Selective inhibition of MCL1 overcomes venetoclax resistance in a murine model of myelodysplastic syndromes

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Supplementary Methods

In vitro growth inhibition assays and determination of GI_{50} values. Cells were plated into a 384-well dish with IMDM + 10% FBS + 1% Pennicillin/Streptomycin + cytokines and supplements (10ng/ml hIL3, 10ng/ml hFLT3 ligand, 10ng/ml hTPO, 10ng/ml hSCF, 5ng/ml hIL6, 4μg/ml hLDL, 10mM 2-Mercaptoethanol, and 1x L-GlutaMAX) and treated for 48 hours with an MCL1 inhibitor (S63845; Chemietek), BCL2 inhibitor (venetoclax; Chemietek), or BCL-xL inhibitor (A-1155463; Chemietek) at concentrations from 3nM to 10μM. For combination studies, 3-fold dilution matrices of each agent were used between 10nM and 3.3μM. Cell viability was measured using CellTiterGlo (Promega), and the relative luminescence units (RLU) were measured with a micro-plate reader (BioTek). Viability was defined as the percent of RLU of each well compared to the RLU of cells treated with DMSO vehicle. The 50% growth inhibition concentration (GI_{50}) values were determined using linear regression of double-log transformed data.

Assessment of apoptosis. Apoptosis was analyzed 24 hours after treatment with DMSO, 100nM VEN, 100nM S63845, or 100nM of each compound for S63845+VEN in the same culture conditions as the growth inhibition assays using a FITC-AnnexinV/Propidium Iodide (PI) Apoptosis Detection kit per manufacter's protocol (BD Biosciences) and the anti-human CD34 antibody (Clone 581, 1:50; Biolegend), as previously described (13). Data were acquired with FACSDiva on an LSR Fortessa (BD Biosciences) equipped with 3 lasers and analyzed with FlowJo V10 software.

Next generation sequencing (NGS) and analysis. NGS results available in clinical records were used when available. When clinical results were not available, NGS was performed on DNA extracted from cryopreserved MDS samples using QIAamp DNA Blood Kit (Qiagen) with the Trusight® Myeloid Panel (Illumina). Importantly, this panel is the backbone of the clinically utilized panel, OnkoSight™ (Bio-reference Laboratories), which the sequences the identical genetic regions of interest. Alignment to hg19 and variant calling was performed using Illumina Enrichment app in BaseSpace, with restriction to minimum depth of 500x and variant allele frequency of 5%, in accordance with the manufacturer's parameters. Variant annotation was done with a modified version of vcf2maf.pl (https://github.com/mskcc/vcf2maf), a wrapper around ensembl's Variant Effect Predictor. Multiple annotation packages, including dbNSFP, ExAC, COSMIC, dbSNP, ClinVar, SIFT, Polyphen2, GERP, and CADD were used. Variants designated "common", following the protocol established by AACR GENIE(43), were considered common polymorphisms.

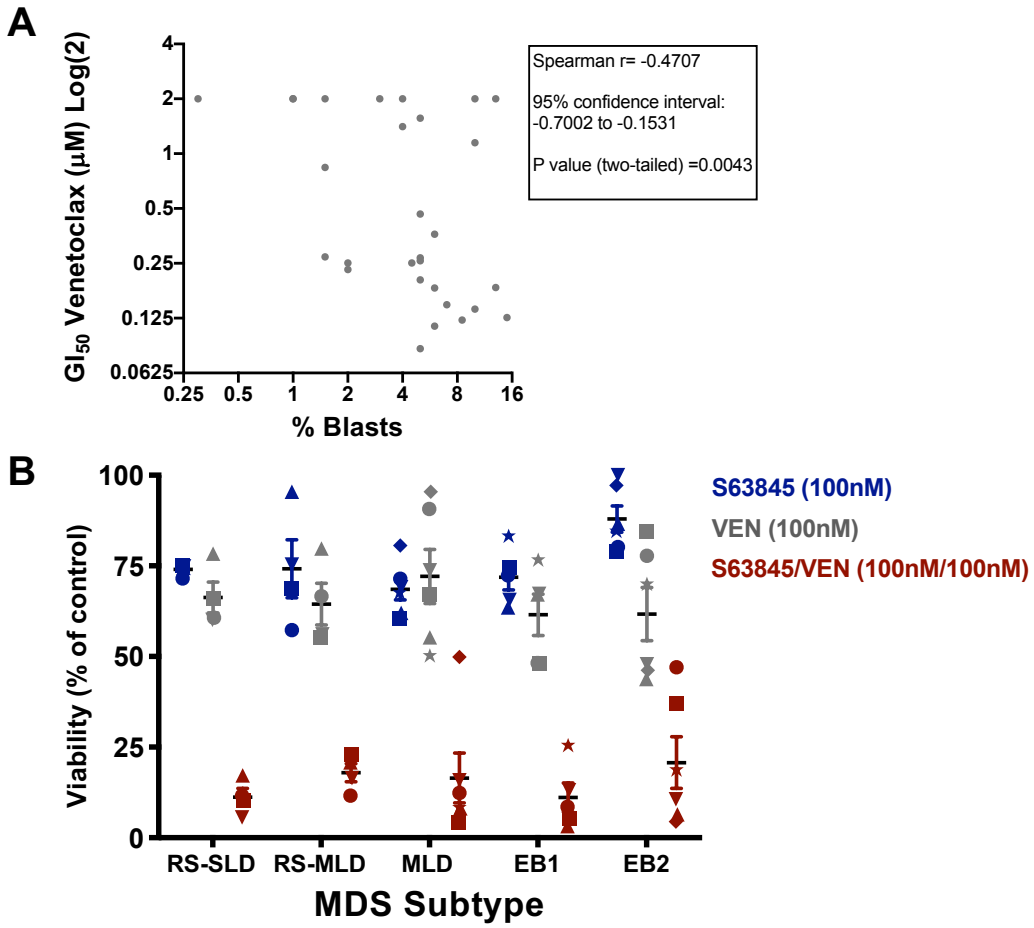
Analysis of MDS PDX. Flow cytometric analysis: Engraftment of human $CD45^+$ cells and their subsets were determined by flow cytometry. In brief, cells were isolated from engrafted mice, blocked with human/murine Fc block (BD Pharmingen), and stained with combinations of antibodies all purchased from Biolegend: HSPC panel: APC/Cy7 mCD45 (30-F11, 1:300), APC/Cy7 mTer119 (Ter-119, 1:300), BV510 hCD45 (HI30, 1:100), BV421 huCD38 (HIT2, 1:100), PE huCD34 (561, 1:100), PE/Cy7 huCD10 (HI10a, 1:100); human engraftment panel: APC/Cy7 mCD45 (30-F11, 1:300), APC/Cy7 mTer119 (Ter-119, 1:300), BV510 hCD45 (HI30, 1:100), FITC huCD3 (OKT3, 1:100), PE/Cy7 huCD19 (HIB19, 1:100), APC huCD33 (WM53, 1:100), PE huCD34 (561, 1:100). Data were acquired with FACSDiva on an LSR Fortessa (BD Biosciences) equipped with 5 lasers and analyzed with FlowJo V10 software.
Histologic analysis: Tissues were fixed in BBC Biochemical B-PLUS FIX Fixative solution (Thermo Fisher Scientific) and embedded in paraffin. Femurs were decalcified with Formic Acid Bone Decalcifier (Decal Chemical). Paraffin blocks were sectioned at 4 μm and stained with hematoxylin and eosin (H&E) and antigen-specific antibodies by the Yale Clinical Pathology and Yale Pathology Tissue Services. Antibody: human CD34 Class II, clone QBEnd 10, Dako. Images were acquired using the Nikon Eclipse 80i microscope.

Supplementary Figures

Supplementary Table 1. Detailed NGS mutation information from patient samples. (-), not detected; NA, not available. Clinically relevant mutations were called by Bio-reference (^) or clinical pathology at Vanderbilt (+) based on the OnkoSight™ NGS Myeloid Malignancies gene panel; other genes tested that were not detected in any of the samples include ABL1, BRAF, CALR, CDKN2A, CSF3R, ETV6, FBXW7, GATA2, HRAS, JAK2, KIT, KRAS, MPL, MYD88, NRAS, PHF6, PTEN, ZRSR2. # Sample was not tested for molecular diagnostics. * NGS mutation analysis was conducted.

		Molecular Diagnostics																				
MDS WHO 2016 Classification	Sample ID	ASXL1	BCOR	BCORL1	CBL	DNMT3A	EZH2	FLT3	IDH1	IDH2	NPM1	PTPN11	RUNX1	SETBP1	SF3B1	SRSF2	TET2	TP53	U2AF1	WT1
MDS-RS-SLD	MDS001^	-	-	-	-	c.2246G>A; p.Arg749His; 44%	c.1947+27>A; p.?; 7%	-	-	-	-	-	-	-	c.2098A>G; p.Lys700Glu; 31%	-	c.5600_5612del; p.Thr1857Ilefs*16; 6%	-	-	-
	MDS002^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.2230_2231delG ClnsTA; p.Ala744Tyr; 46%	-	-	-	-	-
	MDS003^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.1874G>T; p.Arg625Leu; 17%	-	-	-	-	-
	MDS004^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.1873C>T; p.Arg625Cys; 33%	-	c.3909C>g; p.Ser1303Arg; 8%	-	-	-
MDS-RS-MLD	MDS005^	c.2309del; p.Ser770*; 16%	-	c.1380_1382dup; p.Asp460dup; 40%	-	-	-	-	-	-	-	-	-	-	c.1984C>G; p.His662Asp; 39%	-	c.5688del; p.Ile1897Serfs*11; 37%	-	c.470A>G; p.Gln157Arg; 8%	-
	MDS006^	-	-	c.2117del; p.Ser706*; 14%	-	c.2645_2646delG ClnsCT; p.Arg882Pro; 46%	-	c.1828_1829ins80; p.Asn609_Leu610insZn; 7%	-	-	-	-	-	c.609-2A>G; p.?; 44%	c.2098A>G; p.Lys700Glu; 48%	-	-	-	-	-
	MDS007^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.2098A>G; p.Lys700Glu; 36%	-	c.1576C>T; p.Gln526*; 40%	-	-	-
	MDS008^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.1998G>T; p.Lys666Asn; 25%	-	-	c.750_756dup; p.Thr253Hisfs*13; 27.3% and c.698_699del; p.Val233Glyfs*5; 29%	-	-
	MDS009^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.2098A>G; p.Lys700Glu; 36%	-	c.961C>T; p.Gln321*; 38.3% and c.4750>T; p.Arg1281Cys; 44%	-	-	-
MDS-MLD	MDS010^	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.284C>A; p.Pro95His; 39%	c.4546C>T; p.Arg1516*; 46%	-	-	-
	MDS011^	c.1900_1922del; p.Glu634Asnfs*15; 39%	-	-	-	-	-	-	-	-	-	-	c.136_139dup; p.Leu47Argfs*92; 11%	-	c.1774G>A; p.Glu592Lys; 49%	-	c.4748C>A; p.Ser1583*; 42%	-	-	-
	MDS012**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS013**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS014*+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS015^	c.1907_1908del; p.Ala636Glyfs*21; 40%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS016^	-	-	-	-	-	-	-	-	-	-	-	-	-	c.1998G>C; p.Lys666Asn; 38%	c.283C>G; p.Pro95Ala; 41%	-	-	-	-
	MDS017#	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MDS-EB1	MDS018*	-	-	-	-	-	-	-	-	-	-	-	c.484A>G; p.Arg162Gly; 39%	-	-	-	-	-	-	-
	MDS019^	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.469G>T; p.Val157Phe; 7%	-	-
	MDS020*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.5650A>G; p.Thr1884Ala; 28.5% and c.5704T>C; p.Tyr1902His; 29%	c.394A>C; p.Lys132Gln; 28%	-	-
	MDS021*	c.1934dupG; p.Gly646Trpfs*12; 21%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.101C>T; p.Ser34Phe; 24%	-
	MDS022^	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.653T>C; p.Tyr222His; 73%	-	-
	MDS023^	c.2693G>A; p.Trp898*; 17%	-	-	-	c.1148C>G; p.Thr383Arg; 10%	-	-	-	-	-	-	-	-	-	-	c.4317dup; p.Arg1440Thrfs*38; 7%	-	-	-
	MDS024*+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS025^	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.757A>G; p.Thr253Ala; 40.3% and c.526T>G; p.Cys176Gly; 42%	-	-
	MDS026^	-	-	-	-	-	-	c.2503G>C; p.Asp835His; 15%	-	-	c.863_864insCATG; p.Trp288Cysfs*12; 41%	c.211T>C; p.Phe71Leu; 6%	-	-	-	-	-	-	-	C.509_508dup; p.Ala170Valfs*4; 38%
	MDS027^	-	-	-	-	-	-	-	-	-	-	-	c.610C>T; p.Arg204*; 9%	-	-	c.284C>T; p.Pro95Leu; 6%	-	-	-	-
	MDS028^	c.2077C>T; p.Arg693*; 44%	-	-	-	-	-	-	c.394C>T; p.Arg132Cys; 52%	-	c.863_864insCATG; p.Trp288Cysfs*12; 49%	-	-	-	-	c.284C>G; p.Pro95Arg; 47%	-	-	-	-
MDS-EB2	MDS029^	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.748C>T; p.Arg250Trp; 48%
	MDS030**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS031*	-	-	-	c.1247G>C; p.Cys416Ser; 66%	-	-	-	-	-	-	-	c.474T>G; p.Phe158Leu; 46%	c.2608G>A; p.Gly870Ser; 45.8%	-	-	-	-	-	-
	MDS032**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MDS033^	-	c.717_718ins16; p.Asn240240Phefs*66; 26%	-	-	-	-	-	-	-	-	c.179G>T; p.Gly60Val; 32.4% and c.215C>T; p.Ala72Val; 7%	-	-	-	-	-	-	-	-
	MDS034^	-	c.4936dup; p.Leu1646Profs*6; 17%	-	-	c.2645G>A; p.Arg882His; 47%	-	-	-	c.419G>A; p.Arg140Gln; 49%	-	-	-	-	-	c.284C>A; p.Pro95His; 52%	-	-	-	-
	MDS035*	c.1934dupG; p.Gly646Trpfs*12; 26%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Figure 1

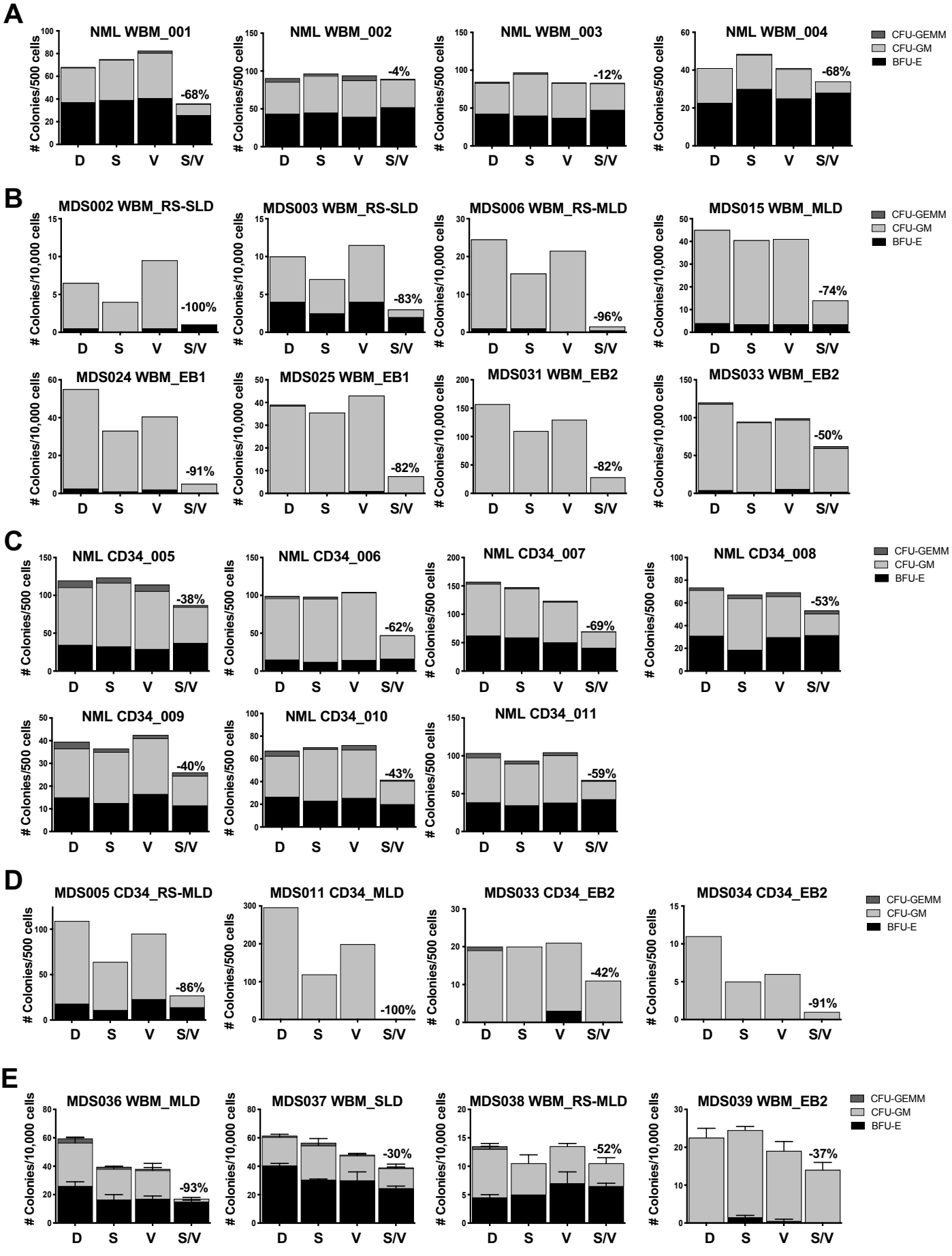


Supplementary Figure 1. Venetoclax sensitivity is correlated with blast percentage in MDS patient samples, and dual inhibition of MCL1 and BCL2 results in significant loss in cell viability. A Correlation analysis of GI_{50} with venetoclax and blast percent in MDS patient samples. Blast percent in the bone marrow by IHC recorded in the clinical record, when available, or blast percent counted on morphologic assessment of the aspirate were used. GI_{50} values greater than 2μM were capped at 2μM as this is the maximum concentration likely achievable *in vivo*. Spearman correlation and p value is displayed. **B** Cell viability was measured by CellTiter-Glo at 48 hr after treatment with 100nM of S63845, 100nM VEN, or 100nM S63845 + 100nM VEN and normalized to DMSO control.

Supplementary Table 2. Combination of BCL2+MCL1 inhibition is synergistic across all subtypes of MDS. Average ZIP δ synergy score (based on the entire range of doses tested) and maximum δ score (based on the specific concentrations that have the highest synergy for each sample) for each MDS patient sample tested. Red shading indicates synergistic ZIP scores >10, with darker shading indicating higher synergy.

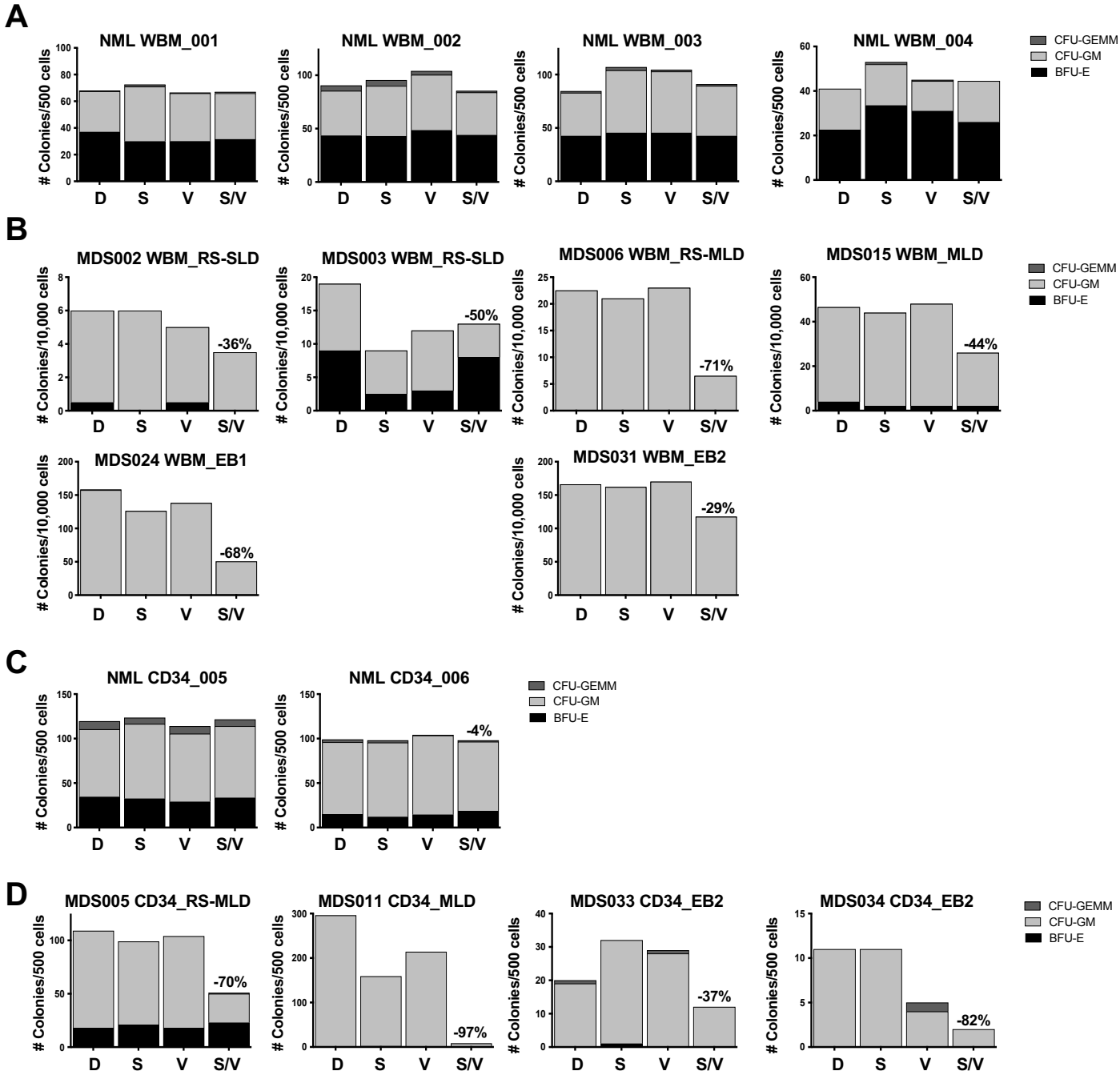
Sample ID	MDS Subtype	Average δ Score	Maximum δ Score
MDS001	RS-SLD	29.01	42.99
MDS002	RS-SLD	34.15	49.49
MDS003	RS-SLD	30.11	48.96
MDS004	RS-SLD	22.22	32.63
MDS005	RS-MLD	23.17	32.84
MDS006	RS-MLD	25.71	40.02
MDS008	RS-MLD	14.77	21.27
MDS009	RS-MLD	20.2	29.21
MDS010	MLD	22.98	37.55
MDS011	MLD	20.81	41.71
MDS013	MLD	19.72	31.01
MDS015	MLD	20.18	27.82
MDS016	MLD	32.23	43.13
MDS019	EB1	21.64	34.93
MDS025	EB1	32.55	48.44
MDS026	EB1	12.54	19.53
MDS027	EB1	19.54	32.61
MDS028	EB1	20.89	35.01
MDS029	EB2	25.9	38.47
MDS031	EB2	19.07	26.57
MDS032	EB2	27.19	41.87
MDS033	EB2	17.98	32.71
MDS034	EB2	22.65	37.57
MDS035	EB2	11.01	16.09

Supplementary Figure 2



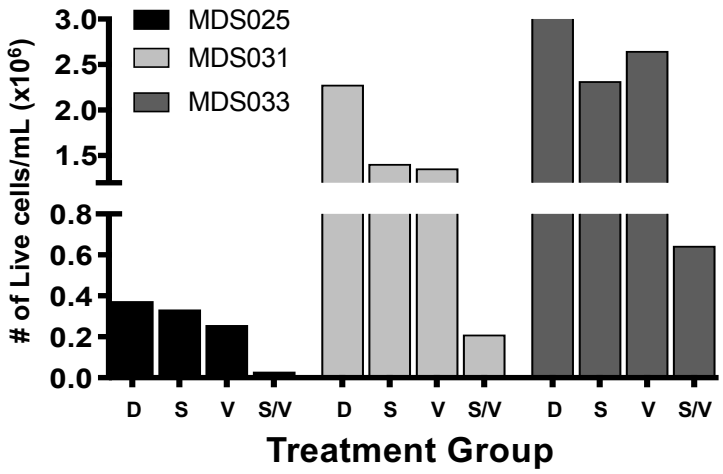
Supplementary Figure 2. Clonogenic assays reveal decreased CFU-GM colonies after BCL2+MCL1 treatment in primary MDS stem and progenitor cells. Colony forming assays of normal (NML) whole bone marrow (WBM) (**A**), MDS WBM (**B**), NML $CD34^+$ (**C**), MDS $CD34^+$ (**D**), or freshly-obtained MDS (**E**) primary cells treated with 100nM S63845 (S) + 100nM VEN (V). The number of colonies per 1 x 10^4 cells plated for WBM samples or per 500 cells plated for $CD34^+$ samples were calculated for each of the treated samples and for a DMSO control. The percent reduction of CFU-GM colonies in the combination treatment group (S/V) compared to the DMSO (D) control group is listed above each combination bar. Data is represented as the mean of two replicates. MDS-RS-SLD is MDS with ring sideroblast with single lineage dysplasia; MLD is multi-lineage dysplasia; EB1 is excess blasts 5-9%; EB2 is 10-19% blasts; CFU-GEMM is colony-forming unit – granulocyte, erythroid, macrophage, megakaryocyte; CFU-GM is colony-forming unit – granulocyte, macrophage; BFU-E is burst-forming unit – erythroid.

Supplementary Figure 3



Supplementary Fig. 3. Clonogenic assays reveal decreased CFU-GM colonies after low-dose BCL2+MCL1 treatment in primary MDS stem and progenitor cells. Colony forming assays of frozen normal (NML) whole bone marrow (WBM) (**A**), MDS WBM (**B**), NML CD34$^+$ (**C**), or MDS CD34$^+$ (**D**) primary cells treated with 50nM S63845 (S) + 50nM VEN (V). The number of colonies per 1 x 10^4 cells plated for WBM samples or per 500 cells plated for CD34$^+$ samples were calculated for each of the treated samples and for a DMSO control. The percent reduction of CFU-GM colonies in the combination treatment group (S/V) compared to the DMSO (D) control group is listed above each combination bar. Data is represented as the mean of two replicates. MDS-RS-SLD is MDS with ring sideroblast with single lineage dysplasia; MLD is multi-lineage dysplasia; EB1 is excess blasts 5-9%; EB2 is 10-19% blasts; CFU-GEMM is colony-forming unit – granulocyte, erythroid, macrophage, megakaryocyte; CFU-GM is colony-forming unit – granulocyte, macrophage; BFU-E is burst-forming unit – erythroid.

Supplementary Figure 4

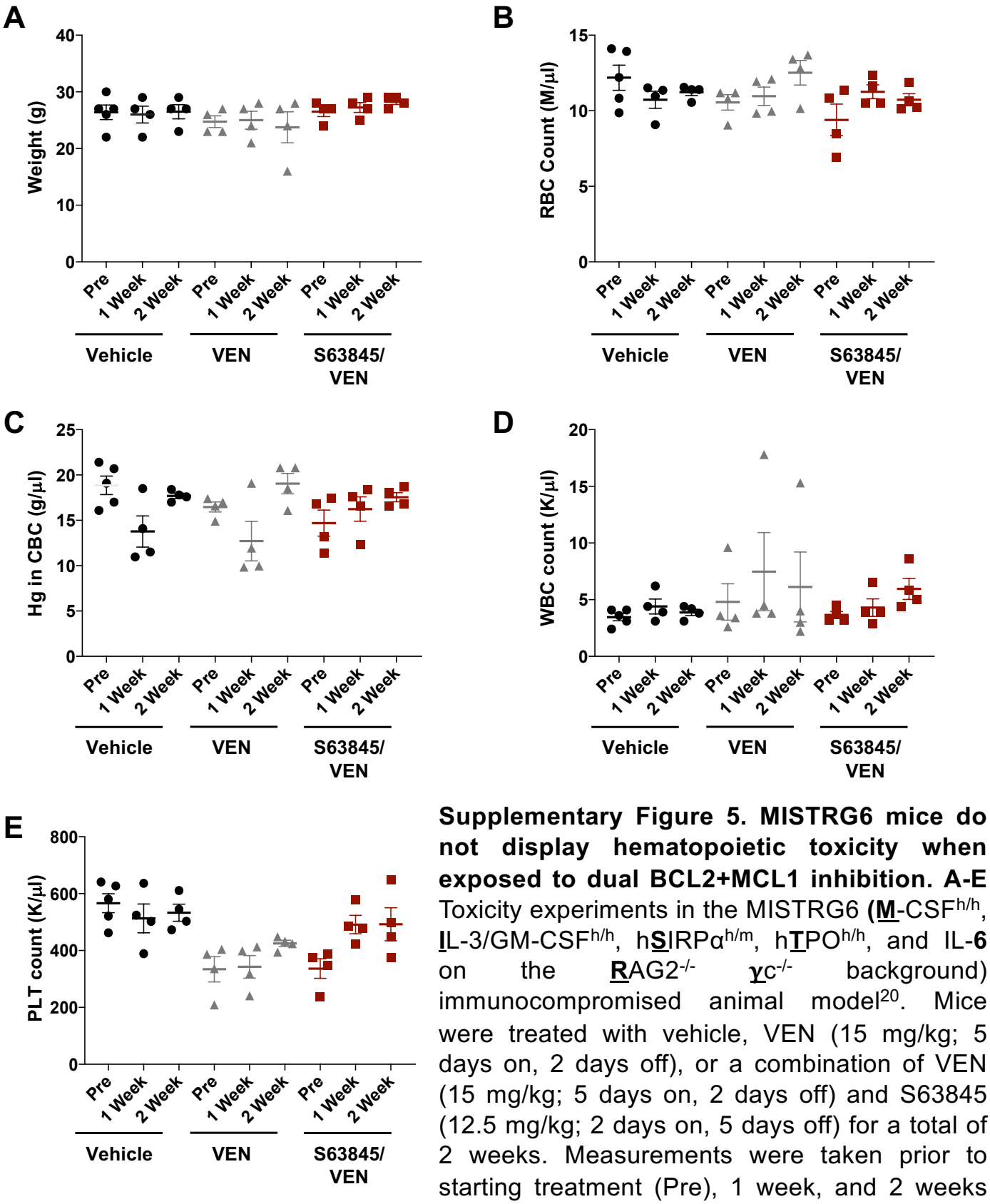


Supplementary Figure 4. Dual inhibition of MCL1 and BCL2 results in significant loss of viable cells in CFU assays. After colony forming units were counted, replicate plates were washed and the number of live cells/mL from the combined plates were determined via trypan blue exclusion for each sample tested.

Supplementary Table 3. Combination of BCL2+MCL1 inhibition reduces CFU-GM colony formation in VEN-resistant samples. The VEN GI_{50} and percent reduction of CFU-GM colonies in the combination treatment group (S63845 + VEN) compared to the DMSO control group were calculated for whole bone marrow (WBM) and CD34+ cells. Treatment groups were 100nM S63845 + 100nM VEN (100/100 SV) or 50nM S63845 + 50nM VEN (50/50 SV). CFU-GM is colony-forming unit – granulocyte, macrophage.

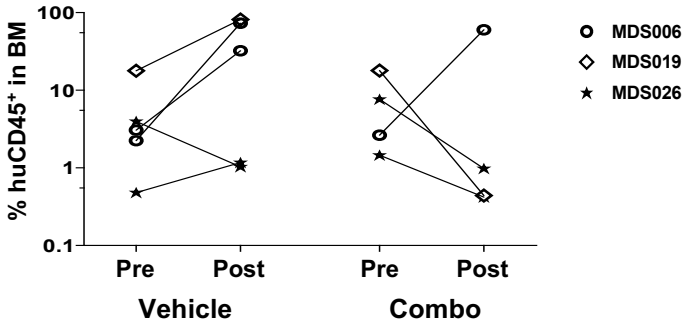
			WBM cells		$CD34^+$ cells	
		VEN GI_{50} (uM)	% Reduction from DMSO in CFU-GM with 100/100 SV	% Reduction from DMSO in CFU-GM with 50/50 SV	% Reduction from DMSO in CFU-GM with 100/100 SV	% Reduction from DMSO in CFU-GM with 50/50 SV
MDS-RS-SLD	**MDS002**	2.5	100	36		
	MDS003	>5	83	50		
MDS-RS-MLD	**MDS005**	>5			86	70
	MDS006	1	96	71		
MDS-MLD	**MDS011**	>5			100	97
	MDS015	0.3	74	44		
MDS-EB1	**MDS024**	0.1	91	68		
	MDS025	0.4	82			
MDS-EB2	**MDS031**	>5	82	29		
	MDS033	>5	50		42	37
	MDS034	0.2			91	82

Supplementary Figure 5



Supplementary Figure 5. MISTRG6 mice do not display hematopoietic toxicity when exposed to dual BCL2+MCL1 inhibition. A-E Toxicity experiments in the MISTRG6 (**M**-CSF$^{h/h}$, **I**L-3/GM-CSF$^{h/h}$, h**S**IRPα$^{h/m}$, h**T**PO$^{h/h}$, and IL-**6** on the **R**AG2$^{-/-}$ **γ**c$^{-/-}$ background) immunocompromised animal model[20]. Mice were treated with vehicle, VEN (15 mg/kg; 5 days on, 2 days off), or a combination of VEN (15 mg/kg; 5 days on, 2 days off) and S63845 (12.5 mg/kg; 2 days on, 5 days off) for a total of 2 weeks. Measurements were taken prior to starting treatment (Pre), 1 week, and 2 weeks after treatment to determine weight (**A**), red blood cell (RBC) count (**B**), hemoglobin (Hg) in complete blood count (CBC) (**C**), white blood cell (WBC) count (**D**), and platelet (PLT) count (**E**).

Supplementary Figure 6



Supplementary Figure 6. Dual BCL2+ MCL1 inhibition diminishes human MDS in bone marrow. After baseline engraftment was determined (pre), the mice from Fig. 2A-C were treated with vehicle, or a combination of VEN (15 mg/kg; 5 days on, 2 days off) and S63845 (12.5 mg/kg; 2 days on, 5 days off) for a total of 4 weeks before determining the percent of huCD45$^+$ engraftment (post) in the bone marrow (BM).