SUPPLEMENTARY APPENDIX

Proteolysis targeting chimeric molecules as therapy for multiple myeloma: efficacy, biomarker and drug combinations

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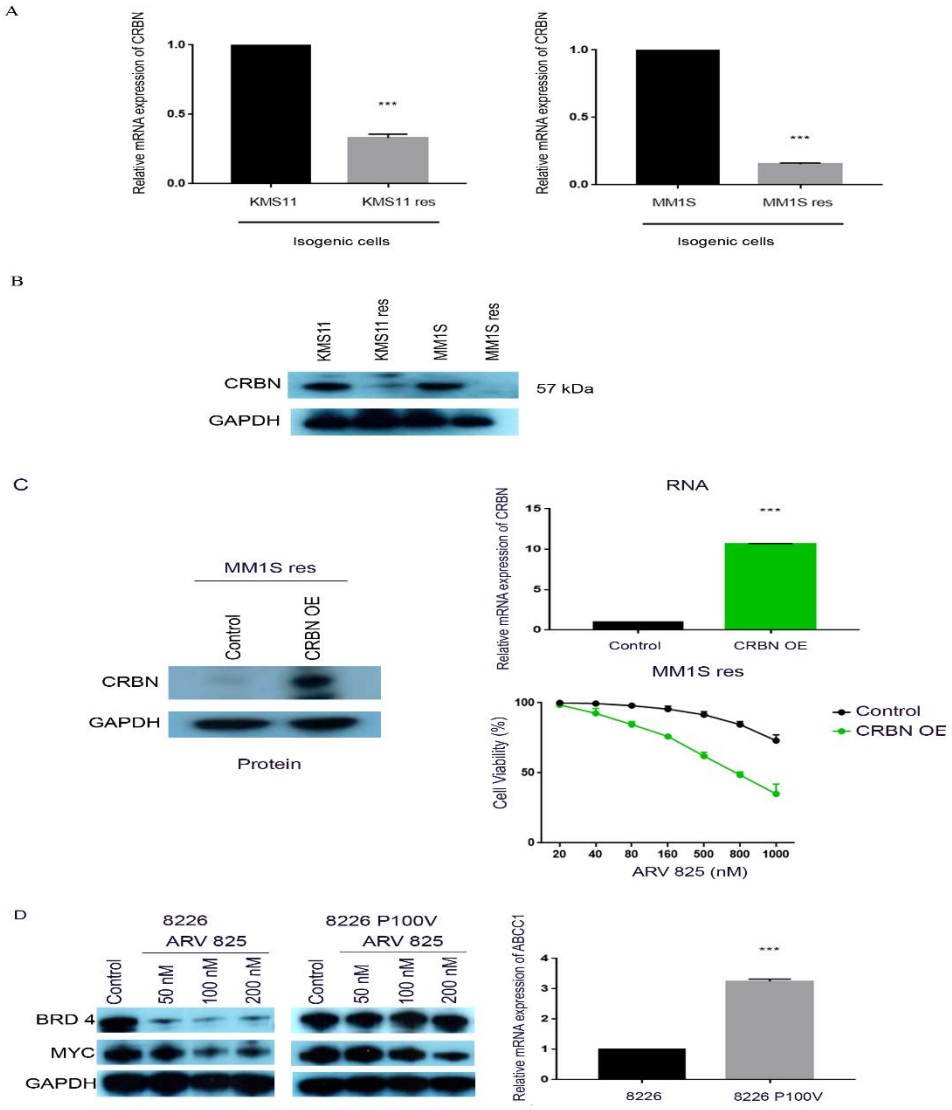
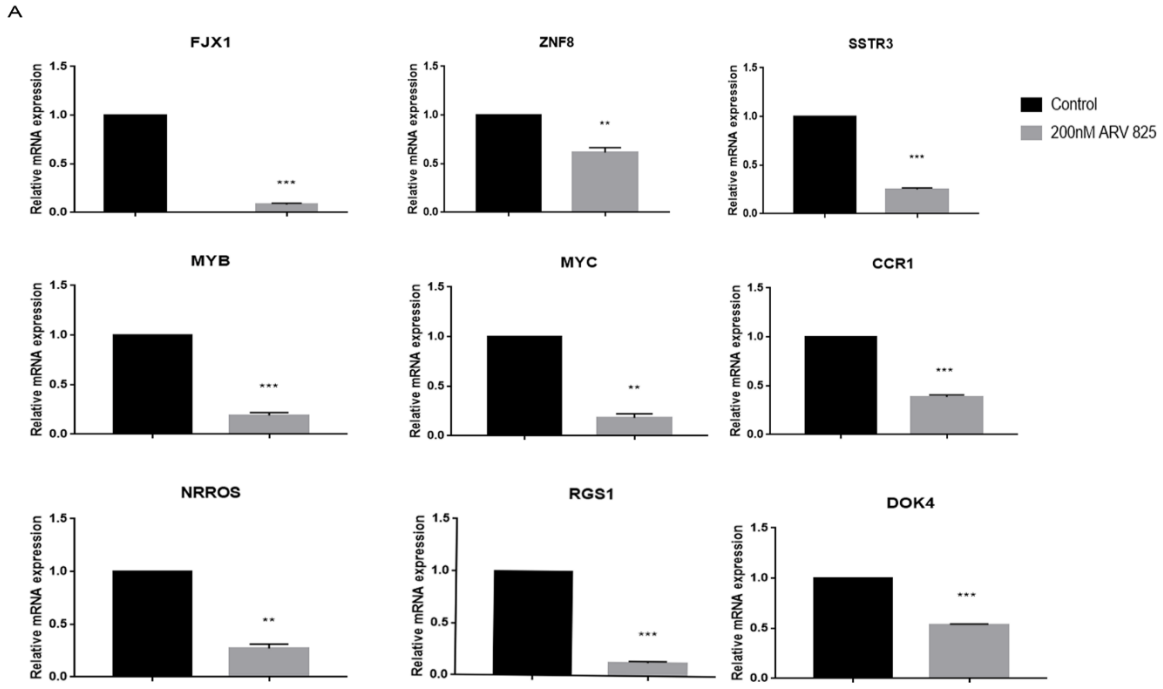
Supplemental data:

Supplementary Figure 1



Supplementary Figure 1. (A) CRBN mRNA expression and (B) CRBN protein expression in parental (KMS11, MM1S) and isogenic lenalidomide resistant (KMS11 res and MM1S res) MM cells. (C) Levels of CRBN mRNA (right upper panel) and protein (left panel) present in MM1S res cells and their viability cultured with ARV 825 (1 nM to 1000 nM, 72 h) (right lower panel) after overexpression. OE: Overexpression. (D) Levels of BRD 4 and MYC protein expression (left panel) in 8226 and 8226 P100V, cultured with ARV 825 (50 nM, 100 nM, 200 nM, 4h). GAPDH, internal control.

Supplementary Figure 2

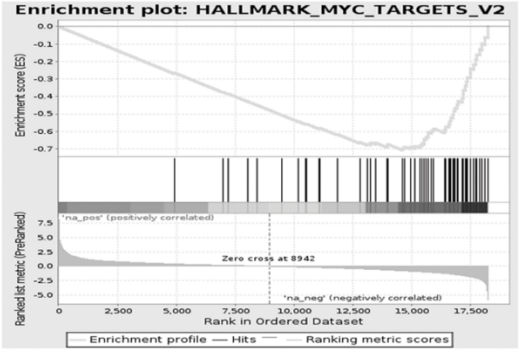


Gene Set enriched among genes downregulated by ARV 825 in KMS11 cells derived from RNA seq data

B

Rank	MSigDB Gene Set	n	NES	FDR q-val
1	MYC TARGETS	57	-2.47	<0.001
2	INFLAMMATORY RESPONSE	108	-1.95	<0.001
3	UV RESPONSE	106	-1.94	<0.001
4	ESTROGEN RESPONSE EARLY	143	-1.93	<0.001
5	TNFA SIGNALING VIA NFKB	138	-1.74	0.005
6	ANDROGEN RESPONSE	87	-1.68	0.008
7	WNT BETA CATENIN SIGNALING	31	-1.63	0.011
8	IL2 STAT5 SIGNALING	152	-1.59	0.018
9	INTERFERON ALPHA RESPONSE	77	-1.58	0.017
10	INTERFERON GAMMA RESPONSE	156	-1.58	0.016

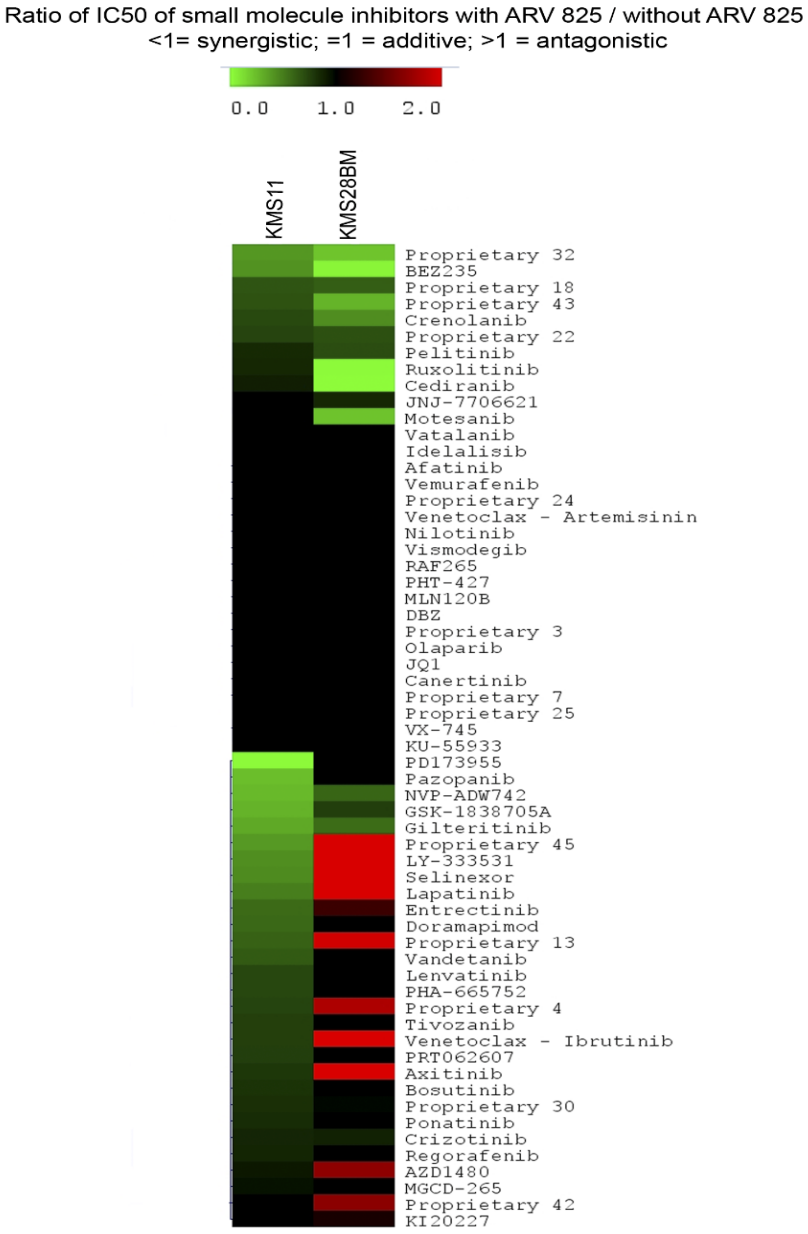
C

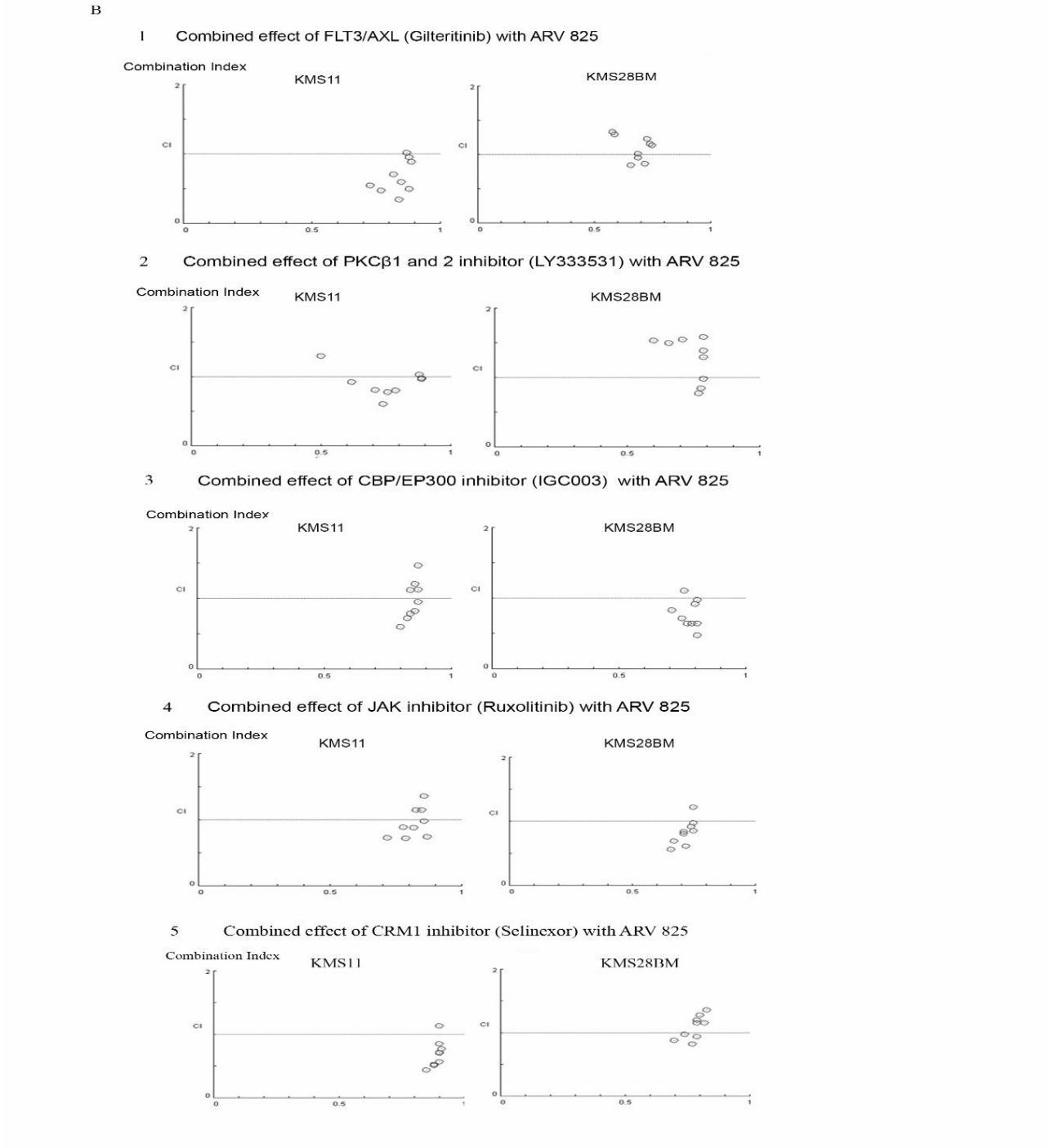


Supplementary Figure 2. (A) mRNA levels of 9 selected genes by qRT-PCR after ARV 825 treatment (200 nM ARV 825, 8 h) of KMS28BM cells. Expression of each gene was normalized to β-actin as a reference (control value converted to the value of 1). Data represent mean ± SD of 3 independent experiments, each done in triplicate, $^{**}p \leq 0.001$, $^{***}p \leq 0.0001$. (B) Table of gene sets enriched among genes downregulated by ARV 825 (20 nM, 8 h) in KMS11 MM cells derived from RNA seq data. Number of genes in each set (n), the normalized enrichment score (NES), and test of statistical significance (FDR q value). (C) GSEA of MYC-dependent gene sets.

Supplementary Figure 3

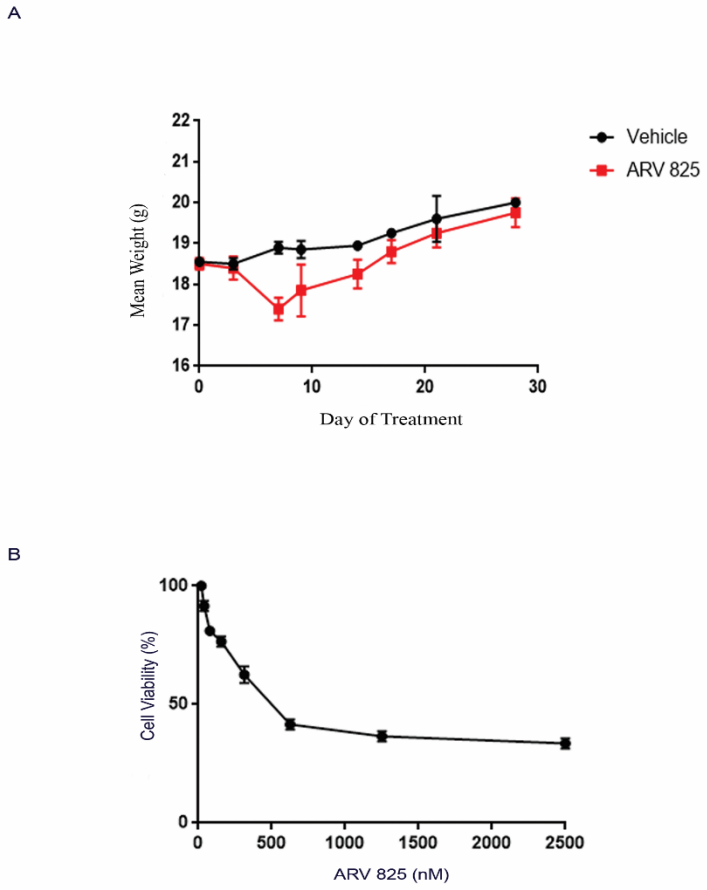
A





Supplementary Figure 3. High throughput screening of small molecule inhibitors. (A) KMS11 and KMS28BM MM cells: Heatmap of ratio (IC50 of small molecule inhibitor with ARV 825 divide by IC50 of small molecule inhibitor without ARV 825) using KMS11 and KMS28BM cells. Ratio < 1, =1 and > 1 indicates synergistic, additive and antagonistic activity, respectively. Shown are 60 out of the 170 small molecule inhibitors tested. (B 1-5) Combination Index plot of 5 small molecule inhibitors with synergistic activity with ARV 825 on either KMS11 or KMS28BM cells.

Supplementary Figure 4



Supplementary Figure 4. (A) Comparison of weight of mice during and after treatment with either ARV 825 or diluent control. Mean ± SD of 9 mice in each group. (B) Mouse bone marrow cells (2 X 10^5 cells/well) treated with PROTAC ARV 825 (1 nM-2500 nM, 72 h, and measured by MTT assay). IC50 = 500 nM ± 2. Results represent the mean ± SD of 3 experiments done in triplicate.

Supplementary Table 1: List of inhibitors

Inhibitor	Manufacturer
Bortezomib	Selleckchem Catalog No S1013
Cediranib	Selleckchem Catalog No S1017
Crenolanib	Selleckchem Catalog No S2730
Gilteritinib (ASP2215)	Selleckchem Catalog No S7754
GSK-1838705A	Selleckchem Catalog No S2703
IGC001	Wuxi App Tech Co.
Lapatinib	Selleckchem Catalog No S2111
LY333531 (hydrochloride)	Cayman Chemical Item No 13964
LY3023414	Selleckchem Catalog No S8322
Melphalan	Selleckchem Catalog No S8266
Motesanib Diphosphate (AMG-706)	Selleckchem Catalog No S1032
Pomalidomide	Selleckchem Catalog No S1567
Ruxolitinib	Selleckchem Catalog No S1378
Selinexor (KPT330)	Selleckchem Catalog No S7252

Supplementary Table 2: Sequences of qRT-PCR primers

Gene	**Forward (5' → 3')**	**Reverse (5' → 3')**
FJX1	TAGCAGGCATGTGGGACAAG	AATGTGCTTGGCGAGGAAGT
ZNF8	TCTACCGTGACGTGATGCTG	GTGGTTCCTCTCTCAGCCAC
SSTR3	TGTCCACGACCTCAGAACCT	ATGACCAGCGAGTTACCCAG
KCNJ12	CATCGTGTCATCGGAGGAGG	GTCCACACAGGTGGTGAACA
CCR1	CACAGGCTTGTACAGCGAGA	CTGCAGGTGTGGTGAGTGAA
MYB	GACCCTGAGAAGGAAAAGCGA	CATGAGGTCTGGTGTGGTCG
NRROS	CGGGACCTGTACAACACCTC	ATGTGAAGCGTCATCAGGCA
MYC	TAGTGGAAAACCAGCAGCCT	AGAAATACGGCTGCACCGAG
RGS1	GAGTTCTGGCTGGCTTGTGA	ATTCTCGAGTGCGGAAGTCA
DOK4	CTCTGGGACATCCACAACCC	CCTTCCCCAGCATCACACAT
ABCB1	CCCATCATTGCAATAGCAGG	TGTTCAAACTTCTGCTCCTGA
ABCC1	ATGTCACGTGGAATACCAGC	GAAGACTGAACTCCCTTCCT

Supplementary Table 3: IC50s of ARV 825 against MM cells, 72 h

Cell lines	ARV 825 IC50 ± SD (nM), 72 h
KMS11	9 ± 1.9
MM1R (Steroid resistant)	10 ± 1.8
KMS12BM	11 ± 1.3
MM1S	11 ± 1.8
H929	16 ± 1.6
KMS18	17 ± 1.1
8226 LR 5 (Melphalan resistant)	20 ± 1.9
KMS11 res (Lenalidomide resistant)	70 ± 1.4
U266	71 ± 1.8
8226	84 ± 1.4
KMS28BM	137 ± 1.1
8226 P100V (Bortezomib resistant)	500 ± 0.6
MM1S res (Lenalidomide resistant)	>500

Supplementary Table 4: IC50s of MZ1 against MM cells, 72 h

Cell lines	MZ1 IC50 ± SD (nM), 72 h
H929	3 ± 1.2
MM1S res (Lenalidomide resistant)	7 ± 0.75
KMS11 res (Lenalidomide resistant)	15 ± 1.6
MM1S	18.3 ± 1.2
MM1R (Steroid resistant)	37 ± 1.2
KMS12BM	60 ± 0.8
KMS28BM	69 ± 1.2
KMS11	104 ± 1.2
U266	385 ± 1.4
KMS18	700 ± 1.2
8226 LR5 (Melphalan resistant)	2300 ± 1.4
8226	2600 ± 1.4
8226 P100V (Bortezomib resistant)	> 3000

Supplementary Table 5: Combination Index of ARV 825 synergistic with small molecule inhibitors (CI < 1, CI = 1 and CI > 1 represent synergism, additive, and antagonism respectively).

KMS11			
	ARV 825 (nM)		
	5	10	20
Cediranib (nM)			
200	0.57	0.61	0.78
400	0.46	0.50	0.73
800	0.46	0.48	0.71
Crenolanib (nM)			
500	0.63	0.7	1.0
1000	0.64	0.7	1.0
2000	0.63	0.7	1.0
GSK 1904529A (nM)			
150	0.73	0.74	0.84
300	0.3	0.57	0.77
600	0.5	0.46	0.71

LY3023414 (nM)			
60	0.55	0.62	0.85
120	0.56	0.58	0.88
240	0.63	0.76	1.02
Motesanib (µM)			
10	0.55	0.70	1.0
20	0.48	0.60	0.95
40	0.35	0.49	0.89
Selinexor (nM)			
50	0.44	0.52	0.71
100	0.51	0.57	0.85
200	0.71	0.77	1.1
Gilteritinib (nM)			
300	0.77	0.85	1.1
600	0.55	0.66	1.0
1200	0.85	0.58	0.82
LY333531 (µM)			
1	1.3	0.93	0.6
3	0.81	0.78	0.8

6	0.97	0.98	1.0
IGC003 (µM)			
1	0.6	0.72	1.1
3	0.78	0.82	1.2
6	0.95	1.1	1.5
Ruxolitinib (µM)			
5	0.65	0.74	1.1
10	0.65	0.8	1.1
20	0.72	0.92	1.4

KMS28BM			
	ARV 825 (nM)		
	50	**100**	**200**
Cediranib (µM)			
3	0.69	0.62	0.8
6	0.63	0.62	0.78
12	0.45	0.48	0.71
Crenolanib (nM)			
500	0.6	0.56	0.79

1000	0.45	0.48	0.70
2000	0.59	0.6	0.81
GSK1904529A (nM)			
150	0.38	0.20	0.72
300	0.51	0.45	0.45
600	0.29	0.29	0.51
LY3023414 (nM)			
120	0.68	0.66	0.88
240	0.64	0.43	0.74
480	0.75	0.82	0.96
Motesanib (µM)			
10	0.46	0.54	0.51
20	0.42	0.35	0.41
40	0.24	0.22	0.27
Selinexor (nM)			
100	0.88	0.82	1.15
200	0.98	0.94	1.28
400	1.2	1.15	1.36

Gilteritinib (nM)			
300	0.87	0.76	1.00
600	0.6	0.54	0.76
1200	0.59	0.58	0.75
LY333531 (µM)			
2.5	1.5	1.5	1.55
5	0.77	0.84	0.98
10	1.29	1.39	1.58
IGC003 (µM)			
1	0.83	0.72	1.1
3	0.64	0.64	0.91
6	0.48	0.64	0.97
Ruxolitinib (µM)			
5	0.56	0.62	0.85
10	0.69	0.81	0.97
20	0.83	0.91	1.22

8226			
	ARV 825 (nM)		
	40	**80**	**160**
Cediranib (µM)			
1.5	0.49	0.63	0.7
3	0.63	0.74	0.81
6	0.89	0.91	0.91
Crenolanib (nM)			
1000	0.74	0.8	0.82
2000	0.88	0.9	0.89
4000	0.91	0.91	0.91
GSK1904529A (nM)			
5000	0.46	0.55	0.89
10000	0.57	0.69	0.98
20000	0.85	0.96	1.21
LY3023414 (nM)			
20	0.23	0.44	0.48
40	0.46	0.59	0.62
80	0.66	0.71	0.73

Motesanib (μM)			
10	NA	NA	0.57
20	NA	NA	0.46
40	0.15	0.28	0.42

Supplementary Table 6: Combination Index of MZ1 synergistic with small molecule inhibitors (CI < 1, CI = 1 and CI > 1 represent synergism, additive, and antagonism respectively).

KMS11			
	MZ1 (nM)		
	50	**100**	**200**
Cediranib (nM)			
200	0.44	0.55	0.71
400	0.39	0.50	0.66
800	0.37	0.47	0.68
Crenolanib (nM)			
500	0.99	0.87	0.91
1000	0.92	0.87	0.82
2000	0.98	1.05	0.93
GSK1904529A (nM)			
150	0.67	0.68	0.86
300	0.55	0.60	0.75
600	0.53	0.54	0.69
LY3023414 (nM)			
60	0.86	0.85	0.88

120	0.90	0.76	0.86
240	0.86	0.71	0.89
Motesanib (µM)			
10	0.64	0.57	0.77
20	0.42	0.42	0.67
40	0.23	0.32	0.56

KMS28BM			
	MZ1 (nM)		
	35	**70**	**140**
Cediranib (nM)			
3000	0.64	0.59	0.78
6000	0.69	0.85	0.97
12000	0.68	1.22	0.91
Crenolanib (nM)			
500	1.64	0.85	0.96
1000	1.31	0.75	0.99
2000	0.94	0.99	1.31
GSK1904529A (nM)			
300	0.88	0.73	0.78

600	0.60	0.56	0.67
1200	0.42	0.44	0.58
LY3023414 (nM)			
120	1.05	1.25	1.10
240	1.11	1.02	1.10
480	1.14	1.22	1.07
Motesanib (µM)			
10	0.81	0.80	0.94
20	1.05	0.72	0.95
40	0.79	0.70	0.93

Online Supplemental Method:

Cell culture

Human MM cell lines: KMS11, KMS28BM, KMS18, KMS12BM, MM1S, MM1R, H929, 8226, 8226 LR5, 8226 P100V and U266 were kind gift from Dr. W.J. Chng (Cancer Science Institute, Singapore) and KMS11 res and MM1S res were generous gift from Dr. A.K. Stewart (Mayo Clinic, Arizona).

Cell proliferation assay

Twenty thousand cells were seeded in 96-well plates followed by drug treatment. After culture, 10 µl of MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the wells and cultured at 37°C for an additional 4 h followed by addition of 100 µl stop solution (10% Sodium Dodecyl Sulphate). Plates were measured with a spectrophotometer at 570 nm absorbance. IC50 values were calculated using Graph pad Prism.

Soft agar colony formation

Soft agar colony assay was performed to evaluate the anchorage-independent growth of MM cells. 500 µl of 0.5% agarose (dissolved in RPMI with 10% FBS) was added to 12-well plates as a bottom layer. 1,000 cells/well were seeded in 500 µl of top layer (0.35% agarose in RPMI with 10% FBS) either with or without drug for 21 days. Plates were kept at 37 °C in a 5% CO_2 incubator before analysis. The colonies were examined and counted using an inverted light microscope.

Annexin V and propidium iodide (Annexin V-PI) apoptosis analysis

Cells were treated with different concentrations of ARV 825 for 48 h. Staining was performed using Apoptosis Detection Kit II (BD Biosciences, USA). Cells were harvested and washed twice with phosphate-buffered saline (PBS, Life technologies, USA), suspended in 1X binding buffer with 5 µl of FITC conjugated Annexin V and 5 µl of PI for 15 min in the dark at room temperature. Samples were analyzed using flow cytometric analysis (Sony SA3800).

Cell cycle analysis

Cells were treated with different concentrations of ARV 825 (48 h), fixed with 70% chilled ethanol, washed with PBS two times and stained with PI solution [40 µg/ml PI, Triton X-100 (1%), 20 ug/ml DNase-free RNase A in PBS] for 30 min at 37 °C in the dark followed by flow cytometric analysis (Sony SA3800). Cells which were Annexin and PI positive were defined as apoptotic cells.

Screening of small molecule inhibitors

Inhibitors were provided by or purchased from several manufacturers (Supplementary Table 1). Graded concentrations of small molecule inhibitors were applied to 384-well plates containing 5,000 cells per well and incubated for 3 days at 37°C, 5% CO_2. Relative number of viable cells were assayed with tetrazolium-based cell viability assay and were normalized to cells containing no-drug control. A third order polynomial curve fit was used to calculate IC50 values for each drug.

Drug combination studies

Results from MTT assays with different combinations of drugs were evaluated by CompuSyn (ComboSyn, Inc, Paramus, NJ). A combination index (CI) plot is a Fa-CI plot in which CI<1, =1, >1 indicate synergism, additive and antagonism, respectively. Fa: fraction of proliferation inhibition by the drug.

Reagent and antibodies

ARV 825 was developed by the C.M. Crew's laboratory (Department of Chemistry, Yale University, New Haven, CT, USA). We obtained the drug from Chemietek (Indianapolis, IN, USA). For *in vitro* administration, ARV 825 was dissolved in dimethyl sulfoxide (Sigma-Aldrich) (20 mM) and stored at -20°C. Antibody against CRBN (cat no. HPA045910) was purchased from Sigma-Aldrich. Antibodies against BRD 2, BRD 4 (cat no. 5848S, 13440S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against BRD 3 (cat no. 11859-1-AP) was purchased from Proteintech (Chicago, USA). Antibody against GAPDH (cat no. 2118) was from Cell Signaling Technology (Danvers, MA, USA). List of inhibitors are showed in Supplementary Table 1.

Western blot analysis

Cell lysates were prepared using M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, USA) containing 1X protease cocktail inhibitor (Roche, Switzerland). After 20 min incubation on ice, lysates were centrifuged at 13,000g for 20 min at 4°C. Total protein concentrations was measured by Pierce Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific). Twenty micrograms of protein were loaded on SDS-PAGE gel and resolved at 80

voltages, followed by transfer to PVDF (Millipore, Massachusetts). Membranes were blocked with 5% non-fat milk and incubated with antibodies.

RNA sequencing and quantitative PCR of KMS11 cells

mRNA expression profiling was done on biological duplicates (KMS11 control versus drug treated). After treatment with 20nM ARV 825 for 8 h, total RNA was extracted using RNeasy Isolation Kit (Qiagen, Germany). Sequencing libraries were prepared using TruSeq RNA Library Prep Kit (Illumina, San Diego) according to the manufacturer's protocol and were sequenced on HiSeq 2000 sequencer (Illumina). Paired-end reads (100 bp) were aligned to hg19 reference genome with Ensembl gtf (version 75) provided as a known junction file, using splice-aware STAR aligner. Expression levels were measured as FPKM using Stringtie software against Ensembl v75gtf. qRT-PCR was performed with standard procedures. cDNA was generated using Maxima H Minus First Strand cDNA synthesis kit (Thermo Scientific), and qRT-PCR was performed on CFX96 qPCR System (Bio-Rad). Expression of each gene was normalized to GAPDH and quantified using $2^{-\Delta(Ct)}$ method. Primers for qRT-PCR are listed in Supplementary Table 2.