Steroid-free combination of 5-azacytidine and venetoclax for the treatment of multiple myeloma

Lyndsey Flanagan,[1] Aisling Coughlan,[1] Nicola Cosgrove,[1] Andrew Roe,[1] Yu Wang,[1] Stephanie Gilmore,[1] Izabela Drozdz,[2] Claire Comerford,[2,3] Jeremy Ryan,[4] Emma Minihane,[4] Salma Parvin,[4] Michael O'Dwyer,[5] John Quinn,[2,3] Philip Murphy,[2,3] Simon Furney,[1] Siobhan Glavey[2,3#] and Tríona Ní Chonghaile[1#]

[1]Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland; [2]Department of Pathology, Royal College of Surgeons in Ireland, Dublin, Ireland; [3]Department of Haematology, Beaumont Hospital, Dublin, Ireland; [4]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA and [5]Department of Medicine/Haematology, University of Galway, Galway, Ireland

[#]*SGl and TNC contributed equally as senior authors.*

Correspondence: T. Ní Chonghaile
tnichonghaile@rcsi.com

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Supplementary Materials and Methods

siRNA Transfection

JJN3 cells were counted, washed in PBS and resuspended in OptiMeM (Gibco Cat# 31985-047), 400,000 cells were combined with a mix of 4 μL Lipofectamine (Invitrogen Cat# L3000-008) and 4 μL of siRNA targeting PKR, NOXA or non-targeting control (siRNAs purchased from Dharmacon reagents, Horizon Discovery), and plated in each well a 6-well plate. RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 1% L-Glutamine (2 mM) (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% v/v penicillin/streptomycin (50 units/ml) (Gibco, Invitrogen, Carlsbad, CA, USA) was added after 2 hours (hrs) to promote cell growth. Following 24 hrs after transfection, cells were treated with drug or DMSO control for an additional 24 hrs, then cell viability was determined using flow cytometry on the BD LSR II flow cytometer. The remaining cells were harvested for western blot analysis.

BH3 Profiling

BH3 profiling was performed using whole cell (JC-1) plate-based fluorimetry as described in the following papers(1-3). BH3 peptides (see **Table S3**) in DTEP buffer (300 mM Trehalose, 10 mM HEPES-KOH, 0.1 % w/v BSA, 1 mM EDTA, 1 mM EGTA, 80 mM KCl, 5 mM succinate, final pH 7.4) were plated at 70 μM/L (unless otherwise stated) in triplicate in a black 384-well plate. The BH3 peptides used and their binding affinities are listed in Table 1. MM cell lines were harvested, washed and resuspended in DTEP buffer. An equal volume of dye Mastermix (1 μM JC-1, 0.005% digitonin, 10 μg/ul oligomycin, 5 mM B-mercaptoethanol in DTEP buffer) was added and after 10 minutes (min) at room temperature, the cells were plated on top of the peptide

template at the concentration of 40,000 cells per well. Mitochondrial potential loss was measured using the Varioskan™ kinetic plate reader at excitation 545 nm and emission 590 nm for 3 hrs at the temperature control to 27 degrees (kinetic measurements every 5 min). Mitochondrial depolarization was normalized to DMSO control (0%) and positive control FCCP (100%) (carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone).

Dynamic BH3 profiling

JJN3 cells were seeded at 3 x 10^6 cells in a T25 flask for 24 hrs, cells were treated with 3 μM 5-azacytidine for 20 hrs. Cells were collected, washed 1x with PBS and resuspended in 100 μL of FACS buffer (1% FBS 0.4% EDTA (2 mM) in PBS, final pH 7.4). Samples were blocked with Human Fc Block solution for 5 min (1:100) (BD Biosciences, Pharmingen, San Diego, CA cat #564219). Samples were incubated with live/dead far red stain (Invitrogen) (1:100) on ice in the dark for 30 min. Cells were pelleted at 500 x g for 5 min at 4^{o}C, washed with PBS, resuspended in DTEB buffer (300 mM Trehalose, 10 mM HEPES-KOH, 0.1% w/v BSA, 1 mM EDTA, 1 mM EGTA, 80 mM KCl, 5 mM succinate, final pH 7.4), and incubated with a panel of BH3 peptides (please see Table 1 for list of BH3 peptides and binding affinities). DTEB was added to each eppendorf tube containing twice the final concentration of each peptide treatment in 100 μL of DTEB with 0.002% w/v digitonin. The permeabilized cells were exposed to peptides for 60 min at 21 ^{o}C in the dark. Following peptide exposure, the samples were fixed with 200 μL 8% formaldehyde in PBS for 15 min at room temperature and quenched with 50 μL of 100 mM Tris / 2.5 M glycine pH 8.2 for 10 min. Cells were pelleted 1500 x g for 5 min at RT and were stained with anti-cytochrome c-FITC (#610324, Biolegend, San Diego, CA, USA) 1:100 in 0.1%

Saponin/1% BSA/PBS overnight and in dark at 4°C. The samples were resuspended in 400 μL PBS. MOMP was measured by assessing the loss of cytochrome *c* using FACS Canto II and the data was normalized to controls.

Annexin V/ Propidium Iodide staining

Apoptosis following BH3 mimetic treatment in a panel of MM cells using Annexin V-FITC/ Propidium Iodide (PI) staining. Cell lines were seeded at 3 x 10^4 cells in a 24-well plate for 24 hrs, cells were treated with venetoclax (ven), ABT-263, AMG-176, and WEHI-539 for 24 hrs. Following treatment, the cells were harvested and centrifuged at 500 g for 5 min and then washed with PBS. Each sample was re-suspended in 250 μL of Annexin V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) Annexin V-FITC (0.25 mg/ml) and propidium iodide (PI) (1 mg/ml). Cell viability was determined using flow cytometry on the BD LSR II flow cytometer. The results were normalized to the DMSO-only control and dose-response curves were graphed using GraphPad Prism.

Western blot analysis

The cells were harvested and centrifuged at 500 rcf for 5 min. The supernatant was discarded, and the pellet was washed with PBS, before the addition of the lysis buffer. 100 μL of RIPA buffer (150 nM NaCl, 20 μM Tris, 0.5% Triton, 1 mM PMSF) was added to each sample, vortexed and incubated for 30 min on ice. The samples were then centrifuged at 12,000 RPM for 10 min. To quantify the amount of protein in each sample, the BCA assay was used, as per the manufacturer's instructions (Pierce). A total of 20 μg of protein from each sample was separated with 12% SDS-PAGE gels and transferred to nitrocellulose membranes at 40 V for 2 hrs. The blots were

incubated with the following antibodies: anti-BCL-2 (Cell Signaling Technology Cat# 15071S), anti-BCL-xL (Cell Signaling Technology Cat# 2764S), anti-MCL-1 (Cell Signaling Technology Cat# 94296), anti-NOXA (Abcam Cat# ab13654), anti-eIF2α (Cell Signaling Technology Cat# 5324S) anti-Phospho-eIF2α (Cell Signaling Technology Cat# 9721S), anti-DNMT1 (Cell Signaling Technology Cat# 5032S), anti-PKR (Invitrogen Cat#700286), anti-ATF4 (Cell Signaling Technology Cat# 11815) and anti-β-actin was used as a loading control (Sigma-Aldrich Cat# A2228). The blots were imaged using the ImageQuant LAS 4000 and analyzed using Image J software.

Immunoprecipitation

Cells were plated at 5 x 10^6 for 24 hrs and treated with DMSO or 3 μM 5-azacytidine for 24 hrs. Cellular proteins were extracted and cell lysates (500 μg) were pre-cleared with 20 μl of agarose beads (Piece Protein A Agarose, Thermofisher cat no. 20333) and rotated for 1 hr at 4°C at 3 RPM. Cell lysates (500 μg) were then subjected to immunoprecipitation by incubating with 40 μl of agarose beads and 20 μl of MCL-1 antibody (Cell Signaling Technology Cat# 94296) or IgG control in the presence of protease inhibitors overnight at 4°C rotating at 3 RPM. The immunocomplexes were applied to 18% SDS-polyacrylamide gels and blotted to a nitrocellulose membrane for Western blotting.

Co-culture with fibroblasts

JJN3, MM1S, HS-5 and MM-BMSC were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 1% L-Glutamine (2 mM) (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% v/v penicillin/streptomycin (50 units/ml) (Gibco, Invitrogen, Carlsbad, CA, USA). For the

co-culture system, HS-5 or MM-BMSC cells were seeded in a 24-well plate at 5 x 10^4 cells for 24 hrs. The day after 1 x 10^5 JJN3 or MM1S cells were seeded on top of the confluent monolayer for 24 hrs. Cells were treated with 5-azacytidine (5-aza), ven or 5-aza and ven for 24 hrs and cell viability was assessed using Annexin V/PI staining.

CD138$^+$ cell isolation from primary MM bone marrow samples

Ethical approval was granted from the Beaumont hospital and RCSI ethics committee, study number 19/32. Upon receipt of a primary patient sample, red cell lysis buffer (Roche) is added at 3 times the volume of the patient sample and incubated at room temperature for 10 min. The sample is then centrifuged at 1500 rpm for 5 min and the supernatant is discarded. The pellet is resuspended in 20 mL PBS for the total cell count. The sample is centrifuged again and resuspended in the appropriate amount of CD138 magnetic beads and MACS buffer (Miltenyi Biotec) 20 μL of beads per 10 million cells, 80 μL of buffer per 10 million cells. The sample is incubated on ice for 15 min before 20 mL of MACS buffer is added and the sample is centrifuged. The supernatant is removed, and the sample is resuspended in 1 mL of MACS buffer. The sample is run through the magnetic LS column, followed by 3 x 3 mL washes with MACS buffer. The CD138$^+$ cells are collected and counted. Once counted, CD138$^+$ cells were seeded at 1 x 10^4 cells per well in a 96-well plate. Cells were treated with BH3 mimetics (ABT-199, A-1331852, AMG-176) at doses ranging from 30 nM to 3 μM, 5-aza 3 μM +/- ven 100 nM or 300 nM, for 16 hrs. Cell viability was assessed using Annexin V/PI staining as described above. The MM-BMSC cells were grown by harvesting the negative fraction of cells that ran through the CD138$^+$ column. The cells were washed with PBS then seeded into a T25 with RPMI media and grown for two

weeks until a confluent layer of fibroblasts was cultured. The MM-BMSC cells were then seeded for further experiments.

Epigenetic modifier screen using CellTiter-Glo®

Cells were seeded in a 96-well plate at a density of 1 x 10^4 cells per well for 24 hrs and treated with 2 doses (500 nM, 100 nM) of epigenetic modifier (see Table S1) alone and in combination with ven (1 μM for 24 hrs). Following treatment, 20 μL of CellTiter-Glo® is added to each well at RT for 30 min. Luminescence is read on the ClarioStar plate reader as per the manufacturer's instructions. The results were normalized to the DMSO-only control and heatmaps were graphed using Graphpad Prism.

Live cell microscopy

JJN3 cells were seeded at 15,000 cells/well in a μ-Slide 8-well high chambers (Ibidi, cat no. 80801) for 24 hrs and treated with DMSO, 5-azacytidine 3 μM, ven 1 μM and 5-azacytidine and ven for 24 hrs. PKMO(4) (Spirochrome, cat no. SC053) and Picogreen staining (0.1%) (Thermofisher, cat no. P11495) was added at 300 nM concentration for 20 min in RPMI 1640 cell culture medium at 37°C. Cells were washed twice with warmed medium and imaged via confocal microscopy on a Leica Stellaris 8 system. A minimum of 5 images were taken for each condition and analysed using Fiji software.

dsRNA staining

For immunofluorescence microscopy, JJN3 cells were seeded at 3 x 10^4 cells in a 24-well plate for 24 hrs. Following this, the cells were treated with DMSO, 5-azacytidine 3 μM, ven 1 μM or 5-azacytidine and ven for 24 hrs. The cells were harvested and

washed in PBS. The cells were incubated with 200 nM mitotracker red (Invitrogen, cat no. M22425) for 30 min protected from light at 37°C. The cells were washed twice with PBS and cytospun at 400 x rcf for 6 minutes onto glass slides. The cells were fixed with ice-cold 4% paraformaldehyde (PFA) for 15 min protected from light. The cells were washed 3 times with ice-cold PBS and permeabilized with Triton-X100 (0.5%) for 15 minutes. The cells were washed 3 times with PBS and blocked with 5% BSA/Hoechst (1:1000) for 1 hr. The cells were washed in PBS before being incubated with anti-dsRNA (1:700) overnight at 4°C (Sigma-Aldrich, cat no. MABE1134). The cells with incubated for 2 hrs in secondary antibody (Goat anti-mouse IgM, Alexa Fluor 594, Invitrogen, cat no. A-21044) diluted in 5% BSA at room temperature. After washing the cells with PBS, the coverslip was mounted to the glass slide using mounting medium and allowed to dry at room temperature for a 2 hrs. The slides were imaged via confocal microscopy on a Leica Stellaris 8 system. 5 images were taken per treatment and images were analysed using Fiji software.

Peripheral Blood Mononuclear Cells (PBMC) Annexin V/Propidium Iodide staining

Apoptosis following treatment with 5-azacytidine/ven and bortezomib, dexamethasone and ven in PBMCs using AnnexinV-FITC/ Propidium Iodide (PI) staining. PBMCs were seeded at 3 x 10^4 cells in a 96-well plate, cells were treated with 5-azacytidine alone, ven alone or in combination for 16 hrs. Additionally, cells were treated with bortezomib alone, dexamethasone alone, ven alone or in combination for 16 hrs. Following treatment, the cells were stained with cell surface markers CD19 (Miltenyi; cat no. 561295) and CD3 (Miltenyi; cat no. 317317) for 30 min on ice protected from light. The cells were then centrifuged at 500 g for 5 min and then washed with PBS. Each sample was assessed for Annexin V/Pi staining as described previously. Cell viability was

determined using flow cytometry on the BD LSR II flow cytometer. The results were normalized to the DMSO-only control and dose-response curves were graphed using GraphPad Prism.

Intracellular BCL-2 staining

PBMCs were seeded at 1 x 10^5 cells in a 96-well plate and treated with increasing concentrations of dexamethasone for 16 hrs. PBMCs were harvested and resuspended in 100 uL of 1% BSA/PBS and blocked with a human FcR blocker (1:100) for 10 min on ice. Cells were then stained with anti-CD3-APC (cat no. 555335) at 1:100 dilutions for 30 min on ice protected from light. Cells were fixed in 2% formaldehyde for 15 min protected from light and neutralized with 1M Tris for 5 min. Cells were resuspended in staining buffer (1% BSA, 2% FBS, 0.1% Saponin and 1.5 mM NaN3 in PBS) and stained with BCL-2 FITC (cat no. 130-114-339) and IgG control overnight at 4 ^{o}C. Following washing, the cells were resuspended in PBS and BCL-2 protein expression was determined using flow cytometry on the BD LSR II flow cytometer.

Data and statistical analyses

GraphPad Prism 9.0 software was used for all statistical analyses. Dose-response curves and IC_{50} values were calculated using linear regression curve fit (Log inhibitor vs. normalized response, variable slope). Unless otherwise stated, the results are expressed as the mean $\pm$ standard error of the mean (SEM) of three independent experiments.

Supplementary Figure Legends

Figure S1. BH3 profiling and BH3 mimetics identify diverse anti-apoptotic dependence in MM cell lines.

A) The induction of cell death by BH3 mimetics ABT-199, ABT-263, WEHI-529 and AMG-176 in the JJN3 cells was measured by annexin V/PI. Graphed in the dose-response curves are the mean +/- SEM of three biological repeats. The IC_{50} values are listed below the graph. B) similar to A) for the KMS18 cell line. C) Cell viability following treatment with BH3 mimetics (left) and BH3 profile (right) for C) MM1S D) KMS12-BM E) KMS-27 F) H929 G) RPMI-8266 H) U266 cells. Cells were treated with increasing doses of ABT-199, ABT-263, WEHI-539 or A-1331852 and AMG-176 for 24 hrs and cell viability was assessed by annexin V/PI staining. Cells were treated with a series of BH3 peptides at the listed concentrations. The mitochondrial membrane potential was measured using JC-1. Graphed is the normalized mitochondrial potential (based on positive and negative controls) mean ± SEM of three independent experiments.

Figure S2. Assessing potential hits from the initial epigenetic screen in JJN3 MCL-1 dependent cells.

Cell viability was assessed in JJN3 cells using annexin V/PI staining following 24 hr treatment with or without the following epigenetic modifiers A) JIB-04, B) GSK-J4 C) Panobinostat D) 5-aza E) Belinostat F) Curcumin G) CPI023 H) Decitabine I) BAS-2 J) Citarinostat K) UNC-1215 L) I-BET151 +/- 1 μM ven. Graphed are the mean values ± SEM from three independent experiments. The green dot shows the viability of 1 μM ven.

Figure S3. Assessing potential hits from the initial epigenetic screen in KMS-18 a BCL-2/BCL-xL dependent cells.

Cell viability was assessed in KMS18 cells using annexin V/PI staining following 24 hr treatment with the following epigenetic modifiers A) JIB-04, B) Panobinostat C) 5-aza D) GSK-J4 E) Citarinostat F) Belinostat G) BAS-2 H) Curcumin I) Decitabine J) UNC-1215 K) CPI-023 L) I-BET151 +/- 500 nM ven. Graphed are the mean values ± SEM from three independent experiments. The green dot shows the viability of 500 nM ven.

Figure S4. Validating the combination of ven and 5-aza in a panel of MM cell lines.

Cell viability was assessed in A) MM1S) B) H929 C) KMS-27 D) KMS-18 E) KMS12-BM cells using annexin V/PI staining following 24-hour treatment with listed doses of ven with or without 3 μM 5-aza. Graphed are the mean values ± SEM from three independent experiments, and the IC_{50} values are listed on the graph. F) Based on the dose-response curves shown in A-E the area under the curve of ven alone or ven + 5-aza 3 μM was calculated and the ratio was graphed. G) The combination index was calculated for a series of ven doses with 5-aza 3 μM using Webb's fractional product and is visualized by a heatmap with the combination index listed for each treatment. H) The combination index for KMS-27 was graphed in a heatmap separately due to the lower dose of ven. Cell viability was assessed in I) MM1S) J) KMS-189 K) RPMI-8266 L) JJN3 M) U266 cells using annexin V/PI staining following 24-hour treatment with listed doses of 5-aza with or without 1 μM ven (500 nM ven for KMS-18). N) the ratio of the area under the curve of each dose-response curve is

graphed O) The combination index of different doses of 5-aza with 1 μM ven (500 nM ven for KMS-18) is represented by a heatmap.

Figure S5. Molecular analysis of 5-azacytidine treatment in cells.

(A) Western blot analysis of DNMT1 expression following a dose-response with 5-azacytidine treatment at 24 hr. Actin blot demonstrates loading control. B) Densitometry of BIM and NOXA bound to MCL-1 following DMSO or 5-AZA 3 μM treatment. C) Densitometry of PKR siRNA Western blots shown in Figure 3H, where the data was normalized to actin densitometry. The densitometry was then normalized to each treatment to show that a downregulation by the siRNA (A mean of two independent Western blots are graphed). D) Densitometry of NOXA siRNA Western blots shown in Figure 3I, where the data was normalized to actin densitometry. The densitometry was then normalized to each treatment to show that a downregulation by the siRNA (A mean of two independent Western blots are graphed).

Figure S6. Ven and 5-azacytidine combination enhances cell death in primary MM samples *ex-vivo.* A-K) Individual graphs of cell viability following 16 hr treatment with (left) BH3 mimetics or (right) 5-azacytidine, ven and in combination. Error bars indicate duplicate measurements. L) Protein expression analysis of primary samples and MM cell lines.

Figure S7. Non-normalized ven and 5-azacytidine combination in primary MM samples *ex-vivo.* A-K) Individual graphs of cell viability following 16 hr treatment with (left) BH3 mimetics or (right) 5-azacytidine, ven and in combination. Error bars indicate duplicate measurements.

Patient number	Date of recruitment	M/F	Age	Cytogenetics	No. of prior treatments	Stage of MM
1	13/01/2022	M	60	Normal cytogenetics	NDMM, no previous treatment	ISS 2
2	26/07/2022	F	55	Cytogenetics unavailable	NDMM, no previous treatment	Data unavailable
3	19/05/2021	M	55	Normal cytogenetics	NDMM, no previous treatment	ISS 2
4	23/09/2021	M	58	P53 deletion	Five prior lines – CVD, R, IRD, DPD, KD	ISS 3
5	05/05/2021	F	78	Normal cytogenetics	First relapse – previous CVD	ISS 2
6	14/02/2022	M	59	1q amplification	Six prior lines – RVD, CVD, KD, PD, DVD, CPD-Dara clinical trial, IxaDex	ISS 3
7	20/04/2021	M	91	Cytogenetics available	NDMM, no previous treatment	Data unavailable
8	03/03/2022	M	54	Normal cytogenetics	NDMM, no previous treatment	ISS 2
9	02/09/2021	F	58	P53 deletion, t(11;14) translocation	Five prior lines – RVD, KD, DPD, IRD, PD	ISS 2
10	10/03/2021	M	69	P53 deletion, loss of CDKN2C/CKS18, t(11;14) translocation	Two prior lines – CVD, KRD	ISS 3
11	28/10/2021	F	76	IGH/CCN1 rearrangement, t(11;14) translocation	First relapse – previous RVD	ISS 2

Table S1. Patient clinical database.

Listed in the table are the characteristics including, sex, age, cytogenetics, staging and prior treatments for each of the patients who generously provided a sample for analysis.

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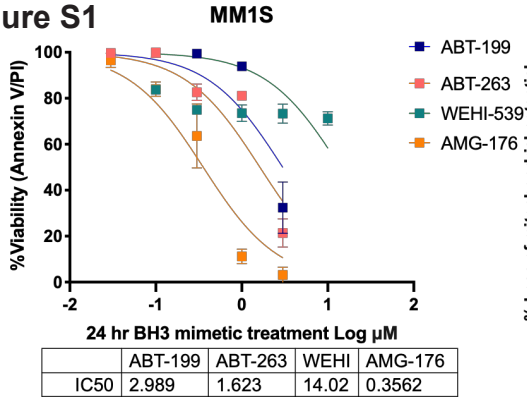
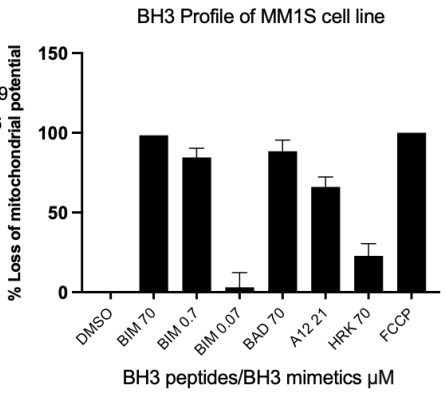
Figure S1

A



	ABT-199	ABT-263	WEHI	AMG-176
IC50	2.989	1.623	14.02	0.3562



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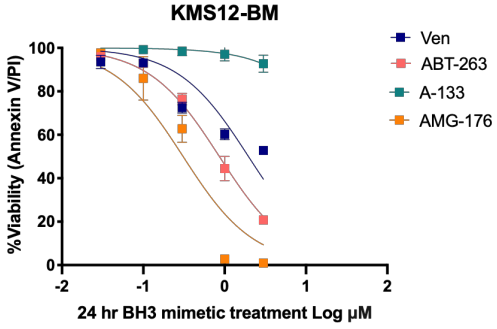

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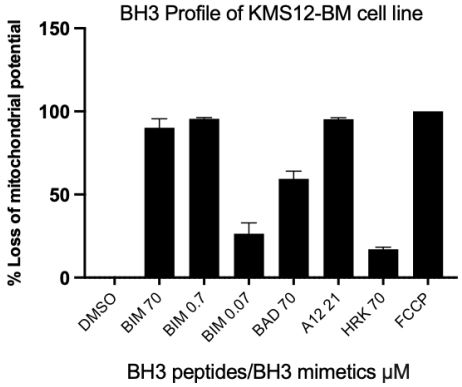
IC50	1.963	0.8612	37.21	0.3086



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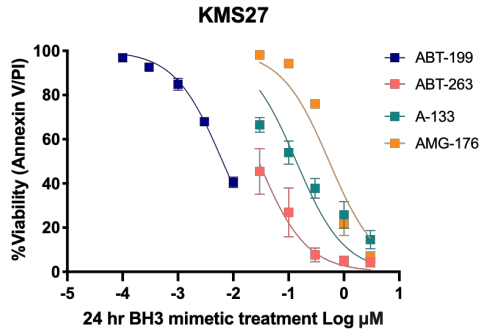

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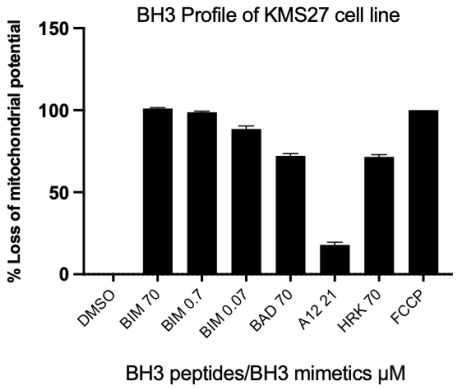
IC50	0.006395	0.02848	0.5388	0.1376



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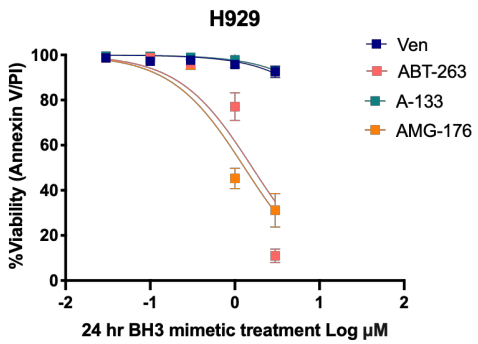

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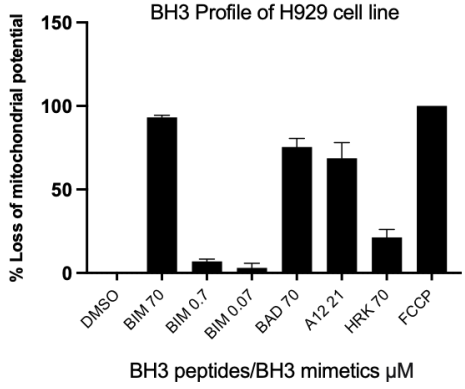
IC50	33.23	1.613	40.58	1.303



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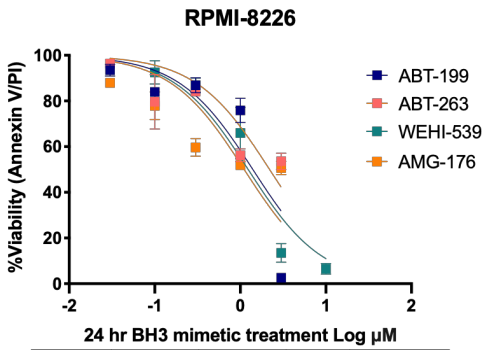

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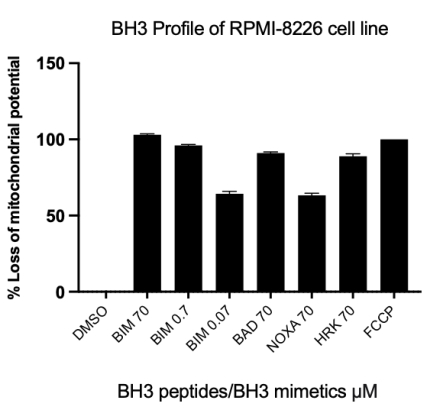
IC50	1.401	2.198	1.235	1.109



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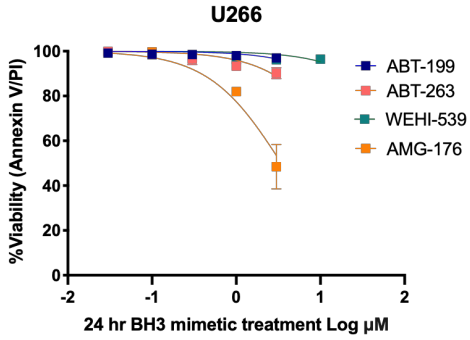

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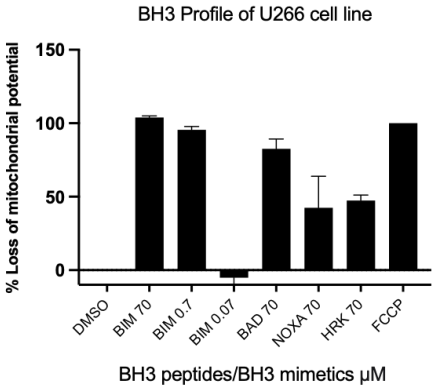
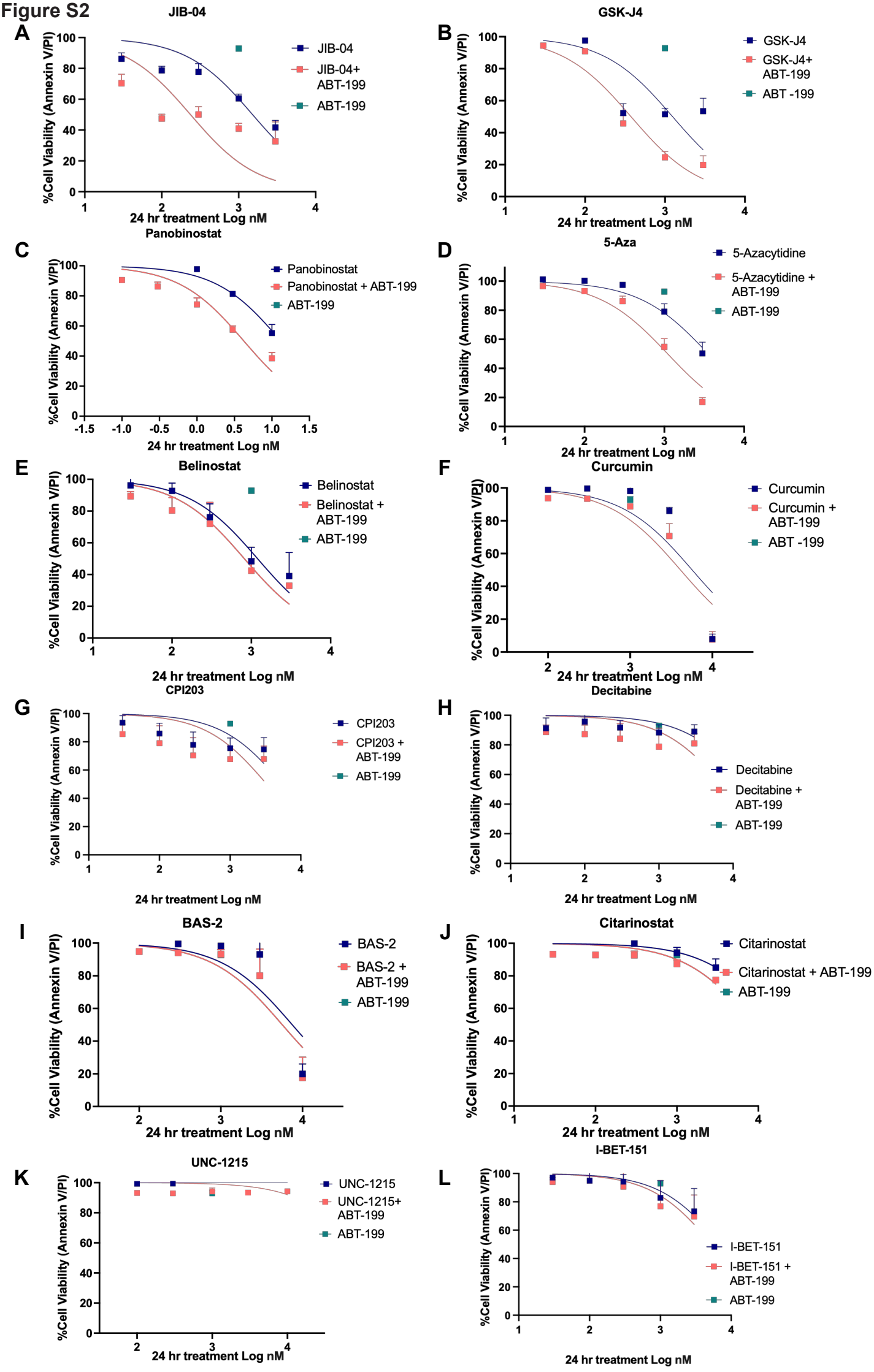
IC50	84.28	23.85	201.2	3.442



H

Figure S2


A
JIB-04
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24 hr treatment Log nM
JIB-04
JIB-04+ ABT-199
ABT-199
B
GSK-J4
GSK-J4
GSK-J4+ ABT-199
ABT -199
C
Panobinostat
Panobinostat
Panobinostat + ABT-199
ABT-199
D
5-Aza
5-Azacytidine
5-Azacytidine + ABT-199
ABT-199
E
Belinostat
Belinostat
Belinostat + ABT-199
ABT-199
F
Curcumin
Curcumin
Curcumin + ABT-199
ABT -199
G
CPI203
CPI203
CPI203 + ABT-199
ABT-199
H
Decitabine
Decitabine
Decitabine + ABT-199
ABT-199
I
BAS-2
BAS-2
BAS-2 + ABT-199
ABT-199
J
Citarinostat
Citarinostat
Citarinostat + ABT-199
ABT-199
K
UNC-1215
UNC-1215
UNC-1215+ ABT-199
ABT-199
L
I-BET-151
I-BET-151
I-BET-151 + ABT-199
ABT-199

Figure S3

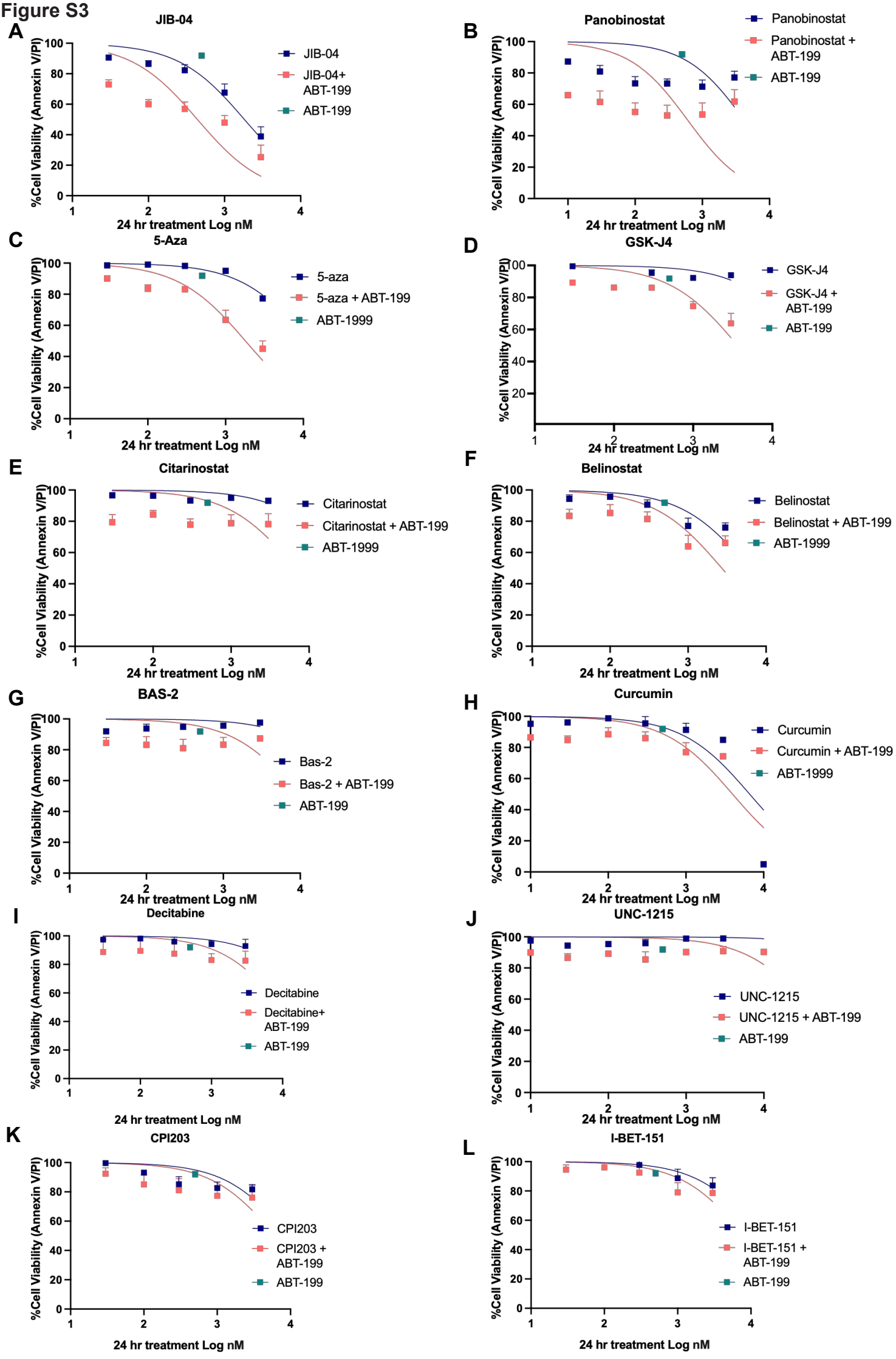

A
JIB-04
%Cell Viability (Annexin V/PI)
24 hr treatment Log nM
JIB-04
JIB-04+ ABT-199
ABT-199
B
Panobinostat
Panobinostat
Panobinostat + ABT-199
ABT-199
C
5-Aza
5-aza
5-aza + ABT-199
ABT-1999
D
GSK-J4
GSK-J4
GSK-J4 + ABT-199
ABT-199
E
Citarinostat
Citarinostat
Citarinostat + ABT-199
ABT-1999
F
Belinostat
Belinostat
Belinostat + ABT-199
ABT-1999
G
BAS-2
Bas-2
Bas-2 + ABT-199
ABT-199
H
Curcumin
Curcumin
Curcumin + ABT-199
ABT-1999
I
Decitabine
Decitabine
Decitabine+ ABT-199
ABT-199
J
UNC-1215
UNC-1215
UNC-1215 + ABT-199
ABT-199
K
CPI203
CPI203
CPI203 + ABT-199
ABT-199
L
I-BET-151
I-BET-151
I-BET-151 + ABT-199
ABT-199

Figure S4

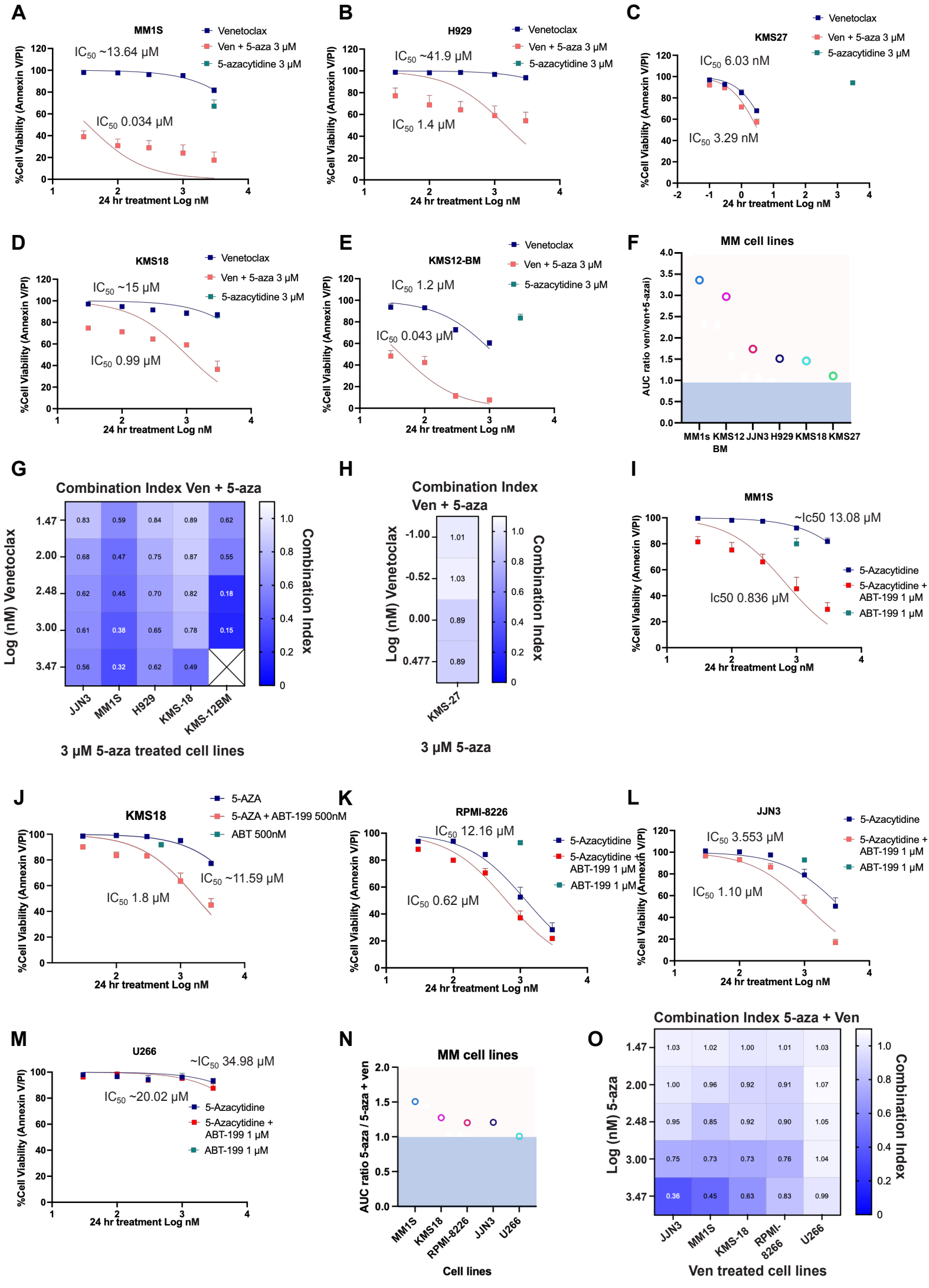
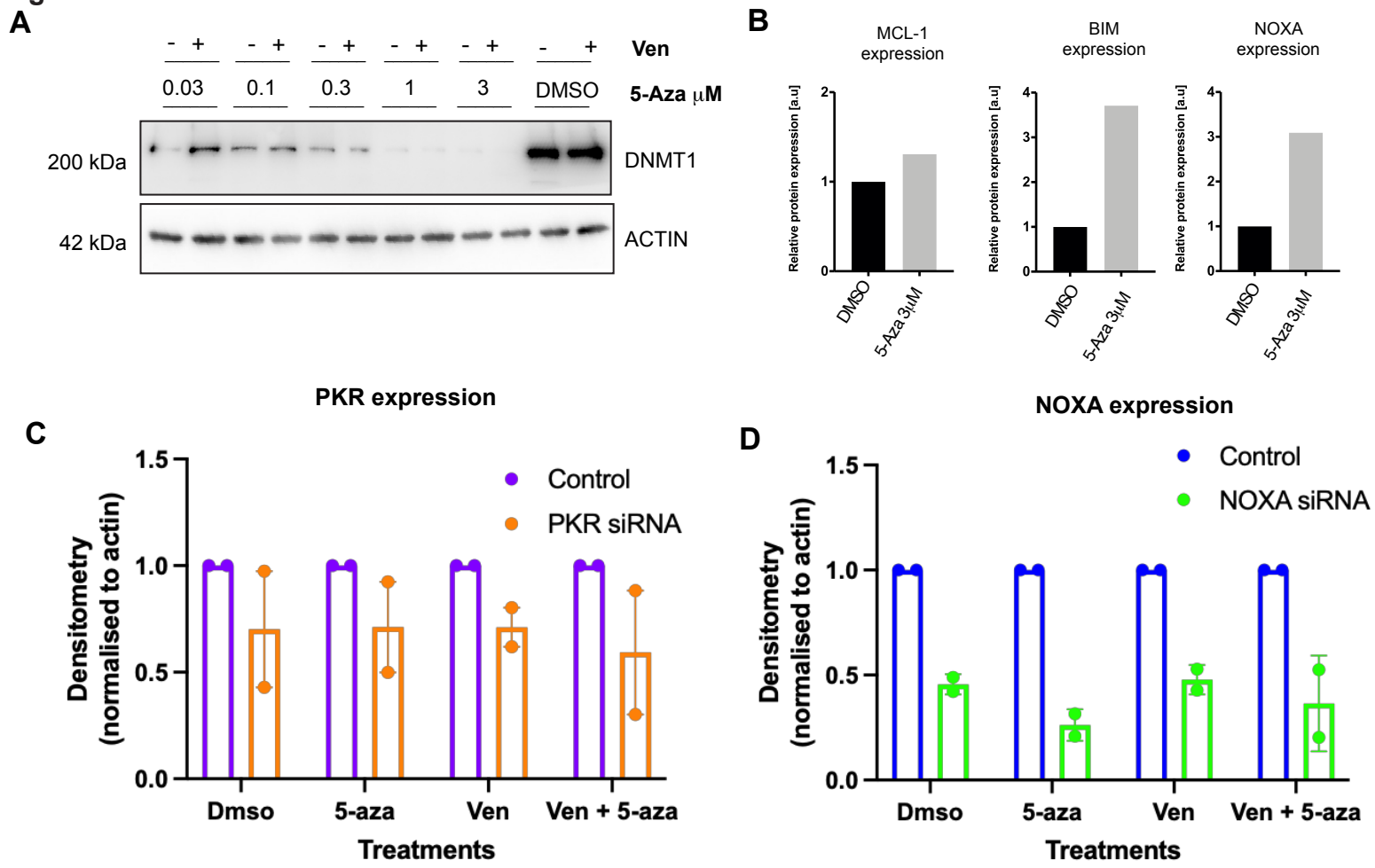

A
MM1S
Venetoclax
Ven + 5-aza 3 μM
5-azacytidine 3 μM
IC50 ~13.64 μM
IC50 0.034 μM
%Cell Viability (Annexin V/PI)
24 hr treatment Log nM
B
H929
IC50 ~41.9 μM
IC50 1.4 μM
C
KMS27
IC50 6.03 nM
IC50 3.29 nM
D
KMS18
IC50 ~15 μM
IC50 0.99 μM
E
KMS12-BM
IC50 1.2 μM
IC50 0.043 μM
F
MM cell lines
AUC ratio ven/ven+5-azai
MM1s
KMS12 BM
JJN3
H929
KMS18
KMS27
G
Combination Index Ven + 5-aza
Log (nM) Venetoclax
1.47
2.00
2.48
3.00
3.47
0.83 0.59 0.84 0.89 0.62
0.68 0.47 0.75 0.87 0.55
0.62 0.45 0.70 0.82 0.18
0.61 0.38 0.65 0.78 0.15
0.56 0.32 0.62 0.49
JJN3
MM1S
H929
KMS-18
KMS-12BM
Combination Index
3 μM 5-aza treated cell lines
H
Combination Index
Ven + 5-aza
-1.00
-0.52
0.00
0.477
1.01
1.03
0.89
0.89
KMS-27
3 μM 5-aza
I
MM1S
~Ic50 13.08 μM
Ic50 0.836 μM
5-Azacytidine
5-Azacytidine + ABT-199 1 μM
ABT-199 1 μM
J
KMS18
5-AZA
5-AZA + ABT-199 500nM
ABT 500nM
IC50 ~11.59 μM
IC50 1.8 μM
K
RPMI-8226
IC50 12.16 μM
IC50 0.62 μM
L
JJN3
IC50 3.553 μM
IC50 1.10 μM
M
U266
~IC50 34.98 μM
IC50 ~20.02 μM
N
MM cell lines
AUC ratio 5-aza / 5-aza + ven
MM1S
KMS18
RPMI-8226
JJN3
U266
Cell lines
O
Combination Index 5-aza + Ven
Log (nM) 5-aza
1.47
2.00
2.48
3.00
3.47
1.03 1.02 1.00 1.01 1.03
1.00 0.96 0.92 0.91 1.07
0.95 0.85 0.92 0.90 1.05
0.75 0.73 0.73 0.76 1.04
0.36 0.45 0.63 0.83 0.99
JJN3
MM1S
KMS-18
RPMI-8266
U266
Ven treated cell lines

Figure S5


A
- + - + - + - + - + - + Ven
0.03 0.1 0.3 1 3 DMSO 5-Aza μM
200 kDa
DNMT1
42 kDa
ACTIN
B
MCL-1 expression
BIM expression
NOXA expression
Relative protein expression [a.u]
DMSO
5-Aza 3μM

C
PKR expression
Control
PKR siRNA
Densitometry (normalised to actin)
Dmso
5-aza
Ven
Ven + 5-aza
Treatments

D
NOXA expression
Control
NOXA siRNA
Densitometry (normalised to actin)
Dmso
5-aza
Ven
Ven + 5-aza
Treatments

Figure S6

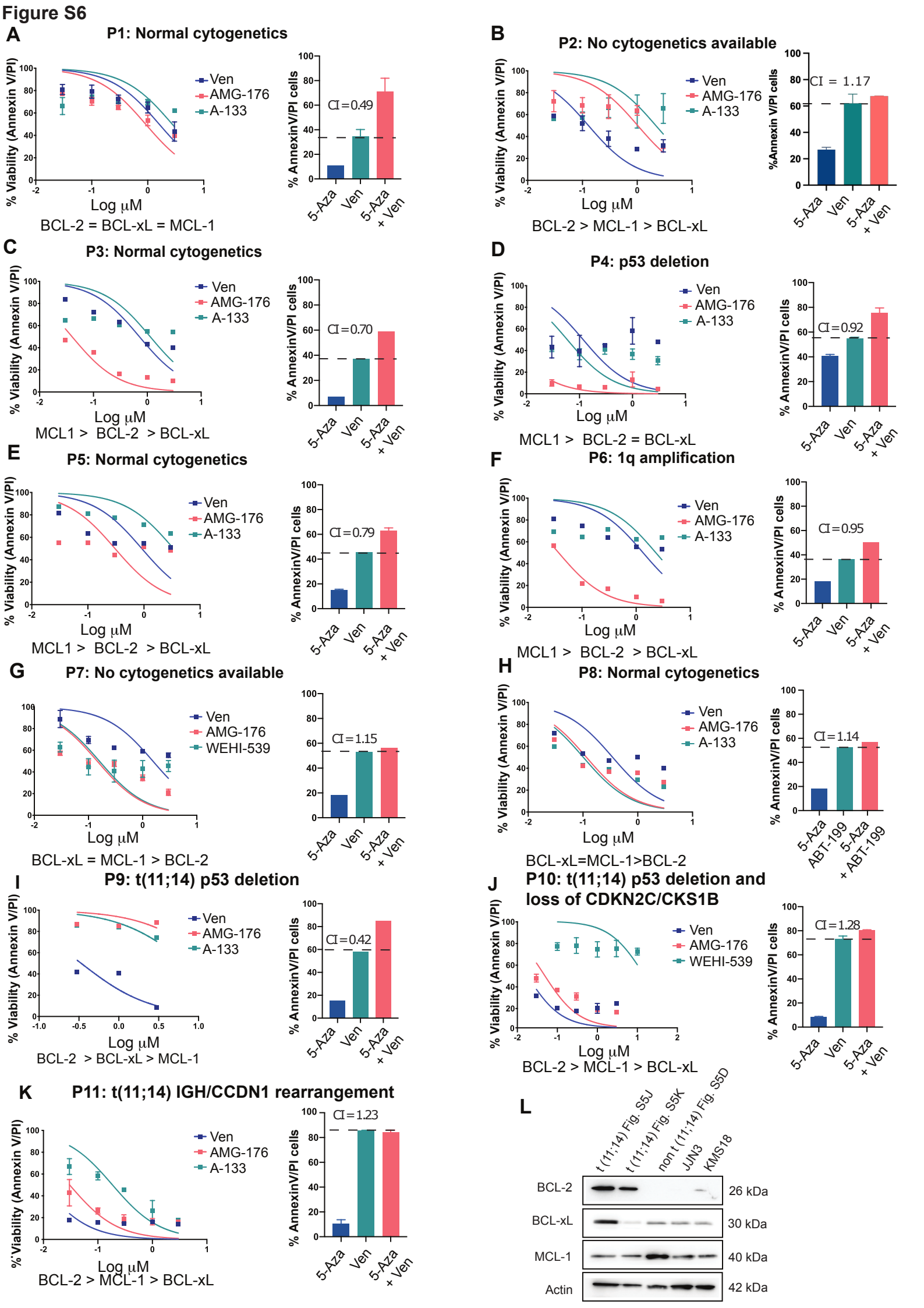
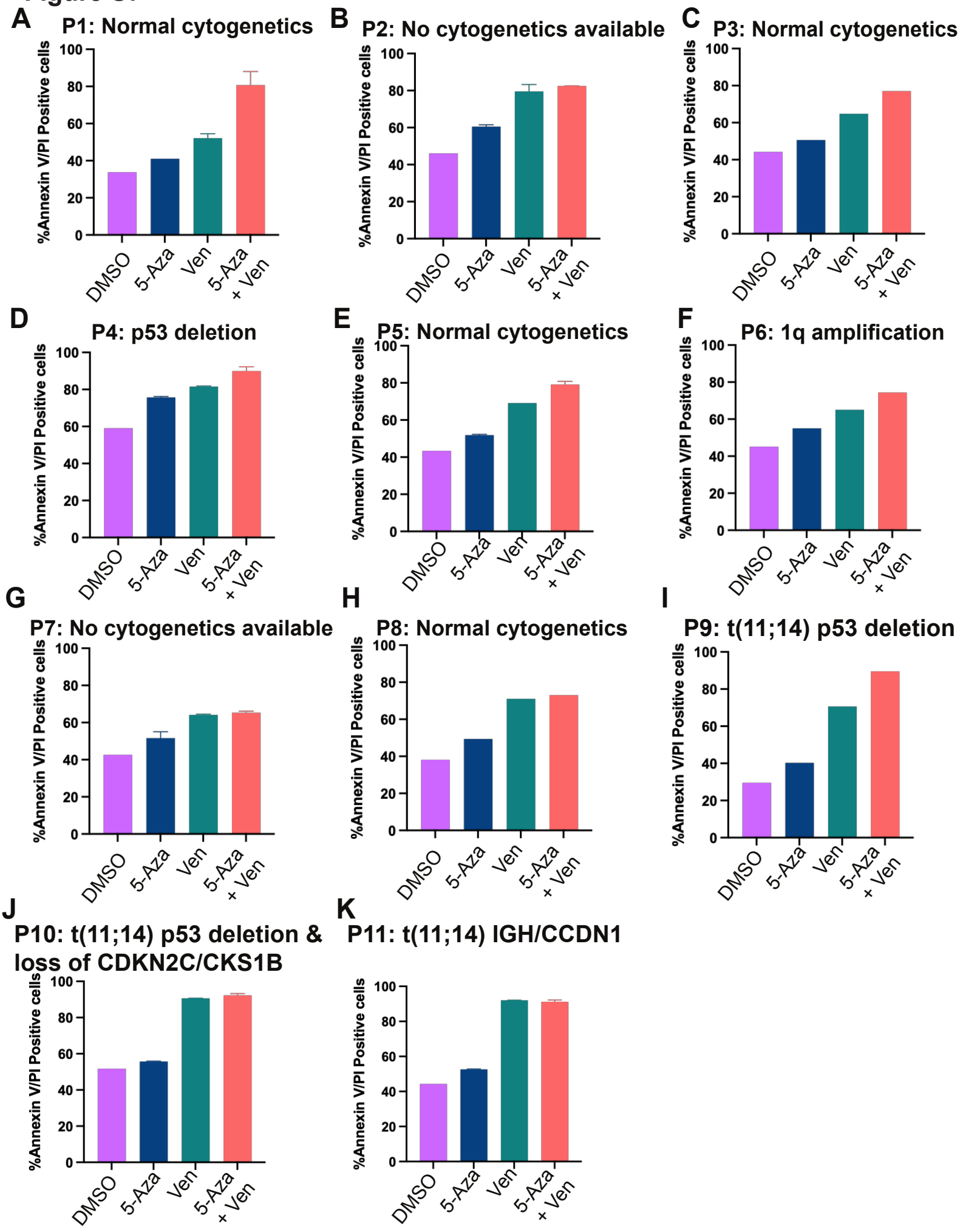

A
P1: Normal cytogenetics
% Viability (Annexin V/PI)
Log μM
Ven
AMG-176
A-133
BCL-2 = BCL-xL = MCL-1
% AnnexinV/PI cells
CI=0.49
5-Aza
Ven
5-Aza + Ven
B
P2: No cytogenetics available
BCL-2 > MCL-1 > BCL-xL
CI = 1.17
C
P3: Normal cytogenetics
MCL1 > BCL-2 > BCL-xL
CI=0.70
D
P4: p53 deletion
MCL1 > BCL-2 = BCL-xL
CI=0.92
E
P5: Normal cytogenetics
MCL1 > BCL-2 > BCL-xL
CI=0.79
F
P6: 1q amplification
MCL1 > BCL-2 > BCL-xL
CI=0.95
G
P7: No cytogenetics available
WEHI-539
BCL-xL = MCL-1 > BCL-2
CI=1.15
H
P8: Normal cytogenetics
BCL-xL=MCL-1>BCL-2
CI=1.14
5-Aza
ABT-199
5-Aza + ABT-199
I
P9: t(11;14) p53 deletion
BCL-2 > BCL-xL > MCL-1
CI=0.42
J
P10: t(11;14) p53 deletion and loss of CDKN2C/CKS1B
BCL-2 > MCL-1 > BCL-xL
CI=1.28
K
P11: t(11;14) IGH/CCDN1 rearrangement
BCL-2 > MCL-1 > BCL-xL
CI=1.23
L
t (11;14) Fig. S5J
t (11;14) Fig. S5K
non t (11;14) Fig. S5D
JJN3
KMS18
BCL-2
BCL-xL
MCL-1
Actin
26 kDa
30 kDa
40 kDa
42 kDa

Figure S7


A
P1: Normal cytogenetics
B
P2: No cytogenetics available
C
P3: Normal cytogenetics
D
P4: p53 deletion
E
P5: Normal cytogenetics
F
P6: 1q amplification
G
P7: No cytogenetics available
H
P8: Normal cytogenetics
I
P9: t(11;14) p53 deletion
J
P10: t(11;14) p53 deletion & loss of CDKN2C/CKS1B
K
P11: t(11;14) IGH/CCDN1
%Annexin V/PI Positive cells
0
20
40
60
80
100
DMSO
5-Aza
Ven
5-Aza + Ven