Inhibition of eIF2α Dephosphorylation Maximizes Bortezomib Efficiency and Eliminates Quiescent Multiple Myeloma Cells Surviving Proteasome Inhibitor Therapy

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Abstract

The proteasome inhibitor bortezomib (Velcade) effectively eradicates multiple myeloma (MM) cells, partly by activating endoplasmic reticulum (ER) stress apoptotic signaling. However, MM recurrences in bortezomib-treated patients are invariable. We have shown that ER stress signaling can also induce growth arrest and survival in cancer cells. Thus, we hypothesized that bortezomib therapy could induce quiescence and survival of residual MM cells, contributing to disease recurrence. Here, we report that in MM cells, proteasome inhibition with MG-132 or bortezomib results in a surviving cell fraction that enters a prolonged quiescent state (G0-G1 arrest). Mechanism analysis revealed that bortezomib-surviving quiescent cells attenuate eIF2α phosphorylation and induction of the ER stress proapoptotic gene GADD153. This occurs independently of the eIF2α upstream kinases PERK, GCN2, and PKR. In contrast, the prosurvival ER-chaperone BiP/Gpr78 was persistently induced. The bortezomib-surviving quiescent fraction could be eradicated by a simultaneous or sequential combination therapy with salubrinal, an inhibitor of GADD34-PP1C phosphatase complex, and, in consequence, eIF2α dephosphorylation. This effect was mimicked by expression of a phosphorylated mimetic eIF2α-S51D mutant. Our data indicate that bortezomib can induce growth arrest in therapy-surviving MM cells and that attenuation of eIF2α phosphorylation contributes to this survival. Most importantly, this survival mechanism can be blocked by inhibiting eIF2α dephosphorylation. Thus, strategies that maintain eIF2α in a hyperphosphorylated state may be a novel therapeutic approach to maximize bortezomib-induced apoptosis and reduce residual disease and recurrences in this type of cancer.

Introduction

Proteasome inhibitors induce multiple myeloma (MM) cell apoptosis by modulating several pathways, including endoplasmic reticulum (ER) stress signaling (1). In the clinic, bortezomib is now widely used in patients with refractory or relapsed MM and is also being studied as an adjunct to first-line therapies (1). However, the use of prolonged bortezomib therapy has lead to the development of drug resistance (1). Drug resistance has been linked to enhanced ER stress signaling and increased chaperone expression (1, 2), which may be an adaptive capacity of MM cells.

Proteasome inhibitors induce an ER stress response in MM cells contributing to apoptosis (2, 3). However, a primary function of ER stress signaling is also to adapt to exogenous stress and induce growth arrest and survival. This is achieved in part by reducing cyclin D1 levels (4) and inducing protein folding and degradation genes that alleviate the damage caused by unfolded proteins. Thus, it could be anticipated that growth arrest and survival may be a response to proteasome inhibitor therapy.

Depending on the activation intensity and cellular context, eIF2α phosphorylation by upstream kinases, like PERK or GCN2, can induce survival, growth arrest, and/or apoptosis in response to ER stress (5, 6). We showed that eIF2α signaling was linked to the induction and maintenance of HEp3 head and neck squamous carcinoma cell dormancy and survival (5, 7, 8). Growth arrest (i.e., dormancy) is in part due to PERK-dependent phosphorylation of eIF2α, which leads to the down-regulation of cyclins D1/D3 and cyclin-dependent kinase 4 (CDK4). Survival and resistance to chemotherapy, on the other hand, is due to induction of BiP, ATF6 activation, and also eIF2α phosphorylation (5, 8). In other cases, very intense eIF2α phosphorylation can activate apoptotic programs (9). The capacity of eIF2α signaling to decide cell fate in response to stress might be particularly important for MM patients treated with proteasome inhibitors, as different levels of stress may impinge on the cells during this therapy.

We set out to explore the link between proteasome inhibition, activation of ER stress, and growth arrest in MM cells. We hypothesized that proteasome inhibition with MG-132 or bortezomib in clinically relevant concentrations may force cells into a growth arrest/survival program due to an ER stress adaptation response. This could be a potential contributor to therapy resistance and disease recurrence. We further hypothesized that maximizing ER stress signaling could enhance sensitivity to proteasome inhibitors by tipping the balance from signaling for growth arrest/survival to apoptosis. Here, we report that a fraction of MM cells, surviving a single treatment with bortezomib, down-regulate eIF2α phosphorylation and enter a prolonged G0-G1 arrest. More importantly, the quiescent surviving fraction of MM cells with different genetic abnormalities could be almost completely eradicated by enhancing eIF2α phosphorylation with salubrinal (an inhibitor of GADD34-PP1c complex assembly) or by expression of a phosphorylated mimetic eIF2αS51D protein. Our findings reveal that enhancement of ER stress signaling can be exploited as a strategy to maximize efficiency of proteasome inhibitor therapy.

Materials and Methods

Reagents, antibodies, reverse transcription–PCR, cell lines, and tissue culture plasmids. MG-132 and salubrinal were purchased from...
Calbiochem; bortezomib (Velcade) was kindly provided by Millenium Pharmaceuticals. Proteasome inhibitor treatments were performed using 400 nmol/L MG-132 or 4 nmol/L bortezomib (Velcade) for 24 h. The drug was then removed by serial washes with PBS, and the remaining viable cells were replated. Salubrinal treatments were performed using the drug at 5 or 10 μg/mL, as indicated, for 24 h. RPMI 8226 cells [c-MYC insertion on t(16;22)(q32;q11):der16] were kindly provided by Dr. Douglas Conklin (State University of New York), U266B1 cells [t(11;14)] were from American Type Culture Collection (ATCC). Cells were cultured according to ATCC recommendations. Antibodies used were p-Rb, Rb, p-eIF2α, eIF2α, GCN2, p-PKR, and PKR from Cell Signaling; p-PERK and PERK from Santa Cruz Biotechnology; BiP from BD; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Calbiochem; and β-tubulin from Abcam. Total RNA was extracted using Trizol (Invitrogen). Primer sequences were published previously (8, 10). The pCLBabe-eIF2αS51D plasmid was kindly provided by Dr. David Schubert (Salk Institute). Transfection of RPMI 8226 cells was performed using Amaxa technology according to the manufacturer’s instructions.

Cell cycle analysis and label retention assay. Cell cycle analysis was performed using propidium iodine (PI)/Rnase staining buffer from BD according to the manufacturer’s instruction. For label retention and viability quantification, cells were treated with proteasome inhibitors or DMSO for 24 h, then stained with 50 nmol/L CFDA/SE (Molecular Probes) for 15 min, and replated. Before analysis, cells were stained using Hoechst-33342 (10 μg/mL) for 15 min at 4°C to determine cell viability. Cells were then fixed using 3% paraformaldehyde for 20 min and analyzed by flow cytometry (LSR-II from BD).

Viability assay and immunoblotting. Cell viability was assessed using trypan blue. Immunoblotting was performed as described previously (7).

Image quantification and statistical analysis. Image semiquantification was performed using ImageJ from NIH. Quantifications of all Western blots and reverse transcription–PCR (RT-PCR) experiments in Figs. 3–5 showing changes in expression levels are given in Supplementary Figs. S1 and S2. Western blots showing protein cleavage were not quantified. Statistical analysis was performed using Prism 4.0 from GraphPad Software and Student’s t test.

Results

Bortezomib induces G₀-G₁ arrest in a fraction of surviving cells. Treatment of RPMI 8226 MM cells with MG-132 (400 nmol/L)
and bortezomib (4 nmol/L) strongly induced cell death (sub-G0 population) in a time-dependent fashion (Fig. 1A). This may be due to an initial (4–8 hours) G2-M arrest, as determined by fluorescence-activated cell sorting (data not shown) and in accordance with the data in other systems (11). We next explored the fate of MM cells surviving proteasome inhibitor therapy. RPMI 8226 cells treated with 400 nmol/L MG-132 for 24 hours were washed to remove the drug. We found that the absolute number of viable cells that were then replated remained constant for 7 days. In contrast, vehicle-treated cells initiated exponential growth after 48 hours (Fig. 1B). The lack of expansion in the surviving cells was not due to a balance between proliferating and dying cells but to a growth arrest as shown by PI staining followed by flow cytometry (Fig. 1C). Cell cycle analysis revealed that the number of cells in G0-G1 increased significantly from ~35% in the control population to ~55% to 65% in the cells surviving proteasome inhibition (Fig. 1C). Similar results were obtained using 4 nmol/L bortezomib, a dose that is pharmacodynamically achievable in patients (Fig. 1D, top; ref. 12). The arrest was due to a failure to transit the G1-S boundary because, 72 hours after washout of proteasome inhibitors, there was an intermediate (Ser65) to strong reduction (Ser35/65/70/90) in phosphorylated Rb protein levels. These Ser residues are phosphorylated to promote G1-S transition (Fig. 1D, bottom). Our results show that two proteasome inhibitors are able to induce G0-G1 arrest in RPMI 8226 MM cells after a single treatment.

To corroborate our cell cycle analysis, we performed double labeling with CFSE and Hoechst-33342 to measure viable CFSE retaining cells and to gate out sub-G0 cells. CFSE, due to its irreversible esterification onto cellular proteins and its equal distribution within daughter cells, can be used to monitor cell division (13). We found a highly significant ~4-fold to 7-fold increase in the percentage of CFSE-positive cells at 72 hours in cells surviving both MG-132 and bortezomib (Fig. 2A and B), suggesting that at 72 hours, after the washout of proteasome inhibitors, the surviving cells divide to a lesser extent. These results indicate that, whereas proteasome inhibition can cause acute apoptosis, the surviving MM cells rapidly enter a G0-G1 arrest. Given that pharmacologic treatments do not reach maximal lethal doses in all targeted cells, the induction of a growth-arrested surviving fraction adapting to the treatment may be a possible side effect of proteasome inhibition.

**Bortezomib-surviving cells attenuate eIF2α phosphorylation and unouple the induction of proapoptotic from prosurvival ER stress genes.** We next investigated if the cells surviving proteasome inhibitors undergo any specific changes in ER stress signaling. Proteasome inhibitors are known to induce an unfolded protein response (UPR) in MM cells by inhibition of the ER-associated degradation pathway (2, 14). Furthermore, UPR mediates cell cycle arrest and cell survival resistance in several models, including MM (5, 8, 15). We were interested in the phosphorylation of eIF2α, which attenuates translation and induces a selective gene expression program as an adaptive response to ER stress. We also monitored the expression of the prosurvival chaperone BiP/Grp78 (5, 8), the transcription factor XBP-1 (5), and the proapoptotic transcription factor CHOP (10). MG-132 treatment caused increased eIF2α phosphorylation (Fig. 3A and B), XBP-1 splicing (Fig. 3C), BiP (Fig. 3D), and CHOP (Fig. 3C) up-regulation in the acute phase (8–24 hours). This was not associated with changes in the phosphorylation of the eIF2α upstream kinases PERK, GCN2, and PKR (Fig. 3F). With the exception of BiP, which was strongly induced, the expression of CHOP mRNA, spliced XBP-1, or increased eIF2α phosphorylation did not persist in the surviving cells (Fig. 3A–D). Phosphorylation of p38 was also activated both by MG-132 and bortezomib, and p38 remained phosphorylated in the surviving quiescent cells (data not shown). This suggests that, as reported (16), p38 activation can have a prosurvival role in MM cells. BiP can induce survival in other cancer cells (5, 17–19), suggesting that these functions might be preserved during quiescence induced by proteasome inhibitors in MM cells.
Interestingly, both CHOP induction and XBP-1 splicing are only observed during the acute phase and both are then down-regulated in the surviving cells. The functional role for the down-regulation of XBP-1 splicing is not clear. However, it is possible that down-regulation of CHOP eliminates the proapoptotic function of this gene (20), further promoting survival of the residual MM cells. Although the levels of phosphorylated eIF2α kinases did not change in acute treatments or after proteasome inhibitor washout, eIF2α phosphorylation was consistently down-regulated. This suggests that other mechanisms may be responsible for this event.

**Pharmacologic or genetic enhancement of eIF2α phosphorylation potentiates bortezomib-induced cell death minimizing the surviving cell fraction.** Loss of eIF2α phosphorylation and of its downstream target CHOP in the surviving fraction may be associated with an evasion of apoptosis, as enhanced phosphorylation of eIF2α can induce cell death (21). Thus, we tested whether sustained eIF2α phosphorylation in the surviving cells would limit their survival after bortezomib treatment. To this end, we used salubrinal, an inhibitor of GADD34-PP1c complex assembly and, therefore, eIF2α-dephosphorylation, identified in a screen for small molecules protecting from ER stress (22). We tested whether salubrinal would enhance eIF2α phosphorylation in the surviving cells greater than salubrinal alone, resulting in increased phosphorylation of eIF2α and of its downstream target CHOP in the surviving cells. Although the levels of phosphorylated eIF2α kinases did not change in acute treatments or after proteasome inhibitor washout, eIF2α phosphorylation was consistently down-regulated. This suggests that other mechanisms may be responsible for this event.

While in both RPMI 8226 and U266B1 cells, MG-132 or bortezomib alone caused roughly 40% to 50% cell death at 24 hours, salubrinal could further augment this effect to roughly 70% to 90% (Fig. 5A), which is in agreement with the activation of apoptotic signals. It is unknown if salubrinal can also inhibit other PP1c complexes, but the initial study argues for this compound being highly specific for the GADD34-PP1c complex (22). We cannot rule out that other targets of GADD34-PP1c might contribute to the observed effect. However, our data, obtained by transfecting RPMI 8226 cells with a phosphorylated mimetic eIF2αS51D mutant (25), strongly suggest that the effect is specific for GADD34-PP1c activity on eIF2α (Fig. 5B). Transient transfection...
efficiency in these cells using electroporation is ~50% (data not shown), and cell death was enhanced by around the same magnitude when combining eIF2αS51D overexpression with proteasome inhibition (Fig. 5B). Thus, it can be deduced that majority of MM cells expressing the eIF2αS51D mutant were more sensitive to bortezomib.

Finally and most importantly, we tested whether salubrinal treatment of the cells that are arrested but still surviving proteasome inhibition will affect their viability. Treatment of this MM cell population with 5 μmol/L salubrinal, which does not affect the control population, caused a 10-fold reduction in the number of viable cells after 24 hours (Fig. 5C). This was comparable with the effect observed using the combination therapy (Fig. 5C). The effect could still be observed in quiescent cells 5 days after the bortezomib treatment (Fig. 5D). Thus, these cells, while still growth arrested, are in a high ER stress condition, evidenced by the high Grp78 levels, and remain highly sensitive to salubrinal. These results further suggest that the reduction in p-eIF2α observed after bortezomib treatment is indeed a mechanism to evade apoptosis.

Discussion
Exploring mechanisms that might induce quiescence in cancer cells led us to test the hypothesis that therapies known to induce the UPR might be the cause of a protective response in cancer cells (5, 7, 8). We had previously shown that head and neck cancer cells (HNSCC) entered a state of cellular dormancy, which was linked to the activation of ER stress pathways that, in a concomitant fashion, promoted both growth arrest and survival (5, 7, 8). These are the same pathways that can be activated by proteasome inhibitors (1, 2, 14), which led us to reason that proteasome inhibition with
MG-132 and the clinically used drug bortezomib (Velcade) might induce quiescence and persistence of cells surviving the therapy. Several findings are worth discussing. We discovered that, whereas a portion of MM cells treated with proteasome inhibitors die, a significant fraction (30–50% of the cells) could adapt to the stress and survive by entering a protracted state of quiescence. The fact that ~30% to 50% of the cells survive suggests that the G0-G1 arrest is more likely due to treatment adaptation than to selection of a cell population genetically predisposed to undergo quiescence. This response is plausible as one function of ER stress signaling is to allow cells to pause proliferation while unfolding of proteins in the ER is being corrected (6, 26). This ultimately leads to cell survival and, in the context of MM, may allow for recurrence. We cannot rule out that this response may also be operational in MM cells with preexistent mutations that confer resistance to bortezomib (27). If true, this adaptive response, which seems to be functional in a large proportion of cells, may further accelerate the selection of these underrepresented clones. However, such hypothesis needs to be tested.

We also found that survival of the residual quiescent cells hinges on the down-regulation of eIF2α phosphorylation, which allows these cells to silence proapoptotic signals downstream of eIF2α signaling. This was primarily evidenced by the loss of CHOP mRNA expression. The silencing of CHOP might be an important apoptosis escape mechanism, as this transcription factor was shown to repress Bcl-2 expression and at the same time induce expression of BimEL (24), a powerful proapoptotic protein.

First and importantly, we found a potential solution to the problem by combining bortezomib treatment with salubrinal, an inhibitor of GADD34-PP1c complex assembly. Maintaining eIF2α phosphorylation through drugs like salubrinal can potentiate killing of MM cells, and they may serve as an adjuvant therapy to proteasome inhibitors when given simultaneously. Second and more importantly, salubrinal can virtually eliminate the fraction of quiescent MM cells surviving proteasome inhibition. We therefore propose that salubrinal or similar compounds affecting phosphorylation of eIF2α may serve as a potential strategy to maximize proteasome inhibitor efficiency and decrease MM cell resistance to this substance class. The ability of salubrinal to potentiate killing of MM cells by enhancing eIF2α phosphorylation is in accordance with previous models hypothesizing a sigmoidal relationship between stress intensity and eIF2α activation leading to survival at very low and apoptosis at very high levels (9). Thus, the enhancement of eIF2α phosphorylation during MG-132 or bortezomib treatment prevents MM cells to modulate this pathway to adapt to and survive ER stress.

This response was observed in two MM cell lines with different genetic alterations [c-MYC insertion on t(16;22)(q32;q11):der16 in Figure 5. A, percentage of dead cells in RPMI 8226 (left) and U266B1 (right) after 24 h of the indicated treatments with salubrinal (SAL) and/or proteasome inhibitors (MG or Velcade, VEL, left). B, RPMI 8226 cells transfected with a phosphomimetic S51D eIF2α mutant showed enhanced Velcade (VEL) sensitivity compared with the empty vector control. The inset shows increased total protein levels of eIF2α in the cells expressing the construct. C, viability of RPMI 8226 cells after 24 h of the indicated treatments. Cells were treated with either DMSO or Velcade (VEL, 4 nmol/L) for 24 h, then washed, and treated with DMSO or salubrinal (SAL) after that. The right two columns are combination treatments with proteasome inhibitors and salubrinal for 24 h as controls. D, the enhanced sensitivity to salubrinal observed 3 d posttreatment could still be observed at 5 d after proteasome inhibitor washout, suggesting that the sensitivity to salubrinal postproteasome inhibition is long lasting.
Salubrinal Potentiates Bortezomib Therapy in MM Cells

RPMI 8226 and [11:14] in U266B1, suggesting that it may not be linked to a given genotype. Still, as discussed above, this does not rule out that it may, in the long-term, favor the expansion of clones predisposed to resist bortezomib therapy. We also found that, while diffuse B-cell lymphoma U937 cells were highly sensitive to 4 nmol/L bortezomib and K-562 chronic myeloid leukemia cells were intermediate sensitive, neither of these cell lines were susceptible to an enhancement of bortezomib-induced killing by salubrinal (data not shown). Furthermore, neither bortezomib alone nor its combination with salubrinal were effective in inducing killing of HEp3 HNSCC or Raji Burkitt’s lymphoma cells (data not shown). This suggests that the combination therapy using a proteasome inhibitor and salubrinal may be mostly suited for the treatment of MM.

We hypothesize that the induction of XBP-1 during the acute phase of bortezomib treatment is most likely associated with a survival/adaptation response (5, 15, 28). It is possible that, whereas XBP-1 may function to protect cells during the acute phase, its ability to lead to the prolonged induction of chaperones, such as BiP/grp78, may prevent apoptosis in the cells surviving the treatment. This response may be related to the inherent capacity of the UPR to protect from subsequent insults to the ER (9). Thus, prolonged survival of the quiescent fraction may be linked to the abundant induction of BiP/grp78. Previous studies provided compelling evidence for a prosurvival function for this chaperone in cancer (5, 17–19). Thus, it is possible that, in addition to the inhibition of GADD34-PP1c and enhancement of p-eIF2a signaling, blockade of Grp78 expression and/or function may be an additional strategy to prevent adaptation and survival of MM and other cancer cells to bortezomib treatment. Similar strategies applied to identify HSP90 inhibitors (29, 30) may be successful in identifying small molecule inhibitors specific for Grp78.

Several upstream kinases, such as PERK, PKR, and GCN2, can phosphorylate eIF2a (26). Our study revealed that during the acute phase there was only a marginal modulation of PERK, PKR, and GCN2 activation. Thus, their functional contribution to the growth arrest remains to be elucidated. It is also unclear whether the down-regulation of eIF2a phosphorylation is solely due to GADD34-PP1c expression/activity. Still, our data support that this is the most plausible option. What seems to be evident from our results is that in both acute and post–bortezomib treatments with salubrinal the strong phosphorylation of eIF2a is associated with apoptosis. Studies in HNSCC cells showed that, whereas PERK and eIF2a signaling can contribute to survival, persistent eIF2a signaling is also linked to the quiescent state (5). In the case of MM cells, phosphorylation of eIF2a does not persist posttreatment during the quiescence period. This suggests that the growth arrest is linked to other mechanisms or that persistent eIF2a phosphorylation is not needed for this effect.

 Whereas Rb phosphorylation in sites that are indicative of G1 exit was reduced after proteasome inhibition, we failed to detect significant changes in CDK or cyclin protein levels (data not shown). Only p21 induction was observed in the acute phase but not in the surviving fraction of arrested cells (data not shown). We speculate that this acute induction is sufficient to induce the long-term MM cell arrest and that this or other mechanisms (i.e., p15 and p16 induction) maintain cyclin/CDK complexes inactive for prolonged periods.

In summary, we report that the induction of MM tumor cell quiescence and survival can be an undesirable side effect of proteasome inhibition. We also show that, by blocking eIF2a dephosphorylation, proteasome inhibitor efficiency can be maximized during acute treatment and that residual cells can be eliminated by nontoxic doses of salubrinal as a monotherapy for MM minimal residual disease after proteasome inhibition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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