SUPPLEMENTARY APPENDIX

Histone deacetylase inhibition in combination with MEK or BCL-2 inhibition in multiple myeloma

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Supplementary Methods

Multiple Myeloma Cell Lines and Patient Cells

MM1S, MM1R, OPM2, RPMI8226 and U266 cell lines were kindly provided by Dr. Jonathan Keats (TGen, Phoenix, AZ, USA). KMS11, KMS18, KMS28BM, OPM-1 and OPM-2 were obtained from Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ, USA). DOX40 was kindly provided by Dr. William Dalton's laboratory (Moffitt Cancer Center, Tampa, FL, USA). H929 was purchased from ATCC (Manassas, VA, USA). All cell lines, except OPM-1 and OPM-2, were cultured in RPMI 1640 media (Mediatech Inc., Manassas, VA, USA) containing 10% fetal bovine serum (FBS) (Mediatech, Inc.), 2 mM L-glutamine (Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen); OPM-1 and OPM-2 were cultured in the same type of media except instead 20% FBS was used. Likewise, freshly obtained bone marrow aspirates from patients with MM were collected after informed consent, and cultured in RPMI 1640 media that contained 20% fetal bovine serum, 2 mM L-glutamine (GIBCO), 100 U/mL penicillin, and 100 µg/mL streptomycin.

Supplementary Table 1. Clinical characteristics of the patients with multiple myeloma (MM), smoldering multiple myeloma (SMM) and monoclonal gammopathy of underdetermined significance (MGUS) from which bone marrow plasma cells were sorted, cultured and exposed to drugs of interest at indicated doses (Supplementary Tables 2-4).

Patient #	Sex	Diagnosis	Age at diagnosis	ISS at diagnosis	Disease status at time of biopsy	Age at time of biopsy	Cytogenetic/ FISH risk status	Cytogenetics/FISH	# of prior lines of therapy	Prior ASCT?
MC1	M	MGUS	62	n/a	n/a	62	Normal	trisomy 3, 7, 9, 15 and trisomy/tetrasomy 11	0	N
MC2	M	MM	86	n/a	Newly diagnosed	86	Normal	trisomy 3, 7, 9, 11, and 15	0	N
MC3	F	MM	56	II	Relapsed	58	High	*TP53* deletion, t(4;14) and monosomy 13	1	Y-1
MC4	F	MM	70	n/a	Day +100 post ASCT, in VGPR	71	High	*TP53* deletion, 1q duplication, monosomy 13 and 16, and trisomy 7 and 9	2	Y-1
MC5	M	MM	70	III	Newly diagnosed	70	High	*TP53* deletion, and monosomy 13 and 14	0	N
MC6	M	MM	60	III	Relapsed	64	Normal	hyperdiploidy, with trisomies 3, 7, 9. 11 and 15	4	Y-1
MC7	M	MM	61	III	Relapsed	62	Normal	t(11;14)	1	N
MC8	M	MM	59	III	Newly diagnosed	59	High	1q duplication, trisomy 9 and 15	0	N
MC9	F	MM	78	n/a	Newly diagnosed	78	Normal	t(11;14)	0	N
MC10	M	MM	63	I	Newly diagnosed	63	High	1q duplication and monosomy 14	0	N
MC11	F	MM	65	III	Relapsed and refractory	76	High	1q duplication, trisomy 3, 7, 9, trisomy/tetrasomy 15, and monosomy 13	12	Y-2
MC12	M	MM	62	II	Newly diagnosed	62	Normal	trisomy 3, 9, 11, and trisomy/tetrasomy 15	0	N
MC13	M	MM	54	III	Relapsed	61	High	1q duplication and trisomies 3, 7, 11 and 17	2	Y-1
MC14	M	MM	60	III	Newly diagnosed	60	High	t(4;14) and 1q duplication	1	N
MC15	M	MM	73	II	Newly diagnosed	73	Standard	trisomy 3, 7, 9, 11, 15	0	N
MC16	F	MM	79	III	Newly diagnosed	79	High	1q duplication 13q deletion; trisomies 5, 9, and 15	0	N
MC17	M	SMM	58	n/a	Stable (evaluated for neuropathy)	61	Standard	t(11;14)	0	N
MC18	M	MM	70	n/a	Relapsed and refractory	75	High	*TP53* deletion, 1q duplication, trisomies 3, 7, 9, 11, and 15, and monosomy 13	4	Y-1
MC19	M	MM	71	I	Newly diagnosed	71	Standard	monosomy 13, and trisomies 3, 7, and 9	0	N

ISS: International Staging System. ASCT: Autologous stem cell transplantation. VGPR: Very good partial remission.

Supplementary Table 2. Plasma cells from patients with multiple myeloma were exposed to either AZD6244 or ABT-199 alone, or in combination with either MS275 (entinostat) or FK228 (romidepsin) for 72 hours. The proportion of cells undergoing apoptosis was measured using flow cytometry, and the relative fold change in apoptosis is indicated. The mean fold change in apoptosis, summarizing the results from the n=2, n=2, and n=4 patients is also shown. Few patient samples were available, so choice of particular drug combinations was arbitrary and based on limited availability.

	Drug Dose (nM)		***Relative fold change in apoptosis***			
Patient #	AZD6244 or ABT-199	MS275 or FK228	Control	AZD6244	MS275	AZD6244+ MS275
AZD6244+MS275						
MC13	500 AZD6244	200 MS275	1	2	4.25	8
MC16	500 AZD6244	200 MS275	1	4	3.67	15.33
Mean fold change in apoptosis			**1**	**3**	**3.96**	**11.67**
AZD6244+FK228						
MC7	500 AZD6244	2.5 FK228	1	1.67	2.9	10.78
MC9	500 AZD6244	2.5 FK228	1	1	1.77	2.62
Mean fold change in apoptosis			**1**	**1.34**	**2.34**	**6.70**
ABT-199+MS275						
MC13	500 ABT-199	200 MS275	1	3	4.25	14
MC16	500 ABT-199	200 MS275	1	2.33	3.67	12.67
MC17	500 ABT-199	200 MS275	1	7.71	2.71	10.57
MC19	500 ABT-199	200 MS275	1	2.38	2.62	6.62
Mean fold change in apoptosis			**1**	**3.86**	**3.31**	**10.97**

Supplementary Figure Legends

Supplementary Figure 1.

Single-agent MEK inhibition does not effectively induce cell death in MM cell lines. (A) Cellular viability at 72h, assayed with MTT, after single-agent MEK inhibition with AZD6244 in a panel of human MM cell lines, shown as % of control in the Y-axis. (B) Proliferation arrest by 72h in the same cell lines, assayed using ^{3}H-thymidine incorporation into DNA, after single agent AZD6244 treatment, shown as % of control in the Y-axis. (C) Viability of the *RAS*/*RAF* mutated human MM cell lines H929 and MM1S, measured as the proportion of annexin (-)/PI (-) cells, assessed using flow cytometry, after 24, 48 and 72h of AZD6244 (5000 nM) treatment, shown as % of control in the Y-axis. (D) Viability of the human MM cell line MM1S, assayed with MTT, after 72h of treatment with AZD6244/LBH589, MEK162/LBH589, or SCH772984/LBH589 at the indicated doses, shown as % of control in the Y-axis. Error bars represent the standard error of the mean (SEM) of triplicate experiments. Differences between groups were calculated with the Student t test, where ** denotes P <0.001 and ## denotes P <0.01. All experiments were performed in triplicate.

Supplementary Figure 2.

BCL-2+HDAC inhibition induces enhanced apoptosis in BCL-2 primed MM cell lines. (A) Cell viability, assessed using flow cytometry by analyzing the proportion of annexin (-)/PI (-) cells, shown as a percentage, after ABT-199/LBH589 treatment at 72h in KMS18 and OPM2. All experiments were performed in triplicate.

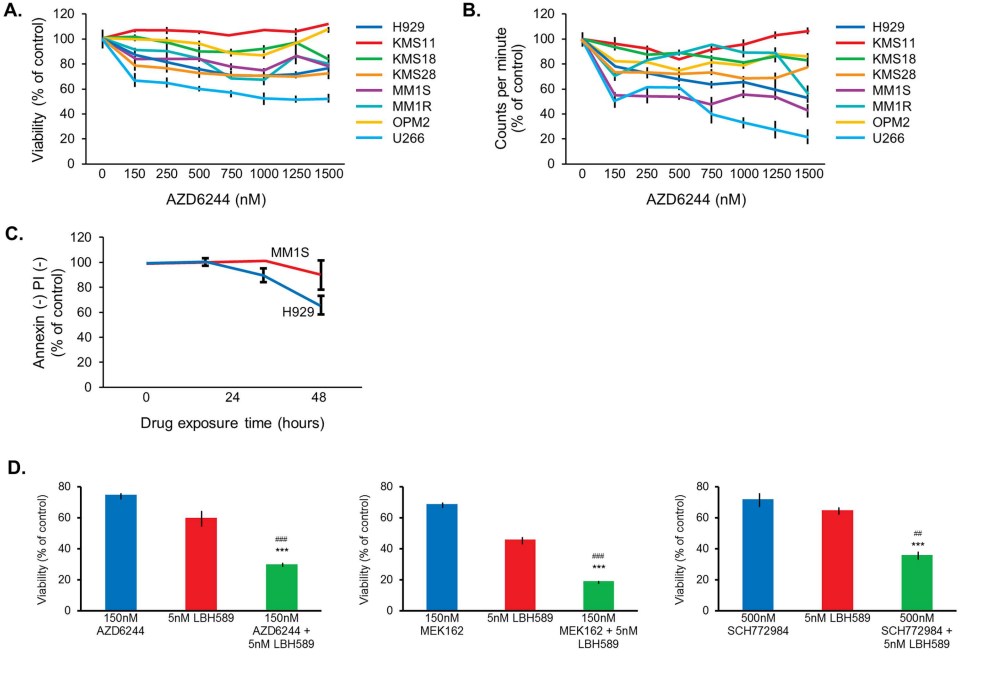
Supplementary Figure 3.

HDAC6 inhibition does not enhance apoptosis induced by MEK inhibition. (A) The HDAC6 inhibitor tubacin and AZD6244 were combined in increasing doses. Cellular viability was assessed using MTT at 72h in the human MM cell lines MM1S and H929. Viability is shown as % of control in the Y-axis. Both experiments were performed in triplicate. (B) MM1S was electroporated with scrambled siRNA or HDAC6 siRNA, then left untreated or treated with 150 nM AZD6244. At 72h, cell viability was assessed using flow cytometry by analyzing the proportion of annexin (-)/PI (-) cells, shown as % of control in the Y-axis. Whole-cell lysates were separated using SDS-PAGE and subject to western blotting for the indicated proteins to confirm silencing. Error bars represent the SEM of triplicate experiments.

Supplementary Figure 4.

Class I HDAC inhibition replicates synergy with MEK or BCL-2 inhibition in MM cell lines. (A) The HDAC1, 2 and 3 inhibitor MS275 (entinostat) was combined with AZD6244 in increasing doses. Cellular viability was assessed using MTT at 72h in the human MM cell lines MM1S, RPMI8226 and MM1R. Viability is shown as % of control in the Y-axis. Combination index (CI) values <1.0, indicating synergy, are shown for each cell line. (B) MS275 was combined with ABT-199 in increasing doses in the human MM cell lines KMS18, KMS28 and OPM2. Cellular viability was assessed using MTT at 72h, shown as % of control in the Y-axis. (C) H929 was treated with 250 nM AZD6244 and 1 nM of the HDAC1 and 2 inhibitor FK228 for 24h, then immunopreciptates for BIM, BCL-2, MCL-1, BCL-X_L or whole cell lysates (input) were separated using SDS-PAGE and probed for the indicated proteins. All experiments were performed in triplicate.

Supplementary Figure 1.



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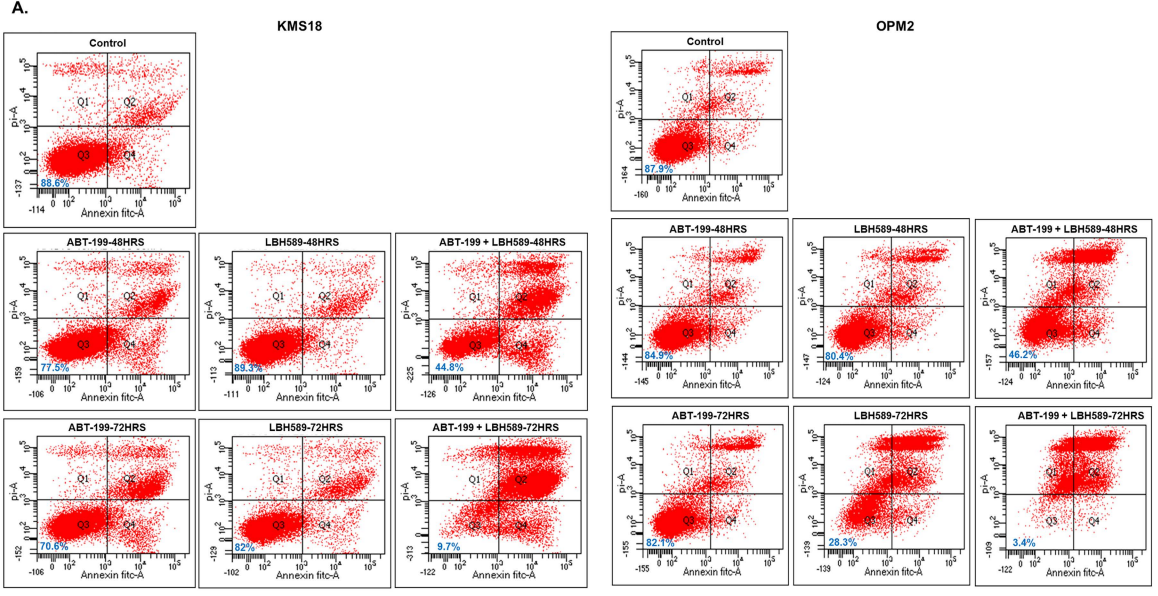
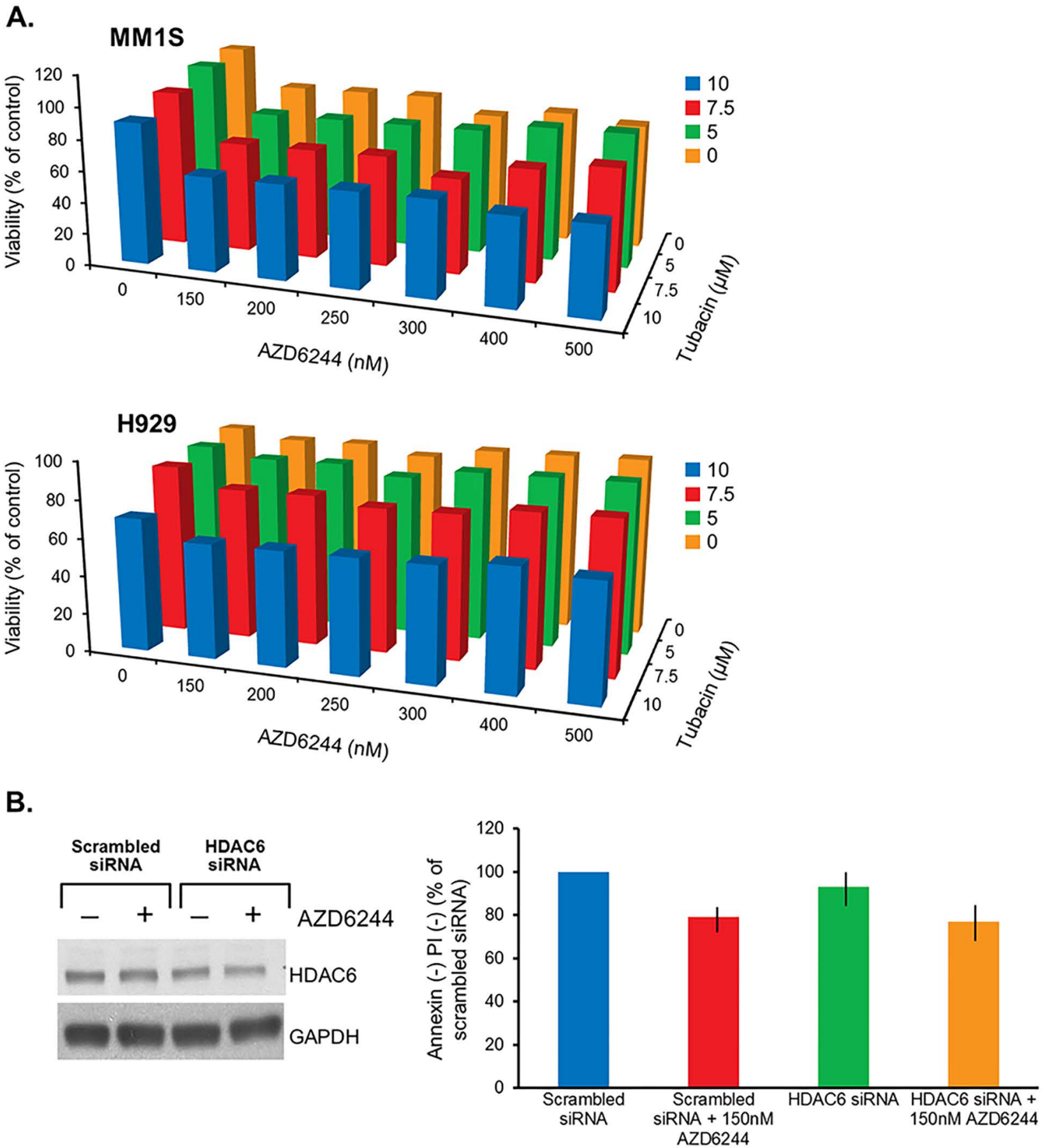


Supplementary Figure 3.



Supplementary Figure 4.