

A regimen combining the Wee1 inhibitor AZD1775 with HDAC inhibitors targets human acute myeloid leukemia cells harboring various genetic mutations

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Supplemental Materials and Methods

Analysis of cell death

Apoptosis was evaluated by flow cytometry utilizing Annexin V-FITC/PI staining as before (Dai Y, et al. Mol Cancer Ther 12:878, 2013). Loss of mitochondrial membrane potential and cell death were assessed by double staining with 3,3-dihexyloxycarbocyanine (DiOC₆) and 7-AAD as before (Dai Y, et al. Mol Cancer Ther 12:878, 2013).

For CD34⁺/CD38⁻/CD123⁺ analysis, mononuclear cells isolated from AML patient bone marrows were blocked by TruStain FcX on ice for 10min, stained with C34-PE, CD38-PE/Cy7 and/or CD123-APC (Biolegend, San Diego, CA) on ice for 30min followed by staining with Annexin V- FITC and 7AAD at room temperature for 15min. The percentage of apoptotic (Annexin V⁺) cells in the CD34⁺/CD38⁻/CD123⁺ population was determined using a FACSCanto flow cytometer (BD Biosciences).

RNA interference

SureSilencing shRNA plasmids (neomycin resistance) targeting human Wee1 (shWee1) or scrambled sequence as negative control (shNC) were purchased from SABioscience (Frederick, MD). Cells were stably transfected using the Amaxa Nucleofector (Amaxa GmbH, Cologne, Germany), and clones with Wee1 knock down were selected by G418.

Western blot analysis

Samples from whole-cell lysates were prepared, and 30 μ g of protein per condition were subjected to Western blot analysis as previously described (Dai Y, et al. *Mol Cancer Ther* 12:878, 2013). Blots were reprobed with anti- β -actin (Sigma) or anti- α -tubulin (Oncogene Inc., San Diego, CA) to ensure equal protein loading. Primary antibodies included: anti-p53, anti-phospho-cdc2/Cdk1 (Y15 or T14), and anti-phospho-Cdk2 (Y15/T14), anti-cdc2/Cdk1, and anti-Wee1 (Santa Cruz Biotech, Santa Cruz, CA); anti-phospho-Wee1 (S642), anti-cleaved caspase 9 (Asp315), anti-cleaved caspase 3 (Asp175), anti-phospho-p53 (S15 or S20), anti-phospho-histone H3 (S10), anti-phospho-Chk1 (S296, S317, or S345), and anti-Chk1 (Cell Signaling); anti-PARP (Biomol, Plymouth Meeting, PA); anti-phospho-histone H2A.X (S139; Millipore, Billerica, MA).

Supplemental Data

Table S1. Patient clinical, molecular, and cytogenetic characteristics.

Figure S1. HDACIs diminish Chk1 activation and cdc2/Cdk1 T14 phosphorylation induced by the Wee1 inhibitor AZD1775, potentiating lethality in human leukemia cells. (A) Human leukemia U937 cells were incubated with the indicated concentrations of AZD1775 +/- 1.5 μ M Vorinostat (Vor) for 8 h, after which Western blot analysis was performed to monitor phosphorylation of cdc2/Cdk1 (Y15 and T14) and Chk1 (S317), as well as expression of γ H2A.X, a marker for DNA double strand breaks. (B and C) Human leukemia U937 (B) and MV4-11 (C) cells were exposed to the indicated concentrations of AZD1775 +/- Vorinostat (Vor) or SBHA for 24 h, after which flow cytometry was performed to determine the percentage of apoptotic (Annexin V⁺) cells. (D) Time-course responses were examined at 8-48 h by Annexin V-FITC/PI staining and flow cytometry. (E) U937 and MV-4-11 cells were incubated with the indicated concentrations of AZD1775 +/- Vorinostat or SBHA, after which Western blot analysis was performed to monitor cleavage of caspase 9 and PARP. CF = cleaved fragment.

Figure S2. HDACIs block AZD1775-induced Chk1 activation and cdc2/Cdk1 T14 phosphorylation, accompanied by DNA damage. (A) U937 cells were incubated with the indicated concentrations of AZD1775 +/- Vorinostat or SBHA for 24 h, after which Western blot analysis was performed to monitor phosphorylation (S83) and total protein levels of Myt1. (B-C) U937 (B) and MV4-11 (C) cells were exposed to the indicated concentrations of AZD1775 +/- SBHA for 24 h, followed by Western blot analysis to detect phosphorylation (Chk1: S296, S317, S345; cdc2/Cdk1: T14) and total protein levels of Chk1 and cdc2/Cdk1, as well as expression of γ H2A.X. (D) After p53

wild type OCI-AML-3 cells were treated with 250 nM AZD1775 +/- 1.5 μ M Vorinostat or 20 μ M SBHA for 24 h, Western blot analysis was performed to monitor phosphorylation (S10) and total protein levels of histone H3 and expression of γ H2A.X.

Figure S3. Co-treatment for 8 h with AZD1775 and HDACIs triggers premature mitotic entry. (A) U937 cells were treated with 250 nM AZD1775 +/- 1.5 μ M Vorinostat or 15 μ M SBHA for 8 h, after which cell cycle analysis was performed by PI staining and flow cytometry. Values indicate the percentage of cells in G0/G1, S, or G2/M phases. (B-C) Alternatively, cells were pulse labeled with EdU for 30 min, followed by flow cytometry to determine the percentage of EdU⁺ cells. Cells were also double stained for p-H3 and EdU, followed by flow cytometry (upper, values indicate fold increases in p-H3-positive cells and EdU-positive cells vs untreated control, respectively) or immunofluorescence microscopy (lower, red = p-H3, green = EdU). Treatment with 10 μ M Taxol as control.

Figure S4. Co-exposure to AZD1775 and HDACIs for 16 h results in increased newly synthesized DNA and persistence of premature mitotic entry. (A) Cell cycle distribution of U937 cells was analyzed by PI staining and flow cytometry at 16 h after treatment with 250 nM AZD1775 +/- 1.5 μ M Vorinostat or 15 μ M SBHA. Values indicate the percentage of cells in G0/G1, S, or G2/M phases. (B-C) Alternatively, cells were also analyzed for EdU incorporation (B), p-H3 mitotic index (C), and double staining for p-H3 and EdU (D). Treatment with 10 μ M Taxol as control.

Figure S5. Combined treatment with AZD1775 and SBHA induces apoptosis in primary AML cells and the CD34⁺/CD38⁻/CD123⁺ primitive population, while sparing normal CD34⁺ cells. (A) Primary blasts from a patient with AML (upper) and normal cord blood CD34⁺ cells (lower) were exposed to 250 nM AZD1775 +/- 15 μ M Vorinostat for 24 h, after which cells were stained with Annexin-V/PI and analyzed by flow cytometry. Values indicate the percentage of Annexin V⁺/PI and Annexin V⁺/PI⁺ cells (B) Alternatively, the percentage of apoptotic cells was determined in the primitive CD34⁺/CD38⁻/CD123⁺ population.

Figure S6. A regimen combining AZD1775 and SBHA is active against primary AML cells. (A) Primary AML blasts were treated with 250 nM AZD1775 +/- 1.5 μ M Vorinostat or 15 μ M SBHA for 24 or 48 h, after which flow cytometry was performed to determine the percentage of cell death as determined by DiOC₆/7AAD double staining.

Figure S7. Co-administration of AZD1775 and Vorinostat is tolerable in a murine xenograft model. NSG mice were subcutaneously inoculated in the right rear flank with 5x10⁶ U937 cells stably expressing luciferase. Treatment was initiated after luciferase activity was detected (5 days after injection of tumor cells). Mice were treated with AZD1775 (50 mg/kg p.o., bid) \pm Vorinostat (100 mg/kg i.p., daily). Control animals were given equal volumes of vehicle. Body weight was measured every other day.

Table S1. Patient clinical, molecular, and cytogenetic characteristics

Pt #	Clinical Dx	Molecular Feature	Karyotype	Cytogenetics	
				FISH	FISH
Pt #1	MDS →AML (RAEB-2)	FLT3(-), NPM1(-), CEBPA(-)	43-44, XY, -3, -3, der(5)t(3;5)(q11.1;q23), del(6)(q21), -7, -21, +22, +1-3mar[cp7]/43-45, XY, sl, +3, t(4;6)(q21;p23), -del(6)(q21)[cp12]	MDS Probe Set* (1) A loss of signal for the EGR1 (5p15.2) locus in 95.5% of cells (consistent with a structural abnormality involving the long arm of chromosome 5); (2) A loss of signal for both the pericentromeric region and 7q31 in 92.5% of cells (consistent with monosomy 7 or a large deletion involving chromosome 7); AML Probe Set** (3) A gain of 22q11.2 (possible trisomy 22 or structural alteration involving for the BCR locus (22q11.2) in 50% of cells.	
Pt #2	T cells AML, biophenotypic	na*	na*	AML Probe Set** (1) A loss of signal for RUNX1 (21q22) in 82.5% of cells, with doubling of aberrant stemline with 4 signals for RUNX1T1 (8q22) and 2 for RUNX1 in 10% of cells; (2) A gain of signal for the ABL1 (9q34) and BCR (22q11.2) loci in 10.5% of cells; (3) A gain of signal for the PML (15q22) and RARA (17q21.1) loci in 7.5% of cells; (4) A gain of signal for the CBFB (16q22) locus in 7.5% of cells. (5) A gain of signal for the MLL (11q23) in 4% of cells; ALL Probe Set*** (6) A gain of signal for the MYC (8q24) locus in 8% of cells; (6) A gain of signal for the CDKN2A (9p21) locus in 6% of cells; (7) A loss of signal for 21q22 in 89.3% of cells, with doubling of aberrant stemline with 4 signals for ETV6 (12q13) and 2 for RUNX1 in 5.3% of cells.	
Pt #3	AML, monocytic	FLT3(-), NPM1(-), CEBPA(-)	47, XY, +8[9]/46, XY[11]	MDS Probe Set*: (1) a gain of signal for the pericentromeric region of chromosome 8 in 65% of cells; AML Probe Set** (2) a gain of signal for the RUNX1T1 (8q22) locus in 64.5% of cells.	
Pt #4	AML	FLT3(+), NPM1(+), CEBPA (unknown)	46, XY, der(16)(pter→p13::q22→q271::?→q22::p13→q12-13::q24)[cp2]/46, sl, t(5;10)(q31;q26)[12]/46, sl, t(9;14)(q24;q11.2)[3]/46, sl, add(7)(q32), add(11)(p15)[2]/46, XY[1].ish der(16)(pter→p13::q22→16q271::16p13→16q12-13::16q24)(3[CBFB+, 5[CBFB+][6/11]	AML Probe Set** A gain of signal for the CBFB (16q22) locus in 69.5% of cells.	
Pt #5	AML	na*		AML Probe Set** (1) A gain of signal for the MLL (11q23) locus in 96% of cells; ALL Probe Set*** (2) A loss of signal for the ETV6 (12q13) locus in 98% of cells	
Pt #6	AML (New Dx)	FLT3(-), NPM1(+), CEBPA(-)	46, XX[20].ish 7q36.3(VYJyRM2000x2)[7], 9q34.3 (D9S325x2)[8]	AML Probe Set** No anomalies for the 8 chromosomal regions evaluated.	

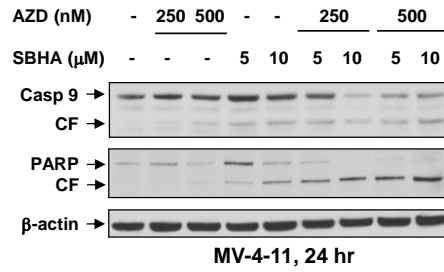
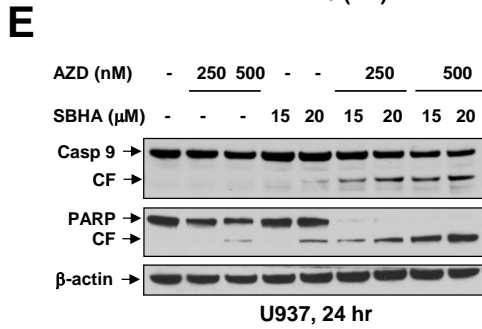
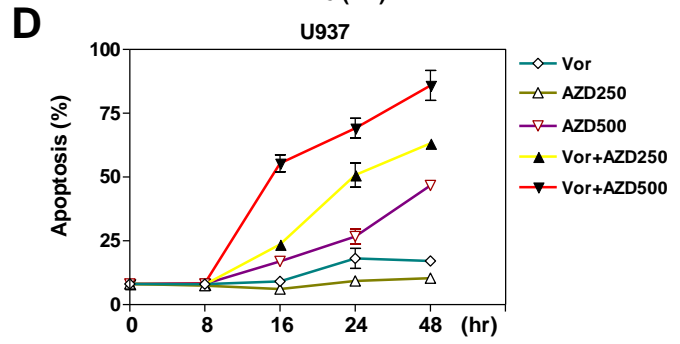
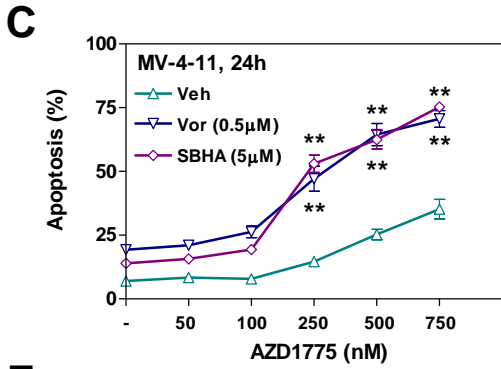
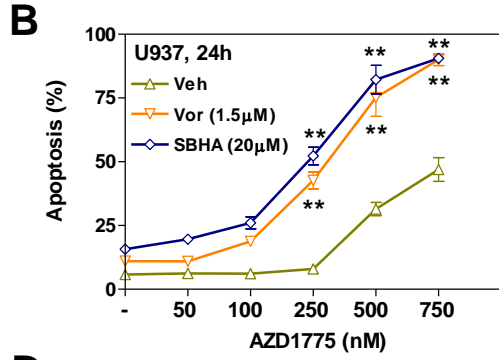
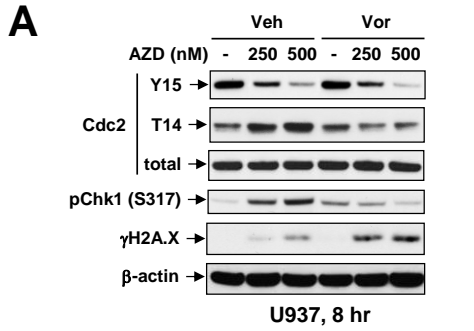
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* MDS Probe Set: D5S23x2, EGR1x1; D7Z1, D7S522; D8Z2, D20S108

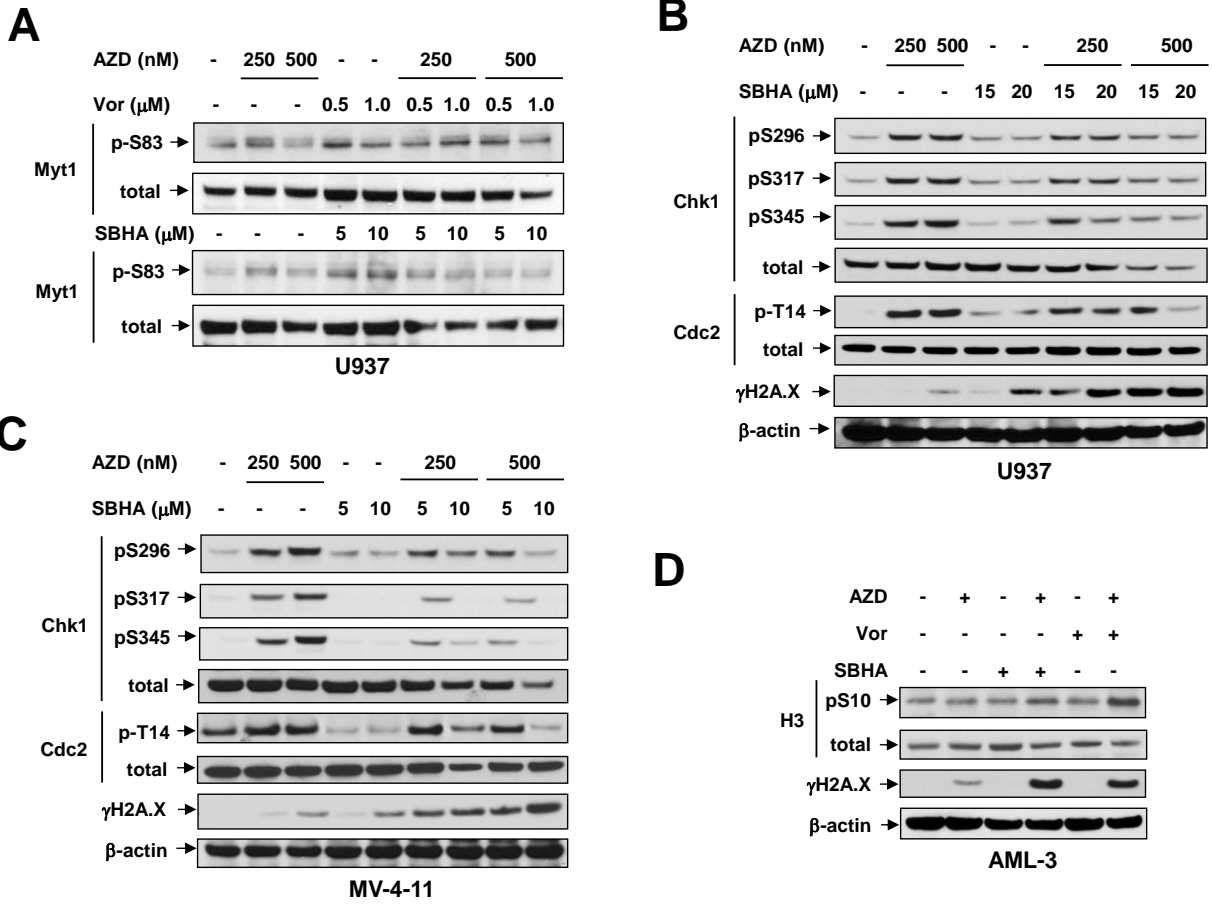
** AML Probe Set: RUNX1T1, RUNX1; ABL1x2, BCRx3; PML, RARA; CBFBx2; MLLx2

*** ALL Probe Set: MYC; CDKN2A; ABL1/BCR; MLL; ETV6/RUNX1

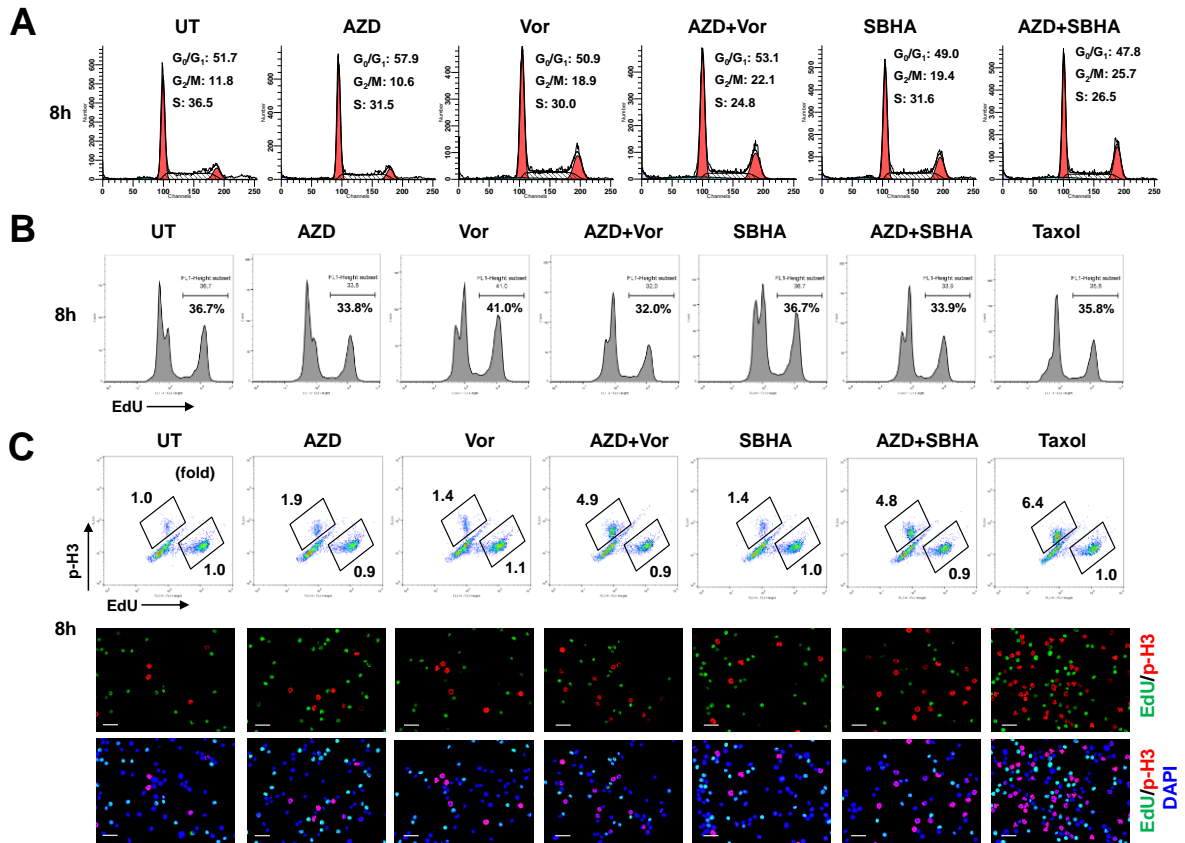
Supplemental Figure S1



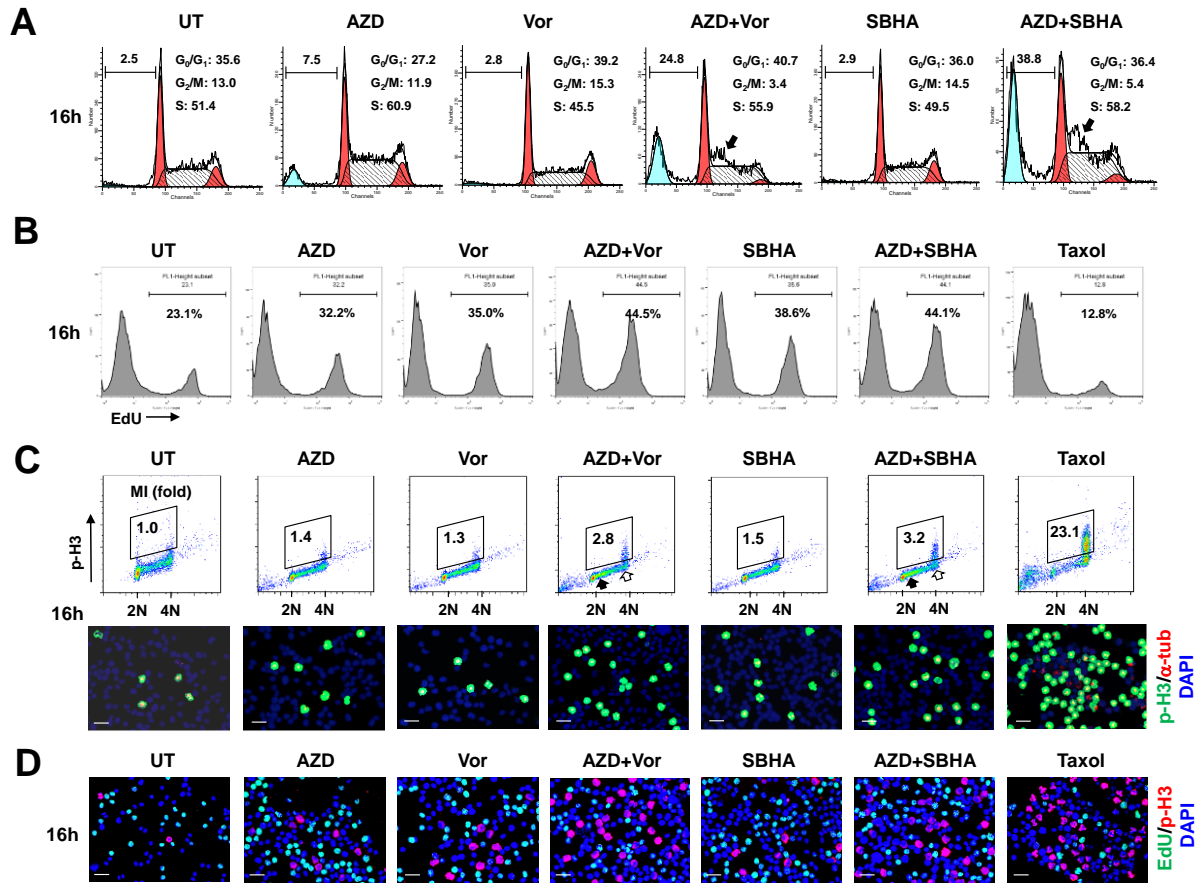
Supplemental Figure S2



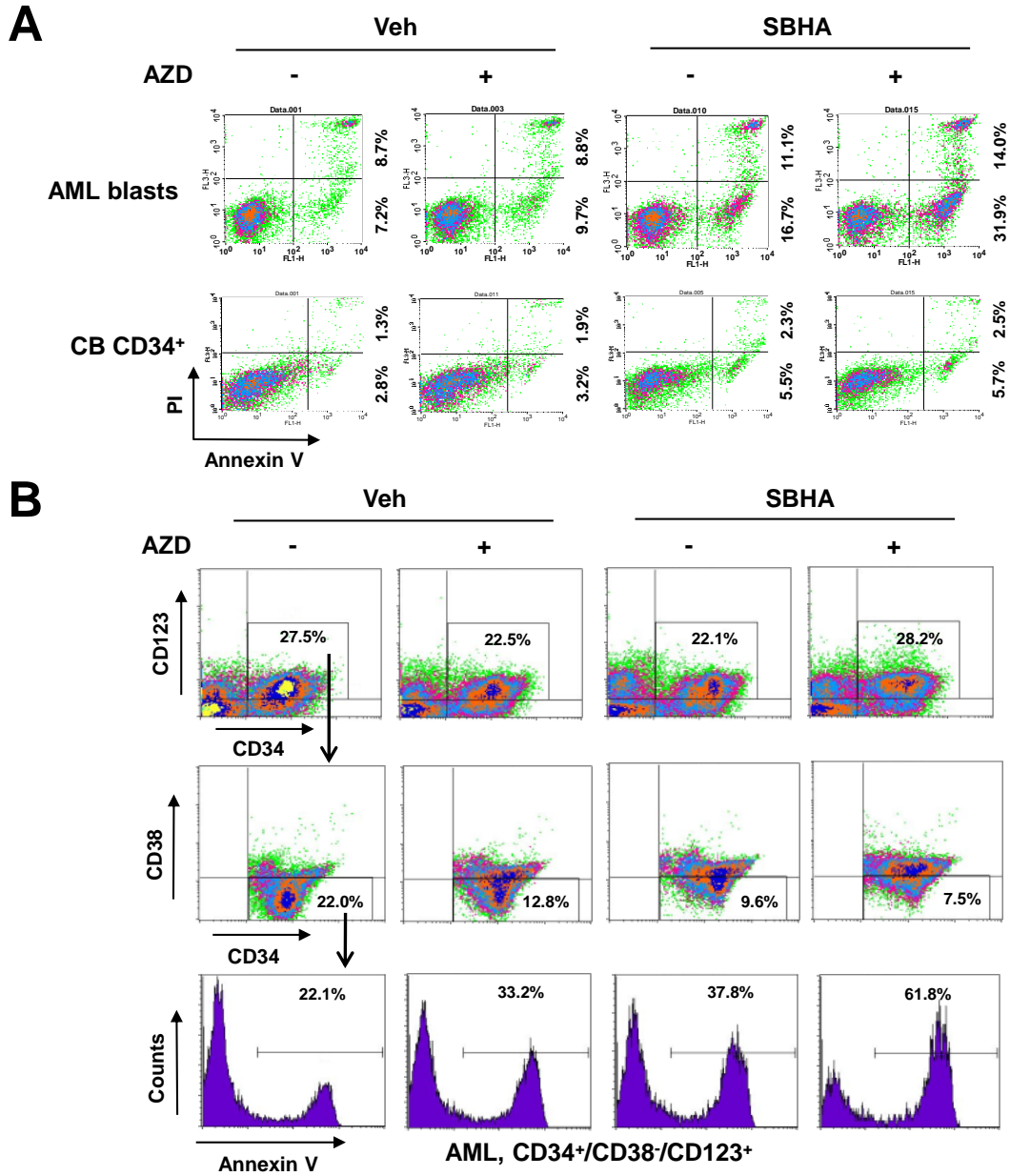
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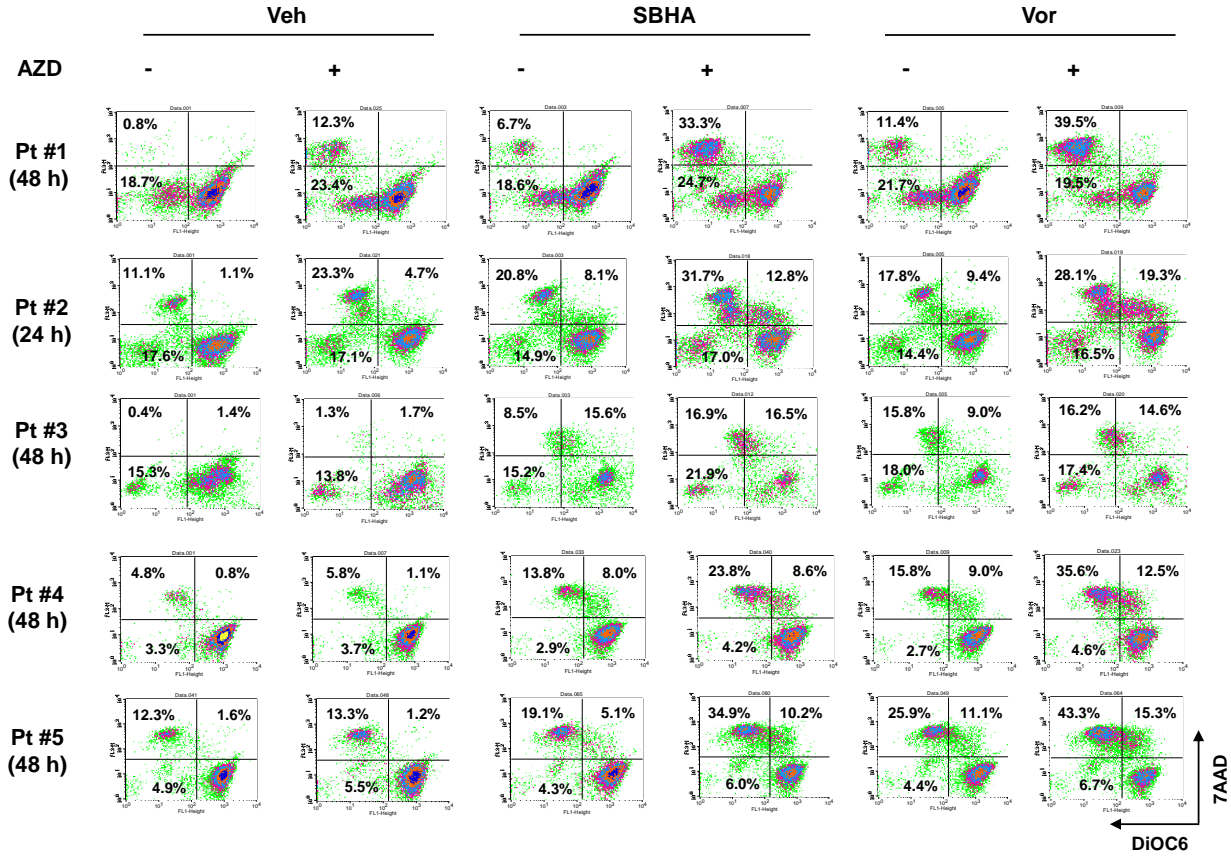
Supplemental Figure S4



Supplemental Figure S5



Supplemental Figure S6



Pt #6 (48 h)	Veh		SBHA		Vor	
	AZD -	AZD +	AZD -	AZD +	AZD -	AZD +
Cell death (%)	10.6	14.5	14.1	15.6	12.9	20.5

Supplemental Figure S7

