The HDAC and PI3K dual inhibitor CUDC-907 synergistically enhances the antileukemic activity of venetoclax in preclinical models of acute myeloid leukemia

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METHODS

Drugs

CUDC-907, venetoclax, hydroxyurea (HU), MK-1775, MG132, daunorubicin and 10058-F4 were purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

MV4-11, THP-1, and U937 cell lines were purchased from the American Type Culture Collection (2006, 2014, 2002, respectively; Manassas, VA, USA). OCI-AML3 was purchased from the German Collection of Microorganisms and Cell Cultures in 2011 (DSMZ, Braunschweig, Germany). CTS was a gift from Dr. A Fuse from the National Institute of Infectious Diseases, Tokyo, Japan (2004). MOLM-13 was purchased from AddexBio (2012; San Diego, CA, USA). HS-5 was purchased from ATCC. The cell lines were authenticated in 2017 at the Genomics Core at Karmanos Cancer Institute using the PowerPlex® 16 System from Promega (Madison, WI, USA). Mycoplasma testing was performed on a monthly basis using the PCR method [1]. AML cell lines were cultured as previously described.[2, 3]

Diagnostic blast samples were purified by standard Ficoll-Hypaque density centrifugation, then cultured in RPMI 1640/20% fetal bovine serum (Thermo Fisher Inc., Rockford, IL, USA) supplemented with ITS solution (Sigma-Aldrich, St. Louis, MO, USA) and 20% supernatant of the 5637 bladder cancer cell line (as a source of granulocyte-macrophage colony-stimulating factor).[4] Normal bone marrow mononuclear cells (BMMNCs) were purchased from Lonza (Walkersville, MD, USA).

Annexin V/PI staining

AML cells were treated with CUDC-907, venetoclax, HU, MK-1775, MG132, 10058-F4, or a combination for up to 24 h and then underwent Annexin V-fluorescein isothiocyanate

(FITC)/propidium iodide (PI) staining (Beckman Coulter; Brea, CA, USA). Samples were analyzed by flow cytometry, as previously described.[5, 6] All experiments utilizing the AML cell lines were performed three times in triplicate independently, and experiments using primary patient samples were performed once in triplicate due to limited sample. Patient samples were selected based on availability of adequate sample for the assay. Apoptotic events are displayed as mean percentage of Annexin V positive/PI negative (early apoptotic) and Annexin V positive/PI positive (late apoptotic and/or dead) cells ± the standard error from one representative experiment. Combination index values (CI) were calculated using CompuSyn software (Combosyn Inc. Paramus, NJ, USA). CI<1, CI=1, CI>1 indicate synergistic, additive, and antagonistic effects, respectively.

HS-5 co-culture

AML cells from freshly isolated primary patient samples (n=3) were seeded in the absence or presence of the human bone marrow stromal HS-5 cells (1:1 ratio) for 24 h in RPMI 1640/20% fetal bovine serum (Thermo Fisher Inc.) supplemented with ITS solution (Sigma-Aldrich) and 20% supernatant of the 5637 bladder cancer cell line (as a source of granulocyte-macrophage colony-stimulating factor).[4] The cells were then treated with CUDC-907, venetoclax, or in combination for 24 h. Cells were trypsinized, stained with CD45 (to distinguish AML cells from HS-5 cells), CD34, and Annexin V-FITC and analyzed by flow cytometry. This experiment was performed once in triplicate due to limited sample.

Western blot analysis

AML cells were lysed by sonication in 10 mM Tris-Cl, pH 7.0, containing 1% SDS, protease inhibitors, and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, electrophoretically

transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc., Rockford, IL, USA) and immunoblotted with antibodies. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described by the manufacturer. For cell lines, three independent experiments were performed. Due to limited number of cells obtained from primary patient samples, Western blots of patient samples were from one independent experiment. Patient samples were selected based on availability of adequate sample for the analysis. Anti-PARP, -Mcl-1, -Bcl-2, -β-actin, -CHK1 (Proteintech, Chicago, IL, USA), -Bim, -p-Mcl-1 (T163), -Wee1, -RPA32, -cleaved (cf) caspase 3, -c-Myc (Cell Signaling Technologies, Danvers, MA, USA), -RRM1, -H4 (Abcam, Cambridge, MA, USA), and -γH2AX (Millipore, Billerica, MA, USA) antibodies were used for Western blot analysis.

shRNA knockdown of Bim and overexpression of Mcl-1 and c-Myc

Lentivirus production and transduction were carried out as previously described.[7] The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at Tulane University. Bim and non-target control (NTC) shRNA lentiviral vectors were purchased from Sigma-Aldrich. Red fluorescent protein (RFP), c-Myc, and Mcl-1 cDNA constructs were purchased from Thermo Fisher Scientific Biosciences (Lafayette, CO, USA).

Immunoprecipitation

AML cells were treated with CUDC-907 and venetoclax, alone or in combination, for 24 hours and then lysed using 1% CHAPS, 5 mM MgCl2, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM Tris, and 0.05% Tween-20 in the presence of protease inhibitors (Roche Diagnostics). Immunoprecipitation of Bim and Mcl-1 were performed using 2 μg of anti-Bim (2819, Cell Signaling Technologies) or anti-Mcl-1 (16225-1-AP, Proteintech), 1 mg of protein lysate, and

Protein A agarose beads (Roche Diagnostics) as previously described.[8] Proteins were eluted using 50 mM glycine, pH 2.0, and then analyzed by Western blotting.

Alkaline comet assay

Following treatment with CUDC-907, venetoclax, HU, MK-1775, 10058-F4, or in combination for up to 28 hours, AML cells were subjected to alkaline comet assays, as previously described [7]. Using SYBR Gold (Life Technologies, Grand Island, NY, USA), slides were stained and then subsequently imaged using an Olympus BX-40 microscope equipped with a DP72 microscope camera and Olympus CellSens Dimension software (Olympus America Inc., Center Valley, PA, USA). Approximately 50 comets were scored per gel, using CometScore (TriTek Corp, Sumerduck, VA, USA). Median percentage of DNA in the tail was calculated and graphed as mean ± the standard error. Primary patient samples were chosen for this assay based on adequate sample availability.

Real-time PCR

Total RNA was extracted using TRIzol (Life Technologies), cDNAs were prepared from 2 µg of total RNA using random hexamer primers and a RT-PCR kit (Thermo Fisher Scientific), and then purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD, USA), as described previously.[2, 6, 9] *Mcl-1* (Hs01050896_m1), *Bim* (Hs00708019_s1), *CHK1* (Hs00967506_m1), and *Wee1* (Hs01119384_g1) transcripts were quantitated using TaqMan probes (Thermo Fisher Scientific) and a LightCycler 480 real-time PCR machine (Roche Diagnostics), based on the manufacturer's instructions. *RRM1* transcripts were quantified using forward (5'-ACTAAGCACCCTGACTATGCTATCC-3') and reverse (5'-CTTCCATCACATCACTGAACACTTT-3') primers and SYBR green, and the above-mentioned real-time PCR machine. *c-Myc* transcripts were quantified using forward (5'-

GTGGTCTTCCCCTACCCTCT-3’) and reverse (5’-CGAGGAGAGCAGAGAATCCG-3’) primers. Real-time PCR results are expressed as means from three independent experiments and normalized to GAPDH transcripts measured by either TaqMan probe (Hs02786624_g1) or forward (5’-AGCCACATCGCTCAGACA-3’) and reverse (5’-GCCCAATACGACCAAATCC-3’) primers and SYBR green. Fold changes were calculated using the comparative Ct method.[10]

Chromatin fractionation

Cells were treated with CUDC-907 and venetoclax, alone or in combination, for 16 or 24 hours. Chromatin fractionation was carried out as described previously.[11] These experiments were repeated three independent times.

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Table S1. Patient characteristics of the primary AML patient samples used in this study

Patient	Gender	Age (year)	Disease status	FAB subtype	Cytogenetics	Blast purity (%)	Gene mutation
AML#1	Female	74	Newly diagnosed	M1	46, XX	69	
AML#2	Male	48	Newly diagnosed	M2	46, XY	79.5	FLT-3 ITD
AML#3	Male	50	Newly diagnosed	M2	46, XY	88.0	
AML#4	Female	5	Newly diagnosed	M4	NA	49.0	
AML#5	Female	13	Newly diagnosed	M2	NA	66.0	NA
AML#6	Male	65	Newly diagnosed	M4	46, XY	86.0	FLT3-ITD, DNMT3A, IDH2
AML#7	Male	27	Newly diagnosed	M3	46, XY, t(15;17)(q22;q21)	94.0	FLT3-ITD, PML-RARα
AML#8	Male	46	Newly diagnosed	M1	46, XY	92.0	CEBPA double mutation
AML#9	Female	10	Newly diagnosed	M5	46, XX	97.0	
AML#10	Female	60	Relapsed	M2	46, XX	69.0	CEBPA double mutation
AML#11	Male	40	Newly diagnosed	M2	46, XY	88.5	CEBPA double mutation
AML#12	Female	51	Relapsed	M5	46, XX	94	FLT3-ITD, NPM1, DNMT3A
AML#13	Male	49	Relapsed	M2	46, XY	79.5	FLT3-ITD, NPM1
AML#14	Male	66	Newly diagnosed	M5	46, XY	41	
AML#15	Male	2	Newly diagnosed	M5	50, XY, +8, +11, +14, +19	66.4	
AML#16	Male	66	Newly diagnosed	M2	46, XY	NA	CEBPA double mutation
AML#17	Female	40	Newly diagnosed	M4	46, XY	53.5	NPM-1, IDH-1
AML#18	Female	48	Newly diagnosed	M2	NA	61	NA
AML#19	Female	59	Newly diagnosed	M2	47, XX,+8	60.5	FLT3-ITD, DNMT3A
AML#20	Female	54	Newly diagnosed	M2	46, XX	58.5	CEBPA double mutation, IDH2, N-RAS
AML#21	Male	68	Relapsed	M5	46, XY	96.5	FLT3-ITD, NPM1, DNMT3A
AML#22	Female	51	Newly diagnosed	M4	NA	94	NA
AML#23	Female	42	Newly diagnosed	M5	46, XX	64	NPM1, DNMT3A, IDH1, FLT3-TKD
AML#24	Female	61	Newly diagnosed	M3	46, XX, t(15;17)(q22;q21)	90.2	PML-RARα, ATRX, FLT3-ITD
AML#25	Female	33	Newly diagnosed	M4/M5	46, XX	87	NPM1
AML#26	Male	56	Relapsed	M4/M5	46, XY	88	NA
AML#27	Male	9	Newly diagnosed	M4/M5	46, XY	83.5	CEBPA, FLT3-TKD, WT1

NA: not available

Table S2. Characteristics of the AML cell lines used in this study

Cell line	Gender	Age (year)	Disease Statue	FAB subtype	Cytogenetics	Gene fusion/mutation	Doubling time	CUDC-907 EC50 (nM)
MOLM-13	Male	20	At relapse	M5	Ins(11;9)(q23;p22p23	FLT3-ITD, MLL-AF9	24	42.4
U937	Male	37	Refractory	M5	t(10;11)(p13;q14)	CALM-AF10	24	74.0
CTS	Female	13	At relapse	M1	t(6;11)(q27;q23)	MLL-AF6	72	40.4
MV4-11	Male	10	At relapse	M5	t(4;11)(q21;q23)	FLT3-ITD, MLL-AF4	32	24.0
OCI-AML3	Male	57	At relapse	M5		NPM1	35-40	44.8
THP-1	Male	1	At relapse	M5	t(9;11)(p21;q23)	MLL-AF9	35-40	323.9

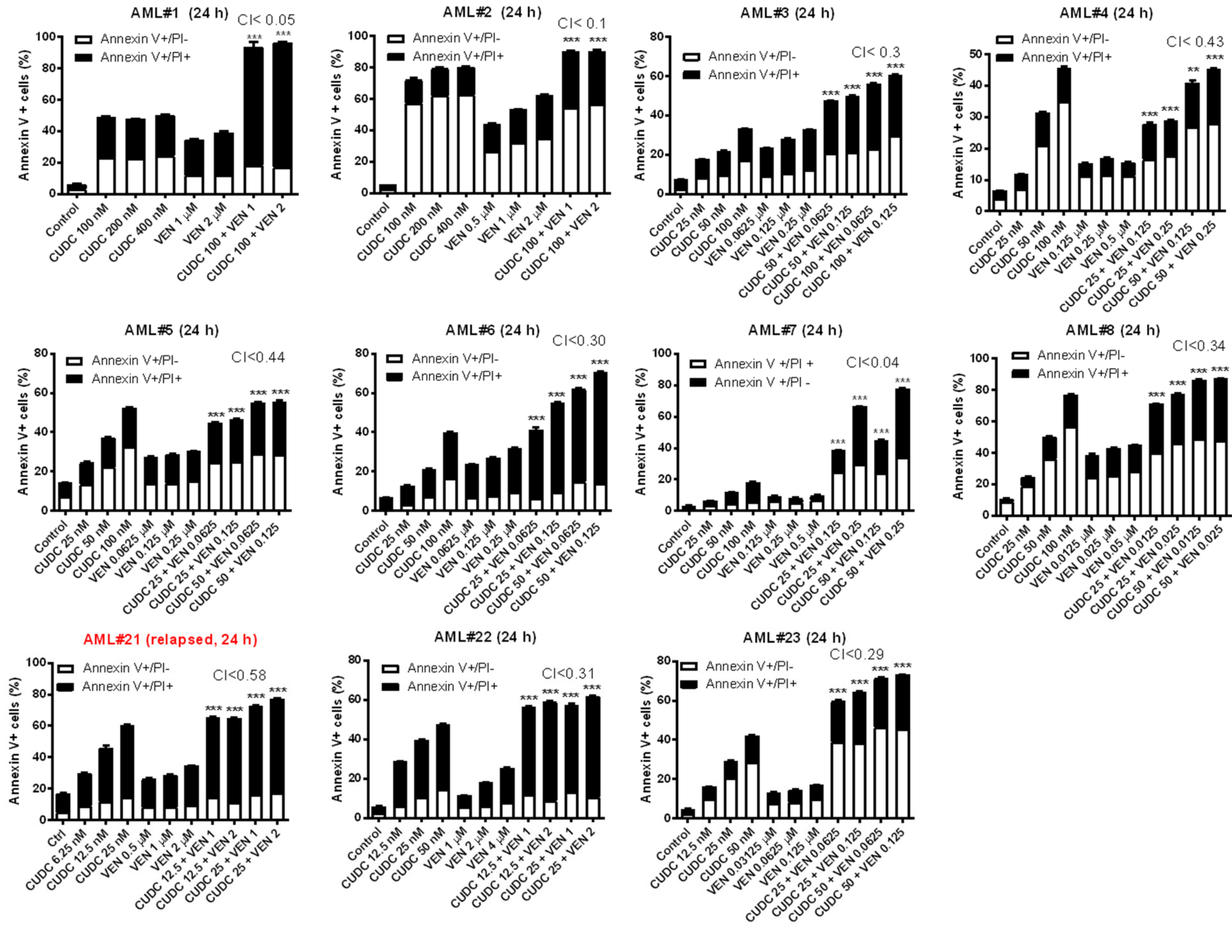

Figure S1: Primary AML patient samples were treated with vehicle control, venetoclax (VEN), CUDC-907 (CUDC), or in combination for 24 h. Flow cytometry analysis of Annexin-V-FITC/PI staining was performed. Results are shown as mean percent Annexin V+ cells ± SEM. Combination index (CI) values were calculated using CompuSyn software. **indicates $p<0.01$ and ***indicates $p<0.001$ compared to single drug treatments.

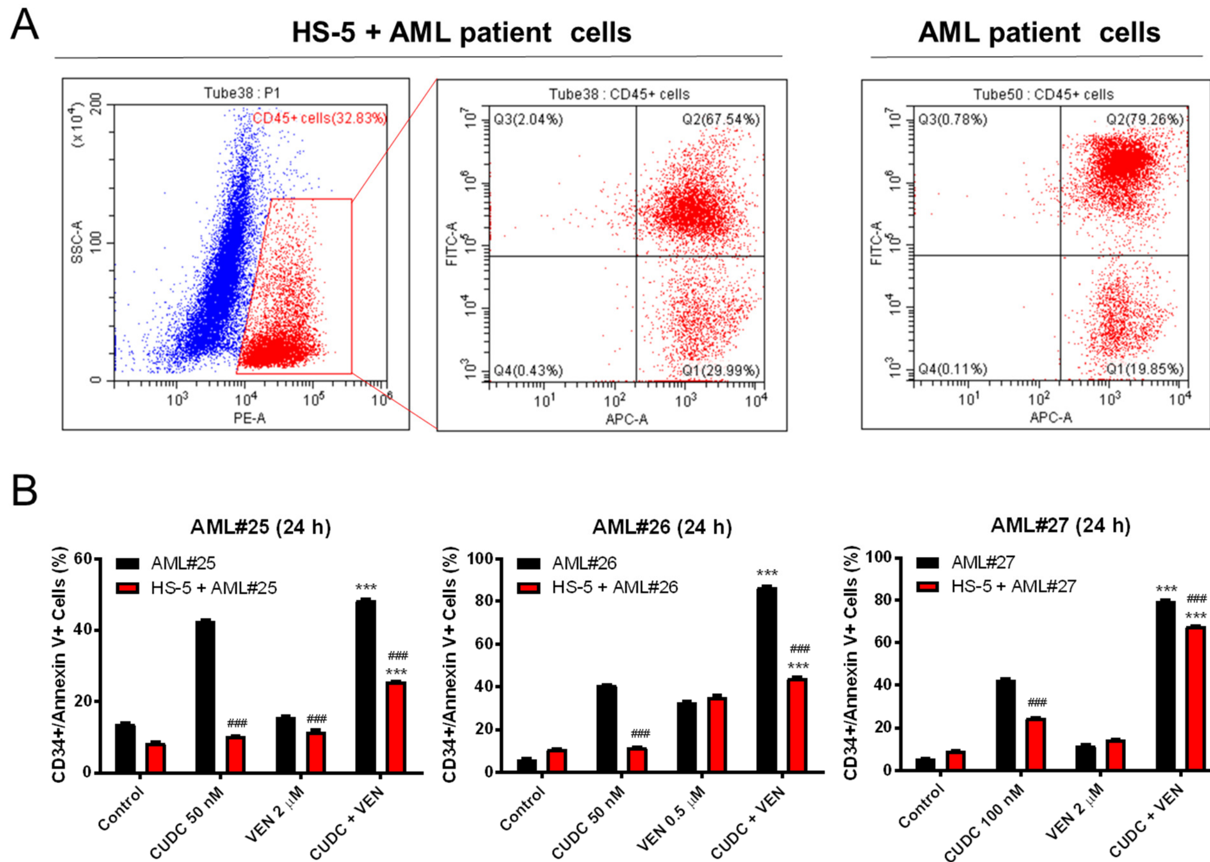

Figure S2: (A&B) Primary AML patient samples were treated with vehicle control, venetoclax (VEN), CUDC-907 (CUDC), or in combination for 24 h in the absence or presence of human bone marrow stromal HS-5 cells. The cells were harvested and then subjected to flow cytometry analysis. Within the CD45+ cell population, CD34+ and Annexin V+ cells were determined. The gating strategy is shown in panel A. Results are shown as mean percent CD34+/Annexin V+ cells ± SEM. ***indicates p<0.001 compared to control, CUDC-907, and venetoclax single drug treatment, for either the patient sample or the patient sample grown in the presence of HS-5. ###indicates p<0.001 compared to the same drug treatment, in the absence of HS-5 cells.

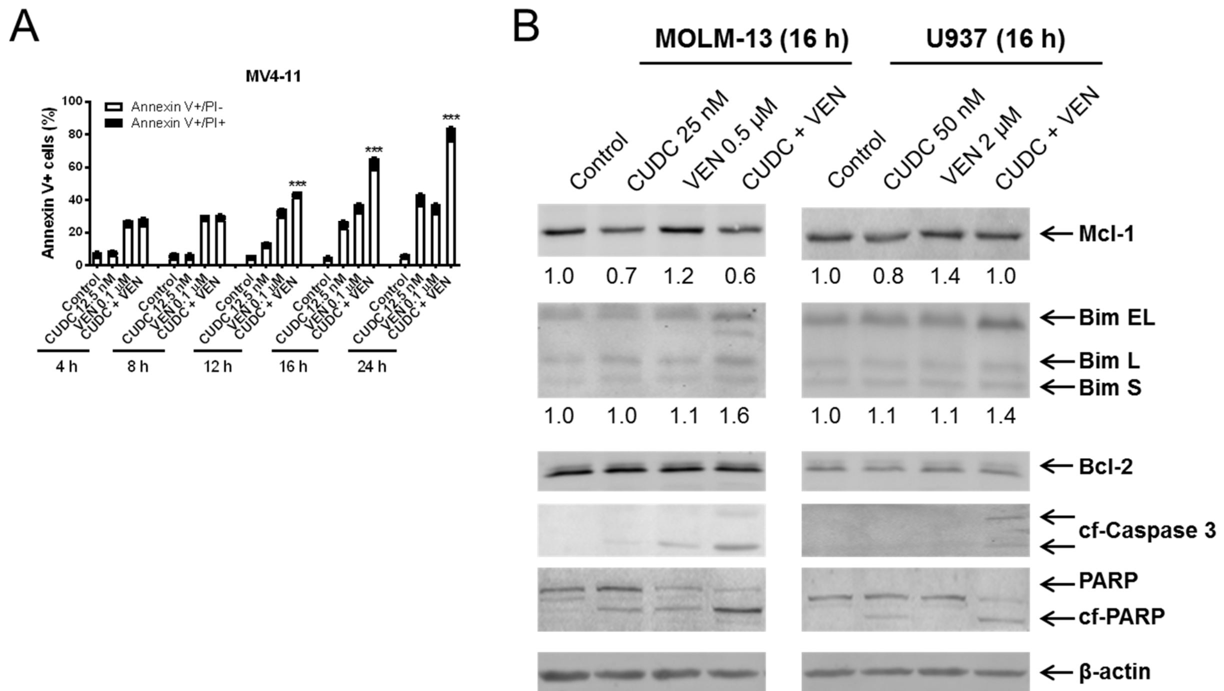

Figure S3: (A) MV4-11 cells were treated with vehicle control, venetoclax (VEN), CUDC-907 (CUDC), or in combination for up to 24. Flow cytometry analysis of Annexin-V-FITC/PI staining was performed. Results are shown as mean percent Annexin V+ cells ± SEM. ***indicates p<0.001 compared to single drug treatments. (B) AML cells were treated with vehicle control, venetoclax, CUDC-907, or in combination for 16 hours, and whole cell lysates were subjected to Western blotting. Representative Western blots are shown. The fold changes for the densitometry measurements, normalized to β-actin and then compared to no drug control, are indicated below the corresponding blot. Bim S, L, and EL indicate Bim short, long, and extra-long isoforms, respectively.

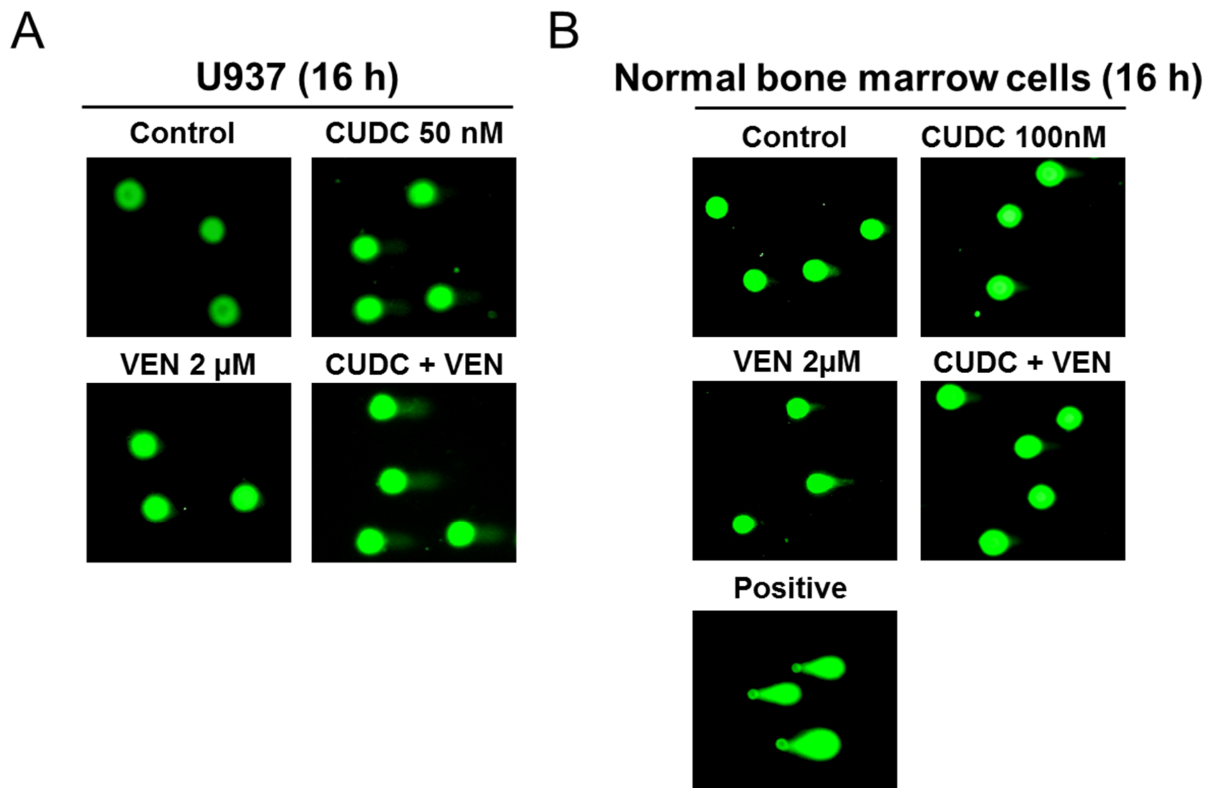

Figure S4: AML cells and normal bone marrow cells were treated for 16 hours with vehicle control, venetoclax (VEN), CUDC-907 (CUDC), venetoclax + CUDC-907, or a positive control (20 μM daunorubicin for 4 hours), and then subjected to alkaline comet assay analysis. Representative alkaline comet assay images for U937 and normal bone marrow cells are shown.

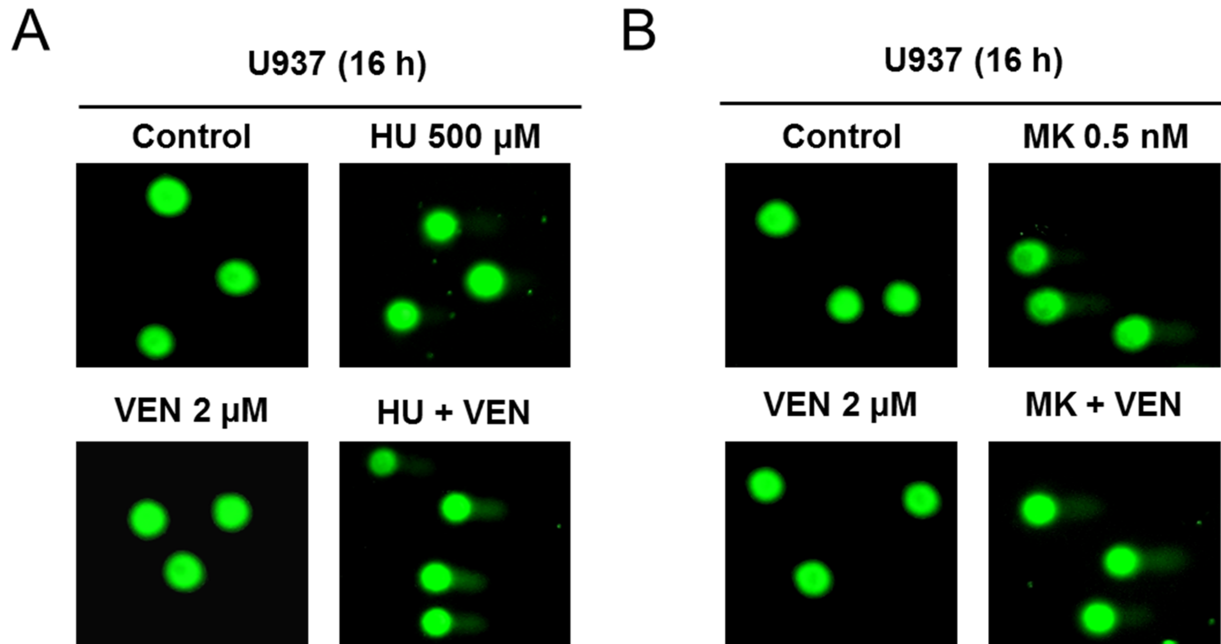

Figure S5: U937 cells were treated with venetoclax (VEN) in the presence or absence of MK-1775 (MK) or hydroxyurea (HU) for 16 h, and then subjected to alkaline comet assay analysis. Representative images are shown.

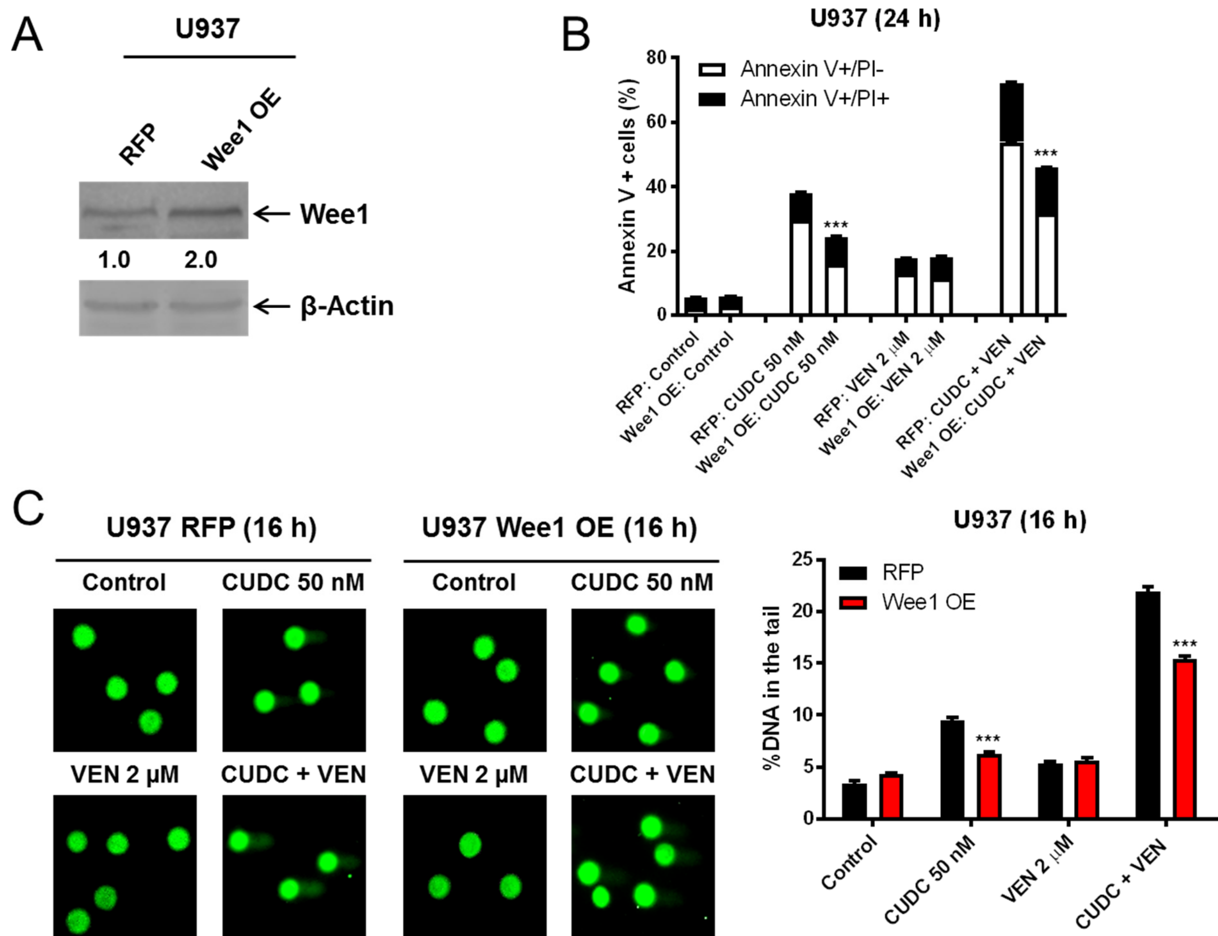

Figure S6: U937 cells were infected with Precision LentiORF Wee1 (U937/Wee1) or RFP control (U937/RFP) lentivirus overnight, then washed and incubated for 48 h prior to adding blasticidin to the culture medium. Whole cell lysates of the antibiotic resistant cells were subjected to Western blotting. The fold changes for the Wee1 densitometry measurements, normalized to β-actin and then compared to no drug treatment control, are indicated (panel A). The cells were treated with CUDC-907 (CUDC) or/and venetoclax (VEN) for 24 hours, and then subjected to Annexin V/PI staining and flow cytometry analysis. ***indicates $p<0.001$ (panel B). The antibiotic-resistant cells were treated with CUDC-907 or/and venetoclax for 16 hours and then subjected to alkaline comet assay analysis. Representative images are shown (panel C, left panel). Data are graphed as mean percent DNA in the tail from 3 replicate gels ± SEM. ***indicates $p<0.001$ (panel C, right panel).

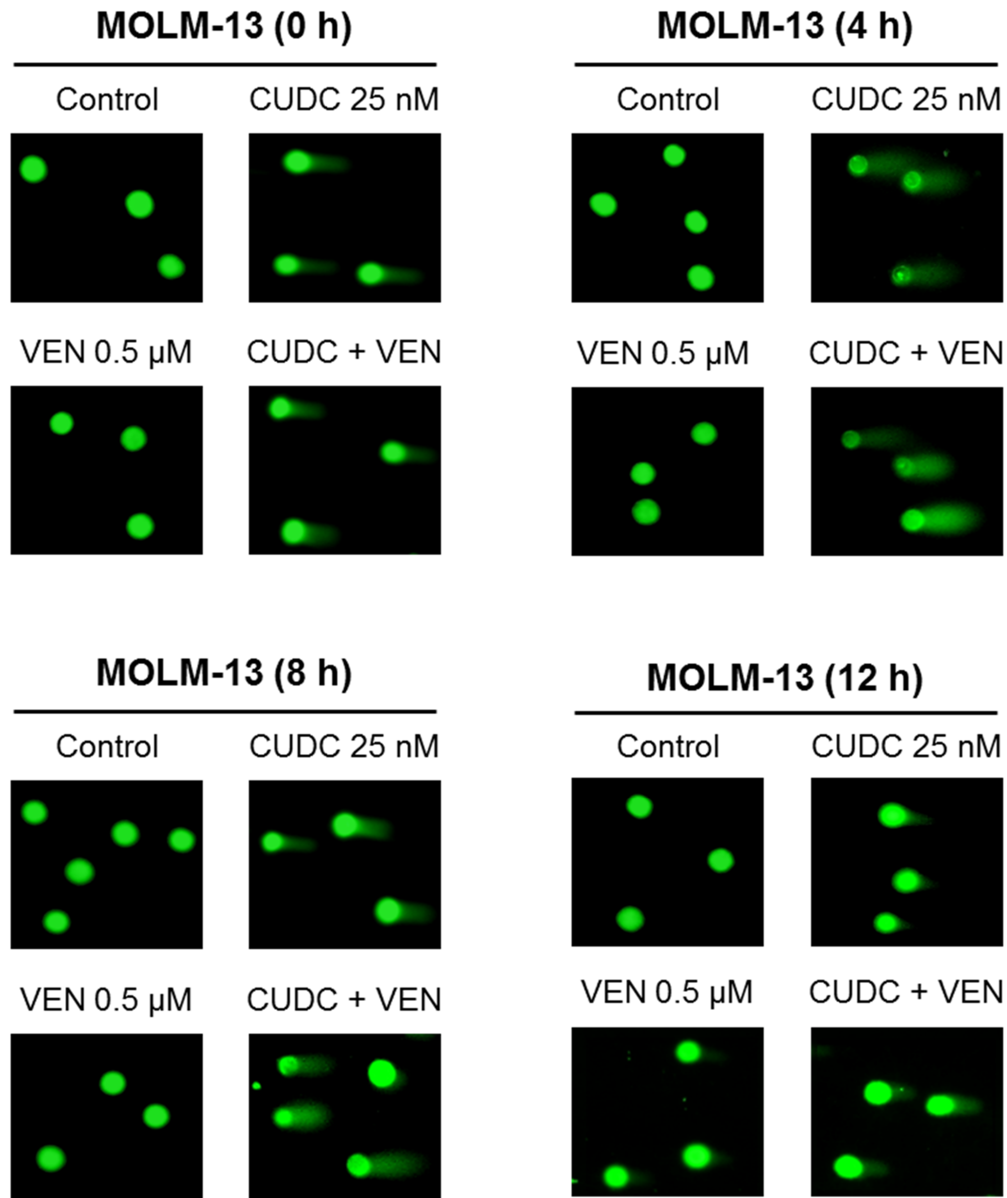

Figure S7: MOLM-13 cells were treated with or without 25 nM CUDC-907 (CUDC) for up to 16 hours. The cells were washed with PBS three times and then treated with or without venetoclax (VEN) for up to 12 h. The cells were subjected to alkaline comet assay analysis. Representative images are shown.