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PROTEASOME INHIBITION IN MULTIPLE MYELOMA: Therapeutic Implication

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■ **Abstract** Normal cellular functioning requires processing of proteins regulating cell cycle, growth, and apoptosis. The ubiquitin-proteasome pathway (UBP) modulates intracellular protein degradation. Specifically, the 26S proteasome is a multienzyme protease that degrades misfolded or redundant proteins; conversely, blockade of the proteasomal degradation pathways results in accumulation of unwanted proteins and cell death. Because cancer cells are more highly proliferative than normal cells, their rate of protein translation and degradation is also higher. This notion led to the development of proteasome inhibitors as therapeutics in cancer. The FDA recently approved the first proteasome inhibitor bortezomib (Velcade^{TM}), formerly known as PS-341, for the treatment of newly diagnosed and relapsed/refractory multiple myeloma (MM). Ongoing studies are examining other novel proteasome inhibitors, in addition to bortezomib, for the treatment of MM and other cancers.

INTRODUCTION

Multiple myeloma (MM) remains fatal despite all available therapies (1, 2), and novel approaches that target mechanisms regulating MM cell growth, survival, and apoptosis are urgently needed. Apoptosis is the primary means by which most radio- and chemotherapy modalities kill cancer cells (3); conversely, resistance to apoptosis is one potential mechanism whereby tumor cells evade cytotoxic drug-induced and immune-mediated cell death (4). Our studies to date have delineated apoptotic signaling triggered by various conventional and novel anti-MM agents (5). Importantly, recent studies show remarkable anti-MM activity of the proteasome inhibitor PS-341/bortezomib (Velcade^{TM}) even in MM cells refractory to multiple prior therapies, including dexamethasone (Dex), melphalan, and thalidomide (6, 7). In addition to directly inducing apoptosis of MM cells, multiple other lines of evidence provided rationale for the use of proteasome inhibitors (PIs) to

treat MM. First, adhesion of MM cells to bone marrow stromal cells (BMSCs) triggers transcription and secretion of MM growth factors, such as interleukin-6 (IL-6) or IGF-1, which stimulate the growth of MM cells and also block the cytotoxic effects of chemotherapy (8, 9). Inhibition of proteasomes downregulates adhesion molecules and secretion of cytokines, thereby abrogating bone marrow (BM)-dependent growth of MM cells (10–12). Second, angiogenesis plays a role in MM pathogenesis (13, 14), and bortezomib is an antiangiogenic agent (15–17). Finally, in vitro studies showed that bortezomib adds to the cytotoxicity of conventional anti-MM agents including Dex- and DNA-damaging agents (6, 18). Indeed, based on our preclinical (6) and phase II clinical studies (19), the FDA recently approved bortezomib for the treatment of relapsed/refractory MM. This successful development of bortezomib therapy for MM has established proteasome inhibition as an effective therapeutic strategy for the treatment of cancer.

PROTEIN DEGRADATION VIA UBIQUITIN-PROTEASOME PATHWAY

Major intracellular processes are regulated by transcription, translation, and protein degradation (20). Specifically, recent reports show that degradation of proteins is critical not only for maintaining normal cell functions but also for response to various chemotherapeutic agents (21, 22). Protein ubiquitination and degradation regulate various cellular processes, including cell cycle progression from G1 to S phase, tumor suppression, transcription, DNA replication, inflammation, and apoptosis (23–26); conversely, mutations or alterations in the ubiquitination and/or proteasomal degradation cascades result in defective transition from G1 to S phase (24, 27). The ubiquitin-proteasome pathway (UBP) degrades the majority of damaged/misfolded, short (half-lives less than three hours), or long-lived regulatory proteins in the cell (28); conversely, blockade of protein degradation by proteasome inhibitors causes accumulation of ubiquitin-bound misfolded/damaged proteins, which in turn triggers heat-shock response and cell death (28, 29). Indeed, proteasome inhibitors do not target specific cellular proteins or associated functions, but rather, affect a wide spectrum of proteins with diverse functions.

Proteasomal protein degradation occurs through these sequential events: Protein is first marked with a chain of small polypeptides called ubiquitin; E1 ubiquitin enzyme then activates ubiquitin and links it to the ubiquitin-conjugating enzyme E2 in an ATP-dependent manner; E3 ubiquitin ligase then links the ubiquitin molecule to the protein; a long polypeptide chain of ubiquitin moieties is formed; and finally, proteasomes degrade protein into small fragments and free ubiquitin for recycling (29, 30).

Proteasomes are key regulators of protein degradation (31): The human cell contains approximately 30,000 proteasomes, each equipped with protein-digesting proteases. Proteasomes regulate diverse cellular functions, including transcription, stress response, viral infection, cell cycle, oncogenesis, ribosome biogenesis,

abnormal protein catabolism, neural and muscular degeneration, cellular differentiation, antigen processing, and DNA repair (31). The 26S proteasome complex, which constitutes up to 2%–3% of the total protein in cells, has two 19S units flanking a barrel-shaped 20S proteasome core (24, 29, 32) (Figure 1*A*). Four stacked rings comprise the 20S structure: two central β rings are surrounded by two α rings, each composed of seven proteins. Most action occurs at six sites located in the β rings: Two sites act like chymotrypsin, which cleaves after hydrophobic residues; two trypsin-like sites cleave after basic residues; and two are like caspase, cleaving after acidic residues (33, 34) (Figure 1*B*).

The 19S units regulate entry into the 20S core chamber of only those proteins marked for degradation (29, 35). Each 19S unit contains binding sites for ubiquitinated protein, enzymes to depolymerize the ubiquitin chain, and six ATPases that unfold the proteins, thereby preparing them for entry into the proteasome (Figure 1*C*). Attachment of ubiquitin to a target protein is the principal mechanism whereby proteins are marked for degradation by the proteasome. Importantly, blocking proteasome activity leads to stabilization of inhibitory proteins, thereby abrogating growth, survival, and triggering apoptosis (Figure 1*D*).

Most proteasome inhibitors fall in three categories: peptide aldehydes, peptide boronates, and nonpeptide inhibitors such as lactacystin. Peptide aldehydes (MG-132, MG-115, ALLN, or PSI) potently, but reversibly, block the chymotrypsin-like activity; however, they also inhibit lysosmal cysteine and serine proteases and calpains. The peptide boronates, such as bortezomib/PS-341, are reversible and more potent and selective than peptide aldehydes. Finally, lactacystin is a natural, irreversible, nonpeptide inhibitor that is more selective than peptide aldehydes but less selective than peptide boronates.

RATIONALE FOR TARGETING THE PROTEASOME FOR CANCER THERAPY

Given that the proteasome is involved in various distinct cellular functions, it was difficult to predict whether proteasome inhibition could be used as a target for chemotherapy with an acceptable therapeutic index. However, multiple lines of evidence suggest that proteasome inhibitors are more cytotoxic to proliferating malignant cells to quiescent normal cells: (*a*) Proteasome inhibitor lactacystin triggers apoptosis even in gamma-radiation-resistant CLL cells without affecting the viability of normal lymphocytes (36); (*b*) proteasome inhibitor induces cell death in contact-inhibited primary endothelial cells but not quiescent cells (37); (*c*) lactacystin induces apoptosis in oral squamous carcinoma cells but not in oral epithelial cells (38); (*d*) HL60 leukemic cells are significantly more sensitive to proteasome inhibitor than quiescent cells (39); and (*e*) bortezomib triggers apoptosis of MM cells at doses that do not affect the viability of normal lymphocytes (6) (Figure 2). The mechanism whereby cancer cells are more susceptible to proteasome inhibitors than normal counterparts is unclear (12). One possibility is that

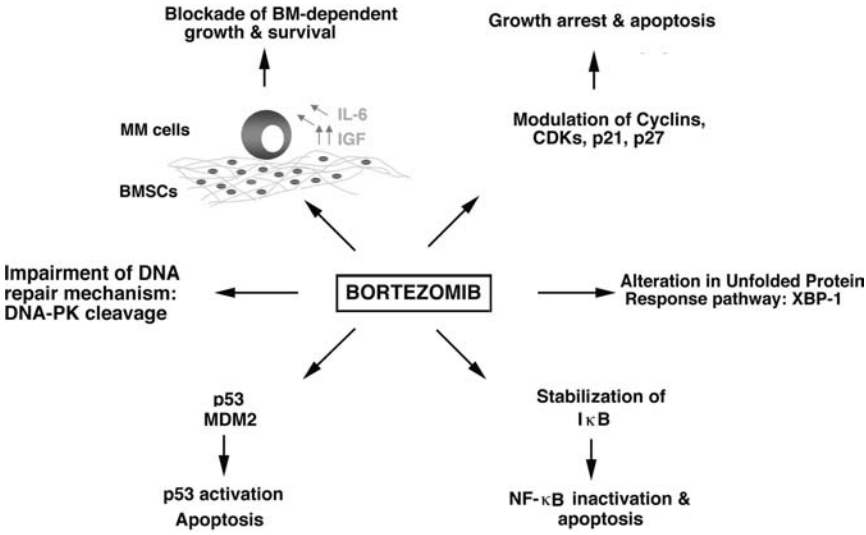

Figure 2 Bortezomib targets various growth and survival signaling mechanisms in MM cells and abrogates protection from bone marrow stroma cells (BMSCs).

malignant cells have altered or defective cell cycle proteins, which leads to an increased proliferation rate. These cells therefore accumulate damaged proteins at a much higher rate than do normal cells, which in turn increases dependency on the proteasomal degradation. In contrast, another study showed that quiescent cancer cells are more susceptible to proteasome inhibition than are normal counterparts (40). NF-κB is linked to proliferation and drug-resistance in cancer cells (41, 42), and PIs downregulate NF-κB activation, thereby enhancing the cytotoxic effects of chemotherapy (43). Together, these findings suggest that the proteasome is a valid target for chemotherapy with a tolerable therapeutic index.

BORTEZOMIB/PROTEASOME INHIBITOR PS-341 TARGETS NF-κB IN MM CELLS

As noted above, one of the major mechanisms whereby proteasome inhibitors exert their growth inhibitory effects in cancer cells is by blocking NF-κB signaling (28). Multiple studies have linked constitutive activation of NF-κB to growth/proliferation and drug-resistance, thereby conferring differential sensitivity to proteasome inhibitors in cancer versus normal cells (43). Activation of NF-κB occurs via the following sequential events: activation of IκB kinase (IKK), IκB phosphorylation, ubiquitination and degradation of IκB, and nuclear translocation of p50/65 NF-κB (44, 45). Once in the nucleus, NF-κB promotes the production of cytokines (IL-6, TNF-α), survival factors (IAPs, Bcl-Xl), and cell

adhesion molecules [intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and E-selectin] (45).

In the context of MM, NF-κB mediates key cellular functions, including immune responses, as well as growth, survival, and apoptosis (46, 47). Intrinsic activation of NF-κB is associated with growth and survival of MM cells. Specifically, adhesion of MM cells to BMSCs triggers NF-κB-mediated transcription and secretion of IL-6 and insulin-like growth factor-I (46–48); both IL-6 and IGF-1 promote the survival of MM cells in the BM by blocking apoptosis triggered by conventional agents such as Dex (49) (Figure 2). Patient MM-derived primary cells and BMSCs have upregulated NF-κB activity relative to normal cells (50). Furthermore, drug-sensitive MM cells show lower NF-κB activity than drug-resistant MM cells, suggesting that NF-κB confers chemoresistance (50). Elevated NF-κB levels have also been reported in MM cells derived from patients relapsing after chemotherapy (47). These findings indicate that NF-κB is a key regulator of growth and survival of MM cells in the BM milieu. Importantly, treatment of MM with bortezomib prevents degradation of IκB, thereby blocking not only NF-κB activation but also related cytokine production and the survival advantage for MM cells conferred by BMSCs (Figure 2).

Bortezomib downregulates NF-κB; however, our recent work shows that NF-κB inhibition alone is unlikely to account for the total anti-MM activity of bortezomib (51, 52). The evidence for this finding is derived from the experiments using a specific inhibitor of IκB, PS-1145. Both PS-1145 and bortezomib blocked TNF-α-induced NF-κB activation by inhibiting phosphorylation and degradation of IκB-α. Dex, a conventional anti-MM agent, increases IκB-α protein and thereby enhances blockade of NF-κB activation by PS-1145. Importantly, both bortezomib and PS-1145 block NF-κB activation; however, in contrast to bortezomib, PS-1145 only partially inhibits MM cell growth (20%–40% inhibition by PS-1145 versus 80%–90% inhibition by bortezomib) (51), suggesting that NF-κB inhibition cannot account for the overall anti-MM activity of bortezomib.

Multiple genomics and proteomic studies have now established that besides modulating NF-κB, bortezomib indeed affects various other signaling pathways. For example bortezomib-induced apoptosis is associated with initiation of the following additional events: (*a*) activation of classical stress response proteins such as heat shock proteins, Hsp27, Hsp70, and Hsp90 (17, 53); (*b*) upregulation of c-Jun-NH2-terminal kinase (JNK) (54) (Figure 3); (*c*) alteration of mitochondrial membrane potential and generation of reactive oxygen species (ROS) (55) (Figure 3); (*d*) induction of intrinsic cell death pathway, i.e., the release of mitochondrial proteins cytochrome-c/Smac into cytosol and activation of caspase-9 > caspase-3 cascade (Figure 3) (49); (*e*) activation of extrinsic apoptotic signaling through Bid and caspase-8 cleavage (17) (Figure 3); (*f*) inactivation of DNA-dependent protein kinase (DNA-PK) (18) (Figure 2), which is essential for the repair of DNA double-strand breaks; (*g*) inhibition of MM to BMSCs-host interaction, thereby blocking of associated MM growth factor transcription and secretion from BMSCs (56) (Figure 2); and (*h*) inhibition of MM cell

growth factor–triggered signaling: MAPK and PI3-kinase/Akt (57). Although many of these cellular events may appear correlative and common to other apoptotic agents, our studies have directly established an obligatory role of JNK using dominant-negative strategies or specific biochemical inhibitors of JNK (54). The role of IκB is under evaluation using dominant-negative constructs and/or IκB knockout cells. Bortezomib may have additional substrates that mediate normal cell growth and survival. It is very likely that all the above signaling cascades mutually interact and contribute toward the overall response to bortezomib in MM cells.

MECHANISMS MEDIATING BORTEZOMIB-RESISTANCE AND THERAPEUTIC STRATEGIES TO OVERCOME BORTEZOMIB-RESISTANCE

Bortezomib kills MM cells; however, prolonged exposure is associated with toxicity and development of bortezomib-resistance. Mechanisms mediating bortezomib-resistance have now been delineated. For example, our recent study showed that treatment with bortezomib induces apoptosis in SUDHL6 (DHL6), but not SUDHL4 (DHL4), lymphoma cells (53). Microarray analysis showed high RNA levels for heat shock protein-27 (Hsp27) in DHL4 versus DHL6 cells, which correlated with Hsp27 protein expression. Importantly, blocking Hsp27 using an antisense (AS) strategy restores the apoptotic response to bortezomib in DHL4 cells; conversely, ectopic expression of wild-type (WT) Hsp27 renders bortezomib-sensitive DHL6 cells resistant to bortezomib. These findings provide the first evidence that Hsp27 confers bortezomib resistance. Moreover, MM cells obtained from patients refractory to bortezomib treatment also show high levels of Hsp-27 expression. The mechanism(s) whereby Hsp-27 mediates bortezomib-resistance are unclear. We and others have shown that Hsp-27 negatively regulates the release of mitochondrial protein cytochrome-c and Smac, thereby blocking the intrinsic cell death-signaling pathway (58–60). Further studies are required to determine whether inhibition of Hsp-27 using clinical grade–specific inhibitors enhances bortezomib anti-MM activity and overcomes drug-resistance. Besides Hsp-27, Bcl2 protein family members also confer drug-resistance in many cell types (61), and bortezomib-triggered apoptosis in MM cells is also partially abrogated by Bcl2 expression (17). Upregulated expression of inhibitors of apoptosis proteins (IAPs), such as XIAP, may also contribute to bortezomib resistance (17). Indeed, it is unlikely that one specific mechanism confers bortezomib resistance, suggesting that combinations of bortezomib with other conventional and/or novel agents will be required to overcome drug resistance.

To address this issue, in vitro studies showed that combining bortezomib with other conventional agents, such as Dex, doxorubicin, melphalan, or mitoxantrone, triggers additive and/or synergistic anti-MM activity (6, 18, 50). Moreover, combined treatment of MM cells and of MM patient cells with bortezomib and novel

agents, such as relvimid or triterpenoids CDDO-Im, induces synergistic anti-MM activity (18, 62). For example, bortezomib + CDDO-Im triggers synergistic apoptosis, even in bortezomib-resistant MM cells from patients, thereby providing the basis for clinical protocols using this treatment regimen (62). Besides MM, combined bortezomib and irinotecan treatment also induces apoptosis in pancreatic tumor xenografts (63). Another study showed that bortezomib prevents irinotecan-induced NF-κB activation, thereby increasing chemosensitivity and apoptosis in colorectal cancer cells in a xenograft model (64). Together, these combination strategies will reduce attendant toxicity and overcome and/or prevent the development of drug-resistance.

Bortezomib in Clinic

It is known that bortezomib mediates its effects by inhibiting cellular proteasomes; however, whether proteasome inhibition is universally required for bortezomib-triggered apoptosis is unclear. Our findings showed that treatment with bortezomib led to 82% and 88% inhibition of proteasome activity in bortezomib-resistant SUDHL4 and bortezomib-sensitive SUDHL6 lymphoma cells, respectively (53). Together, these data confirm that (*a*) the proteasome inhibition pathway is not defective in bortezomib-resistant DHL4 cells, and (*b*) proteasome inhibition is not correlated with apoptosis.

Direct determination of proteasome inhibition in patient blood and tissue samples was examined in phase I studies. Bortezomib was well tolerated at doses, resulting in up to 80% proteasome inhibition (65). Furthermore, extended dosing did not further reduce sensitivity to proteasome inhibition. These data suggest that proteasome inhibition is the main function of the proteasome inhibitor, but that proteasome blockade may not correlate with degree of cytotoxicity in cancer cells.

PHASE I TRIALS OF BORTEZOMIB

Phase I trials of bortezomib in hematologic and solid tumors confirmed the antineoplastic activity of bortezomib observed in preclinical in vitro studies (66, 67). During an initial dose-ranging trial in patients with refractory MM, lymphoma, and leukemia, patients received bortezomib twice weekly for 4 weeks followed by 2 weeks of no therapy. The maximum tolerated dose (MTD) was 1.04 mg/m^2 (66). Dose-limiting toxicities (DLTs) were fatigue and malaise, electrolyte imbalances, and thrombocytopenia. Patients with lower than normal platelet counts at study entry were at higher risk for the development of thrombocytopenia. Even in phase I studies, encouraging responses were observed in MM patients: one complete response (CR), evidenced by immunofixation-negativity, and eight responses with reduction in serum monoclonal protein and marrow plasmacytosis. Bortezomib antitumor activity in these phase I studies was also noted in non-Hodgkin's lymphoma (NHL).

The efficacy of bortezomib was evaluated in another phase I trial in advanced solid tumors, using a 3-week dose cycle (twice weekly for 2 weeks followed by 1 week of no therapy) (67). The MTD was 1.56 mg/m^2, indicating that the 3-week cycle may allow administration of higher doses than the 6-week cycle. No hematologic DLT was observed; and nonhematologic DLTs included grade-3 diarrhea and neuropathy. Of note, grade-3 neuropathy was observed predominantly in patients with preexisting neuropathy, and improved after drug discontinuation. Bortezomib also showed antitumor activity in other malignancies including non-small cell lung cancer, nasopharyngeal carcinoma, malignant melanoma, and renal cell carcinoma (67).

PHASE II STUDIES IN MM

A phase II bortezomib study was conducted in MM patients who had relapsed and were refractory to multiple prior therapies. Each cycle of therapy included bortezomib (1.3 mg/m^2) administered twice weekly for 2 weeks, with 1 week off (19). Oral dexamethasone was given to patients with a suboptimal response after two cycles, and eight cycles of therapy were given to responders. Two hundred and two patients were enrolled, all of whom received corticosteroids, 92% alkylating agents, 81% anthracyclines, 83% thalidomide, and 64% stem-cell transplant; the median number of prior therapies was six. Poor prognostic factors at enrollment included elevated beta-2-microglobulin and abnormal cytogenetics. Of 193 patients, 4% achieved a CR, evidenced by MM protein undetectable by both electrophoresis and immunofixation; 6% achieved showed a near CR, evidenced by MM protein detectable only by immunofixation. Partial response (PR) was noted in 18% and minimal response (MR) in 7% patients. Overall response rate (CR + PR + MR) was 35%.

Response to bortezomib was examined relative to prognostic factors, including the patient's age; gender; type of MM; beta-microgloblin levels; extent of disease, i.e., plasma cells in BM; chromosomal abnormalities, i.e., deletion of chromosome 13; and intensity of prior therapies. Statistical analysis (univariate) indicated that >50% plasma cells in BM significantly predicted a lower response rate. Multivariate analysis suggested that older age (65 years or older) and >50% plasma cells in BM significantly predicted for lower response rate. Major responses (CR and PR) were associated with improved hemoglobin and nonmyeloma immunoglobulin levels, decreased transfusion requirements, increased platelet counts, and improvements in global quality of life and disease symptoms. Median time to disease progression (TTP) for all patients was 7 months, compared with TTP of 3 months for the last treatment before enrollment, and 13 months of TTP for patients achieving a CR or PR. Responses were durable: Median response duration was 12 months among patients achieving an objective response (CR + PR + MR) and 15 months among those achieving a CR or near CR. The median survival for the entire population (n = 202) was 16 months and patients achieving a major response (CR + PR)

survived significantly longer than those who did not ($P = 0.007$). Of 74 patients who did not achieve at least a MR and therefore received dexamethasone in combination with bortezomib, 18% improved; these included 6 patients with steroid-refractory disease, indicating that bortezomib can overcome resistance to steroids. Most commonly reported adverse events were nausea, vomiting, diarrhea, fatigue, loss of appetite including anorexia, constipation, anemia, thrombocytopenia, peripheral neuropathy, and pyrexia.

CONCLUSIONS

The proteasome is a promising target in the treatment of cancer. Ongoing research in this field will unveil the complex mechanisms whereby proteasome inhibitors impact a wide array of cellular functions with a differential sensitivity of normal versus cancer cells. More specific therapeutic targets may be the E2 and E3 ubiquitin enzymes, which target unique proteins. Proteasomes have six active sites, and blocking the chymotrypsin-like site decreases protein degradation significantly, whereas inhibition of trypsin- or caspase-like sites is less effective. Whether simultaneous inhibition of all three activities is more cytotoxic to cancer versus normal cells remains to be examined. The proteasome inhibitor bortezomib/PS-341 has shown potent preclinical activity in vitro as well as therapeutic activity in hematologic malignancies, especially MM. Importantly, bortezomib is the first treatment in more than a decade to be FDA approved for patients with MM, and the European Commission also granted marketing authorization for bortezomib for the treatment of patients with MM who have received at least two prior therapies and have demonstrated disease progression on their last therapy. Ongoing clinical trials are examining its efficacy in other hematologic malignancies and solid tumors.

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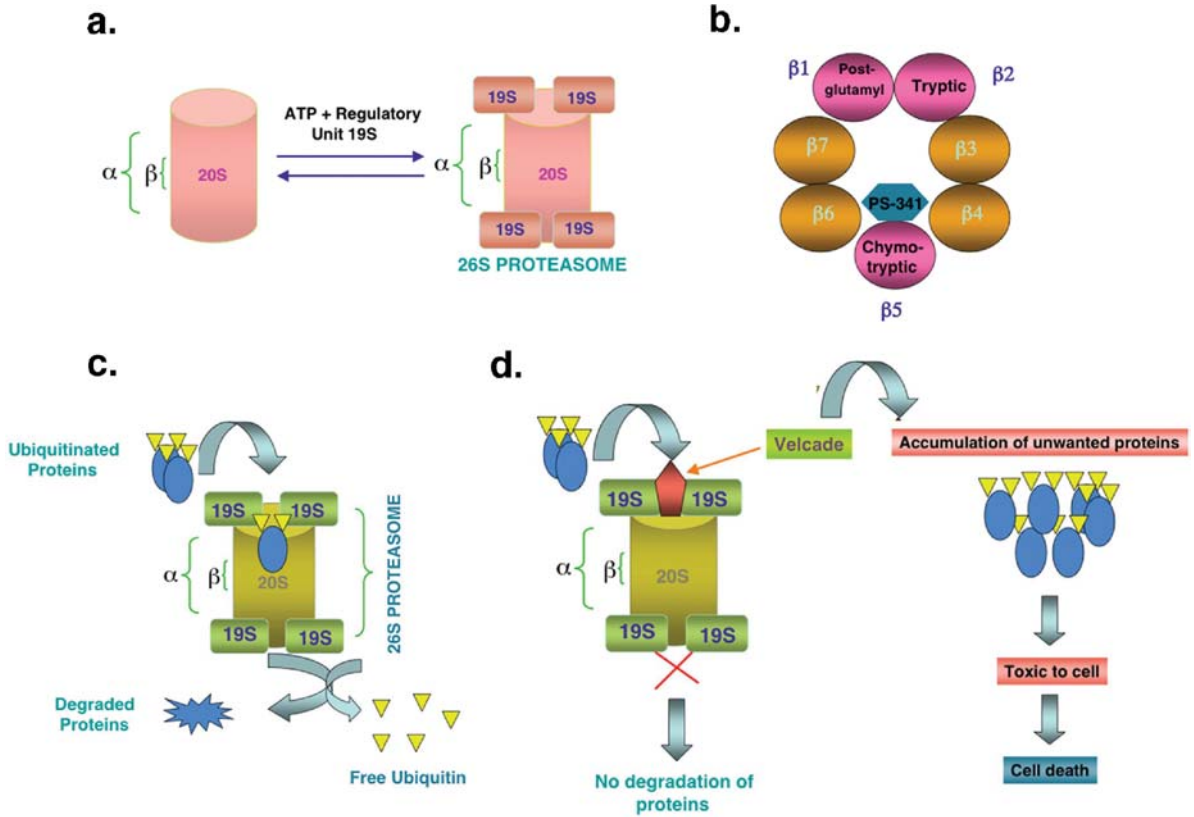

Figure 1 (*A*) Structure and function of proteasomes; (*B*) cross-sectional view of 26S proteasome complex; (*C*) process of degradation of ubiquitinated proteins by proteasome complex; and (*D*) bortezomib/velcade blocks the proteasomal protein degradation resulting in accumulation of cytotoxic proteins.

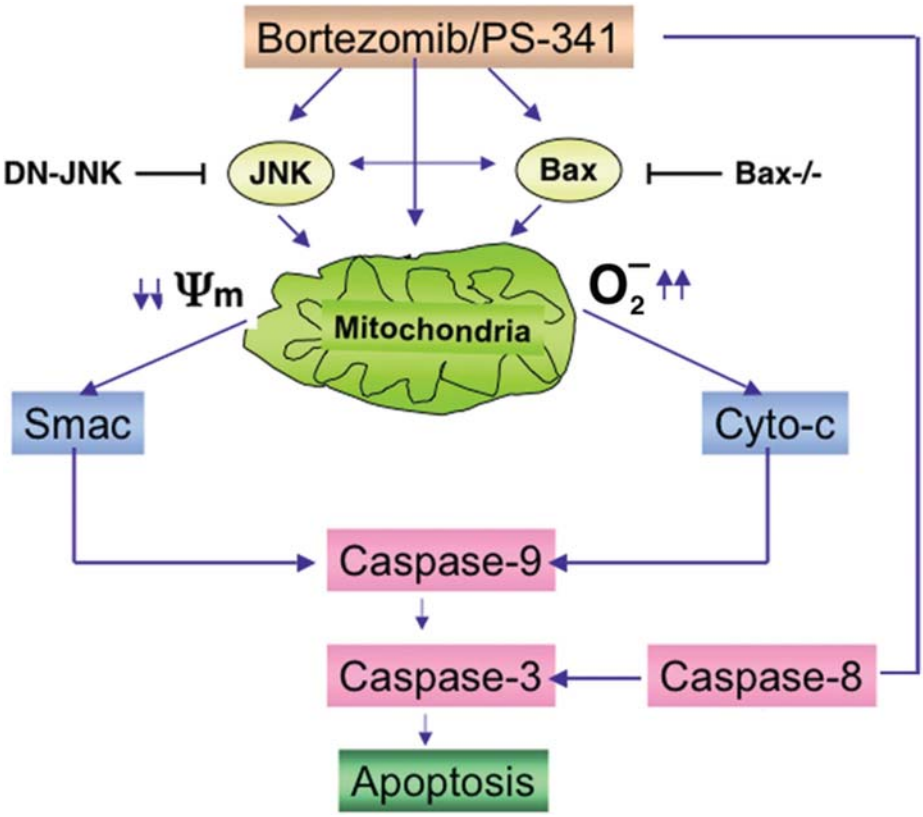

Figure 3 Apoptotic signaling triggered by bortezomib in MM cells.

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