

# Concurrent Inhibition of Pim and FLT3 Kinases Enhances Apoptosis of FLT3-ITD Acute Myeloid Leukemia Cells through Increased Mcl-1 Proteasomal Degradation



Shivani Kapoor<sup>1</sup>, Karthika Natarajan<sup>1,2</sup>, Patrick R. Baldwin<sup>1,2</sup>, Kshama A. Doshi<sup>1</sup>, Rena G. Lapidus<sup>1,2</sup>, Trevor J. Mathias<sup>1</sup>, Mario Scarpa<sup>1</sup>, Rossana Trotta<sup>1,3</sup>, Eduardo Davila<sup>1,3,4</sup>, Manfred Kraus<sup>5</sup>, Dennis Huszar<sup>5</sup>, Adriana E. Tron<sup>5</sup>, Danilo Perrotti<sup>1,2</sup>, and Maria R. Baer<sup>1,2,4</sup>

## Abstract

**Purpose:** *fms*-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) is present in 30% of acute myeloid leukemia (AML), and these patients have short disease-free survival. FLT3 inhibitors have limited and transient clinical activity, and concurrent treatment with inhibitors of parallel or downstream signaling may improve responses. The oncogenic serine/threonine kinase Pim-1 is upregulated downstream of FLT3-ITD and also promotes its signaling in a positive feedback loop, suggesting benefit of combined Pim and FLT3 inhibition.

**Experimental Design:** Combinations of clinically active Pim and FLT3 inhibitors were studied *in vitro* and *in vivo*.

**Results:** Concurrent treatment with the pan-Pim inhibitor AZD1208 and FLT3 inhibitors at clinically applicable concentrations abrogated *in vitro* growth of FLT3-ITD, but not wild-type FLT3 (FLT3-WT), cell lines. AZD1208 cotreatment increased FLT3 inhibitor-induced apoptosis of FLT3-ITD, but not FLT3-WT, cells

measured by sub-G<sub>1</sub> fraction, annexin V labeling, mitochondrial membrane potential, and PARP and caspase-3 cleavage. Concurrent treatment with AZD1208 and the FLT3 inhibitor quizartinib decreased growth of MV4-11 cells, with FLT3-ITD, in mouse xenografts, and prolonged survival, enhanced apoptosis of FLT3-ITD primary AML blasts, but not FLT3-WT blasts or remission marrow cells, and decreased FLT3-ITD AML blast colony formation. Mechanistically, AZD1208 and quizartinib cotreatment decreased expression of the antiapoptotic protein Mcl-1. Decrease in Mcl-1 protein expression was abrogated by treatment with the proteasome inhibitor MG132, and was preceded by downregulation of the Mcl-1 deubiquitinase USP9X, a novel mechanism of Mcl-1 regulation in AML.

**Conclusions:** The data support clinical testing of Pim and FLT3 inhibitor combination therapy for FLT3-ITD AML. *Clin Cancer Res*; 24(1); 234–47. ©2017 AACR.

## Introduction

Internal tandem duplication (ITD) of *fms*-like tyrosine kinase 3 (FLT3) is present in acute myeloid leukemia (AML) cells of 30% of patients (1), and these patients have short disease-free survival after chemotherapy (1) and also after

allogeneic hematopoietic stem cell transplantation (2). Relapse results at least in part from constitutive growth signaling by FLT3-ITD (3), but FLT3 inhibitors have demonstrated only limited and transient clinical activity (4).

FLT3-ITD activates signal transducer and activation of transcription (STAT) 5 (5), which transcriptionally upregulates the oncogenic serine threonine kinase Pim-1 (6). Pim-1 contributes directly to the proliferative and antiapoptotic effects of FLT3-ITD (6), and also phosphorylates and stabilizes FLT3, promoting STAT5 signaling in a positive feedback loop in cells with FLT3-ITD (7, 8). Pim-1 (7–9) and the Pim kinase isoform Pim-2 (10) have been proposed as therapeutic targets in FLT3-ITD AML. Notably, upregulation of Pim-1 has been shown to be a mechanism of resistance to FLT3 inhibitors (7). Pan-Pim kinase inhibitors have entered clinical trials (11, 12).

Here we demonstrate that concurrent treatment with clinically active pan-Pim and FLT3 inhibitors at pharmacologically relevant concentrations enhances induction of apoptosis in cells with FLT3-ITD, but not wild-type FLT3 (FLT3-WT), *in vitro* and has efficacy *in vivo*. Mechanistically, concurrent Pim and FLT3 inhibitor treatment increases proteasomal degradation of the antiapoptotic protein Mcl-1, a novel mechanism. The data support clinical testing of Pim and FLT3 inhibitor combination therapy in patients with FLT3-ITD AML.

<sup>1</sup>University of Maryland Greenebaum Comprehensive Cancer Center, Baltimore, Maryland. <sup>2</sup>Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland. <sup>3</sup>Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland. <sup>4</sup>Veterans Affairs Medical Center, Baltimore, Maryland. <sup>5</sup>Oncology iMED, AstraZeneca, Waltham, Massachusetts.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

S. Kapoor and K. Natarajan are the co-first authors of this article.

Current address for M. Kraus: Pfizer Oncology Research Unit, San Diego, California; and current address for D. Huszar: Oncology Drug Discovery Unit, Takeda Pharmaceuticals International Co, Cambridge, Massachusetts.

**Corresponding Author:** Maria R. Baer, University of Maryland Greenebaum Comprehensive Cancer Center, 22 South Greene Street, Baltimore, MD 21201. Phone: 410-328-8708; Fax: 410-328-6896; E-mail: mbaer@umm.edu

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### Translational Relevance

Internal tandem duplication of the *fms*-like tyrosine kinase receptor 3 receptor tyrosine kinase (FLT3-ITD) is present in acute myeloid leukemia (AML) cells of 30% of patients, and these patients have short disease-free survival following therapy. The oncogenic serine/threonine kinase Pim-1, upregulated downstream of FLT3-ITD, contributes directly to the proliferative and antiapoptotic effects of FLT3-ITD and also phosphorylates and stabilizes FLT3 and thereby promotes its signaling in a positive feedback loop in cells with FLT3-ITD, suggesting benefit of combined Pim and FLT3 inhibition. We demonstrate that Pim inhibition enhances FLT3-ITD AML cell apoptosis induction by clinically applicable FLT3 inhibitors and that combination therapy has efficacy in *in vivo* models of FLT3-ITD AML. Mechanistically, Pim and FLT3 inhibitor cotreatment increases proteasome-dependent degradation of the antiapoptotic protein Mcl-1, a novel mechanism of Mcl-1 regulation in AML cells. The data support clinical testing of Pim and FLT3 inhibitor combination therapy for FLT3-ITD AML.

## Materials and Methods

### Cell lines

Ba/F3-ITD, Ba/F3-WT, 32D/ITD, 32D/WT, MV4-11, and MOLM-14 cells were obtained and cultured as described previously (13). KG-1a human leukemia cells (14), with FLT3-WT, were obtained from the ATCC. MV4-11-luc cells (15) used in the orthotopic *in vivo* model, gift from Dr. Sharyn Baker, the Ohio State University, formerly St. Jude Children's Research Hospital, were grown in RPMI1640 with 10% FBS and 1% glutamine.

### Lentiviral and retroviral infection of Ba/F3-ITD cells

Ba/F3-ITD cells were infected using a pMX-puro retroviral vector encoding FLAG-K67M kinase-dead (KD) Pim-1 (13). They were also transduced with Mcl-1-specific and scrambled shRNA lentiviral particles (Sigma-Aldrich) and selected with puromycin (8). Mcl-1 knockdown was confirmed by immunoblotting. Finally, Mcl-1 cDNA (FLAG-tagged) in a pMSCV-puro-Flag-mMcl-1 vector and pMSCV-puro control vector (Addgene) were transfected into Phoenix-AMPHO cells. Ba/F3-ITD cells were transduced with the lentiviral particles collected after 48-hour culture, and transduced cells were selected with puromycin (Sigma-Aldrich). FLAG expression and Mcl-1 overexpression were confirmed by immunoblotting.

### AML patient samples

Pretreatment AML bone marrow and blood and remission bone marrow samples were obtained on a University of Maryland Baltimore Institutional Review Board-approved protocol. Written informed consent was obtained. The studies were conducted in accordance with the Declaration of Helsinki. Mononuclear cells isolated by density centrifugation over Ficoll-Paque (Sigma-Aldrich) were studied without prior cryopreservation. FLT3-ITD and FLT3-WT AML cells from 3 patients each and remission bone marrow cells from 3 patients were cultured in RPMI 1640 with 10% FBS, without cytokine supplementation.

### Reagents

AZD1208, an orally bioavailable highly selective inhibitor with single nanomolar potency against all three Pim kinases, Pim-1, Pim-2, and Pim-3 (14), provided by AstraZeneca, was used at 1  $\mu$ mol/L based on inhibition of BAD phosphorylation at serine 112 as a pharmacodynamic endpoint (16) and on phase I clinical trial data (11). The FLT3 inhibitors quizartinib and crenolanib (Selleck Chemicals), sorafenib (LC Laboratories), and gilteritinib (Active Biochem), all clinically active in FLT3-ITD AML, were used at pharmacologically relevant concentrations (17–20). The proteasome inhibitor MG132 and the USP9X inhibitor WP1130 were purchased from EMD Millipore.

### Cytotoxicity assay

Cytotoxicity was measured using the WST-1 assay (13). IC<sub>50</sub> values were determined by nonlinear curve fitting to a dose-response curve using Prism V software (GraphPad).

### Cell proliferation assay

Cultured cells were collected at serial time points and live cells were counted after Trypan blue dye exclusion (13).

### Cell-cycle analysis

Percentages of cells in sub-G<sub>1</sub> and in different phases of the cell cycle were measured using FlowJo software (Tree Star; ref. 13).

### Measurement of apoptosis by annexin V-PI staining

Cells were stained with annexin V-FITC and propidium iodide (PI; Trevigen), acquired on a FACSCanto II (BD Biosciences) and analyzed using FlowJo (13). Percent total annexin V<sup>+</sup>/PI<sup>-</sup> and annexin V<sup>+</sup>/PI<sup>+</sup> cells was compared by two-way ANOVA with *post hoc* Bonferroni testing.

### Measurement of mitochondrial membrane potential

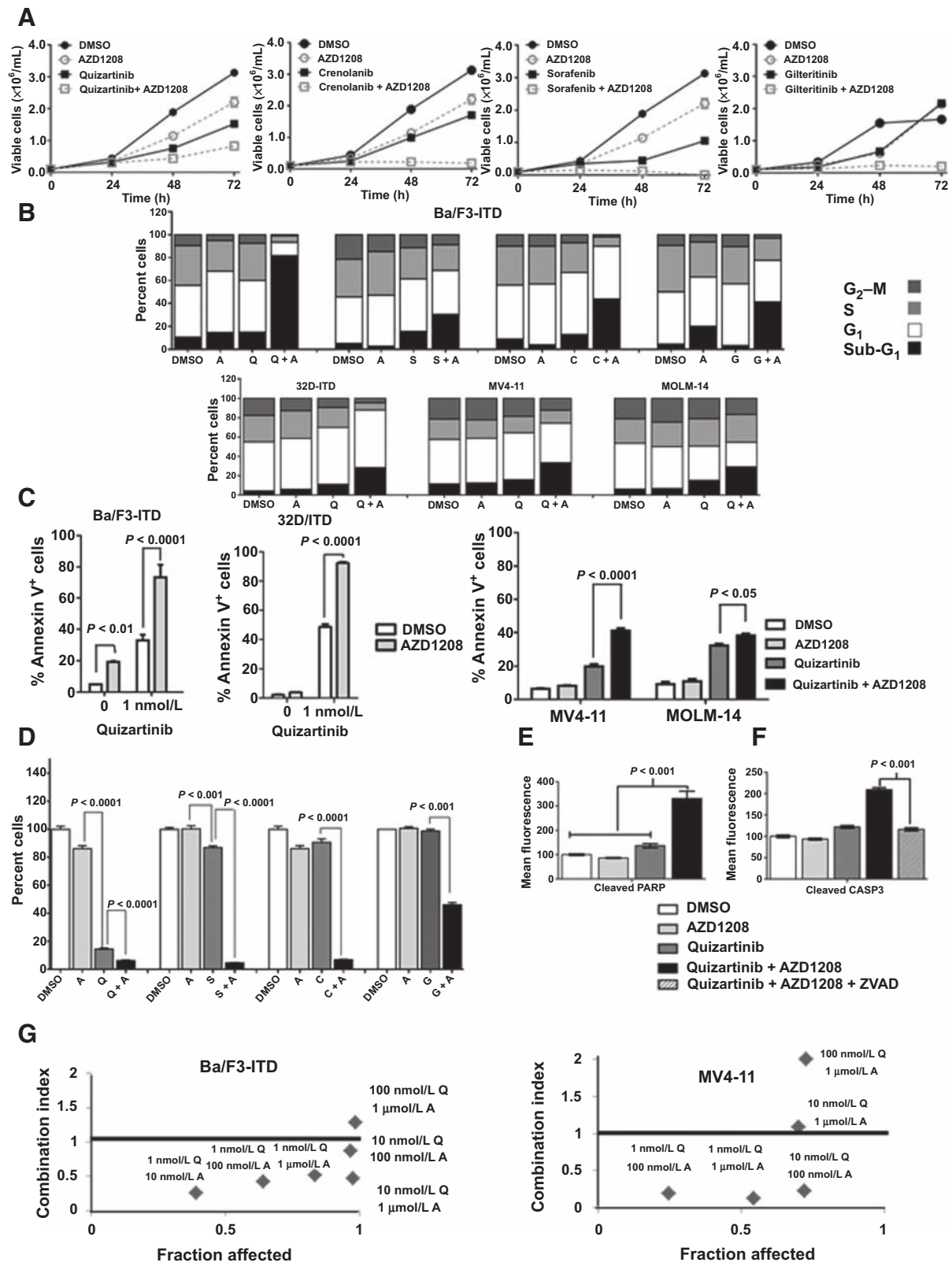
Mitochondrial membrane potential (MMP) was measured using the MitoProbe JC-1 Assay Kit (Life Technologies; ref. 13). Median red fluorescence was measured on a FACSCanto II, analyzed using FlowJo and compared by two-way ANOVA.

### Cleaved PARP and caspase-3 by flow cytometry

Cells treated with drugs with and without the pan-caspase inhibitor Z-VAD-FMK (Enzo) were washed with ice-cold PBS, resuspended, and fixed in 100  $\mu$ L 4% paraformaldehyde at 4°C for 20 minutes, then washed in 2% FBS in PBS, resuspended in 10% DMSO in FBS, and cryopreserved at -80°C. Cells were thawed at 37°C, washed with cold PBS, resuspended, and incubated in BD Perm/Wash buffer at room temperature for 15 minutes. They were then pelleted, resuspended in 100  $\mu$ L BD Perm/Wash buffer containing 10  $\mu$ L Alexa Fluor 647-labeled anti-Cleaved PARP (Asp 214) antibody (BD Biosciences) and 20  $\mu$ L FITC-labeled anti-active caspase-3 antibody (BD Biosciences), incubated at room temperature for 30 minutes, then washed, resuspended in BD Perm/Wash buffer and acquired on a FACSCanto II. Mean fluorescence was compared by one-way ANOVA.

### Determination of synergy

Cells plated in triplicate on 96-well plates were treated with drugs at various concentrations alone and in combinations. Assays were terminated after 48 hours and combination indexes were determined according to the Chou-Talalay method using CompuSyn software (21).



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### Immunoblotting

Cells were lysed in buffer with protease and phosphatase inhibitors (Roche Applied Science). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific), and 30 µg from each sample was electrophoresed (8). Immunoblots were incubated with polyclonal antibodies to Pim-1, PARP, Mcl-1, Bim, phospho-BAD<sup>Ser112</sup>, BAD, Bax, Bak, Bcl-2, Bcl-xL, USP9X, phospho-STAT5<sup>Tyr694</sup> (Cell Signaling Technology), USP24 (Abcam), vinculin and Trim17 (Sigma-Aldrich), SCFβ-TRCP and ARF-BP1 (Abcam) and mouse mAbs to β-actin (Santa Cruz Biotechnology), GAPDH (EMD Millipore), and FLAG (Sigma-Aldrich) overnight at 4°C, then with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Band intensities at serial time points, measured by densitometry (VisionWorks LS, UVP), were compared with pretreatment intensity, defined as 100%. Measurements of Mcl-1 and USP9X expression in cells treated with inhibitors and combinations were repeated in at least four separate replicate experiments.

### In vivo studies

**Subcutaneous model.** Female CB17 SCID mice (Charles River) were maintained under specific pathogen-free conditions and used in compliance with protocols approved by the AstraZeneca Institutional Animal Care and Use Committee and conforming to institutional and national regulatory standards on experimental animal usage. MV4-11 ( $10 \times 10^6$ ) or KG-1a ( $5 \times 10^6$ ) cells were implanted with Matrigel subcutaneously into the right flanks of the mice. Tumor length and width were measured twice weekly with calipers. When tumor volume, calculated as  $(\text{length} \times \text{width}^2) \times 0.5$  (22), reached 150–200 mm<sup>3</sup>, groups of 9 mice were randomly assigned to daily oral gavage with vehicle (22% Captisol), AZD1208 (in 0.5% HPMC/0.1% Tween80), quizartinib (in 22% Captisol) or both, dosed 20 minutes apart. Log-transformed tumor volume fold change from treatment start was compared using a two-tailed Student *t* test, paired.

**Orthotopic model.** The orthotopic model was described previously (23). The University of Maryland IACUC approved the study. Mice sorted into 4 treatment groups with equal mean signal intensity, 5 mice in each, were treated by oral gavage with vehicle or AZD1208 and/or quizartinib, as above. They were

observed daily and weighed 5 days per week. Leukemia burden was assessed weekly by noninvasive luciferin imaging (23). Initial quizartinib dosing was 1 mg/kg 5 days/week based on the subcutaneous model experiment, but it was then reduced to 0.25 mg/kg 3×/week, with or without AZD1208, based on response.

### Colony formation assay

A total of  $1 \times 10^4$  cells/plate seeded in triplicate in MethoCult H4435 Enriched methylcellulose-based medium (StemCell Technologies) with 100 nmol/L quizartinib and/or 1 µmol/L AZD1208 or DMSO control were incubated for 16 days. Colonies were stained with 1 mg/mL 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (Sigma-Aldrich) for 48 hours, then counted with an automated image analysis system (Omnicon FAS IV, BIOSYS GmbH).

### Measurement of reactive oxygen species

Cellular reactive oxygen species (ROS) were measured using the redox-sensitive dye CM-H<sub>2</sub>DCFDA (Invitrogen; ref. 13), with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-treated cells as a positive control. Mitochondrial ROS were measured with MitoPY1 fluorescent probe (Tocris) as per manufacturer instructions.

### Real-time RT-PCR

Cells were stored at –80°C in RNAlater solution (Life Technologies). RNA isolated using the nucleospin RNA kit (Macherey-Nagel) was measured using a NanoDrop Lite Spectrophotometer (Thermo Scientific). One-hundred nanograms RNA from each sample, spiked with 1/10th concentration of an exogenous luciferase vector control, were reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). *Mcl-1*, *GAPDH*, and luciferase were amplified in triplicate from 2 µL reverse-transcribed RNA using the iQ SYBR Green Supermix in the CFX Connect RT-PCR system (Bio-Rad). Primer sequences were: *Mcl-1* F 5'-AAACTCGGGCAGGATTGTGA-3', *Mcl-1* R 5'-CCAGTCCCGTTTCGTCCTTA-3', *GAPDH* F 5'-GAGAGTGTTCCTCGTCCCG-3' and *GAPDH* R 5'-ATGAAGGGTCCGTTGATGCC-3'. *Mcl-1* and *GAPDH* mRNA levels relative to exogenous control were compared with pretreatment levels, defined as 100%. For analysis of miR-29b expression, extracted total RNA was reverse-

### Figure 1.

Pim kinase inhibition enhances FLT3 inhibitor induction of apoptosis in cells with FLT3-ITD. **A**, Combined treatment with Pim and FLT3 inhibitors abrogates growth of Ba/F3-ITD cells. Ba/F3-ITD cells were cultured at  $1 \times 10^5$ /mL with the Pim kinase inhibitor AZD1208 at 1 µmol/L and/or the FLT3 inhibitors quizartinib at 1 nmol/L, sorafenib at 2.5 nmol/L, crenolanib at 20 nmol/L or gilteritinib at 15 nmol/L, or DMSO control. Cell counts measured at 24, 48, and 72 hours were normalized to 0-hour control. Line graphs represent means ± SEM of triplicate values. **B**, Pim and FLT3 inhibitor cotreatment increases percentages of FLT3-ITD cells in sub-G<sub>1</sub> phase. Ba/F3-ITD cells were cultured with DMSO control or AZD1208 (A) and/or quizartinib (Q), sorafenib (S), crenolanib (G) or gilteritinib (G), as above, and 32D/ITD, MV4-11 and MOLM-14 cells were cultured with 1 µmol/L AZD1208 (AZD) and/or 1 nmol/L quizartinib (Q), or DMSO control. Cells were collected at serial time points, fixed overnight, and cell cycle was analyzed by flow cytometry. Representative 72-hour data from triplicate experiments are shown. **C**, AZD1208 and quizartinib cotreatment increases annexin V labeling of cells with FLT3-ITD. Ba/F3-ITD, 32D/ITD, MV4-11, and MOLM-14 cells were cultured with AZD1208 and/or quizartinib, as above. Cells were stained with annexin V/PI and analyzed by flow cytometry, and percentages of annexin V-positive cells were compared by two-way ANOVA. Means ± SEM of triplicate values are shown. **D**, AZD1208 and quizartinib cotreatment decreases mitochondrial membrane potential in Ba/F3-ITD cells. Ba/F3-ITD cells were cultured as above. Mitochondrial membrane potential was measured by flow cytometry as increased red fluorescence of JC-1 dye and compared by two-way ANOVA. Means ± SEM of triplicate values are represented in bar graphs. **E**, AZD1208 and quizartinib cotreatment increases PARP cleavage in Ba/F3-ITD cells. Ba/F3-ITD cells were cultured as above. Cleaved PARP measured by flow cytometry at 48 hours under the different conditions in triplicate experiments was compared by one-way ANOVA. **F**, AZD1208 and quizartinib cotreatment increases caspase-3 cleavage in Ba/F3-ITD cells. Ba/F3-ITD cells were cultured as above, and cleaved caspase-3 (CASP3) measured by flow cytometry at 48 hours in triplicate experiments in the presence and absence of the pan-caspase inhibitor Z-VAD-FMK (ZVAD) at 20 µmol/L was compared by one-way ANOVA. **G**, AZD1208 and quizartinib are synergistic at the concentrations studied. Ba/F3-ITD cells were treated for 48 hours with quizartinib (Q) and AZD1208 (A) alone and in combinations at the indicated concentrations, followed by WST-1 assay to determine cytotoxicity (x-axis, fraction of cells affected) and determination of synergy using the Chou-Talalay method (y-axis, combination index). Means of quadruplicate values are shown.

transcribed using a TaqMan MicroRNA reverse transcription kit (Life Technologies), with primers specific for miR29b and snoRNA202 as control. miR-29b was measured using the Taqman Fast Universal PCR Master Mix (Life Technologies) on the RT-PCR system. Data were analyzed with the comparative  $C_t$  method using internal control snoRNA202 RNA levels to normalize differences in sample loading, and graphed with Prism V.

### Polysome assay

Cells were lysed in buffer containing 100  $\mu$ g/mL cycloheximide (Sigma-Aldrich). Nuclei and mitochondria were removed. Supernatants were layered onto 10%–50% sucrose gradients and spun at 38,000 rpm for 2 hours at 4°C in an SW 40 rotor (Beckman Coulter). Centrifuged gradients were fractionated into twenty-one 500- $\mu$ L fractions and the polysome profile was determined via ultraviolet absorbance at 260 nm, followed by RNA extraction, reverse transcription in the presence of an external standard (luciferase vector), and quantification of *Mcl-1*, *GAPDH*, and luciferase in each fraction by qRT-PCR. Expression of *Mcl-1* and *GAPDH* relative to luciferase was determined in each fraction. *Mcl-1* and *GAPDH* percent in each fraction was then calculated in relation to total *Mcl-1* and *GAPDH*, respectively, in all fractions and plotted in its respective sucrose density fraction.

## Results

### Pim kinase inhibition enhances FLT3 inhibitor induction of apoptosis of FLT3-ITD cells

The effect of cotreatment with clinically active Pim and FLT3 inhibitors on Ba/F3-ITD and Ba/F3-WT cell growth was studied first. Ba/F3/ITD cells were treated with 1  $\mu$ mol/L AZD1208 and/or quizartinib, sorafenib, crenolanib, or gilteritinib at their  $IC_{50}$  concentrations (Supplementary Fig. S1), and viable cells were counted at 24, 48, and 72 hours. While treatment of Ba/F3-ITD cells with quizartinib, sorafenib, crenolanib, gilteritinib, or, to a lesser extent, AZD1208 decreased viable cell numbers at each time point in relation to DMSO control, cotreatment with AZD1208 and quizartinib, sorafenib, crenolanib, or gilteritinib reduced cell numbers, compared with each drug alone (Fig. 1A).

To understand whether Ba/F3-ITD cell number reduction with Pim and FLT3 inhibitor cotreatment was a cytostatic or a cytotoxic effect, cell cycle was analyzed. Cotreatment with AZD1208 and quizartinib, sorafenib, crenolanib, or gilteritinib caused accumulation of cells in sub- $G_1$ , compared with treatment with each drug alone, with no other changes in cell-cycle distribution (Fig. 1B). Similar results were seen in 32D/ITD, MV4-11, and MOLM-14 FLT3-ITD cells (Fig. 1B).

Induction of apoptosis by AZD1208 and FLT3 inhibitor cotreatment was confirmed by increased cell surface phosphatidylserine exposure, measured by annexin V labeling. Ba/F3-ITD, 32D/ITD, MV4-11, and MOLM-14 cells were cultured with 1 nmol/L quizartinib and/or 1  $\mu$ mol/L AZD1208 and total annexin V-positive cells were measured by flow cytometry after 48 hours. Annexin V-positive cells increased significantly with quizartinib and AZD1208 cotreatment, compared with either drug alone (Fig. 1C). Annexin V-positive Ba/F3-ITD and 32D/ITD cells also increased significantly with AZD1208 in combination with sorafenib, crenolanib, or gilteritinib, as well as quizartinib, in a concentration-dependent manner (Supplementary Fig. S2).

AZD1208 and FLT3 inhibitor cotreatment reduced the MMP in FLT3-ITD cells, also indicating induction of apoptosis (Fig. 1D). Quizartinib alone markedly reduced MMP, and quizartinib and AZD1208 cotreatment reduced it further, while single-agent AZD1208 had little effect. Sorafenib, crenolanib, and gilteritinib did not reduce MMP as single agents, but combination with AZD1208 caused significant MMP decrease (Fig. 1D).

Combined treatment with AZD1208 and quizartinib also enhanced PARP and caspase-3 cleavage in Ba/F3-ITD cells. Single-agent quizartinib increased PARP and caspase-3 cleavage slightly, relative to DMSO control, while single-agent AZD1208 had no effect, and quizartinib and AZD1208 cotreatment produced a marked increase in PARP and caspase-3 cleavage, detected by flow cytometry (Fig. 1E and F). Caspase cleavage was blocked by the pan-caspase inhibitor Z-VAD-FMK, highlighting the role of caspase activation in the enhanced apoptosis induced by the combination treatment (Fig. 1F).

Synergy between AZD1208 and quizartinib was analyzed according to the Chou-Talalay method (Fig. 1G). Combination indexes for 1 nmol/L quizartinib with 1  $\mu$ mol/L AZD1208 were 0.5, 0.1, and 0.3 in Ba/F3-ITD cells, MV4-11 and MOLM-14 (not shown) cells. Synergy was also seen at other concentration combinations, but 100 nmol/L quizartinib and 1  $\mu$ mol/L AZD1208 were antagonistic in all three cell lines.

In contrast to findings in cells with FLT3-ITD, cotreatment of Ba/F3-WT cells with AZD1208 and quizartinib at 1 nmol/L or 1  $\mu$ mol/L, its  $IC_{50}$  concentration in these cells when cultured with 10 ng/mL IL3 (Supplementary Fig. S1), produced only minimal additional growth suppression relative to each drug alone (Supplementary Fig. S3A). Moreover cotreatment of Ba/F3-WT and/or 32D/WT cells with quizartinib and AZD1208 did not increase sub- $G_1$  cells (Supplementary Fig. S3B) or annexin V labeling (Supplementary Fig. S3C) or decrease MMP (Supplementary Fig. S3D).

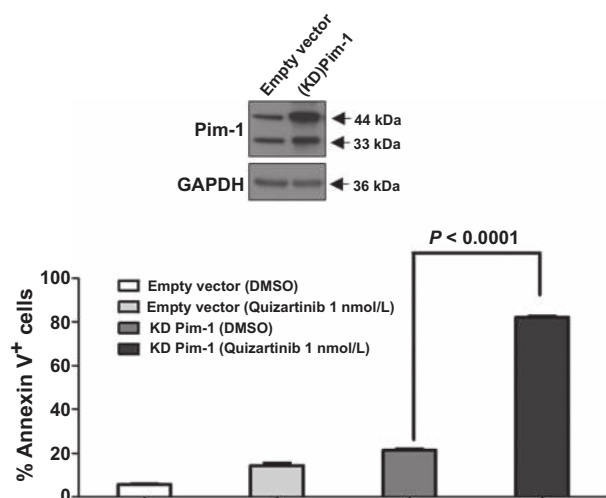
### Expression of kinase-dead mutant Pim-1 kinase sensitizes Ba/F3-ITD cells to apoptosis induction by quizartinib

To demonstrate that the enhanced apoptosis seen with AZD1208 in conjunction with FLT3 inhibitors was dependent on inhibition of Pim-1-generated survival signals, induction of apoptosis by FLT3 inhibitor was studied in Ba/F3-ITD cells expressing a Pim-1 kinase-dead mutant with reported dominant-negative activity (13). A highly significant increase in annexin V labeling was seen with quizartinib treatment of Ba/F3-ITD cells ectopically expressing kinase-dead mutant Pim-1, but not empty vector (Fig. 2).

### AZD1208 and quizartinib cotreatment causes tumor regression in FLT3-ITD *in vivo* models

The *in vitro* data prompted us to test whether AZD1208 in combination with quizartinib inhibits growth of cells with FLT3-ITD *in vivo* and provides a therapeutic antitumor benefit.

CB-17 SCID mice engrafted subcutaneously with MV4-11 cells were treated with AZD1208 and/or quizartinib, or vehicle control. Quizartinib monotherapy substantially decreased tumor growth, while AZD1208 had no effect, but the quizartinib and AZD1208 combination provided a significant benefit versus quizartinib (Fig. 3A). While all tumors eventually became resistant to treatment with quizartinib and quizartinib and AZD1208, the quizartinib and AZD1208 combination substantially prolonged survival (Fig. 3B). In a parallel study in mice engrafted with



**Figure 2.**

Expression of kinase-dead mutant Pim-1 kinase sensitizes Ba/F3-ITD cells to apoptosis induction by quizartinib. Ba/F3-ITD cells transduced with a pMX-puro retroviral construct containing a FLAG-tagged kinase-defective (KD) K67M mutant Pim-1 or control empty vector were selected with puromycin. Pim-1 expression was measured by immunoblotting (top). Percentages of annexin V-positive cells analyzed by flow cytometry following 48-hour culture with quizartinib or DMSO control were compared by one-way ANOVA. Means  $\pm$  SEM of triplicate values are shown. A highly significant increase in annexin V-labeled cells was seen with quizartinib treatment of Ba/F3-ITD cells ectopically expressing kinase-dead mutant Pim-1, but not empty vector.

KG-1a cells, with FLT3-WT, quizartinib did not inhibit tumor growth, AZD1208 monotherapy showed modest inhibition (36% on day 33), and the quizartinib and AZD1208 combination provided no additional benefit (Supplementary Fig. S4).

As in the subcutaneous model, mice injected intravenously with MV4-11 cells also exhibited delayed tumor growth (Fig. 3C and D) and prolonged survival (Fig. 3E) with AZD1208 and quizartinib compared with quizartinib alone, while AZD1208 alone had no effect. Combination therapy was well tolerated (Supplementary Fig. S5).

#### AZD1208 and quizartinib cotreatment enhances apoptosis and significantly reduces colony formation in primary patient AML cells with FLT3-ITD, but not FLT3-WT

*Ex vivo* bone marrow or blood blasts from three FLT3-ITD and three FLT3-WT AML patients were treated with quizartinib at a range of concentrations and/or 1 mmol/L AZD1208 for 48 hours, and apoptosis was measured by annexin V labeling. Cotreatment with AZD1208 increased concentration-dependent quizartinib-induced apoptosis in FLT3-ITD, but not FLT3-WT, AML cells (Fig. 4A). The degree of increase was variable, possibly reflecting differences in FLT3-ITD allelic ratios (0.66, 0.21, and 0.29 in patients 1, 2, and 3, respectively). Bone marrow mononuclear cells from three patients in remission also did not show increased apoptosis with AZD1208 and quizartinib cotreatment (Fig. 4A).

The effect of treatment with AZD1208 and/or quizartinib on colony formation by bone marrow blasts from patients with FLT3-ITD and FLT3-WT AML was also studied. AZD1208 and quizartinib cotreatment markedly decreased colony formation by FLT3-ITD AML cells, in relation to each drug alone, but had a much smaller effect on FLT3-WT AML cells (Fig. 4B).

#### AZD1208 and quizartinib cotreatment does not increase cellular ROS generation, but increases mitochondrial ROS generation

We previously demonstrated that Pim inhibition sensitizes cells with FLT3-ITD, but not FLT3-WT, to induction of apoptosis by topoisomerase inhibitors via enhanced induction of cellular ROS (18). We therefore measured cellular ROS at serial time points in Ba/F3-ITD cells treated with 1 nmol/L quizartinib and/or 1  $\mu$ mol/L AZD1208, DMSO control or H<sub>2</sub>O<sub>2</sub> control. Increased cellular ROS generation was not seen with quizartinib and AZD1208 cotreatment (Supplementary Fig. S6A). We also then measured mitochondrial ROS production, which was previously shown to increase with quizartinib treatment of cells with FLT3-ITD in a concentration-dependent manner (24). The combination of quizartinib at 1 nmol/L and 1  $\mu$ mol/L AZD1208 increased mitochondrial ROS generation, in relation to quizartinib alone (Supplementary Fig. S6B).

#### AZD1208 and quizartinib cotreatment downregulates Mcl-1 protein

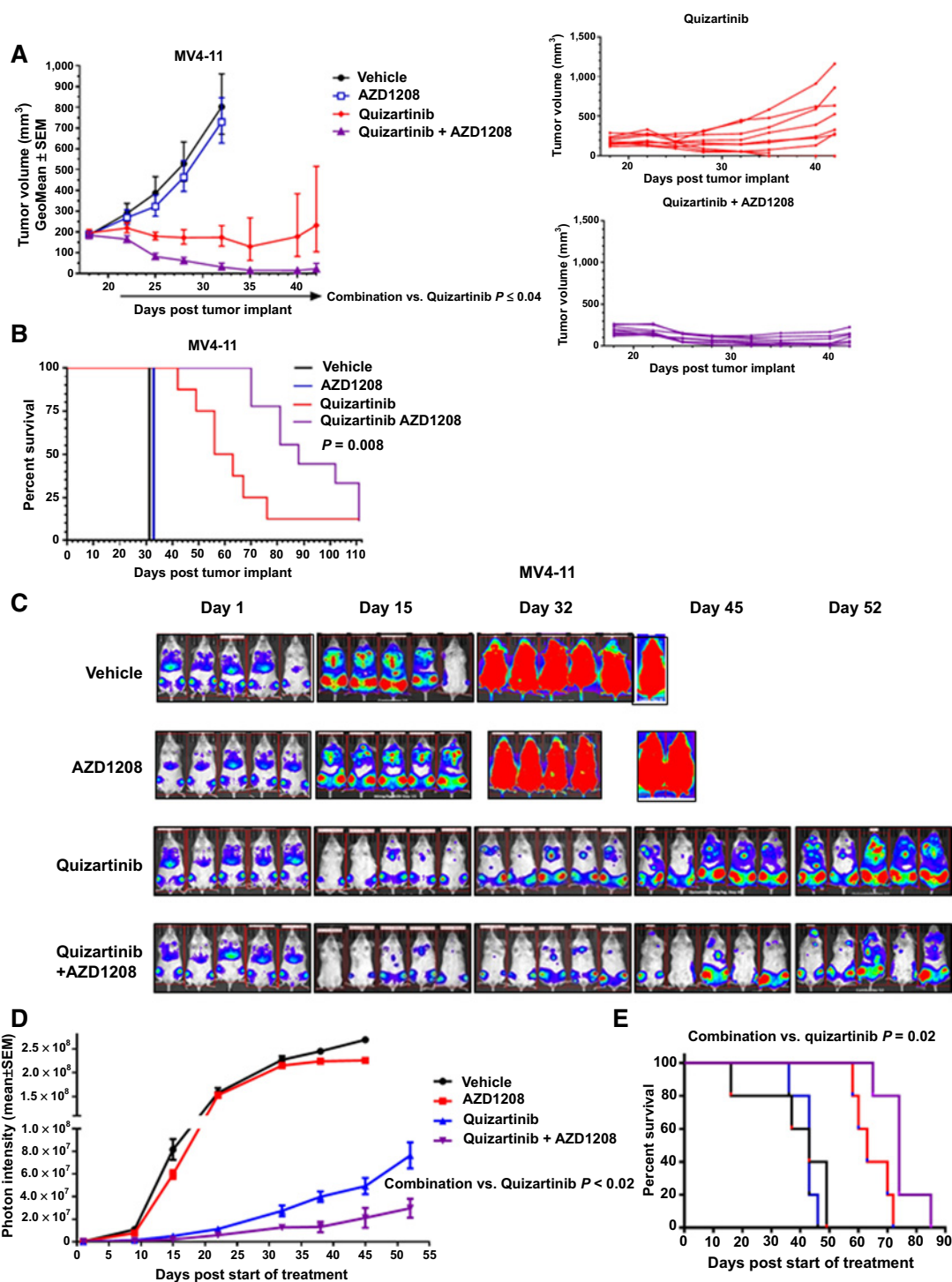
We next sought to elucidate the mechanism underlying enhanced apoptosis with Pim and FLT3 inhibitor cotreatment. Expression of the antiapoptotic proteins Mcl-1, Bcl-2, and Bcl-xL and the proapoptotic proteins BAD/p-BAD<sup>S112</sup>, BAK, BAX and Bim was measured by immunoblotting at serial time points in Ba/F3-ITD cells treated with quizartinib and/or AZD1208. Mcl-1 expression decreased in a time-dependent manner with quizartinib and AZD1208 cotreatment, relative to quizartinib alone (Fig. 5A), confirmed by densitometry (Fig. 5D), while levels of the other proteins did not change significantly with combination treatment, relative to quizartinib (Fig. 5A). Of note, Bim expression increased markedly, but the increase was equal with quizartinib and AZD1208 and with quizartinib alone. Moreover phospho-BAD<sup>Ser112</sup> decreased with AZD1208 treatment, but not with quizartinib or with quizartinib and AZD1208. In addition, phospho-STAT5 decreased similarly with quizartinib and AZD1208 as with quizartinib alone (Fig. 5B).

To further study the role of Mcl-1 in the response to AZD1208 and quizartinib cotreatment, apoptosis induction was studied in Ba/F3-ITD cells with Mcl-1 knocked down with targeted shRNA. Depletion of Mcl-1 markedly increased apoptosis induction by AZD1208 and quizartinib cotreatment at 24 hours (Fig. 5C), while a smaller effect was seen with quizartinib alone and no effect was seen with AZD1208 alone.

#### AZD1208 and quizartinib cotreatment reduces Mcl-1 protein levels posttranslationally

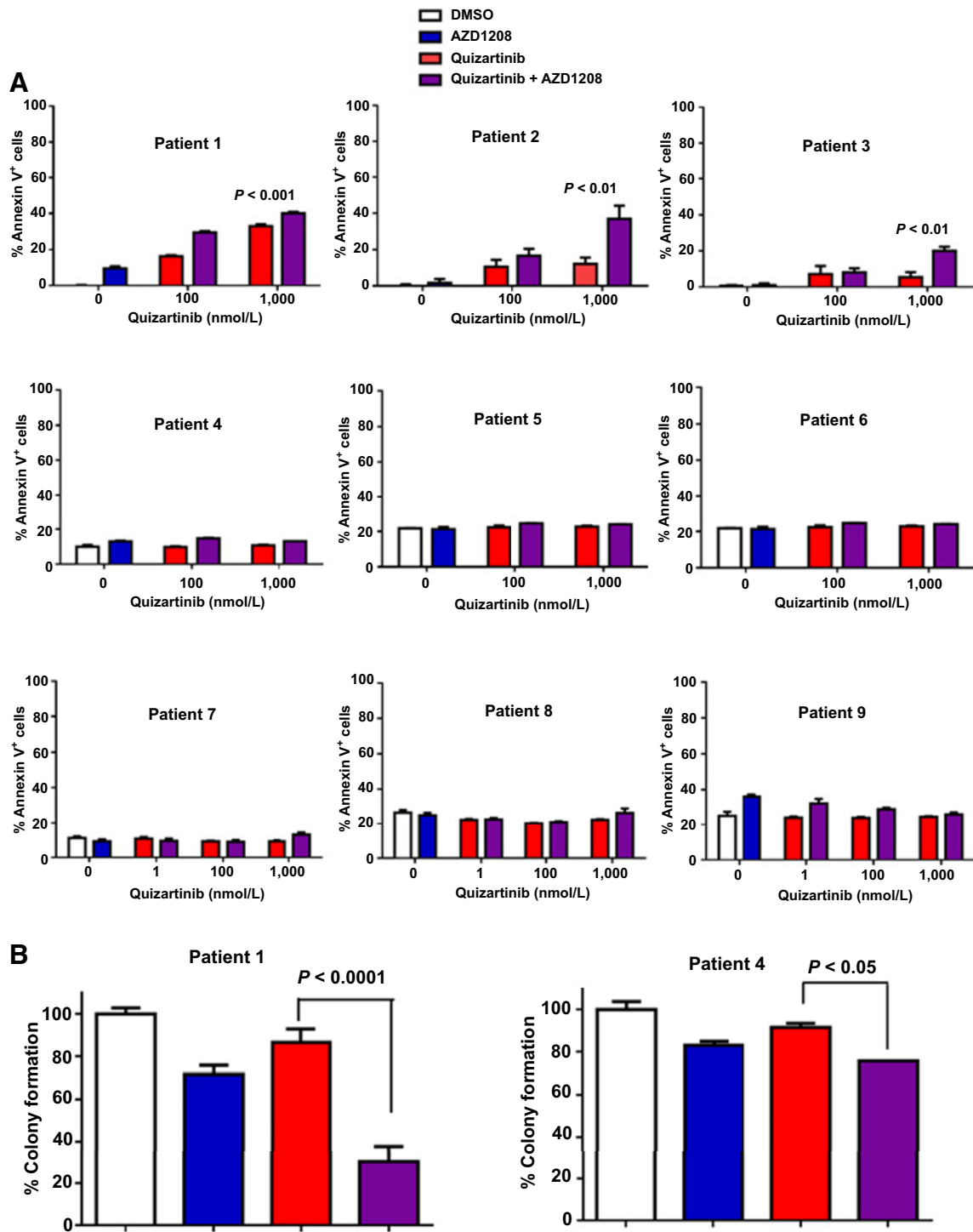
We next sought to determine the mechanism(s) by which AZD1208 and quizartinib cotreatment downregulates Mcl-1 expression. We first measured *Mcl-1* mRNA in conjunction with protein. While Mcl-1 protein levels decreased in AZD1208 and quizartinib cotreated cells, *Mcl-1* mRNA levels did not change relative to *GAPDH* (Fig. 5D) or to an exogenous luciferase vector control (not shown). Therefore Mcl-1 downregulation by AZD1208 and quizartinib cotreatment occurs at a posttranscriptional level.

As miR-29b is a negative regulator of Mcl-1 translation (25), we determined whether miR-29b levels were altered in Ba/F3-ITD cells treated with AZD1208 and quizartinib, compared with quizartinib alone, using qRT-PCR. miR-29b levels decreased with quizartinib treatment alone and decreased similarly with



**Figure 3.**

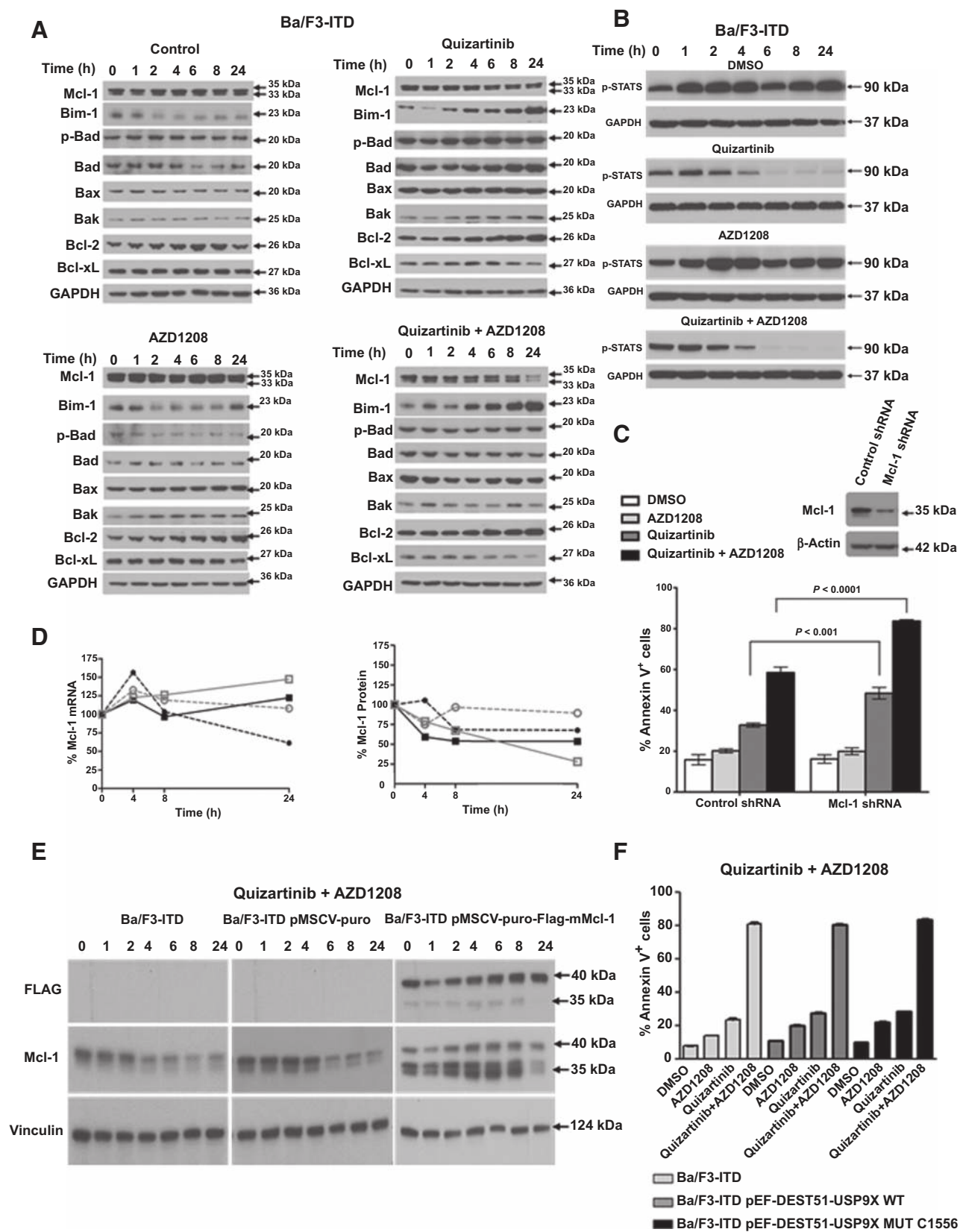
Combined AZD1208 and quizartinib treatment abrogates growth of MV4-11 cells and prolongs survival in subcutaneous and orthotopic *in vivo* models. Doses and schedules were based on efficacy in the cell line and tolerability in the mouse models. **A**, Subcutaneous tumor growth. Mice injected subcutaneously with MV4-11 cells were treated with 30 mg/kg AZD1208 and/or 1 mg/kg quizartinib, or with vehicle control, and tumor volumes measured at serial time points were graphed. Means  $\pm$  SEM values are shown in the graph on the left, and effects of quizartinib alone or in combination with AZD1208 on individual MV4-11 tumor growth are shown in the graphs on the right. Quizartinib in combination with AZD1208 significantly decreased tumor growth ( $P \leq 0.04$  beginning on day 22; Student *t* test, two-tailed, paired). **B**, Survival in subcutaneous model. Survival was significantly longer in mice treated with quizartinib in combination with AZD1208, compared with quizartinib alone ( $P = 0.008$ ; Student *t* test, two-tailed, paired). Mice treated with vehicle control and with AZD1208 were sacrificed on day 32. **C**, Orthotopic model. Mice injected intravenously with MV4-11-luc cells were treated three days per week with 30 mg/kg AZD1208 and/or 0.25 mg/kg quizartinib or vehicle control and imaged at serial time points. **D**, Photon intensity. Photon intensity means  $\pm$  SEM versus time are shown graphically. Decreased photon intensity was seen with quizartinib in combination with AZD1208, compared with quizartinib alone ( $P < 0.002$ ; Student *t* test, two-tailed, paired). **E**, Survival in orthotopic model. Survival was significantly longer in mice treated with quizartinib in combination with AZD1208, compared with quizartinib alone ( $P = 0.02$ ; Student *t* test, two-tailed, paired).



**Figure 4.** **A**, Combined AZD1208 and quizartinib treatment increases apoptosis in FLT3-ITD AML patient blasts, but not FLT3-WT AML patient blasts or remission marrow cells. Bone marrow or blood blasts from AML patients with FLT3-ITD (top) or FLT3-WT (middle) and bone marrow mononuclear cells from patients in remission (bottom) were treated with 1  $\mu$ mol/L AZD1208 and/or quizartinib at increasing concentrations. Percentages of cells labeled with annexin V at 48 hours were measured by flow cytometry and compared by two-way ANOVA. Means  $\pm$  SEM of triplicate experiments are shown. **B**, Combined AZD1208 and quizartinib treatment reduces FLT3-ITD blast colony formation. Blasts from Patient 1, with FLT3-ITD, and Patient 4, with FLT3-WT, were seeded in methylcellulose with 1  $\mu$ mol/L AZD1208 and/or 100 nmol/L quizartinib or DMSO control and incubated for 16 days. Colonies were counted following 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride staining for 48 hours. Data were normalized to those from DMSO-treated cells and compared by one-way ANOVA. Means  $\pm$  SEM of triplicate experiments are shown.

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combination treatment (Supplementary Fig. S7A), indicating that miR-29b is not driving the effect of the combination treatment on Mcl-1 protein expression.

Next, we tested the effect of AZD1208 and quizartinib cotreatment on Mcl-1 translation by analyzing the polysome profile from Ba/F3-ITD cells treated with AZD1208 and/or quizartinib for 24 hours. The polysomal RNA (P) to uninitiated/untranslated (U) RNA ratio was lower with cotreatment, compared with single-drug or DMSO control treatment, indicating decreased total mRNA translation. However qRT-PCR analysis showed decreased association of *GAPDH*, but not *Mcl-1*, mRNA with polysomes upon cotreatment, indicating that *Mcl-1* translation is not selectively reduced (Supplementary Fig. S7B).

In addition, Mcl-1 overexpression in Ba/F3-ITD cells did not abrogate Mcl-1 downregulation (Fig. 5E) nor induction of apoptosis (Fig. 5F) by quizartinib and AZD1208 cotreatment, which is also consistent with a posttranslational effect of quizartinib and AZD1208 cotreatment on Mcl-1 expression.

#### Mcl-1 protein expression is downregulated via enhanced Mcl-1 proteasomal degradation

We then investigated whether quizartinib and AZD1208 cotreatment causes increased Mcl-1 protein proteasomal degradation. To test this, we analyzed Mcl-1 protein expression in Ba/F3-ITD cells treated with 1  $\mu\text{mol/L}$  AZD1208 and/or 1 nmol/L quizartinib in the absence and presence of the proteasome inhibitor MG132. The progressive decrease in Mcl-1 protein expression at 4 and 8 hours of quizartinib and AZD1208 cotreatment was rescued by addition of the proteasome inhibitor MG132 (Fig. 6A). This finding is consistent with enhanced Mcl-1 proteasomal degradation as the mechanism for Mcl-1 protein downregulation upon AZD1208 and quizartinib cotreatment. Expression of the deubiquitinase ubiquitin-specific peptidase 9 X-linked (USP9X), which plays a prominent role in controlling Mcl-1 degradation by the proteasome (26), also decreased with quizartinib and AZD1208 cotreatment, and the decrease was also rescued by MG132.

We then determined the timing of the decrease in USP9X and Mcl-1 expression with quizartinib and AZD1208 cotreatment. Ba/F3-ITD cells were treated with 1 nmol/L quizartinib and/or 1  $\mu\text{mol/L}$  AZD1208, harvested at serial time points and analyzed by immunoblotting. USP9X protein levels initially decreased after 3 hours of treatment with quizartinib and AZD1208 (Fig. 6B), but not with either drug alone or with DMSO control (not shown),

and the decrease in USP9X protein level preceded the decrease in Mcl-1 protein level. In contrast, expression of the deubiquitinase USP24, an additional regulator of Mcl-1 proteasomal degradation (27), was not altered by cotreatment with quizartinib and AZD1208 (Fig. 6B). In addition, no changes in expression of the ubiquitin E3 ligases ARF-BP1, SCF $\beta$ -TRCP, and Trim17 preceded Mcl-1 downregulation (Fig. 6B).

To confirm that posttranslational Mcl-1 protein downregulation is a consistent finding with AZD1208 and FLT3 inhibitor cotreatment of cells with FLT3-ITD, the FLT3-ITD human AML cell lines MV4-11 and MOLM-14 were studied. Consistent with the findings in Ba/F3-ITD cells, MV4-11 and MOLM-14 cells cotreated with AZD1208 and quizartinib showed reduced Mcl-1 protein levels, and decreased USP9X expression preceded decreased Mcl-1 expression (Fig. 6C). Moreover, the decrease in Mcl-1 expression was abrogated by treatment with the proteasome inhibitor MG132 (Fig. 6D), consistent with decrease via proteasomal degradation. Finally, as in Ba/F3-ITD cells, the effect of quizartinib and AZD1208 combination therapy was not attributable to greater decreases in phospho-STAT5 or phospho-BAD (Fig. 6E).

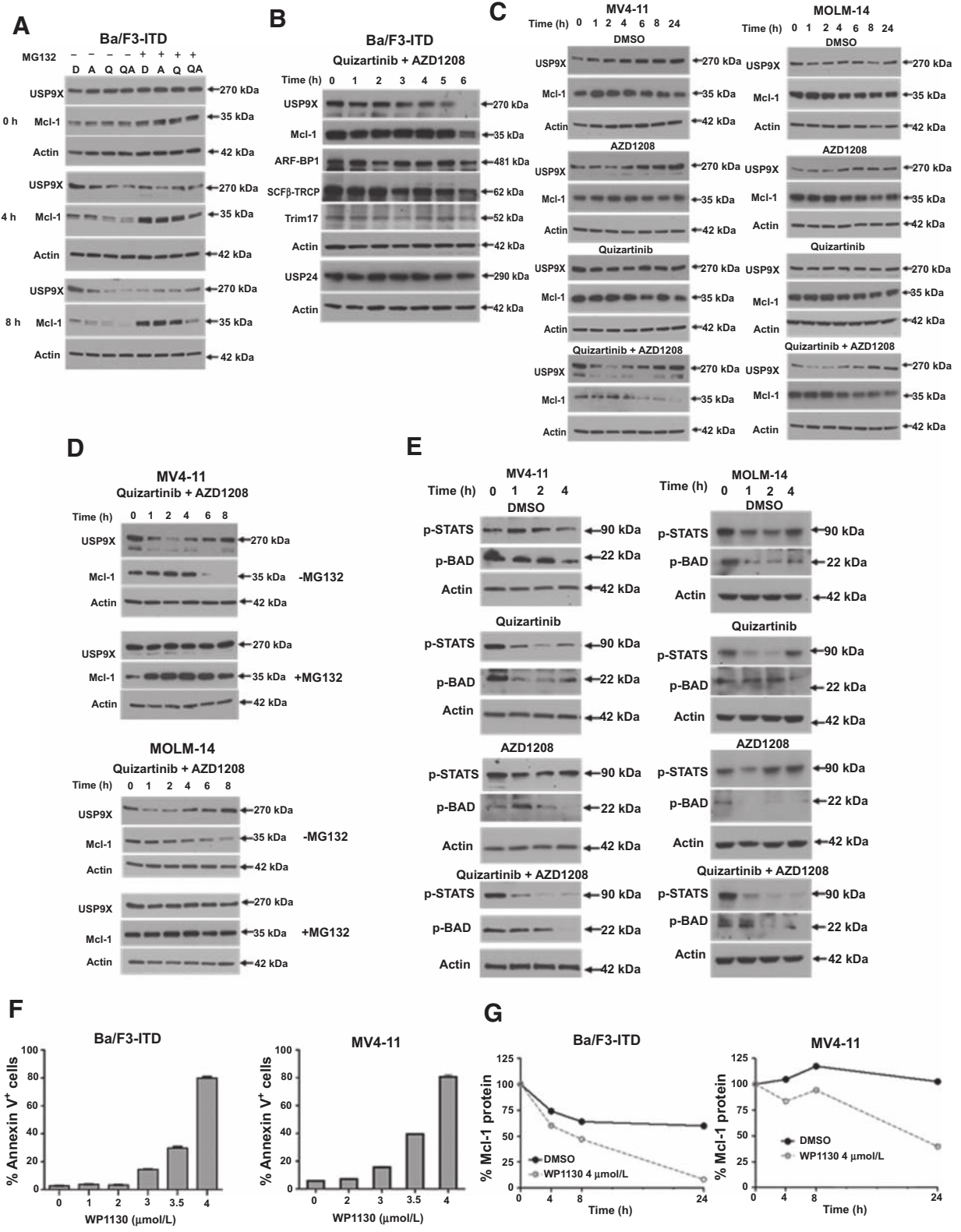
Treatment with the USP9X inhibitor WP1130 induced apoptosis of Ba/F3-ITD cells in a concentration-dependent manner (Fig. 6F), consistent with USP9X as a mediator of apoptosis induction by concurrent Pim and FLT3 inhibition. Downregulation of Mcl-1 expression by WP1130 at a concentration that induced apoptosis was also demonstrated (Fig. 6G). Finally, WP1130 enhanced induction of apoptosis of Ba/F3-ITD and MV4-11 cells by quizartinib in a concentration-dependent manner (Supplementary Fig. S8).

## Discussion

FLT3-ITD is present in AML cells in 30% of patients (1) and these patients have short disease-free survival following chemotherapy (1) and following transplant (2). FLT3 inhibitors have limited and transient clinical activity (4), and responses might be augmented by combining them with inhibitors of parallel or downstream signaling. The oncogenic prosurvival kinase Pim-1 is overexpressed downstream of FLT3-ITD (6) and also promotes FLT3-ITD signaling in a positive feedback loop (7, 8). We demonstrate here that Pim inhibition enhances apoptosis induction by the clinically active FLT3 inhibitors quizartinib, sorafenib, crenolanib, and gilteritinib in FLT3-ITD cell lines *in vitro* and *in vivo* and in FLT3-ITD AML patient samples, and that this effect is

#### Figure 5.

Apoptosis induction by AZD1208 and quizartinib cotreatment is associated with posttranscriptional downregulation of Mcl-1 protein expression. **A**, Expression of proapoptotic and prosurvival proteins at serial time points, showing Mcl-1 downregulation by cotreatment. Expression of pro- and antiapoptotic proteins in Ba/F3-ITD cells treated with 1  $\mu\text{mol/L}$  AZD1208 and/or 1 nmol/L quizartinib, or DMSO control for 0, 1, 2, 4, 6, 8, and 24 hours was measured by immunoblotting. **B**, Expression of p-STAT5 at serial time points, showing similar decrease with combination and quizartinib. Expression of p-STAT5 in Ba/F3-ITD cells treated with 1  $\mu\text{mol/L}$  AZD1208 and/or 1 nmol/L quizartinib, or DMSO control, for 0, 1, 2, 4, 6, 8, and 24 hours was measured by immunoblotting. **C**, Mcl-1 gene knockdown accelerates apoptosis induction by AZD1208 and quizartinib cotreatment. Mcl-1 gene expression was knocked down in Ba/F3-ITD cells by shRNA (top). Cells were then treated with 1  $\mu\text{mol/L}$  AZD1208 and/or 1 nmol/L quizartinib, or DMSO control, for 24 hours, labeled with annexin V/PI and analyzed by flow cytometry. Percentages of annexin V-positive cells were compared by two-way ANOVA. Means  $\pm$  SEM of triplicate values are shown. **D**, Downregulation of Mcl-1 expression by AZD1208 and quizartinib cotreatment is posttranscriptional. *Mcl-1* mRNA levels (left) were measured at serial time points in conjunction with protein levels (right) and normalized to GAPDH expression in Ba/F3-ITD cells treated with AZD1208 and/or quizartinib, or DMSO control. **E**, Mcl-1 overexpression does not inhibit Mcl-1 downregulation by cotreatment, consistent with posttranslational downregulation. Expression of Mcl-1 and vinculin, as a loading control, was measured by immunoblotting at serial time points in Ba/F3-ITD cells expressing Mcl-1 cDNA (FLAG-tagged) in a pMSCV-puro-Flag-mMcl-1 vector (right) and pMSCVpuro control vector (center) cotreated with quizartinib and AZD1208. FLAG expression and Mcl-1 overexpression were confirmed by immunoblotting (right). **F**, Mcl-1 overexpression does not inhibit apoptosis induction by cotreatment. Annexin-V labeling was measured on Ba/F3-ITD cells expressing Mcl-1 cDNA (FLAG-tagged) in a pMSCV-puro-Flag-mMcl-1 vector (right) and pMSCVpuro control vector (center) cotreated with quizartinib and AZD1208 for 48 hours.



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associated with reduction in Mcl-1 protein levels resulting from increased Mcl-1 proteasomal degradation.

We used the Pim inhibitor AZD1208 to test the effect of Pim kinase inhibition in combination with FLT3 inhibition. AZD1208 was the first selective pan-Pim inhibitor tested clinically in AML, based on preclinical activity (14). It was well tolerated and clinically active in a phase I trial in AML patients (11), but was withdrawn from clinical studies due to highly variable pharmacokinetics and time-dependent decrease in exposure in patients, despite favorable pharmacokinetics in mice. The pan-Pim inhibitors PIM447 (formerly LGH447; ref. 12) and INCB053914 (28) are currently in clinical trials, and we have also shown *in vitro* efficacy of PIM447 in conjunction with FLT3 inhibitors in abrogating growth and enhancing apoptosis of cells with FLT3-ITD (29).

Single-agent AZD1208 did not induce apoptosis of FLT3-ITD cells, nor of the FLT3-WT cells that we studied. Similarly, Keeton and colleagues found that, among 14 AML cell lines including MV4-11 and MOLM13 with FLT3-ITD, AZD1208 only induced apoptosis of MOLM16 cells (14), with FLT3-WT but rearrangement of the non-receptor tyrosine kinase *Tyk2* (30). In contrast, the Pim-1/Pim-3 inhibitor SGI-1776 induced apoptosis in the FLT3-ITD cell lines MV4-11 and MOLM-13 (31), but also inhibits FLT3, and apoptosis was likely due to concurrent FLT3 inhibition (32).

Increased apoptosis induction with AZD1208 and FLT3 inhibitor cotreatment was not associated with enhanced effects on phospho-STAT5 or phospho-BAD, but rather was associated with downregulation of Mcl-1, previously identified as an important target in FLT3-ITD AML. AML cells and stem cells with FLT3-ITD express high levels of Mcl-1, and Mcl-1 depletion by shRNA induces apoptosis, while Mcl-1 overexpression promotes cell survival (33, 34). Moreover, the Pim-1 and FLT3 inhibitor SGI-1776 induced apoptosis of FLT3-ITD AML cells by downregulating Mcl-1 expression (31).

Mechanistically, AZD1208 and quizartinib cotreatment decreased Mcl-1 protein levels through enhanced proteasome-dependent Mcl-1 degradation. The Pim-1 and FLT3 inhibitor SGI-1776 also induced apoptosis of FLT3-ITD AML cells by downregulating Mcl-1 expression, as noted above, but Mcl-1 downregulation occurred via decreased Mcl-1 transcription and translation (31). Enhanced proteasomal degradation is a novel mechanism of Mcl-1 downregulation in FLT3-ITD cells.

Treatment of FLT3-ITD cells with AZD1208 alone did not affect Mcl-1 protein levels. Pim phosphorylation protects a number of substrate proteins from proteasomal degradation (35–37), but Mcl-1 is not a known Pim-1 substrate. In contrast, the Pim inhibitor SMI-4a downregulated Mcl-1 in prostate cancer cells via both globally decreased translation and increased Mcl-1 proteasomal degradation (38). Differential effects of AZD1208 and SMI-4a on Mcl-1 proteasomal degradation might reflect different effects on proteins regulating Mcl-1 degradation, rather than direct effects on Mcl-1.

Mcl-1 proteasomal degradation is regulated by GSK3 phosphorylation, and, in different tissues, by the ubiquitin E3 ligases Mule/ARF-BP1, SCF $\beta$ -TrCP, SCFFbw7 and, in neurons, Trim17, which promote its degradation (39), and the deubiquitinases USP9X (26) and USP24 (26), which protect it from degradation. We demonstrated time-dependent downregulation of USP9X in Ba/F3-ITD cells cotreated with quizartinib and AZD1208, preceding the reduction in Mcl-1 protein levels, while Mule/ARF-BP1, SCF $\beta$ -TrCP, Trim17, and USP24 levels did not change. These data suggest that USP9X downregulation plays a key role in the increase in Mcl-1 proteasomal degradation induced by concurrent Pim and FLT3 inhibition. We also demonstrated that treatment of Ba/F3-ITD cells with the USP9X inhibitor WP1130 decreased Mcl-1 protein levels and induced apoptosis in a concentration-dependent manner, reproducing the effect of concurrent Pim and FLT3 inhibition. Of note, USP9X is increasingly recognized as a therapeutic target in diverse malignancies (26, 27, 40–43), but, to our knowledge, has not been previously studied in AML.

There are several explanations for the efficacy of Pim and FLT3 inhibitor cotreatment in cells with FLT3-ITD, but not FLT3-WT. First, Pim-1 is upregulated downstream of FLT3-ITD and is an important mediator (6) and potentiator (7, 8) of FLT3-ITD signaling. Second, FLT3 inhibitors more potently induce apoptosis in cells with FLT3-ITD, and apoptosis is therefore likely potentiated more effectively. Third, we found that AZD1208 and FLT3 inhibitor cotreatment downregulates Mcl-1 expression and, as discussed above, Mcl-1 is an important target in FLT3-ITD AML (31, 33, 34).

Pim inhibition sensitizes FLT3-ITD cells to apoptosis induction by FLT3 inhibitors and by topoisomerase inhibitor chemotherapy drugs by different mechanisms. We previously demonstrated that Pim inhibition sensitizes cells with FLT3-ITD, but not FLT3-WT, to induction of apoptosis by topoisomerase inhibitors via enhanced induction of cellular ROS and increased DNA damage, without effect on expression of Mcl-1 or other

#### Figure 6.

Mechanism of posttranslational Mcl-1 downregulation in FLT3-ITD cells cotreated with quizartinib and AZD1208. **A**, Proteasome inhibition abrogates Mcl-1 downregulation by AZD1208 and quizartinib cotreatment. Ba/F3-ITD cells treated with 1  $\mu$ M AZD1208 and/or 1 nM quizartinib (A, Q, QA), or DMSO control (D), were studied for Mcl-1 expression at serial time points in the presence and absence of the proteasome inhibitor MG132 (20  $\mu$ M/L), added 30 minutes before drug treatment. The Mcl-1 deubiquitinase USP9X is also shown, also demonstrating downregulation by proteasomal degradation. **B**, Expression of the Mcl-1 deubiquitinase USP9X decreases prior to the decrease in Mcl-1 expression in Ba/F3-ITD cells cotreated with AZD1208 and quizartinib. Expression of USP9X and Mcl-1 in Ba/F3-ITD cells cotreated with quizartinib and AZD1208 was measured by immunoblotting. In contrast, expression of the Mcl-1 deubiquitinase USP24 and the ubiquitin E3 ligases ARF-BP1, SCF $\beta$ -TRCP and Trim17 did not change. **C**, AZD1208 and quizartinib cotreatment downregulates Mcl-1 in MV4-11 and MOLM-14 cells, preceded by USP9X downregulation. Serial expression of Mcl-1 and USP9X protein is shown in cells treated with AZD1208 and/or quizartinib, or DMSO control. **D**, Proteasome inhibition abrogates Mcl-1 downregulation in MV4-11 and MOLM-14 cells cotreated with quizartinib and AZD1208. Mcl-1 and USP9X expression was measured at serial time points in MV4-11 and MOLM-14 cells cotreated with quizartinib and AZD1208 in the absence and presence of MG132. Mcl-1 downregulation was preceded by USP9X downregulation in the absence of MG132, but neither was downregulated in the presence of MG132. **E**, Quizartinib and AZD1208 cotreatment of MV4-11 and MOLM-14 cells does not decrease p-STAT5 or p-BAD, in relation to each drug alone. p-STAT5 and p-BAD<sup>S112</sup> expression is shown in MV4-11 and MOLM-14 cells treated with AZD1208 and/or quizartinib, or DMSO control. **F**, Treatment with the USP9X inhibitor WP1130 induces apoptosis of Ba/F3-ITD and MV4-11 cells. Ba/F3-ITD and MV4-11 cells were treated in triplicate with WP1130 in increasing concentrations and annexin V labeling was measured by flow cytometry, demonstrating concentration-dependent induction of apoptosis. **G**, Treatment with the USP9X inhibitor WP1130 decreases Mcl-1 expression in Ba/F3-ITD and MV4-11 cells. WP1130 at 4  $\mu$ M/L, a concentration that induced apoptosis, downregulated Mcl-1 expression in both cell lines.

antiapoptotic or proapoptotic proteins (13). Here we found that cotreatment with Pim and FLT3 inhibitors did not induce cellular ROS, but downregulated Mcl-1 expression. Topoisomerase inhibitors induce cellular ROS and DNA damage by themselves (13), while FLT3 inhibitors do not induce cellular ROS, but downregulate Mcl-1 expression in FLT3-ITD cells (33, 34). Of note, we did find increased induction of mitochondrial ROS with quizartinib and AZD1208 cotreatment as a contributor to apoptosis (24). Pim inhibition appears to enhance apoptosis via the mechanism associated with its partner drug. A similar phenomenon was reported in combination with the Bcl-2 inhibitor ABT-737 (38).

Our *in vitro* and *in vivo* data support clinical testing of concurrent treatment with Pim and FLT3 inhibitors in patients with FLT3-ITD AML, and, as noted above, we recently reproduced the *in vitro* findings reported here with PIM447 (29), which has undergone successful clinical testing (12). Of note, Pim inhibitors have been well tolerated in clinical trials to date (11, 12), as have FLT3 inhibitors (4, 17, 19, 20), and they have not had overlapping toxicities. As with effective BCR-ABL inhibition in chronic myelogenous leukemia, enhanced cytotoxicity of FLT3 inhibitors in conjunction with a Pim inhibitor may reduce induction of FLT3 point mutations conferring resistance to FLT3 inhibitors (44). Pim expression also increases at relapse following FLT3 inhibitor therapy (7), and Pim inhibitors may abrogate the effects of Pim upregulation. Finally, as enhanced apoptosis is specific for FLT3-ITD cells and, in particular, was not seen in remission bone marrow cells, a favorable therapeutic index is expected for concurrent FLT3 and Pim kinase inhibitor therapy in AML with FLT3-ITD, in relation to effects on normal hematopoietic cells.

### Disclosure of Potential Conflicts of Interest

M. Kraus is an employee of Pfizer. D. Huszar and A.E. Tron have ownership interests (including patents) at AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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### Authors' Contributions

**Conception and design:** K. Natarajan, P.R. Baldwin, M. Kraus, D. Huszar, M.R. Baer

**Development of methodology:** S. Kapoor, K. Natarajan, T.J. Mathias, M. Scarpa, R. Trotta, M.R. Baer

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Kapoor, P.R. Baldwin, K.A. Doshi, R.G. Lapidus, T.J. Mathias, D. Huszar

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Kapoor, K. Natarajan, P.R. Baldwin, K.A. Doshi, R.G. Lapidus, M. Scarpa, M. Kraus

**Writing, review, and/or revision of the manuscript:** S. Kapoor, K. Natarajan, P.R. Baldwin, T.J. Mathias, M. Scarpa, R. Trotta, E. Davila, D. Huszar, A.E. Tron, D. Perrotti, M.R. Baer

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S. Kapoor, K. Natarajan, A.E. Tron

**Study supervision:** M. Kraus, M.R. Baer

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