

Curriculum Vitae

JONELLE K. LEE

jonelle.lee88@gmail.com

SUMMARY

Motivated PhD candidate in molecular medicine with a concentration in cancer biology. My research focuses on treatment of acute myeloid leukemia (AML) with small molecule inhibitor combinations and elucidating mechanisms of action.

RESEARCH TECHNIQUES

- Performed wet laboratory, biochemical and molecular research using techniques including western blot analysis, RT-qPCR, flow cytometry, molecular cloning, agarose gel electrophoresis, cell viability assays, drug combination cytotoxicity assays, processing of patient blood and bone marrow samples, culture and transfection of mammalian cell lines (adherent and non-adherent), CAR-T cell development, spheroid formation assay and migration assays.
- Analyzed data using bioinformatic techniques with basic programming skills in Perl and R
- Bred and maintained populations of drosophila

EDUCATION

Bachelor of Science in Biology

Longwood University, Farmville, VA

Dec 2010

Master of Science in Biology

Virginia State University, Petersburg, VA

Dec 2015

Doctor of Philosophy in Molecular Medicine

University of Maryland Baltimore, Baltimore, MD

Aug 2016 – May 2023

RESEARCH EXPERIENCE

Graduate Trainee Research Assistantship

Virginia State University, Petersburg, VA

August 2014-Dec 2015

- Performed independent research on type 2 diabetes under the direction of a VSU faculty members
- Identified type 2 diabetes-related SNPs that disproportionately affect different ethnicities for my thesis project

- Developed a comprehensive overview of type 2 diabetes, Genome Wide Association Studies (GWAS), and population-specific genomics
- Performed data analysis and compiled previously published data to quantify research results

Graduate Research Assistant

Marlene and Stewart Greenebaum Comprehensive Cancer Center

University of Maryland, Baltimore, MD

Oct 2017-present

- Conducted research on acute myeloid leukemia (AML) with internal tandem duplication of fms-like tyrosine kinase 3 receptor tyrosine kinase (FLT3-ITD), a common and unfavorable AML subtype, under the mentorship of Dr. Maria Baer
- Used combination targeted strategies to improve the efficacy of treatment for this AML subtype in preclinical models.
- Explored the molecular signaling pathway resulting from concurrent FLT3 and Pim kinase inhibition in mouse/human cell lines (Ba/F3-ITD, Ba/F3-WT, MV4-11, MOLM-14) using RT-qPCR, western blot analysis, cytotoxicity assays, drug combination cytotoxicity assays and flow cytometry.
- Isolated and treated PBMCs from primary patient blood and bone marrow samples.
- Used genome editing techniques to knock down and overexpress genes of interest.

PROFESSIONAL EXPERIENCE

Genetics Laboratory Assistant, Department of Biology

Longwood University, Farmville, VA

Jan 2008-May 2008

- Maintained living laboratory specimens (*Drosophila melanogaster*)
- Successfully mated specimens and isolated progeny with double mutations
- Kept accurate and precise notes on day-to-day lab activities

Teaching Assistant, Genetics Laboratory

Longwood University, Farmville, VA

August 2008-Dec 2008

- Assisted in the setup, maintenance and cleanup of laboratory experiments including PCR, electrophoresis, and ELISA
- Maintained living laboratory specimens
- Developed and evaluated laboratory assignments
- Ensured compliance with laboratory processes and safe handling practices

General Biology Laboratory Instructor, Department of Biology

Virginia State University, Petersburg, VA

Jan 2015- May 2015

- Taught assigned laboratory sections of General Biology by preparing laboratory materials and activities, as specified by course syllabus and instructor course outline
- Maintained appropriate record keeping for assigned classes and submitted assessment data to the appropriate lead or chair, consistent with departmental guidelines
- Mentored students to help them achieve their educational goals and objectives
- Developed, assigned and graded assessments of student learning appropriate for the specific course content

- Created a classroom environment that enhanced learning
- Maintained currency of knowledge, technical skills and teaching methodologies in the area of instruction through professional development from adjunct faculty
- Ensured compliance with college policies, guidelines and processes

LEADERSHIP EXPERIENCE

- Mentored rotating undergraduate and graduate students in the laboratory
- Trained new hires on relevant laboratory techniques and procedures
- Developed and managed independent research project

HONORS/AWARDS

- Geneva Schwartz Scholarship recipient
 - Aug 2006-May 2008
- Hull Scholarship recipient
 - Aug 2007-May 2009
- University of Maryland Meyerhoff Fellowship
 - Aug 2016-May 2017
- University of Maryland Institute for Clinical and Translational Research Scholar
 - Aug 2018-May 2019
- American Association for Cancer Research Minority in Cancer Research Scholar Travel Award
 - April 2019
- University of Maryland, Baltimore Presidential Scholarship recipient
 - Aug 2019-May 2020
- American Society of Hematology Abstract Achievement Award
 - Dec 2021
- American Association for Cancer Research Minority in Cancer Research Scholar Travel Award
 - April 2022

ORGANIZATIONS/MEMBERSHIPS

- Beta Beta Beta Biology Honor Society
 - 2007-2009
- Delta Sigma Theta Sorority, Inc.
 - Nov 2008-present
- University of Maryland Meyerhoff Graduate Fellow
 - Aug 2016-present

- University of Maryland Graduate Program in Life Sciences Student Advisory Council member
 - April 2017-May 2022
- Society for the Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS) member
 - Sept 2017-present
- University of Maryland, Baltimore Continuing Umbrella of Research (CURE) Mentor
 - Sept 2017-May 2019
- American Association for Cancer Research
 - Jan 2019-present
- American Society of Hematology
 - Oct 2021-present

PUBLICATIONS

Scarpa M, Singh P, Bailey C, **Lee JK**, Kapoor S, Lapidus RG, Niyongere S, Sangodkar J, Wang Y, Perrotti D, Narla G, Baer MR. PP2A-activating drugs enhance FLT3 inhibitor efficacy through AKT inhibition-dependent GSK-3 β -mediated c-Myc and Pim-1 proteasomal degradation. *Molecular Cancer Therapeutics* 20:676-690, 2021. *Epub 2021 Feb 10*.

Scarpa M, Kapoor S, Tvedte ES, Doshi KA, Zou YS, Singh P, **Lee JK**, Chatterjee A, Mustafa Ali M, Bromley RE, Hotopp JCD, Rassool FV, Baer MR. Pim kinase inhibitor co-treatment decreases alternative non-homologous end-joining DNA repair and genomic instability induced by topoisomerase 2 inhibitors in cells with FLT3 internal tandem duplication. *Oncotarget* 12:1763-1779, 2021. *Epub 2021 Aug 31*.

MANUSCRIPT

Lee JK, Chatterjee A, Scarpa M, Bailey CM, Niyongere S, Singh P, Mustafa Ali MK, Kapoor S, Wang Y, Baer MR. Pim kinase inhibitor enhances FLT3 inhibitor gilteritinib efficacy through GSK-3 β activation and GSK-3 β -mediated c-Myc and Mcl-1 proteasomal degradation. In preparation, Leukemia.

ABSTRACTS/PRESENTATIONS

Lee JK, Scarpa M, Kapoor S, Baer MR. Combined FLT3 and Pim kinase inhibitor treatment downregulates c-Myc early in apoptosis induction in acute myeloid leukemia with FLT3-ITD. American Association for Cancer Research, 2019. (2019 AACR Minority Scholar in Cancer Research Award).

Scarpa M, Singh P, Kapoor S, **Lee JK**, Niyongere S, Narla G, Perrotti D, Baer MR. PP2A activators enhance efficacy of FLT3 inhibitors in FLT3-ITD acute myeloid leukemia cells

through AKT inactivation-dependent Pim-1 and c-Myc proteasomal degradation. American Society of Hematology, 2019.

Lee JK, Scarpa M, Chatterjee A, Mustafa Ali M, Singh P, Kapoor S, Trotta R, Baer MS. Pim Kinase Inhibitor Enhances FLT3 Inhibitor Efficacy through GSK-3 β Activation and GSK-3 β -Mediated Proteasomal Degradation of c-Myc. American Society of Hematology Annual Meeting and Exposition, 2021. (ASH Abstract Achievement Award).

Lee JK, Scarpa M, Chatterjee A, Mustafa Ali MK, Singh P, Kapoor S, Trotta R, Baer MR. Concurrent treatment with Pim kinase inhibitor enhances efficacy of FLT3 inhibitors in AML with FLT3-ITD through GSK-3 β activation and GSK-3 β -mediated enhanced proteasomal degradation of c-Myc and Mcl-1. American Association for Cancer Research, 2022. (2022 AACR Minority Scholar in Cancer Research Award).

Mustafa Ali MK, Chatterjee A, **Lee JK**, Scarpa M, Baer MR. Effects of PP2A-activating drugs on FLT3 inhibitor resistance mediated by diverse mechanisms in acute myeloid leukemia with *FLT3*-ITD. American Association for Cancer Research Meeting, 2023.

ABSTRACT

Title: Pim kinase inhibitor enhances FLT3 inhibitor gilteritinib efficacy through GSK-3 β activation and GSK-3 β -mediated c-Myc and Mcl-1 proteasomal degradation

Jonelle Lee, Doctor of Philosophy, 2023

Dissertation Directed by: Maria Baer, MD, Professor, School of Medicine

Acute myeloid leukemia (AML) with *fms*-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) has poor outcomes. FLT3-ITD drives constitutive and aberrant FLT3 signaling, activating STAT5 and upregulating the downstream oncogenic serine/threonine kinase Pim-1. FLT3 inhibitors have limited clinical efficacy. We previously showed that concurrent Pim and FLT3 inhibition increases apoptosis induction in FLT3-ITD-expressing cells through post-translational downregulation of Mcl-1. Here we further elucidate the mechanisms of action of this dual targeting strategy. Protein expression and turnover, cytotoxicity and apoptosis were measured in FLT3-ITD-expressing cell lines and AML blasts treated with FLT3 inhibitor gilteritinib and/or Pim inhibitors AZD1208 or TP-3654. Pim and FLT3 inhibitor co-treatment decreased c-Myc protein, prior to Mcl-1, increased turnover of both proteins, rescued by proteasome inhibition, dephosphorylated (activated) GSK-3 β , and increased apoptosis and *in vivo* efficacy. GSK-3 β inhibition prevented c-Myc and Mcl-1 downregulation and apoptosis. Pim and FLT3 inhibitor co-treatment of Ba/F3-ITD cells infected with T58A c-Myc or S159A Mcl-1 plasmids, preventing phosphorylation at these sites, did not downregulate these proteins, increase their turnover or induce apoptosis, consistent with GSK-3 β activation and c-Myc T58 and Mcl-1 S159 phosphorylation as the mechanism of

combination treatment. These data further support GSK-3 β activation as a therapeutic strategy in FLT3-ITD AML.

Pim kinase inhibitor enhances FLT3 inhibitor gilteritinib efficacy through GSK-3 β activation and GSK-3 β -mediated c-Myc and Mcl-1 proteasomal degradation

by
Jonelle K Lee

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2023

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Dedication

To my daughter Arwen. Thanks for being my motivation and my biggest supporter. Everything that I do is for you. I hope that this serves as an example of how anything is possible if you put your mind to it. I hope this also inspires you to follow your dreams and live a life full of purpose.

Acknowledgments

I would like to thank my advisor and mentor, Dr. Maria Baer, for her support during my graduate work. It has been a long 6 years, but I am grateful to have been given the opportunity to work with you and for all that I have learned under your leadership.

Thank you to my committee members, Dr. Rassool, Dr. Kingsbury, Dr. Bieberich, Dr. Shapiro, and Dr. Qiu for serving on my committee. I appreciate all of the time and consideration that you have given to me throughout my thesis research project.

I also want to acknowledge all the wonderful people that I had the pleasure of working with in the Baer lab. A special thank you to Dr. Mario Scarpa and Shivani Kapoor for taking me under their wings and literally teaching me most of the techniques that I know today. Thanks to the rest of the lab members, past and present, Perna Sign, Dr. Moaath Mustafa-Ali, Dr. Aditi Chatterjee, Dr. Sandrine Niyongere, and Dr. Jake Liu. You guys are more than just my co-workers, you are my friends. Thanks for all of your help.

I would like to give an extra special thank you to the past and present members of the Rassool lab. Thank you Anna, Lena, Aksi, Lora, Rachel, and Julia for always being willing to help me with a protocol, lend me a reagent, or just listen to me complain. Your friendships and support have been invaluable. I must give an extra special shout out to Anna for helping me throughout my entire program. You have always been willing to help me since Core Course and I appreciate you more than you know.

Thank you to everyone on the 9th and 8th floor of Bressler who offered advice and help to me throughout my degree. Thank you to Dr. Rossana Trotta for all of her help and advice with my project. Thank you, Dr. Kavita, for always giving me suggestions and helping me troubleshoot my issues. Thanks to the members of Tim Luetkens' lab,

especially Dr. Luetkens, for always answering all of my random questions and Kenny for being kind enough to teach me new techniques. A special thank you to my good friend Aerielle for being so supportive.

I also have to acknowledge my friends. They have been so patient, kind, and supportive during my long graduate career. Sitesha, Brittany, Angela, and Nicole, thanks for always having my back. Dr. Sade Johns, thank you for keeping me focused and uplifted. Nicole, Dede, Tila, and Mimi, thank you for being the sisters I never had. RJ thank you for your love and support. Dr. Smith Jean, thank you for always being an extended mentor to me, someone I can rely on for advice, and for your help in getting through the end of my project.

Lastly, I would like to thank my family. My mother, Rosemary, for moving to Baltimore to help with my daughter. To my brothers, Michael, Dyrell, and Marquis, and my sister-in-law, Tasha, for their love and support. To father Osker, and my grandparents, Osker and Sandra, thank you for believing in me. To all my aunts and uncles that offered their encouragement, thank you. I would also like to give a special acknowledgment to my foster mother, Alberta Bolden, for always keeping me lifted in prayer.

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List of Abbreviations

4E-BP1	4E-binding protein 1
ABC	ATP binding cassette
AKT	Pro-kinase B
ALL	Acute lymphocytic leukemia
Alt-NHEJ	Alternative non-homologous end joining
AML	Acute myeloid leukemia
BAD	BCL-2 associated agonist of cell death
BCL-2	Protein B-cell lymphoma 2
BET	Bromodomain and extra-terminal
CBFB	Core-binding factor beta
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
C/EBP β	CCAAT-enhancer-binding protein
c-Kit	Stem cell factor receptor
c-Myc	Cellular myelocytomatosis oncogene
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CSF1R	Colony stimulating factor 1
CXC4	Chemokine receptor 4
CXCL12	Chemokine ligand 12
DNMT	DNA methyltransferase inhibitors
eIF4E	Eukaryotic translation initiation factor

ERK	Extracellular signal-regulated kinase
FDA	Federal Drug Administration
FL	FLT3 ligand
FLK-2	Fetal liver kinase-2
FLT3	Fms-like tyrosine kinase 3
Grb2	Growth factor receptor-bound protein 2
HiDAC	High-dose cytarabine
HOXA9	Homeobox A9
HSP	Heat shock protein
HSCT	Hematopoietic stem cell transplantation
ITD	Internal tandem duplications
MAPK	Mitogen activated protein
Mcl-1	Myeloid cell leukemia-1
miRs	MicroRNAs
mTOR	Mammalian target of rapamycin
MYH1	Myosin heavy chain 1
NPM1	Nucleophosmin
PDGFR	Platelet derived growth factor receptor α/β ?
PI3K	Phosphoinositide 3-kinase kinase
Pim	Proviral integration site for Moloney murine leukemia virus
PP2A	Phosphatase protein phosphatase 2A
PUMA	p53 upregulated modulator of apoptosis
RTK	Receptor tyrosine kinase

RUNX	Runt related transcription factor
SHIP	SH2-containing Inositol 5'-phosphatase
STAT5	Signal transducer and activator of transcription 5
Src	Non-receptor protein tyrosine kinase
STK-1	Human stem cell kinase-1
TK	Tyrosine kinase
WT	Wild type

Chapter 1: Background

1.1 Acute Myeloid Leukemia

Leukemia is a term used to designate several types of cancers that differentially affect blood-forming cells or hematopoietic stem-progenitor cells (**Fig. 1.1**).

Figure 1.1 Hematopoiesis

By A. Rad and M. Häggström. CC-BY-SA 3.0 license

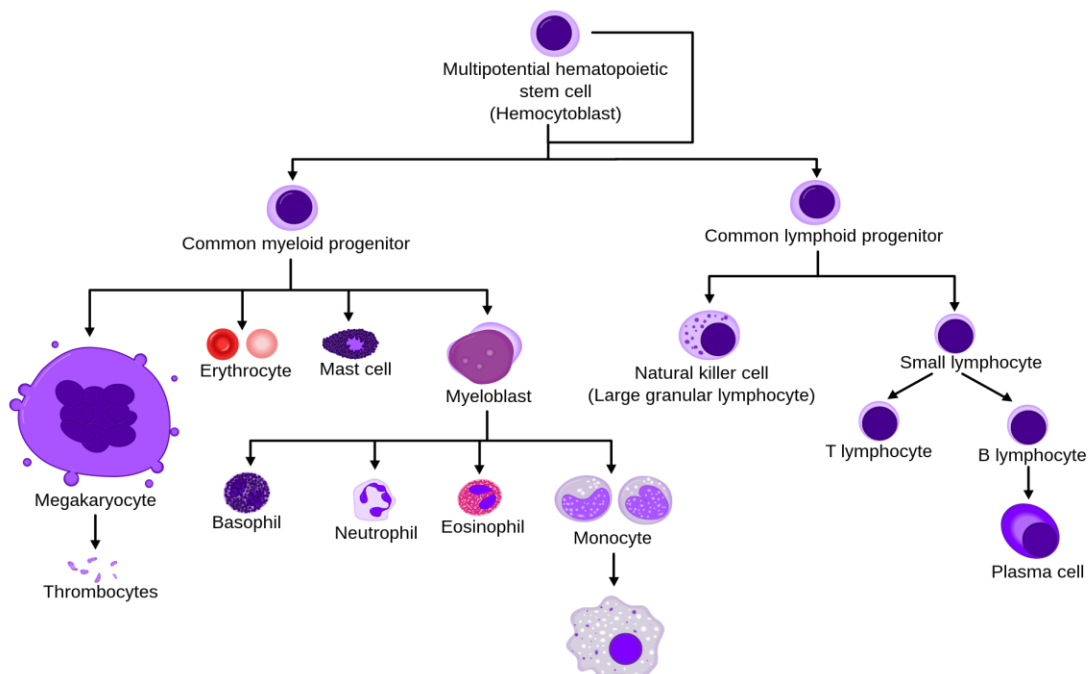


Diagram displaying the development of blood cells from hematopoietic stem cells through both the myeloid and lymphoid lineages.

Leukemias are grouped into two main sub-categories based on lineage (myeloid versus lymphoid) and further subdivided based on maturation of the cells (acute versus chronic). This results in four major categories of leukemia, which include: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL). Acute leukemias are characterized by maturation arrest of

malignant hematopoietic cells, and are associated with morbidity and mortality, while chronic leukemias have preserved cellular maturation and have a chronic or subacute presentation. Acute leukemias have more rapid progression than chronic leukemias. Altogether, there were an estimated 60,650 new cases of leukemia and ~22,000 deaths due to leukemia in 2022 (**Table 1.1**)¹. AML represented 33% of all new leukemia cases but disproportionately caused ~50% of leukemia deaths.

Table 1.1 Leukemia Incidence Rates and 5-Year Survival
Adapted from SEER Cancer Facts & Figures 2022

	Estimated New Cases	Estimated New Deaths	5-Year Survival (20 years and older)
Leukemia	60,650	24,000	N/A
Chronic lymphocytic leukemia	20,160	4,410	87%
Acute myeloid leukemia	20,050	11,540	27%
Chronic myeloid leukemia	8,860	1,220	70%
Acute lymphocytic leukemia	6,660	1,560	40%
Other leukemias	4,920	5,270	N/A

The estimated 5-year survival rate is 69% for AML patients 0-19 years old, but only 27% for patients 20 years and older. The median age of patients at diagnosis of AML is 68 years.¹

AML is caused by maturation arrest of a malignant clone of myeloid progenitor cells, the cells that differentiate into neutrophils, monocytes, red blood cells and megakaryocytes. It is characterized by accumulation of acquired cytogenetic abnormalities

and gene mutations over time. These genetic changes are considered recurrent because they are commonly identified in AML. These clonal genetic changes cause dysregulation of differentiation and proliferation of myeloid progenitor cells in the bone marrow.²

1.2 Classifications, cytogenetics, and mutations

Classification Systems

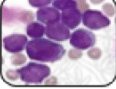
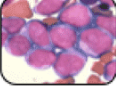
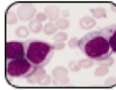
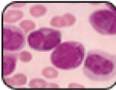
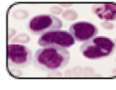
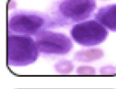

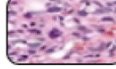
The treatment and prognostic outcomes of AML vary greatly based on disease subtypes. A number of parameters can be used to classify AML, including morphology, immunophenotype, cytogenetics, and gene mutations. Currently two systems are used to classify AML, including the World Health Organization (WHO) classification system³ and the International Consensus Classification (ICC).⁴ The WHO classification has had serial versions and is more widely used. The current WHO classification system divides AML into numerous subcategories based on genetic abnormalities or on differentiation (**Table 1.2**).³

Table 1.2 WHO AML Classification³

Acute myeloid leukaemia with defining genetic abnormalities
Acute promyelocytic leukaemia with <i>PML::RARA</i> fusion
Acute myeloid leukaemia with <i>RUNX1::RUNX1T1</i> fusion
Acute myeloid leukaemia with <i>CBFB::MYH11</i> fusion
Acute myeloid leukaemia with <i>DEK::NUP214</i> fusion
Acute myeloid leukaemia with <i>RBM15::MRTFA</i> fusion
Acute myeloid leukaemia with <i>BCR::ABL1</i> fusion
Acute myeloid leukaemia with <i>KMT2A</i> rearrangement
Acute myeloid leukaemia with <i>MECOM</i> rearrangement
Acute myeloid leukaemia with <i>NUP98</i> rearrangement
Acute myeloid leukaemia with <i>NPM1</i> mutation
Acute myeloid leukaemia with <i>CEBPA</i> mutation
Acute myeloid leukaemia, myelodysplasia-related
Acute myeloid leukaemia with other defined genetic alterations
Acute myeloid leukaemia, defined by differentiation
Acute myeloid leukaemia with minimal differentiation
Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia

The original classification of AML was the French-American British (FAB) classification system, based on cell maturation determined by morphology. It classified AML into 9 sub-categories (M0-M7) (Fig. 1.2).⁵

Figure 1:2 FAB Classification ⁶
Adapted from Khan et al., 2020

FAB CLASSIFICATION	
	M0: Undifferentiated acute myeloblastic leukemia (5%)
	M1: Greater number of myeloblasts with <10% granulocytic differentiation.
	M2: Myeloblasts in great number with granulocytic differentiation >10% , NSE <20%.
	M3: Promyelocytes that are hyper granular with many Auer rods on CAE or Wright-stain and variant form cells with reniform nuclei, multilobed or bibbed, primeval cells with multiple Auer rods or relative scarcity of Hypergranular promyelocytes.
	M4: >20% but <80% NSE-butyrate positivity in Monocytic cells
	M5: Monocytic cells with >80% NSE positivity. (a) Monocytic differentiated (b) Monocytic, differentiated.
	M6: >30% myeloblasts with more than 50% erythroblasts eliminating the erythroid cells.
	M7: Acute megakaryoblastic leukemia <5%

Description and pictures of FAB subtypes using non-specific esterase (NSE) cyto-chemical staining as an indicator of moncytic differentiation.

Numerous cytogenetic changes and gene mutations can lead to or contribute to leukemogenesis in AML. Furthermore, cytogenetic changes and gene mutations have prognostic significance, and guide treatment decisions.^{7, 8} Cytogenetic and molecular risk stratification is summarized in **(Table 1.3)**.

Table 1:3 AML Genetic Risk Stratification ⁷

Adapted from Dohner et al. Blood, 2017

Prognostic Risk	Cytogenetics	Molecular Abnormalities
Favorable	t(8;21)(q22;22);RUNX1-RUNX1T1	Biallelic CEBPA
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CFBF-MYH11	Mutated NPM1 without FLT3-ITD or with low FLT3-ITD
Intermediate	t(9;11)(p21.3;q23.3); MLLT3-KMT2A	Mutated NPM1 and high FLT3-ITD
	Cytogenetic abnormalities not classified as favorable or adverse	Wild-type NPM1 without FLT3-ITD or low FLT3-ITD
Adverse	t(6;9)(p23;q34.1); <i>DEK-NUP214</i>	Wild-type NPM1 with high FLT3-ITD
	t(v;11q23.3); <i>KMT2A</i> rearranged	Mutated RUNX1
	t(9;22)(q34.1;q11.2); <i>BCR-ABL1</i>	Mutated ASXL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); <i>GATA2,MECOM(EVII)</i>	Mutated P53
	-5 or del(5q); -7; -17/abn(17p)	
	Complex karyotype monosomal karyotype	

Cytogenetics

Cytogenetic abnormalities include structural or numerical changes in chromosomes. Structural changes can include, but are not limited to, translocations, inversions, deletions, and insertions of portions of chromosomes. Cytogenetic findings are the strongest prognostic factor used to predict patient outcomes in AML. Risk status can

be stratified into three categories, favorable, intermediate, and adverse, based on cytogenetic changes.^{7,8} These categories predict patients' responses to treatment and their survival. In a validation study of the ELN guidelines, patients with favorable cytogenetics had a 54 % 5-year overall survival, compared to intermediate (30.6%) and adverse (12.2%).⁷ Translocations and inversions in chromosome 16, which result in fusion of the *CBFB* and *MYH11* genes, are favorable and patients have a high response rate and cure rate with chemotherapy.⁹ t(8;21)(q22;q22), resulting in *RUNX1::RUNX1T1* (previously called *AML1::ETO*) fusion, is also a prognostically favorable chromosome abnormality.¹⁰ Patients with normal karyotypes are typically classified as intermediate-risk, while those with complex karyotypes (three or more unrelated chromosome abnormalities) are classified as adverse-risk.

Mutations

AML is a highly heterogeneous disease, with numerous mutations occurring in a number of important genes that regulate signaling pathways and other cellular processes. Much like cytogenetic findings, these mutations serve as prognostic factors and stratify AML into prognostic groups based on expected outcomes (**Table 1.3**). Favorable mutations include mutations in nucleophosmin (*NPM1*), in the absence of fms-like tyrosine kinase 3 internal tandem duplication (*FLT3-ITD*), and bi-allelic mutations in CCAAT enhancer-binding protein A (*CEBPA*).⁷ In contrast, *FLT3-ITD* or mutations in *RUNX1*, *ASXL1* or *TP53* (the latter usually associated with a complex karyotype) are all considered adverse, with unfavorable prognoses.⁷

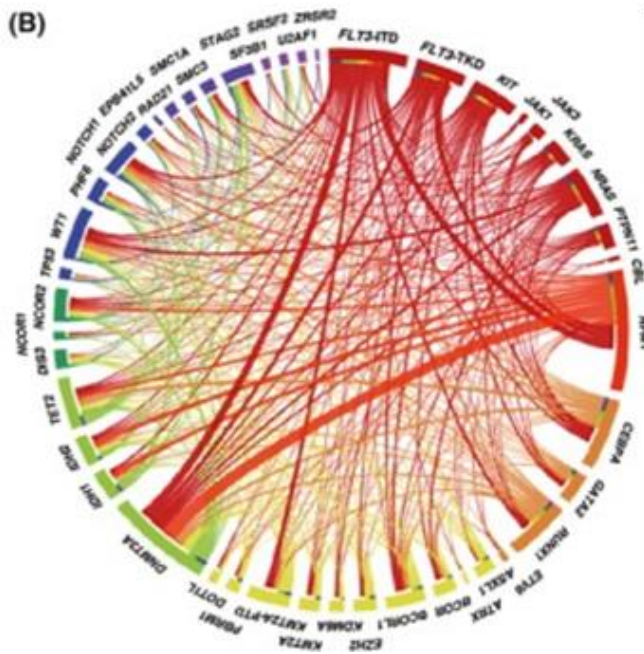
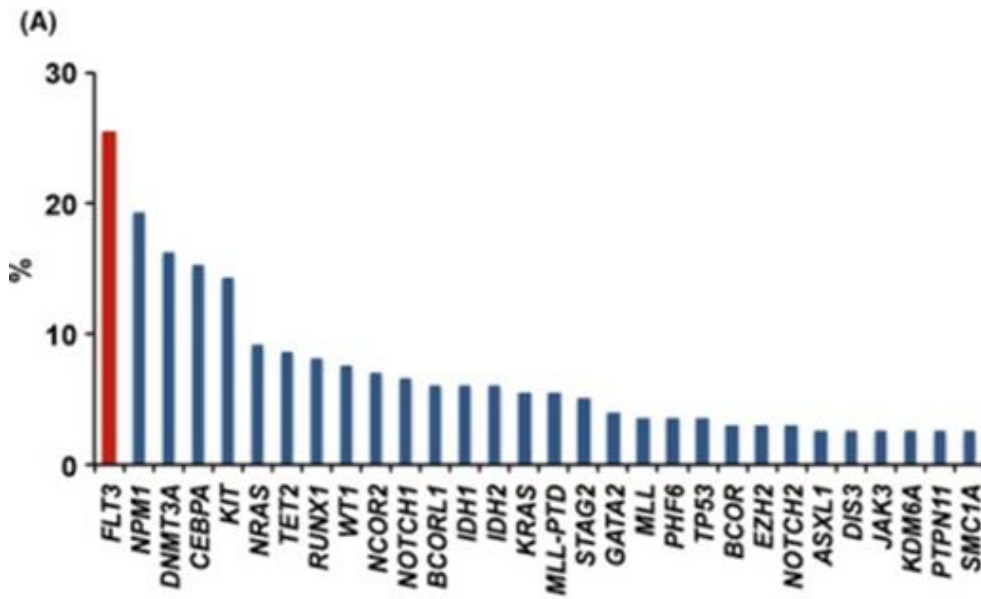
Table 1.4 Functional Classes of AML Mutations¹¹

Adapted from DiNardo and Cortes, Blood, 2016

Functional Class	Examples of genes with mutations
Signaling and kinase pathways	<i>FLT3, KRAS, NRAS, KIT, PTPN11, and NF1</i>
Epigenetic modifiers (DNA Methylation and DNA Modifiers)	<i>DNMT3A, IDH1, IDH2, TET2, ASXL1, EZH2, and MLL/KMT2A</i>
Nucleophosmin	<i>NPM1</i>
Transcription factors	<i>CEBPA, RUNX1, and GATA2</i>
Tumor suppressors	<i>TP53</i>
Spliceosome complex	<i>SRSF2, U2AF1, SF3B1, and ZRSR2</i>
Cohesin complex	<i>RAD21, STAG1, STAG2, SMC1A, and SMC3</i>

Mutated genes can also be divided into functional classes, which include kinase/signaling pathways, transcription factors, epigenetic modifiers and tumor suppressors, among others (**Table 1.4**).^{11, 10} Patients often have mutations in multiple genes in different functional classes (**Fig. 1.3**).^{12, 11} Most mutations in AML occur in genes involved in cell signaling and kinase pathways, including *FLT3*, V-Ki-RAS2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and neuroblastoma RAS viral oncogene homolog (*NRAS*), and contribute to increased proliferation through ligand-independent activation of proteins in pathways such as pro-kinase B/phosphoinositide 3-kinase (AKT/PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK).¹¹ Other commonly mutated genes in AML include nucleophosmin (*NPM1*) and the epigenetic modifier DNA methyltransferase 3A (*DNMT3A*) (**Fig. 1.3**).

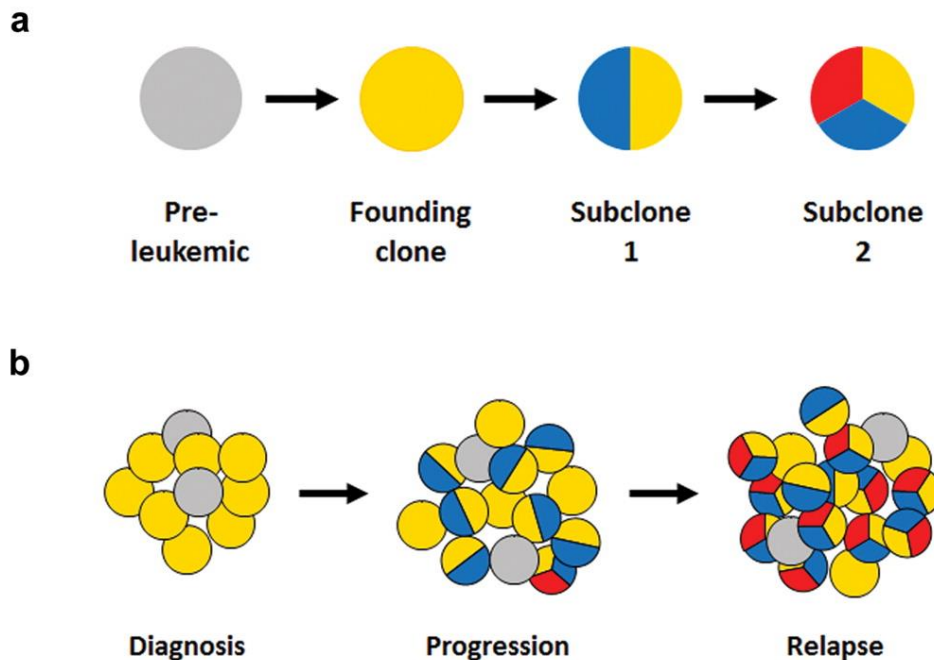
Figure 1:3 Mutational Landscape of AML ¹²
 Adapted from Kiyoi et al., 2020



(A) Graphs of most commonly mutated genes in AML and (B) the mutual exclusivity of the commonly co-mutated genes in AML.

In addition, heterogeneity can exist within the subclonal populations of AML.¹³ With the emergence of new sequencing techniques, populations of cancer cells with different genetic, epigenetic and chromosomal changes can be found within one patient.¹⁴ Progenitor cells are thought to undergo a sequential series of mutations in conjunction with clonal expansion, which results in multiple subclonal populations within a tumor that offer unique benefits to the tumor, such as increased survival, proliferation, drug resistance and/or self-renewal (**Fig.1.4**).^{13,15} It is important to note that while most tumors harbor multiple mutations, certain mutations are mutually exclusive due to redundancy in function. (**Fig.1.3**).^{12,11}

Figure 1.4 Clonal Evolution in AML¹⁵
From Grauber et al., 2014



Schematic of clonal evolution in AML. (A) Mutations can be acquired over time in pre-leukemic cells (gray) leading to a leukemia-initiating mutation (yellow). Subsequent mutations are acquired (blue and red) leading to the establishment and clonal expansion of different leukemic sub-clones. (B). At diagnosis most patients have a mixture of both pre-leukemic cells and founding clones. As the disease progresses, new clones emerge which may offer survival benefits. At relapse after remission, these minor subclonal populations may emerge as dominant.

1.3 AML Treatments

Standard treatment for patients with newly diagnosed AML consists of induction chemotherapy on a 7+3 schedule (cytarabine by 7-day continuous intravenous infusion, with an anthracycline, either daunorubicin or idarubicin, intravenously daily on the first 3 days).¹⁶ The complete remission (CR) rate has increased over the past several decades,¹⁷ with up to 75% of patients 18-60 years old now achieving CR.¹⁷ Targeted therapies such as midostaurin, a FLT3 inhibitor, or gemtuzumab ozogamicin, a toxin conjugated to a monoclonal antibody targeted to CD33, can also be incorporated into induction chemotherapy.^{18,19} As previously mentioned, AML patients are commonly older than 60 years (median age, 68 years) and may not be fit for intensive chemotherapy regimens. Fitness is determined by age, performance status (PS), or ability to perform daily tasks, and comorbidities.²⁰ In addition, as noted above, disease characteristics, including cytogenetics and molecular abnormalities, factor into treatment choices, as they predict likelihood of response. For patients who are unable to undergo intensive chemotherapy or are unlikely to respond, other options for treatment include DNA methyltransferase inhibitors (DNMTis), which work through epigenetic modification of dysregulated genes, given by themselves or with targeted treatments. Some FDA-approved first-line targeted treatments used in older unfit patients, include the isocitrate dehydrogenase (IDH) inhibitors ivosidenib and enasidenib, which target IDH1 and IDH2, respectively, venetoclax, a proapoptotic protein B-cell lymphoma 2 (BCL-2) inhibitor, and glasdegib, an inhibitor of the hedgehog signaling pathway, the latter two administered with DNMTis.²⁰

After achieving remission, patients undergo consolidation treatment with the goal of eradicating or decreasing residual AML cells and preventing or delaying relapse. One form of consolidation therapy is chemotherapy using high-dose cytarabine (HiDAC), which is mainly given to “fit” patients with more favorable prognostic factors. Allogeneic hematopoietic stem cell transplantation (HSCT) may be used as a consolidation treatment after, or in lieu of, HiDAC in “fit” patients with less favorable prognosis with chemotherapy alone.²¹ Altogether, treatment is curative in 35-40% of patients 60 years of age and younger and less than 15% of patients over 60 years.¹⁶ Unfortunately, most patients will relapse after achieving CR, or will have refractory, unresponsive, disease. Options for these patients include salvage chemotherapy, targeted therapies if available, or clinical trials of new treatments.²²

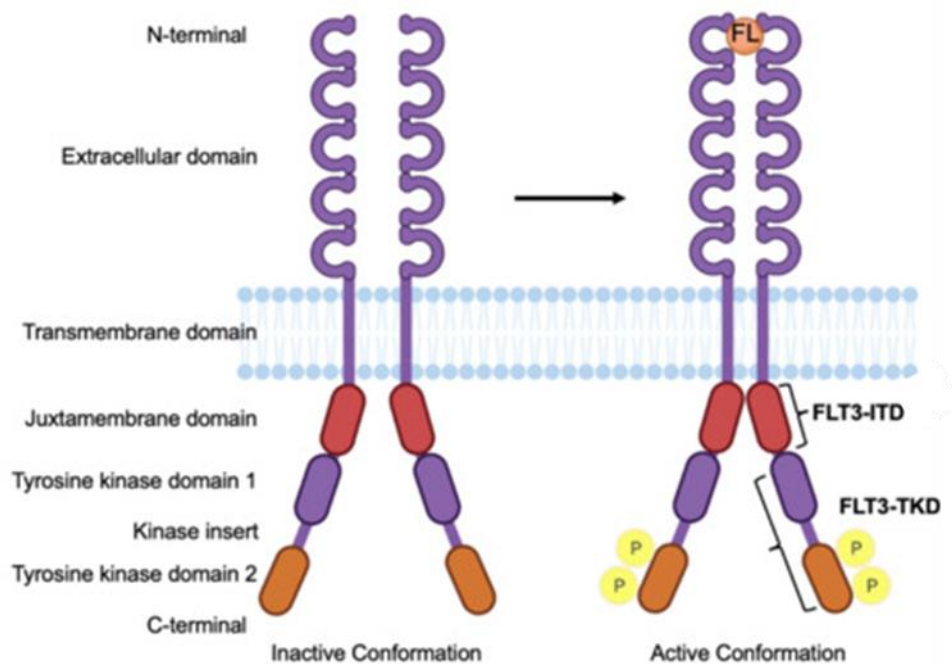
1.4 FLT3 Receptor and Mutations

FLT3 Receptor and Ligand

fms-like tyrosine kinase 3 (FLT3) is a type III receptor tyrosine kinase (RTK) expressed on hematopoietic progenitor cells and dendritic cells.²³ It is also known as fetal liver kinase-2 (FLK-2) and human stem cell kinase-1 (STK-1).²⁴ Other members of the type III receptor class include stem cell factor receptor (c-Kit), platelet-derived growth factor receptor (PDGFR) α/β and colony-stimulating factor 1 receptor (CSF1R). FLT3 signaling supports cell survival, proliferation and differentiation. The FLT3 gene is on chromosome 13q12, and the protein is 993 amino acids in length.²⁵ FLT3 is a transmembrane protein composed of an immunoglobulin-like extracellular domain to which FLT3 ligand (FL) binds, a transmembrane domain, a juxtamembrane domain, and

an intracellular kinase domain (**Fig.1.5**).²⁶ Glycosylation of the extracellular domain is an important post-translational modification that allows for proper transport of the wild-type (WT) receptor to the plasma membrane and signaling.²⁷ FLT3 is approximately 158-169 kD in size when glycosylated and 130-143 kD unglycosylated.²⁷

Figure 1.5 FLT3 Receptor ²⁸
Adapted from Wei et al., Cancer, 2022.

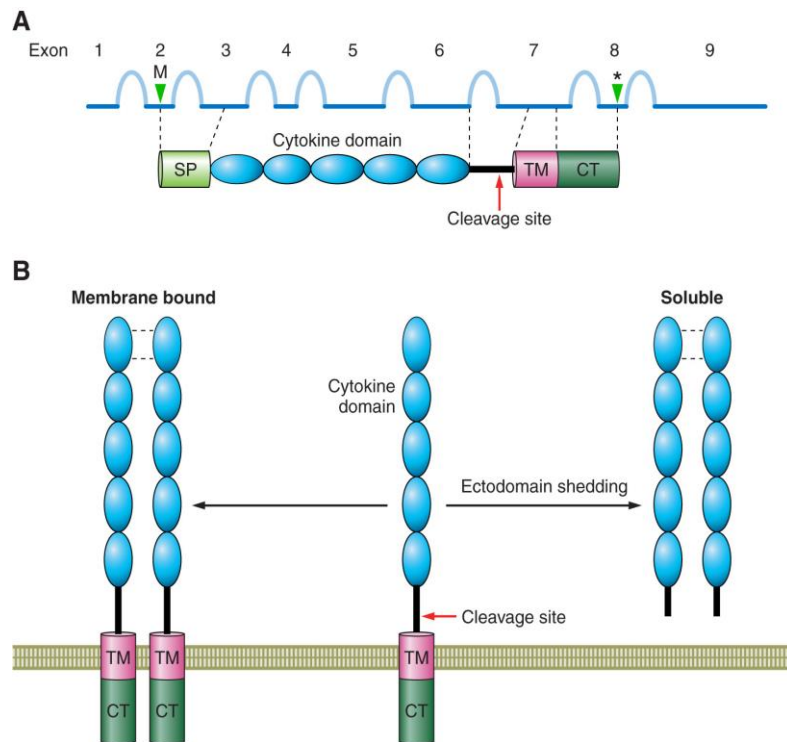


FLT3 receptor, highlighting the main structural domains and the domains in which FLT3-ITD and TKD mutations occur.

The helical cytokine FLT3 ligand (FL) is a non-covalently linked homodimer; it has structural homology and overlapping functions with stem cell factor (SCF) and

macrophage colony-stimulating factor (M-CSF).^{29,30} The human FL ligand gene is located on chromosome 19 and encodes a protein 235 amino acids in length that stimulates proliferation and differentiation of hematopoietic progenitor cells.²⁵ It is the only known ligand for FLT3. FL is expressed as a transmembrane protein on the surface of hematopoietic cells, type-2 conventional dendritic cells (CDC2)³¹ and T-cells³² and can be cleaved to produce a soluble ligand (**Fig. 1.6**).²

Figure 1.6 FLT3 Ligand³³
From Kazi and Roonerstrand, 2019



(A) Comparison of FLT3 ligand transcript and protein structure. (B) Membrane-bound FLT3 ligand can be cleaved to form soluble ligand. Both membrane-bound and soluble ligands can form dimers and induce signaling through binding to the FLT3 receptor.

Upon binding of FL, the FLT3 receptor, which exists as a monomer, undergoes conformational change and homodimerization, followed by autophosphorylation, resulting in activation and a cascade of downstream phosphorylation and activation events.²⁶ FLT3 primarily activates PI3K/AKT and MAPK/ERK signaling through adaptor proteins such as non-receptor protein tyrosine kinase (SRC), growth factor receptor-bound protein 2 (GRB2), and SH2-containing inositol 5'-phosphatase (SHIP).³⁴ After activation, the receptor-ligand complex is rapidly internalized and degraded, with detectable degradation products within 20 minutes.³⁴

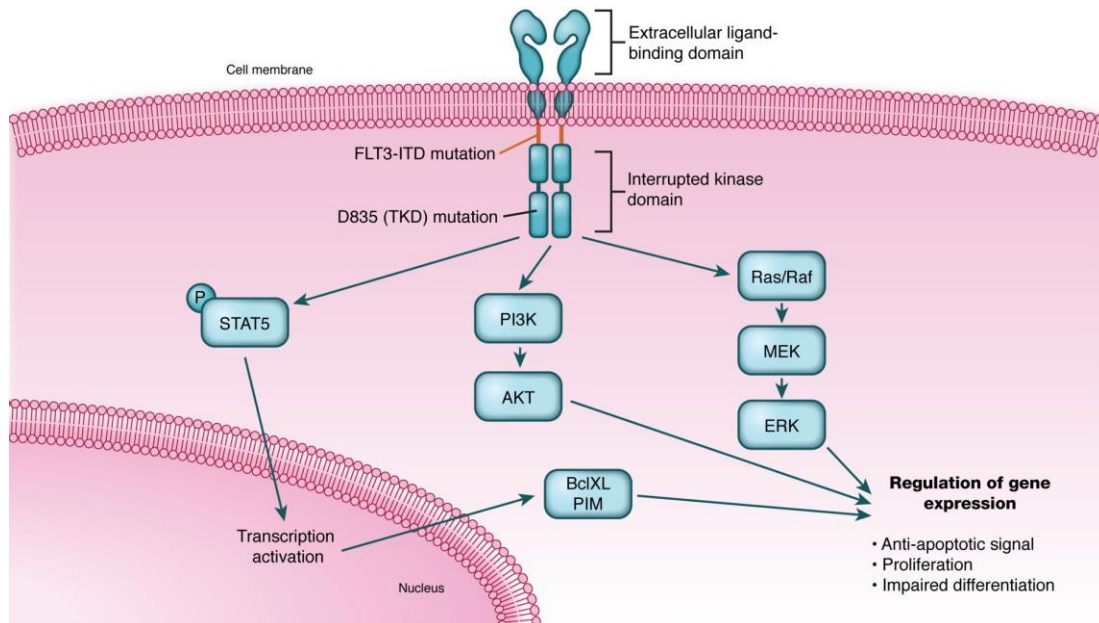
FLT3 Mutations

Mutations in the *FLT3* gene, encoding the receptor, are found in AML cells in approximately 30% of patients. Internal tandem duplications (FLT3-ITD) account for 25%, while point mutations within the tyrosine kinase domain (TKD) account for 5%.³⁵ In FLT3-ITD, nucleotide repeats are inserted within the juxtamembrane domain of the FLT3 receptor (**Fig. 1.5**). The most common TKD mutations are point mutations at D835, accounting for 50% of TKD mutations, resulting in stabilization of the active conformation of the FLT3 receptor.³⁶ Both ITD and TKD mutations lead to constitutive, ligand-independent, activation of FLT3 and activation/upregulation of downstream signaling. FLT3-ITD is a negative prognostic factor in patients, associated with rapid relapse following chemotherapy response, while TKD mutations are associated with an intermediate prognosis.³⁷ In a retrospective study, 5-year overall survival (OS) for patients with FLT3-ITD, TKD mutations, and WT FLT3 was 22.6%, 46.1%, and 42.4%, respectively.³⁷

It is also important to note that in cells with FLT3-ITD, the FLT3 receptor is unable to be packaged and transported to the cell membrane, and remains in the Golgi apparatus as a monomer. This allows for activation and preferential signaling through signal transducer and activator of transcription 5 (STAT5), in addition to signaling through PI3K and MAPK, which may account for the prognostic difference between FLT3-ITD and TKD mutations (**Fig 1.7**).²⁷

Figure 1.7 Mutated FLT3 Signaling³⁸

From Kavanagh et al., 2019



FLT3 receptor signals through PI3K and ERK. FLT3-ITD causes the receptor to also signal through STAT5, which leads to an increase in transcription, survival and proliferation.

1.5 Pim Kinases

Structure

Constitutive FLT3 signaling upregulates and/or activates many other kinases and signaling pathways, contributing to leukemogenesis associated with FLT3-ITD. One important kinase that is transcriptionally upregulated downstream of STAT5 in cells with FLT3-ITD is the proviral integration site for Moloney murine leukemia virus (Pim) kinase Pim-1. Pim kinases are a family of serine/threonine protein kinases that are classified as proto-oncogenes because of their ability to promote cell survival and enhance proliferation. The family consists of three members, Pim-1, Pim-2, and Pim-3, with highly conserved homology. The three Pim kinase genes are on different chromosomes. All are expressed in a wide variety of tissues and malignancies. Pim-1 and Pim-2 are expressed in normal hematopoietic and lymphoid cells and are overexpressed in AML cells.³⁹

The *Pim 1* transcript produces two distinct protein isoforms, 34 kD and 44 kD in size, designated short and long Pim-1, or Pim-1S and Pim-1L, respectively, due to alternative translation initiation sites. Pim-1S and Pim-1L are functionally distinct, with different sub-cellular localization patterns and substrate specificities. Pim-1L is localized in cytosol and is associated with the plasma membrane; it is also more stable, with a longer half-life than Pim-1S. Pim-1S is mainly localized in the nucleus and is less stable, with a shorter half-life.⁴⁰ *Pim-2* is also translated from multiple initiation sites, resulting in three isoforms. Pim-2 has been shown to synergize with cellular myelocytomatosis oncogene (c-Myc) to aid in the development of lymphomas.³⁹ Pim-3 is primarily expressed in brain, kidney and breast and is localized in the cytoplasm; it is expressed in many solid tumors.⁴⁰

Although expression levels and function of each isoform vary by tissue type, the Pim kinases have physiological redundancy, as demonstrated in several knockout models.³⁹

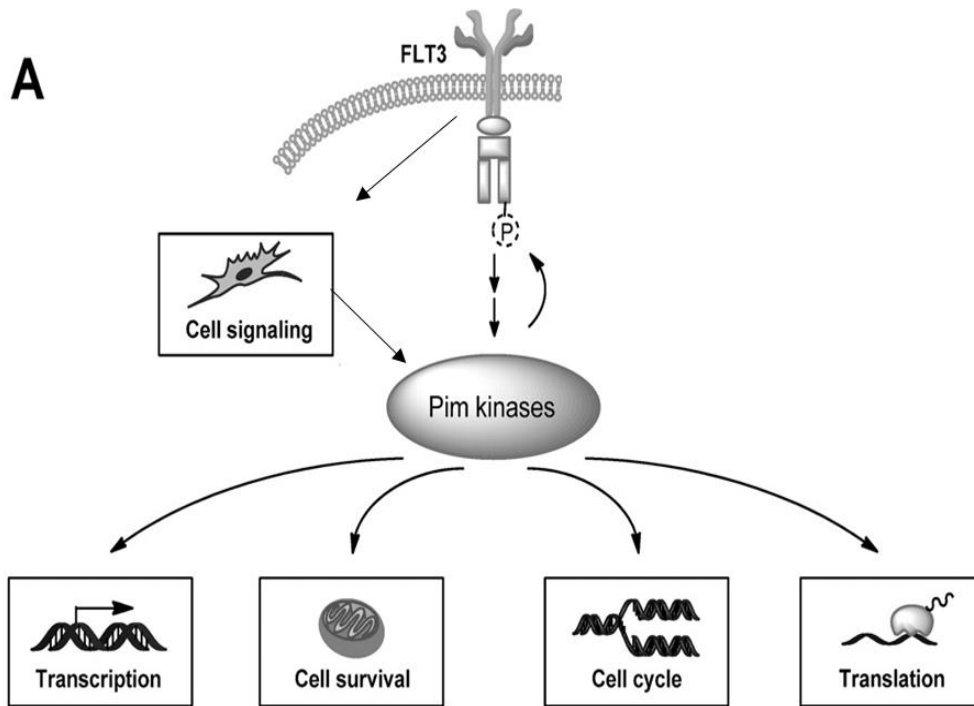
Pim kinases have a unique hinge structure that results in constitutive activation. Thus their activity reflects their level of expression, which is regulated by transcription rate and by both mRNA and protein stability. Growth factors and cytokines increase expression of Pim kinases mainly through upregulation of transcription factors such as STAT, runt-related transcription factor (RUNX), and homeobox A9 (HOXA9).⁴¹ Pim mRNA species are labile, and turnover is regulated by microRNAs (miRs); MiR-16, specifically, has been implicated in regulation of Pim-1 in cell lines harboring FLT3-ITD.⁴² Pim kinase proteins undergo ubiquitination and proteasomal degradation, and are regulated post-transcriptionally by binding to the cellular chaperones heat shock protein (HSP) 70 and 90, and by phosphorylation and dephosphorylation. In particular, dephosphorylation of Pim kinases by the serine/threonine phosphatase protein phosphatase 2A (PP2A) promotes their ubiquitination and proteasomal degradation.⁴³

Function

Pim kinases phosphorylate a wide range of substrates involved in many oncogenic processes including proliferation, cell cycle, apoptosis, cap-dependent protein translation, drug resistance and homing (**Fig. 1.8**). Pim kinases regulate proliferation at the transcriptional level through phosphorylation and stabilization of the oncogenic transcription factor c-Myc.⁴⁴ Other Pim kinase substrates include proteins involved in cell cycle checkpoints, such as cyclin-dependent kinases (CDKs) and cell division cycle (cdc) homologs, both of which promote cell cycle progression upon phosphorylation.⁴⁴ Another major function of Pim kinases is inhibition of apoptosis and consequent promotion of cell

survival by phosphorylation of BCL-2-associated agonist of cell death (BAD). This initiates binding of BAD to the regulatory protein 14-3-3, which prevents BAD from signaling to cells to undergo apoptosis.⁴⁴ Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which controls cap-dependent translation, is a target of Pim kinases as well. Phosphorylation of 4E-BP1 releases eukaryotic translation initiation factor (eIF4E), the initiator of cap-dependent translation, leading to translation of several early response genes that can also be involved in tumorigenesis, such as c-Myc, myeloid cell leukemia-1 (Mcl-1), and even Pim kinases.⁴⁴ Pim kinases post-translationally modify and stabilize p-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), which are ATP-binding cassette (ABC) drug resistance proteins and drug transporters that mediate resistance to cancer therapies.^{45,46} Pim kinases have also been shown to modulate expression of CXC chemokine receptor 4 (CXCR4), which regulates homing and migration.⁴⁰

Figure 1.8 Pim Kinase Function ⁴⁷
Adapted from Chen et al., 2011



Pim kinases are transcriptionally upregulated downstream of FLT3-ITD and lead to upregulation of proteins and processes that promote transcription, cell survival, cell cycle progression and translation. Pim kinases can also phosphorylate the FLT3 receptor and further stabilize it in a positive feedback loop in cells with FLT3-ITD.

Pim-1 in AML with FLT3-ITD

Pim-1 plays an important dual role in AML with FLT3-ITD. First, it is transcriptionally upregulated downstream of STAT5 in cells with FLT3-ITD and directly stimulates proliferation and inhibits apoptosis.⁴⁸⁻⁵⁰ In addition, our laboratory⁵¹ and another laboratory⁵² showed that Pim-1 also phosphorylates and thereby stabilize FLT3, enhancing FLT3 signaling in a positive feedback loop in cells with FLT3-ITD. Higher levels of Pim-1 mRNA were observed in patients with FLT3-ITD compared to healthy individuals and

those with AML in remission. Furthermore, AML patients with high Pim-1 mRNA expression had lower complete remission rates and took longer to achieve complete remission.⁵³ Pim-1 upregulation in FLT3-ITD cell lines also increased resistance to PI3K/AKT inhibitors⁵⁴ and FLT3 inhibitors.⁵²

1.6 Inhibitors

FLT3 Inhibitors

FLT3 inhibitors are small molecule tyrosine kinase inhibitors targeting the FLT3 receptor. Two FLT3 inhibitors, midostaurin and gilteritinib, are currently approved by the United States Food and Drug Administration, and several others are in development and in clinical trials. Midostaurin was approved in 2017 for treatment of newly diagnosed AML in combination with chemotherapy in patients with FLT3 mutations,¹⁸ and gilteritinib was approved in 2018 as monotherapy for patients with relapsed and refractory AML with FLT3 mutations.⁵⁵

FLT3 inhibitors can be divided into first- and second-generation based on their specificity for the FLT3 receptor. First-generation inhibitors are less specific for FLT3 and additionally target other kinases and other signaling pathways. First-generation inhibitors may have greater overall anti-leukemic effect by virtue of targeting diverse kinases, but are generally also associated with increased toxicities due to off-target effects.⁵⁶ Conversely, second-generation inhibitors are more specifically targeted to FLT3, are more potent and have fewer off-target effects, but do not inhibit other kinases or pathways.

FLT3 inhibitors can also be divided into two types, type I and type II, based on the nature of inhibitor binding to the receptor. Type I inhibitors bind directly in the ATP pocket of the active receptor (DFG-in conformation), while type II inhibitors bind to the inactive

receptor (DFG-out conformation) in a pocket adjacent to the ATP-binding site, preventing activation.^{57,58} Type I inhibitors show efficacy against FLT3 with both ITD and TKD mutations; in contrast, type II inhibitors are only effective against FLT3 with ITD, but not with TKD mutations, due to the active conformation conferred by TKD mutations.^{56,59} The table below summarizes FLT3 inhibitors in current use or currently or previously in clinical development (**Table 1.5**).

Table 1.5 FLT3 Inhibitors ²⁸
Adapted from Wei et al, Cancer, 2022.

FLT3 Inhibitor	Generation	Type	FLT3 Target	Other Targets	Phase of Development
Sunitinib*	First	I	ITD, TKD	VEGFR1, VEGFR2, KIT, PDGFR α/β , RET, CSF1R	II
Lestaurtinib	First	I	ITD, TKD	JAK2/3, trkA,B,C AURKA, AURKB	Terminated
Midostaurin	First	I	ITD, TKD	EGFR2, KIT, PGDFR, PKC α , VEGFR, AKT	Approved for FLT3m AML
Crenolanib	Second	I	ITD, TKD	PDGFR α/β	III
Gilteritinib	Second	I	ITD, TKD	ALK, AXL	Approved for FLT3m AML
Sorafenib*	First	II	ITD	VEGFR, PDGFR, c-Kit and RET, RAF	III
Quizartinib	Second	II	ITD	PDGFR α/β , RET, Kit, CSF1R	III
Ponatinib*	First	II	ITD	Abl, c-Kit, c-Src, FGFR1, PDGFR1 α , VEGFR2, LYN	II
Pexidartinib	First	II	ITD	KIT, CSF1R	I/II
Tandutinib	First	II	ITD	PDGFR α/β , c-Kit	Terminated
FF-10101	Novel	I	ITD, TKD	N/A	II
FN-1501	First	I	ITD, TKD	CDK4/6, KIT, PDGFR, ALK, RET	I
ETH-155008	Novel	I	ITD, TKD	CDK4/6	I

*Approved for other indications

FLT3 Inhibitor Resistance

Advances have been made in the development of targeted treatments for AML with FLT3 mutations in the past decade, but the efficacy of FLT3 inhibitors is still limited due to both incomplete responses and relapse due to development of resistance. FLT3 inhibitor resistance can be intrinsic, involving the tumor, or extrinsic, not involving the AML cells directly. Intrinsic resistance can be described as either primary (present prior to treatment) or secondary (acquired).

Primary resistance can occur due to mutations within the receptor, usually in the TKD, that prevent FLT3 inhibitors from binding to the receptor.⁵⁶ Another mechanism of primary resistance is upregulation of other signaling pathways that promote proliferation and cell survival. For example, Mcl-1 and Bcl-XL are upregulated in FLT3-ITD627E and FLT3-ITD/TKD mutated cells, respectively, leading to increased cell survival.^{56,60,61} One of the most notable primary resistance mechanisms is lack of addiction to FLT3 signaling because of low FLT3 mutational burden. These cells contain more WT FLT3, which is less sensitive to FLT3 inhibitors, thus allowing activation and signaling of the receptor through FL binding even in the presence of inhibitors.^{56,62}

Secondary resistance occurs after treatment with FLT3 inhibitors. A common mechanism of secondary resistance is acquired mutations within the FLT3 receptor that prevent drugs from binding. For example, D835 and Y842 mutations in the activation loop and F691, a gatekeeper mutation, are acquired mutations that lead to quizartinib resistance.⁶³ Additionally, newly acquired mutations within the RAS/MAPK pathway have been observed in patients with acquired gilteritinib resistance.⁶⁴ FLT3-ITD-expressing cells also have a higher incidence of error-prone DNA repair through alternative non-

homologous end joining (Alt-NHEJ); this contributes to genomic instability and acquisition of mutations that may lead to cancer progression and resistance.⁶⁵ Another mechanism of resistance noted in sorafenib-resistant cells is upregulation of Pim kinases, and the addition of a Pim kinase inhibitor restored FLT3 inhibitor sensitivity.⁵² Other kinases such as tyrosine-protein kinase receptor UFO (AXL) and spleen tyrosine kinase (SYK) have increased activation following FLT3 inhibitor treatment, contributing to FLT3 inhibitor resistance.^{66,67}

Extrinsic resistance can also significantly impact the efficacy of FLT3 inhibitors, and may occur through mechanisms mediated by the bone marrow microenvironment.⁵⁶ MAPK signaling can be upregulated due to an increase in fibroblast growth factor receptor 1 (FGFR1) activation through fibroblast growth factor 2 (FGF2) secretion from bone marrow.⁶⁸ Stromal cells within the bone marrow express FGF2 which binds to FGFR1, leading to an increase in proliferation and cell survival through activation of MAPK.⁶⁸ Similarly, ERK may be activated within the bone marrow stroma, contributing to FLT3 inhibitor resistance by upregulating other compensatory signaling pathways. Another extrinsic resistance mechanism is through the CXCL12/CXCR4 signaling axis; FLT3-ITD mutated cells migrate towards CXCL12, which is heavily expressed in the bone marrow and provides a protective niche against therapeutics.⁶⁹ The availability of FLT3 inhibitors can also be compromised due to an increase in metabolism in the liver.⁷⁰ Lastly, chemotherapy can increase induction of FL, leading to upregulation of FLT3 signaling even in the presence of FLT3 inhibitors.⁷¹ The table below gives examples of the different types of resistance mechanisms (**Table 1.6**). In an effort to overcome FLT3 resistance, new

treatment strategies must be developed, including combination treatments with other targeted kinase inhibitors.

Table 1.6 FLT3 Inhibitor Resistance Mechanism⁵⁶

Adapted from Larrosa Garcia and Baer, 2017

Intrinsic Resistance	
Primary	Secondary
Lack of addiction to FLT3 signaling	Newly acquired FLT3 mutations
Point mutations that confer resistance to specific inhibitor(s)	Genomic Instability
Upregulation of Mcl-1 by FLT3-ITD627E	Upregulation of Pim kinases
Bcl-XL upregulation in FLT3-ITD TKD dual mutants	Activation of AXL and SYK
FLT3-independent Bcl-2 upregulation	RAS/MAPK activation
Extrinsic Resistance	
Induction of FLT3 ligand by chemotherapy	
Induction of fibroblast growth factor 2 (FGF2)	
Enhanced CXCL12 and CXCR4-mediated homing	
Activation of ERK by bone marrow	
Induced hepatic metabolism of FLT3 inhibitor	

Pim Inhibitors

Over the years several Pim inhibitors have been developed and tested in preclinical and clinical studies. The structure of Pim kinases has been resolved through crystallography and includes an N-terminal lobe and C-terminal lobe connected by a hinge region and an ATP-binding site. Small molecule inhibitors have been developed based on this structure to selectively inhibit Pim kinases. Some inhibitors target Pim-1 specifically,

while others have been developed as pan-Pim inhibitors, targeting all three isoforms. Pim kinase inhibitors have been studied in a variety of AML cell lines and mouse models.

SGI-1776, ETP-45299, and ETP-39010 are Pim inhibitors that belong to the imidazopyridine class of drugs. ETP-45299 inhibits Pim-1 and also Pim-3 at higher concentrations. SGI-1776 and ETP-39010 inhibit all three Pim isoforms as well as FLT3 at micromolar concentrations. Treatment of MV4-11 AML cells with ETP-45299 or ETP-39010 decreased phosphorylation of the Pim substrate BAD and inhibited proliferation, while treatment with SGI-1776 downregulated c-Myc and decreased phosphorylation of 4EBP-1 in the same cell line.³⁹

Other pan-Pim inhibitors include AZD1208, GNE-652, and Pim447. AZD1208 belongs to the thiazolidine drug class and inhibits the growth of both primary samples and several AML cell lines; sensitivity has been linked to increased STAT5 phosphorylation and expression of Pim-1.³⁹ AZD1208 inhibited proliferation but had limited efficacy in inducing apoptosis as a single agent in a panel of AML cell lines.⁷³ Pim447 is a highly selective inhibitor that contains a pyridylamide scaffold which allows it to bind into the ATP pocket of Pim kinases with great efficiency. It is most effective in AML cell lines with high Pim expression such as MOLM16, KG1 and EOL1.⁷⁴ Another pan-Pim inhibitor is SEL-24-B58, a benzimidazole, which also inhibits other kinases including FLT3, Haspin, HIPK and CLK 1. SEL-24-B58, which also inhibits FLT3, reduced MCL1 protein levels *in vitro* and inhibited tumor growth in an MV4:11 xenograft mouse model.³⁹ TP-3654 is a selective second-generation inhibitor of Pim-1 and Pim-3. TP-3654 sensitized cells overexpressing ABCG2 to cytotoxic treatments.⁷⁵ TP-3654 also reduced bone marrow fibrosis, splenomegaly and leukocytosis in a murine myelofibrosis model.⁷⁶ Altogether,

preclinical findings for Pim inhibition in AML are promising and have prompted exploration of the efficacy of these drugs in clinical trials.

Unfortunately, most clinical trials of Pim inhibitors have failed due to lack of response and/or toxicities. Currently, only SEL24 and TP-3654 are in ongoing clinical trials. SEL24 is being explored in patients with AML with *IDH1* or *IDH2* mutations⁷⁷ based on empiric observation of efficacy in a phase 1 trial. *FLT3* and *IDH* are also commonly co-mutated in AML.⁷⁸ TP-3654, on the other hand, is being assessed in patients with myelofibrosis, a rare cancer that affects the bone marrow and can transform into AML.⁷⁹ Below is a table of Pim inhibitors that have been under clinical development (**Table 1.7**).

Table 1.7 Pim Inhibitors

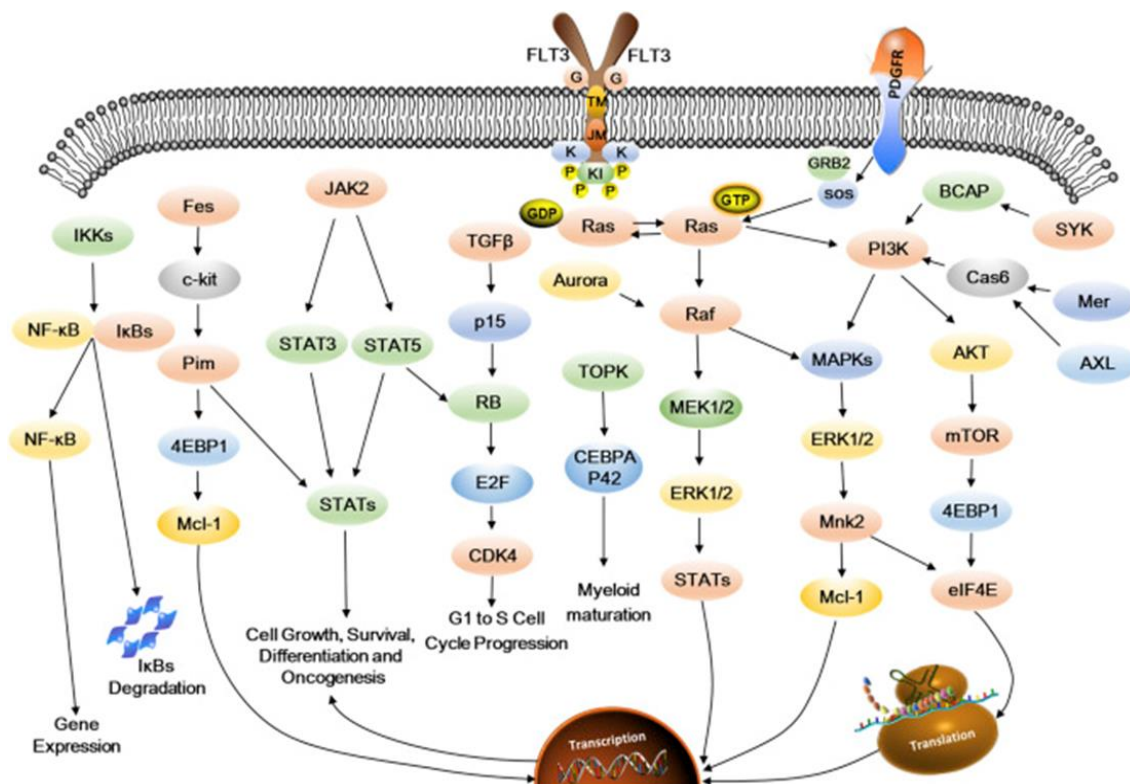
Inhibitor	Class	Pim Inhibition Selectivity	Other Targets
SGI-1776	Imidazopyridazine	Pim 1 IC ₅₀ : 7 nM Pim 2 IC ₅₀ : 363 nM Pim 3 IC ₅₀ : 69 nM	FLT3 IC ₅₀ : 44 nM Haspin IC ₅₀ : 34nM
AZD1208	Thiazolidine	Pim 1 IC ₅₀ : 0.4 nM Pim 2 IC ₅₀ : 5 nM Pim 3 IC ₅₀ : 1.9 nM	None
PIM447	Fluoropicolinamide-based inhibitor	Pim 1 IC ₅₀ : 6 pM Pim 2 IC ₅₀ : 18 pM Pim 3 IC ₅₀ : 9 pM	GSK-3β, PKN1, PI3K: IC ₅₀ s range from 1-5 mM
INCB053914	Not disclosed	Pan Pim inhibitor: IC ₅₀ s range from 3-300 nM	None
SEL24	Benzimidazole	Pim 1 IC ₅₀ : 31 nM Pim 2 IC ₅₀ : 154 nM Pim 3 IC ₅₀ : 152 nM	FLT3, HIPK, Haspin, CLK
TP-3654	Pyrazolopyrimidine-based inhibitor	Pim 1 IC ₅₀ : 5 nM Pim 3 IC ₅₀ : 42 nM	None

1.7 Rationale and Goals of the Study

AML is a very heterogeneous disease, with many cytogenetic and molecular abnormalities, making it a very complicated cancer to treat. AML with FLT3-ITD is a relatively frequent (25%) AML subtype that responds poorly to standard treatments. FLT3 inhibitors have been incorporated into treatment for AML with FLT3-ITD, but with limited success. Multiple signaling pathways can be upregulated as a result of, and in conjunction with, FLT3 mutations, pictured in **Fig.1.9**. Targeting multiple proteins amongst this signaling paradigm is a way to increase drug efficacy and overcome resistance.

Figure 1.9 Dual Inhibition Strategies for AML with Mutated FLT3 ⁸⁰

From Yuan et al., 2019



Schematic of the many signaling pathways that are activated due to and alongside FLT3 signaling. This image highlights the many possible druggable targets in AML.

Pim-1 is an attractive therapeutic target for combination treatment with FLT3 inhibitors because, as noted above, in addition to being transcriptionally upregulated downstream of FLT3-ITD and directly stimulating proliferation and inhibiting apoptosis, it also phosphorylates and thereby stabilizes FLT3, enhancing FLT3 signaling in a positive feedback loop in cells with FLT3-ITD.⁵¹ Pim kinase inhibitors on their own have little cytotoxicity, but our laboratory and others have shown that concurrent targeting of Pim-1 kinase increases cytotoxicity of FLT3 inhibitor therapy in FLT3-ITD-expressing cells.^{51,52,81} Additionally, overexpression of either Pim-1 or Pim-2 in MOLM-14 cells led to resistance to the FLT3 inhibitor sorafenib.⁵²

Our laboratory and several others have studied Pim kinase and FLT3 inhibitors in combination as a novel approach to treatment of AML with FLT3-ITD. The overall goal of this study was to understand the mechanisms of increased cytotoxicity of this combination therapy in AML with FLT3-ITD. Our laboratory is the first to show that c-Myc and Mcl-1 degradation and GSK-3 activation play key roles in the increased efficacy of Pim inhibitor co-treatment with FLT3 inhibitors.

Chapter 2: Concurrent treatment with the FLT3 inhibitor gilteritinib and Pim inhibitor increases cytotoxicity and downregulates c-Myc and Mcl-1 post-translationally through increased proteasomal degradation

2.1 Introduction

FLT3-ITD is present in AML in 30% of patients.³⁵ These patients have initial responses to chemotherapy, but relapse rapidly and have poor outcomes.⁸³ While outcomes have improved with incorporation of FLT3 inhibitors and allogeneic hematopoietic stem cell transplantation into treatment,⁸⁴ efficacy of FLT3 inhibitors is limited by incomplete responses and rapid onset of resistance, suggesting that outcomes may be enhanced by dual targeting of FLT3-ITD signaling pathways.⁵⁶ FLT3-ITD drives constitutive and aberrant FLT3 signaling. WT FLT3 receptor signals through the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathways upon binding of FL.⁸⁵ FLT3-ITD additionally and aberrantly activates STAT5, upregulating the downstream oncogenic serine/threonine kinase Pim-1,^{48,50} the c-Myc oncogene⁵⁰ and the anti-apoptotic protein Mcl-1.⁸⁶ Pim-1 not only contributes directly to the proliferative and anti-apoptotic effects of FLT3-ITD, but also phosphorylates and stabilizes FLT3 in a positive feedback loop in cells with FLT3-ITD.^{51,52} Pim kinase inhibitors are in clinical development.⁸⁷ Our laboratory and others have shown that dual inhibition of Pim and FLT3 kinases enhances apoptosis induction in cell lines with FLT3-ITD *in vitro* and *in vivo* and in primary AML cells with FLT3-ITD.^{51,52,82} Combining Pim kinase and FLT3 inhibitors is a promising treatment strategy for AML with FLT3-ITD.

c-Myc is a “master regulator” transcription factor that dimerizes with its co-activator, Max, and transcriptionally activates a number of gene families, driving

proliferation and resistance to apoptosis induction,⁸⁸ increasing protein translation, and promoting drug resistance.⁸⁹ In addition to being transcriptionally upregulated in AML with FLT3-ITD,⁵⁰ c-Myc is also regulated post-translationally by Pim-1.⁹⁰ Pim-1-mediated phosphorylation of c-Myc at S62 and decreased phosphorylation at T58 result in c-Myc upregulation via post-translational upregulation through protein stabilization.⁹⁰

We previously showed that concurrent Pim and FLT3 inhibition increases apoptosis induction in cells with FLT3-ITD through post-translational downregulation of the anti-apoptotic protein Mcl-1.⁸² Mcl-1 is a pro-survival oncogene upregulated in AML. Here we demonstrate that Pim and FLT3 inhibitor combination treatment leads to post-translational downregulation of both c-Myc and Mcl-1.

2.2 Rationale

The landscape for treatment of AML with FLT3-ITD has changed significantly over the past several years with the FDA approval of targeted therapies such as the FLT3 inhibitors midostaurin and gilteritinib. However, resistance and relapse still remain a major problem and more effective treatment is needed. Combination treatment strategies are being explored to prevent and overcome resistance through targeting multiple proteins or signaling pathways. Pim inhibitors have little efficacy as single agents but have been shown to enhance the efficacy of other therapeutics. Here we examine the signaling mechanisms that contribute to the enhanced cytotoxicity resulting from concurrent Pim and FLT3 inhibitor treatment of AML cells with FLT3-ITD.

2.3 Materials and Methods

Cell lines

Cell lines studied included Ba/F3-ITD (Ba/F3 murine pro-B cells stably transfected with human FLT3-ITD) and MV4-11 and MOLM-14 human AML cells, which harbor homozygous and heterozygous FLT3-ITD, respectively. FLT3-ITD-transfected BaF3 (Ba/F3-ITD) cells were from Dr. Mark Levis, Johns Hopkins University School of Medicine, Baltimore, MD).⁹¹ MV4-11 and MOLM-14 human AML cells with FLT3-ITD⁹² were from the American Type Culture Collection (ATCC), Manassas, VA. Cells were maintained in RPMI 1640 with 10 percent fetal bovine serum (FBS).

Patient samples

Blood samples were obtained from patients with AML with FLT3-ITD with peripheral blasts on a University of Maryland School of Medicine Institutional Review Board-approved tissue procurement protocol, following written informed consent. Studies were conducted in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll-Paque (Sigma-Aldrich). Fresh samples were studied, without prior cryopreservation. Patient demographics are shown in the appendix (**Table S2.1**).

Retroviral infection of Ba/F3-ITD cells

Ba/F3-ITD cells were infected with pMSCVpuro-Flag-cMyc-T58A plasmid (Addgene #20076)⁹³ containing c-Myc with a mutation changing threonine to alanine at

residue 58, preventing phosphorylation, or pMSCVpuro empty vector control, as previously described⁹⁴ and detailed below.

Cells were also infected with Mcl-1 with S159A pBabe-Flag hMcl-1(S159A) containing Mcl-1 with a mutation changing serine to alanine at residue 159, preventing phosphorylation, or pBabe-puro empty vector control.⁹⁵

Approximately 80% confluent Phoenix-AMPHO packaging cells (ATCC CRL-3213) were incubated in 25 $\mu\text{mol/L}$ chloroquine for 1 hour and then transfected with 20 μg retroviral plasmid DNA by the calcium phosphate method. Ba/F3-ITD cells were infected with virus-containing medium collected after 24 hours in the presence of polybrene (4 $\mu\text{g/mL}$). Cells were seeded in 2 mL virus-containing medium, centrifuged at 1,800 rpm at 32°C for 45 minutes, then incubated at 32°C for 4 hours, centrifuged at 1,800 rpm at 32°C for 45 minutes, and then incubated in fresh virus-containing medium at 32°C for 2 hours. The cells were then incubated at 37°C for 24 hours, infected with virus-containing medium, and incubated at 32°C for 5 hours. Infected cells were incubated in fresh virus-free medium overnight and then cultured with 1 mg/mL puromycin for 14 days.

Materials

The FLT3 inhibitor gilteritinib (ASP2215) (Active Biochem, Maplewood, NJ) was used at the biologically⁹⁴ and clinically⁵⁵ relevant concentration of 15 nM. The pan-Pim inhibitors AZD1208 (Tocris Bioscience, Minneapolis, MN) and TP-3654 (Medchem Express, Monmouth Junction, NJ) were used at 1 μM based on inhibition of p-BAD (S112) at this concentration.^{96, 97}

Measurement of apoptosis

Cells were stained with Annexin V and propidium iodine (PI) and analyzed on a FACS Canto II flow cytometer as previously described.⁸² Briefly, cells were incubated with drugs or DMSO control for 48 hours, and apoptosis and necrosis were measured by staining with Annexin V–FITC and PI, respectively. After treatment, cells (2×10^5 – 3×10^5) were washed with PBS, resuspended in Annexin V binding buffer (1x), stained with Annexin V–FITC (1 μ L) and PI (2 μ L), incubated at room temperature in the dark, then washed and acquired on a FACSCanto II (BD Biosciences, San Diego, CA) and analyzed with FlowJo™ Software (BD Biosciences).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated using Nucleospin® RNA (Macherey-Nagel, Bethlehem, PA) and cDNA was synthesized using Superscript IV Reverse Transcriptase (Thermo Fisher Scientific). RNA was quantified by qRT-PCR using SYBER® Green (Millipore Sigma, St. Louis, MO). Human c-Myc primers (Forward 5'- CTG AGG AGG AAC AAG AAG ATG AG -3' and reverse 5'- TGT GAG GAG GTT TGC TGT G -3' and Mouse c-Myc primers (Forward 5'-CTG GAG ATG ATG ACC GAG TTA C -3' and reverse 5' -GAG AAA CCG CTC CAC ATA CA-3') and mouse Mcl-1 primers (Forward 5'-AAA CTG GGG CAG GAT TGT GA-3' and reverse 5'-CCA GTC CCG TTT CGT CCT TA-3') were from Integrated DNA Technologies, Coralville, IA.⁸² Mouse and human GAPDH primers were previously published.⁹⁴ Relative c-Myc and Mcl-1 mRNA expression after normalization to GAPDH mRNA was determined using the Δ Ct method.⁹⁸

Drug combination studies

Cells were seeded on a 96-well plate and treated in triplicate with FLT3 inhibitor and Pim inhibitor alone or in combination at constant or variable ratios. WST-1 Cell Proliferation Reagent (Millipore Sigma, St. Louis, MO) was added after 48 hours to terminate the assay. Drug combination effects were determined using the Chou-Talalay method, analyzed with CompuSyn software.⁹⁹ Synergy was defined by combination index values less than 1.

***in vivo* study**

Exponentially growing MV4-11-luc cells (1×10^6) were injected intravenously into the lateral tail veins of restrained female NOD-Rag1null IL2rgnull, NOD rag gamma (NRG) mice (6-8 weeks old). Cell engraftment was assessed 7 days later on a Xenogen IVIS-2-Imaging System (Alameda, CA) after injection of D-luciferin (150 mg/kg intraperitoneally). Mice were sorted into 4 treatment groups, 5 mice in each, with equal mean signal intensity. Treatment was initiated with gilteritinib 7.5 mg/kg and/or TP-3654 50 mg/kg in 5% DMSO, 40% polyethylene glycol 300 (PEG 300), 5% polysorbate 80 (Tween 80) and 50% water, or vehicle control, all by oral gavage, once every other day for three doses, followed by two days rest each week. Mice were weighed prior to each treatment. Leukemia burden was assessed weekly by non-invasive luciferin imaging. Endpoints were 20% body weight loss, hind limb paralysis or lack of mobility to eat/drink. The University of Maryland Institutional Animal Care and Use Committee approved the study.

Immunoblotting

Cells were lysed in 150 mM NaCl lysis buffer with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). Protein was measured using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) and 15 µg (cell lines) or 50 or 75 µg (primary AML cells) were immunoblotted for each sample. Immunoblots were incubated with polyclonal primary antibodies to c-Myc and Mcl-1 (Cell Signaling Technology, Danvers, MA) and monoclonal antibodies to vinculin (Santa Cruz Biotechnology, Dallas, TX) or β-actin (Sigma-Aldrich) loading control protein at 1:1000 dilution overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. Band intensities were measured by densitometry with Visonworks®LS, UVP (Upland, CA). Band intensities at serial time points, normalized to vinculin controls, were compared to intensities at time 0, defined as 100%.

Protein turnover and proteasomal degradation

To study protein turnover, cells were treated with 100 µg/mL cycloheximide (CHX) (Sigma-Aldrich) for 60 minutes to block new protein translation prior to treatment with FLT3 inhibitor and/or Pim inhibitor, or DMSO control. Protein expression was measured at serial time points by immunoblotting. Band intensities were measured by densitometry, as above, and 50% protein turnover time points were determined using the line of best fit.

To study the effect of proteasomal degradation, cells were treated with CHX as above, with or without addition of the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132; Calbiochem, San Diego, CA) (20 µM) 30 minutes after

addition of CHX and 30 minutes prior to treatment with FLT3 inhibitor and/or Pim inhibitor, or DMSO control. Protein expression was measured at serial time points by immunoblotting. Band intensities were measured by densitometry and 50% protein turnover time points were determined using the line of best fit, as above.

Statistical analysis

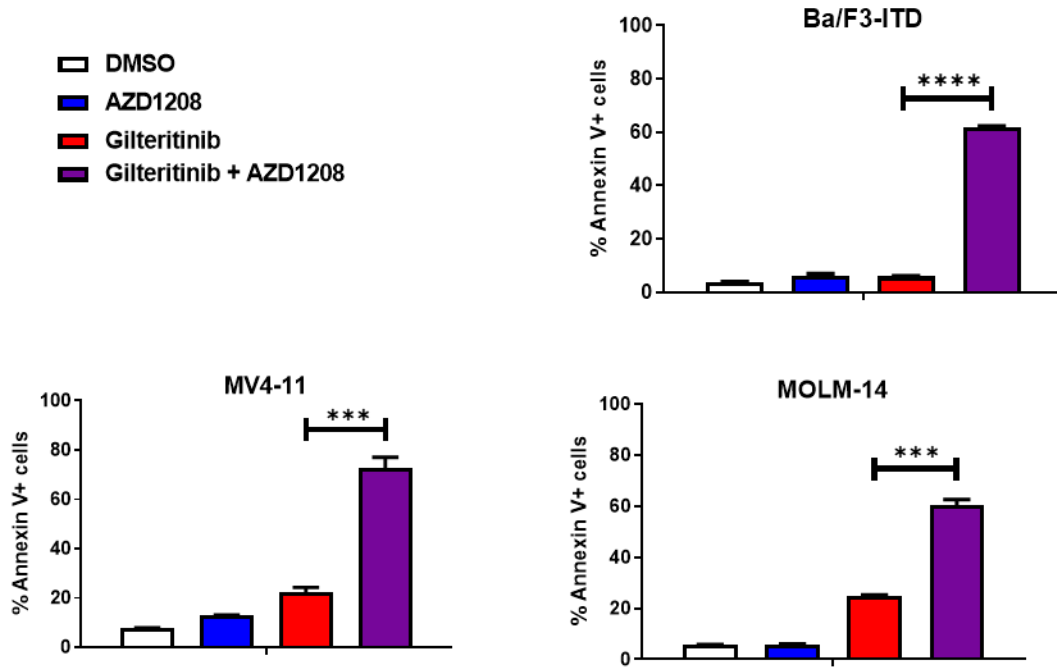
All data were derived from at least three independent experiments, with error bars in figures representing standard error of the mean (SEM). Statistical analyses for *in vitro* analyses were performed by unpaired t-test, using Prism 9 (GraphPad, San Diego, CA). For the *in vivo* experiment, statistical analysis was performed by 2-way ANOVA with multiple comparisons test.

2.4 Results

FLT3 and Pim inhibitor combination treatment is synergistic in cells with FLT3-ITD

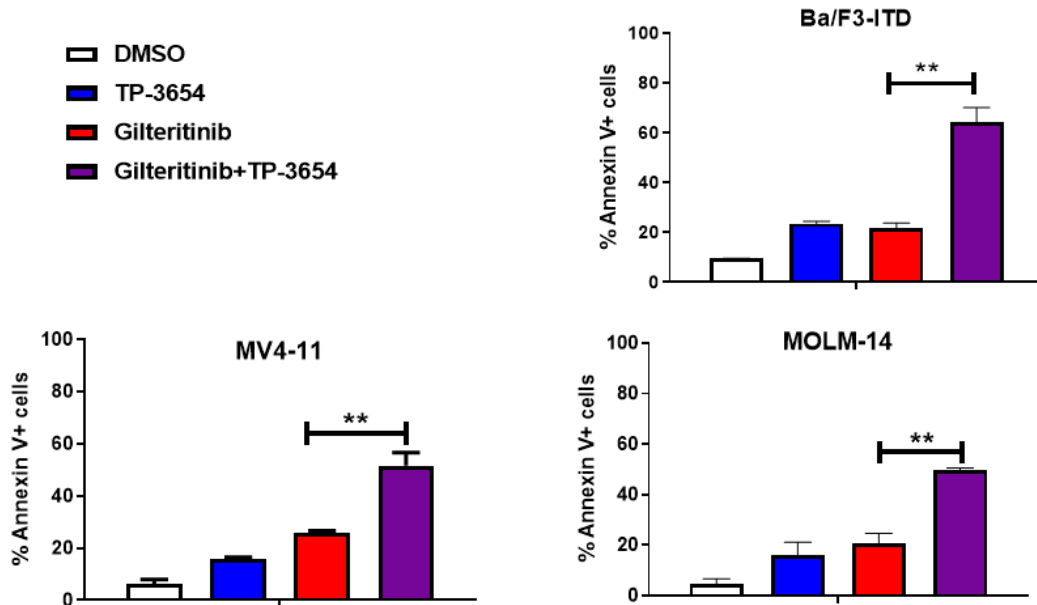
We previously showed that combination treatment with the pan-Pim inhibitor AZD1208 and the FLT3 inhibitor quizartinib enhanced cytotoxicity in FLT3-ITD cells, compared to single drugs, *in vitro* and *in vivo*.⁸² To determine whether synergy was also seen with the Food and Drug Administration (FDA)-approved FLT3 inhibitor gilteritinib and AZD1208 or the Pim-1 inhibitor TP-3654, currently in clinical trials, Ba/F3-ITD, MV4-11 and MOLM-14 cells, with FLT3-ITD, were treated with gilteritinib and/or AZD1208 (**Fig. 2.1**) or TP-3654 (**Fig. 2.2**) or DMSO control and apoptosis was measured by Annexin V/propidium iodine (PI) staining. Both combinations significantly increased apoptosis induction, compared to single drugs, in all three cell lines.

Figure 2.1 Gilteritinib and AZD1208 combination treatment increases apoptosis induction.



Ba/F3-ITD, MV4-11 and MOLM-14 cells were treated with the FLT3 inhibitor gilteritinib (15 nM for Ba/F3-ITD and 10 nM for MV4-11 and MOLM-14) and/or the Pim inhibitor AZD1208 (1 μ M) or DMSO control for 48 hours. Cells were analyzed for apoptosis by Annexin V and PI staining, measured by flow cytometry. ****= $p < 0.0001$; ***= $p < 0.001$; **= $p < 0.01$.

Figure 2.2 Gilteritinib and TP-3654 combination treatment increases apoptosis induction.

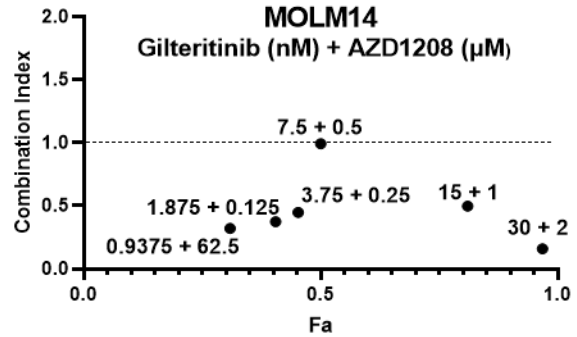
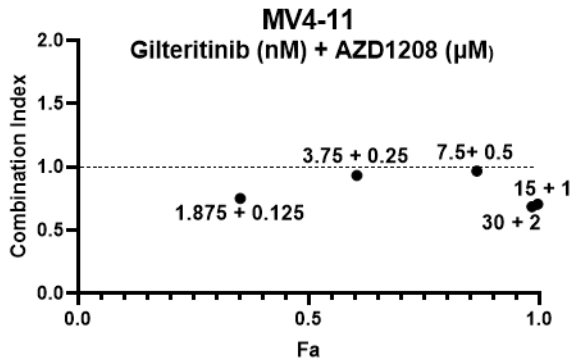
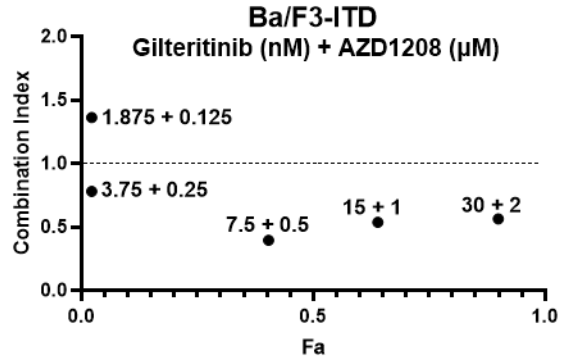


Ba/F3-ITD, MV4-11 and MOLM-14 cells were treated with the FLT3 inhibitor gilteritinib (15 nM for Ba/F3-ITD and 10 nM for MV4-11 and MOLM-14) and/or the TP-3654 (1 μ M) or DMSO control for 48 hours. Cells were analyzed for apoptosis by Annexin V and PI staining, measured by flow cytometry. ****= $p < 0.0001$; ***= $p < 0.001$; **= $p < 0.01$.

Ba/F3-ITD, MV4-11 and MOLM-14 cells were also treated with single drugs or combinations of gilteritinib with AZD1208 (**Fig. 2.3**) or TP-3654 (**Fig. 2.4**) at different concentrations in a cytotoxicity assay. Synergistic effects of both drug combinations were demonstrated by Chou-Talalay analysis in all three cell lines.

Figure 2.3 Combination treatment with gilteritinib and AZD1208 produces synergistic cytotoxicity

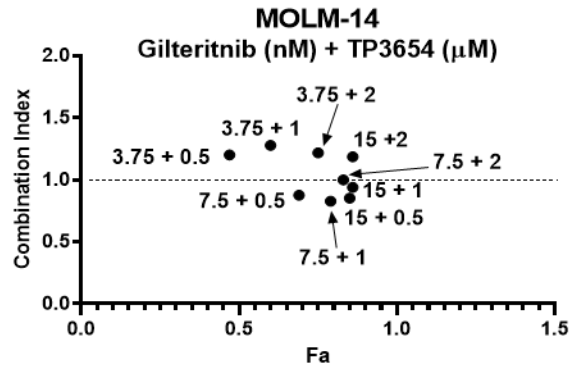
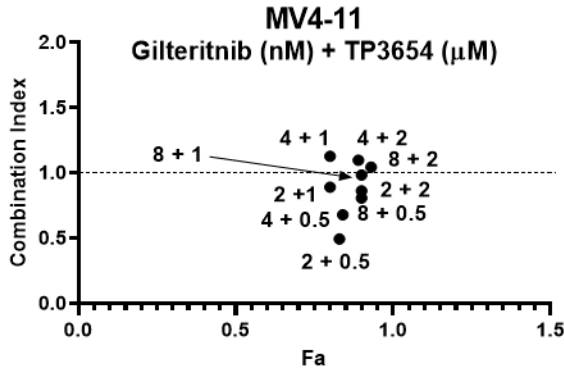
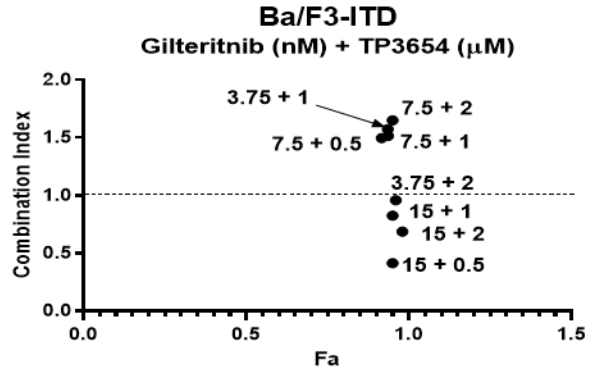
	CI Values
Synergistic	<1
Additive	1
Antagonistic	>1



Ba/F3-ITD cells seeded in 96-well plates at 5000 cells/well and MV4-11 and MOLM-14 cells at 10000 cells/well were treated for 48 hours with gilteritinib and/or AZD1208 as single drugs and in combinations at the concentrations shown. Cytotoxicity was measured by WST-1 assay, and drug combination effects were determined by Chou-Talalay analysis. Synergism was defined by combination index values <1.

Figure 2.4 Combination treatment with gilteritinib and TP-3654 produces synergistic cytotoxicity.

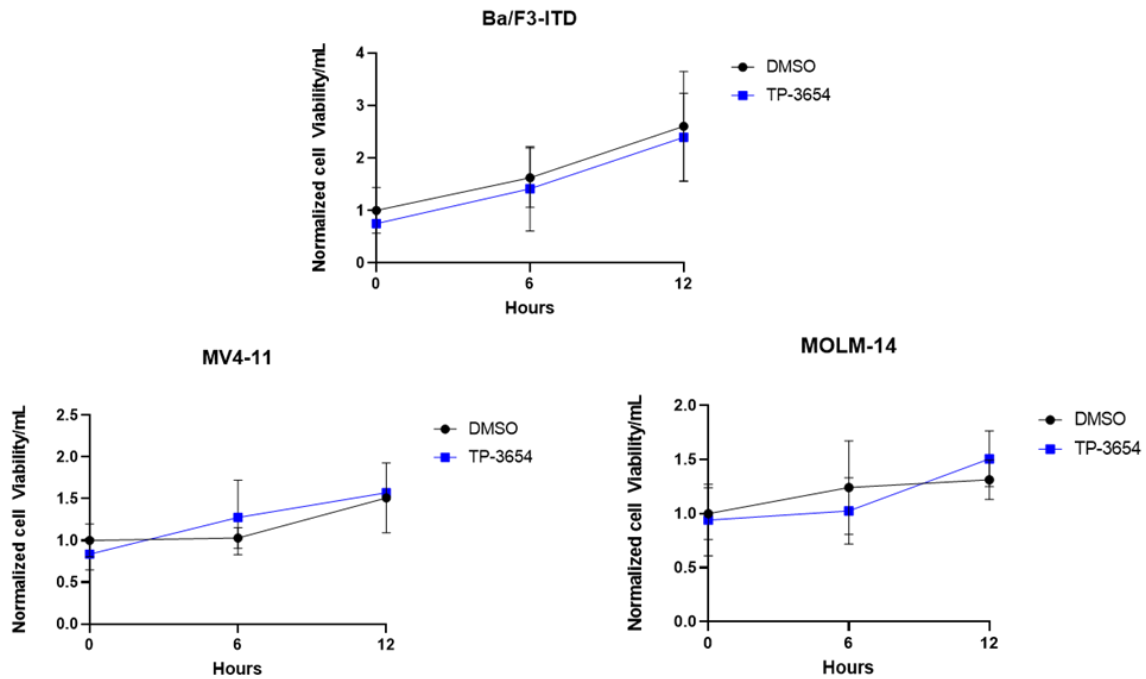
	CI Values
Synergistic	<1
Additive	1
Antagonistic	>1



Ba/F3-ITD cells seeded in 96-well plates at 5,000 cells/well and MV4-11 and MOLM-14 cells at 10,000 cells/well were treated for 48 hours with gilteritinib and/or TP-3654 as single drugs and in combinations at the concentrations shown. Cytotoxicity was measured by WST-1 assay, and drug combination effects were determined by Chou-Talalay analysis. Synergism was defined by combination index values <1.

TP-3654 as a single agent induced slightly more apoptosis than AZD1208 (**Fig. 2.1, 2.2**). To ensure that TP-3654 did not significantly affect cell viability, cells were treated with TP-3654 or DMSO and viable cells were counted up to 12 hours using trypan blue dye exclusion. There were no differences in cell viability between cells treated with TP-3654 or DMSO control (**Fig. 2.5**).

Figure 2.5 TP-3654 does not affect cell viability



Ba/F3-ITD, MV4-11 and MOLM-14 were plated at 1×10^5 cells/mL and treated with $1 \mu\text{M}$ TP-3654. Samples were collected at the time points shown and viable cells were counted using trypan blue dye exclusion and normalized to time 0.

***in vivo* efficacy of TP-3654 and gilteritinib combination treatment**

To study effects of TP-3654 and gilteritinib co-treatment *in vivo*, NSG mice with MV4-11-luc cells injected intravenously and allowed to engraft were treated with gilteritinib and/or TP-3654 or vehicle control, five mice per treatment group, and leukemia burden was measured by luciferin imaging. By day 39, leukemia burden was significantly lower in mice treated with TP-3654 and gilteritinib combination, compared with gilteritinib alone (**Fig. 2.6**).

Figure 2.6 Gilteritinib and TP-3654 combination treatment reduced tumor burden in an orthotopic mouse model

NRG mice injected intravenously with MV4-11-luc cells were treated with gilteritinib and/or TP-3654, or vehicle control, beginning on Day 1. (A) Graphical treatment schema. (B) Serial images of mice from all treatment groups. (C) Change in photon intensity, measured by bioluminescence imaging, over time, with $P=0.0043$ by 2-way ANOVA comparing TP-3654 and gilteritinib combination versus gilteritinib alone on day 39. (D) Survival curve over time, with $P=0.0027$.

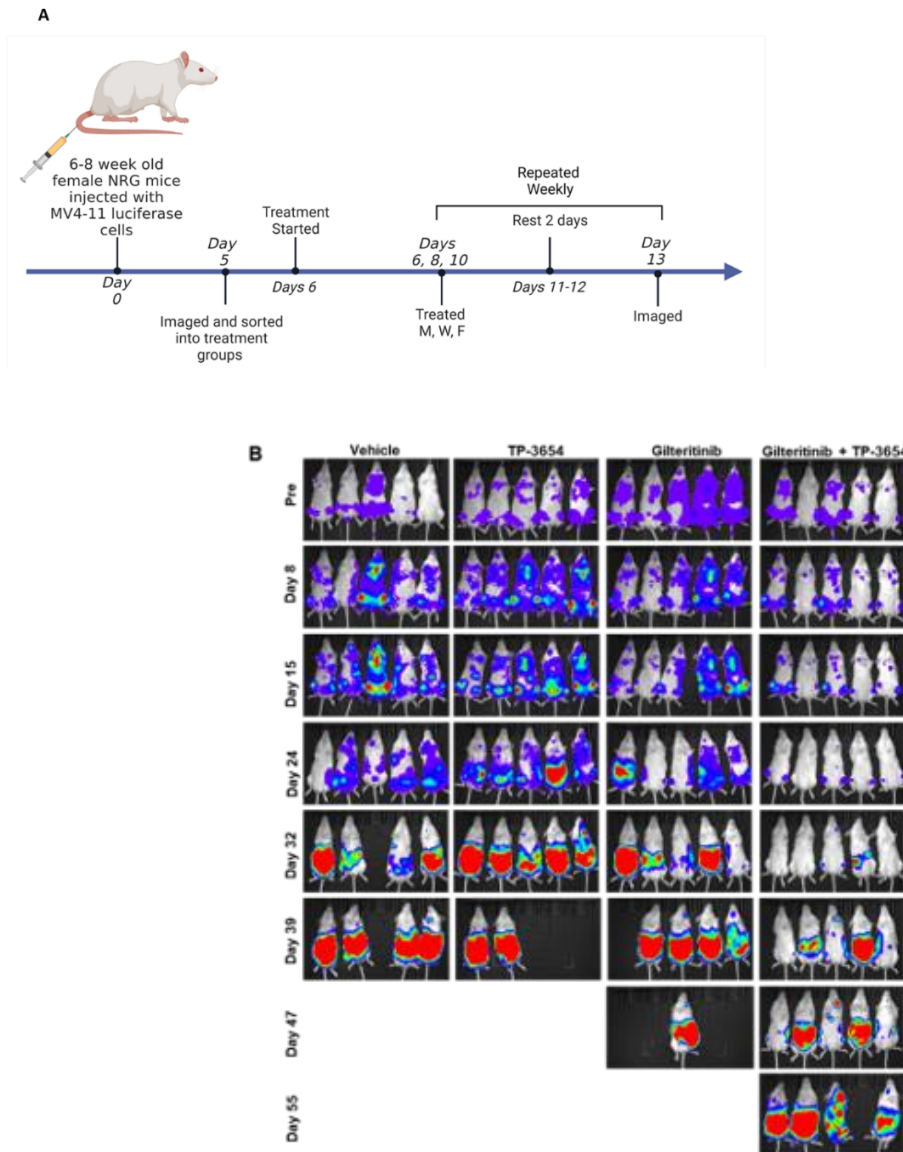
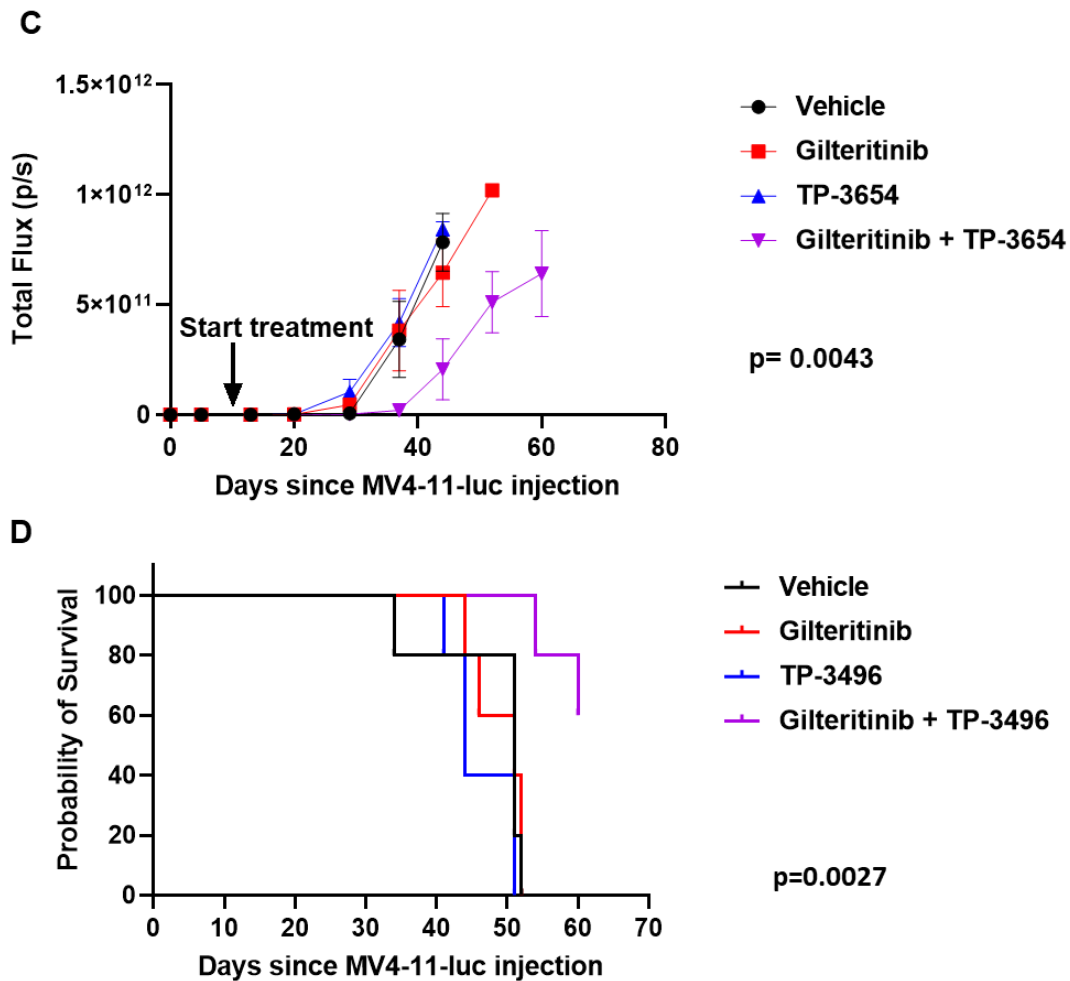


Figure 2.6 continued



FLT3 and Pim inhibitor combination treatment downregulates c-Myc prior to Mcl-1 protein.

To determine the effects of FLT3 and Pim inhibitor combination treatment on expression of c-Myc, relative to Mcl-1, Ba/F3-ITD, MV4-11 and MOLM-14 cells and blasts from a patient with AML with FLT3-ITD (Patient 1) were treated with the FLT3 inhibitor gilteritinib and/or the Pim inhibitor AZD1208, or DMSO control (**Fig. 2.7**), and Ba/F3-ITD, MV4-11 and MOLM-14 cells were also treated with gilteritinib and/or the Pim

inhibitor TP-3654, or DMSO control (**Fig. 2.8**), and samples collected at serial time points were analyzed by immunoblotting. c-Myc was rapidly downregulated in FLT3-ITD-expressing cell lines and patient blasts treated with AZD1208 or TP-3654 and gilteritinib combinations, relative to single drugs, and c-Myc downregulation occurred prior to Mcl-1 downregulation (**Fig. 2.7 and 2.8**).

Figure 2.7 c-Myc downregulation precedes Mcl-1 in cells co-treated with gilteritinib and AZD1208.

Ba/F3-ITD (**A**), MV4-11 (**B**) and MOLM-14 (**C**) cells and blasts from a patient with FLT3-ITD AML (**D**) plated at 1×10^5 cells/mL were treated with gilteritinib (15 nM for Ba/F3-ITD and patient blasts and 10 nM for MV4-11 and MOLM-14) and/or 1 μ M AZD1208, or DMSO control. c-Myc, Mcl-1, and vinculin loading control protein levels were measured by immunoblotting in samples collected at serial time points. Data are also shown graphically.

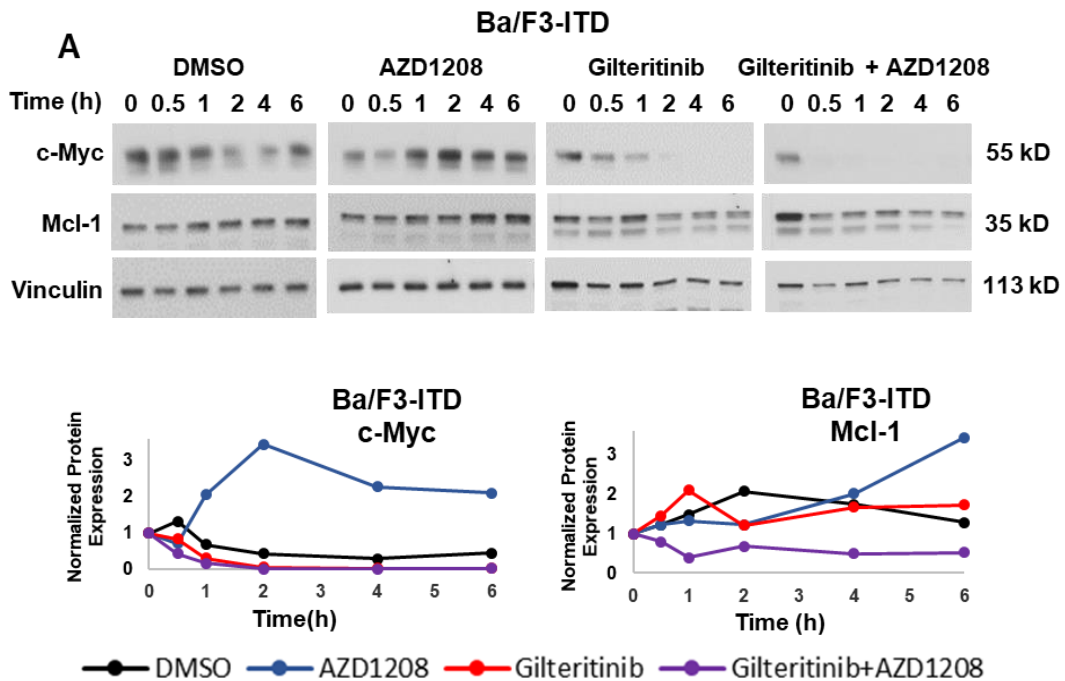
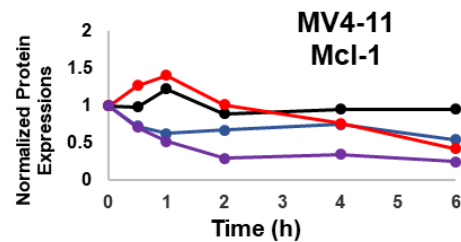
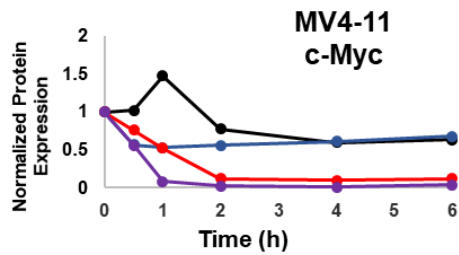
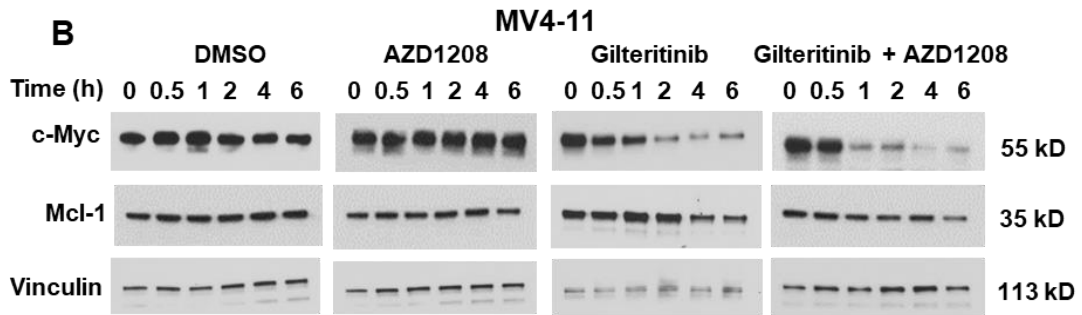
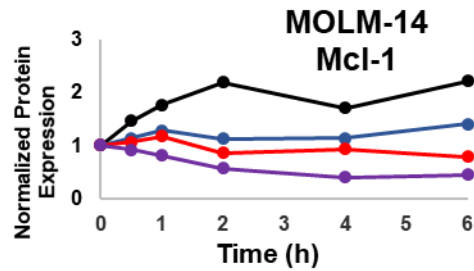
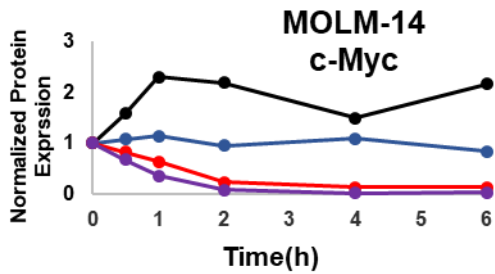
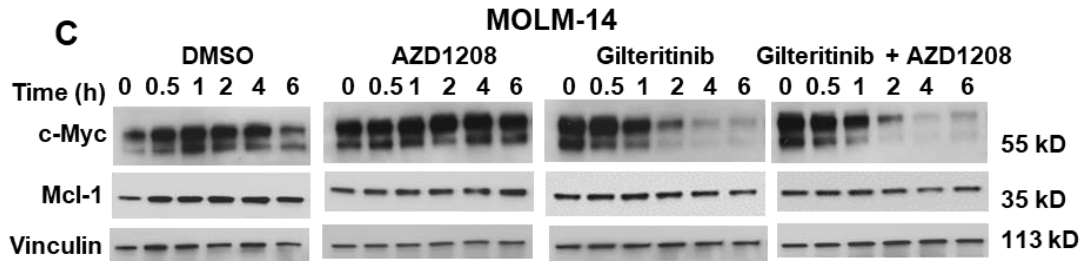


Figure 2.7 continued



● DMSO ● AZD1208 ● Gilteritinib ● Gilteritinib+AZD1208



● DMSO ● AZD1208 ● Gilteritinib ● Gilteritinib+AZD1208

Figure 2.7 continued

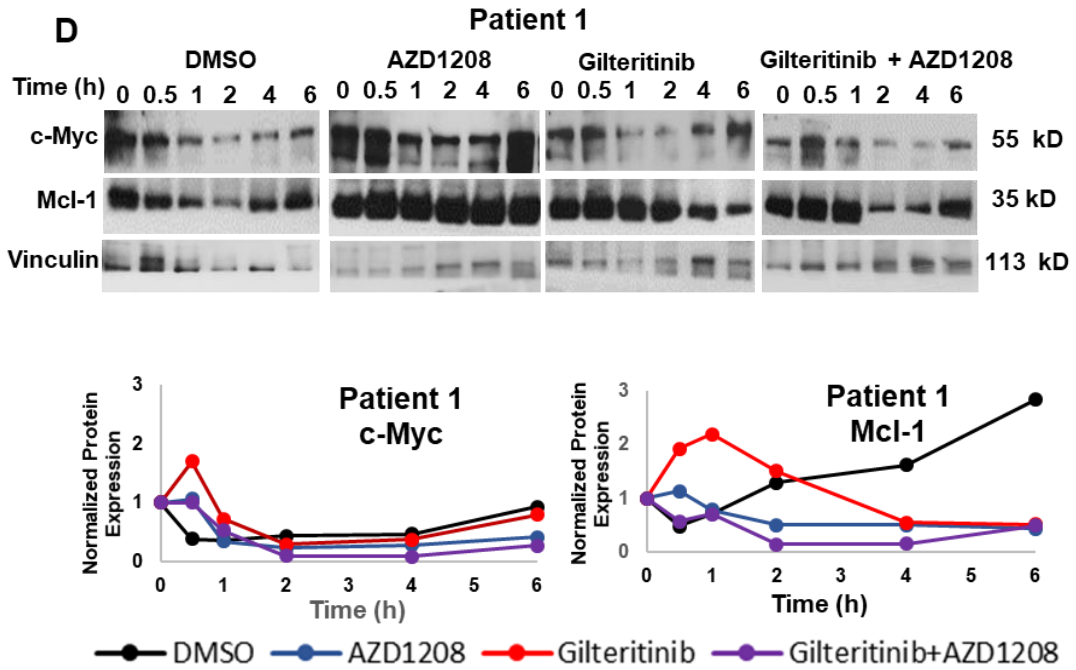


Figure 2.8 c-Myc downregulation precedes Mcl-1 in cells co-treated with gilteritinib and TP-3654

Ba/F3-ITD (A), MV4-11 (B) and MOLM-14 (C) plated at 1×10^5 cells/mL were treated with gilteritinib (15 nM for Ba/F3-ITD and patient blasts and 10 nM for MV4-11 and MOLM-14) and/or 1 μ M TP-3654, or DMSO control. c-Myc, Mcl-1, and vinculin loading control protein levels were measured by immunoblotting in samples collected at serial time points. Data are also shown graphically.

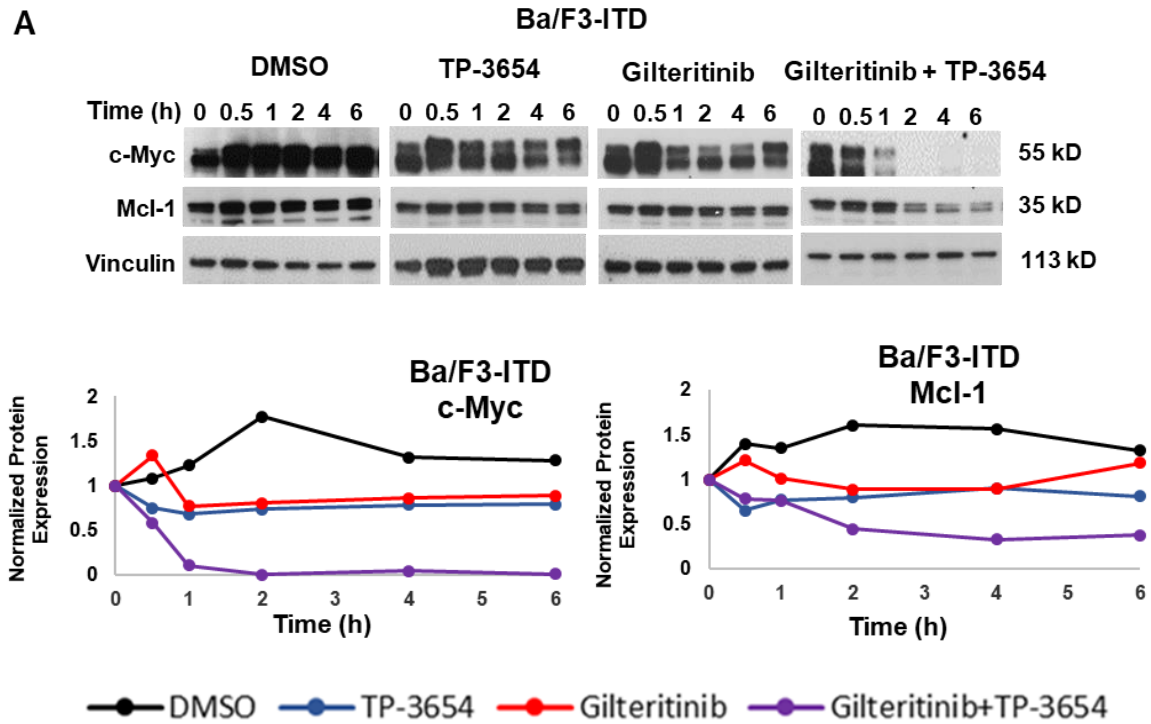
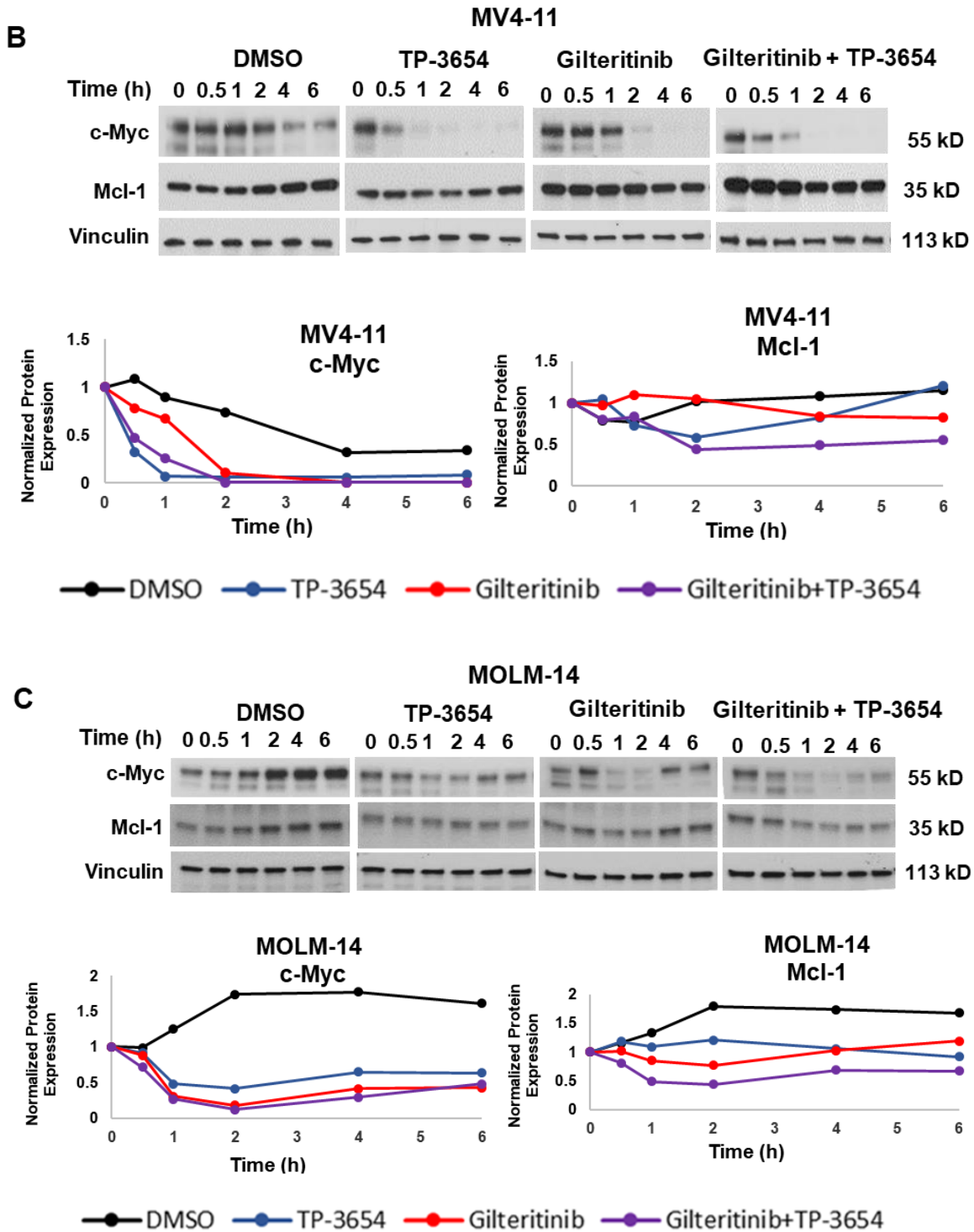


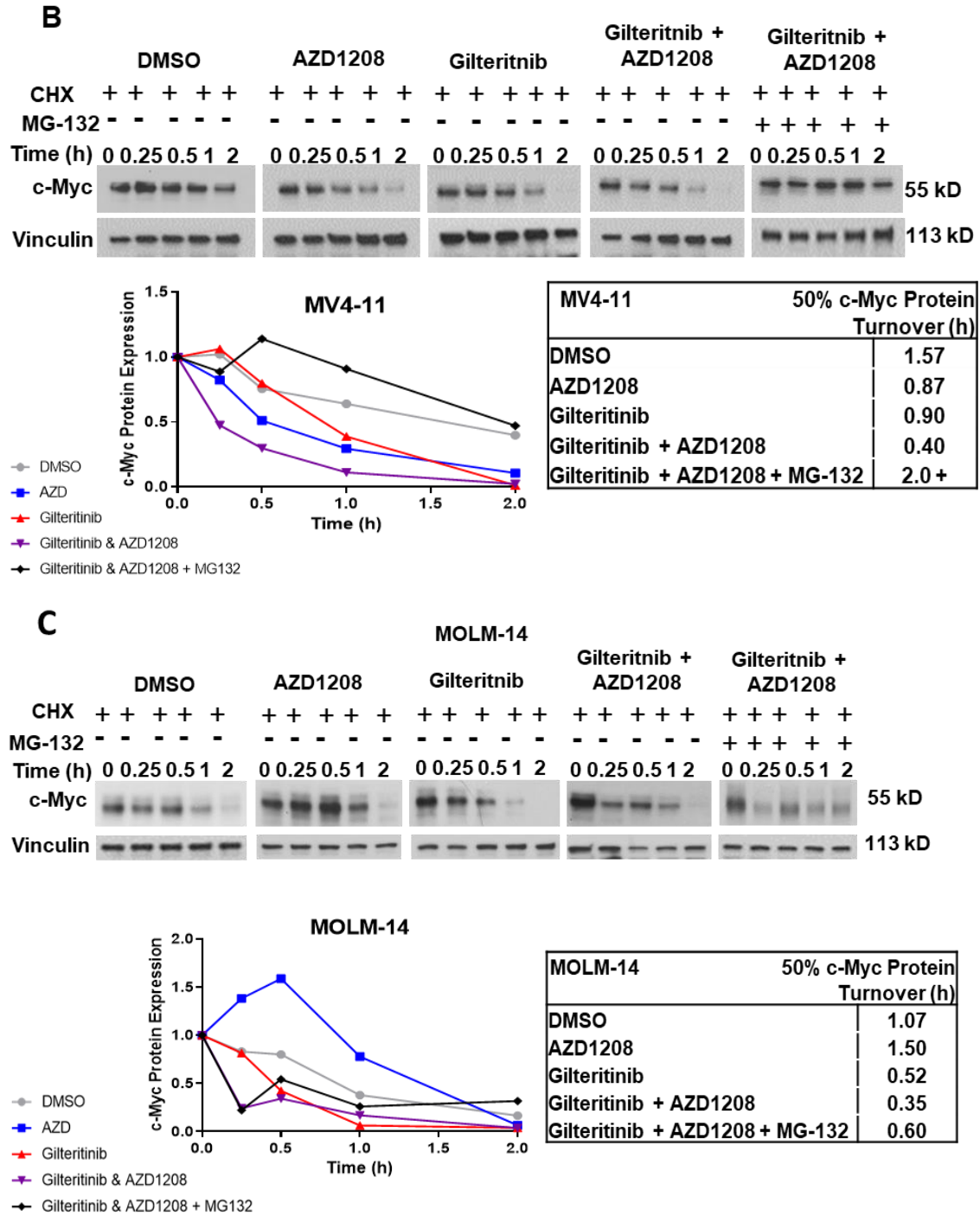
Figure 2.8 continued



Pim and FLT3 inhibitor combination treatment downregulates c-Myc and Mcl-1 protein post-translationally, through increased proteasomal degradation

To determine the mechanism(s) by which Pim and FLT3 inhibitor combination treatment downregulates c-Myc, Ba/F3-ITD, MV4-11 and MOLM-14 cells were pretreated for 1 hour with CHX with or without addition of the proteasome inhibitor MG-132 after 30 minutes, then with gilteritinib and/or AZD1208, or DMSO control. c-Myc protein was measured by immunoblotting at serial time points, along with vinculin loading control. AZD1208 and gilteritinib combination treatment accelerated c-Myc protein turnover, relative to single drugs or DMSO control, in all three cell lines (**Fig. 2.9**). In Ba/F3-ITD cells, 50% c-Myc protein turnover occurred in 0.48 hours with combination treatment, compared to 0.78 hours with DMSO control treatment, and this decrease was abrogated by pre-treatment with MG-132 (**Fig. 2.9A**). In MV4-11 cells, 50% c-Myc protein turnover occurred in 0.40 hours with combination treatment, compared to 1.57 hours with DMSO control treatment, and this decrease was abrogated by pre-treatment with MG-132 (**Fig. 2.9B**). MOLM-14 cells similarly showed more rapid 50% c-Myc protein turnover with combination treatment (0.35 hours) compared to DMSO (1.07 hours), also rescued by pre-treatment with MG-132 (**Fig. 2.9C**).

Figure 2.9 continued



Mcl-1 protein turnover was also accelerated in combination-treated, compared to single-drug- and DMSO-treated, Ba/F3-ITD (0.57 versus 1.05 hours), MV4-11 (0.27

versus 0.93 hours) and MOLM-14 (0.32 versus 0.92 minutes) cells, and turnover was slower in cells pre-treated with MG-132 (**Fig. 2.10**). These data are consistent with our previously published results⁸² and demonstrate post-translational downregulation of both c-Myc and Mcl-1 by FLT3 and Pim inhibitor combination treatment through increased proteasomal degradation in cells with FLT3-ITD.

Figure 2.10 Combination treatment increases Mcl-1 protein turnover through proteasomal degradation

Ba/F3-ITD (**A**), MV4-11 (**B**) and MOLM-14 (**C**) cells plated at 1×10^5 cells/mL were treated with 100 μ g/mL cycloheximide for one hour to inhibit protein synthesis, with or without addition of the proteasome inhibitor MG-132 after 30 minutes, prior to treatment with gilteritinib and/or AZD1208, or DMSO control. Samples collected at serial time points were studied for expression of Mcl-1. Data were graphed and 50% protein turnover was determined using the line of best fit.

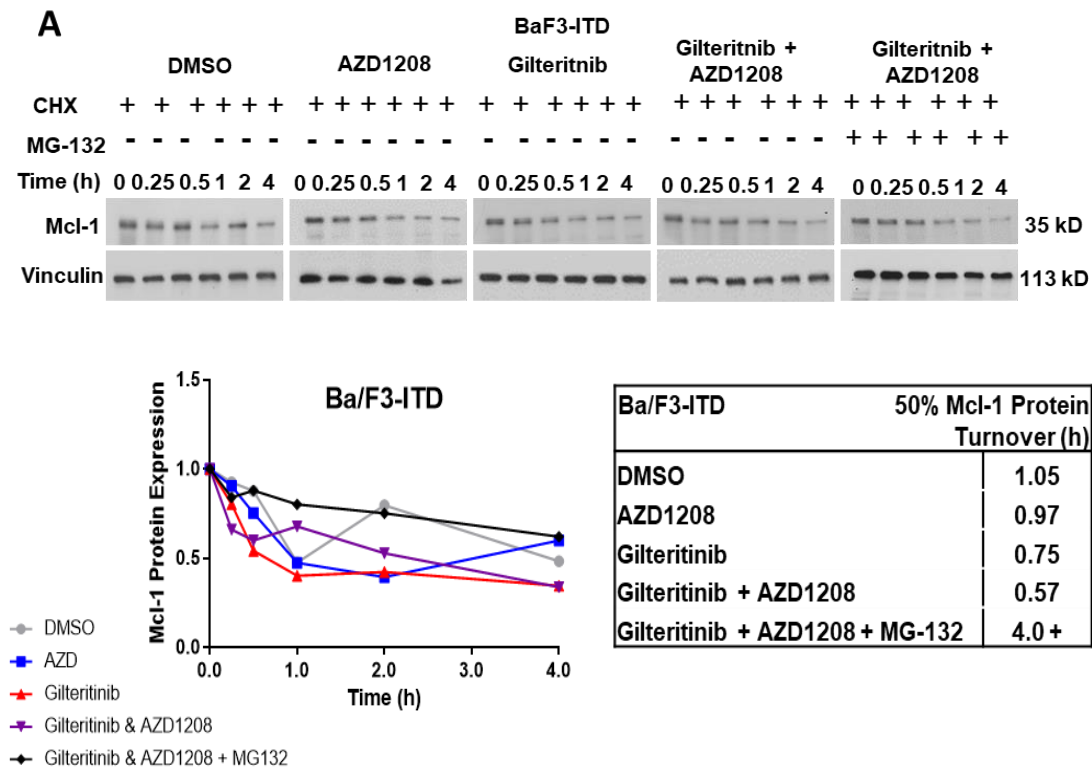
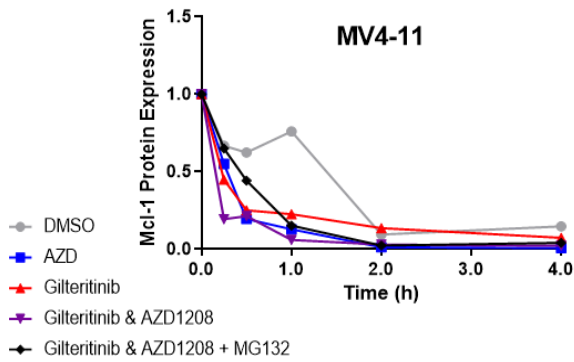
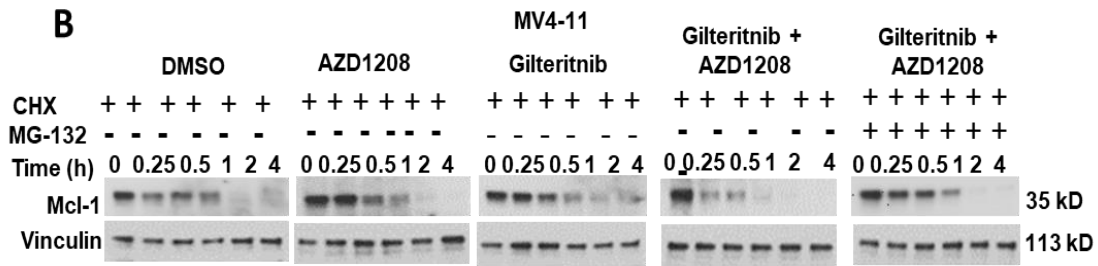
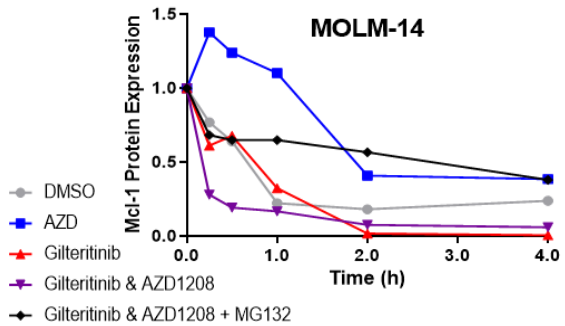
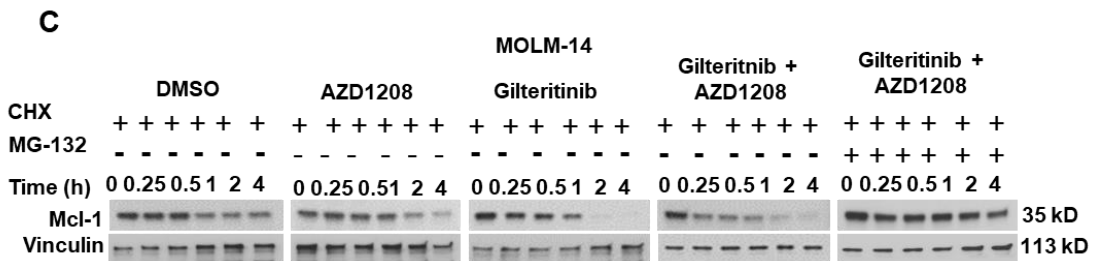


Figure 2.10 continued



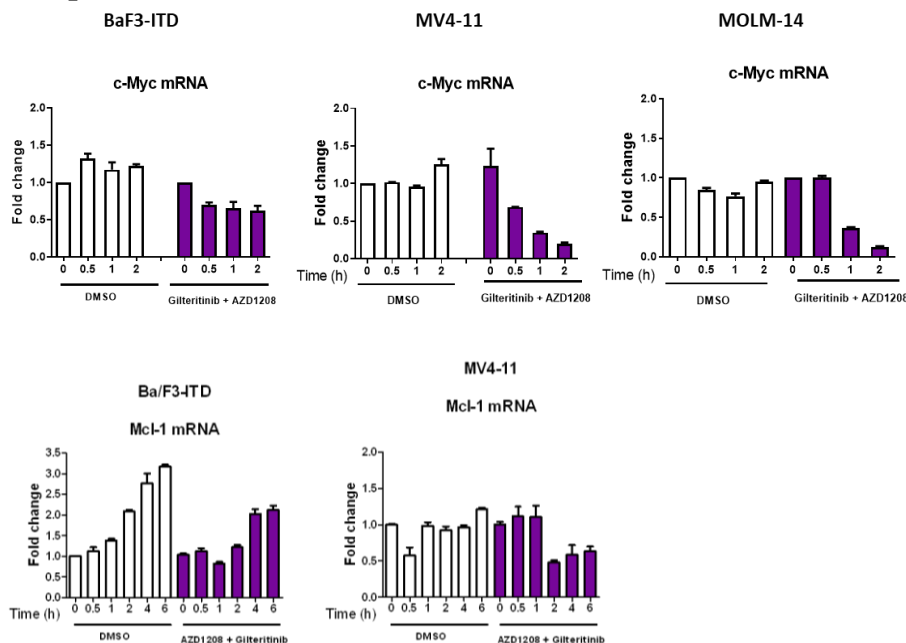
MV4-11	50% Mcl-1 Protein Turnover (h)
DMSO	0.93
AZD1208	0.40
Gilteritinib	0.48
Gilteritinib + AZD1208	0.27
Gilteritinib + AZD1208 + MG-132	0.65



MOLM-14	50% Mcl-1 Protein Turnover (h)
DMSO	0.92
AZD1208	2.12
Gilteritinib	0.82
Gilteritinib + AZD1208	0.32
Gilteritinib + AZD1208 + MG-132	2.65

To assess whether downregulation of c-Myc and Mcl-1 by co-treatment is also transcriptional, qRT-PCR was performed. RNA was isolated from Ba/F3-ITD, MV4-11 and MOLM-14 cells treated with FLT3 and Pim inhibitor combinations or DMSO control at serial time points, up to 2 hours. c-Myc mRNA expression, normalized to GAPDH mRNA expression, did not change significantly in Ba/F3-ITD cells treated with AZD1208 and gilteritinib (**Fig. 2.11**). In contrast, AZD1208 and gilteritinib combination treatment downregulated c-Myc mRNA in MV4-11 and MOLM-14 cells (**Fig. 2.11**). Therefore, changes in c-Myc mRNA with combination treatment are cell line-specific and may vary based on species, in contrast to consistent post-translational downregulation due to increased proteasomal degradation in all cells studied. Mcl-1 mRNA expression did not change with combination treatment, as previously published.⁸²

Figure 2.11 c-Myc and Mcl-1 mRNA is downregulated by combination treatment in cell-specific manner



Ba/F3-ITD, MV4-11, and MOLM-14 cell were treated with DMSO or AZD1208 and gilteritinib. Cells were collected at the time points shown and RT-qPCR was performed.

FLT3 and Pim inhibitor combination treatment downregulates c-Myc through phosphorylation at T58.

c-Myc degradation can be regulated by diverse processes, including calpain-dependent cleavage¹⁰⁰ and proteasomal degradation.¹⁰¹ A majority of c-Myc degradation occurs through the ubiquitin proteasome system.¹⁰¹ Fbw7 is a well-studied ubiquitin ligase that is targeted to phosphorylated T58 in c-Myc; it is a part of a complex that ubiquitinates c-Myc, thus targeting it for degradation.¹⁰¹

To test whether phosphorylation of c-Myc at T58 contributes to c-Myc downregulation by FLT3 and Pim inhibitor combination treatment, parental Ba/F3-ITD cells and Ba/F3-ITD cells infected with pMSCVpuro-Flag-cMyc-T58A plasmid, containing c-Myc with a mutation changing threonine to alanine at residue 58, preventing phosphorylation, or with pMSCVpuro empty vector control were treated with gilteritinib and AZD1208 or with DMSO control, and c-Myc protein expression was measured by immunoblotting at serial time points, along with vinculin as a protein loading control. c-Myc protein levels did not decrease with treatment of cells infected with pMSCVpuro-Flag-cMyc-T58A plasmid, in contrast to cells infected with empty vector (**Fig 2.12A**). c-Myc protein turnover was then studied in cells treated with gilteritinib and AZD1208 combination or DMSO control, demonstrating slower c-Myc protein turnover in cells infected with pMSCVpuro-Flag-cMyc-T58A treated with gilteritinib and AZD1208 combination, relative to cells infected with empty vector, with 50% c-Myc protein turnover in 1.97 hours in cells infected with pMSCVpuro-Flag-cMyc-T58A, versus 0.47 hours in cells with empty vector (**Fig. 2.12B**).

To determine the role of c-Myc T58 phosphorylation in apoptosis induction by combination treatment, cells infected with pMSCV-puro-Flag-cMyc-T58A or pMSCV-puro control were treated with gilteritinib and AZD1208 combination or DMSO control and apoptosis was measured after 48 hours. Apoptosis was reduced by 30% in cells infected with pMSCVpuro-Flag-cMyc-T58A, compared to empty vector (**Fig. 2.12C**), demonstrating that c-Myc T58 phosphorylation contributes to apoptosis induction by gilteritinib and AZD1208 combination.

Figure 2.12 c-Myc is downregulated through phosphorylation at T58

(A) Ba/F3-ITD cells infected with pMSCV-puro-Flag-cMyc T58A or pMSCV-puro empty vector were treated with either gilteritinib and AZD1208 or DMSO control, and serial samples were immunoblotted for c-Myc and vinculin loading control. (B) To measure c-Myc protein turnover, cells were pretreated with CHX for 1 hour and then treated with gilteritinib and AZD1208 (+) or DMSO control (-) and lysates were immunoblotted for c-Myc and vinculin loading control. Densitometry was performed, c-Myc was normalized to vinculin and 50% protein turnover time points were determined. (C) Cells infected with pMSCV-puro-Flag-cMyc T58A or pMSCV-puro empty vector control were treated with gilteritinib and AZD1208, or DMSO control, for 48 hours, and apoptosis was measured.

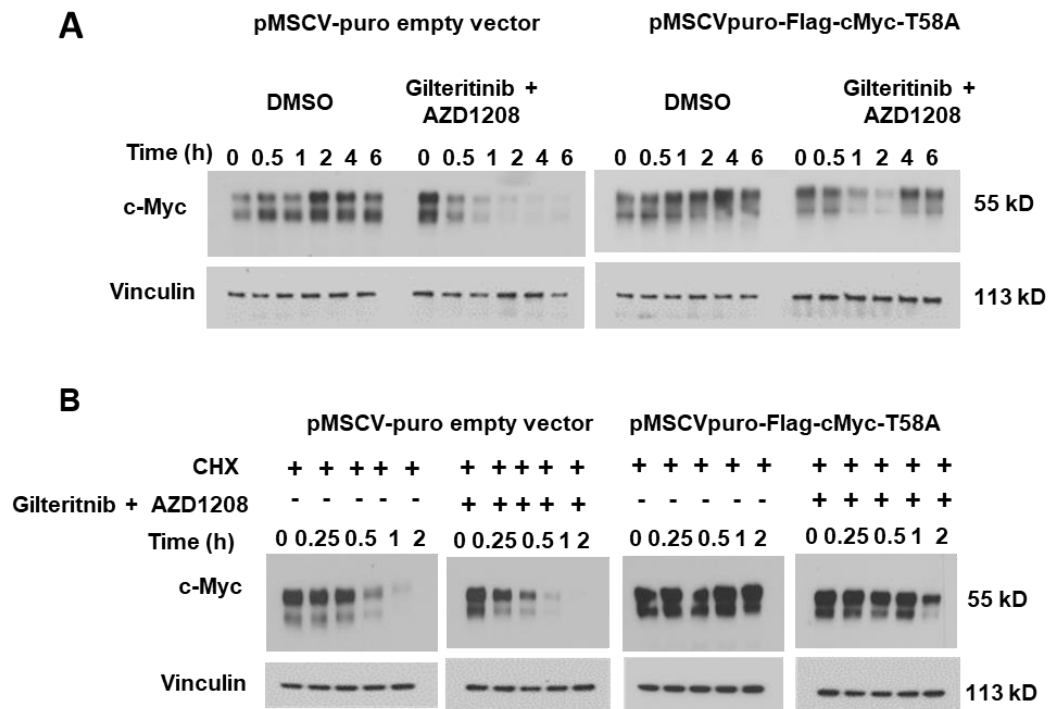
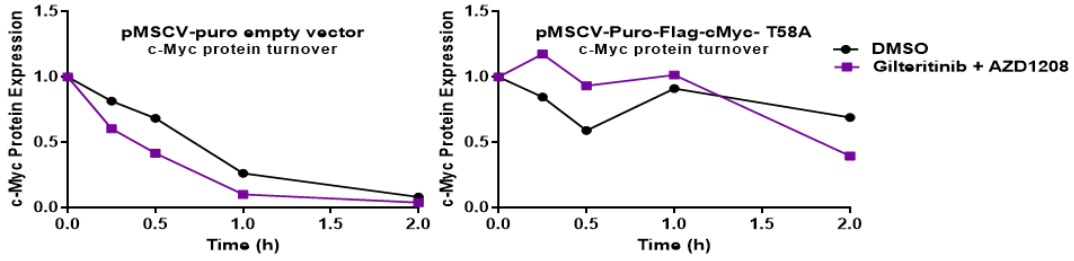
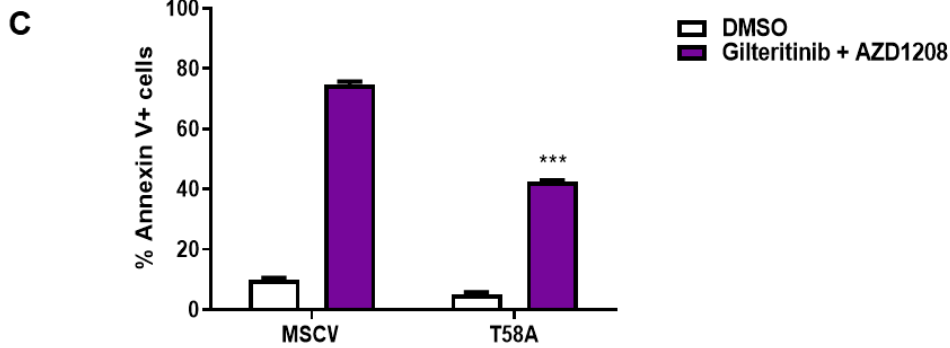


Figure 2.12 continued



50% Protein Turnover (h)		
	DMSO	Gilteritinib + AZD1208
pMSCV-puro-empty vector	0.70	0.47
pMSCV-puro-flag-cMyc-T58A	2.0 +	1.97



FLT3 and Pim inhibitor combination treatment downregulates Mcl-1 through phosphorylation at S159

Mcl-1 is a short-lived and highly regulated protein with multiple phosphorylation sites that promote proteasomal degradation.¹⁰² Mutations to residue S159, specifically, are important to Mcl-1 stability,¹⁰³ lymphoma development *in vivo*,¹⁰⁴ and resistance to apoptosis.¹⁰⁵ To test the role of S159 phosphorylation in Mcl-1 downregulation by FLT3

and Pim inhibitor combination treatment, Ba/F3-ITD cells infected with pBabe-puro-S159A plasmid, containing Mcl-1 with a mutation changing serine to alanine at residue 159, preventing phosphorylation, or pBabe-puro empty vector control were treated with gilteritinib and AZD1208 combination or DMSO control. Cells infected with pBabe-puro-S159A plasmid, compared to empty vector, showed a decrease in Mcl-1 protein downregulation (**Fig. 2.13A**) and reduction in Mcl-1 protein turnover, with 50% Mcl-1 protein turnover in 2+ hours in cells infected with pMSCV-puro-Flag-cMyc-T58A, versus 1.32 hours in cells with empty vector (**Fig. 2.13B**). Apoptosis was reduced by 20% in cells infected with pBabe-puro-S159A compared to empty vector (**Fig. 2.13C**), demonstrating that Mcl-1 S159 phosphorylation contributes to apoptosis induction by gilteritinib and AZD1208 combination.

Figure 2.13 Mcl-1 downregulated through phosphorylation at S159

(A) Ba/F3-ITD cells infected with pBABE-puroS159A, containing Mcl-1 with a mutation changing serine to alanine at residue 159, preventing phosphorylation, or pBABE-puro empty vector were treated with either gilteritinib and AZD1208 or DMSO control, and serial samples were immunoblotted for Mcl-1 and vinculin loading control. (B) To measure Mcl-1 protein turnover, cells were pretreated with CHX for 1 hour and then treated with gilteritinib and AZD1208 (+) or DMSO control (-) and lysates were immunoblotted for Mcl-1 and vinculin loading control. Densitometry was performed, Mcl-1 was normalized to vinculin and 50% protein turnover time points were determined. (C) Cells infected with pBABE-puroS159A, or pBABE-puro empty vector control were treated with gilteritinib and AZD1208, or DMSO control, for 48 hours, and apoptosis was measured.

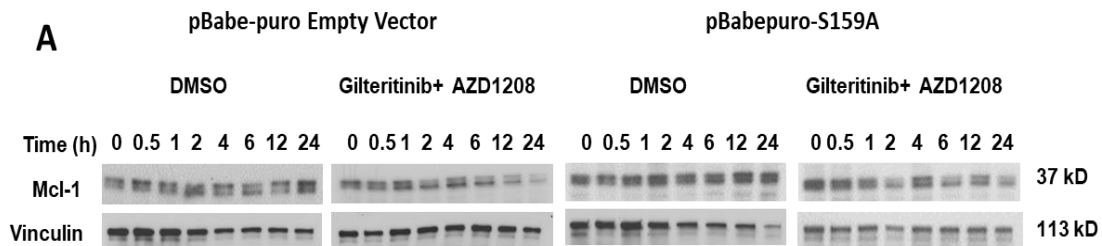
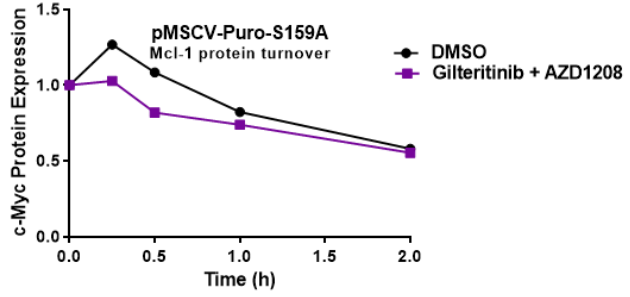
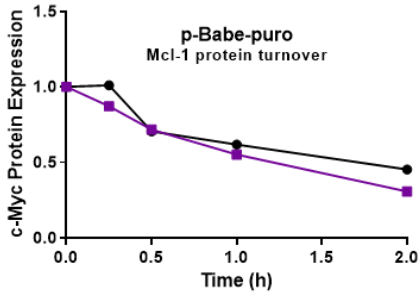
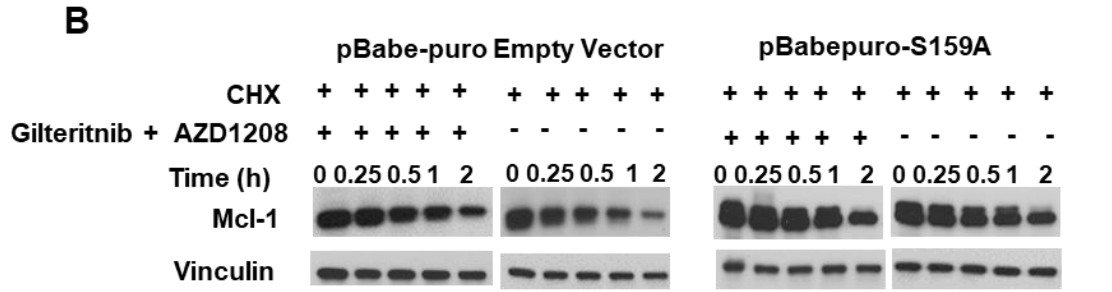
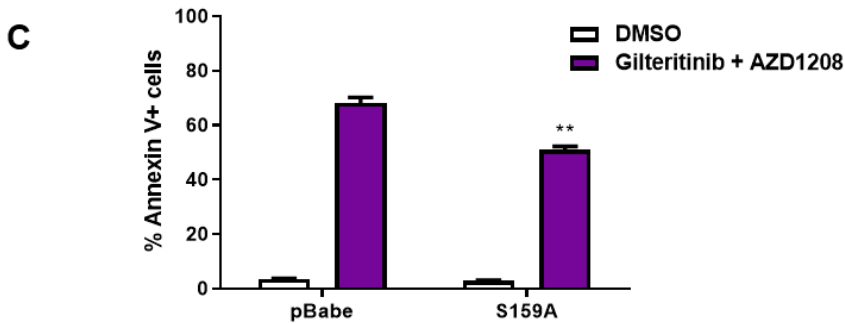


Figure 2.13 continued



50% Protein Turnover (h)		
	DMSO	Gilteritinib + AZD1208
pMSCV-puro-empty vector	1.67	1.32
pMSCV-puro-S159A	2.0+	2.0+



2.5 Discussion

Our data demonstrate that combining the FLT3 inhibitor gilteritinib with the Pim kinase inhibitors AZD-1208 or TP-3654 increases apoptosis induction and produces synergistic cytotoxicity in cell lines with FLT3-ITD, compared to single drugs. Combination treatment also reduced tumor burden and prolonged survival in an orthotopic FLT3-ITD AML mouse model, compared to gilteritinib alone. Our combination treatment also downregulates two major proteins, c-Myc and Mcl-1, which are involved in tumorigenicity and response to FLT3 inhibitors.

c-Myc is overexpressed in AML with FLT3-ITD through both transcriptional upregulation⁵⁰ and post-translational upregulation by Pim-1, which stabilizes c-Myc protein through increased phosphorylation at S62 and decreased phosphorylation at T58.⁹⁰ FLT3 inhibitors, as single agents, downregulate c-Myc in AML with FLT3-ITD,⁵⁰ which we also confirmed here. c-Myc overexpression contributes to leukemogenesis in cells with FLT3-ITD, as demonstrated by marked reduction in cell proliferation following c-Myc siRNA knock-down.⁵⁰

The anti-apoptotic protein Mcl-1 is also a key target in AML with FLT3-ITD. Inhibition of Mcl-1 induces apoptotic cell death, while enforced Mcl-1 expression inhibits apoptosis induction.⁸⁶ FLT3-ITD-expressing cells express Mcl-1 at high levels. FLT3 inhibition downregulates Mcl-1, and blocking STAT5 activation completely abrogates Mcl-1 expression.⁸⁶ Additionally, suppression of endogenous Mcl-1 by siRNA or by flavopiridol treatment sensitizes FLT3-ITD-expressing hematopoietic cells to cytotoxic and targeted therapeutics.¹⁰⁶

Currently there are no clinically approved treatments that inhibit c-Myc or Mcl-1 directly. These proteins, however, remain prime therapeutic targets in the treatment of many cancers and hematological malignancies. Furthermore, c-Myc and Mcl-1 have been shown to work cooperatively to induce resistance in breast cancer¹⁰⁷ and worsen outcomes in non-small lung cancer.¹⁰⁸ Examining other combination strategies that simultaneously downregulate c-Myc and Mcl-1 may be of great value in the treatment of many different cancers.

Chapter 3: Concurrent Pim and FLT3 inhibitor treatment downregulates c-Myc and Mcl-1 post-translationally through phosphorylation by GSK-3 α/β

3.1 Introduction

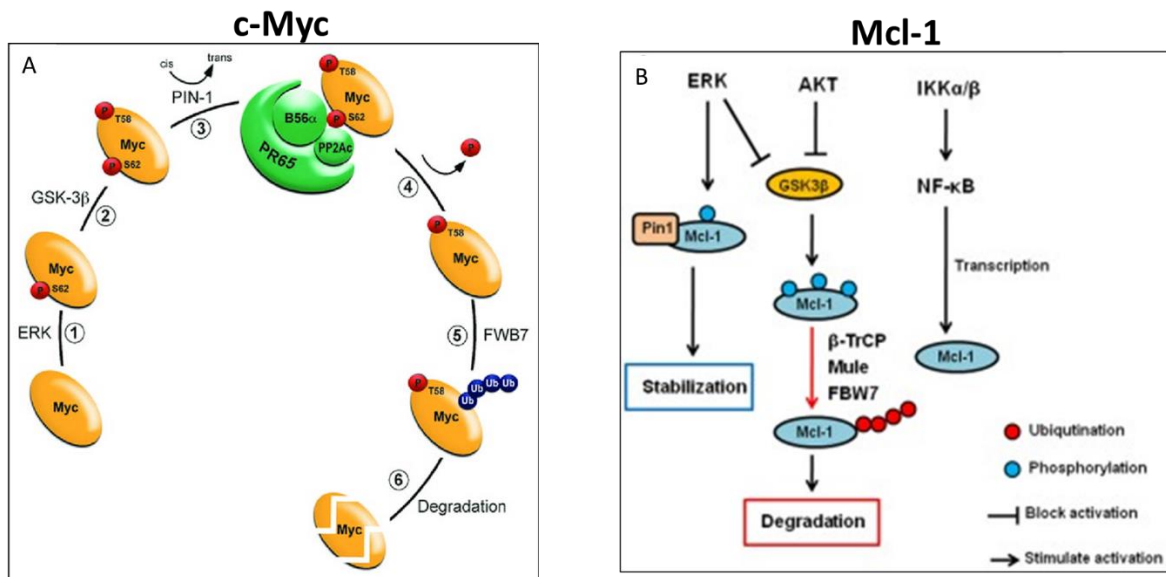
Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase with more than 100 known substrates.¹⁰⁹ It was first discovered for its regulation of energy metabolism through glycogen synthase, but was subsequently found to be involved in many signaling pathways including Wnt, PI3K/AKT, MAPK and hedgehog.¹¹⁰ GSK-3 is implicated in several diseases, including psychiatric diseases such as bipolar disorder, neurological disorders such as Alzheimer's disease, inflammatory diseases, and a variety of cancers.¹¹¹

GSK-3 has two paralogs, GSK3 α and GSK3 β , encoded by two different genes. The isoforms have a highly conserved catalytic domain with unique C and N terminal domains.¹¹⁰ GSK-3 α and GSK-3 β may have overlapping functions in many tissue types but have been shown to have distinct roles in processes including neocortex development, a number of cancers, including leukemias,¹¹² and regulation of beta-catenin.¹¹³ Unlike most kinases, GSK-3 is constitutively active, with autophosphorylation¹¹⁴ at Tyr-279 in GSK3- α and Tyr-216 in GSK3- β during translation.^{115,116} Inactivation is regulated competitively through phosphorylation of GSK-3 α/β at S9/S21, respectively.¹¹⁵

Specificity of GSK-3 for its wide range of targets is regulated by the requirement for its substrates to be primed by a phosphorylation event prior to GSK-3 phosphorylation. Notable substrates of GSK that are regulated through phosphorylation priming include glycogen synthase, beta-catenin, APC, p53, c-Myc and Mcl-1.¹¹⁸ GSK-3 also maintains specificity through its subcellular localization, association with protein complexes and regulation of substrate availability, as well as phosphorylation events.^{119,111}

AKT, a serine/threonine kinase, is a major component of the PI3K signaling axis and is often dysregulated in many cancers, including AML. AKT phosphorylates GSK3- α/β at S9 and S21, thus causing inactivation of GSK-3. In most cancers, AKT signaling is upregulated or activated, resulting in inactivation of GSK-3. In Chapter 2 we demonstrated that concurrent Pim and FLT3 inhibitor treatment downregulates c-Myc through T58 phosphorylation and Mcl-1 through S159 phosphorylation. Both of these residues can be phosphorylated by GSK-3 (Fig 3.1). Here we further elucidate the role that GSK-3 and AKT may play in downregulation of c-Myc and Mcl-1 by concurrent Pim and FLT3 inhibition.

Figure 3.1 GSK-3 regulation of c-Myc and Mcl-1



c-Myc (A) and Mcl-1 (B) can both be phosphorylated by GSK-3 β at T58 and S159, respectively, to target these proteins for ubiquitination and proteasomal degradation.

3.2 Rationale

c-Myc is transcriptionally upregulated in AML with FLT3-ITD⁵⁰ and is also regulated post-translationally by Pim-1⁹⁰ and GSK-3 β .⁹³ Pim-1-mediated phosphorylation of c-Myc at S62 and decreased phosphorylation at T58 result in post-translational upregulation via stabilization,⁹⁰ while GSK-3 β phosphorylation of c-Myc at T58 promotes its proteasomal degradation.⁹³ GSK-3 β , in turn, is a substrate of both AKT¹²⁰ and Pim-1 kinase,¹²¹ both of which phosphorylate it at S9, thereby rendering it catalytically inactive.^{120,122} Additionally, we recently showed that GSK-3 β also phosphorylates Pim-1, resulting in its post-translational downregulation, creating a negative feedback loop between these two kinases.⁹⁴

The pro-survival oncogene Mcl-1, which is upregulated in AML with FLT3-ITD, is also phosphorylated by GSK-3 β , at S159, and thereby tagged for degradation.¹⁰³ Here we hypothesize that Pim and FLT3 inhibitor combination treatment activates GSK-3 β , resulting in post-translational downregulation of both c-Myc and Mcl-1.

3.3 Materials and Methods

Cell lines

Cell lines studied included Ba/F3-ITD (Ba/F3 murine pro-B cells stably transfected with human FLT3-ITD) and MV4-11 and MOLM-14 human AML cells, with homozygous and heterozygous FLT3-ITD, respectively. Cell lines were obtained and cultured as previously described.⁸² FLT3-ITD-transfected BaF3 (Ba/F3-ITD) cells were from Dr. Mark Levis, Johns Hopkins University School of Medicine, Baltimore, MD). MV4-11 and MOLM-14 human AML cells with FLT3-ITD⁹² were from the American Type Culture

Collection (ATCC), Manassas, VA. Cells were maintained in RPMI 1640 with 10 percent fetal bovine serum (FBS).

Retroviral infection of Ba/F3-ITD cells

Ba/F3-ITD cells were infected with constitutively active myristoylated AKT (Myr-AKT), pBabe-Puro-Myr-Flag-AKT1 (Addgene plasmid #15294)¹²³ or pBABE-puro empty vector control, as previously described.⁹⁴

Approximately 80% confluent Phoenix-AMPHO packaging cells (ATCC CRL-3213) were incubated in 25 $\mu\text{mol/L}$ chloroquine for 1 hour and then transfected with 20 μg retroviral plasmid DNA by the calcium phosphate method. Ba/F3-ITD cells were infected with virus-containing medium collected after 24 hours in the presence of polybrene (4 $\mu\text{g/mL}$). Cells were seeded in 2 mL virus-containing medium, centrifuged at 1,800 rpm at 32°C for 45 minutes, then incubated at 32°C for 4 hours, centrifuged at 1,800 rpm at 32°C for 45 minutes, and then incubated in fresh virus-containing medium at 32°C for 2 hours. The cells were then incubated at 37°C for 24 hours, infected with virus-containing medium, and incubated at 32°C for 5 hours. Infected cells were incubated in fresh virus-free medium overnight and then cultured with 1 mg/mL puromycin for 14 days. Myc overexpression was confirmed by immunoblotting.

Overexpression of p-AKT (S473) in cells infected with pBabe-Puro-Myr-Flag-AKT1 was confirmed by immunoblotting.

Patient samples

Blood samples were obtained from patients with AML with FLT3-ITD with peripheral blasts on a University of Maryland School of Medicine Institutional Review Board-approved tissue procurement protocol, following written informed consent. Studies were conducted in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll-Paque (Millipore Sigma, St. Louis, MO). Fresh samples were studied, without prior cryopreservation.

Materials

The FLT3 inhibitor gilteritinib (ASP2215) (Active Biochem, Maplewood, NJ) was used at the biologically⁹⁴ and clinically⁵⁵ relevant concentration of 15 nM. The pan-Pim inhibitor AZD1208 (Tocris Bioscience, Minneapolis, MN) was used at 1 μ M based on inhibition of p-BAD (S112) at this concentration.^{96,97} The GSK-3 β inhibitor TC-G 24, used at 17 nM,⁹⁴ was also from Tocris.

Measurement of apoptosis

Cells were stained with Annexin V and propidium iodine (PI) and analyzed on a FACS Canto II flow cytometer as previously described.⁸² Briefly, cells were incubated with drugs or DMSO control for 48 hours, and apoptosis and necrosis were measured by staining with Annexin V-FITC and PI, respectively. After treatment, cells (2×10^5 – 3×10^5) were washed with PBS, resuspended in Annexin V binding buffer (1x), stained with Annexin

V-FITC (1 μ L) and PI (2 μ L), incubated at room temperature in the dark, then washed and acquired on a FACSCanto II and analyzed with FlowJo.

Immunoblotting

Cells were lysed in 150 mM NaCl lysis buffer with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). Protein was measured using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) and 15 μ g (cell lines) or 50 or 75 μ g (primary AML cells) were immunoblotted for each sample. Immunoblots were incubated with polyclonal primary antibodies to c-Myc and Mcl-1 and monoclonal primary antibodies to p-AKT (S473) (4060), p-AKT (T308) (2965), AKT (9272), p-GSK-3 α / β (S9/S21) (8566), GSK-3 α / β (5676), p-GSK-3 β (S9) (9322), GSK-3 β (9832) (Cell Signaling Technology, Danvers, MA), vinculin (Santa Cruz Biotechnology, Dallas, TX) and β -actin (Sigma-Aldrich) at 1:1000 dilution overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. Band intensities were measured by densitometry with Visionworks@LS, UVP (Upland, CA). Band intensities at serial time points, normalized to vinculin controls, were compared to intensities at time 0, defined as 100%.

Statistical analysis

All data were derived from at least three independent experiments, with error bars in the figures representing standard error of the mean (SEM). Statistical analysis was performed by unpaired t-test, using Prism 9 (GraphPad, San Diego, CA).

3.4 Results

Pim and FLT3 inhibitor combination treatment activates GSK-3

The serine/threonine kinase GSK-3 β phosphorylates c-Myc and Mcl-1 at T58 and S159, respectively, to promote their proteasomal degradation. To understand whether Pim and FLT3 inhibitor combination treatment enhances c-Myc and Mcl-1 proteasomal degradation via GSK-3 β activation, Ba/F3-ITD cells and FLT3-ITD AML patient blasts (Patient 2) were treated with gilteritinib and/or AZD1208 or DMSO control. Combination treatment decreased p-GSK-3 β (S9) expression in Ba/F3-ITD cells and the primary patient sample (Fig. 3.2). Downregulation of p-GSK-3 β , or GSK3 activation, preceded c-Myc and Mcl-1 downregulation in combination-treated cells (Fig. 3.2).

Figure 3.2 GSK-3 is activated by Pim and FLT3 inhibition

Ba/F3-ITD cells (A) and FLT3-ITD AML patient blasts (B) were treated with gilteritinib and/or AZD1208, or DMSO control, and expression of c-Myc, Mcl-1, p-GSK-3 α/β (S9/S21), GSK-3 α/β and β -actin or vinculin loading control at serial time points was measured by immunoblotting.

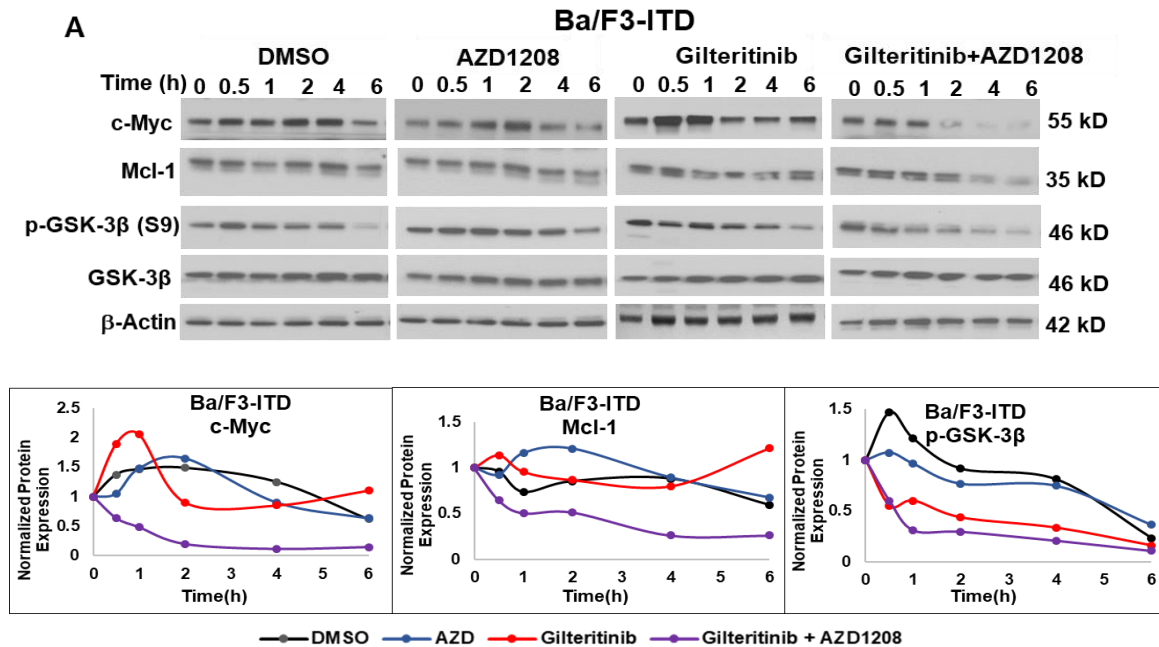
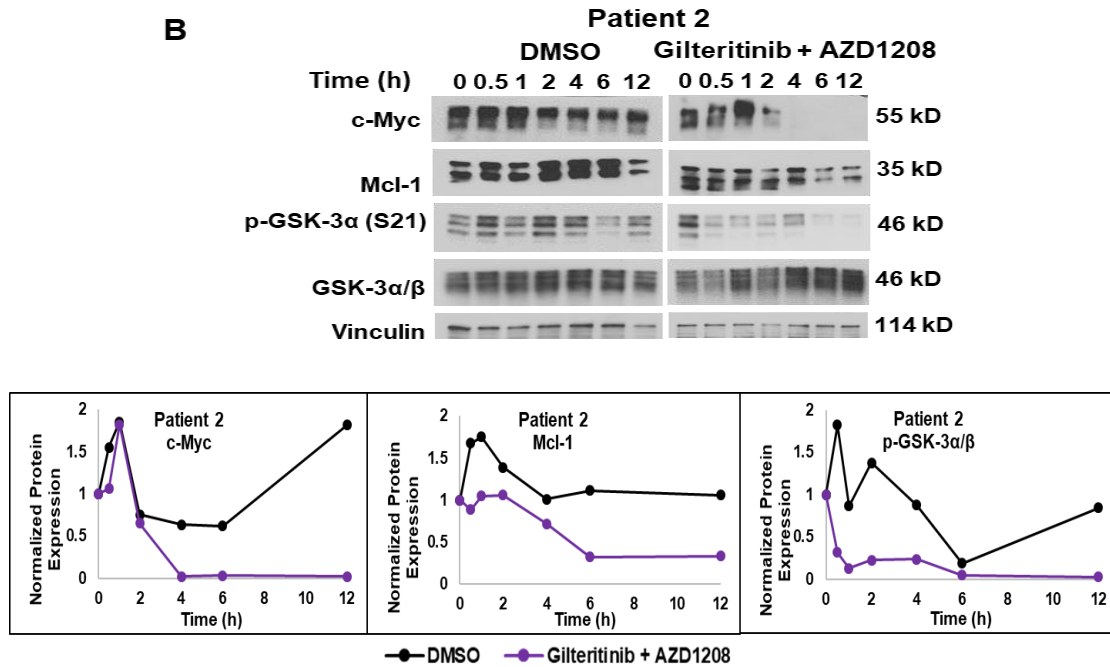


Figure 3.2 continued



GSK-3β inhibition abrogates c-Myc and Mcl-1 downregulation by FLT3 and Pim inhibitor combination treatment

To determine whether downregulation of c-Myc and Mcl-1 is caused by GSK-3β activation, Ba/F3-ITD and MV4-11 cells and FLT3-ITD AML patient blasts (Patient 3) were treated with FLT3 and Pim inhibitors in the presence and absence of the GSK-3β inhibitor TG-C 24, and expression of c-Myc and Mcl-1 was measured by immunoblotting at serial time points. Treatment with the GSK-3β inhibitor TC-G 24 markedly inhibited c-Myc downregulation by combination treatment, and also inhibited Mc-1 downregulation in Ba/F3-ITD and MV4-11 cells and primary AML blasts (**Fig. 3.3**), indicating that downregulation of c-Myc and Mc-1 by combination treatment results, at least in part, from GSK-3β activation.

Figure 3.3 GSK inhibition by TC-G 24 abrogates c-Myc and Mcl-1 downregulation by FLT3 and Pim inhibitor combination treatment

Ba/F3-ITD (A) and MV411 (B) cells and FLT3-ITD AML patient blasts (C) were treated with gilteritinib and/or AZD1208, or DMSO control, with and without the GSK-3 β inhibitor TC-G 24 at 20nM, and expression of c-Myc, Mcl-1, p-GSK-3 α/β , (S9/S21), GSK-3 α/β , and β -actin or vinculin loading control was measured at serial time points by immunoblotting.

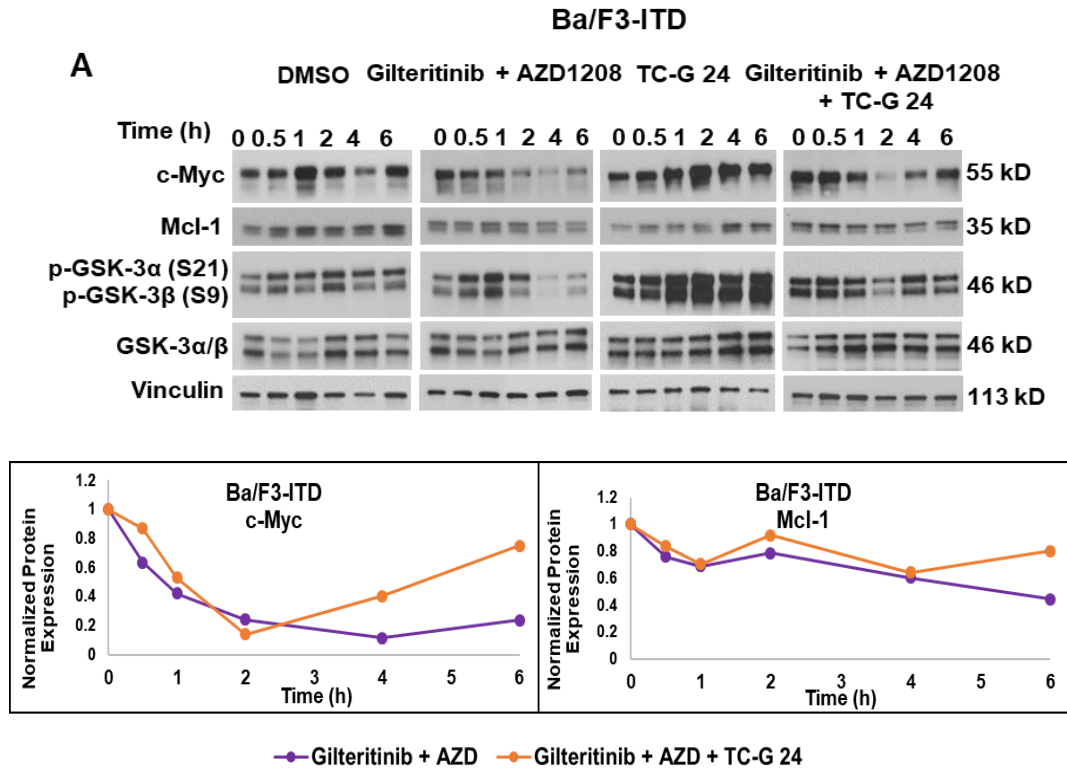
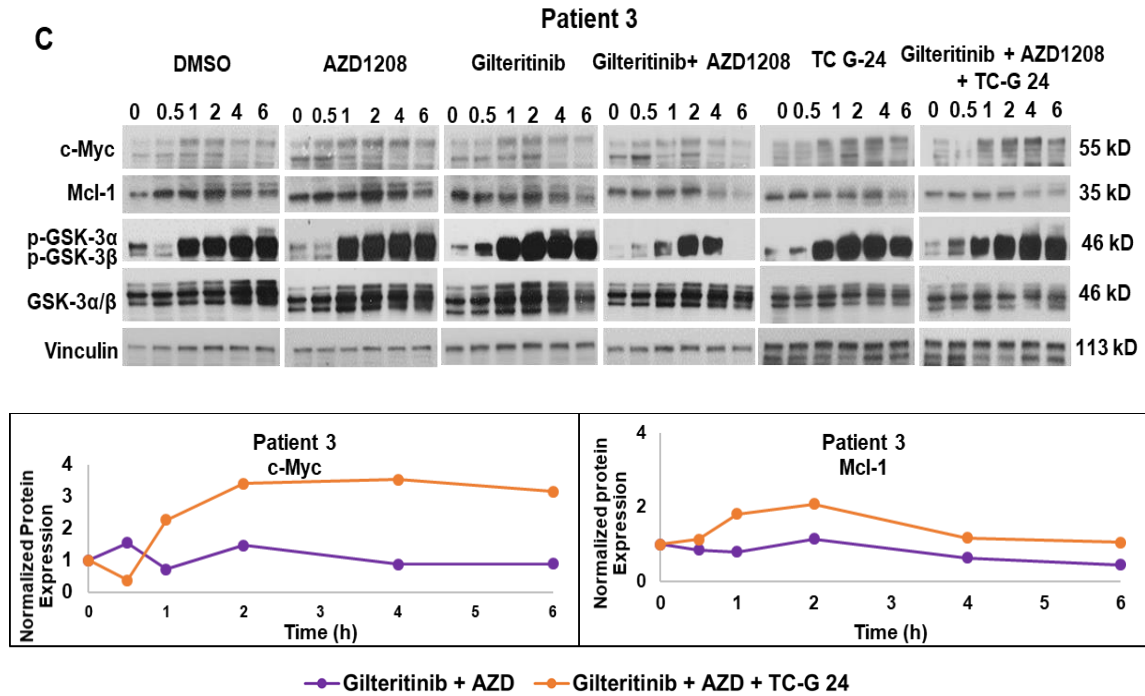
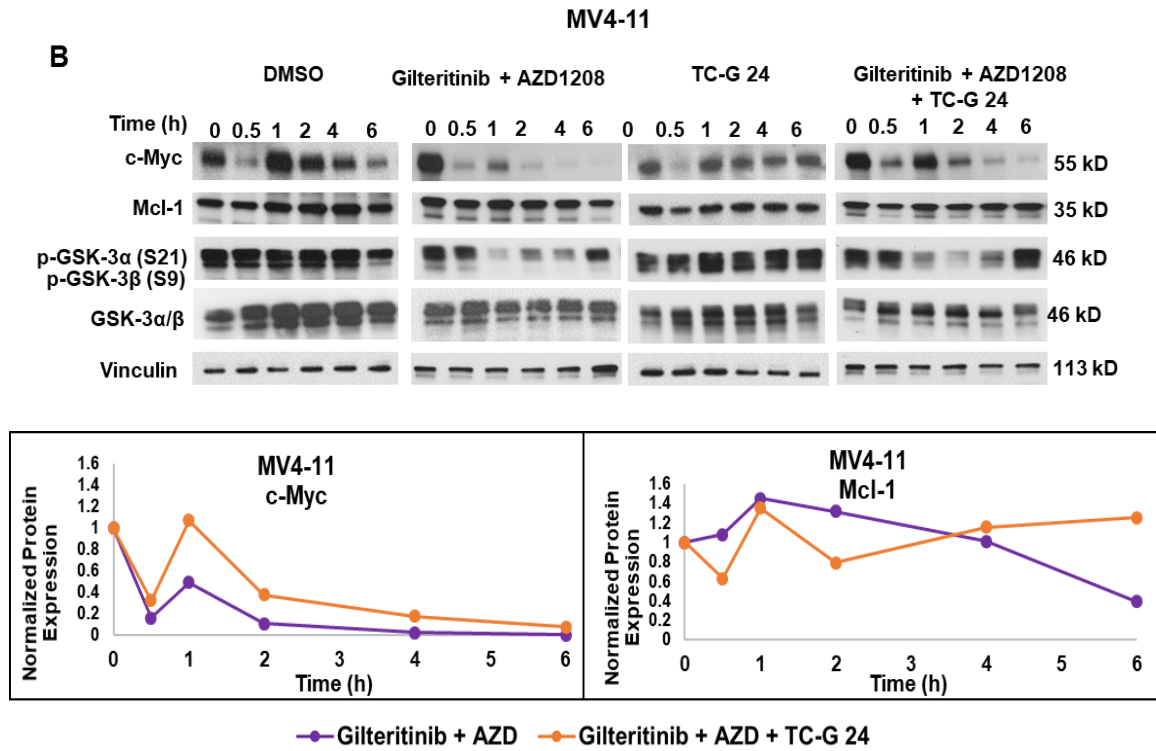


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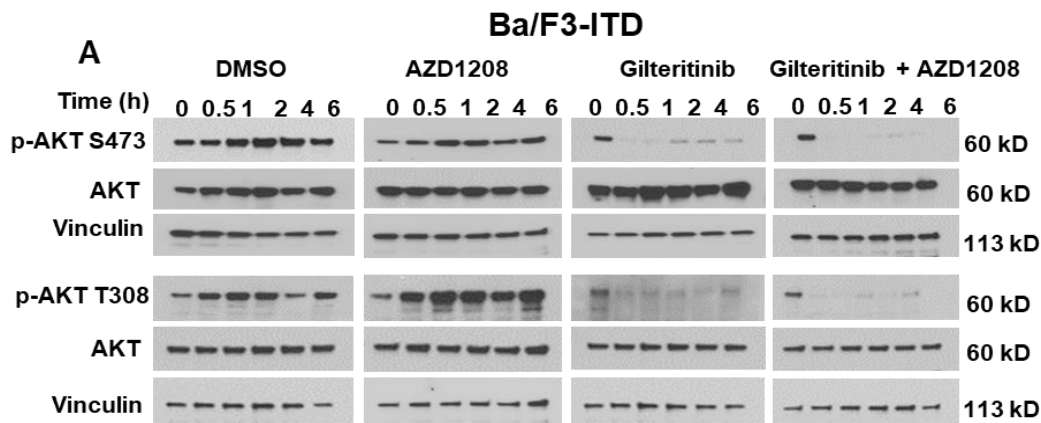


Pim and FLT3 inhibitor combination rapidly inactivates AKT, but AKT inactivation is not necessary for c-Myc or Mcl-1 downregulation or apoptosis induction

Our laboratory recently showed that PP2A-activating drugs enhance FLT3 inhibitor efficacy in cells with FLT3-ITD through AKT inhibition, which activates GSK-3 β , resulting in GSK-3 β -mediated enhanced c-Myc and Pim-1 proteasomal degradation.⁹⁴ We therefore studied the possible role of AKT inhibition in GSK-3 β activation and post-translational c-Myc and Mcl-1 downregulation in cells with FLT3-ITD treated with Pim and FLT3 inhibitor combination.

Pim and FLT3 inhibitor combination treatment rapidly inactivated AKT, decreasing both p-AKT (S473) and (T308) expression. p-AKT decreased by 30 minutes in Ba/F3-ITD cells treated with AZD1208 and gilteritinib, compared to single drugs or DMSO control, while total AKT levels remained unchanged (**Fig. 3.4**).

Figure 3.4 Combination treatment activates AKT



Ba/F3-ITD cells were treated with gilteritinib and/or AZD1208, or DMSO control, and c-Myc, p-AKT (S473 and T308), total AKT and vinculin protein levels were measured in serial samples by immunoblotting.

To test whether AKT inactivation is necessary for c-Myc downregulation, Ba/F3-ITD cells infected with a myristoylated AKT (Myr-AKT) plasmid, which renders AKT constitutively active, or empty vector control (Clones A and B; **Fig. 3.5 A**), were treated with AZD1208 and/or gilteritinib, or DMSO control. AZD1208 and gilteritinib combination treatment downregulated c-Myc and Mcl-1 (**Fig. 3.5 B**) and induced apoptosis (**Fig. 3.5 C**) similarly in cells infected with myristoylated AKT or with empty vector. Therefore, while Pim and FLT3 inhibitor combination rapidly inactivates AKT, AKT inactivation is not necessary for c-Myc or Mcl-1 downregulation or for apoptosis induction by combination treatment. Furthermore, GSK3- α/β was activated similarly in both Myr-AKT and empty vector expressing cells, also demonstrating a lack of regulation of GSK-3 via AKT activation (**Fig. 3.5 B**).

Figure 3.5 GSK-3 β activation, c-Myc and Mcl-1 downregulation and apoptosis induction are independent of AKT inhibition

(A) Ba/F3-ITD cells were infected with p-Babe-Puro-Myr-Flag-AKT1, with myristoylated (constitutively active) AKT, or pBabe-Puro empty vector control. (B) Cells were treated with AZD1208 and/or gilteritinib, or DMSO control, and c-Myc, p-AKT (S473), AKT, p-GSK-3 α/β , GSK-3 α/β , Mcl-1 and β -actin or vinculin loading control protein levels were measured in serial samples by immunoblotting. C. Apoptosis was measured in parental Ba/F3-ITD cells and Ba/F3-ITD cells infected with p-Babe-Puro-Myr-Flag-AKT1, or pBabe-Puro empty vector control treated with AZD1208 and/or gilteritinib, or DMSO control, for 48 hours.

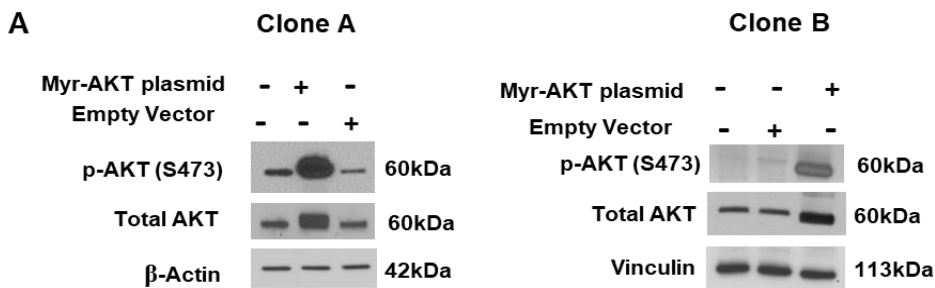


Figure 3.5 continued

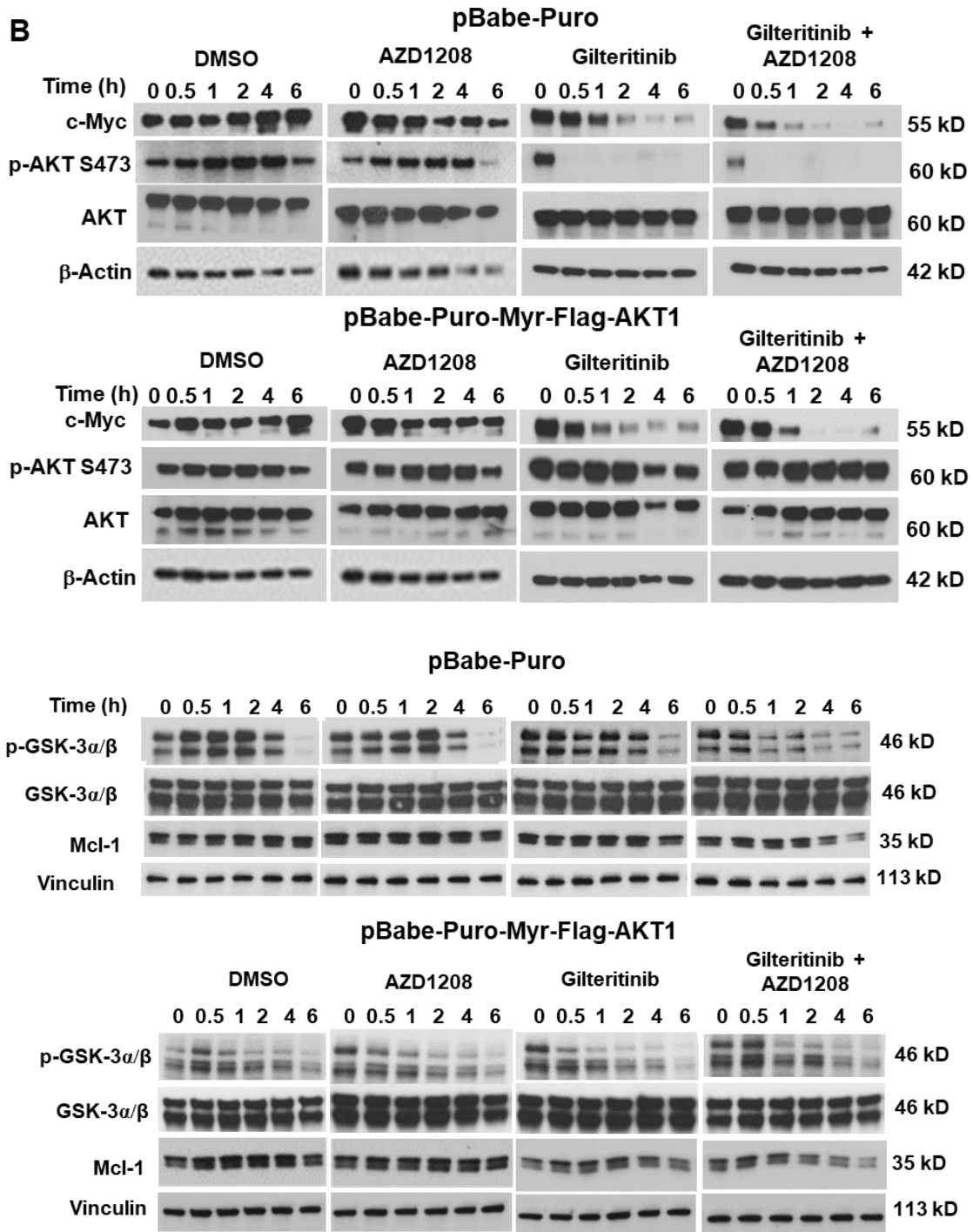
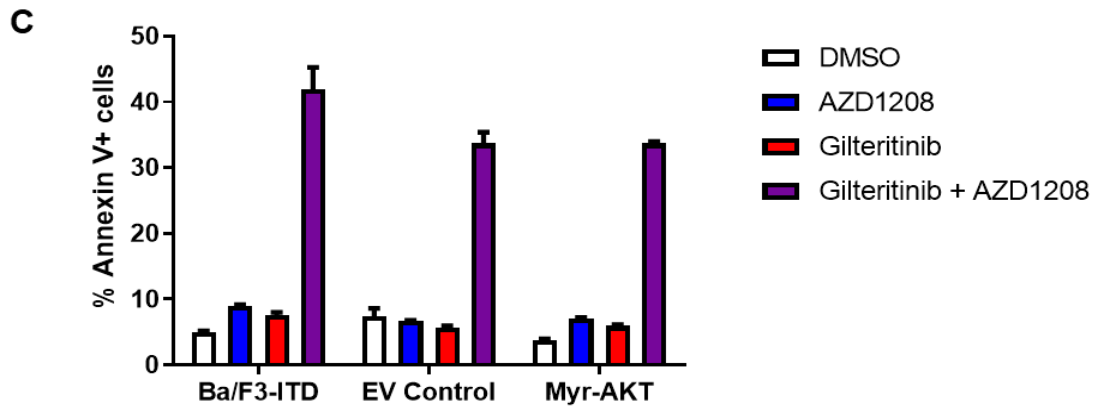


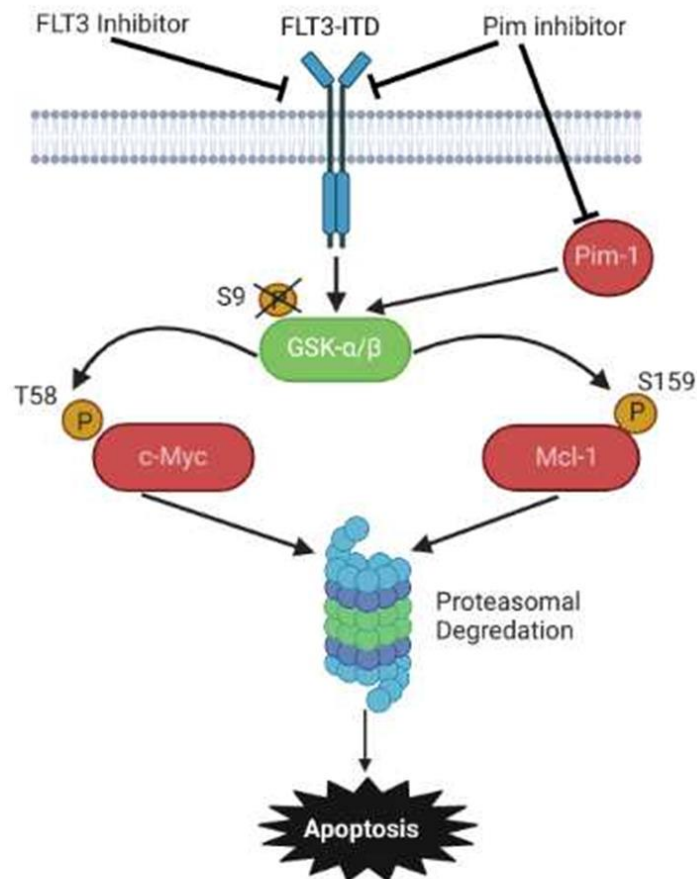
Figure 3.5 continued



3.6 Discussion

We found that post-translational downregulation of both c-Myc and Mcl-1 resulted from activation of GSK-3 β , which phosphorylates the two proteins at T58 and S159, respectively, and thereby tags them for proteasomal degradation (Fig. 3.6).

Figure 3.6 Mechanism Schematic



We previously found that PP2A-activating drugs also enhance efficacy of FLT3 inhibitors through activation of GSK-3 β , which phosphorylates c-Myc and Pim-1, resulting in post-translational downregulation of both proteins and enhanced apoptosis.⁹⁴ We did not study Mcl-1, which was likely also downregulated.

GSK-3 is a substrate of AKT in the (PI3K)-Akt-mTOR cell survival pathway.¹²⁰ In our work here, FLT3 inhibitor and Pim inhibitor combination treatment caused both AKT

inhibition and GSK-3 activation, but GSK-3 activation was independent of AKT inhibition. Combination treatment with the Pim inhibitor AZD1208 and the FLT3 inhibitor gilteritinib downregulated c-Myc and Mcl-1 and induced apoptosis identically in cells infected with myristoylated (constitutively activated) AKT and with empty vector, indicating that downregulation of c-Myc by AZD1208 and gilteritinib combination treatment was not dependent on inhibition of AKT. p-GSK-3 was also downregulated similarly in cells infected with myristoylated AKT and with empty vector. In contrast, we previously showed that combined PP2A-activating drug and FLT3 inhibitor treatment rapidly inactivated AKT in FLT3-ITD-expressing cells through dephosphorylation at both S473 and T308, and that AKT inactivation by treatment with the AKT inhibitor MK-2206 caused GSK-3 β activation.⁹⁴ Also, PP2A-activating drug and FLT3 inhibitor combination treatment caused GSK-3 β activation in parental Ba/F3-ITD cells and Ba/F3-ITD cells infected with empty vector, but not with myristoylated AKT.⁹⁴ Thus GSK-3 activation occurred through AKT inhibition in FLT3-ITD-expressing cells treated with PP2A-activating drug and FLT3 inhibitor combination, but not with Pim inhibitor and FLT3 inhibitor combination.

The two GSK-3 paralogs, GSK-3 α and GSK-3 β , have variably been reported to have tumor suppressor and oncogenic properties in different acute leukemia types and subtypes.^{124,125} GSK-3 α or GSK-3 β was found to be necessary for survival and proliferation of cells with *KMT2A* (*MLL*) gene rearrangements *in vitro* and *in vivo*, through destabilization of p27^{Kip1}, a cyclin-dependent kinase inhibitor that is a tumor suppressor protein.¹²⁶ Treatment with selective GSK-3 inhibitors, knockdown of GSK-3 α or genetic ablation of GSK-3 β inhibited proliferation of cells with *KMT2A* rearrangements, and this anti-proliferative effect was inhibited by p27Kip1 knockdown.¹²⁶ In contrast, GSK-3

inhibition did not produce anti-proliferative effects and did not increase expression of p27Kip1 in acute leukemia cells with other gene rearrangements, including *TEL-AML1*, *E2A-HLF* and *E2A-PBX1*.¹²⁶

The prognostic significance of both GSK-3 phosphorylation and localization has also been reported in AML. Lower levels of p-GSK3 α/β in AML cells, measured by reverse phase protein analysis, correlated with longer remission duration and overall survival in patients in the intermediate, but not the unfavorable, cytogenetic group.¹²⁵ Notably, in patients in the intermediate cytogenetic group with FLT3 mutations, lower p-GSK3 α/β levels were strongly correlated with longer remission duration.¹²⁵ Aberrant nuclear localization of GSK-3 β has also been described in AML, correlating with AML growth *in vitro* and *in vivo*, with drug (daunorubicin and cytarabine) resistance and with shorter patient survival.¹²⁷ A proposed mechanism is that nuclear GSK-3 β may promote nuclear localization of the NF- κ B subunit p65, increasing transcription of NF- κ B target genes.¹²⁸

These data and previously published data support an important role for GSK-3 in leukemias. Our work specifically highlights the tumor suppressive role that GSK-3 plays in AML with FLT3-ITD, with GSK-3 activation downregulating several proteins that promote cell survival and proliferation.

Chapter 4: Conclusion and Next Steps

Standard treatment for AML has remained relatively constant for the past five decades. Even with the introduction of new targeted therapies over the past 10 years, survival rates for AML patients have increased only slightly. This remains especially true for AML with FLT3-ITD, which is prognostically unfavorable, with low 5-year survival. The introduction of FLT3 inhibitors into the clinic has been promising, but due to incomplete responses and rapid onset of resistance, the need to develop better treatment strategies still exists. Combination treatments have emerged as a strategy to increase drug efficacy and to prevent and overcome resistance in a plethora of cancer types. Combinations of FLT3 inhibitors with Pim inhibitors and with PP2A-activating drugs have been extensively studied in our lab and have been shown to have greater efficacy than FLT3 inhibitors as single drugs.^{52,82,94} Other research has shown efficacy of combining FLT3 inhibitors with BCL-2 inhibitors, PARP inhibitors, JAK/STAT inhibitors, and oxidative phosphorylation inhibitors, to name a few.¹²⁹ Here, we combined Pim inhibitors and FLT3 inhibitors and further elucidated the mechanisms by which this combination regulates key proteins involved in the maintenance and tumorigenicity of AML. There are also implications for overcoming drug resistance.

Chapter 2 explored the efficacy of the Pim kinase inhibitors AZD1208 and TP-3654 in combination with the FLT3 inhibitor gilterinib in several FLT3-ITD-expressing cell lines. The data confirmed that the combination treatments produce synergistic cytotoxicity and apoptosis induction. We previously showed that AZD1208 and quizartinib combination treatment reduced tumor burden in both subcutaneous and orthotopic mouse models of FLT3-ITD AML.⁸² In our study here, we studied the efficacy of TP-3654 and

gilteritinib in an *in vivo* orthotopic mouse model of FLT3-ITD AML; this combination was markedly more effective than gilteritinib alone in reducing tumor burden. It is important to note that the *in vivo* data in this project use two clinically applicable drugs and can be more readily translated into clinical trials. While AZD1208 is no longer in clinical development due to increased CYP3A4 activity after multiple dosing in a clinical trial, resulting in increased drug clearance,¹³⁰ the pan-Pim inhibitor TP-3654 remains in clinical development, with promising clinical trial results to date,^{131,132} and gilteritinib has gained FDA approval as monotherapy for refractory or relapsed AML with FLT3 mutations.⁵⁵

We confirmed Pim and FLT3 inhibitor combination treatment regulation of c-Myc and Mcl-1 through proteasomal degradation in Chapter 2. c-Myc is aberrantly activated, amplified, or mutated in a variety of cancers and is regulated transcriptionally by other transcription factors and miRNAs, and post-translationally through proteasomal degradation.¹³³ c-Myc has been shown to be very important in the induction and maintenance of AML. c-Myc downregulates miR-26a through EZH2 in AML, and overexpression of miR-26a induced myeloid differentiation and a decrease in proliferation.¹³⁴ c-Myc overexpression in AML cell lines caused increased resistance to chemotherapeutics which was overcome by c-Myc inhibition; c-Myc inhibition also induced differentiation via upregulation of C/EBP β .¹³⁵

These studies and many others highlight the many roles that c-Myc plays in AML and why it is an important therapeutic target. Several approaches have been explored to inhibit c-Myc. One approach targets the dimerization of c-Myc and its co-factor Max, which is required for translocation into the nucleus and transactivation of genes.¹³⁶ Another approach involves the use of bromodomain and extra-terminal motif (BET) inhibitors,

which inhibit the interactions between transcription factors, histones, and proteins that contain the BET domain. BET inhibition downregulates c-Myc transcription, increases apoptosis induction, and reduces tumor burden in leukemia cell lines and mouse models.¹³⁷

There are no direct c-Myc inhibitors available or in clinical development, and Pim inhibitors have been proposed as indirect c-Myc inhibitors in other malignancies, including prostate and breast cancers.^{138,139,140} In our studies, Pim inhibitors alone did not downregulate c-Myc, but combination Pim and FLT3 inhibitor treatment downregulated c-Myc more markedly and more rapidly than FLT3 inhibitor treatment alone, likely contributing to the enhanced apoptosis induction seen with combination therapy, demonstrated by us previously^{51,82} and here, as well as by others.^{52,81}

Mcl-1 inhibitors are also not used in the clinic. Direct Mcl-1 inhibitors have been associated with cardiotoxicity¹⁴² and indirect approaches to Mcl-1 inhibition or downregulation¹²⁴ appear preferable. We show here and showed previously⁸² that FLT3 and Pim inhibitor co-treatment effectively downregulates Mcl-1 in cells with FLT3-ITD more markedly and more rapidly than FLT3 inhibitors alone.

In Chapter 3 we explored regulation of c-Myc and Mcl-1 by GSK-3 and AKT in cells with FLT3-ITD treated with Pim and FLT3 kinase inhibitors. We found that c-Myc and Mcl-1 downregulation was abrogated by GSK-3 inhibition. We also found that constitutive AKT activation did not affect c-Myc and Mcl-1 downregulation by Pim and FLT3 inhibitor co-treatment and did not affect GSK-3 activation.

One limitation of our study is that we did not separate the functions of GSK-3 β from GSK-3 α . For example, all three isoforms of AKT can phosphorylate GSK-3 β , while GSK-3 α can only be phosphorylated by AKT2.¹⁴³ We specifically looked at the role of

AKT1, which is most relevant to AML, and its ability to regulate GSK-3 β . Pim and FLT3 kinase inhibitor combination treatment in our study activated both GSK-3 α and GSK-3 β , even in cells with constitutively active AKT1. However, to better understand the role of each isoform, it would be of interest to study downstream signaling following GSK-3 α or GSK-3 β knockdown and in a GSK-3 β -mutated mouse model. It may also be of interest to further understand what may be contributing to the activation of GSK-3 in lieu of AKT. GSK-3 is activated by a number of pathways, and it would be of interest to further explore the upstream signaling that may contribute to its activation and consequent regulation of c-Myc and Mcl-1.

As previously discussed, resistance remains an obstacle to the success of FLT3 inhibitors. To better understand how well Pim and FLT3 kinase inhibitor combination treatment works in preventing and overcoming resistance, more research is needed. The addition of Pim kinase inhibitors to FLT3 inhibitors has been shown to increase apoptosis in FLT3 inhibitor-resistant cell lines.⁵² Furthermore, our laboratory has shown that PP2A inhibitors increase sensitivity to FLT3 inhibitors in certain FLT3 inhibitor-resistant cell lines.¹⁴⁴ More *in vitro* and *in vivo* studies are needed to understand the molecular landscape and signaling pathways that are being augmented by combination therapies and which subsets of patients will benefit. It will also be interesting to further explore the role of GSK-3 in FLT3 inhibitor resistance in cells with FLT3-ITD. A recent study also confirmed that GSK-3 β activation and Mcl-1 inhibition effectively induced apoptosis in sorafenib-resistant MOLM-13 cells, with FLT3-ITD.¹⁴⁵

Based on our work here and our previous work,⁹⁴ activation of GSK-3 appears to be a key strategy for optimizing response to FLT3 inhibitors, through post-translational

downregulation of c-Myc, Mcl-1 and Pim-1, key proteins driving proliferation and resistance to apoptosis in AML cells with FLT3-ITD. This pathway also appears to be relevant to other tyrosine kinase-driven leukemias, as well as solid tumors.

GSK-3 was reported to be inactivated in chronic myeloid leukemia (CML), which is driven by the oncogenic tyrosine kinase BCR-ABL, and enforced expression of constitutively active GSK-3 reduced proliferation and potentiated BCR-ABL inhibitor-induced apoptosis in both BCR-ABL inhibitor-sensitive and -resistant cells.¹⁴⁷ This work suggested therapeutic efficacy of GSK-3 activation in CML. Indeed, the PIM-1 kinase inhibitor SMI-4a was subsequently found to exert antitumor effects in both imatinib-sensitive and -resistant CML cells by increasing GSK-3 β activity.¹⁴⁶

GSK-3 α/β activation has also been described in response of colorectal cancer cells to kinase inhibitors. In one study, treatment with the multi-kinase inhibitor sorafenib inactivated ERK1/2 by dephosphorylation at T202/Y204, resulting in GSK3- β activation by dephosphorylation at S9, promoting p65 phosphorylation and expression of the pro-apoptotic Bcl-2 protein family member PUMA.¹⁴⁸ In a subsequent study, treatment of colorectal cancer cells with the tyrosine kinase inhibitor gilteritinib, which inhibits AXL as well as FLT3, caused AKT inhibition and consequent GSK-3 α/β activation, which resulted in nuclear translocation of p65 and induction of PUMA as a mechanism of apoptosis induction, and GSK3 β knockdown suppressed gilteritinib-induced p65 phosphorylation and induction of PUMA.¹⁴⁹ The same mechanism was demonstrated for the multi-kinase inhibitor regorafenib¹⁵⁰ in colorectal cancer cells.

Finally, as noted above, gilteritinib is FDA-approved for relapsed and refractory FLT3-mutated AML, and TP-3654 has been well tolerated and has shown efficacy in

clinical trials.^{131,132} Our data here provide groundwork for a potential future clinical trial combining TP-3654 and gilteritinib in AML with FLT3-ITD, with correlative laboratory studies.

Appendix

Table S2.1 Patient Sample Demographics

Patient	Age, sex	WBC (x10⁹/L)	Blasts (%)	Karyotype	FLT3-ITD allelic burden (%)
1	45F	38.5	66	46,XX,t(4;17)(q21;q21),t(6;14)(p21;q24)[20]	22
2	85F	32.2	45	46,XX[20]	52
3	55M	153.7	75	46,XY	71

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