SUPPLEMENTARY APPENDIX

CHK1 and WEE1 inhibition combine synergistically to enhance therapeutic efficacy in acute myeloid leukemia *ex vivo*

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Supplementary Material

SUPPLEMENTAL METHODS

Cell Culture and Reagents: The human cell lines TF-1, THP-1, HEL and U937 were obtained from ATCC (Manassas, VA). HL-60 (Robert T. Abraham, Pfizer) and ML-1 (Michael Kastan, Duke University) were authenticated by short tandem repeat profiling in the Mayo Advanced Genomics Technology Center. MDS-L is a MDS cell line transformed to AML and was kindly provided by Dr. Kaoru Tohyama, Japan. Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). Cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37˚C under 5% CO_2 atmosphere.

High-Throughput siRNA Screens: The transfection conditions and siRNA assay were adapted to myeloid suspension cells from our previously reported study.[1] Custom designed siRNA plates were obtained from Qiagen. A custom designed siRNA library directed against 41 genes with 2 different siRNA sequences per kinase was printed onto 384-well plates (Fisher Scientific, Pittsburgh, PA). Negative controls on the plate included buffer and non-silencing (NS) siRNA, whereas a universally lethal siRNA served as positive control. The 'universally lethal' siRNA is a custom designed sequence from Qiagen against ubiquitin. This siRNA is widely used by us and other groups in publications. Opti-MEM (Invitrogen, Carlsbad, CA) was used to dilute cationic lipid-based transfection reagents, which were then added to 384 well plates using a μFill liquid dispenser (Bio-Tek, Winooski, VA). siRNA screens were performed by reverse transfection of cells. Briefly, diluted RNAiMAX (Invitrogen, Grand Island, NY) or Dharmafect 3 (Fisher Scientific, Pittsburg, PA) was added to the wells containing siRNAs and allowed to complex for 30 mins at room temperature. TF-1, THP-1, HEL or MDS-L cells were added and incubated with siRNA for 48 hours. Following which MK1775 at 0 nM, 250 nM, 750 nM and 1500 nM were

added for each individual cell line. After a total of 96 hours, relative cell viability was measured using Cell Titer Glo (CTG, Promega, Madison, WI). Relative luminescence units (RLU) were measured using an EnVision plate reader (Perkin-Elmer, Waltham, MA). Screens in TF-1, THP-1 and HEL cells were performed in duplicate. Transfection efficiency was measured as an indirect readout of proliferation and was determined by reduction in relative cell number after transfection of the lethal siRNA compared with the median relative cell number of all kinase siRNAs.

Dual Drug Response Studies: For dual drug dosing, the two agents were added simultaneously and relative cell viability was determined with CTG at 96 h post drug additions. Using GraphPad Prism 5 software, the EC_{50} for MK1775 was calculated at various MK8776 concentrations and the fold change versus MK1755 alone was determined. Whether the effects met the formal definition of synergy was determined by calculating Combination Index (CI) values with CalcuSyn software according to the Chou and Talalay model under the assumption that effects are mutually exclusive, which is equivalent to isobologram analysis.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: RNAi screen and quality parameters. (A) Transfection efficiency for TF-1, THP-1, HEL and MDS-L cell lines used in the RNAi screen. Transfection efficiency was calculated as percentage of the ratio for median value for all universal lethal control over the median value for all siRNAs on the plate. (B) Non-specific toxicity effects of the siRNAs are plotted for TF-, THP-1, HEL and MDS-L used in the RNAi screens. Non-specific toxicity was calculated as percentage of the ratio for median value for all non-silencing siRNA over the median value for all buffer controls on the plate. (C) Graphical representation of RNAi screen is shown for THP-1 and MDS-L cells treated with 250 nM MK1775. Data is plotted as the delta between the median of each (siRNA + 250 nM MK1775)/NS and siRNA/NS. Each graph is representative from one screen.

Supplementary Figure 2: CHK1 siRNA silencing reduces protein. TF-1 cells were left untreated (Lane 1) or treated with siRNA to CHK1 and cell pellets collected at 24-72 hours post transfection (Lane 3-5). Non-silencing siRNA (NS) was transfected to account for non-specific toxicity effects (Lane 2). Solubilized samples were separated on SDS-PAGE and probed with Chk1 antibody. Tubulin antibody was used as loading control.

Supplementary Figure 3. MK8776 enhances MK1775-induced apoptosis as assessed by annexin V binding. (A**)** U937 cells were treated for 48 h with diluent, 100 nM MK8776, 100 nM MK1775, or the combination of 100 nM MK8776 + MK1775; stained with allophycocyanin-coupled annexin V; and subjected to flow microfluorimetry. (B**),** summary of results obtained when U937 cells were treated for 48 h with the indicated concentrations of MK1775 in the absence or presence of 100 nM MK 8776, stained with allophycocyanin-coupled annexin V, subjected to flow microfluorimetry, and analyzed as illustrated in panel A. Error bars, ± SEM from 3 independent experiments.

Supplementary Table 1: ATR inhibition sensitizes and CDK inhibition antagonizes WEE1 inhibiition by MK1775. (A) EC50 values for MK1775 upon co-treatment with 0-8 uM VE-821. Fold change of MK1775 EC50 upon co-treatment with VE-821 is shown in parenthesis. (B) EC50 values for MK1775 upon co-treatment with 0-8 uM Roscovitine. Fold change of MK1775 EC50 upon co-treatment with Roscovitine is shown in parenthesis. Representative result is shown from two independent experiments, each conducted in quadruplicate.

Supplementary Table 2: CHK1 kinase inhibition sensitizes to WEE1 kinase inhibition in AML in vitro. (A) Fold change in EC50 for MK1775 upon co-treatment with 625 nM MK8776

Supplementary Figure 4: MK8776 enhances MK1775-induced antiproliferative effects in colony forming assays of AML cell lines and primary AML specimens. (A) AML samples were plated in Methocult® methycellulose containing the indicated concentrations of MK1775 in the absence or presence of 100 nM MK8776. Leukemic colonies were counted at 14 d and compared to samples containing diluent (0.2% DMSO). (B-C)**,** peripheral blood mononuclear cells from a normal conctrol were plated in Methocult® methycellulose containing the indicated concentrations of MK1775 in the absence or presence of 100 nM MK8776. Myeloid colonies (CFU-G, CFU-GM and BFU-E) were counted at 14 d and compared to samples containing diluent (0.2% DMSO). Error bars in A-C, range of values from duplicate plates..

REFERENCE

1. Tibes R, Bogenberger JM, Chaudhuri L, Hagelstrom RT, Chow D, Buechel ME, et al. RNAi screening of the kinome with cytarabine in leukemias. Blood. 2012;119(12):2863-72.

Online Supplementary Table S1

A

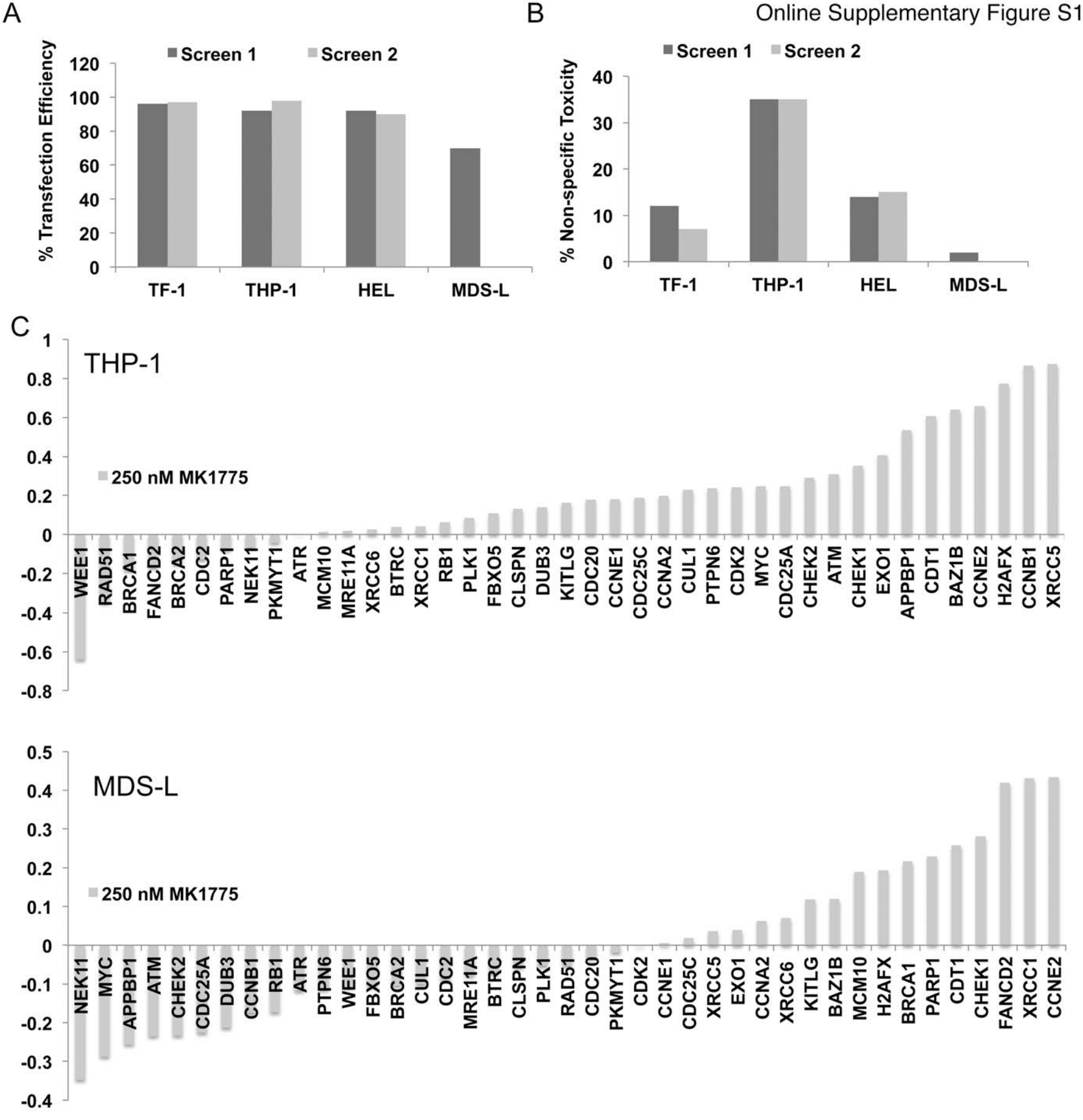
VE-821 (uM)	MK1775 EC_{50} (uM)		
	TF-1	HEL	THP-1
0.00	0.09 (1.0)	0.13 (1.0)	0.25 (1.0)
0.10	0.09 (1.0)	0.11 (1.2)	0.29 (0.9)
0.29	0.1 (0.9)	0.05 (2.6)	0.3 (0.8)
0.89	0.08 (1.1)	0.04 (3.3)	0.31 (0.8)
2.67	0.03 (3.0)	0.025 (5.2)	0.05 (5.0)
8.00	0.01 (9.0)	0.006 (21.0)	0.005 (50.0)

B

Roscovitine (uM)	MK1775 EC_{50} (uM)		
	TF-1	HEL	THP-1
0.00	0.10 (1.0)	0.10 (1.0)	0.22 (1.0)
0.10	0.11 (0.9)	0.12 (0.8)	0.23 (0.9)
0.29	0.12 (0.8)	0.13 (0.7)	0.27 (0.8)
0.89	0.13 (0.7)	0.16 (0.6)	0.35 (0.6)
2.67	0.19 (0.5)	0.27 (0.4)	0.70 (0.3)
8.00	0.50 (0.2)	0.37 (0.3)	1.32 (0.16)

Online Supplementary Table S2

Cell Line	MK1775 EC_{50} (uM)	MK1775 EC_{50} (uM) with 625 nM MK8776	Fold change
TF-1	0.14	0.07	2
HEL	0.12	0.03	4
MDS-L	0.71	0.59	1.2
THP-1	0.23	0.02	11.5
M07E	0.19	0.08	2.4
SupB15	0.26	0.17	2.2


A
% Transfection Efficiency
Screen 1
Screen 2
120
100
80
60
40
20
0
TF-1
THP-1
HEL
MDS-L
B
% Non-specific Toxicity
Screen 1
Screen 2
40
30
20
10
0
TF-1
THP-1
HEL
MDS-L
C
THP-1
250 nM MK1775
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
WEE1
RAD51
BRCA1
FANCD2
BRCA2
CDC2
PARP1
NEK11
PKMYT1
ATR
MCM10
MRE11A
XRCC6
BTRC
XRCC1
RB1
PLK1
FBXO5
CLSPN
DUB3
KITLG
CDC20
CCNE1
CDC25C
CCNA2
CUL1
PTPN6
CDK2
MYC
CDC25A
CHEK2
ATM
CHEK1
EXO1
APPBP1
CDT1
BAZ1B
CCNE2
H2AFX
CCNB1
XRCC5
MDS-L
250 nM MK1775
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
NEK11
MYC
APPBP1
ATM
CHEK2
CDC25A
DUB3
CCNB1
RB1
ATR
PTPN6
WEE1
FBXO5
BRCA2
CUL1
CDC2
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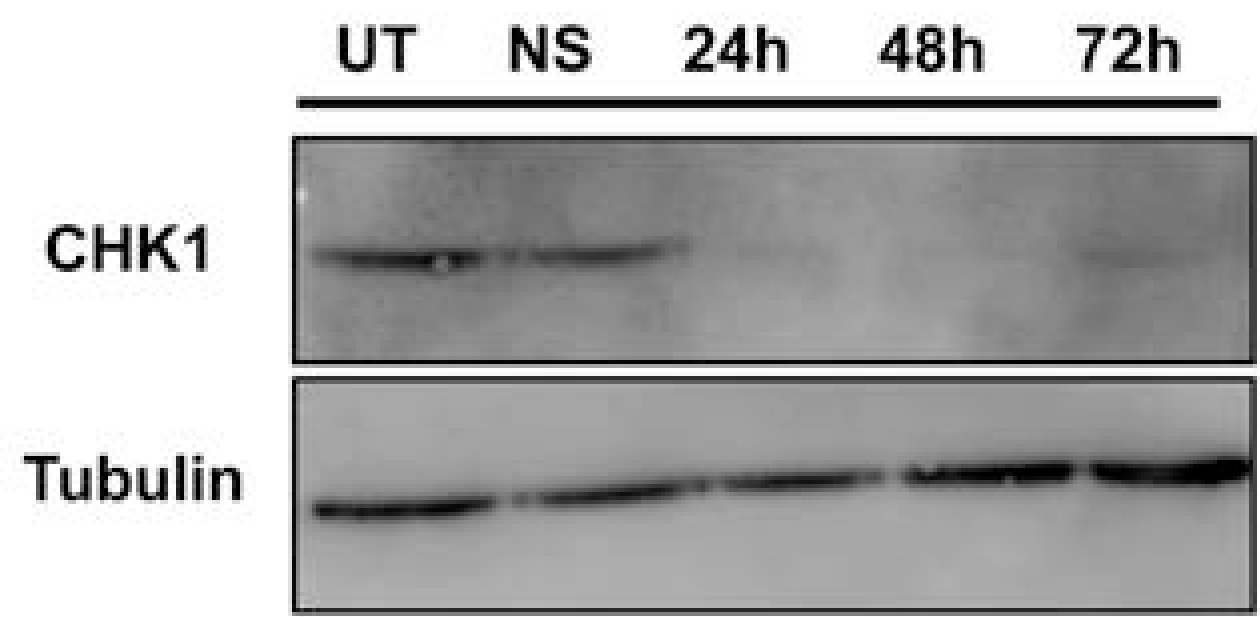
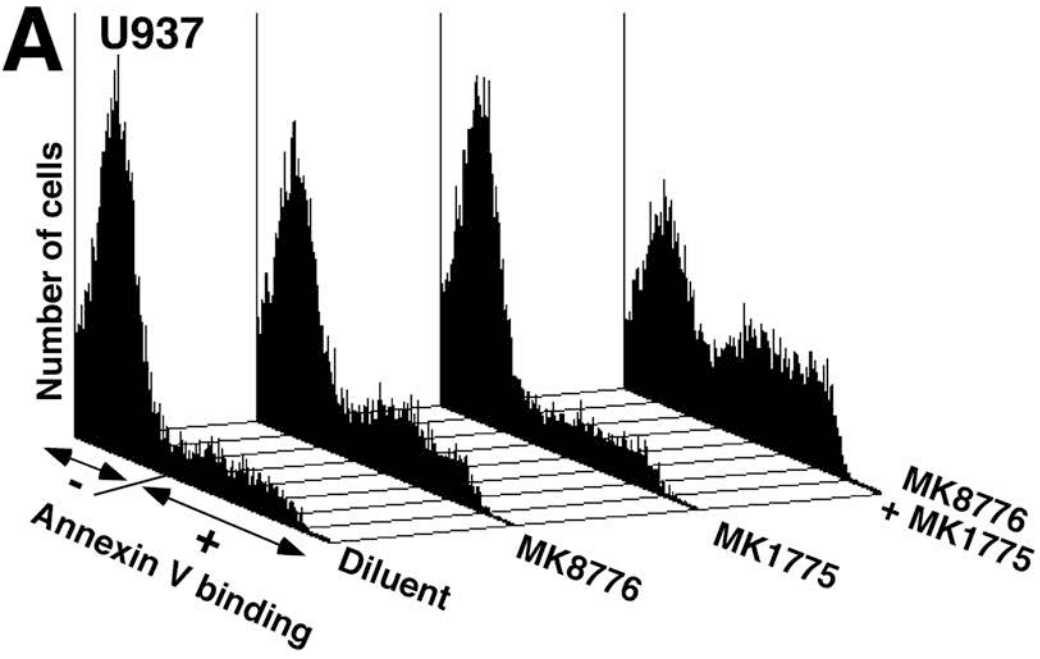
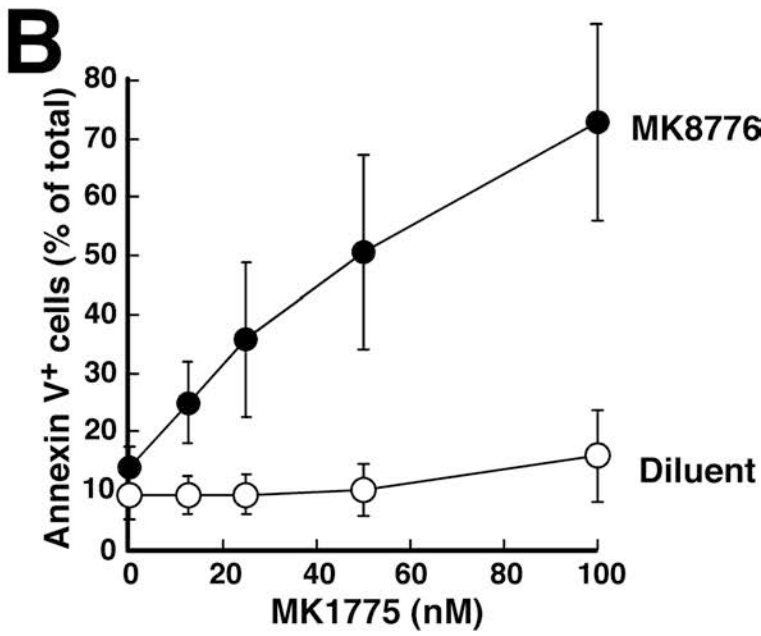
Online Supplementary Figure S2



Online Supplementary Figure S3





Online Supplementary Figure S4