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Resistance of Multiple Myeloma to Proteasome Inhibitors

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26S proteasomes degrade mostly proteins marked by poly-ubiquitination and is responsible for the majority of protein degradation in the cells. Consequently, proteasome inhibitors are toxic to many cells and proteasome inhibitors have become a preferred therapy for the blood cancer multiple myeloma. Bortezomib has been used to treat more than 400,000 myeloma patients since its FDA approval in 2004, and in 2012, another proteasome inhibitor, Carfilzomib, was also approved to treat refractory myeloma patients. Despite these encouraging progresses, drug resistance remains a prominent challenge to myeloma therapy using these inhibitors. In fact, all patients will eventually relapse with a highly advanced lethal disease. Therefore, it is important to understand the mechanisms of such resistance and to develop therapeutic approaches. There have been tremendous research effort on this topic, and it is currently clear that resistance to the proteasome inhibitor treatment can occur by several diverse mechanisms, all rescuing a particular step in the killing pathway elicited by proteasome inhibition. This editorial aims at reviewing how proteasome inhibition kills myeloma cells and how cells may evolve methods to inactivate critical steps of the killing mechanism and develop drug resistance. This information will be useful to identify crucial targets for drug development to overcome such resistance.

Proteasome inhibitors elicit many mechanisms to kill myeloma cells, and these mechanisms largely belong to two categories: (1) blocking the degradation of abnormal proteins, thus triggering ER stress and apoptosis; and (2) blocking the degradation of key regulatory proteins, thus directly altering pro-survival or pro-apoptotic signaling cascades. Myelomacells are abnormal B plasma cells that continuously synthesize and secret large amounts of immunoglobulins, and most of them abnormal and are rapidly cleared by proteasomes. Consequently, blocking their degradation easily triggers ER stress (the accumulation of unfolded protein in the endoplasmic reticulum). The cellular response to ER stress involves transcription of molecular chaperones to assist protein folding, and ERAD (ER-Associated Degradation) components to degrade abnormal proteins. However, in case the unfolded proteins are not readily cleared, as in myeloma cells with abundant misfolded immunoglobulins loaded in the ER, the ER stress persists, and the same transcription factors will also induce the expression of pro-apoptotic gene CHOP to downregulate Bcl2 expression. In addition, the accumulation of misfolded proteins upon proteasome inhibition can also cause Reactive Oxygen Species (ROS) production that directly cause mitochondrial injury and activate the mitochondrial apoptotic pathway [1,2]. On the other hand, proteasome inhibition can directly block the degradation of pro-apoptotic regulatory proteins to cause cell death. Examples of these regulatory proteins are IκB, which needs to be degraded after ubiquitination by β-TRCP in order to turn on the pro-survival NF-κB pathway [3] and subsequently the expression and secretion of interleukin-6 (IL-6 [4]). Other pro-apoptotic proteins that are stabilized by proteasome inhibitor include Noxa, a BH3-onlyBcl2 family protein that is degraded after ubiquitination [5] and the tumor suppressor p53, which is degraded after ubiquitination by Mdm2. Although proteasome inhibitor can trigger many different mechanisms to kill myeloma cells, unfortunately drug resistance mechanisms have emerged in almost every step to promote cell survival under proteasome inhibitor treatment.

Since the primary target of proteasome inhibitors is the proteasome, it is not surprising that cells can elicit mechanisms to compensate for loss of protein degradation in various ways.

First, cells can elicit a compensatory increase of proteasomal degradation by two main mechanisms: (1) mutation of the proteasome subunit β5, rendering it to be insensitive to Bortezomib and (2) inducing the production of more proteasomes. Studies have demonstrated that in vitro culture of myeloma cells or monocytes with a sublethal concentration of Bortezomib can selection cells that carry mutations in β5, the catalytic proteasome subunit that is the primary target of Bortezomib. Consequently, the mutant β5 becomes much more refractory to inhibition by Bortezomib [6]. Interestingly, these studies performed by different groups with different cells come to very similar mutation sites, indicating that Bortezomib treatment may indeed favor the selection of these mutants. However, so far there is no evidence that such mutations occur in patients. On the other hand, cells can induce the production of 26S proteasomes upon proteasome inhibitor treatment. In human cells, the transcription factor Nrf1 appears to be responsible for coordinately inducing all 26S proteasomes upon proteasome inhibition, and this induction promotes cell surviva [7,8]. In addition to Nrf1, there are other transcription factors that can also regulate the transcription of 26S subunits. Nrf2 is a related transcription factor to Nrf1 that regulates 26S expression upon oxidative stress [9], and in hypoxic tumor tissues [10]. Recently, Vilchez et al. [11,12] reported that FoxO4 is a key transcription factor that induces the expression of only Rpn6, but not other proteasome subunits, to greatly elevate cellular proteasome activity in multipotent cells. Interestingly, prolonged incubation of monocytes with a sub-lethal concentration of proteasome inhibitors can cause preferential induction of β5 above other proteasome subunits, indicating that an unidentified transcription factor that may induce only β5 [6]. The relative contribution of these transcriptional mechanisms to the compensatory production of 26S proteasomes upon proteasome inhibition remains to be determined. Since newly-produced proteasomes will still be subject to inhibition by proteasome inhibitors, this transcriptional response is unlikely to become a long-term drug resistance mechanism, but would greatly affect the efficiency of myeloma cell killing. Currently many other proteasome inhibitors are under clinical trials [13] to test for their improved therapeutic potential. However, most of these inhibitors were developed to achieve a higher affinity for the chymotrypsin-like site (β5), or as an irreversible inhibitor of β5. It would be equally important to develop proteasome inhibitors that also blocks the β1 (caspase-like)

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and β2 (trypsin-like) sites since inhibition of at least two active sites was required to substantially inhibit cellular protein degradation [14] thus to achieve more efficient proteasome inhibition.

Second, cells can activate the other protein degradation machinery, the autophagy-lysosome system. In autophagy degradation pathway, substrates were first enclosed by membrane structures called autophagosomes. Autophagosome then fuse with lysosomes to cause the degradation of the substrates. Although initially characterized as a non-selective protein degradation, there is growing evidence that autophagy can mediate the degradation of poly-ubiquitinated proteins through a class of ubiquitin receptor proteins that can also bind LC3 family proteins on the growing autophagosome membranes, thereby sorting the ubiquitin conjugates to autophagosomes for degradation [15]. It has been firmly established that upon proteasome inhibition, cell scan active autophagy. However it is under debate whether autophagy induction rescues cells from killing by Bortezomib. There are equally strong evidences to suggest that autophagy can either promote cell survival or autophagic cell death of Bortezomib treated cells. Co-treatment of BTZ and autophagy inhibitors such as 3-MA, CQ, Baf can cause synergistic killing in many cells [16-21] via apoptosis and necrosis. However, in myeloma cells, inhibition of autophagy promotes survival under BTZ [22]. In Human Umbilical Vein Endothelial Cells (HUVEC), co treatment of 3-MA with BTZ increase cell survival at 20 hrs, but causes necrosis after 48 hrs [20]. It appears that autophagy in general appear to be cytoprotective. However, autophagy can also mediate cell death in certain scenario, as prolonged autophagy can kill cells. In myeloma cells, autophagy can easily promote cell death, probably due to higher basal level of ER stress and autophagy in myeloma cells, which limit their ability to tolerate autophagic response. Therefore, although hydroxychloroquine and bortezomib combinational therapy is currently under Phase 1 trial to treat refractory multiple myeloma and has yielded minor effects [23], further investigation is absolutely necessary to determine the therapeutic window that maximizes the killing of myeloma cells by co-inhibition of proteasome and autophagy.

Autophagy is responsible for degrading many proteins as well as organelles. Therefore, instead of inhibit global autophagy; it is more beneficial to inhibit crucial autophagy components that function specifically in the clearance of ubiquitinated proteins. One such candidate is histone deacetylase-6 (HDAC6), which plays several important roles in the degradation of aggregated ubiquitin conjugates by autophagy. First, HDAC6 can bind ubiquitin conjugates via its UBA domain and recruit these conjugates to the Microtubule Organization Center (MTOC) to facilitate the formation of aggresomes. Second, HDAC6 promotes the clearance of these aggresomes. The second step may involve the de-acetylation of cortactin to remodel the actinomyosin system [24] and cause the fusion of the aggresomes with lysosomes [25]. These properties make HDAC6 an ideal therapeutic target to block specifically ubiquitin aggregate clearance without affecting global autophagy. Indeed, both the pan-HDAC inhibitor Romidepsin [26] and as selective HDAC6 inhibitor, ACY-1215 [27], were shown to promote killing of myeloma cells by Bortezomib in preclinical assays. Other autophagic components that are key to degrade poly-ubiquitinated conjugates are autophagic ubiquitin receptors (p62, Nbr1, NDP52, and Optineurin) and their binding partner, the Atg8 family autophagy proteins (LC3A, B, C, GABARAP, GABARAPL1, L2). The presence of multiple members of these protein families indicates functional specificity. For example, p62 and Nbr1 promote the degradation of poly-ubiquitinated protein aggregates, while NDP52 and Optineurin may specialize in autophagic destruction of pathogenic bacteria. Identification of the particular autophagic components that are activated (transcriptionally or post translationally) in response to proteasome inhibition will provide valuable therapeutic targets to allow specific blockage of the autophagy pathway that help clear ubiquitinated proteins following proteasome inhibition.

In addition to degrading abnormal proteins, cells can also limit the damage caused by these proteins without degrading them. These mechanisms can also be exploited by the cells to promote survival under proteasome inhibition. Protein chaperones including heat shock proteins and ER chaperones binds misfolded or damaged proteins and facilitates their re-folding while prevents the formation of aggregations. Intriguingly, several of these chaperones have been shown to play a role in the resistance to proteasome inhibitors, including HSP90 (help refolding of heat damaged proteins, and stabilizes several key oncoproteins such as Her2 and Raf1), Hsp27 (a small heat shock proteins that not only functions in thermotolerance, but also inhibits procaspase-9 and can even activate ubiquitin-dependent degradation by the proteasome), and grp78/bip (ER chaperone that promotes refolding of newly synthesized proteins in the ER lumen). Geldanamycin (17-AAG) has been developed to block the ATPase activity of HSP90, and preclinical data suggests that a combination of Geldanamycin and Bortezomib enhance killing of multiple myeloma cells [28].

In addition to degrading abnormal proteins, proteasomes can also degrade key regulatory proteins that functions in both pro-survival and pro-apoptotic pathways. Therefore, reactivation of these regulatory proteins has become a useful way of overcoming cell killing by proteasome inhibitors. On the one hand, cells can down regulate anti-proliferative or pro-apoptotic factors such as the repression of the expression of pro-apoptotic Bcl2 family proteins such as Bim [29,30], mutational silencing of the tumor suppressor p53, and down regulation of cell cycle inhibitor p27, frequently via post-translational modifications [31,32]. On the other hand, cells can up regulate pro-survival signaling cascades such as the emergence of Bortezomib-resistant NF-κB signaling that is consistently active and also promote the secretion of interleukin-6 [33,34], and the activation of the Akt pro-survival pathway [35,36]. Based on these findings, clinical and pre-clinical trials are ongoing to determine whether inhibitors of Bcl2, NF-κB, IL-6, and Akt could enhance the therapeutic effect of Bortezomib. Since cells can evolve many different mechanisms to alter the downstream pro-survival and pro-apoptotic pathways of proteasome inhibition, it is key to precisely diagnose the underlying drug resistance mechanisms in order to select the right therapeutic agents.

The survival of myeloma cells upon proteasome inhibitor treatment can also be promoted by their contact with Bone Marrow Stromal Cells (BMSCs) [37] and vascular endothelial cells [38]. Such microenvironment turn on several anti-apoptotic and pro-proliferative signaling cascades (PI3K/Akt/mTOR/S6K, NF-κB, Ras/Raf/MAPK JAK/STAT3) in myeloma cells via either direct cell-cell contact or via cytokine receptors such as IL-6R, IGF-1R, c-met, IL-1R, and IL-21R [37]. Also upregulated are the caspase inhibitors (FLIP, c-IAP2, survivin), anti-apoptotic Bcl2 family proteins, telomerase activity, and HIF1α [37]. Intriguingly, BMSCs may induce the emergence of Bortezomib-resistant NF-κB activity in myeloma cells via cytokines such as IL-8 [39]. Recently, de Haart et al. demonstrated that such micro-environment is able to trigger Cell Adhesion-Mediated Immune Resistance (CAM-IR) against CTL lysis, via downregulation of Fas and upregulation of the caspase 3 inhibitor survivin in myeloma cells [38], and therefore proposed CAM-IR modulation as a useful way of overcoming drug resistance.

Another important drug resistance mechanism is due to the presence of myeloma stem cells or progenitor cells. These cells are not immunoglobulin-secreting plasma cells and are therefore less sensitive to killing by proteasome inhibitors. Matsui et al. defined myeloma stem cells as a population of cells that do not express the cell surface antigen syndecan-1 (CD138) [40], and subsequent studies showed that these CD138 cells are resistant to Bortezomib as well as other chemotherapeutic agents [41,42]. Recently, Leung-Hagesteijn et al. reported that a population of myeloma progenitor cells is characterized with not only lack of CD138, but also low expression of Xbp1 [43]. Xbp1 is a transcription factor that is part of the unfolded protein response. It not only regulates the expression of chaperones and ERAD components, but is also an important factor in B cell maturation. Leung-Hagesteijn et al. showed that these Xbp1- myeloma progenitor cells have decreased ER front loading and consequently lower sensitivity to the cytotoxicity caused by Bortezomib treatment. Intriguingly, Xbp1 inactivating mutations were found in 2/20 myeloma tumors, clearly indicating its clinical relevance [44].

In summary, the drug resistance mechanism in multiple myeloma is heterogeneous. Consequently, there is no single method to overcome drug resistance, and the therapeutic solution depends heavily on diagnosis of drug resistance mechanisms. With the development of powerful molecular diagnostic approaches such as high throughput proteomics and genome sequencing techniques, diagnosis of the drug resistance mechanism via "personalized medicine" should greatly improve our treatment of multiple myeloma. However, before such advanced techniques become affordable and widely-used practice, efforts should also be made in other aspects to improve the therapy of myeloma patients. Most crucial to combating drug resistance is early diagnosis. Myeloma is notoriously hard to diagnose early because unlike other solid tumors, early-stage myeloma has almost no prominent symptoms. Common symptoms such as bone pain and elevated blood protein level are frequently neglected and not followed up seriously. Late diagnosis means that at the time of treatment, the cancer is already in an advanced stage, and many oncogenic mutations, such as the silencing of p53 and the activation of Akt and Ras, have already taken place and will contribute to the failure of the Bortezomib therapy. On the other hand, successful therapy with proteasome inhibitors would rely heavily on a highly efficient inhibition of protein degradation in order to achieve maximal killing of cancer cells. New proteasome inhibitors that target the trypsin-like and caspase-like activities of the proteasome should be developed to achieve a more complete inhibition of the proteasome. In the meantime, proteasome inhibition should be combined with other therapeutic agents that prevent the cellular compensatory mechanisms to clear abnormal proteins via autophagy, such as with HDAC6 inhibitors. Finally, it would be essential to target myeloma progenitor cells that are refractory to killing by proteasome inhibitors, and to block the pro-survival signals provided by accessory cells in the myeloma micro-environment, which would require a more complete understanding the signaling mechanisms.

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