Strategies To Enhance The Anti-Leukemic Activity Of Venetoclax (abt-199) In Aml Through Targeting Of Mcl-1

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STRATEGIES TO ENHANCE THE ANTI-LEUKEMIC ACTIVITY OF VENETOCLAX (ABT-199) IN AML THROUGH TARGETING OF MCL-1

by

DANIEL ALLEN LUEDTKE

DISSERTATION

Submitted to the Graduate School
of Wayne State University
Detroit, Michigan

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for the degree of

DOCTOR OF PHILOSOPHY

2019

MAJOR: CANCER BIOLOGY

Approved By:

_____________________________________
Advisor Date
DEDICATION

To all those who provided me the opportunities and supportive environment to develop and utilize the gifts I have been given to improve the lives of others
ACKNOWLEDGEMENTS

I am grateful for my mentor, Dr. Yubin Ge, who has been supportive in my transition from a lab technician to an independent scientist. As a researcher, he has guided this progression through the training exercise of my dissertation research. As I have progressed in my studies, he has emphasized the continuous improvement of my technical skills (e.g. laboratory work, writing) as well as critical thinking and planning skills necessary for me to evaluate the research of myself and others in order to develop novel directions derived from those evaluations. His focus has been to prepare me for the next step in my career and has been amicable in advocating for those goals. He has worked towards preparing me for a job in industry research through the careful maintenance of risk and scope of my research project as well as the various internal and external opportunities to hone my technical and critical thinking skillsets.

I would also thank the members of the lab and others who have helped in my research over the years, especially Holly Pitman, whose technical expertise and feedback on manuscripts and presentations has been very helpful. Her kindness and experience helped smooth over any difficulties I had during my studies. Dr. Polin, Juiwanna Kushner, Dr. Sijana H. Dzinic, and AMTEC (Animal Model and Therapeutic Evaluation Core) aided in my training in animal models and the in vivo studies that increased the clinical relevance of our studies. Dr. Hai Lin helped acquire primary AML (acute myeloid leukemia) patient samples, improving the impact of our studies. My dissertation committee (Dr. Jeffrey W. Taub, Dr. Stephan Patrick, Dr. Youming Xie, and Dr. Gerard Madlambayan) helped guide my research and my personal development. I would like to thank my rotation advisors Dr. Izabela Podgorski and Dr. Lori Pile for providing my first training and experiences in biological science research and acclimating me to an academic environment from my previous role in industry. I would like to thank the Wayne State
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I would like to thank my sources of funding that provided the resources to complete my dissertation. These studies were supported by grants from the DeRoy Testamentary Foundation, MEI Pharma Inc., Children’s Hospital of Michigan Foundation, Kids Without Cancer, Hyundai Hope On Wheels, the Ring Screw Textron Endowed Chair for Pediatric Cancer Research, Lafontaine Family/U Can-Cer Vive Foundation, the Decerchio/Guisewite Family, Justin’s Gift, Elana Fund, and the Ginopolis/Karmanos Endowment and supported by the Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine.

I would also like to thank my many friends who supported me through my studies. Their continued support was instrumental in helping me survive the marathon of graduate school. Whether it was through sports (e.g. soccer, kickball, volleyball, basketball, broomball), potluck dinners, bonfires, etc.; their support helped to balance and enrich my life. I am thankful for all of the wonderful memories shared.

Lastly, I will like to thank my family for their, past, present, and continued support. Throughout my life, my parents, Buck and Cindy, have developed my curiosity about the world as well as developing my character and independence. Books and encyclopedias were always available. As a consequence of not having cable television, educational Public Broadcasting Station programming held a disproportionate amount of my television viewing time. My parents worked hard to send me and my siblings to private middle and high school. While my siblings
and I were not forced to do activities, we were encouraged to expand our horizons and exercise our decision making skills. Having chores like gardening and doing the dishes that we would need to complete on our own schedule taught us self-management skills, the importance of deadlines and the consequences of stressing out due to procrastination. My parents worked hard to provide me and my siblings with the opportunities to succeed which has come to fruition for all of us. I would also like to thank my sister Sara and brother Karl in their continued guidance and friendship which has been an important part of guiding me in life’s journey.
LAY ABSTRACT

Cancer is where cells in the body grow in an uncontrolled manner and can potentially spread and take over the normal cells. My dissertation covers Acute Myeloid Leukemia (AML), a type of blood cancer. It is the most common type of leukemia cancer while also having the poorest survival rates (21450 cases and 10920 deaths per year in the United States). Treatment has been relatively unchanged from the 1970’s until 2017. Even with the new treatment approvals in 2017 and 2018, survival improvements have been modest at best. New treatments are urgently needed to improve long term survival of patients with AML.

My research focuses on apoptosis, also known as programmed cell death. This is a way a cell chooses to kill itself. Normal cells die if they get damaged or abnormal. Cancer cells survive when they are able to avoid this. As cancer cells are more abnormal and stressed than normal cells, cancer cells need to avoid cell death more so than normal cells. Inducing apoptosis can preferentially kill cancer cells over normal cells. The goal of any cancer treatment is to kill as many cancer cells and as few normal cells as possible.

Venetoclax is a drug that targets a protein called Bcl-2, which prevents apoptosis. The drug works well in the lab and the clinic but resistance to the drug quickly occurs. Our lab and others found that a second protein called Mcl-1, which is related to Bcl-2, causes this resistance. In chapter 2, using lab tools, I show that direct targeting of Mcl-1 overcomes resistance to venetoclax. In chapters 3 and 4, I show that using drugs that both target blood cancer cells and decrease Mcl-1 can enhance venetoclax treatment. In chapter 5, I discuss the future directions and implications of the research.
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LIST OF ABBREVIATIONS

5hmC 5-hydroxymethylcytosine
5mC 5-methylcytosine
AE Adverse event
ALL Acute lymphocytic leukemia
AMKL Acute megakaryoblastic leukemia
AML Acute myeloid leukemia
AML-MRC Acute myeloid leukemia - myelodysplasia related changes
AMTEC Animal Model and Therapeutics Evaluation Core
ANOVA Analysis of variance
APAF-1 Apoptotic peptidase activating factor 1
APL Acute promyelocytic leukemia
Ara-C Cytarabine (cytosine arabinoside)
ATP Adenosine triphosphate
ATRA All-trans retinoic acid
Bak Bcl-2 homologous antagonist killer
Bax Bcl-2 associated X protein
Bcl-2 B-cell lymphoma 2
Bcl-xL B-cell lymphoma-extra large
BH1/2/3/4 Bcl-2 homology domain 1/2/3/4
B.I.D. Twice daily (Latin “bis in die”)
Bid BH3 interacting domain death agonist
Bim Bcl-2-like protein 11
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>BMT</td>
<td>Bone marrow transplant</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSC</td>
<td>Best supportive care</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>Cas</td>
<td>Caspase (cysteine aspartate proteases)</td>
</tr>
<tr>
<td>CBFB</td>
<td>Core-binding factor subunit beta</td>
</tr>
<tr>
<td>CBGP</td>
<td>Cancer Biology Graduate Program</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CEBPA/CEBPα</td>
<td>CCAAT/enhancer binding protein alpha</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CHAPS</td>
<td>(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate</td>
</tr>
<tr>
<td>CHIP</td>
<td>Clonal hematopoiesis of indeterminate potential</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CI</td>
<td>Combination Index</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CR</td>
<td>Complete response</td>
</tr>
<tr>
<td>CRADD</td>
<td>Caspase and RIP adapter with death domain (see TRADD)</td>
</tr>
<tr>
<td>CRh</td>
<td>Complete response with partial hematologic recovery</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>CRM1</td>
<td>Chromosomal maintenance 1 (see XPO1)</td>
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<tr>
<td>CRR</td>
<td>Complete response rate</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
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<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
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<tr>
<td>DED</td>
<td>Death effector domain</td>
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<tr>
<td>DIABLO</td>
<td>see SMAC (direct IAP binding protein with low pi)</td>
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<tr>
<td>DLBCL</td>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>DNA Methyltransferase 3 Alpha</td>
</tr>
<tr>
<td>DOR</td>
<td>Duration of response</td>
</tr>
<tr>
<td>DR4/5</td>
<td>Death receptor 4/5</td>
</tr>
<tr>
<td>DS</td>
<td>Down syndrome</td>
</tr>
<tr>
<td>Dup</td>
<td>Duplication</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFS</td>
<td>Event free survival</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’'-tetraacetic acid or egtazic acid</td>
</tr>
<tr>
<td>ELN</td>
<td>European Leukemia Net</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
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<td>Definition</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FLT3-ITD</td>
<td>FMS-like related tyrosine kinase 3-internal tandem duplication</td>
</tr>
<tr>
<td>FLT3-TKD</td>
<td>FMS-like related tyrosine kinase 3-tyrosine kinase domain mutation</td>
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<tr>
<td>FS</td>
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<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor, colony-stimulating factor 3</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor, colony-stimulating factor 2</td>
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<tr>
<td>GO</td>
<td>Gemtuzumab ozogamicin</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin’s Lymphoma</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplant</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
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<td>Acronym</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-3/6</td>
<td>Interleukin 3/6</td>
</tr>
<tr>
<td>ILS</td>
<td>Increased lifespan</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space (mitochondrial)</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin, transferrin, and sodium selenite</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KCI</td>
<td>Karmanos Cancer Institute</td>
</tr>
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<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KMT2A</td>
<td>Lysine methyltransferase 2A (also known as MLL)</td>
</tr>
<tr>
<td>KPT</td>
<td>KPT-330 (selinexor) or KPT-8602 (eltanexor, Fig 3.3-3.4)</td>
</tr>
<tr>
<td>LDAC</td>
<td>Low dose cytarabine (ara-C)</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemic stem cell</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle cell lymphoma or Mast cell leukemia</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid Cell Leukemia 1</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>MLL</td>
<td>See KMT2A</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane potential</td>
</tr>
<tr>
<td>MPN</td>
<td>Myeloproliferative neoplasm</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOXA</td>
<td>Phorbol-12-myristate-13-acetate-induced protein 1, Latin for damage</td>
</tr>
<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
</tr>
<tr>
<td>NSG-SGM3/NSGS</td>
<td>NOD-scid IL2Rg null-3/GM/SF murine model</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-target control</td>
</tr>
<tr>
<td>OE</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Omi</td>
<td>Omi stress-regulated endoprotease</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>ORR</td>
<td>Overall response rate</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>p-TEFb</td>
<td>Positive transcription elongation factor b</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Palmo-ara-C</td>
<td>Palmitate ara-C</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>PO</td>
<td>Per os, oral administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PTD</td>
<td>Partial tandem duplication</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>QD</td>
<td>Daily (Latin “quaque die”)</td>
</tr>
<tr>
<td>Q2D</td>
<td>Every 2 days</td>
</tr>
<tr>
<td>Q3D</td>
<td>Every 3 days</td>
</tr>
<tr>
<td>R/R</td>
<td>Relapsed/refractory</td>
</tr>
<tr>
<td>RAEB</td>
<td>Refractory anemia excessive blasts</td>
</tr>
<tr>
<td>RAIDD</td>
<td>RIP-Associated Protein with a Death Domain</td>
</tr>
<tr>
<td>RARA</td>
<td>Retinoic acid receptor alpha</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAP II</td>
<td>RNA polymerase 2</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Stem cell</td>
</tr>
<tr>
<td>SCFβ</td>
<td>Stem cell factor β</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SM-AHN</td>
<td>Systemic mastocytosis with associated hematological neoplasm</td>
</tr>
<tr>
<td>SMAC</td>
<td>(see DIABLO) second mitochondrial-derived activator of caspases</td>
</tr>
<tr>
<td>SODD</td>
<td>Silencer of death domain</td>
</tr>
<tr>
<td>SOM</td>
<td>School of Medicine</td>
</tr>
<tr>
<td>SS</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SSA</td>
<td>Social security administration</td>
</tr>
<tr>
<td>t-AML</td>
<td>Therapy related AML</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRADD</td>
<td>(see CRADD) Tumor necrosis factor receptor type 1-associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEN</td>
<td>Venetoclax</td>
</tr>
<tr>
<td>VOR</td>
<td>Voruciclib</td>
</tr>
<tr>
<td>WC/WCL</td>
<td>Whole cell lysate</td>
</tr>
<tr>
<td>WD-40</td>
<td>WD-40 repeat domain which is 40 amino acids long and ends in a tryptophan-aspartic acid (W-D) dipeptide</td>
</tr>
<tr>
<td>WES</td>
<td>Whole exome sequencing</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
WIT  WT1, IDH1/2, TET2 pathway

WSU  Wayne State University

WT  Wild type

XIAP  X-linked inhibitor of apoptosis

XPO1  Exportin 1 (see CRM1)

Z-VAD-FMK  (Z-VAD) N-Benzylloxycarbonyl-Val-Ala-Asp(O-methyl) fluoromethyl ketone

β-TrCP  Beta-transducin repeat containing E3 ubiquitin protein ligase

γH2AX  Phosphorylated H2A histone family member X
CHAPTER 1: INTRODUCTION

1.1 Clinical Pathology of Acute Myeloid Leukemia

1.1.1 Normal hematopoiesis

In healthy individuals, the production of blood is tightly controlled by the process of hematopoiesis (from the Greek “haima” for “blood” and “poiein” “to make”) as the hematopoietic stem cell (HSC) differentiates into the various blood cell progenitors and differentiated blood cells in a hierarchical manner (Figure 1.1) (1). Turnover is constant for most blood cells (except for a few long lived immune cells), which requires constant replenishment of these cell components (2). With only 10,000 to 20,000 HSCs and over $10^{11}$ cells that need to be made each day; this process requires coordination and delegation (3). The multipotent HSCs undergo symmetric division to replenish the stem cell (SC) pool and asymmetric division to generate committed progenitor cells. The HSCs divide more slowly than committed progenitors and as a result acquire fewer mutations than other cancer types (4).

These HSCs generate intermediate cells that have limited self-renewal, which in turn generate the terminal blood cells (e.g. red blood cells [erythrocytes, RBCs], thrombocytes [platelets], plasma cells) (2). The lymphoid lineage is derived from the common lymphoid progenitor and plays a critical role in the generation of natural killer (NK) cells, B cells (including plasma cells), and T cells. The myeloid lineage is derived from the common myeloid lineage and plays an important role in the generation of RBCs, platelets, and other blood cell types. The extent to which cells are produced in the various lineages is dependent on the various supportive signals which balance the number of cells. For example, in higher elevation or anemia, more blood cells are needed. Erythropoietin is secreted by the kidney to increase red
blood cell (RBC) production. Dysregulation in these hierarchical processes can lead to disease. Too many platelets can lead to excess clotting of the blood. Too few platelets or RBCs can lead to excessive bleeding and anemia, respectively. Too many or too few immune cells can lead to autoimmune disorders or a weakened ability to fight infections. Regulation of hematopoiesis is critical for homeostasis.

Figure 1.1 Diagram of hematopoiesis. (From Wikimedia Commons by Mikael Häggström et.al.)

1.1.2 Epidemiology and prognosis of leukemia

Leukemia is a blood disorder that is the result of arrested differentiation and uncontrolled proliferation of the blood progenitor cells (2). When a clonal population dominates, the other types of cells produced by hematopoiesis are diminished. The term leukemia comes from the observation of patients with excess white blood cells and the Greek words “leukos” which means white and “haima” which means blood. The white blood cell clonal population had overtaken
and crowded out the other blood types, which created blood that was milky in appearance. The result of this clonal dominance is anemia, a weakened immune system, excess bleeding, and other associated symptoms from lacking the critical types of blood cells.

This clonal nature of leukemia was determined by researchers looking at the chromosomal status of patients. Early studies focused on chromosomal changes for females, as one parental X chromosome is randomly inactivated in each cell (5). Leukemia, being a clonal disease, leads to a disproportionate amount of one allele being activated over the other as the blood cells are disproportionately derived from a single clone. This was demonstrated by the X-linked gene \textit{G6PD} [glucose-6-phosphate dehydrogenase] in heterozygous women where one allele had a near complete dominance, providing early proof of the clonal nature of leukemia and cancer in general (6, 7).

Leukemias are also classified by their differentiation and their cell lineage (8). Acute leukemias (common in pediatric cancer) are the result of the uncontrolled proliferation of immature blood cells with a more rapid onset, while chronic leukemias (very rare in children) are a result of the uncontrolled proliferation of more mature blood cells. Acute leukemias will kill the patient in weeks to months if left untreated, while chronic leukemias will kill the patients in years if left untreated (8). Lymphoblastic leukemias are a disorder of the common lymphoid progenitor lineage which normally produces natural killer cells, T cells, and B cells, while myeloid leukemias are a disorder of the common myeloid progenitor lineage which normally produces thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, macrophages, and dendritic cells. The four main subtypes of leukemia are distinguished by these two pairs of criteria; acute lymphoblastic leukemia (ALL, more common in children), acute
myeloid leukemia (AML, found in children but more common in adults), chronic lymphoblastic leukemia (CLL), and chronic myeloid leukemia (CML).

AML is the most common form of acute leukemia in the United States with 21,450 cases expected in 2019 (9). Compared to other leukemia subtypes, AML has by far the most deaths (10,920 projected for 2019) and the poorest survival (9). 5 year survival is 67% for those 19 and under and 24% for those 20 and older with the prognosis declining with age (10). A further challenge is the 68 year median age of onset, which means treating older patients with comorbidities (11). Many patients are unable to tolerate standard chemotherapy and bone marrow transplant, leading to mortality and poor outcomes (12, 13). Only 40% of those above 65 years old are treated with chemotherapy within 3 months of diagnosis (14). For treated patients over untreated patients, outcomes remain poor (65-69 years old 10 versus 4 months, 70 to 74 years old 8 versus 3 months, 75-79 years old 6 versus 2 months) (15-17). Leukemic stem cells (LSCs) and their evasion of therapy are believed to be responsible for relapse (18, 19). Similar to other cancers as well as normal tissues, this LSC population maintains the overall bulk leukemia and due to its less proliferative (quiescent) nature does not respond to anti-proliferative chemotherapeutics agents (20-22). In summary, little has changed in the standard of care over the last several decades leading to only modest improvements in patient outcomes compared to other cancers and leukemia subtypes (23).

1.1.3 FAB and WHO Classification Systems

The first classification system for leukemia was developed by the French-American-British (FAB) co-operative group (24). The group independently and cooperatively looked at the physical appearance of stained leukemia samples. The goal was to standardize leukemia subtypes to better compare clinical trial data among different clinical trials and institutions, and reduce
discrepancies in the classification of leukemia cases. The first distinction was between lymphoblastic and myeloid leukemias. For the myeloid leukemias, six subtypes were defined (M1-M6). Two additional subtypes, M0 for minimally differentiated AML and M7 for megakaryoblastic leukemia (AMKL), were later added (25, 26). While an improvement, these classifications were superficial and often non-specific to the functional properties of the leukemias.

In a partial move from phenotypic to genotypic classification, the World Health Organization (WHO) devised a system that includes genetic, treatment, and other causal backgrounds for AML subtypes (Table 1.1, (27)). Some FAB subtypes, like APL (acute promyelocytic leukemia, M3, all cases related to retinoic acid receptor alpha [RARA] fusions) and AMKL (M7, some cases are due to down syndrome [DS]), are strongly correlated to genetic alterations (28). Genetic alterations are able to predict the behavior of the disease and guide treatment, including the use of targeted therapies, aggressive chemotherapy, and the value proposition of a bone marrow transplant (BMT) (29). With the advent of biologic tools including sequencing technologies, our ability to probe the genetic subtypes has exponentially grown. The understanding of the different behavior of specific subtypes led to the re-classification of AML subtypes in 2002 and later 2016 by the WHO (27, 28). As the study of AML progresses, the classification of AML will move away from surface level appearances to functional and genetic properties of the cancer that are more relevant for treatment decision making.
### World Health Organization (WHO) 2016 classification of acute myeloid leukemia and related neoplasms

**AML with recurrent genetic abnormalities**

- AML with t(8;21)(q22;q22.1); *RUNX1-RUNXIT1*
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* (M4eo)
- APL with *PML-RARA* (M3)
- AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* AML with t(6;9)(p23;q34.1); *DEK-NUP214*
- AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*, MECOM
- AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1* Provisional entity
- AML with *BCR-ABL1* AML with mutated NPM1
- AML with biallelic mutations of *CEBPA* Provisional entity
- AML with mutated *RUNX1*  

**AML with myelodysplasia-related changes**

**Therapy-related myeloid neoplasms**

**AML, Not otherwise specified**

- AML with minimal differentiation (M0)
- AML without maturation (M1)
- AML with maturation (M2)
- Acute myelomonocytic leukemia (M4)
- Acute monoblastic/monocytic leukemia(M5)
- Pure erythroid leukemia (M6)
- Acute megakaryoblastic leukemia(M7)
- Acute basophilic leukemia (M8)
- Acute panmyelosis with myelofibrosis

**Myeloid sarcoma**

**Myeloid proliferations related to Down syndrome**

- Transient abnormal myelopoiesis
- Myeloid leukemia associated with Down syndrome

**Table 1.1. World Health Organization (WHO) 2016 classification of acute myeloid leukemia. (27)**
1.1.4 Genetics of AML

Biologically, AML is a very heterogeneous disease. AML has one of the lowest mutational rates among cancer types and even the most common AML mutations appear less than 30% of the time (4, 30). The mutations that do occur have broad acting functions, such as signal transduction, transcription factors, differentiation, and epigenetic regulation (31). As a result, the AML cells are difficult to distinguish, both by the host’s immune system, and by targeted therapy.

AML has been theorized to be due to a “two-hit” model where mutations in one class of genes provide a proliferative advantage and mutations in a second class of genes impair differentiation (32). Both of these hits alone do not lead to leukemia. In combination, they lead to cancerous blood, i.e. leukemia. As people age, HSCs can gain a proliferation advantage either by chance (stochastic) or by driver mutations (e.g. DNMT3A, TET2, ASXL1) which confer a proliferative advantage (31, 33). Over time, HSCs acquire mutations and clonal hematopoiesis of indeterminate potential (clonal dominance, CHIP), increasing the risk of leukemia (34). Myelodysplastic syndromes (MDS) occur when blood precursors fail to properly differentiate, leading to a lack of differentiated blood products (35). Myeloproliferative neoplasms (MPN) occur when excess proliferation causes pathology (35). Both MDS and MPN can lead to AML, particularly in older adults. Patients with genetic predispositions (e.g. Li Fraumeni [TP53 mutation], Down syndrome [trisomy 21], Fanconi anemia or BRCA1/2 mutations [impaired DNA repair]) may already have a “hit” or be prone to acquiring mutational “hits” (36-38). Regardless of the genetic, environmental, or random cause, it is when both the lack of differentiation and excess proliferation occur that leads to leukemia.
These AML “hits” led to the classification of AML mutations into Class I Signal Activation (which confers proliferation advantages, e.g. FLT3, NRAS, KRAS, c-Kit) and Class II Transcription and Differentiation (which prevents differentiation and confers stemness, e.g. RUNX1, CEBPA, NPM1) classes (39). A newer third class, Class III Epigenetic factors, has been added (which affects general cellular programming, e.g. IDH1, IDH2, TET2, DMNT3A) (40). Despite these categories being listed separately for clarity, AML alterations often overlap several categories in their various roles (41).

A common theme of AML mutations is the synthesis of fusion proteins, which can occur in any of these groups (also noted in the WHO classification of AML). APL (acute promyelocytic leukemia) is the result of the PML-RARA fusion found in 13% of AML (often excluded from AML statistics as a whole due to its unique treatment and good overall survival rate) (42). The MLL gene (also known as KMT2A), has dozens of fusion partners such as AF6 (MLLT4), AF9 (MLLT3), AF10 (MLLT10), ELL, ENL (MLLT1), and partial tandem duplication (PTD) being the most common partners (43). As a positive global regulator of transcription, the pathways of these fusion proteins vary. MLL fusions are more common in infant acute leukemia patients (70%) than adult leukemia patients (10%). MYH11-CBFB, present in 16% of AML, is caused by either inv(16)(p13.1q22) or t(16;16)(p13.1;q22), results in blocked differentiation and is one of the many alterations in AML that affects the CBF transcription complex (44, 45). RUNX1-RUNX1T1, present in 7% of AML (mostly M2) and is caused by t(8;21)(q22;q22.1), associates with CBF to alter transcription. BCR-ABL1 should be noted as unlike in CML where it is more common, in AML it usually lacks IGH/VDJ and TARP regions, which means less mutations and a more durable response to targeted inhibitors (46). Other fusion proteins (e.g. RBM-MKL1) and non-fusion proteins (CEBPA whose loss of function leads to excess blasts)
function in a similar transcriptional manner, maintaining the theme of hematopoiesis and its
dysregulation being a result of altered transcriptional control (47, 48).

Activation of proliferative signaling can help AML maintain a clonal advantage over
non-transformed cells. FLT3-ITD (inter tandem duplication) and FLT3-TKD (tyrosine kinase
domain mutation) activate downstream RAS/RAF/MEK/ERK (proliferation) and
PI3K/AKT/mTOR (survival) signaling pathways (49). NRAS (and occasionally KRAS) can also
be mutated as well as c-KIT (upstream of STAT3 and RAS) (50). Mutated PTPN11 (also known
as SHP2) is an oncogene whose loss of regulation over the RAS pathway leads to excess
proliferative signaling. Similar to the “two hit” model of leukemogenesis, these mutations often
coop-occur with chromosomal abnormalities in hematopoietic transcription factors to gain both a
proliferative advantage while becoming more stem-like and resistant to cell death.

Tumor suppressor mutations can help AML maintain a proliferative advantage (41, 50).
TP53 mutations are common in most solid tumors but rare in AML cases (~8-10%). They are
more common in the elderly and provide the most dismal outcomes (51). WT1 is mutated in
~6% of AML and is a transcription factor which regulates proliferation and differentiation.
NPM1 is often mutated in ~20-25% of AML cases. In normal physiology, it is located in the
nucleus where it acts as a chaperone for tumor suppressor proteins like ARF and p53 in the DNA
damage response. It also plays a regulatory role in histone chaperoning, centrosome duplication,
ribosomal biogenesis, among its many roles. In aberrant cells, NPM1 is truncated and localizes
to the cytosol, losing these nuclear roles, taking the “brakes” off of the cell.

Similar to the partial role of tumor suppressors, the cohesin complex helps maintain
chromosomal integrity (52). The complex is responsible for the equal separation of sister
chromatids during anaphase. Disruptions in members of these complexes (e.g. STAG1/2,
SMC1/3A, and Rad21) can lead to genetic instability that can lead to further gene mutations with the potential to become oncogenic (53).

Epigenetic DNA methylation of histones can alter how open the chromatin structure is with a more open structure leading to more transcription (41). DNMT3A is the most commonly mutated member of this class (~20-28% of AML) and loss of function leads to impaired differentiation (41). Epigenetic alterations are often mutually exclusive of each other as they often share the same pathway (e.g. TET2, WT1, IDH1, and IDH2 in the WIT pathway). WT1, IDH1/2, and TET1/2/3 are a part of the tumor suppressor WIT pathway (54). WT1 binds to the DNA and recruits TET1/2/3 to help demethylate 5-methylcytosine (5mC), with TET2 being the first of the three enzymes in this conversion changing 5mC to 5-hydroxymethylcytosine (5hmC). Mutant WT1 does not bind to the DNA or TET2, halting enzymatic activity. Mutant TET2 prevents binding of TET2 to either WT1 or α-KG (alpha-ketoglutarate), preventing the conversion of 5mC to 5hmC. IDH1/2 generates α-KG which binds and promotes TET2 enzymatic activity. This is disrupted by gain of function IDH1/2 mutants that generate the oncometabolite 2-HG (alpha-hydroxyglutaric acid), which inhibits TET2 enzymatic activity. Other chromatin remodelers often dysregulated in AML include ASXL1 (more common in the elderly), EZH2, and KMD6A.

The spliceosome processes pre-mRNA, removing introns and sometimes exons (55). Mutations in the spliceosome (e.g. SF3B1, SRSF2, U2AF1, and ZRSR2) can affect how RNA is processed leading to alternatively spliced exons or non-functional proteins. This can affect many downstream processes including chromatin structure, cell cycle control, and DNA repair. These mutations are more common in older patients and are associated with mutations of chromatin modifying genes (56).
While age is a major risk factor in treatment, certain mutations of AML have worse outcomes than others (57, 58). This may be due to the limited treatment options available and may change as new targeted therapies become available and data is generated from the newly approved AML therapies. The European Leukemia Net (ELN) generated an updated 2017 risk stratification based on 1540 AML patients who were screened for 111 myeloid cancer genes and chromosomal abnormalities (58). (Table 1.2) AML patients harboring TP53 mutations have by far the worse prognosis. Half of AML cases are cytogenetically normal, having no chromosomal abnormalities, while those with chromosomal loss or complex karyotypes (3 or more chromosomal abnormalities) are at a higher risk. MDS and MPN can be considered as pre-leukemia as the cells are partly transformed. Knowing how aggressive the AML is can guide treatment decisions. A more aggressive treatment (e.g. chemotherapy, hematopoietic stem cell transplant [HSCT]) may be necessary to cure a high risk patient but would provide excess toxicity in a lower risk patient. Alternatively, a higher risk patient may have a better quality of life with non-curative treatment if curative treatment is unlikely to succeed.
<table>
<thead>
<tr>
<th>Risk Category</th>
<th>Status</th>
</tr>
</thead>
</table>
| Favorable     | -(t(8;21)(q22;q22.1); RUNX1-RUNX1T1  
- inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); CBFB-MYH11  
- Mutated NPM1 without FLT3-ITD or with low allelic ratio (<0.5) of FLT3-ITD  
- Biallelic mutated CEBPA |
| Intermediate  | - Mutated NPM1 and high allelic ratio (>0.5) of FLT3-ITD  
- Wild-type NPM1 without FLT3-ITD or with low allelic ratio (<0.5) of FLT3-ITD (without adverse-risk genetic lesions)  
- t(9;11)(p21.3;q23.3); MLL3-KMT2A  
- Cytogenetic abnormalities not classified as favorable or adverse |
| Poor          | -(t(6;9)(p23;q34.1); DEK-NUP214  
- t(v;11q23.3); KMT2A rearranged  
- t(9;22)(q34.1;q11.2); BCR-ABL1  
- inv(3)(q21.3;q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM (EVI1)  
- Monosomy 5 or del(5q); monosomy 7; monosomy 17/abn(17p)  
- Complex karyotype, monosomal karyotype  
- Wild-type NPM1 and high allelic ratio (>0.5) of FLT3-ITD  
- Mutant RUNX1, ASXL1, or TP53  
- Advanced age  
- Poor performance Status  
- Prior radiation or chemotherapy  
- Prior MDS or MPN |

Table 1.2. ELN risk stratification of molecular, genetic and cytogenetic alterations. (58)
1.1.5 Standard of Care

For the last several decades the standard of care in AML has been the “7 + 3” cytarabine (ara-C) and daunorubicin regimen developed in 1973 (59). Four years later, bone marrow transplant was found to cure some patients but many patients are unable to tolerate this intensive regimen (60). The only advancements from 1977 until 2017 were the approval and removal of a single drug (gemtuzumab ozogamicin known as GO), improvements in the use of standard chemotherapy and bone marrow transplant, and the use of arsenic trioxide in 2000 and all-trans retinoic acid in 2004 for APL (61-64). It should be noted that my dissertation research started in early 2016 when considering past and future research directions (especially toward the development of putative Mcl-1 inhibitors).

Older patients remain difficult to treat, as only 40% undergo chemotherapy within 3 months of diagnosis (14). (Note: the definition of older patients varies widely in AML and cancer literature.) This is because curative treatment involves intense chemotherapy followed by HSCT which also carries high risks. As patients age, the performance status worsens, leading to more treatment related mortality. Despite these challenges, treatment outcomes have improved from the 1970’s, with 5 year survival for those over 60 years of age improving from 8% with a median of 1.8 months to 13% with a median of 11.5 months (65). Supportive care has made HSCT more tolerable, even to patients in their 70’s (something unthinkable in years prior) (66). Growth factors and transfusions help combat cytopenias (low blood cell counts of specific blood cell types). Antibiotics and antifungals help patients with suppressed immune systems handle pathogens. The outcome is grimmer as age increases, with 5 year survival for those over 75 years of age remaining under 5%. In phase III trials of R/R (relapsed/refractory) AML of HDAC (histone deacetylase) inhibitor or investigator choice, CR (complete response) rates of 12-18%
were achieved with 3.3-6.3 month OS (overall survival) (67, 68). These patients may benefit from less intensive, leukemic reduction regimens, but the goal of treatment is less curative and more supportive. Outside of the relatively curable APL patients, new treatments are needed that address the genetic foundation of AML and the frailty of the patients both from the disease and advanced age.

**Figure 1.2. Recent history of AML treatment and classification.** A simplified timeline of the recent history of AML. 7+3: cytarabine and daunorubicin, BMT: Bone marrow transplant, FAB: French-American-British, WHO: World Health Organization, GO: gemtuzumab ozogamicin, As$_2$O$_3$: Aresenic trioxide, ATRA: all-trans retinoic acid

### 1.1.6 Recent FDA Approvals

The decades of research into the genetic foundations of AML has finally paid off with the approval of 4 new drugs in 2017 and another 4 new drugs in 2018 (timeline in Figure 1.2). Due to the poor outcomes of older AML patients, the FDA (Food and Drug Administration) considers
CR or CR with partial/incomplete hematological recovery (CRh) an acceptable outcome for improving patient outcomes for those patients where the intent of therapy is not curative.

2017 started with the approval of a targeted therapy, the oral kinase inhibitor midostaurin (trade name Rydapt, molecule name PKC412) for FLT3-mutated AML (69). FLT3 mutations are associated with poor survival and are present in about ~30% of AML cases (30, 70, 71). The FLT3 status (WT [wild type], ITD, TKD D835 or I836) is tested by the companion FDA approved LeukoStrat CDx FLT3 Mutation Assay. The name PKC412 comes from initial studies of its inhibition of Protein Kinase C (PKC). Midostaurin’s activity against mutated c-KIT led to its approval for ASM, SM-AHN (systemic mastocytosis with associated hematologic neoplasm), and MCL (mast cell leukemia) (72). Several other more specific FLT3 inhibitors are at various stages of development with the broadly acting midostaurin being the first to gain FDA approval. The FDA approval for AML was based on the Phase III clinical trial NCT00651261. 717 FLT3-mutated patients from a pool of 3277 patients aged 18-59 received standard daunorubicin and cytarabine induction and consolidation therapy. One of the randomized cohorts received 50 mg BID (twice daily) midostaurin on days 8-21 of induction and consolidation and for an additional 12 months. The survival curves quickly separated but remained parallel, meaning there was efficacy for induction therapy but not for maintenance therapy. The 4 years survival benefit was 51% vs 44% and CR in 59% vs 53% of patients in the midostaurin plus 7+3 and 7+3 arms, respectively. 56% of patients had to interrupt dosing due to an adverse event and 21% had to discontinue the drug entirely. 59% of patients were able to progress to allogenic HSCT. FLT3 has proven to be a viable target for AML but the outcome improvements are meager and may be due to off target effects. Future FLT3 inhibitors and study of current generation drugs to use in combination with FLT3 inhibitors will guide and potentially improve these treatments.
A second targeted therapy, the oral IDH2 inhibitor enasidenib (trade name Idhifa, molecule name AG-221) was approved for R/R AML with IDH2 mutations based on the single arm phase I/II NCT01915498 trial (73). IDH2 mutations were tested by the RealTime IDH2 Assay to detect R140 and R172 mutations that are present in ~10% of adult AML cases (30). The recommended dose based on dose escalation studies was 100 mg QD (daily) until disease progression or unacceptable toxicity. 199 patients from 2 cohorts 19 to 100 years of age were selected. 40% of patients responded to treatment and 23% of patients had CR/CRh with a median duration of response (DOR) of 8.2 months. 34% of the patients became red blood cell (RBC) or platelet transfusion independent. 43% of the patients had to interrupt dosing due to an adverse event and 17% had to discontinue the drug entirely. For the patients who responded to the drug, median survival was 9.3 months. Again, meager outcome improvements are promising for an AML targeted therapy but treatment outcome improvements are urgently needed.

Mylotarg (also known as gemtuzumab ozogamicin) is an antibody drug conjugate delivered by IV (intravenous) which was initially approved for CD33+ AML (85-90% of cases) in 2000, but was withdrawn after failing to show clinical benefit while also having excess mortality in 2010 (phase III NCT00085709) (74). After a re-evaluation of the dosing schedule, toxicity was found to be reduced with more frequent doses at lower concentrations while retaining efficacy in subsets of patients. In the phase III study NCT00927498, 271 patients aged 50 to 70 years old were randomized into two arms of 7+3 with or without mylotarg at 3mg/m² over 2 h on days 1, 4, and 7 of induction and day 1 of 1st and 2nd consolidations. Mylotarg improved EFS (event free survival, 17.3 v 9.5 months) but not overall survival (27.5 v 21.8 months, not significant). Other trials have shown efficacy as single agent over BSC (best supportive care). Overall, mylotarg is another case of an incremental success in AML targeted
therapies as well as a reminder that optimization of dosing schedules and patient subsets to treat can improve patient outcomes.

Vyxeos is a liposomal formulation with cytarabine and daunorubicin at a 5:1 molar ratio to optimize synergy of the 7+3 standard of care. It was FDA approved for therapy related AML (t-AML) and AML with myelodysplasia related changes (AML-MRC). The phase III NCT01696084 trial, randomized 309 newly diagnosed t-AML and AML-MRC patients aged 60-75 to Vyxeos or 7+3. Vyxeos was administered by IV over 90 minutes on days 1, 3, and 5 at 44 mg/m² daunorubicin and 100 mg/m² cytarabine and days 1 and 3 for the second induction, and at 29 and 65 mg/m² on days 1 and 3 of consolidation. Vyxeos improved survival against 7+3 (9.56 v 5.95 months) and reduced 30 and 60 day mortality (75).

2018 continued with 4 more AML drug approvals starting with the approval of oral IDH1 inhibitor ivosidenib (Tibsovo) for patients with IDH1 mutated R/R AML. Ivosidenib preferentially inhibits R132 mutated IDH1 over wild type IDH1 and does not inhibit IDH2 at physically relevant concentrations. The RealTime IDH1 Assay companion test determines IDH1 R132 mutational status, which is a gain of function mutation present in about ~7% of patients (30, 76). The approval came after the results of a phase I single arm 3+3 dose escalation trial to determine the phase II recommended dose. 258 IDH1 mutated patients including 179 with R/R AML received 500 mg of ivosidenib daily. 33% had a CR/CRh with a median DOR of 8.2 months. The drug was well tolerated with 38% of patients experiencing a dose interruption and 13% discontinuing treatment due to adverse events (AEs). As mutant IDH1 prevents blood cell differentiation, differentiation syndrome must be monitored (experienced in 20% of patients). In 2019, ivosidenib was approved for de novo AML for patients with an IDH1 mutation and are
either over 75 or chemotherapy ineligible. As with many of the other new AML therapies, OS is poor and long term OS benefits are yet to be determined.

Glasdegib (trade name Daurismo, also known as PF-04449913), which inhibits Smoothened in the sonic hedgehog signaling (SHH) pathway, was approved in combination with low dose cytarabine (LDAC) for patients older than 75 or chemotherapy ineligible (77). SHH signaling and subsequent transcription is important for regulation of differentiation and development, is often activated in AML, and is associated with poor survival and persistence of cancer stem cells (CSCs) (78). The phase Ib/II trial NCT01546038 recruited 114 patients who were either older than 75 years or older than 55 years with an ECOG (Eastern Cooperative Oncology Group) performance status of at least 2. The phase I MTD (maximum tolerated dose) of glasdegib was 400 mg but 100 mg was chosen for the Ib/II study. At 100 mg, glasdegib inhibited 90% of smoothened. 20 mg of LDAC was administered daily on days 1-10 of the cycle and 100 mg glasdegib was given in one of the two arms on days 1-28 of the cycle. This continued until disease progression or unacceptable toxicity. Despite the low statistical power of the study (single sided α=0.1) the results were striking. Glasdegib and LDAC improved OS 8.3 vs 4.3 months in comparison to LDAC. Future trials with greater statistical power will further determine OS differences and potential benefit in MDS.

The oral Bcl-2 inhibitor venetoclax was also approved for patients older than 75 or were chemotherapy ineligible in combination with either azacitidine or decitabine or LDAC (79). Bcl-2 is an anti-apoptotic protein overexpressed in AML that prevents cells death, increases chemoresistance, and is associated with poor outcomes (80, 81). Venetoclax was approved in CLL in 2016 and is well tolerated in older patients and patients with poor performance status. In AML, venetoclax has shown promise but response rates as a single agent are relatively low (82).
The approval of venetoclax was the result of two trials, the phase Ib NCT02203773 and the phase I/II NCT02287233. Enrolled patients were newly diagnosed AML patients older than 75 years and patients older than 55 who are chemotherapy ineligible. Due to the risk of tumor lysis syndrome (TLS, where too many tumor cells dying causes cellular contents to enter the bloodstream with potentially toxic levels of metabolites and electrolytes), a dose escalation schedule was used for venetoclax. The azacitidine arm was given azacitidine on days 1-7 of each 28-day cycle at 75mg/m². The decitabine arm was given decitabine on days 1-5 of each cycle at 20 mg/m². The LDAC arm was given LDAC on days 1-10 of each cycle on days 1-10 of each cycle. On days 1, 2, and 3; 100, 200, and 400 mg of venetoclax were given respectively. Day 4 and beyond, 400 mg of venetoclax was given in the azacitidine or decitabine arms and 600 mg of venetoclax in the LDAC arm. 25 of 67 patients in the azacitidine arm had a CR with a median DOR of 5.5 months. 7 of 13 patients in the decitabine arm had a CR with a median DOR of 4.7 months. 13 of 61 patients in the LDAC arm had a CR with a median DOR of 6 months. Efficacy will be further determined in the phase II NCT02993523 and NCT03069352 trials. While the results are an improvement for the sensitive, older population, further improvements with venetoclax combination therapies are needed.

The latest approval for AML is the oral FLT3 inhibitor gilteritinib (XOSPATA), which was approved for FLT3-mutanted R/R AML (83). FLT3 status was determined by the FDA approved LeukoStrat CDx FLT3 Mutation Assay. The approval was based on the phase III trial NCT02421939 in which 138 R/R AML patients with a FLT3-ITD or FLT3-TKD mutation received 120 mg of gilteritinib daily. 12% of patients had a CR with an 8.6 month median DOR (21% CR/CRh, 4.6 month DOR). Ongoing clinical trial data after approval with additional patients showed a survival benefit versus doctor’s choice (OS 9.3 v 5.6 months) and 8% had to
discontinue the drug due to AEs (83). Further studies with gilteritinib combinations and other FLT3 inhibitors are ongoing (84).

Despite these advances, many patients do not have the targetable mutations (e.g. FLT3, IDH1/2), do not respond to, or relapse on current therapies. Even for those who do respond to therapy, response rates and survival remain frustratingly low. In addition, many elderly patients cannot tolerate intensive chemotherapy and forego treatment all together (14). In summary, there remains an unmet need for more effective treatments that are tolerated by more patients in AML.

### 1.2 Apoptosis Pathways

All multicellular organisms depend not only on the controlled proliferation of cells, but also on the removal and turnover of old and damaged cells (2). This process is important for many cellular processes, including but not limited to: neuronal pruning, turnover of blood cells, and the removal of non-functional and autoimmune lymphocytes (85). Unlike necrosis, which is unregulated and involves the spilling of cell contents and subsequent damage to surrounding tissues, apoptosis (programmed cell death [PCD]) is a highly regulated process that ends in neatly packaged cell components being engulfed by phagocytes. Apoptosis is initiated by two main pathways: internal signals (intrinsic apoptosis) and external signals and receptors (extrinsic apoptosis) (86). In cancers including AML, avoiding apoptosis allows the cells to survive, even when overcrowded, stressed, damaged, and/or abnormal (87).
1.2.1 Extrinsic Apoptosis

The extrinsic apoptotic process starts with signaling from the tumor necrosis factor (TNF) family on the surface of the cell (85) (Figure 1.3). The Fas ligand (FasL) binds to Fas, TNFα binds to TNFR (TNF receptor), and TNF-related apoptosis inducing ligand (TRAIL) binds to death receptors 4 and 5 (DR4/5) (88). These receptors and ligands are expressed in response to cellular and environmental signals. Once one of these receptors is bound, the intracellular death domain (DD) undergoes a conformational change to release the inhibitory silencer of death domain (SODD) bound to the DD. The DD of the cellular membrane receptor can then bind to DD of the Fas-associated protein with death domain (FADD). The death effector domain (DED) of FADD can either be inhibited by proteins such as c-FLIP (cellular FLICE [FADD-like IL-1β-converting enzyme]-inhibitory protein) or bind to the DED of pro-caspases 8 or 10. Caspases start as inactive zymogens (pro-caspases) which are activated by cleavage of the protein (89). TNFα can also bind to Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD, also known as CRADD), which binds to Receptor Interacting Protein (RIP), which binds to RIP-associated protein with a death domain (RAIDD) to bind to and activate pro-caspase 2 through the caspase activation and recruitment domain (CARD). Activated “initiator” caspases 2, 8, and 10 subsequently activate “executioner” caspases 3, 6, and 7 as well as activating pro-apoptotic Bid of the intrinsic apoptotic pathway through cleavage.
Figure 1.3. **Extrinsic apoptotic pathway.** FasL is Fas ligand. DR4/5 is Death Receptor 4/5. TRAIL is tumor necrosis factor-related apoptosis inducing ligand. TNF-α is tumor necrosis factor α. TNFR is tumor necrosis factor receptor. DD is death domain. FADD is Fas-associated protein with death domain. DED is death effector domain. Pro-cas is pro-caspase and cas is caspase. Bid is BH3 Interacting Domain Death Agonist and t-Bid is truncated Bid. L and S are the large and small subunits of caspase. c-FLIP is cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein. TRADD is Tumor necrosis factor receptor type 1-associated DEATH domain. RIP is receptor interacting protein. RAIDD is RIP-associated protein with a death domain. CARD is caspase activation and recruitment domain. (85, 88)
1.2.2 Intrinsic Apoptosis

The intrinsic apoptotic pathway responds to internal cellular stresses and is a caspase and mitochondrial dependent process (85). The balance towards survival or death can be directed by many factors, such as the loss of growth signaling, loss of apoptotic suppression, radiation, toxins, DNA damage, viral infections, free radicals, and hypo/hyper-thermia. The initiation of apoptosis starts with a shift in favor of the pro-apoptotic Bcl-2 (B cell lymphoma 2) family proteins over the anti-apoptotic Bcl-2 family members that normally sequester them (these interactions are explained further in section 1.2.4). Eventually, pro-apoptotic family effectors such as Bak and Bax are activated by excess pro-apoptotic activators, leading to pores on the outer mitochondrial membrane (OMM). This leads to the release of cytochrome c (cyt c) from the intermembrane space (IMS) of the mitochondria and the decoupling of the energy gradient (mitochondrial outer membrane potential, MOMP) in the mitochondria (Figure 1.4). The loss of the energy generation potential of the powerhouse of the cell, signals that the cell is starting programmed cell death. APAF-1 (Apoptotic peptidase activating factor 1) binds to cyt c through its WD-40 domains (repeat domain which is 40 amino acids long and ends in a tryptophan-aspartic acid [W-D] dipeptide) and binds to and activates procaspase 9 through its CARD domain, forming a heptameric complex. Initiator caspase 9 cleaves pro-caspases 3, 6, and 7 into active caspases. SMAC (second mitochondria-derived activator of caspases, also known as DIABLO) and Omi (Omi stress-regulated endoprotease, released by the mitochondrial pores like cyt c) sequester XIAP (X-linked inhibitor of apoptosis) and other IAPs (inhibitors of apoptosis) from inhibiting activator caspase activation. Subsequent steps of apoptosis include the degradation and packing of the cellular contents, including but not limited to the cleavage of DNA into fragments, the cleavage and degradation of DNA repair proteins such as PARP (poly
[ADP-ribose] polymerase), and the localization of phosphatidylserine (PS) to the outer membrane to promote immune engulfment (90-92). The localization of PS to the inner membrane during normal cell conditions is controlled by the ATP (adenosine triphosphate) dependent flippase, which loses its ATP supply during apoptosis (93).

Figure 1.4. Intrinsic apoptotic pathway. cyt c is cytochrome c. SMAC is second mitochondria-derived activator of caspases. Omi is Omi stress-regulated endoprotease. CARD is caspase activation and recruitment domain. L and S are the large and small subunits of caspase. WD-40 is the WD-40 repeat domain which is 40 amino acids long and ends in a tryptophan-aspartic acid (W-D) dipeptide. XIAP is X-linked inhibitor of apoptosis. (85)
1.2.3 Detecting Apoptosis

The cellular changes that occur during apoptosis can be used to observe apoptosis. Visually, the cells will have irregular membranes that eventually fragment. These alterations in the membrane only occur well after apoptosis has been initiated (94). DNA fragmentation between centrosomes creates DNA “ladder” fragments of multiples of the length between centrosomes during late apoptosis. During apoptosis and before the DNA ladder, DNA is cut in preparation for fragmentation. These cuts can be observed by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay. These cuts can also be the result of DNA damage, so the assay is non-specific (95). Cleavage of the DNA repair protein PARP can also indicate that the cell is giving up on repairing DNA and undergoing apoptosis. (Both cleavage of caspase 3 (an execution apoptotic caspase) and PARP are shown throughout the dissertation work.) Phosphatidylserine (PS) is normally on the inside of the cell membrane but can be expressed on the outside of the membrane during early apoptosis. This is recognized by phagocytes which engulf the cell. Annexin V (AnnV) can bind to PS and be quantified by flow cytometry to measure apoptotic cells (used often in the dissertation work) (96). Charged dyes such as propidium iodide (PI) can penetrate cell membranes that are not intact (e.g. dead cells). Mitochondrial assays can measure the maintenance of the voltage gradient necessary for energy generation and the release of cytochrome c (97). A caveat of these assays is many of the observations of late apoptosis coincide with dead cells in general. After cells die, they degrade and are hard to detect in vitro (often leading to an underestimate of the number of dead cells).
Figure 1.5. Bcl-2 Family proteins. BH is the Bcl-2 homology domain. TM is the transmembrane domain. Bad is Bcl-2 associated agonist of cell death. PUMA is p53 upregulated modulator of apoptosis. NOXA is Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA is Latin for damage). Bid is BH3 interacting-domain death agonist. Bim is Bcl-2-like protein 11. Bcl-2 is B cell lymphoma 2. Bcl-xL is B-cell lymphoma-extra large. Mcl-1 is myeloid cell leukemia 1. Bak is Bcl-2 antagonist/killer 1. Bax is Bcl-2-associated X protein. (98)
1.2.4 The Bcl-2 Family of proteins

The intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins which are grouped into four categories (98). While there are over 20 Bcl-2 family proteins with a variety of roles, we will discuss the members most relevant to AML. They are categorized by their Bcl-2 homology domains (BH) and their roles in intrinsic apoptosis (Figure 1.5).

The effectors, such as Bak (Bcl-2 Antagonist/Killer 1) and Bax (Bcl-2-associated X protein), form oligomers, which form larger pores in the outer mitochondrial membrane which release cytochrome c to activate apoptosis. Both Bax and Bak have BH1, BH2, and BH3 domains which form a pocket that can bind to proteins with a BH3 domain (99). BH3 only proteins can activate effectors through a conformational change to form pores. Alternatively, anti-apoptotic proteins can sequester effectors and inhibit apoptosis. Bak is localized to the mitochondria and binds to Mcl-1 and Bcl-xL (B-cell lymphoma-extra large) (100). Bax is localized in the cytosol but moves to the mitochondria upon apoptotic signaling. Bax binds to Bcl-2, Bcl-xL, and Mcl-1 (myeloid cell leukemia 1).

The anti-apoptotic members, such as Bcl-2, Mcl-1, and Bcl-xL, can bind to and inhibit the effectors, as well as bind to BH3 only proteins, that promote apoptosis and have BH1, BH2, BH3, and BH4 domains (101). These proteins are often overexpressed in cancers as a way to avoid cell death and overcome the proliferative oncogenic stresses. Bcl-2 was first implicated in lymphomas with chromosome 14 to 18 translocations which place the highly expressed immunoglobulin (IgG) heavy chain locus next to the Bcl-2 gene, increasing Bcl-2 protein levels (102). Bcl-xL, like Bcl-2, is inhibited by Bad and is necessary for platelet survival (103). Mcl-1 is inhibited by NOXA and also plays a role in the DNA damage response (DDR) (104).
The activators, such as Bim (Bcl-2-like protein 11) and Bid (BH3 interacting-domain death agonist), possess a BH3 domain, and can activate the effectors or sequester the anti-apoptotic proteins. This is also where the intrinsic and extrinsic apoptotic pathway crosstalk. Bim binds strongly to multi-domain Bcl-2 family proteins while Bid binds less strongly to Mcl-1. Truncated Bid (t-Bid) as a result of extrinsic or intrinsic apoptotic signaling translocates to the mitochondria to enhance its pro-apoptotic activity (105). PUMA (p53 upregulated modulator of apoptosis, sometime considered a direct activator) binds to Mcl-1, Bcl-2, and Bcl-xL and is upregulated in response to stress signaling in addition to p53 (106).

The sensitizers, such as Bad (Bcl-2 associated agonist of cell death), PUMA (may be considered an activator), and NOXA (Phorbol-12-myristate-13-acetate-induced protein 1, NOXA is Latin for damage), also possess only a BH3 domain but are not potent enough to directly activate the effectors at physically relevant concentrations (107). They can, however, bind to the anti-apoptotic proteins and indirectly free up effectors and activators to induce apoptosis. Bad binds to Bcl-2 and Bcl-xL. When phosphorylated by growth factors or other signals, Bad becomes anti-apoptotic and acts as a link between growth and apoptotic signaling (108). NOXA binds to Mcl-1 and can promote its degradation (109). It can compete with Mcl-1 binding to Bak and with Bim binding to Mcl-1, preventing apoptosis inhibition (110). PUMA and NOXA may be considered activators but are much weaker than Bim and Bid (98).

Several models have been proposed for interactions between the anti-apoptotic and pro-apoptotic Bcl-2 family members (Figure 1.6) (107). In the displacement model, BH3 only proteins inhibit anti-apoptotic proteins. This allows the effectors to induce apoptosis. In the direct activation model, sensitizers inhibit anti-apoptotic proteins which inhibit activators, resulting in activation of effectors. The most recent embedded together model, takes into account
reciprocal inhibition between anti-apoptotic proteins with sensitizers, activators, and effectors. It is the culmination of affinity and concentration of these members that determine the availability of apoptotic proteins to induce cell death.

The Bcl-2 family members also have many non-apoptotic roles, as well (111). Some of these roles, such as Mcl-1 localizing to the nucleus to aid in DNA repair responses, relate tangentially to the stress and damage versus survival decision that underlie the apoptotic response. The localization of Bcl-2 family proteins can affect their function. Cytosolic proteins such as Bax require activation to enter the OMM preventing their spurious activation as well as providing a mechanism of control for its function. Mcl-1 can be truncated and translocated to the mitochondrial intermembrane space (IMS) or inner mitochondrial membrane (IMM) to modulate oxidative phosphorylation (112). Other roles for Bcl-2 family proteins include mitochondrial fusion/fission required for mitochondrial health, the unfolded protein response (UPR), Ca^{2+} regulation in the endoplasmic reticulum (ER), and autophagy which involves non-apoptotic BH3 domain containing proteins such as Beclin-1 (111).
Figure 1.6. Interaction models for Bcl-2 family proteins. (107)

1.2.5 Dysregulation of the Bcl-2 family

Too little or too much cell death can have negative effects for the organism. Excess programmed cell death is seen in degenerative disorders, and human immunodeficiency virus (HIV) which cleaves anti-apoptotic Bcl-2, upregulates pro-apoptotic Bax, as well as activates extrinsic apoptosis (113, 114). A lack of apoptosis can cause excess proliferation, not only in cancer, but also in autoimmune diseases (115). In Crohn’s disease, excess Bcl-2 leads to less T cell death, T cell accumulation, and chronic inflammation (116). The gene *Bcl-2* (*B cell lymphoma 2*) is associated with diffuse large B cell lymphoma (DLBCL) and was later found to be associated with many hematological malignancies including but not limited to CLL, multiple myeloma (MM), ALL, and AML (80, 117-121). As cancer cells are more stressed (e.g. reliance on growth factors due to excess proliferation, excess DNA damage and replication leading to
increased reliance on DNA repair) they are closer to being lethally stressed. The evasion of programmed cell death becomes more important relative to less stressed non-malignant cells.

**1.2.6 Targeting the Bcl-2 family**

The anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 proteins have been known to play an important role in apoptosis dysregulation in AML (80, 117-120). Bcl-2 is a particularly attractive therapeutic target as it is overexpressed in bulk AML and LSCs responsible for relapse and is associated with poor prognosis (18). While inhibitors of the Bcl-2 family have been promising in AML and other hematological malignancies, inhibition of Bcl-xL has been associated with platelet death and thrombocytopenia (reduced platelet count) (117).

Oblimersen (G3139) was the first drug developed to target Bcl-2 to reach clinical trials in 2003 (NCT00039117) (122). This was the culmination of years of progress: the discovery of the t(14;18) translocation in 1978, the discovery of its pro-survival function in 1988, the mechanistic studies of intrinsic apoptosis and the development of inhibitors preclinically in the 1990’s (123). The drug worked by delivering anti-sense RNA to bind to Bcl-2 mRNA and block translation of the Bcl-2 protein (124). Due to a lack of efficacy, clinical trials of this drug have been stopped in lieu of more promising agents.

ABT-737 was one of the earlier BH3 mimetics which sought to mimic the BH3 only proteins to bind to the binding pocket formed by the BH1, BH2, and BH3 domains of the anti-apoptotic proteins (125, 126). It was developed based on the crystal structure of Bcl-xL and how it binds to its target substrates. It was able to bind to Bcl-2 and Bcl-xL with a much higher affinity than previous BH3 mimetics. While showing good preclinical efficacy, ABT-737 was not orally bioavailable, leading to reformulation (127).
Navitoclax (ABT-263), a result of that reformulation, is an oral inhibitor of Bcl-2 and Bcl-xL. However, due to its inhibition of Bcl-xL, it depleted platelets leading to dose limiting toxicity (128). It entered the clinic in 2009 but did not gain FDA approval. It has been tested in clinical trials in solid tumors which tend to be more Bcl-xL dependent (129, 130).

A new Bcl-2 specific inhibitor venetoclax (also known as ABT-199), improves upon its predecessor navitoclax by not targeting Bcl-xL (131). [Note: Venetoclax was in phase I-II trials in AML and was FDA approved for 17p deletion CLL at the start of the dissertation work. It was approved in combination with other agents in AML on November 21, 2018. However, poor overall survival with monotherapy remains a concern at the writing of this dissertation.] Our group and others have reported that venetoclax has promising activity against preclinical models of AML and other cancers (131-137). While venetoclax alone has promising clinical activity, relapse remains a concern (average time to relapse is 2.5 months) (82, 138). Combination therapies (e.g. with decitabine, azacitidine, or LDAC) increased remission rates and had low early mortality but long term improvements have yet to be determined (139, 140). Survival improvements have been modest and new therapies are needed to target AML and the LSCs responsible for relapse.

1.2.7 Resistance to Bcl-2 inhibition through Mcl-1

Our lab and others have shown that resistant AML cell lines and patient samples upregulate Mcl-1 in response to venetoclax treatment (86, 141). While venetoclax treatment disrupts Bcl-2 binding to Bim, Mcl-1 compensates by binding to Bim, preventing Bim from inducing apoptosis. We found that CRISPR (clustered regularly interspaced short palindromic repeats) knockdown of Mcl-1 enhanced venetoclax activity in venetoclax resistant AML cells. Mcl-1 was found to be upregulated after venetoclax treatment through increase of its protein
stability rather than transcription. This increase in Mcl-1 protein was found to be Bim-dependent (141).

Mcl-1 is potentially a therapeutic target for overcoming resistance to venetoclax in AML. This can be achieved by direct targeting of Mcl-1, which is discussed in chapter 2. A-1210477 was one of the first putative Mcl-1 inhibitors available at the start of my dissertation work (142, 143). Other Mcl-1 inhibitors developed preclinically during the course of my study have progressed into Phase I studies and are discussed in Chapter 5, future directions. Mcl-1 can be targeted indirectly such as through protein stability which is discussed in Chapter 3. Mcl-1 is a short-lived protein and changes in protein half-life can significantly and quickly alter its protein levels (144, 145). Inhibition of XPO1 (exportin 1, chromosomal maintenance 1 [CRM1]) has anti-leukemic activity and downregulates Mcl-1 which could potentially further enhance venetoclax activity (146, 147). Mcl-1 can also be indirectly targeted transcriptionally, which is discussed in chapter 4. Inhibition of CDK9 (cyclin dependent kinase 9) has anti-leukemic activity and downregulates Mcl-1 transcription which could potentially enhance venetoclax activity (148-150).

1.3 Hypothesis and Specific Aims

To test our hypothesis that targeting Mcl-1 can overcome resistance to venetoclax in AML, my thesis work was split into three aims in chapters 2-4, respectively:

**Specific Aim 1: To determine if direct targeting of Mcl-1 with A-1210477 can synergize with venetoclax to induce apoptosis in AML cells.** Our working hypothesis was that targeting both Mcl-1 and Bcl-2 will prevent Bim sequestration, allowing Bim to activate Bak and Bax and induce apoptosis. (Daniel A. Luedtke et al. Inhibition of Mcl-1 enhances cell death...
induced by the Bcl-2-selective inhibitor venetoclax in acute myeloid leukemia cells. *Signal Transduction and Targeted Therapy*. 2017 Apr 7; 2: 17012)

**Specific Aim 2: To determine if the XPO1 selective inhibitor KPT-330 can synergize with venetoclax to induce apoptosis in AML through downregulation of Mcl-1 protein.** Our working hypothesis was that XPO1 (exportin 1) inhibition selectively targets leukemic cells and decreases Mcl-1 protein levels; therefore targeting XPO1 can synergize with venetoclax treatment. (*Daniel A. Luedtke et al. Inhibition of XPO1 enhances cell death induced by venetoclax in acute myeloid leukaemia via Mcl-1. Journal of Cellular and Molecular Medicine*. 2018; 22: 6099-6111)

**Specific Aim 3: To determine if the CDK9 selective inhibitor voruciclib can synergize with venetoclax to induce apoptosis in AML through decrease of Mcl-1 gene transcription.** Our working hypothesis is that CDK9 (cyclin-dependent kinase 9) inhibition selectively targets AML cells and decreases Mcl-1 levels through transcriptional means; therefore targeting CDK9 can synergize with venetoclax treatment. (*Pending publication by Daniel A. Luedtke et al. Inhibition of CDK9 by Voruciclib Synergistically Enhances Cell Death Induced by the Bcl-2 Selective Inhibitor Venetoclax in Preclinical Models of Acute Myeloid Leukemia. Signal Transduction and Targeted Therapy. Under review*)

Our studies seek to understand the mechanisms of resistance to venetoclax treatment to lead to novel approaches to overcome this resistance in AML. The proposed experiments provide an improved understanding of the efficacy and molecular mechanisms of targeting Bcl-2 family members for the treatment of AML.
CHAPTER 2: DIRECT TARGETING OF MCL-1 VIA A-1210477 ENHANCES VENETOLCAHX ACTIVITY

This section is partly modified from the publication by Daniel A. Luedtke et al. Inhibition of Mcl-1 enhances cell death induced by the Bcl-2-selective inhibitor venetoclax in acute myeloid leukemia cells. Signal Transduction and Targeted Therapy. 2017 Apr 7; 2: 17012

2.1 Introduction

Our previous studies showed that venetoclax treatment increased Mcl-1 levels (but not Bcl-2 and Bcl-xL) in venetoclax-resistant AML cell lines and an AML primary patient sample ex vivo (cells from an organism cultured in the lab) (141). Knockdown of Bim by shRNA (short hairpin RNA) partially abolished the increase in Mcl-1 induced by venetoclax and CRISPR knockdown of Mcl-1 enhanced venetoclax activity. This prompted our working hypothesis that targeting both Mcl-1 and Bcl-2 will prevent Bim sequestration, allowing Bim to activate Bak and Bax and induce apoptosis of AML cells.

Development of Mcl-1 inhibitors has been challenging due to its rigid and hydrophobic BH3 binding pocket. Despite this, some breakthroughs were made before the start of my thesis work in the development of Mcl-1 specific inhibitors (including A-1210477) (142). In this chapter, we tested whether direct targeting of Mcl-1 with the specific small molecule inhibitor A-1210477 could overcome venetoclax resistance in both AML cell lines and primary patient samples.
2.2 Materials and Methods

2.2.1 Drugs

Venetoclax and A-1210477 were purchased from Selleck Chemicals (Houston, TX).

2.2.2 Cell Culture

THP-1 and U937 cell lines were purchased from the American Type Culture Collection (Manassas, VA). MOLM-13 was purchased from AddexBio (San Diego, CA, USA). The cell lines were authenticated in August of 2017. The cell lines were cultured in RPMI 1640 media with 10-20% fetal bovine serum (FBS, heat treated at 56°C for 45min and stored at 4°C, Life Technologies, Carlsbad, CA) and 2 mM L-glutamine, plus 100 U/ml penicillin and 100 μg/ml streptomycin, in a 37 °C humidified atmosphere containing 5% CO2/95% air, at 1-10 E5 cells/mL counted manually by hemocytometer from Recihert (Buffalo, NY) and trypan blue (from Gibco to count dead cells, Grand Island, NY) staining. Cell lines were tested for the presence of mycoplasma 2-3 days after thawing, monthly, and before making new freezes (151). Cell lines were frozen in 1 mL aliquots of complete media plus 10% DMSO (dimethyl sulfoxide) with 5 E6 cells in Nalgene cryogenic vials (Thermo Fischer, Waltham, MA) in the Mr. Frosty Freezing container (Thermo Fischer, Waltham, MA) at -80°C to cool slowly before long term storage in liquid nitrogen. Cell line freezes were quickly thawed in a 37°C water bath, then 9 mL of complete media added to dilute DMSO, spun, and resuspended in fresh media.

Diagnostic AML blast samples derived from patients were purified by standard Ficoll-Hypaque density centrifugation, then cultured in RPMI 1640 with 20% fetal bovine serum, ITS (insulin, transferrin, sodium selenite) Solution (Sigma-Aldrich, St. Louis, MO) and 20% supernatant of the 5637 bladder cancer cell line [as a source of granulocyte-macrophage colony-
stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin-1 beta, macrophage colony-stimulating factor (M-CSF), and stem cell factor β (SCFβ)] (134, 152, 153).

2.2.3 Clinical Samples

Diagnostic blast samples were obtained from the First Hospital of Jilin University. Written informed consent was provided according to the Declaration of Helsinki. This study was approved by the Human Ethics Committee of The First Hospital of Jilin University. Clinical samples were screened for FLT3-ITD, NPM1, C-kit, CEBPA, IDH1, IDH2 and DNMT3A gene mutations and the following fusion genes by real-time polymerase chain reaction (RT-PCR): PML-RARα, BCR-ABL, AML1-MDS1, MLL-AF10, MLL-AF4, MLL-ELL, SET-CAN, TLS-ERG, NPM-RARα, E2A-PBX1, AML1-EAP, MLL-AF17, MLL-AF6, MLL-ENL, SIL-TAL1, HOX11, PLZF-RARα, TEL-AML1, DEK-CAN, MLL-AF1p, MLL-AF9, NPM-ALK, TEL-ABL, EIP1L1-PDGFA, AML1-ETO, CBFB-MYH11, E2A-HLF, MLL-AF1q, MLL-AFX, NPM-MLF1, dupMLL, and TEL-PDGFB. Normal peripheral blood mononuclear cells (PBMCs) were derived from healthy donors (154, 155).

2.2.4 Western Blot Analysis

Cells were collected and spun at 1800 rpm for 5 min (Jouan CR4i centrifuge, Thermo Electron Industries SAS, Chateau-Gontier, France) to remove most of the media and transferred to a 1.5 mL tube to be spun on a microcentrifuge to remove the remaining media (Eppendorf 5415R, Hamburg, Germany). Cells were lysed (Heat Systems Ultrasonics W-375 Sonicator, Qsonica, Newtown, CT) in the presence of protease (cOmplete) and phosphatase (PhosSTOP) inhibitors (Roche Diagnostics, Indianapolis, IN) in 10 mM Tris-Cl pH 7.0 (Fisher Bioreagents, Fair Lawn, NJ) and 0.5% sodium-dodecyl-sulfate (SDS, Sigma Life Science, St. Louis, MO). After lysing, samples rested on ice for 20 min and were spun at 12,000 rpm for 10 min by
microcentrifuge. Protein concentrations were measured using the DC Protein Assay kit from BioRad (Hercules, CA) and a µQuant plate reader and the Gen5 software (BioTek Instruments, Windowski, VT).

Western blot samples were prepared with equal protein concentrations of the whole cell lysates and loading dye and heat treated (95°C for 5 min, PTC-200 Peltier Thermocycler, MJ Research, Watertown, MA). A molecular weight ladder was prepared with either MagicMark XP (Invitrogen, Carlsbad, CA) or prestained SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis) standards low range (BioRad, Hercules, CA). Samples were subjected to 7.5-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 30 min and 90V for the stacking gel phase followed by 180V for a variable time depending on the protein of interest and gel percentage. Proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc., Rockford, IL) for 250 mA for 90 min. Membranes were dried, rehydrated with methanol and water, blocked with either 5% milk or BSA, and immunoblotted with anti-Bcl-2 (ab692, Abcam, Cambridge, MA), -Bcl-xL (2764), -Mcl-1 (4572), -PARP (9542), -Bim (2819), -γH2AX (2577), -Bak (3814), -Bax (2774), -cleaved caspase-3 (9661, designated -cf caspase-3, Cell Signaling Technology, Danvers, MA), or -β-actin (A2228, Sigma-Aldrich) antibody at 1:1000 dilution (except for β-actin at 1:10,000) overnight at 4°C or 1 h at room temperature and washed off. Secondary antibody (IRDye 680RD Goat antimouse or IRDye 800CW goat anti-rabbit, LiCor, Lincoln, NE) was applied overnight at 4°C or 1 h at room temperature and washed off. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE), as described by the manufacturer. Western blots were repeated at least 3 times and one representative blot is shown. Densitometry
measurements were made using Odyssey V3.0 (Li-Cor), normalized to β-actin on the same blot, and calculated as the fold change compared to the corresponding no drug treatment control.

2.2.5 Annexin V/PI Staining and Flow Cytometry Analysis

AML cells were treated with venetoclax or A-1210477, alone or in combination, for 4 or 24 h and subjected to flow cytometry analysis using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Kit (Beckman Coulter; Brea, CA). Briefly, 50 μL resuspended cells were transferred to clean 12 × 75 culture tubes containing 50 μL Annexin-V FITC/PI reagents in 1× binding buffer and incubated in the dark for 15 minutes. At the end of incubation, an additional 0.4 mL of 1× binding buffer was added to each tube, vortexed, and analyzed with the use of a Coulter XL Flow Cytometer (Coulter) equipped with an Argon laser. Cells were gated to include the main viable cell population based on forward scatter (FS) /side scatter (SS) characteristics (62). Cellular events with low FS/high SS character were not included in the gate because these represent a mixture of overtly necrotic cells and apoptotic bodies that can artificially elevate apoptosis levels. Apoptotic events from this intact cell gate were recorded as a combination of the Annexin-V+/PI− (early apoptotic) and Annexin-V+/PI+ (late apoptotic/dead) events. Results are expressed as percent Annexin V+. Experiments were performed 3 independent times in triplicate. For the AML cell lines, data presented are from one representative experiment, while patient sample experiments were performed once in triplicate due to limited sample. Patient samples were chosen based on availability of adequate sample for the assay. The extent and direction of anti-leukemic interaction was determined by calculating the combination index (CI) values using CompuSyn software (Combosyn Inc., Paramus, NJ). CI<1, CI=1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively (156, 157).
2.2.6 Co-immunoprecipitation (Co-IP)

AML cell lines were treated for 4 h and then the cells were lysed at 4°C for 3 h or overnight using 1% CHAPS, 5 mM MgCl$_2$, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris, and 0.05% Tween-20 in the presence of protease inhibitors as previously described (158). After determination of protein concentrations by the DC protein assay as described in the Western Blotting section, 2 μg of anti-Bim (2819, Cell Signaling Technology) or anti-Mcl-1 (SC-819, Santa Cruz Biotechnology, Santa Cruz, CA) antibody, 1 mg protein lysate, and Protein A agarose beads (Roche Diagnostics) were incubated overnight at 4°C. After washing, proteins were eluted from the Protein A agarose beads using 50 mM glycine, pH 2.0, and then analyzed by Western blotting.

2.2.7 shRNA Knockdown

The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at Tulane University. Bim and non-target control (NTC) shRNA lentiviral vectors were purchased from Aldrich. Lentivirus production and transduction were carried out according to the manufacturer’s instructions. Briefly, TLA-HEK293T cells were transfected with pMD-VSV-G, delta 8.2, and lentiviral shRNA constructs using Lipofectamine and Plus reagents (Life Technologies) according to the manufacturer’s instructions. Virus containing culture medium was harvested 48 h post transfection. Cells were transduced overnight using 1 mL of virus supernatant and 4 μg of polybrene and then cultured for an additional 48 h prior to selection with puromycin from Invivogen (San Diego, CA). The resulting puromycin-resistant cells were used to determine the effect of the genes of interest on AML cell sensitivity to venetoclax and/or A-1210477.

2.2.8 Alkaline Comet Assay
U937 cells were treated for 4 h with venetoclax and/or A-1210477 and subjected to alkaline comet assay. The harvested cells were mixed with 1.0% low melting point agarose in PBS (phosphate buffered saline, Mg and Ca free) at 37°C. Approximately 5000 cells were layered onto pre-coated microscope slides (pre-coated slides were coated with a thin layer of 1% low melting point agarose and allowed to dry for 90 min), a glass coverslip was placed on top of the cell/agarose suspension and the gels were allowed to gel for 10 min at 4°C. The slides were then placed in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO and 1% Triton X-100, pH 10.0) overnight at 4°C. The slides were placed in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13.0) for 40 min at 4°C to allow unwinding of the DNA. Electrophoresis was conducted for 30 min at 30 V (1.25 V/cm). After electrophoresis, the slides were rinsed in 400 mL distilled water, dipped in 95% ethanol and dried. DNA was stained with 1:30,000 SYBR Gold (Life Technologies) in 10 mM Tris, pH 7.5 plus 1 mM EDTA for 40 min at room temperature. The slides were rinsed in distilled water and imaged on an Olympus BX-40 microscope with an Olympus DP72 microscope camera and Olympus cellSens Dimension software (Olympus America Inc., Center Valley, PA). 50 comets per gel were scored using CometScore (TriTek Corp, Sumerduck, VA). The median percent DNA in the tail from at least three replicate gels were averaged. The error bars indicate standard errors of the means.

2.2.9 Statistical Analysis

Differences were compared using the two-sample t-test. Statistical analyses were performed with GraphPad Prism 5.0. Error bars represent ± s.e.m. (standard error of the mean). The level of significance was set at p<0.05.
2.3 Results

2.3.1 A-1210477 synergizes with venetoclax to induce apoptosis in venetoclax-resistant AML cells

To begin to test our hypothesis that Mcl-1 inhibition through A-1210477 (A) can synergize with venetoclax (VEN), we tested various concentrations of venetoclax and A-1210477, alone or in combination, in venetoclax-resistant AML cell lines (THP-1 IC₅₀ [half maximal inhibitory concentration] 2.4µM, and U937 IC₅₀ 13.5 µM) and a relapsed primary AML patient sample (154). The concentrations of venetoclax used in our studies are clinically achievable (FDA application 208573Orig1s000). The combination index (CI) was used to determine synergy. CI=1 denotes an additive effect while CI<0.9 denotes synergy, and CI<0.3 denotes strong synergy (157). At 24 h, synergy was observed between the two drugs for THP-1 (CI<0.30) and U937 (CI<0.70) cell lines (Figure 2.1 A&B). Annexin V positive cells were largely propidium iodide (PI) positive as well, indicating that the cells were late apoptotic or dead (159). Annexin V/PI staining was assessed after 4 h treatment to determine if cells may have undergone early apoptosis as venetoclax is known to show anti-leukemic activity early (160). At 4 h, synergy was still observed for THP-1 (CI<0.002) and U937 (CI<0.74) cell lines and a majority of the Annexin V positive cells were PI negative, indicating that the cells underwent early apoptosis (Figure 2.1 C&D). Corroborating this, cleavage of PARP and caspase 3 was strongly enhanced in the combination treatment when compared to venetoclax or A-1210477 alone in THP-1 and U937 cells (Figure 2.2). Similar results were achieved in a primary patient sample ex vivo (Figure 2.3, patient characteristics are in Table 2.1, collect by Dr. Hai Lin, analyzed by Xiaojia Niu). In summary, A-1210477 is able to synergize with venetoclax to induce apoptosis in otherwise venetoclax-resistant AML cells.
Figure 2.1. A-1210477 synergizes with venetoclax to induce apoptosis in venetoclax-resistant AML cell lines. (A-D) THP-1 and U937 cells were treated with venetoclax (VEN) and A-1210477 (A), alone or in combination, for 4 h or 24 h and then subjected to Annexin V/PI staining and flow cytometry analyses. ***indicates p<0.001. Combination index (CI) values were calculated using CompuSyn software. VEN, venetoclax. A, A-1210477. These abbreviations are used throughout this chapter.
Figure 2.2. A-1210477 cooperates with venetoclax to induce cleavage of caspase 3 and PARP in venetoclax-resistant AML cell lines. (A&B) THP-1 and U937 cells were treated with venetoclax or A-1210477 alone or in combination for 4 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies.
Figure 2.3. A-1210477 synergizes with venetoclax to induce apoptosis in a venetoclax-resistant relapsed primary AML patient sample ex vivo. (A&B) Primary AML patient sample AML#110 was treated with venetoclax and A-1210477, alone or in combination, for 24 h and then subjected to Annexin V/PI staining and flow cytometry analyses (panel A). Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies (panel B). ***indicates p<0.001. CI values were calculated using CompuSyn software. Patient characteristics are shown in Table 2.1.
2.3.2 A-1210477 treatment increases Mcl-1 protein levels but releases Bim from Mcl-1

Having observed the synergy between venetoclax and A-1210477, the next question was to determine how treatment affected levels of relevant Bcl-2 proteins and their interactions. Protein levels of Bcl-2, Mcl-1, Bcl-xL, and Bim were determined by Western blotting. Individual treatment with venetoclax or A-1210477 caused increased levels of Mcl-1 without changing the levels of Bcl-2, Bim, and Bcl-xL in THP-1, U937, and a primary AML patient sample derived at relapse (Figure 2.4 A-C). The occupation of the BH3 binding pocket by Bim or A-1210477 can prevent Mcl-1 degradation and ubiquitination through competition with E3 ligases (141, 161). Combination treatment appeared to decrease Mcl-1 levels compared to A-1210477 alone, though levels remained similar to or higher than that in vehicle control treated cells, suggesting that disruption of the interaction of Mcl-1 with Bcl-2 family proteins played a critical role in the synergistic effects. To determine if interaction of Bcl-2 family members with Bim were disrupted by drug treatments, co-IP was performed. A-1210477 disrupted the interaction between Bim and Mcl-1 (Figure 2.4 D), which surprisingly occurred despite the presence of elevated Mcl-1. The binding of Bim with Bcl-2 was disrupted by venetoclax and not A-1210477 (Figure 2.4 E). The binding of Bim with Bcl-2 and Mcl-1 was disrupted by the combination treatment. Bcl-xL, another anti-apoptotic Bcl-2 family member which binds to Bim, did not compensate for this disruption, indicating that Bim was possibly unbound, freeing it to carry out its pro-apoptotic role.
Figure 2.4. A-1210477 treatment increases Mcl-1 protein levels but releases Bim from Mcl-1. (A-C) THP-1, U937, and primary AML patient sample cells (AML#110) were treated with venetoclax and A-1210477, alone or in combination, for 4 h or 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements of Mcl-1 expression were measured using Odyssey Software V3.0 and normalized to actin and the no drug control. (D&E) U937 cells were treated with venetoclax and A-1210477, alone or in combination, for 4 h. Mcl-1 (panel D) or Bim (panel E) was immunoprecipitated from whole cell lysates and then subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements of Mcl-1, Bim, and Bcl-2 were measured using Odyssey Software V3.0 and normalized to actin and the no drug control (panels A-C) or to no drug control (panels D&E). *indicates the light chain of the anti-Mcl-1 or -Bim antibody.
2.3.3 The effect of Bim, Bax, and Bak knockdown on apoptosis induced by venetoclax and A-1210477 in AML cells

To confirm the contribution of Bim to apoptosis induced by venetoclax and A-1210477 combined treatment, shRNA knockdown was performed. Knockdown of Bim in U937 and THP-1 (Figure 2.5 A&C) significantly reduced apoptosis in response to A-1210477 and combined treatment in both cell lines (Figure 2.5 B&D). Bim knockdown also significantly reduced apoptosis induced by venetoclax in THP-1 cells (Figure 2.5 D). Bak and Bax individual knockdowns were performed in U937 cells due to the critical roles they play in the execution of the intrinsic apoptotic pathway (Figure 2.6 A). As expected, knockdown of Bax or Bak (especially Bak, which binds preferentially to Mcl-1) also reduced apoptosis significantly in the A-1210477 and combined treatments (Figure 2.6 B) (107). These results demonstrate that the canonical pathway of intrinsic apoptosis including Bim, Bak, and Bax plays an important role in induction of apoptosis in response to A-1210477 treatment alone or in combination with venetoclax.
Figure 2.5. The effect of Bim knockdown on apoptosis induced by venetoclax and A-1210477 in AML cell lines. (A&C) U937 (panel A) or THP-1 (panel C) cells were infected with non-template control (NTC) or Bim-shRNA lentivirus. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the knockdown. Relative densitometry measurements of Bim protein were measured using Odyssey Software V3.0 and normalized to actin and NTC. (B&D) U937 (panel B) or THP-1 (panel D) shRNA knockdown (KD) cells were treated with venetoclax and A-1210477, alone or in combination, for 4 h and then subjected to Annexin V/PI staining and flow cytometry analyses. *indicates p<0.05, **indicates p< 0.01, and ***indicates p<0.001.
Figure 2.6. The effect of Bax and Bak knockdown on apoptosis induced by venetoclax and A-1210477 in AML cell lines. (A) U937 cells were infected with non-template control (NTC-shRNA)-, Bax-, or Bak-shRNA lentivirus. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the knockdowns. (B) The U937 Bax and Bak knockdown (KD) cells were treated with venetoclax and A-1210477, alone or in combination, for 4 h and then subjected to Annexin V/PI staining and flow cytometry analyses. ***indicates p<0.001.
2.3.4 A-1210477 induces DNA damage in AML cells

Mcl-1 has a known role in both apoptosis and the DNA damage response (104). Our lab previously demonstrated that venetoclax treatment enhances DNA damage induced by DNA damaging agents (141, 160), thus the question remained whether the synergistic action of A-1210477 combined with venetoclax was due to apoptosis and/or DNA damage. γ-H2AX (phosphorylated H2A histone family member X), a surrogate marker for DNA damage was measured by Western blot (162). The level of γ-H2AX increased following venetoclax or A-1210477 treatment, and increased after combined treatment in THP-1, U937, and a patient sample (Figure 2.7 A). However γ-H2AX levels can indicate late apoptosis, as well. To further elucidate the extent of DNA damage, the comet assay was performed. DNA damage, as measured by alkaline comet assay (Figure 2.7 B&C), increased dramatically with A-1210477 treatment but not venetoclax treatment. Interestingly, combination treatment did not further increase DNA damage compared to A-1210477 treatment. While A-1210477 may induce some apoptosis by DNA damage, its synergistic interaction with venetoclax is not due to DNA damage and is likely due to the apoptotic function of Mcl-1.
Figure 2.7. A-1210477 induces DNA damage in AML cells. (A) THP-1, U937, and a primary AML patient sample (AML#110) were treated with venetoclax and A-1210477, alone or in combination, for 4 h or 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibody. Relative densitometry measurements of γH2AX protein were measured using Odyssey Software V3.0 and normalized to actin and the no drug control. (B) U937 cells were treated with venetoclax and A-1210477, alone or in combination, for 4 h and then subjected to alkaline comet analyses. Representative images are shown. (C) Comet assay results are graphed as median percent DNA in the tail from 4 replicate gels ± s.e.m. *indicates p<0.05 and **indicates p<0.01.
2.3.5 A-1210477 synergizes with venetoclax in venetoclax-sensitive AML cells

To further increase the clinical relevance of the study, A-1210477 and venetoclax combination treatment was performed in venetoclax-sensitive MOLM-13 cells, newly diagnosed AML patient samples, and normal peripheral blood mononuclear cells (PBMCs). Treatment of MOLM-13 with venetoclax and A-1210477 synergistically induced apoptosis (Figure 2.8 A, CI<0.16), accompanied by caspase 3 and PARP cleavage (Figure 2.8 B). Similar to resistant cell lines, Bcl-2 and Bim levels were unchanged and Mcl-1 was induced by A-1210477 treatment alone (Figure 2.8 C). However, Mcl-1 was not detected after combined treatment, potentially due to Mcl-1 degradation resulting from caspase activation. Treatment of three newly diagnosed AML patient samples with A-1210477 and venetoclax synergistically induced apoptosis, as well (CI<0.40, CI<0.49, CI<0.05, respectively, Figure 2.9 A&B, work by Dr. Hai Lin and Xiaoji Niu). In several relatively venetoclax-sensitive and -resistant AML cell lines and patient samples, venetoclax and A-1210477 combination treatment synergistically induced apoptosis, suggesting that this combination works regardless of venetoclax sensitivity.

Lastly, to test the effects of combined treatment on normal cells in addition to AML cells, PBMCs derived from 5 healthy donors were subjected to single drug treatment of either venetoclax or A-1210477 to determine IC\textsubscript{50} values. Venetoclax IC\textsubscript{50} values ranged from 5.5 µM to 48.3 µM, while A-1210477 IC\textsubscript{50} values ranged from 3.3 µM to 7.1 µM (Figure 2.10 A). IC\textsubscript{50} values were also determined for A-1210477 in the presence of set concentrations of venetoclax (Figure 2.10 B). It was found that A-1210477 and venetoclax act synergistically to reduce viable normal PBMCs albeit at higher concentrations than in AML (Figure 2.11), indicating that a potential therapeutic window could be found.
Figure 2.8. A-1210477 synergizes with venetoclax in venetoclax-sensitive MOLM-13 AML cell line. (A) MOLM-13 cells were treated with venetoclax and A-1210477, alone or in combination, for 4 h and then subjected to Annexin V/PI staining and flow cytometry analyses. ***indicates p<0.001. CI values were calculated using CompuSyn software. (B&C) MOLM-13 cells were treated with venetoclax and A-1210477, alone or in combination, for 4 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements of Mcl-1 expression were measured using Odyssey Software V3.0 and normalized to actin and the no drug control.
Figure 2.9. A-1210477 synergizes with venetoclax in venetoclax-sensitive primary AML patient samples *ex vivo*. Newly diagnosed primary AML patient sample cells (AML#111-113) were treated with venetoclax and A-1210477, alone or in combination, for 24 h and then subjected to Annexin V/PI staining and flow cytometry analyses. ***indicates p<0.001. CI values were calculated using CompuSyn software. Patient characteristics are shown in Table 2.1.
Figure 2.10. Effect of A-1210477 or venetoclax on normal PBMCs ex vivo. MTT assays were performed on normal PBMCs at the indicated concentrations of venetoclax (panel A) or A-1210477 (panel B) for 72 h. Patient sample data are means of duplicates due to limited sample.
Figure 2.11. A-1210477 synergizes with venetoclax in normal PBMCs ex vivo. MTT assays were performed on normal PBMCs at the indicated concentrations of A-1210477 and venetoclax, alone or in combination, for 72 h. Patient sample data are means of duplicates due to limited sample. Standard isobologram analyses of anti-leukemic interactions were performed to determine the extent and direction of the anti-leukemic interactions. The IC₅₀ values of each drug are plotted on the axes; the solid line represents the additive effect, while the points represent the concentrations of each drug resulting in 50% inhibition of proliferation. Points falling below the line indicate synergism whereas those above the line indicate antagonism.
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<th>Blast purity (%)</th>
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Table 2.1. Patient characteristics for primary AML patient samples.
2.4 Discussion

Relapse in cancer is due to remaining cells that are not targeted by therapy and are able to reconstitute the tumor. In AML, it is believed that the largely quiescent leukemic stem cells are able to avoid chemotherapy and cause relapse (18). Bcl-2 was found to be overexpressed in bulk AML and LSCs, prompting the clinical development of the Bcl-2 inhibitor venetoclax (163, 164). However, relapse remains a concern (median time to relapse was 2.5 months) (82, 138).

We previously reported that the Chk1 inhibitor LY2603618 induces DNA damage, decreases Mcl-1 levels alone and in combination with venetoclax (160). We have also shown that DNA damage induced by chemotherapy is enhanced by venetoclax (141, 165). Mcl-1 plays a role in the DNA damage response (166). While it is plausible that DNA damage induced by A-1210477 could be enhanced by venetoclax, enhanced DNA damage was not observed in the combination, ruling out the role of DNA damage in the synergy of the combination.

While targeting of both Bcl-2 and Mcl-1 synergized to induce apoptosis in AML cells, the treatment also synergistically decreased viable PBMCs, albeit at higher concentrations. While direct inhibition of Mcl-1 may be toxic, indirect inhibition of Mcl-1 may prove to be more effective. Multiple anti-leukemic therapies can also downregulate Mcl-1 and can potentially synergize with venetoclax. As Mcl-1 has a short protein half-life compare to other Bcl-2 family members, targeting Mcl-1 protein stability or transcript levels can be used to effectively enhance the anti-leukemic activity of venetoclax.

In summary, we found that inhibition of Mcl-1 by A-1210477 enhanced venetoclax-induced apoptosis in venetoclax-sensitive and -resistant AML cell lines in vitro and primary patient samples ex vivo. The enhancement in activity was at least partially dependent on the intrinsic apoptotic pathway. Venetoclax prevents the binding of Bim to Bcl-2. In venetoclax-
sensitive cells it is plausible that without increases in Mcl-1 there is not enough Mcl-1 to fully sequester Bim. This results in free Bim which can activate Bak/Bax and lead to apoptosis. In venetoclax-resistant cells, Mcl-1 binds to Bim freed by venetoclax. A-1201477 disrupts the interaction between Bim and Mcl-1, freeing Bim to activate Bak/Bax and induce apoptosis. A caveat is that A-1210477 is merely a research tool to validate the target of Mcl-1. With the validation of the efficacy of targeting both Bcl-2 and Mcl-1, the question remains as to how to target Mcl-1 in a clinically relevant and leukemic specific manner.
Figure 2.12. Proposed mechanism for the synergy between A-1210477 and venetoclax in AML cells. Venetoclax treatment (black arrows) releases Bim from Bcl-2. In venetoclax-sensitive cells, there is an inadequate amount of Mcl-1 to sequester all of the released Bim, resulting in free Bim, which can then activate Bak/Bax, leading to apoptosis. In venetoclax-resistant cells, the Bim released from Bcl-2 is sequestered by Mcl-1 (blue arrows), leading to stabilization of Mcl-1, and ultimately resulting in survival. In the combined drug treatment, addition of A-1210477 abolishes sequestration of Bim by Mcl-1 (red arrows), allowing Bim to activate Bax/Bak, resulting in enhanced apoptosis.
CHAPTER 3: INHIBITION OF XPO1 DOWNREGULATES MCL-1 AND ENHANCES VENETOLCAX ACTIVITY

This section is partly modified from the publication by Daniel A. Luedtke et al. Inhibition of XPO1 enhances cell death induced by venetoclax in acute myeloid leukaemia via Mcl-1. Journal of Cellular Molecular Medicine 2018; 22: 6099-6111.

3.1 Introduction

After having shown the efficacy of direct targeting Bcl-2 and Mcl-1 in Chapter 2, we sought to target Mcl-1 using clinically tested compounds through indirect mechanisms. Exportin 1 (XPO1), also known as Chromosome Region Maintenance 1 (CRM1), is a protein which regulates the nuclear export of client proteins and has been found to play a critical role in many cancers including prostate cancer, ovarian cancer, osteosarcoma, glioma, cervical cancer, CLL, Non-Hodgkin’s lymphoma (NHL), MM, and AML (146, 167-170).

As a nuclear exporter, XPO1 plays a critical role in the localization of many proteins as well as some mRNA transcripts, and has been shown to be required for survival of solid tumors and hematological malignancies (146, 147, 171-175). XPO1 is also the sole exporter for many tumor suppressor proteins including, but not limited to, p53, p73, FOXO, IκB, Rb, p21, and NPM (176). XPO1 inhibition has also been shown to synergize with DNA damaging agents, which potentially could synergize with venetoclax, which is known to weaken the DNA damage response (177, 178). KPT-330 (also known as selinexor) is a XPO1-selective inhibitor that is currently being tested in phase I and phase II clinical trials for hematological malignancies and solid tumors (NCT02091245, NCT02530476, NCT02249091, NCT02419495, NCT02343042, and NCT02178436). After the submission of this paper, selinexor was approved for penta-
refractory MM (179). Preclinical results show that KPT-330 induces responses at well-tolerated doses (180) and decreases Mcl-1 protein levels (171). Higher XPO1 levels are associated with poor prognosis and targeting XPO1 by KPT-330 has shown promise in clinical trials in targeting AML (146, 147). Our working hypothesis was that because XPO1 inhibition decreases Mcl-1 levels, targeting XPO1 can synergize with venetoclax treatment, which is known to synergize with Mcl-1 inhibition. In addition, KPT-330 has been shown to not only eliminate the rapidly proliferating bulk AML cells but also LSCs (180). Therefore, it was conceivable that KPT-330 may synergize with venetoclax in eradicating both bulk AML cells and LSCs.

3.2 Materials and Methods

3.2.1 Drugs

Venetoclax, KPT-330 (selinexor), KPT-8602 (eltanexor) and MG-132 were purchased from Selleck Chemicals (Houston, TX). Cycloheximide was purchased from Sigma-Aldrich (St Louis, MO, USA).

3.2.2 Cell Culture

MV4-11 and THP-1 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). CTS was a gift from Dr. A Fuse from the National Institute of Infectious Diseases (Tokyo, Japan). OCI-AML3 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). MOLM-13 was purchased from AddexBio (San Diego, CA, USA). Cell lines were authenticated in August 2017 at the Genomics Core at Karmanos Cancer Institute using the PowerPlex® 16 System from Promega (Madison, WI, USA). The cell lines were cultured as described in section 2.2.2.

Diagnostic AML blast samples derived from patients either at initial diagnosis or at relapse were purified by standard Ficoll-Hypaque density centrifugation, then cultured in RPMI
1640 with 20% fetal bovine serum, ITS Solution (Sigma-Aldrich, St. Louis, MO) and 20% supernatant of the 5637 bladder cancer cell line (as a source of GM-CSF, G-CSF, interleukin-1 beta, M-CSF, and SCFβ) (134, 152, 153).

3.2.3 Clinical Samples

Diagnostic AML blast samples were obtained from the First Hospital of Jilin University. Written informed consent was provided according to the Declaration of Helsinki. This study was approved by the Human Ethics Committee of The First Hospital of Jilin University. Clinical samples were screened for gene mutations by automated DNA sequencing and fusion genes by real-time RT-PCR, as described in section 2.2.3 (154, 155).

3.2.4 Western Blot Analysis

See section 2.2.4 for complete details. Anti-Bcl-2 (ab692, Abcam, Cambridge, MA), -Bcl-xL (2764), -Mcl-1 (4572), -PARP (9542), -Bim (2819), -Bak (3814), -Bax (2774), -cleaved caspase-3 (9661, designated –cf-Cas3, Cell Signaling Technology, Danvers, MA), or -β-actin (A2228, Sigma-Aldrich) antibody at 1:1000 dilution (except for anti-β-actin antibody at 1:10,000) antibodies were used.

3.2.5 Annexin V/PI Staining and Flow Cytometry Analysis

AML cells were treated with venetoclax, KPT-330, or KPT-8602, alone or in combination, and subjected to flow cytometry analysis using the Annexin V-FITC/PI Apoptosis Kit (Beckman Coulter; Brea, CA). See section 2.2.5 for details.

3.2.6 shRNA Knockdown

Please see section 2.2.7.

3.2.7 Co-Immunoprecipitation (Co-IP)

See section 2.2.6.
3.2.8 CRISPR knockdown

The lentiCRISPRv2 plasmid was a gift from Feng Zhang at the Broad Institute of MIT and Harvard [Addgene plasmid #52961 (29)]. Guide RNAs were designed using the CRISPR design tool (http://CRISPR.mit.edu). The NTC and Mcl-1 vectors were generated using Feng Zhang's protocol, which is available on Addgene's website (www.addgene.org). Lentivirus production and transduction were carried out as described above in "shRNA Knockdown," except that psPAX2 (gift from Didier Trono at the Swiss Institute of Technology, Addgene plasmid # 12260) was used instead of delta 8.2.

3.2.9 Colony Forming Assay

Primary AML patient samples were treated with either venetoclax or KPT-330, alone or in combination, for 24 h. The cells were washed three times with PBS and then the cells were plated in methylcellulose in media and allowed to incubate at 37 °C humidified atmosphere containing 5% CO2/95% air for 14-16 days. Colonies (colony forming units [CFUs], more than 50 cells/CFU) were manually counted under a microscope. Technical triplicates were performed.

3.2.10 Real-time (RT-PCR)

Total RNA was extracted using TRIzol (Life Technologies) and cDNAs (complementary DNA) were prepared from 2 μg total RNA using random hexamer primers and a RT-PCR Kit (Life Technologies), and then purified using the QIAquick PCR (polymerase chain reaction) Purification Kit (Qiagen) as described previously (181). Mcl-1 (Hx01050896_m1) and GAPDH (Hs99999905_m1) transcripts were quantitated using TaqMan probes (Life Technologies) and a LightCycler 480 real-time PCR machine (Roche Diagnostics), based on the manufacturer's instructions. Real-time PCR results were expressed as means from three independent
experiments and were normalized to GAPDH transcripts. Fold changes were calculated using the comparative Ct method (182).

### 3.2.11 Statistical Analysis

All data was checked graphically for conformity with the assumptions of the normal theory linear model and transformations applied if necessary. Statistical significance was evaluated with Welch’s t-test to compare two groups and Analysis of Variance (ANOVA) (183) for comparing groups of 3 or more samples with Tukey or Bonferroni correction to control the type 1 error rate. After analysis, models were evaluated for fit and the possible existence of influential points. Statistical analyses were performed with GraphPad Prism 5.0. Error bars represent ± s.e.m. The level of significance was set at p<0.05.

### 3.3 Results

#### 3.3.1 Inhibition of XPO1 synergizes with venetoclax in AML cell lines

To begin to test our hypothesis that KPT-330 can synergize with venetoclax to induce apoptosis, we tested various clinically achievable concentrations of venetoclax and KPT-330, alone and in combination, in five AML cell lines (FDA application 212306Orig1s000). The CI was used to determine drug synergy (157). At 24 h, synergy was observed between the two drugs in THP-1 (CI<0.02), OCI-AML3 (CI<0.31), MV4-11 (CI<0.12), MOLM-13 (CI<0.55), and CTS (CI<0.27) cell lines (Figure 3.1). Corroborating this, cleavage of PARP and caspase 3 was strongly enhanced in the combination treatment when compared to venetoclax or KPT-330 alone in THP-1, OCI-AML3, and MV4-11 cells (Figure 3.2). To further confirm our results, we used a second generation XPO1 inhibitor and KPT-330 analog, KPT-8602. At 24 h, synergy was observed between KPT-8602 and venetoclax in THP-1 (CI<0.30), OCI-AML3 (CI<0.16), and MV4-11 (CI<0.04) cell lines (Figure 3.3). Consistent with KPT-330, cleavage of PARP and
caspase 3 was strongly enhanced in the combination treatment when compared to venetoclax or KPT-8602 alone in the cell lines (Figure 3.4). In summary, XPO1 inhibition synergizes with venetoclax to induce apoptosis in AML cell lines.
Figure 3.1. Inhibition of XPO1 by KPT-330 synergizes with venetoclax in AML cell lines. THP-1 (A), MV4-11 (B), OCI-AML3 (C), MOLM-13 (D), and CTS (E) cells were treated with venetoclax or KPT-330, alone or in combination, for 24 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001. Combination index (CI) values were calculated using CompuSyn software.
Figure 3.2. Inhibition of XPO1 by KPT-330 cooperates with venetoclax in inducing caspase 3 and PARP cleavage in AML cell lines. THP-1 (A), OCI-AML3 (B), and MV4-11 (C) cells were treated with venetoclax or KPT-330, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies.
Figure 3.3. Inhibition of XPO1 by KPT-8602 synergizes with venetoclax in AML cell lines. THP-1 (A), OCI-AML3 (B), and MV4-11 (C) cells were treated with venetoclax or KPT-8602, alone or in combination, for 24 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001. Combination index (CI) values were calculated using CompuSyn software.
Figure 3.4. Inhibition of XPO1 by KPT-8602 cooperates with venetoclax in inducing caspase 3 and PARP cleavage in AML cell lines. THP-1 (A), OCI-AML3 (B), and MV4-11 (C) cells were treated with venetoclax or KPT-8602, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies.
3.3.2 KPT-330 downregulates Mcl-1 and disrupts its interaction with Bim

Having observed the synergy between venetoclax and KPT-330 or KPT-8602, we sought to determine how the combination treatment affected levels of Bcl-2 family proteins. In agreement with our previous studies, Mcl-1 levels increased in response to venetoclax treatment in the venetoclax-resistant cell lines (THP-1 and OCI-AML3), but not the venetoclax-sensitive cell line (MV4-11; Figure 3.5) (141, 143). In concordance with our hypothesis, KPT-330 treatment decreased Mcl-1 levels and was able to prevent upregulation of Mcl-1 induced by venetoclax. In contrast, the levels of Bcl-2, Bak, Bax, and Bcl-xL remained largely unchanged. Curiously, KPT-330 treatment alone or in combination with venetoclax decreased levels of Bim. This would be expected to reduce apoptosis. However, based on the previous figure, the overall effect is the induction of apoptosis. Thus, the effects of Mcl-1 downregulation likely predominate. KPT-8602 had similar effects as KPT-330 on Mcl-1 levels alone and in combination with venetoclax (Figure 3.6). At the time when this project was ongoing, KPT-330 was more advanced in clinical development than KPT-8602, therefore KPT-330 was used in the rest of experiments in this chapter and is labeled as KPT.
Figure 3.5. Modulation of Bcl-2 family proteins after Bcl-2 inhibition by venetoclax and XPO1 inhibition by KPT-330. THP-1 (A), OCI-AML3 (B), and MV4-11 (C) cells were treated with venetoclax or KPT-330, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were measured using Odyssey Software V3.0 and normalized to actin and no drug control.
Figure 3.6. Modulation of Bcl-2 family proteins after Bcl-2 inhibition by venetoclax and XPO1 inhibition by KPT-8602. THP-1 (A), OCI-AML3 (B), and MV4-11 (C) cells were treated with venetoclax or KPT-8602 alone or in combination for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were measured using Odyssey Software V3.0 and normalized to actin and no drug control.
To further understand the role of Bcl-2 family proteins in the apoptosis induced drug treatment, a timecourse experiment was performed. Apoptosis, determined by flow cytometry, started between 4-8 h for the combination treatment in THP-1 cells (Figure 3.7 A). Curiously, at 8 h, Mcl-1 levels were largely unchanged in response to KPT-330 treatment (Figure 3.7 B). However, co-IP of Bim shows that at 8 h KPT-330 prevents increased Mcl-1 binding to Bim in response to venetoclax treatment (Figure 3.8 A). This was further confirmed by reciprocal Mcl-1 co-IP (Figure 3.8 B). Bim levels remained unchanged by drug treatment (Figure 3.8 A). Further, Mcl-1 levels were increased in both venetoclax treatment alone and in combination with KPT-330. This shows that before total protein levels of Mcl-1 and Bim change, their interaction is disrupted by KPT-330 when in combination with venetoclax. Knockdown of Bim, partially rescued the THP-1 cells from apoptosis, confirming the role of Bim in response to venetoclax and KPT-330 treatment (Figure 3.8 C&D).
Figure 3.7. Time dependent effect of XPO1 and Bcl-2 inhibition on Bcl-2 family proteins and cell apoptosis. (A) THP-1 cells were treated with venetoclax and KPT-330, alone or in combination, for the indicated time and then subjected to Annexin V/PI staining and flow cytometry analyses. * indicates p<0.05 and *** indicates p<0.001. (B) THP-1 cells were treated with venetoclax or KPT-330, alone or in combination, for the indicated time. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were measured using Odyssey Software V3.0 and normalized to actin and no drug control.
Figure 3.8. Interaction between Bim, Bcl-2, and Mcl-1 in response to XPO1 and Bcl-2 inhibition at 8 h. (A&B) THP-1 cells were treated with venetoclax and KPT-330, alone or in combination, for 8 h. Bim or Mcl-1 were immunoprecipitated from whole cell lysates and then subjected to Western blotting and probed with the indicated antibodies (n=2). Relative densitometry measurements of Mcl-1, Bim, and Bcl-2 were measured using Odyssey Software V3.0 and normalized to no drug control. *indicates the light chain of IgG. WC lysate indicates whole cell lysate. (C&D) shRNA knockdown of Bim was generated with the indicated non-template control (NTC). Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the knockdown. For panel C, relative densitometry measurements measured using Odyssey Software V3.0 and normalized to actin and NTC. * indicates p<0.05 (n=3) compared to the control for densitometry measurements. For panel D, shRNA knockdown cells were treated with venetoclax and KPT-330, alone or in combination, for 24 h and then subjected to Annexin V/PI staining and flow cytometry analyses. ** indicates p<0.01 and *** indicates p<0.001.
Consistent with the 8 h treatment and previous studies from our lab, 24 h venetoclax treatment substantially increased binding of Mcl-1 to Bim, which was prevented by the addition of KPT-330 (Figure 3.9 A). These results were confirmed by reciprocal immunoprecipitation with a Bim antibody (Figure 3.9 B). Interestingly, KPT-330 treatment resulted in increased binding of Bim to Bcl-2, which was abolished by combination with venetoclax. To further determine the role of Mcl-1 in venetoclax- and KPT-330-induced apoptosis, a CRISPR knockdown of Mcl-1 and pLOC overexpression (OE) of Mcl-1 were developed in the THP-1 cell line (Figure 3.10 A&C). Consistent with our previous studies and our hypothesis, Mcl-1 knockdown significantly enhanced venetoclax activity (Figure 3.10 B). Interestingly, it also enhanced combination treatment of venetoclax and KPT-330. Mcl-1 overexpression partially rescued the cells from drug treatment (Figure 3.10 D). The incomplete rescue may be due to the Mcl-1 overexpression not being high enough and/or Mcl-1-independent mechanisms through which KPT-330 acts. Mcl-1 transcript levels were not decreased by venetoclax or KPT-330 treatment in both THP-1 and OCI-AML3 cells (Figure 3.11 A&B). To test Mcl-1 protein half-life, cells were treated with or without KPT-330 for 12 h, then washed and cultured in the presence of the protein translation inhibitor cycloheximide (CHX) for up to 120 minutes. KPT-330 treatment resulted in significantly shorter Mcl-1 half-life in both THP-1 and OCI-AML3 cells (Figure 3.11 C&D). Treatment with the proteasome inhibitor MG-132 prevented downregulation of Mcl-1 by KPT-330 (Figure 3.11 E&F). In summary, Mcl-1 is modulated by KPT-330 treatment alone and in combination with venetoclax, and this modulation of Mcl-1 plays an important role in apoptosis in response to drug treatment.
Figure 3.9. Interaction between Bim and Bcl-2 or Mcl-1 in response to XPO1 and Bcl-2 inhibition at 24 h. THP-1 cells were treated with venetoclax and KPT-330, alone or in combination, for 24 h. Bim (A) or Mcl-1 (B) were immunoprecipitated from whole cell lysates and then subjected to Western blotting and probed with the indicated antibodies (n=2). Relative densitometry measurements of Mcl-1, Bim, and Bcl-2 were measured using Odyssey Software V3.0 and normalized to the no drug control. *indicates the light chain of IgG. WC lysate, whole cell lysate.
Figure 3.10. Role of Mcl-1 in apoptosis induced by KPT-330 and venetoclax, alone or combined. (A&C) CRISPR knockdown of Mcl-1 and PLOC overexpression of Mcl-1 were generated with indicated controls (NTC and RFP [red fluorescent protein]). Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the knockdown or overexpression. Relative densitometry measurements of Mcl-1 expression were measured using Odyssey Software V3.0 and normalized to actin and the control. (B&D) The cells were treated with venetoclax or KPT-330, alone or in combination, for 24 h and then subjected to Annexin V/PI staining and flow cytometry analyses. *** indicates p<0.001.
Figure 3.11. Regulation of Mcl-1 protein levels by XPO1 inhibition. (A&B) THP-1 and OCI-AML3 cells were treated with venetoclax and KPT-330, alone or in combination for 24 h. RNA was extracted with TRIzol and subjected to RT-PCR analysis and normalized to the GAPDH rRNA control and the no drug control. (C&D) THP-1 and OCI-AML3 cells were treated with or without KPT-330 for 12 h followed by CHX treatment (10 μg/mL) and collected at the indicated times. Whole cell lysates were subjected to Western blotting and probed with Mcl-1 and actin antibodies. (E&F) THP-1 and OCI-AML3 cells were treated with MG-132 or KPT-330, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements of Mcl-1 and were measured using Odyssey Software V3.0 and normalized to actin and the no drug control.
3.3.3 KPT-330 synergizes with venetoclax in primary AML patient samples

To determine if KPT-330 can synergize with venetoclax to induce apoptosis in primary AML patient samples ex vivo, we tested various concentrations of venetoclax and KPT-330, alone and in combination, in ten primary AML patient samples (Patient characteristics are shown in Table 3.1, samples were collected by Dr. Hai Lin and analyzed by Yongwei Su). At 24 h, synergy was observed between the two drugs for all samples (Figure 3.12 and 3.13). Cleavage of PARP and caspase 3 was strongly enhanced in the combination treatment when compared to venetoclax or KPT-330 alone (Figure 3.14). Similar to AML cell lines, KPT-330 alone and in combination with venetoclax decreased Mcl-1 levels. Next, we performed colony forming assays on primary AML patient samples post drug treatment and found that combination treatment significantly enhanced inhibition of colony formation (AML-CFUs, colony forming units) when compared to single drug treatment (Figure 3.15), indicating that the combined drug treatment decreased leukemia progenitor cells. In summary, venetoclax and KPT-330 can cooperate to induce apoptosis in AML cell lines in vitro and primary patient sample bulk AML cells ex vivo. The combination also cooperates to inhibit AML progenitor cells ex vivo.
Figure 3.12. KPT-330 synergizes with venetoclax to induce apoptosis in relapsed primary AML patient samples \textit{ex vivo}. Primary AML patient samples derived from patients at relapse were treated with venetoclax and/or KPT-330 for 24 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. *** indicates \(p<0.001\). Combination index (CI) values were calculated using CompuSyn software. Patient characteristics are shown in Table 3.1.
Figure 3.13. KPT-330 synergizes with venetoclax to induce apoptosis in newly diagnosed primary AML patient samples ex vivo. Primary AML patient samples derived from patients at initial diagnosis were treated with venetoclax and/or KPT-330 for 24 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001. Combination index (CI) values were calculated using CompuSyn software. Patient characteristics are shown in Table 3.1.
Figure 3.14. KPT-330 cooperates with venetoclax to induce apoptosis in primary AML patient samples *ex vivo*. Primary AML patient samples were treated with venetoclax or KPT-330, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were measured using Odyssey Software V3.0 and normalized to actin and no drug control. Patient characteristics are shown in Table 3.1.
Figure 3.15: KPT-330 synergizes with venetoclax to reduce colony formation in primary AML patient samples *ex vivo*. Primary AML patient samples were treated with venetoclax and KPT-330, alone or in combination, for 24 h and then plated in methylcellulose (n=3). Colonies (CFU) were counted manually. # indicates p<0.05 versus control. ## indicates p<0.01 versus control. ### indicates p<0.001 versus control. ** indicates p<0.01 versus single drug treatment. *** indicates p<0.001 versus single drug treatment. Patient characteristics are shown in Table 3.1.
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**Table 3.1. Patient characteristics of primary AML patient samples.** NA, not available; ND, not detected.
3.4 Discussion

For the Chapter 3 aim, we sought to expand upon the work of Chapter 2 by targeting Mcl-1 through indirect means in a clinically relevant manner. Inhibition of XPO1 by KPT-330 and KPT-8602 was previously found to downregulate Mcl-1, target leukemic stem cells, and spare normal hematopoietic stem cells (180, 184). It was theorized that the Mcl-1 downregulation could synergize with Bcl-2 inhibition in AML.

XPO1 is the best studied nuclear exporter. Its aberrant overexpression is associated with poor prognosis and drug resistance in many cancers including but not limited to prostate, ovarian, osteosarcoma, glioma, cervical, CLL, NHL, MM, and AML. It is believed that export of growth promoters to the cytoplasm and export of tumor suppressor proteins that function by altering transcription in the nucleus can cause excess proliferation. Inhibition of XPO1 through early inhibitors like leptomycin B proved to have anti-tumor efficacy but were toxic due to non-reversible inhibition of XPO1 (185).

The next generation selective inhibitors of nuclear export (SINEs) like KPT-330 (selinexor) and KPT-8602 (eltanexor, developed after our studies were underway) were developed to address this potential therapeutic target. Due to their slowly reversible binding to their target, the compounds remained effective while also being less toxic. It was previously reported that KPT-330 in CLL decreased Mcl-1 by decreasing mRNA levels. In contrast, we found that in AML KPT-330 did not decrease Mcl-1 through mRNA but rather through decreased Mcl-1 protein stability. Surprisingly, Mcl-1 downregulation happened after the timepoint where elevated apoptosis was found in the combination. However, at 8 h we still found disruption of Bim binding to Mcl-1 post drug treatment, even without decreased Mcl-1 levels. KPT-330 treatment also resulted in increased binding of Bim to Bcl-2 indicating that venetoclax
and KPT-330 reciprocally overcome resistance to single drug treatment. It is unknown how KPT-330 disrupts Bim binding to Mcl-1 but it may be due to post-translational modifications in Bim or Mcl-1, which warrants future study.

Both venetoclax and KPT-330 modulate Mcl-1 through protein stability. Stability of Mcl-1 is affected by phosphorylation sites (altered by CDK1, CDK2, GSK3β, p38, JNK, ERK, etc.) and binding to Bim, both of which alter the binding of E3 ligases (such as Mule, β-TrCP, FBW7, Trim17, SCFβ) to Mcl-1 (144). Bim binds to the BH3 binding domain used by E3 ligases. Deubiquitinases (such as USP9X and USP28) can also counteract E3 ligases by removing ubiquitin. Also, while the bulk of the combination’s activity was recapitulated through Mcl-1 knockdown, Mcl-1 overexpression did not completely rescue the cells. As is the case with small molecule inhibitors and the targeting of broad nuclear export activity, other factors may play a role in drug activity and resistance that could guide clinical use.

In addition to questions about the mechanism of action of the drugs alone and in combination, questions about tolerability and selectivity remain. The concentrations used in this study were clinically achievable, increasing the likelihood of future success. Bcl-2 and XPO1 are also both upregulated in leukemic stem cells and their inhibition has been found to selectively target these cells. The combination enhanced single drug treatment in reducing both bulk AML patient blasts and reducing the leukemic progenitor cells ex vivo. Further tolerability and efficacy could be tested in in vivo studies and selectivity could be tested against normal peripheral blood cells and hematopoietic stem cells.

In summary, KPT-330 synergizes with venetoclax to induce apoptosis in AML cell lines and primary patient samples ex vivo at clinically relevant concentrations. The combination enhances single drug activity to target leukemic progenitor cells of primary patient samples ex
in vivo. Further studies to determine the mechanism in vitro and efficacy and tolerability in vivo are warranted.
CHAPTER 4: INHIBITION OF CDK9 DOWNREGULATES MCL-1 THROUGH DECREASED TRANSCRIPTION AND ENHANCES VENETOCLAX ACTIVITY

This section is partly modified from the pending publication by Daniel A. Luedtke et al. Inhibition of CDK9 by Voruciclib Synergistically Enhances Cell Death Induced by the Bcl-2 Selective Inhibitor Venetoclax in Preclinical Models of Acute Myeloid Leukemia. Signal Transduction and Targeted Therapy. Under review.

4.1 Introduction

In chapters 2 and 3 we demonstrated that targeting Mcl-1 directly or targeting Mcl-1 indirectly through downregulation of its protein stability can enhance venetoclax activity. As Mcl-1 has a short protein half-life, inhibition of Mcl-1 transcript levels could plausibly be used to enhance venetoclax activity. CDK9, which regulates the positive transcription elongation factor complex b (p-TEFb) through phosphorylation of RNA polymerase II (RNAP II), regulates many pro-survival genes including but not limited to Mcl-1, Cyclin D1, and c-Myc (186, 187). Inhibition of CDK9 with flavopiridol (alvocidib, a pan-CDK inhibitor in phase II trials in AML and tolerable in elderly patients) can downregulate Mcl-1 and enhance venetoclax treatment in AML (148-150). However, as a pan-CDK inhibitor, off target toxicity remains a concern. A second generation CDK9 selective inhibitor, voruciclib, was developed. Voruciclib has greater specificity for CDK9 than flavopiridol (188). Voruciclib was also found to downregulate Mcl-1 and synergize with venetoclax in DLBCL in vitro and in vivo (in life [e.g. mouse model]) (188). However, the molecular mechanisms underlying the synergistic interaction between venetoclax and CDK9 inhibition are not fully understood.
In this chapter we found that voruciclib synergizes with venetoclax to induce apoptosis in both AML cell lines and primary patient samples. Further, voruciclib transiently downregulates Mcl-1, which plays a role in the synergy between voruciclib and venetoclax. Interestingly, downregulation of c-Myc by voruciclib also contributes to its synergy with venetoclax. Simultaneous inhibition of c-Myc and knockdown of Mcl-1 show further enhancement of apoptosis induced by venetoclax than inhibition of c-Myc or knockdown of Mcl-1 alone. Furthermore, an every other day schedule seems optimal for the combination of venetoclax and voruciclib in an AML cell lined-derived xenograft model.

4.2 Materials and Methods

4.2.1 Drugs

Venetoclax (ABT-199), flavopiridol (alvocidib), Z-VAD-FMK (a pan-caspase inhibitor), and 10058-F4 (a c-Myc inhibitor) were purchased from Selleck Chemicals (Houston, TX). Voruciclib was provided by MEI Pharma Inc. (San Diego, CA, USA).

4.2.2 Cell Culture

See section 2.2.2 for details.

4.2.2 Clinical Samples

See section 2.2.3 for details.

4.2.3 Western Blot Analysis

See section 2.2.4 for details.

anti-Mcl-1 (4572), -PARP (9542), -Bim (2819), -Bak (3814), -Bax (2774), -c-Myc (5605S), -cleaved caspase-3 (9661, designated –cf-Cas3, Cell Signaling Technology, Danvers,
MA), or -β-actin (A2228, Sigma-Aldrich) antibody at 1:1000 dilution (except for the anti-β-actin antibody at 1:10,000) were used.

4.2.4 Annexin V-FITC/PI Staining and Flow Cytometry Analysis

AML cells were treated with venetoclax, flavopiridol, voruciclib, Z-VAD-FMK, or 10058-F4, alone or in combination, and subjected to flow cytometry analysis using the Annexin V-FITC/PI Apoptosis Kit (Beckman Coulter; Brea, CA). See section 2.2.5 for details.

4.2.5 Co-Immunoprecipitation (Co-IP)

See section 2.2.6 for details.

4.2.6 shRNA Knockdown and pLOC overexpression

shRNA knockdown and pLOC overexpression were performed as described in section 2.2.7 with the following changes. Precision LentiORF Mcl-1 and RFP lentivirus vector were purchased from Dharmaco (Lafayette, CO, USA). Selection for overexpression was done using blasticidin.

4.2.7 CRISPR knockdown

See section 3.2.8 for details.

4.2.8 Real-time (RT-PCR)

Total RNA was extracted and cDNAs were prepared, and then purified) as described in section 3.2.10. c-Myc transcripts were quantified using forward (5’-GTGGTCTTCCCCCTACCCTCT-3’) and reverse (5’-CGAGGAGAGCAGAATCCG-3’) primers. Real-time PCR results are expressed as means from three independent experiments and normalized to GAPDH transcripts measured by forward (5’-AGCCACATCGCTCAGACA-3’).
and reverse (5’-GCCCAATACGACCAAATCC-3’) primers and SYBR green. Fold changes were calculated using the comparative Ct method. (182)

### 4.2.9 Cell line derived xenograft

NSG-SGM3 mice (NSGS, JAX#103062; non-obese diabetic scid gamma (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup> Tg(CMV-IL3, CSF2, KITLG)1Eav/MloySzJ; Jackson Laboratory, Bar Harbor ME, USA) were injected by IV (intravenous) through tail vein with 1 E6 MV4-11 cells/mouse (day 0) and treated Q2D (every other day) from day 3 through day 31 (first day of leukemia symptoms detected in control mice) for a total of fifteen injections. Treatment cohorts consisted of 25 mg/kg/inj venetoclax p.o. (per os, oral) and/or 200 mg/kg/inj voruciclib p.o.. A group of vehicle treated mice was included as a control and there were 5 mice included in all arms. Average mouse body weights for the study was 22.4 +/- 1 g at treatment start with body weight and condition monitored 1-2 times daily for the duration of the study.

In a separate trial, NSGS mice were injected by IV through tail vein with 1 E6 MV4-11 cells/mouse (day 0) and treated from day 3 through day 27 (first day of leukemia symptoms detected in control mice). Treatment cohorts consisted of 25 mg/kg/inj venetoclax p.o. (daily for 5 days, one day off, followed by 19 days, for a total of 24 injections) and/or 16 mg/kg/inj Palmo-Ara-C i.p. Q3D (every three days, for a total of 9 injections [inj] intraperitoneal [i.p.]). A group of vehicle treated mice was included as a control and there were 5 mice included in all arms. Average mouse body weights for the study was 24.2 +/- 1 g at treatment start with body weight and condition monitored 1-2 times daily for the duration of the study.

Experimental endpoint and efficacy response for both trials was determined for each group based on the median day for development of leukemic symptoms (hind leg weakness, >15% weight loss, metastatic spread to internal organs). % increase in lifespan (%ILS) was
calculated: % ILS = [T-C/C] x 100 where “T” = treated and “C” = control median day of death. All mice were provided food and water ad libitum, given supportive fluids and supplements as needed, and housed within an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited animal facility with 24/7 veterinary care and were sacrificed by CO₂ asphyxiation when they showed signs of terminal illness, including hind limb paralysis, inability to eat or drink, and/or moribund. All animal experiments were approved by the Institutional Animal Care and Use Committee of Wayne State University.

4.2.10 Statistical Analysis

Differences were compared utilizing two-sample t-test. Error bars represent ± s.e.m.; significance level was set at p<0.05 and adjusted for multiple comparisons with the Bonferroni correction. One-way ANOVA (analysis of variance) was used to compare differences between 3 or more groups with Dunnett correction when compared to control or Bonferroni correction when compared to single drug treatments. Overall survival probability was estimated using the Kaplan-Meier method and statistical analysis was performed using the Mantel-Cox test. All the statistical analyses were performed utilizing GraphPad Prism 5.0.

4.3 Results

4.3.1 Voruciclib induces apoptosis in AML cell lines and primary patient samples

To begin to test the anti-leukemic activity of voruciclib (VOR), we tested various clinically achievable concentrations (189, 190) in 5 AML cell lines and 3 primary AML patient samples (Figure 4.1 A). Single drug treatment induced high levels of Annexin V+ cells (determined by Annexin V/PI staining and flow cytometry analyses) in all 5 AML cell lines and 2 out of the 3 primary patient samples (patient characteristics are in Table 4.1), with moderate
activity in the third primary patient sample. Voruciclib treatment caused substantially increased cleavage of caspase 3 and PARP at higher concentrations (Figure 4.1 B), confirming the flow cytometry data and demonstrating that voruciclib induces apoptosis of AML cells.

4.3.2 Voruciclib synergizes with venetoclax in AML cell lines

Having shown the anti-leukemic activity of single drug treatment, we sought to determine the effect of voruciclib on apoptosis induced by venetoclax. AML cell lines were treated with various concentrations of venetoclax and voruciclib, alone or in combination, for 24 h. Apoptosis induced by the drug treatments was determined by Annexin V/PI staining and flow cytometry analyses. The CI was used to determine the extent and direction of anti-leukemic interactions (34). MV4-11, U937, THP-1, and MOLM-13 cell lines treated with venetoclax plus voruciclib at clinically achievable concentrations resulted in significantly increased Annexin V+ cells compared to single drug treatment (CI<0.73, Figure 4.2), demonstrating the efficacy and synergy of the combination treatment.
Figure 4.1. CDK9 inhibition induces apoptosis in AML cell lines and primary patient samples. (A) THP-1, U937, MOLM-13, MV4-11, and OCI-AML3 AML cell lines and primary patient samples were treated with voruciclib for 24 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. (B) Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. VOR, voruciclib; cf, cleaved form; and Cas3, caspase 3. These abbreviations are used throughout this chapter. Patient characteristics are in Table 4.1.
Figure 4.2. Voruciclib synergizes with venetoclax to induce apoptosis in AML cells. MV4-11 (A), U937 (B), THP-1 (C), and MOLM-13 (D) cell lines were treated with venetoclax or voruciclib, alone or in combination, for 24 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. CI values were calculated using CompuSyn software. *** indicates p<0.001.
4.3.3 Transient Mcl-1 downregulation by CDK9 inhibition enhances venetoclax-induced apoptosis

Next, we began to determine the mechanism by which the combination induced apoptosis, particularly modulation of Mcl-1. In concordance with previous studies by our lab and others (3, 17), venetoclax-resistant cells (THP-1) but not venetoclax-sensitive cells (MV4-11) showed an increase of Mcl-1 protein in response to venetoclax treatment (Figure 4.3). Interestingly, CDK9 inhibition by voruciclib resulted in a transient downregulation of Mcl-1 protein, which rebounded after 12 h of drug treatment in both MV4-11 and THP-1 cell lines. However, this transient downregulation was sufficient to keep Mcl-1 levels below the control when the cells were treated with the combination of voruciclib and venetoclax (Figure 4.3 A&B, upper panels). Similar results were also obtained with flavopiridol (FLV) in both cell lines (Figure 4.3 A&B, lower panels). Mcl-1 transcript levels also decreased after treatment with voruciclib alone, which was further decreased by venetoclax treatment, potentially due to apoptosis induced by the drug combination. Curiously, in THP-1, but not MV4-11, Mcl-1 transcripts rebounded in response to voruciclib treatment indicating that besides the transcriptional mechanism, the rebound of Mcl-1 protein may also involve modulation of protein stability (Figure 4.4).
Figure 4.3. CDK9 inhibition induces transient Mcl-1 protein downregulation. MV4-11 (A) and THP-1 (B) cells were treated with venetoclax, voruciclib (upper panels) or flavopiridol (lower panels), alone or in combination, for the indicated times. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were determined using Odyssey Software V3.0 and normalized to actin and the no drug control. FLV: flavopiridol.
Figure 4.4. CDK9 inhibition induces transient Mcl-1 transcript downregulation. MV4-11 (A) and THP-1 (B) cells were treated with venetoclax or voruciclib, alone or combined, for 6 or 24 h. RNA was extracted with TRIzol and subjected to RT-PCR analysis and normalized to the 18S rRNA control and the no drug control. *** indicates p<0.001 versus control and ### indicates p<0.001 versus single drug treatment.
Given the important role Mcl-1 plays in the intrinsic resistance to venetoclax in AML cells (17); we sought to determine if the transient downregulation of Mcl-1 would enhance venetoclax-induced apoptosis. At 6 h, voruciclib significantly and greatly enhanced apoptosis induced by venetoclax (Figure 4.5 A&B). This was accompanied by greatly enhanced cleavage of caspase 3 and PARP in the combination treatment (Figure 4.5 C&D). To further increase the clinical relevance of our study, we tested three primary AML patient samples *ex vivo* and striking synergy was observed between venetoclax and voruciclib (Figure 4.6, CI<0.07). Taken together, our results show that voruciclib transiently downregulates Mcl-1, which may contribute to the synergy between voruciclib and venetoclax.
Figure 4.5. Voruciclib enhances apoptosis induced by venetoclax in AML cell lines. (A&B) MV4-11 and THP-1 cells were treated with venetoclax or voruciclib, alone or in combination, for 6 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001. (C&D) MV4-11 and THP-1 cells were treated with venetoclax or voruciclib, alone or in combination, for 6 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies.
Figure 4.6. Voruciclib enhances apoptosis induced by venetoclax in primary AML patient blasts *ex vivo*. Primary AML patient samples were treated with venetoclax or voruciclib, alone or in combination, for 6 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001. CI values were calculated using CompuSyn software. Patient characteristics are in Table 4.1.
4.3.4 The intrinsic apoptotic pathway is partially responsible for the cooperation of voruciclib and venetoclax to induce apoptosis

To further determine the role of Mcl-1 and the intrinsic apoptotic pathway in apoptosis induced by the combination, we performed co-immunoprecipitation of Mcl-1 in AML cell lines. Voruciclib alone or in combination with venetoclax was able to greatly decrease the binding of Mcl-1 to Bim in MV4-11 and THP-1 cells at 6 h (Figure 4.7). Mcl-1 knockdown (~80% compared to NTC) was able to moderately but significantly enhance single drug treatment, but did not significantly affect the highly efficacious combination treatment (Figure 4.8 A&B). Inversely, Mcl-1 overexpression was able to partially rescue MV4-11 cells from single and combination treatment (Figure 4.8 C&D). To further test the role of the intrinsic apoptotic pathway, Bax or Bak individual knockdown and Bax/Bak double knockdown were performed. Knockdown of Bak, which has a higher affinity to Mcl-1 than Bax, was able to completely rescue the cells from voruciclib treatment (Figure 4.9). Knockdown of Bax or Bak led to a modest rescue of the cells from venetoclax treatment and its combination with voruciclib. Bax/Bak double knockdown was able to completely rescue the cells from voruciclib and venetoclax treatment, while partially rescuing the cells from the combination treatment (Figure 4.9). These results demonstrate that apoptosis induced by the single and combination treatment was at least partially dependent upon the intrinsic apoptotic pathway.
Figure 4.7. CDK9 inhibition overcomes enhanced Bim binding to Mcl-1 induced by venetoclax. MV4-11 (A) and THP-1 (B) cells were treated with venetoclax or voruciclib, alone or in combination, for 6 h. Cell lysates were immunoprecipitated from whole cell lysates and then subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements of Mcl-1 and Bim were measured using Odyssey Software V3.0 and normalized to the no drug control. * indicates the light chain of IgG. # indicates a non-specific band.
Figure 4.8. Apoptosis induced by voruciclib and venetoclax, alone or combined, is at least partially through downregulation of Mcl-1. (A&B) CRISPR knockdown (KD) of Mcl-1 and pLOC overexpression (OE) of Mcl-1 were generated with the indicated control (non-target control [NTC] and red fluorescent protein [RFP]) in the indicated cell lines. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the KD or OE. Relative densitometry measurements were determined using Odyssey Software V3.0 and normalized to the control and actin. (C&D) Cells were treated with venetoclax or voruciclib, alone or in combination, for 6 h and then subjected to Annexin V-FITC/PI staining and flow cytometry analyses. ** indicates p<0.01 and *** indicates p<0.001.
Figure 4.9. Apoptosis induced by voruciclib and venetoclax, alone or combined, is at least partially through the intrinsic apoptotic pathway. (A) shRNA KD of Bax, Bak, and Bax/Bak were generated with the indicated control (non-target control [NTC]) in THP-1 cells cell lines. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the KD. Relative densitometry measurements were determined using Odyssey Software V3.0 and compared normalized to the control and actin. (B) Cells were treated with venetoclax or voruciclib, alone or in combination, for 6 h and then subjected to annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001, and ns indicates not significant.
4.3.5 Downregulation of c-Myc and Mcl-1 enhances venetoclax-induced apoptosis

CDK9 promotes transcription of genes encoding pro-survival factors, including c-Myc, which plays a critical role in leukemogenesis and drug resistance in AML (149, 191, 192). Thus, it is conceivable that CDK9 inhibition downregulates c-Myc leading to enhancement of apoptosis induced by venetoclax. CDK9 inhibition for 6 h with voruciclib alone or in combination with venetoclax caused major downregulation of c-Myc protein in both MV4-11 and THP-1 cells (Figure 4.10 A&B). Unlike the effect on Mcl-1, c-Myc protein levels did not rebound after extended treatment with voruciclib (Figure 4.10 C&D). Transcripts of c-Myc were also greatly decreased by voruciclib alone and in combination with venetoclax (Figure 4.10 E-F), suggesting a transcriptional mechanism by which voruciclib downregulates c-Myc expression. The pan-caspase inhibitor Z-VAD-FMK (Z-VAD) showed that induction of apoptosis by voruciclib, alone or in combination with venetoclax was entirely caspase dependent (Figure 4.11). To determine the functional contribution of c-Myc downregulation to the anti-leukemic activity of venetoclax, we used the c-Myc inhibitor 10058-F4. Treatment of MV4-11 and THP-1 cells with 10058-F4 modestly decreased c-Myc protein, but significantly and greatly enhanced venetoclax induced apoptosis (Figure 4.12), which was further enhanced when Mcl-1 was knocked down in the cells (Figure 4.13). These results demonstrate that both c-Myc and Mcl-1 are important factors involved in the synergy between venetoclax and voruciclib. Further, to achieve maximal efficacy for the combination of voruciclib and venetoclax, both c-Myc and Mcl-1 need to be downregulated.
Figure 4.10. Voruciclib downregulates c-Myc in AML cells. (A-D) MV4-11 and THP-1 cells were treated with venetoclax or voruciclib, alone or in combination, for 6 h or 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were determined using Odyssey Software V3.0 and normalized to the no drug control and actin. (E&F) RNA was extracted with TRIzol and subjected to RT-PCR analysis and normalized to the 18S rRNA control and the no drug control.
Figure 4.1. CDK9 inhibition enhances venetoclax activity in a caspase dependent manner. (A-B) MV4-11 and THP-1 cells were treated with venetoclax, voruciclib, or Z-VAD-FMK, alone or in combination, for 6 h and were subjected to annexin V/PI staining and flow cytometry analyses. ** indicates p<0.01, *** indicates p<0.001 by t-test compared to treatment without Z-VAD-FMK with Bonferroni correction. Z-VAD is Z-VAD-FMK. This abbreviation is used throughout the manuscript.
Figure 4.12. Inhibition of c-Myc enhances venetoclax-induced apoptosis. (A&B) MV4-11 and THP-1 cells were treated with venetoclax or 10058-F4, alone or in combination, for 6 h and then subjected to Annexin V/PI staining and flow cytometry analyses. *** indicates p<0.001. (C&D) Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were determined using Odyssey Software V3.0 and normalized to the no drug control and β-actin.
Figure 4.13. Downregulation of both c-Myc and Mcl-1 maximally enhances venetoclax-induced apoptosis. (A) CRISPR knockdown (KD) of Mcl-1 was generated with the indicated control (NTC) in THP-1 cells. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies to confirm the KD. Relative densitometry measurements were determined using Odyssey Software V3.0 and normalized to NTC and actin. (B) Cells were treated with venetoclax and 10058-F4, alone or in combination, for 6 h and then subjected to annexin V-FITC/PI staining and flow cytometry analyses. *** indicates p<0.001.
4.3.6 Intermittent CDK9 inhibition enhances venetoclax activity *in vitro* and *in vivo*

To determine if retreatment of AML cells with voruciclib will cause re-downregulation of Mcl-1, we designed experiments as shown in Figure 4.14 A. We started by treating MV4-11 cells for 24 h with 2000 nM voruciclib. Then, the cells were washed and resuspended in fresh media. The pretreated cells were treated with venetoclax and voruciclib alone or in combination for 6 h immediately after cell wash or after a 24 h waiting period to allow the cells to “recover”. Retreatment of the pretreated cells with voruciclib immediately after cell wash neither caused obvious change of Mcl-1 protein levels nor significantly enhanced apoptosis induced by venetoclax (0 h wait, 6 h retreatment; Figure 4.14 B&C). Conversely, pretreated cells that had a 24 h treatment “break” were more responsive to CDK9 inhibition alone, and in combination with venetoclax based on the changes of Mcl-1 protein levels and extent of apoptosis induction. These results were found to be similar with flavopiridol (data not shown).
Figure 4.14. Intermittent CDK9 inhibition enhances venetoclax activity in vitro. (A) Schematic for the rechallenge treatments. (B) MV4-11 cells were treated with voruciclib alone for 24 h. The cells were spun and washed with PBS. Cells were cultured in drug-free medium for 0 or 24 h, then treated as indicated for 6 h. Whole cell lysates from retreated cells were subjected to Western blotting and probed with the indicated antibodies. Relative densitometry measurements were determined using Odyssey Software V3.0 and normalized to the no drug control and actin. (C) Cells treated as shown in panel A were subjected to annexin V/PI staining and flow cytometry analyses. *** indicates p<0.001.
Based on our \textit{in vitro} voruciclib data, a Q2D dosing schedule was chosen for our \textit{in vivo} studies (performed by Dr. Lisa Polin and AMTEC). 1 E6 MV4-11 cells were injected into NSGS mice via tail vein on day 0. Treatment started on day 3 and continued every other day until day 31 (the first day leukemia symptoms in control mice were detected). A total of fifteen injections were given for each single agent as monotherapy or in combination with mice receiving either: 25 mg/kg/inj venetoclax p.o., 200 mg/kg/inj voruciclib p.o., or in combination (Figure 4.15 A). Body weight loss nadir for all treatment groups was $\leq 2.7\%$, indicative of well-tolerated treatment regimens (Figure 4.15 B). Venetoclax or voruciclib treatment did not extend median survival (43 days) compared to vehicle control treatment (43 days), however the combination of voruciclib and venetoclax modestly improved median survival (52 days, p<0.05, Mantel-Cox; 20.9\% ILS; Figure 4.15 C).
Figure 4.15. Intermittent CDK9 inhibition enhances venetoclax activity in vivo. (A) In vivo dosing schedule. NSGS mice were injected with 1E6 MV4-11 cells and treated Q2D starting on day 3 with 25 mg/kg venetoclax p.o. and/or 200 mg/kg Vorucilibr p.o. (B) Average mouse body weights for the treatment arms were measured on a daily basis. (C) Kaplan-Meier survival curve between the treatment arms (Mantel-Cox statistical test).
Next, we tested low-dose ara-C (palmO-ara-C derivative used in murine studies as ara-C [cytarabine] is not tolerated in mice) in combination with venetoclax, with venetoclax given on a daily basis and ara-C given every third day (Figure 4.16 A). Body weight loss for all treatment groups was ≤ 8% (Figure 4.16 B). Median survival was 40 days for vehicle control and venetoclax treated mice. Median survival of ara-C treated mice was 37.5 days, while combined venetoclax and ara-C had a median survival of 43 days (7.5% ILS; Figure 4.16 C). Taken together, our results demonstrate that CDK9 inhibition enhances the anti-leukemic activity of venetoclax in vivo and the Q2D dosing schedule is effective both in vitro and in vivo.
Figure 4.16. Cytarabine minimally enhances venetoclax activity in vivo. (A) *In vivo* dosing schedule. NSGS mice were injected with 1E6 MV4-11 cells and treated Q2D starting on day 3 with 25 mg/kg venetoclax p.o. and/or 16 mg/kg/inj palmo-ara-C i.p. Q3D. (B) Average mouse body weights for the treatment arms were measured on a daily basis. (C) Kaplan-Meier survival curve between the treatment arms (Mantel-Cox statistical test).
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<td>46, XX, t(15;17): (q22;q21)</td>
<td>90.15</td>
<td>PML-RARα, ATRX, FLT3-ITD</td>
</tr>
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<td>Male</td>
<td>36</td>
<td>Newly diagnosed</td>
<td>M1</td>
<td>46, XY</td>
<td>90.50</td>
<td>DNMT3A, IDH2, NPM1</td>
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</tbody>
</table>

Table 4.1. Patient characteristics of primary AML patient samples. NA, not available

### 4.4 Discussion

In this chapter we confirmed the role of Mcl-1 downregulation by CDK9 inhibition to enhance venetoclax-induced apoptosis in AML cells. Similar to findings in DLBCL, voruciclib treatment was found to quickly downregulate Mcl-1 in AML (188). Also similar to their study, voruciclib was found to synergize with venetoclax in AML. Flavopiridol was also found to synergize with venetoclax in AML cells, matching previous findings in primary AML cells (148). The combination treatment was found to be caspase dependent (Figure 4.11). However, Bax/Bak knockdown did not fully rescue the cells from combination treatment. The Bax/Bak knockdown may not have been sufficient as residual Bax and/or Bak may have been sufficient to induce apoptosis. Alternatively, the extrinsic apoptotic may have been activated as downregulation of c-Myc has been shown to increase TRAIL expression (193).
In addition to the confirmed hypothesized mechanism of Mcl-1 downregulation by CDK9 inhibition synergizing with venetoclax in AML, we found previously unrecognized novel mechanisms that will add to preclinical development as well as guide the use of CDK9 inhibitors in the clinic. While other studies have shown that Mcl-1 plays a role in the anti-leukemic activity of CDK9 inhibition in combination with venetoclax (148, 188, 194), we found that CDK9 inhibitor downregulation of Mcl-1 was transient (Figure 4.3&4.4) and that continuous CDK9 inhibition no longer reduced Mcl-1 protein nor enhanced venetoclax activity (Figure 4.14). However, an intermittent schedule was found to resume downregulation of Mcl-1 and enhance venetoclax activity. Further, a short intermittent administration schedule, e.g. every other day, showed significant survival benefit for voruciclib in combination with venetoclax (Figure 4.15 C). Voruciclib in combination with venetoclax resulted in a 20.9% ILS, while low-dose ara-C in combination with venetoclax only increased the lifespan by 7.5% (Figure 4.16). MV4-11 cells are relatively resistant to ara-C in vitro (2 μM treatment for 24 h only results in 20% Annexin V positive cells (141)). Thus, our data suggests that voruciclib in combination with venetoclax may be a potential promising option for ara-C resistant AML cases.

Another novel finding was the role of c-Myc in the synergistic activity of voruciclib in combination with venetoclax in AML. Mcl-1 overexpression only partially rescued AML cells from venetoclax in combination with voruciclib treatment (Figure 4.8 B&D), indicating that downregulation of Mcl-1 was only part of the mechanism of action of the combination treatment. c-Myc inhibition significantly and greatly enhanced venetoclax activity (Figure 4.11 A-B), especially when combined with the knockdown of Mcl-1 (Figure 4.12). These results suggest that while downregulation of both c-Myc and Mcl-1 are important contributors to voruciclib’s enhancement of venetoclax activity in AML cells, their effects alone in enhancing venetoclax
activity are moderate. Further studies to determine how downregulation of c-Myc enhances venetoclax activity are needed.

In summary, voruciclib synergizes with venetoclax in AML cells. Voruciclib treatment results in downregulation of c-Myc and Mcl-1, which both contribute to venetoclax activity (Figure 4.18). Voruciclib alone and in combination with venetoclax were observed as more effective with an intermittent drug administration schedule \textit{in vitro} and a Q2D schedule of voruciclib was effective \textit{in vivo}. Based on our study, continuous combination treatment shows efficacy, though enhancement from downregulation of Mcl-1 is lost, suggesting that for optimal results, an intermittent schedule should be considered. Such a schedule may reduce toxicity, as time between treatments will likely be prolonged in order to account for Mcl-1 rebound. In conclusion, our results support further clinical development of voruciclib in combination with venetoclax for the treatment of AML and provide evidence to suggest that CDK9 inhibitors should be administered with an intermittent schedule.
Venetoclax (VEN) treatment prevents Bim from binding to Bcl-2. In sensitive cells, there is an inadequate amount of Mcl-1 to sequester all of the excess Bim, resulting in free Bim, which can then activate the canonical intrinsic apoptosis pathway (black arrows). In venetoclax-resistant cells, the Bim not bound to Bcl-2 is sequestered by Mcl-1, stabilizing Mcl-1, and ultimately resulting in survival (blue arrows). CDK9 inhibition by voruciclib, reduces Mcl-1 protein levels by decreasing transcription, leading to reduced sequestration of Bim by Mcl-1. Additional, pro-survival c-Myc is decreased by CDK9 inhibition (green arrows) and enhances venetoclax activity by an unknown means. This, in combination with venetoclax, can free Bim to bind to Bak and/or Bax, induce loss of mitochondrial membrane potential and induce apoptosis.
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Significance

The goal of this dissertation project was to provide proof-of-concept strategies for overcoming resistance to venetoclax in AML. Preliminary studies by our lab and others have shown the efficacy and limitations of Bcl-2 inhibition in AML and other malignancies. In the context of AML, Bcl-2 inhibition by venetoclax has good overall response rates and is well tolerated but also has a high rate of relapse (82). Combination therapies (with azacitidine, decititabine, or LDAC) proved to be slightly more effective than the standard of care for older and chemotherapy unfit patients, leading to the approval of venetoclax for use in combination therapies in late 2018 (79, 195). Our lab and others found that venetoclax-resistant AML cells compensate the decreased binding of Bim to Bcl-2 with increased binding of Bim to Mcl-1, preventing Bim from inducing apoptosis (86, 141). Due to the shallow, charged, hydrophobic, and rigid nature of the Mcl-1 binding site, Mcl-1 inhibitors had remained elusive (charged and hydrophobic molecules do not penetrate the cell well) (196).

In Chapter 2, we tested whether the recently developed Mcl-1-specific inhibitor, A-1210477, could overcome resistance to venetoclax treatment (142, 143). The combination of venetoclax and A-1210477 synergistically induced apoptosis in AML cell lines and primary patient samples. A-1210477 was able to disrupt increased binding of Bim to Mcl-1 induced by venetoclax. While A-1210477 has poor bioavailability and cell penetrance, it was still useful in studying the mechanistic role of Mcl-1 inhibition in a proof-of-concept manner (197). At the time, no other putative Mcl-1-specific small molecule inhibitors were available and even now, Mcl-1 inhibitors are only just beginning to enter clinical trials (198, 199).
In Chapter 3, we tested whether inhibition of XPO1, whose overexpression is associated with poor prognosis and whose inhibition downregulates Mcl-1, could overcome resistance to venetoclax treatment (145). KPT-330, also known as selinexor, inhibits XPO1, downregulates Mcl-1, and preferentially targets leukemic initiating cells over normal hematopoietic stem cells (180). KPT-330 was in phase I and II trials in multiple hematological and solid tumors at the time of the Chapter 3 research and was approved in July 2019 for R/R MM (NCT02336815). While KPT-330 decreased Mcl-1 protein levels through decreased Mcl-1 transcripts in CLL, we found that KPT-330 decreased Mcl-1 protein stability but not transcript levels in AML. While, Mcl-1 levels decreased after apoptosis induction, at apoptosis induction, Bim-Mcl-1 binding was disrupted. Bim to Mcl-1 binding as well as protein stability can be altered by post-translational modifications and may play a role in the mechanism of action of KPT-330 disruption of Bim-Mcl-1 interaction, decrease in Mcl-1 stability, and synergy with venetoclax.

During the course of our studies, KPT-8602 (also known as eltanexor), was developed to improve tolerability (reduced blood brain barrier penetration) and efficacy compared to KPT-330 (184). While the mechanism was less explored due to this late timing, we were able to confirm similar effects as KPT-330: synergy with venetoclax in apoptosis induction and Mcl-1 downregulation. Overall, the combination of XPO1 and Bcl-2 inhibition is effective in AML. Questions remain on Bim and Mcl-1 modifications and stability, in vivo efficacy and tolerability, and selectivity versus normal cells.

Having shown that Mcl-1 can be downregulated through decreased protein stability to synergize with venetoclax, in Chapter 4 we explored whether transcriptional downregulation of Mcl-1 could have similar effects. Mcl-1 is a short lived, transiently expressed protein.
Transcriptional levels could affect Mcl-1 protein levels. Many stress response genes also turn on Mcl-1 transcription as a survival mechanism to fix damage or overcome stresses (144).

CDK9, which is part of the positive transcription elongation factor complex b (pTEFb), regulates transiently expressed pro-survival genes including but not limited to Mcl-1, Cyclin D1, and c-Myc (186, 187, 200). Inhibition of CDK9 by the pan-CDK inhibitor flavopiridol (in phase II trials in AML) can decrease Mcl-1 transcript levels, has anti-leukemic activity, and can synergize with venetoclax in AML (148-150). There are concerns about the off target effects and toxicity of flavopiridol. A second-generation CDK9-selective inhibitor voruciclib has greater CDK9 selectivity than flavopiridol and in the diffuse large B cell lymphoma (DLBCL) model synergized with venetoclax to kill lymphoma cells in vitro and in vivo. We found that voruciclib synergizes with venetoclax in AML cell lines and primary patient samples and effective in vivo. Several novel mechanisms were found including that c-Myc downregulation (not just Mcl-1 downregulation) by CDK9 inhibition contributed to the efficacy of the combination treatment. Mcl-1 downregulation by CDK9 inhibition was found to be transient. Despite the treatment effect on apoptosis being entirely caspase dependent, Bax/Bak knockdown did not fully rescue the cells from treatment. A complete Bak/Bax knockout may rescue the cells or the extrinsic pathway may be involved (201). Another novel finding was that repeated CDK9 inhibition was found to be less effective than interrupted CDK9 inhibition, likely a result of transient compensation of the cells in response to CDK9 inhibition. The interrupted schedule was found to be effective in vivo. In summary, the results support the clinical development of combined CDK9 and Bcl-2 inhibition with an interrupted dosing scheduling that may improve efficacy while reducing toxicity.
In addition to venetoclax, which targets Bcl-2, 7 other drugs received FDA approval in AML, with targets such as CD33, IDH1/2, FLT3, and SHH (Figure 1.2). AML is a well-studied and characterized disease with targeted therapies finally coming to fruition. Despite these advances, relapse and tolerability remain a concern. Single agent treatments run the risk of selecting for a more resistant leukemic population (202). Our studies seek to target these difficult to target cells using well-tolerated therapies that are clinically relevant in the hopes of overcoming these challenges.

5.2 Future Directions

The insights provided by this dissertation and the field of Bcl-2 inhibition in AML, leave many open questions. Venetoclax seems to enhance many chemotherapeutic agents through reduced sequestration of pro-apoptotic Bim. While my dissertation studies outline the efficacy of co-targeting Bcl-2 and Mcl-1 and the efficacy of targeting Mcl-1 through indirect means, questions remain of the efficacy, selectivity, and mechanisms of these treatments.

The first major question that remains is how Mcl-1 stability and binding is affected by our treatments. Venetoclax alone is known to increase Mcl-1 protein levels through increased protein stability but not through increased Mcl-1 transcripts. This stabilization is also Bim dependent. The BH3 binding site of Mcl-1 is used by E3 ligases (e.g. Mule, β-TrCP, FBW7, Trim17, and SCFβ) to ubiquitinate Mcl-1 and promotes its degradation (203-205). A-1210477, like Bim, occupies the BH3 binding pocket of Mcl-1, increasing Mcl-1 levels. It is possible that competition for E3 ligases affects Mcl-1 protein stability. Deubiquitinases (e.g. USP9X and USP28) or E3 ligase levels may be modulated as well.

Alternatively, XPO1 inhibition affects Bim-Mcl-1 binding followed by decreased Mcl-1 stability. Post-translational modifications can affect Bim-Mcl-1 binding as well as their binding
to E3 ligases and deubiquitinases (206). Mcl-1 can be phosphorylated by many signaling molecules (e.g. GSK3β, p38, JNK, ERK) which are known to affect protein stability (144).

During the course of my dissertation work, developments were made in the development of small molecule inhibitors. Several Mcl-1 inhibitors (e.g. AMG176, AZD5991, VU661013, and S64315) have reached phase I trials, although no data has been reported yet (198, 207, 208). It remains to be seen in the clinic whether this on target activity improves upon more broad acting nature of the inhibitors (e.g. pan CDK inhibition of CDK inhibitors, broad activity of FLT3 inhibitors) (49, 188). This was shown in chapter 4, where CDK9 inhibition activity relied on targeting both Mcl-1 and c-Myc. Additionally, the alternative roles of Mcl-1, such as in DNA damage repair, oxidative phosphorylation regulation, and the UPR may contribute to single drug and combination efficacy (104, 111, 112).

In addition to new therapies in preclinical and clinical development, much is to be learned about recent drug approvals in AML. Half of the recent approvals were approved in phase I and II trials (section 1.1.6) and the longer term efficacy and tolerability have yet to be determined. In addition, most approvals were for cohorts of patients that failed or were unable to tolerate other therapies (e.g. R/R AML, older than 75) with the exception of mylotarg (CD33+ AML) and midostaurin (FLT3 mutant AML). Future trials are focusing on the benefit of these new drugs for a broader set of patients and/or in combination with other therapies, including venetoclax (209, 210). Questions remain on which patients will benefit from these therapies and potential patient selection to find patients who will benefit most from these therapies (211, 212).

A major caveat of in vitro and in vivo studies is their applicability to the clinic. In vitro models generally lack cellular crosstalk found in the marrow stroma. Leukemic cells are known to share mitochondria with stromal marrow cells to enhance their survival in addition to
benefitting from supporting growth factors (213). In addition, most cell culture is performed at 21% O₂ while the peripheral blood and marrow have much lower oxygen levels (214). Hypoxia is known to affect stromal and hypoxia inducible factor (26) signaling which can drastically alter drug efficacy (215, 216). Preclinical models are necessary for the timely and cost effective testing of theories but ultimately the true test of a drug’s tolerability, selectivity, and efficacy is in the clinic.

In summary, co-targeting of Mcl-1 is effective in overcoming resistance to venetoclax in AML. The question remains open whether recently developed Mcl-1 inhibitors will be effective in the clinic. Alternatively, many chemotherapeutic anti-leukemic agents can indirectly target Mcl-1, which allows them to enhance venetoclax activity. Future studies in the regulation of Mcl-1 and mechanisms of efficacy and resistance can guide the clinical development and use of combination therapies to continue to improve patient outcomes.
APPENDIX

Author contributions

Dan Luedtke is the principal author of this dissertation and the manuscripts associated with chapters 2-4. All primary patient samples were collected by Hai Lin and analyzed by Xiaojia Niu or Yongwei Su. Dan Luedtke performed the vast majority of the *in vitro* experiments while Yongwei Su also performed a minor portion of the *in vitro* experiments. All of the *in vivo* experiments were performed in conjunction with the Animal Model and Therapeutic Evaluation Core and Dr. Lisa Polin.
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<th>Cytogenetics</th>
<th>Properties</th>
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<td>U937</td>
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<td>n=63 (58-69) 3n xxy 3n, -2, -4, -6, +7, -9, -20, -21, +3mar t(1;12)(q21;p13), 3n, der(5)(1;5)(p22;q35), add(9)p(22), 3n, t(10;11)(p14;q23), i(11q), 3n, i(12p), add(16)(q22), 3n, add(19)(q13)</td>
<td>1st continuous monocytic cell line p53 null CALM-AF10 fusion Diffuse histiocytic lymphoma, from refractory pleural effusion</td>
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<td>2nd oldest monocytic cell line p53 null Mutant NRAS P15INK4B and P16INK4A deletions RB1 rearrangement Well differentiated At relapse</td>
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<td>NPM1c type A DNMT3A R882C Peripheral blood at diagnosis</td>
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<td>Yes</td>
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<td>FLT3-ITD heterozygous and only constitutively phosphorylated line Monocytic at diagnosis</td>
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<td>n=51(48-52)&lt;2n&gt;XY, +8, +8, +8, +13 del(8)(p1,p2), ins(11;9)(q23;p22p23)</td>
<td>P15INK4B and P16INK4A deletions, MLL-AF9 fusion FLT3-ITD (gene level only); no FLT3 expression Monocytic MDS/RAEB to M5a</td>
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<td>Peripheral blood post-BMT Myelocytic</td>
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Table A1. Cell lines used in the dissertation work. (217)
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<th>WGS/ WES</th>
<th>Total %</th>
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**Table A.2. AML mutation frequencies from multiple Next Gen Sequencing manuscripts.**

This table was derived from “Next Gen Sequencing in AML” by Leisch et. Al. (30, 42) This review tabulated the results of AML mutation frequencies from numerous NGS (next generation sequencing) studies of varying properties. The rightmost column is the percentage of mutated patients across the studies that tested for that mutation. Mutations under 2% were excluded to save space on a single page. WGS is whole genome sequencing. WES is whole exome sequencing.
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<th>Target</th>
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<td>Antibody-drug conjugate</td>
<td>CD33</td>
<td>CD33+ AML</td>
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<td>Enasidenib</td>
<td>Small molecule</td>
<td>IDH2</td>
<td>R/R AML w/ IDH2 mutation</td>
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<tr>
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<td>Midostaurin</td>
<td>Small molecule</td>
<td>FLT3</td>
<td>FLT3 mutant AML</td>
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<tr>
<td></td>
<td>Vyxeos</td>
<td>Liposome w/ Ara-C and DNR at 5:1</td>
<td></td>
<td>t-AML, AML-MRC</td>
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<td>FLT3, AXL</td>
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<td>75+ years old or inductive chemotherapy ineligible</td>
</tr>
</tbody>
</table>

Table A.3. Summary of recent FDA approvals in AML.
Figure A.4. Life expectancy curves derived from the United States Social Security Administration (SSA) actuarial life tables. The life expectancy for healthy adults in the US for comparison to the poor survival of aged leukemic patients (218).
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ABSTRACT

STRATEGIES TO ENHANCE THE ANTI-LEUKEMIC ACTIVITY OF VENETOCLAX (ABT-199) IN AML THROUGH TARGETING OF MCL-1

by

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Acute Myeloid Leukemia (AML) is a frustratingly difficult to treat disease (67% 5 year survival for children and 24% for adults). The standard of care, similar to outcomes, has seen few improvements over the last several decades. The Bcl-2 family, which controls cell survival and apoptosis, is dysregulated in AML. Bcl-2, which is overexpressed in AML and associated with chemoresistance, is a promising therapeutic target. The now FDA approved venetoclax (ABT-199) is a BH3 mimetic that is able to bind to anti-apoptotic Bcl-2 and prevent it from sequestering pro-apoptotic Bim. While overall response rates are promising, our lab and others had found that resistance often occurs through anti-apoptotic Mcl-1 sequestering Bim and preventing apoptosis.

In this dissertation, we used the molecular tool and Mcl-1 inhibitor A-1210477 and found that inhibition of Mcl-1 can synergize with venetoclax to induce apoptosis in AML cells. To inhibit Mcl-1 in a more clinically relevant manner, we sought to target Mcl-1 transcription or protein stability using clinically available drugs. XPO1 is overexpressed in AML cells and its inhibition decreases Mcl-1 levels and shows anti-leukemic activity. XPO1 inhibition by KPT-330 (selinexor) or KPT-8602 (eltanexor) was able to synergistically enhance apoptosis induced
by venetoclax. XPO1 inhibition decreased Mcl-1 by decreasing protein half-life but not transcript levels. XPO1 inhibition and venetoclax were able to disrupt Bim binding to both Bcl-2 and Mcl-1.

CDK9 inhibition transcriptionally decreases levels of Mcl-1 and c-Myc, and shows anti-leukemic activity. CDK9 inhibition by flavopiridol or voruciclib decreases Mcl-1 transcripts in a transient manner. CDK9 inhibition was able to disrupt Bim binding to Mcl-1 alone and in combination with venetoclax. The efficacy of the combination was dependent upon downregulation of Mcl-1 and c-Myc. Interrupted dosing of CDK9 inhibition was effective in vitro and in vivo.

Overall, Mcl-1 inhibition is a valid strategy to overcome resistance to venetoclax in AML. This strategy has been validated through direct targeting of Mcl-1 and clinically relevant indirect targeting of transcript levels or protein stability of Mcl-1 to decrease Mcl-1 protein levels.
AUTOBIOGRAPHICAL STATEMENT

Education

**Wayne State University, Detroit MI**
Cancer Biology Graduate Program: Doctor of Philosophy: Cancer Biology

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Grants, Awards, Scholarships:

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2016 C.P. Lee Graduate Student Research Day, Wayne State University, 2nd place poster presentation
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Work Experience

2015-2019 Graduate Research Assistant
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