Combination of Proteasome Inhibitors Bortezomib and NPI-0052 Trigger in vivo Synergistic Cytotoxicity in Multiple Myeloma

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Running title: Proteasome inhibitors as myeloma therapy

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Abstract

Our recent study demonstrated that a novel proteasome inhibitor NPI-0052 triggers apoptosis in multiple myeloma (MM) cells; and importantly, is distinct from bortezomib (Velcade™) in its chemical structure, effects on proteasome activities, and mechanisms of action. Here, we demonstrate that combining NPI-0052 and bortezomib induces synergistic anti-MM activity both in vitro using MM cell lines or patient CD138+ MM cells and in vivo in a human plasmacytoma xenograft mouse model. NPI-0052 + bortezomib-induced synergistic apoptosis is associated with: 1) activation of caspase-8, caspase-9, caspase-3, and PARP; 2) induction of ER-stress response and JNK; 3) inhibition of migration of MM cells and angiogenesis; 4) suppression of chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) proteolytic activities; and 5) blockade of NF-κB signaling. Studies in a xenograft model show that low dose combination of NPI-0052 and bortezomib is well tolerated, triggers synergistic inhibition of tumor growth, and CT-L, C-L and T-L proteasome activities in tumor cells. Immununostaining of MM tumors from NPI-0052 + bortezomib-treated mice showed growth inhibition, apoptosis, and a decrease in associated angiogenesis. Taken together, our study provides the preclinical rationale for clinical protocols evaluating bortezomib together with NPI-0052 to improve patient outcome in MM.
Introduction

Normal cellular homeostasis requires balanced regulation of protein synthesis and degradation. Intracellular protein degradation occurs majorly via a multi-subunit complex called the proteasome \[^1-4\]. Earlier studies by Ciechanover, Hershko and Rose et al., demonstrated that ATP-dependent conjugation of proteins with polypeptide (ubiquitin) mediates protein degradation. \[^5-11\]

The 26S multi-subunit proteasome complex \[^12-15\] containing 19S units flanking a barrel-shaped 20S proteasome core \[^16-18\]. The 19S units of the 26S proteasome complex regulate entry of ubiquitinated proteins into the 20S core chamber \[^2,19,20\]. Protein ubiquitination is facilitated through several enzymatic reactions involving E1 and E2 ubiquitin enzymes as well as E3 ubiquitin ligase. \[^21,22\] Once the ubiquitinated proteins are recognized by the 19S regulatory subunits of the proteasome complex, they are degraded into small peptides by three major proteasomal activities residing within the 20S core complex i.e., chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) activities \[^23-26\].

Proteasomes regulate many normal cellular processes, such as cell cycle, inflammation, transcription, DNA replication, and apoptosis via proteolysis of key enzymes and regulatory proteins. Deregulation of the Ubiquitin-Proteasome Signaling (UPS) \[^27\] pathway is linked to the pathogenesis of various human diseases, \[^4,24,29\] and therefore proteasome inhibitors offer great promise as therapeutic agents.
The dipeptidyl boronic acid bortezomib/PS-341 (Velcade\textsuperscript{TM}) is a reversible inhibitor of CT-L\textsuperscript{[30]} exhibited remarkable anti-tumor activity against the 60 NCI tumor cell line panel. Bortezomib is the first in class proteasome inhibitor, approved by FDA for the treatment of relapsed and relapsed/refractory MM and Mantle cell lymphoma.\textsuperscript{[4,31-33]} Even though bortezomib therapy is a major advance (43% objective response rates)\textsuperscript{[32,33]}, it has been associated with possible off-target toxicities and the development of drug-resistance.\textsuperscript{[34-38]}

Our recent study\textsuperscript{[39]} characterized a novel proteasome inhibitor NPI-0052, a small molecule derived from the fermentation of a marine gram-positive actinomycete Salinospora tropica.\textsuperscript{[40]} NPI-0052 induces apoptosis in MM cells resistant to conventional and bortezomib therapies, without significantly affecting normal lymphocyte viability.\textsuperscript{[39]} Importantly, NPI-0052 is distinct from bortezomib in its chemical structure, effects on proteasome inhibition profiles and mechanisms of action.\textsuperscript{[39]} For example, biochemical and genetic studies showed that NPI-0052, in contrast to bortezomib, relies more on FADD-caspase-8-mediated cell death signaling in MM cells. In vivo studies using human MM-xenografts show that NPI-0052 is well tolerated, prolongs survival, and reduces tumor recurrence.\textsuperscript{[39]} Similar anti-tumor activity of NPI-0052 has been reported in CLL and colon cancer cells.\textsuperscript{[41,42]} Our preclinical data provided the basis for the ongoing phase-I clinical trial of NPI-0052 in relapsed/refractory MM patients.
A recent series of elegant studies using an in vitro protein model system demonstrate that simultaneous inhibition of multiple proteasome activities is a prerequisite for significant (i.e., > 50%) proteolysis. Moreover, MM and leukemia cell lines exhibit a differential pattern of qualitative and quantitative constitutive proteasome activity, suggesting a differential requirement to inhibit proteasome activity. Our prior study showed that bortezomib predominantly inhibits proteasome CT-L and more recently defined C-L activities. A recent study showed that the epoxyketone-based irreversible proteasome inhibitor PR-171, like bortezomib, predominantly blocks CT-L activity. Importantly, NPI-0052 blocks all three i.e., CT-L, T-L, and C-L proteasome activities. It is therefore likely that NPI-0052, by virtue of its ability to block all three proteasome activities, can be combined with bortezomib or PR-171 to confer a broader proteasome inhibition at lower and potentially, safer doses. These findings provide the rationale for combining NPI-0052 with bortezomib to achieve optimal proteasome inhibition and potent anti-tumor activity, while allowing for the use of lower doses of bortezomib to reduced toxicity.

In the present study, we characterized the effects of NPI-0052 and bortezomib combinations against MM cell lines and primary patient cells resistant to conventional and novel therapies. Both in vitro and in an in vivo MM xenograft model, combined NPI-0052 and bortezomib inhibits growth of MM cells and overcomes drug resistance, setting the stage for potential
clinical trials of combination therapy to improve patient outcome in MM.

**Material and Methods**

**Cell culture** MM.1S (Dexamethasone-(Dex) sensitive), MM.1R (Dex-resistant), RPMI-8226, Doxorubicin (Dox)-resistant (Dox-40), U266, OPM2 and INA6-6 (IL-6-dependent) human MM cell lines were maintained as previously described. MM cells were freshly isolated from patients relapsing after multiple prior therapies including Dex, Melphalan, Thalidomide or bortezomib. Tumor cells were purified by CD138 positive selection using the Auto MACS magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA). Informed consent was obtained from all patients in accordance with the Helsinki protocol. Cells were treated with NPI-0052 (Nereus Pharmaceuticals, Inc., San Diego, CA), and bortezomib (Millennium Pharmaceuticals, Cambridge, MA). PBMNCs from normal healthy donors were maintained in culture medium.

**Cell viability and apoptosis assays** Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, CA) assay, as previously described. Cell death was quantified using ‘Live and dead cell assay’ (Molecular Probes, Carlsbad, CA). Briefly, 1 x 10^6 cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or their combination; stained with ethidium homodimer and calcein-AM; and then analyzed under a fluorescence microscope. Annexin V/Propidium iodide (PI) staining assays were
performed as previously described.  TUNEL apoptosis detection kit (Upstate/Millipore, Billerica, MA) was utilized to measure apoptosis in murine tumor sections.

**In vitro migration and capillary-like tube structure formation assays** Migration was assessed by Transwell Boyden chamber (Chemicon, Billerica, MA), as previously described.  In vitro angiogenesis was determined by Matrigel capillary-like tube structure formation assay.  

**Western blotting** Immunoblot analysis was performed using antibodies to Caspase-8, Caspase-9, Caspase-3 (Cell Signaling, Beverly, MA), PARP, Bcl-2, BIM, Hsp-27, Hsp-70, Hsp-90, CHOP, phospho-eIF2-α, phospho-JNK, Actin or Tubulin (BD Bioscience Pharmingen, San Diego, CA).  Blots were then developed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

**In vitro and in vivo proteasome activity assays** 20S proteasome activity assays were performed using fluorogenic peptide substrates, as previously described.  In vivo comparative analysis of proteasome activities was performed in tumors from treated mice.  Ninety minutes after administration of drugs, the animals were anesthetized and tumors were excised, followed by determination of ex vivo proteasome activity, as detailed above.

**Human plasmacytoma xenograft model** All animal studies were approved by DFCI Institutional Animal Care and Use Committee.  The xenograft tumor model was performed as previously described.  CB-17 SCID-mice (n = 24) (Taconic, Gemantown, NY) were subcutaneously inoculated with 5.0 x 10^6 MM.1S cells in 100 µl of...
serum free RPMI-1640 medium. When tumors were measurable approximately three weeks after MM cell injection, mice were treated with vehicle or drugs at various doses on a twice a week schedule for four consecutive weeks. Tumor size was measured every third day in two dimensions using calipers; tumor volume was calculated using the formula: $V = 0.5 \times a \times b^2$, where $a$ and $b$ are the long and short diameter of the tumor, respectively. Animals were sacrificed when their tumors reached 2 cm$^3$.

**In situ detection of apoptosis, immunohistochemical (IHC) determination of proliferation, and assessment of microvessel density (MVD)** Apoptotic cells in xenografted MM tumors were identified by TUNEL assays, using the ApopTag in situ apoptosis detection system (Intergen, NY) and IHC staining for caspase-3 activation and H&E staining. Ki-67 was determined by automated IHC staining to quantify proliferation. MVD, a marker for tumor angiogenesis, was quantified by IHC staining of factor VIII. VEGFR1/FLT-1 expression was examined by IHC staining with specific VEGFR1 Abs (Abcam, Cambridge, MA), as previously described.

**Statistical Analysis** Wilcoxon's signed rank test was performed to compare proliferation in untreated and treated patient cells, and the Jonckheere-Terpstra (J-T) trend test was used to measure viability of lymphocytes and cell lines. Statistical significance of differences observed in NPI-0052, bortezomib or NPI-0052 + bortezomib-treated mice compared with control groups was determined using a Student $t$ test. The minimal level of significance was $P < 0.05$. Tumor growth
inhibition was determined using the SigmaPlot analysis software. Isobologram analysis was performed using “CalcuSyn” software program (Biosoft, Ferguson, MO and Cambridge, UK). A combination index (CI) < 1.0 indicates synergism, and CI = 1 indicates additive activity. [52]

Results

Combined low doses of NPI-0052 and bortezomib trigger synergistic anti-MM activity For these studies, we utilized NPI-0052 and bortezomib at concentrations lower than their respective IC₅₀ for each cell line. MM.1S, U266, RPMI-8226, INA-6, OPM-2, Dox-40 and MM.1R cells were treated for 24h with low doses of NPI-0052, bortezomib or the combination and analyzed for viability by MTT assay. A more significant decrease in viability of all cell lines was noted in response to treatment with combined low doses of NPI-0052 and bortezomib than with either agent alone (P < 0.05; n = 3) (Fig 1A-1G). Isobologram analysis confirmed that combined NPI-0052 + bortezomib triggered synergistic anti-MM activity in MM cell lines, albeit with differential kinetics. The combination ratio of agents varied for each cell line to achieve a similar degree of cell death. Shown in Fig 1 are representative results from minimally toxic and maximally additive concentrations of each agent: for example, treatment of U266 MM cells with low doses of 7 nM NPI-0052 (IC₅₀: 24 nM) and 1 nM bortezomib (IC₅₀: 30 nM) triggers 43% growth inhibition, whereas no significant growth inhibition was observed using either of these agents alone at these low concentrations.
(Fig 1B). These data confirm and extend our prior findings demonstrating synergistic anti-MM activity of NPI-0052 + bortezomib.

We next examined whether NPI-0052 + bortezomib-induced decrease in viability is due to apoptosis. Combined NPI-0052 + bortezomib, but not either agent alone, triggered significant apoptosis in MM.1S cells, as determined by Annexin V/PI staining assays. (P < 0.05, n=3) (Fig 1H). Assessment of cell death by “Live and dead cell” assays demonstrated similar results (Fig 1I).

To determine whether NPI-0052 + bortezomib similarly affect purified patient MM cells, tumor cells from five MM patients relapsing after multiple prior therapies including thalidomide (Pt# 1), bortezomib (Pt# 2 and 3), and Dex/Melphalan (Pt# 4 and 5) were treated for 24h with NPI-0052 (1 nM) + bortezomib (3 nM), and then analyzed for viability. A significant decrease in viability of all patient MM cells was noted (P < 0.005) (Fig 2A). Of note, two of five patients studied were refractory to bortezomib therapy, and two were resistant to Dex and Melphalan therapies. Patients were considered refractory to bortezomib therapy when they have relapsed within six months after or progressed on bortezomib therapy. NPI-0052 + bortezomib-triggered decrease in viability of MM patient cells was due to apoptosis, as evidenced by DNA fragmentation (data not shown). Taken together, these findings suggest that NPI-0052 + bortezomib induce synergistic anti-MM activity and overcome drug resistance in both MM cell lines and purified patient MM cells.
Importantly, the low dose combination of NPI-0052 (1 nM) + bortezomib (3 nM) did not trigger significant decrease in viability of normal PBMNCs (P = 0.22 from J-T trend test) or PHA/IL-2-stimulated normal PBMCs (Fig 2A). Higher concentrations of bortezomib (10 nM), but not of NPI-0052 (10 nM), induced a modest (20%) decrease in normal cell viability, as previously reported. 

Combination of NPI-0052 and bortezomib trigger apoptosis in leukemic (Molm-14), lymphoma (SUDHL-4), pancreatic (Panc-1) and breast cancer (MCF-7) cells As seen in Fig 2B, NPI-0052 + bortezomib decreased the viability of Molm-14, SUDHL-4, Panc-1 and MCF-7 cells. The decrease in viability was due to apoptosis, as determined by Annexin V/PI staining and flow cytometry. The percentage of apoptotic cell population (Annex V+/PI-) in each case were as follows: For Molm-14- NPI-0052 alone=7.2%, bortezomib alone=10.5% and NPI-0052 + bortezomib=26.7%; For SUDHL-4 cells- NPI-0052 alone=5.5%, bortezomib alone=4.8% and NPI-0052 + bortezomib=20.9%; For Panc-1 cells- NPI-0052 alone=10.9%, bortezomib alone=9.8% and NPI-0052 + bortezomib=25.6%; and finally, for MCF-7 cells- NPI-0052 alone=8.3%, bortezomib alone=13.4%, NPI-0052 + bortezomib=32.8% (CI < 1 in all cell lines; n=3).

Combined low doses of NPI-0052 and bortezomib blocks both migration of MM cells and angiogenesis We and others have shown that migration and angiogenesis play an important role in progression of MM. The effect of NPI-0052 + bortezomib was examined on these events using Transwell insert systems and in
vitro tubule formation assays. Serum alone markedly increased MM.1S cell migration; importantly, NPI-0052 (1 nM) + bortezomib (3 nM) significantly inhibit serum-dependent MM.1S cell migration, as reflected by a decrease in the number of crystal violet-stained cells (Fig 3A). These results were further confirmed by quantification of cell migration in response to treatment with serum (58 X 10^4) versus NPI-0052 + bortezomib (12 X 10^4) (P < 0.05, n=2). No significant inhibition of migration occurred in cells treated with low doses of either agent alone (Fig 3A). NPI-0052 + bortezomib at the concentrations tested in the migration assays did not affect survival of MM cells (viability > 95%). Similarly, combination of NPI-0052 + bortezomib, but not either agent alone, significantly blocked rVEGF-induced migration of MM.1S cells (Fig 3B; P < 0.05, n = 3).

We next utilized in vitro capillary-like tube structure formation assays to determine whether NPI-0052 + bortezomib triggers anti-angiogenic effects. In vitro angiogenesis was measured using Matrigel capillary-like tube structure formation assays: human vascular endothelial cells (HUVECs) seeded onto Matrigel differentiate and form capillary-like tube structures similar to in vivo neovascularization, dependent upon cell-matrix interaction, cellular communication, and cellular motility. This assay therefore provides evidence for anti-angiogenic effects of drugs/agents. HUVECs were seeded in 96-well culture plates precoated with Matrigel; treated with vehicle (DMSO), NPI-0052 (1 nM), bortezomib (3 nM), or NPI-0052 (1 nM) + bortezomib (3 nM) for 8 h; and then examined for tube formation using an
inverted microscope. As seen in Fig 3C, tubule formation was markedly decreased in the NPI-0052 + bortezomib-treated cells, but not after treatment with either agent alone. In order to clearly demonstrate the magnitude of changes in tubule formation, we compared the effects of NPI-0052 (1 nM) + bortezomib (3 nM) with a known anti-angiogenic agent Revlimid (5 μM). Our data show that combination of low doses of bortezomib + NPI-0052 trigger more potent inhibition of in vitro capillary-like tube formation than Revlimid alone (Fig 3D, P = 0.021 for NPI-0052 + bortezomib- versus Revlimid-treated cells; n = 2). Taken together, these findings suggest that combination of low doses of NPI-0052 and bortezomib block migration and angiogenesis.

Mechanisms mediating anti-MM activity of NPI-0052 + bortezomib Our earlier studies showed that higher doses of NPI-0052 (7-24 nM) and bortezomib (5 nM-15 nM) efficiently triggered both intrinsic and extrinsic cell death pathways in various MM cell lines. Here we asked whether the combination of low concentrations of each agent retain the ability to induce extrinsic and/or intrinsic apoptotic signaling pathways. Our results show that NPI-0052 (1 nM) + bortezomib (3 nM), but not NPI-0052 (1 nM) or bortezomib (3 nM) alone, induces activation of caspase-9 (intrinsic), caspase-8 (extrinsic), caspase-3, followed by cleavage of PARP, a hallmark of apoptosis (Fig 4A). Importantly, pretreatment of MM.1S cells with the pan-caspase inhibitor (z-VAD-fmk) significantly blocked NPI-0052 + bortezomib-induced apoptosis (P < 0.05, n = 3) (Fig 4B).

Prior studies have established that BH3-only Bcl-2 family
protein BIM regulates caspase-9 activation by modulating Cyto-
c/Smac release from mitochondria to the cytosol. Our data show that combined NPI-0052 + bortezomib, but not either agent alone, significantly upregulates BIM protein levels (3 fold increase) without altering Bcl-2 protein levels (Fig 4C). To determine whether BIM mediates NPI-0052 + bortezomib-induced apoptosis, we knocked down BIM expression using siRNA strategy. The functional specificity of BIM siRNA was evident from a marked decrease in protein levels of all three isoforms of BIM (Fig 4D). Importantly, transfection of siRNA BIM, but not negative-control (scrambled) siRNA, significantly inhibits NPI-0052 + bortezomib-induced apoptosis in MM.1S cells (Fig 4E). These findings suggest that NPI-0052 + bortezomib-triggered apoptosis is mediated, at least in part, via BIM.

Stress-induced apoptosis is associated with activation of c-
Jun NH(2)-terminal kinase (JNK/SAPK). As seen in Fig 5A, treatment of MM.1S cells with low concentrations of NPI-0052 (1 nM) + bortezomib (3 nM) for 12h triggered JNK activation. The extent of JNK activation was similar to that observed with IC_{50} concentrations of each agent at 24h (7 nM of NPI-0052 or 5 nM for bortezomib). We also compared JNK activation at concentrations equivalent to the combined doses i.e., 1 nM of NPI-0052 + 3 nM of bortezomib versus 4 nM of each agent. No significant JNK activity was observed in response to 4 nM of each agent (data not shown). Together, these findings demonstrate that NPI-0052 + bortezomib-induced apoptosis is associated with the activation of caspase cascade, BIM and JNK.
Effects of NPI-0052 + bortezomib on endoplasmic reticulum stress response, heat shock proteins and NF-κB Previous studies have shown that bortezomib or NPI-0052 alone at higher concentrations induces unfolded protein response (UPR). We asked whether combined low doses of NPI-0052 and bortezomib similarly induce UPR. Our results show that even at low doses, the combination of NPI-0052 and bortezomib retained its ability to induce ER-stress response, evidenced by increased eIf2-α kinase activity and protein levels of its target protein CHOP/GADD153 (Fig 5B).

Multiple prior studies link bortezomib-induced apoptosis with the upregulation of heat shock proteins (Hsp’s). We therefore next examined the effect of combined low doses of NPI-0052 and bortezomib on three Hsps: Hsp-27, Hsp-70 and Hsp-90. MM.1S cells were treated with NPI-0052 (1 nM) + bortezomib (3 nM) for 24h, 48h, and 72h and protein lysates were subjected to immunoblot analysis with anti-Hsp-90, anti-Hsp-27 and anti-Hsp-70 Abs. NPI-0052 + bortezomib triggered an increase in Hsp-70, but not Hsp-27 or Hsp-90 protein levels even at longer exposure time (48h and 72h) (Fig 5C). Similarly, a shorter exposure time (12h) triggered Hsp-70, but not Hsp-27 or Hsp-90 (data not shown). Our prior studies showed that IC₅₀ concentrations of bortezomib (5 nM) trigger all three Hsp’s. Thus, while higher dose of bortezomib alone is able to induce Hsp-27, Hsp-70 and Hsp-90, a lower dose combination of bortezomib with NPI-0052 only triggers Hsp-70. These findings suggest that apoptosis triggered by combination of low doses of bortezomib and NPI-0052 involves only Hsp-70 and not
Hsp-90 or Hsp-27. The lack of Hsp-90 or Hsp-27 induction may be due to differential signaling pathways triggered by a higher dose of bortezomib alone versus its combination with NPI-0052. Furthermore, NPI-0052 + bortezomib-induced ER stress response (as shown in Fig 5B) and apoptosis likely involves Hsp-70, but not Hsp-27 and Hsp-90. These findings provide evidence of distinct mechanism of action of combined NPI-0052 + bortezomib versus each agent alone. Our findings have clinical implications: Hsp’s confer drug resistance, and prior studies showed that higher concentrations of bortezomib induce all three Hsp’s. Based on these findings, bortezomib has been combined with Hsp-90 inhibitor in clinical trials to overcome Hsp-mediated drug resistance. Our present finding that combined low dose bortezomib with NPI-0052 does not induce two of the three Hsp’s, suggests that drug resistance may be less frequent in patients given combined low dose regimens.

We and others have shown that proteasome inhibitors block NF-κB, a major growth and survival pathway in MM cells. Combined low doses of NPI-0052 (1 nM) and bortezomib (3 nM), but not either agent alone, showed synergistic inhibition of NF-κB activity, evidenced by decrease in nuclear translocation of p65 subunit (CI = 0.35; n =3) (Fig 5D).

**Effects of NPI-0052 + bortezomib on CT-L, C-L and T-L proteolytic activities** Although proteasome inhibitors induce apoptotic signaling cascades, the primary target of these agents is the proteasome. The proteolytic activity of proteasomes is mediated by three active sites: CT-L, T-L and C-L.
prior study showed that NPI-0052 blocks all 20S proteasomal activities in MM cells, while bortezomib primarily inhibits CT-L and to a lesser degree C-L activity. \[^{39}\] Treatment of MM.1S cells for 30 mins with a low dose (1 nM) NPI-0052 inhibits 13% CT-L, 8% C-L and 6% T-L activity, whereas bortezomib (3 nM) only inhibits 3% CT-L and 7% C-L activities, as measured by activity specific fluorogenic peptide substrates. \[^{49}\] Combined low doses of these agents result in a significant block in all three proteasomal activities: 40%, 43% and 20% inhibition in CT-L, C-L and T-L activities, respectively (Fig 5E). Longer (3 h) exposure also induced synergistic inhibition in CT-L, C-L and T-L activities (data not shown). Our data suggest that the combination of even low doses of NPI-0052 and bortezomib can effectively target all three proteasome activities and trigger potent anti-MM activity.

**NPI-0052 and bortezomib synergize to suppress human MM cell growth in vivo** Having shown that combined NPI-0052 + bortezomib induces synergistic apoptosis in MM cells in vitro, we next examined the in vivo efficacy of low dose combination NPI-0052 and bortezomib treatment using a human plasmacytoma xenograft mouse model. \[^{46,50}\] Our prior studies have shown that either NPI-0052 or bortezomib alone are effective anti-MM agents in this xenograft model. \[^{39,50}\] To assess for synergistic cytotoxicity in vivo, we first used low doses of either NPI-0052 (0.025 mg/kg, 0.050 mg/kg or 0.075 mg/kg) or bortezomib (0.25 mg/kg or 0.50 mg/kg). As seen in Fig 6A, low doses of either agent had minimal effect on the growth of tumors, which increased as in control mice. Importantly, when NPI-0052 was combined with bortezomib,
there was a significant (75%) reduction \( (P = 0.03) \) in tumor growth relative to untreated mice (Fig 6A and inset). As an additional control, we also treated mice with MTD of NPI-0052 and bortezomib (0.15 mg/kg and 1 mg/kg, respectively). As in our previous study, \[39\] a significant reduction in tumor growth was noted in these cohorts (data not shown). Importantly, the extent of tumor growth inhibition was similar in mice treated with low dose combination NPI-0052 + bortezomib versus mice treated with MTD of either NPI-0052 or bortezomib. Furthermore, the combination did not have increased toxicity, evidenced by lack of weight loss and neurological changes even after 4 weeks of NPI-0052 + bortezomib-treatment (data not shown). These findings suggest that combining NPI-0052 with bortezomib markedly reduces tumor growth and is well tolerated in vivo.

We next investigated the effect of the drug combination on in vivo apoptosis using TUNEL staining, cleavage of caspase-3, and H&E staining of paraffin-embedded sections of xenografted tumors. The peripheral necrotic regions of the sections from the xenografts were excluded for quantification of cell death. As seen in Fig 6B (upper and lower panels), either NPI-0052 or bortezomib alone at low doses triggered a very modest increase in the number of TUNEL-positive cells (Brown color) compared to tumors from control cohorts. However, the combination dramatically increased the number of TUNEL-positive cells compared to either treatment alone (Fig 6B). Similarly, NPI-0052 + bortezomib triggered robust caspase activation in tumors (Fig 6C). In agreement with these data, a significant decrease in Ki-
67-positive cells was noted in tumor sections from NPI-0052 + bortezomib-treated mice relative to tumors from mice receiving either treatment alone (Fig 6D).

In vitro data indicate anti-angiogenic activity of NPI-0052 + bortezomib, and we therefore next evaluated paraffin-embedded sections of xenografted tumors harvested from mice treated with NPI-0052 and bortezomib for Factor VIII staining, a marker of angiogenesis. As seen in Fig 7A low doses of NPI-0052 or bortezomib alone triggered a very modest decrease in the number of Factor VIII-positive cells compared to sections from control-treated tumors, whereas the combination dramatically decreased the number of Factor VIII-positive cells compared to treatment with either agent alone (Fig 7A). Since VEGFR1/FLT-1 is highly expressed in MM cells and plays a crucial role in angiogenesis in MM [48, 54], we examined the sections of xenografted tumors harvested from mice treated with NPI-0052 and bortezomib for VEGFR1 expression. IHC analysis showed that low doses of NPI-0052 or bortezomib markedly decrease the number of VEGFR1-positive cells compared to sections from control-treated tumors (Fig 7B). These findings suggest a likely mechanism mediating NPI-0052 + bortezomib-triggered inhibition of migration and angiogenesis.

Since combined low doses of NPI-0052 and bortezomib inhibits proteasome activities in MM cell lines, we next asked whether this combination alter the proteasome activity profile in the xenografted tumors. Tumors were excised after 90 mins of the last dose administration and examined for CT-L, C-L and T-L proteasome activity. Treatment with combined low doses of NPI-
NPI-0052 (0.025 mg/kg, 0.050 mg/kg or 0.075 mg/kg) and bortezomib (0.25 mg/kg or 0.50 mg/kg), but not with either agent alone, significantly inhibited all three proteasome activities (Fig 7B), confirming that the ability of NPI-0052 to synergize with bortezomib in vivo is associated with inhibition of proteasome activity in tumors. Together, these findings demonstrate potent in vivo anti-MM activity of NPI-0052 combined with bortezomib at doses that are well tolerated in a murine model, supporting the potential clinical evaluation of combined NPI-0052 + bortezomib treatment in MM.

Discussion

Our preclinical and clinical studies led to the FDA approval of bortezomib (Velcade™) for the treatment of relapsed and relapsed/refractory MM. However, as with other agents, dose-limiting toxicities and the development of resistance limits its long term utility. Our recent study showed another novel proteasome inhibitor NPI-0052 to also have anti-MM cytotoxicity. NPI-0052 is currently being evaluated in MM patients in a phase-I clinical trial. Here, we examined whether two proteasome inhibitors that are distinct in their chemical structure, mechanisms of action, and effects on proteasomal activities, can be combined at low doses to enhance anti-MM activity, reduce toxicity, and overcome drug resistance.

We first show that the combination of low doses of NPI-0052 and bortezomib induces apoptosis even in MM cells resistant to conventional and bortezomib therapies, without affecting normal
lymphocyte viability. For example, combined low doses of NPI-0052 (5 nM) and bortezomib (7 nM) triggers a degree of apoptosis in OPM-2 MM cells that is only achievable at much higher doses of either agent alone (26 nM and 20 nM for NPI-0052 and bortezomib, respectively). Likewise, we observed synergistic growth inhibition induced by NPI-0052 and bortezomib in MM cell lines and patient cells, including those resistant to anti-MM therapies such as Dex or Doxorubicin. Mechanistic studies show that NPI-0052 + bortezomib-induced apoptosis is associated with activation of the caspase cascade, BIM, JNK, Hsp-70 and ER stress response. However, the overall effective cell killing by NPI-0052 + bortezomib may involve activation of other apoptotic signaling pathways. Our data also demonstrate synergistic cytotoxicity of NPI-0052 and bortezomib on lymphoma, leukemic, and solid tumor cells that are relatively resistant to bortezomib, thereby suggesting clinical applicability of this therapeutic regimen beyond MM.

Importantly, the synergistic anti-MM activity of NPI-0052 and bortezomib is also observed in vivo in a human MM xenograft mouse model. NPI-0052 and bortezomib were utilized in vivo at doses six and four times lower, respectively, than has been reported to be inhibitory on tumor growth in order to demonstrate synergistic anti-MM activity. No significant tumor growth inhibition was noted at these low doses for either agent alone, but a marked growth inhibitory effect was observed with combined NPI-0052 and bortezomib. The combination of low dose NPI-0052 and bortezomib is not associated with any toxicity, since no
differences in body weight and overall appearance was noted in mice. The remarkable anti-MM activity of NPI-0052 + bortezomib in vivo was confirmed by IHC analysis of tumors harvested from control and NPI-0052 + bortezomib-treated mice using several molecular markers of growth inhibition (Ki67), apoptosis (TUNEL, caspase-3 cleavage and H&E staining) and associated angiogenesis (Factor VIII staining and VEGFR1 expression). Therefore, these findings demonstrate a dual effect of combining NPI-0052 and bortezomib: decreased proliferation and increased apoptosis.

Direct analysis of tumor cells from mice showed synergistic blockade of all three proteasome activities in NPI-0052 + bortezomib-treated mice, but not in mice treated with low doses of either agent alone. Similar to our earlier results \textsuperscript{[50]}, examination of whole blood and spleen showed a greater degree of proteasome inhibition than observed in tumors. These in vivo findings, coupled with our in vitro data showing minimal toxicity of combined NPI-0052 + bortezomib against normal cells (Fig 2A), confirm that MM cells are more sensitive to proteasome inhibition than normal cells.

Interestingly, while only NPI-0052 is known to inhibit T-L activity, its combination with bortezomib blocks T-L activity even higher. In this context, prior studies have reported that proteasome active sites allosterically regulate each other \textsuperscript{[69,70]} For example, a biochemical study showed that occupancy of C-L sites induces the T-L activity of proteasomes. \textsuperscript{[69]} It is likely that blockade of one proteasomal activity may affect the function of other proteasomal activity. While a definitive evidence for
the role of individual proteasomal activity awaits further
genetic studies involving specific inhibition of beta subunits,
it is clear from our in vivo data that even 30-40% proteasome
inhibition, albeit of all three activities, is sufficient to
trigger significant anti-MM activity.

The clinical observation that bortezomib therapy can be
associated with toxicity and resistance, coupled with our present
preclinical findings demonstrating that low doses of bortezomib
together with NPI-0052 trigger a similar or more potent anti-MM
effect in vitro and in vivo without increased toxicity, suggests
the promise of combination treatment strategies.

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Figure Legends

Figure 1. Combination of low doses of NPI-0052 and bortezomib induces synergistic MM cell death. (A-G) MM.1S (A), U266 (B), RPMI-8226 (C), INA-6 (D), OPM-2 (E), Dox-40 (F) or MM.1R (G) cells were treated with NPI-0052, bortezomib or combined NPI-0052 + bortezomib and assessed for viability using MTT assays. The concentrations of drugs, either alone or in combination, were as follows: For MM.1S cells: 1 nM NPI-0052, 3 nM bortezomib or NPI-0052 (1 nM) + bortezomib (3 nM); for U266 cells: 7 nM NPI-0052, 1 nM bortezomib or NPI-0052 (7 nM) + bortezomib (1 nM); for RPMI-8226 cells: 10 nM NPI-0052, 10 nM bortezomib or NPI-0052 (10 nM) + bortezomib (10 nM); for INA-6 cells: 5 nM NPI-0052, 5 nM bortezomib or NPI-0052 (5 nM) + bortezomib (5 nM); for OPM-2 cells: 5 nM NPI-0052, 7 nM bortezomib or NPI-0052 (5 nM) + bortezomib (7 nM); for Dox-40 cells: 5 nM NPI-0052, 10 nM bortezomib or NPI-0052 (5 nM) + bortezomib (10 nM); and for MM.1R cells: 5 nM NPI-0052, 3 nM bortezomib or NPI-0052 (5 nM) + bortezomib (3 nM) (mean ± SD; n = 3, P < 0.005 for all cell lines). Combination index (CI) < 1 indicates synergy. (H) MM.1S cells were treated with the indicated concentrations of NPI-0052, bortezomib or combined NPI-0052 + bortezomib for 12h and assessed for apoptosis using Annexin V/PI staining assays by flow cytometry. Percentage of apoptotic cells (Annexin V+/PI-) were as follows: For control cells: 3.5%; for NPI-0052-treated cells: 3.6%; for bortezomib-treated cells: 5%; and for NPI-0052 + bortezomib-treated cells: 25% (mean ± SD; n = 2, P < 0.04). (I) MM.1S cells were treated with the indicated concentrations of
NPI-0052, bortezomib or combined NPI-0052 + bortezomib for 12h and assessed for cell death using ‘Live and dead cell assay’ (live cells are Green colored and Dead cells are red colored) (mean + SD; n = 3, P < 0.05).

Figure 2 Combined NPI-0052 and bortezomib trigger synergistic anti-tumor activity in MM patient (CD138-positive) cells and other cancer cell types (A) Purified patient MM cells (CD138-positive) were treated with the indicated concentrations of NPI-0052, bortezomib (3 nM) or combined NPI-0052 + bortezomib and assessed for viability using MTT assays. Data represent mean + SD of triplicate samples, P < 0.05 for all patient samples). Combination index (CI) < 1 indicates synergy. PBMNCs from healthy donors were treated with indicated concentrations of NPI-0052, bortezomib or NPI-0052 + bortezomib and then analyzed for viability. Data are mean + SD of three independent experiments (P = 0.25 from Jonckheere-Tepstra test for trend). (B) MTT assays were performed after incubation of leukemia, lymphoma, pancreatic and breast cancer cells with NPI-0052, bortezomib or combined NPI-0052 + bortezomib. The concentrations of agents for cell lines were as follows: For Molm-14, SUDHL-4 and Panc-1 cells: 7 nM bortezomib, 7 nM NPI-0052 or NPI-0052 (7 nM) + bortezomib (7 nM); and for MCF-7 cells: 10 nM bortezomib, 3 nM NPI-0052 or NPI-0052 (3 nM) + bortezomib (10 nM) (mean + SD; n = 2, P < 0.05 for all cell lines).

Figure 3 Combined low doses of NPI-0052 and bortezomib block migration and tubule formation (A) Growth-factor-deprived MM.1S cells were either pretreated with indicated concentrations of
NPI-0052, bortezomib, or combination of NPI-0052 and bortezomib. Cells were then plated on a fibronectin-coated polycarbonate membrane in a modified Boyden chamber and exposed for 8 hours to serum containing medium in the lower chamber. The migrated cells on the bottom face of the membrane were fixed with 90% ethanol and stained with crystal violet. Three randomly selected fields were examined for cells that had migrated from upper to lower chambers. Figure 3A is a representative image from two experiments with similar results. (B) MM.1S cells were treated with the indicated concentrations of NPI-0052, bortezomib or NPI-0052 + bortezomib for 4h (viability > 95%), washed, and then treated for 24h with rVEGF (10 ng/ml), followed by analysis in a Transwell migration assay. The bar graph represents quantification of migrated cells. Data represents mean ± SD of two independent experiments (P < 0.05 for samples treated with rVEGF alone versus NPI-0052 + bortezomib-treated cells). (C) Human vascular endothelial cells were treated with the indicated concentrations of NPI-0052, bortezomib or combined NPI-0052 + bortezomib and assessed for in vitro angiogenesis using Matrigel capillary-like tube structure formation assays (mean ± SD; n = 3, P < 0.05). The in vitro angiogenesis is reflected by capillary tube branch formation (dark brown). Figure 3C is a representative image from three experiments with similar results. (D) HUVECs were treated with the indicated concentrations of NPI-0052, bortezomib, combined NPI-0052 + bortezomib, or Revlimid (5 μM) and assessed for in vitro angiogenesis using Matrigel capillary-like tube structure formation assays as in (C). The
bar graph represents quantification of in capillary-like tube structure formation in response to indicated agents: Branch points in several random view fields/well were counted; values were averaged; and statistically significance differences were measured using student t-test.

**Figure 4** Mechanisms of NPI-0052 + bortezomib-induced apoptosis

(A) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 12h; harvested; and total proteins were subjected to immunoblot analysis with anti-caspase-9, caspase-8, caspase-3 or PARP Abs. “FL” indicates full length; “CF” denotes cleaved fragment. Blots shown are representative of three independent experiments. (B) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 24h in the presence or absence of pan-caspase inhibitor z-VAD-fmk and then assessed for viability. Shown is mean ± SD (error bar) of three independent experiments (P <0.005). (C) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 12h; harvested; and total proteins were subjected to immunoblot analysis with anti-BIM or anti-Bcl-2 Abs. (D) MM.1S cells were transfected with siRNA BIM or scrambled siRNA for 48h; harvested; and total protein extracts were subjected to immunoblot analysis with anti-BIM or anti-Tubulin Abs. Blots shown are representative of two independent experiments. (E) MM.1S cells were transfected with siRNA BIM or scrambled siRNA for 48h; followed by 12h treatment with indicated agents and analysis for apoptosis by Annexin V/PI staining.
Figure 5 Effects of NPI-0052 + bortezomib on heat shock proteins and proteasomal activities (A) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 12h; harvested; and total proteins were subjected to immunoblot analysis with anti-phospho-JNK or actin Abs. Blots shown are representative of three independent experiments. (B) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 12h; harvested; and total proteins were subjected to immunoblot analysis with anti-phospho-eIF2-α, CHOP/GADD153 or actin Abs. Blots shown are representative of three independent experiments. (C) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 24h, 48h and 72h; harvested; and total proteins were subjected to immunoblot analysis with anti-Hsp-90, Hsp-27, or Hsp-70 Abs. Lysates from HeLA cells served a positive control in immunoblotting with Hsp Abs. Blots shown are representative of two independent experiments. (D) MM.1S cells were treated with NPI-0052 (1 nM), bortezomib (3 nM) or combined NPI-0052 (1 nM) + bortezomib (3 nM) for 24h; harvested; and nuclear extracts were then analyzed for NF-κB activity by using p65 ELISA kit. (E) MM.1S cells were treated with indicated concentrations of NPI-0052, bortezomib or combined NPI-0052 + bortezomib for 30 mins; harvested; and cytosolic extracts were then analyzed for CT-L, C-L and T-L proteasomal activities. The data are represented as % inhibition compared to vehicle control (mean ± SD; n = 3, P < 0.05).
Figure 6 Combination of low doses of NPI-0052 + bortezomib inhibits human plasmacytoma growth in immune deficient beige-nude-xid (BNX) mice. (A) Average and standard deviation of tumor volume (mm$^3$) from group of mice (n = 3/group) versus time (days) when tumor was measured. Mice were treated with vehicle, NPI-0052 (i.v), bortezomib (i.v), or NPI-0052 + bortezomib (i.v) at the indicated doses on a twice a week schedule for 30 days. A significant delay in tumor growth in NPI-0052 + bortezomib-treated mice was noted compared to vehicle-treated control mice ($P = .04$). Bars indicate mean ± standard error (SE). Inset: Photograph represents tumors resected from control (vehicle) and NPI-0052 (0.025 mg/kg) + bortezomib (0.25 mg/kg)-treated mice. Figure B-D (photographs) show apoptotic cells in tumors sectioned on day 30 (endpoint) from untreated- or NPI-0052 (0.025 mg/kg) + bortezomib (0.25 mg/kg)-treated mice as identified by a TUNEL assay (TUNEL-positive cells: dark brown), caspase-3 cleavage (dark brown cells), as well as H&E and Ki-67 staining. Shown photographs are representative of similar observations in three different mice receiving same treatment. The Bar graphs (B and C) show the quantification of TUNEL-positive cells and caspase-3 cleavage activity in tumors from NPI-0052 + bortezomib-treated mice. Apoptotic tumor cells were enumerated in non-necrotic areas of each section using light microscopy (X400).

Figure 7 Combination of low doses of NPI-0052 + bortezomib inhibits neovascularization and proteasomal activities in vivo in xenografted MM tumors (A) Figure 7A (upper panel) shows blood vessels in tumor sections from untreated- or NPI-0052 (0.025
mg/kg) + bortezomib (0.25 mg/kg)-treated mice, as identified by staining with a marker for angiogenesis Factor VIII. Shown photograph is representative of similar observations in three different mice receiving same treatment. The Bar graph (Figure 7A, lower panel) shows the quantification of blood vessels in tumor sections from mice treated with indicated doses of NPI-0052, bortezomib or combined NPI-0052 + bortezomib. Blood vessels were enumerated in non-necrotic areas of each section using light microscopy (X400). (B) Figure 7B shows VEGFR1 expression in tumor sections from untreated- or NPI-0052 (0.025 mg/kg) + bortezomib (0.25 mg/kg)-treated mice, as identified by IHC staining with VEGFR1 Abs. Shown photograph is representative of similar observations in two different mice receiving same treatment. (C) NPI-0052 + bortezomib trigger synergistic inhibition of CT-L, C-L and T-L proteasomal activities in vivo in xenografted MM tumors. For the analysis of proteasome activities in tumors, mice were treated with NPI-0052 (i.v), bortezomib (i.v) or NPI-0052 + bortezomib (as in "A"); sacrificed after 90 mins of drug administration; and tumors were examined for CT-L, C-L and T-L proteasome activity. The data represents percent inhibition compared to vehicle control-treated animals from two independent experiments with similar results. Combination index (CI) < 1 indicates synergy.
Figure 1
Figure 2

A. Viable cells (% Control) for Patient cells and Normal cells treated with NPI-0052 (1 nM) and Bortezomib (3 nM) for different patients (Pt#1 to Pt#5) and PBMNCs.

B. % Cell death for different tumor types (Molm-14 (AML), SUDHL-4 (Lymphoma), Panc-1 (Pancreatic), MCF-7 (Breast)) treated with NPI-0052 and Bortezomib.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Combination of proteasome inhibitors bortezomib and NPI-0052 trigger in vivo synergistic cytotoxicity in multiple myeloma

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