Venetoclax combines synergistically with FLT3 inhibition to effectively target leukemic cells in FLT3-ITD+ acute myeloid leukemia models

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 doi:10.3324/haematol.2019.244020

Received: December 5, 2019.

Accepted: May 14, 2020.

Pre-published: May 15, 2020.

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Supplemental Material

Extended Experimental Methods

Materials

A1331852 and navitoclax were provided by AbbVie Inc. (North Chicago, IL). Venetoclax was provided by AbbVie Inc. or purchased from Selleckchem (Houston, TX). Pictisilib and cobimetinib were provided by Genentech. Quizartinib was synthesized by Bellen Chemistry Company (Beijing, China) or purchased from Selleckchem. AMG 176 was synthesized by Pharmaron (Beijing, China). Compounds used for *in vivo* studies were formulated in 60% Phosal 50 PG/30% PEG 400/10% ethanol and dosed by oral gavage (PO). MG132 was purchased from Sigma-Aldrich (St. Louis, MO) and Z-VAD-FMK was purchased from BD Pharmingen (San Jose, CA). Anti-BCL-2 antibody was purchased from BD Pharmingen and all other antibodies were obtained from Cell Signaling Technology (Danvers, MA).

Colony forming (CFU) assay

Patient samples were collected at the University of California-San Francisco (UCSF) under informed consent in accord with the Declaration of Helsinki using Institutional Review Board (IRB)-approved protocols. Triplicate samples were cultured in methylcellulose enriched media (#HSC005, R&D Systems, Minneapolis, MN) with inhibitors for 14 days.

Cell Lines

Cell lines were obtained from the Genentech in-house tissue culture cell bank and authenticated by short tandem repeat (STR) analysis and used within 6 months. Cell lines were grown in RPMI1640 medium with 10% FBS, glutamine (2 mmol/L), penicillin

(100 units/ml) and streptomycin (100 µg/ml), and incubated at 37 °C with 5% CO_2. Luciferase-GFP expressing cell lines were generated by lentiviral infection of CMV-Luciferase-EF1a-copGFP plasmid with pPACKH1 Packaging Plasmid mix from System Biosciences (Palo Alto, CA).

BLISS Analysis

Cells were seeded in 384-well plates and treated at indicated concentration of compound for 48 hours. Each treatment was done in quadruplicate and ATP content was determined with CellTiter-Glo (Promega, Madison, WI). Bliss sums were determined as described[1].

BH3 profiling.

MV4;11 cells were pre-treated with 5 nM quizartinib or 20 nM sorafenib for 6 hours in RPMI growth media followed by 1 hour with indicated peptides or venetoclax and cytochrome c release was measured as described[2].

Western blotting and immunoprecipitation

Cells were lysed in cold 1% CHAPS lysis buffer (Bioworld, Dublin, OH) supplemented with protease (Roche, Mannheim, Germany) and phosphatase inhibitor cocktails (Sigma, St. Louis, MO), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). For Western blotting, equal amounts of protein was separated on SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes. For immunoprecipitation, equal amounts of protein was pre-cleared with protein G dynabeads (Invitrogen) and incubated with antibodies overnight at 4°C. Immunoprecipitates were recovered by protein G dynabeads beads and transferred to

nitrocellulose membranes. Blots were visualized by LI-COR Odyssey Imaging System and quantified with Odyssey software (LI-COR Biosciences, Lincoln, NE).

Quantitative PCR

TaqMan gene expression assays. for *BCL2*, *MCL1*, *BCL2L1*, *BIM* and *ACTIN* were purchased from Applied Biosystems. The thermal cycling conditions were: 48°C for 20 minutes, 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. *ACTIN* was used as a reference housekeeping gene and expression levels for *BCL-2, BCL-XL, MCL-1 and BIM* were determined using the $\Delta\Delta C_t$ method.

Apoptosis assay

Cells were starved in RPMI containing 0.2% BSA for 6 hours then plated in growth media containing indicated inhibitors for 48 hours. Apoptosis was determined by annexin V and 7-AAD staining per manufacturer's recommendations (BD Pharmingen) by flow cytometry and data was analyzed with FlowJo software (version 9; FlowJo LLC, Ashland, OR).

Bioluminescence imaging

Mice were anesthetized using isoflurane and injected with 250 mg/kg d-luciferin (Goldbio, St. Louis, MO) and imaged using PhotonIMAGER (Biospace Lab, Nesles la Vallée, France). Bioluminescence was analyzed using M3 Vision software (Biospace Lab).

Pharmacokinetic Studies

Pharmacokinetic studies were studies were approved by Genentech's Institutional Animal Care and Use Committee (IACUC) and adhere to the Eighth Edition of the Guide for the

Care and Use of Laboratory Animals (NRC 2011). NSG mice were obtained from Jackson Laboratory and housed in autoclaved individually ventilated cages. Mice were dosed with AMG 176 or quizartinib formulated in 60% Phosal 50/30%PEG 400/ 10% ethanol via oral administration. Serial blood samples were collected at various time interval over a period of up to 24 h post-dose. Concentrations of compound were determined by a non-validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assay. The plasma samples were prepared for analysis by placing an aliquot of blood or plasma into a 96-well plate followed by the addition of internal standard. The samples were vortexed and centrifuged at 1600 g for 15 min at room temperature, 50µL of the supernatant was diluted with 150 µL of water and 5 µL of the solution of the solution was injected onto an analytical column. Data was acquired using multiple reaction monitoring (MRM) with specific transitions monitored for each compound. Pharmacokinetic (PK) parameters were calculated by non-compartmental methods as described in Gibaldi and Perrier (*Pharmacokinetics* 2nd edition, 1982) using Phoenix™ WinNonlin®, version 6.3.0 software (Pharsight Corporation, Mountain View, CA) All PK parameters are presented as mean ± standard deviation (SD).

References

1. Wallin JJ, Guan J, Prior WW, et al. GDC-0941, a novel class I selective PI3K inhibitor, enhances the efficacy of docetaxel in human breast cancer models by increasing cell death in vitro and in vivo. Clin Cancer Res. 2012;18(14):3901-3911.

2. Ryan J, Montero J, Rocco J, Letai A. iBH3: simple, fixable BH3 profiling to determine apoptotic priming in primary tissue by flow cytometry. Biol Chem. 2016;397(7):671-678.

Supplemental Figure Legends

Supplemental Figure 1. The combination of venetoclax and quizartinib is well tolerated in NSG mice. (A) Individual body weight data for NSG mice engrafted with luciferase positive MV4;11 cells during treatment period as indicated. (B) Individual body weight data for NSG mice engrafted with luciferase positive Molm13 cells during treatment period as indicated.

Supplemental Figure 2. Murine pharmacokinetic assessment of quizartinib and AMG 176. Total blood concentration in peripheral blood samples from NSG mice following a single, oral dose of 5 mg/kg quizartinib or 10 mg/kg AMG 176 at indicated time points.

Supplemental Figure 3. The combination of venetoclax and quizartinib is well tolerated in primary AML PDX models. (A) Cytogenetics and mutations identified in primary patient samples. (B) Western blot analysis and quantified BCL-2, BCL-X_L and MCL-1 protein expression from human CD45+ leukemia cells harvested from vehicle-treated FLT3-WT or FLT3-ITD+ PDX models. (C) Flow cytometry analysis of human and murine CD45 positive cells in bone marrow samples from vehicle, venetoclax, quizartinib and venetoclax + quizartinib treated mice at the end of dosing period. (D) Individual body weight data for NSG mice engrafted FLT3-WT or FLT3-ITD+ primary AML cells as indicated during treatment period.

Supplemental Figure 4. Combined inhibition of FLT3-ITD and BCL-2 family members is well tolerated in FLT3-ITD cell line xenograft models. Individual body weight data for NSG mice engrafted with luciferase positive MV4;11 cells during treatment period as indicated.

Supplemental Figure 5. Synergistic combination of venetoclax and quizartinib only in FLT3-ITD+ cell lines. (A) Cell lines were treated for 48 hours with dose range of quizartinib and venetoclax at indicated concentrations. ATP content was determined by CellTiter-Glo and delta Bliss values were calculated and plotted as a dose range for each compound. Positive values indicate synergistic effect. (B) Cell lines were treated for 48 hours with indicated concentrations of venetoclax and quizartinib and apoptosis was assessed by annexin V staining. Values represent the percent apoptotic cells (annexin V positive) per sample for the FLT3-ITD+ cell lines. Error bars represent s.d. within the experiment. (C) Cell lines were treated for 48 hours with venetoclax in combination with sorafenib (top panel) or midostaurin (bottom panel) as indicated and cell viability was assessed by CellTiter-Glo. Values are normalized to the average of the untreated samples for each cell line.

Supplemental Figure 6. Synergistic combination of BCL-2, BCL-X_L and MCL-1 demonstrated in FLT3-ITD+ cell lines. (A) Cell lines were treated for 48 hours with dose range of AMG 176 + navitoclax, AMG 176 + A1331852, A1331852 + venetoclax or AMG 176 + venetoclax as indicated. ATP content was determined by CellTiter-Glo and delta Bliss values were calculated and plotted as a dose range for each compound. Positive values indicate synergistic effect. (B) Western blot analysis (left panel) of BCL-2 family members in MV4;11 cell grown *in vivo* vs. *in vitro* as indicated. Protein expression was normalized to β-actin for each sample and values are plotted in the right panel.

Supplemental Figure 7. BCL-X_L and MCL-1 expression are regulated post-transcriptionally by FLT3-ITD inhibition. (A) Quantified protein expression for BCL-2

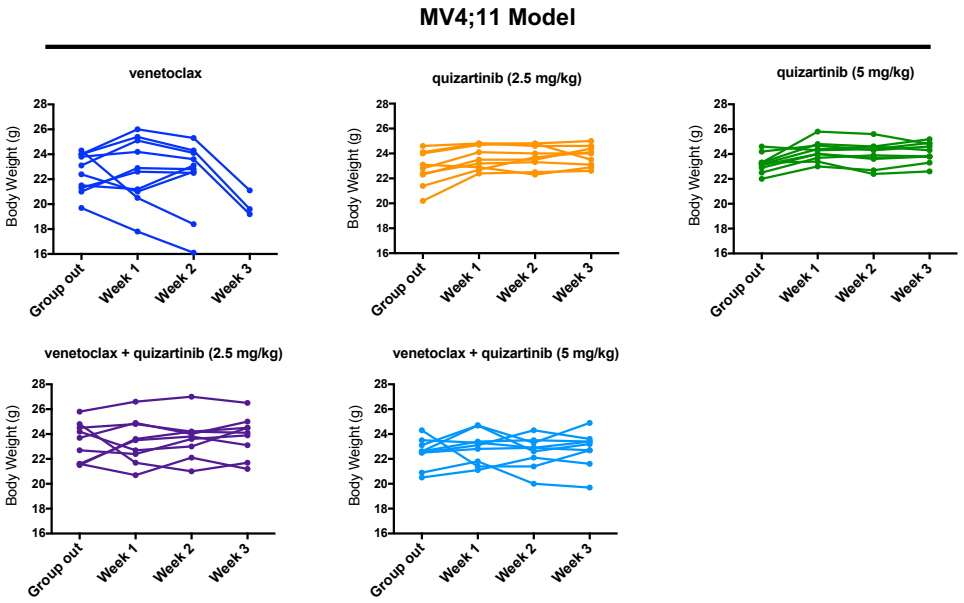
family members in indicated cell lines. Protein expression was first normalized to β-actin then normalized to the untreated sample for each cell line per experiment. Data represents average fold change in protein expression relative to untreated ± s.e.m. across experiments (n=5 independent experiments). For the MV4;11 cell line, * p = 0.0024 and 0.0031 for BCL-X_L following 5 nM and 10 nM quizartinib treatment vs. untreated, respectively; ** p < 0.0001 for MCL-1 following 5 nM and 10nM quizartinib treatment vs. untreated by one-way ANOVA with Tukey post-test. For the Molm13 cell line, * p = 0.0005 and <0.0001 for BCL-X_L following 5 nM or 10 nM quizartinib treatment vs. untreated, respectively; ** p <0.0001 for MCL-1 following 5nM and 10 nM quizartinib treatment vs. untreated; and *** p = 0.0055 for MCL-1 following 10 nM quizartinib treatment vs. 5 nM treatment by one-way ANOVA with Tukey post-test. (B) Cells were treated for 24 hours with 10 nM quizartinib, 100 nM midostaurin or 10 nM sorafenib as indicated and MCL-1 protein was assessed by Western blot. (C) Quantified protein expression for MCL-1 following 10 nM quizartinib treatment for the indicated time point from panel B. Protein expression was first normalized to β-actin then normalized to the untreated sample for each cell line per experiment. Data represents average fold change in protein expression relative to untreated ± s.e.m. across experiments (n=4 independent experiments). For both the MV4;11 and Molm13 cell lines, * p < 0.0001 for 8, 16 and 24 hour treatment vs. untreated by one-way ANOVA with Tukey post-test. (D) Fold change in gene expression of *BCL-2*, *BCL-X_L (BCL2L1)*, *MCL-1* and *BIM* (*BCL2L11*) at 4 and 24 hours post 10 nM quizartinib treatment. Data represent the mean fold change relative to untreated ± s.d. within the experiment. Data representative of 2 independent experiments. (E) MV4;11 cells were treated for 8 hours with 2 μM MG132, 10 μM Z-VAD-FMK or 10 nM quizartinib alone or in combination as indicated. Cell lysates were analyzed for MCL-1 and β-actin by Western blot (left panel). Data is representative of 3 independent experiments.

Quantification of MCL-1 protein expression for indicated treatment (right panel). MCL-1 protein expression was normalized to β-actin first then to the vehicle-treated sample for each experiment. Data represents average fold change in MCL-1 protein expression relative to untreated ± s.e.m. across experiments (n=3). * p=0.0147 for quizartinib + MG132 vs. quizartinib and ** p=0.0462 for quizartinib + MG132 vs. quizartinib + Z-VAD-FMK by one-way ANOVA with Tukey post-test.

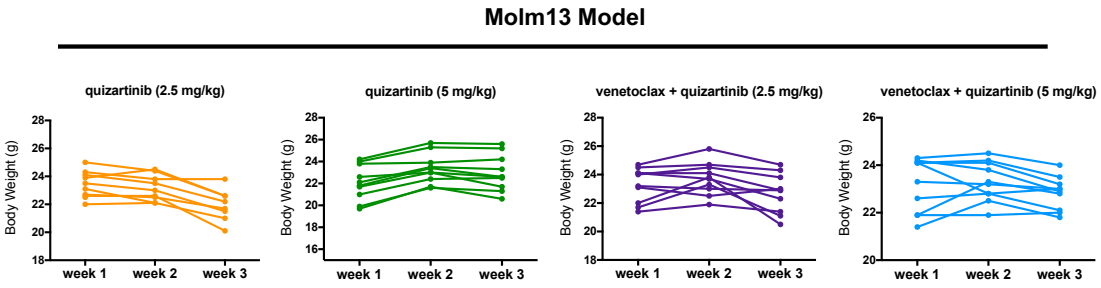
Supplemental Figure 8. PI3K, MEK and JAK-STAT signaling contribute to survival of FLT3-ITD+ cell lines *in vitro*. (A) Cell lines were treated with 1 μM pictisilib, 100 nM cobimetinib or 1 μM ruxolitinib for 24 hours and cell lysates were assessed by Western blot as indicated. (B) Quantified protein expression for BCL-2 family members in indicated cell lines. Protein expression was first normalized to β-actin then normalized to the untreated sample for each cell line per experiment. Data represents average fold change in protein expression relative to untreated ± s.e.m. across experiments (n=3 independent experiments). For BCL-2, *p=0.0288 for cobimetinib vs. vehicle in the HL60 cell line. For MCL-1, *p=0.0344 and 0.0231 for cobimetinib vs. vehicle in the HL60 and OCI-AML3 cell lines, respectively; and **p=0.0149 for ruxolitinib vs. vehicle in the MV4;11 cell line by one-way ANOVA with Dunnett post-test. (C) Cells were treated for 48 hours with a dose range of venetoclax and pictisilib, cobimetinib or ruxolitinib as indicated and Bliss sums were calculated and plotted.

Supplemental Figure 1

1A



1B



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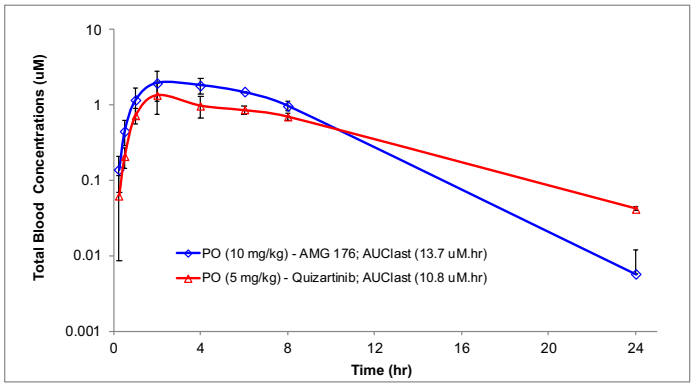
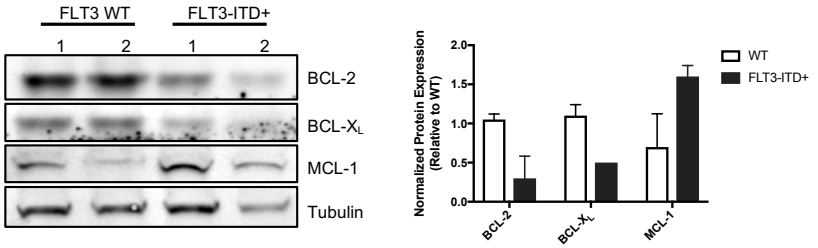


Supplemental Figure 3

3A

FLT3 WT PDX	FLT3-ITD PDX
cytogenetics -Y; EZH2, NRAS, TET3	cytogenetics diploid; DNMT3A, IDH1, NPM1

3B



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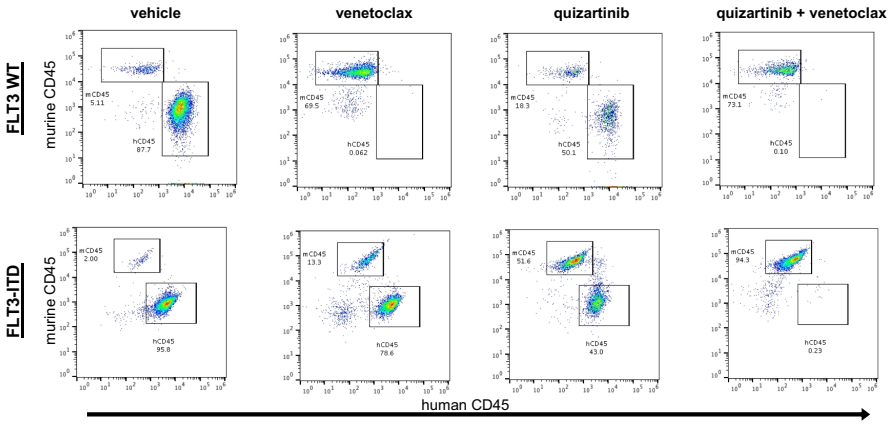
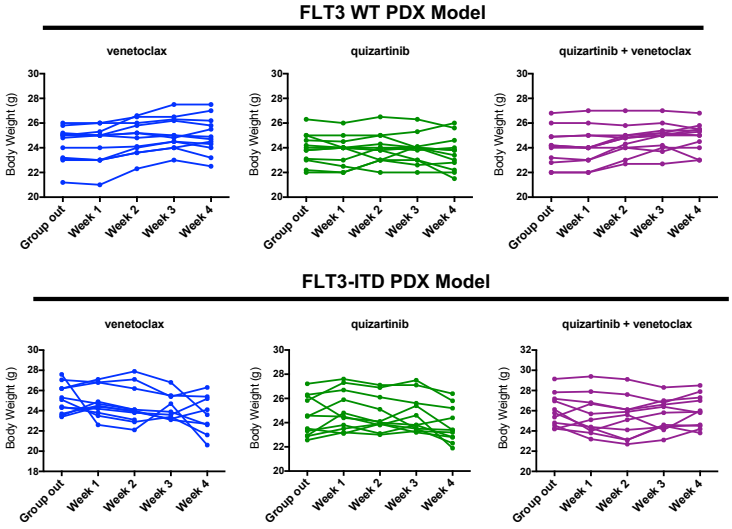
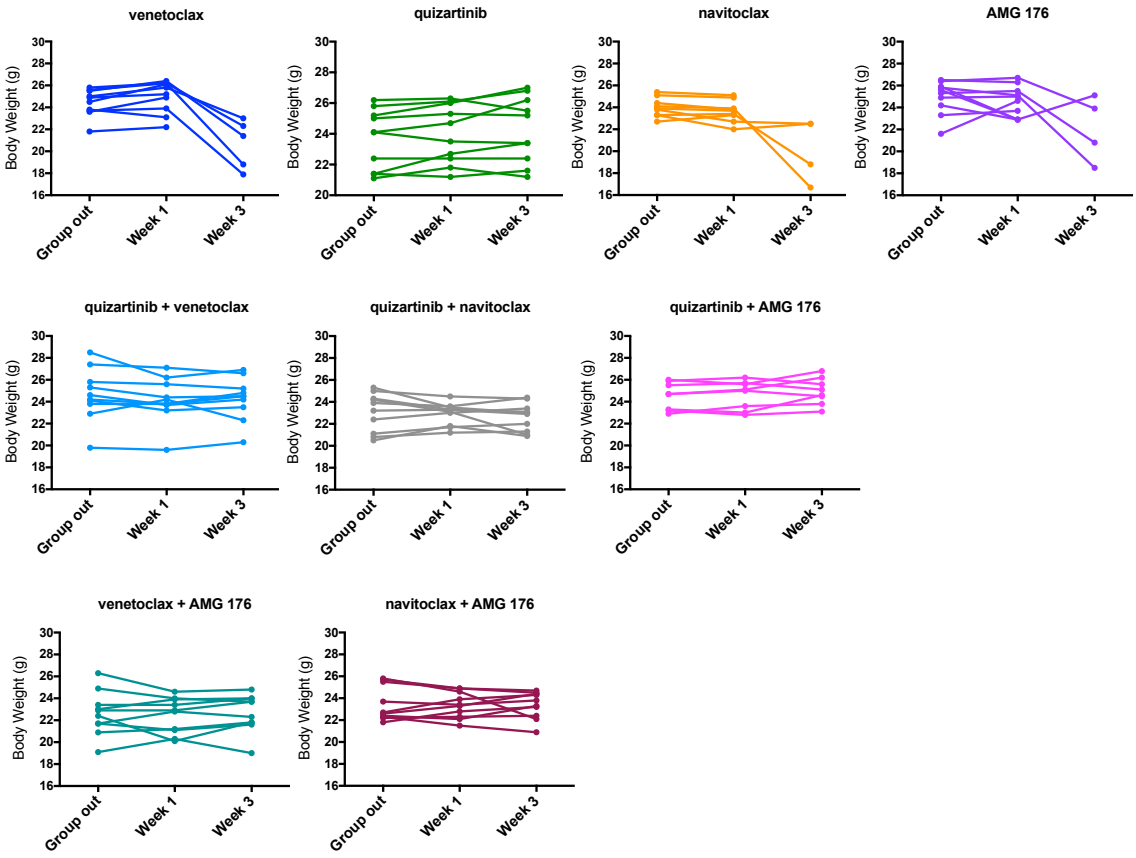


3D

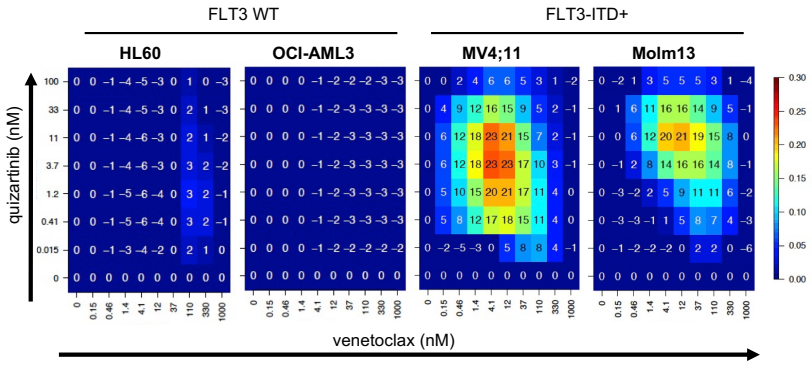


Supplemental Figure 4



Supplemental Figure 5

5A



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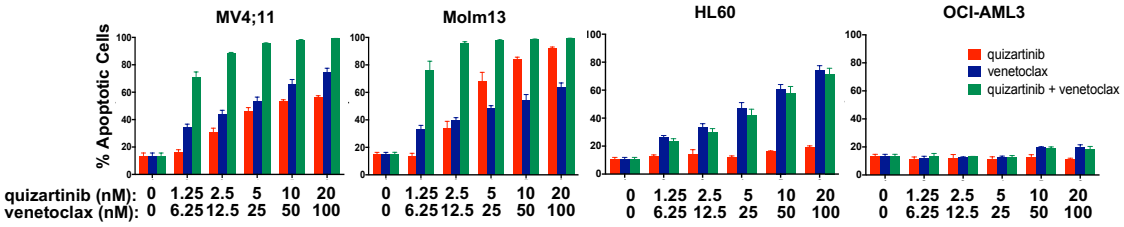

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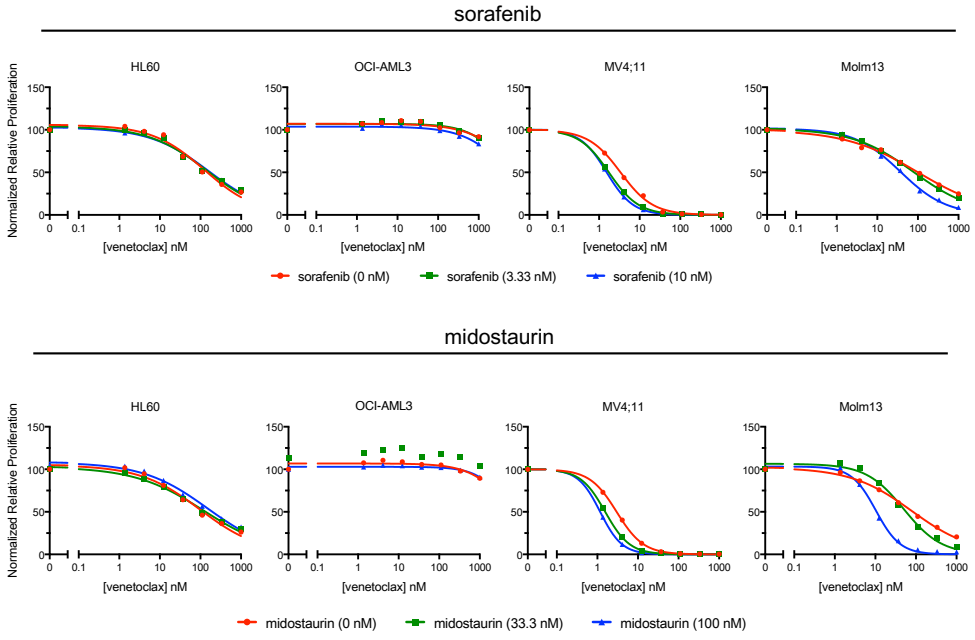
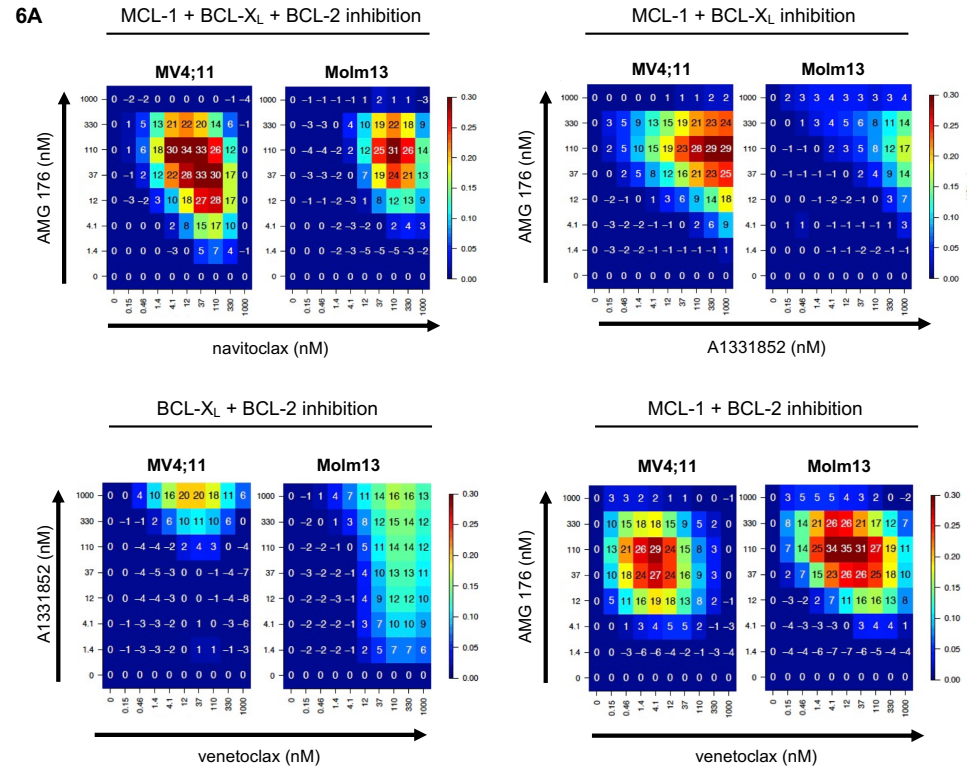


Supplemental Figure 6



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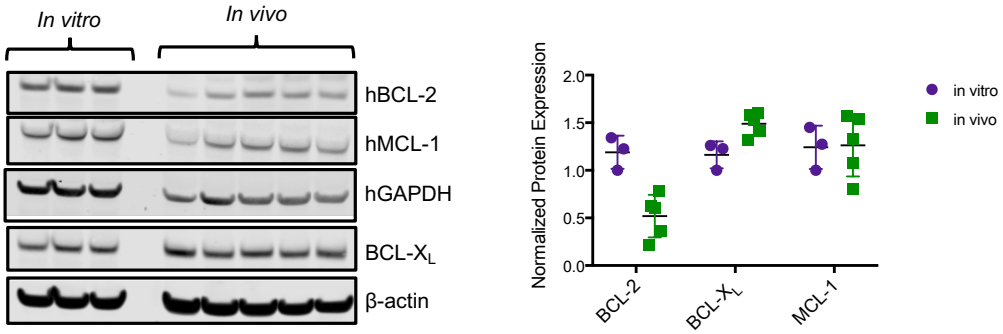
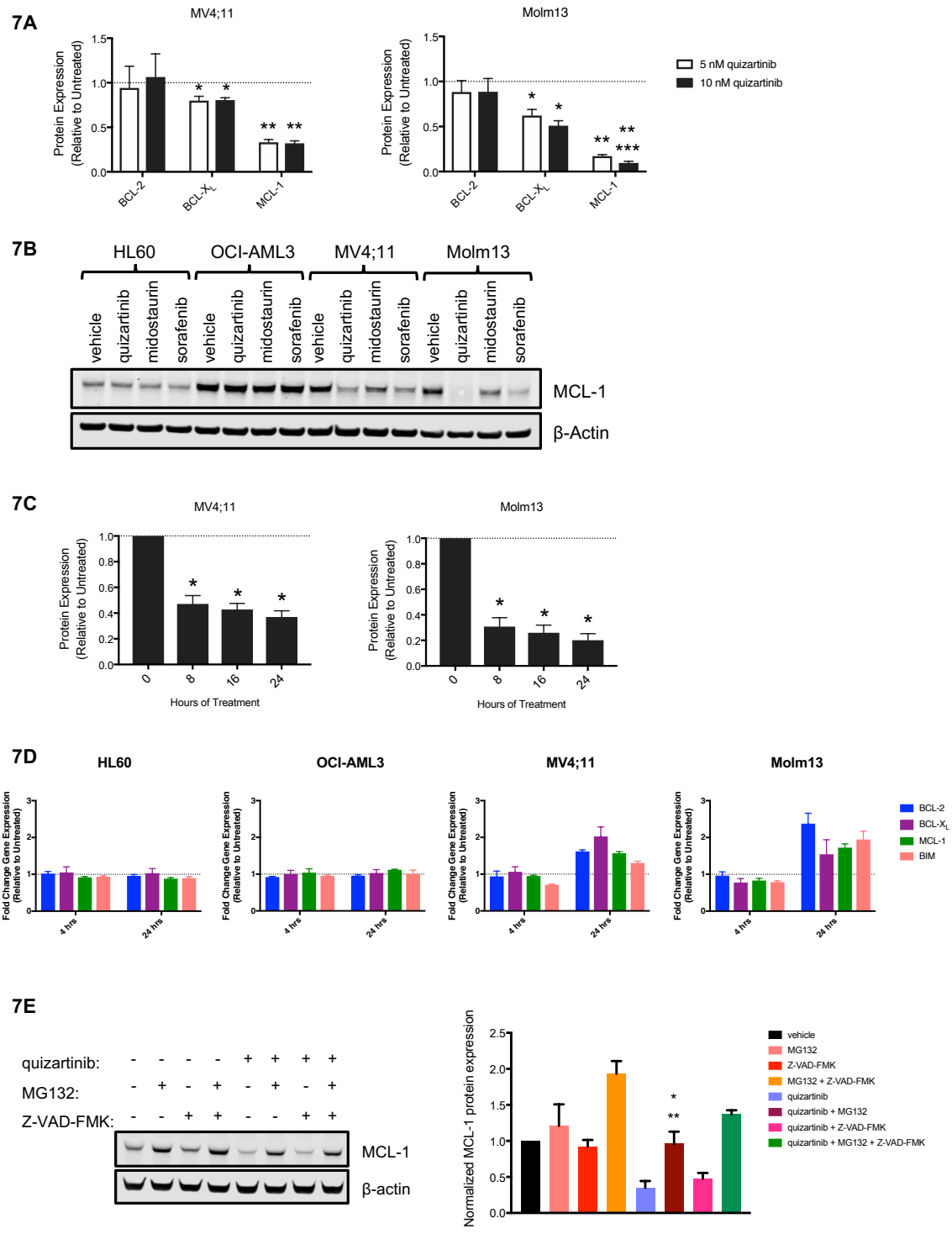


Supplemental Figure 7



Supplemental Figure 8