Therapeutic enhancement of ER stress with Insulin Like Growth Factor 1 (IGF-1) sensitizes myeloma cells to proteasomal inhibitors

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Translational relevance

The proteasome inhibitor bortezomib is used in clinic for the treatment of multiple myeloma (MM), a disease in which the insulin-like growth factor type 1 (IGF-1) is playing an important role. We performed a study to elucidate the combined effects of bortezomib and IGF-1 on MM cells in vitro, in vivo and ex vivo and showed that IGF-1 enhances the cytotoxic effect of bortezomib as well as other proteasome inhibitors on MM cells. These results can be used to develop association strategies in MM patients in order to increase therapeutic response.
Abstract

Purpose: Multiple myeloma (MM) 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 1 (IGF-1). Bortezomib, the first-in-class proteasome inhibitor, has displayed significant antitumor activity in MM.

Experimental design: We analyzed the impact of IGF-1 combined with proteasome inhibitors on MM cell lines in vivo and in vitro as well as on fresh human myeloma cells.

Results: Our study shows that IGF-1 enhances the cytotoxic effect of proteasome inhibitors against myeloma cells. The effect of bortezomib on the content of pro-apoptotic proteins such as Bax, Bad, Bak and Bim S and anti-apoptotic proteins such as Bcl-2, Bcl-XL, XIAP, Bfl-1 and survivin was enhanced by IGF-1. The addition of IGF-1 to bortezomib had minor effect on NF-κB signalling 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-1 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-1, which possesses a “priming” effect on myeloma cells for this family of compounds.
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 (MM) 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 1 (IGF-1) and have prompted the evaluation of targeted therapies aiming to block the activity of these proteins (2, 3). IGF-1 has been shown to behave as an important growth factor for MM cells (4). IGF-1 signalling in these cells involves the PI3-kinase/Akt pathway with downstream activation of mTOR/P70S6K pathways and has also been shown to activate NF-κB signalling (5-8). In vivo and in vitro studies have shown that IGF-1 increases the content of anti-apoptotic proteins such as Bcl-2, Bcl-XL, cIAP-1, cIAP-2 and FLIP, and decreases the content of pro-apoptotic proteins such as caspase 3, caspase 8 and caspase 9 in MM cells thus protecting them from drug-induced apoptosis (9, 10). In addition, serum IGF-1 concentrations have been reported to be associated with prognosis in myeloma patients (11). As a result, the IGF-1 signaling cascade has been studied as a potential therapeutic target in patients with MM, either by interfering with IGF-1 receptor (IGF-1R) activity or by inhibiting downstream effectors (7, 12, 13).

MM 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 MM cells to therapeutic intervention at the level of protein folding and/or degradation through the induction of an excessive unfolded protein response (UPR) (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 murine transgenic model (17).
addition, activation of the ER pathway has been suggested to be required for survival of MM cells (18). Thus, myeloma represents an interesting model of a neoplastic disease in which there is an intrinsically hyperactive metabolic pathway which constitutes a potential therapeutic target.

Therapeutic targeting of protein metabolism in cancer has essentially consisted in strategies 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 MM (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 MM and is now indicated for first line therapy (20, 22-24). Bortezomib down-regulates the expression of several antiapoptotic factors and induces caspase-dependent apoptosis both in MM cell lines and ex vivo in cells from MM patients (25-29). Bortezomib has also been found to affect the bone marrow microenvironment, reducing the production of growth and survival factors (30, 31). While the mechanisms of cytotoxicity of proteasome inhibitors in MM cells have not been definitely determined, the two main mechanisms described to date include inhibition of the NF-κB 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 which 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-1 on the cytotoxicity of bortezomib in human MM cell lines, fresh MM cells from patients and in a murine xenotransplant model. We analysed 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 an UPR. Our results
suggest that IGF-1 signalling enhances the cytotoxicity of proteasome inhibitors in MM cells via several mechanisms, including the induction of an UPR.
Material and Methods

Reagents

Human recombinant IGF-1 was generously provided by Tercica Inc (CA, USA). Bortezomib was obtained from Janssen Cilag (Issy-Les-Moulineaux, France), MG115 (C6706), MG132 (C2211), PSI (S3922), epoxomicin (E3652), tunicamycin (T7765), thapsigargin (T9033), PP2424 (P0037) and cycloheximide were obtained by Sigma-Aldrich (St Quentin Fallavier, France). The monoclonal antibody directed against IGF-1R (CD221) was from BD Biosciences. Polyclonal antibodies and dilutions used in this study are described in Supplementary Table 1. Annexin-V-FLUOS staining kit was obtained from Roche (Meylan, France), 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)) from Invitrogen (Cergy Pontoise, France) and the 20S proteasome assay kit from Cayman Chemical Company (Ann Arbor, MI, USA).

MM cell lines and survival assays

Human MM cell lines MM.1S, LP1, RPMI8226, U266 and AMO-1 were cultured in RPMI1640 + L-Glutamine (Invitrogen, Cergy Pontoise, France) containing 10% fetal bovine serum and 1% penicillin streptomycin (Invitrogen) at 37 °C in humidified 95% air and 5% CO₂. Bortezomib-resistant cells (BR) 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 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma Aldrich, St Louis, USA) as described elsewhere (36). Cells (30,000 per well) were cultured for 72 h in the presence of various concentrations of proteasome inhibitors and a fixed concentration of 200 ng/mL of IGF-1 before assessment of cell survival.
RNA interference assays

A desalted duplex siRNA targeting GADD153 (5'-GGUAUGAGGAUCUGCAGGAUU-3’) and a corresponding scrambled sequence were obtained from Sigma-Aldrich (St Quentin Fallavier, France). MM.1S cells (2.10⁶ cells/mL) were transfected with siRNAs (200 nM) using sonoporation as previously described (37). Cells were incubated for 48 h before protein analyses was performed by western blot.
Western blot analysis

Cells (1.10^7) were exposed to 1.5 nM bortezomib, 200 ng/mL human recombinant IGF-1 or both for different times (1, 4, 8, 16, 24, 48, 72 or 96 hours). Proteins were extracted and analysed by western blot as described elsewhere (38) using 50 µg of proteins per lane and β-actin as internal control for loading.

Pangenomic analyses

Total RNA from MM.1S cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations and subsequently quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was verified using the BioAnalyser 2100™ (Agilent Technologies, Palo Alto, CA, USA). One-color labeled cRNAs were generated from 200 ng of total RNA using the Low RNA Input Amplification Kit (Agilent Technologies) according to the instructions of the manufacturer. Labeled cRNA were hybridized overnight to Whole Human Genome 4 x 44K microarrays. After washing, microarrays were scanned using the Agilent model G2505B microarray scanner and data extracted by Feature Extraction software (version 9.5). The default settings were used to scan the microarrays. The quantile normalization method was used to normalize microarray data.

Animals and xenotransplant experiments

Female SCID mice (4 weeks old, Charles River, Arbresle, France) were housed and treated according to local institutional guidelines. Before initiating the experiment, we acclimatized all mice to a pulverized diet for one week. None of the mice exhibited any lesions and all were tested pathogen-free. Our experimental protocols were reviewed and approved by the Animal Ethics Committee of the University of Lyon. For xenotransplant
experiments, $3.10^6$ cells from subconfluent cultures were suspended in 200 µL and injected subcutaneously into the right flank of the mice on day 1. Mice were then randomly divided into 4 groups containing each 5 mice: one control group receiving vehicle, one group treated with IGF-1 alone (0.03 mg/Kg), one group treated with bortezomib alone (0.5 mg/Kg), and one group treated with a combination of bortezomib (0.5 mg/Kg) and IGF-1 (0.03 mg/Kg). All treatments were performed 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 $V = 2/3 \pi r^3$ (r: radius). Mice were euthanized when tumor volume reached 2,000 mm$^3$. The tumors were carefully excised and used for the study of protein expression by western blot.

Flow cytometry analysis

For FACS assays, cells were incubated as indicated and analyzed on a FACS Canto II flow cytometer (BD Biosciences Europe, Erembodegem, Belgium). Data analysis was performed with Modfit LT 2.0™ software (Veritysoftware Inc, Topsham, USA). For apoptosis, incubated cells were washed with PBS, suspended in 100 µL Annexin buffer with 1 µl Annexin-V FITC and incubated for 15 minutes at room temperature in the dark before FACS analysis. For cell cycle determination, incubated cells were washed with PBS and incubated in 800 µl of propidium iodide solution (0.05 mg/mL) containing Nonidet-P40 (0.05%) in the dark for 1 h at 4 °C before FACS analysis. For light chain expression, incubated cells were washed with PBS, fixed, permeabilized and incubated for 15 minutes at room temperature with monoclonal antibody anti-lambda (FR481, Dako, Trappes, France) before FACS analysis.

Analysis of fresh human myeloma samples
Fresh bone marrow samples were obtained from 9 MM patients at diagnosis after having obtained written informed consent. This study was approved by the Lyon ethics committee. Erythrocytes were lysed for 10 minutes at room temperature using Lysing Buffer (BD Biosciences) and washed cells were suspended in 300 µL of PBS. Cells were cultured with RPMI 1640 in 24-well plates (Costar, NY, USA) at 37 °C with 15 nM bortezomib, 200 ng/mL IGF-1, bortezomib + IGF-1 or bortezomib + IGF-1 + 10 µg/mL anti-IGF-1R antibody. After 24 h, cells were washed and incubated with monoclonal antibody directed against CD38, CD138 and CD45, washed, suspended in 100 µL Annexin buffer with 1 µL Annexin-V FITC and analysed by flow cytometry as described above.

**Proteasome 20S activity**

Cells (5.10^5 cells/well) were incubated overnight in 100 µL of culture medium in a 96 well plate and thereafter exposed to 15 nM bortezomib, 200 ng/mL IGF-1 or bortezomib + IGF-1 for 15, 30, 120 or 360 minutes. After incubation, cells were washed with 200 µL of the 20S proteasome assay buffer and incubated for 30 min with 100 µL proteasome lysis buffer. The plate was centrifuged at 1,000 g for 10 min. Supernatant (90 µL) was transferred from each well to a black 96 well plate and mixed with 10 µL of the substrate solution. After incubation at 37 °C for 1 h, the fluorescence intensity was read on a Chameleon II microplate reader (Hidex, Finland) with excitation at 360 nm and emission at 480 nm.

**Statistical analyses**

The statistical significance of the data was determined with a Student's t-test. \( p < 0.05 \), \( p < 0.001 \) and \( p < 0.0001 \) indicate a statistically significant (*), very significant (**) and highly significant difference (***) respectively. Student's t-test was used to identify
differences between unexposed and exposed cells, or between cells exposed to bortezomib alone or in combination with IGF-1. No correction for multiple testing was performed.
Results

IGF-1 potentiates the apoptotic effects of bortezomib and other proteasome inhibitors in vitro, ex vivo and in vivo

We exposed five different human MM cell lines to the proteasomal inhibitor bortezomib. As shown in Figure 1A, the addition of recombinant human IGF-1 to bortezomib potentiates activity of bortezomib on cell viability. This effect was observed with the four IGF-1R positive human MM cell lines MM1.S, LP-1, U266 and RPMI8226, but not with the IGF-1R negative AMO.1 cell line. MM cells incubated with 200 μg/ml IGF-1 alone did not show any modification in cell viability (data not shown).

IGF-1 also sensitized MM.1S and U266 cells to the antiproliferative effect of other proteasome inhibitors (MG-115, MG132, PSI, epoximicin) (Figure 1B). Sensitization was not dependent on the level of gene or protein expression of IGF-1R in these tumor cell lines (Figures S1a and S1b). The potentiating effect of IGF-1 on bortezomib induced cytotoxicity was abolished by the addition of an anti-IGF-1R antibody (Figure 1C) (39). IL-6, another well described MM growth factor, was not found to enhance bortezomib induced cytotoxicity (Figure 1D). This may be due to the fact that IL-6 does not have an as potent effect as IGF-1 on the mTor pathway and protein synthesis. As previously reported we found that IGF-1 protected cells from the cytotoxic effect of dexamethasone (Figure S1c).

Toxicity of bortezomib on fresh human MM cells was also evaluated by FACS analysis with annexin V staining. MM cells were gated on the basis of CD38 and CD138 positivity. In a series of samples obtained from 9 MM patients at diagnosis, exposure to IGF-1 alone slightly reduced the percentage of annexin V positive cells whereas bortezomib increased this percentage by 15 % (Figure 1E). Apoptosis in cells exposed to bortezomib in combination with IGF-1 was significantly greater than that of cells exposed to bortezomib alone (p<0.001), and this was partially reversed by the anti-IGF-1R antibody.
To determine whether IGF-1 enhanced the antitumor effects of bortezomib against MM in vivo, we studied human MM xenografts models in scid mice using MM.1S and LP1 cells. While 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-1 and bortezomib (Figures 1F and S2). For MM.1S-carrying mice, animals in the control group, IGF-1 group, bortezomib group and IGF-1 + bortezomib group were euthanized on days 45, 53, 68 and 107, respectively. This experiment was performed twice on groups of five mice. These results show that recombinant IGF-1 enhances the antitymoma effect of bortezomib in vivo in this model.

Furthermore, exposure of a bortezomib-resistant MM.1S variant (MM.1S BR) to IGF-1 allowed partial sensitization to 100 nM bortezomib (Figure 1G), a concentration which induced maximal toxicity in the sensitive parental line.

Effect of IGF-1 on apoptotic signalling induced by bortezomib and cell cycle distribution in MM cells

To determine whether IGF-1 could influence apoptotic signalling in MM.1S and U266 cells, we studied the content of pro-apoptotic and anti-apoptotic proteins by western blot analysis. The addition of IGF-1 to bortezomib significantly increased the content of pro-apoptotic proteins Bax, Bad, Bak and Bim S in both cell lines (Figure 2A). Furthermore, the content of anti-apoptotic 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-1 than in those exposed to bortezomib alone (Figure 2B). The expression of Mcl-1 did not vary between the different exposures (Figure S3). Of note, exposure to IGF-1 alone tended to generate pro-survival signalling 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 (Figure 2C). Cell cycle analysis of MM.1S cells by flow cytometry showed that IGF-1 increased the proportion of cells in S phase while 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-1 (Figure 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 co-exposure to IGF-1 (Figure S3).

**Effect of bortezomib and IGF-1 on the NF-κB pathway in MM cells**

Given previous reports implicating the role of NF-κB signalling in the antmyeloma effect of bortezomib (29) we determined whether IGF-1 increased the effect of bortezomib on the NF-κB pathway in MM.1S cells. We found that the content of IκBα, an inhibitor of nuclear translocation of p65, was up-regulated by IGF-1 and down-regulated by bortezomib, with a more pronounced reduction in presence of the combination of IGF-1 and bortezomib (Figure S4A). This was associated with a concurrent increase in pIκBα. 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-1 to bortezomib (Figure S4B). To evaluate the transcriptional activity of NF-κB, we performed a pangenomic analysis of MM.1S cells exposed to bortezomib in the presence or absence of IGF-1 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 2). These results suggest that the potentiating effect of bortezomib by IGF-1 does not involve NF-κB signalling.
**Effect of IGF-1 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-1 on protein synthesis and proteasomal activity in MM.1S and U266 cells exposed to IGF-1, bortezomib or their combination *in vitro*. IGF-1 was found to increase the synthesis of the monoclonal component, an effect which was not inhibited by bortezomib (Figure 3A). IGF-1 increased the content of phospho-P70S6K and phospho-AKT, an observation consistent with increased protein synthesis, and this effect was enhanced by bortezomib (Figure 3B). The effect of IGF-1 on proteasomal activity was biphasic with an initial decrease during the first 30 minutes followed by an important rebound effect (Figure 3C). Bortezomib alone or combined with IGF-1 were associated with reduced proteasomal activity. Thus, IGF-1 did not appear to alter the inhibitory effect of bortezomib on the proteasome. To determine the importance of protein synthesis on the effect of IGF-1 on bortezomib, cells were exposed to cycloheximide, a protein synthesis inhibitor, concurrently with bortezomib and IGF-1. Co-incubation with 1 or 10 µM cycloheximide effectively prevented the enhancement of the cytotoxicity of bortezomib by IGF-1 thus supporting the role of protein synthesis (Figure 3D).

Bortezomib has been shown to synergize with PP242 which is a selective inhibitor of TORC1 and TORC2 (40). As mTORC1 activation induces phosphorylation of P70S6k, we studied this protein as a marker for mTORC1 activity (13, 19). PP242 suppressed the effect of IGF-1 on the phosphorylation of P70S6k (Figure 3E) and the effect of IGF-1 on the apoptosis induced by bortezomib (Figures 3F) in MM.1S and U266 cells.

These results suggest that IGF-1 as a single agent stimulates protein turnover by increasing both protein synthesis and degradation in MM cells. Conversely, the combination
of IGF-1 with bortezomib is associated with increased protein synthesis and reduced proteasomal degradation, which could lead to enhanced ER stress.

**IGF-1 increases ER stress and potentiates the unfolded protein response caused by bortezomib**

To determine the level of unfolded protein response in MM.1S and U266 cells exposed to bortezomib and IGF-1, we performed 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-1 as compared to one of the compounds alone in both U266 and MM.1S cells, and the same was observed for spliced Xbp1 in MM.1S cells (Figure 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 (Figure 4B). Interestingly, IGF-1 induced a UPR response alone on MM.1S after 16 hours incubation, which might explain why pharmacological 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 (Figure 4C).

To determine whether the UPR was required for IGF-1-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 (Figure S6). Forty eight hours after transfection with siRNA, cells were exposed to IGF-1 and/or bortezomib for another 24 hours before apoptosis was analyzed by flow cytometry. Cells transfected with siRNA against GADD153 did not demonstrate enhancement of sensitivity to bortezomib by IGF-1 while cells transfected with scrambled siRNA were sensitized, confirming that GADD153 was required for the potentiating effect (Figure 4D). These data
suggest that the potentiation of the cytotoxicity of proteasome inhibitor by IGF-1 involves enhanced ER stress and requires an unfolded protein response.
Discussion

“Priming” of tumor cells is a strategy consisting in 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-1, a bona fide growth factor for MM 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 appear to be complex as recently suggested by a genome-wide screen of potential modulators which involve protein translation or DNA damage repair, among other pathways (42). In our experiments we found that the addition of IGF-1 to bortezomib potently increased pro-apoptotic signalling and the unfolded protein response, explaining the specificity of the potentiation of bortezomib by IGF-1.

Activation of the NF-κB pathway has been suggested to be important for the survival both of normal and neoplastic plasmocytes (43). More than 50% of MM cell lines and most primary samples have a high level of NF-κB activity, as demonstrated by the transcription signature of 11 genes (44). Abnormal NF-κB activity may be due to extrinsic signalling involving BAFF and APRIL or may be due to mutations in the NF-κB pathway (32). Additionally, several studies have shown that treatment resistance in MM is related to increased activation of NF-κB, while 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-1 to bortezomib reduced the content of IκBα with a simultaneous increase of phosphorylated IκBα but was not associated with an increase in NF-κB signalling. A pangenomic gene expression study failed to demonstrate 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 et al (32). Overall, our data suggest that the effect of IGF-1 on the cytotoxicity of bortezomib in this model does not involve an effect on NF-κB signalling.

IGF-1 strongly enhanced the apoptotic signalling consecutive to bortezomib exposure, with an enhanced content of the pro-apoptotic proteins Bax, Bad, Bak and Bim S and a decreased content of the anti-apoptotic proteins Bcl-2, Bcl-XL, XIAP, Bfl-1 and survivin. Conversely, IGF-1 alone provided a pro-survival signal with a reduced content of Bax and an increased content of Bcl-2, Bcl-XL and XIAP. Mcl-1, a key regulator of survival of MM cells, remained unchanged under our experimental conditions. Overall, addition of IGF-1 to cells exposed to bortezomib strongly enhanced the apoptotic signalling of bortezomib. IGF-1 also enhanced mitochondrial membrane depolymerisation as well as caspase 3 and PARP cleavage induced by bortezomib in MM cells.

The UPR has come to be considered as a potential therapeutic target in cancer since it may lead to cell death in response to increased ER stress. While UPR has been described in several models (15), MM cells appear 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 an 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-1 and bortezomib potently increases ER stress by increasing protein synthesis while blocking protein degradation leads to an enhanced UPR (Figure 5). This observation confirms the differences in the role of GADD153 in MM cells and primary B cells (15, 48).
Furthermore, we observed that inhibition of GADD153 protein using a specific siRNA inhibited the potentiation of bortezomib by IGF-1, 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 MM cells. Finally, IGF-1 increases the frequency of MM cell cycling, and this might play a role in the increased cytotoxicity of bortezomib when associated to IGF-1 as bortezomib also modifies the cell cycle in cells that subsequently undergo cell death.

In conclusion, our results support the hypothesis that IGF-1 sensitizes MM cells to the cytotoxic activity of proteasome inhibitors such as bortezomib, as a consequence of enhanced level of ER-stress and the induction of an UPR. This is in contrast to the protective effect of IGF-1 against certain agents such as dexamethasone previously reported in the literature and which we have confirmed in our study. This observation raises the question of the impact of local concentrations of IGF-1 in the bone marrow environment in patients receiving bortezomib therapy for MM. Another aspect concerns the possibility of evaluating the combination of recombinant IGF1 and bortezomib in patients who have developed resistance to bortezomib, since recombinant IGF-1 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.
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References


Figure legends

**Figure 1.** IGF-1 potentiates MTT reducing activity of bortezomib and other proteasome inhibitors against MM cell lines and fresh human MM 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’s *t*-test.

Fig. 1A. IGF-1 potentiates MTT reducing activity effect of bortezomib against 4 human myeloma lines *in vitro*. Cells were cultured in 96 well plates for 72 h in the presence of various concentrations of bortezomib alone (▲) or with a fixed concentration of 200 ng/mL of IGF-1 (■). LP1, MM.1S, U266 and RPMI8226 express IGF-1R. Amo-1 cell line does not expresses IGF-1R. Data are mean values from 3 independent experiments Bars are SD.

Fig. 1B. IGF-1 potentiates MTT reducing activity of other proteasome inhibitors in MM.1S and U266 cells *in vitro*. Cells were cultured in 96 well plates for 72 h in the presence of various concentrations of proteasome inhibitors alone (▲) or with a fixed concentration of 200 ng/mL of IGF-1 (■). Data are mean values from 3 independent experiments Bars are SD.

Fig. 1C. Anti-IGF1-R antibody reverses the effect of IGF-1 on the MTT reducing activity of bortezomib in MM.1S cells *in vitro*. Cells were cultured in 96 well plates for 72 h in the presence of bortezomib (1 nM), IGF-1 (200 ng/mL) and/or monoclonal antibody anti-IGF-1R (10 µg/mL). Data are mean values from 3 independent experiments Bars are SD.

Fig. 1D. 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 h in the presence of bortezomib (1 nM), IGF-1 (200 ng/mL) and/or IL-6 (10 ng/mL). Data are mean values from 3 independent experiments Bars are SD.
Fig. 1E. IGF-1 potentiates the apoptotic effect of bortezomib on fresh samples from 9 MM patients at diagnostic. Cells were cultured with RPMI 1640 in 24-well plates at 37 °C with 1.5 nM bortezomib, 200 ng/mL IGF-1, bortezomib + IGF-1 or bortezomib + IGF-1 + 10 μg/mL anti-IGF-1R 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 MM cells under different conditions.

Fig. 1F. In vivo effect of IGF-1 and bortezomib in a SCID mice model of human MM.1S cells. 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-1 0.03 mg/kg; (■): bortezomib 0.5 mg/kg; (●) bortezomib 0.5 mg/kg + IGF-1 0.03 mg/kg. These are the results of one out of two similar experiments.

Fig. 1G. Cytotoxicity of the IGF-1 + bortezomib combination in MM.1S and a bortezomib-resistant MM.1S cell line (MM.1S BR). Cells were incubated for 24h with 10 nM bortezomib and IGF-1 (200 ng/mL). Graphs show the percentage of annexin V-positive cells after incubation of MM.1S cells in different conditions.

**Figure 2.** Effect of the IGF-1+bortezomib on apoptotic signalling in MM.1S and U266 cells. Results shown are representative of three separate experiments (A-D and F) and mean values from 3 independent experiments +/- SD (E).

Fig. 2A. Content of pro-apoptotic proteins analysed by immunoblotting in MM.1S and U266 cells exposed for 8 hours to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination.
Fig. 2B. Content of anti-apoptotic proteins analysed by immunoblotting in MM.1S and U266 cells exposed for 8 hours to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination.

Fig. 2C. Content of PARP, caspase 8 and caspase 3 analysed by immunoblotting in MM.1S cells exposed for 8 hours to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination.

Fig. 2D. Cell cycle of MM.1S cell line. Cells were incubated with IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination for 24 hours.

Fig. 2E. Content of cyclin B1, cyclin G1 and p21 analysed by immunoblotting in MM.1S cells exposed for 8 hours to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination.

**Figure 3.** mTor pathway regulates the cytotoxicity effect of the combination bortezomib + IGF-1. Fig 3: Results shown are representative of three separate experiments (B and E) and mean values from 3 independent experiments +/- SD (A, C, D and F).

Fig. 3A. Median Fluorescence (MFI) of monoclonal lambda chain component as determined by FACS analysis in MM.1S cells exposed to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination for 8 hours.

Fig. 3B. Protein content of AKT, phospho-AKT, P70S6K and phospho-P70S6K analysed by immunoblotting in MM.1S and U266 cells exposed for 1 hour to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination. Time and concentration dependent induction of phospho-AKT is shown in figure S5.

Fig. 3C. Kinetic study of proteasome activity in MM.1S cells. Cells were exposed to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination. The fluorescent intensity was read with excitation at 360 nm and emission at 480 nm.
Fig. 3D. Effect of cycloheximide on apoptotic inducing activity of bortezomib +/- IGF-1 in MM.1S. Cells were exposed to IGF-1 (200 ng/mL), bortezomib (1.5 nM) and different concentration of cycloheximide (0 µM; 0.1 µM; 1 µM and 10 µM) 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.

Fig. 3E. Protein content of phospho-p70S6K analysed by immunoblotting in MM.1S cells exposed to 100 nM pp242 for one hour followed by 15 minutes exposure to 200 ng/mL IGF-1.

Fig. 3F. Inhibition of the effect of IGF-1 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-1 (20 ng/mL), bortezomib (1.5 nM) and PP242 (100 nM) alone or in combination for 24 hours.

Figure 4. Induction of an unfolded protein response in MM1.S and U266 cells and in patient samples by IGF-1 + bortezomib. Results shown are representative of three separate experiments (A-D and E) and mean values from 3 independent experiments with one data point +/- SD (D).

Fig. 4A. Protein content of GADD 153 (CHOP), GRP-78 (Bip) and spliced Xbp1 analysed by immunoblotting in MM.1S and U266 cells exposed to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination for 8 h. Tunicamycin (Tuni) (1µg/mL) and thapsigargin (Thap) (1µM) were used as positive controls.

Fig. 4B. Kinetics of GADD 153 (CHOP), GRP-78 (Bip) and spliced Xbp1 proteins in MM.1S. Cells were exposed to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination.
Fig. 4C. Protein content of GADD 153 (CHOP) and GRP-78 (Bip) analysed by immunoblotting in a fresh human MM sample exposed to IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination for 16 hours.

Fig. 4D. Inhibition of the potentiating effect of IGF-1 on the cytotoxicity of bortezomib in MM1.S cells by anti-GADD153 siRNA. siRNA-transfected cells were incubated with IGF-1 (200 ng/mL) and bortezomib (1.5 nM) alone or in combination for 24 h and assessed for induction of apoptosis. Graphs show the percentage of annexin V-positive cells after incubation of MM.1S cells in different conditions.

**Figure 5.** Suggested mechanism of the combination IGF-1 and bortezomib in MM.1S. IGF-1 enhances protein synthesis by the activation of mTor pathway. Bortezomib inhibits proteasome activity and the degradation of proteins. The accumulation of proteins induces an unfolded protein response which induces release of calcium store, thereby activating mitochondrial-mediated apoptosis and activation of caspases.
Therapeutic enhancement of ER stress with Insulin Like Growth Factor 1 (IGF-1) sensitizes myeloma cells to proteasomal inhibitors

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