SUPPLEMENTAL METHODS AND FIGURES

Cells and Reagents

The following human MM cell lines were used: MM.1S (dexamethasone [Dex]-sensitive), MM.1R (dexamethasone [Dex]-resistant), RPMI-8226/S, U266 (obtained from ATCC); ANBL-6-bortezomib–sensitive (ANBL-6.WT) and -resistant (ANBL-6.BR) cell lines were kindly provided by Dr. Robert Orlowski (M.D. Anderson Cancer Centre, Houston, TX). All MM cell lines were cultured in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L l-glutamine. Blood samples collected from healthy volunteers were processed by Ficoll-Paque gradient to obtain peripheral blood mononuclear cells (PBMCs). Patient MM cells and BMSCs were obtained from BM samples after informed consent was obtained, in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Dana-Farber Cancer Institute. BM mononuclear cells were separated using Ficoll Paque density sedimentation, and MM cells were purified by positive selection with anti-CD138 magnetic activated cell separation microbeads (Miltenyi Biotec, Auburn, CA). Residual CD138-negative BM mononuclear cells were cultured in Dulbecco Modified Eagle medium (DMEM) with 20% FCS for 3 to 6 weeks to generate BMSCs, as previously described. (1) The Nampt inhibitor FK866 was generously provided by the NIMH Chemical Synthesis and Drug Supply Program. It was dissolved in dimethyl sulphoxide at 10mM and stored at -80°C for in vitro study. For in vivo studies in mice, FK866 was formulated in 0.9% saline solution and stored at -20ºC. Bortezomib was purchased from Selleck Chemicals LLC. The pan-caspase inhibitor (z-VAD-fmk) and recombinant IL6 and IGF1 were obtained from Promega and R&D Systems, respectively

Cell viability and apoptosis assays

Dead cells were quantified by propidium iodide (PI) staining (2mg/ml) and flow cytometry (FACS Canto II, Becton Dickinson, BD), as previously described. (2) Apoptosis was quantified using Annexin V/PI staining assays kit, as per manufacturer`s instructions (BD Bioscences, San Jose, CA, USA), followed by analysis on FACS Calibur (BD Bioscences). Caspase 3 activation was assessed using Caspase-Glo 3/7 Assay, according to manufacturer's procedure (Promega).

Determination of the intracellular NAD^+ levels

The intracellular content of NAD^+ was assessed with a sensitive enzyme cyclic assay (BioVision, Mountain View, CA), according to the manufacturer`s procedure. The value was normalized to total cell number.

In vitro proteasome activity

The proteasome activities assays were performed using fluorogenic peptide substrates, as previously described. (3)

Immunofluorescence

Viable MM cell lines as well as primary MM cells (2.5 x 104) were pelleted on glass slides by centrifugation at 250 rpm for 5 minutes using a cytospin system (Thermo Shandon) and fixed in cold absolute acetone and methanol for 10 minutes. After fixation, cells were washed in PBS and blocked for 60 minutes with 5% fetal bovine serum in PBS. Slides were then incubated with anti-Nampt (Bethyl Laboratories) at 4°C for 24 hours, washed in PBS, incubated with goat anti–rabbit IgG-fluorescein isothiocyanate for 1 hour at 4°C, and analyzed.

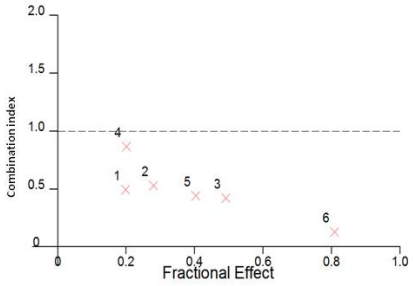
The effect of drug combination on TNF-α–induced nuclear translocation of p65 and RelB was examined by an immunocytochemical method. Briefly, MM1S cells were cultured in the presence or absence of FK866 (3nM) for 24 h and bortezomib (2 nM) for an additional 4 h, or with either agent alone. Cells were then stimulated with TNF-α (10 ng/mL) during the last 20 min of culture. Immunocytochemical analysis was conducted using a fluorescence microscope (Nikon Eclipse E800; Nikon) and a Photometrics Coolsnap CF color camera (Nikon), as previously described. Similarly, evaluation of ubiquitin was evaluated by using immunofluorescence on MM1S cells treated with FK866 (3 nM), bortezomib (2 nM), or the combination. (4, 5)

In *situ* detection of apoptosis, proliferation and angiogenesis

Mice tumor sections were subjected to immunohistochemical (ICH) staining for caspase-3 activation to detect apoptotic cell death. *Ki-67* was assessed to quantify proliferation, and tumor angiogenesis was evaluated by ICH staining for CD31 expression, as previously described. (6) Immunostained tissues were imaged using a Canon IXY digital 700 camera.

A

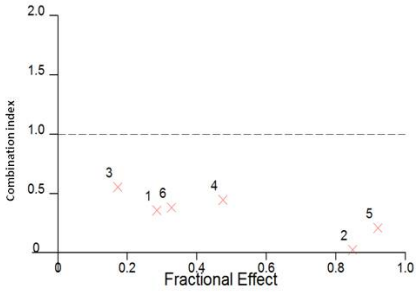
RPMI-8226/S



FK866 (nM)	Bortezomib (nM)	F.A.	C.I.
1	2.5	0.1982	0.496
1	5	0.2794	0.531
1	10	0.4911	0.424
3	2.5	0.2003	0.866
3	5	0.4032	0.442
3	10	0.8086	0.132

B

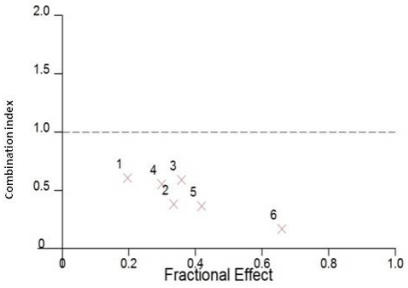
U266



FK866 (nM)	Bortezomib (nM)	F.A.	C.I.
1	2.5	0.2851	0.359
1	5	0.8483	0.027
1	10	0.1723	0.556
3	2.5	0.3273	0.383
3	5	0.4756	0.448
3	10	0.9212	0.209

C

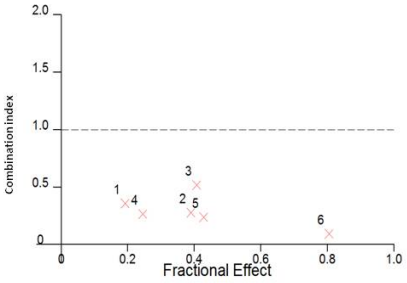
MM1R



FK866 (nM)	Bortezomib (nM)	F.A.	C.I.
1	2.5	0.1956	0.609
1	5	0.3346	0.383
1	10	0.3576	0.593
3	2.5	0.2986	0.554
3	5	0.4186	0.367
3	10	0.659	0.169

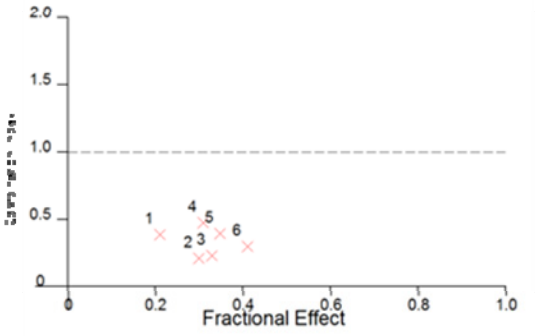
D

MM1S

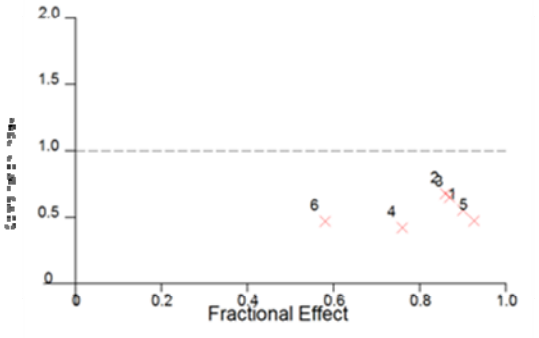


FK866 (nM)	Bortezomib (nM)	F.A.	C.I.
1	2.5	0.1922	0.360
1	5	0.3869	0.279
1	10	0.4076	0.520
3	2.5	0.2456	0.267
3	5	0.428	0.240
3	10	0.805	0.093

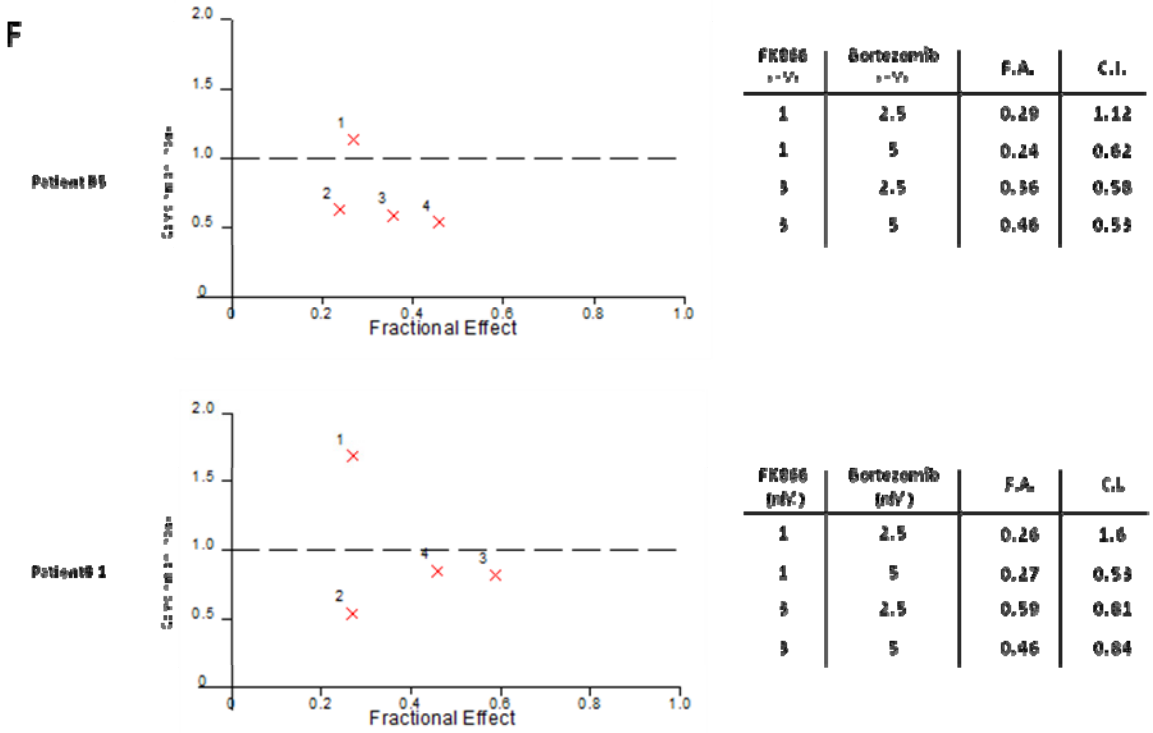
E



FK866 (nM)	Bortezomib (nM)	F.A.	C.I.
1	1.25	0.2108	0.387
1	2	0.2999	0.211
1	5	0.3294	0.231
5	1.25	0.3097	0.474
5	2	0.3474	0.393
5	5	0.4116	0.300



FK866 (nM)	Bortezomib (nM)	F.A.	C.I.
1	1.25	0.9	0.55
1	2	0.86	0.68
1	5	0.87	0.65
5	1.25	0.76	0.42
5	2	0.92	0.47
5	5	0.58	0.47



FK866	Bortezomib	F.A.	C.I.
1	2.5	0.29	1.12
1	5	0.24	0.62
3	2.5	0.36	0.58
3	5	0.46	0.53

FK866	Bortezomib	F.A.	C.I.
1	2.5	0.26	1.6
1	5	0.27	0.53
3	2.5	0.59	0.61
3	5	0.46	0.84

Figure 1S. FK866 with bortezomib triggers synergistic MM cells cytotoxicity. (A) RPMI-8226/S, (B) U266, (C) MM1R, (D) MM1S, (E) ANBL6/BR and ANBL6/WT cells and (F) $CD138^{+}$ cells from Bortezomib-resistant (#5) and -sensistive (#1) patients were treated with FK866 (1-3nM) for 96 h, with bortezomib (1.25-10nM) added for the last 48 h. (*Right panel*) CalcuSyn software: isobologram analysis with combination indices (CI; y axis) plotted versus the fractions affected (FA; x axis) achieved by the combination are shown. CI < 1 indicates synergy. All experiments were repeated in triplicate. *(Left panel)* CI and FA for each dose of drug combination are represented.

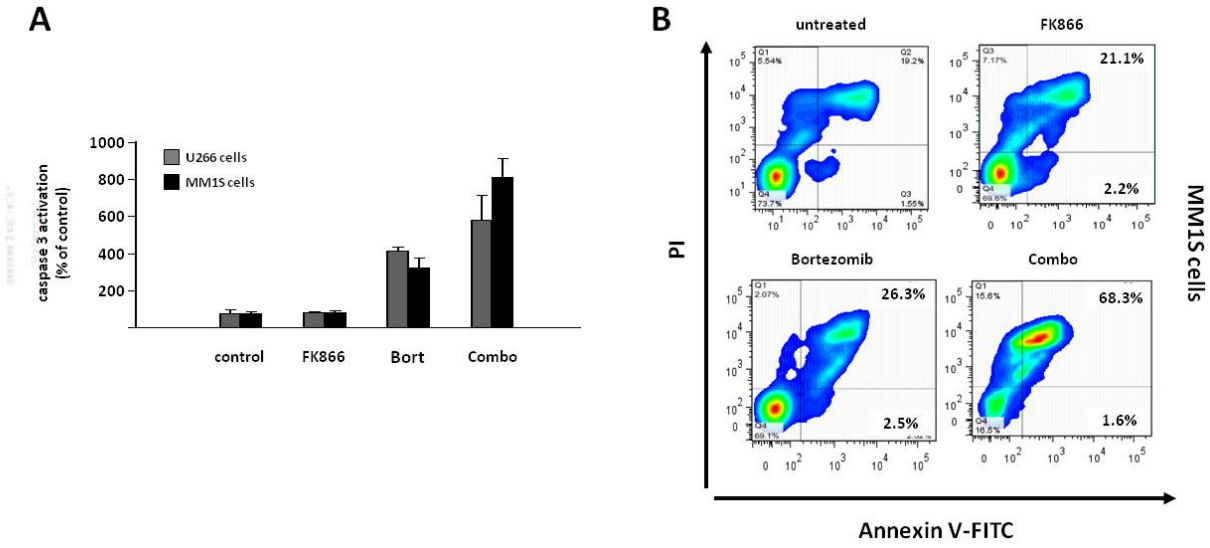


Figure 2S. Anti-MM activity of combination is dependent on caspase activation. (A) MM1S and U266 cells were pretreated with or without low dose FK866 (3 nM) for 24 h, and then bortezomib (2 nM) was added for an additional 24 h; caspase-3 activation was tested using Caspase-Glo assay. (B) MM1S cells were treated with FK866 (3 nM), bortezomib (2 nM) or combined therapy for 72 h, followed by Annexin V/PI staining and flow cytometry analysis.

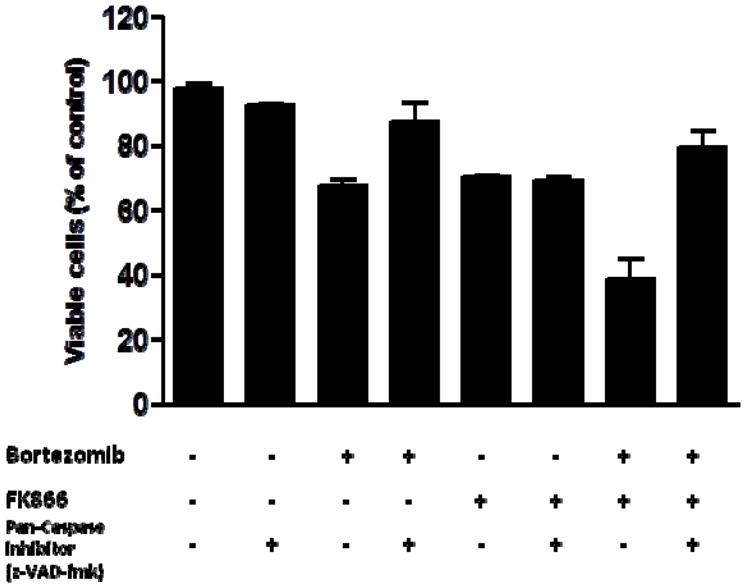


Figure 3S. Anti-MM activity of combination is dependent on caspase activation. MM1S cells were treated with FK866 (3nM), bortezomib (2 nM) or the combination for 96 h, in the presence or absence of pan-caspase inhibitor zVAD-fmk. Specific cell death was measured with PI staining and cytometry analysis. Shown are the means ± SD (error bar) of 3 independent experiments.

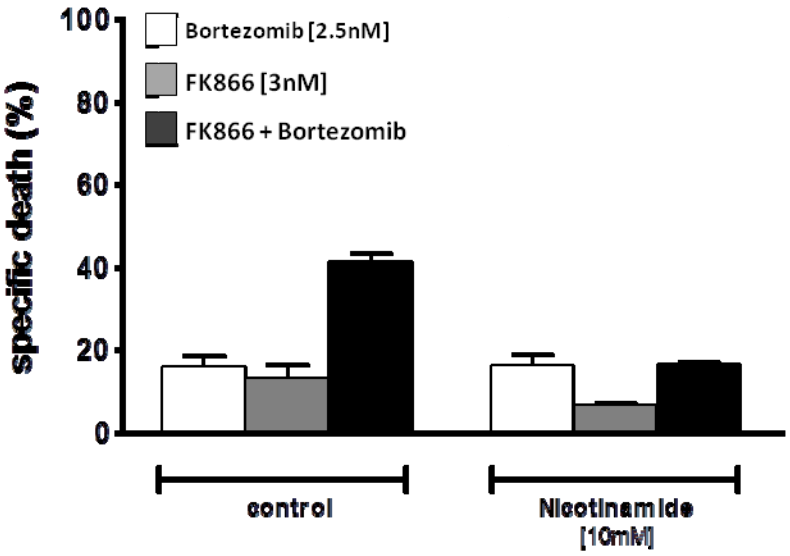


Figure 4S. Anti-MM activity of combination treatment is rescued by Nicotinamide. MM1S cells were treated with FK866 (3nM), Bortezomib (2nM) or the combination, in the presence or absence of 10 mM Nam. After 72 h, specific cell death was determined by PI staining and flow cytometry. Each treatment was tested in triplicate wells, and results are means ± SD of three independent experiments.

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