Therapeutic Enhancement of ER Stress by Insulin-Like Growth Factor I Sensitizes Myeloma Cells to Proteasomal Inhibitors

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Abstract

**Purpose:** Multiple myeloma is a clonal plasma cell disorder in which growth and proliferation are linked to a variety of growth factors, including insulin-like growth factor type I (IGF-I). Bortezomib, the first-in-class proteasome inhibitor, has displayed significant antitumor activity in multiple myeloma.

**Experimental Design:** We analyzed the impact of IGF-I combined with proteasome inhibitors on multiple myeloma cell lines in vivo and in vitro as well as on fresh human myeloma cells.

**Results:** Our study shows that IGF-I enhances the cytotoxic effect of proteasome inhibitors against myeloma cells. The effect of bortezomib on the content of proapoptotic proteins such as Bax, Bad, Bak, and BimS and antiapoptotic proteins such as Bcl-2, Bcl-XL, XIAP, Bfl-1, and survivin was enhanced by IGF-I. The addition of IGF-I to bortezomib had a minor effect on NF-kB signaling in MM.1S cells while strongly enhancing reticulum stress. This resulted in an unfolded protein response (UPR), which was required for the potentiating effect of IGF-I on bortezomib cytotoxicity as shown by siRNA-mediated inhibition of GADD153 expression.

**Conclusions:** These results suggest that the high baseline level of protein synthesis in myeloma can be exploited therapeutically by combining proteasome inhibitors with IGF-I, which possesses a "priming" effect on myeloma cells for this family of compounds. Clin Cancer Res; 19(13); 3556–66. ©2013 AACR.

Introduction

Cancer cells differ from normal cells by the abnormal use or activation of certain metabolic pathways such as anaerobic glycolysis (1). The bone marrow microenvironment plays an important role in the proliferation, survival, and migration of multiple myeloma cells as well as in their resistance to therapy. These stimulatory and protective roles of the microenvironment partly rely on the local production of growth factors such as interleukin-6 (IL-6) and insulin-like growth factor I (IGF-I) and have prompted the evaluation of targeted therapies aiming to block the activity of IGF-I receptor (IGF-IR) and/or to activate NF-kB signaling (5–8). In vivo and in vitro studies have shown that IGF-I increases the content of proapoptotic proteins, such as Bcl-2, Bcl-XL, cIAP-1, cIAP-2, and FLIP, and decreases the content of proapoptotic proteins, such as caspase-3, -8, and -9 in multiple myeloma cells, thus protecting them from drug-induced apoptosis (9, 10). In addition, serum IGF-I concentrations have been reported to be associated with prognosis in patients with myeloma (11).

As a result, the IGF-I signaling cascade has been studied as a potential therapeutic target in patients with multiple myeloma, either by interfering with IGF-I receptor (IGF-IR) activity or by inhibiting downstream effectors (7, 12, 13). Multiple myeloma cells are characterized by a very high overall level of protein synthesis due to production of a monoclonal immunoglobulin (14). This intense protein synthesis is associated with high baseline endoplasmic reticulum (ER) stress, which predisposes multiple myeloma cells to therapeutic intervention at the level of protein folding and/or degradation through the induction of an excessive unfolded protein response (UPR; refs. 15, 16). Of interest, the induction of Xbp1 splicing, a differentiation and stress response factor activated during UPR, has been reported to be associated with myeloma pathogenesis in a...
IGF-I and Bortezomib in Myeloma

Translational Relevance

The proteasome inhibitor bortezomib is used in the clinic for the treatment of multiple myeloma, a disease in which the insulin-like growth factor type I (IGF-I) plays an important role. We conducted a study to elucidate the combined effects of bortezomib and IGF-I on multiple myeloma cells in vitro, in vivo, and ex vivo and showed that IGF-I enhances the cytotoxic effect of bortezomib as well as other proteasome inhibitors on multiple myeloma cells. These results could be used to develop association strategies in patients with multiple myeloma to increase therapeutic response.

murine transgenic model (17). In addition, activation of the ER pathway has been suggested to be required for survival of multiple myeloma cells (18). Thus, myeloma represents an interesting model of a neoplastic disease in which there is an intrinsically hyperactive metabolic pathway that constitutes a potential therapeutic target.

Therapeutic targeting of protein metabolism in cancer has essentially consisted of strategies aiming to inhibit protein synthesis, for example by inhibiting mTOR (19). Proteasomal inhibitors constitute the first class of agents specifically altering protein catabolism to have been introduced in the clinic, with spectacular results in patients with multiple myeloma (20). Bortezomib is a boronic acid dipeptide inhibitor of the 26S proteasome (21). It has become an essential component in the treatment of patients with multiple myeloma and is now indicated for first-line therapy (20, 22–24). Bortezomib downregulates the expression of several antiapoptotic factors and induces caspase-dependent apoptosis both in multiple myeloma cell lines and ex vivo in cells from patients with multiple myeloma (25–29). Bortezomib has also been found to affect the bone marrow microenvironment, reducing the production of growth and survival factors (30, 31). Although the mechanisms of cytotoxicity of proteasome inhibitors in multiple myeloma cells have not been definitely determined, the two main mechanisms described to date include inhibition of the NF-kB pathway, which is abnormally activated in myeloma (32) as well as a terminal UPR due to enhanced ER stress (15). Other related therapeutic targets include protein chaperones associated with increased protein misfolding (33), ubiquitin binding enzymes (34), and histone deacetylases that are involved both in gene expression and protein degradation through shuttling of polyubiquitinated misfolded proteins (35).

In this study, we examined the effects of IGF-I on the cytotoxicity of bortezomib in human multiple myeloma cell lines, fresh multiple myeloma cells from patients, and in a murine xenotransplant model. We analyzed the effects of this combination on the mTOR pathway, on the content of pro- and antiapoptotic proteins, as well as on the ER stress and occurrence of a UPR. Our results suggest that IGF-I signaling enhances the cytotoxicity of proteasome inhibitors in multiple myeloma cells via several mechanisms, including the induction of a UPR.

Materials and Methods

Reagents

Human recombinant IGF-I was generously provided by Terica Inc. Bortezomib was obtained from Janssen Cilag, MG115 (C6706), MG132 (C2211), PSI (S3922), epoxomicin (ES652), tunicamycin (T7765), thapsigargin (T9033), PP2424 (P0037), and cycloheximide were obtained from Sigma-Aldrich. The monoclonal antibody directed against IGF-IR (CD221) was obtained from Roche. Polyclonal antibodies and dilutions used in this study are described in Supplementary Table S1. The Annexin-V-FITC/PI staining kit was obtained from Roche, 3,3′-Dihexyloxacarbocyanine iodide [DiOC6(3)] was from Invitrogen, and the 2OS Proteasome Assay Kit was from Cayman Chemical Company.

Multiple myeloma cell lines and survival assays

Human multiple myeloma cell lines MM.1S, LP1, RPMI8226, U266, and AMO-1 were cultured in RPMI-1640 + 1-glutamine (Invitrogen) containing 10% FBS and 1% penicillin–streptomycin (Invitrogen) at 37°C in humidified 95% air and 5% CO2. Bortezomib-resistant cells were developed in our laboratory by prolonged in vitro exposure to incrementally increased concentrations of bortezomib for 6 weeks.

In vitro cell survival in the presence of various compounds was estimated using the MTT assay (Sigma-Aldrich) as described elsewhere (36). Cells (30,000/well) were cultured for 72 hours in the presence of various concentrations of proteasome inhibitors and a fixed concentration of 200 ng/mL of IGF-I before assessment of cell survival.

RNA interference assays

A desalted duplex siRNA-targeting GADD153 (5′-GGUGAUGAGGAUCUGCAGGAU-3′) and a corresponding scrambled sequence were obtained from Sigma-Aldrich. MM.1S cells (2 × 10⁶ cells/mL) were transfected with siRNAs (200 nmol/L) using sonoporation as previously described (37). Cells were incubated for 48 hours before protein analyses were conducted by Western blot analysis.

Western blot analysis

Cells (1 × 10⁷) were exposed to 1.5 nmol/L of bortezomib, 200 ng/mL of human recombinant IGF-I, or both for different times (1, 4, 8, 16, 24, 48, 72, or 96 hours). Proteins were extracted and analyzed by Western blot analysis as described elsewhere (38) using 50 μg of proteins per lane and β-actin as internal control for loading.

Pan-genomic analyses

Total RNA from MM.1S cells was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations and subsequently quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was verified using the BioAnalyzer 2100...
Using the formula and tumor volume was measured every 4 days and derived (mg/kg). All treatments were carried out intraperitoneally alone (0.5 mg/kg), and one group was treated with a combination of bortezomib (0.5 mg/kg) and IGF-I (0.03 mg/kg), one group was treated with bortezomib alone (0.03 mg/kg), one group was treated with vehicle, one group was treated with IGF-I alone (0.5 mg/kg), and one group was treated with a combination of bortezomib (0.5 mg/kg) and IGF-I (0.03 mg/kg). All treatments were carried out intraperitoneally once a week for 4 weeks. Treatments were started on day 1, and tumor volume was measured every 4 days and derived using the formula \[ V = \frac{4}{3} \pi r^3 \] (\( r \), radius). Mice were euthanized when tumor volume reached 2,000 mm\(^3\). The tumors were carefully excised and used for the study of apoptosis, or between cells exposed to bortezomib and exposed cells, or between cells exposed to 15 nmol/L of bortezomib, 200 ng/mL of IGF-I, or bortezomib + IGF-I for 15, 30, 120, or 360 minutes. After incubation, cells were washed with 200 mL of the 20S proteasome assay buffer and incubated for 30 minutes with 100 mL of proteasome lysis buffer. The plate was centrifuged at 1,000 \( \times g \) for 10 minutes. Supernatant (90 mL) was transferred from each well to a black 96-well plate and mixed with 10 mL of the substrate solution. After incubation at 37 \(^\circ\)C for 1 hour, the fluorescence intensity was read on a Chameleon II microplate reader (Hidex) with excitation at 360 nm and emission at 480 nm.

Statistical analyses
The statistical significance of the data was determined with a Student \( t \)-test. \( P < 0.05 \), \( P < 0.001 \), and \( P < 0.0001 \) indicate a statistically significant (‘‘), very significant (‘‘’’), and highly significant difference (‘‘‘‘’’’, respectively. Student \( t \)-test was used to identify differences between unexposed and exposed cells, or between cells exposed to bortezomib alone or in combination with IGF-I. No correction for multiple testing was carried out.

Results

IGF-I potentiates the apoptotic effects of bortezomib and other proteasome inhibitors in vitro, ex vivo, and in vivo
We exposed 5 different human multiple myeloma cell lines to the proteasomal inhibitor bortezomib. As shown in Fig. 1A, the addition of recombinant human IGF-I to bortezomib potentiates activity of bortezomib on cell viability. This effect was observed with the 4 IGF-IR-positive human multiple myeloma cell lines MM1.S, LP-1, U266, and RPMI8226, but not with the IGF-IR-negative AMO.1 cell line. Multiple myeloma cells incubated with 200 mg/mL of IGF-I alone did not show any modification in cell viability (data not shown).

IGF-I also sensitized MM.1S and U266 cells to the antiproliferative effect of other proteasome inhibitors (MG-115, MG132, PSI, and epoxomicin; Fig. 1B). Sensitization was not dependent on the level of gene or protein expression of IGF-IR in these tumor cell lines.
Cells were incubated for 24 hours with 10 nmol/L bortezomib and IGF-I (200 ng/mL). Graphs show the percentage of annexin V-suspended in 200
1.5 nmol/L bortezomib, 200 ng/mL IGF-I, bortezomib myeloma cells under different conditions. F, antibodies directed against CD38, CD138, and CD45. Graphs show the percentage of annexin V
myeloma samples. Results shown are representative of three separate experiments for cell lines
in vitro. Cells were cultured in 96-well plates for 72 hours in the presence of various concentrations of bortezomib alone (●) or with a fixed concentration of 200 ng/mL IGF-I (■); LP1, MM.1S, U266, and RPMI8226 express IGF-IR. AMO-1 cell line does not express IGF-IR. Data are mean values from three independent experiments; bars are SD. C, anti-IGF-IR antibody reverses the effect of IGF-I on the MTT reducing activity of bortezomib in MM.1S cells in vitro. Cells were cultured in 96-well plates for 72 hours in the presence of bortezomib (1 nmol/L), IGF-I (200 ng/mL), and/or IL-6 (10 ng/mL). Data are mean values from 3 independent experiments; bars are SD. D, IL-6 does not sensitize MM.1S cells to bortezomib in vitro. Graphs represent MTT reducing activity obtained from MTT assay after incubation of cells 72 hours in the presence of bortezomib (1 nmol/L), IGF-I (200 ng/mL), and/or IL-6 (10 ng/mL). Data are mean values from 3 independent experiments; bars are SD. E, IGF-I potentiates the apoptotic effect of bortezomib on fresh samples from 9 patients with multiple myeloma at diagnostic. Cells were cultured with RPMI-1640 in 24-well plates at 37° C with 1.5 mmol/L bortezomib, 200 ng/mL IGF-I, bortezomib + IGF-I, or bortezomib + IGF-I + 10 µg/mL anti-IGF-IR antibody. Plasma cells were gated with monoclonal antibodies directed against CD38, CD138, and CD45. Graphs show the percentage of annexin V-positive cells after incubation of human multiple myeloma cells under different conditions. F, in vivo effect of IGF-I and bortezomib in a SCID mice model of human MM.1S cells. A total of 3 × 10^6 cells were suspended in 200 µL and injected subcutaneously into the right flank of the mice on day 0, and mice were treated once a week for 4 weeks as indicated. Each group contained 5 mice. ○, untreated controls; ■, IGF-I 0.03 mg/kg; ●, bortezomib 0.5 mg/kg; ◇, bortezomib 0.5 mg/kg + IGF-I 0.03 mg/kg. These are the results of 1 of 2 similar experiments. G, cytotoxicity of the IGF-I + bortezomib combination in MM.1S and a bortezomib-resistant MM.1S cell line (MM.1S bortezomib-resistant). Cells were incubated for 24 hours with 10 nmol/L bortezomib and IGF-I (200 ng/mL). Graphs show the percentage of annexin V-positive cells after incubation of MM.1S cells in different conditions.

Figure 1. IGF-I potentiates MTT reducing activity of bortezomib and other proteasome inhibitors against multiple myeloma cell lines and fresh human multiple myeloma samples. Results shown are representative of three separate experiments for cell lines in vitro and two experiments for the in vivo model. *, P < 0.05; **, P < 0.01; and $$$, P < 0.001 as determined by Student t test. A, IGF-I potentiates MTT reducing activity effect of bortezomib against four human myeloma cell lines in vitro. Cells were cultured in 96-well plates for 72 hours in the presence of various concentrations of bortezomib alone (●) or with a fixed concentration of 200 ng/mL IGF-I (■); LP1, MM.1S, U266, and RPMI8226 express IGF-IR. AMO-1 cell line does not expresses IGF-IR. Data are mean values from three independent experiments; bars are SD. B, IGF-I potentiates MTT reducing activity of other proteasome inhibitors in MM.1S and U266 cells in vitro.
The toxicity of bortezomib on fresh human multiple myeloma cells was also evaluated by FACS analysis with Annexin V staining. Multiple myeloma cells were gated on the basis of CD38 and CD138 positivity. In a series of samples obtained from 9 patients with multiple myeloma at diagnosis, exposure to IGF-I alone slightly reduced the percentage of Annexin V–positive cells, whereas bortezomib increased this percentage by 15% (Fig. 1E). Apoptosis in cells exposed to bortezomib in combination with IGF-I was significantly greater than that of cells exposed to bortezomib alone (P < 0.001), and this was partially reversed by the anti-IGF-IR antibody.

To determine whether IGF-I enhanced the antitumor effects of bortezomib against multiple myeloma in vivo, we studied human multiple myeloma xenograft models in SCID mice using MM.1S and LP1 cells. Although bortezomib as a single agent significantly delayed tumor growth, the largest delay in tumor growth was observed in animals having received the combination of IGF-I and bortezomib (Figs. 1F and Supplementary Fig. S2). For MM.1S-carrying mice, animals in the control group, the IGF-I group, the bortezomib group, and the IGF-I + bortezomib group were euthanized on days 45, 53, 68, and 107, respectively. This experiment was carried out twice on groups of 5 mice. These results show that recombinant IGF-I enhances the antmyeloma effect of bortezomib in vivo in this model.

Furthermore, exposure of a bortezomib-resistant MM.1S variant (MM.1S bortezomib-resistant) to IGF-I allowed partial sensitization to 100 nmol/L of bortezomib (Fig. 1G), a concentration that induced maximal toxicity in the sensitive parental line.

**Effect of IGF-I on apoptotic signaling induced by bortezomib and cell-cycle distribution in multiple myeloma cells**

To determine whether IGF-I could influence apoptotic signaling in MM.1S and U266 cells, we studied the content of proapoptotic and antiapoptotic proteins by Western blot analysis. The addition of IGF-I to bortezomib significantly increased the content of proapoptotic proteins Bax, Bad, Bak, and BimS in both cell lines (Fig. 2A). Furthermore, the content of antiapoptotic proteins Bcl-2, Bcl-XL, Bfl1, XIAP, and survivin was lower in MM.1S and U266 cells exposed to the combination of bortezomib and IGF-I than in those exposed to bortezomib alone (Fig. 2B). The expression of Mcl-1 did not vary between the different exposures (Supplementary Fig. S3). Of note, exposure to IGF-I alone tended to generate prosurvival signaling with decreased Bax, Bad, and Bak and increased Bcl-2, Bcl-xL, A1/Bfl-1, and XIAP specifically on MM.1S cell line. The analysis of effector proteins in MM.1S cells showed that the combination induced a greater content of cleaved PARP and cleaved caspase-3 than bortezomib alone, with a similar content of cleaved caspase-8 (Fig. 2C). Cell-cycle analysis of MM.1S cells by flow cytometry showed that IGF-I increased the proportion of cells in S-phase, whereas bortezomib decreased the percentage of cells in S-phase and increased the G0–G1 population, an effect which was not antagonized but rather increased by the addition of IGF-I (Fig. 2D). However, the combination of both agents was associated with a greater content in p21 protein than that observed after exposure to bortezomib alone (Fig. 2E). Finally, the expression of c-Myc increased in cells exposed to bortezomib and this increase was inhibited by the coexposure to IGF-I (Supplementary Fig. S3).

**Effect of bortezomib and IGF-I on the NF-κB pathway in multiple myeloma cells**

Given previous reports implicating the role of NF-κB signaling in the antimyeloma effect of bortezomib (29), we determined whether IGF-I increased the effect of bortezomib on the NF-κB pathway in MM.1S cells. We found that the content of IkBa, an inhibitor of nuclear translocation of p65, was upregulated by IGF-I and downregulated by bortezomib, with a more pronounced reduction in presence of the combination of IGF-I and bortezomib (Supplementary Fig. S4A). This was associated with a concurrent increase in pIkBα. However, p65 and phospho-p65 content remained unchanged and nuclear translocation of NF-κB protein p65 was not significantly modified by the addition of IGF-I to bortezomib (Supplementary Fig. S4B). To evaluate the transcriptional activity of NF-κB, we conducted a pan-genomic analysis of MM.1S cells exposed to bortezomib in the presence or absence or IGF-I and did not observe any variation in the 11 genes of the NF-κB score previously described (32) nor in other NF-κB–related genes (Supplementary Table S2). These results suggest that the potentiating effect of bortezomib by IGF-I does not involve NF-κB signaling.

**Effect of IGF-I alone and in combination with bortezomib on proteasomal activity and protein synthesis**

The proteasome 26S subunit controls the degradation of unfolded proteins. The inhibition of proteasome 26S by proteasome inhibitors may increase the accumulation of misfolded proteins, resulting in enhanced ER stress (15). We examined the effect of IGF-I on protein synthesis and proteasomal activity in MM.1S and U266 cells exposed to IGF-I, bortezomib, or their combination in vitro. IGF-I was found to increase the synthesis of the monoclonal component, an effect that was not inhibited by bortezomib (Fig. 3A). IGF-I increased the content of phospho-P70S6K and phospho-AKT, an observation consistent with increased protein synthesis, and this effect was enhanced by bortezomib (Fig. 3B). The effect of IGF-I on proteasomal activity...
was biphasic with an initial decrease during the first 30 minutes followed by an important rebound effect (Fig. 3C). Bortezomib alone or combined with IGF-I were associated with reduced proteasomal activity. Thus, IGF-I did not seem to alter the inhibitory effect of bortezomib on the proteasome. To determine the importance of protein synthesis on the effect of IGF-I on bortezomib, cells were exposed to cycloheximide, a protein synthesis inhibitor, concurrently with bortezomib and IGF-I. Coincubation with 1 or 10 μmol/L of cycloheximide effectively prevented the enhancement of the cytotoxicity of bortezomib by IGF-I, thus supporting the role of protein synthesis (Fig. 3D).

Bortezomib has been shown to synergize with PP242, which is a selective inhibitor of TORC1 and TORC2 (40). Because mTORC1 activation induces phosphorylation of P70S6K, we studied this protein as a marker for mTORC1 activity (13, 19). PP242 suppressed the effect of IGF-I on the phosphorylation of P70S6K (Fig. 3E) and the effect of IGF-I on the apoptosis induced by bortezomib (Fig. 3F) in MM.1S and U266 cells.

These results suggest that IGF-I as a single agent stimulates protein turnover by increasing both protein synthesis and degradation in multiple myeloma cells. Conversely, the combination of IGF-I with bortezomib is associated with increased protein synthesis and reduced proteasomal degradation, which could lead to enhanced ER stress.

**IGF-I increases ER stress and potentiates the UPR caused by bortezomib**

To determine the level of UPR in MM.1S and U266 cells exposed to bortezomib and IGF-I, we conducted Western blot analysis of ER stress–associated proteins GRP-78 (Bip), GADD153 (CHOP), and spliced Xbp1. GADD153 (CHOP) was strongly increased by the combination of bortezomib and IGF-I as compared with one of the compounds alone in both U266 and MM.1S cells, and the same was observed for
spliced Xbp1 in MM.1S cells (Fig. 4A). A kinetic study of these parameters showed that each of the agents could induce ER stress but that the combination caused earlier and more profound ER stress (Fig. 4B). Interestingly, IGF-I induced a UPR response alone on MM.1S after 16 hours of incubation, which might explain why pharmacologic doses displayed a slight antitumor effect in vivo in the SCID model. The baseline content of the expression of GRP-78 (Bip) remained unchanged, possibly due to the long half-life of this protein. A similar effect on GADD153 (CHOP) was observed on a fresh myeloma sample (Fig. 4C).

To determine whether the UPR was required for IGF-I–mediated enhancement of the cytotoxicity of bortezomib, we transfected siRNA directed against GADD153 into MM.1S cells. This transfection efficiently decreased GADD153 (CHOP) protein expression (Supplementary Fig. S6). Forty-eight hours after transfection with siRNA, cells were exposed to IGF-I and/or bortezomib for another 24 hours before apoptosis was analyzed by flow cytometry. Cells transfected with siRNA against GADD153 did not show enhancement of sensitivity to bortezomib by IGF-I, whereas cells transfected with scrambled siRNA were sensitized, confirming that GADD153 was required for the

Figure 3. The mTOR pathway regulates the cytotoxicity effect of the combination of bortezomib + IGF-I. Results shown are representative of three separate experiments (B and E) and mean values from three independent experiments ± SD (A, C, D, and F). A, mean fluorescence intensity (MFI) of monoclonal lambda chain component as determined by FACS analysis in MM.1S cells exposed to IGF-I (200 ng/mL) and bortezomib (1.5 nmol/L) alone or in combination for 8 hours. B, protein content of AKT, phospho-AKT, P70S6K, and phospho-P70S6K analyzed by immunoblotting in MM.1S and U266 cells exposed for 1 hour to IGF-I (200 ng/mL) and bortezomib (1.5 nmol/L) alone or in combination. Time- and concentration-dependent induction of phospho-AKT is shown in Supplementary Fig. S5. C, kinetic study of proteasome activity in MM.1S cells. Cells were exposed to IGF-I (200 ng/mL) and bortezomib (1.5 nmol/L) alone or in combination. The fluorescent intensity was read with excitation at 360 nm and emission at 480 nm. D, effect of cycloheximide on apoptotic inducing activity of bortezomib + IGF-I in MM.1S. Cells were exposed to IGF-I (200 ng/mL), bortezomib (1.5 nmol/L), and different concentrations of cycloheximide (0, 0.1, 1, and 10 μmol/L) alone or in combination for 24 hours. Graphs show the percentage of Annexin V–positive cells after incubation of MM.1S cells in different conditions. E, protein content of phospho-p70S6K analyzed by immunoblotting in MM.1S cells exposed to 100 nmol/L of pp242 for 1 hour followed by 15 minutes of exposure to 200 ng/mL of IGF-I. F, inhibition of the effect of IGF-I on bortezomib cytotoxicity in MM1.S cells by mTOR inhibitor PP242. Graphs show the percentage of Annexin V–positive cells after incubation of MM.1S cells with IGF-I (20 ng/mL), bortezomib (1.5 nmol/L), and PP242 (100 nmol/L) alone or in combination for 24 hours.

To determine whether the UPR was required for IGF-I–mediated enhancement of the cytotoxicity of bortezomib, we transfected siRNA directed against GADD153 into MM.1S cells. This transfection efficiently decreased GADD153 (CHOP) protein expression (Supplementary Fig. S6). Forty-eight hours after transfection with siRNA, cells were exposed to IGF-I and/or bortezomib for another 24 hours before apoptosis was analyzed by flow cytometry. Cells transfected with siRNA against GADD153 did not show enhancement of sensitivity to bortezomib by IGF-I, whereas cells transfected with scrambled siRNA were sensitized, confirming that GADD153 was required for the
potentiating effect (Fig. 4D). These data suggest that the potentiation of the cytotoxicity of proteasome inhibitor by IGF-I involves enhanced ER stress and requires an UPR.

Discussion

‘Priming’ of tumor cells is a strategy consisting of the sensitization of tumor cells to anticancer agents by a specific growth factor. This approach is currently evaluated in the treatment of acute myeloid leukemia but not yet in myeloma (41). Proteasome inhibitors such as bortezomib constitute a novel and original family of anticancer agents. In the current study, we report that IGF-I, a bona fide growth factor for multiple myeloma cells, potently sensitizes these cells to proteasome inhibitor–induced decrease in cell survival. This effect was observed on cell lines in vitro, on xenografts in vivo, as well as on fresh human myeloma cells ex vivo. In addition, this potentiation was observed with other proteasome inhibitors, suggesting a common mechanism involving protein degradation. The mechanisms of cytotoxicity of bortezomib against cancer cells seem to be complex, as recently suggested by a genome-wide screen of potential modulators that involve protein translation or DNA damage repair, among other pathways (42). In our experiments, we found that the addition of IGF-I to bortezomib potently increased proapoptotic signaling and the UPR, explaining the specificity of the potentiation of bortezomib by IGF-I.

Activation of the NF-κB pathway has been suggested to be important for the survival of both normal and neoplastic plasmocytes (43). More than 50% of multiple myeloma cell lines and most primary samples have a high level of NF-κB activity, as shown by the transcription signature of 11 genes (44). Abnormal NF-κB activity may be due to extrinsic signaling involving BAFF and APRIL or may be due to mutations in the NF-κB pathway (32). In addition, several studies have shown that treatment resistance in multiple myeloma is related to increased activation of NF-κB, whereas inhibition of NF-κB-activation by specific inhibitors or by blocking the activator of this pathway may reduce resistance to treatment (13, 45). NF-κB activates genes involved in proliferation, cytokine synthesis, and adhesion molecules such as ICAM1 and VCAM1 (30) and regulates...
antiapoptotic proteins. In our study, the addition of IGF-I to bortezomib reduced the content of IκBα with a simultaneous increase of phosphorylated IκBε but was not associated with an increase in NF-κB signaling. A pan-genomic gene expression study failed to show an increase in NF-κB transcriptional activity, with no significant variations in any of the 11 genes of the "NF-κB index" previously reported by Annunziata and colleagues (32). Overall, our data suggest that the effect of IGF-I on the cytotoxicity of bortezomib in this model does not involve an effect on NF-κB signaling.

IGF-I strongly enhanced the apoptotic signaling consecutive to bortezomib exposure, with enhanced content of the proapoptotic proteins Bax, Bad, Bak, and BimS and decreased content of the antiapoptotic proteins Bcl-2, Bcl-XL, XIAP, Bfl-1, and survivin. Conversely, IGF-I alone provided a prosurvival signal with reduced content of Bax and increased content of Bcl-2, Bcl-XL, and XIAP. Mcl-1, a key regulator of survival of multiple myeloma cells, remained unchanged under our experimental conditions. Overall, addition of IGF-I to cells exposed to bortezomib strongly enhanced the apoptotic signaling of bortezomib. IGF-I also enhanced mitochondrial membrane depolymerization as well as caspase-3 and PARP cleavage induced by bortezomib in multiple myeloma cells.

The UPR has come to be considered as a potential therapeutic target in cancer as it may lead to cell death in response to increased ER stress. Although UPR has been described in several models (15), multiple myeloma cells seem to be particularly sensitive to increased ER stress due to the high level of protein synthesis associated with monoclonal component production. It has been shown that proteasome inhibitors induced ER stress resulting in a UPR. Although there is no single unequivocal marker of UPR, results obtained in our model showing increased content of GADD153 (CHOP) protein and spliced Xbp1 mRNA suggest that the combination of IGF-I and bortezomib potently increases ER stress by increasing protein synthesis, whereas blocking protein degradation leads to an enhanced UPR (Fig. 5). This observation confirms the differences in the role of GADD153 in multiple myeloma cells and primary B cells (15, 46). Furthermore, we observed that inhibition of GADD153 protein using a specific siRNA inhibited the potentiation of bortezomib by IGF-I, suggesting that the UPR response is required for this effect. Our results suggest that it is possible to specifically sensitize tumor cells to proteasome inhibitors by increasing the baseline level of ER stress. This can be assimilated to a tumor cell priming effect specific for cells with a high level of ER stress, such as multiple myeloma cells. Finally, IGF-I increases the frequency of multiple myeloma cell cycling, and this might play a role in the increased cytotoxicity of bortezomib when it is associated with IGF-I because bortezomib also modifies the cell cycle in cells that subsequently undergo cell death.

In conclusion, our results support the hypothesis that IGF-I sensitizes multiple myeloma cells to the cytotoxic activity of proteasome inhibitors, such as bortezomib, as a consequence of the enhanced level of ER stress and the induction of an UPR. This is in contrast to the protective effect of IGF-I against certain agents, such as dexamethasone, previously reported in the literature and that we have
confirmed in our study. This observation raises the question of the impact of local concentrations of IGF-I in the bone marrow environment in patients receiving bortezomib therapy for multiple myeloma. Another aspect concerns the possibility of evaluating the combination of recombinant IGF-I and bortezomib in patients who have developed resistance to bortezomib, as recombinant IGF-I is currently used in the clinic for the treatment of growth deficiency. More generally, these results suggest that tumor “growth factors” may have unexpected effects in the therapeutic setting, including those of potentiating the cytotoxicity of anticancer agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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