Supplemental Figure 1B

B

% Annexin V +

0 8 24 30 50 72

Time (hours)

PBS
IFN-α
Bortezomib
Bortezomib + IFN-α
Supplemental Figure 1C

% Apoptotic Cells

PBS/PBS  PBS/IFN  Bort/IFN  IFN/Bort  Bort + IFN (Combined)

Treatment #1/Treatment #2
Supplemental Figure 2

B

1  2  3  4

**PARP**

- 89 KDa

**Caspase-3**

- 35 KDa
- 17 KDa

**β-actin**
Supplemental Figure 2

C

% Dead Cells

IFN-γ:
- 1ng 10ng - 1ng 10ng - 1ng 10ng

Bortezomib: 0 10nM 15nM

Bortezomib: 0 10nM 15nM
Supplemental Figure 5

A

- % Dead Cells
- Ctrl siRNA
- Fas siRNA

Treatment:
- PBS
- IFN-alpha
- Bortezomib
- B + IFN

B

- Control siRNA
- Fas siRNA

- PBS
- IFN-α
- Bort.
- B + IFN

Proteins:
- Fas: 45 kDa
- β-Actin: 44 kDa
Supplemental Figure 6

Mean Weight (grams) vs. Time (Days)

- Bortezomib
- B + IFN
Supplemental Figure 8

Noxa

β-Actin
Figure Legends of Supplemental Information.

Supplemental Fig. 1. Bortezomib and IFN-α induce synergistic apoptosis. (A) The pro-apoptotic effects of IFN-α (10^4 U/mL), bortezomib (10 nM), or both agents combined were evaluated in human melanoma (18105 MEL, MEL 39) and RCC cell lines (RC-45) by Annexin V/PI staining at 48 hours post treatment. The percentage of Annexin V-positive cells is indicated on each dot plot. (B) A representative time course experiment in the B16F1 melanoma cell line demonstrated that maximal apoptosis was observed following treatment with bortezomib (10 nM) plus IFN-α (10^4 U/ml) for extended periods of time (>24 hours). Variability of apoptotic cells within triplicate conditions for this experiment was < 5%. (C) A375 melanoma cells were pre-treated for 24 hours with one agent, washed, and subsequently treated for an additional 24 hours with the other agent. The percentage of apoptotic A375 melanoma cells was examined by Annexin V/PI staining. Cells treated with media containing PBS served as negative controls, while cells treated with bortezomib and IFN-α combined for 48 hours served as a positive control in this assay.

Supplemental Fig. 2. Bortezomib and IFN-β but not IFN-γ induce synergistic apoptosis. (A) Apoptosis measured by Annexin V/PI staining in A375 cells following treatment with IFN-β (10^3 U/mL) and bortezomib (Bort.; 20nM). (B) PARP cleavage and caspase-3 processing in response to treatment with bortezomib alone (Lane2) or bort. + IFN-β (Lane4). PBS and IFN-β treated cells (Lane 1, 3). Arrows = cleaved proteins. (C) Apoptosis was measured by AnnV/PI staining in A375 following treatment with IFN-γ plus bortezomib.

Supplemental Fig. 3. Treatment with bortezomib and IFN-α results in cleavage of PARP in human melanoma cell lines. Various human melanoma cell lines were treated for 48 hours with PBS (P), IFN-α (α; 10^4 U/mL), bortezomib (B; 10 nM) or both agents combined (C) and evaluated by immunoblot analysis for cleaved PARP (arrows). Membranes were also probed with an anti-β-actin Ab to serve as a loading control.

Supplemental Fig. 4. The pro-apoptotic effects of bortezomib and IFN-α treatment are dependent upon caspase activation. HT144 cells were treated with PBS, IFN-α (10^4 U/mL), bortezomib (10 nM) or both agents combined in the presence of the pan-caspase inhibitor Z-VAD-FMK or a negative control compound (Z-F-A-FMK) at a 50 μM concentration for 48 hours. The ability of Z-VAD-FMK to inhibit cell death in response to bortezomib and IFN-α was reproducible and confirmed in multiple experiments. Error bars represent the standard deviation of data obtained by annexin V/PI staining in n = 3 separate experiments with the HT144 melanoma cell line.

Supplemental Figure 5. The pro-apoptotic effects of bortezomib and IFN-α treatment are inhibited by Fas-specific siRNA. A375 cells were transfected with Fas-specific siRNA or negative control siRNA (20 μM; Santa Cruz Biotechnology, Inc.) using Lipofectamine 2000 (Invitrogen). The following day, cells were treated for 48 hours with PBS, IFN-α (2 x 10^4 U/mL), bortezomib (15nM) or both agents combined.
(A) Cell viability in three independent experiments was evaluated via trypan blue exclusion and (B) knockdown of Fas was confirmed by immunoblot analysis. Membranes were probed with an anti-β-actin Ab to serve as a loading control.

Supplemental Figure 6. Bortezomib and IFN-α are not excessively toxic in mice. The body weight was measured in C57BL/6 mice receiving either bortezomib alone or bortezomib and IFN-α combined (B + IFN) twice weekly immediately prior to each administration of bortezomib. Data presented represent the mean total body weight in grams (n = 6 mice/group). Error bars = standard deviation within groups.

Supplemental Figure 7. Microvessel density is unchanged by bortezomib and IFN-α. Sections from tumors were stained for CD31 and microvessel density (MVD) was quantitated manually. Columns, mean (n = 4-6 tumors per group); bars, standard deviation.

Supplemental Figure 8. Bortezomib-mediated induction of Noxa is not further enhanced by IFN-α. A375 melanoma cells were treated for 16 hours (prior to the induction of apoptosis) with PBS, IFN-α (10^4 U/mL), bortezomib (Bort.), or both agents combined (B + IFN). The level of Noxa protein was evaluated by immunoblot. Membranes were re-probed with an anti-β-actin Ab to serve as a loading control. Positive control = MUM2B melanoma cells treated for 16 hr with bortezomib (Ref. #49).