

# A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib

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## Summary

**Bortezomib therapy has proven successful for the treatment of relapsed and/or refractory multiple myeloma (MM); however, prolonged treatment is associated with toxicity and development of drug resistance. Here, we show that the novel proteasome inhibitor NPI-0052 induces apoptosis in MM cells resistant to conventional and Bortezomib therapies. NPI-0052 is distinct from Bortezomib in its chemical structure, effects on proteasome activities, mechanisms of action, and toxicity profile against normal cells. Moreover, NPI-0052 is orally bioactive. In animal tumor model studies, NPI-0052 is well tolerated and prolongs survival, with significantly reduced tumor recurrence. Combining NPI-0052 and Bortezomib induces synergistic anti-MM activity. Our study therefore provides the rationale for clinical protocols evaluating NPI-0052, alone and together with Bortezomib, to improve patient outcome in MM.**

## Introduction

The successful development of Bortezomib/PS-341 Velcade therapy for treatment of relapsed/refractory multiple myeloma (MM) has established proteasome inhibition as an effective therapeutic strategy. The dipeptide boronic acid analog Bortezomib is a potent, highly selective, and reversible proteasome inhibitor that targets the 26S proteasome complex and inhibits its function (Adams et al., 1999). Besides inhibiting NF- $\kappa$ B, Bortezomib has pleiotropic effects on MM biology by targeting (1) cell cycle regulatory proteins; (2) the unfolded protein response (UPR) pathway; (3) p53-mediated apoptosis; and (4) DNA repair mechanisms; as well as (5) classical stress response pathways via both intrinsic (caspase-9-mediated) and extrinsic (caspase-8-mediated) cell death cascades. Specifically, Bortezomib activates c-Jun amino-terminal kinase (JNK) (Chauhan et al., 2003), which triggers mitochondrial apoptotic signaling;

release of cytochrome-c (cyto-c) and second mitochondrial activator of caspases (Smac) from mitochondria to cytosol, followed by activation of caspase-9 and caspase-3 (Chauhan and Anderson, 2003). Despite the potent anti-MM activity of Bortezomib, both intrinsic and acquired resistance has already been observed in MM patients (Anderson, 2004). The mechanisms conferring Bortezomib resistance are now being defined (Chauhan et al., 2005). Nonetheless, the combination of Bortezomib with other novel and conventional agents can overcome Bortezomib resistance (Chauhan et al., 2005).

Recent studies have focused on developing other proteasome inhibitors as therapeutics in cancer. NPI-0052 is one such molecule derived from fermentation of *Salinospora*, a new marine gram-positive actinomycete (Macherla et al., 2005). In the present study, we show that both NPI-0052 and Bortezomib can be distinguished by chemical structure, their effects on proteasomal activities, and differential toxicity profiles

## SIGNIFICANCE

The ubiquitin-proteasome pathway modulates intracellular protein degradation. The multienzyme protease 26S proteasome degrades misfolded proteins; conversely, blockade of the proteasomal degradation pathways results in accumulation of unwanted proteins and cell death. Because cancer cells proliferate to a greater extent than normal cells, the rate of protein translation and degradation is also higher. This notion led to the development of proteasome inhibitors as cancer therapeutics. Recently, the FDA approved the first proteasome inhibitor, Bortezomib (Velcade), for treatment of relapsed/refractory multiple myeloma (MM). This study shows that the orally bioavailable novel proteasome inhibitor NPI-0052 is cytotoxic to MM cells, with reduced toxicity against normal cells compared to Bortezomib, providing the framework for clinical trials of NPI-0052 in MM.

against normal cells. NPI-0052 induces apoptosis in MM cell lines and patient cells. Both biochemical and genetic studies show that NPI-0052-induced cell death, in contrast to that triggered by Bortezomib, relies primarily on caspase-8-mediated signaling pathways. In vivo studies show that NPI-0052 significantly inhibits tumor growth in mice, is well tolerated, and prolongs survival. Based on their structural and mechanistic differences, NPI-0052 and Bortezomib show synergistic anti-MM activity. Finally, current Bortezomib therapy requires intravenous (i.v.) administration, whereas NPI-0052 is orally bioactive. Overall, our study provides the rationale for clinical protocols evaluating NPI-0052 to inhibit tumor cell growth, overcome conventional and Bortezomib resistance, limit toxicity profiles, and improve patient outcome in MM.

## Results and discussion

### NPI-0052 is structurally distinct from Bortezomib and inhibits all three protease activities within the proteasome both in vitro and in vivo

The naturally occurring and synthetic inhibitors of the ubiquitin-proteasome pathway include peptide aldehydes, peptide boronates, nonpeptide inhibitors, peptide vinyl sulfones, and peptide epoxyketones (Adams, 2004; Adams et al., 1999; Chauhan et al., 2005). Bortezomib/PS-341/Velcade is a boronic acid dipeptide derivative and inhibits proteasome function via interaction of boronic acid at the C terminus of Bortezomib with an active threonine site in the proteasome (Adams et al., 1999) (Figure 1A). NPI-0052 is a nonpeptide proteasome inhibitor and shares structural features with another proteasome inhibitor, Omuralide (Figure 1A), which inhibits proteasome activity by covalently modifying the active site threonine residues of the proteasome (Corey and Li, 1999). Omuralide is clasto-lactacystin  $\beta$ -lactone, the active form of the well-known proteasome inhibitor lactacystin (Corey and Li, 1999). Despite the structural similarity with Omuralide, NPI-0052 can be distinguished by the presence of a uniquely methylated C3 ring juncture, chlorinated alkyl group at C2, and cyclohexene ring at C5 (Figure 1A). Evaluation of the effects of NPI-0052, Omuralide, and Bortezomib on cathepsin A activity show that Omuralide is a more specific inhibitor of cathepsin A ( $IC_{50} = 65 \pm 6$  nM). Both NPI-0052 and Bortezomib also inhibited cathepsin A with different  $IC_{50}$  (NPI-0052,  $1.4 \pm 0.1$   $\mu$ M; Bortezomib,  $14 \pm 5$   $\mu$ M).

Omuralide is known to inhibit all three protease activities in the proteasome: the chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) activities (Corey and Li, 1999); however, the effect of NPI-0052 on these proteasome activities is undefined. We therefore examined whether NPI-0052 affects these proteasome activities using human erythrocyte 20S proteasomes and fluorogenic peptide substrates. We also simultaneously compared the effect of Bortezomib on proteasome activities. Both NPI-0052 and Bortezomib inhibit all three proteasome activities, albeit at different concentrations (Figures 1B–1D). Results show that (1) NPI-0052 inhibits CT-L and T-L activities at lower concentrations than Bortezomib, and (2) higher concentrations of NPI-0052 than Bortezomib are required to inhibit C-L activity. Our in vitro data suggest that NPI-0052, like Bortezomib, targets proteasomes.

We next compared the effects of NPI-0052 and Bortezomib on all three proteasome activities in vivo in mice. For these studies we selected the MTD dose for each agent. Based on

our previous study (LeBlanc et al., 2002), the MTD of Bortezomib is 1.0 mg/kg (i.v.) given twice weekly. Additional experimentation in beige-nude-xid (BNX) mice established the MTD of NPI-0052 at 0.15 mg/kg (i.v.) twice weekly (data not shown). Mice were treated with a single dose of NPI-0052 (0.15 mg/kg i.v.) or Bortezomib (1 mg/kg i.v.); blood samples were collected at 90 min, 24 hr, 48 hr, 72 hr, or 168 hr; and whole blood cells were then analyzed for proteasome activity (Figure 2). NPI-0052 completely inhibited CT-L activity by 90 min, which was recoverable by 168 hr (Figure 2A), whereas Bortezomib-inhibited CT-L activity was markedly restored at 24 hr (Figure 2B). T-L activity is significantly inhibited (50% inhibition) by NPI-0052 at 90 min, 24 hr, 48 hr, and 72 hr and is restored by 168 hr (Figure 2C); in contrast, Bortezomib enhances T-L activity, which remains elevated even at 168 hr (Figure 2D). Finally, NPI-0052 inhibits C-L activity at 90 min, 24 hr, 48 hr, and 72 hr, and this activity recovered at 168 hr (Figure 2E), whereas Bortezomib significantly inhibits C-L activity at 90 min, 24 hr, 48 hr, and 72 hr and is similarly recoverable at 168 hr (Figure 2F). Therefore, after NPI-0052 treatment, all three proteasome activities remain inhibited at 72 hr and were restored to significant levels by 168 hr. These data suggest that NPI-0052 and Bortezomib differentially affect all three proteasome activities.

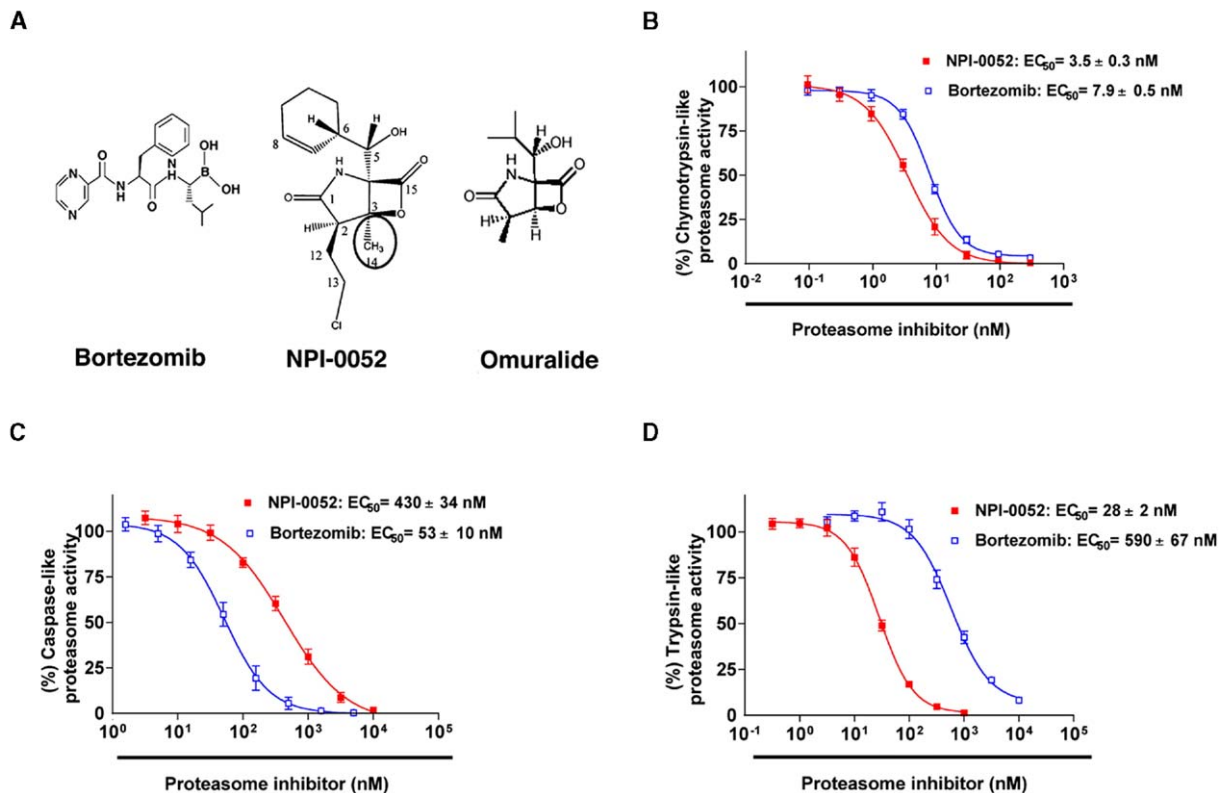
### NPI-0052 is orally bioactive

Previous studies showed that Bortezomib is orally active (Pallombella et al., 1998; Teicher et al., 1999); however, the current Bortezomib therapy in MM is administered i.v. The rationale for using the i.v. route instead of oral administration may include bioavailability, rapid distribution (Adams et al., 1999), solubility, and reversibility/half-life issues. We therefore next examined whether NPI-0052 is also orally bioactive. Mice were treated with various oral and i.v. concentrations of NPI-0052; at 90 min whole-blood lysates (WBL) were analyzed for proteasome activity. Oral administration of NPI-0052 significantly inhibits CT-L activity of 20S proteasomes in a dose-dependent manner between 0.025 mg/kg and 0.50 mg/kg (Figure 3A). Both T-L and C-L activities were also inhibited, albeit to lesser extent (data not shown). These findings show that NPI-0052 given orally inhibits proteasome activity in vivo.

### NPI-0052 targets nuclear factor- $\kappa$ B

A major rationale for using Bortezomib therapeutically is its ability to inhibit nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation (Adams, 2002; Hideshima et al., 2002; Russo et al., 2001). We therefore next asked whether NPI-0052 similarly affects NF- $\kappa$ B activation. To address this issue, a stable HEK-293 clone was generated carrying a luciferase reporter gene under the regulation of 5 $\times$  NF- $\kappa$ B binding sites. Stimulation of these cells with human TNF- $\alpha$  leads to increased luciferase activity due to NF- $\kappa$ B activation. Pretreatment of NF- $\kappa$ B/luc HEK-293 cells with NPI-0052 results in a significant ( $p < 0.001$ ) dose-dependent decrease of luciferase activity after TNF- $\alpha$  stimulation (Figure 3B), indicating inhibition of NF- $\kappa$ B. Bortezomib also downregulates NF- $\kappa$ B activity, but at higher concentrations than NPI-0052 (Figure 3B). These findings indicate that NPI-0052 is a more potent inhibitor of NF- $\kappa$ B than Bortezomib.

Activation of NF- $\kappa$ B triggers transcription and secretion of various proinflammatory cytokines, such as TNF- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6), which mediate the growth and survival of tumor cells (Adams, 2002; Hideshima et al.,



**Figure 1.** The proteasome inhibitor NPI-0052 is structurally distinct from Bortezomib/PS-341 and attenuates proteasome activity in vitro

**A:** Chemical structures of the proteasome inhibitors Bortezomib, NPI-0052, and Omuralide.

**B–D:** NPI-0052 and Bortezomib inhibit CT-L, C-L, and T-L proteasome activities in human erythrocyte-derived 20S proteasomes. The  $EC_{50}$  values are the drug concentration at which 50% maximal relative fluorescence is inhibited.

2002; Russo et al., 2001). Inhibition of cytokine synthesis and function by means of proteasome inhibition thereby has clinical benefit. Since both NPI-0052 and Bortezomib inhibit NF- $\kappa$ B, we examined the effect of these agents on LPS-induced cytokine production in human peripheral blood mononuclear cells (PBMNCs). NPI-0052 inhibited LPS-triggered secretion of all three cytokines at lower concentrations than Bortezomib (Figure 3C). These findings show that NPI-0052, like Bortezomib, targets NF- $\kappa$ B and related cytokine secretion.

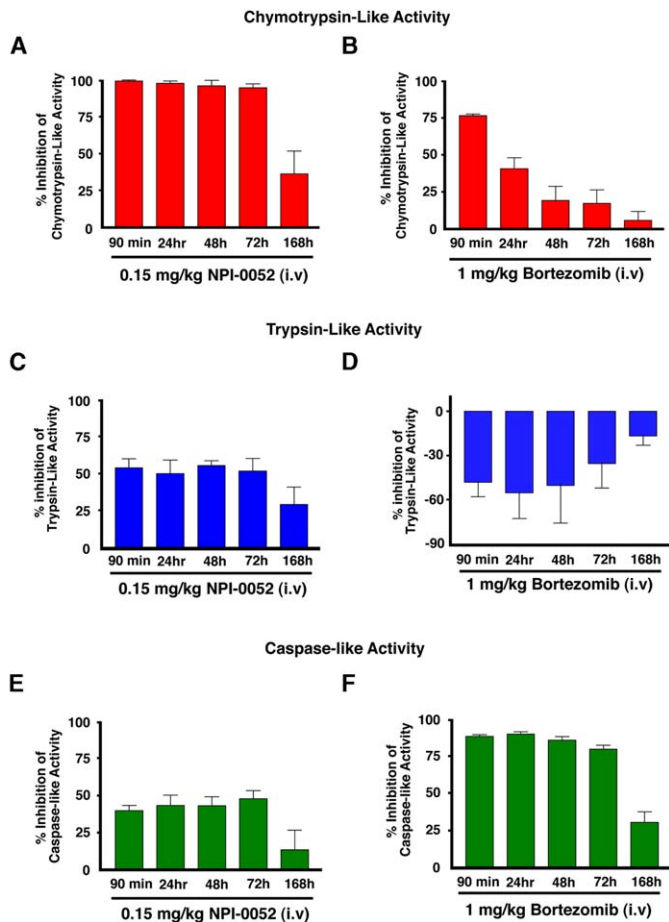
#### NPI-0052 blocks proteasome activity in MM cells

Our preclinical and clinical studies have already shown Bortezomib to be an effective therapy in MM (Hideshima and Anderson, 2002; Richardson et al., 2005). Our recent study showed the composition of active proteasome in MM.1S cell line and the proteasome targets of Bortezomib in these cells (Berkers et al., 2005). In that study, we utilized a novel methodology to measure proteasome activity by immunoblotting using DansylAhx<sub>3</sub>L<sub>3</sub>VS as a probe (Berkers et al., 2005). The results from this method correlated with those obtained using fluorogenic substrates and, in addition, allow for determining subunit specificity of a given proteasome inhibitor (details provided in the Experimental Procedures). In the present study, we examined the effects of NPI-0052 and Bortezomib on the catalytic activities of proteasome subunits. Cells were cultured in the

presence or absence of various concentrations of either NPI-0052 (2 nM, low toxic dose; 7 nM,  $IC_{50}$ ; and 20 nM, highly toxic to MM.1S cells), and compared these effects with those triggered by Bortezomib (2 nM, low toxic dose; 5 nM,  $IC_{50}$ ; and 20 nM, highly toxic to MM.1S cells). Competition experiments between either NPI-0052 or Bortezomib and DansylAhx<sub>3</sub>L<sub>3</sub>VS revealed that NPI-0052 (7 nM) markedly inhibits the CT-L activity as represented by  $\beta$ -5 ( $\beta$ -5 subunit of the proteasome) (Figure 3D). Furthermore, NPI-0052 also decreased the DansylAhx<sub>3</sub>L<sub>3</sub>VS labeling of the  $\beta$ -1 (C-L activity) and  $\beta$ -2 (T-L activity) in a dose-dependent manner. Slightly higher concentrations of Bortezomib are necessary to markedly inhibit  $\beta$ -5 and  $\beta$ -1 subunits, whereas  $\beta$ -2 subunits are not inhibited. Together, these findings demonstrate the ability of NPI-0052 to inhibit all three proteasome activities in MM cells and are consistent with in vitro results using fluorogenic substrates shown in Figures 1 and 2.

#### NPI-0052 inhibits growth and triggers apoptosis in MM cells

We next asked whether NPI-0052-induced proteasome inhibition correlates with cytotoxicity in MM cells. Treatment for 24 hr of MM cell lines (MM.1S, RPMI-8226, OPM2, U266), including those that are resistant to the conventional anti-MM agents Dexamethasone (Dex) (MM.1R) and Doxorubicin (Dox-40), with



**Figure 2.** NPI-0052 and Bortezomib differentially affect proteasome activities in vivo

Mice ( $n = 5$ ) were treated with either NPI-0052 (A, C, and E) or Bortezomib (B, D, and F) at their respective MTD (1 mg/kg for Bortezomib and 0.15 mg/kg for NPI-0052) for the indicated times, and blood samples were analyzed for CT-L, C-L, and T-L proteasome activities. The data represent percent inhibition compared to vehicle control-treated animals from two independent experiments with similar results.

NPI-0052 induces a dose-dependent significant ( $p < 0.005$ ) decrease in viability of all cell lines ( $IC_{50}$  range 7–24 nM) (Figure 4A). To determine whether NPI-0052 similarly affects purified patient cells, tumor cells from nine MM patients relapsing after multiple prior therapies including Dex, melphalan, Bortezomib, and Thalidomide were treated for 24 hr with NPI-0052 (10 nM) and then analyzed for apoptosis. NPI-0052 induced significant apoptosis in these cells, as measured by DNA fragmentation assays ( $p < 0.005$ ) (Figure 4B). Importantly, of nine patients examined four were refractory to Bortezomib therapy, and five were resistant to Thalidomide and Dex therapies. Patients were deemed to be refractory to Bortezomib when they had progressive disease while on Bortezomib therapy. In addition, data from the phase II clinical studies in MM show that only 35% of patients with relapsed/refractory MM respond to the treatment with Bortezomib; and in this context, the 65% of patients who were nonresponders were considered to be a Bortezomib-resistant patient population. We next examined whether cells

from such patient populations are affected by Bortezomib treatment in vitro and whether NPI-0052 exerts a cytotoxic effect in these cells. CD138-positive cells from seven MM patients were treated with Bortezomib (10 nM) and NPI-0052 (10 nM) in vitro and then analyzed for viability (Figure 4C). Results demonstrate a varying sensitivity to Bortezomib in vitro, with a 15%–50% decrease in viability; however, all patient cells were significantly more sensitive to NPI-0052 ( $IC_{50} \leq 10$  nM in 6 of 7 patients) (Figure 4C).

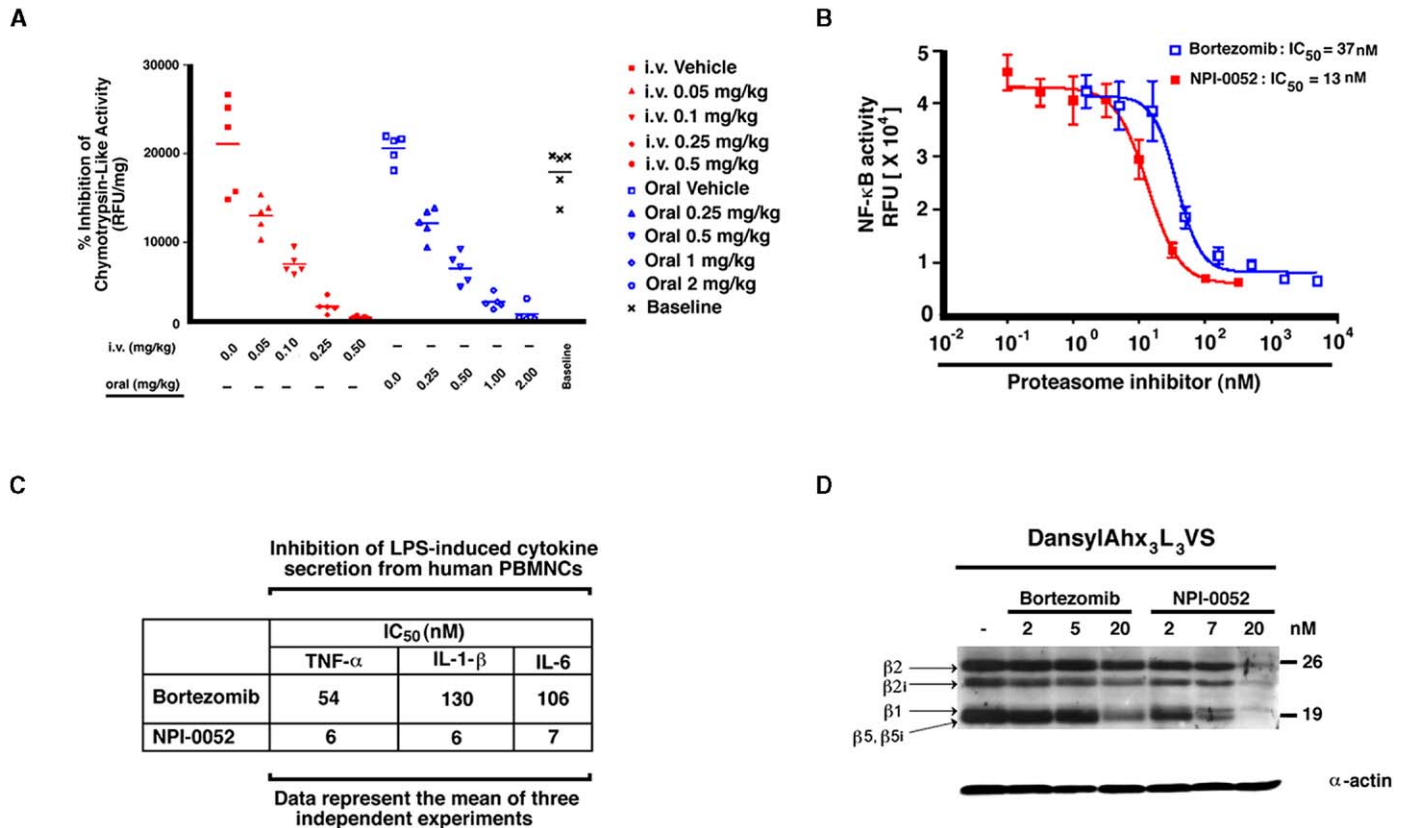
The finding that cells from Bortezomib-refractory patients are sensitive to the treatment with Bortezomib in vitro suggests that extracellular factors, such as the BM microenvironment, play a role in conferring drug resistance in vivo. Absence of the BM milieu under the ex vivo culture conditions may restore sensitivity of MM cells to Bortezomib; however, the activation of intrinsic cellular drug mechanisms cannot be excluded. Additionally, as noted above, NPI-0052 and Bortezomib differentially affect proteasome activities, which may explain their variable in vitro cytotoxicity. Furthermore, it is unclear at present whether alteration in any proteasome subunit confers Bortezomib resistance versus sensitivity. Our data obtained from viability studies suggest that NPI-0052 is a more potent inducer of MM cell apoptosis than Bortezomib in tumor cells obtained from Bortezomib-refractory MM patients.

#### NPI-0052 does not affect viability of patient MM-derived bone marrow stromal cells

Interaction of MM cells with bone marrow stromal cells (BMSCs) induces cytokine secretion, which mediates paracrine growth of MM cells, as well as protects against drug-induced apoptosis (Hideshima and Anderson, 2002). We examined whether NPI-0052 affects viability of BMSCs. Treatment of BMSCs (patients 1–5) for 24 hr with NPI-0052 (20 nM) does not induce apoptosis in these cells, whereas NPI-0052 triggered a significant (10- to 12-fold) increase in apoptosis of purified patient MM cells (Figure 4D). Importantly, NPI-0052 significantly decreased the secretion of IL-6 triggered by adhesion of MM cells to BMSCs ( $IC_{50}$ , 80–100 nM;  $p < 0.05$ ), as is observed using Bortezomib (Anderson, 2004). Together, these results suggest that NPI-0052 does not directly affect BMSC viability, but blocks the secretion of BMSC-derived MM cell growth factor IL-6 within the BM milieu.

#### NPI-0052 overcomes recombinant human interleukin-6- and recombinant human insulin-like growth factor-I-mediated antiapoptotic effects

Both IL-6 and IGF-I trigger growth and protect against chemotherapy-induced apoptosis in MM cells (Chauhan and Anderson, 2003; Xu et al., 1997). We therefore next examined whether NPI-0052 overcomes this protective effect of IL-6 or IGF-I. Neither recombinant human interleukin-6 (rhIL-6) nor recombinant human insulin-like growth factor-I (rhIGF-I) blocks NPI-0052-triggered cytotoxicity in MM.1S cells (Figure 4E). As in our prior studies (Chauhan and Anderson, 2003; Mitsiades et al., 2002a), both rhIL-6 and rhIGF-I block Dex-induced decreases in MM.1S cell viability. High serum levels of IL-6 or IGF-I contribute to clinical chemoresistance and treatment failure (Hideshima and Anderson, 2002), and our data therefore suggest a remarkable ability of NPI-0052 to induce MM cell apoptosis even in the presence of IL-6 or IGF-I.



**Figure 3.** Orally bioactive NPI-0052 inhibits cytokine secretion, NF- $\kappa$ B, and proteasome activities

**A:** NPI-0052 is orally bioactive. Mice ( $n = 5$ ) were administered NPI-0052 i.v. or orally, and CT-L activity of the 20S proteasome in WBC lysates was determined. Relative fluorescence units (RFU) were normalized for protein concentrations of the cell lysates. The 20S proteasome activity of the individual mice is shown, with the horizontal bar representing the average activity. Baseline represents 20S proteasome activity observed in WBC lysates prepared from untreated mice.

**B and C:** NPI-0052 inhibits NF- $\kappa$ B and the cytokine secretion. Effect of NPI-0052 and Bortezomib on NF- $\kappa$ B activity in NF- $\kappa$ B/Luc HEK-293 cells and LPS-triggered cytokine secretion in PBMCs.

**D:** Competition experiment between NPI-0052 or Bortezomib and DansylAhx<sub>3</sub>L<sub>3</sub>VS (upper panel). The three distinct catalytic activities of proteasome are represented by  $\beta$ -1 for caspase-like,  $\beta$ -2 for Trypsin-like, and activity  $\beta$ -5 for chymotrypsin-like activities. The  $\beta$ -2i and  $\beta$ -5i are the interferon-inducible subunits to form immunoproteasomes with altered catalytic specificity that help production of antigenic peptides. Filters were reprobbed with anti- $\alpha$ -actin Ab to show equal protein loading. Blots shown are representative of two independent experiments.

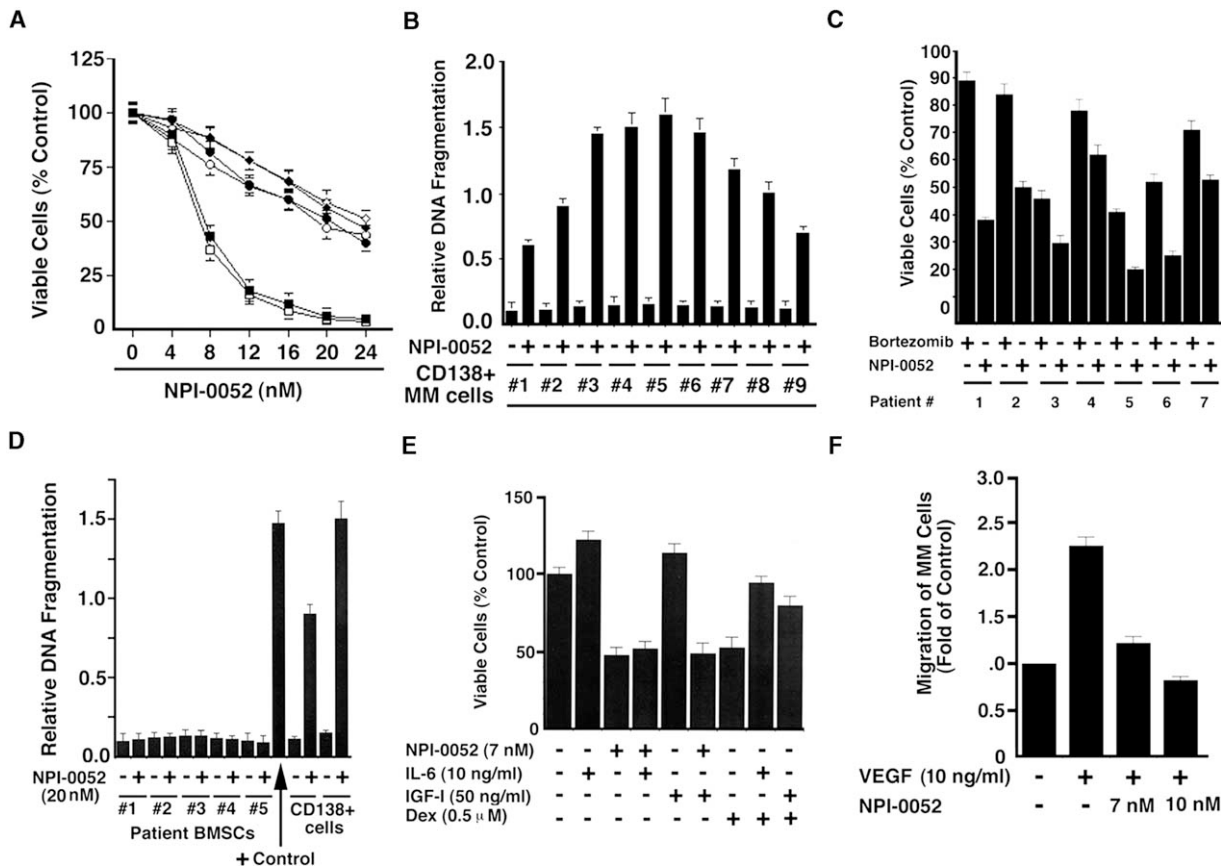
### NPI-0052 blocks vascular endothelial growth factor-induced migration of MM cells

Vascular endothelial growth factor (VEGF) is elevated in the MM BM microenvironment, and our studies showed that VEGF triggers growth, migration, and angiogenesis in MM (Podar et al., 2002). We therefore asked whether NPI-0052 affects VEGF-triggered MM cell migration. VEGF alone markedly increases MM.1S cell migration; conversely, NPI-0052 significantly ( $p < 0.05$ ) inhibits VEGF-dependent MM cell migration (Figure 4F). The short exposure time of MM.1S cells to NPI-0052 did not affect survival of MM cells (viability  $> 95\%$ ). These findings indicate that NPI-0052 may negatively regulate homing of MM cells to the BM, as well as their egress into the peripheral blood.

### NPI-0052 inhibits human MM cell growth in vivo and prolongs survival in a murine model

Having shown that NPI-0052 induces apoptosis in MM cells in vitro, we next examined the in vivo efficacy of NPI-0052

using our human plasmacytoma xenograft mouse model (LeBlanc et al., 2002). As noted above, NPI-0052 has achieved therapeutically effective levels of proteasome inhibition after oral administration in mice, and we therefore administered NPI-0052 orally. Treatment of tumor-bearing mice with NPI-0052, but not vehicle alone, significantly ( $p < 0.001$ ) inhibits MM tumor growth and prolongs survival ( $p < 0.001$ ) of these mice (Figures 5A–5C). The concentrations of NPI-0052 administered were well tolerated by mice, without significant weight loss (see Figure S1 in the Supplemental Data available with this article online). Moreover, no neurological behavioral changes were observed even after 12 weeks of NPI-0052 treatment. Analysis at day 300 showed no recurrence of tumor in 57% of the NPI-0052-treated mice (Figure 5C). In addition, histologic analysis performed on the inoculation sites confirmed the disappearance of plasma cells in the NPI-0052- versus vehicle-treated mice (Figure 5D, left and right panels, respectively). These data demonstrate the potent antitumor activity of NPI-0052 in vivo. Importantly, these findings also show that NPI-0052 is orally



**Figure 4.** NPI-0052 induces apoptosis in human MM cells

**A:** MTT assays were performed after incubation of MM.1S (black squares), MM.1R (white boxes), RPMI-8226 (black circles), Dox-40 (black squares), OPM2 (white circles), and U266 (white diamonds) human MM cells with indicated concentrations of NPI-0052 for 24 hr. Data represent mean  $\pm$  SD (error bars) from three independent experiments ( $p < 0.005$  for all cell lines).

**B:** Purified MM cells from patients were treated for 24 hr with NPI-0052 (10 nM) and then analyzed for apoptosis. Data represent mean  $\pm$  SD (error bars) of triplicate samples ( $p < 0.005$  for all patient MM cells).

**C:** Tumor cells from MM patients refractory (patients 1, 2, 4, and 7) or sensitive (patients 3, 5, and 6) to Bortezomib therapy were treated with either NPI-0052 (10 nM) or Bortezomib (10 nM) for 24 hr and then analyzed for viability. Shown is mean  $\pm$  SD (error bars) of duplicate samples ( $p < 0.004$  for all samples).

**D:** Effect of NPI-0052 on bone marrow (BM) microenvironment. Patient MM-derived BM stromal cells (BMSCs) were treated for 24 hr with NPI-0052 (20 nM) and then analyzed for apoptosis. Purified MM cells from two of these five MM patients were similarly examined. Data represent mean  $\pm$  SD (error bars) from triplicate samples ( $p < 0.005$ ).

**E:** MM.1S cells were treated for 24 hr with the indicated concentrations of NPI-0052 or Dex, in the presence or absence of rhIL-6 or rhIGF-I, and then analyzed for viability. Shown is mean  $\pm$  SD (error bars) of three independent experiments ( $p < 0.04$  for all samples).

**F:** MM.1S MM cells were treated with indicated concentrations of NPI-0052 for 4 hr (viability  $> 95\%$ ), washed, and then treated for 24 hr with rhVEGF (10 ng/ml), followed by analysis in a transwell migration assay. Data represent mean  $\pm$  SD (error bars) of two independent experiments ( $p < 0.005$  for all samples).

bioactive, which together with the *in vivo* proteasome inhibition studies (Figures 2 and 3A), provides the preclinical framework for its evaluation as an oral agent in phase I trials in MM.

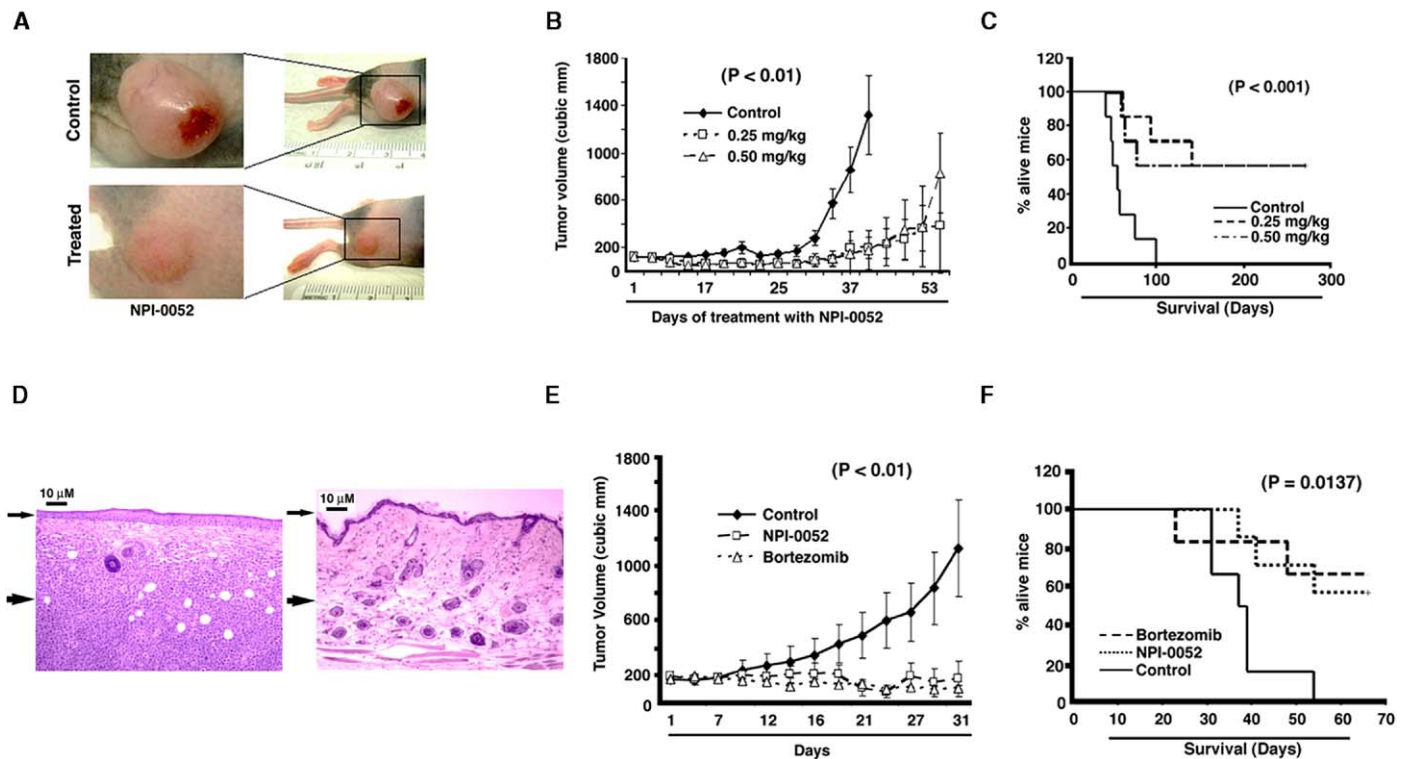
#### Comparative analysis of *in vivo* antitumor activity of NPI-0052 and Bortezomib

Our prior study using the human plasmacytoma xenograft mouse model established the MTD of Bortezomib at 1 mg/kg (*i.v.*) (LeBlanc *et al.*, 2002). Using the same animal model, we obtained the MTD of NPI-0052 at 0.15 mg/kg (*i.v.*) (data not shown). For the comparative study, we therefore selected the MTD of each agent. Mice were treated with NPI-0052 (0.15 mg/kg *i.v.*) or Bortezomib (1.0 mg/kg *i.v.*) twice weekly, the optimal dose and schedule reported for Bortezomib (LeBlanc *et al.*,

2002). Both agents significantly reduced the tumor progression ( $p < 0.01$ ) and prolonged survival ( $p = 0.0137$ ) (Figures 5E and 5F).

#### Mechanisms mediating anti-MM activity of NPI-0052

We first studied mitochondria, given their critical role in apoptosis (Bossy-Wetzel and Green, 1999). NPI-0052 decreases  $\Delta\Psi_m$ , evidenced by an increased number of CMXRos-negative cells ( $p < 0.005$ ) (Figure 6A), and also triggers  $O_2^-$  production in MM.1S cells (Figure 6B). Moreover, NPI-0052 decreases mitochondrial cyto-c and Smac (Figure 6C, upper and middle left panels), coupled with a concurrent accumulation of these proteins in the cytosol (Figure 6C, upper and middle right panels). As in previous studies (Chauhan *et al.*, 2001; Chauhan *et al.*,



**Figure 5.** NPI-0052 inhibits human plasmacytoma growth and increases survival in immune-deficient BNX mice

**A:** Photographs. The mouse on the upper panel received oral doses of vehicle alone, whereas the mouse in the lower panel received oral NPI-0052 (0.25 mg/kg twice weekly). Left panels are enlargements of subcutaneous plasmacytomas growing on the flanks of the mice.

**B:** Kinetics of myeloma xenograft growth. Mice ( $n = 7$ ) received NPI-0052 orally at the indicated doses twice weekly. A significant delay in tumor growth in NPI-0052-treated mice was noted compared to vehicle-treated control mice, as early as day 15 ( $p < 0.001$ ). No significant differences between the NPI-0052 treatment groups were observed.

**C:** Kaplan-Meier plots showing survival for mice treated with NPI-0052 at the indicated concentrations. NPI-0052-treated mice show significantly increased survival ( $p < 0.001$ ) compared to the untreated group. The mean overall survival (OS) was 58 days (95% confidence interval [CI], 44–73 days) in the control cohort versus 196 days (95% CI, 130–262 days) and 182 days (95% CI, 107–258 days) in groups treated with 0.25 and 0.5 mg/kg NPI-0052, respectively. A statistically significant prolongation in mean OS compared with control mice was observed in animals treated with 0.25 mg/kg ( $p = 0.0019$ ) and 0.5 mg/kg ( $p = 0.0074$ ).

**D:** Histology. Tissue sections of inoculation sites from a treated and a control mouse were examined under an Olympus BX60 inverted microscope (Olympus, Lake Success, NY) equipped with Hoffman objective lenses ( $\times 10/\times 20/\times 40$ ; Diagnostic Instruments, Sterling Heights, MI) connected to a SPOT One Digital Camera (Diagnostic Instruments). Images were then processed using Adobe Photoshop software (Adobe, San Jose, CA). Left panel shows extensive subcutaneous infiltration with plasma cells replacing the subcutaneous connective tissue and fat. Right panel shows disappearance of the subcutaneous tumor in a mouse treated with NPI-0052. The thin arrow indicates the cutaneous layer, and the thick arrow shows subcutaneous tissue. Scale bar, 10  $\mu\text{M}$  (micrometers).

**E:** Comparative analysis of NPI-0052- and Bortezomib-triggered effects on myeloma xenograft growth. Treatment with either 0.15 mg/kg NPI-0052 or 1.0 mg/kg Bortezomib ( $n = 7$  per group) significantly delayed tumor growth versus control group ( $p < 0.01$ ).

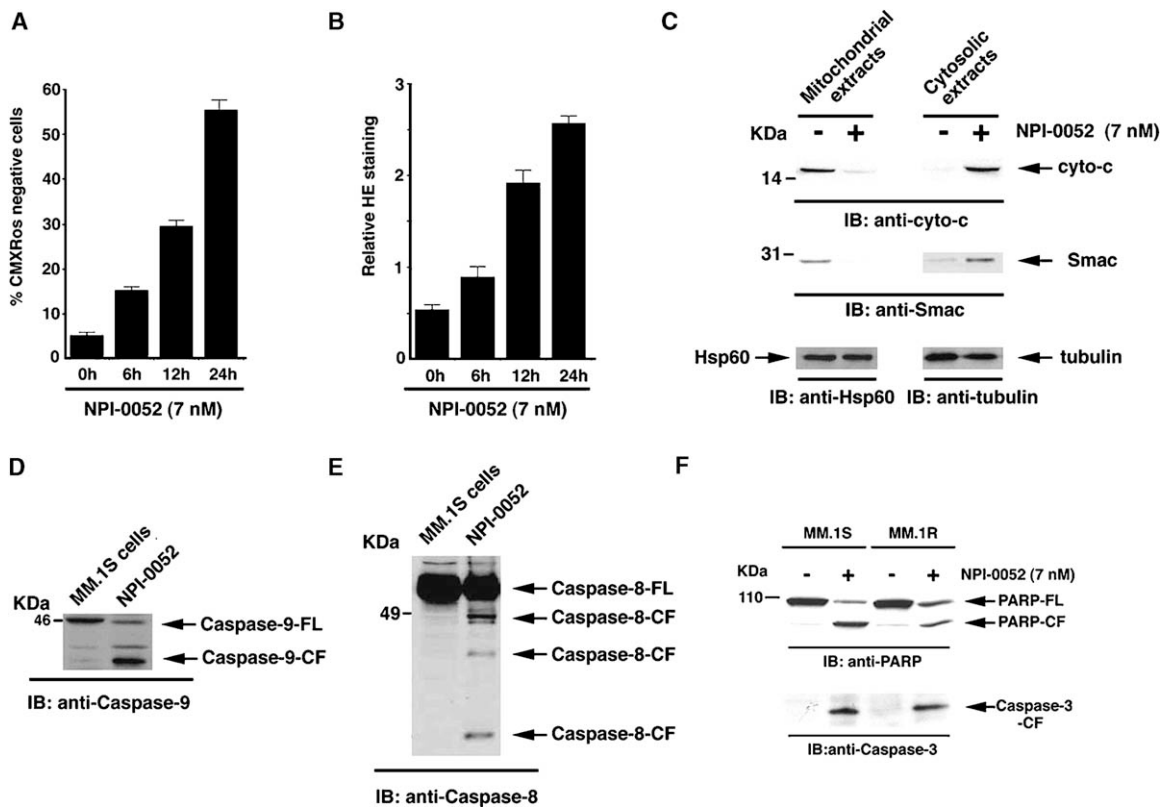
**F:** Treatment with either 0.15 mg/kg NPI-0052 or 1.0 mg/kg Bortezomib significantly increases survival ( $p < 0.0137$ ) compared to treatment with either vehicle alone. A statistically significant prolongation in mean OS compared with control mice was observed in animals treated with Bortezomib ( $p = 0.003$ ) and NPI-0052 ( $p = 0.01$ ).

al., 1997), the release of cyto-c and Smac from mitochondria to cytosol correlates with caspase-9 activation (Figure 6D). Besides caspase-9, NPI-0052 also activates caspase-8 (Figure 6E), followed by activation of downstream effector caspase-3 (Miller, 1999), and proteolytic cleavage of PARP (Figure 6F, lower and upper panels). Together, these findings show that NPI-0052, like Bortezomib, triggers both mitochondria-dependent and -independent signaling pathways.

#### Differential requirement of caspases and mitochondrial signaling during NPI-0052- and Bortezomib-induced MM cell apoptosis

We next defined the requirement for caspase-8 versus caspase-9 during NPI-0052- and Bortezomib-induced apoptosis.

Incubation of MM.1S cells with pan-caspase inhibitor (Z-VAD-FMK) markedly abrogates both NPI-0052- and Bortezomib-induced apoptosis (Figure 7A). Inhibition of caspase-8 (IETD-FMK) led to a significant decrease in NPI-0052-triggered cell death, and inhibition of caspase-9 (LEHD-FMK) only moderately blocked NPI-0052-triggered decreased viability in MM.1S cells ( $p < 0.005$ ;  $n = 4$ ), whereas Bortezomib-induced decreases in viability are equally blocked by either caspase-8 or caspase-9 inhibitor ( $p < 0.005$ ;  $n = 4$ ) (Figure 7A). These biochemical data were confirmed by genetic studies using dominant-negative (DN) strategies. Treatment of DN-caspase-8-transfected MM cells with NPI-0052 ( $\text{IC}_{50}$ , 7 nM) markedly increases survival of these cells, compared to the cells transfected with DN-caspase-9 (Figure 7B). In contrast, treatment of either DN-caspase-8 or DN-caspase-9-



**Figure 6.** NPI-0052-induced apoptotic signal transduction

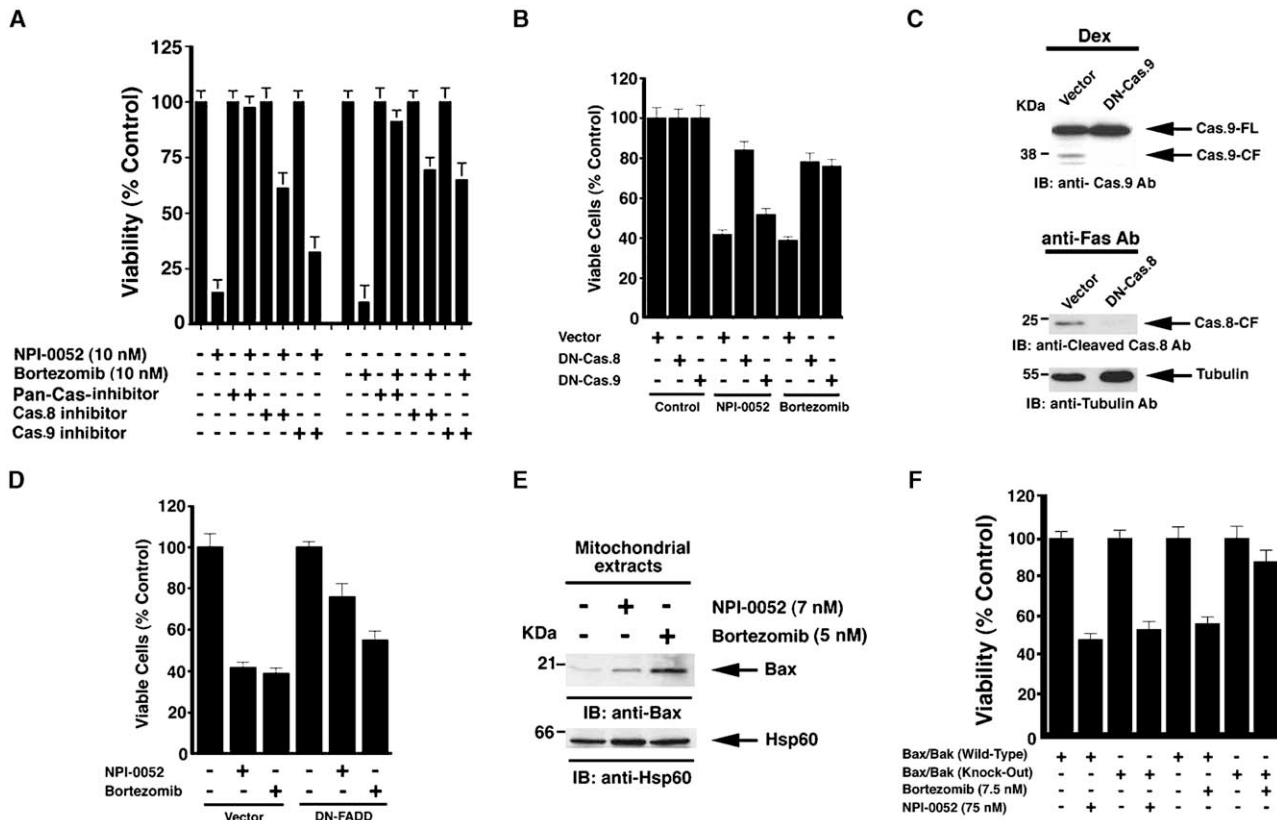
**A:** MM.1S cells were treated with NPI-0052 (7 nM) for the indicated times and then analyzed by flow cytometry for alterations in mitochondrial membrane potential ( $\Delta\Psi_m$ ). Data are mean  $\pm$  SD (error bars) of two independent experiments ( $p < 0.005$ ).  
**B:** MM.1S cells were treated with NPI-0052 for the indicated times, stained with dihydroethidium, and analyzed by flow cytometry. Shown is mean  $\pm$  SD (error bars) of two independent experiments ( $p < 0.005$ ).  
**C:** Immunoblot analysis of NPI-0052-treated MM.1S cells showing alterations of cyto-c and Smac levels (upper and middle panels). Blots were reprobated with anti-Hsp60 and anti-tubulin Abs (lower left and lower right panels).  
**D-F:** MM.1S cells were treated for 24 hr with NPI-0052 (7 nM) and harvested; cytosolic proteins were subjected to immunoblot analysis using anti-caspase-9, -caspase-8, and -caspase-3 and PARP Abs. "FL" indicates full length; "CF" denotes cleaved fragment. Blots shown are representative of three independent experiments.

transfected MM.1S cells with Bortezomib ( $IC_{50}$ , 5 nM) increased the survival to a similar extent. The functional specificity of DN-caspase-8 and DN-caspase-9 was confirmed by treatment of MM.1S cells with known inducers of caspase-9 (Dex) and caspase-8 in these cells (anti-Fas MoAb) (Chauhan et al., 1997) (Figure 7C). These data suggest that (1) NPI-0052-induced MM cell apoptosis is predominantly mediated by caspase-8; and (2) Bortezomib-induced apoptosis requires both caspase-8 and caspase-9 activation.

We next determined whether inhibition of an upstream signaling pathway that leads to caspase-8 activation affects the response to NPI-0052 or Bortezomib. The Fas-associated death domain (FADD) protein is an essential part of the death-inducing signaling complexes (DISCs) that assemble upon engagement of TNF receptor family members, such as Fas (Strasser et al., 2000), resulting in proteolytic processing and autoactivation of pro-caspase-8 (Strasser et al., 2000). Since both NPI-0052 and Bortezomib trigger caspase-8 activation, we examined the role of FADD during this event in MM cells using DN-FADD. Blockade of FADD with DN-FADD significantly

attenuated NPI-0052-induced cytotoxicity compared to the empty vector-transfected MM.1S cells ( $42\% \pm 2.0\%$  viable cells in vector-transfected cells versus  $76\% \pm 5.1\%$  viable cells in DN-FADD-transfected cells;  $p < 0.05$ ) (Figure 7D). DN-FADD decreases NPI-0052-induced caspase-8 activation; however, minimal caspase-8 activation is still noted (data not shown), which may be due to upstream activators of caspase-8 other than FADD. Importantly, treatment of DN-FADD-transfected MM.1S cells with Bortezomib results in only a 16% increase in survival compared to vector-transfected cells ( $39\% \pm 2.4\%$  viable cells in vector-transfected cells versus  $55\% \pm 4.1\%$  viable cells in DN-FADD-transfected cells;  $p < 0.05$ ) (Figure 7D). These data, coupled with caspase-8 or caspase-9 inhibition studies, suggest that NPI-0052 relies more on FADD-caspase-8 signaling axis than does Bortezomib, further confirming differential mechanism of action of NPI-0052 versus Bortezomib in MM cells.

To further address this issue, we examined the alterations in Bax, a proapoptotic protein that translocates from cytosol to mitochondria during apoptosis, inhibits Bcl-2, and facilitates



**Figure 7.** Role of caspase-8 versus caspase-9 during NPI-0052- and Bortezomib-induced apoptosis

**A:** MM.1S cells were treated for 24 hr with NPI-0052 (10 nM) or Bortezomib (10 nM) in the presence or absence of pan-caspase, caspase-8, or caspase-9 inhibitors and then assessed for viability. Shown is mean  $\pm$  SD (error bars) of four independent experiments ( $p < 0.004$ ).

**B:** MM.1S cells transfected with vector alone, DN-caspase-8 (Cas.8), or DN-Cas.9 were treated for 48 hr with NPI-0052 (7 nM) or Bortezomib (5 nM) and then analyzed for cell viability.

**C:** Control for functional specificity of DN-Cas.9 and DN-Cas.8. MM.1S cells were transiently transfected with vector alone, DN-Cas.9, or DN-Cas.8 and treated for 24 hr with either Dexamethasone (Dex; 5  $\mu$ M) (upper panel) or anti-Fas MoAb (lower panels). Cytosolic extracts were analyzed by immunoblotting with anti-Cas.9, anti-Cleaved Cas.8, or anti-tubulin Abs. Blots shown are representative of two independent experiments.

**D:** Vector- or DN-FADD-transfected MM.1S cells were treated for 48 hr with NPI-0052 (7 nM) or Bortezomib (5 nM) and then analyzed for cell viability.

**E:** Mitochondrial protein extracts from NPI-0052- or Bortezomib-treated MM.1S cells were subjected to immunoblot analysis with anti-Bax (upper panel) or anti-Hsp60 (lower panel) Abs.

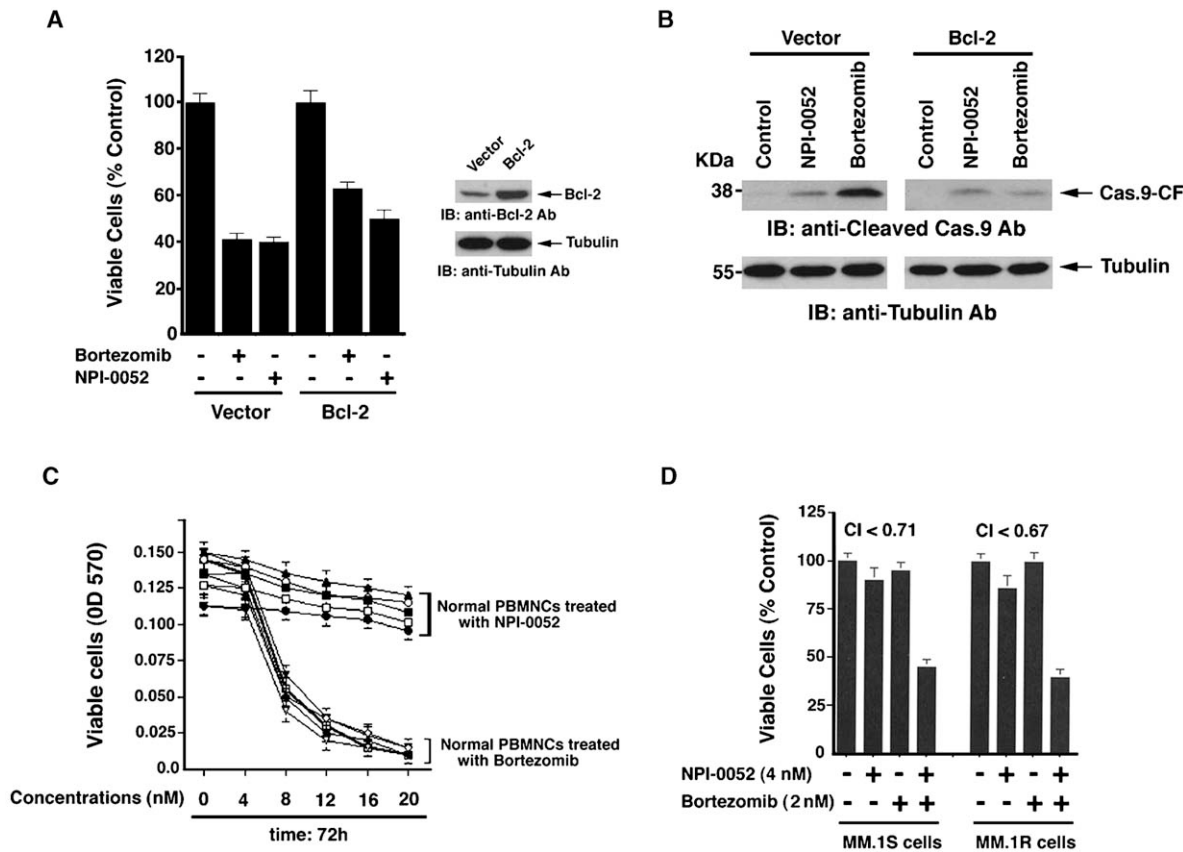
**F:** The MEFs Bax and Bak (wild-type or double knockouts) were treated with NPI-0052 or Bortezomib and analyzed for viability. Data represent mean  $\pm$  SD (error bars) of three independent experiments ( $p < 0.05$ ).

release of cyto-c and activation of caspase-9 (Guo et al., 2003; Wei et al., 2001). NPI-0052 induces little, if any, increase in Bax levels in mitochondria, whereas Bortezomib triggers a significant accumulation of Bax in mitochondria (Figure 7E, upper panel). Previous studies showed that oligomerization of Bax with another proapoptotic Bcl-2 family member, Bak, facilitates release of cyto-c from mitochondria to the cytosol, resulting in caspase-9 activation and cell death (Wei et al., 2001). Reports in Bax/Bak knockout mice showed that these proteins are required for mitochondria-mediated cell death (Wei et al., 2001). We therefore examined the effects of Bortezomib and NPI-0052 on DKO mouse embryonic fibroblasts lacking both Bax and Bak. Deletion of Bax and Bak markedly inhibits Bortezomib- but not NPI-0052-mediated cytotoxicity (Figure 7F). These findings indicate that, in contrast to NPI-0052-, Bortezomib-mediated cell death requires mitochondrial apoptotic signaling via Bax and Bak.

A recent study showed that Bax and Bak can also localize in the endoplasmic reticulum (ER) and that ER stress can trigger caspase-12 activation (Zong et al., 2003). Bortezomib induces ER stress (Mitsiades et al., 2002b) and activates ER-resident caspase-12 in MM cells (Landowski et al., 2005). Another study showed that caspase-12 and caspase-4 are not required for caspase-dependent ER stress-induced apoptosis (Obeng and Boise, 2005). Whether caspase-12 or caspase-4 has an obligate role during NPI-0052-triggered cell death remains to be examined.

#### Differential effects of NPI-0052 and Bortezomib on Bcl-2-overexpressing MM cells

As noted above, during apoptosis Bax neutralizes the anti-apoptotic function of Bcl-2, thereby facilitating the cyto-c release and caspase-9 activation (Rathmell and Thompson, 2002; Willis et al., 2003). Bcl-2 also confers drug resistance in



**Figure 8.** NPI-0052 decreases survival in Bcl-2-overexpressing MM.1S cells

**A:** Left panel: empty vector- or Bcl-2-transfected MM.1S cells were treated for 48 hr with NPI-0052 (10 nM) or Bortezomib (10 nM) and then analyzed for viability. Results are mean  $\pm$  SD (error bars) of three independent experiments. Right panel: Bcl-2 protein levels in MM.1S cells transfected with Bcl-2 or empty (neo) vector.

**B:** Vector- or Bcl-2-transfected MM.1S cells were treated for 48 hr with NPI-0052 (7 nM) or Bortezomib (5 nM), and cytosolic extracts were analyzed by immunoblotting with anti-Cleaved Cas.9 or anti-tubulin Abs. Blots shown are representative of two independent experiments.

**C:** Differential cytotoxicity of NPI-0052 and Bortezomib against lymphocytes from healthy donors. Normal lymphocytes from five healthy donors were treated with indicated concentrations of NPI-0052 or Bortezomib and then analyzed for viability. Data are mean  $\pm$  SD (error bars) of three independent experiments ( $p = 0.27$  from Jonckheere-Terpstra test for trend).

**D:** NPI-0052 and Bortezomib trigger synergistic anti-MM activity. Low doses of NPI-0052 and Bortezomib trigger synergistic anti-MM activity in MM cells. MM.1S and MM.1R cells were treated for 24 hr with indicated concentrations of NPI-0052, Bortezomib, or NPI-0052 + Bortezomib and then assessed for viability. Shown is mean  $\pm$  SD (error bars) of three independent experiments ( $p < 0.005$ ). Combination index (CI) of  $< 1$  indicates synergy.

cancer cells, including MM (Hideshima and Anderson, 2002), and provides partial protection against Bortezomib-induced killing (Mitsiades et al., 2002b). We therefore next asked whether ectopic expression of Bcl-2 in MM.1S cells affects the ability of NPI-0052 or Bortezomib to trigger cytotoxicity and postmitochondrial apoptotic signaling in MM cells. Overexpression of Bcl-2 promotes a modest increase in viability of cells treated with both agents: for NPI-0052,  $50\% \pm 2.6\%$  viability in Bcl-2-transfected cells versus  $39\% \pm 1.5\%$  viability in vector-transfected cells ( $p < 0.05$ ); and for Bortezomib,  $61\% \pm 2.9\%$  viability in Bcl-2-transfected cells versus  $40\% \pm 2.1\%$  viability in vector-transfected cells ( $p < 0.05$ ) (Figure 8A). Importantly, the increased survival of Bcl-2 transfectants in response to Bortezomib was greater (21%) than that in response to NPI-0052 (11%) ( $p < 0.04$ ;  $n = 3$ ) (Figure 8A). Moreover, Bortezomib triggers significant caspase-9 cleavage in control vector-transfected cells, which is markedly attenuated (3-fold decrease by

densitometry) in Bcl-2-transfected cells; in contrast, NPI-0052-induced caspase-9 cleavage is minimally affected by Bcl-2 overexpression (Figure 8B). These findings, together with the viability results, suggest that Bcl-2 provides more protection against Bortezomib than NPI-0052.

#### NPI-0052 and Bortezomib have differential effects on normal lymphocytes

Our data demonstrate that NPI-0052 and Bortezomib proteasome inhibitors have differential mechanisms of action. It is also possible, due to differences in their chemical structure, specificity, or signaling mechanisms, that these agents may have different cytotoxic activities against normal cells. To address this issue, we compared the effects of NPI-0052 and Bortezomib on normal lymphocytes. NPI-0052 does not significantly decrease viability of normal lymphocytes ( $p = 0.27$  from Jonckheere-Terpstra [J-T] trend test) even at the increased

doses (20 nM) (Figure 8C). Higher concentrations of NPI-0052 at 50 and 100 nM decrease the viability of lymphocytes by 25% and 50%, respectively. These data suggest that normal cells are not completely refractory to NPI-0052. In contrast, Bortezomib significantly decreases the survival of lymphocytes even at the low concentrations of 6–10 nM (Figure 8C), as is observed in clinical studies (Adams, 2002; Richardson, 2004). Importantly, the  $IC_{50}$  for patient MM cells of NPI-0052 or Bortezomib is at concentrations that do not affect viability of normal lymphocytes; however, dose escalation of each agent suggests a larger therapeutic index for NPI-0052 than for Bortezomib. Moreover, a 50% decrease in viability of normal CCD-27sk fibroblasts is observed at  $317 \pm 17$  nM NPI-0052 versus  $15 \pm 3$  nM Bortezomib ( $p < 0.05$ ), suggesting that NPI-0052 has significantly reduced cytotoxicity against normal cells than Bortezomib.

We further examined whether NPI-0052 or Bortezomib alters proteasome activity in normal lymphocytes and skin fibroblasts. Both NPI-0052 and Bortezomib significantly inhibit proteasome activity in these cells: 20 nM NPI-0052 or Bortezomib triggers 99% or  $59 \pm 11\%$  inhibition of CT-L proteasome activity, respectively (data not shown). Thus, although 20 nM NPI-0052 does not trigger significant cytotoxicity in normal lymphocytes, it reduces CT-L proteasome activity in these cells. Similarly, treatment of normal CCD-27sk fibroblasts at the  $IC_{50}$  for NPI-0052 (317 nM) or Bortezomib (15 nM) also inhibits proteasome activity (data not shown). Bortezomib inhibits 20S proteasome activity in murine WBCs at 1 hr post-i.v. injections (Adams et al., 1999). Furthermore, these data are consistent with the clinical observation showing a similar degree of proteasome inhibition in blood from responders versus nonresponders to Bortezomib therapy (Adams, 2002; Richardson, 2004). Nonetheless, these findings demonstrate a selective anti-MM activity of NPI-0052.

### Combined Bortezomib and NPI-0052 treatment triggers synergistic apoptosis in MM cells

The proteasome inhibitors Bortezomib and NPI-0052 are distinct, providing the rationale for combining these agents to enhance anti-MM activity. MM.1S or MM.1R MM cells were treated with both NPI-0052 and Bortezomib simultaneously across a range of concentrations and analyzed for viability. Results demonstrate that NPI-0052 and Bortezomib induce synergistic cytotoxicity (Figure 8D; shown are the representative results from minimally toxic and maximally synergistic concentrations of each agent). Isobologram analysis confirmed synergistic anti-MM activity of Bortezomib with NPI-0052 (combination index  $< 1.0$ ). Importantly, combining low doses of these two agents does not significantly affect the viability of normal lymphocytes (Figure S2). While the definitive demonstration of decreased toxicity of combination therapy awaits results of careful clinical trials, the synergy observed in vitro may allow for use of lower doses and decreased toxicity.

The mechanisms mediating the enhanced cytotoxicity of the combination may simply reflect higher levels of proteasome inhibition with the two-drug regimen, and our ongoing studies are examining this issue in MM animal models using various parameters to determine the proteasome activity profiles of combination therapy, such as drug route, dose, and sequence of administration. Nonetheless, our present study shows that these two agents trigger differential apoptotic signaling path-

ways, which may account for the enhanced cytotoxicity upon combined treatment. Activation of different apoptotic signaling cascades provides the basis for combining two drugs to enhance cell death (Chauhan and Anderson, 2003). Another recent study also showed that Bortezomib sensitizes to TRAIL-induced apoptosis in genitourinary cancer cells via p21 accumulation and enhanced caspase-8 activation (Lashinger et al., 2005). Combination therapy with Bortezomib and NPI-0052 therefore may (1) allow use of subtoxic concentrations of each agent and (2) permit escalating synergistic doses of these agents to increase the apoptotic threshold, thereby enhancing anti-MM activity.

Collectively, our study shows the following: (1) a novel proteasome inhibitor, NPI-0052, inhibits proteasome activity both in vitro and in vivo at pharmacologically achievable concentrations and exhibits a different proteasome inhibition profile than Bortezomib; (2) NPI-0052 is a more potent inhibitor of NF- $\kappa$ B and related cytokine secretion than Bortezomib; (3) NPI-0052 induces apoptosis in MM cells resistant to conventional and Bortezomib therapies, without affecting normal lymphocyte viability; (4) NPI-0052 does not affect viability of BMSCs; (5) NPI-0052 induces MM cell apoptosis even in the presence of MM growth factors, such as IL-6 or IGF-1; (6) NPI-0052 blocks VEGF-induced migration of MM cells, confirming its antiangiogenic activity; (7) NPI-0052 overcomes drug resistance conferred by the antiapoptotic protein Bcl-2; (8) NPI-0052-induced apoptosis in MM cells is associated with loss of  $\Delta\Psi_m$ , increase in  $O_2^-$  production, release of cyto-c/Smac, and activation of caspase-8, caspase-9, and caspase-3; (9) both biochemical and genetic evidence indicate that NPI-0052, in contrast to Bortezomib, relies more on the FADD-caspase-8-mediated cell death signaling pathway; (10) combinations of low doses of NPI-0052 and Bortezomib trigger synergistic anti-MM activity; (11) NPI-0052 inhibits MM tumor growth in vivo, as well as prolongs survival without reoccurrence of tumor in 57% mice; and finally, (12) NPI-0052 is orally bioactive. Together, these findings provide the framework for clinical trials of NPI-0052, either alone or in combination with Bortezomib, to enhance clinical efficacy, reduce toxicity, and overcome drug resistance in patients with relapsed/refractory MM.

### Experimental procedures

#### In vitro and in vivo proteasome activity assays

Proteasome activity assays were performed using purified human erythrocyte-derived 20S proteasomes, as previously described (Lightcap et al., 2000). In vivo comparative analysis of proteasome activities were performed as follows. Single i.v. administration of NPI-0052: NPI-0052 was dissolved in 100% DMSO and serially diluted with 5% Solutol (Solutol HS 15; polyethylene glycol 660 12-hydroxystearate; BASF, Shreveport, LA), yielding a final concentration of 2% DMSO. The vehicle control consisted of 2% DMSO and 98% (5% Solutol). Male Swiss-Webster mice ( $n = 5$ ) were treated with a single dose of NPI-0052 (0.15 mg/kg) or Bortezomib (1 mg/kg); blood samples were collected at 90 min, 24 hr, 48 hr, 72 hr, or 168 hr; and whole blood cells were then analyzed for proteasome activity. Further details are found in the Supplemental Data.

#### Assaying proteasome activity by immunoblotting

The method has been described previously (Berkers et al., 2005). Briefly, the principle is as follows: DansylAhx<sub>3</sub>L<sub>3</sub>VS is a proteasome inhibitor that covalently modifies all active proteasome subunits with comparable affinity (Bogyo et al., 1998; Kessler et al., 2001). This proteasome probe is equipped with a dansyl-sulfonamido-hexanoyl hapten and can be visualized by immunoblotting using polyclonal antibodies against the dansyl moiety.

In the assay, sites that are not targeted by NPI-0052 or Bortezomib are labeled by DansylAhx<sub>3</sub>L<sub>3</sub>VS and visualized on Western blot, while sites that are targeted by NPI-0052 or Bortezomib cannot be seen. Equal amounts of protein (60 µg) from Bortezomib- or NPI-0052-treated MM.1S cells were incubated with 0.8 µM DansylAhx<sub>3</sub>L<sub>3</sub>VS at 37°C and subjected to immunoblotting using an anti-dansyl-sulfonamidohexanoyl polyclonal Ab (1:500; rabbit; Molecular Probes, Carlsbad, CA).

#### Human plasmacytoma xenograft models

All experiments involving animals were approved by an institutional Animal Care and Use Committee. The xenograft tumor formation was performed as previously described (LeBlanc et al., 2002). Briefly, mice (n = 7/group) were inoculated subcutaneously in the flank with 3 × 10<sup>7</sup> MM.1S MM cells in 100 µl of RPMI-1640 media. NPI-0052 treatment was started after the development of measurable tumor (120–180 mm<sup>3</sup> size). NPI-0052 (0.25 mg/kg or 0.5 mg/kg) was given orally twice a week. For comparative studies, mice were treated by the i.v. route with NPI-0052 (0.15 mg/kg) or Bortezomib (1 mg/kg) twice a week. Tumor measurements were taken on alternate days with a caliper, and tumor volume was calculated according to the equation 1/2 (length [mm] × width [mm])<sup>2</sup>. Animals were sacrificed if the tumor was ≥2 cm or exhibited visible signs of necrosis.

#### Expression vectors and transfections

MM.1S cells were transiently transfected using Cell line Nucleofecto kit V, according to the manufacturer's instructions (Amaxa Biosystems, Germany), with vector alone, DN-caspase-8, DN-caspase-9, or DN-FADD and cotransfected with vector containing green fluorescence protein (GFP) alone. Following transfections, GFP-positive cells were selected by flow cytometry, treated with NPI-0052 or Bortezomib, and analyzed for viability.

#### Statistical analysis

Nonparametric tests and mixed models were used to analyze the data. This includes the Wilcoxon signed rank test to compare proliferation in untreated and treated patient cells and the J-T trend test for measuring the viability of lymphocytes and cell lines resistant to conventional therapy. Wilcoxon rank-sum test was used for measuring change in the tumor volume, the Kaplan-Meier method was used for survival analysis, and log-rank analysis was used for statistical significance.

#### Isobologram analysis

Isobologram analysis was performed using the CalcuSyn software program (Biosoft, Ferguson, MO and Cambridge, UK), and a combination index (CI) of <1.0 indicates synergism (Chou and Talalay, 1984).

#### Supplemental data

Information on MM cell lines and culture medium, reagents, purification of CD138-positive MM patient BM samples, PBMC preparation from normal healthy donors, cytotoxicity and apoptosis assays, transwell migration assays, protein extraction, mitochondrial assays, immunoblotting, and antibodies can be found in the Supplemental Data. The Supplemental Data include two supplemental figures as well as the [Supplemental Experimental Procedures](#) and can be found with this article online at <http://www.cancerell.org/cgi/content/full/8/5/407/DC1/>.

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