Menin inhibitor DS-1594b drives differentiation and induces synergistic lethality in combination with venetoclax in acute myeloid leukemia cells with rearranged mixed-lineage leukemia and mutated nucleophosmin-1

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SUPPLEMENTARY METHODS

Cell lines and patient samples

AML cell lines OCI-AML2, OCI-AML3, HL60, MOLM-13, MOLM-14, MV4-11, MOLM-13 ven-res, and U937 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. All cells were authenticated by short tandem repeat DNA fingerprinting in September 2016 by the Cytogenetics and Cell Authentication Core Facility at The University of Texas MD Anderson Cancer Center. IMS-M2 and NPM1 luc PDX were received from the University of Perugia under MTA agreement 25854. Primary AML patient bone marrow or peripheral blood samples (n = 6) were collected under MD Anderson IRB protocol LAB-01-473. All cell lines were cultured in suspension in RPMI-1640 medium (Sigma) and supplemented with 10% fetal bovine serum (Sigma), L-glutamine, and penicillin/streptomycin (Invitrogen).

Fresh whole blood samples from patients were mixed 1:1 with phosphate-buffered saline (PBS) without calcium and magnesium and added to 15 mL Lymphocyte Separation Medium (Sigma). Cells were centrifuged (1800 rpm for 20 min), and the mononuclear cell layer was extracted. Ammonium chloride solution (5 mL) was used to lyse residual red blood cells for 5 minutes while shaking. Primary AML patient cells were plated at 0.2-1.5 x 10^5 cells/mL in Stemline II (Milliporesigma), SCF (100 ng), FLT3L (100 ng), TPO (100 ng), IL3 (20 ng), and IL6 (20 ng) (Peptrotech).

Reagents

Venetoclax was purchased from LC Laboratories; DS-1594b was received from Daiichi SankyoCo., Ltd.; stock solutions for in vitro studies were prepared with DMSO (Sigma-Aldrich). For animal studies, DS-1594b was prepared in 0.1% methylcellulose and venetoclax was prepared in 10% ethanol/30% phosphal 50/60% PEG 400. Western blot antibodies were purchased from Cell Signaling (Table S1).

Generation of ven-res cell lines

OCI-AML2, MV4-11 and MOLM-13 AML cell lines were cultured in suspension in RPMI-1640 medium and supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. Venetoclax resistance was induced by continuously exposing the parental cells to increasing concentrations of Venetoclax, starting at 10 nM and increasing to 1 μM. The medium with venetoclax was changed every 2 days. After cells achieved stable viability (above 90%) at a certain venetoclax dose, the dose was increased until it reached 1 μM.

Western Blotting

Cells (approximately 1-2 million per well) were plated in 6-well dishes. After undergoing treatment for the specified period, we extracted total proteins using Laemmli buffer and performed a Bicinchoninic acid assay (BCA). About 30 micrograms of these proteins were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes provided by Millipore Sigma. Post-blocking with Li-COR Odyssey blocking buffer, these membranes were left to incubate with primary antibodies overnight at 4°C. Following this, they were washed and incubated for 2 hours with suitable infrared fluorochrome-conjugated secondary antibodies - Odyssey Irdye 680 RD anti-mouse and Odyssey Irdye 800 CW goat anti-rabbit. The protein signals were made visible using a LI-COR Odyssey imaging system.

BH3 profiling

BH3 profiling relies on the exposure of mitochondrial BCL-2 family of proteins to peptides that mimic endogenous pro-apoptotic BH3 activators and sensitizers that selectively bind to antiapoptotic BCL-2 family members. The release of cytochrome c from mitochondria in response to a given BH3 peptide therefore allows us to estimate the level of mitochondrial apoptotic priming (susceptibility of cells to commit apoptosis) and dependence on selective BCL-2 family members for survival. Cells were pelleted at 400 g and resuspended in DTEB (Derived from Trehalose

Experimental Buffer). Cells were permeabilized with digitonin and exposed to BH3 peptides (hBIM, PUMA, hBID-Y, Bmf-Y, mNoxaA, MS1; New England Peptide). The mitochondrial outer membrane permeabilization was monitored by cytochrome C release. Dimethyl sulfoxide (DMSO) and Ala were used as the negative and positive controls, respectively.

RNA sequencing

RNA was extracted from ~ 5 million cells using the Qiagen RNeasy Kit for RNA (74104) purification following the manufacturer's instructions. mRNA was purified using the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490S) using 1 µg of input RNA. Library preparation for sequencing was done using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760S). Samples were sequenced by paired-end sequencing on NextSeq500.

CyTOF protocol

Antibodies for time-of-flight mass cytometry (CyTOF) were either purchased from Fluidigm or conjugated in house as follows. Purified, carrier-free antibodies were conjugated with lanthanide isotopes using a MaxPar Antibody Labeling Kit (Fluidigm) following the manufacturer's instructions. Protein concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and metal contents of the conjugated antibodies were determined by CyTOF using a CyTOF mass cytometer (Fluidigm) in solution mode using Claritas PPT Grade Multi-Element Solution 1 (SPEX CertiPrep) with 0.5 ppb as a standard. Table S1 lists the antibodies used. All antibodies were labeled with heavy metals using Maxpar-X8 labeling reagent kits (Fluidigm) according to the manufacturer's instructions and titrated to determine the optimal concentration.

For each sample evaluated, 3×10^6 cells were aliquoted into separate fluorescence-activated cell sorting tubes and washed twice with Maxpar PBS buffer (Fluidigm; cat. 201058). For live/dead cell discrimination, cells were resuspended in 200 µL of 5 µM cisplatin (Fluidigm; cat. 201064) for

5 minutes on an orbital shaker, followed immediately by 3 washes in Maxpar cell staining buffer (CSB; Fluidigm; cat. 201068). Cells were then fixated for 10 minutes in 1 mL of 1× Fix I buffer (Fluidigm; cat. 201065), followed by 2 washes with 1× barcode perm buffer (Fluidigm; cat. 201057). Each unique palladium barcode (Fluidigm; Cell-ID 20-Plex Pd Barcoding Kit, cat. 201060) was suspended in 100 µL of barcode perm buffer and immediately transferred to cells in 800 µL of barcode perm buffer. Cells were incubated with barcodes for 30 minutes, then washed twice with 2 mL CSB. Barcoded cells were then combined into a single tube for CyTOF staining. The staining factor was calculated as the total number of barcoded cells/3 × 106 cells. Cells were blocked with 5 µL× staining factor of anti-human Fc receptor binding inhibitor (eBioscience Invitrogen) in 45 µL × staining factor of CSB for 15 minutes at room temperature. An appropriate amount of surface antibody master mix was added directly to the tube and incubated for 1 hour at room temperature before it was washed with CSB twice.

Next, cells were washed twice with PBS and stained for 30 minutes with antibodies against intracellular markers in a final reaction volume of 50 µL, washed twice with wash buffer and once with PBS, and stained for 20 minutes in 500 µL of 1:1000 iridium intercalator (Fluidigm; cat. 201192A) diluted in 1.6% PFA in PBS. Cells were then washed twice with wash buffer and filtered through blue-capped tubes. Each sample pellet was resuspended in 50 µL deionized water and transferred to a 96–deep-well plate containing 50 µL Eu151/153 calibration beads (Cat. 201073, Fluidigm). Samples were analyzed on a CyTOF mass cytometer using an AS5 Autosampler (Fluidigm); 0.4 mL deionized water was added to each sample just before injection. The data were saved in FCS3.0 format, de-barcoded by a Fluidigm de-barcoder, and analyzed by spanning-tree progression analysis of density-normalized events (SPADE) software (version 3) or Cytofkit for Bioconductor.

Data were initially filtered with FlowJo to eliminate normalization beads, debris, doublets, and non-viable cells. Subsequently, dimensionality reduction was conducted using the t-distributed stochastic neighbor embedding technique. Processed information underwent a transformation using the negative value pruned inverse hyperbolic sine method and was then clustered employing the PhenoGraph algorithm (with a parameter k set to 22), utilizing the cell surface markers detailed in Table S2.

Statistical analyses

In vitro experiments were conducted in technical triplicate. The combination index (CI), based on the Chou–Talalay method (CalcuSyn version 2.0), was obtained at the effective doses of 50%, 75%, and 90% in the population exposed to the different agents (CI < 1, synergistic; CI = 1 additive; CI > 1, antagonistic). Bliss Synergy scores of the drugs combination treatment on cell lines were determined using Combenefit software. Statistical differences between groups were determined using a Student t-test or 2-way ANOVA with the Dunnett test. P less than or equal to 0.05 was considered statistically significant. Overall survival rate was estimated by the Kaplan–Meier method and compared using the log-rank test. Dose-response curves were analyzed using a curve-fitting routine based on nonlinear regression. All preceding analyses were performed utilizing GraphPad Prism 9 software.

Table S1.

Company	Cat. Number	Antibody
Cell Signaling	6891S	Menin (D45B1) XP® Rabbit mAb
Cell Signaling	49420S	CD11b/ITGAM (D6X1N) Rabbit mAb
Cell Signaling	3700S	β-Actin (8H10D10) Mouse mAb
Cell Signaling	9662S	Caspase-3 Antibody
Cell Signaling	9664S	Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb
Cell Signaling	3686S	p27 Kip1 (D69C12) XP® Rabbit mAb
Cell Signaling	15071S	Bcl-2 (124) Mouse mAb
Cell Signaling	5625S	Cleaved PARP (Asp214) (D64E10) XP® Rabbit mAb
Cell Signaling	94296S	Mcl-1 (D2W9E) Rabbit mAb #

Table S2.

ID	Tagged Abs	Target	Label	Intracellular Staining	Clone	Specificities	Source	Cat
1	CD11c 159Tb	CD11c	159Tb	FALSE	Bu15	Hu	DVS-Fluidigm	3159001B
6	CD19 142Nd	CD19	142Nd	FALSE	HIB19	Hu	DVS-Fluidigm	3142001B
9	CD33 158Gd	CD33	158Gd	FALSE	WM53	Hu	DVS-Fluidigm	3158001B
11	CD11b 144Nd	CD11b	144Nd	FALSE	ICRF44	Hu	DVS-Fluidigm	3144001B
14	CD34 148Nd	CD34	148Nd	FALSE	581	Hu	DVS-Fluidigm	3148001B
17	CD44 166Er	CD44	166Er	FALSE	BJ18	Hu	DVS-Fluidigm	3166001B
41	CD90 171Yb	CD90	171Yb	FALSE	5.00E+10	Hu	BioLegend	328102
43	CXCR4 172Yb	CD184, CXCR4	172Yb	FALSE	12G5	Hu	BioLegend	306502
125	CD133/2 153Eu	CD133/2	153Eu	FALSE	293C3	Hu	Miltenyi	130-090-851
145	CD13 152Sm	CD13	152Sm	FALSE	WM15	Hu	DVS-Fluidigm	3152003B
175	HLA-E 145Nd	HLA-E	145Nd	FALSE	3D12	Hu	BioLegend	342602
183	CD49d 141Pr	CD49d	141Pr	FALSE	9F10	Hu	DVS-Fluidigm	3141004B
257	CD3 175Lu	CD3	175Lu	FALSE	UCHT1	Hu	BioLegend	300443
309	CD14 160Gd (MDA)	CD14	160Gd	FALSE	M5E2	Hu	BioLegend	301802
356	CD8a 176Yb	CD8a	176Yb	FALSE	HIT8a	Hu, Ch	BioLegend	300902
375	CD15 173Yb	CD15	173Yb	FALSE	W6D3	Hu	BioLegend	323002
394	CD64 146Nd	CD64	146Nd	FALSE	10.1	Hu	DVS-Fluidigm	3146006B
416	TIM-3 156Gd	TIM-3	156Gd	FALSE	F38-2E2	Hu, Rh	BioLegend	345002
455	CD4 161Dy	CD4	161Dy	FALSE	RPA-T4	Hu, Ch	BioLegend	300502
459	CD45 89Y	CD45	89Y	FALSE	HI30	Hu, Ch	DVS-Fluidigm	3089003B
467	CD56 163Dy	CD56	163Dy	FALSE	NCAM16.2	Hu	DVS-Fluidigm	3163007B
536	CD123 151Eu	CD123	151Eu	FALSE	7G3	Hu	BD	554527
538	CD38 167Er (MDA)	CD38	167Er	FALSE	HIT2	Hu	BioLegend	303502
557	Ki67 168Er	Ki67	168Er	TRUE	B56	Ms, Hu	BD	556003
558	CD85j 154Sm	CD85j, ILT2, LILRB1	154Sm	FALSE	HP-F1	Hu	BC	A07408
647	CD117 155Gd	CD117(c-kit)	155Gd	FALSE	104D2	Hu	BioLegend	313202
698	CD16 209Bi	CD16	209Bi	FALSE	3G8	Hu, Rh	DVS-Fluidigm	3209002B
824	CLL-1 174Yb (BL)	CD371, CLL-1, CLEC12A	174Yb	FALSE	50C1	Hu	BioLegend	353602
937	CD7 115In	CD7	115In	FALSE	CD7-6B7	Hu	BioLegend	343102
1094	CD85k 165Ho	CD85k, ILT3, LILRB4	165Ho	FALSE	ZM4.1	Hu	BioLegend	333002
1100	HLA-DR 143Nd	HLA-DR	143Nd	FALSE	L243	Hu	DVS-Fluidigm	3143013B

Figure S1

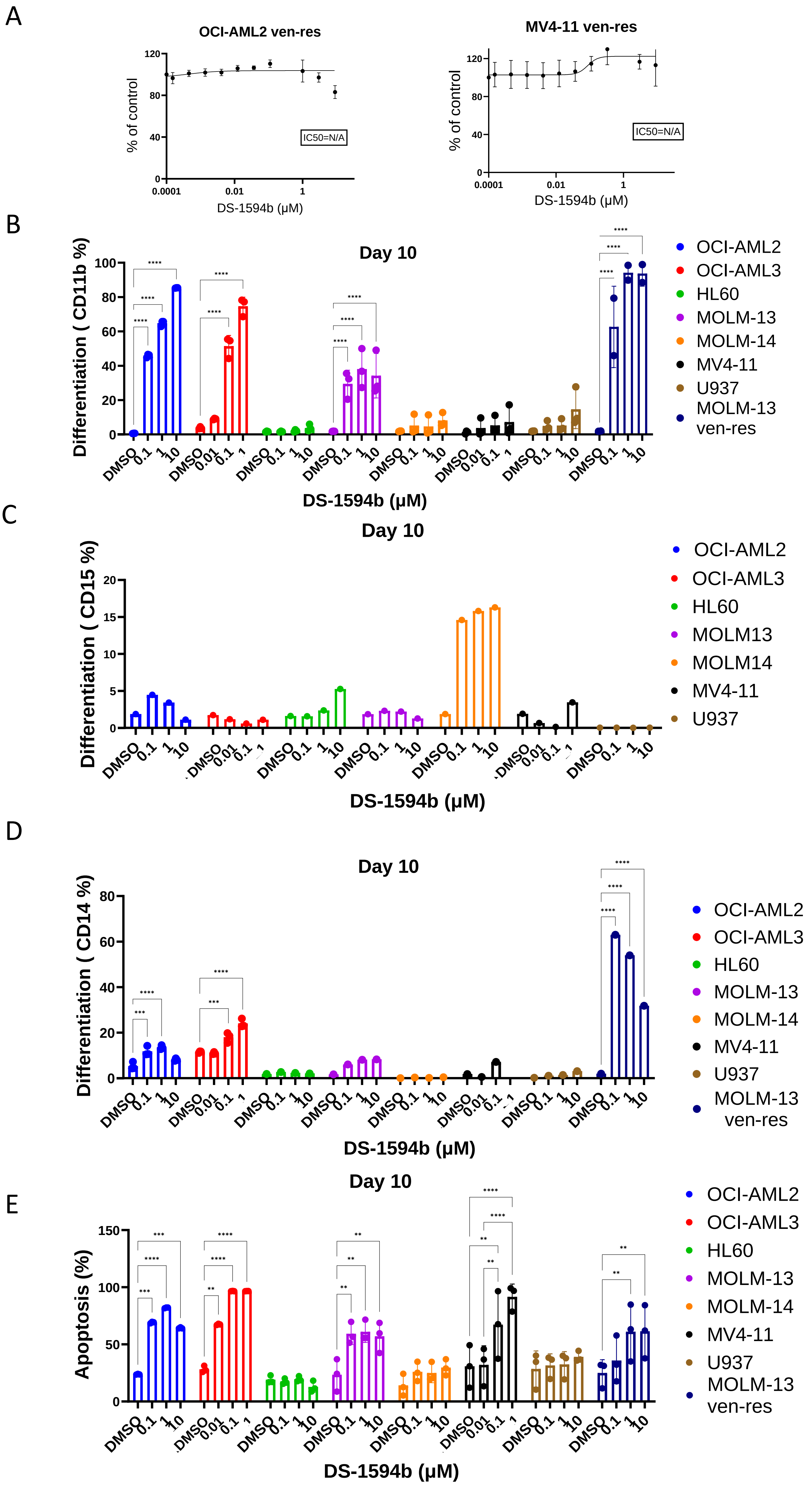
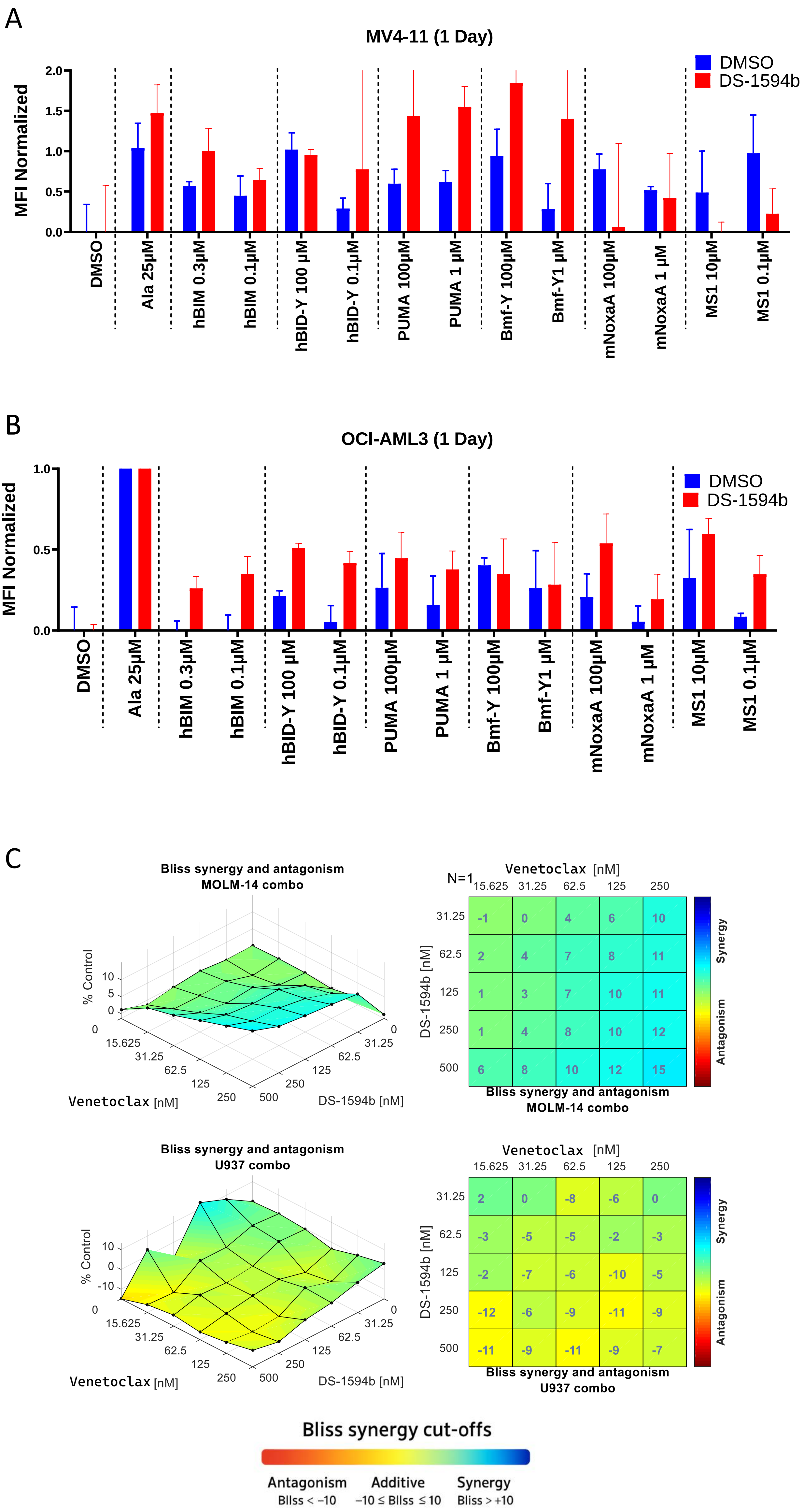

A
OCI-AML2 ven-res
% of control
120
80
40
0
0.0001
0.01
1
DS-1594b (µM)
IC50=N/A
MV4-11 ven-res
% of control
120
80
40
0
0.0001
0.01
1
DS-1594b (µM)
IC50=N/A
B
Day 10
Differentiation (CD11b %)
100
80
60
40
20
0
****
DMSO
0.1
1
10
0.01
DS-1594b (µM)
OCI-AML2
OCI-AML3
HL60
MOLM-13
MOLM-14
MV4-11
U937
MOLM-13 ven-res
C
Day 10
Differentiation (CD15 %)
20
15
10
5
0
DS-1594b (µM)
OCI-AML2
OCI-AML3
HL60
MOLM13
MOLM14
MV4-11
U937
D
Day 10
Differentiation (CD14 %)
80
60
40
20
0
****
***
DS-1594b (µM)
OCI-AML2
OCI-AML3
HL60
MOLM-13
MOLM-14
MV4-11
U937
MOLM-13 ven-res
E
Day 10
Apoptosis (%)
150
100
50
0
****
***
**
DS-1594b (µM)
OCI-AML2
OCI-AML3
HL60
MOLM-13
MOLM-14
MV4-11
U937
MOLM-13 ven-res

Figure S2



Bliss synergy and antagonism MOLM-14 combo

N=1

DS-1594b [nM] \ Venetoclax [nM]	15.625	31.25	62.5	125	250
31.25	-1	0	4	6	10
62.5	2	4	7	8	11
125	1	3	7	10	11
250	1	4	8	10	12
500	6	8	10	12	15

Bliss synergy and antagonism U937 combo

DS-1594b [nM] \ Venetoclax [nM]	15.625	31.25	62.5	125	250
31.25	2	0	-8	-6	0
62.5	-3	-5	-5	-2	-3
125	-2	-7	-6	-10	-5
250	-12	-6	-9	-11	-9
500	-11	-9	-11	-9	-7

Figure S3

A

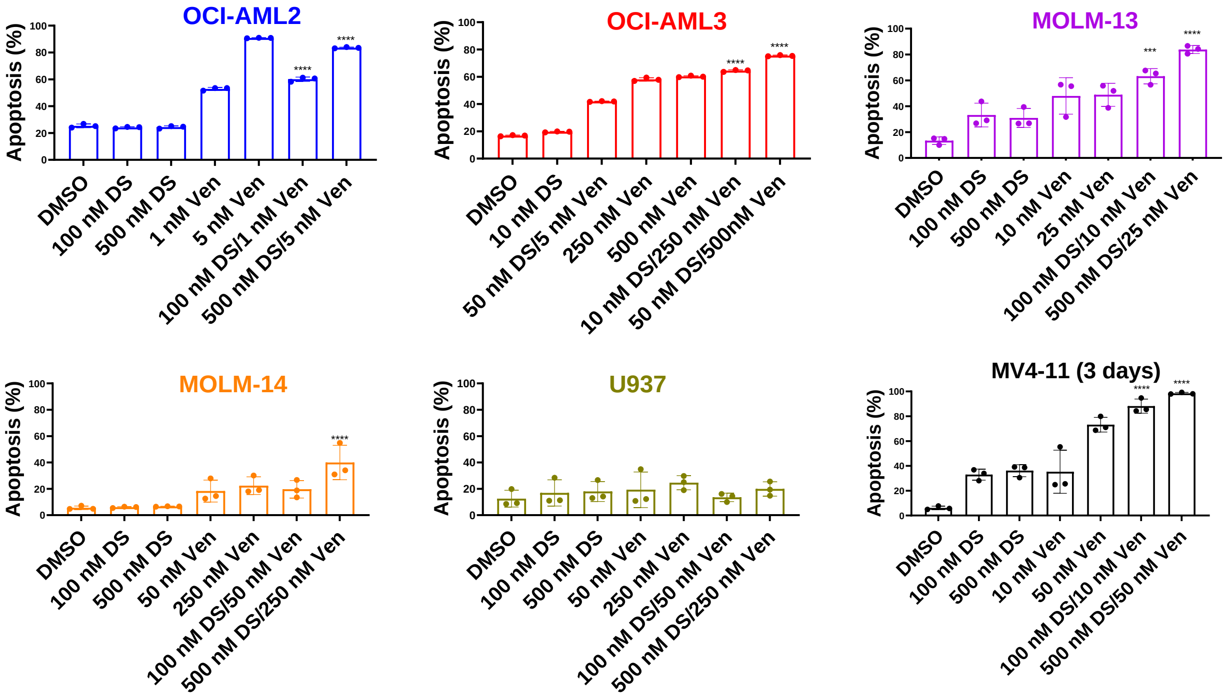
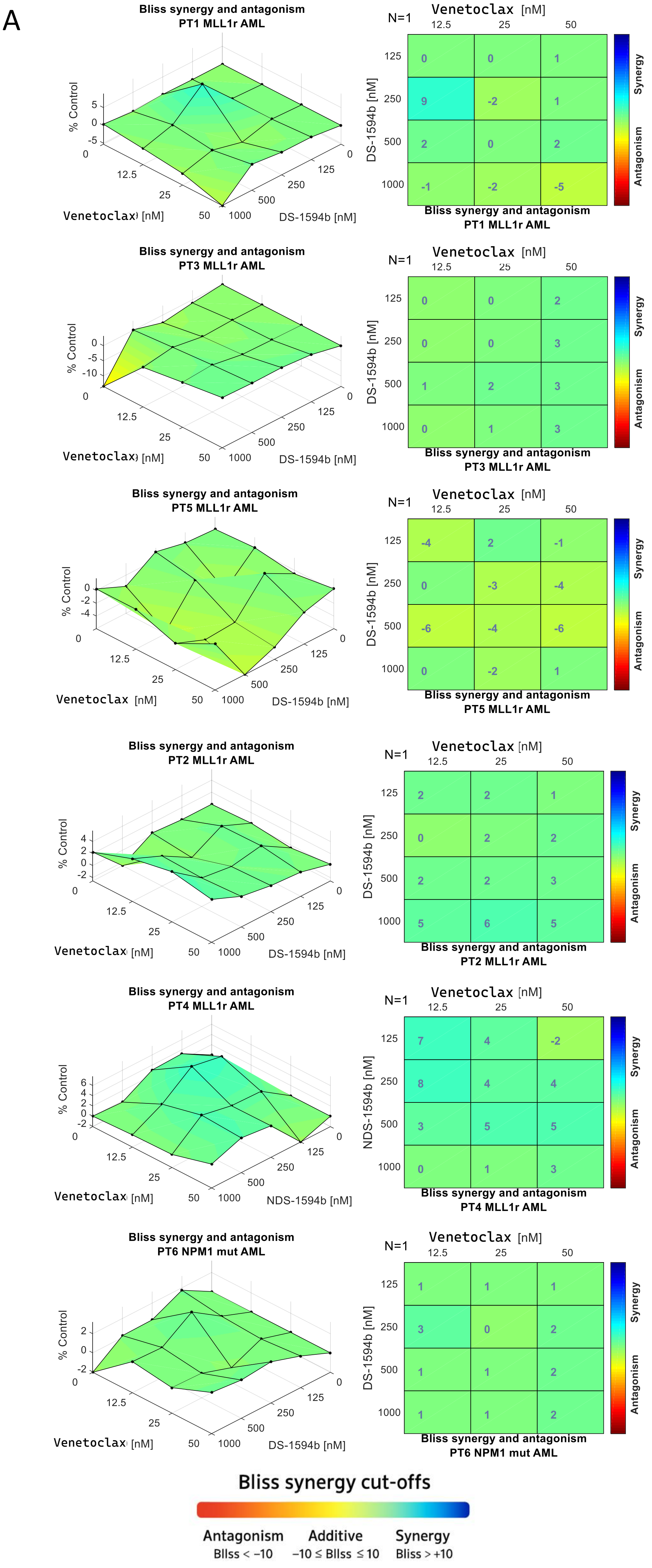

OCI-AML2
Apoptosis (%)
0
20
40
60
80
100
****
****
DMSO
100 nM DS
500 nM DS
1 nM Ven
5 nM Ven
100 nM DS/1 nM Ven
500 nM DS/5 nM Ven
OCI-AML3
Apoptosis (%)
0
20
40
60
80
100
****
****
DMSO
10 nM DS
50 nM DS/5 nM Ven
250 nM Ven
500 nM Ven
10 nM DS/250 nM Ven
50 nM DS/500nM Ven
MOLM-13
Apoptosis (%)
0
20
40
60
80
100
***
****
DMSO
100 nM DS
500 nM DS
10 nM Ven
25 nM Ven
100 nM DS/10 nM Ven
500 nM DS/25 nM Ven
MOLM-14
Apoptosis (%)
0
20
40
60
80
100
****
DMSO
100 nM DS
500 nM DS
50 nM Ven
250 nM Ven
100 nM DS/50 nM Ven
500 nM DS/250 nM Ven
U937
Apoptosis (%)
0
20
40
60
80
100
DMSO
100 nM DS
500 nM DS
50 nM Ven
250 nM Ven
100 nM DS/50 nM Ven
500 nM DS/250 nM Ven
MV4-11 (3 days)
Apoptosis (%)
0
20
40
60
80
100
****
****
DMSO
100 nM DS
500 nM DS
10 nM Ven
50 nM Ven
100 nM DS/10 nM Ven
500 nM DS/50 nM Ven

Figure S4



Bliss synergy and antagonism
PT1 MLL1r AML

DS-1594b [nM] \ Venetoclax [nM]	12.5	25	50
125	0	0	1
250	9	-2	1
500	2	0	2
1000	-1	-2	-5

Bliss synergy and antagonism
PT3 MLL1r AML

DS-1594b [nM] \ Venetoclax [nM]	12.5	25	50
125	0	0	2
250	0	0	3
500	1	2	3
1000	0	1	3

Bliss synergy and antagonism
PT5 MLL1r AML

DS-1594b [nM] \ Venetoclax [nM]	12.5	25	50
125	-4	2	-1
250	0	-3	-4
500	-6	-4	-6
1000	0	-2	1

Bliss synergy and antagonism
PT2 MLL1r AML

DS-1594b [nM] \ Venetoclax [nM]	12.5	25	50
125	2	2	1
250	0	2	2
500	2	2	3
1000	5	6	5

Bliss synergy and antagonism
PT4 MLL1r AML

NDS-1594b [nM] \ Venetoclax [nM]	12.5	25	50
125	7	4	-2
250	8	4	4
500	3	5	5
1000	0	1	3

Bliss synergy and antagonism
PT6 NPM1 mut AML

DS-1594b [nM] \ Venetoclax [nM]	12.5	25	50
125	1	1	1
250	3	0	2
500	1	1	2
1000	1	1	2

Figure S5

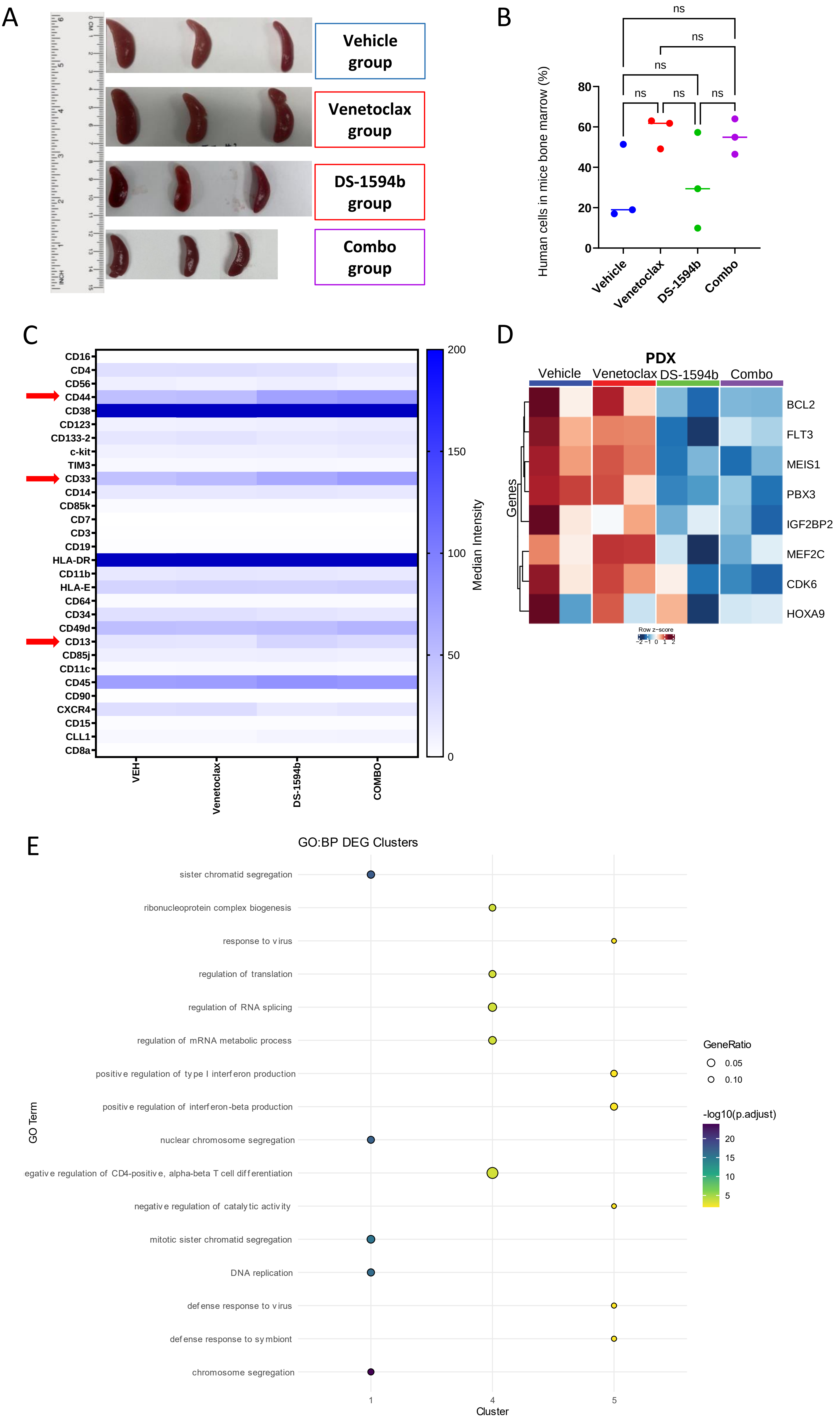

A
Vehicle group
Venetoclax group
DS-1594b group
Combo group
B
ns
ns
ns
ns
ns
ns
Human cells in mice bone marrow (%)
0
20
40
60
80
Vehicle
Venetoclax
DS-1594b
Combo
C
CD16
CD4
CD56
CD44
CD38
CD123
CD133-2
c-kit
TIM3
CD33
CD14
CD85k
CD7
CD3
CD19
HLA-DR
CD11b
HLA-E
CD64
CD34
CD49d
CD13
CD85j
CD11c
CD45
CD90
CXCR4
CD15
CLL1
CD8a
VEH
Venetoclax
DS-1594b
COMBO
Median Intensity
200
150
100
50
0
D
PDX
Vehicle
Venetoclax
DS-1594b
Combo
Genes
BCL2
FLT3
MEIS1
PBX3
IGF2BP2
MEF2C
CDK6
HOXA9
Row z-score
−2 −1 0 1 2
E
GO:BP DEG Clusters
sister chromatid segregation
ribonucleoprotein complex biogenesis
response to virus
regulation of translation
regulation of RNA splicing
regulation of mRNA metabolic process
positive regulation of type I interferon production
positive regulation of interferon-beta production
nuclear chromosome segregation
egative regulation of CD4-positive, alpha-beta T cell differentiation
negative regulation of catalytic activity
mitotic sister chromatid segregation
DNA replication
defense response to virus
defense response to symbiont
chromosome segregation
GO Term
1
4
5
Cluster
GeneRatio
0.05
0.10
-log10(p.adjust)
20
15
10
5

Figure S6

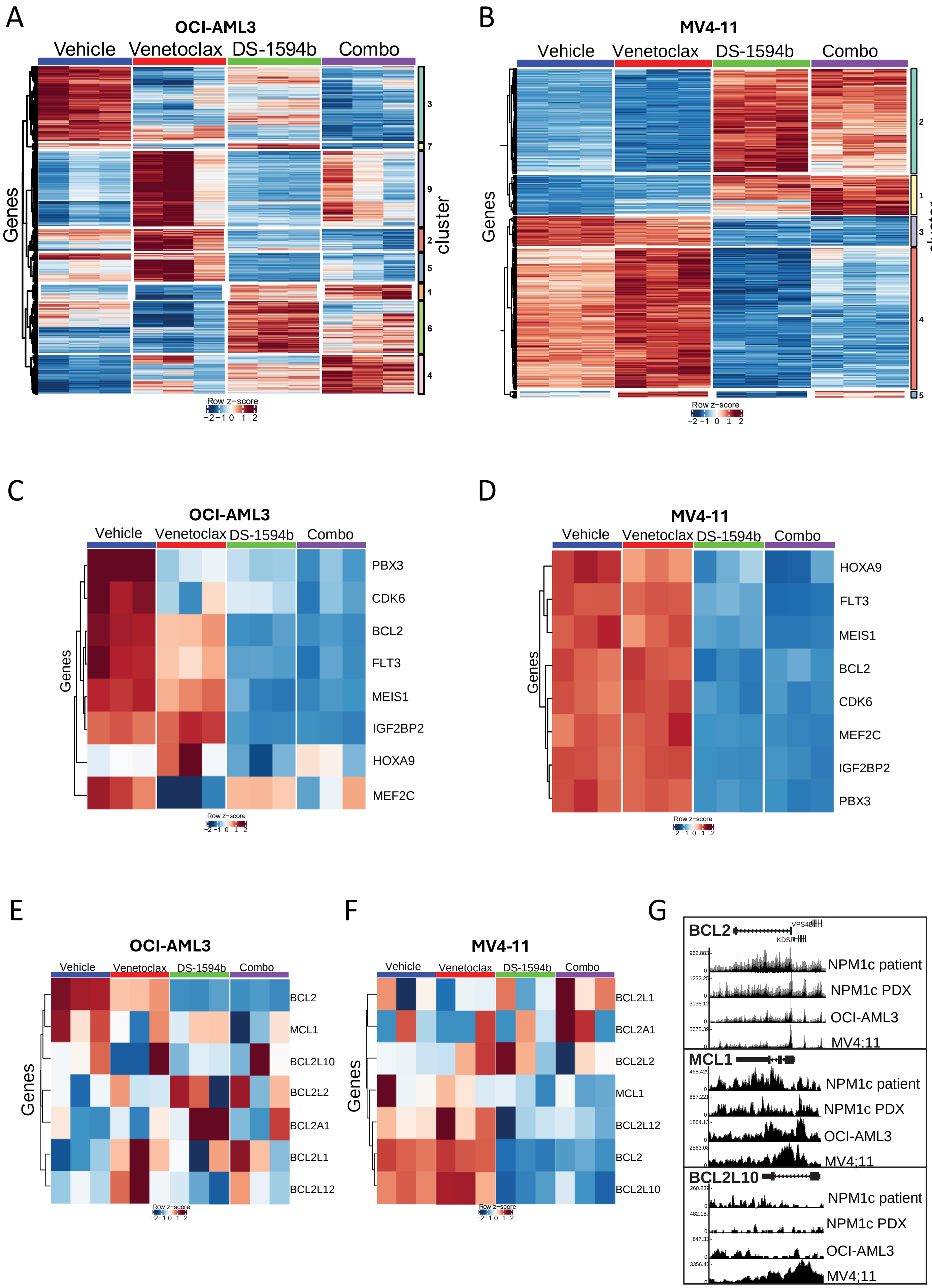
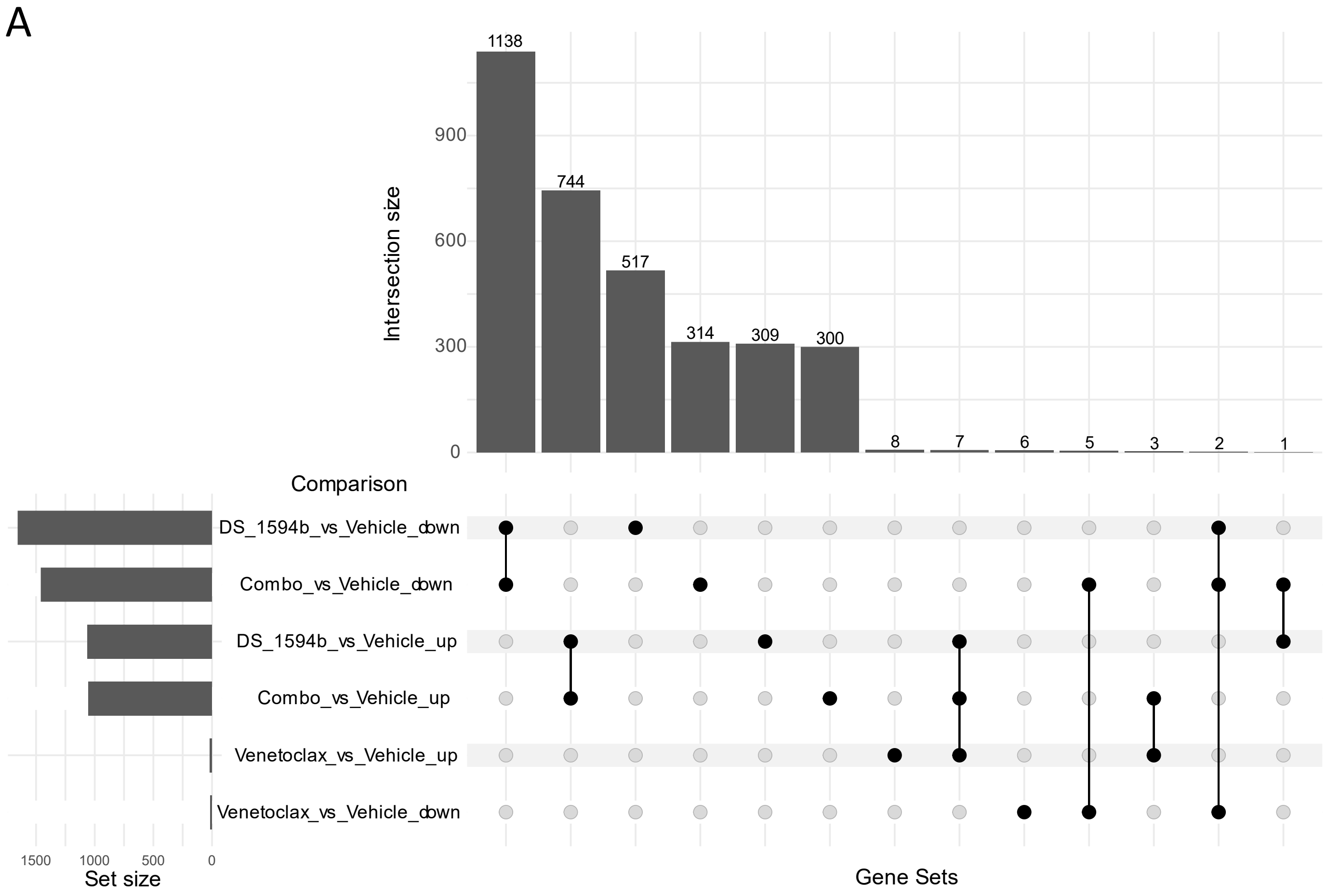

A
OCI-AML3
Vehicle
Venetoclax
DS-1594b
Combo
Genes
cluster
3
7
9
2
5
1
6
4
Row z−score
−2 −1 0 1 2
B
MV4-11
Vehicle
Venetoclax
DS-1594b
Combo
Genes
cluster
2
1
3
4
5
Row z−score
−2 −1 0 1 2
C
OCI-AML3
Vehicle
Venetoclax
DS-1594b
Combo
Genes
PBX3
CDK6
BCL2
FLT3
MEIS1
IGF2BP2
HOXA9
MEF2C
Row z−score
−2 −1 0 1 2
D
MV4-11
Vehicle
Venetoclax
DS-1594b
Combo
Genes
HOXA9
FLT3
MEIS1
BCL2
CDK6
MEF2C
IGF2BP2
PBX3
Row z−score
−2 −1 0 1 2
E
OCI-AML3
Vehicle
Venetoclax
DS-1594b
Combo
Genes
BCL2
MCL1
BCL2L10
BCL2L2
BCL2A1
BCL2L1
BCL2L12
Row z−score
−2 −1 0 1 2
F
MV4-11
Vehicle
Venetoclax
DS-1594b
Combo
Genes
BCL2L1
BCL2A1
BCL2L2
MCL1
BCL2L12
BCL2
BCL2L10
Row z−score
−2 −1 0 1 2
G
BCL2
VPS4B
KDSR
NPM1c patient
NPM1c PDX
OCI-AML3
MV4;11
MCL1
NPM1c patient
NPM1c PDX
OCI-AML3
MV4;11
BCL2L10
NPM1c patient
NPM1c PDX
OCI-AML3
MV4;11

Figure S7


A
Intersection size
0
300
600
900
1138
744
517
314
309
300
8
7
6
5
3
2
1
Comparison
DS_1594b_vs_Vehicle_down
Combo_vs_Vehicle_down
DS_1594b_vs_Vehicle_up
Combo_vs_Vehicle_up
Venetoclax_vs_Vehicle_up
Venetoclax_vs_Vehicle_down
1500
1000
500
0
Set size
Gene Sets

Supplementary Table 1. Antibodies used for Western blot analysis.

Supplementary Table 2. Antibodies used for CyTOF analysis.

Supplementary Figure 1. Effects of treatment with DS-1594b alone and in combination with venetoclax on various cell lines.

A. Proliferation assays were conducted by treating OCI-AML2 ven-res and MV4-11 ven-res cell lines for 7 days with DS-1594b alone at the indicated concentrations. Dose-response curves were analyzed using a curve-fitting routine based on nonlinear regression to compute the IC_{50} value. **B.** Cell lines were treated with vehicle (0.2% DMSO) or 0.1, 1, or 10 μM (or 0.01, 0.1, and 1 μM) DS-1594b for 10 days. Differentiation effects were determined by flow cytometry using the CD11b marker. Two-way ANOVA was performed to determine statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$). **C.** Differentiation effects in all cell lines was determined by flow cytometry using the CD15 marker. Two-way ANOVA was performed to determine statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$). **D.** Differentiation effects in all cell lines were determined by flow cytometry using the CD14 marker on all cell lines. Two-way ANOVA was performed to determine statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$). **E.** Apoptosis assay was conducted by treating cell lines for 10 days with the indicated concentrations. Apoptotic cells were determined by flow cytometry using counting beads, Annexin V, and DAPI. Two-way ANOVA was performed to determine statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Supplementary Figure 2. BH3 profiling and bliss synergy scores in cell lines treated with Venetoclax and/or DS-1594b.

A. MV4-11 and **B.** OCI-AML3 cell lines were treated for 24 hours and analyzed for BH3 profiling. Cells were then permeabilized with digitonin and exposed to BH3 peptides (hBIM, hBID-Y, PUMA, Bmf-Y, mNoxaA, MS1; synthesized by New England Peptide). The mitochondrial transmembrane potential loss was monitored by cytochrome C release. DMSO and Ala were used as the negative and positive controls, respectively, for cytochrome C release. MFI Normalization was calculated following this equation: 1-(MFI(log)-(DMSO-Ala)/(DMSO-Ala). **C.** U937 and MOLM-14 were treated with the indicated concentrations of DS-1594b and venetoclax for 5 days. Bliss synergy scores of the combination treatment on cell lines were determined using Combenefit software 2.0. Values > +10 indicate synergy, between -10 and +10 indicate additive effects, and < -10 indicate antagonism.

Supplementary Figure 3. Apoptosis assay on AML cell lines after treatment with DS-1594b in combination with venetoclax

A. Cell lines listed above were treated with vehicle (0.2% DMSO) or 0.1, 1, or 10 μM (or 0.01, 0.1 and 1 μM) DS-1594b for 5 days. Differentiation effects were determined by flow cytometry using the CD11b marker. Two-way ANOVA was performed to determine statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$).

Supplementary Figure 4. Bliss synergy scores of AML patient samples treated with DS-1594b and Venetoclax.

A. Patient samples were treated with the indicated concentrations of DS-1594b and venetoclax for 3 days. Bliss synergy scores of the combination treatment on cell lines were determined using Combenefit software 2.0. Values > +10 indicate synergy, between -10 and +10 indicate additive effects, and < -10 indicate antagonism.

Supplementary Figure 5. Effects of treatment with DS-1594b and Venetoclax on PDX mouse model.

A. Photographs of the spleens from three mice within each treatment group. **B.** Percentage of human cells in the bone marrow of three mice from each treatment group. **C.** The expression levels of each protein within every treatment group. **D.** Heatmaps showing z-scored expression of canonical Menin-MLL transcriptional target genes following treatment with Venetoclax, DS-1594b, or a combination, in PDX cells. **E.** GO:BP analysis, filtered to the top 5 significantly enriched terms, in the clusters of differentially expressed genes in the PDX model. Cluster numbers refer to the clusters in Fig. 5A.

Supplementary Figure 6. Effects of treatment with DS-1594b and Venetoclax on AML cell lines.

A. Clustered heatmaps of differentially expressed genes following treatment with Venetoclax, DS-1594b, or a combination, in OCI-AML3 cells. **B.** Clustered heatmaps of differentially expressed genes following treatment with Venetoclax, DS-1594b, or a combination, in MV4-11 cells. **C.** Heatmaps showing z-scored expression of canonical Menin-MLL transcriptional target genes following treatment with Venetoclax, DS-1594b, or a combination, in OCI-AML3. **D.** Heatmaps showing z-scored expression of canonical Menin-MLL transcriptional target genes following treatment with Venetoclax, DS-1594b, or a combination, in MV4-11. **E.** Clustered heatmaps showing z-scored expression of anti-apoptosis genes following treatment with Venetoclax, DS1594b, or a combination, in OCI-AML3. **F.** Clustered heatmaps showing z-scored expression of anti-apoptosis genes following treatment with Venetoclax, DS-1594b, or a combination, in MV4-11 cells. **G.** Menin ChIP-seq and ChIpmentation in OCI-AML3 and MV4-11 cells and small cell number ChIPmentation for Menin in an NPM1-mutant primary AML patient sample and an NPM1-mutant PDX sample at the promoters of key anti-apoptotic genes. MV4-11 data were obtained from publicly available GEO dataset GSE196036.

Supplementary Figure 7. Upset plot in PDX model.

A. Upset plot comparing significant (p.adj <0.05) up-regulated and down-regulated genes across treatment conditions in PDX model.