

Curriculum Vitae

SHIVANI KAPOOR

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Summary of qualifications

- Three years of research experience with proficiency in vast array of molecular biology techniques.
- Experience in conducting, optimizing, and developing cell culture-based assays.
- Multiple years of experience in laboratory management.
- Excellent team worker.

EDUCATION:

- Master of Science in Biomedical Research, University of Maryland Baltimore 01/15-12/16
- Bachelor of Science in Biotechnology and Genetic Engineering, Merit International Institute of Technology, India 08/2007 - 07/2011

RESEARCH EXPERIENCE:

- Currently working on my Master's thesis in Dr. Maria R. Baer's lab at University of Maryland Baltimore. 01/2016- Present
- **Research Assistant, JS YOON Memorial Cancer Research Institute, University of Maryland, BioPark** 01/2014 – 01/2015
 - Was responsible for applied molecular biology techniques (e.g. RNA and DNA isolation methods, genotyping, gel electrophoresis, western blots, PCR) Supervised routine laboratory maintenance including preparing media/buffers and purchasing laboratory supplies.
 - Maintaining laboratory cell cultures.
- **Laboratory Technician in Department of Microbiology Medicine, University of Maryland Baltimore** 11/ 2013 - 05/2014
 - Applied molecular biology techniques toward investigating tissue hypoxia and heart morphogenesis research in mice
 - Managed daily operations of laboratory including ordering of laboratory supplies, monitoring mouse colonies and mouse genotyping.
- **Laboratory Assistant in Department of Microbiology and Immunology, University of Maryland-Baltimore** September 2012-January 2013
 - Applied molecular biology techniques toward investigating innate immune responses and role of signaling kinases.

- Conducted cell-culturing methods for maintaining human monocytic cell lines and Western blots.
- Internship at Carnegie Institution for Science, Department of Embryology, Johns Hopkins University. (12/2011-6/2012).
 - During this period of time, I learned about mouse genotyping, microtome sectioning and staining.
- **Internship at Ranbaxy Pharmaceuticals Inc, New Delhi, India. (1/2011-5/2011)**
- **DNA Research Center (12/2009-1/2010)**
 Worked on Vaccine development approach for Mycobacterium Leprae in the Department of Bioinformatics

SKILLS:

- **Cell culture:** primary cells and cell lines (suspension and adherent)
- **Molecular biology techniques:** DNA isolation, real time PCR, SDS-PAGE, western blot, Bradford protein estimation, BCA protein estimation, cytotoxicity assays, immunohistochemistry (IHC), H&E staining, multi-colored flow cytometry assays to study apoptosis, ROS detection.
- **Animal work:** Experienced in maintaining and monitoring experimental mice, mouse genotyping
- **Software skills:** Flowjo, Graphpad, Adobe Photoshop, MEDLINE, Blast, Microsoft Office

Poster Presentation:

- **Shivani Kapoor**, Patrick R. Baldwin, Kshama A. Doshi and Maria R. Baer. The Pim kinase inhibitor PIM447 sensitizes AML cells with FLT3-ITD to apoptosis induction by FLT3 inhibitors and topoisomerase 2 inhibitors. Cancer research retreat, University of Maryland Baltimore

Abstracts:

- Doshi KA, Baldwin PR, **Kapoor S**, Baer MR. The clinically applicable pan-Pim kinase inhibitor PIM447 sensitizes acute myeloid leukemia cells with FLT3-ITD to induction of apoptosis by FLT3 inhibitors and by topoisomerase 2 inhibitors. American Association for Cancer Research, 2016 (poster presentation).
- Baldwin PR, **Kapoor S**, Natarajan K, Trotta R, Tron AE, Huszar D, Davila E, Perrotti D, Baer MR. Concurrent inhibition of Pim-1 and FLT3 kinases in FLT3-ITD acute myeloid leukemia post-translationally downregulates the anti-apoptotic protein Mcl-1 through downregulation of the Mcl-1 deubiquitinase USP9X. American Society of Hematology, 2016. Abstract 35 (oral presentation).

Manuscript in preparation

- Baldwin, P.R., K. Natarajan, **Shivani Kapoor**, T.J. Mathias, K.A. Doshi, R. Trotta, E. Davila, D. Perrotti, M. Kraus, A.E. Tron, D. Huszar, and M.R. Baer. Concurrent inhibition of Pim and FLT3 kinases increase apoptosis of FLT3-ITD acute myeloid leukemia cells through enhanced degradation of Mcl-1. In preparation for submission.

LEADERSHIP ROLES

- **Graduate Student Association Officer, UMB**
 - **Biomedical Research Representative (January 2016-Present).**

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Abstract

Title of Thesis: Effect of inhibiting Pim-1 kinase in acute myeloid leukemia with FLT3 internal tandem duplication

Shivani Kapoor: Master of Science, 2016

Thesis Advisor: Maria Baer, M.D. Professor Division of Hematology/Oncology
Department of Medicine University of Maryland, Baltimore MD 21201

The project is focused on combinatorial treatment involving downstream inhibitors of FLT3 signaling, representing a promising approach for treating AML patients with FLT3-ITD. Pim-1 kinase is upregulated downstream of FLT3-ITD pathway by a positive feedback loop. The goal of this work was to characterize Pim kinase inhibitor (PIM447, currently in clinical trials) sensitization of cells FLT3-ITD to apoptosis induction by combination of FLT3 inhibitors and chemotherapy drugs. PIM447 sensitizes FLT3-ITD cells to FLT3 inhibitors and topoisomerase-2 inhibitors. Co-treatment with the Pim kinase inhibitor AZD1208 and FLT3 inhibitor quizartinib was found to enhance apoptosis of cells FLT3-ITD post-translationally, decreasing expression of the anti-apoptotic protein Mcl-1 through increased proteasomal degradation and in association with decreased expression of deubiquitinase USP9X. PIM447 in combination with FLT3 inhibitors as quizartinib, sorafenib and midostaurin and topoisomerase-2 inhibitors as daunorubicin, mitoxantrone and etoposide in FLT3-ITD cells, induced a dose response dependent increasing apoptosis.

Effect of inhibiting Pim-1 kinase in acute myeloid leukemia with FLT3 internal tandem duplication

By:
Shivani Kapoor

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2016

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I would like to acknowledge all Dr. Baer's lab members Dr. Patrick Baldwin and Kshama Doshi for helping me in the lab and also for his guidance whenever needed.

Finally, I would like to thank my husband Arun Kapoor who has been a constant source of support and encouragement during the different phases of graduate school

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INTRODUCTION

Acute myeloid leukemia (AML) is a life-threatening blood cancer that mostly affects adults. It is characterized by accumulation of immature myeloid cells, or myeloblasts, in the bone marrow and frequently also in the blood, and impairment of normal blood cell production^{1, 2}. In the United States, the annual incidence of AML is around 2.4 per 100,000 men and women, and it increases with age¹. Current treatment for AML patients consists of chemotherapy with or without hematopoietic stem cell transplantation, depending on patients' age and health and the chromosomal and molecular abnormalities in their leukemia cells. Overall survival in AML patients needs improvement. Hence, there is a need for an improved therapy for AML^{1, 3}.

As a result of developments in science and technology, research in AML has been advancing, and the biology of AML is becoming better understood, resulting in development of new targeted therapies³. Chromosomal and molecular abnormalities in AML involve genes that regulate cell survival, proliferation, and maturation⁴. They predict treatment outcome, guide treatment choices, and are the basis for design of targeted therapies.

The most common molecular abnormalities identified in AML include mutations in the NPM1, CEBP α , KIT, MLL, IDH1/2, DNMT3A, ASXL1 and FLT3 genes⁵.

NPM1 mutation

The nucleophosmin gene is located on chromosome 5q35. It encodes a nuclear-cytoplasmic shuttling protein, and is also responsible for duplication of centrosomes during the cell cycle⁶. Mutations in the NPM1 gene cause abnormal accumulation of NPM1 protein in the cytoplasm. NPM1 mutations are more common in females^{6, 7}. AML with NPM1 mutations is sensitive to chemotherapy, and patients have favorable outcomes⁷, with longer disease-free survival and lower incidence of relapse⁸.

DNA methyltransferase 3A (DNMT3A) mutation

DNA methyltransferase (DNMT) 3A is an enzyme that catalyzes the addition of methyl groups to the cytosine residue of the CpG dinucleotide in DNA. DNMT3A mutations present in AML are usually heterozygous, with a majority of missense alterations in the R882 residue⁹. DNMT3A mutations in AML patients are associated with DNA hypomethylation, which is associated with short patient overall survival⁹.

CEBP α mutation

The transcription factor CCAAT/enhancer binding protein alpha (CEBP α) promotes granulocytic differentiation, and mutation in CEBP α impairs differentiation¹⁰. CEBP α mutations occur in 7-10% of AML patients, and are associated with favorable treatment outcomes¹⁰. Research is ongoing to better understand the pathways and signaling involved in this mutation¹⁰.

C-KIT mutation

KIT, a proto-oncogene located on chromosome 4q11–12, encodes a 145 kD type III receptor tyrosine kinase¹¹. Kit regulates a number of cell types, including hematopoietic stem cells, mast cells, melanocytes and germ cells¹². KIT is also expressed on AML blasts¹³.

MLL translocations

The mixed lineage leukemia (MLL) or KMT2A gene encodes a protein with histone methyltransferase activity. Translocations affecting the MLL gene are associated with aggressive acute lymphoblastic and myeloid leukemia characterized by the overexpression of HOX genes¹⁴.

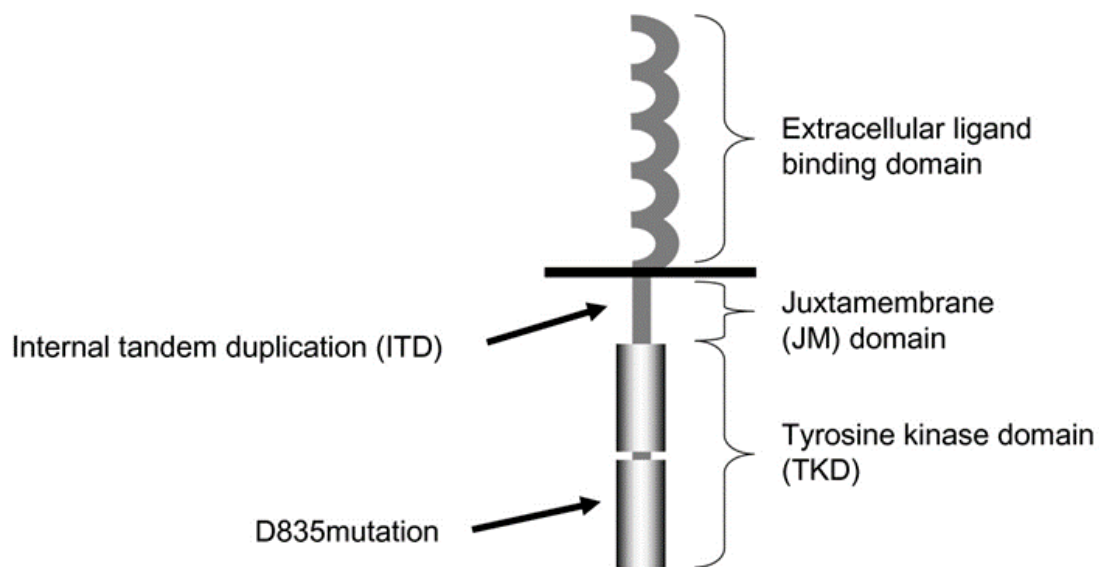
IDH1/2 mutations

Mutations in the genes encoding the isocitrate dehydrogenase (IDH) enzymes IDH1 and IDH2 cause loss of physiological functions of IDH enzymes. These mutations are found in approximately 20% of cases of AML^{14, 15}. Mutations in IDH have been identified as potential therapeutic targets in AML. AGI-6780 has been identified as a small molecule inhibitor of the IDH2 mutation (R140Q)¹⁵.

FLT3 mutations

FMS-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase family, and is important for the development of hematopoietic stem cells and the immune response¹⁶. AML cells have FLT3 mutations in approximately 30% of patients.

There are two types of FLT3 mutations. The first type is an internal tandem duplication (ITD) in the juxtamembrane domain, which is associated with a high percentage of bone marrow blast cells and a high blood blast count, an increased relapse rate, and short disease-free survival¹⁶. In cells with wild-type FLT3 (FLT3-WT) receptor, ligand binds to FLT3, resulting in receptor dimerization, phosphorylation of the tyrosine kinase domain and activation of downstream signaling. However, ITD mutation in the juxtamembrane domain of FLT3 leads to ligand-independent receptor dimerization and constitutive activation of downstream signaling pathways^{16, 17}. The second type of mutation is FLT3-TKD (tyrosine kinase domain) mutation, a point mutation in the tyrosine kinase domain (TKD), most commonly at D835¹⁸. It also constitutively activates the FLT3 receptor. FLT3 is one of the most commonly mutated genes in AML. FLT3 inhibitors are being developed to treat AML patients with FLT3 mutations.



Takahashi et al., *Journal of Hematology & Oncology* (2011)

FLT3 TKD point mutations cause substitution of one amino acid in the kinase domain, which produces a change in the activation loop that favors the active kinase conformation¹⁹, resulting in receptor activation in the absence of the ligand. Both types of mutation lead to constitutive activation of FLT3, which promotes cell survival and growth²⁰.

Current treatment of AML

- Chemotherapy

Chemotherapy is the main treatment for patients with AML. Patients are given cytarabine (ara-C) at a dose of 100 mg/m² every day for 7 days and daunorubicin or idarubicin for 3 days, a regimen commonly known as “7+3”^{5, 6}. Most patients receive 2 to 4 cycles of consolidation chemotherapy as post-remission therapy. Many patients relapse after chemotherapy.

- Allogeneic hematopoietic stem cell transplantation (alloSCT).

Post-remission therapy consists of additional chemotherapy and/or allogeneic hematopoietic stem cell transplantation (alloSCT). Patients receive stem cells from tissue type-identical, or alternative, donors.

New inhibitors for the treatment of AML

- FLT3 inhibitors in clinical trials:

A number of FLT3 inhibitors have been tested in clinical trials. FLT3 inhibitors bind to the ATP-binding site of the intracellular TK (tyrosine kinase) domain of FLT3, competitively inhibiting ATP binding and preventing autophosphorylation of the receptor, and thereby blocking downstream signalling activation²¹.

First-generation inhibitors were not specifically designed for FLT3 inhibition. They were developed to block oncogenic receptor tyrosine kinase (RTK) signalling in various solid and liquid malignancies^{21, 22}. Responses to first-generation inhibitor are limited and not durable²¹. Subsequently there was more interest in developing specific inhibitors, known as second-generation inhibitors, which are highly potent and specific. Quizartinib is one of the most potent and selective FLT3 tyrosine kinase inhibitors, with promising clinical activity in a Phase 2 trial in FLT3-ITD AML patients²¹.

Inhibitors are also classified based on the nature of their binding to the receptor. The FLT3 activation loop undergoes a conformational change that involves the flipping of three specific residues, Asp-Phe-Gly, also called DFG; active and inactive conformations are called DFG-in and DFG-out, respectively. Type I inhibitors bind to the ATP-binding site when the receptor is active, while type II inhibitors interact with a hydrophobic region immediately adjacent to the ATP-binding site that is only accessible when the receptor is in the inactive conformation, and prevent its activation. Type I inhibitors include midostaurin, crenolanib, sunitinib, lestaurtinib and gilteritinib^{22, 23} Type II inhibitors include sorafenib, quizartinib and ponatinib²².

Second-generation inhibitors potently inhibit FLT3, but their clinical activity is transient, mainly related to development of resistance after a short period of time with single-agent inhibition. Thus, combining inhibitors of downstream signalling with FLT3 inhibitors has the potential to improve responses.

PIM (provirus integration site for Moloney murine leukemia virus) proto-oncogenes encode a family of short-lived serine/threonine/tyrosine kinases (Pim-1, Pim-2 and Pim-3)^{24, 25}. There is high homology between the three members of Pim kinase family, as Pim-1 and Pim-2 share 61% amino acid homology, and Pim-1 and Pim-3 have 71% amino acid homology. Pim kinases regulate a number of signaling pathways by phosphorylating several proteins involved in cell cycle regulation, apoptosis, invasion and metastasis, and senescence. Pim kinases are upregulated in several types of cancers, including AML²⁶. Pim kinases promote proliferation and differentiation of hematopoietic cells in response to hematopoietic growth factors²⁵. Pim-1 kinase is upregulated downstream of FLT3-ITD and it phosphorylates and stabilizes FLT3 in a positive feedback loop in cells with FLT3-ITD²⁶⁻²⁹. Therefore, Pim kinases represent a promising target for treatment of AML with FLT3-ITD. Our laboratory also recently demonstrated that Pim kinase inhibition sensitizes AML cells with FLT3-ITD to topoisomerase 2 inhibitor chemotherapy drugs, including daunorubicin, a mainstay of AML chemotherapy³⁰. Several small molecule inhibitors of the Pim kinase family (SGI-1776, AZD1208, SMI-4a, DHPCC-9, SEL24-B58, M-110, GNE-652, PIM447, etc.) have been identified and are at different stages of preclinical research and development testing in clinical trials¹⁴.

Materials and Methods

Cell Lines

Ba/F3-ITD and Ba/F3-WT cells, transfected with FLT3-ITD and FLT3-WT, respectively, were generously provided by Dr. M. Levis, Johns Hopkins University School of Medicine, Baltimore. MV4-11 and MOLM-14 human AML cell lines, with FLT3-ITD, were

obtained from the American Type Culture Collection (ATCC), Gaithersburg, MD. Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY), pH 7.4, with 10% fetal bovine serum (FBS) at 37°C. Cell lines were split every other day.

Reagents

The pan - kinase inhibitor AZD1208 was provided by AstraZeneca (Waltham, MA). AZD1208 was used at 1 μ M concentration³¹. The FLT3 inhibitor Quizartinib was purchased from Selleck Chemicals (Houston, TX). The pan-Pim kinase inhibitor PIM447 (formerly LGH447) was provided by Novartis Pharmaceuticals. The USP9X inhibitor WP1130 was purchased from Sigma Aldrich and the Inhibitors were dissolved in dimethyl sulfoxide (DMSO) as per manufacturers' instructions.

Cytotoxicity assay

Viability of drug-treated cells was measured using the WST-1 cell viability assay from Roche Diagnostics (Indianapolis, IN). Cells were plated in 96-well tissue culture plates with 100 μ l of complete medium with drugs at the desired concentrations at 37°C in 5% CO₂ for 48 hours. 10 μ l of the tetrazolium salt WST-1 was added to cells after 48-hour incubation. The cells were incubated for 2 to 4 additional hours at 37°C. Cellular viability was determined by measuring absorbance at 450 nM using a spectrophotometer. IC₅₀ concentrations (Table-1), or concentrations at which 50% of cells remained viable, were calculated by non-linear curve fitting to dose response curves using GraphPad Prism software (La Jolla, CA).

TABLE-1: IC₅₀

Cell lines	Mutation	Daunorubicin IC ₅₀	Mitoxantrone IC ₅₀	PIM447 IC ₅₀	Quizartinib IC ₅₀
Ba/F3-ITD	FLT3-ITD	7.7nM	32nM	3.8nM	1nM
Ba/F3-WT	FLT3-WT	8.5nM			1 μM
32D-ITD	FLT3-ITD	2.8nM	43.2nM		
MV4-11	FLT3-ITD	38.3nM	36.3nM	541nM	
MOLM-14	FLT3-ITD	7.1nM	220nM		

Table1: IC₅₀ Inhibitory concentration of Daunorubicin, Mitoxandrone, PIM447, Quizartinib in Ba/F3-ITD, 32D-ITD, MV411, MOLM-14.

SDS PAGE and Western blotting

Cells were treated with different drugs and samples were collected at serial time points. Cells were washed with phosphate-buffered saline (PBS) twice and were centrifuged at 1400 rpm for 5 minutes. Cell pellets were lysed using RIPA buffer (Sigma-Aldrich) containing the protease and phosphatase inhibitors sodium orthovanadate (Roche Applied Science, Indianapolis, IN) and sodium fluoride (New England Biolabs). After adding lysis buffer to the samples, pellets were incubated on ice for 30 min and centrifuged at 8000 g for 10 minutes at 4° Celsius. Supernatants were collected and pellets were discarded. Protein was quantified using the bicinchoninic acid (BCA) protein assay kit method from Pierce Thermo Fisher Scientific (Waltham, MA). 10 μg of protein was loaded in each gel. Transfer was performed using a Bio-Rad semi-dry transfer apparatus. A polyvinylidene fluoride (PVDF) membrane was used to transfer

the protein from the gel. After 30 minutes of transfer, the membranes were incubated in 5% BSA for one hour at room temperature and then incubated overnight with primary antibody at 4° Celsius. Membranes were washed three times for 10 minutes using Tris-Buffered Saline with Tween twenty (TBST). Secondary antibody was added for one hour at room temperature. Membranes were then washed again three times for 10 minutes. Membranes were developed using Pierce ECL Western Blotting Substrate. Bands were quantified by densitometry using VisionWorks LS Image Acquisition and Analysis Software (UVP, Upland, CA).

Apoptosis assay by Annexin V staining

Apoptosis was measured by flow cytometric detection of Annexin V-APC and IR dye or Annexin V-FITC and propidium iodide (PI) (Trevigen, Gaithersburg, MD) staining. Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Measurement of reactive oxygen species

Reactive oxygen species (ROS) were measured using the redox-sensitive dye CM-H₂DCFDA. Cells were incubated with CM-H₂DCFDA for 30 minutes prior to treatment with chemotherapy and/or Pim kinase inhibitor or DMSO control. Samples were harvested at 24, 48 and 72 hours and fluorescence was measured on a FACSCanto II and analyzed using FlowJo software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Percentages of apoptotic cells were compared by two-way ANOVA.

Specific Aim 1:

To evaluate the mechanism of synergistic apoptosis induction resulting from combining Pim kinase and FLT3 inhibitors in cells with FLT3-ITD.

Hypothesis:

Concurrent treatment with Pim kinase and FLT3 inhibitors enhances apoptosis of cells with FLT3-ITD by decreasing expression of the anti-apoptotic protein Mcl-1.

INTRODUCTION:

Our laboratory has previously studied the effect of combination treatment with Pim inhibitor (AZD1208) and FLT3 inhibitors (quizartinib, sorafenib and crenolanib) on growth of the Ba/F3-ITD and Ba/F3-WT cell lines and observed that Pim and FLT3 inhibitor co-treatment abrogates growth of cells with FLT3-ITD. Additionally, results from Annexin V PI staining showed enhanced apoptosis in Ba/F3-ITD and 32D-ITD cells following combination treatment. Enhanced apoptosis induced by combination treatment was also seen in MV4-11 and MOLM-14 (human AML cell lines expressing FLT3-ITD) and primary AML cells expressing FLT3-ITD. This effect was specific for cells with

FLT3-ITD, as Ba/F3 cells with WT-FLT3 as well as primary AML cells expressing WT-FLT3 did not show enhanced apoptosis induction by combination treatment.

The following work focuses on the mechanism of enhanced apoptosis induction by concurrent AZD1208 and FLT3 inhibitor treatment in AML cells with FLT3-ITD.

RESULTS

Combination treatment with FLT3 inhibitor (quizartinib) and Pim inhibitor (AZD1208) causes Mcl-1 downregulation

We measured expression of the Bcl2 family including pro-survival proteins (Mcl-1, Bcl2 and Bcl-xL) and pro-apoptotic proteins (BAD/S112 p-BAD, BAK, BAX and Bim) at serial time points at serial time points at serial time points in Ba/F3-ITD cells treated with quizartinib and/or AZD1208. Expression of the pro-survival protein Mcl-1 decreased in a time-dependent manner with quizartinib and AZD1208 co-treatment, as compared to treatment with either drug alone (Figure 1A). There was no change in expression of the other proteins. Densitometric analysis also confirmed downregulation of Mcl-1 expression. We also analyzed expression of Mcl-1 protein in the human AML cell lines MV4-11 and MOLM-14 co-treated with quizartinib and AZD1208 and saw reduced Mcl-1 expression in these cells (Figure 1, B and C).

Figure 1A

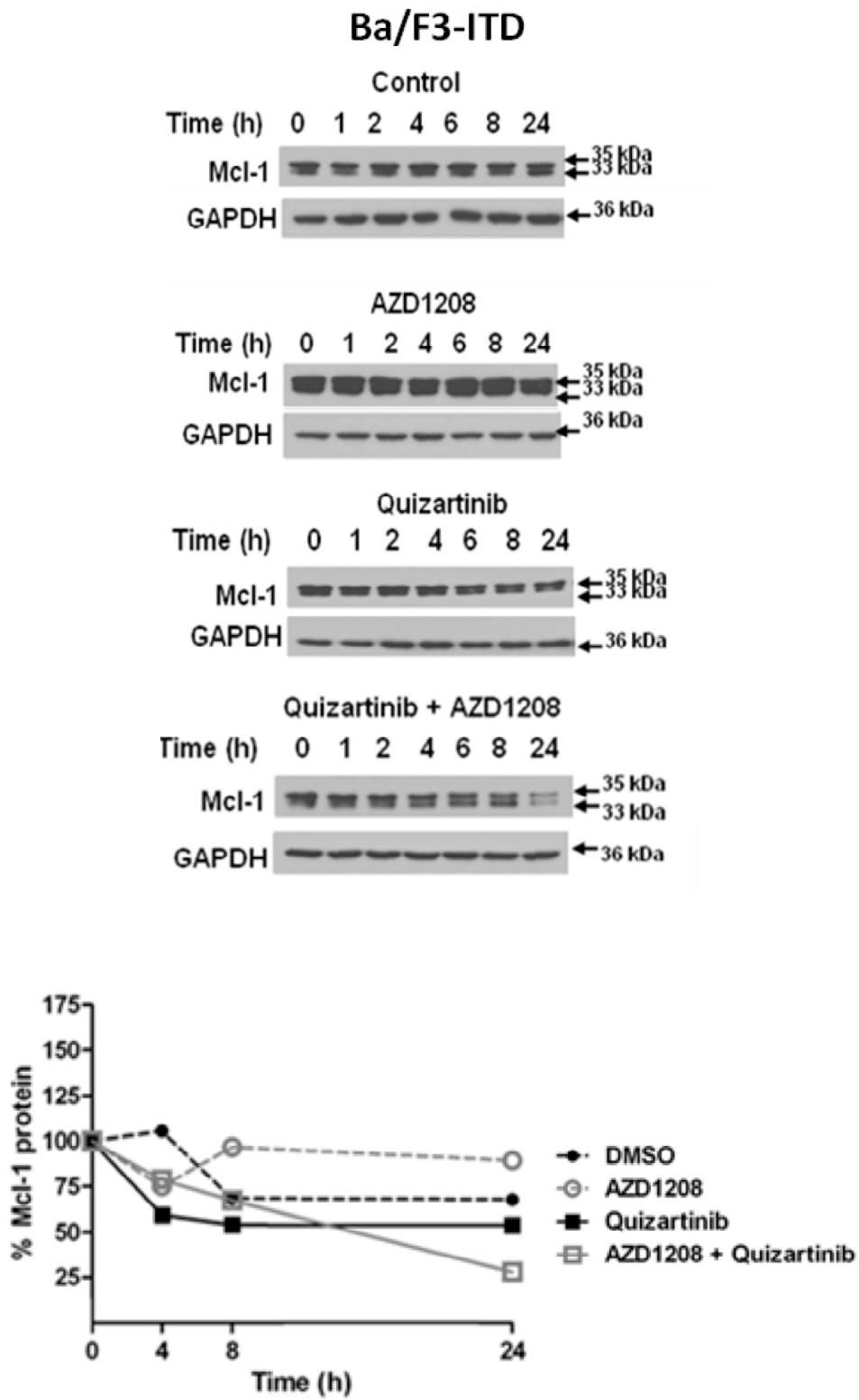


Figure 1 Continued: 1B

MOLM-14

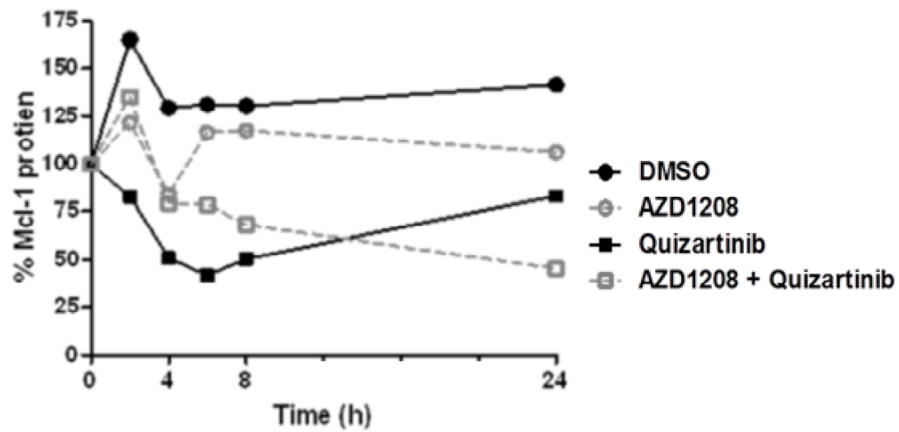
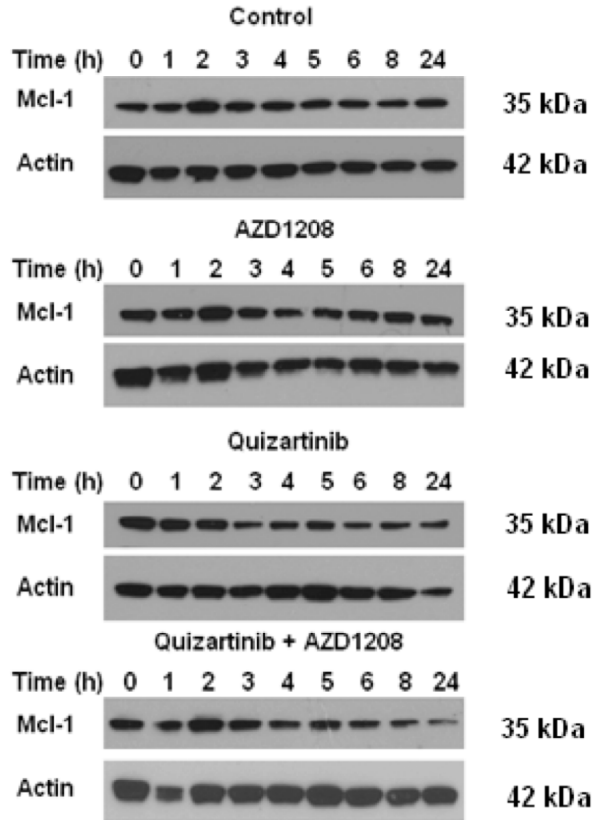


Figure 1 Continued: 1C

MV4-11

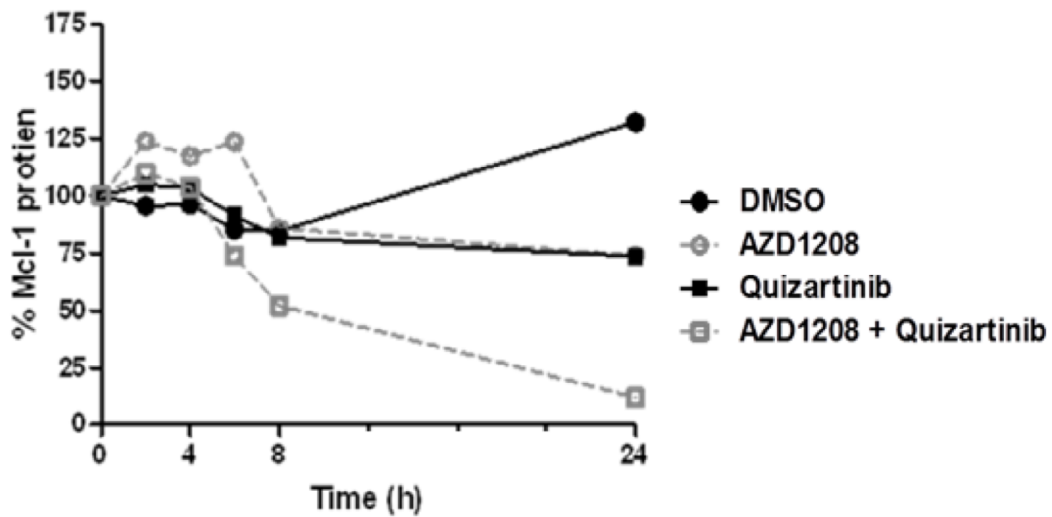
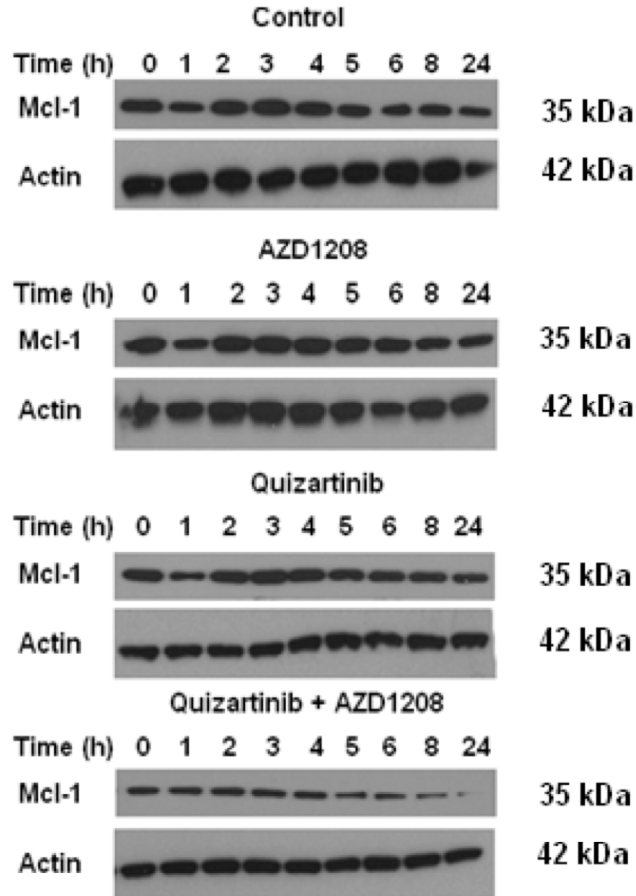


Figure 1. Combination treatment with FLT3 inhibitor (quizartinib) and Pim inhibitor (AZD1208) causes Mcl-1 downregulation in cells with FLT3-ITD. Ba/F3-ITD (A), MOLM-14 (B) and MV4-11 (C) cells were treated with quizartinib and/or AZD1208. Immunoblotting was performed using SDS-PAGE and Mcl-1 primary antibody. Time-dependent decrease in Mcl-1 expression was observed with combination treatment in Ba/F3-ITD, MV4-11 and MOLM-14 cells.

Decreased expression of Mcl-1 with combination treatment is mediated by increased proteasomal degradation of Mcl-1

We next studied the mechanism underlying decreased Mcl-1 expression. Mcl-1 is degraded by the proteasome. Hence, we studied the effect of the proteasome inhibitor MG-132 in altering the expression of Mcl-1 in FLT3-ITD cells treated with FLT3 and Pim inhibitors. As seen in Figure 6, combination treatment with Pim and FLT3 inhibitors reduced Mcl-1 expression, but this decrease is rescued in the presence of MG132. These results suggest that decreased expression of Mcl-1 induced by combination treatment is mediated by increased proteasomal degradation.

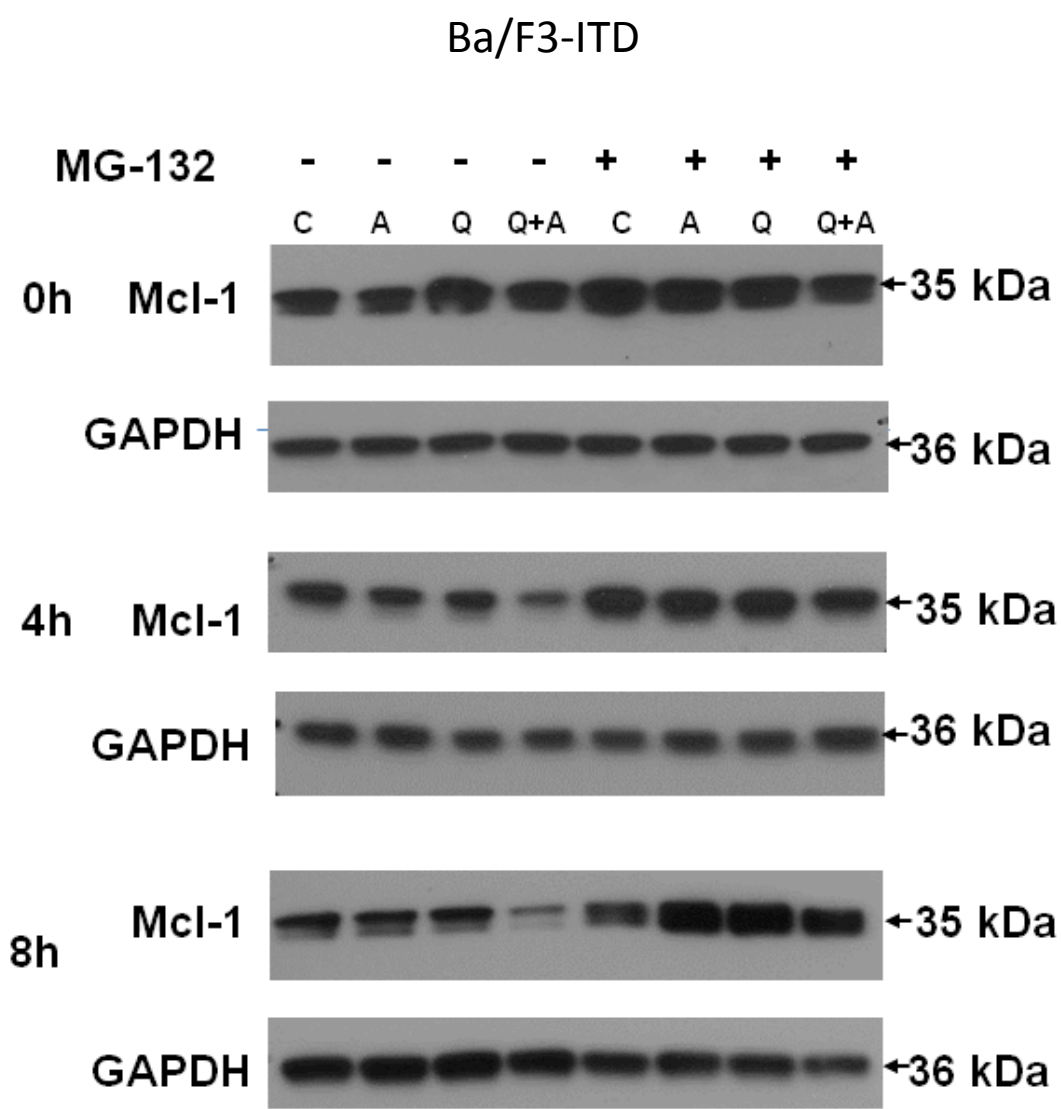


Figure 2. Decreased expression of Mcl-1 with combination treatment is mediated by increased proteasomal degradation of Mcl-1. Cells were treated with DMSO control, or with quizartinib and/or AZD1208 in the absence or presence of MG-132 and cells were harvested at the indicated time points. Mcl-1 protein expression was measured by SDS-PAGE and western blot. MG-132 treatment abrogated the decrease in Mcl-1 expression with quizartinib and AZD1208 combination treatment.

Combination treatment decreases expression of the Mcl-1 deubiquitinase USP9X

Mcl-1 is deubiquitinated by deubiquitinases including USP9X. We hypothesized that expression of Mcl-1 deubiquitinase enzymes may be altered by FLT3 and Pim-1 inhibitor combination treatment. We studied the expression of the deubiquitinase USP9X in cells treated with FLT3 and/or Pim inhibitors. USP9X expression decreased with combination treatment with FLT3 and Pim inhibitors, and this decrease preceded downregulation of Mcl-1 (Figure 3A).

3A

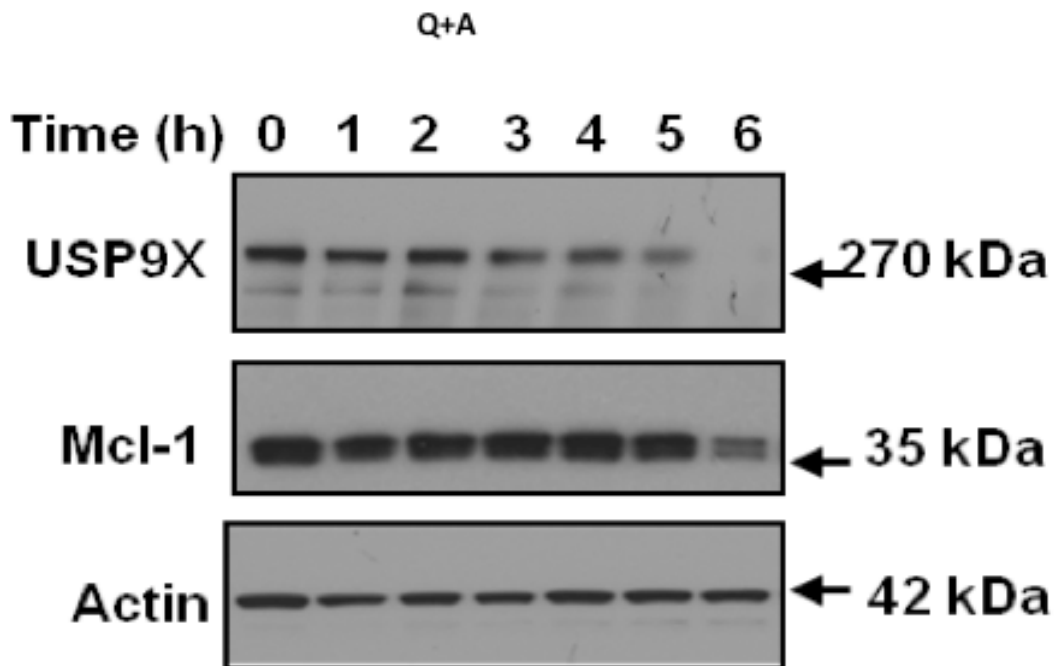
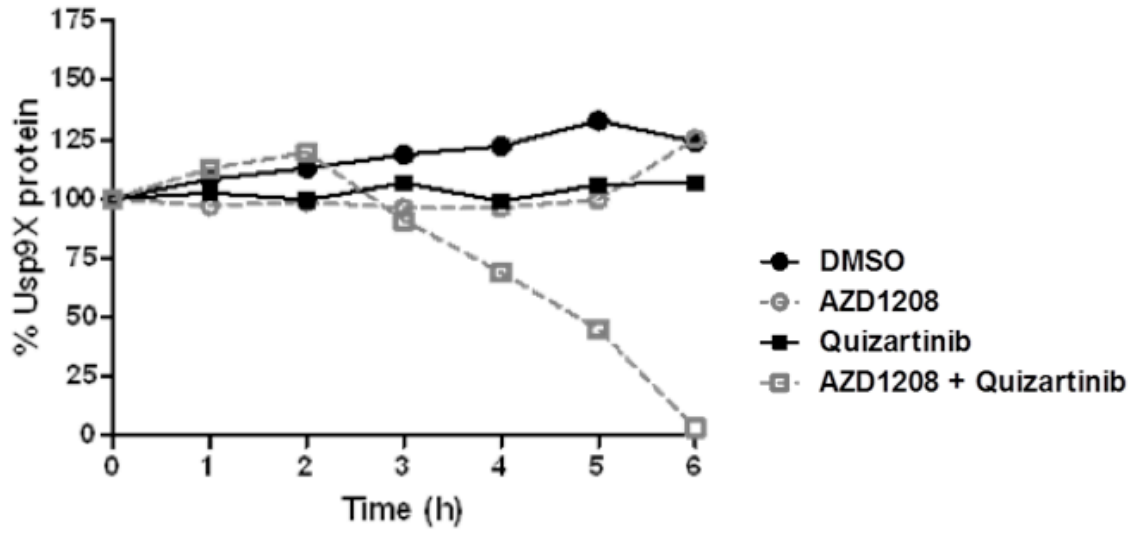


Figure 3 Continued: 3B



3C

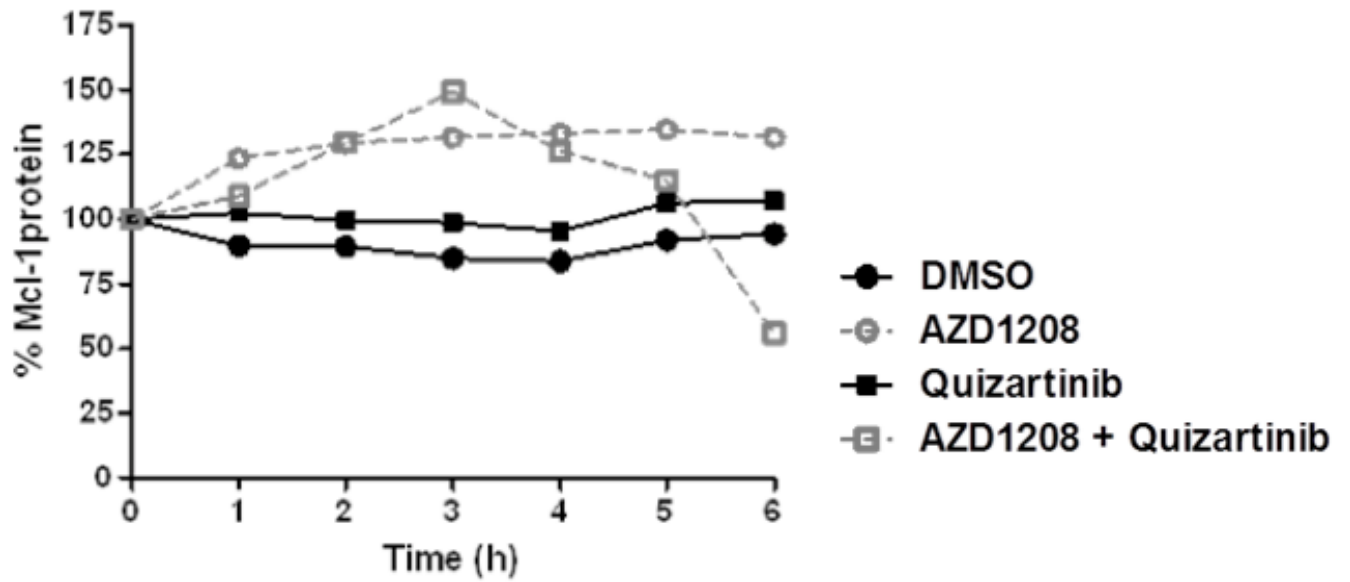


Figure 3. Decreased expression of the deubiquitinase USP9X preceding the decrease in Mcl-1 expression with combination treatment. **A**, Cells were treated with quizartinib and AZD1208 for the indicated time intervals, and expression of USP9X and Mcl-1, and actin as a loading control, was measured by immunoblotting. Changes in expression of USP9X and Mcl-1 were measured by densitometry (Figure 3 B, C).

Inhibition of the deubiquitinase USP9X reduces Mcl-1 expression and induces apoptosis

To confirm the effect of the deubiquitinase USP9X on Mcl-1 expression, we treated cells with FLT3-ITD with the USP9X inhibitor WP1130 and measured Mcl-1 protein expression by immunoblotting. WP1130 at 4uM caused a time-dependent decrease in Mcl-1 expression (Figure 4A). We also observed a concentration-dependent increase in apoptosis induced by treatment with WP1130 (4C).

4A

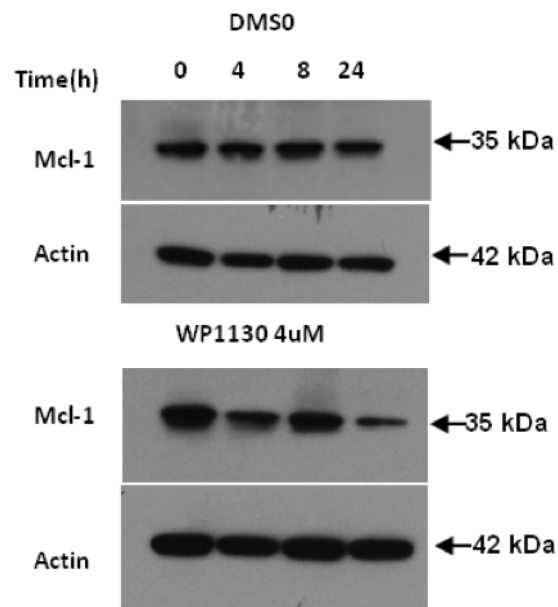
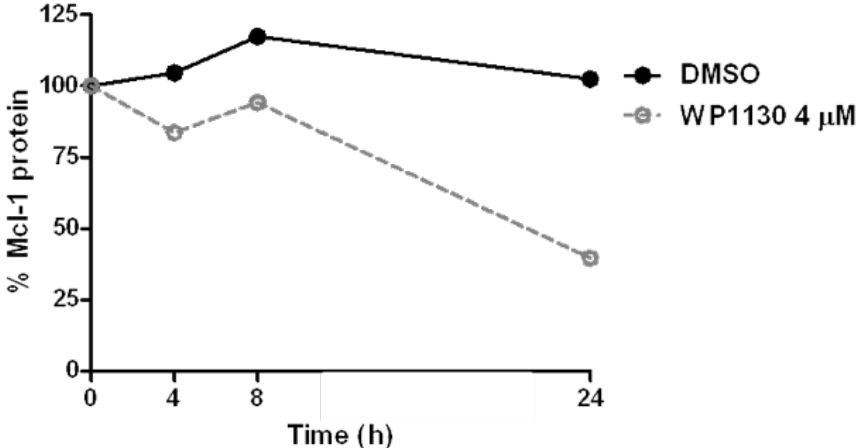


Figure 4 Continued:

4B



4C

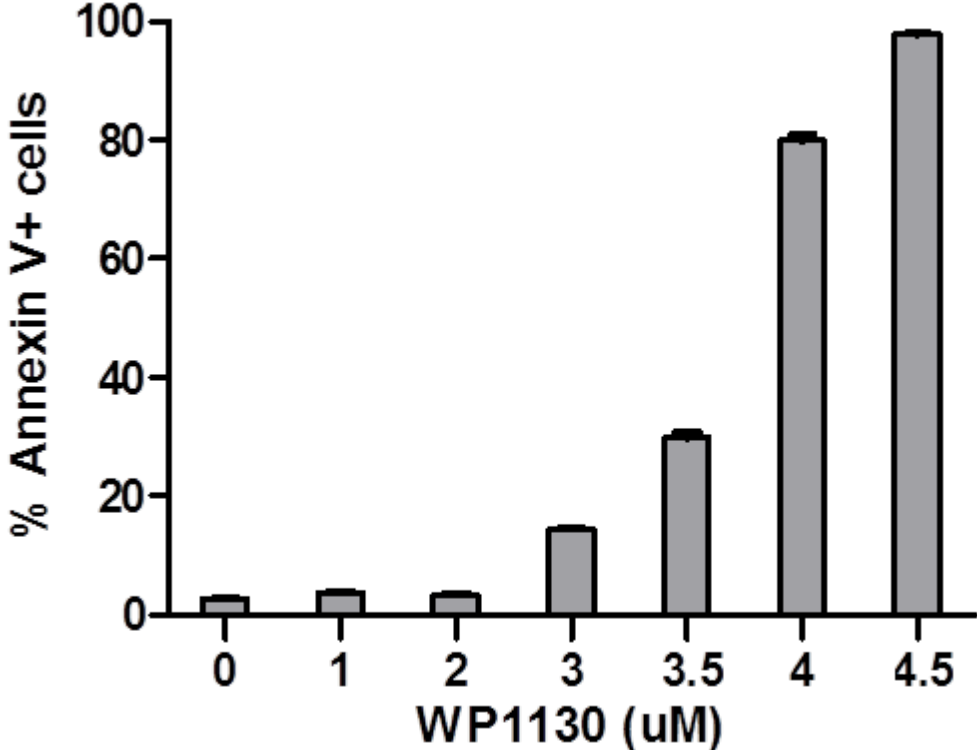


Figure 4. The USP9X inhibitor WP1130 decreases Mcl-1 expression and induces apoptosis in Ba/F3-ITD cells. A, B. Cells were treated with DMSO control or WP1130 and harvested at the indicated time points. Mcl-1 expression was measured by SDS-PAGE and western blot. **C,** Cells were treated with WP1130 at serial concentrations and flow cytometric analysis was performed following Annexin V-FITC and PI staining.

Specific Aim-2

Specific aim 2 was to evaluate the effect of PIM447, a clinically applicable Pim kinase inhibitor, in combination with topoisomerase 2 inhibitors in AML cells expressing FLT3-ITD.

Hypothesis:

The clinically applicable pan-Pim kinase inhibitor PIM447 sensitizes cells with FLT3-ITD to induction of apoptosis by topoisomerase 2 inhibitors.

Introduction:

Patients with AML with FLT3-ITD have poor outcomes with current therapies³². Current treatment of AML patients consists of induction chemotherapy, including a nucleoside analog cytarabine (AraC) and a topoisomerase 2 inhibitors, daunorubicin (DNR), mitoxandrone (MXR) or etoposide (VP-16)¹. We have previously seen that Inhibition of Pim kinase sensitizes cells with FLT3-ITD to apoptosis induction by topoisomerase 2 inhibitor chemotherapy drugs. Enhanced apoptosis with combination treatment with Pim kinase (AZD1208) and topoisomerase 2 (DNR, MXR and VP-16) inhibitors results from increased DNA damage and induction of reactive oxygen species³⁰. AZD1208 is no

longer in clinical trial due to variability in pharmacokinetic properties (personal communication).

PIM447 (formerly LGH447; Novartis Pharmaceuticals), is a pan-Pim kinase inhibitor that is currently being tested in a Phase I clinical trial. In this study, we elucidated the effect of PIM447 on apoptosis induction by topoisomerase 2 inhibitors in cells expressing FLT3-ITD.

RESULTS

Co-treatment with PIM447 induces a concentration-dependent increase in apoptosis by TOP-2 inhibitors in cells with FLT3-ITD

We first studied the effect of treatment with Pim kinase inhibitor and topoisomerase-2 inhibitors in Ba/F3-ITD and 32D-ITD cells, with FLT3-ITD, and Ba/F3-WT, with wild-type FLT3, as a control. The IC₅₀ concentrations of the topoisomerase-2 inhibitors daunorubicin (DNR) and mitoxantrone (MXR) in FLT3-ITD- and FLT3- WT-expressing cell lines were first determined using the WST colorimetric assay. We then performed an apoptosis assay on Ba/F3-ITD cells with daunorubicin 10 nM and different concentration of PIM447 (10nM, 100nM, 500nM). Flow cytometric analysis was performed using Annexin V-APC and PI staining in cells treated with DNR and Annexin V-FITC and IR staining in cells treated with MXR, to avoid spectral overlap. We observed a concentration-dependent increase in apoptosis with combination treatment, compared to single-agent treatment (Figure 1 A, B). We performed the same apoptosis assay on BA/F3-WT cells and did not observe any change in apoptosis with combination treatment, compared to single-agent treatment (Figure 1C). Thus, we

conclude that enhanced topoisomerase 2-induced apoptosis in the presence of PIM447 is specific for cells with FLT3-ITD.

5A

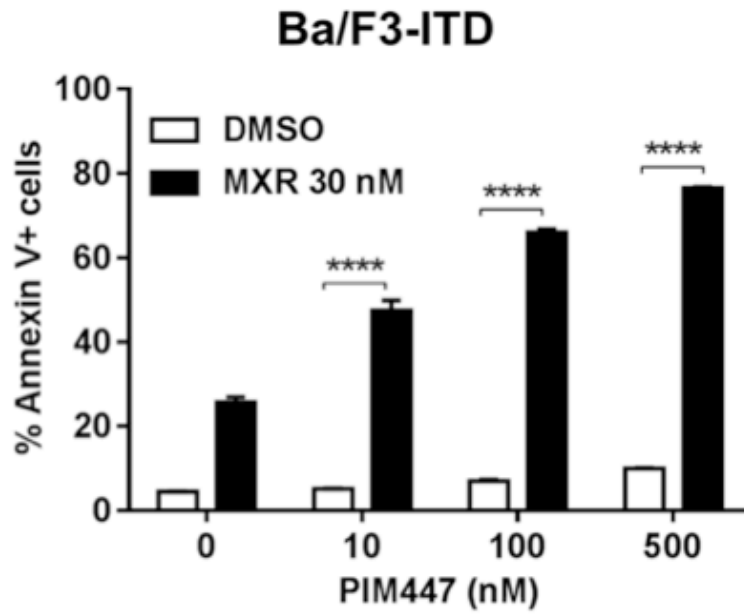
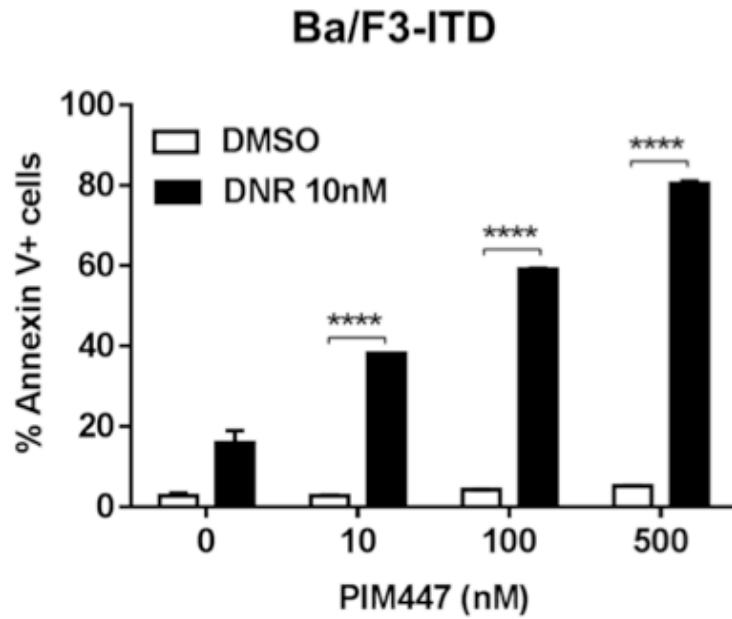


Figure 5 Continued: 5B

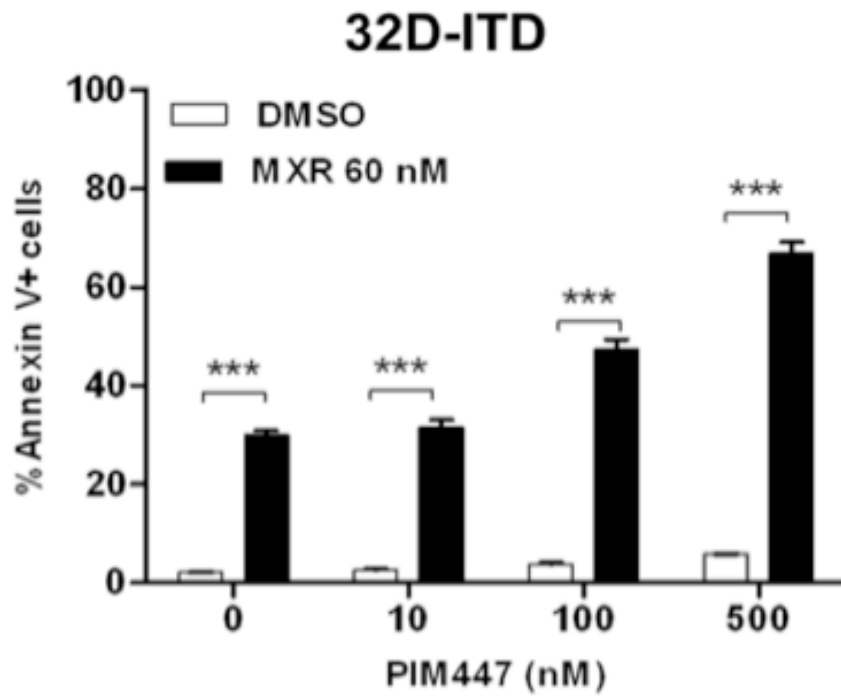
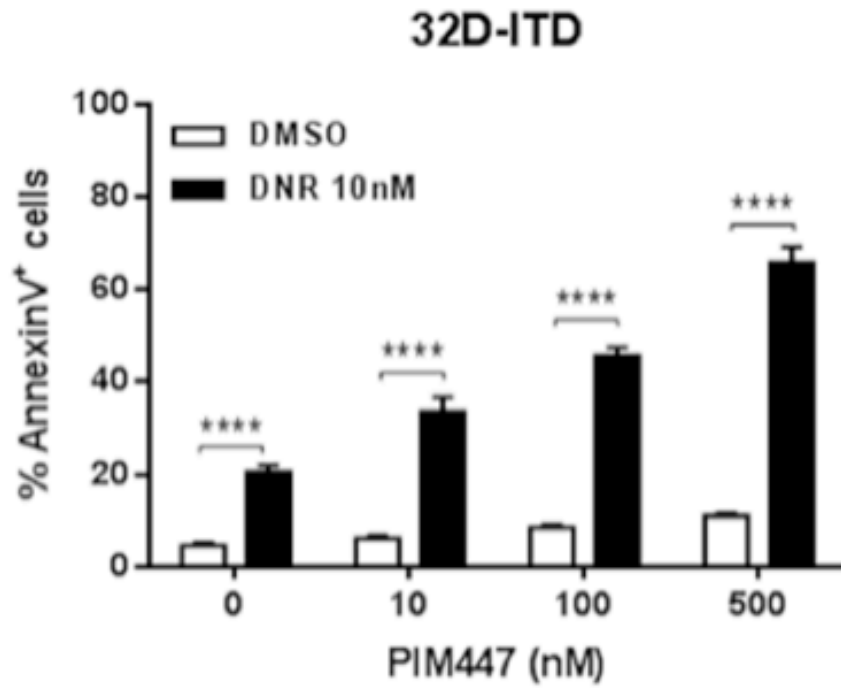


Figure 5 Continued: 5C

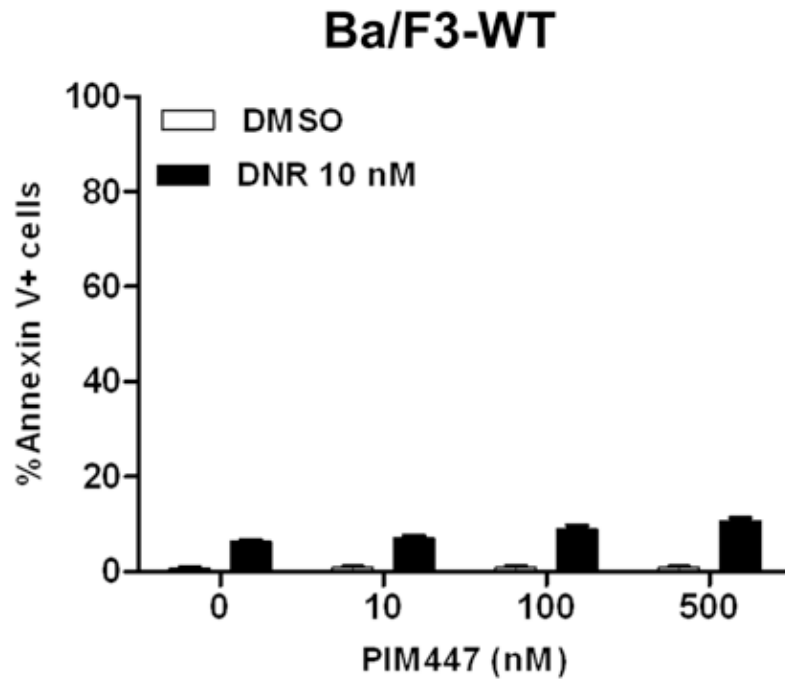


Figure 5. Co-treatment with PIM447 induces a concentration-dependent increase in apoptosis induction by topoisomerase 2 inhibitors in cells with FLT3-ITD. A, B. PIM447 and topoisomerase 2 inhibitor co-treatment increases Annexin V labelling in Ba/F3-ITD and 32D-ITD cells. Cells were cultured with PIM447 and DNR or MXR, and apoptosis was measured at 48 hours by flow cytometric analysis of Annexin V-APC and IR dye labelling in cells treated with DNR and Annexin V-FITC and PI labelling cells treated with MXR and PIM447. **C.** Enhanced apoptosis was not seen in Ba/F3-WT cells treated with DNR and PIM447.

PIM447 enhances DNA double-strand breaks induced by topoisomerase 2

Topoisomerase 2 inhibitors are known to induce DNA damage. Hence, we studied DNA damage following our combination treatment. γ -H2ax is a marker for DNA double-strand breaks. DNA double-strand breaks increased with in a time-dependent manner with daunorubicin (DNR) and PIM447 combination treatment, as compared to single-agent treatment (Figure 6).

6

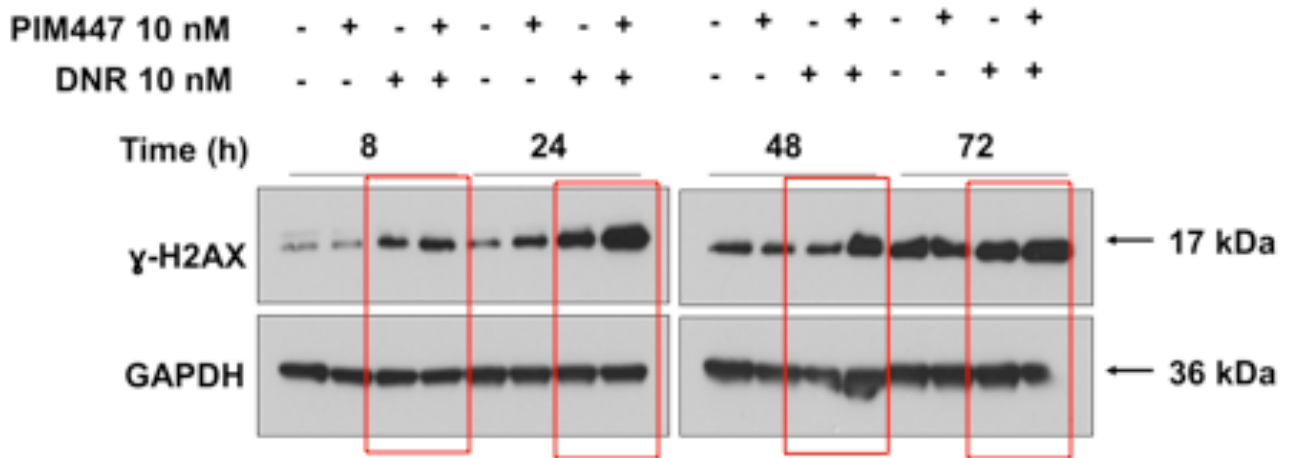


Figure 6. PIM447 enhances DNA double-strand break induction by topoisomerase 2 inhibitor. Ba/F3-ITD cells were treated with PIM 447 and/or DNR for the indicated time intervals. Expression of γ -H2AX, a marker for DNA double-strand breaks, was measured by immunoblotting using primary antibodies against γ -H2AX, and levels of GAPDH were determined as a loading control.

Combination treatment with PIM447 and topoisomerase 2 inhibitor results in increased oxidative stress

DNA damage causes an increase in oxidative stress, which in turn enhances DNA damage. Hence, we looked at reactive oxygen species (ROS) production in cells treated with PIM447 and the topoisomerase 2 inhibitor DNR. As compared to treatment with DNR alone, combination treatment with PIM447 causes increased ROS production (Figure 7A). To study the mechanistic role of ROS generation in enhanced apoptosis, we used a ROS scavenger, N-acetyl cysteine (NAC). Cells treated with PIM447 and DNR showed a time-dependent increase in apoptosis from 24 hours to 72 hours in the absence of NAC (Figure 11A), but this increase did not occur in the presence of NAC (Figure 7B). Thus we conclude that enhanced apoptosis with PIM447 and topoisomerase 2 inhibitor combination treatment is mediated by increased ROS generation.

7A

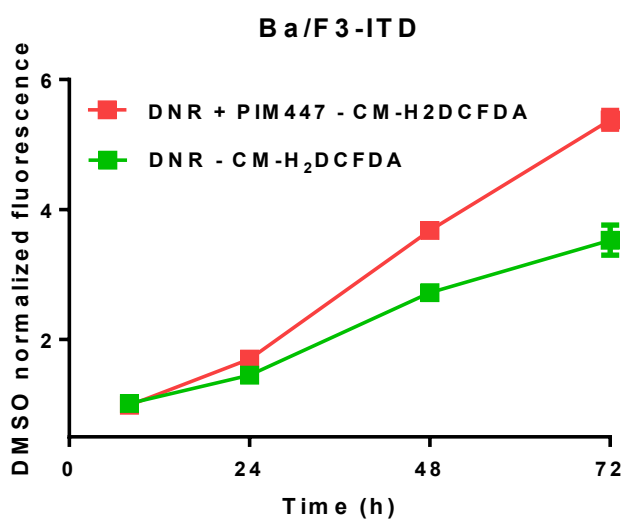


Figure 7 Continued:7B

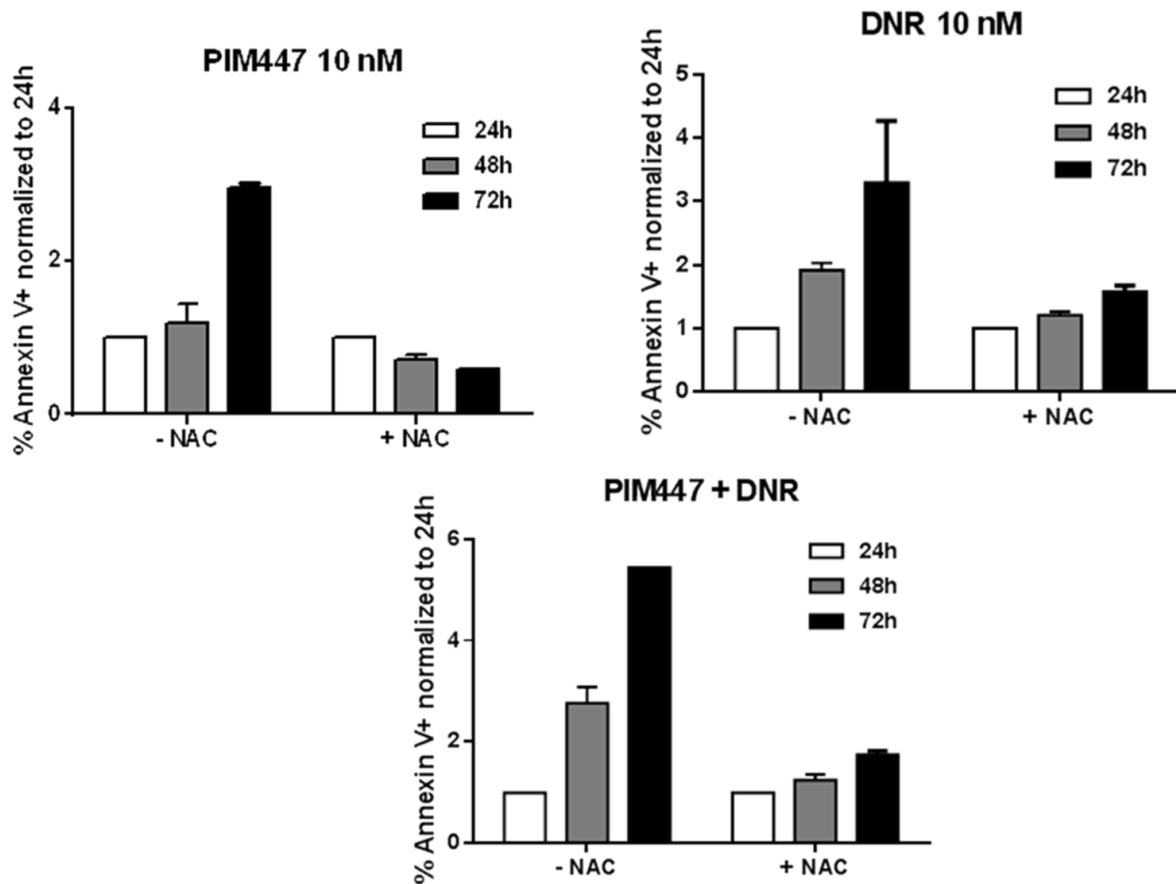


Figure 7 A. Co-treatment with Pim kinase inhibitor and topoisomerase 2 inhibitor

increases ROS production. Ba/F3-ITD cells were incubated with CM-H2DCFDA to measure intracellular ROS levels. Cells were treated with DNR and/or PIM447.

Fluorescence normalized to DMSO was plotted at different time points. **B Enhanced**

apoptosis with PIM447 and topoisomerase 2 inhibitor combination treatment is

caused by increased oxidative stress. The ROS scavenger NAC reduces apoptosis

induction by PIM447 and topoisomerase 2 inhibitor co-treatment. Ba/F3-ITD cells were

treated with PIM447 and DNR in the presence and absence of NAC (N-acetyl cysteine).

Apoptosis was measured by flow cytometric analysis of Annexin V-APC and IR dye

labelling at 24, 48 and 72 hours. Fold apoptosis at serial time points was plotted relative to apoptosis at 24 hours, defined as 1. Means \pm S.E.M are shown.

DISCUSSION

Patients with AML with FLT3-ITD generally achieve remission with chemotherapy, but have high rates of relapse and short disease free survival. The FLT3-ITD mutation occurs in approximately 30% of AML patients. A number of FLT3 inhibitors have been tested in clinical trials in AML with FLT3-ITD, but their activity has been limited and transient³². There is still a need to develop new FLT3 inhibitors with increased efficacy in AML with FLT3-ITD². Additionally, inhibitors targeting signalling downstream of FLT3-ITD may also have a role in treating AML with FLT3-ITD, alone or combined with FLT3 inhibitors.

The serine/threonine kinase Pim-1 is transcriptionally upregulated downstream of FLT3-ITD³⁷. Additionally, Pim-1 phosphorylates FLT3 and enhances its signalling² in a positive feedback loop in cells with FLT3-ITD^{16, 28}. Our laboratory has previously shown that Pim kinase inhibition using AZD1208 enhances apoptosis induction by FLT3 inhibitors (quizartinib and sorafenib) in cell lines and AML patient cells expressing FLT3-ITD. This effect was selective for cells expressing FLT3-ITD, as enhanced apoptosis was not seen with combination treatment in cells with FLT3-WT receptor.

Here we found that enhanced apoptosis of cells with FLT3-ITD with Pim kinase inhibitor and FLT3 inhibitor combination treatment is associated with downregulation of Mcl-1. Mcl-1 is a pro-survival protein belonging to the Bcl2 family of proteins. Decreased Mcl-1

protein expression was found to be due to its increased proteasomal degradation. We further studied expression of Mcl-1 deubiquitinase USP9X in Ba/F3-ITD cells treated with AZD1208 and quizartinib, alone or in combination. We observed decreased expression of the deubiquitinase USP9X in a time-dependent manner with combination treatment, preceding the decrease in Mcl-1 expression. To confirm the effect of USP9X on Mcl-1 expression, we also used the USP9X-specific inhibitor WP1130 and observed time-dependent decrease in Mcl-1 expression, as well as induction of apoptosis. This strongly suggests that downregulation of USP9X is the mechanism for the enhanced Mcl-1 degradation that promotes apoptosis.

First-line treatment of AML consists of chemotherapy, including topoisomerase 2 inhibitors¹. Our laboratory has previously shown that Pim kinase inhibition using AZD1208 enhances apoptosis induction by topoisomerase 2 inhibitors in cell lines and primary AML samples with FLT3-ITD³⁰. AZD1208 was previously in Phase I clinical trials and had clinical activity at well-tolerated doses³³ but its clinical development was discontinued due to variability in its pharmacokinetic properties (personal communication). The pan-Pim kinase inhibitors PIM447³⁴ and INCB53914³⁵ are currently being tested in clinical trials, with favourable pharmacokinetics to date. We wished to demonstrate sensitization of FLT3-ITD cells to topoisomerase 2 inhibitors by a clinically applicable Pim kinase inhibitor, in support of future clinical trials. Here we studied the effect of the Pim kinase inhibitor PIM447 on apoptosis induction by the topoisomerase 2 inhibitors daunorubicin (DNR) and mitoxantrone (MXR) in cells expressing FLT3-ITD.

As with AZD1208, we observed that topoisomerase 2 inhibitor-induced apoptosis was enhanced by combination treatment with PIM447. Topoisomerase 2 inhibitors are known to induce DNA damage³⁶. Mechanistically, we observed enhanced DNA double-strand breaks in a time-dependent manner within 8 hours of DNR and PIM447 co-treatment, as compared to DNR treatment alone. DNA damage is known to induce increased oxidative stress³⁷. We observed increased induction of reactive oxygen species (ROS) following induction of DNA DSBs in cells treated with both PIM447 and DNR. To study the mechanistic role of ROS induction in combination treatment-induced enhanced apoptosis, we used a ROS scavenger, NAC. We observed decreased apoptosis with combination treatment in the presence of NAC. This suggests that oxidative stress is mechanistically involved in the enhanced apoptosis induced by PIM447 and DNR in cells with FLT3-ITD.

Our data show that concurrent treatment with the Pim kinase inhibitor AZD1208 enhances apoptosis induction by FLT3 inhibitors in cells with FLT3-ITD occurs via increased Mcl-1 degradation mediated by USP9X inhibition. We have also seen that PIM447, a clinically applicable Pim kinase inhibitor, works similarly to AZD1208 to enhance apoptosis induction by topoisomerase 2 inhibitor chemotherapy drugs in cells expressing FLT3-ITD. Overall our data support clinical testing of Pim kinase inhibitors with FLT3 inhibitors and with topoisomerase 2 inhibitors in order to achieve better therapeutic outcomes.

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