Targeting MCL-1 dysregulates cell metabolism and leukemia-stroma interactions and re-sensitizes acute myeloid leukemia to BCL-2 inhibition

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 doi:10.3324/haematol.2020.260331

Received: May 21, 2020

Accepted: December 14, 2020.

Pre-published: December 23, 2020.

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Supplemental Materials

Methods

Cells. AML cell lines Molm13 and MV4-11 were purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and American Type Culture Collection (Manassas, VA), respectively and OCI-AML3 was kindly provided by Dr. M. Minden (the Ontario Cancer Institute, Toronto, ON, Canada). AML patient samples (Supplementary Table 1) were obtained after acquiring written informed consent following MD Anderson Cancer Center IRB approved protocol and in accordance with the Declaration of Helsinki. Mononuclear cells were isolated from patient samples by density-gradient centrifugation using Lymphocyte Separation Medium (Corning; Manassas, VA).

Western blot analysis and co-immunoprecipitation. Antibodies against MCL-1 (Santa Cruz Biotechnology; Dallas, TX), and c-MYC (Cell Signaling Technology; Danvers, MA) were used for western blot analysis. For co-immunoprecipitation, BCL-2 (#15071s), MCL-1 (#94296), or BCL-XL (#2764) (all from Cell Signaling Technology) was pulled down with the respective antibody using the Dynabeads™ Protein G Immunoprecipitation Kit following the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA). IgG was used as a control. BCL-2 (#M0887, Dako/Agilent or #4223, Cell Signaling Technology), MCL-1 (#sc-12756, Santa Cruz Biotechnology), BCL-XL (#NBP1-28559, Novus Biologicals; Centennial, CO), BIM (#ab32158, Abcam; Cambridge, UK), BAX (#B8554, Sigma; St. Louis, MO), and BAK (#NBP1-28559, Novus Biologicals or #12105, Cell Signaling Technology) levels were determined by western blot analysis. β-ACTIN was used as a loading control.

Protein determination by flow cytometry. Cells were incubated with Fc Blocker (Miltenyi Biotec), stained with Ghost Dye Violate 510 (Tonbo Biosciences; San Diego, CA), then with PE-labeled anti-CD44 antibody or APC-labeled anti-CXCR4 antibody (both from BioLegend; San Diego, CA)

for 30 min. Cells stained with the anti-CXCR4 antibody were fixed with 4% paraformaldehyde/PBS, permeabilized with 100% methanol at 20°C overnight, and then stained with PE-labeled anti–MCL-1 antibody (Cell Signaling Technology) for 1 h. The stained cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter Life Science) and quantified using the FlowJo analytic platform (BD Bioscience). Protein levels were presented as mean fluorescence intensity differences between antibody-stained and IgG-stained cells.

$^{13}C_2$-1,2-glucose and $^{13}C_5$-glutamine tracing. MCL-1–KD or vector control OCI-AML3 cells and OCI-AML3 cells treated with AZD5991 (50 nM) or vehicle (DMSO) were cultured in a custom formulation of glucose- and glutamine-free RPMI-1640 medium (based on cat#SH30096, Hyclone, Logan, UT) containing 10% dialyzed fetal bovine serum (ThermoFisher Scientific) plus 10 mM $^{13}C_2$-1,2-glucose or 2 mM $^{13}C_5$-glutamine, respectively, for isotopic tracing. $^{13}C_2$-1,2-glucose and $^{13}C_5$-glutamine were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Cells were seeded by adding 3 ml of cell suspension ($0.8x10^6$ cells/ml) to 6-cm-diameter plates. After a 6-h incubation for drug treatment and isotopic tracing, cells were isolated by centrifugation at 250 g for 2 min at 4°C, and washed with PBS. After the removal of PBS by centrifugation, polar metabolites were extracted by adding 500 μl of a 4:1 mix of LC-MS grade methanol (ThermoFisher Scientific) and water. For measuring extracellular lactate, 400 μl of methanol was added to 100 μl of media. Media and cell extracts were vortexed for 2 min using a Disruptor Genie (Scientific Industries Inc., Bohemia, NY). Proteins were removed by centrifugation at 20,000 g for 5 min at 0°C. The supernatants were dried and stored at -80°C until further processing.

Ion chromatography-mass spectrometry metabolite analysis. Samples were analyzed with ion chromatography-mass spectrometry (IC-MS) as described previously(1) to broadly assess

metabolites in cellular energetic pathways, including glycolysis, PPP, the TCA cycle, and nucleotide metabolism. Dried polar fractions were reconstituted in 50 µl of ultrapure water (Barnstead Nanopure Diamond, ThermoFisher Scientific), and 5 µl of the reconstituted fractions were subjected to IC-MS analysis with a Dionex ICS-5000+ system interfaced with an Orbitrap Fusion Tribrid mass spectrometer (both from ThermoFisher Scientific) operating at a resolution of 240,000 (FWHM at m/z 200) for MS1 acquisition (m/z range of 80–800). Peak areas were integrated using the TraceFinder 4.1 software package (ThermoFisher Scientific) and exported to a tabular format for further analysis. Peak areas and fractional enrichments were corrected for natural abundance using the ElemCor software tool (2). Replicates were normalized using summed intensity across all metabolites and adjusted for the amount of cells during cell harvest. Differences in metabolites between AZD5991-treated or MCL-1–KD cells and their respective control cells were assessed with a two-tailed equal variance Student t-test in Excel 2013 (Microsoft, Redmond, WA). Relative PPP flux was calculated as the lactate secretion rate x [M+1 lactate]/([M+2 lactate] +[M+1 lactate]), where [M+1 lactate] and [M+2 lactate] are fractional ^{13}C enrichments in secreted lactate and the lactate secretion rate is the total cell normalized lactate ion intensity after 6 h of cell culture. Flux was normalized to that of control cells (i.e. untreated parental cells or vector control cells) by setting the control average as 1.

In vivo experiments. Mouse care and mouse experiments were performed in accordance with protocols approved by MD Anderson Cancer Center Animal Care and Use Committee. For the AML xenograft model, MCL-1–OE and vector control Molm13 cells ($0.4x10^6$ cells/mouse, female) or MCL-1–KD and vector control OCI-AML3 cells ($1x10^6$ cells/mouse, male) were injected via tail vein into 10- to 12-wk-old NSGS mice (n=5/group; The Jackson Laboratory, Bar Harbor, ME). The PDX model was developed in NSGS mice using a BM sample (73% blasts) from an AML patient (Supplementary Table 1) whose disease initially responded to venetoclax plus

decitabine but relapsed after 3 cycles of therapy. The PDX cells ($0.5x10^6$ cells/mouse) collected from the mouse spleen (>90% human $CD45^+$) were injected into 6- to 8-wk-old male NSGS mice (The Jackson Laboratory). After engraftment, the mice (10 mice/group) were treated for 6 wk with vehicle; venetoclax (50 mg/kg daily by gavage); AZD5991 (60 mg/kg, once/wk by intravenous injection); AZD4573 (15 mg/kg twice daily by intraperitoneal injections [2 h apart], for 2 consecutive d/wk); venetoclax+AZD5991; and venetoclax+AZD4573. In the cases of drug combinations, venetoclax was given first. Leukemia engraftment and progression were assessed by flow cytometric measurement of human $CD45^+$ cells in the peripheral blood, BM, or spleen. BM cells were collected for CyTOF analyses. Survival was followed.

Supplementary Table 1. Patient Characteristics

no.	Source	Blast %	Treatments/ responses	Cytogenetics	Mutations
3	BM	75	Relapsed AML. Subsequently was treated with venetoclax. Responded, then relapsed	complex	NOTCH1, WT1
4	BM	90	Untreated AML. Subsequently was treated with chemotherapy and responded	diploid	CEBPA, BCORL1, NRAS, U2AF1, DNMT3A
5	PB	83	Newly diagnosed. Subsequently was treated with venetoclax+decitabine and responded	46,XX,t(11;19)(q23;p13.3)[20] 9 Representative metaphases photographed/Karyotyped	no mutation
7	PB	89	Resistance to decitabine+venetoclax, venetoclax (oral), AMG-673 etc.	complex	TET2, STAT5A, TP53
8	BM	98	Relapsed/Refractory AML. Resistance to multiple agents including venetoclax+decitabine, venetoclax+sorafenib	complex	BCORL1, PTPN11, FLT3-ITD
9	PB	88	Resistance to multiple agents.	diploid	SRSF2, RUNX1, IDH2, FLT3-ITD
10	PB	88	Resistance to multiple agents. Subsequently was treated with decitabine+venetoclax, midostaurin+venetoclax and deceased	complex	ETV6, RUNX1, FLT3-ITD
11	PB	96	Resistance to multiple agents including decitabine+venetoclax. Subsequently was treated with multiple agents and deceased	diploid	CEBPA, IDH2, DDX41
12	PB	88	Relapsed AML Resistance to multiple agents including venetoclax	complex	CEBPA, KRAS, NRAS, WT1
PDX	BM	73	Relapsed after 3 cycles of venetoclax+decitabine treatment	complex	FLT3-ITD, NRAS, GATA2

Supplementary Table 2. Antibody Panel for CyTOF Analysis

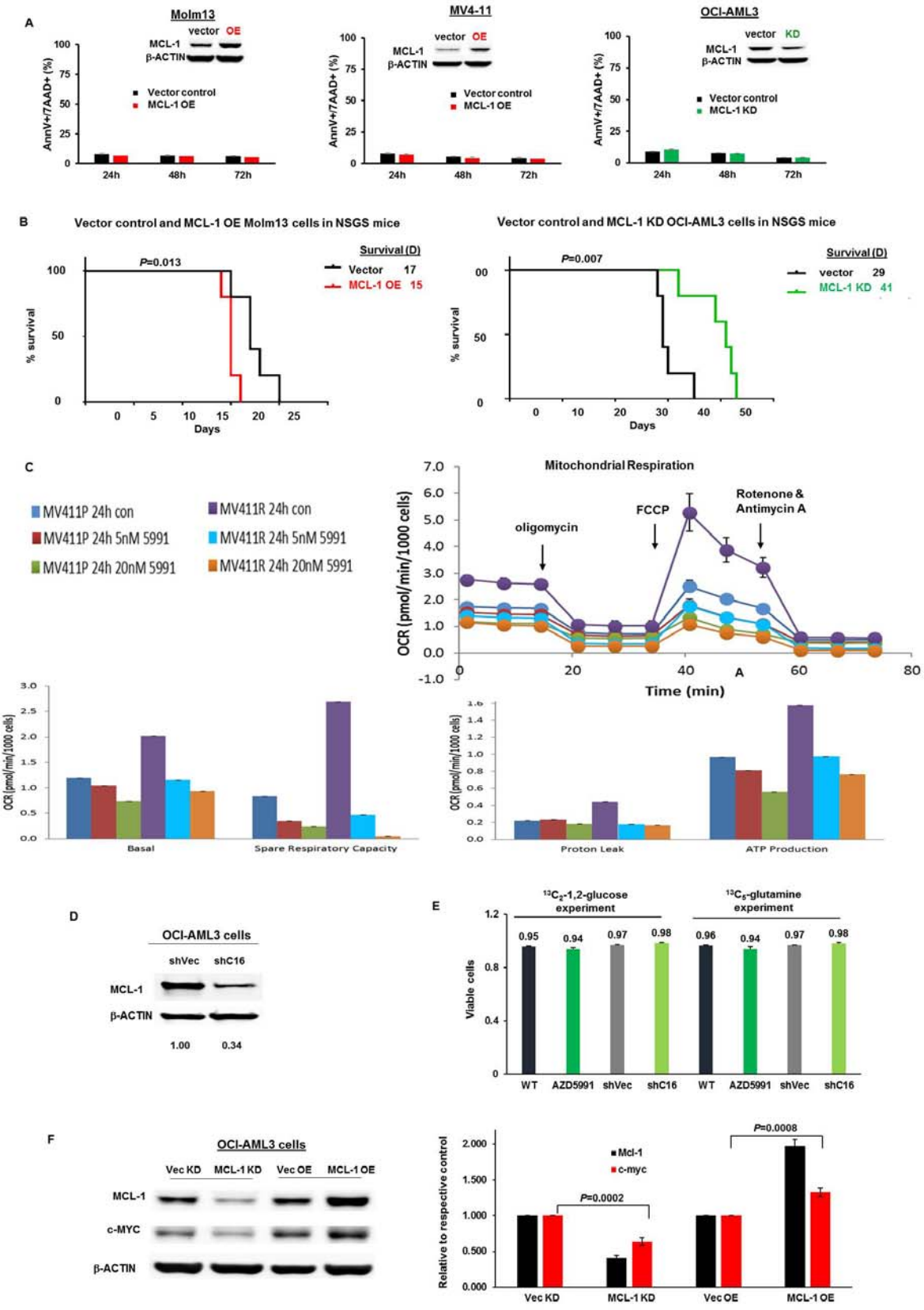
Target	label	clone	Vendor	Cat#
FAK	139La	D2R2E	CST	13009BF
BCL-XL	141Pr	54H6	CST	2764BF
CASPASE 3, Cleaved	142Nd	D3E9	DVS-Fluidigm	3142004A
BCL-2	144Nd	100	BioLegend	658702
CD123	145Nd	7G3	BD	554527
β-CATENIN	147Sm	D10A8	DVS-Fluidigm	3147005A
CD34	148Nd	581	DVS-Fluidigm	3148001B
p-AKT	159Tb		DVS-Fluidigm	
c-MYC	163Dy	D84C12	CST	5605BF
CD44	166Er	BJ18	DVS-Fluidigm	3166001B
p-ERK1/2	167Er	D13.14.4E	DVS-Fluidigm	3167005A
CD38	168Er	HIT2	BioLegend	303502
CD90	171Yb	5E10	BioLegend	328102
p-FAK(Y397)	175Lu	D20B1	CST	8556BF
MCL-1	176Yb	22/MCL-1	BD	559027
CD45	89Y	HI30	DVS-Fluidigm	3089003B
CD184, CXCR4	172Yb	12G5	BioLegend	306502

Supplementary Table 3. CI Values of Combination Treatment of Venetoclax with AZD5991 or AZD4573 in Various Cell populations of AML Patient Samples. VEN, venetoclax.

With co-culture	$CD45^+$		$CD34^+$		$CD34^+CD38^+$		$CD34^+CD38^-$		$CD34^+CD38^+CD123^+$		$CD34^+CD38^-CD123^+$	
AML #	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573
3	0.034	0.686	0.121	0.507	0.112	0.520	0.844	0.507	0.127	0.522	0.675	0.392
4	0.493	0.525	0.348	0.978	0.234	0.343	0.546	0.145	0.157	0.239	0.529	0.289
5	0.217	0.674	0.199	0.559	0.190	0.303			0.224	0.597		
7	0.053	0.030	0.037	0.114	0.037	0.114			0.035	0.021		
8	0.124	0.316	0.143	0.277	0.129	0.436	0.218	0.228	0.147	0.392	0.173	0.163
9	0.216	0.554	0.082	0.590	0.221	0.225			0.208	0.773		
10	0.309	0.128	0.316	0.301	0.206	0.125	0.860	0.408	0.100	0.214	0.106	0.363
11	0.363	0.403										
12	0.023	0.322	0.039	0.432	0.011	0.299	0.102	0.905	0.025	0.249	0.270	0.691

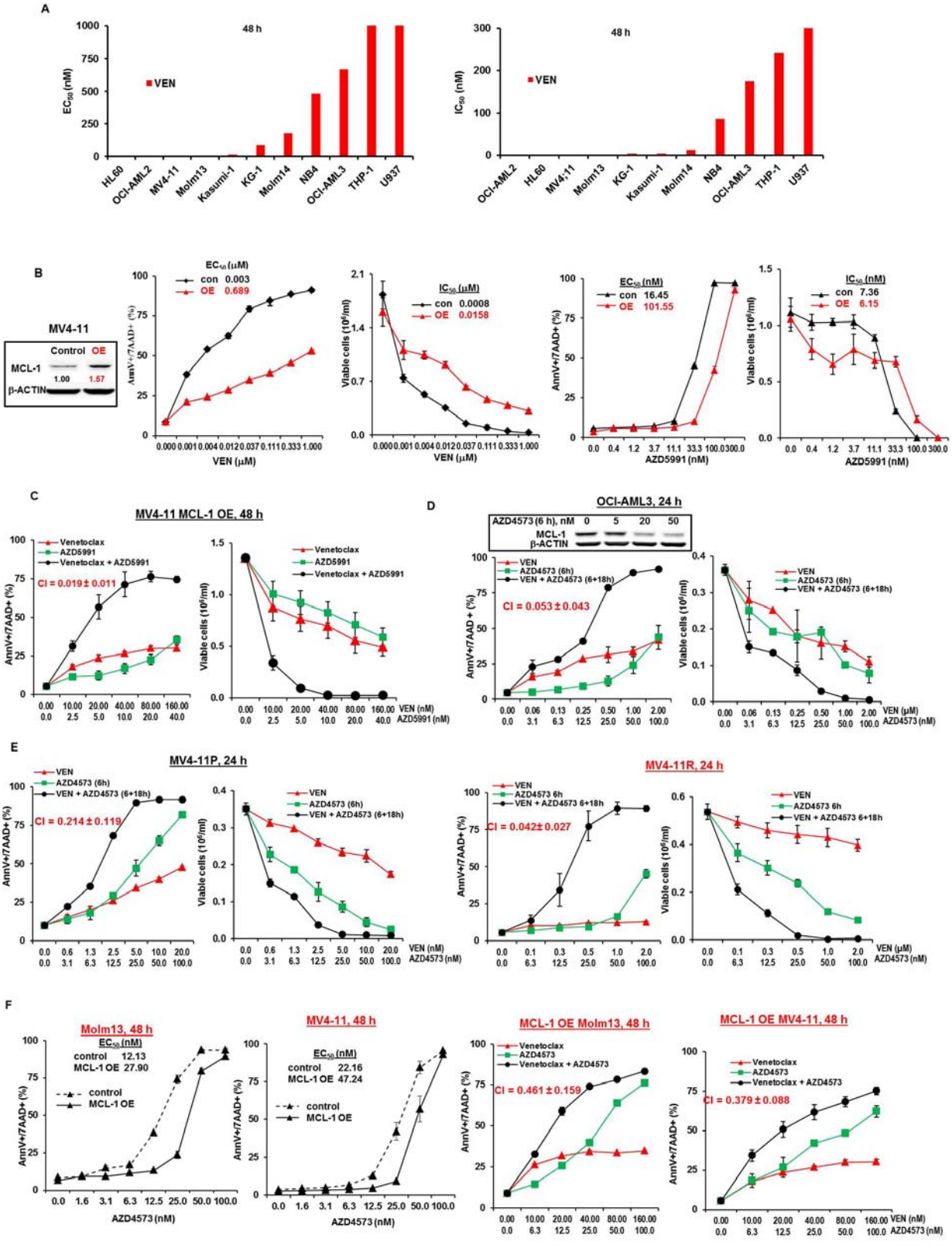
No co-culture	$CD45^+$		$CD34^+$		$CD34^+CD38^+$		$CD34^+CD38^-$		$CD34^+CD38^+CD123^+$		$CD34^+CD38^-CD123^+$	
AML #	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573	VEN + AZD5991	VEN + AZD4573
3	0.113	0.323	0.181	0.658	0.193	0.868	0.236	0.299	0.216	0.715	0.212	0.291
4	0.454	0.534	0.387	0.326	0.278	0.585	0.412	0.465	0.403	0.338	0.434	0.556
5	0.284	0.511	0.318	0.598	0.271	0.469			0.270	0.542		
7	0.283	0.681	0.229	0.590	0.229	0.590			0.202	0.733		
8												
9	0.138	0.377	0.240	0.475	0.374	0.297						
10												
11	0.287	0.181										
12	0.078	0.554	0.120	0.485	0.197	0.486	0.089	0.512	0.144	0.357	0.268	0.620

Supplementary Figure 1



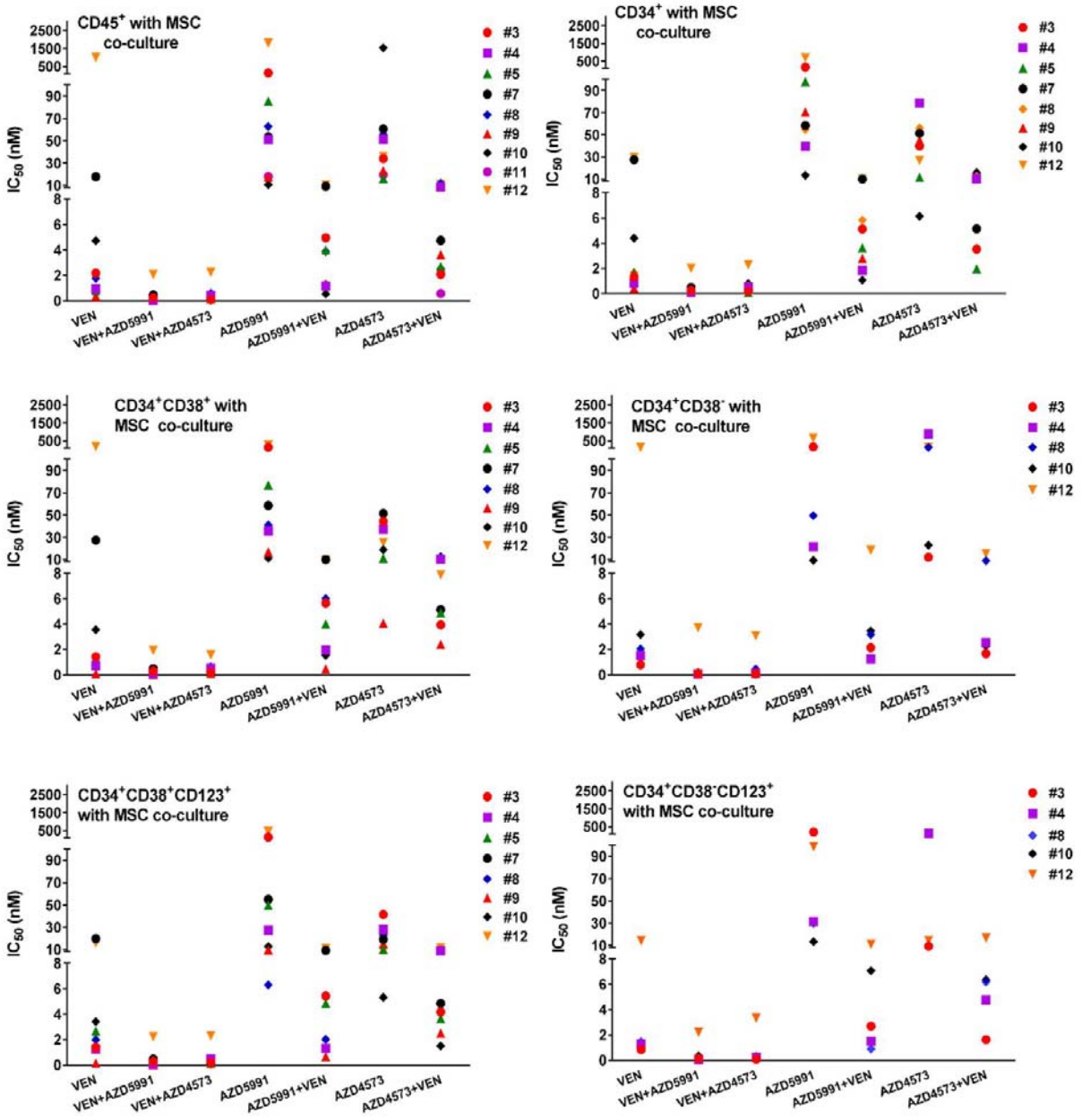
Supplementary Figure S1. MCL-1 regulates metabolic activity and affects c-MYC levels in AML cells. A. Spontaneous apoptosis of MCL-1−OE and −KD AML cells. MCL-1 levels were determined by Western blot. B. Survival curves of NSGS mice harboring vector control or MCL-1−OE Molm13 cells (left) or vector control or MCL-1−KD OCI-AML3 cells (right) (n=5/group). Survival data were analyzed using the log-rank test. C. Seahorse analysis of mitochondrial respiration in parental MV4-11 cells (MV4-11P) and venetoclax-resistant MV4-11 cells (MV4-11R) untreated or treated with AZD5991 (5991). All Seahorse experiments were performed in triplicates, with duplicates for each experiment. The results are expressed as mean±SEM of the 6 repeats. D. Western blot analysis of MCL-1 expression in control (shVec) and MCL-1−KD (shC16) OCI-AML3 cells. E. Viability of OCI-AML3 cells used in the $^{13}C_2$-1,2-glucose and $^{13}C_5$-glutamine tracing experiments. F. Representative Western blotting (left) and quantitative analysis (n=5) of c-MYC levels in MCL-1−OE and MCL-1−KD OCI-AML3 cells. Results are expressed as the mean±SEM.

Supplementary Figure 2



Supplementary Figure S2. MCL-1 expression contributes to venetoclax resistance, and BCL-2 inhibition with venetoclax plus MCL-1 inhibition with AZD5991 or AZD4573 synergistically induces apoptosis in AML cell lines with intrinsic or acquired resistance to venetoclax. A. EC_{50} and IC_{50} values of venetoclax (VEN) treatment for 48 h in leukemia cell lines. B. MCL-1−OE and control MV4-11 cells were treated with venetoclax or AZD5991 for 48 h. C. MCL-1−OE MV4-11 cells were treated with venetoclax and/or AZD5991 for 48 h. D-F. OCI-AML3 cells (D), control parental MV4-11 cells (MV4-11P) and venetoclax-resistant MV4-11 cells (MV4-11R) (E), and vector controls and MCL-1−OE Molm13 and MV4-11 cells (F) cells were treated with AZD4573 or with venetoclax and/or AZD4573 for the indicated times. For the AZD4573 treatments, cells were treated for 6 h, and then the drug was washed out. Cells were continuously cultured until they were analyzed. Apoptosis and viable cell counts were assessed by flow cytometry. Protein levels were measured by Western blotting. con, control; VEN, venetoclax. All experiments were performed in triplicates. Results are expressed as the mean±SEM.

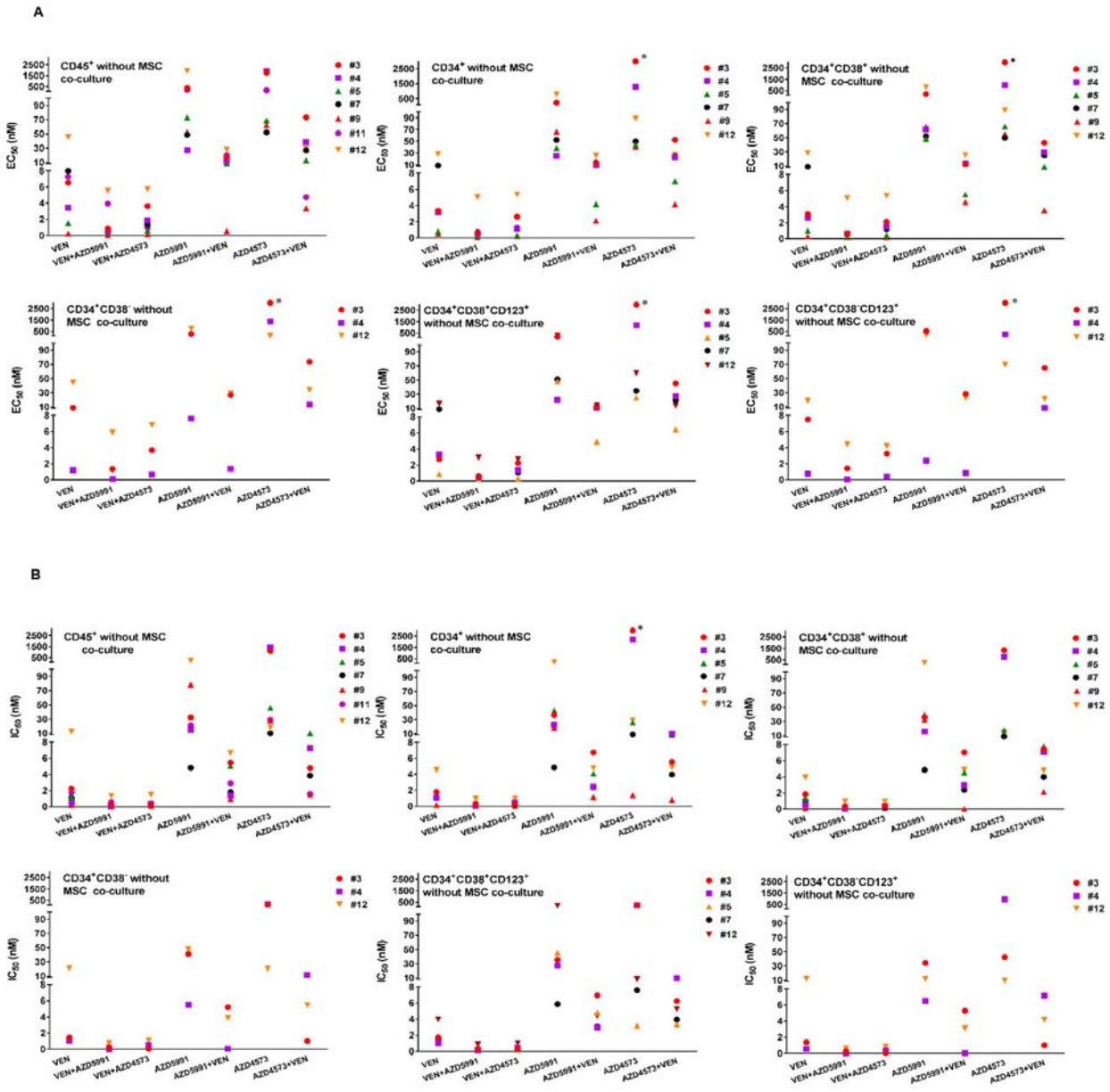
Supplementary Figure 3



Supplementary Figure S3. Combined inhibition of BCL-2 and MCL-1 synergistically decreased cell viability in primary AML cells and AML stem/progenitor cells under MSC co-culture conditions. Primary AML cells co-cultured with MSCs were treated with venetoclax, AZD5991, AZD4573, venetoclax+AZD5991, or venetoclax+AZD4573 for 48 h. The viable cell numbers of blast cells and stem/progenitor cells were assessed by flow cytometry. IC_{50} values of venetoclax

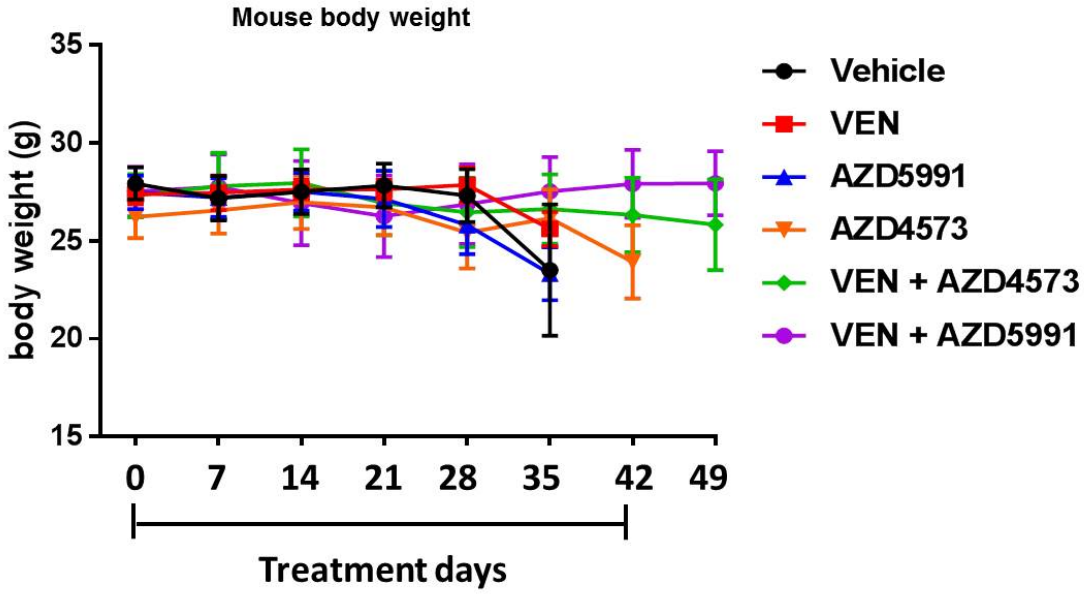
alone or combined with AZD5991 or AZD4573, of AZD5991 alone or combined with venetoclax, and of AZD4573 alone or combined with venetoclax in blast cells and stem/progenitor cells from individual patient samples are shown. Samples lacking the indicated cell populations or that had excessively high spontaneous apoptosis are not represented.

Supplementary Figure 4



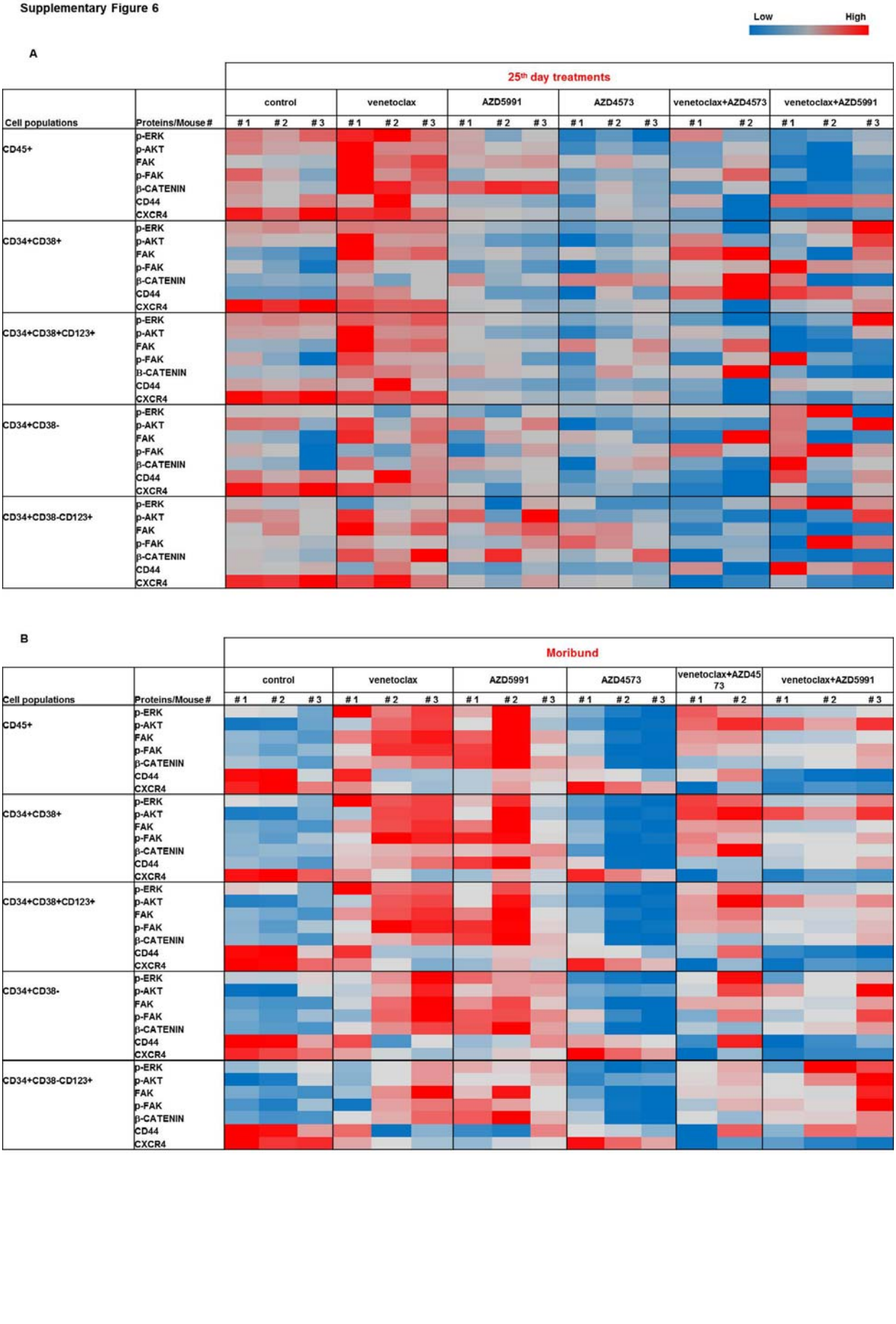
Supplementary Figure S4. Combined inhibition of BCL-2 and MCL-1 synergistically induces apoptosis and decreases viable cells in primary AML cells and AML stem/progenitor cells. Primary AML cells were treated with venetoclax (VEN), AZD5991, AZD4573, venetoclax+AZD5991, or venetoclax+AZD4573 for 48 h. The apoptosis and viable cell numbers of blast cells and stem/progenitor cells were determined by flow cytometry. EC_{50} (A) and IC_{50} (B) values of venetoclax alone or combined with AZD5991 or AZD4573, of AZD5991 alone or combined with venetoclax, and of AZD4573 alone or combined with venetoclax in blast cells and stem/progenitor cells from individual patient samples are shown. VEN, venetoclax; *, >3000 nM. Samples lacking the indicated cell populations or that had excessively high spontaneous apoptosis are not represented.

Supplementary Figure 5



Supplementary Figure S5. Body weight of the PDX cell-bearing mice during treatments with venetoclax (VEN), AZD5991, AZD4573, and the combinations.

Supplementary Figure 6

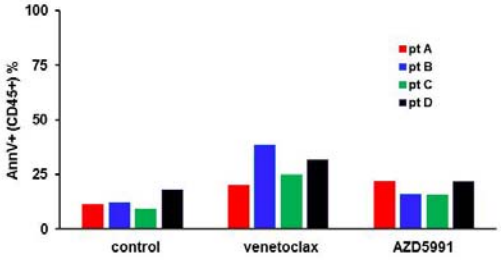


Supplementary Figure S6. The expression of signaling proteins in various leukemia cell populations isolated from mouse BM as determined by CyTOF. A. Cells from BM samples collected on treatment d 25. B. Cells from BM samples of moribund mice. After stained with antibodies against cell surface markers, all BM samples collected at d 25 treatment were barcoded, pooled into the same tube, stained, and run concomitantly and all BM samples collected from moribund mice were barcoded, pooled into the same tube, stained, and run concomitantly.

Supplementary Figure 7

A

pt sample	blast	treatments and responses	cytogenetics	mutation
A	78%	Resistance to azacitidine, Gemtuzumab+venetoclax	complex	NF1, DNMT3A, GATA2
B	77%	Resistance to chemotherapy	46,XX,add(1)(p36.1)[cp2]/46,XX[18]	NRAS, WT1
C	90%	Resistance to BP1001+Decitabine	complex	TP53, NF1, DNMT3A
D	89%	Resistance to Quizartinib+venetoclax, gilteritinib, Enasidenib, Cladribine and Low-Dose cytarabine with gilteritinib.	complex	RUNX1, IDH2, FLT3-ITD, DNMT3A, ZRSR2



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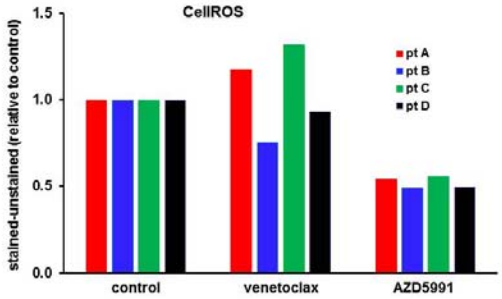

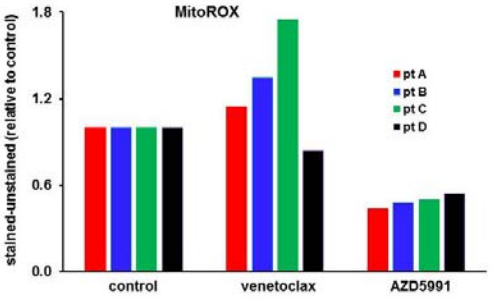

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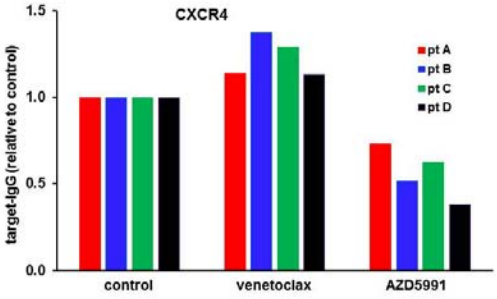
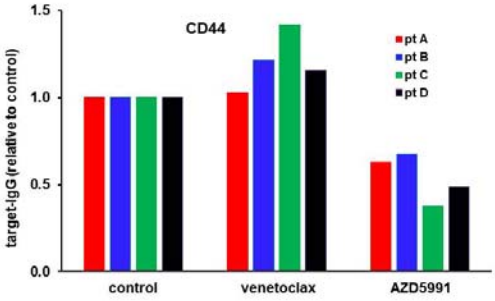
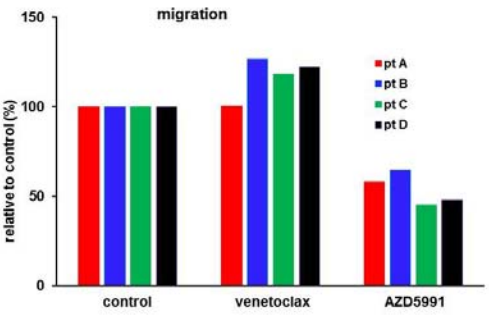
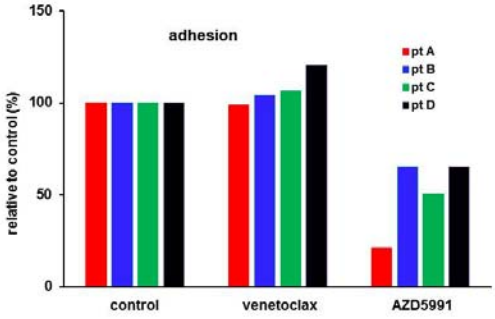




D





Supplementary Figure S7. Inhibition of MCL-1, but not BCL-2 suppresses cellular and mitochondrial ROS generation, the expression of stromal microenvironmental factors, and migration and adhesion of AML cells to MSCs in primary AML samples. A. Primary AML patient

peripheral blood samples (n = 4) were treated with venetoclax (2.5 nM) or AZD5991 (12.5 nM) for 24 h. Apoptosis in $CD45^+$ cells were determined by flow cytometry. B. Cellular and mitochondrial ROS of live cells ($CD45^+$AnnV-/DAPI-) and C. Cell surface expression of CXCR4 and CD44 were determined by flow cytometry and compared with untreated cells. D. Migration and adhesion of AML patient cells to MSCs were determined 24 h after BH3 mimetic-treated cells were incubated with MSCs and compared with untreated cells.

References

1. Sun RC, Fan TW, Deng P, Higashi RM, Lane AN, Le AT, et al. Noninvasive liquid diet delivery of stable isotopes into mouse models for deep metabolic network tracing. Nat Commun. 2017 Nov 21;8(1):1646.

2. Du D, Tan L, Wang YM, Peng B, Weinstein JN, Wondisford FE, et al. ElemCor: accurate data analysis and enrichment calculation for high-resolution LC-MS stable isotope labeling experiments. Bmc Bioinformatics. 2019 Feb 19;20.