicin to deliver the drugs in a more prolonged and concentrated manner (*118*). A phase II trial of CPX-351 in patients diagnosed with AML over 60 years of age showed that CPX-351 improved drug retention in the body and improved overall response and overall survival compared to free drug (*119*).

Immunotherapies

Immune based cancer therapies have received extensive attention for their potential to cure cancer (*120*). Monoclonal antibodies are a subset of immunotherapies, which recognize cell surface

antigens that are highly expressed on cancer cells. CD33, or Siglec-3, is a transmembrane receptor expressed on 85-95% of AML cells (*121, 122*). CD33 is also expressed on myeloid progenitor cells as well as other cells of the myeloid lineage; however, expression of CD33 is highest in leukemic blast cells (*121, 122*). Gemtuzumab ozogamicin (GO) is an immunoconjugate consisting of humanized anti-CD33 antibody linked to a derivative of calicheamicin, a potent anthracycline antibiotic (*123*). Treatment of AML cells with GO results in the rapid internalization of calicheamicin into the target cell causing DNA double stranded cleavage followed by target cell death (*124*). Early clinical trials with GO in combination with conventional chemotherapy showed uncertain clinical benefit and increased mortality with patients treated with GO (*125*). These results led to its voluntary withdrawal in 2010 (*126*); however, recent meta-analysis of additional GO clinical trials indicated that there may be some clinical benefit of GO in combination with conventional chemotherapy (*127, 128*). To decrease the treatment related mortality associated with GO, CD33 linked to a less toxic conjugant, pyrrolobenzodiazepine dimer are now in clinical trials (*129*). Further, new CD33-CD3 T-cell-engaging BiTE (bi-specific T-cell engagers) in which CD33 is linked to a cytotoxic T-cell rather than a cytotoxic therapeutic to enhance cancer cell killing have proven effective in the pre-clinical trial setting (*130-132*).

Monoclonal antibodies targeting CD123 are also in clinical trials. As mentioned previously, CD123 is an interleukin 3 receptor expressed on acute myeloid leukemia stem cells (LSCs) (*113*). LSCs are resistant to chemotherapy and contribute to the relapsed disease (*112*); therefore, it is essential to eradicate these resistant cells. Pre-clinical results with CD123 monoclonal antibodies showed potent killing of LSCs (*133, 134*). To move these results to the clinic SL-401 was developed, which is an anti-CD123 antibody conjugated to diphtheria toxin. Although SL-401 was effective in blastic plasmacytoid dendritic cell neoplasm (BPDCN), only two AML patients achieved a CR out of seventy patients treated (five additional patients achieved a partial response) (*135*).

Kinase inhibitors

As mentioned earlier, FLT3 is one of the most commonly mutated kinases in AML and FLT3-ITD status is an indicator of intermediate prognosis. First generation FLT3 inhibitors, including midostaurin and sorafenib, entered clinical trials as monotherapies in the early 2000s and showed transient reductions in

Table 1.1. Novel targeted therapies for AML currently in pre-clinical and clinical trials

Class of drug	Target	Drug	Notes	Phase of trial	Ref
Immunotherapy	CD33	Gemtuzumab ozogamicin	CD33 conjugated to calicheamicin	In Phase1/2 combo trials	(*136*)
		SGN-CD33A	CD33 conjugated to pyrrolobenzodiazepine	Phase 1	(*129*)
		AMG 330	CD3-CD33 BiTE	Pre-clinical	(*130*)
	CD123	SL-401	CD123 conjugated to diphtheria toxin	Phase 1/2	(*137*)
	CXCR4	Ulocuplumab	Inhibits binding of CXCR4 from binding to CXCR12, mobilizing leukocytes	Phase 1	(*138*)
Kinase inhibitor	Aurora a kinase	Alisertib	In combination with induction therapy with newly diagnosed patients	Phase 2	(*139*)
	cKIT	Dasatinib	In combination with chemo in patients with CBF mutations	Phase 2	(*140*)
	FLT3-ITD (mutated in 20-30% AML cases)	Sorafenib	FLT3 binding activity Kd, = 13 ± 6 nm	Phase 2*	(*141*)
		Quizartinib	FLT3 binding activity Kd = 1.6 ± 0.7 nm	Phase 2	(*142*)
Epigenetic inhibitor	IDH1	AGI-120	Promotes AML differentiation	Phase 1	(*143*)
	IDH2	AGI-221	Promotes AML differentiation	Phase 1	(*144*)
Other	CRM1	KPT-330	Inhibits the nuclear export activity of CRM1	Phase 1/2	(*145*)
Apoptosis inducer	MDM2	RG7388	In combination trials with AraC	Phase1/1b	(*146*)
	BCL-2	ABT-199	Specific inhibitor of BCL-2; now in combination with hypomethylating agents	Phase II results discussed in Chapter 3	Chapter 3

* Multiple phase 1 and 2 trials are ongoing with the FLT3 inhibitors in combination with conventional chemotherapies.

peripheral blasts, but failed to produce meaningful clinical responses (*147-150*). The early FLT3 inhibitors were not successful because of their non-specificity and concomitant toxicity, and high serum binding in plasma (*151-153*). These results prompted the development of second generation FLT3 inhibitors including quizartinib which offers increased specificity and lower serum binding, effectively increasing the drug's plasma half-life (*142*). In a Phase I clinical trial of monotherapy quizartinib in relapsed or refractory AML patients with and without FLT3-ITD mutations, quizartinib treated patients had a 30% response rate, including 13% complete responses. Interestingly, 14% of FLT3 negative or FLT3 indeterminate patients had CRs or PRs suggesting that quizartinib may be beneficial for a cohort of FLT3 WT patients (*142*). Due to its success as a monotherapy in Phase I trials, quizartinib is now in combination trials with conventional chemotherapies to improve upon its initial monotherapy results.

Epigenetic regulators

Mutations in IDH1 and IDH2 are found in approximately 15-20% of AML cases. Recently, AG-120 and AG-221, potent inhibitors of IDH1 and IDH2 respectively, have shown impressive pre-clinical activity with an interesting mechanism of action. Instead of causing robust apoptosis in cancer cells, both AG-120 and AG-221 cause the immature AML blasts to differentiate into more mature cells (*143, 154*). Early results in the Phase I clinical trial of monotherapy AG-221 indicates that the drug is well tolerated and has impressive results. Of the forty-eight IDH2 mutant patients enrolled in the trial, there were 8 CRs, and several complete remissions with incomplete bone marrow reconstitution (CRi) (*144*).

Nuclear export inhibitors

KPT-330 (Selinexor) is a potent inhibitor of the nuclear export protein chromatin maintenance protein 1 (CRM1) currently in Phase I clinical trials of AML. CRM1 is a nuclear export protein responsible for translocation of a multitude of tumor suppressor proteins, including p53, from the nucleus to the cytoplasm. High expression of CRM1 is associated with poor prognosis, and inhibition of CRM1 by KPT-330 potently kills AML cell lines, xenografts and leukemic initiating cells in pre-clinical analysis (*155-157*). Early Phase I clinical results show that single agent KPT-330 is effective in a cohort of heavily pre-treated AML patients with four out of thirty-two patients achieving a complete response (*158*).

Apoptotic inducers

As discussed in the previous section on mitochondrial apoptosis, the BCL-2 family controls commitment to the mitochondrial apoptotic pathway. Therefore, one method to treat cancer cells is to directly inhibit anti-apoptotic proteins or enhance the function of pro-apoptotic proteins. BCL-2 is an anti-apoptotic protein which inhibits cell death by binding to the activator and effector proteins of mitochondrial apoptosis. Some AML cells are dependent on the anti-apoptotic protein BCL-2 for survival and inhibition of BCL-2 by shRNA or small molecule inhibitors causes cell death in a subset of AML cell lines primary patient cells *ex vivo* (*56, 159, 160*). Furthermore, increased expression of BCL-2 has been associated with poor prognosis in AML (*161, 162*). Therefore, there has been interest in inhibiting the anti-apoptotic BCL-2 in AML.

Early iterations of BCL-2 inhibitors included the antisense oligonucleotide oblimersen sodium. Oblimersen is an antisense oligodeoxyribonucleotide directed against the first six codons of BCL-2 mRNA. The DNA/RNA complex formed by oblimersen and BCL-2 mRNA is recognized as foreign material and destroyed, presumably decreasing the levels of BCL-2 protein (*163*). A Phase III clinical trial of induction chemotherapy followed by consolidation chemotherapy ± oblimersen sodium did not exhibit superior survival of patients treated with oblimersen compared to patients receiving consolidation alone (*164*). The lack of *in vivo* efficacy was attributed to short half-life of the DNA as the majority of patients did not show decreased of BCL-2 in AML blasts.

In contrast to the BCL-2 targeted oblimersen, pan-BCL-2 inhibitors have also entered clinical trials. AT-101 is derived from the natural product gossypol and is a presumptive potent inhibitor of BCL-2, BCL-XL, BCL-w and MCL-1. AT-101 did not exhibit single agent killing in clinical trials in AML, but did have synergistic killing efficacy in combination with rituximab in chronic lymphocytic leukemia (*165, 166*). Obatoclax mesylate is also considered to be a pan-BCL-2 inhibitor; however it may have BAX/BAK independent cytotoxic activities (*167*). As with AT-101, single agent obatoclax was not efficient in clinical trials. In a Phase I/II clinical trial of obatoclax mesylate in previously untreated AML patients showed no evaluable responses in the patients treated with the maximal tolerated dose (*168*).

More recently, the ABT series of compounds which selectively inhibit the BCL-2 family members have been developed. The first compounds in the series were ABT-737 and the orally available derivative

navitoclax (ABT-263). These two compounds are small molecule mimetics of the BH3-only sensitizer protein BAD which efficiently bind to BCL-2, BCL-XL and BCL-w, releasing the pro-apoptotic activator proteins and causing MOMP in cancer cells (*169*). In early clinical trials, navitoclax showed potency in the treatment of chronic lymphocytic leukemia and small-cell lung cancer (*170, 171*). However, treatment with navitoclax causes on-target, dose-limiting thrombocytopenia because platelets are dependent on the anti-apoptotic protein BCL-XL for survival (*61*). This observation prompted development of ABT-199, a modified BH3-only mimetic which maintains specificity for BCL-2, but lacks affinity for BCL-XL (*172*). The remodeled drug has shown cancer killing efficacy in CLL *in vivo* and myc driven lymphomas in mice, while sparing platelets (*172, 173*). Chapters two and three discuss pre-clinical and Phase 2 clinical results of using ABT-199 in AML and how BH3 profiling can be used to direct treatment of ABT-199 to patients who will be most sensitive BCL-2 inhibition by ABT-199.

IV. PHYSIOLOGICAL HEMATOPOIESIS

Aging is associated with an increase in incidence of hematological malignancies, including AML (**Figure 1.1**). In order to understand why aging is accompanied with an increase in hematological malignancies it is necessary to understand how the cell from which all hematological cells are derived is maintained throughout an organism's life. An average adult human requires the production of approximately 10^{11} hematopoietic cells per day. A rare subset of cells, the hematopoietic stem cells (HSCs), which reside in specific niches in the bone marrow, are responsible for making all of these cells. This process requires strict control of proliferation, differentiation and apoptosis in order to maintain a steady equilibrium of cells throughout the lifespan of an organism. Most of the time, this process occurs seamlessly. However, during aging and disease, this process becomes deregulated leading to unwanted conditions including anemia, immunosenescence, cancer and even death. This section reviews the role of the HSC in normal physiological hematopoiesis and how the process if transformed with organismal aging.

At the pinnacle of the hematopoietic hierarchy is the HSC (**Figure 1.5**). The HSC possesses the unique properties of self-renewal and multi-lineage potency. Self-renewal results in the HSC producing

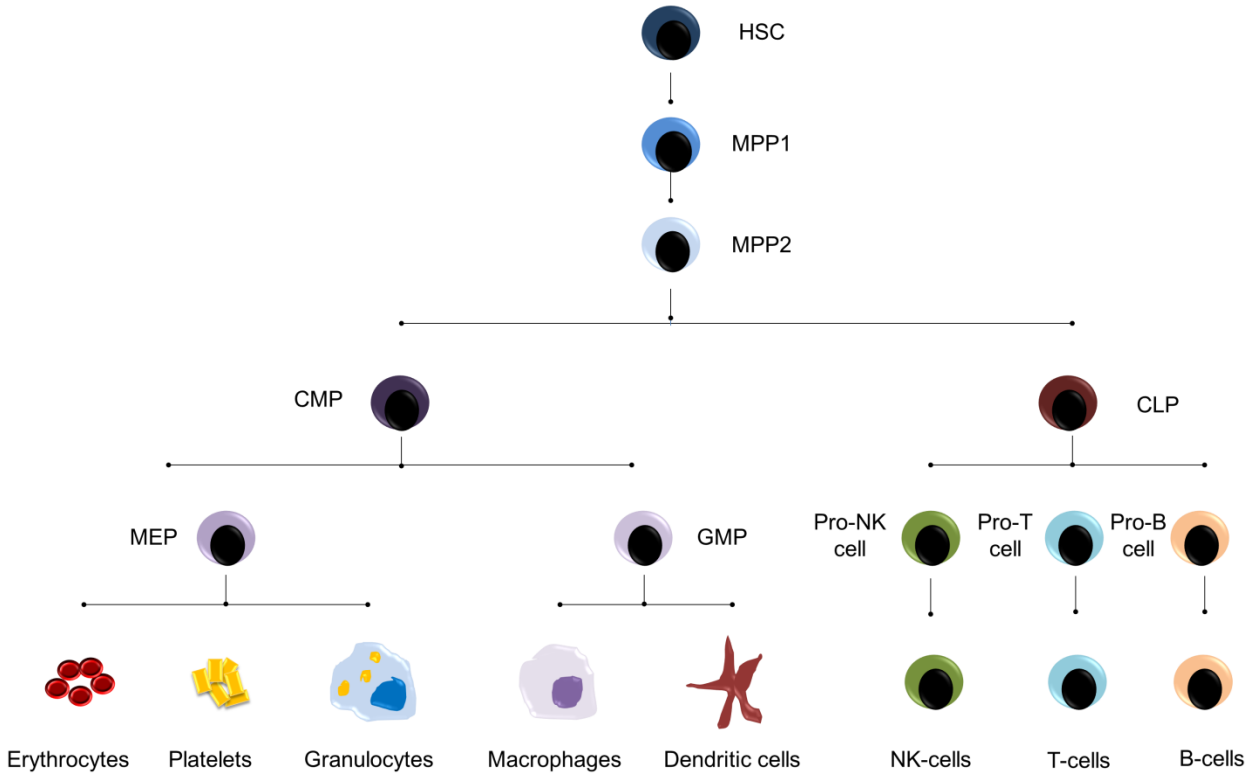


Figure 1.5. Hematopoiesis is a hierarchal process. All of the mature cells found in the murine and human blood are derived from the hematopoietic stem cell through successive differentiation steps.

identical copies of itself; and, multi-lineage potency allows the HSC the ability to generate committed progenitors that, through subsequent divisions, differentiate into all of the lineages found in the blood. Human and mouse blood consists of at least ten distinct cell types including erythrocytes, platelets, macrophages, granulocytes, dendritic cells, T-cells, B-cells and natural killer cells (*174*). These cell subsets can further be divided into more differentiated subsets such as CD4+ T cells or activated macrophages, and each has a unique and complicated role in the protection of the human body from infection and disease as well as maintenance of homeostasis.

HSCs produce all of the cells found in the human body by giving rise to multipotent progenitors which in turn generate more committee myeloid and lymphoid progenitors. HSCs are functionally defined by their ability to reconstitute the bone marrow of a lethally irradiated recipient and are further able to replenish secondary recipients (*175*). Phenotypically, HSCs have been defined by specific cell surface markers which are detailed for both mice and humans in **Table 1.2**. In response to external stimuli such as infection, irradiation or chemotherapy, HSCs are stimulated to produce a multitude of progenitor cells to quickly reclaim blood homeostasis (*176*). HSCs first differentiate into multi-potent progenitors (MPPs). MPPs retain the ability to differentiate into multiple lineages, but have finite, short-term self-renewal capabilities (*177-179*). MPPs can be further subdivided into MPP1 and MPP2 based of the expression of Flk2. MPP FLK2+ cells consist of cells with more transient multi-lineage reconstitution potential compared to MPP FLK2- subsets (*179*). MPPs differentiate into the oligopotent, lineage restricted common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). The CLPs give rise to cells of the lymphoid lineage including B, T and NK cells which are necessary for the adaptive immune response. CMPs are responsible for producing the cells of the innate immune system and differentiate into more restricted progenitors including granulocyte-monocyte progenitors (GMP) which give rise to macrophages and granulocytes, and megakaryocyte-erythroid progenitors (MEPs) which eventually give rise to platelets and red blood cells (*174*). Throughout an organism's lifespan HSCs must maintain strict control over cell fate – whether the cell undergoes self-renewal, differentiation or apoptosis in response to external stimuli. Incorrect cell fate decisions can lead to hematological malignancies. Therefore, there is a complicated network of genes, transcription factors and cytokines which regulate HSC cell fate (*174, 180, 181*).

Table 1.2 Cell surface markers used for this thesis

	Markers	
Subset	Mice	Humans
HSC	$Lin^-Sca^+cKit^+FLK2^-CD34^-CD150^{+/-}$	$Lin^-CD34^+CD38^-CD90^+CD45RA^-$
MPP1	$Lin^-Sca^+cKit^+FLK2^-CD34^+CD150^-$	$Lin^-CD34^+CD38^-CD90^-CD45RA^-$
MPP2	$Lin^-Sca^+cKit^+FLK2^+CD34^+CD150^-$	
CLP	$Lin^-FLK2^+IL7R\alpha+CD34+$	$Lin^-CD34^+CD38^+CD10^+$
CMP	$Lin^-Sca\text{-}cKit^+CD34^+FcγRlow$	$Lin^-CD34^+CD38^+IL3Ra^{low}CD45RA^-$
GMP	$Lin^-Sca\text{-}cKit^+CD34^+FcγR^+$	$Lin^-CD34^+CD38^+IL3Ra+CD45RA^-$
MEP	$Lin^-Sca\text{-}cKit^+CD34^-FcγR^-$	$Lin^-CD34^+CD38^+IL3Ra\text{-}CD45RA^-$

Control of the apoptosis within HSCs

Various methodologies from gene deletion, gene overexpression to more systems biology approaches of gene expression, and epigenome analysis have identified a large set of genes that are differently regulated in HSCs compared to downstream progenitor cells (*174, 181-183*). Various transcription factors, signaling modulators, epigenetic modifiers and cell cycle regulators have been implicated in regulating stem cell fate and are preferentially up and down-regulated in HSCs compared to more differentiated cells (*184, 185*). Beyond intrinsic regulators of cell fate, the bone marrow niche has also been implicated in stem cell fate maintenance. A complete review of the hundreds of genes and various cytokines which have been implicated in stem cell fate is beyond the scope of this introduction; therefore, the focus of the next section is specifically on how the stem cell fate of apoptosis is controlled in the HSC niche.

It is hypothesized that the more mature hematopoietic cells are rather dispensable following genotoxic stress because HSCs and progenitors can regenerate the tissue rather quickly. Therefore, the apoptotic response in mature cells can be activated rather easily, but the response must be curtailed in HSCs. Since stem cell activity needs to persist throughout an organism's life, it is believed that both the extrinsic and intrinsic apoptotic pathways are maintained in an anti-apoptotic state in HSCs. Human CD34+ cells cultured with Fas ligand, which stimulates the extrinsic apoptotic pathway, do not show appreciable apoptosis induction (*186*). Further, CD34+ cells express higher levels FLICE inhibitory protein (FLIP) and lower levels of caspase 8 which prevents HSCs from undergoing erroneous apoptosis compared to more mature cells (*186*).

The intrinsic apoptotic pathway has also been implicated in promoting cell survival of HSCs. Irradiated murine hematopoietic stem and progenitor cells (HSPCs, Lin/c-Kit+/Sca-1+/Flk2-) are less sensitive to irradiation compared to downstream, lineage committed progenitors as measured by cleaved caspase 3 (*187*). The decreased sensitivity of HSPCs is attributed to decreased mRNA expression of pro-apoptotic BCL-2 family members BAX, BAK BID and NOXA, and increased expression of MCL-1 and BCL-XL in the HSPC compartment compared to the CMP and GMP cells. Consistent with these findings, mice treated with genotoxic stress upregulate Slug, a zinc-finger transcriptional repressor that inhibits the p53 dependent translation of PUMA, a BH3-only pro-apoptotic member in myeloid progenitor cells (*188*).

The repression of PUMA inhibits DNA damaged progenitor cells from undergoing apoptosis which is thought to promote their extended survival throughout an organism's life.

The importance of the mitochondrial apoptotic pathway to HSC survival is supported by gene over expression and gene deletion studies. The anti-apoptotic protein MCL-1 is understood to be essential for maintaining homeostasis of early HSC progenitors in both humans and mice. Inducible deletion of the anti-apoptotic MCL-1 in LSK cells (LSK consists of both HSCs and MPPs) causes bone marrow ablation in mice (*189*). Similarly, si-RNA knock-down of MCL-1 in human HSCs disrupts their ability to self-renew in *in vivo* reconstitution assay into irradiated NOD/SCID mice (*190*). Recently, it was revealed that MCL-1 can be post-translationally modified into a truncated protein that localizes to the inner mitochondrial matrix where it controls mitochondrial fusion and respiration, distinct from its cytoplasmic role in apoptosis (*191, 192*). Since the knock-down experiments performed in the HSCs affect both the full and truncated forms of MCL-1, it is unclear whether the role of MCL-1 in the survival of HSCs is due to its anti-apoptotic or mitochondrial fusion and respiration roles.

The anti-apoptotic protein BCL-2 has also been implicated as an important regulator of HSC and MPP cell number (*193-195*). Murine HSCs that overexpress human BCL-2 out-compete wild-type HSCs in a competitive reconstitution experiment (*193*). BCL-2 over expressing HSCs also exhibit higher viability when challenged with growth factor withdrawal compared to WT cells (*193*). However, mice deficient for BCL-2 do not seem to exhibit overt difficulties in HSC function except for impaired lymphopoiesis (*196*). Therefore, it is unclear what the physiological role of BCL-2 is in stem cell maintenance. Thus far, the role of the BCL-2 family in maintaining stem cell fate has been studied with overexpression and knock-out models. Furthermore, many of the aforementioned studies were performed in heterogeneous populations consisting of HSCs and MPPs. Therefore, the goal of the project described in Chapter 4 is to examine the mitochondrial priming and anti-apoptotic dependencies of HSCs and downstream progenitors.

HEMATOPOIESIS DURING AGING

The above discussion describes how regulation of the apoptotic HSC cell fate is controlled in normal adult HSCs. However, it is appreciated that HSCs isolated from organisms of different ages are not equal. Aging of the HSC compartment is associated with a variety of phenotypes in both humans and

mice including increased absolute numbers of HSCs, decreased lymphopoiesis, increased auto-immunity and increased frequency of myeloproliferative disorders (*197-199*) (**Figure 1.6**). The next section describes the phenotypic changes observed in aging HSCs as well as the intrinsic and extrinsic factors regulating these phenotypes.

HSCs increase in absolute number but decrease in function with age

There is an expansion of the absolute number of HSCs within the bone marrow with age in both humans and mice (*198, 200, 201*). In young mice (two-months old), HSCs represent approximately 8% of the total LSK cells, whereas in old mice, HSCs represent approximately 26% of LSK cells (23-24 month old) (*184*). Similarly in young humans, HSCs comprise approximately 5% of CD34+ cells (consisting of HSCs and MPPs), whereas in the elderly, HSCs represent 11% of CD34+ cells (*198*). The HSC population within the mouse can be further subdivided into $CD150^{hi}$, $CD150^{lo}$ and $CD150^{neg}$ populations. Where the distribution of HSCs isolated from young mice is rather balanced with its expression of CD150, HSCs isolated from old mice have a great expansion within the $CD150^{hi}$ population with a concomitant decrease in the abundance of $CD150^{lo}$ and $CD150^{neg}$ cells (*199*).

Although the absolute number of HSC in the bone marrow increases with age, a second phenotype of aging HSCs is the diminished function of aged HSC. The diminished HSC function has been measured in several ways. First, HSCs and MPPs isolated from old mice exhibit decreased colony formation in stromal co-culture *in vitro* as well as spleen colony forming units abilities *in vivo* compared to young HSCs (*202, 203*). Since multi-potent and lineage committed progenitors are capable of producing colony forming units in the spleen, a more rigorous assay of HSC function is to test the ability of the aged HSCs to engraft and repopulate the bone marrow of an irradiated mouse in primary and secondary transplantations. Consistent with the colony formation assays, HSCs isolated from old mice and competitively transplanted into young recipients do not re-populate the marrow as efficiently as young HSCs in primary and secondary engraftment assays (*184, 202, 204*).

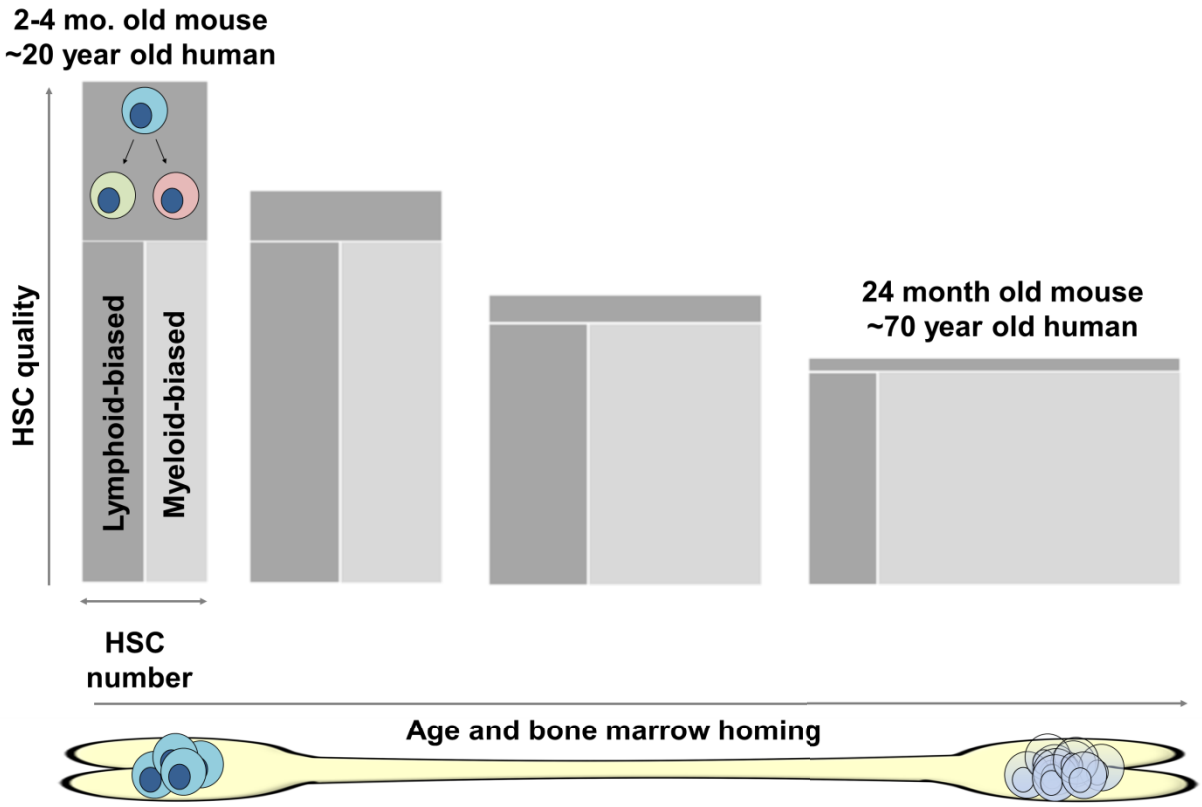


Figure 1.6. The hallmarks of aging in the hematopoietic system. HSC aging is associated with an increase in absolute cell number, a decrease in quality, decreased bone marrow homing and a myeloid skewing. Modified from Woolthuis, de Haan, Huls, Current Opinion in Immunology 2011.

Old human HSCs also have decreased engraftment capabilities when injected into mice (*198*). Additionally, the inferior engraftment of old human HSCs is also observed in the clinic in the setting of bone marrow transplantation. Recipients of bone marrow transplants from older human donors experience an increase transplant-related mortality compared to recipients of young donor bone marrow (*205*). In fact, bone marrow registries request that donors be between 18-44 years of age due to the better long term survival of recipients who receive marrow from younger donors compared to older donors (bethematch.org). This reduced engraftment function of old human HSCs suggest that HSCs lose their self-renewal capabilities with age.

Myeloid skewing with age

A third hallmark of aging HSCs in humans and mice is the preferential production of myeloid cells and loss of lymphoid potential (*198, 199*). As mentioned previously, aging is associated with an increase in the $CD150^{hi}$ HSC population. $CD150^{hi}$ HSCs isolated from both young and old mice and transplanted into irradiated recipients preferentially give rise to myeloid cells, whereas $CD150^{lo}$ HSCs preferentially give rise to lymphoid cells (*199*). Therefore, the preferential expansion of myeloid skewed $CD150^{hi}$ cells compared to the $CD150^{lo}$ cells is thought to contribute to the myeloid skewing of the bone marrow with age. Further, it is thought that the myeloid skewing observed with HSC aging may contribute to the increased incidence of myeloid malignancies with age.

Decreased bone marrow homing with age

A fourth phenotype of aging HSCs is decreased bone marrow homing function. Homing is the process of traveling from the peripheral blood to the bone marrow niche (*206*). Fluorescently labeled old HSCs injected into irradiated recipient mice and assessed approximately 20 hours post-injection show an almost two-fold decreased homing to the bone marrow compared with young HSCs (*202*). The diminished short-term homing efficiency to the bone marrow may contribute to the decrease in transplantation efficiency observed in HSCs isolated from old mice since HSCs that cannot properly home to the bone marrow cannot efficiently re-populate the bone marrow. In contrast, old HSCs exhibit increased mobilization from the bone marrow to the peripheral blood upon cytokine stimulation (*207*). This

decreased homing and increase in mobilization may be driven by decreased cell adhesion of old HSCs to stromal cells. Old HSCs have increased expression of cdc42 which is a Rho GTP-ase involved in the regulation of adhesion signaling (*207*). Young HSCs that are genetically engineered for increased expression of cdc42 have decreased ability to attach to fibronectin *in vitro* as well as lower engraftment capabilities *in vivo* (*208-210*). Therefore, the expression of adhesion molecules may also contribute to the decreased homing of old HSCs.

INTRINSIC AND EXTRINSIC FACTORS EFFECTING AGING HSCS

Due to the decline of function of aged HSCs with the concomitant increase in hematological malignancies, there is a keen interest in understanding the molecular mechanisms governing the above mentioned aging phenotypes of HSCs. This section outlines the various extrinsic and intrinsic mechanisms affecting the HSC aging phenotypes.

Accumulation of DNA damage

Aging of the HSC is associated with the accumulation of DNA damage in both humans and mice. When there is DNA damage in the form double stranded DNA breaks, the DNA repair machinery is recruited to the sites of DNA damage by the phosphorylation of serine 139 of histone H2AX. The phosphorylated H2AX is termed γH2AX and can be visualized with immunostaining (*211*). Thorough analysis of freshly isolated, unstimulated HSCs show that old mouse HSCs exhibit higher levels of γH2AX staining compared to young HSCs (*212, 213*). Increases in γH2AX staining is also observed in old human stem cells (*214*). Since γH2AX staining is mostly a measure of DNA double stranded breaks, a more rigorous analysis of other forms of DNA damage is analysis by comet tail moments which measures both single and double strand breaks. In a recent analysis of comet tail moments in which over 4,900 young HSCs and 3,100 old HSCs were isolated and analyzed for DNA damage by comet tail moments it was observed that old HSCs had significantly more DNA damage compared to young HSCs (*215*).

An increase in DNA damage with age is also observed in human blood cells as γH2AX staining is higher in HSCs isolated from older individuals compared to younger individuals (*214*). Moreover, analysis of whole blood exomes isolated from seemingly healthy individuals (donors who had no evidence of

hematological malignancies) found an increase in clonal hemopoiesis in which clones exhibited genetic mutations in cancer associated genes. Mutations were rarely found in people under the age of 40, but mutation incidence rose significantly with age in (*216-218*). The most common genetic mutations occurred in DNMT3A, TET2 and ASXL which are genes described above as being commonly mutated in hematological malignancies, including AML (*91, 219, 220*).

Above describes evidence that DNA damage is accumulated with HSC age; however, it does not answer the question why do HSCs accumulate DNA damage with age. Recent analysis of HSCs found that the quiescent nature of HSCs may contribute to the accumulation of DNA damage (*187, 215*). As described above, old HSCs at steady state have more DNA damage measured by comet tail moments and γH2AX staining compared to young HSCs. However, both young and old HSCs induced to enter the cell cycle either by cytokine stimulation *ex vivo,* or 5FU treatment *in vivo* repair double stranded DNA breaks as measured by comet tail moments. Examination of the transcriptional genome of the DNA damage repair pathways including non-homologous end joining (NHEJ), nucleotide excision repair (NER), homologous recombination (HR), base excision repair (BER), DNA damage repair and checkpoints (DDRC) find that all of these pathways are attenuated in both young and old HSCs, and these pathways are upregulated in more differentiated, cycling progenitor cells (*215*). Therefore, both old and young HSCs may accumulate DNA damage because they lack expression of repair machinery in their quiescent state.

Mitochondrial DNA damage accumulation

In addition to nuclear DNA damage accumulation with age, an increase of mutations in mitochondrial DNA (mtDNA) is evident in many aging tissues. Transgenic mice harboring an error prone mitochondrial DNA polymerase (POLG) have a similar phenotype to aged mice including increased hematopoiesis in the spleen and liver which are commonly found in aged mice (*221, 222*). Although pathologically POLG mutant mice may reflect the phenotype of aging blood, transgenic POLG mice do not exhibit all the hallmarks of aged HSCs. HSCs isolated from young mice deficient for POLG preferentially give rise to myeloid cells upon transplantation, similar to old WT HSCs, but the young POLG deficient HSCs do not share the same CD150 expression patterns of WT old HSCs. Furthermore, POLG

transgenic mice do not exhibit an increase in absolute HSC cell number (*223*). Therefore, mitochondrial mutations accumulated with age may contribute to some of the observed phenotypes of aged HSCs (myeloid skewing), but do not account for all of the phenotypes.

Gene expression and epigenetics alterations

To determine what genes regulate the phenotype changes observed in young and old mice, several studies have performed gene expression analysis on young and old HSCs (*184, 204*). Genes associated with the myeloid lineage tend to be over-expressed, whereas genes associated with the lymphoid lineage tend to be down regulated in old murine HSCs compared to young HSCs (*184*). This change in expression likely contributes to the myeloid skewing of HSCs with age. Furthermore, additional studies identified genes associated DNA repair and histone modification were down-regulated with age; while genes associated with the stress response, protein folding and the inflammatory response were overexpressed in old HSCs (*185, 204*).

Age-related epigenetic alterations likely contribute to the gene expression changes described above (*204*). Both alterations in DNA methylation as well as histone modifications have been observed with HSC aging. In general, HSC aging is associated with a slight increase in global methylation with site specific regions of significant hypermethylation of HSC DNA (*183*). Methylome analysis of specific regions of DNA found genes associated with lymphoid lineage potential are hypermethylated, whereas genes associated with myeloid cells are generally hypomethylated (*183*). Since methylation suppresses expression of genes found at that region of DNA, this pattern of methylation likely contributes to the gene expression alterations leading to the myeloid skewing of aged HSCs. Interestingly, expression of enzymes which manage DNA methylation, such as DNMT3A and TET2 decrease with age, and deletions of these enzymes in murine mouse models recapitulate some of the hallmarks of HSC aging (*185*). Loss of DNMT3A in the murine bone marrow results in greater self-renewal of HSCs and inhibition of differentiation (*224*), and silencing of TET2 in the hematopoietic compartment results in increased self-renewal and increased myeloproliferation (*225*).

Modifications to chromatin have also been associated with HSC aging. Recent analysis identified differences in repressive and activating histone marks in HSCs isolated from young and old mice (*185*).

There is a broad accumulation of methylation of H3K4 (H3K4me3), a marker of active chromatin, and an accumulation of H3K27me3, a repressive marker. The Slamf1 gene (CD150) is marked by the activating histone mark H3K4me and the $CD150^{hi}$ population expands within the aging HSC population, whereas, the FLT3 gene is marked with H3K27me3 and has decreased expression in old HSCs which may contribute to the decreased lymphoid potential with age (*185, 226*). Together, these data suggest that epigenetic alterations modulate the functional decline of aged HSCs.

Microenvironment niche alterations

The bone marrow microenvironment in which HSCs reside undergoes functional changes with age. Aging of the bone marrow niche is accompanied with decreased bone formation, decreased bone density, increased adipogenesis and changes to the extracellular matrix (*227, 228*). However, the role of the aging BM niche in regulating the aging phenotypes of aged HSCs is unclear. Transplantation of old HSCs into young recipients does not rescue the old cell phenotype, rather the cells remain myeloid skewed and have decreased transplantation efficiencies (*184*). However analysis of pre-leukemic cells instead of normal aged HSCs found that pre-leukemic cells injected into old murine recipients preferentially expand in the old niche compared to cells injected into young mice (*229, 230*). The specific cell types and cytokines that regulate the preferential expansion of pre-leukemic cells in the old bone marrow niche are yet to be fully identified. The inflammatory cytokine, Rantes, has been implicated in the myeloid skewing of old cells. Ex-vivo exposure of bone marrow cells to Rantes increases the output of myeloid cells and decreases the lymphoid progenitor output, and Rantes knockout mice have deficient myeloid cell output compared to WT mice suggesting that the cytokine may be important in the myeloid skewing of aged HSCs (*231*). Furthermore, proteins involved in the TGFβ pathway are likely candidates to regulate the stem cell niche as these genes are deregulated with age (*185, 232*).

Role of Inflammation

The free radical theory of aging, developed in the 1950s, suggests that aging is due to the accumulation of DNA damage caused by reactive oxygen species (*233*). It is unclear if and how ROS contributes to the aging HSC phenotype. The stem cell niche is relatively hypoxic, and it is thought that

the low oxygen state of the niche uniquely protects HSCs from the production of free radicals (*234*). Furthermore, HSCs are quiescent and metabolically inactive, effectively reducing their exposure to oxygen radicals. However, treatment of HSCs with the antioxidant N-acetyl-L-cysteine prevents reduction of reconstitution capabilities in serial transplantation assays of HSCs (*235*). This finding suggests that the accumulation of ROS with age may contribute to the decreased reconstitution abilities of old HSCs.

Role of mitochondrial apoptosis in aging HSCs?

One significant gap in understanding the aging phenotypes of increased HSC number and myeloid skewing is how apoptosis is regulated in aging HSCs. Apoptosis is a cell fate of HSCs, yet there is a dearth of information regarding how apoptosis is controlled during normal stem cell differentiation as well as aging. As is stated above, old HSCs accumulate DNA damage and epigenetic alterations with age; however, it is not known how these alterations may contribute to the functional decline of HSCs. It is possible that the accumulation of these changes in aged HSCs may affect the ability of the cells to respond to apoptotic stimuli. Initial evidence using gene expression analysis has identified that the pro-apoptotic activator protein BIM is significantly down regulated in old HSCs and progenitor cells compared to young cells (*215*). However, these studies lack a thorough investigation into all of the BCL-2 apoptotic family members and fail to functionally test whether aging is associated with a decrease in apoptosis in aging HSCs. Therefore, the second goal of the project presented in chapter four is to determine whether HSCs are differently primed and how this affects the ability of the cells to respond to DNA damage. The results of this study have implications as to why there is an increase in stem cell number and myeloid skewing with age.

CHAPTER 2: SELECTIVE BCL-2 INHIBITION BY ABT-199 CAUSES ON-TARGET CELL DEATH IN PRE-CLINICAL MODELS OF ACUTE MYELOID LEUKEMIA

Copyright disclosure:

This work was published in Cancer Discovery, 2014 (*54*), and re-printed with permission from the journal.

Attributions:

This work was the result of a productive collaboration between myself and Aaron Pan, a graduate student in Dr. Marina Konopleva's lab at University of Texas MD Anderson Cancer Center. All data was collected and compiled by myself with the exception of: Figure 1-2, 4C-G: 6A-B.

I. ABSTRACT

B-cell leukemia/lymphoma 2 (BCL-2) prevents commitment to programmed cell death at the mitochondrion. It remains a challenge to identify those tumors that are best treated by inhibition of BCL-2. Here we demonstrate that acute myeloid leukemia (AML) cell lines, primary patient samples, and murine primary xenografts are very sensitive to treatment with the selective BCL-2 antagonist ABT-199. In primary patient cells, the median IC50 was approximately 10 nM, and cell death occurred within 2 h. Our *ex vivo* sensitivity results compare favorably with those observed for chronic lymphocytic leukemia (CLL), a disease for which ABT-199 has demonstrated consistent activity in clinical trials. Moreover, mitochondrial studies using BH3 profiling demonstrate activity at the mitochondrion that correlates well with cytotoxicity, supporting an on-target mitochondrial mechanism of action. Our protein and BH3 profiling studies provide promising tools that can be tested as predictive biomarkers in any clinical trial of ABT-199 in AML.

II. INTRODUCTION

Acute myelogenous leukemia (AML) is a hematopoietic neoplasia characterized by the rapid, clonal growth of the myeloid lineage of blood cells. The disease affects approximately 14,000 adults in the United States each year and unfortunately, despite recent advances in the treatment of AML, 10,400 people die from their disease (*236*). Most AML patients become resistant to chemotherapy at some point

in their course and succumb to their disease. Therefore, it is necessary to prevent chemo-resistance or enhance chemosensitivity in a selective fashion to lead to a higher cure rate and a lower toxic burden.

A novel strategy to treat cancer cells is to directly stimulate the mitochondrial apoptotic pathway in them. The mitochondrial apoptotic pathway is regulated by the B-cell leukemia/ lymphoma 2 (BCL-2) family of proteins. These proteins respond to upstream apoptotic signals that control mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c, a hallmark of mitochondrial apoptosis (*7*). The family consists of pro-apoptotic BH3-only proteins, pro-apoptotic multi-domain effector proteins and anti-apoptotic proteins which together act as a rheostat to control MOMP. The BH3-only proteins are further subdivided into two groups based on function - the "activators" (BID, BIM and PUMA) and "sensitizers" (such as BAD, BIK, HRK and NOXA) (*14, 237, 238*). The activators bind to and inhibit the anti-apoptotics (such as BCL-2, BCL-XL and MCL-1), and also directly interact with the effector proteins causing the proteins to oligomerize at the mitochondria and cause MOMP (*29, 239*). However, the BH3-only sensitizers can only bind to the anti-apoptotics which allows for the release of the activator and effector proteins to drive MOMP (*14*).

Navitoclax (ABT-263), which functions as a small molecule mimetic of the BH3 domain of the BH3-only sensitizer protein BAD, efficiently binds to BCL-2, BCL-XL and BCL-W, releasing bound pro-apoptotic proteins and causing MOMP in BCL-2 dependent cancer cells (*169*). In early clinical trials, navitoclax showed potency in the treatment of chronic lymphocytic leukemia (CLL) and small-cell lung cancer (*170, 171*). However, treatment with navitoclax causes on-target, dose-limiting thrombocytopenia because platelets are dependent on the anti-apoptotic protein BCL-XL for their survival (*61*). The dose-dependent thrombocytopenia limited navitoclax's use in many malignancies, particularly leukemias where patients often present with pre-existing thrombocytopenia. This prompted the development of ABT-199, a modified BH3-mimetic derivative of ABT-263 which maintains specificity for BCL-2, but lacks affinity for BCL-XL (*172*). The remodeled drug has shown cancer killing efficacy in CLL *in vivo,* myc-driven lymphomas in mice and estrogen receptor-positive breast cancer while sparing platelets (*172, 173, 240*).

AML bulk and stem cells are dependent on BCL-2 for survival and BCL-2 inhibition by ABT-737 (an *in vitro* tool compound with activity very similar to navitoclax) causes cell death in AML cells (*160*). Importantly, BCL-2 inhibition relatively spares normal hematopoietic stem cells which are more dependent

on MCL-1 for their survival (*56, 189*). Thus, the first goal of the present study is to evaluate the anti-cancer effects of ABT-199 on AML and compare its efficacy with ABT-737/navitoclax, drugs that have both shown activity in the *ex vivo* treatment of AML cell lines and AML primary patient samples and in human clinical trials (*160*). The second goal is to determine if BH3-profiling can be used as a tool to predict cellular response to ABT-199 treatment.

BH3-profiling is a method to determine the mitochondrial priming level of a cell by exposing cellular mitochondria with standardized amounts of peptides derived from the BH3 domains of BH3-only proteins and determining the rate of MOMP, as measured by either cytochrome c release or depolarization across the inner mitochondrial membrane (*39*). Previously, we have shown that the priming status of the cell is predictive of the cell's chemo-responsiveness in that the more primed the cell is, the more sensitive the cell is to various chemotherapeutics (*52, 56*). Furthermore, BH3-profiling can also identify anti-apoptotic addictions (*52, 56, 241*). For instance, the BAD BH3-only peptide binds with high affinity with BCL-2, BCL-XL and BCL-W, while the HRK BH3 peptide binds with high affinity only to BCL-XL. Thus, MOMP following BAD peptide incubation suggests an anti-apoptotic dependency on BCL-2, BCL-XL or BCL-W, while MOMP following HRK peptide incubation indicated dependency on BCL-XL. Using this tool, we can identify AML cells which depend on BCL-2 for survival and that are more likely to die following BCL-2 inhibition. Thus, we hypothesize that cells that are addicted to BCL-2 for survival will be sensitive to ABT-199 and that we can predict this response by BH3 profiling.

III. METHODS

Cell lines

The AML cell lines were purchased from the American Type Culture Collection (Manassas, VA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) or were kindly provided by Dr. James Griffin (Dana-Farber Cancer Institute, Boston, MA). HL-60, MOLM-13, OCI-AML2, OCI-AML3, KG-1, U937 and Kasumi-1 were validated in September 2010 by short tandem repeat DNA fingerprinting using the AmpFlSTR Identifiler kit according to manufacturer's instructions (Applied Biosystems). HL-60, MOLM-13, OCI-AML2, OCI-AML3, KG-1, U937 and Kasumi-1 were validated in September 2010 by short tandem repeat DNA fingerprinting using the AmpFlSTR Identifiler kit according

to manufacturer's instructions (Applied Biosystems). HL-60 cell lines with stable overexpression of BCL-2 or BCL-XL, and the control cell line with empty vector, were kindly provided by Dr. Kapil N. Bhalla (The Methodist Hospital Research Institute, Houston, TX). AML cell lines were cultured in RPMI 1640 medium supplemented with 10% or 20% fetal bovine serum, 10mM L-glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2.

Treatment of AML Cell Lines with ABT-737 and ABT-199

AML cells were incubated for appropriate time in RPMI 1640- medium supplemented with 10% FBS and titrated concentrations of ABT-199 or ABT-737. Viable AML cells were enumerated by flow cytometry using counting beads with concurrent Annexin-V and propidium iodide (PI) staining. IC50 values were calculated using Calcusyn software (Biosoft, Cambridge, MA) based on the number of live cells (Annexin-V^-/PI^-).

Quantitative Western Blot

Cell lysates were prepared and immunoblotted as previously described (*242*). Antibodies used for quantitative Western blot were: BCL-2 (#M0887, Dako, Carpinteria, CA), BCL-XL (# 2764, Cell Signaling Technology, Danvers, MA), MCL-1 (#559027, BD Biosciences, San Diego, CA), α-Tubulin and β-Actin (loading controls, #T6199 and #A5441, Sigma-Aldrich, St. Louis, MO). Blots were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The band intensity was quantified using Odyssey software v2.0. The ratio of band intensity of BCL-2 proteins relative to that of loading control was normalized to the ratio in untreated OCI-AML3 cells.

Gene Knockdown by shRNA

MCL1 was knocked down by lentiviral transduction using a MCL1-specific shRNA transfer vector targeting residues 2421-2440 on RefSeq NM_021960.4. Lentivirus was prepared by co-transfection of HEK293T cells (ATCC) with an equal molar mix of transfer vector and packaging plasmid (psPAX2 and pMD2.G, Addgene, Cambridge, MA) using JetPrime transfection reagent as directed by the manufacturer (Polyplus, Illkirch, France). Fresh lentiviral supernatants were passed through 0.45 μm surfactant-free

cellulose acetate membranes; polybrene was added to 8 µg/mL, and the virus stock was used at once to spinoculate OCI-AML3 cells as described before (*243*). Infected cells were selected with 0.5 µg/mL puromycin. In parallel, control cells were transduced using lentivirus delivering a hairpin targeting GFP in pLKO.1 (Addgene). Knockdown was verified by Western blot analysis.

Selection of Resistant MOLM-13 Cells and Measurements of BCL-2 Protein by Intracellular Flow Cytometry

Resistant MOLM-13 cells were selected in RPMI 1640 medium supplemented with 10% FBS and 50 nM of ABT-199. Every two days, the cells were pelleted by centrifugation and resuspended in fresh medium with 50 nM ABT-199. Cell viability was monitored by Vi-CELL viability analyzer (Beckman Coulter, Irving, TX) until MOLM-13 cells reached a viability higher than 90%. The cellular content of BCL-2 protein was measured in both parental and resistant MOLM-13 cells by flow cytometry. Briefly, 1 million highly viable cells were washed twice with PBS and fixed in 1 mL of 4% paraformaldehyde for 15 minutes on ice, followed by washing with PBS and permeabilization with 1 mL of 0.1% Triton X-100 in PBS-buffered bovine serum albumin (BSA, 1% w/v). After incubation on ice for 10 minutes, cells were then washed with washing buffer (1% BSA in 1 x PBS), resuspended in 90 µL of washing buffer and stained with 10 µL of FITC-conjugated BCL-2 antibody or FITC-conjugated IgG1 isotype control (#F7053 and #X0927, DAKO, Carpinteria, CA). After incubation in dark at room temperature for 30 minutes, the cells were washed again with washing buffer and analyzed by flow cytometry using Gallios^{TM} flow cytometer (Beckman Coulter). The intensity of BCL-2–associated fluorescence was measured on a logarithmic scale. For each sample, 20 000 cells were analyzed for Median Fluorescence Intensity (MFI) using Kaluza flow analysis software (Beckman Coulter).

BH3 Profiling of Cell Lines

AML cell lines were seeded at a density of 4 x 10^5 cells/mL in 10% FBS RPMI media supplemented with 10 mM L-glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin 24 h before BH3 profiling. Two million cells of each cell line were pelleted at 400 xg for 5 minutes at RT and resuspended in 2 mL DTEB (135 mM Trehalose, 10 mM HEPES-KOH, 0.1% w/v BSA, 20 µM EDTA, 20 µM EGTA, 50 mM KCl, 5 mM

succinate, final pH 7.4). Cell lines were profiled by using the plate-based JC-1 BH3 profiling assay previously described (*16*). Cells were permeabilized with digitonin, exposed to BH3 peptides, and mitochondrial transmembrane potential loss was monitored using the ratiometric dye JC-1.

Isolation and Treatment of Primary AML Cells with ABT-263, ABT-737 and ABT-199

Primary AML cells were obtained by informed consent from the Dana-Farber Cancer Institute, Leukemia Group, the Pasquarello Tissue Bank at the Dana-Farber Cancer Institute, the University of Texas MD Anderson Cancer Center, Leukemia Tissue Bank Shared Resource from the Ohio State University Comprehensive Cancer Center and the Germany-Austrian Study Group according to protocols approved by the Institute's Institutional Review Board. Samples were Ficoll purified, used immediately or viably frozen in 90% FBS/10%DMSO.

Fresh (**Figures 2.4A, Figure 2.4D** and **Figure 2.4E**) or thawed (Figures **Figure 4.4B**, **Figure 4.5**) mononuclear cells were resuspended in culture medium supplemented with cytokines. Cells were treated with ABT-199, ABT-263 or ABT-737 for appropriate time. Cells were then washed with PBS and resuspended in Annexin binding buffer. Cell viability was assessed by FACS analysis following concurrent Annexin V and PI (or 7-AAD) staining.

Apoptosis of AML Stem/Progenitor Cells

AML mononuclear cells were isolated by Ficoll density centrifugation and cultured with 100 nM ABT-199 or ABT-737 as described above. After 24 h, AML cells were washed twice in Annexin binding buffer (ABB) and resuspended in 100 µL ABB containing 1:100 dilution of Annexin-V-APC (#550475), 1:50 dilution of CD45-APC-Cy7 (#557833), CD34-FITC (#555821), CD38-PE-Cy7 (#335790) and CD123-PercP-Cy5.5 (#58714) (all from BD Biosciences) for 20 minutes at room temperature in dark. Following staining, cells were washed with ABB and resuspended in 95 µL ABB containing 5 µL DAPI. Cells were analyzed by Gallios Flow Cytometer (Beckman Coulter). Results were expressed as percentage of specific apoptosis calculated as: (% AnnV+ cells sample-%AnnV+ cells control)/(100-%AnnV+ cells control).

In vivo Study of ABT-199 Efficacy in AML Mouse Models

All animal studies were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committees at the University of Texas MD Anderson Cancer Center. Twenty female NOD SCID gamma (NSG) mice (6-wk old, Jackson Laboratory, Bar Harbor, MA) were intravenously injected with luciferase-labeled MOLM-13 cells (0.7×10^6 cells/100 µL) and randomly divided into two groups. Four days post injection, the mice were treated with vehicle or ABT-199 (100 mg/kg body weight) daily by oral gavage for 2 weeks. For oral dosing, ABT-199 (10 mg/mL) was formulated in 60% phosal 50 propylene glycol, 30% polyethyleneglycol-400, and 10% ethanol. Bioluminescence imaging (BLI) was used to monitor tumor burden on different time points. Briefly, mice were anaesthetized and injected intraperitoneally with firefly luciferase substrate D-luciferin and then imaged noninvasively using IVIS-200 *in vivo* imaging system (PerkinElmer, Waltham, MA). Three mice from each group were sacrificed by CO_2 asphyxiation after 15 d. Bone marrow, spleen, and liver were collected for H&E and immunohistochemical staining. The remaining seven mice in each group were followed for survival.

For primary AML derived xenograft models, NSG mice were sub-lethally irradiated (250 cGy) the day prior to intravenous injection of 10^5 PDX21 patient-derived AML cells. Three weeks following injection and after confirmation of AML engraftment, the mice were randomly divided into two groups and treated with 100 mg/kg ABT-199 or vehicle via gavage daily for 2 weeks. All mice were then sacrificed and femur bone marrows were analyzed for leukemia burden by CD45 flow cytometry (using anti-human CD45-PE antibody #555483, BD Biosciences, San Jose, CA).

Immunohistochemistry Analysis

Immunohistochemistry was performed as described previously (*244*). Briefly, the tissue was formalin-fixed, paraffin-embedded, sectioned into 5-µm thickness and mounted onto microscope slides. Tissue sections were then deparaffinized and rehydrated using xylene and ethanol in decreasing concentration. Samples were stained with hematoxylin and eosin (H&E) for histopathological evaluation. For immunohistochemical staining, the tissue sections were incubated with primary antibody against human CD45 (#555480, BD Biosciences, San Jose, CA), followed by sequential incubation with biotinylated secondary antibody, peroxidase labeled streptavidin and 3,3' diaminobenzidine tetrahydrochloride/H_2O_2

(Dako), which resulted in a brown precipitate at the antigen site. Images were taken using an optical microscope under the same magnification.

Microarray-Based Gene Expression Profiling in AML

The expression of BCL-2 family genes was determined using oligonucleotide microarrays (HG-U133 Plus 2.0, Affymetrix) in 288 AML samples comprising all cytogenetic groups, and in 103 normal samples (healthy BM and non-leukemia conditions) as described in Haferlach et al (*245*). All samples in this study were obtained from untreated patients at the time of diagnosis. Cells used for microarray analysis were collected from the purified fraction of mononuclear cells after Ficoll density centrifugation. The study design adhered to the tenets of the Declaration of Helsinki and was approved by the ethics committees of the participating institutions before its initiation. The analysis is conducted at logarithm-2 transformed gene expression intensities. Correlation analysis based on Pearson correlation coefficient and Spearman's rank correlation coefficient was performed to identify probe sets that have consistent expression pattern corresponding to a common gene. Two-sample t-test was performed for each two-group comparison, and the *P* value threshold of 0.005 was used to moderately control for multiple testing.

iBH3 of Primary AML Patient Cells

Thawed cells were washed 1x with PBS and stained with 1:100 Invitrogen Live/Dead – near IR stain (#10119, Life Technologies, Grand Island, NY) in PBS for 20 min on ice, washed with PBS and subsequently stained with CD45-V450 (#642275; BD Biosciences; San Jose, CA) 1:100 FACS buffer on ice for 20 min. Cells were pelleted at 400 x g for 5 min at RT and resuspended in DTEB. 100 uL of cells in DTEB was added to each tube containing twice the final concentration of each peptide treatment in 100 uL of DTEB with 0.002% w/v digitonin. Mitochondria in the permeabilized cells were exposed to peptides for 60 minutes at ambient temperature before the addition of 200 uL 4% v/v formaldehyde at RT for 15 minutes, quenched with 50 uL of 100 mM Tris / 2.5 M glycine pH 8.2 for 5 minutes at RT, and pelleted at 1500 xg for 5 minutes at RT. Cells were stained with anti-cytochrome c-Alexa488 (#560263, BD Bioscience) 1:100 in 0.1% Saponin/1%BSA/PBS overnight at 4^{o}C and diluted 1:5 in PBS an hour before

FACS on a LSR Fortessa flow cytometer (BD Bioscience) to quantify cytochrome c loss calculated from the median fluorescence intensity (MFI) as

% cyto c loss = 100 x (1 – $\underline{([MFI_{sample} – MFI_{Isotype}]}$ ÷ [MFI_{DMSO}-MDI $_{Isotype}$]))

AML blasts were identified by low-mid CD45/low SSC-A.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software v6.0 (GraphPad, La Jolla, CA). Unless otherwise indicated, the results are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Differences with *P* values ≤ 0.05 were considered statistically significant.

III. RESULTS

ABT-199 Kills AML Cell Lines Potently and Quickly in vitro and in vivo

As an initial test of the potential utility of ABT-199 in AML, we exposed AML cell lines to increasing concentrations of ABT-199 for 48 h and then determined the IC50 values. Comparisons were made with ABT-737. As shown in **Figure 2.1A**, the IC50 of ABT-199 ranged from <10 nM to >1000 nM, and sensitivity to ABT-737 roughly tracked sensitivity to ABT-199. It is notable that in sensitive AML cell lines (IC50 < 0.1 μM), ABT-199 is more potent than ABT-737, probably due to ABT-199's 5-fold higher affinity to BCL-2 protein (*172*). Prior experience with CLL, a disease for which excellent clinical activity of ABT-199 has been observed, has revealed that CLL cells are killed in an on-target fashion, and that the killing was evident within 4 h (*60*). Therefore, we tested whether ABT-199 could rapidly induce apoptosis in a sensitive AML cell line - MOLM-13. In **Figure 2.1B** and **Figure 2.1C**, it can be seen that cell growth is inhibited and cell apoptosis is observed within just a few hours of exposure to ABT-199.

To demonstrate that the efficacy seen was consistent with tolerable *in vivo* delivery of ABT-199, we tested the effect of ABT-199 on an aggressive mouse xenograft model of MOLM-13. NOD SCID gamma (NSG) mice were injected with luciferase-labeled MOLM-13 cells and monitored by bioluminescence imaging (BLI) for tumor development. After confirmation of AML engraftment in the bone marrow (**Figure 2.1D**, day 4), the mice were treated with ABT-199 (100 mg/kg) by daily oral gavage for 2

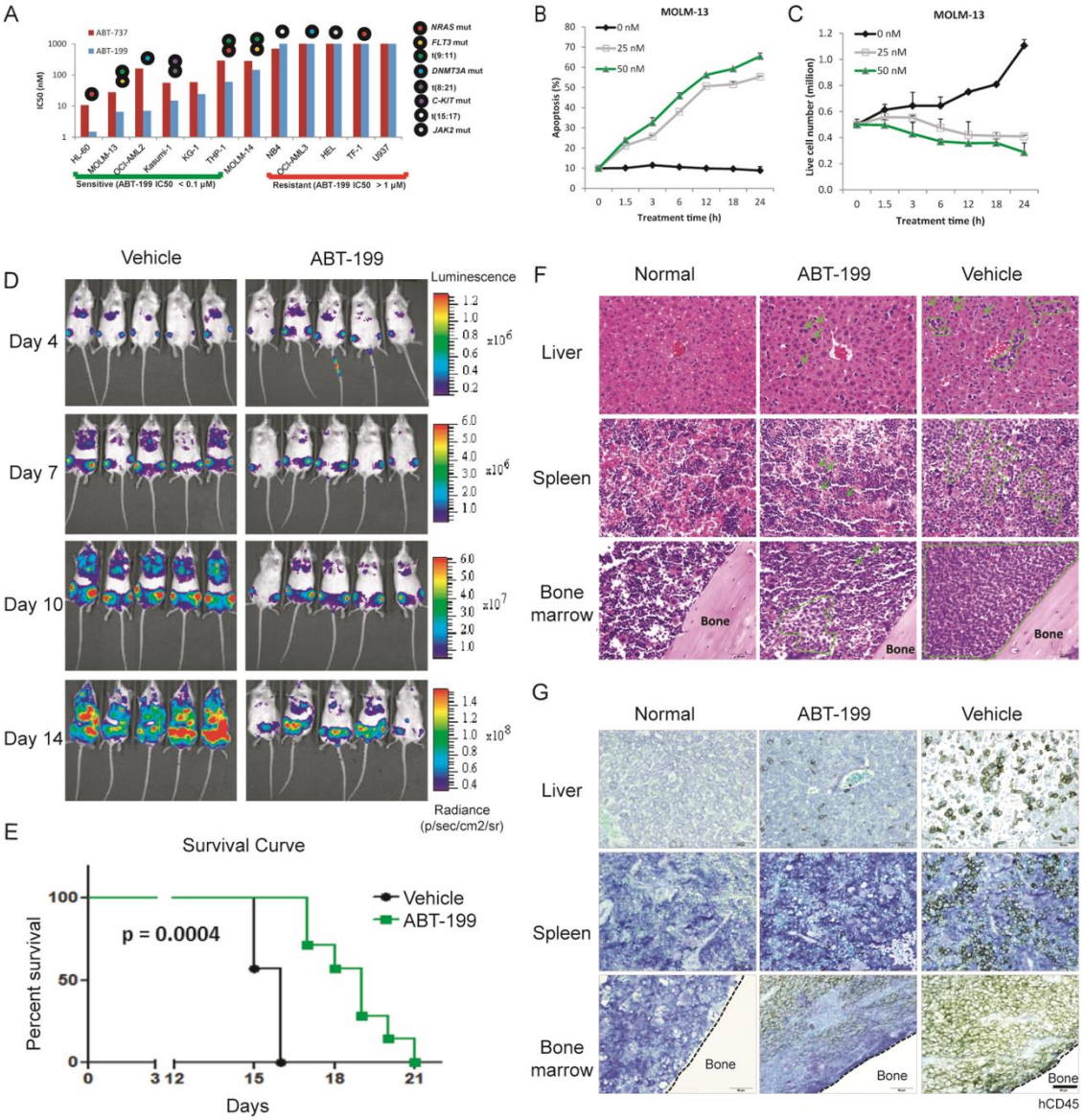


Figure 2.1. Selective inhibition of BCL-2 by ABT-199 kills AML cell lines quickly and effectively.

A). AML cell lines were treated with ABT-199 or ABT-737 for 48 h. Calcusyn software was used to calculate the IC50 values based on the number of viable cells (i.e., Annexin V^-/PI^-) determined by FACS analysis. **B).** MOLM-13 AML cells were treated with indicated concentrations of ABT-199. Apoptosis induction was determined by Annexin V/PI flow cytometry. **C).** Viable (i.e., Annexin V^-/PI^-) cell counts were quantified by FACS analysis using CountBright counting beads. **D).** Serial bioluminescence images of mice bearing MOLM-13 tumors treated with the vehicle or ABT-199 (treatment started on day 4, administered by oral gavage at dose of 100 mg/kg). **E).** Kaplan-Meier survival curves for mice treated as described in E (n = 7 per arm). Statistical significance was calculated using Log-rank (Mantel-Cox) test ($p < 0.0004$). **F).** H&E staining of histological sections of liver, spleen, and bone marrow 15 d post leukemia cell injection. Age- and sex-matched mice without tumor were used as controls. Representative MOLM-13 cells are indicated by arrows. Representative engraftment areas are circled in green. All pictures were taken under the same magnification; scale bar equals 50 μm. **G).** Immunohistochemical staining of histological sections of liver, spleen, and bone marrow with human CD45 antibody 15 d post leukemia cell injection. Scale bar equals 50 μm.

weeks. Serial BLI images showed that ABT-199 treatment markedly inhibited leukemia progression, which translated into prolonged overall survival when compared to vehicle-treated mice ($p = 0.0004$, **Figure 4.1E**). ABT-199 treated mice also carried significantly lower leukemia burden in bone marrow, spleen and liver as indicated by hematoxylin and eosin staining (H&E, **Figure 2.1F**) and immunohistochemical analysis of human CD45 (**Figure 2.1G**).

ABT-199 Sensitivity Correlates with BCL-2 Protein Level

Next we tested whether there were correlates of cell line sensitivity to ABT-199 that supported an on-target action of killing via competition for the BH3 binding site selectively of BCL-2. Relative levels of several BCL-2 family proteins were measured by Western blot and densitometry (**Figure 2.2A**). Spearman analysis was performed to evaluate the correlation between IC50 values and protein expression. Levels of BCL-2 correlated with sensitivity to ABT-199, while levels of BCL-XL inversely correlated with ABT-199 sensitivity (**Figure 2.2B**). Levels of MCL-1 demonstrated a trend to anti-correlation with sensitivity to ABT-199, but the trend was not statistically significant (**Figure 2.2B**). These observations supported the on-target effects of ABT-199.

The OCI-AML3 cell line was relatively insensitive to ABT-199 and ABT-737 (**Figure 2.1A**). A quantitative immunoblot showed that OCI-AML3 cells had high expression of BCL-2 and MCL-1 and relatively low level of BCL-XL (**Figure 2.2A**). If ABT-199 is a BH3 mimetic specific for BCL-2, then MCL-1 knockdown should significantly sensitize OCI-AML3 cells to this compound. To test this, MCL-1 protein level was reduced by 85% in OCI-AML3 cells by lentiviral transduction using a previously validated MCL1-specific shRNA, without affecting BCL-2 protein expression (**Figure 2.2C**). Indeed, MCL-1 knockdown greatly increased sensitivity to ABT-199 (**Figure 2.2D**) as well as to ABT-737. HL-60 cells with high levels of BCL-2 protein and relatively low BCL-XL and MCL-1 expression are very sensitive to ABT-199 (**Figure 2.1A**). BCL-XL overexpression conferred resistance to ABT-199 in HL-60 cells, while BCL-2 overexpression made HL-60 cells moderately resistant to ABT-199 (**Figure 2.2E-F**). All these results are consistent with a killing mechanism operating via selective targeting of BCL-2 in AML cells.

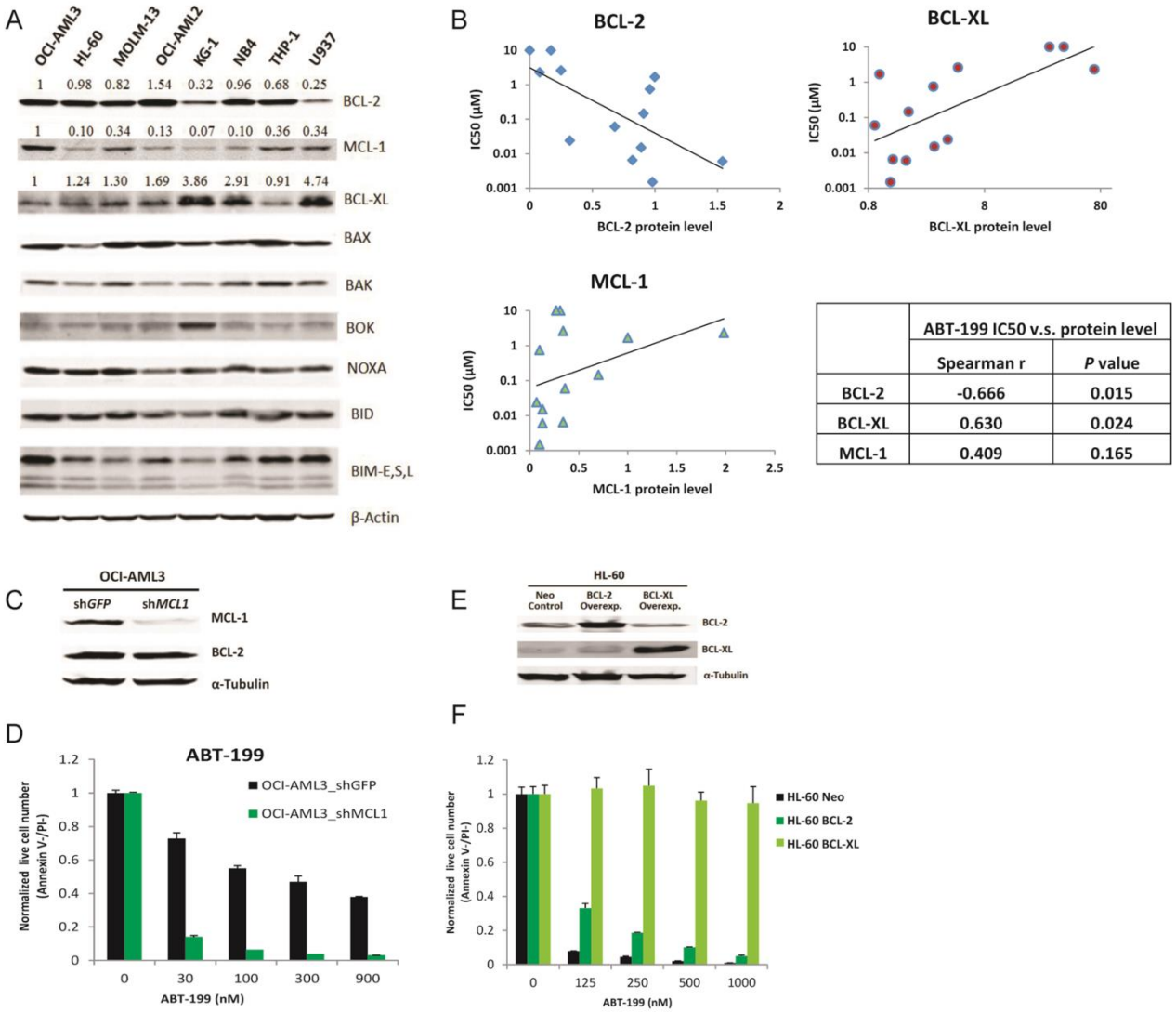


Figure 2.2. Sensitivity to ABT-199 positively correlates with endogenous BCL-2 protein level and negatively correlates with BCL-XL protein level in AML cell lines.
A). Western blot analysis of BCL-2 family proteins in untreated AML cells. The band intensity was quantified using Odyssey v2.0 software, and displayed numerically as a ratio of the band intensity detected in the OCI-AML3 cells. **B).** Significant correlations were observed between ABT-199 IC50 values and BCL-2/BCL-XL protein levels. The non-parametric one-tailed Spearman test was used to determine the correlation coefficient. The p values provided are nominal p values not corrected for multiple comparisons. **C).** MCL-1 knockdown by 85% was achieved by lentiviral shRNA. **D).** MCL-1 knockdown significantly sensitized OCI-AML3 cells to ABT-199. **E).** Western blot analysis showing HL-60 AML cells transfected to stably overexpress BCL-XL or BCL-2. **F).** Overexpression of BCL-XL or BCL-2 in HL-60 cells confers complete resistance to ABT-199-induced apoptosis.

ABT-199-resistant MOLM-13 cells express lower BCL-2 levels compared to the parental cells

Although ABT-199 effectively induced apoptosis in MOLM-13 cells, a fraction of MOLM-13 cells remained alive after 24 h treatment with 50 nM ABT-199 (**Figure 2.2B**). To determine whether surviving cells represent a sub-clone with low target (Bcl-2) expression levels, we selected ABT-199-resistant MOLM-13 cells by culturing parental cells in medium containing 50nM ABT-199. Intracellular flow cytometry was performed to measure cellular BCL-2 protein in the parental and ABT199-resistant MOLM-13 cells. Although no distinct sub-populations were observed, the BCL-2 associated MFI of the parental MOLM-13 cells distributed between ~1 to ~20, indicating heterogeneity of BCL-2 expression in the parental cells. In sum, resistant cells expressed lower levels of BCL-2 protein compared to the parental cells (resistant cells: MFI = 3.48; parental cells: MFI = 5.51; $P < 0.0001$).

ABT-199 works selectively on BCL-2 dependent mitochondria

If ABT-199 is killing cancer cells via displacement of pro-apoptotic proteins from BCL-2, it should be operating on mitochondria. As one would expect if this were the case, we observed a correlation between direct mitochondrial toxicity and cellular toxicity for ABT-199 and ABT-737 in the 12 cell lines studied in **Figure 2.1A** (**Figure 2.3A, B**). We also tested whether detection of BCL-2 dependence using mitochondrial exposure to the BAD BH3 peptide correlated with cellular sensitivity to these agents. We found that while there was a good correlation between mitochondrial sensitivity to the BAD peptide and cellular drug sensitivity for the most sensitive cell lines, there was a group of relatively drug -resistant cell lines that still demonstrated mitochondrial sensitivity to the BAD BH3 peptide (**Figure 2.3 C, D**). A clue to the reason for this was revealed by the tendency of these cell lines to have mitochondria that were also quite sensitive to the HRK peptide, an indicator of BCL-XL dependence. To ensure that we were studying BCL-2 dependence specifically, especially in these less drug-sensitive cells, we made a correction, by subtracting the HRK signal from the BAD signal. In **Figure 2.3E** and **Figure 2.3F**, we used this modified metric to observe a good correlation between mitochondrial BCL-2 dependence and cellular sensitivity.

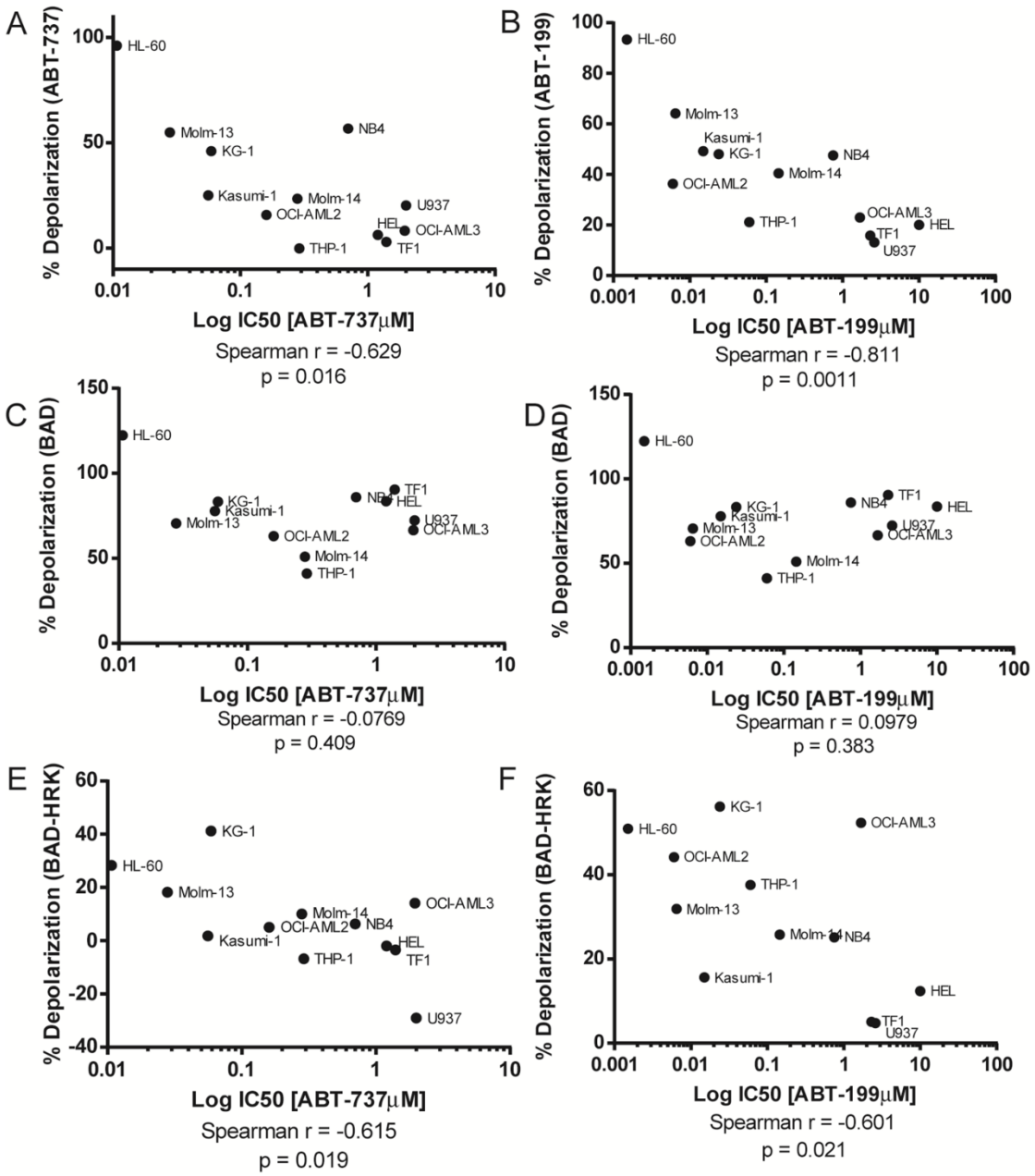


Figure 2.3. ABT-199 functions selectively on BCL-2 dependent mitochondria in AML cell lines. **A).** The IC50 values of AML cell lines treated with ABT-737 from Figure 2.1A were correlated with the mitochondrial response of ABT-737 (1μM). Mitochondrial response was measured by JC1 based BH3 profiling. **B).** IC50 values of cell lines treated with ABT-199 from Figure 2.1A were correlated with the mitochondrial response of mitochondrial ABT-199 (0.1μM). **C).** IC50 values of AML cells treated with ABT-737 were correlated with the response to the BAD BH3 (80μM). **D).** IC50 values of AML cell treated with ABT-199 from Figure 2.1A were correlated with the mitochondrial response of the BAD BH3 (80uM) peptide. **E).** IC50 values of AML cells treated with ABT-737 were correlated with the response to the BAD BH3 (80μM) – HRK BH3 (80μM). **F).** IC50 values of AML cells treated with ABT-199 from Figure 2.1A were correlated with the mitochondrial response of the BAD BH3 (80uM) – HRK BH3 (80uM) peptide. Statistical correlation was performed using a one-tailed Spearman r using GraphPad Prism 6.

ABT-199 Efficiently Kills Primary AML Myeloblasts

We observed ABT-199 selectively kills BCL-2 dependent cell lines; therefore, we next wanted to test the sensitivity of primary patient AML samples to treatment with ABT-199. AML myeloblasts from patient bone marrow or peripheral blood were exposed to ABT-199 for 48 h in minimum essential medium alpha supplemented with cytokines. It is notable that the primary cells were quite sensitive, with median IC50 values less than 10 nM (**Figure 2.4A**). Note that this is significantly lower than the IC50 observed in the AML cell lines (**Figure 2.1A**). Note also that this is comparable to the sensitivity observed for *ex vivo* exposure of CLL cells, a disease in which ABT-199 has shown clinical activity in most patients treated *(25).* Because prolonged *ex vivo* culture by itself can promote sensitivity to apoptosis of AML myeloblasts, we wanted to test whether we would see sensitivity at shorter time points as well. Another potential confounding element is that the ABT class of compounds tend to be highly bound by serum proteins (*246*). We found that culturing AML cell lines or primary cells in the absence of serum for 8 h did not alter the mitochondrial priming or BCL-2 dependence, compared to culture in the presence of serum (**Supplemental Figure S2.1**). Therefore, we tested sensitivity of AML myeloblasts to ABT-199 at 8 h in the absence of serum (**Figure 2.4B**). Clinical and genetic data for both sets of AML samples is available in **Supplemental Table 2.1**. Again, AML myeloblasts proved to be sensitive to ABT-199 with a median IC50 of 20 nm. Indeed, when we reduced exposure times further, to 2 hours, we could still see induction of apoptosis by ABT-199 in AML myeloblasts (**Figure 2.4C**). Similarly rapid induction of cell death has been observed for the clinically sensitive CLL, consistent with a direct action of ABT-199 on AML myeloblast mitochondria, promoting apoptosis in the absence of a requirement for additional cell signaling extrinsic to the mitochondria.

Upon testing of additional cryopreserved AML patient samples, including AML cells with diploid cytogenetics and mutations in *FLT3*, *NRAS*, and *NPM1* genes, 20 out of 25 (80%) were sensitive to ABT-199 (100 nM), while 5 samples were resistant to both ABT-199 and ABT-737 (**Supplemental Table S2.2**). However, samples from patients with complex cytogenetics and *JAK2* mutation (n = 9) were largely insensitive to ABT-199 (1 of the 9, or 11.1% response rate, p = 0.0005 by two-tailed Fisher exact test). Further we found no correlation between ABT-199 sensitivity and FAB classification (**Supplemental Figure S2.2A**) or NPM1 (**Supplemental Figure S2.2B**) or FLT-3 mutational status (**Supplemental**

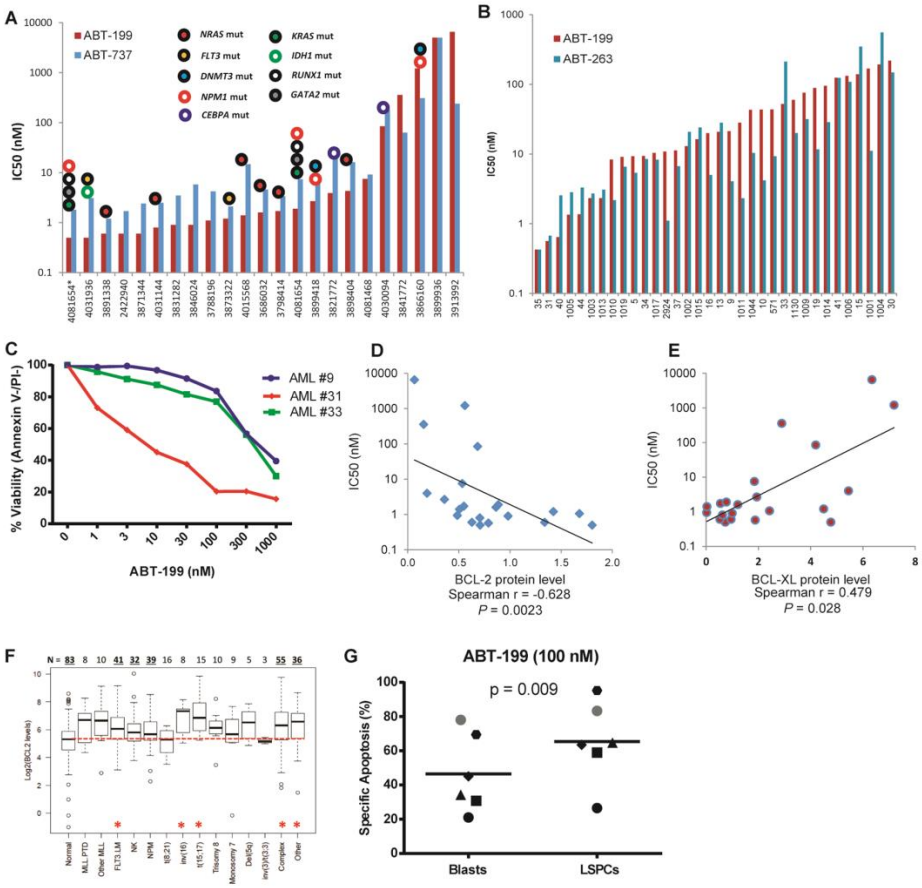


Figure 2.4. ABT-199 efficiently kills primary AML myeloblasts as a single agent. A). IC50 determination for ABT-199 and ABT-737 treatment of primary AML samples. Fresh mononuclear cells from AML patients were isolated from bone marrow or peripheral blood and treated with ABT-199 and ABT-737 for 48 h. The IC50 values were calculated based on viable (i.e., Annexin V^-/PI^-) cell numbers determined by FACS analysis. Samples with ABT-199 IC50 < 0.1 µM were defined as "sensitive", while those with ABT-199 IC50 > 1 µM were defined as "resistant". **B).** Frozen primary AML myeloblasts were thawed treated with ABT-199 and ABT-263 for 8 h in the absence of fetal bovine serum. Viability was assessed by Annexin-/PI- via FACS analysis and IC50 values were calculated using GraphPad Prism software. **C).** Thawed primary AML samples were treated for 2 h with 1-1000 nm of ABT-199 and viability was assessed by Annexin V-PI- by FACS analysis **D).** Nonparametric Spearman correlation analysis shows a significant (p = 0.017) negative correlation between ABT-199 IC50 values and BCL-2 protein levels. **E)** A non-significant (p = 0.069) positive correlation was observed between ABT-199 IC50 values and BCL-XL protein levels. **F).** Boxplots represent the quartiles and range of log2 values of mRNA expression for BCL-2 genes in different subgroups of AML and normal bone marrows. The median is indicated by the black line in each box. Numbers on top indicate number of patients in each specified subgroup. Differences in gene expression with P values ≤ 0.005 were considered statistically significant, as denoted by *. **G).** Patient AML samples treated with 100 nM ABT-199 for 24 hours were subjected to FACS analysis of specific apoptosis based on Annexin V staining in the bulk AML myeloblast and CD34+/CD38-/CD123+ LSC-containing population. P value determined via paired t-test.

Figure S2.2C). There was no difference in ABT-199 sensitivity between samples sensitive or resistant to conventional induction chemotherapy (**Supplemental Figure S2.2D**), consistent with prior findings with ABT-737 (16).

We next tested whether sensitivity to ABT-199 correlated with protein expression for primary AML myeloblasts. As we found with AML cell lines, sensitivity to ABT-199 correlated directly with BCL-2 expression and inversely with BCL-XL expression as measured by quantitative Western blot (**Figure 2.4D-E**). No significant relationship was observed between MCL-1 level and sensitivity to ABT-199 in primary AML myeloblasts. AML myeloblasts also demonstrate higher BCL-2 mRNA expression than normal bone marrow (**Figure 2.4F**). Higher expression of mRNA for MCL-1, BCL-XL, and BIM in AML myeloblasts was not observed.

ABT-199 Induces Apoptosis in AML Stem/Progenitor Cells (LSPCs)

We next tested whether ABT-199 is capable of inducing cell death not only in AML blasts, but also in the phenotypically defined AML stem/progenitor cells characterized by $CD34^{+}CD38^{-}$ $CD123^{+}$ immunophenotype (*27*). Samples from six ABT-199-sensitive AML patients with high blast counts were incubated with ABT-199 or ABT-737 for 24 h, and apoptosis induction was determined by Annexin V flow cytometry in electronically gated AML blasts ($CD45^{dim}SSC^{low}$) and AML stem/progenitor cells ($CD45^{dim}SSC^{low}$ $CD34^{+}CD38^{-}$ $CD123^{+}$). ABT-199 (**Figure 2.4G**) and ABT-737 induced apoptotic cell death in both bulk AML blasts and AML stem/progenitor cells.

BH3 Profiling Predicts AML Myeloblast Killing by ABT-199

We next tested whether killing of primary AML myeloblasts by ABT-199 acted as a true BH3 mimetic in an on-target fashion on BCL-2 dependent mitochondria. If this is the case, we would expect that mitochondria sensitive to the BAD BH3 peptide should also be sensitive to the ABT-199 peptide. Indeed, we found an extremely tight correlation between mitochondrial sensitivity to BAD BH3 and ABT-199 across 30 independent patient samples (**Figure 2.5A**). Supporting the on-target effect of this class of drugs, a similar correlation was found for ABT-263. No such correlation was observed for the comparison of the BCL-XL selective peptide HRK BH3 and the IC50 of ABT-199, supporting BCL-2 selective action of

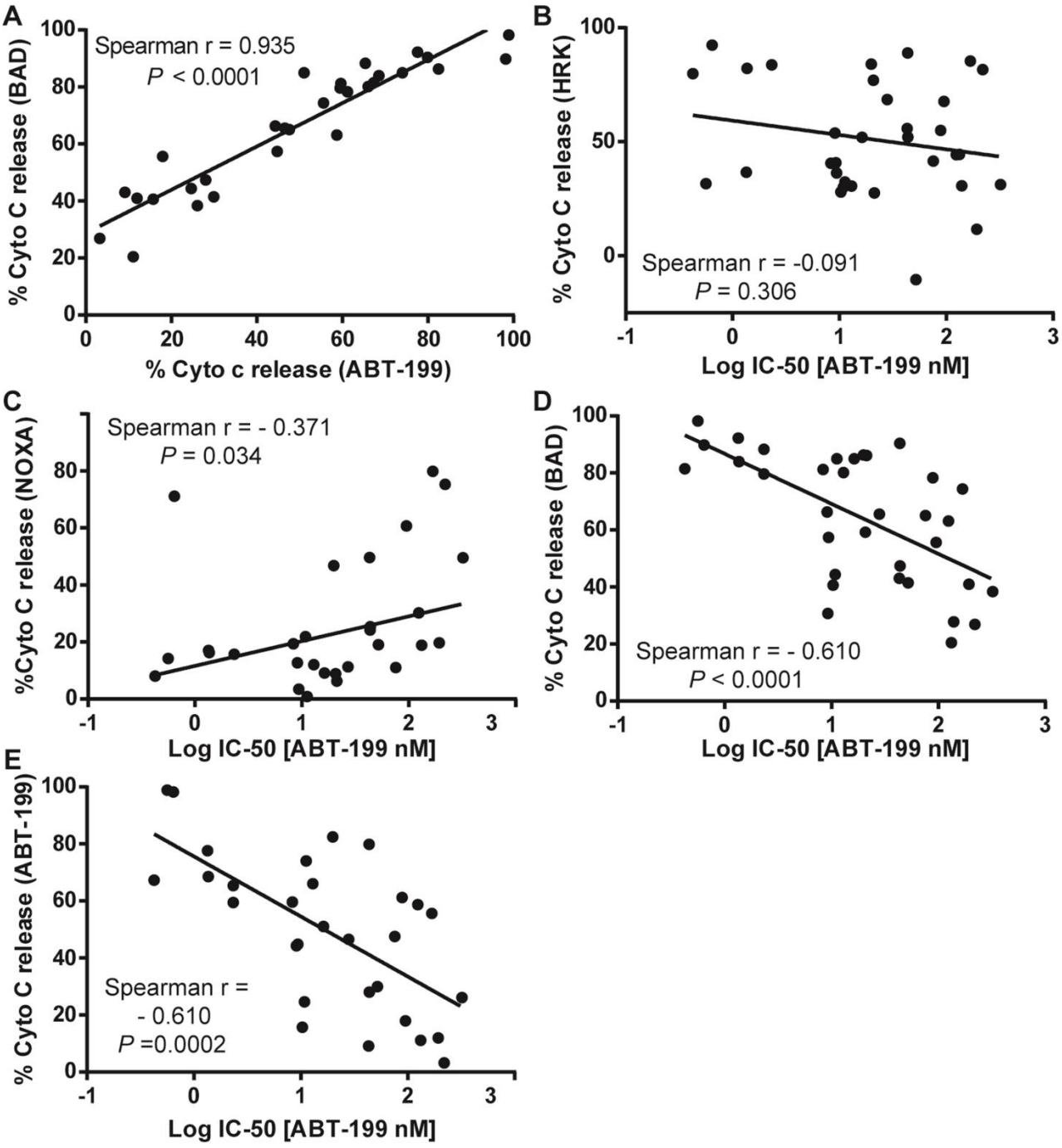


Figure 2.5. BH3 profiling predicts AML myeloblast killing by ABT-199.
A). Intracellular BH3 (iBH3) profiling was performed on thawed primary AML cells using the BAD BH3 (80 μM) and ABT-199 (1μM). The mitochondrial sensitivity to BAD BH3 and ABT-199 were positively correlated. **B).** There is no correlation between the IC50 of primary AML samples from Figure 2.4B with the BCL-XL specific BH3 peptide HRK (80 μM). **C).** The IC50 of primary AML samples from Figure 2.4B were correlated with the NOXA (80μM), a MCL-1 specific NOXA BH3 peptide. **D).** The ABT-199 IC50 of primary AML samples from Figure 2.4B were correlated with the BAD BH3 peptide (80uM). **E).** The ABT-199 IC50 from Figure 2.4B was correlated with the ABT-199 mitochondrial response (1μM). All correlations were tested using a one-tailed Spearman r correlation using GraphPad Prism software.

ABT-199 (**Figure 2.5B**). We observed a weak anti-correlation between cellular sensitivity to ABT-199 and sensitivity to the MCL-1 selective peptide NOXA BH3 (**Figure 2.5C**). This suggests that there is a minor tendency for MCL-1 dependent mitochondria to be less sensitive to ABT-199.

In other diseases, BH3 profiling has proven a useful tool for predicting the cytotoxic effect of BH3 mimetic small molecules (*59, 247*). Here we tested whether BH3 profiling using the BAD BH3 peptide predicted cytotoxicity from ABT-199, and found that the correlation was very good (**Figure 2.5D**). In addition, the mitochondrial effect of ABT-199 correlated well with the cytotoxic effect (**Figure 2.5E**), again supporting a direct mitochondrial effect of ABT-199, consistent with a mechanism of action of direct competition for the BH3 binding site of BCL-2 on mitochondria.

BH3 Profiling Predicts Response to ABT-199 in an AML Xenograft Model

Tumor xenograft models established by inoculation of cancer cell lines into immunodeficient mice have been used widely for testing novel therapies. However, cultured tumor cells can undergo changes in their gene expression patterns after prolonged passage in *in vitro* culture. Therefore, the preclinical results obtained from patient-tumor derived xenograft (PDX) models may offer superior modeling of the human disease, especially for testing target-oriented therapies. We have shown that ABT-199 was very effective in a murine AML cell line xenograft model (**Figure 2.1E**). As a more clinically relevant test of ABT-199's anti-leukemic efficacy *in vivo*, NSG mice were injected with primary AML cells from two different patients (R and S) and monitored for leukemia engraftment by measurements of human $CD45^+$ cells in peripheral blood. After confirmation of AML engraftment, the mice were randomly divided into vehicle and treatment groups. Treated mice received ABT-199 for 2 weeks, after which all the mice were sacrificed, and bone marrows were examined for AML tumor burden by human CD45 flow cytometry. FACS analysis showed that 2-wks of ABT-199 treatment significantly reduced leukemia burden in murine bone marrows in mice injected with cells from patient S (mean, 70 ± 16% human $CD45^+$ cells in bone marrow of control mice (n = 9) and 32.7 ± 12% in ABT-199 treated mice (n = 11, p = 0.0004, **Figure 2.6A**). We did not observe a decrease in tumor burden in mice injected with cells from patient R (mean 70.3 ± 8.1% human $CD45^+$ cells in bone marrow of control mice (n = 8) and 74.3 ± 6.4% in ABT-199 treated mice (n = 8, p = 0.1930, **Figure 2.6B**).

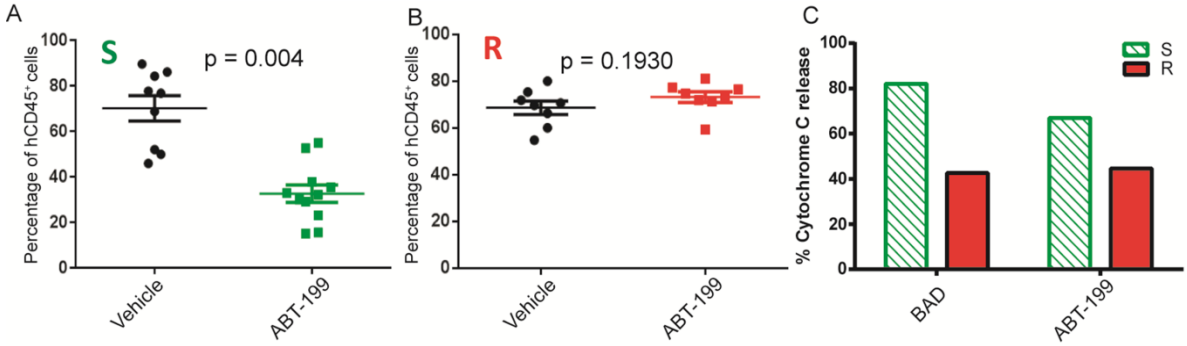


Figure 2.6. BH3 profiling predicts AML progression in a primary AML xenograft model.
A-B). NSG mice were injected with primary AML cells as described under Methods. Mice were treated with ABT-199 100 mg/kg oral daily dose starting 3 weeks after AML cell injection, for two weeks. The graph represents % of human $CD45^+$ leukemic cells in the murine bone marrow in mice sacrificed upon completion of the therapy. A non-parametric, unpaired, two-tailed *t*-test was used to evaluate the significance of mean difference. **C).** Intracellular BH3 profiling using the BAD BH3 (80 μM) and ABT-199 (10 μM) was performed on pre-treatment patient samples.

Since we observed a difference in response in the xenograft model following ABT-199 treatment, we asked whether the response to ABT-199 could be predicted by BH3 profiling. In blinded fashion, pre-treatment AML myeloblasts from each model were subjected to BH3 profiling in which mitochondria were exposed to the BAD BH3 peptide as well as ABT-199 itself. We found that mitochondria from AML myeloblasts from patient S released more cytochrome c following incubation with the BAD peptide or ABT-199 compared to patient R (**Figure 2.6C**). These results provide evidence that ABT-199 kills AML myeloblasts by the expected mechanism of inhibition of mitochondrial BCL-2. Furthermore, these results suggest that BH3 profiling might predict the response of AML primary cells to ABT-199 *in vivo*.

IV. DISCUSSION

Relapsed AML is a difficult cancer to treat effectively; therefore, there is need for improved treatment options for refractory AML. Here, we show that selective inhibition of BCL-2 by ABT-199 kills AML cell lines and primary patient cells both *ex vivo* and in *in vivo* mouse xenografts as a single agent in the low nano-molar range. The concentrations used in our studies here are in the 0.001-1 μg/ml range, a range readily achievable in clinical trials where serum concentrations of 3-4 μg/ml have been observed (*25*). Moreover, the drug acts very quickly *in vitro*, killing cells within 2 h of drug exposure. We also show that as in CLL, ABT-199 functions on-target at the mitochondria. This is consistent with the observation that AML myeloblasts from chemorefractory patients showed no difference in their BCL-2 dependence, as measured by BH3 profiling, or sensitivity to ABT-199 compared to chemosensitive cells (*56*) .

Our *in vitro* results suggest there will be heterogeneity in clinical response (IC50s ranged from 0.43 to >1000 nm), so that a predictive biomarker would be of great utility. Here we present four methods that may be predictive of clinical response to ABT-199. The first method is cytogenetics. The cellular death response to ABT-199 appears to be largely independent of cytogenetic and genetic mutation status, except perhaps for complex karyotype and *JAK2* mutant patients, suggesting that treatment with ABT-199 could be useful for patients who have poor prognostic factors. The utility of cytogenetics as a more general predictive biomarker for response to ABT-199 needs to be examined across many more samples.

A second method is *ex vivo* short term culture of the primary patient samples with ABT-199. The disadvantage of this method is that it is difficult to reliably culture primary AML cells for the requisite time frame to observe cell death. We observed that even after a short 8 h culture there could be upwards of 60% spontaneous apoptotic death in the control un-treated primary AML cells. Therefore, it would not be ideal to rely on an *in vitro* cell death assay where many samples could be potentially lost due to spontaneous cell death during culture.

The third predictive biomarker method is to measure BCL-2 levels by Western blot. We show that increased expression of BCL-2 is associated with increased sensitivity to ABT-199. However, given the complex interactions of the BCL-2 family members, individual measurements of the various anti-apoptotics alone may not provide accurate data on the *in vivo* biology of the anti-apoptotic dependencies in AML. Many of the BCL-2 family members are regulated by post-translational modifications and interactions with other proteins. These types of interactions are difficult to capture in static Western blot measurements.

The fourth method, BH3 profiling, may prove useful as a predictive biomarker. BH3 profiling is a functional assay which accounts for the relative amounts and interactions of all of the BCL-2 family members. We show here that the mitochondrial response to the BAD peptide as well as mitochondrial ABT-199 correlates with the *ex vivo* drug treatment. Most significantly, BH3 profiling could discriminate *in vivo* sensitivity of human AML cells to ABT-199 (**Figure 2.6**). Thus, we may be able to use BH3 profiling of pre-treatment AML samples to direct ABT-199 treatment to AML cases that are most BCL-2 dependent. While the assay is less familiar to many, it is a straightforward protocol using reagents and equipment available in most clinical and research laboratories. Moreover, results are available the same day the sample is acquired. As for the other putative biomarkers, empiric testing in the clinical setting is the only way to truly validate BH3 profiling as a useful predictive biomarker.

Although we focused largely on the expression of BCL-2 and BCL-XL in AML, we also detected the expression of MCL-1. It has been recently reported that the anti-apoptotic MCL-1 is necessary for the development and sustained growth of AML (*248*). Since ABT-199 does not inhibit MCL-1, increased expression of MCL-1 could be a potential source of upfront resistance to BCL-2 inhibition by ABT-199. However, we show that the majority of AML patient samples tested did not show MCL-1 dependence (as

indicated by the NOXA response). This suggests that although MCL-1 may be necessary for the development of AML, most cases of AML may not depend on MCL-1 for survival as much as on BCL-2. Indeed, in a pertinent direct comparison, we have found that most AML myeloblasts are more BCL-2 dependent and less MCL-1 dependent than HSC, though there are about 20% exceptions (*56*). It may well be that dependence on individual anti-apoptotic proteins varies with myeloid differentiation state.

BCL-2 was discovered in lymphoid cancer cells, and much of the research on this protein has been conducted in lymphoid cells, where it is highly expressed (*8*). It is therefore understandable that clinical testing of ABT-199 has so far focused on lymphomas and CLL. Here we demonstrate that selective, on-target BCL-2 inhibition using a clinically active drug is a promising avenue for clinical investigation in the myeloid malignancy AML. It is particularly important to recognize that even AML myeloblasts that are resistant to conventional therapies appear to be quite sensitive to BCL-2 inhibition. Thus, BCL-2 inhibition by ABT-199 offers hope to those AML cases that most need novel therapeutic intervention. Our results strongly support the testing of ABT-199 for treatment of AML patients as the majority of patient samples were sensitive to the drug in *ex vivo* culture. Furthermore, our results support the testing of BH3-profiling as a predictive biomarker for ABT-199 response in the clinic.

CHAPTER 3: BH3 PROFILING PREDICTS CLINICAL RESPONSE IN A PHASE II CLINICAL TRIAL OF ABT-199 IN ACUTE MYELOID LEUKEMIA

Attributions

This project was a collaboration between clinical and research colleagues at Abbvie, MD Anderson, the clinical trial sites and myself. Clinical tables were compiled from data acquired from the clinical sites, and compiled by Abbvie. Clinical information and mutation status was provided by Abbvie and clinical collaborators. BCL-2 family levels were collected at a central location. All BH3 profiling (Figure 3.2-3.5) were compiled by data collected by myself.

I. ABSTRACT

BCL-2 dependence is found in many hematological malignancies, including acute myeloid leukemia. As we described in chapter 2, ABT-199 is a potent and selective inhibitor of BCL-2, which promotes apoptosis in acute myeloid leukemia (AML) cells by displacing pre-bound activator BH3-only proteins from BCL-2, freeing the activator proteins to bind to and activate the effectors of apoptosis, BAX and BAK. In this Phase II clinical trial (NCT01994837), AML patients with relapsed/refractory disease, or AML patients unfit for induction chemotherapy were treated with a dose escalation of ABT-199. ABT-199 treatment was generally well tolerated, with the most serious adverse events being febrile neutropenia and pneumonia. There was an overall response rate of 19%. BH3 profiling, an assay that identifies anti-apoptotic dependencies was tested as a predictive biomarker of ABT-199 clinical response. We found that patients who had pre-treatment mitochondria dependent on MCL-1 or BCL-XL, anti-apoptotic proteins that are not inhibited by ABT-199, predicted failure of the drug to reduce myeloblast counts. This data suggests that BH3 profiling may be used to direct treatment of ABT-199 to AML patients who will most benefit from BCL-2 inhibition.

II. INTRODUCTION

ABT-199 is a potent and selective inhibitor of the B-cell lymphoma 2 protein (BCL-2) which has shown potent cell killing in clinical trials of chronic lymphocytic leukemia and in the pre-clinical model of AML described in Chapter 2 (*249*). In Chapter 2 we showed that ABT-199 potently killed AML cell lines,

primary patient samples and AML xenografts in as a single agent in the nanomolar range. These data directly led to the initiation of the Phase II multi-center clinical trial of ABT-199 in patients diagnosed with relapsed refractory AML and/or patients unfit for induction chemotherapy described in this chapter.

Importantly, our pre-clinical analysis of ABT-199 in AML cell lines and patient primary samples suggested that AML patients would have a heterogeneous response to ABT-199 monotherapy *in vivo as* the IC50 of cell killing varied over 3 logs in the *in vitro* setting (**Figures 2.4**). Therefore, it would be beneficial to identify predictive biomarker(s) to select for patients who will be most sensitive to ABT-199 treatment. We showed in chapter two that BCL-2 dependence as measured by the BAD BH3-only peptide as well as mitochondrial ABT-199 correlated well with cellular toxicity *in vitro* with AML cell lines and primary patient samples as well as cytotoxicity of AML mouse xenografts (**Figure 2.5**). Therefore, BH3 profiling was integrated into this Phase II clinical trial to test the ability of the assay to retrospectively identify patients who will be most sensitive to ABT-199 treatment.

In addition to BH3 profiling two other predictive biomarkers were utilized in this study. The second prognostic indicator used was isocitrate dehydrogenase I (IDH1) or isocitrate dehydrogenase 2 (IDH2) mutation status. IDH mutations occur in approximately 20% of AML patients and it was recently reported that AML blasts harboring IDH mutations tend to be more BCL-2 dependent and sensitive to ABT-199 treatment *in vitro* and in xenograft models of AML (*250*). The oncometabolite produced by IDH1 and IDH2 mutations, 2-HG, inhibits cytochrome c oxidase leading to increased BCL-2 dependency. Therefore, in this clinical trial, it was tested if the presence of IDH mutation correlates with superior clinical response to ABT-199 treatment. The third predictive biomarker tested was measurement of pre-treatment protein levels of BCL-2 and BCL-XL within AML blasts. As we showed in chapter two, increased protein expression of BCL-2 measure by Western blot was associated with increased cellular sensitivity to ABT-199 treatment; whereas high expression of BCL-XL was associated with decreased sensitivity to ABT-199. Therefore, in this trial, we tested if the relative expression of these proteins correlated with clinical response.

Thus, there were two goals for this clinical trial of ABT-199 in AML. The first goal was to identify the safety profile and assess the efficacy of ABT-199 monotherapy in relapsed/refractory AML patients as well as patients unfit for standard induction chemotherapy. A second goal was to assess the utility of the

three aforementioned prognostic/predictive biomarkers to identify the patients who will be most sensitive to ABT-199 monotherapy treatment.

III. METHODS

Study Design

This phase 2 open-label, dose-escalation study was conducted at four centers in the United States. The primary objective was to evaluate overall response rate of ABT-199 administered daily to patients with relapsed or refractory AML or patients with newly diagnosed AML unfit for induction chemotherapy. The secondary objectives were to assess the effectives of potential therapeutic biomarkers. The protocol was approved by the institutional review boards at all participating centers, and all patients signed an informed consent document according to the Declaration of Helsinki.

Patients were admitted to the hospital Day-1 and received prophylactic treatment for tumor lysis syndrome since two fatalities due to tumor lysis syndrome had occurred in CLL trials (*249*). Patients were hospitalized through Day 7 to monitor for tumor lysis syndrome. ABT-199 was administered at a starting dose of 20mg/kg of ABT-199 on Wk1/Day 1 with daily dose escalation until a maximum dose of 800mg/kg was achieved. Patients who experienced no adverse effects and did not enter a complete response were allowed to escalate to 1200mg/kg.

Patients

Inclusion criteria included patients aged ≥ 18 years diagnosed with relapsed or refractory AML, or unfit for intensive chemotherapy, ECOG performance score ≤ 2, adequate renal (creatinine clearance ≥ 50 mL/min) and liver function. Exclusion criteria included receiving anti-cancer therapy within 14 days of the first dose of ABT-199, receiving monoclonal antibody for anti-neoplastic intent within 8 wks of first dose of ABT-199, receiving CYP3A inducer within 7 days of first dose of ABT-199, receiving CYP3A inhibitors within 5 days of first dose of ABT-199, white blood cell count > 25 x 10^9/L, diagnosed with APL, patient diagnosed with CNS disease.

Tolerability and Safety Assessments

Adverse events (AEs) were graded in accordance with the National Cancer Institute Common Terminology Criteria, version 4.0. Physical examination, vital signs, solicitation of AEs, and urinalyses were performed at baseline and throughout the study.

Response to Treatment

Patients underwent bone marrow aspirates and/or biopsies at screening and on day 28 and every eight weeks thereafter. Responses were evaluated using the revised guidelines by the International Working Group for AML (*74*).

BH3 Profiling of patient samples

Pre-treatment bone marrow aspirates or peripheral blood samples were obtained from patients on trial with consent and shipped at ambient temperature to a central location to isolate the mononuclear cells using a ficoll gradient and viably frozen. Frozen samples were sent to the Letai laboratory for BH3 profiling. Samples were BH3 profiled as described in Chapter 2 with the following cell surface staining changes: Cells were stained with 1:100 Invitrogen Live/Dead – aqua stain (#423102, BioLegend, San Diego, CA) in PBS for 20 min on ice, washed with PBS and subsequently stained with 1: 100 CD45-BV421 (clone HI30, #563879; BD Biosciences; San Jose, CA) and 1:100 CD33-PE (clone WM53,

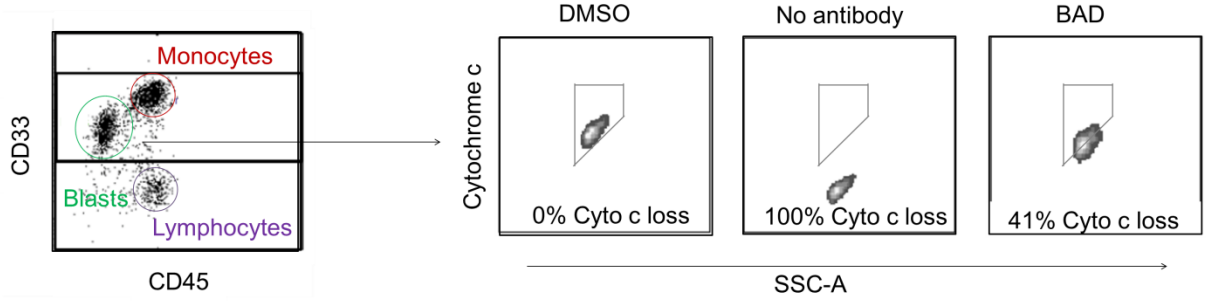


Figure 3.1 Gating strategy used for BH3 profiling of clinical trial patient samples. AML patient samples were thawed, washed with PBS and stained with CD33 and CD45. Following cell surface staining, cells were BH3 profiled by permeabilizing cells with digitonin and adding peptides, or DMSO as a control. Cells were fixed and stained with a cytochrome c antibody, except in the negative control which received no antibody. AML blasts were identified by $CD33^{mid}$ and $CD45^{lo}$ by flow cytometry. For BH3 profiling analysis, a gate around the DMSO treated cells was drawn to depict 0% cyto c loss.

#561816; BD Biosciences; San Jose, CA) in FACS buffer (2% FBS in PBS) on ice for 20 min with 1:100 human FcR block (#130-059-901; Miltenyi Biotec; San Diego, CA). Cytochrome c loss was measured by a gating strategy illustrated in **Figure 3.1**. DMSO was used as a negative control for cytochrome c retention, whereas a no-antibody (Cytochrome c) was used a positive control for 100% cytochrome c release. Cytochrome c loss was calculated using the equation:

Cytochrome c loss = 100 – (% of cells within cytochrome c gate)

AML blasts were identified by $CD45^{low\text{-}mid}$/ $CD33^{mid\text{-}high}$/$SSC\text{-}A^{low}$. Monocytes were identified as $CD33^{hi}$/$CD45^{hi}$ and lymphocytes were identified as $CD33^{lo}$/$CD45^{hi}$/$SSC\text{-}A^{low}$. Cells from the DHL4 cell line were BH3 profiled with the AML patient samples as an internal control for peptide function. In addition to the pre-treatment samples, three WK4 on-treatment bone marrow aspirates were obtained from unique patients and BH3 profiled to test effects of *in vivo* ABT-199 on the myeloblast BH3 profile.

Statistical analysis

Comparisons between AML blasts, monocytes and lymphocytes were performed using a two-tailed paired parametric T-test. Correlation of pre-treatment anti-apoptotic dependencies with clinical response to single agent ABT-199 treatment was measured in several ways. A one-tailed parametric Pearson correlation was used to test for correlation between anti-apoptotic dependencies and % blasts reduction and time on trial. For ROC curve analysis for length of time on trial, patients were dichotomized into patients who were on trial for < or > 30 days, for analysis of blast count reduction, patients were dichotomized into those who had < or > 0% blast reduction at WK4. Fisher's exact test was used to examine the significance of the association with BCL-2 family levels and IDH mutation status with achieving a complete response (CR) or complete response with incomplete bone marrow reconstitution (CRi). All statistical analysis was performed using GraphPad Prism Software version 6.05.

Table 3.1. Clinical characteristics of AML patients on ABT-199 clinical trial.

Characteristic	N=32 (%)
Age, median (range)	71 (19–84)
Sex	
Male	16 (50)
Diagnosis	
Relapsed/refractory	30 (94)
Unfit	2 (6)
Baseline ECOG	
0	3 (10)
1	16 (50)
2	13 (40)
Prior standard induction (3+7) therapy	17 (53)
Prior number of regimens ≥ 3	14 (44)
Prior Hypomethylating agents	24 (75)
Prior allogeneic stem cell transplant	4 (13)
None (Treatment naïve)	2 (6)
Prior myeloid disorder	
Prior myelodysplastic syndrome, MDS	11 (34)
Molecular Markers	**N=32 (%)**
IDH mutations	11 (35)
FLT3-ITD	6 (18)
BCR/ABL	1 (3)
JAK2	1 (3)
KRAS	1 (3)
MLL	1 (3)
NPM1	4 (12)
CEBPα	2 (6)
Cytogenetics	**N=32 (%)**
del 7	10 (31)
Complex	10 (31)
t(8:12)	2 (6)

IV. RESULTS

Patient characteristics

Thirty-two patients were enrolled in the study of monotherapy ABT-199 treatment. The median age was 70 years (**Table 3.1**). Eleven patients (36%) had prior myelodysplastic syndrome. All but two patients received prior chemotherapy and nearly half of the patients had received greater than three rounds of prior chemotherapy. The most abundant molecular mutation was an IDH1 or IDH2 mutation (35%) followed by FLT-3-ITD mutation (18%). Other mutations were also represented. Ten patients (31%) had del 7 and ten patients had complex cytogenetic karyotype.

Safety and tolerability

ABT-199 was generally well tolerated. Grade 3/4 adverse events (AE) that occurred in > 3 patients included febrile neutropenia (28%), pneumonia (18%), hypokalemia (16%), hypotension (13%), hypocalcemia (9%), hypophosphatemia (9%), urinary tract infection (9%). Serious AEs in >3 patients included febrile neutropenia (25%) and pneumonia (19%). There were no reported events of clinical or laboratory tumor lysis syndrome.

ABT-199 is effective as a monotherapy for some AML patients

As expected from the pre-clinical analysis, there was significant but heterogeneous clinical activity of ABT-199 (**Figure 3.2**). Objective responses were observed in 6 (19%) of 32 patients, with two patients experiencing a complete response (CR), and four additional patients experiencing a complete response with incomplete bone marrow re-constitution (CRi) at first assessment (end of WK4). One of the four CRi patients achieved a CR by week 20. Six additional patients (19%) showed anti-leukemic activity (**Table 3.2**). Twenty patients (63%) had progressive disease. The median time on trial study was 63 days with a range of 23-246 days, and the median CR duration was 47.5 days. Four (36%) of eleven IDH mutation positive patients had an objective response; 2 CRs and 2 CRis. Two additional IDH-positive patients experienced stable disease. IDH mutation status was not statistically associated with achieving a CR or No CR (Fisher's exact test, $p = 0.15$), although the sample size was small.

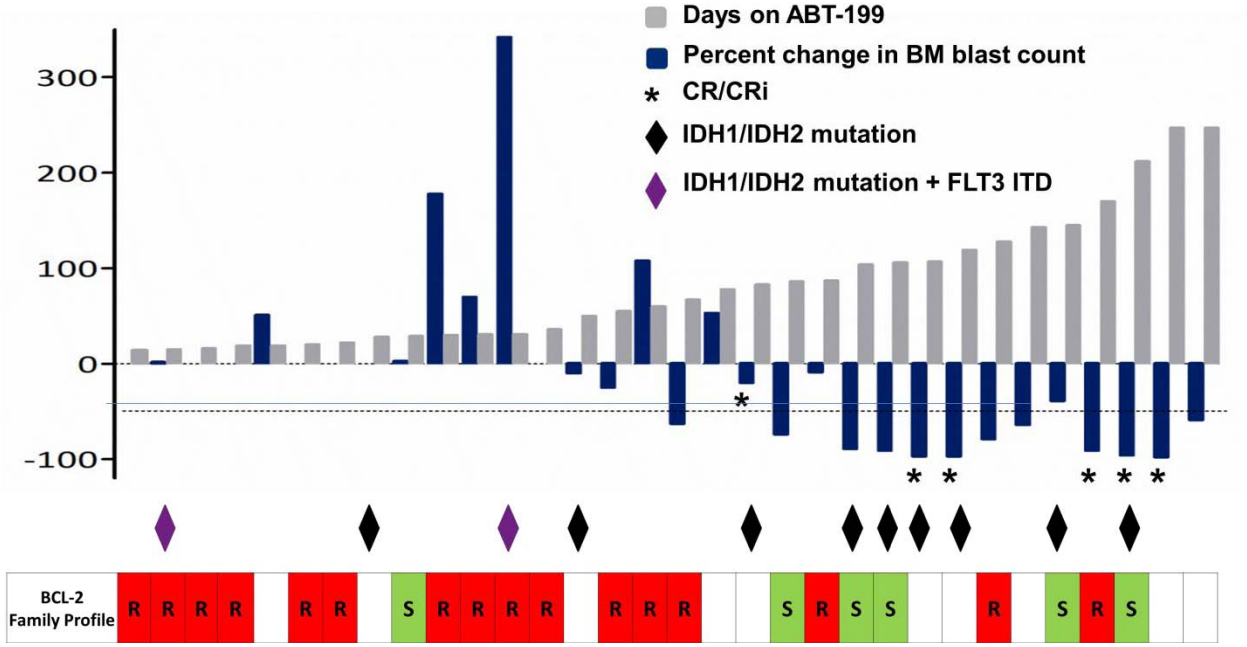


Figure 3.2. Clinical response to monotherapy ABT-199. Light purple depicts the number of days on ABT-199 treatment. Dark purple shows the % change in BM blast count from baseline to WK4 of ABT-199 treatment. IDH positive mutant patients is indicated with diamonds. For the BCL-2 family profile: R = BCL-2 is expressed in less than 35% of the tumor population or BCL-XL is expressed in greater than 40% of the tumor population. S = BCL-2 is expressed in greater than 35% of the tumor population and BCL-XL is expressed in less than 40% of the tumor population.

Table 3.2 Objective responses observed in Phase II clinical trial of ABT-199 in AML

Response	N=32 (%)
Objective Response (CR + CRi)	6 (19)
Complete Response, CR	2 (6)
Complete Response with Incomplete Blood Count Recovery, CRi *	4 (13)
Anti-leukemic activity	6 (19)
≥ 50% blast reduction with two cell line recovery transfusion independence	2 (6)
≥ 50% blast reduction with one cell line recovery	2 (6)
≥ 50% blast reduction with no hematologic recovery	2 (6)
Treatment failure (Progressive disease and less than PR)	20 (63)

BCL-2 family levels

Myeloblasts from twenty-two pre-treatment AML blasts were analyzed for BCL-2 family expression levels. Pre-treatment samples were characterized as BCL-2 family resistant (R) if BCL-2 protein was expressed < 35% or BCL-XL was expressed in > 40% of the major tumor population. Alternatively, samples were characterized BCL-2 family sensitive if BCL-2 was expressed in > 35% and BCL-xL was expressed < 40% of the major tumor population. Of the six patients who achieved a CR or a CRi, one sample was classified as BCL-2 family resistant and one was classified as BCL-2 sensitive (the remaining four CR samples were not tested with this method). BCL-2 family levels was not statistically associated with a CR or No CR (Fisher's exact test, $p = 0.47$); however, sample sizes were limiting.

ABT-199 trial patients have heterogeneous anti-apoptotic dependencies

BH3 profiling was performed on eighteen pre-treatment bone marrow biopsies or peripheral blood samples. Of the eighteen samples, twelve samples produced analyzable results (>50% viability on thaw, >5% AML blasts). The samples were BH3 profiled using a panel of peptides which included BAD (measures BCL-2, BCL-XL, BCL-W dependence), MS1 (MCL-1 dependence (*251*)), HRK (BCL-XL dependence) and mitochondrial ABT-199 (BCL-2 dependence). We found that patients exhibited heterogeneous anti-apoptotic dependencies as predicted by our pre-clinical studies (**Figure 3.3A-C**). Similar to our pre-clinical BH3 profiling data, BCL-2 dependence as measured by mitochondrial ABT-199 correlated well with the BAD response (binds specifically to BCL-2, BCL-XL and BCL-w), which is expected if ABT-199 is functioning as a true BH3 mimetic (**Figure 3.3D**). We found no correlation between BCL-2 dependent and BCL-XL dependent mitochondria (**Figure 3.3E**). We did observed a trend of MCL-1 dependent mitochondria to be less dependent on BCL-2 (**Figure 3.3F**).

We next asked if other cell populations exhibited similar anti-apoptotic dependencies as the AML blasts as our gating strategy allowed for the investigation of the mitochondrial priming and anti-apoptotic dependencies of other blood cells in the bone marrow and peripheral blood samples. For this analysis, AML patient samples acquired from additional sources other than the clinical trial were used to increase sample numbers. We found that normal monocytes, defined as either $CD33^{hi}/CD45^{hi}$ (or $CD45^{hi}$ $SSC\text{-}A^{hi}$ if cells were not stained with CD33), were more primed than AML blasts and less dependent on BCL-2

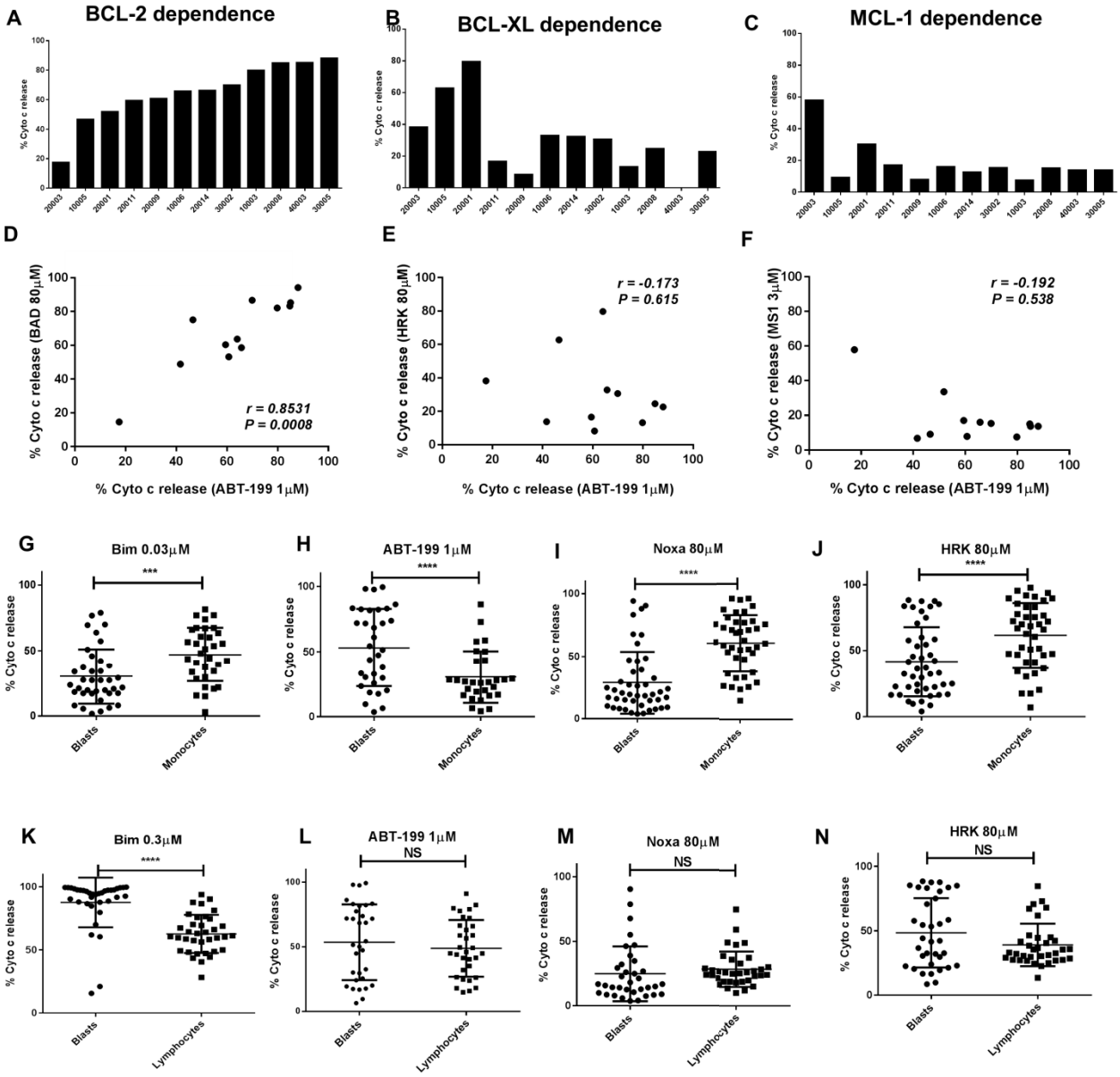


Figure 3.3 AML blasts from different patients on ABT-199 trial have heterogeneous BH3 profiles. **A – C).** BH3 profiling of AML samples using mitochondrial ABT-199 (1μM, **A**), HRK (80μM, **B),** sample 40003 was not tested with HRK due to lack of sample quantity) and MS1 (3μM; **C**). **D).** Correlation between mitochondrial ABT-199 (1μM) and BAD (80μM). **E).** Correlation between MS1 (3μM) and ABT-199 (1μM). **F).** Correlation between HRK (80μM) and ABT-199 (1μM). **G-J).** Paired analysis comparing AML blasts and monocytes using the Bim 0.03μM (**G**), mitochondrial ABT-199 1μM (**H**), NOXA 80μM (**I**) and HRK 80μM (**J**). **K-N).** Paired analysis comparing AML blasts and lymphocytes monocytes using the BIM 0.3μM (**K**), mitochondrial ABT-199 1μM (**L**), NOXA 80μM (**M**) and HRK 80μM (**N**). **** = p <0.0001, *** = p < 0.001 - 0.05

compared to AML blasts cells (**Figure 3.3G-H**). Further the monocytes were more dependent on MCL-1 and BCL-XL compared to blasts cells (**Figure 3.3I-J**). This suggests that normal monocytes should be spared from ABT-199 treatment due to the dependence on MCL-1 and BCL-XL. Lymphocytes identified as $CD33^{lo}/CD45^{hi}/SSC\text{-}A^{low}$ (or $CD45^{hi}/SSC\text{-}A^{low}$ if CD33 was not used) were less primed compared to blasts and did not differ in their anti-apoptotic dependencies compared to AML blast cells (**Figure 3.3K-N**). The dependency of lymphocytes on BCL-2 and BCL-XL correlates with the lymphopenia that was observed in a clinical trial of ABT-263 in non-Hodgkin's lymphoma.

BH3 profiling predicts clinical response to ABT-199 monotherapy

A good predicative biomarker is particularly useful in such instances where clinical response is heterogeneous as was observed with ABT-199 in AML. Therefore, we asked if pre-treatment anti-apoptotic dependence of AML blasts as measured by BH3 profiling could be used as a predictive biomarker for ABT-199 clinical response. We used two analysis methods to test if BH3 profiling could be predictive of clinical response. In one, biologically–driven approach, we compared the correlation of the parameter of peptide induced cytochrome c loss with the parameter of % blast reduction using a Pearson correlation test. In an alternative approach, we examined the receiver operating characteristic (ROC) to test prediction of the binary result of >0% vs. <0% blast reduction at 4 weeks. The ROC curve is a way to test the diagnostic value of a predictive biomarker such as BH3 profiling by assessing the sensitivity and selectivity of the assay. A perfect predictive biomarker will be perfectly sensitive and perfectly selective and have an area under the curve of 1.0. In addition to examining reduction in blast count, we also examined time on trial as a clinical endpoint. We used this metric as a surrogate end point because patients were removed from the trial when their disease progressed. For ROC analysis, we tested the ability of BH3 profiling to predict the binary result of >30 days vs < 30 days on trial. Thirty days was chosen as a cut-off in the binary analysis because it was an adequate time point which separated patients who experienced stable disease or CR/CRi patients from patients who experienced progressive disease. We correlated these clinical metrics with anti-apoptotic dependency on BCL-2, BCL-XL and MCL-1.

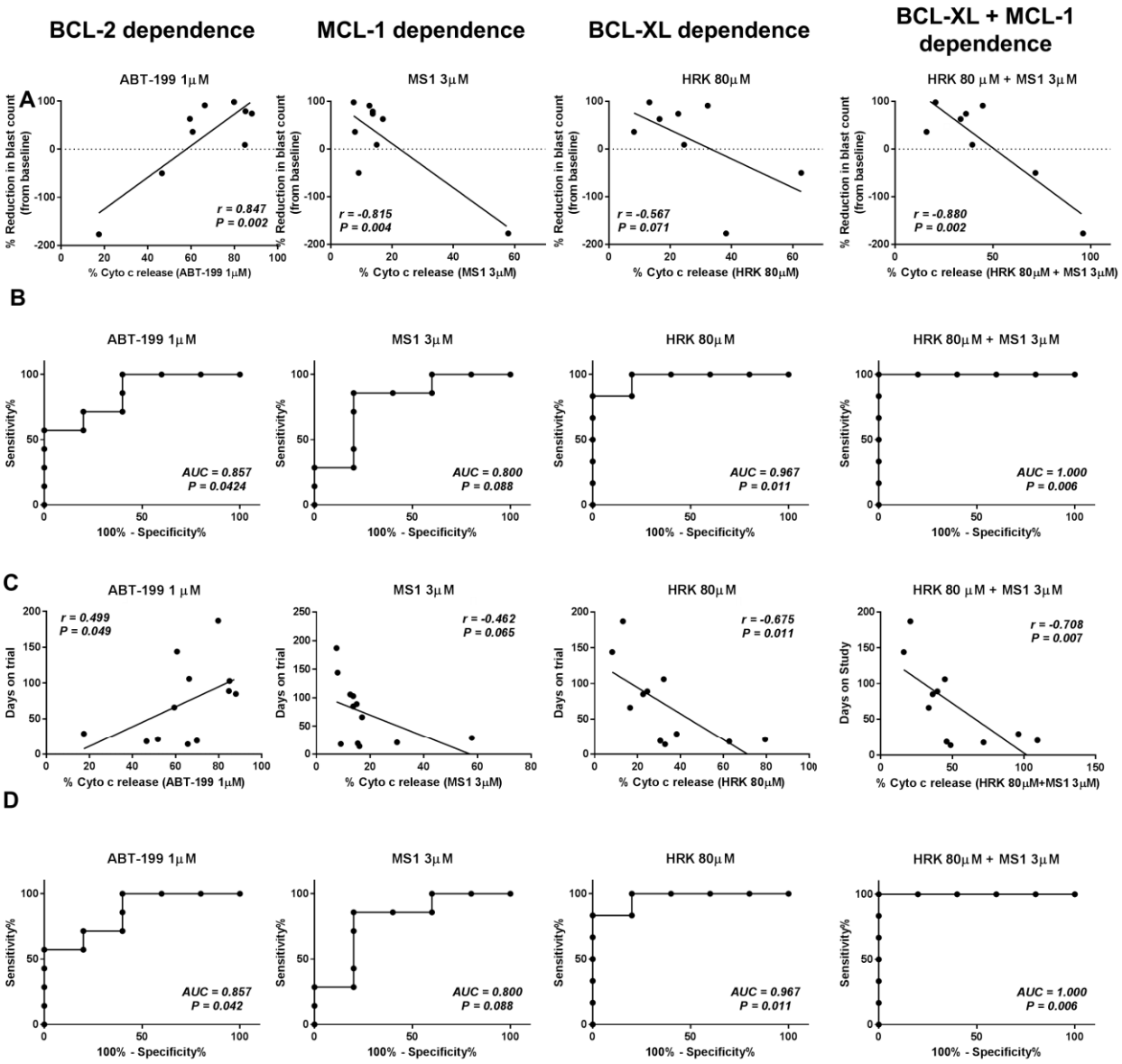


Figure 3.4 Anti-apoptotic dependencies measured by BH3 profiling correlated with % Reduction in blast count at WK4 of trial and duration on trial. The % blast reduction from baseline to WK4 were correlated with the BH3 profiling results from Figure 3.3. **A).** Parametric analysis of mitochondrial ABT-199, MS1 (3uM), HRK (80uM) and MS1 (3uM) + HRK (80uM) response vs. % reduction in blast count from baseline. **B).** Samples were divided into >/< 0% blast reduction after 4 WKs of treatment for ROC analysis. **C).** Parametric analysis of mitochondrial ABT-199, MS1 (3uM), HRK (80uM) and MS1 (3uM) + HRK (80uM) response vs. days on trial. **D).** Samples were divided into >/< 30 days on trial for ROC analysis. Correlations were tested using a one-tailed Pearson correlation using GraphPad Prism software.

We found that BH3 profiling correlated with clinical response in several ways. BCL-2 dependence, measured by mitochondrial response to ABT-199, predicted greater than 0% blast at week 4 with an AUC of the ROC of 0.86 (first column, **Figure 3.4A-B**). Further, AML blast dependence on the anti-apoptotic protein MCL-1 (using the MS1 peptide) predicted blast reduction greater than 0% with an AUC of the ROC of 0.80 (second column, **Figure 3.4A-B**). Myeloblast dependence on the anti-apoptotic protein BCL-XL (using the HRK peptide) was the best sole predictor of blast reduction with an AUC of the ROC of 0.97 (third column, **Figure 3.4A-B**). Since dependence on MCL-1 or BCL-XL both predicted poor clinical performance, we asked whether we could combine the information to make a superior predictor by arithmetically adding response to HRK to that of MS1. We found that this metric was a perfect binary predictor of greater than 0% blast reduction at week 4 (AUC = 1.0, fourth column, **Figure 3.4A-B**). As mentioned above, this reflects a diagnostic assay which is perfectly sensitive and selective. Similar results were obtained analyzing the data by days on trial (**Figures 3.4C-D**)

ABT-199 is functioning on-target, at the mitochondria

We next asked if ABT-199 exhibited an on-target mechanism of action *in vivo*. To answer this question, we obtained and BH3 profiled three bone marrow aspirates following four weeks of ABT-199 treatment. If ABT-199 was acting on-target, we would expect to observe an increase in overall priming of myeloblasts following four weeks of ABT-199 treatment. If ABT-199 is acting on-target at the mitochondria, it should bind to any unbound BCL-2, and/or bind and displace any pre-bound BIM or BID to effector proteins effectively increasing the overall apoptotic reserve (on-target mechanism of action schematic shown in **Figure 3.5A**) (*56*). To test if ABT-199 was functioning on-target, we measured the cytochrome c release in response to the BIM and PUMA peptides in the BH3 profiling assay and compared the results to our pre-treatment samples to measure the overall priming of the cells as BIM and PUMA bind to all of the anti-apoptotics. We found that the WK4 samples had an increase in overall priming in response to the BIM and PUMA peptides when the patient was on ABT-199 treatment compared to pre-treatment samples (**Figure 3.5B-C**), suggesting that ABT-199 was functioning on target at the mitochondria.

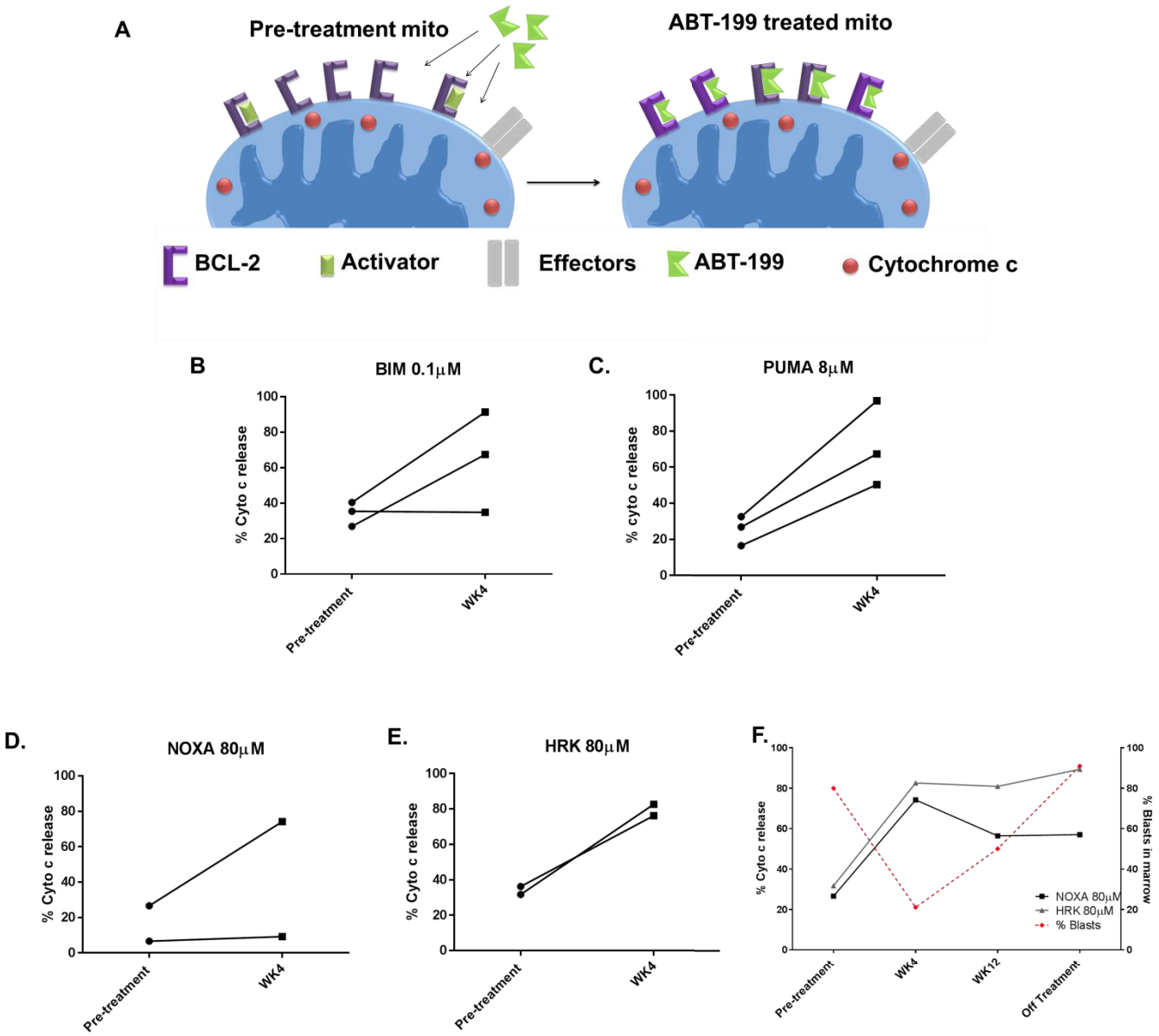


Figure 3.5 ABT-199 is functioning on-target in vivo.
A). On-target mechanism of action of ABT-199 on BCL-2 dependent mitochondria increasing overall priming. **B-E).** Pre-treatment and WK4 treatment samples iBH3 profiled. Each line represents a unique patient sample. Samples were profiled with BIM 0.1μM (**B**), PUMA 8μM (**C**) NOXA 80μM (**D**) and HRK 80μM (**E**). **F).** Changes in MCL-1 and BCL-XL dependence in pre-treatment and on-treatment samples for one patient in which we acquired serial samples. Changes in % blasts within the bone marrow or shown in red.

The second change that we observed in the on-treatment BH3 profiles was an increase in MCL-1 and BCL-XL dependence while the patients were on trial (**Figure 3.5D-E**). We found that the AML blasts remaining following four weeks of ABT-199 treatment exhibited an increased cytochrome c release following treatment with HRK (measures BCL-XL dependence) and MS1 (measures MCL-1 dependence) compared to pre-treatment samples. Furthermore, for one patient we had the opportunity to test serial samples (bone marrow sample at WK12 and a peripheral blood sample seven days after the patient was removed from the study). The patient exhibited an increase in MCL-1 and BCL-XL dependence while the patient was on trial (WK4 and WK12). Moreover, when the patient was removed from the trial due to progressive disease, the patient still exhibited dependence on BCL-XL and MCL-1 (**Figure 3.4F**). This suggests that ABT-199 treatment may have selected for BCL-XL and MCL-1 dependent blasts, or treatment with ABT-199 results in an adaptive response of increased BCL-XL and MCL-1 dependence in AML blasts.

V. DISCUSSION

This Phase II clinical trial represents the first trial of a BH3 memetic in patients with AML. The trial demonstrated that ABT-199 was generally well tolerated and provided encouraging evidence that ABT-199 could be effective in some AML patients as a monotherapy. In this first set of patients who had been heavily treated with prior rounds of chemotherapy, the overall response rate was 19%, and responses were observed relatively quickly with responses observed within the first month of treatment. Furthermore, at least two patients were able to continue to transplant after achieving a complete response from ABT-199 monotherapy. The most frequent grade 3 treatment-related AEs were pneumonia and febrile neutropenia. Therefore ABT-199 as a monotherapy is much better tolerated compared with standard induction chemotherapy which has considerably higher treatment related mortality (*252*).

The response rates to ABT-199 in AML were heterogeneous, as was predicted by pre-clinical trial analysis described in chapter 2 (*54*). Therefore, establishing a predictive or prognostic biomarker to identify patients who will be most sensitive to ABT-199 treatment would be particularly useful in this setting. IDH mutation status, BCL-2 family profile and BH3 profiling were tested as predictive biomarkers in this cohort of patients. Previously, it was reported that mutations in IDH1 or IDH2 promote BCL-2

dependence due to inhibition of COX by (R)-2-HG, an oncometabolite produced by the neomorphic activity of IDH mutants (*250*). Approximately 15-20% of all AML patients have mutations in either IDH1 or IDH2 (*99, 253*). This study was slightly enriched for IDH mutant patients as eleven patients (35%) exhibited an IDH1 or IDH2 mutation. Patients harboring an IDH mutation tended to have greater responses to ABT-199 treatment. Four patients with IDH mutation had a CR or CRi following ABT-199 monotherapy; however, two patients who had IDH mutations experienced progressive disease. Furthermore, two patients who were IDH WT experienced a CR or CRi. Therefore, IDH mutant patients tend to have a better clinical response than IDH WT patients, but an IDH mutation does not guarantee response to ABT-199 monotherapy treatment.

The second predictive biomarker of ABT-199 clinical response tested was BCL-2 family profile. This analysis involved measuring protein levels of BCL-2 and BCL-XL with the hypothesis that AML cells which express low levels of BCL-2 OR high levels of BCL-XL will be refractory to monotherapy ABT-199 treatment. Patients who had BCL-2 family resistant profiles tended to be removed from the trial earlier than patients who had BCL-2 sensitive samples, but the BCL-2 family profiles lacked sensitivity to identify the samples that responded to ABT-199 treatment. This could be due to not including MCL-1 expression into the analysis as expression of MCL-1 is an important resistance factor for BCL-2 treatment as measured by our BH3 profiling (*254*).

The third biomarker assay tested was BH3 profiling. Although there were small numbers of samples tested, BH3 profiling identified patient samples that had a greater than zero reduction in blast count and patient samples that remained on trial for more than thirty days. Interestingly, the factors that mediate resistance to BCL-2 inhibition, BCL-XL and MCL-1, were better predictors of blast reduction compared to measuring BCL-2 dependence alone. Analysis using ABT-737, an inhibitor of BCL-2, BCL-XL and BCL-w, showed that the drug is most efficient in tumors in which MCL-1 is neutralized (*255*). Taken together, this data suggests that expression of the target protein alone (BCL-2), is not sufficient for sensitivity to ABT-199; rather, AML blasts also need to lack dependence on resistance factors (BCL-XL and MCL-1). Novel agents specifically inhibiting MCL-1 and BCL-XL are in development; therefore, AML blasts dependent on these anti-apoptotics may be sensitive to the novel inhibitors (*256-258*).

Potential for resistance to ABT-199

In addition to upfront resistance to ABT-199 treatment, acquired resistance to the inhibitor was also observed in the trial. The median CR duration was 47.5 days suggesting that resistance is acquired relatively quickly. In pre-clinical analysis in diffuse large B cell lymphoma (DLBCL) cell lines, acquired resistance to ABT-199 is due to increased expression of BCL-XL and MCL-1 following chronic exposure to ABT-199 (*259*). We also observed an increase in BCL-XL and MCL-1 dependence in our BH3 profiling analysis comparing pre-treatment and WK4 blast samples. The daily dosing schedule of ABT-199 may contribute to the acquired resistance. Daily exposure of MCF10A (mammary epithelial cells) to TRAIL, an agonist of the extrinsic cell death pathway, at a dose which causes a fractional kill of the cell population promotes resistance. However, removal of TRAIL for a few days "re-sets" the cells and they become as sensitive to the ligand as treatment naïve cells (*260*). Although these TRAIL experiments were performed on a genetically homogenous population, and it is appreciated that AML is composed of functionally and genetically distinct subpopulations, it is plausible that the daily dose escalation protocol used in this trial may promote resistance by selecting for resistant AML blasts by selecting for BCL-XL/MCL-1 resistant sub-clones (*104, 111*). A periodic dose schedule in which the AML cells are allowed to 're-set' to the treatment naïve state may be more beneficial than daily dosing.

Future or ABT-199 in AML

The single agent ABT-199 in AML did not meet pre-established clinical end points for continuation of this ABT-199 trial to a larger subset of patients. Nevertheless, these Phase II ABT-199 monotherapy results have provided useful information of BCL-2 inhibition in AML patients. It is possible that monotherapy ABT-199 treatment can be successful in a subset of relapsed/refractory patients or patients unfit for induction therapy. Using IDH status, BH3 profiling or a combination of both to identify patients who will be most sensitive to ABT-199 monotherapy is a possibility. The relative low toxicity of the drug as a monotherapy makes it an attractive option for AML patients.

The exciting biological activity and low toxicity of ABT-199 provoked further testing and ABT-199 is currently in clinical trial as a combination therapy to improve upon its single agent killing activity. Pre-clinical analysis of ABT-199 with the hypomethylating agent 5-azacytidine show that the drugs act

synergistically to kill AML cells (*261*), and a Phase 1b trial of a combination of 5-azacytidine or decitabine with ABT-199 are actively recruiting. A second clinical trial of ABT-199 in combination with low-dose Ara-C is also recruiting for AML treatment naïve patients over the age of sixty-five (clinicaltrials.gov). Additionally, since we have previously found that the level of mitochondrial apoptotic priming predicts clinical response to conventional chemotherapy (*52, 56*), we speculate that ABT-199 could facilitate myeloblast killing in combination with conventional chemotherapy *in vivo*.

CHAPTER 4: MITOCHONDRIAL PRIMING INCREASES DURING DIFFERENTIATION AND DECREASES WITH AGING IN HEMATOPOIETIC STEM CELLS

Attributions: This work was initiated by myself and data was generated through a productive collaboration between Dr. Paula Gutierrez-Martinez, a postdoctoral fellow in Dr. Derrick Rossi's laboratory at Harvard Medical School. I performed all of the BH3 profiling studies and Dr. Gutierrez-Martinez led the DNA damage functional experiments (Figures 4.5 and 4.6).

I. ABSTRACT

Preservation of hematopoietic stem cell (HSC) number and function is essential for homeostatic maintenance throughout an organism's life. Thus, the choice of stem cell fate, whether a HSC undergoes differentiation, self-renewal or apoptosis is strictly controlled. However, it is not well understood how apoptosis is controlled in HSCs during normal differentiation or with aging. In this chapter, we utilize BH3 profiling to probe the mitochondrial apoptotic pathway in HSCs and downstream progenitors during normal differentiation and in aging (**Figure 4.1**). We observed that HSCs are less primed than downstream progenitors and this correlates with their decreased sensitivity to DNA damaging agents. Further, we observed that HSCs isolated from aged mice are less primed than HSCs isolated from young mice. Interestingly, when we examined the myeloid skewed $CD150^{hi}$ cells which accumulate with old age in mice, we observed that these were the least primed of all cells tested. To extend these findings, we treated cells with several DNA damaging agents and found that old HSCs are resistant to DNA damage. Together, this data suggests that the increases in HSC cell number and myeloid skewing of HSCs with age may be partially driven by the persistence of low primed, apoptotic resistant myeloid skewed HSCs.

II. INTRODUCTION

Within the hematopoietic system, hematopoietic stem cells (HSCs) possess unique long-term multi-lineage potential and self-renewal capabilities. They regenerate the entire hematopoietic system by progressively differentiating into multi-potent progenitor cells (MPPs) which ultimately develop into all of the mature blood sets found in the blood circulation. Since the HSC lies at the pinnacle of the

hematopoietic hierarchy, it is pivotal that the cell retains its function with fidelity throughout an organism's lifespan in order to thwart hematopoietic dysfunction (*262*). The disadvantage of a long lived HSC is the potential for exposure to environmental and intracellular hazards such as gamma irradiation, reactive oxygen species (ROS) and chemotherapy (*263*). As the HSC function is long lived, the response to such stimuli must be tightly controlled as there is a tradeoff between increased cell death which decreases the size of the HSC pool reducing the capacity to respond to stress and injury, and persistence of DNA damaged HSCs which can result in mutation accumulation and transmission to progeny which may result in hematological malignancies. However, it is unknown how the HSC achieves a balance between life and death.

One hypothesis is that HSCs are particularly sensitive to DNA damage and undergo apoptosis more readily than downstream progenitors in order to immediately cull damaged HSCs from the population. This strategy would be beneficial in removing damaged HSCs before they are able to contribute to malignancy, as mutated HSCs have been implicated in contributing to hematological

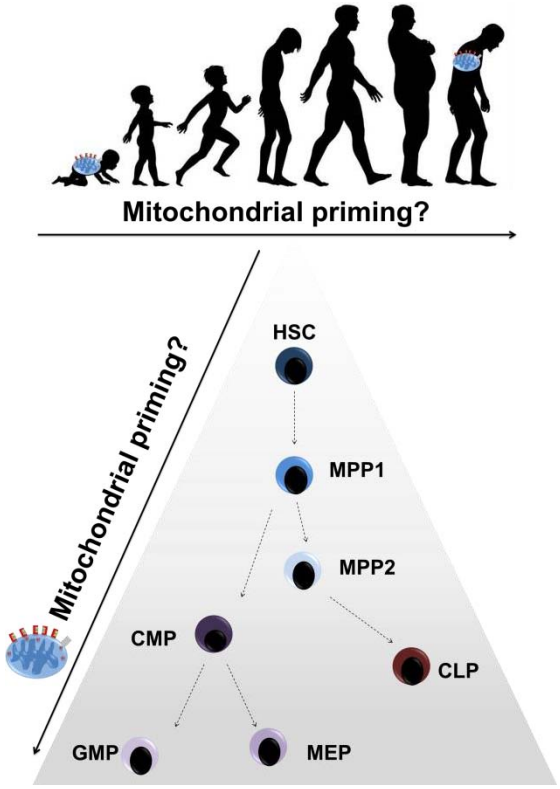


Figure 4.1. Two questions addressed in Chapter 4. 1. Are HSCs more or less primed than differentiated cells? 2. Does mitochondrial priming change with age in HSCs?

malignancy (*110, 264*). Human HSCs and progenitor cells (HSPCs; CD34+ CD38+) have been reported to be more sensitive to chemicals which induce ER stress, and are also more sensitive to irradiation compared to lineage restricted progenitor cells *ex vivo* (*265, 266*).

An alternative hypothesis is that HSCs are resistant to apoptosis because of the need for long lived cells to sustain hematopoiesis throughout the life of an organism. Analysis in mice suggest that HSPCs (Lin/c-Kit+/Sca-1+/Flk2-) are less sensitive to irradiation compared to downstream progenitors (*187*). Furthermore, this hypothesis is supported by the observation that HSCs persist through radiation therapy and are able to re-populate the bone marrow when most circulating cells are killed (*267*). These inconsistent results in mice and humans have been measured in impure populations which consist of HSCs and multi-potent progenitors. Important differences exist between HSCs and MPPs such as their proliferation rate and expression of various DNA damage response factors (*183*). Thus, the first goal of this study was to perform a thorough analysis of apoptotic sensitivity of highly pure populations of HSCs and downstream progenitors by BH3 profiling.

The experiments discussed above have mainly focused analysis of HSCs to those isolated from young humans (cord blood) or young mice (2-4 month old). It is known, however, that organismal aging is accompanied with a host of unwanted clinical pathologies derived from the HSC compartment including immunosenescence, increased incidence of myelodysplasias and myeloid leukemias (*197, 262, 268*) suggesting that control of stem cell fate may be attenuated with age. The molecular underpinnings driving these aging phenotypes are complex and numerous, and include epigenetic dysregulation, altered bone marrow homing, dominance of myeloid skewed HSC clones and loss of cell polarity (*198, 201, 204, 262*). However, it is not known how these alterations affect the ability of the HSCs to respond to DNA damage and undergo apoptosis with age. As appropriate response to apoptotic stimuli and DNA damage is necessary to prevent the accumulation of damaged HSCs with age, it is important to understand how apoptosis is regulated in young and old HSCs. Therefore, the second goal of this study was to examine the mitochondrial priming of old and young HSCs to determine if the mitochondrial apoptotic pathway is controlled differently with age.

We utilized BH3 profiling to probe apoptotic sensitivity and anti-apoptotic dependencies of HSCs and downstream progenitors to assess how differentiation and aging of the HSC affects mitochondrial

priming. BH3 profiling is a particularly useful tool for probing mitochondrial apoptosis in HSCs because it is a fast, functional assay that can be performed with relatively few cells and without the need for using a fluorescent assisted cell sorter for isolation of HSCs. Moreover, BH3 profiling does not require *ex vivo* culturing of HSCs as is the case with many assays which probe the mitochondrial apoptotic pathway. Culturing HSCs in a fashion which maintains the self-renewal property has deemed to be exceedingly difficult (*269*). Most HSC culturing techniques require a complex cytokine milieu which often promotes lineage commitment and/or the media lacks the factors found in the bone marrow niche. Therefore, this may promote undue stress on the HSCs potentially affecting the readout of the BCL-2 family proteins as they are particularly sensitive to cellular stress.

BH3 profiling also offers a sensitive and direct way to assess the function of the BCL-2 family members. Previous analysis of the role of BCL-2 family members in HSC survival has often relied on overexpression or knockout murine models (*189, 193*). Although useful to understand the affect certain proteins may have in the setting of gain or loss of a protein, these types of models often do not address how BCL-2 family members contribute to apoptosis maintenance at physiologic levels. For instances, overexpression of BCL-2 in stem cells may suggest that the anti-apoptotic protein may prevent apoptosis following chemotherapeutic treatment (*270*), but it does not address whether the protein is important for cell survival at physiological levels. Furthermore, as HSCs are rare cells, it is exceedingly difficult to perform protein quantification by Western blot analysis on highly pure population of HSCs; therefore, many investigations of mitochondrial apoptosis in HSCs have been performed by analyzing mRNA expression levels. However, since there are important post-translational modifications to the BCL-2 proteins which can alter the proteins' function as well as interactions among the various BCL-2 family members which affect function (*271*), it is important to measure the functional capabilities of the expressed BCL-2 family members to promote apoptosis. Therefore, the goals this this project were to further understand how mitochondrial apoptosis is controlled during physiological differentiation and during aging of HSCs.

III. METHODS

Mice

All mice used were C57BL/6 males. Young mice were 3–4 months old, and old mice, obtained from the National Institute on Aging were 24–26 months old. All mice were maintained according to protocols approved by Harvard Medical School Animal Facility and all procedures were performed with consent from the local ethics committees.

Purification of Cells

Bone marrow cells were extracted by crushing the bones of mice. cKit enrichment was performed by staining the cells with magnetically labeled antibodies against cKit and using Miltenyi columns for enrichment. Enriched cells were stained to identify the following cell-surface phenotypes: HSCs, Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)-, c-Kit+ Sca-1+ CD34- Flk2- ; MPP1, Lin-c-Kit+ Sca1+ CD34+ Flk2-; MPP2, Lin- c-Kit+ Sca-1+ CD34+ Flk2+ ; GMP, Lin- c-Kit+ Sca1+ CD34+ FcγR+ ; CLP, Lin- CD19 CD11 cB220- CD27+ c-Kitmid Flk2+ IL7Rα+ Ly6d; CMP, Lin-c-Kit+ Sca-1 CD34+ FcγRlow; MEP, Lin-c-Kit+ Sca-1CD34 FcγR. FACS data were analyzed with FlowJo.

BH3 profiling of mouse hematopoietic stem cells

Bone marrow cells were isolated, cKit enriched, split into three populations and stained with antibody cocktail to identify LSKs, CLPs and myeloid progenitors as described above. For BH3 profiling, cells were seeded at $2x10^6$ cells/mL and incubated with 20 µg/ml oligomycin, 0.0005% digitonin and BH3 peptides (amino acid peptide sequences for BH3 peptides can be found in (*272*)) in DTEB buffer (135 mM Trehalose, 10 mM HEPES-KOH, 0.1% w/v BSA, 20 µM EDTA, 20 µM EGTA, 50 mM KCl, 5 mM succinate, final pH 7.4) for 30 min, except for cell cycle induction experiments and experiments with sensitizer peptides in which cells were incubated for 1 h. Fifteen minutes prior to the completion of the peptide incubation, Tetramethylrhodamine (TMRE) was added to a final concentration of 20nM. Cells were analyzed by flow cytometry to assess mitochondrial depolarization on BD Canto II Analyzer. In order to ensure that each mitochondrial reaction was run for exactly 30 minutes, cells were added to the peptides every two minutes in a staggered fashion. Peptide and TMRE incubation was performed on heating blocks at 22°C. The % depolarization, was calculated from the median fluorescence intensity (MFI) from the PE peak

$$\% \text{ Depolarization/Priming} = 100 \times (1 - ([MFI_{Peptide} - MFI_{FCCP}] \div [MFI_{DMSO} - MFI_{FCCP}]))$$

Heatmaps were produced using plotly.

Treatment of cells

Using the single-cell mode of the FACSAria and double sorting for purity, we sorted cells individually into a single well of a 96-well round-bottom plate. For each experiment, 24-48 individual cells of each animal were used. Each well contained either Dulbecco's modified Eagle's medium and F-12 medium (Gibco and Invitrogen) supplemented with 10% fetal calf serum (Hyclone and Thermo Scientific), 1x penicillin/streptomycin, 2 mM GlutaMAX, 50 mM 2-mercaptoethanol (Invitrogen), and the following cytokines: 20 ng/ml mouse stem cell factor, 20 ng/ml mouse thrombopoietin, 20 ng/ml mouse IL-3, 20 ng/ml mouse granulocyte macrophage colony-stimulating factor (all purchased from PeproTech) or SClone (Iwai North America Inc.) supplemented with 0.75% AlbuMAX-I (Gibco), 1x penicillin/streptomycin, 50 mM 2-mercaptoethanol (Invitrogen) and the following cytokines: 20 ng/ml mouse stem cell factor, 20 ng/ml mouse thrombopoietin, 20 ng/ml mouse IL-12. Cells were either irradiated with 2Gy or treated for 18 hours using the following DNA damaging agents: 0.2 mg/ml ENU (Sigma), 0.2 mg/ml EMS (Sigma) or 250ng/ml Doxorubicin (Sigma). After the incubation time, most media was removed and replaced with fresh complete SClone or fresh complete F12 media. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. At 8 days of culture, colonies were counted under the microscope. For the proliferation experiments, BM cells were isolated, ckit enriched, and plated in F12 complete media described above for indicated time points.

Transplantation methods

Five-hundred HSCs from young and old donors (CD45.2) were isolated and treated with 2Gy irradiation or 200ug/mL ENU for 18h, or no treatment as a control in culture media as described above. Cells were then competitively transplanted against 300,000 whole bone marrow cells (CD45.1) into lethally irradiated (10Gy) young recipient mice (CD45.1). Peripheral blood analysis was performed at 4 week intervals post-transplant with antibodies against Ter119, B220, Mac1, Gr1, CD3, CD45.1, CD45.2, and PI.

IV. RESULTS

Hematopoietic differentiation is associated with an increase in mitochondrial priming and sensitivity to irradiation

HSCs are reported to be relatively resistant to genotoxic stress as compared to mature hematopoietic cells (*267*). To test if the mitochondrial apoptotic pathway contributes to the resistance, we asked if HSCs are less primed compared to more mature downstream progenitors. To discriminate among the various cell populations within in the bone marrow, we used cell surface staining to identify the following populations: HSCs, multi-potent progenitors (MPPs), common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), granulocyte/monocyte progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). Following cell surface staining, we BH3 profiled the cells using the activator peptide BIM (gating strategy shown in **Figure 4.2A**). BIM binds to all of the anti-apoptotic proteins, and also binds to the effector proteins BAX and BAK; therefore, measures the overall priming of a cell (*14*). We found that the mitochondria in differentiated cells (consisting of the CLP, CMP, GMP, MEP) were more sensitive to the BIM peptide compared to cells within the primitive compartment (consisting of HSCs, MPP1 and MPP2) (**Figure 4.2B**). This suggested that the HSCs and MPPs are less mitochondrially primed compared to the differentiated cells.

The HSC population is composed of functionally and phenotypically distinct subtypes that can be further divided into the $CD150^{hi}$, $CD150^{low}$ and $CD150^{neg}$ populations (*273, 274*). $CD150^{hi}$ cells appear to have superior self-renewal capabilities and are more adept to produce successful primary and secondary transplantations compared to $CD150^{lo}$ and $CD150^{neg}$ cells (*274*). Further, the $CD150^{hi}$ subset is more myeloid skewed, meaning that it preferentially gives rise to myeloid lineage of cells than the $CD150^{low}$ and $CD150^{neg}$ populations (*199*). Given this information, we next asked if the distinct CD150 populations within the HSC pool are differently primed. To test this, we included a stain for CD150 within the HSC stain and BH3 profiled the cells. We found that the $CD150^{hi}$ cells, considered to be the most primitive HSC, are the least primed subset of all of the cells that we examined, and that overall priming increases as the HSCs lose the CD150 marker (**Figure 4.2C**).

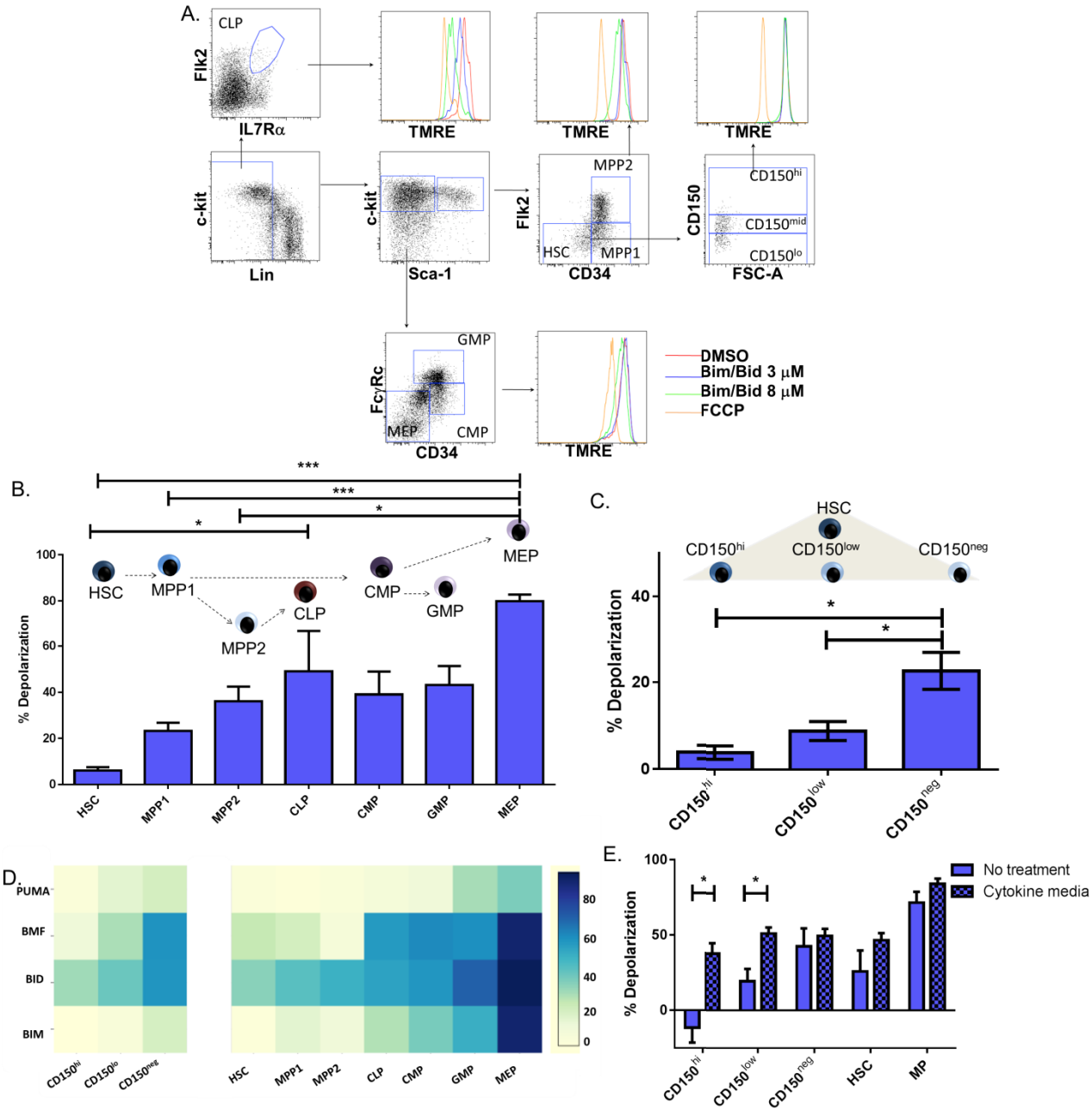


Figure 4.2. Differentiation is associated with an increase in mitochondrial priming. **A).** Gating schematic to identify various cell populations analyzed in BH3 profiling. TMRE peaks are shown for control conditions (DMSO and FCCP) and experimental peptides. **B).** Bone marrow cells were isolated from mice, enriched for ckit+ cells, stained for indicated cell populations and BH3 profiled with BIM 3 μM (n = 3, mean ± SEM is graphed. **C).** BH3 profiling using BIM 8μM of HSCs subdivided into $CD150^{hi}$, $CD150^{lo}$ and $CD150^{neg}$ cell populations (n = 3, mean ± SEM is graphed). **D).** BH3 profiles using Puma 8μM, BMF 3μM, Bid 3μM and Bim 3μM. Heatmap was produced using plotly. **E.)** BH3 profiling was performed following culturing ckit+ enriched cells for 4.5 h. * = p < 0.01 - 0.05, ** = p < 0.001 - 0.01, *** = p < 0.001.

We also BH3 profiled the cell populations with BID, an additional BH3 only activator. This peptide, like BIM, also provides a measure of overall priming of the various cell populations, but it also provides additional information as BID preferentially binds to BAK, whereas BIM preferentially binds to BAX (*275*). Using the BID BH3 only peptide we also observed an increase in priming during differentiation suggesting that BAX and BAK are important for the priming phenotype observed in the differentiation of HSCs. In addition to BIM and BID, we also used the pan sensitizers, PUMA and BMF to probe overall priming during differentiation. Using these sensitizers, we also observed an increase in mitochondrial priming from the most primitive $CD150^{hi}$ cells to the MEP populations (**Figure 4.2D**).

Previously, our lab has shown that decreased mitochondrial priming is associated with insensitivity to apoptotic stimuli (*52, 56*). Therefore, we asked if the most primitive HSCs were less sensitive to DNA damage. We hypothesized that $CD150^{hi}$ cells would be most resistant to DNA damage because they are the least primed of all cells that we tested. To test our hypothesis, we single cell sorted $CD150^{hi}$, $CD150^{low}$ and $CD150^{neg}$ cells, and treated the cells with the irradiation (IR), N-ethyl-N-nitrosourea (ENU) or doxorubicin (dox) for 18h and counted the number of clones which grew following 8 days of culture. We found that $CD150^{hi}$ cells were resistant to the DNA damaging agents compared to $CD150^{lo}$ cells in all three treatments tested (**Supplemental Figure S4.1**). Together, this data suggests that the low primed phenotype of HSCs causes the $CD150^{hi}$ cells to be resistant to genotoxic stress, and furthermore, differentiation of HSCs into lineage committed progenitor cells is associated with increased mitochondrial priming.

Induced proliferation increases mitochondrial priming

HSCs are quiescent and the estimated frequency of cell cycle entry is ~1 every 36-145 days (*276*); whereas more differentiated progenitor cells are cycling more frequently (*277*). We hypothesized that cell cycling may contribute to the low primed phenotype observed in HSCs. One way to induce entry into the cell cycle is to culture HSCs in a rich media that supports the health of cycling HSCs. Previous research has shown that culturing HSCs for 3 h stimulates exit from G0 and up-regulation of DNA damage checkpoint genes, and importantly, at this time point, the cells are pushed into cycle, but it is not enough time for HSCs to differentiate (*215*). To test if entry into the cell cycle increases priming, we

enriched for c-kit expressing cells, and following 4.5 h of culture in a cytokine media, cells were collected, stained for the various cell populations and BH3 profiled using the BIM peptide. We found that the $CD150^{hi}$ and $CD150^{low}$ cells cultured for 4.5 h were more primed compared to cells that were profiled immediately following bone marrow harvest (**Figure 4.2E**). This suggests that the low primed HSC phenotype may be associated with the quiescent state of the HSCs, and forcing cells to cycle increases the priming of the cell.

Anti-apoptotic dependencies of murine HSCs

We next examined if HSCs and downstream progenitors are dependent on specific anti-apoptotics as previous analysis suggests that certain anti-apoptotics are necessary for HCS survival. mRNA analysis show that the HSPCs express higher levels of the anti-apoptotic proteins BCL-XL and MCL-1 compared to downstream progenitors (*187*). Further, knockdown of MCL-1 depletes the HSPC pool, and overexpression of BCL-2 increases HSC cell numbers, improves re-populating potential and protects HSPCs from chemotoxic stress (*189, 193, 270*). However, these studies do not examine the physiological role of these proteins in HSCs as knockdown and overexpression can modulate compensatory mechanisms. Therefore, we tested whether there is a difference in functional anti-apoptotic dependencies in HSCs and more differentiated cells in freshly isolated bone marrow.

To test for anti-apoptotic dependencies within the primitive compartment, we exposed bone marrow cells to BAD (specific for BCL-2, BCL-XL and BCL-w), NOXA (specific for MCL-1) and HRK (specific for BCL-XL) peptides for one hour and measured mitochondrial depolarization in HSCs, MPPs and myeloid progenitors (consists of CMPs, GMPs and MEPs). We used the DHL4, DHL10 and 2643 cell lines as internal controls for peptide function as they have known dependencies on BCL-2 (DHL4), MCL-1 (2643), or are entirely resistant to mitochondrial depolarization due to deletion of BAX and having non-functional BAK (DHL10) (*39, 241*). We observed that HSCs and MPPs depolarized following incubation with the BAD peptide and not to the HRK or NOXA peptide suggesting that murine HSCs are more dependent on BCL-2 than MCL-1 and BCL-XL (as we did not observe mitochondrial depolarization from the HRK peptide, we conclude that the BAD response is indicative of mostly BCL-2 dependence).

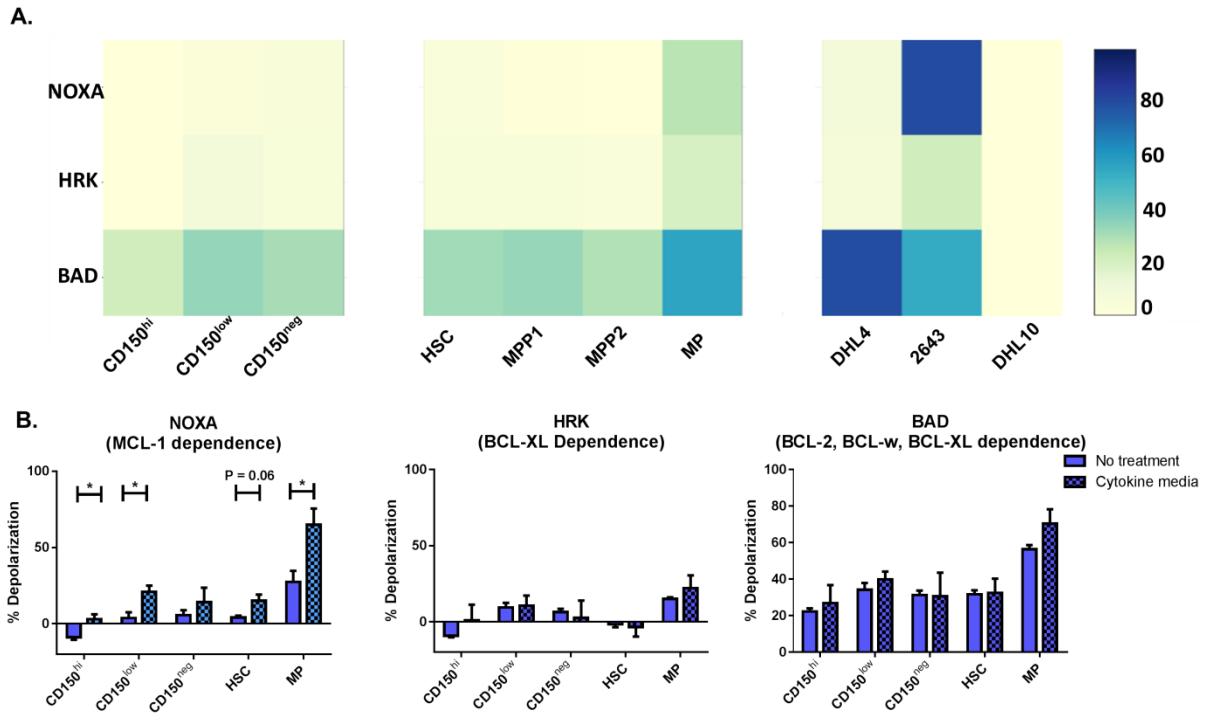


Figure 4.3. Anti-apoptotic dependencies during HSC differentiation. A). Bone marrow cells were isolated, enriched for c-Kit and stained to identify the indicated populations. Cells were then BH3 profiled Noxa (80μM), HRK (80μM) and BAD (80μM) for 1hr. **B).** Bone marrow cells were c-kit enriched and split into two groups. One group was stained immediately following bone marrow isolation and BH3 profiled, the second cohort was cultured in a cytokine rich media for 4.5 h to promote HSC cell cycle entry and then BH3 profiling was performed * = $p < 0.01 - 0.05$

Further, there was an increase in BCL-2 dependence in the more differentiated myeloid cells (**Figure 4.3A**).

Previous studies have reported murine HSCs are dependent on MCL-1 for survival (*189*); however, we did not observe this in our studies. In order to observe a latent MCL-1 dependence we increased the peptide exposure time to two hours, and in a separate experiment increased the temperature of the BH3 profile assay to 28°C in order to detect any suppressed MCL-1 response. Even with the increased time and temperature exposure, we did not observe an increase in mitochondrial depolarization in response to NOXA, the MCL-1 specific peptide. It is possible that in the quiescent state, HSCs are not dependent on MCL-1, but the anti-apoptotic may be necessary during proliferation of HSCs and progenitor cells. To test this hypothesis, we isolated murine bone marrow cells, enriched for cKit expressing cells and cultured the cells in cytokine rich media described above and then performed BH3 profiling. We found after 4.5 h of culturing that there is a small, but significant increase in MCL-1 dependence as measured by depolarization in $CD150^{hi}$ and $CD150^{low}$ cells. HSCs forced into the cell cycle tend to be more MCL-1 dependent, but the finding was not significant ($p = 0.06$; **Figure 4.3B**). We did not observe an increase in BCL-XL or BCL-2 dependence. This data suggests that MCL-1 is not necessary for survival during HSC quiescence, but may be necessary during cell cycle entry to inhibit apoptosis as the cells become more primed.

Old HSCs are less primed than young HSCs

The previous experiments were performed with bone marrow cells collected from young mice (2-4 months). As aging is associated with an increase in the number of HSCs, we next asked if old cells were less primed which may account with their retention with age. To assess this, we collected bone marrow cells from young and old mice, stained the cells to identify the populations described in **Figure 4.2A** and performed BH3 profiling on the cells using the BIM peptide. We found that old HSCs have less mitochondrial depolarization when exposed to the BIM compared to young HSCs (**Figure 4.4A**), meaning that old HSCs are farther away from the apoptotic threshold and are less primed.

We also examined the CD150 subpopulation within the HSC population. The relative frequency of CD150 subpopulations cells changes with age. The $CD150^{hi}$ population expands with organismal aging

and composes approximately 80% of the HSC compartment of aged organisms, whereas young HSCs have balanced expression of the CD150 marker. Additionally, the $CD150^{hi}$ cells promote the differentiation of myeloid cells and the accumulation of this population is thought to contribute to the myeloid skewing in aged individuals (*199*). In our BH3 profiling assays, we observed that the $CD150^{hi}$ cells isolated from old mice were less primed than the $CD150^{hi}$ cells isolated from young mice (**Figure 4.4A**, first three column sets). Therefore, our BH3 profiling data suggests that the accumulation of $CD150^{hi}$ cells may be due to the low primed mitochondrial phenotype of the population of cells.

We previously found that inducing cell cycle entry of young HSCs increases mitochondrial priming; therefore, we next asked if old HSCs shared the same phenotype. To this end, we cultured old HSCs in a cytokine rich media for 4.5 h to induce cell cycle entry as described above. We found that inducing cell cycle entry slightly increased priming in old HSCs, but not significantly (**Figure 4.5B**). Previous results have shown that old HSCs stimulated to enter the cell cycle by culturing cells show a stronger induction of checkpoint genes approximately 3h following stimulation (*215*). Therefore, the attenuated increase in priming of old HSCs may be connected to the delayed entry into the cell cycle compared to young HSCs.

We next asked if the overall decrease in apoptotic priming may be due to a concomitant difference in anti-apoptotic dependencies within the old HSCs compared to the young HSCs. To examine this, we exposed the old and young HSCs to the sensitizer peptides, NOXA, HRK and BAD to probe anti-apoptotic dependencies. We did not observe a difference between young and old cells in response to the MCL-1 specific peptide NOXA, and observed a slight decrease in BCL-XL dependence as measured by the depolarization caused by the HRK peptide (**Figure 4.4B-C**). However, we did observe a significant decrease in mitochondrial depolarization following incubation with the BAD peptide in the HSCs, MPPs and MP cell populations suggesting that old cell hematopoietic populations have reduced dependency on BCL-2 compared to young cells (**Figure 4.4D**). The decrease in BCL-2 dependence suggests that there is a decrease of BCL-2:BIM/BID complexes at the mitochondria in old HSCs. This could be due to a down-regulation of BCL-2, a down-regulation of BIM and BID, a down-regulation of BAX and BAK or a combination of all three.

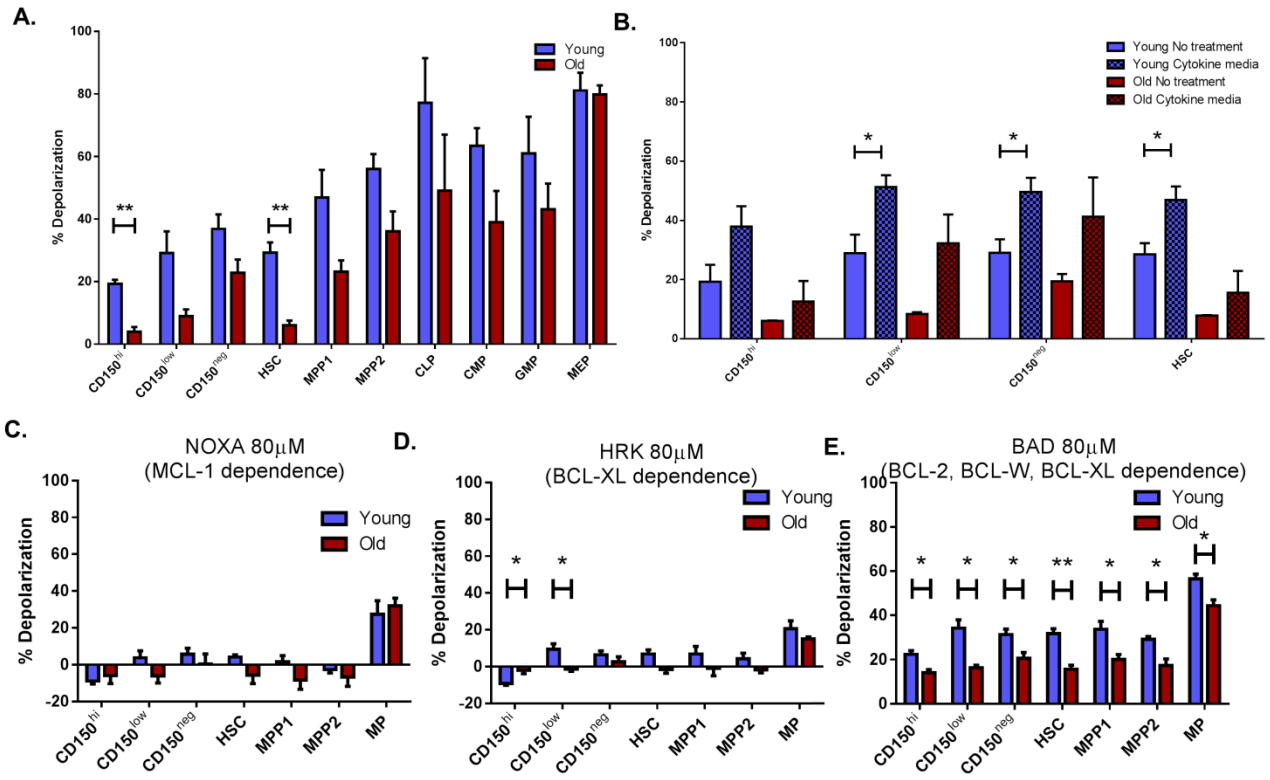


Figure 4.4. Old HSCs are less primed than young HSCs A). Bone marrow cells from young (2-4months) and old (24 mo old) mice were isolated, stained for the indicated populations and BH3 profiled using the BIM 8μM peptide (n = 3, mean ± SEM is graphed). **B).** BH3 profiling was performed following culturing ckit+ enriched cells for 4.5h. BH3 profiling was performed with BIM 0.8μM following 1h peptide incubation. **C-E).** BH3 profiles indicated cell population treated with NOXA 80μM (C) HRK 80μM (D) or BAD 80μM (E) for 1hr. . * = p < 0.01 - 0.05, ** = p < 0.001 - 0.01.

Old HSCs are less sensitive to DNA damaging agents

Based off of our BH3 profiling data, we hypothesized that old HSCs would be more resistant to DNA damage and apoptotic stimuli compared to young HSCs. To test this hypothesis, we measured the functional response of young and old HSCs to apoptotic stimuli in two ways; first by measuring the ability of single sorted HSCs exposed to DNA damaged to form colonies *in vitro*, and in the second functional assay we tested the ability of DNA damaged HSCs to repopulate the bone marrow of irradiated recipients *in vivo*.

To test if unprimed, old HSCs were resistant to DNA damage compared to young HSCs, we single cell sorted HSCs from young (2-4 month old) and old (24-26 months old) mice into plates containing an HSC supportive media. We then exposed the single cells to various DNA damaging agents including N-ethyl-N-nitrosourea (ENU), ethyl methanesulfonate (EMS), doxorubicin (dox), hydroxyurea (HU) and irradiation (IR) and assessed cell viability by colony formation following 8 days of culture. We tested these five agents because they have different mechanisms in which they damage DNA. ENU and EMS are alkylating agents, doxorubicin is an intercalating agent, HU inhibits ribonucleotide synthase and blocks dNTP production causing replicative stress, and IR produces double stranded DNA breaks. We found that, regardless of DNA damage stimulus used, that old HSCs produce more colonies following DNA damage compared to young HSCs (**Figure 4.5**).

Self-renewal and differentiation are important attributes of HSCs which allow the cells to maintain and repair deficiencies in the hematopoiesis throughout the lifespan of the organism. These capabilities of HSCs can be assessed by the ability of sorted HSCs to repopulate the bone marrow of irradiated recipient mice. Thus, to further assess the self-renewal potential of DNA damaged young and old HSCs *in vivo*, untreated or HSCs treated with ENU for 18 h were transplanted into lethally irradiated congenic recipients. Chimerism was followed by peripheral blood reconstitution at four week intervals. As was observed in previous reports, the transplant efficiency of untreated young HSCs was greater than that of untreated old HSCs at 16 weeks (peripheral blood reconstitution in untreated young HSCs (% of donor) = 73%; untreated, old HSCs = 21%) (*184, 200*). In accordance with our colony formation assays, young HSCs that were treated with a sub-lethal dose of ENU and transplanted into irradiated recipients were statistically inferior to the young untreated HSCs at engrafting (peripheral blood reconstitution in ENU

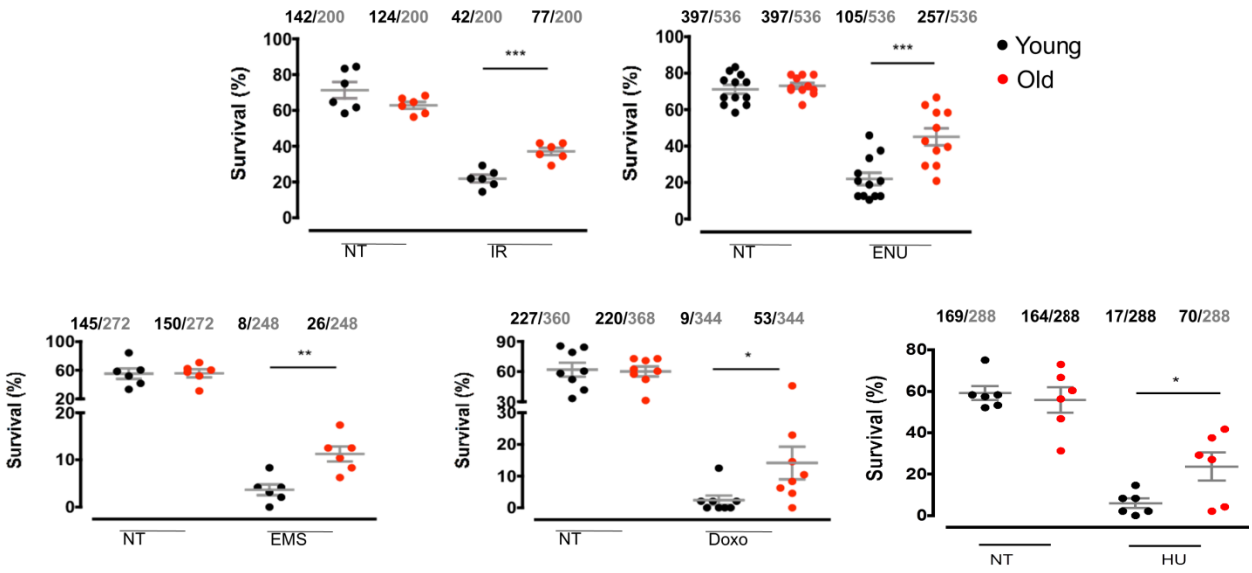


Figure 4.5 Old mouse HSCs are less sensitive to DNA damaging agents compared to young HSCs. Single cell sorted HSCs were exposed to 2Gy irradiation, 200 µg/ml ENU, 200 µg/ml EMS, 250 µg/ml doxorubicin, hydroxyurea or no treatment (NT) for 18 h. Cells were washed and colony formation was counted 8 days post-plating. One dot represents one mouse. Numbers above each group represent the number of single cells that grew into colonies per single cell that were plated. Red dots indicate cells isolated from young mice, black dots indicate cells isolated from old mice. * = $p < 0.01 - 0.05$, ** = $p < 0.001 - 0.01$, *** = $p < 0.001$.

transplant efficiency as untreated old HSCs (peripheral reconstitution in ENU treated old mice; % of donor = 20%) (**Figure 4.6A-B**). Regardless if the HSCs were treated with ENU or not, they gave rise to similar differentiated progeny with old cells preferentially producing myeloid progeny and young cells producing balanced myeloid and lymphoid progeny (**Figure 4.6C**). In order to ensure that this observation was not specific to alkylating DNA damage, we repeated this transplantation experiment with HSCs treated with IR, and observed similar results (**Figure 4.6D-E**). Together with the colony formation assays, the transplantation assays show that old HSCs are more resistant to DNA damage compared to young HSCs. As others have shown that the there is no difference between the ability of young and old HSCs to upregulate DNA damage response genes (*215*), we hypothesize that mitochondrial priming is a major contributor to the decreased sensitivity of old HSCs to DNA damage compared to young HSCs .

V. DISCUSSION

As HSC function needs to persist throughout the life of an organism, understanding how stem cells respond to apoptotic stimuli throughout an organism's life is necessary. Using BH3 profiling as a tool to investigate mitochondrial priming, we found that HSCs were unprimed compared to downstream progenitors which correlated with decreased sensitivity to irradiation. Furthermore, HSCs isolated from old mice were less primed compared to HSCs isolated from young mice (**Figure 4.7**). Interestingly the $CD150^{hi}$, myeloid skewed cells which contribute to the myeloid skewing and increase in number with age, were the least primed of all cells tested. Our BH3 profiling results correlated with the decreased sensitivity of old HSCs to DNA damage compared to young HSCs.

In the introduction of this chapter, we distinguished between two plausible hypotheses for the sensitivity of HSCs to DNA damage. Our data supported our second hypothesis, that HSCs are less sensitive to DNA damage compared to more mature progenitor cells. Previous data showed that murine HSPCs are resistant to irradiation compared to lineage committed progenitors; however in the present study, we have extended these findings to more refined HSC sub-populations (187). Clinically, it has been observed that HSCs are relatively resistant to chemotherapy treatment (278). For instance in acute myeloid leukemia, induction chemotherapy ablates the bone marrow, but the hematopoietic system is

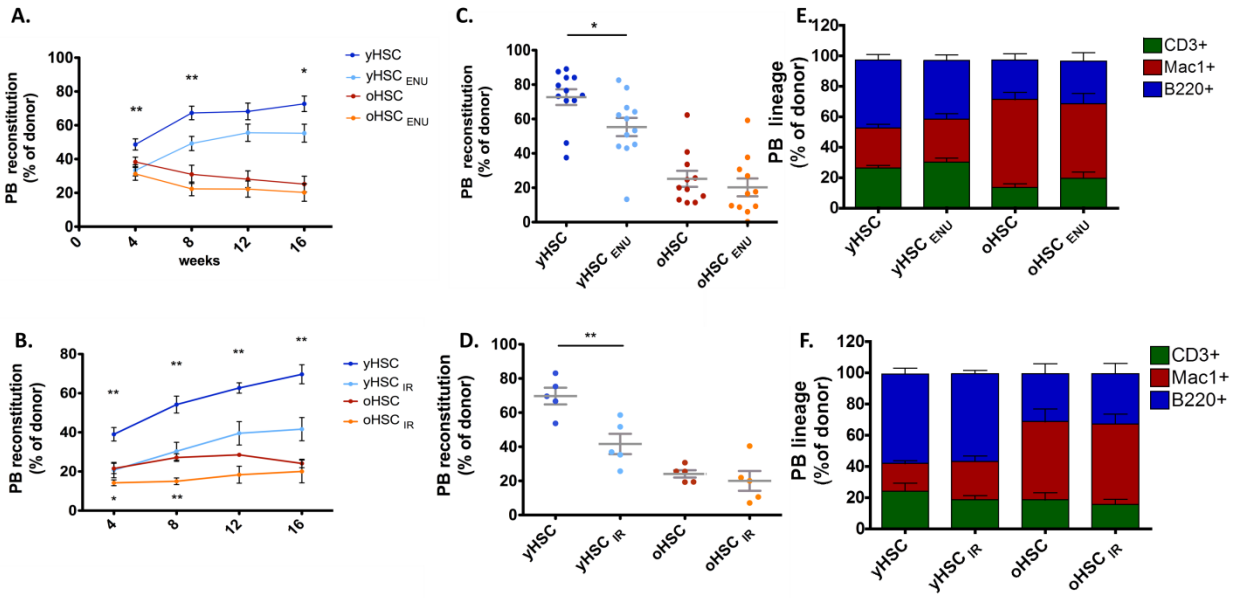


Figure 4.6. The repopulation potential of old HSCs is less affected by DNA damage compared to young HSCs. **A-B).** 500 young and old HSCs (CD45.2) were sorted and treated with DMSO, 200ug/mL ENU (A), or 2Gy irradiation (B) for 18h. Cells were washed and competitively transplanted against 300,000 whole bone marrow cells (CD45.1) into a lethally irradiated recipient (CD45.1). PB reconstitution in 4 WK intervals. **C-D)**. Total reconstitution at 16 WKs for cells treated with ENU or IR. **E-F)**. Contribution of B cells (B220+) myeloid (Mac1+) cells and T cells (CD3+).

eventually reconstituted. Yet, it has been unclear how HSCs are able to survive myeloablative therapy to repopulate the marrow. Here, we show that the most primitive HSCs are less primed and insensitive to irradiation; thus, it is possible that this phenotype allows the cells to persist during chemotherapy treatment.

The low primed HSC phenotype may be associated with the quiescent nature of the HSC. It has been established that HSCs are quiescent and that progenitor cells are cycling more than HSCs (*278-280*). Here we show that HSCs forced into the cell cycle by incubation with a cytokine rich media increases priming. We also show that more differentiated, higher cycling progenitors are more primed. The low primed phenotype of HSCs may protect tissues from stem cell exhaustion. Chronic exposure to proliferation stimulants, such as IFNα, results in increased cell cycle activity at the cost of reduced transplantation efficiency (*281*). Therefore, by being low primed, the HSCs are apoptotic resistant and the need to re-populate the HSC pool following hematopoietic injury is diminished which may protect the HSC pool from premature stem cell exhaustion.

Previous studies have implicated MCL-1 as an important anti-apoptotic necessary for HSC survival (*56, 189*). In the present study we did not observe a dependence on MCL-1; rather we find HSCs tend to be more BCL-2 dependent at steady state. However, when cells were forced into cycle, there was an increase in MCL-1 dependence. Interestingly, the HSCs studied in Vo et al. were isolated from GM-CSF mobilized blood collars. GM-CSF induces efficient proliferation and mobilization of HSCs from the bone marrow (*282*). Furthermore, the MCL-1 knockout HSCs studied in Opferman et al. were conditionally deleted by type 1 IFN-inducible MX-1 Cre locus (*189*). IFNα signaling can promote HSC cell cycling (*283*). Together, this suggests that MCL-1 may be important in HSC proliferation and self-renewal. Alternatively, beyond its role as an anti-apoptotic protein, MCL-1 can be post-translationally modified into a truncated protein that localizes to the inner mitochondrial matrix where it controls mitochondrial fusion and respiration, distinct from its cytoplasmic role in apoptosis (*191, 192*). The aforementioned MCL-1 studies relied on knockdown experiments which affect both the long and truncated form of MCL-1. Thus, it is also possible that the dependence of HSCs on MCL-1 may be distinct from its apoptotic role.

In our studies of aging HSCs, we found that old HSCs are less apoptotically primed compared to young HSCs and this correlates with a decreased sensitivity to DNA damage of old HSCs. It is possible

that old and young HSCs have different exhibit different DNA responses; however, recent gene expression analysis suggests that both young and old HSCs up-regulate similar levels of DNA damage response genes (*215*). This suggests that when exposed to equal amounts of DNA damage young cells die because they are more primed and old HSCs survive because of their low primed phenotype. In addition to a decrease in overall priming, there is a decrease in BCL-2 dependence in old cells. There are three possibilities which could promote these observations at the mitochondria. 1. Old HSCs have less pre-bound activators to anti-apoptotic proteins, 2. Old HSCs have similar concentrations of activators, but higher expression of anti-apoptotics and/or 3. Old HSCs have decreased BAX and BAK expression compared to young HSCs. In order to determine which hypothesis contributes to the decreased priming phenotype, protein expression in the pure HSC population will need to be performed.

In humans and mice there has been an observed clonal expansion of HSCs with age (*199, 216, 217, 284*). In humans, oligoclonality has been identified by specific mutations within the HSC compartment of individuals lacking hematological malignancies. In mice expansion of the $CD150^{hi}$ population with age has been observed. In both organisms, it is unclear as to why these particular cells dominate the stem cell pool in aged organisms. In our experiments, we found that the $CD150^{hi}$ cells were the most unprimed cells of all of the populations examined in the hematopoietic system. The low primed phenotype provides these cells a survival advantage over other cell subsets as was confirmed with our colony formation assays. As it is known that murine HSC aging is associated with the accumulation of DNA damage as well as epigenetic alterations, it will be important to investigate how these alterations may promote the low primed phenotype.

Preliminary evidence in human HSPCs suggests that primitive HSPCs are more sensitive to tunicamycin and IR compared to lineage committed progenitors (*265, 266*). This discrepancy with our study may be due to technical limitations of defining HSCs in humans, as HSC marker identification in mice is more refined. Alternatively, the differences may be due to different ways in which murines and humans respond to DNA damage and ER stress. Even though humans share many of the HSC aging phenotypes of mouse HSCs including increases in HSC cell number, accumulating of DNA damage and myeloid skewing, there are also obvious differences between mouse and man (*198*). The average lifespan of laboratory mouse is approximately 2 years (research.jax.org), whereas the average human

lifespan is 78 years (census.org). Therefore, the maintenance of HSCs in the two systems may be regulated differently due to organism lifespan. BH3 profiling of bone marrow from young and old humans will provide insight into how mitochondrial apoptosis is controlled in human HSCs.

Finally, it is tempting to speculate that our results may have implications on the observed increase in cancer incidence with age. In our studies we find that when exposed to similar amount of DNA damage, young HSCs die more readily than old HSCs due to their more primed phenotype. The potential contribution of DNA mutation accrual of old HSCs in combination with their low primed phenotype may predispose the old HSCs and their progeny to development of hematological malignancy. Experiments in which old and young DNA damaged HSCs are followed in primary and secondary transplantations to detect the emergence of malignancy are ongoing.

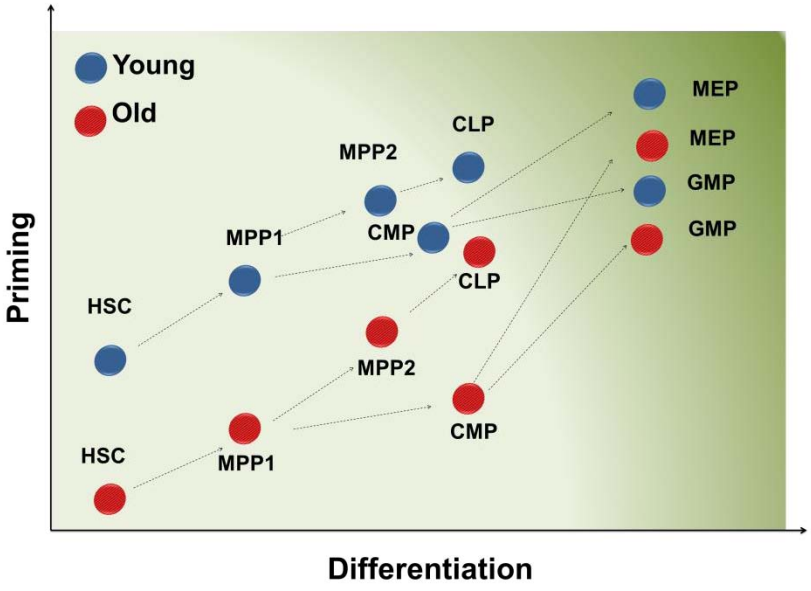


Figure 4.7. Graphical summary of differentiation and aging mitochondrial priming in the hematopoietic system. 1. HSCs are less primed than downstream, more differentiated progenitors. 2. Young HSCs are more primed than old HSCs**.**

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Aging of the hematopoietic system is associated with an increase in incidence of hematological malignancies, including acute myeloid leukemia. This thesis detailed the use of a novel BCL-2 inhibitor to improve survival outcomes for relapsed refractory AML and tested the utility of BH3 profiling, a functional biomarker, to identify AML samples that will be most sensitive to BCL-2 inhibition in pre-clinical and clinical trial settings. Furthermore, by analyzing the mitochondrial priming of young and old HSCs, we found that old HSCs are less mitochondrial primed and are less sensitive to DNA damage. We hypothesized that the low primed phenotype of aged, myeloid skewed HSCs may lead to the expansion of DNA damaged myeloid skewed HSCs with age. Although this thesis answered important questions regarding improvements to AML therapy and stem cell aging biology, each biological question answered raises several additional questions. This chapter explores the major conclusions of this work and discusses future directions of this research.

ABT-199 in AML: Important results and unanswered questions

Use of functional biomarkers in a genetic biomarker world

With the emergence of novel targeted therapies in cancer treatment, there is a need to identify patients who will be best treated with these drugs. Thus far, most predictive biomarkers have relied on the presence of particular genetic lesions in the tumor to guide therapy. The Philadelphia chromosome for Gleevec treatment in chronic myeloid leukemia, and the presence of the $BRAF^{V600E}$ mutations for treatment with Vemurafenib are two of the most well-known and successful predictive biomarkers.

However, the dependence of AML cells on BCL-2 is not due to an identifiable mutation. Therefore, a non-genetic biomarker is needed to identify patients who will be most sensitive to ABT-199 treatment. The Phase II clinical trial of ABT-199 in relapsed/refractory AML was the first time that the functional biomarker, BH3 profiling, has been integrated into a clinical trial setting to test for correlation between anti-apoptotic dependence and clinical response. The assay identified patient samples that were most sensitive and most resistant to ABT-199 treatment *in vivo*. The best measure of clinical success was the sum of the resistance factors of ABT-199: BCL-2 and MCL-1. This measurement was produced post hoc; therefore, a larger sample set of AML patients will need to be tested prospectively to assess the

robustness of this measurement as a predictive biomarker. Together, the data presented here suggests that BCL-XL and MCL-1 dependence measured by BH3 profiling could be a potential method to recruit patients who will be most successfully treated by single agent ABT-199. The relative low toxicity profile of ABT-199 makes this therapy particularly attractive for patients diagnosed with AML.

Identifying effective combination therapies with ABT-199

Although two patients in the ABT-199 clinical trial were able to receive a stem cell transplant following remissions induced by ABT-199 treatment, the other patient responses to ABT-199 monotherapy were not as durable. Thus, it is necessary to explore methods to improve depth and durability of ABT-199 clinical response in AML As discussed above, one method to improve response rates is to direct ABT-199 treatment to patients who have high BCL-2 dependence, and low MCL-1 and BCL-XL dependence. An additional method is to combine ABT-199 with other chemotherapies. There are two types of therapies that are likely to synergize with ABT-199 treatment. The first is combining with conventional chemotherapies, and the second is combination with therapies which either increase BCL-2 dependence, or decrease MCL-1 and BCL-XL dependence in AML cells.

We have shown previously that mitochondrial primed cancers are more sensitive to conventional chemotherapies compared to unprimed tumors (*52, 56*). Thus, increasing the overall priming of tumors would be expected to enhance therapeutic success of conventional chemotherapeutics. We show here that ABT-199 increases the mitochondrial priming of myeloblasts remaining in the bone marrow following four weeks of ABT-199 treatment (**Figure 3.5A**). Thus, ABT-199 increases the priming and treating with an additional therapy may eliminate remaining blasts. We have previously shown that increased priming correlates sensitivity to topoisomerase II inhibitors such as daunorubicin and idarubicin in AML cell lines; therefore, we hypothesize that combination treatments with ABT-199 with these drugs will be effective (*52*). Furthermore, these drugs are FDA approved for treatment of AML; so, it will be easier to move these combinations into trial than non-approved drugs. Once effective drug combinations are identified, optimal dosing schedules will need to be identified for combination therapies. It may be beneficial to pre-treat the patients with ABT-199 to increase the priming of the myeloblasts and then treat with additional therapies to further eliminate the AML blasts, or it may be most effective to treat with both drugs at once.

A separate method to increase AML cell sensitivity to ABT-199 treatment is to selectively increase BCL-2 dependence in AML cells. In CLL, effective combinations of the Bruton's tyrosine kinase inhibitor, ibrutinib, and ABT-199 have been observed in clinical trials (*285*). Preliminary BH3 profiling results from our lab show that treatment of primary CLL cells with ibrutinib selectively increases dependence on BCL-2, and does not alter BCL-XL and MCL-1 dependence. Although ibrutinib has not been tested in clinical trials for AML yet, and AML cells lack the B-cell receptor which ibrutinib is thought to inhibit, pre-clinical studies show that ibrutinib is effective in killing a subset of AML primary patient cells and AML cell lines (*286*). Thus, ibrutinib treated AML cells should be BH3 profiled to determine if the cells have increased BCL-2 dependence post-ibrutinib treatment. Through a collaboration with Dr. Fumi Ishikawa at the Riken Institute, we have also identified that the hematopoietic cell kinase inhibitor RK20449 (*287*) selectively increases AML cell dependence on BCL-2. Following a short 16h treatment of AML cell lines with RK20449 we observe an increase BCL-2 dependence as measured by BH3 profiling. Importantly, cell lines treated with the drug did not show an increase in dependence on BCL-XL or MCL-1. Thus, a combination of ABT-199 with RK20449 is also a potential effective combination therapy in AML cells. A third possible method to increase sensitivity to ABT-199 is to inhibit BCL-XL and MCL-1 as these were the two most potent resistant factors identified in the clinical trial analysis by BH3 profiling. Recently, selective BCL-XL and MCL-1 inhibitors have been identified, and are described in the next section.

Emergence of ABT-199 resistance

As with any targeted therapy, the emergence of resistance to ABT-199 is a concern. In Chapter 3 we report that, in the limited patient set tested, there is an increase in MCL-1 and BCL-XL dependence while the patients are on ABT-199 treatment (**Figure 3.5D-E**). Furthermore, in the one patient in which we had a post-treatment sample, the patient had a sustained increase in BCL-XL and MCL-1 dependence even though the patient was not on ABT-199 treatment (this measurement was taken seven days after the final ABT-199 dose; **Figure 3.5F**). Therefore, this suggests that there a selection for BCL-XL and MCL-1 dependence in this patient's blast population.

There are several mechanisms in which resistance to BCL-2 inhibition may occur. One potential mechanism is the acquisition of mutations in the target protein, BCL-2, or BCL-2 family members

rendering the blast cells resistant to ABT-199. This mechanism of ABT-199 resistance has been observed *in vitro* in mantle cell lymphoma (MCL) cell lines which acquired mutations in the BH3 domain of BCL-2 in a MCL cell line chronically exposed to ABT-199 (*288*). In a separate MCL cell line model of ABT-199 resistance, a mutation in the C-terminal transmembrane domain of BAX was observed which prohibited BAX from oligomerizing at the mitochondrial outer membrane to promote apoptosis. The acquisition of these mutations took seven months of chronic exposure of the MCL cell lines to ABT-199. Thus, it is unclear whether mutational acquisition occurred in the relatively short time frame that patient represented in Figure 3.5 was exposed to the drug.

A second mechanism that could promote resistance is anti-apoptotic heterogeneity within pre-treatment AML subclones and selection for ABT-199 resistant clones. It is possible that there is a sub-fractional kill of AML cells following single-agent ABT-199 treatment *in vivo* due to subclone heterogeneity on different anti-apoptotics. This may allow for the expansion of BCL-XL or MCL-1 subclones within the AML blast population to take over the bone marrow. If there are subclones with in the population that are differentially dependent on BCL-XL or BCL-2, then we may expect to observe a bi-modal cytochrome c release following ABT-199, MS-1/NOXA, or HRK peptide incubation in the pre-treatment samples. This would indicate two populations of AML blasts differently dependent on BCL-2, MCL-1 or BCL-XL. However, this was not observed in any of the 12 patient samples tested in the clinical trial, or in the 30+ AML primary patient samples tested in the pre-clinical setting. It is possible that the subclone population contributing to resistance is such a small percentage of the total blast population that we are unable to detect it with our current BH3 profiling methods.

Adaptive resistance is a third possible mechanism of ABT-199 acquired resistance. Adaptive resistance is caused by adaptive changes to the signaling pathways following inhibition of target pathways by selective inhibitors. For example, selective inhibition of mutant BRAF by BRAF inhibitors results in short term tumor regression followed by relapse due to reactivation of the ERK signaling pathway by degradation the negative feedback loop and/or activation of up-regulation of other pro-growth signaling pathways such as the AKT/PI3K pathways (*289, 290*). Considering this evidence, it is possible that the increased dependence on BCL-XL and MCL-1 of AML blasts following four weeks of ABT-199 treatment is not due to selection of a particular resistant clone, but rather AML cells adapting bypass

signaling pathways which promote resistance. In lymphoma cell lines, it was found that treatment with the BCL-XL, BCL-2 and BCL-w inhibitor ABT-737 dynamically increased expression of MCL-1 mRNA within hours of ABT-737 exposure (*254*). However it remains unclear what signaling pathways are mediating a rapid induction of MCL-1 mRNA following ABT-737 treatment.

Potential strategies to overcome ABT-199 resistance

If BCL-XL or MCL-1 dependent subclones cells are being selected for during continuous ABT-199 treatment, or prolonged exposure to ABT-199 promotes dynamic up-regulation of MCL-1 it may be advantageous to adjust the dosing schedule of single agent ABT-199. In this trial of ABT-199 in AML, patients were treated daily with escalating doses of ABT-199. This dosing schedule was required in order to minimize the risk of tumor lysis syndrome, which was the cause of two mortalities in a clinical trial of ABT-199 in CLL patients. However, continuous dose escalation treatment with ABT-199 is a method to induce resistance in cell lines (*288*). Therefore, this dosing schedule may contribute to resistance. It is worth testing if pulse dosing of ABT-199 once every week, or every other day may be a superior dosing schedule with ABT-199. If an AML blast population consists of mostly BCL-2 dependent cells, this dosing regimen may kill the majority of cells, and allow the BCL-2 dependent cells to recover. Once the cells have recovered, dosing again may kill the majority of cells again. Testing this dosing strategy *in vitro* and in *in vivo* murine models may assess whether it is more effective than dose escalation strategies.

A strategy to potentially combat ABT-199 resistance by the emergence of BCL-XL resistant subclones is to treat the resistant cells with a BCL-XL inhibitor. Recently, potent and selective BCL-XL inhibitors have been developed which have increased affinity for BCL-XL compared to the BCL-XL, BCL-2, BCL-w inhibitor ABT-263 (*291*). The concern for specific BCL-XL inhibitors is the potential for dose limiting thrombocytopenia caused by the BCL-XL dependence of platelets. However, preliminary safety and efficacy profiles of the new BCL-XL inhibitors show that an efficacious dose of the BCL-XL inhibitors can kill non-small cell lung cancer cells without causing thrombocytopenia in rodents (*291*). As some AML patients in our studies presented with BCL-XL dependent blasts, it is important to test these new inhibitors in AML cell lines and patient primary cells.

Furthermore, specific MCL-1 inhibitors are also necessary to combat ABT-199 resistance. Recently, two MCL-1 inhibitors have been identified that kill non-small cell lung cancers and multiple myeloma cell lines. However, Bim:MCL-1 complex disruption was not observed unless treated with micromolar concentrations of the MCL-1 inhibitors and EC50s of cell lines treated with the drug were in the 5-10uM range (*256*). These concentrations are too high to be effective in patients. Thus, more potent MCL-1 inhibitors will need to be identified before they can be transformed into clinically useful drugs.

Identifying other malignancies that will benefit from selective BCL-2 inhibition

Prior to these studies in AML, treatment of ABT-199 in human malignancy was restricted to lymphoid malignancies (chronic lymphocytic leukemia and non-Hodgkin's lymphoma) and multiple myeloma. As BCL-2 stands for B-Cell Lymphoma protein 2, and was cloned from a translocation in a lymphoid leukemia, it is perhaps not surprising that there was a bias in thinking that BCL-2 targeting would be largely restricted to lymphoid leukemias. In chapter 2, we presented compelling evidence that AML cells can be dependent on BCL-2, and that ABT-199 is effective in killing AML cells *in vitro*, which directly led to the Phase II clinical trial of ABT-199 in AML patients.

The relative success of ABT-199 in AML patients suggests that the drug may be useful in other hematological malignancies. Pre-clinical analysis has found that certain subtypes of T-ALL are BCL-2 dependent and would benefit from ABT-199 treatment (*292*). Furthermore, a recent molecular analysis of blastic plasmacytoid dendritic cell neoplasm (BPDCN) found that some BPDCN exhibit increased expression of BCL-2 compared to plasmacytoid dendritic cells (*293*). In collaboration with Dr. Andrew Lane at Dana Farber Cancer Institute, we have tested the sensitivity of several BPDCN xenografts to ABT-199 and also BH3 profiled the cells. The BPDCN were sensitive to ABT-199 in the low nanomolar range, and the BH3 profiling analysis of the samples showed dependence on BCL-2 and virtually no dependence on BCL-XL or MCL-1. As the overall survival for patients diagnosed with BPDCN is approximately 9 months, there is a definite need for improved therapeutic options for these patients, and ABT-199 may be an effective option (*294*).

Thus far, ABT-199 analysis has mostly been restricted to hematological malignancies and its utility in solid cancers has yet to be fully explored. A survey of a large set of solid and hematological cell

lines found that most solid cancer cell lines exhibit higher levels of MCL-1 and intermediate levels of BCL-XL mRNA expression, whereas hematological cancer cell lines exhibited higher levels of BCL-2 mRNA expression (*295*). Nevertheless, in a mouse model of estrogen-receptor positive breast tumors, ABT-199 was effective in improving tumor response in combination with tamoxifen (*240*). Therefore, it is possible that BCL-2 inhibition by ABT-199 may be more effective in solid tumors than previously thought. A thorough functional investigation of anti-apoptotic dependencies in solid tumor cell lines and xenografts will need to be undertaken to identify additional solid tumors that will most benefit from ABT-199 treatment either as a monotherapy or in combination with current chemotherapeutics.

Identifying what dictates anti-apoptotic dependencies in AML

In both the pre-clinical and clinical analysis of anti-apoptotic dependencies in AML, we found that there were heterogeneous dependencies on the anti-apoptotics MCL-1, BCL-2 and BCL-XL. As AML is a diverse disease in terms of pathology, molecular lesions and cytogenetics, it is not surprising that there are various dependencies on the anti-apoptotic proteins. However, it is yet to be understood why particular AML blasts are dependent on certain anti-apoptotics. Identification of the molecular drivers of anti-apoptotic dependencies may potentially identify combination therapies for BH3 mimetics. If we can identify what mutational or epigenetic mechanisms promote BCL-2 dependence, then we may be able to target these pathways to make have more effective combination therapies.

One hypothesis is that certain genetic lesions promote dependencies on distinct anti-apoptotic proteins. As discussed previously, IDH mutations promote BCL-2 dependence (*250*). It is hypothesized that the oncometabolite produced by IDH mutations, 2-(R)- HG, inhibits cytochrome c oxidase which in turn promotes a low oxygen state resulting in an increase in BAX and BAK expression. BAX and BAK are then inhibited by an adaptive increase expression of BCL-2 within the AML blasts (*250*). Although this mechanism of BCL-2 dependence is characterized, there are AML patient samples that are dependent on BCL-2 which do not harbor IDH1 or IDH2 mutations. Thus, other non IDH1 or IDH2 mechanisms promote BCL-2 dependence in AML.

Another hypothesis for the origination of anti-apoptotic dependencies is that the AML blasts are simply dependent on the same anti-apoptotic as the cell in which the cancer originated. In an elegant

study of T-cell maturation and T-ALLs, it was found that the early T-cell progenitor (ETP) subgroup of T-ALLs were dependent on BCL-2, in contrast to more differentiated T-ALLs which were dependent on BCL-XL (*292*). The ETP subgroup of T-ALLs is derived from a more immature T-cell which is also dependent on BCL-2 for survival, whereas, the more mature T-ALLs derived from more mature T-cells switch to a BCL-XL dependency (*296*). Thus in the setting of ETP and more mature T-ALL, cell of origin does seem to dictate anti-apoptotic dependency.

It is possible that cell of origin may dictate AML anti-apoptotic dependency. The French American British (FAB) classification schema categorizes AML cases into different subclasses based off of morphological, maturation and cytogenetic criteria (*297*). The FAB categorizes AML into eight groups, M0-M7, in which M0 and M1 subgroups represent undifferentiated AMLs and M2-M7 represent more mature subtypes. M5 comprises monocytic or monoblastic leukemias. In light of our BH3 profiling results with normal monocytes, which we found to be more dependent on MCL-1 and BCL-XL compared to BCL-2, we hypothesized that M5 AML cases may have increased dependence on MCL-1 or BCL-XL. Of the five M5 classified AMLs tested in our analysis, we found that three out of the five samples exhibited increased dependence on BCL-XL compared to BCL-2. In the remaining two cases, the blasts were similarly dependent on BCL-XL and BCL-2. Thus, it is possible that the differentiation stage also dictates anti-apoptotic dependency in AML. However, this analysis was performed with small sample numbers and a more thorough analysis of normal human cells at different stages of differentiation, as well as more samples which represent each FAB subclass will need to be undertaken for definitive analysis.

Importance of testing hypotheses in the clinical setting

The clinical trial BH3 profiling results exhibited important differences from our pre-clinical tests which highlight the importance of testing hypotheses in the laboratory and in the clinic. In our pre-clinical analysis, we found that BCL-2 dependence, measured by the BAD BH3 peptide, correlated best with the IC50 cellular toxicity of AML patient samples cultured *in vitro* with ABT-199 (**Figure 2.5D**). However, in the clinical trial, we found that MCL-1 and BCL-XL dependence were better predictors of poor clinical response compared to BCL-2 dependence alone (**Figure 3.4**).

One potential explanation for the discrepancy is the time points and assessment of ABT-199 response assayed in the clinical trial vs. the pre-clinical setting. For the *in vitro* analysis, cellular cytotoxicity was measured following 8 h of ABT-199 treatment by Annexin/PI staining, whereas in the clinical setting, response was measured after four weeks of treatment by reduction in blast count (or when the patient was removed from the study). Thus, in the clinical setting, there was ample time for BCL-XL or MCL-1 resistant sub-clones to expand, or for adaptive resistant pathways to emerge. In the larger set of samples tested *in vitro* in Chapter 2, some AML samples were dependent on both BCL-2 and BCL-XL. Following 8 h of ABT-199 treatment, the vast majority of the BCL-2/BCL-XL dependent cells were killed; however, there were a small fraction (0.1-5%) of cells that were Annexin-/PI- after exposure to the highest concentration of ABT-199 (1μM). It is possible that this small fraction of surviving cells, if given time and the appropriate growth conditions, may expand. Therefore, it would be interesting to sort the resistant cells following ABT-199 treatment and perform BH3 profiling on this fraction. It is possible that the resistant cells are more dependent on BCL-XL or MCL-1 and may contribute resistance in the clinic.

Stem cells, aging and cancer stem cells: open questions and future directions

Enhanced proliferation at the cost of increased apoptosis in the hematopoietic system

In our studies of differentiation of HSCs into lineage committed progenitor cells, we found a correlation between entry into the cell cycle and increased mitochondrial priming in the HSC compartment. We found that quiescent HSCs are unprimed, whereas more differentiated cells that have the potential for increased proliferation, are more primed. Furthermore, we found that culturing HSCs to stimulate exit from quiescence also increases mitochondrial priming. This trade-off between enhanced proliferation at the cost of increased likelihood of undergoing apoptosis makes evolutionary sense as unregulated proliferation without the brakes of apoptosis is essentially the definition of cancer. Yet, how these two opposing cell fates – proliferation and apoptosis are controlled in concert is not fully understood. With our observation of enhanced priming with differentiation, the hematopoietic system may be a great, non-malignant model system to study this phenomenon.

Within HSCs, one pathway that may connect proliferation and apoptosis is the DNA damage response pathways. The DNA damage repair (DDR) mechanisms are down regulated in quiescent HSC, whereas these pathways are upregulated in cycling progenitor cells (*215*). Further, upon cell cycle induction by forced proliferation, the DDR mechanisms and response pathways are upregulated in HSCs relatively quickly (between 2 and 24 h) (*215*). Therefore, it is possible that the sentinels which detect DNA damage may not signal to the apoptotic machinery in non-cycling HSC cells which may decrease the overall apoptotic priming of quiescent HSCs. For example, p53 is down-regulated in young HSCs, but has increased expression in cycling lineage committed progenitor cells. p53 is an important transcriptional activator of many genes including the pro-apoptotic pan sensitizer BH3-only protein PUMA as well as NOXA (*18, 298*). As p53 is not expressed in young HSCs, there is no induction of the pro-apoptotic protein PUMA. Therefore, the DNA repair pathway may connect proliferation and apoptosis upon cell cycle entry in HSCs.

The mechanism(s) govern the decreased priming phenotype of aged HSCs

We have yet to address the mechanism(s) which control the phenotype of decreased mitochondrial priming and decreased sensitivity to DNA damage of aged HSCs. As aging in general is a gradual process, the decreased priming observed in aged HSCs is most likely not caused by a single gene or environmental cue, but rather a complex network of cell autonomous alterations as well as modifications to the microenvironmental niche.

One possible explanation for the clonal expansion of low primed HSCs is an adaptive response to increases in reactive oxygen species levels within the HSC compartment with age. Murine HSCs exhibit a decrease in the relative frequency of ROS^{low} cells with age and treatment with antioxidants can enhance engraftment potential of human HSCs (*299*). The accumulation of reactive oxygen species in HSCs with age may select for the most apoptotic resistant HSCs to sustain a pool of stem cells through organismal life. One way to test the hypothesis that poorly primed HSCs are selected for by exposure to ROS, is to treat mice with anti-oxidants and test if this has an effect on the mitochondrial priming of the HSCs. If treatment with anti-oxidants rescues the poorly primed phenotype of aged HSCs this could potentially be a mechanism to rejuvenate low primed, aged HSCs. However, excitement should be tempered because

large scale meta-analysis of antioxidant supplements in the human diet showed that supplementing the diet with anti-oxidants may actually increase mortality rate (*300*).

Beyond the adaptive response to intracellular ROS levels, there are various epigenetic alterations associated with HSC aging that may contribute to the decreased priming phenotype. Recently, it was discovered that blood cells in seemingly normal individuals exhibit clonal hematopoiesis. Approximately 10-20% of humans over the age of 60 exhibit clonal expansion of blood cells (*216, 217, 284*). The most common mutations in the expanded clones are in the epigenetic modulators DNMT3A, TET2 and ASXL1 (*216, 217*). The clonal expansion of these mutated clones suggests that the altered epigenetic landscape afforded by the mutations produces either a growth advantage or promotes an apoptotic resistant phenotype in the blood cells. Loss of DNMT3A in mouse HSCs results in the accumulation of HSCs and loss of differentiation potential following serial competitive transplantation (*224*). TET2 also regulates DNA methylation and deletion of TET2 in hematopoietic progenitor cells results in enhanced repopulation efficiencies (*225*). Furthermore, expression analysis of young and old HSCs has revealed that DNMT3A and TET2 have decreased expression old HSCs compared to young HSCs (*183, 185*). Together, these observations highlight the potential that the methylation landscape of DNA in old HSCs may contribute to the phenotype of aged HSCs. In order to assess the functional contribution of the epigenetic landscape on the priming phenotype, analysis of mitochondrial priming in HSCs derived from mice with various deficiencies in epigenetic machinery will need to be performed.

In addition to cell autonomous mechanisms of DNA damage resistant aged HSCs, cell extrinsic mechanisms may also contribute to the selection of low-primed, DNA damaged aged HSCs. It is known that the old bone marrow niche provides some selection pressure on retro-viral infected hematopoietic progenitor cells (HPCs). When HPCs are exposed to retro-viral mutagenesis and are transplanted into young recipients, the engrafted cells were composed of several distinct clones. However, when the same mutagenized cells were transplanted into old recipients, the resulting chimerisms were mono-clonal (*229*). What the selection pressure is in the old bone marrow that promotes mono-clonality of mutated HPCs is yet to be discovered. Several cytokines have been implicated in the myeloid skewing of HSCs including TGFβ1 and Rantes (*231, 301*). How these cytokines interact with the bone marrow niche and possibly promote survival of low primed HSCs will need to be addressed in future experiments. Heterochronic

transplantation assays in which young cells are injected into old mice or old HSCs are injected into young mice could be performed to assess if aged environmental factors contribute to the priming phenotype.

Is decreased mitochondrial priming a phenotype of all aged stem cells?

Hematopoietic stem cells are the most well characterized tissue stem cell due to the relative ease of isolating the cells and well characterized cell surface markers. Therefore, in this project we focused our studies of aging and differentiation on the hematopoietic stem cells. However, it is worth noting that other organ systems exhibit age related dysfunction and the functional decline of tissue specific stem cells may contribute to this aging phenotype. For instance, aging of neuronal stem cells has been in implicated in decreased cognitive function and aging of satellite muscle cells been implicated in the progressive decline in muscle strength with age.

An interesting observation of tissue specific stem cells with aging is that some stem cells share the same characteristics of aging as HSCs, including increase in stem cell number, decline in self-renewal activity, accumulation of DNA damage, whereas other stem cells do not share these phenotypes. For instance, neuronal stem cells and satellite muscle stem cells decrease in cell number with age (*302, 303*), whereas, intestinal stem cells do not seem to change in cell number with age (*304*). Furthermore, we have observed that old HSCs are resistant to DNA damage. However, the decrease in the number of muscle satellite cells during aging has been attributed to an increase in apoptosis susceptibility of the cells with age (*305, 306*). Additionally, there is increased apoptosis of intestinal cells near the base of the intestinal crypts (where intestinal stem cells are located) in old mice treated with irradiation compared with young mice treated with irradiation (*307*).

Together, these data suggest that it is likely that tissue specific stem cells respond to apoptotic stimuli differently with age. Some stem cells are more susceptible to apoptosis with age and may contribute to the decline in stem cells with age (satellite cells), whereas other stem cells are more resistant to apoptotic stimuli which may contribute to the increase in stem cell number and accumulation of DNA damage with age (HSCs) Therefore, comparative studies between stem cells of different tissues may provide insight into how stem cells regulate apoptosis differently.

Increased therapeutic window in AML treatment with age?

Previous results from our lab indicate that HSCs create the therapeutic window for conventional chemotherapy in AML (*56*). In Vo et al., it was observed that patients whose AML blasts exhibited higher priming than the level of priming of HSCs had higher complete response rates compared to patients who presented with AML blasts that were primed lower than HSCs. Therefore, the level of priming of HSCs defines the therapeutic window for induction therapy success in AML. With this data in mind, together with data presented in chapter 4 showing that old HSCs are less primed than young HSCs, this suggests that older people with AML should have a larger therapeutic window because of their low primed HSCs and have a higher response rate to induction chemotherapy compared to younger patients; however, it is well established that old age is a poor prognostic factor in AML (*79*).

There are several possible explanations for this observation. One potential explanation for this discrepancy is that the older patients harbor AML blasts that are less primed than younger patients, abrogating any potential increased therapeutic window that may have existed. An additional explanation for this discrepancy is that the old HSCs remaining following myeloablative induction chemotherapy are less efficient in re-populating the bone marrow of older individuals. Previously, we described that old HSCs have reduced self-renewal and transplantation efficiencies and this may contribute to morbidity and mortality following chemo-ablative therapy. For instance, it is known that older patients experience prolonged periods of myelosuppression following chemotherapy which renders the patients more susceptible to opportunistic infections (*308*). Therefore, a combination of defective HSCs and lower primed AML blasts may contribute to the increased mortality observed in older AML patients.

Implications for treating cancer stem cells

Our studies of the low primed HSCs have implications in the study of cancer stem cells. It has been observed in many cancers that undifferentiated, immature cancers are typically more resistant to chemotherapy and are associated with poor survival compared to cancers that are more differentiated (*309*). For example, AML blast populations that are comprised of a high proportion of CD34-CD38+ stem-like cells experience worse progression free survival compared to blast populations that are composed of more mature cells (CD34+CD38+) (*309*). If we extend our HSC priming data, it is possible that the more

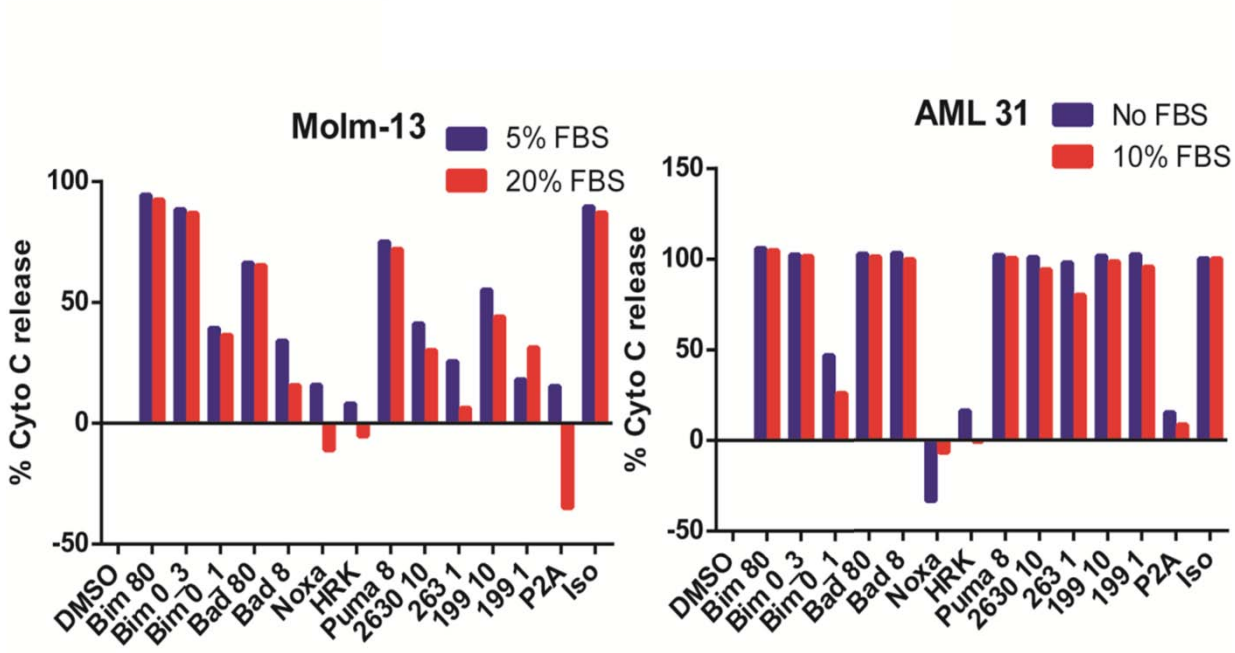
stem-like cancers are comprised of low primed, apoptotic resistant cells, and thus are refractory to chemotherapy, whereas the more mature cells are comprised of higher primed cells. Preliminary data from our lab suggests that AML populations that are comprised of CD34+CD38- cells are less primed than CD34+CD38+ or CD34- CD38+ AML samples; however, a more thorough analysis with a larger set of patient samples needs to be performed for a conclusive answer.

Furthermore, we also observed that the quiescent nature of HSCs may contribute to the low primed phenotype as inducing the cells to enter the cell cycle increased mitochondrial priming. AML LSCs have also been observed to be more quiescent than circulating AML blasts (*310-312*). Since we found a correlation between cell cycle entry and increased priming, a potential method to increase chemosensitivity of LSCs is to induce the blast cells to enter the cell cycle. LSC entry into the cell cycle may increase the overall mitochondrial priming of the cell, and treatment of the cells with conventional chemotherapy may be more effective in treating the cells. Forced entry of LSCs into the cell cycle is not a novel concept. Treating LSCs with granulocyte-colony stimulating factor (G-SCF) forces cells into the cell cycle making the cells more sensitive to AraC treatment (*313*). Furthermore, clinical trials of G-CSF to prime AML cells followed by AraC treatment observed superior survival outcomes for patients treated with an escalated dose of AraC (*314*). The clinical trials did not report on cell cycle status of LSCs or bulk tumor cells following G-CSF treatment, so it would be interesting to assess if treatment with G-CSF increases priming of the AML cells. Further, in most trials of G-CSF to stimulate priming of AML cells, G-CSF was administered during the induction phase; however, it may be more advantageous to use G-CSF as a post remission therapy to coax quiescent LSCs from dormancy to diminish relapse rates of AML.

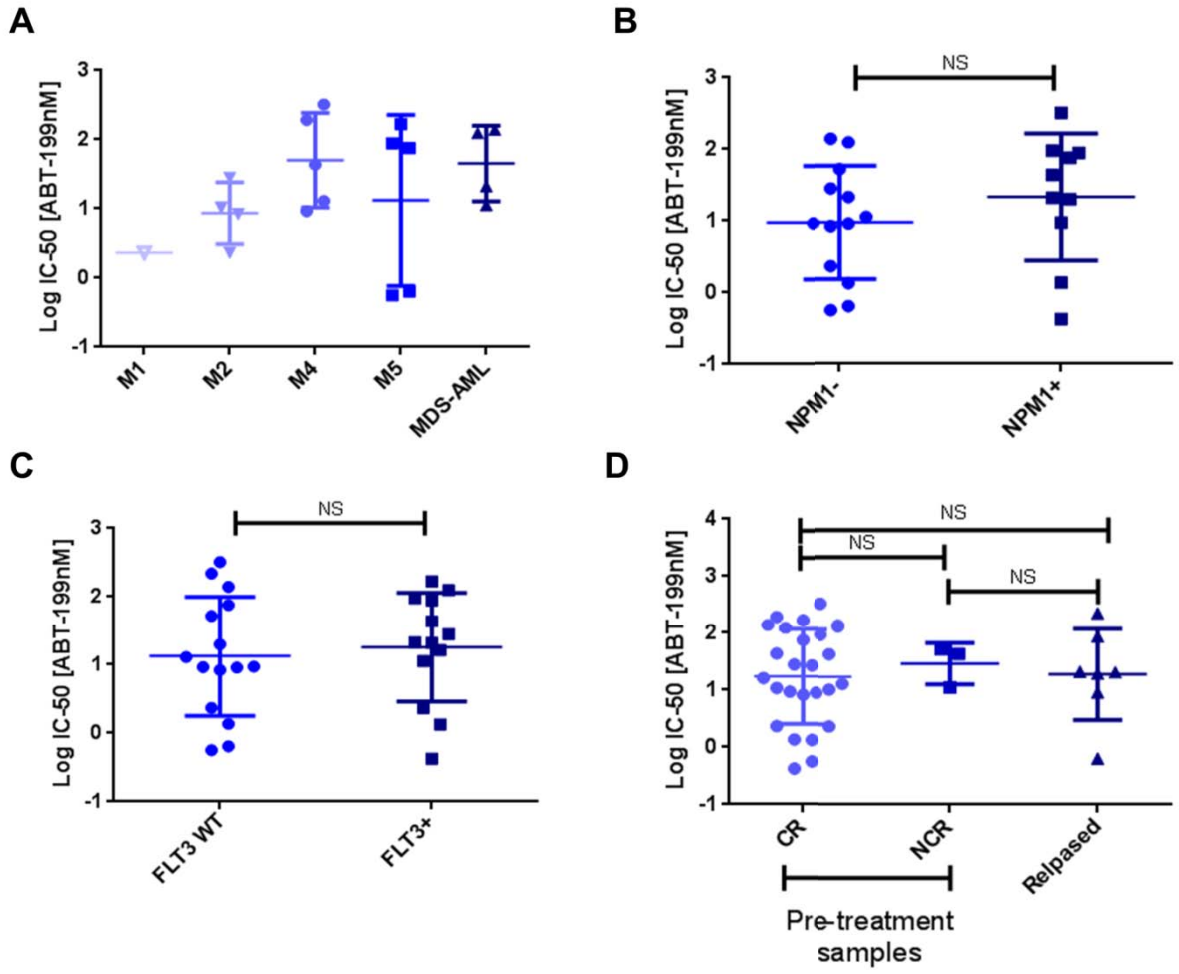
Final remarks

This thesis involved two projects connected by both the mitochondrial apoptotic pathway and organismal aging. In chapter 2 and 3 we showed that BH3 profiling is a successful strategy to guide treatment of the BCL-2 inhibitor ABT-199 in AML. With the development of novel BH3 mimetics in targeting additional anti-apoptotic proteins, identifying patients who will be most successfully treated by such strategies will be important. Secondly, by using BH3 profiling, we were able to detect increases in mitochondrial priming during normal cell differentiation and decreases during organismal aging in

hematopoietic stem cells. This is the first time that the mitochondrial apoptotic pathway has been explored in depth within HSC subpopulations. The results of this study suggests that the accumulation of DNA damaged, low primed HSCs with age may contribute to the increased stem cell numbers, with diminished re-population efficacies with age and we speculate that the accumulation of DNA damaged, apoptotic resistant HSCs with age may contribute to the increase in hematological malignancies observed in human aging.



Supplemental Figure 2.1. Incubation of AML cell lines and AML primary cells with FBS does not influence apoptotic priming.
The AML cell line MOLM-13 and AML primary cells (AML 31) were incubated in the presence or absence of FBS for 8 h. Intracellular BH3 profiling was performed and the % Cytochrome c was measured.



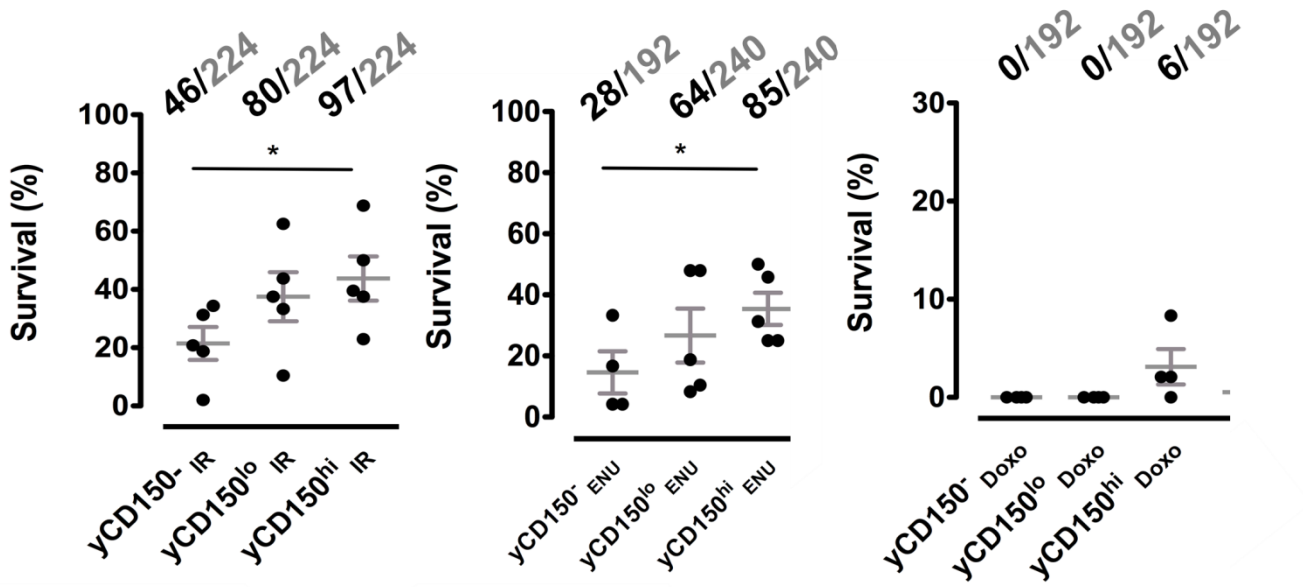
Supplemental Figure 2.2. Clinical characteristics of AML patients do not correlate with ABT-199 sensitivity.
A. Patients from Figure 4B were classified by their FAB status (French-American-British classification). There was no significant difference between the IC50 values between various cohorts (note, M1 was not included in the analysis because there were not enough samples to perform the analysis). B. Patient samples used in Figure 4B were stratified by their nucleophosmin 1 status (an NPM1 mutation is a good prognostic factor). There was no difference in IC50 values between patients who had an NPM1 mutation and those who were WT. C. Patient samples used in Figure 4B were stratified by their FLT3 status (a FLT mutation is a poor prognostic factor). There was no difference in IC50 values between patients who had an FLT3 mutation and those who were WT. D. Patient samples used in Figure 4B were stratified into newly diagnosed patients who achieved a complete response (CR), newly diagnosed patients who did not achieve a CR and relapsed patients. There is no difference in ABT-199 IC50 values between the three groups. A Mann-Whitney t test was used for these comparisons.

Supplemental table 2.1. Clinical characteristics of AML patients

UPIN	DX	Source	Blast (%)	Status	Cyto-genetics	Molecular Mutations					ABT-199 IC50 (nM)	ABT-737 IC50 (nM)	ABT-263 IC50 (nM)
						NPM1	*FLT3*	*NRAS*	*JAK*	Others			
3841772	AML	PB	57	Relapsed Refractory, post-2 SCT	Complex	-	-	-	-	-	356.2	63.3	- -
4031144	AML	PB	98	Primary Refractory	-3, +8	-	-	+ (G12D)	-	-	0.8	2.5	-
3891338	AML	PB	71	Primary Refractory	inv(3)	-	-	+ (G13R)	-	-	0.6	1.2	-
4081468	AML	PB	86	Pre-treatment	Complex incl -5, -7	U	-	U	U	U	7.5	9.2	-
4081654	AML	BM	69	Relapsed Refractory	del(3q)	+	-	-	-	*K-RAS, GATA2, RUNX1*	0.5	1.8	-
		PB	99								1.9	7.4	-
4030094	AML	PB	98	Primary Refractory	t(9;11) and other	-	-	-	-	*CEBPA*	84.8	179.3	-
2422940	AML	BM	80	Refractory	inv(3)	-	-	-	-	-	0.6	1.7	-
4031936	AML	PB	99	Relapsed Refractory	inv(3)	-	+	-	-	*IDH1*	0.5	3.1	-
3913992	AML	PB	82	Primary Refractory	-	-	-	-	-	-	6530.3	238.5	-
3831282	AML	PB	78	Primary Refractory	-	-	-	-	-	-	0.9	3.5	-
3788196	AML	PB	85	Primary Refractory	-	-	-	-	-	-	1.1	4.2	-
3873322	AML	PB	98	Primary Refractory Post-SCT	-	-	+	-	-	-	1.2	2.1	-
3686032	AML	PB	52	New AML from CMML	-	-	-	+	-	-	1.6	4.6	-
3846024	AML	BM	99	Relapsed Refractory	-	-	-	-	-	-	0.9	5.8	-
4015568	AML	PB	87	New therapy-related AML	t(9;11)	-	-	+	-	-	1.4	14.8	-
3866160	AML	BM	77	Relapsed post-SCT	Diploid	+	-	-	-	*DNMT3*	1209.5	308.4	-
3798414	AML	PB	99	AML from MDS, refractory	del(12p)	-	-	+	-	-	1.7	3.4	-
3871344	AML	PB	69	Refractory AMML	Diploid	-	-	-	-	-	0.6	2.4	-
3898404	AML	PB	55	AML from MDS	del(5q), -7	-	-	+	-	-	4.3	16.2	-
3899418	AML	PB	81	Relapsed	Diploid	+	-	-	-	*DNMT3*	2.7	6	-
3899936	AML	PB	74	AML from MDS	N/A	-	-	-	-	-	> 5000	> 5000	-
3821772	AML	PB	93	Refractory, Relapsed post-SCT	Complex	-	-	-	-	*CEBPA*	3.9	21.8	-
5	AML	PB	5	Relapsed	trisomy 11	-	-	U	U	-	9.3	-	5.4
9	AML	PB	10	Relapsed AML from MDS	normal	-	+	U	U	-	21.2	-	4.1
10	AML	BM	83	Pre-treatment	normal	+	+	U	U	-	43.5	-	4.2
13	AML	BM	46	Relapsed Refractory	normal	+	+	U	U	-	20.7	-	28.1
15	AML	BM	16	AML from MDS	trisomy 8	-	-	U	U	-	139.3	-	347.5
16	AML	PB	35	relapsed AML	normal	+	+	U	U	-	19.9	-	5
19	AML	PB	75	Relapsed Refractory	Monosomy 7	U	-	U	U	-	88.7	-	11.7
30	AML	BM	35	Pre-treatment	MLL gene rearrangement	-	-	U	U	-	219.3	-	148.6
31	AML	BM	80	Pre-treatment	Comlplex	-	-	U	U	-	0.6	-	0.7
33	AML	BM	63	Pre-treatment	normal	+	-	U	U	-	52.1	-	212.3
34	AML	BM	80	Pre-treatment	normal	+	+	U	U	-	9.4	-	8.5
35	AML	PB	48	Relapsed, Refractory	normal	+	-	U	U	-	0.4	-	0.4
1002	AML	BM	51	Pre-treatment	t(16;16)	U	U	U	U	-	13	-	20.8
1003	AML	BM	44	Pre-treatment	normal	-	-	U	U	-	2.3	-	2.7
1005	AML	BM	69	Pre-treatment	inversion 16	-	+	U	U	-	1.3	-	2.8
1001	AML	BM	88	Pre-treatment	normal	U	+	U	U	-	168.3	-	11.1
1004	AML	BM	78	Pre-treatment	46,XX,inv(16)(p13q22)[20]	U	U	U	U	-	192.8	-	555.9
37	AML	BM	79	AML from MDS	normal	-	+	U	U	-	11.2	-	6.7
40	AML	BM	73	Relapsed Refractory	complex	-	-	U	U	-	0.6	-	2.5
41	AML	BM	13	Pre-treatment	complex	-	+	U	U	-	124.2	-	123.6
44	AML	BM	69	Pre-treatment	normal	+	-	U	U	-	1.4	-	3.3
1006	AML	BM	U	Pre-treatment	unknown	U	U	U	U	-	132.4	-	108.9
1009	AML	BM	96	Pre-treatment	unknown	+	-	U	U	-	75.7	-	31.8
1010	AML	BM	93	Pre-treatment	normal	-	-	U	U	-	8.3	-	2.2
1011	AML	BM	94	Pre-treatment	normal	-	+	U	U	-	28.1	-	2.3
1013	AML	BM	88	Pre-treatment	normal	U	+	U	U	-	2.3	-	3.1
1015	AML	BM	65	Pre-treatment	46,XY[20].nuc ish(PML,RARA)x2[100]	U	+	U	U	-	16.3	-	24
1017	AML	BM	61	Pre-treatment	inv(16)(p13q22)[20]	U	U	U	U	-	10.3	-	8.3
571	AML	BM	94	Pre-treatment	No info	U	U	U	U	-	43.8	-	9.3
958	AML	BM	95	Pre-treatment	No info	U	U	U	U	-	27.1	-	3
1044	AML	BM	93	Pre-treatment	No info	U	U	U	U	-	43.1	-	10.4
2924	AML	BM	91	Pre-treatment	No info	U	U	U	U	-	10.8	-	1.1
1012	AML	BM	53	Pre-treatment	normal	+	-	U	U	-	321.4	-	2009.1
1019	AML	BM	45	Pre-treatment	normal	-	-	U	U	-	9.1	-	6.6
1014	AML	BM	65	Pre-treatment	normal	+	+	U	U	-	95.3	-	28.6

Supplemental Table 2.2. Sensitivity of genetically distinct subtypes of AML to ABT-199 and ABT-737. Cryopreserved primary AML samples were treated with 125 nM ABT-199 or ABT-737 for 24 h. The number of viable cells (Annexin V-/DAPI- were determined by FACS using counting beads. "Resistant" samples were defined as samples with less than 15% viability loss after drug treatment.

Molecular mutations /cytogenetics	N	737/199 sensitive	737/199 resistant	737 sensitive, 199 resistant
FLT3-ITD	9	7	2	0
NPM1	2	2	0	0
NRAS	4	4	0	0
Diploid	5	3	2	0
11q	2	1	1	0
Misc, *KIT*, CEBPA	3	3	0	0
JAK2	6	1	3	2
Complex	3	0	3	0
Total	34	21	11	2
% of total	100	62	32	6



Supplemental Figure 4.1 Primitive HSCs are less sensitive to DNA damage. Single cell sorted CD150 stained HSCs were exposed to 2Gy irradiation, 200 µg/ml ENU, 250 µg/ml doxorubicin, no treatment for 18h. Cells were washed and colony formation was counted 8 days post-plating. One dot represents one mouse. Numbers above each group represent the number of single cells that grew into colonies per single cell that were plated. Red dots indicate cells isolated from young mice, black dots indicate cells isolated from old mice. * = $p < 0.01 - 0.05$,

REFERENCES

1. C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, G. Kroemer, The Hallmarks of Aging. *Cell* **153**, 1194-1217 (2013).

2. N. N. Danial, S. J. Korsmeyer, Cell death: critical control points. *Cell* **116**, 205-219 (2004).

3. S. W. Tait, D. R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* **11**, 621-632 (2010).

4. A. M. Chinnaiyan, K. O'Rourke, M. Tewari, V. M. Dixit, FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81**, 505-512 (1995).

5. R. M. Locksley, N. Killeen, M. J. Lenardo, The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501 (2001).

6. M. E. Peter, P. H. Krammer, The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* **10**, 26-35 (2003).

7. T. Kuwana, D. D. Newmeyer, Bcl-2-family proteins and the role of mitochondria in apoptosis. *Curr Opin Cell Biol* **15**, 691-699 (2003).

8. Y. Tsujimoto, L. R. Finger, J. Yunis, P. C. Nowell, C. M. Croce, Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* **226**, 1097-1099 (1984).

9. L. Gibson *et al.*, bcl-w, a novel member of the bcl-2 family, promotes cell survival. *Oncogene* **13**, 665-675 (1996).

10. K. M. Kozopas, T. Yang, H. L. Buchan, P. Zhou, R. W. Craig, MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci U S A* **90**, 3516-3520 (1993).

11. L. H. Boise *et al.*, bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597-608 (1993).

12. A. Gross, J. Jockel, M. C. Wei, S. J. Korsmeyer, Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* **17**, 3878-3885 (1998).

13. T. Chittenden *et al.*, Induction of apoptosis by the Bcl-2 homologue Bak. *Nature* **374**, 733-736 (1995).

14. A. Letai *et al.*, Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183-192 (2002).

15. E. Yang *et al.*, Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**, 285-291 (1995).

16. J. M. Boyd *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* **11**, 1921-1928 (1995).

17. N. Inohara, L. Ding, S. Chen, G. Nunez, 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). *EMBO J* **16**, 1686-1694 (1997).

18. E. Oda *et al.*, Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**, 1053-1058 (2000).

19. H. Puthalakath *et al.*, Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* **293**, 1829-1832 (2001).

20. Y. T. Hsu, K. G. Wolter, R. J. Youle, Cytosol-to-membrane redistribution of Bax and Bcl-XL during apoptosis. *Proc Natl Acad Sci U S A* **94**, 3668-3672 (1997).

21. G. J. Griffiths *et al.*, Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *J Cell Biol* **144**, 903-914 (1999).

22. T. Kuwana *et al.*, BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* **17**, 525-535 (2005).

23. M. C. Wei *et al.*, tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* **14**, 2060-2071 (2000).

24. H. Li, H. Zhu, C. J. Xu, J. Yuan, Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491-501 (1998).

25. R. Eskes, S. Desagher, B. Antonsson, J. C. Martinou, Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* **20**, 929-935 (2000).

26. M. Suzuki, R. J. Youle, N. Tjandra, Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* **103**, 645-654 (2000).

27. B. Schafer *et al.*, Mitochondrial outer membrane proteins assist Bid in Bax-mediated lipidic pore formation. *Mol Biol Cell* **20**, 2276-2285 (2009).

28. X. Wang, The expanding role of mitochondria in apoptosis. *Genes Dev* **15**, 2922-2933 (2001).

29. M. Certo *et al.*, Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9**, 351-365 (2006).

30. S. B. Bratton, G. S. Salvesen, Regulation of the Apaf-1-caspase-9 apoptosome. *J Cell Sci* **123**, 3209-3214 (2010).

31. E. N. Shiozaki, J. Chai, Y. Shi, Oligomerization and activation of caspase-9, induced by Apaf-1 CARD. *Proc Natl Acad Sci U S A* **99**, 4197-4202 (2002).

32. S. B. Bratton *et al.*, Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *EMBO J* **20**, 998-1009 (2001).

33. S. M. Srinivasula *et al.*, Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J Biol Chem* **275**, 36152-36157 (2000).

34. L. M. Martins *et al.*, Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol* **24**, 9848-9862 (2004).

35. S. N. Willis *et al.*, Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19**, 1294-1305 (2005).

36. C. L. Day *et al.*, Structure of the BH3 domains from the p53-inducible BH3-only proteins Noxa and Puma in complex with Mcl-1. *J Mol Biol* **380**, 958-971 (2008).

37. D. Merino *et al.*, The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *J Cell Biol* **186**, 355-362 (2009).

38. L. Chen *et al.*, Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17**, 393-403 (2005).

39. J. Deng *et al.*, BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* **12**, 171-185 (2007).

40. A. Villunger *et al.*, p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* **302**, 1036-1038 (2003).

41. B. Wu *et al.*, p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. *Gut* **56**, 645-654 (2007).

42. H. Dai *et al.*, Contribution of Bcl-2 phosphorylation to Bak binding and drug resistance. *Cancer Res* **73**, 6998-7008 (2013).

43. A. Basu, S. Haldar, Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. *FEBS Lett* **538**, 41-47 (2003).

44. M. Upreti *et al.*, Identification of the Major Phosphorylation Site in Bcl-xL Induced by Microtubule Inhibitors and Analysis of Its Functional Significance. *J Biol Chem* **283**, 35517-35525 (2008).

45. J. Zha, H. Harada, E. Yang, J. Jockel, S. J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-628 (1996).

46. M. Enari *et al.*, A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43-50 (1998).

47. X. Liu, H. Zou, C. Slaughter, X. Wang, DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89**, 175-184 (1997).

48. H. Ye *et al.*, DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat Struct Biol* **9**, 680-684 (2002).

49. K. Segawa *et al.*, Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* **344**, 1164-1168 (2014).

50. J. Suzuki, D. P. Denning, E. Imanishi, H. R. Horvitz, S. Nagata, Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science* **341**, 403-406 (2013).

51. V. A. Fadok *et al.*, A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* **405**, 85-90 (2000).

52. T. Ni Chonghaile *et al.*, Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science* **334**, 1129-1133 (2011).

53. A. Letai *et al.*, Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183-192. (2002).

54. R. Pan *et al.*, Selective BCL-2 Inhibition by ABT-199 Causes On-Target Cell Death in Acute Myeloid Leukemia. *Cancer Discov* **4**, 362-375 (2014).

55. R. C. Scaduto, Jr., L. W. Grotyohann, Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys J* **76**, 469-477 (1999).

56. T. T. Vo *et al.*, Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell* **151**, 344-355 (2012).

57. J. A. Ryan, J. K. Brunelle, A. Letai, Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. *Proc Natl Acad Sci U S A* **107**, 12895-12900 (2010).

58. M. Hanada, D. Delia, A. Aiello, E. Stadtmauer, J. C. Reed, bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood* **82**, 1820-1828 (1993).

59. V. Del Gaizo Moore *et al.*, Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest* **117**, 112-121 (2007).

60. J.F Seymour et al. Updated results of a phase I first-in-human study of the BCL-2 inhibitor ABT-199 (GDC-0199) in patients with relapsed/refractory (R/R) chronic lymphocytic leukemia (CLL). (J Clin Oncol 31, 2013 (suppl; abstr 7018), 2013).

61. S. M. Schoenwaelder *et al.*, Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets. *Blood* **118**, 1663-1674 (2011).

62. R. L. Siegel, K. D. Miller, A. Jemal, Cancer statistics, 2015. *CA Cancer J Clin* **65**, 5-29 (2015).

63. J. M. Rowe, M. S. Tallman, How I treat acute myeloid leukemia. *Blood* **116**, 3147-3156 (2010).

64. Seer Fact Sheet. (2015).

65. D. Pulte, A. Gondos, H. Brenner, Expected long-term survival of patients diagnosed with acute myeloblastic leukemia during 2006-2010. *Ann Oncol* **21**, 335-341 (2010).

66. J. W. Vardiman *et al.*, The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **114**, 937-951 (2009).

67. H. Döhner *et al.*, Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474 (2010).

68. Z. Y. Wang, Z. Chen, Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood* **111**, 2505-2515 (2008).

69. Z. X. Shen *et al.*, Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* **89**, 3354-3360 (1997).

70. E. H. Estey, Acute myeloid leukemia: 2014 update on risk-stratification and management. *Am J Hematol* **89**, 1063-1081 (2014).

71. D. Grimwade *et al.*, The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* **92**, 2322-2333 (1998).

72. J. M. Rowe, Important milestones in acute leukemia in 2013. *Best Pract Res Clin Haematol* **26**, 241-244 (2013).

73. E. S. Wang, Treating acute myeloid leukemia in older adults. *Hematology Am Soc Hematol Educ Program* **2014**, 14-20 (2014).

74. B. D. Cheson *et al.*, Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol* **21**, 4642-4649 (2003).

75. Y. Pommier, E. Leo, H. Zhang, C. Marchand, DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol* **17**, 421-433 (2010).

76. W. Hiddemann, W. R. Martin, C. M. Sauerland, A. Heinecke, T. Buchner, Definition of refractoriness against conventional chemotherapy in acute myeloid leukemia: a proposal based on the results of retreatment by thioguanine, cytosine arabinoside, and daunorubicin (TAD 9) in 150 patients with relapse after standardized first line therapy. *Leukemia* **4**, 184-188 (1990).

77. P. A. Cassileth *et al.*, A randomized study of the efficacy of consolidation therapy in adult acute nonlymphocytic leukemia. *Blood* **63**, 843-847 (1984).

78. J. Koreth *et al.*, Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA* **301**, 2349-2361 (2009).

79. D. Grimwade, R. K. Hills, Independent prognostic factors for AML outcome. *Hematology Am Soc Hematol Educ Program*, 385-395 (2009).

80. G. Ossenkoppele, B. Lowenberg, How I treat the older patient with acute myeloid leukemia. *Blood* **125**, 767-774 (2015).

81. K. Dohner, H. Dohner, Molecular characterization of acute myeloid leukemia. *Haematologica* **93**, 976-982 (2008).

82. S. Takahashi, Current findings for recurring mutations in acute myeloid leukemia. *J Hematol Oncol* **4**, 36 (2011).

83. M. Nakao *et al.*, Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* **10**, 1911-1918 (1996).

84. Y. Yamamoto *et al.*, Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* **97**, 2434-2439 (2001).

85. H. Kiyoi, R. Ohno, R. Ueda, H. Saito, T. Naoe, Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain. *Oncogene* **21**, 2555-2563 (2002).

86. P. D. Kottaridis *et al.*, The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* **98**, 1752-1759 (2001).

87. B. Falini *et al.*, Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* **352**, 254-266 (2005).

88. J. L. Cordell *et al.*, Detection of normal and chimeric nucleophosmin in human cells. *Blood* **93**, 632-642 (1999).

89. R. A. Borer, C. F. Lehner, H. M. Eppenberger, E. A. Nigg, Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* **56**, 379-390 (1989).

90. E. Colombo, J. C. Marine, D. Danovi, B. Falini, P. G. Pelicci, Nucleophosmin regulates the stability and transcriptional activity of p53. *Nat Cell Biol* **4**, 529-533 (2002).

91. T. J. Ley *et al.*, DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* **363**, 2424-2433 (2010).

92. M. Okano, S. Xie, E. Li, Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* **19**, 219-220 (1998).

93. L. T.J., M. C., D. L., R. B.J., Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* **368**, 2059-2074 (2013).

94. G. Raddatz, Q. Gao, S. Bender, R. Jaenisch, F. Lyko, Dnmt3a protects active chromosome domains against cancer-associated hypomethylation. *PLoS Genet* **8**, e1003146 (2012).

95. L. I. Shlush *et al.*, Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* **506**, 328-333 (2014).

96. V. I. Gaidzik *et al.*, Clinical impact of DNMT3A mutations in younger adult patients with acute myeloid leukemia: results of the AML Study Group (AMLSG). *Blood* **121**, 4769-4777 (2013).

97. J. P. Patel *et al.*, Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *N Engl J Med* **366**, 1079-1089 (2012).

98. N. Raimundo, B. E. Baysal, G. S. Shadel, Revisiting the TCA cycle: signaling to tumor formation. *Trends Mol Med* **17**, 641-649 (2011).

99. M. E. Figueroa *et al.*, Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18**, 553-567 (2010).

100. W. Xu *et al.*, Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell* **19**, 17-30 (2011).

101. M. Sasaki *et al.*, IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. *Nature* **488**, 656-659 (2012).

102. W. C. Chou *et al.*, The prognostic impact and stability of Isocitrate dehydrogenase 2 mutation in adult patients with acute myeloid leukemia. *Leukemia* **25**, 246-253 (2011).

103. E. Wattel *et al.*, p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood* **84**, 3148-3157 (1994).

104. J. S. Welch *et al.*, The origin and evolution of mutations in Acute Myeloid Leukemia. *Cell* **150**, 264-278 (2012).

105. M. J. Walter *et al.*, Acquired copy number alterations in adult acute myeloid leukemia genomes. *Proc Natl Acad Sci U S A* **106**, 12950-12955 (2009).

106. C. C. Chen *et al.*, Complex karyotypes confer a poor survival in adult acute myeloid leukemia with unfavorable cytogenetic abnormalities. *Cancer Genet Cytogenet* **174**, 138-146 (2007).

107. F. G. Rucker *et al.*, TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Blood* **119**, 2114-2121 (2012).

108. C. Haferlach *et al.*, Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. *Leukemia* **22**, 1539-1541 (2008).

109. L. Ding *et al.*, Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* **481**, 506-510 (2012).

110. M. Jan *et al.*, Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci Transl Med* **4**, 149ra118 (2012).

111. J. M. Klco *et al.*, Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. *Cancer Cell* **25**, 379-392 (2014).

112. T. Reya, S. J. Morrison, M. F. Clarke, I. L. Weissman, Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111 (2001).

113. C. T. Jordan *et al.*, The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* **14**, 1777-1784 (2000).

114. A. J. Gentles, S. K. Plevritis, R. Majeti, A. A. Alizadeh, Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. *JAMA* **304**, 2706-2715 (2010).

115. J. E. Lancet, New agents: great expectations not realized. *Best Pract Res Clin Haematol* **26**, 269-274 (2013).

116. B. A. Carlson, M. M. Dubay, E. A. Sausville, L. Brizuela, P. J. Worland, Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase (CDK) 2 and CDK4 in human breast carcinoma cells. *Cancer Res* **56**, 2973-2978 (1996).

117. J. F. Zeidner *et al.*, Randomized multicenter phase II trial of timed-sequential therapy with flavopiridol (alvocidib), cytarabine, and mitoxantrone (FLAM) versus “7+3” for adults with newly diagnosed acute myeloid leukemia (AML), paper presented at the American Society of Clinical Oncology Annual Meeting, Chicago, IL, 2014.

118. E. J. Feldman *et al.*, First-in-man study of CPX-351: a liposomal carrier containing cytarabine and daunorubicin in a fixed 5:1 molar ratio for the treatment of relapsed and refractory acute myeloid leukemia. *J Clin Oncol* **29**, 979-985 (2011).

119. J. E. Lancet *et al.*, Phase 2 trial of CPX-351, a fixed 5:1 molar ratio of cytarabine/daunorubicin, vs cytarabine/daunorubicin in older adults with untreated AML. *Blood* **123**, 3239-3246 (2014).

120. J. Couzin-Frankel, Breakthrough of the year 2013. Cancer immunotherapy. *Science* **342**, 1432-1433 (2013).

121. P. A. Dinndorf *et al.*, Expression of normal myeloid-associated antigens by acute leukemia cells. *Blood* **67**, 1048-1053 (1986).

122. A. Ehninger *et al.*, Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia. *Blood Cancer J* **4**, e218 (2014).

123. E. L. Sievers *et al.*, Selective ablation of acute myeloid leukemia using antibody-targeted chemotherapy: a phase I study of an anti-CD33 calicheamicin immunoconjugate. *Blood* **93**, 3678-3684 (1999).

124. N. Zein, A. M. Sinha, W. J. McGahren, G. A. Ellestad, Calicheamicin gamma 1I: an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science* **240**, 1198-1201 (1988).

125. S. H. Petersdorf *et al.*, A phase 3 study of gemtuzumab ozogamicin during induction and postconsolidation therapy in younger patients with acute myeloid leukemia. *Blood* **121**, 4854-4860 (2013).

126. A. K. Burnett *et al.*, Attempts to optimize induction and consolidation treatment in acute myeloid leukemia: results of the MRC AML12 trial. *J Clin Oncol* **28**, 586-595 (2010).

127. S. Castaigne *et al.*, Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. *Lancet* **379**, 1508-1516 (2012).

128. A. K. Burnett *et al.*, Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. *J Clin Oncol* **29**, 369-377 (2011).

129. M. S. Kung Sutherland *et al.*, SGN-CD33A: a novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML. *Blood* **122**, 1455-1463 (2013).

130. C. Krupka *et al.*, CD33 target validation and sustained depletion of AML blasts in long-term cultures by the bispecific T-cell-engaging antibody AMG 330. *Blood* **123**, 356-365 (2014).

131. G. S. Laszlo *et al.*, Cellular determinants for preclinical activity of a novel CD33/CD3 bispecific T-cell engager (BiTE) antibody, AMG 330, against human AML. *Blood* **123**, 554-561 (2014).

132. M. Aigner *et al.*, T lymphocytes can be effectively recruited for ex vivo and in vivo lysis of AML blasts by a novel CD33/CD3-bispecific BiTE antibody construct. *Leukemia* **27**, 1107-1115 (2013).

133. C. Stein *et al.*, Novel conjugates of single-chain Fv antibody fragments specific for stem cell antigen CD123 mediate potent death of acute myeloid leukaemia cells. *Br J Haematol* **148**, 879-889 (2010).

134. L. Jin *et al.*, Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. *Cell Stem Cell* **5**, 31-42 (2009).

135. K. M. Frankel et al, Activity and tolerability of SL-401, a targeted therapy directed to the interleukin-3 receptor on cancer stem cells and tumor bulk, as a single agent in patients with advanced hematologic malignancies. *J Clin Oncol 31, 2013 (suppl; abstr TPS3105^)*, (2012).

136. J. M. Rowe, B. Lowenberg, Gemtuzumab ozogamicin in acute myeloid leukemia: a remarkable saga about an active drug. *Blood* **121**, 4838-4841 (2013).

137. K. M. Frankel et al, Activity and tolerability of SL-401, a targeted therapy directed to the interleukin-3 receptor on cancer stem cells and tumor bulk, as a single agent in patients with advanced hematologic malignancies, paper presented at the ASCO, 2013.

138. P. S. Becker *et al.*, Targeting the CXCR4 Pathway: Safety, Tolerability and Clinical Activity of Ulocuplumab (BMS-936564), an Anti-CXCR4 Antibody, in Relapsed/Refractory Acute Myeloid Leukemia paper presented at the American Society of Hematology Annual Meeting, 2014.

139. A. T. Fathi *et al.*, A Phase I Study of the Aurora a Kinase Inhibitor Alisertib in Combination with 7+3 Induction Chemotherapy in Patients with Acute Myeloid Leukemia paper presented at the American Society of Hematology Annual Meeting, 2014.

140. G. Marcucci *et al.*, Adding The KIT Inhibitor Dasatinib (DAS) To Standard Induction and Consolidation Therapy For Newly Diagnosed Patients (pts) With Core Binding Factor (CBF) Acute Myeloid Leukemia (AML): Initial Results Of The CALGB 10801 (Alliance) Study, paper presented at the American Society of Hematology Annual Meeting, 2013.

141. S. A. Wander, M. J. Levis, A. T. Fathi, The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond. *Ther Adv Hematol* **5**, 65-77 (2014).

142. J. E. Cortes *et al.*, Phase I study of quizartinib administered daily to patients with relapsed or refractory acute myeloid leukemia irrespective of FMS-like tyrosine kinase 3-internal tandem duplication status. *J Clin Oncol* **31**, 3681-3687 (2013).

143. E. Hansen *et al.*, AG-120, an Oral, Selective, First-in-Class, Potent Inhibitor of Mutant IDH1, Reduces Intracellular 2HG and Induces Cellular Differentiation in TF-1 R132H Cells and Primary Human IDH1 Mutant AML Patient Samples Treated Ex Vivo paper presented at the American Society of Hematology Annual Meeting, San Francisco, CA, 2014.

144. E. M. Stein *et al.*, AG-221, an Oral, Selective, First-in-Class, Potent Inhibitor of the IDH2 Mutant Metabolic Enzyme, Induces Durable Remissions in a Phase I Study in Patients with IDH2 Mutation Positive Advanced Hematologic Malignancies in *American Society of Hematology Annual Meeting*. (San Francisco, CA, 2014).

145. M. Savona *et al.*, Phase I Trial of Selinexor (KPT-330), A First-In-Class Oral Selective Inhibitor Of Nuclear Export (SINE) In Patients (pts) With Advanced Acute Myelogenous Leukemia (AML) 2013.

146. K. Yee *et al.*, Phase 1/1b Study of RG7388, a Potent MDM2 Antagonist, in Acute Myelogenous Leukemia (AML) Patients (Pts) paper presented at the American Society of Hematology Annual Meeting, 2015.

147. A. M. O'Farrell *et al.*, An innovative phase I clinical study demonstrates inhibition of FLT3 phosphorylation by SU11248 in acute myeloid leukemia patients. *Clin Cancer Res* **9**, 5465-5476 (2003).

148. W. Fiedler *et al.*, A phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. *Blood* **105**, 986-993 (2005).

149. B. D. Smith *et al.*, Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood* **103**, 3669-3676 (2004).

150. W. Zhang *et al.*, Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. *J Natl Cancer Inst* **100**, 184-198 (2008).

151. S. Kayser, M. J. Levis, FLT3 tyrosine kinase inhibitors in acute myeloid leukemia: clinical implications and limitations. *Leuk Lymphoma* **55**, 243-255 (2014).

152. P. P. Zarrinkar *et al.*, AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). *Blood* **114**, 2984-2992 (2009).

153. K. W. Pratz *et al.*, FLT3-mutant allelic burden and clinical status are predictive of response to FLT3 inhibitors in AML. *Blood* **115**, 1425-1432 (2010).

154. F. Wang *et al.*, Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science* **340**, 622-626 (2013).

155. J. Etchin *et al.*, Antileukemic activity of nuclear export inhibitors that spare normal hematopoietic cells. *Leukemia* **27**, 66-74 (2013).

156. J. Etchin *et al.*, KPT-330 inhibitor of CRM1 (XPO1)-mediated nuclear export has selective anti-leukaemic activity in preclinical models of T-cell acute lymphoblastic leukaemia and acute myeloid leukaemia. *Br J Haematol* **161**, 117-127 (2013).

157. J. Etchin *et al.*, Selective Inhibitor of Nuclear Export (SINE), Selinexor (KPT-330), Shows Remarkable Activity Against AML Leukemia-Initiating Cells paper presented at the American Society of Hematology Annual Meeting, San Francisco, CA, 2014.

158. K. W. L. Yee *et al.*, A phase 1 dose-escalation study of the oral selective inhibitor of nuclear export (SINE) KPT-330 (selinexor) in patients (pts) with relapsed/refractory acute myeloid leukemia (AML). paper presented at the 2014 ASCO Annual Meeting, 2014.

159. M. Konopleva *et al.*, Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* **16**, 1713-1724 (2002).

160. M. Konopleva *et al.*, Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10**, 375-388 (2006).

161. L. Campos *et al.*, High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* **81**, 3091-3096 (1993).

162. T. Karakas *et al.*, High expression of bcl-2 mRNA as a determinant of poor prognosis in acute myeloid leukemia. *Ann Oncol* **9**, 159-165 (1998).

163. R. J. Klasa, A. M. Gillum, R. E. Klem, S. R. Frankel, Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense Nucleic Acid Drug Dev* **12**, 193-213 (2002).

164. G. Marcucci *et al.*, A phase III randomized trial of intensive induction and consolidation chemotherapy {+/-} oblimersen, a pro-apoptotic Bcl-2 antisense oligonucleotide in untreated acute myeloid leukemia patients >60 years old paper presented at the ASCO Annual Meeting Proceedings, 06/20/2007 2007.

165. J. C. E. Castro *et al.*, A Phase II, Open Label Study of AT-101 in Combination with Rituximab in Patients with Relapsed or Refractory Chronic Lymphocytic Leukemia. Evaluation of Two Dose Regimens. in *American Society of Hematology Annual Meeting*. (Blood, 2007), vol. 110: Abstract 3119.

166. D. F. James *et al.*, A Phase II, Open Label Study of AT-101 in Combination with Rituximab in Patients with Relapsed or Refractory Chronic Lymphocytic Leukemia. Evaluation of Two Dose Regimens. in *2006 ASCO Annual Meeting Proceedings*. (2006).

167. F. McCoy *et al.*, Obatoclax induces Atg7-dependent autophagy independent of beclin-1 and BAX/BAK. *Cell Death Dis* **1**, e108 (2010).

168. A. D. Schimmer *et al.*, A multicenter phase I/II study of obatoclax mesylate administered as a 3- or 24-hour infusion in older patients with previously untreated acute myeloid leukemia. *PLoS One* **9**, e108694 (2014).

169. C. Tse *et al.*, ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* **68**, 3421-3428 (2008).

170. A. W. Roberts *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. *J Clin Oncol* **30**, 488-496 (2012).

171. L. Gandhi *et al.*, Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. *J Clin Oncol* **29**, 909-916 (2011).

172. A. J. Souers *et al.*, ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* **19**, 202-208 (2013).

173. C. J. Vandenberg, S. Cory, ABT-199, a new Bcl-2-specific BH3 mimetic, has in vivo efficacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia. *Blood* **121**, 2285-2288 (2013).

174. J. Seita, I. L. Weissman, Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* **2**, 640-653 (2010).

175. J. E. Till, C. E. Mc, A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213-222 (1961).

176. C. D. Jude, J. J. Gaudet, N. A. Speck, P. Ernst, Leukemia and hematopoietic stem cells: balancing proliferation and quiescence. *Cell Cycle* **7**, 586-591 (2008).

177. S. J. Morrison, I. L. Weissman, The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1**, 661-673 (1994).

178. J. L. Christensen, I. L. Weissman, Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci U S A* **98**, 14541-14546 (2001).

179. S. W. Boyer, A. V. Schroeder, S. Smith-Berdan, E. C. Forsberg, All hematopoietic cells develop from hematopoietic stem cells through Flk2/Flt3-positive progenitor cells. *Cell Stem Cell* **9**, 64-73 (2011).

180. K. Y., K. S., *Regulation of Hematopoietic Stem Cell Fate: Self-Renewal, Quiescence and Survival; Advances in Hematopoietic Stem Cell Research*. D. R. Pelayo, Ed., (InTech, 2012).

181. M. R. Warr, E. M. Pietras, E. Passegue, Mechanisms controlling hematopoietic stem cell functions during normal hematopoiesis and hematological malignancies. *Wiley Interdiscip Rev Syst Biol Med* **3**, 681-701 (2011).

182. J. Seita *et al.*, Gene Expression Commons: an open platform for absolute gene expression profiling. *PLoS One* **7**, e40321 (2012).

183. I. Beerman *et al.*, Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. *Cell Stem Cell* **12**, 413-425 (2013).

184. D. J. Rossi *et al.*, Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci U S A* **102**, 9194-9199 (2005).

185. D. Sun *et al.*, Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. *Cell Stem Cell* **14**, 673-688 (2014).

186. H. Kim, K. A. Whartenby, R. W. Georgantas, 3rd, J. Wingard, C. I. Civin, Human CD34+ hematopoietic stem/progenitor cells express high levels of FLIP and are resistant to Fas-mediated apoptosis. *Stem Cells* **20**, 174-182 (2002).

187. M. Mohrin *et al.*, Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. *Cell Stem Cell* **7**, 174-185 (2010).

188. W. S. Wu *et al.*, Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. *Cell* **123**, 641-653 (2005).

189. J. T. Opferman *et al.*, Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* **307**, 1101-1104 (2005).

190. C. J. Campbell *et al.*, The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity. *Blood* **116**, 1433-1442 (2010).

191. R. M. Perciavalle *et al.*, Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat Cell Biol* **14**, 575-583 (2012).

192. X. Wang *et al.*, Deletion of MCL-1 causes lethal cardiac failure and mitochondrial dysfunction. *Genes Dev* **27**, 1351-1364 (2013).

193. J. Domen, S. H. Cheshier, I. L. Weissman, The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. *J Exp Med* **191**, 253-264 (2000).

194. J. Domen, I. L. Weissman, Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med* **192**, 1707-1718 (2000).

195. L. J. Fairbairn, G. J. Cowling, B. M. Reipert, T. M. Dexter, Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. *Cell* **74**, 823-832 (1993).

196. D. J. Veis, C. M. Sorenson, J. R. Shutter, S. J. Korsmeyer, Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75**, 229-240 (1993).

197. P. J. Linton, K. Dorshkind, Age-related changes in lymphocyte development and function. *Nat Immunol* **5**, 133-139 (2004).

198. W. W. Pang *et al.*, Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc Natl Acad Sci U S A* **108**, 20012-20017 (2011).

199. I. Beerman *et al.*, Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci U S A* **107**, 5465-5470 (2010).

200. K. Sudo, H. Ema, Y. Morita, H. Nakauchi, Age-Associated Characteristics of Murine Hematopoietic Stem Cells. *The Journal of Experimental Medicine* **192**, 1273-1280 (2000).

201. S. J. Morrison, A. M. Wandycz, K. Akashi, A. Globerson, I. L. Weissman, The aging of hematopoietic stem cells. *Nat Med* **2**, 1011-1016 (1996).

202. B. Dykstra, S. Olthof, J. Schreuder, M. Ritsema, G. de Haan, Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med* **208**, 2691-2703 (2011).

203. M. T. Albright JW, Decline in the growth potential of spleen-colonizing bone marrow stem cells of long-lived aging mice. *J Exp Med* **144**, 1204-1213 (1976).

204. S. M. Chambers *et al.*, Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol* **5**, e201 (2007).

205. S. B. Marley *et al.*, Evidence for a continuous decline in haemopoietic cell function from birth: application to evaluating bone marrow failure in children. *Br J Haematol* **106**, 162-166 (1999).

206. H. Geiger, G. de Haan, M. C. Florian, The ageing haematopoietic stem cell compartment. *Nat Rev Immunol* **13**, 376-389 (2013).

207. Z. Xing *et al.*, Increased hematopoietic stem cell mobilization in aged mice. *Blood* **108**, 2190-2197 (2006).

208. C. C. Sullivan, Yaoyu Shan, Yi Hu, Yiguo Peng, Cong Zhang, Haojian Kong, Linghong Li, Shaoguang, Functional ramifications for the loss of P-selectin expression on hematopoietic and leukemic stem cells. *PLoS One* **6**, e26246 (2011).

209. Y. V. Z. Liang, Gary Szilvassy, Stephen J, Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood* **106**, 1479-1487 (2005).

210. L. Wang, L. Yang, M. Debidda, D. Witte, Y. Zheng, Cdc42 GTPase-activating protein deficiency promotes genomic instability and premature aging-like phenotypes. *Proc Natl Acad Sci U S A* **104**, 1248-1253 (2007).

211. E. P. Rogakou, D. R. Pilch, A. H. Orr, V. S. Ivanova, W. M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* **273**, 5858-5868 (1998).

212. D. J. Rossi *et al.*, Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* **447**, 725-729 (2007).

213. J. Flach *et al.*, Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature* **512**, 198-202 (2014).

214. C. E. Rube *et al.*, Accumulation of DNA damage in hematopoietic stem and progenitor cells during human aging. *PLoS One* **6**, e17487 (2011).

215. I. Beerman, J. Seita, M. A. Inlay, I. L. Weissman, D. J. Rossi, Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell* **15**, 37-50 (2014).

216. S. Jaiswal *et al.*, Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* **371**, 2488-2498 (2014).

217. G. Genovese *et al.*, Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* **371**, 2477-2487 (2014).

218. L. Busque *et al.*, Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet* **44**, 1179-1181 (2012).

219. O. Abdel-Wahab *et al.*, Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood* **114**, 144-147 (2009).

220. J. Boultwood *et al.*, Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. *Leukemia* **24**, 1062-1065 (2010).

221. A. Trifunovic *et al.*, Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417-423 (2004).

222. D. C. Haines, S. Chattopadhyay, J. M. Ward, Pathology of aging B6;129 mice. *Toxicol Pathol* **29**, 653-661 (2001).

223. G. L. Norddahl *et al.*, Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. *Cell Stem Cell* **8**, 499-510 (2011).

224. G. A. Challen *et al.*, Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* **44**, 23-31 (2012).

225. K. Moran-Crusio *et al.*, Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell* **20**, 11-24 (2011).

226. E. Sitnicka *et al.*, Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity* **17**, 463-472 (2002).

227. S. R. Tuljapurkar *et al.*, Changes in human bone marrow fat content associated with changes in hematopoietic stem cell numbers and cytokine levels with aging. *J Anat* **219**, 574-581 (2011).

228. A. J. Freemont, J. A. Hoyland, Morphology, mechanisms and pathology of musculoskeletal ageing. *J Pathol* **211**, 252-259 (2007).

229. V. Vas, K. Senger, K. Dorr, A. Niebel, H. Geiger, Aging of the microenvironment influences clonality in hematopoiesis. *PLoS One* **7**, e42080 (2012).

230. V. Vas, C. Wandhoff, K. Dorr, A. Niebel, H. Geiger, Contribution of an aged microenvironment to aging-associated myeloproliferative disease. *PLoS One* **7**, e31523 (2012).

231. A. V. Ergen, N. C. Boles, M. A. Goodell, Rantes/Ccl5 influences hematopoietic stem cell subtypes and causes myeloid skewing. *Blood* **119**, 2500-2509 (2012).

232. E. J. Moerman, K. Teng, D. A. Lipschitz, B. Lecka-Czernik, Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-

gamma2 transcription factor and TGF-beta/BMP signaling pathways. *Aging Cell* **3**, 379-389 (2004).

233. D. Harman, Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11**, 298-300 (1956).

234. K. Parmar, P. Mauch, J. A. Vergilio, R. Sackstein, J. D. Down, Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci U S A* **104**, 5431-5436 (2007).

235. K. Ito *et al.*, Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* **12**, 446-451 (2006).

236. T. J. Ley *et al.*, DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature* **456**, 66-72 (2008).

237. A. M. Jabbour *et al.*, Puma indirectly activates Bax to cause apoptosis in the absence of Bid or Bim. *Cell Death Differ* **16**, 555-563 (2009).

238. D. Ren *et al.*, BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science* **330**, 1390-1393 (2010).

239. E. H. Cheng *et al.*, BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* **8**, 705-711 (2001).

240. F. Vaillant *et al.*, Targeting BCL-2 with the BH3 Mimetic ABT-199 in Estrogen Receptor-Positive Breast Cancer. *Cancer Cell* **24**, 120-129 (2013).

241. J. K. Brunelle, J. Ryan, D. Yecies, J. T. Opferman, A. Letai, MCL-1-dependent leukemia cells are more sensitive to chemotherapy than BCL-2-dependent counterparts. *J Cell Biol* **187**, 429-442 (2009).

242. Z. Zeng *et al.*, Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood* **113**, 6215-6224 (2009).

243. V. R. Ruvolo *et al.*, Role for PKC δ in Fenretinide-Mediated Apoptosis in Lymphoid Leukemia Cells. *J Signal Transduct* **2010**, 584657 (2010).

244. Y. Chen *et al.*, Human extramedullary bone marrow in mice: a novel in vivo model of genetically controlled hematopoietic microenvironment. *Blood* **119**, 4971-4980 (2012).

245. T. Haferlach *et al.*, Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J Clin Oncol* **28**, 2529-2537 (2010).

246. M. Vogler *et al.*, Diminished sensitivity of chronic lymphocytic leukemia cells to ABT-737 and ABT-263 due to albumin binding in blood. *Clin Cancer Res* **16**, 4217-4225 (2010).

247. V. Del Gaizo Moore, K. D. Schlis, S. E. Sallan, S. A. Armstrong, A. Letai, BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. *Blood* **111**, 2300-2309 (2008).

248. S. P. Glaser *et al.*, Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev* **26**, 120-125 (2012).

249. J. F. Seymour *et al.*, paper presented at the American Society of Hematology Annual Meeting, 2013.

250. S. M. Chan *et al.*, Isocitrate dehydrogenase 1 and 2 mutations induce BCL-2 dependence in acute myeloid leukemia. *Nat Med* **21**, 178-184 (2015).

251. G. W. Foight, J. A. Ryan, S. V. Gulla, A. Letai, A. E. Keating, Designed BH3 peptides with high affinity and specificity for targeting Mcl-1 in cells. *ACS Chem Biol* **9**, 1962-1968 (2014).

252. J. B. Garcia *et al.*, Pneumonia during remission induction chemotherapy in patients with acute leukemia. *Ann Am Thorac Soc* **10**, 432-440 (2013).

253. S. Abbas *et al.*, Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. *Blood* **116**, 2122-2126 (2010).

254. D. Yecies, N. E. Carlson, J. Deng, A. Letai, Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* **115**, 3304-3313 (2010).

255. M. F. van Delft *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* **10**, 389-399 (2006).

256. J. D. Leverson *et al.*, Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). *Cell Death Dis* **6**, e1590 (2015).

257. B. E. Sleebs *et al.*, Discovery of potent and selective benzothiazole hydrazone inhibitors of Bcl-XL. *J Med Chem* **56**, 5514-5540 (2013).

258. Y. Tanaka *et al.*, Discovery of potent Mcl-1/Bcl-xL dual inhibitors by using a hybridization strategy based on structural analysis of target proteins. *J Med Chem* **56**, 9635-9645 (2013).

259. G. S. Choudhary *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* **6**, e1593 (2015).

260. D. A. Flusberg, J. Roux, S. L. Spencer, P. K. Sorger, Cells surviving fractional killing by TRAIL exhibit transient but sustainable resistance and inflammatory phenotypes. *Mol Biol Cell* **24**, 2186-2200 (2013).

261. J. M. Bogenberger *et al.*, Ex vivo activity of BCL-2 family inhibitors ABT-199 and ABT-737 combined with 5-azacytidine in myeloid malignancies. *Leuk Lymphoma* **56**, 226-229 (2015).

262. I. Beerman, W. J. Maloney, I. L. Weissmann, D. J. Rossi, Stem cells and the aging hematopoietic system. *Curr Opin Immunol* **22**, 500-506 (2010).

263. D. J. Rossi, C. H. Jamieson, I. L. Weissman, Stems cells and the pathways to aging and cancer. *Cell* **132**, 681-696 (2008).

264. D. Hanahan, R. A. Weinberg, Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

265. P. van Galen *et al.*, The unfolded protein response governs integrity of the haematopoietic stem-cell pool during stress. *Nature* **510**, 268-272 (2014).

266. M. Milyavsky *et al.*, A distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* **7**, 186-197 (2010).

267. N. Dainiak, Hematologic consequences of exposure to ionizing radiation. *Exp Hematol* **30**, 513-528 (2002).

268. M. A. Lichtman, J. M. Rowe, The relationship of patient age to the pathobiology of the clonal myeloid diseases. *Semin Oncol* **31**, 185-197 (2004).

269. J. Huang, M. Nguyen-McCarty, E. O. Hexner, G. Danet-Desnoyers, P. S. Klein, Maintenance of hematopoietic stem cells through regulation of Wnt and mTOR pathways. *Nat Med* **18**, 1778-1785 (2012).

270. J. Domen, I. L. Weissman, Hematopoietic stem cells and other hematopoietic cells show broad resistance to chemotherapeutic agents in vivo when overexpressing bcl-2. *Exp Hematol* **31**, 631-639 (2003).

271. O. Kutuk, A. Letai, Regulation of Bcl-2 family proteins by posttranslational modifications. *Curr Mol Med* **8**, 102-118 (2008).

272. J. Ryan, A. Letai, BH3 Profiling in Whole Cells by Flourimeter or FACS. *Methods*, (2013).

273. M. J. Kiel, O. H. Yilmaz, T. Iwashita, C. Terhorst, S. J. Morrison, SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121 (2005).

274. Y. Morita, H. Ema, H. Nakauchi, Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med* **207**, 1173-1182 (2010).

275. K. A. Sarosiek *et al.*, BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. *Mol Cell* **51**, 751-765 (2013).

276. E. M. Pietras, M. R. Warr, E. Passegue, Cell cycle regulation in hematopoietic stem cells. *J Cell Biol* **195**, 709-720 (2011).

277. E. Passegué, A. J. Wagers, S. Giuriato, W. C. Anderson, I. L. Weissman, Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med* **202**, 1599-1611 (2005).

278. K. W. Orford, D. T. Scadden, Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet* **9**, 115-128 (2008).

279. A. Foudi *et al.*, Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol* **27**, 84-90 (2009).

280. A. Wilson *et al.*, Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* **135**, 1118-1129 (2008).

281. M. A. Essers *et al.*, IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908 (2009).

282. S. J. Morrison, D. E. Wright, I. L. Weissman, Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci U S A* **94**, 1908-1913 (1997).

283. D. Walter *et al.*, Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature*, (2015).

284. T. McKerrell *et al.*, Leukemia-associated somatic mutations drive distinct patterns of age-related clonal hemopoiesis. *Cell Rep* **10**, 1239-1245 (2015).

285. X. Zhao *et al.*, Combination of ibrutinib with ABT-199: synergistic effects on proliferation inhibition and apoptosis in mantle cell lymphoma cells through perturbation of BTK, AKT and BCL2 pathways. *Br J Haematol* **168**, 765-768 (2015).

286. S. A. Rushworth, M. Y. Murray, L. Zaitseva, K. M. Bowles, D. J. MacEwan, Identification of Bruton's tyrosine kinase as a therapeutic target in acute myeloid leukemia. *Blood* **123**, 1229-1238 (2014).

287. Y. Saito *et al.*, A pyrrolo-pyrimidine derivative targets human primary AML stem cells in vivo. *Sci Transl Med* **5**, 181ra152 (2013).

288. V. Fresquet, M. Rieger, C. Carolis, M. J. Garcia-Barchino, J. A. Martinez-Climent, Acquired mutations in BCL2 family proteins conferring resistance to the BH3 mimetic ABT-199 in lymphoma. *Blood* **123**, 4111-4119 (2014).

289. P. Lito *et al.*, Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. *Cancer Cell* **22**, 668-682 (2012).

290. H. Shi *et al.*, A novel AKT1 mutant amplifies an adaptive melanoma response to BRAF inhibition. *Cancer Discov* **4**, 69-79 (2014).

291. J. D. Leverson *et al.*, Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci Transl Med* **7**, 279ra240 (2015).

292. T. N. Chonghaile *et al.*, Maturation stage of T-cell acute lymphoblastic leukemia determines BCL-2 versus BCL-XL dependence and sensitivity to ABT-199. *Cancer Discov* **4**, 1074-1087 (2014).

293. M. R. Sapienza *et al.*, Molecular profiling of blastic plasmacytoid dendritic cell neoplasm reveals a unique pattern and suggests selective sensitivity to NF-kB pathway inhibition. *Leukemia* **28**, 1606-1616 (2014).

294. L. Pagano *et al.*, Blastic plasmacytoid dendritic cell neoplasm with leukemic presentation: an Italian multicenter study. *Haematologica* **98**, 239-246 (2013).

295. W. J. Placzek *et al.*, 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* **1**, e40- (2010).

296. S. L. Khaw *et al.*, Both leukaemic and normal peripheral B lymphoid cells are highly sensitive to the selective pharmacological inhibition of prosurvival Bcl-2 with ABT-199. *Leukemia* **28**, 1207-1215 (2014).

297. J. M. Bennett *et al.*, Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* **33**, 451-458 (1976).

298. K. Nakano, K. H. Vousden, PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**, 683-694 (2001).

299. T. Yahata *et al.*, Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood* **118**, 2941-2950 (2011).

300. G. Bjelakovic, D. Nikolova, L. L. Gluud, R. G. Simonetti, C. Gluud, Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* **297**, 842-857 (2007).

301. G. A. Challen, N. C. Boles, S. M. Chambers, M. A. Goodell, Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell Stem Cell* **6**, 265-278 (2010).

302. A. Y. Maslov, T. A. Barone, R. J. Plunkett, S. C. Pruitt, Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. *J Neurosci* **24**, 1726-1733 (2004).

303. A. V. Molofsky *et al.*, Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* **443**, 448-452 (2006).

304. A. Giangreco, M. Qin, J. E. Pintar, F. M. Watt, Epidermal stem cells are retained in vivo throughout skin aging. *Aging Cell* **7**, 250-259 (2008).

305. S. S. Jejurikar *et al.*, Aging increases the susceptibility of skeletal muscle derived satellite cells to apoptosis. *Exp Gerontol* **41**, 828-836 (2006).

306. S. Fulle, S. Sancilio, R. Mancinelli, V. Gatta, R. Di Pietro, Dual role of the caspase enzymes in satellite cells from aged and young subjects. *Cell Death Dis* **4**, e955 (2013).

307. K. Martin, T. B. Kirkwood, C. S. Potten, Age changes in stem cells of murine small intestinal crypts. *Exp Cell Res* **241**, 316-323 (1998).

308. F. R. Appelbaum *et al.*, Age and acute myeloid leukemia. *Blood* **107**, 3481-3485 (2006).

309. A. van Rhenen *et al.*, High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. *Clin Cancer Res* **11**, 6520-6527 (2005).

310. Y. Saito *et al.*, Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Sci Transl Med* **2**, 17ra19 (2010).

311. F. Ishikawa *et al.*, Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol* **25**, 1315-1321 (2007).

312. E. D. Lagadinou *et al.*, BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell* **12**, 329-341 (2013).

313. Y. Saito *et al.*, Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat Biotechnol* **28**, (2010).

314. T. Pabst *et al.*, Favorable effect of priming with granulocyte colony-stimulating factor in remission induction of acute myeloid leukemia restricted to dose escalation of cytarabine. *Blood* **119**, 5367-5373 (2012).