Endoplasmic reticulum stress and proteasome inhibitors in multiple myeloma – a room for improvement

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Endoplasmic reticulum stress and proteasome inhibitors in multiple myeloma – a room for improvement

Short title: ER stress and resistance to proteasome inhibitors in multiple myeloma

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Abstract

In the last two decades we witnessed unprecedented progress in the field of multiple myeloma research. Median survival of patients doubled and with the introduction of subsequent new therapeutics we expect even better results in the nearest future. However, the disease still remains incurable. It is attributed to recurring nature of multiple myeloma with reappearance of subclones resistant to previously used therapies. More than 15 years after approval of first-in-class proteasome inhibitor - bortezomib, the mechanisms responsible for resistance to this class of drugs are still not entirely untangled. One of the most promising explanations involves modulation of endoplasmic reticulum stress caused by accumulation of misfolded proteins. Due to excessive monoclonal protein production multiple myeloma cells are particularly susceptible to proteotoxicity. Under normal circumstances they counteract it with activation of adaptive mechanism - the unfolded protein response. This pathway, however, can also lead to cell’s apoptosis when unable to restore proteostasis. It is the expected effect of proteasome inhibition. Resistant cells develop mechanisms that decrease the endoplasmic reticulum stress. This review covers current efforts to understand the nature of this adaptation. It focuses on druggable targets that can potentially enhance proteasome inhibitors activity or re-sensitize resistant patients to this type of therapy.

Key words: er stress; myeloma; proteasome inhibitors; resistance; unfolded protein response
Introduction

Multiple myeloma (MM) is one of the most common hematological malignancies in the western countries, responsible for over 10,000 deaths per year in the United States [1]. Despite tremendous improvement in patients prognosis achieved in the last two decades, the disease is still considered incurable in most cases [2-4]. This situation is attributable mostly to the recurring nature of MM. Using modern drugs, vast majority of patients with newly diagnosed MM (NDMM) responds well to first-line therapy, going into deep remissions that can last few years [5-7], but finally almost all of them experience clinical relapse. Treatment of refractory/relapsed (RR) disease is not as efficient as for the NDMM and every subsequent line of therapy yields poorer results [8, 9]. To overcome this limitation two approaches can be utilized: (i) using classes and types of drugs that were not administered in the previous lines of therapy or (ii) adding agents that can augment activity of previously used medications and re-sensitize MM cells to them. Currently, the first approach is the only available and efficient way to treat RR MM patients [10, 11] whereas the second one is still under development. Nevertheless, recently two drugs have been approved that - at least partially - utilize a concept of synergistic improvement. The first one, panobinostat, a deacetylase inhibitor that disrupts proteins degradation by aggresome pathway, has shown superiority in comparison to placebo when given in combination with bortezomib and dexamethasone in a phase III PANORAMA trial [12]. Intriguingly the drug does not exhibit single-agent activity, underlining the synergistic effect with proteasome inhibition. Similarly, the second drug, selinexor, which inhibits nuclear export through exportin-1 (XPO-1) have recently proved its efficacy in combination with bortezomib and dexamethasone [13]. This is particularly important finding in the context of previously identified role of XPO-1 overexpression in resistance to bortezomib [14]. Thus, with the growing understanding of mechanisms responsible for resistance to the most common classes of anti-MM drugs, it seems reasonable to expect that
also the re-sensitizing/augmenting approaches will become effective and widely used. In the context of resistance to therapy, the most intensively studied class of anti-MM drugs are proteasome inhibitors (PIs) [15, 16]. MM cells are particularly susceptible to proteasome inhibition due to overproduction of monoclonal protein that requires high activity of the ubiquitin-proteasome system (UPS) [17]. When UPS is overwhelmed another adaptive reaction plays important role in maintaining cell’s homeostasis, namely the unfolded protein response (UPR) triggered by endoplasmic reticulum (ER) stress [18, 19]. In this review we summarize current developments in the studies on the role of ER stress modulation in myeloma cells resistance to PIs with special attention to druggable targets that can augment PIs therapeutical efficacy.

**ER stress and unfolded protein response**

Posttranslational modification (i.e. disulfide bond formation, glycosylation) and folding of all secretory proteins occur in the ER [20]. The process is thoroughly controlled by different chaperones and through maintaining appropriate redox conditions and calcium concentration in the ER lumen. Only proteins that are properly folded pass quality control and are allowed to leave the compartment to serve their physiological function. The misfolded proteins are retro-translocated and polyubiquitinated to be directed for degradation in proteasome [21]. 26S proteasomes use multiple proteases to break peptide bonds and degrade unneeded proteins. The process of utilization of misfolded proteins by UPS is called ERAD (ER-associated degradation) and is essential for maintaining cell’s proteostasis [22]. ER stress starts when ER’s capability to fold proteins is lower than the load of polypeptides that enter the compartment. The stress can be triggered by increase in protein influx (pancreatic β-cells during insulin-resistant state, malignant myeloma cells), by lowering of the folding capacity (nutrient deprivation, hypoxia, calcium concentration aberrations, alterations of redox homeostasis) or by interrupting the ERAD (proteasome inhibition) [23]. Because the
accumulation of misfolded proteins induces potentially fatal proteotoxicity, the eucaryotic cells developed highly conservative adaptive response system to counteract it – the UPR [18-20, 24]. It is triggered by ER stress and is regulated by three ER transmembrane proteins: IRE1 (inositol-requiring enzyme 1), PERK (double-stranded RNA-activated protein kinase [PKR]-like ER kinase) and ATF6 (activating transcription factor 6). These enzymes are kept inactive under physiological conditions, without a corresponding increase in ER stress [18]. The state of inactivity is provided by binding of BiP (binding immunoglobulin protein or Grp78 – glucose-regulated protein 78), a heat-shock protein and a chaperone, to the transmembrane proteins. During ER stress caused by the accumulation of unfolded proteins whose hydrophobic fragments have higher affinity to bind BiP, the inhibiting protein dissociates from IRE1, PERK and ATF6, leading to their activation and initiation of the UPR. As a result, activation of certain mechanisms leading to the induced translation of proteins that can restore ER homeostasis is initiated whereas other non-essential proteins translation is stopped [24]. IRE1 dimerizes to induce its endoribonuclease activity and splices XBP1 (x-box binding protein 1) to produce XBP1s (spliced), a transcription factor responsible for initiating processes that lead to increased protein folding and degradation of misfolded proteins in the ER. The endoribonuclease does not splice exclusively XBP1 but can cleave also other RNAs in a process called RIDD (regulated IRE1-dependent decay), attributing to lowering the burden of proteins processed by ER. Activation of PERK leads to eIF2a (eukaryotic translation initiation factor 2) phosphorylation and subsequent repression of global protein translation while simultaneously allowing for selective translation of ATF4 (activating transcription factor 4), transcription factor for proteins necessary in restoring ER homeostasis (anti-oxidants, chaperones, elements of phagosome). ER stress triggers ATF6 dissociation from ER membrane and its further translocation to Golgi apparatus. It is subsequently cleaved to reveal their transcription factor domain – ATF6f through which it regulates expression of
other UPR genes. Despite being an adaptive mechanism to help cells survive under ER stress conditions, in the event of overwhelming ER stress, the same mechanism switches toward apoptosis, leading to cell death [25]. It is also mediated by downstream effects of IRE1 and PERK activation. When ER stress becomes unsustainable CHOP (C/EBP homologous protein) transcription factor is activated by PERK, leading to inhibition of anti-apoptotic proteins from BCL-2 (B-cell lymphoma 2) family and IRE1 endonuclease RIDD activity ultimately results in decay of RNA encoding ER stress relieving proteins. Since the balance between adaptation and apoptosis in response to ER stress is very subtle, it is reasonable to utilize this vulnerability in MM treatment.

**ER stress in myeloma cells and the impact of proteasome inhibitors**

Due to the extensive monoclonal protein synthesis myeloma cells are exposed to significant ER stress and are therefore highly dependent on UPR pathway activity [26, 27]. At least one study has shown that marker of UPR activation, a grp94 (also known as HSP90B1 – heat shock protein 90kDa beta member 1) chaperone is significantly higher expressed in MM patients cells in comparison to MGUS (monoclonal gammopathy of undetermined significance) and healthy population [28] underlining the role of UPR in myeloma cells survival. Under undisturbed conditions the UPR activity sufficiently adapts myeloma cells to intensive protein production [29]. This situation changes when MM cells are exposed to proteasome inhibitors. Drugs from this class bind to different proteasome subunits (β1, β2, β5) to stop their chymotrypsin-like, caspase-like or trypsin-like catalytic activities and disrupt ERAD by blocking the UPS pathway [30]. This process increases ER stress to the point when UPR is not able to restore proteostasis. The overwhelmed UPR stops supporting adaptive mechanisms and activates proapoptotic pathways instead [31, 32]. This can partially explain high efficacy of PIs in the treatment of MM. With three different drugs from this class currently approved (bortezomib, carfilzomib, ixazomib), the PIs are the backbone of a
majority of modern anti-MM therapies [33]. There are some differences among the three drugs - carfilzomib and ixazomib preferentially block β5 subunit, whereas bortezomib blocks β5 and β1, moreover, only carfilzomib inhibits the proteasome irreversibly [30] but they all show similarly high antmyeloma efficacy. However, even using PIs in combination with lenalidomide in NDMM patients, as shown in the recent ENDURANCE trial, is not sufficient to generate response in all of the MM patients [34]. Between 5 to 10% patients are primarily resistant to the therapy and majority of those treated with PIs experience relapse that is resistant to this type of drugs [35].

**Adaptations in PI-refractory cells**

Despite the fact that there are certain resistance mechanisms that appear unique for different members of PI class (e.g. important role of drug efflux pump p-glycoprotein expression in resistance against carfilzomib, not affecting bortezomib-resistant cells [36]), it is postulated that one of the major factors responsible for failure of all 3 approved PIs therapies is that resistant cells are less dependent on UPR, showing lower baseline ER stress level. It was described on cell lines as well as on patients samples. Leung-Hagesteijn et al. [37] described PI-resistant plasma cells population as IRE1/XBP1s negative, making an argument that this arm of UPR is indispensable for bortezomib toxicity. They showed that this adaptation decreases ER stress by plasma cell de-commitment towards earlier progenitors that produce less monoclonal protein. This finding is in line with a known role of XBP1s in plasma cells maturation [38], previous observations on the impact of the level of immunoglobulin production on bortezomib activity [17], as well as with the correlation of the bortezomib sensitivity in mantle cell lymphoma with cells secretory load [39]. Also, clinical observations prove that patients producing higher amounts of monoclonal protein respond better to PI-based therapy [40], that the presence of extramedullary disease is associated with poorer prognosis [41], and that low XBP1s expression is an adverse prognostic factor for
patients undergoing bortezomib-based therapy [42, 43]. Interestingly, the latter conclusion is not true for patients treated with thalidomide, underpinning that this mechanism is PI-specific [44]. In a recent study Zang et al. [45] linked the XBP1s-low phenotype with expression of Cdc37 (cell division cycle 37), a co-chaperone of Hsp90 (heat shock protein 90), another chaperone stabilizing many oncogenes [46], and confirmed its impact on bortezomib resistance. They also provided interesting explanation of discrepancies between high Cdc37 expression in NDMM samples in contrast to low expression in bortezomib-resistant samples from RR MM patients, attributing it to the clonal selection [47] – common phenomenon that includes also evolution of cytogenetic lesions [48]. On the contrary, Borjan et al. [49] studied amounts of secreted proteins, misfolded proteins in ER and markers of UPR activation (BiP and PERK), and were unable to show significant differences between bortezomib-sensitive MM cell lines and solid tumors cells. They confirmed however that bortezomib-sensitive cells show higher activity of IRE1/XBP1 pathway but considered it solely as a marker of plasma cell differentiation, not correlated with ER stress. Soriano et al. [36] investigated differences between PI-sensitive and PI-resistant cells and showed that the latter are characterized by lower UPR activity and proposed a model of metabolic adaptations that enhance protein-folding capacity of ER by maintaining redox homeostasis, leading to decreased ER stress. They further described the changes by metabolomic analysis and showed enrichment in glutathione and NADP(H) renewing pathways, tricarboxylic acid cycle that altogether translated into increased reactive oxygen species buffering capacity leading to more efficient protein folding [50]. Also, a change in lipid synthesis from lysolipids to sphingomyelins was described in this study, consistent with previous reports on the role of lipid composition of ER membrane in ER stress modulation [51, 52].
Targeting ER stress to enhance PI activity

A number of ways to augment the drugs activity in MM cells have been proposed in the recent years, based on the better understanding of ER stress and UPR activation roles in resistance to PIs. Currently explored approaches are discussed in this paragraph and summarized in Table 1. Figure 1 depicts the target molecules places in the context of ER stress and UPR modulation.

Zhang et al. [53] exploited the finding that bortezomib-resistant cells show upregulation of S-glutathionylation level [54]. They were able to identify BiP S-glutathionylation by glutathione S-transferase P (GSTP) as a way to increase the chaperone’s ability to fold proteins and therefore reduce ER stress and burden put on UPS. Its clinical relevance was postulated by confirming higher BiP and S-glutathionylation level in bone marrow samples from patients with MM in comparison to healthy subjects. The study showed that GSTP inhibition by TLK199, a drug studied in a phase 1-2 clinical trials in myelodysplastic syndromes [55] can re-sensitize MM cells to bortezomib.

Hayes et al. [56] identified FAD containing ER oxidoreductin 1 (Ero1) as another target to increase ER stress. The protein enables disulfide bonds formation in ER-processed proteins, leading to maintaining redox homeostasis. It is particularly important in MM cells because their ability to secrete proteins rich in disulfide-bonds is among the highest in mammalian cells [57]. The group proved that blocking Ero1 with specific inhibitor EN-460 increases ER stress and exhibits promising anti-myeloma activity in vitro. Although the study showed that increased Ero1 expression on MM cells is an adverse prognostic factor for patients treated with bortezomib, it remains to be determined if EN-460 can enhance PIs activity. Another approach, exploiting similar vulnerability is inhibition of proteins from protein disulfide isomerase (PDI) group - oxidoreductase enzymes responsible for proper protein folding by creating disulfide bonds. Our proteomic studies confirmed increased
accumulation of proteins from this family in subgroup of bortezomib-resistant patients [58]. Robinson et al. [59] developed new pan-PDI inhibitor E64FC26 that showed promising activity in re-sensitizing bortezomib-resistant cell lines. The effect was attributed to UPR activation and it was able to potentiate also the efficacy of other drugs from PIs group. E64FC26 was also efficient in vivo, in mice xenotransplanted with human MM cells. Interestingly, combination of E64FC26 with bortezomib allowed for bigger increase in median survival, in comparison to vehicle, than bortezomib alone.

Targeting the effector arms of UPR has also been developed as a way to potentiate effect of proteasome inhibition. Bagratuni et al. [60] tested the activity of PERK inhibitor, GSK2606414 and proved its efficacy against human MM cell lines as well as synergistic effect when co-administered with bortezomib. The apoptotic effect was accompanied by increase in expression of survivin – gene associated with ER stress. Also inhibiting the IRE1/XBP1s axis of UPR showed encouraging activity against MM cell lines, mouse myeloma models and patient-derived MM cells [61, 62]. Harnoss et al. [62] comprehensively described effects of IRE1α inhibition. They showed that it causes perturbations in ERAD pathway and decrease in secretion of immunoglobulin light chains and cytokines, enabling anti-myeloma effect. They were able to show that IRE1α blocking by compound 18 significantly reduces viability of CD138+ cells from MM patients while sparing CD138- population and not affecting homeostasis in tested mice. These findings advocate for further evaluation of IRE1α inhibitors in human clinical trials. However, it is important to acknowledge the complex role of IRE1 activation in MM pathogenesis. Goldsmith et al. [63] have recently proved, in large NDMM population (n=768), that high IRE1α expression is associated with significantly worse treatment outcomes - HR 1.37 for progression and HR 1.55 for death. Intriguingly, the study also showed that the expression of IRE1α in post-treatment samples from refractory patients (disease progressed during treatment) is
significantly lower than in paired pre-treatment specimens. The authors, similarly to Zang et al. [45], explain this counter-intuitive finding by clonal selection. Thus, with this complexity in mind, efficacy of IRE1α inhibition in PI-resistant setting remains to be determined.

Grp78, a chaperone with pleiotropic roles in ER stress response, from initiating the UPR to increasing ER folding capacities, is another promising target [64]. Monoclonal IgM antibody against Grp78 was isolated and developed as PAT-SM6. After showing in vitro activity the product entered clinical phase of studies, showed favorable safety profile but failed to induce significant tumor reduction [65]. Even though the drug’s further tests were stopped, a case report of successful treatment of extramedullary, RR disease using combination of PAT-SM6 with bortezomib and lenalidomide [66] may act as a proof of principle for enhancing PI activity in RR MM patients by Grp78 inhibition.

Hoang et al. [67] described serum and glucocorticoid-regulated kinase’s (SGK) protective role in response to ER stress by disrupting IRE1/ASK-1 (apoptosis signal-regulating kinase 1) /JNK (c-Jun N-terminal kinase) pathway – the proapoptotic axis of UPR [68]. The study showed that patients with higher SGK expression achieve worse response to therapy with bortezomib. Recently Tsubaki et al. [69] showed that SGK inhibition by GSK650394 enhances bortezomib and ixazomib toxicity in primarily resistant MM cells.

Genes from RAS/MAPK (mitogen-activated protein kinases) pathway are most commonly mutated in MM [70], with NRAS mutations corresponding with decreased sensitivity to PIs [71]. Shirazi et al. [72] linked activating KRAS, NRAS and BRAF mutations with decreasing ER stress due to enhanced proteasome activity. The findings were supported by the confirmation of additive effect of using MAPK (selumetinib, trametinib) and RAF (TAK-632) kinase inhibitors in combination with bortezomib or carfilzomib. It provides mechanistic rationale for the kinase inhibitors activity in PI-resistant MM patients that has
been recently described in case reports [73, 74] and is investigated in many phase I/II clinical trials [75].

As previously mentioned, UPR can be activated by disturbances in membrane lipid saturation by interacting with ER calcium level and therefore affecting ER folding capabilities [76]. Interestingly, even in cells with mutant IRE1 and PERK, missing their BiP-binding luminal domains, UPR can be initiated by the sensors direct activation through lipid perturbations [77]. In line with these findings, Venkata et al. [78] showed that sphingosine kinase 2 (SK2), one of the key enzymes in sphingolipids metabolism, is overexpressed in patients-derived myeloma cells and that SK2 inhibitor, ABC294640 has antmyeloma activity against cell lines and mouse MM models. A phase Ib/II clinical trial of ABC294640 (NCT02757326), aiming to accrue 13 RR MM patients was initiated in 2016 but terminated in 2019 due to expiration of funding. The results of this study have supposedly been submitted but are not publicly available yet. Wallington-Beddoe et al. [79] were able to connect SK2 inhibitor activity with increasing ER stress and activating UPR. This finding was enforced by synergistic effect of combining bortezomib with another SK2 inhibitor - K145.

As for now, the most compelling data in favor of the idea of re-sensitizing patients to PIs were provided by the Swiss group led by Driessen who combined bortezomib with HIV protease inhibitor – nelfinavir. The drugs from this latter group, apart from being efficient in HIV infection treatment, proved also the antineoplastic activity [80]. Among their biological effects, proteasome inhibition and increasing ER stress were identified. These findings led the investigators to assess anti-MM activity of HIV protease inhibitors. First, they were able to show that, in this class of drugs, nelfinavir has the highest in vitro efficacy and synergistic activity with bortezomib and carfilzomib [81]. Then, a phase I trial of nelfinavir in combination with bortezomib identified 2 x 2500 mg dose as safe and potentially efficient – clinical activity was seen in 5 (3 partial responses, i.e. >50% reduction of monoclonal protein,
2 minor responses, i.e. >25% reduction of monoclonal protein) out of 6 bortezomib- and lenalidomide-refractory patients treated in the extension cohort [82]. The subsequent phase II trial also yielded promising results [83]. Use of combination of nelfinavir, bortezomib and dexamethasone resulted in at least partial response in 65% (22/34) of the heavily-pretreated MM patients (median 5 lines of previous therapies, 100% bortezomib refractory, 100% lenalidomide exposed). The authors underlined that response rates in such population of patients were similar or better than those achieved with recently approved novel drugs, whereas the estimated cost of the therapy does not exceed 15% of the medications currently used in this setting.

Conclusions

With the growing base of evidences supporting crucial interactions between ER stress, UPR and PIs activities, translation of these results into clinically meaningful outcomes is eagerly awaited. Better understanding of myeloma cells biology in the context of proteasome inhibition leads to identification of many vulnerabilities that can potentially be targeted to improve current treatment results. At this moment, among the investigated particles able to target the ER homeostasis, only a minority entered clinical trials phase. However, the example of nelfinavir provides prove of concept that exploiting adaptations present in resistant MM cells is feasible in clinic. It should encourage the research community to further develop the re-sensitizing approach.

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References:


Figure 1. Molecular targets within endoplasmic reticulum currently investigated to modulate endoplasmic reticulum stress. Inhibitory particles presented in red. Detailed description provided in text.
## Table 1. Summary of currently developed approaches to modulate endoplasmic reticulum stress in order to enhance proteasome inhibitors activity.

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BiP – binding immunoglobulin protein; Ero1 - FAD containing ER oxidoreductin 1; ER - endoplasmic reticulum; Grp78 – glucose regulated protein 78; GSTP - glutathione S-transferase P; HIV – human immunodeficiency virus; IRE1α - inositol-requiring enzyme 1α; MAPK - mitogen-activated protein kinase; MM - multiple myeloma; PDI - protein disulphide isomerase; PERK - double-stranded RNA-activated protein kinase [PKR]-like ER kinase; PI - proteasome inhibitors, SGK - serum and glucocorticoid-regulated kinase; SK2 - sphingosine kinase 2; UPR – unfolded protein response