Inhibition of Interleukin-6 Signaling with CNTO 328 Enhances the Activity of Bortezomib in Preclinical Models of Multiple Myeloma

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Abstract

Purpose: Inhibition of the proteasome leads to the activation of survival pathways in addition to those that promote cell death. We hypothesized that down-regulation of interleukin-6 (IL-6) signaling using the monoclonal antibody CNTO 328 would enhance the antitumor activity of the proteasome inhibitor bortezomib in multiple myeloma by attenuating inducible chemoresistance.

Experimental Design: The cytotoxicity of bortezomib, CNTO 328, and the combination, along with the associated molecular changes, was assessed in IL-6–dependent and IL-6–independent multiple myeloma cell lines, both in suspension and in the presence of bone marrow stromal cells and in patient-derived myeloma samples.

Results: Treatment of IL-6–dependent and IL-6–independent multiple myeloma cell lines with CNTO 328 enhanced the cytotoxicity of bortezomib in a sequence-dependent fashion. This effect was additive to synergistic and was preserved in the presence of bone marrow stromal cells and in CD138+ myeloma samples derived from patients with relative clinical resistance to bortezomib. CNTO 328 potentiated bortezomib-mediated activation of caspase-8 and caspase-9 and the common downstream effector caspase-3; attenuated bortezomib-mediated induction of antiapoptotic heat shock protein-70, which correlated with down-regulation of phosphorylated signal transducer and activator of transcription-1; and inhibited bortezomib-mediated accumulation of myeloid cell leukemia-1, an effect that was associated with down-regulation of phosphorylated signal transducer and activator of transcription-3.

Conclusions: Taken together, our results provide a strong preclinical rationale for the clinical development of the bortezomib/CNTO 328 combination for patients with myeloma.

The proteasome has emerged as an important target for the treatment of multiple myeloma. Bortezomib, a dipeptidyl boronate that inhibits the chymotrypsin-like activity of the proteasome, showed notable cytotoxicity in preclinical models of multiple myeloma (1, 2) and in myeloma patients (3–6). However, only 35% of patients with relapsed/refractory multiple myeloma had partial responses or better to bortezomib (6), whereas 40% to 50% of patients with newly diagnosed disease responded, showing the need for strategies aimed at improving myeloma cell responsiveness to proteasome inhibition (7).

The ubiquitin-proteasome pathway is responsible for the degradation of most intracellular proteins (8). Although the net effect of proteasome inhibitors on cancer cells favors the induction of a proapoptotic program in most instances, survival pathways are also activated, thereby attenuating drug efficacy (9). Treatment of the multiple myeloma cell line MM1.S with bortezomib led to activation of the antiapoptotic heat shock protein (HSP) response, as evidenced by increases in HSP-27, HSP-70, and HSP-90 (10). Inhibition of the HSP response with a small-molecule inhibitor of heat shock transcription factor (HSF)-1 or selective inhibition of HSP-90 sensitized MM1.S cells to bortezomib-mediated cell death (10, 11). Similarly, pretreatment of MM1.S and RPMI 8226 multiple myeloma cell lines with an inhibitor of the p38 mitogen-activated protein kinase (MAPK), a known upstream activator of HSP-27 that is induced on inhibition of the proteasome, decreased bortezomib-mediated activation of HSP-27 and enhanced the activity of bortezomib (12).

Expression levels of the antiapoptotic B-cell lymphoma-2 family member myeloid cell leukemia (Mcl)-1, a critical
mediator of myeloma cell survival (13, 14), are tightly regulated by
the proteasome (15, 16). Inhibition of proteasome function
led to accumulation of Mcl-1 in several preclinical cancer
models, including lymphoma and multiple myeloma cell lines,
as well as samples obtained from patients with B-cell chronic
lymphocytic leukemia (13, 17). Pretreatment with proteasome
inhibitors resulted in the accumulation of Mcl-1 in HeLa cells,
inhibited UV light-mediated down-regulation of Mcl-1, and
prevented cell death (18). Similarly, pretreatment of RPMI
8226 cells with proteasome inhibitors led to the accumulation
of Mcl-1, prevented its down-regulation by actinomycin D,
an inhibitor of mRNA synthesis, and abrogated actinomycin D–mediated apoptosis (13). These results suggest that disrupt-
ing the accumulation of Mcl-1 that occurs with proteasome
inhibitors could enhance their antitumor efficacy. In support
of this, down-regulation of Mcl-1 levels using short hairpin
RNAs potentiated the apoptotic activity of the proteasome
inhibitors MG-132 and epoxomicin in HeLa cells (17).
Interleukin-6 (IL-6) plays an important role in myeloma
cell proliferation, survival, and resistance to glucocorticoid-
mediated cell death (19–23) and has several properties that
make its inhibition an attractive means of enhancing the
activity of proteasome inhibitors. Signal transducer and
activator of transcription (STAT)-1, which is activated by IL-6
(24), interacts with HSF-1 to facilitate transcription of HSP-70
and HSP-90 (25). In addition, IL-6 plays an important role in
the transcriptional regulation of Mcl-1 in multiple myeloma
cells (26–28). Based on these observations, we hypothesized
that down-regulation of IL-6 signaling would sensitize preclin-
ical models of myeloma to proteasome inhibitor-mediated
apoptosis by interfering with the induction of the HSP response
and Mcl-1. Using CNTO 328, a chimeric monoclonal antibody
that neutralizes IL-6 function, we show that inhibition of
IL-6 signaling in IL-6–dependent and IL-6–independent
multiple myeloma cell lines, both in suspension and in the
presence of bone marrow stromal cells (BMSC) and in plasma
cells derived from myeloma patients, enhanced the cytotoxicity
of bortezomib, which was associated with attenuation of
bortezomib-mediated accumulation of Mcl-1 and HSP-70.
Taken together, our results support clinical development of
the combination of bortezomib and CNTO 328 for patients
with multiple myeloma.

Materials and Methods

Materials. Bortezomib was provided by Millenium Pharmaceu-
ticals. A stock solution was prepared in DMSO, stored at -20°C,
and diluted in culture medium immediately before use. F105, an antibody
that recognizes the CD4-binding site of HIV type 1 gp120, and CNTO
328 were provided by Centocor and prepared in 0.15 mol/L sodium
chloride and 0.01 mol/L sodium phosphate (pH 7.2). KNK437 was
purchased from Calbiochem and dissolved in DMSO. Final vehicle
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Cancer Therapy: Preclinical

Human multiple myeloma–derived cell lines and patient myeloma
samples. The human multiple myeloma cell lines KAS-6/1 and ANBL-
6 were provided by Dr. Diane Jelinek (Mayo Clinic, Rochester, MN) and
Dr. Beverly Mitchell (Stanford University, Palo Alto, CA), respectively.
MM1.S was provided by Dr. William Dalton (H. Lee Moffitt Cancer
Center, Tampa, FL). RPMI 8226 and H-929 were obtained from the
American Type Culture Collection. HSF-1 knockout and wild-type
mouse embryo fibroblasts, provided by Dr. Ivor Benjamin (University
of Texas Southwestern, Dallas, TX), were grown in DMEM (Life
Technologies), whereas all multiple myeloma cell lines were grown in
RPMI 1640 (Life Technologies) containing 10% fetal bovine serum
(Sigma Chemical Co.). IL-6–dependent cell lines and multiple
myeloma patient samples were supplemented with 1 ng/mL of human
recombinant IL-6 (R&D Systems). For isolation of patient multiple
myeloma cells, mononuclear cells from bone marrow aspirates were
obtained by density gradient centrifugation over Ficoll-Paque Plus
(Amersham Biosciences Corp.). A magnetic-activated cell sorting system
was used with anti-CD138 magnetic beads for positive selection of
plasma cells per the manufacturer’s instructions (Miltenyi Biotech).

BMSC cultures. The mononuclear cell fraction was isolated by
Ficoll-Paque Plus density gradient centrifugation and BMSC cultures
were established as described previously (29). BMSCs were grown in
α-MEM (Life Technologies) and used between passages 2 and 5.
Patient-derived multiple myeloma samples and BMSCs were obtained
under University of North Carolina Institutional Review Board–
approved protocols. Informed consent was obtained in accordance
with the Declaration of Helsinki Protocol.

Cell viability assays. Cells were seeded onto 96-well microtiter
plates and treated as described in the text. Cell viability was measured
using the cell proliferation reagent WST-1 per the manufacturer’s
instructions (Roche Applied Science). For myeloma cell/BMSC cocul-
tures, 1 × 10⁶ myeloma cells were plated onto 1 × 10⁶ BMSCs per well
before drug treatment. Cell viability was recorded as percent viability
relative to vehicle-treated controls.

DNA fragmentation apoptosis assays. Cells were seeded onto 96-well
microtiter plates and treated as indicated in the text. Apoptotic DNA
damage was evaluated using the Cell Death Detection ELISAPLUS kit
(Roche Applied Science) per the manufacturer’s instructions. Induction
of apoptosis was calculated as the fold increase in apoptosis relative to
vehicle-treated controls.

Annexin V/ToPro-3 apoptosis assays. Myeloma cells (2 × 10⁶) were
plated per well of a 12-well plate and treated as indicated in the text.
Apoptosis was evaluated by flow cytometric detection of cell surface
Annexin V staining (BioVision, Inc.) per the manufacturer’s instruc-
tions. The vital dye ToPro-3 (Molecular Probes, Inc.) was used to
distinguish live from dead cells. Events (10,000) were collected on a
FACS Calibur using CellQuest software (Becton Dickinson) and
analyzed using FlowJo software version 6.3.3 (Tree Star, Inc.). For
BMSC/myeloma cell coculture experiments, 2 × 10⁵ myeloma cells
were seeded onto 4 × 10⁵ BMSCs before treatment.

Caspase activation assays. For assessment of caspase-3 activation,
2 × 10⁵ cells were treated as indicated, incubated with the irreversible
caspase-3 inhibitor FITC-DEV-FMK for the last 30 min (BioVision),
resuspended in binding buffer containing phycoerythrin-conjugated
Annexin V, and analyzed by flow cytometry. To evaluate the activation
status of caspase-8 and caspase-9, cellular lysates were prepared from
treated cells and 30 μg of protein per reaction were incubated
with 40 μmol/l of fluorogenic substrate with specificity for activated
caspase-8 (Ac-IETD-AMC) or caspase-9 (Ac-LEHD-AMC, Biomol Inter-
national, L.P.). Measurement of free AMC groups was done at 380/460
nm on a multilabel fluorometer FLUOSTar Optima (BMG Labtech).
Results were expressed as fold activation over vehicle control.

Western blot analysis. Cells (2.5 × 10⁶) were treated as indicated in
the text. Whole-cell lysates were prepared, separated, and transferred
to nitrocellulose membranes as described previously (30). Antibodies
used for immunoblotting included anti-phosphorylated p44/p42 MAPK
(Thr202/Tyr204) and anti-p44/42 (Cell Signaling); anti-phosphorylated
STAT-1 (Tyr701), anti-STAT-1, anti-phosphorylated STAT-3 (Tyr705), and
anti-STAT-3 (New England Biolabs); anti-Mcl-1 (Santa Cruz Biotech-
ology); anti-B-cell lymphoma-2 (BD Pharmingen); and anti-HSP-70
and anti-heat shock cognate-70 (StressGen Bioreagents Corp.). To
quantify protein bands, autoradiographs were scanned with an Agfa
version 1.61.

**Statistical analysis.** Results were calculated and graphed using Excel (Microsoft) and reported as the mean ± SD for typical experiments. Each condition was replicated in triplicate to quintuplicate, and experiments were done at least thrice to ensure reproducibility. Differences between experimental conditions were measured using a Student’s unpaired t test and considered statistically significant at a P value of <0.05. Synergistic activity of the bortezomib/CNTO 328 combination was assessed via isobologram analysis using CalcuSyn software (Biosoft). A combination index of ≤0.9 was considered synergistic.

**Results**

**Cytotoxicity of CNTO 328, bortezomib, and the combination in multiple myeloma cell lines.** The cytotoxicity of CNTO 328, bortezomib, and the combination was evaluated in IL-6–dependent (ANBL-6 and KAS-6/1) and IL-6–independent (H-929, MM1.S, and RPMI 8226) multiple myeloma cell lines using the WST-1 cell viability assay. Treatment of ANBL-6 and KAS-6/1 cells with CNTO 328 for 72 h inhibited proliferation in a dose-dependent manner, with KAS-6/1 cells being more sensitive than ANBL-6 (Fig. 1A). Although dose titration curves were not done in IL-6–independent cell lines, H-929, MM1.S, and RPMI 8226 cells were less sensitive to growth inhibition by CNTO 328 than ANBL-6 and KAS-6/1 cells (Fig. 1B-F). The cytotoxicity of the combination of CNTO 328 and bortezomib was significantly greater than either agent alone in IL-6–dependent multiple myeloma cells (Fig. 1B and C). In ANBL-6 cells, 10 μg/mL CNTO 328 and 2.5 nmol/L bortezomib resulted in cell viabilities of 57% and 71% of controls, respectively, whereas the combination decreased viability further to 25% (Fig. 1B). In IL-6–independent H-929 cells, bortezomib at 2 nmol/L reduced viability to 52%, CNTO 328 at 10 μg/mL to 68%, and the combination to 15% (Fig. 1D). Increased activity with the combination was also seen in the IL-6–independent cell lines MM1.S and RPMI 8226, albeit to a lesser degree than H-929 cells (Fig. 1E and F). For an assessment of the activity of the combination over a broader range of bortezomib dosing, please refer to Supplementary Fig. S1.

Isobologram analysis was done in ANBL-6 cells treated with the CNTO 328/bortezomib combination (Table 1). The activity of the combination was synergistic over a dose range of bortezomib that exhibited single-agent cytotoxicity, with combination indices ranging from 0.505 to 0.758. The combination was additive to synergistic in KAS-6, H-929, MM1.S, and RPMI 8226 cells (data not shown).

![Fig. 1. The cytotoxicity of CNTO 328 and bortezomib in IL-6–dependent and IL-6–independent human multiple myeloma cell lines.](image-url)
Sequence dependence of the interaction between CNTO 328 and bortezomib. Bortezomib has been shown to interfere with IL-6 signaling through caspase-3–mediated cleavage of the IL-6 receptor (31), suggesting that the sequence of drug administration may have an effect on the ability of CNTO 328 to enhance the activity of bortezomib. To evaluate this further, ANBL-6 cells were treated with concurrent CNTO 328 and bortezomib (Fig. 2A), CNTO 328 followed by bortezomib (Fig. 2B), or bortezomib followed by CNTO 328 (Fig. 2C).

<table>
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<tr>
<th>Bortezomib (nmol/L)</th>
<th>Fraction affected</th>
<th>CNTO 328 (µg/mL)</th>
<th>Fraction affected</th>
<th>Fraction affected (combination)</th>
<th>Combination index</th>
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<tr>
<td>2</td>
<td>0.383</td>
<td>2</td>
<td>0.473</td>
<td>0.695</td>
<td>0.758</td>
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<tr>
<td>3</td>
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<td>3</td>
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<td>0.970</td>
<td>0.597</td>
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<tr>
<td>4</td>
<td>0.919</td>
<td>4</td>
<td>0.513</td>
<td>0.995</td>
<td>0.505</td>
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When ANBL-6 cells were treated with CNTO 328 and bortezomib concurrently, or CNTO 328 followed by bortezomib, further cytotoxicity was seen with the addition of CNTO 328 compared with single-agent bortezomib. In contrast, although CNTO 328 had single-agent activity when it was given after vehicle control, no additional cytotoxic effect beyond that seen with single-agent bortezomib was noted when it was administered following bortezomib.

Effect of CNTO 328 in the context of stroma and in patient myeloma samples. Although IL-6 is an important mediator of multiple myeloma cell growth and survival, other growth factors and adhesion molecules in the bone marrow milieu play a similar role (32). To determine if CNTO 328 and bortezomib were active in a model system more closely replicating the bone marrow microenvironment, we tested the activity of the combination in ANBL-6 and KAS-6 cells grown in the presence of patient-derived BMSCs. Although single-agent bortezomib retained activity against ANBL-6 and KAS-6 cells in the presence of BMSCs, CNTO 328 enhanced the cytotoxicity of bortezomib in both cell types, results which were statistically significant (Fig. 3A and B). In KAS-6/1 cells, whereas treatment with single-agent CNTO 328 and bortezomib led to a 19% and 30% decrease in cell viability, respectively, the combination resulted in a 66% reduction in viability ($P < 0.01$, Fig. 3B). Treatment with CNTO 328, bortezomib, or the combination had no effect on the viability of BMSCs (data not shown).

To further validate the CNTO 328/bortezomib combination, we studied the activity of the regimen in patient-derived myeloma cells. The first sample came from a patient who had stable disease on bortezomib and pegylated liposomal doxorubicin and later progressed on thalidomide and dexamethasone as well as lenalidomide and dexamethasone. In this case, CNTO 328 was highly active as a single agent, achieving a 64% reduction in cell viability over 48 h of treatment (Fig. 3C). Although bortezomib alone had only modest activity, with a 35% reduction in cell viability, CNTO 328 significantly enhanced its activity, resulting in a 95% viability reduction with the combination.

Contribution of apoptosis to the enhanced activity of CNTO 328 and bortezomib. To better determine whether the increased cytotoxicity of the CNTO 328/bortezomib combination was the result of enhanced induction of apoptosis, ANBL-6 cells treated with CNTO 328, bortezomib, or the combination were analyzed for the percentage of cells undergoing apoptosis. CNTO 328 and bortezomib alone induced apoptosis in a dose-dependent manner, with the combination inducing a significantly higher percentage of apoptosis than either agent alone. This result is consistent with the findings that the combination is more cytotoxic than either agent alone, and that apoptosis is a major contributor to the enhanced activity of CNTO 328 and bortezomib.
were analyzed by cell surface Annexin V staining. Whereas CNTO 328 and bortezomib led to drug-specific apoptosis of 8% and 56%, respectively, the combination resulted in 80% drug-specific cell death (Fig. 4A). Similarly, although CNTO 328 had no apoptotic activity in IL-6–independent H-929 cells as single-agent therapy, it strongly potentiated bortezomib-mediated apoptosis (Fig. 4C).

Next, we wanted to assess whether CNTO 328 enhanced bortezomib-mediated apoptosis in the context of the bone marrow microenvironment. ANBL-6 and H-929 cells were treated with CNTO 328, bortezomib, or the combination in the presence of BMSCs, and cell surface Annexin V staining was analyzed via flow cytometry. Whereas treatment with CNTO 328 alone had a modest to no effect on drug-specific apoptosis, it strongly potentiated the apoptotic activity of bortezomib in both ANBL-6 and H-929 cells (Fig. 4B and D). Taken together, these results suggested that CNTO 328 enhances the cytotoxicity of bortezomib in suspension culture and in the bone marrow microenvironment, both in IL-6–dependent and IL-6–independent myeloma cell line models, by activating apoptosis.

Increased apoptosis induced by the CNTO 328/bortezomib combination could be the result of caspase-dependent or caspase-independent cell death processes. ANBL-6 and H-929 cells were therefore treated with CNTO 328, bortezomib, or the combination, then incubated with FITC-DEVD-FMK, a fluorescent reagent that binds activated caspase-3 irreversibly, and subjected to flow cytometric analysis. Although CNTO 328 alone did not activate caspase-3, it potentiated induction of caspase-3 by bortezomib in ANBL-6 and H-929 cells (Fig. 5A and B). Bortezomib has been shown to activate the common effector caspase-3 through both the extrinsic and intrinsic apoptotic pathways (1). To determine the effect of CNTO 328 on these bortezomib-mediated processes, ANBL-6 cells treated with CNTO 328, bortezomib, or the combination were incubated with fluorogenic substrates with specificity for activated caspase-8, a marker of extrinsic pathway activation, and caspase-9, a marker of intrinsic pathway activation. Although CNTO 328 alone was not able to activate either caspase-8 or caspase-9 (Fig. 5C and D, respectively), it potentiated bortezomib-mediated activation of both caspases. Thus, CNTO 328 enhanced the apoptotic activity of bortezomib by increasing bortezomib-mediated activation of the intrinsic and extrinsic apoptotic pathways.

Mechanisms underlying the activity of the CNTO 328/bortezomib combination. Ras-dependent activation of the p44/42 MAPK pathway has been shown to play an important role in the proliferative response of multiple myeloma cell lines to IL-6 (24). Furthermore, the cytotoxicity of bortezomib in preclinical myeloma and breast cancer models is in part a function of its ability to down-regulate p44/42 MAPK activity (1, 30). We therefore evaluated the effect of CNTO 328 on IL-6–mediated activation of p44/42 MAPK. Bortezomib alone...
reduced the cellular content of activated and dually phosphorylated p42 and p44 MAPK in a dose-dependent manner, whereas the addition of CNTO 328 inhibited IL-6–induced p44/42 MAPK activation to an even greater extent than was seen with bortezomib alone (Fig. 6A).

STAT-1, a known downstream target of IL-6 signaling (24), binds to HSF-1, thereby enhancing transcription of HSP-70 (25). We therefore sought to determine whether inhibition of IL-6 signaling with CNTO 328 would block induction of antiapoptotic HSP-70 and how this might correlate with suppression of STAT-1 activation. Treatment of ANBL-6 cells with CNTO 328 led to almost complete inhibition of signaling through STAT-1 (Fig. 6B), as determined by a loss of phosphorylated STAT-1 without an effect on total STAT-1 levels. Treatment with bortezomib induced HSP-70 in a dose-dependent fashion, but this effect was blunted by the addition of CNTO 328. To further assess the role of the HSP response in inducible chemoresistance to proteasome inhibitors, we treated ANBL-6 cells with bortezomib alone or in combination with KNK437, a benzylidene lactam compound that inhibits HSP induction (33, 34). Importantly, KNK437 enhanced bortezomib-mediated apoptosis as evidenced by increased Annexin V staining with the combination (Fig. 6C). Furthermore, KNK437 increased bortezomib-mediated DNA fragmentation, a marker of apoptosis, to a much greater extent in wild-type mouse embryo fibroblasts than in HSF-1 knockout mouse embryo fibroblasts, thus showing the relative specificity of KNK437 as an inhibitor of HSP induction (Fig. 6D). Taken together, these results provide support for further evaluation of HSP response modulation as a means of overcoming inducible chemoresistance to bortezomib.

We next evaluated the ability of CNTO 328 to attenuate bortezomib-mediated increases in Mcl-1 levels. Mcl-1 levels increased on exposure of ANBL-6 cells to bortezomib in a dose-dependent fashion by ~2-fold, as determined by densitometry (Fig. 6E). In contrast, levels of B-cell lymphoma-2 were unchanged with CNTO 328, bortezomib, or the combination (data not shown). Interestingly, the accumulation of Mcl-1 on inhibition of the proteasome was blunted in CNTO 328–treated cells. Given the role of STAT-3 in the IL-6–mediated regulation of Mcl-1 levels in myeloma cells, we evaluated the phosphorylation status of STAT-3. As shown in Fig. 5E, CNTO 328 abrogated IL-6–mediated phosphorylation of STAT-3 in ANBL-6 cells. These studies together suggest that CNTO
328–mediated blockade of IL-6 signaling may contribute to the cytotoxicity of bortezomib through several pathways, including modulation of the activity of p44/42 MAPK and STAT-1 and STAT-3, as well as through the expression levels of antiapoptotic proteins, such as HSP-70 and Mcl-1.

Discussion

Although the proteasome inhibitor bortezomib has significantly improved survival for patients with multiple myeloma, most patients with newly diagnosed or relapsed and/or refractory disease do not achieve a partial response or better to single-agent therapy, underscoring the need for the development of strategies aimed at enhancing its activity. Given the role of IL-6 signaling pathways in multiple myeloma cell proliferation, survival, and resistance to glucocorticoids, we sought to evaluate whether inhibition of IL-6 signaling with CNTO 328 could enhance the activity of bortezomib in preclinical models of human multiple myeloma. Treatment of both IL-6–dependent and IL-6–independent human multiple myeloma cell lines with CNTO 328 enhanced the cytotoxicity of bortezomib, an effect that was additive to synergistic. Furthermore, although CNTO 328 alone had modest proapoptotic activity, it was able to potently accelerate bortezomib-mediated induction of programmed cell death via enhanced activation of the intrinsic and extrinsic apoptotic cascades. Importantly, cytotoxicity was seen in the presence of BMSCs and in myeloma samples derived from patients with relative resistance to bortezomib clinically.

Previous studies with the human multiple myeloma cell line MM1.S have shown that bortezomib retains its cytotoxicity in the presence of IL-6, which would suggest that bortezomib overcomes IL-6–mediated drug resistance (1). Our results also confirm that bortezomib is active against IL-6–dependent and IL-6–independent multiple myeloma cell lines in the presence of IL-6. However, previous studies showed that cell proliferation was superior in MM1.S cells treated with bortezomib in the presence of IL-6 compared with those treated in its absence at doses as high as 100 nmol/L (1). Furthermore, it was subsequently shown that incubation of MM1.S cells with IL-6 after a 5-h preincubation with 5 to 10 nmol/L of bortezomib led to improved cellular proliferation compared with pretreatment with bortezomib followed by vehicle control (31). Similarly, our results also showed improved cell viability of bortezomib-treated MM1.S cells in the presence of IL-6 compared with cells in which IL-6 had been effectively neutralized by CNTO 328. Importantly, the combination was synergistic under some conditions, thus supporting the idea that inhibition of IL-6 signaling increases the sensitivity of multiple myeloma cells to bortezomib. Finally, although single-agent CNTO 328 did not possess substantial apoptotic activity, it strongly potentiated bortezomib-mediated cell death in IL-6–dependent and IL-6–independent multiple myeloma cell lines. Therefore, although bortezomib remains active in the presence of IL-6, IL-6 does modulate its activity at a dose range of bortezomib exhibiting single-agent activity, showing the viability of IL-6 as a therapeutic target in combination with proteasome inhibition.
CNTO 328 enhanced the cytotoxicity of bortezomib in ANBL-6 cells when the agents were given concurrently or when the cells were pretreated with CNTO 328 followed by incubation with bortezomib. In fact, very little additional activity was seen with the addition of CNTO 328 when they were pretreated with bortezomib. Interestingly, we observed a dose-dependent decrease in the activation of downstream IL-6 signaling pathways with single-agent bortezomib treatment as evidenced by attenuation of IL-6-mediated phosphorylation of STAT-1, STAT-3, and the p44/42 MAPK. These results are in line with previously published results in which bortezomib treatment of MM1.S cells led to caspase-dependent down-regulation of the IL-6 receptor β subunit gp130 and a decrease in IL-6–mediated phosphorylation of p44/42 MAPK, STAT-3, and AKT (31). The dose of bortezomib used to pretreat ANBL-6 cells in our experiments was one that clearly activated caspase-8, caspase-9, and caspase-3. Therefore, one attractive explanation for the reduced activity of CNTO 328 following bortezomib is that pretreatment with bortezomib down-regulated levels of the IL-6 receptor, rendering neutralization of IL-6 with CNTO 328 less effective. Regardless, our results favor a concurrent dosing strategy for clinical trials, or one in which CNTO 328 is given before bortezomib, rather than the reverse order.
Proteasome inhibitors activate an antiapoptotic HSP response, and strategies aimed at attenuating proteasome inhibitor-mediated HSP-70 induction represent an attractive means of enhancing their activity. STAT-1, which is activated by IL-6 (24), has been shown to interact with the transcription factor HSF-1 to augment the transcription of genes involved in the HSP response (25). Interestingly, the enhanced activity of the CNTO 328/bortezomib combination in ANBL-6 cells was associated with CNTO 328-mediated down-regulation of phosphorylated STAT-1 levels and a decrease in the induction of HSP-70. These results, coupled with our observation that inhibition of the HSP response with KNK437 substantially enhanced bortezomib-mediated apoptosis, support the value of HSP modulation as a means of enhancing the activity of proteasome inhibitors. Although we were able to achieve a reduction in HSP-70 levels with CNTO 328 treatment compared with an isotype control antibody, levels of HSP-70 remained significantly higher with increasing doses of bortezomib compared with vehicle control even in the presence of CNTO 328. Interestingly, others have shown that, under conditions of heat shock, STAT-3 antagonizes the transcriptional activity of HSF-1 at the level of the HSP-90 promoter (35). Therefore, one potential explanation for continued, albeit reduced, activation of HSP-70 by bortezomib in the presence of CNTO 328 is that CNTO 328-mediated decreases in phosphorylated STAT-3 led to the derepression of HSF-1 transcriptional activity. Alternatively, other factors aside from STAT-1 and STAT-3 may play an important role in the transcriptional activity of HSF-1. Further studies evaluating the transcriptional regulators of the HSP response in multiple myeloma cells, and how upstream signaling pathways affect their activity, are clearly warranted and will help facilitate the development of other strategies aimed at interfering with induction of the HSP response.

Mcl-1 plays a crucial role in the survival of myeloma cells. In fact, high levels of Mcl-1 expression in patient myeloma cells have been associated with an inferior outcome (36). Inhibition of proteasome activity has been shown to lead to Mcl-1 accumulation in several model systems, including myeloma cell lines. Our results in the ANBL-6 multiple myeloma cell line further showed a dose-dependent increase in Mcl-1 levels on proteasome inhibition. Furthermore, we showed that inhibition of IL-6 signaling with CNTO 328 was able to down-regulate Mcl-1 expression levels. Although IL-6 has clearly been implicated in the transcriptional up-regulation of Mcl-1, others have reported that Mcl-1 expression is not IL-6 dependent in all cases (37). Clearly, the CNTO 328–mediated changes in Mcl-1 levels were not as dramatic as those seen with phosphorylated STAT-3, supporting the fact that other IL-6–independent factors play a role in Mcl-1 expression. Interestingly, IL-6 inhibition had very little effect on Mcl-1 levels in RPMI 8226 cells, which was the one cell line in which the combination was least active (data not shown). Indeed, it will be interesting to see whether the IL-6 dependence of Mcl-1 expression will correlate with activity of the CNTO 328/bortezomib combination in patients with myeloma. Taken together, the above data provide a strong rationale for clinical evaluation of CNTO 328 and bortezomib in patients with myeloma.

References

31. Hideshima T, Chauhan D, Hayashi T, et al. Proteasome


35. Stephanou A, Isenberg DA, Akira S, Kishimoto T, Latchman DS. The nuclear factor interleukin-6 (NF-IL6) and signal transducer and activator of transcription-3 (STAT-3) signalling pathways co-operate to mediate the activation of the hsp90β gene by interleukin-6 but have opposite effects on its inducibility by heat shock. Biochem J 1998;330:189–95.


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