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Overview of Proteasome Inhibitor-Based Anti-cancer Therapies: Perspective on Bortezomib and Second Generation Proteasome Inhibitors *versus* Future Generation Inhibitors of Ubiquitin-Proteasome System

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Abstract

Over the past ten years, proteasome inhibition has emerged as an effective therapeutic strategy for treating multiple myeloma (MM) and some lymphomas. In 2003, Bortezomib (BTZ) became the first proteasome inhibitor approved by the U.S. Food and Drug Administration (FDA). BTZ-based therapies have become a staple for the treatment of MM at all stages of the disease. The survival rate of MM patients has improved significantly since clinical introduction of BTZ and other immunomodulatory drugs. However, BTZ has several limitations. Not all patients respond to BTZ-based therapies and relapse occurs in many patients who initially responded. Solid tumors, in particular, are often resistant to BTZ. Furthermore, BTZ can induce dose-limiting peripheral neuropathy (PN). The second generation proteasome inhibitor Carfizomib (CFZ; U.S. FDA approved in August 2012) induces responses in a minority of MM patients relapsed from or refractory to BTZ. There is less PN compared to BTZ. Four other second-generation proteasome inhibitors (Ixazomib, Delanzomib, Oprozomib and Marizomib) with different pharmacologic properties and broader anticancer activities, have also shown some clinical activity in bortezomib-resistant cancers. While the mechanism of resistance to bortezomib in human cancers still remains to be fully understood, targeting the immunoproteasome, ubiquitin E3 ligases, the 19S proteasome and deubiquitinases in pre-clinical studies represents possible directions for future generation inhibitors of ubiquitin-proteasome system in the treatment of MM and other cancers.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

Keywords

Bortezomib; carfilzomib; combination therapy; immunoproteasomes; multiple myeloma; proteasome inhibitor; resistance to proteasome inhibitors; ubiquitin-proteasome pathway

INTRODUCTION

The balance of protein synthesis and degradation is tightly regulated in healthy, normal cells. Most intracellular proteins are degraded *via* the ubiquitin-proteasome system (UPS), in which proteins are tagged by ubiquitin and then recognized by the 26S proteasome complex, which degrades them into small peptides (Fig. 1) [1–3]. Since dysfunction of this system is linked to many human diseases, including neurodegenerative disorders, genetic diseases, autoimmue diseases, and many cancers, much work has been conducted by targeting the UPS as a potential treatment of these conditions [4–8]. In the years since the U.S. FDA's 2003 approval of the proteasome inhibitor bortezomib (BTZ; Table 1) for the treatment of multiple myeloma (MM) and mantle cell lymphoma (MCL), the drug has provided ample proof that targeting the UPS is a viable route for the treatment of human cancer. The treatment of MM, in particular, has been revolutionized by the advent of BTZ and immunomodulatory drugs [5–11], with the current overall survival in the MM patients increased by 2 to 3-fold [12]. However, some limitations of BTZ treatment have become evident, including baseline resistance in some patients with MM and virtually all patients with solid tumors, the development of acquired BTZ resistance in many initially-responding MM and MCL patients, and the emergence of potentially permanent peripheral neuropathy (PN) in many of BTZ-treated patients [6–13]. To overcome the limitations of BTZ, several second-generation proteasome inhibitors have been developed with the aim of improving anti-tumor efficacy (by increasing the potency of proteasome inhibition) while reducing toxicity (by improving proteasome binding duration and specificity, thereby reducing "off-target" effects) [14–18].

Herein, we review proteasome function and the status of preclinical and clinical research on first- and second-generation proteasome inhibitors. We will briefly review the development of BTZ, including the clinical efficacy that led to its FDA approval for the treatment of MM and MCL as well as its activity in AL Amyloidosis. We will then review the status of several second-generation proteasome inhibitors, including the recently approved drug carfilzomib (CFZ). We will also describe some important ongoing translational work focused on upstream UPS events, including targeting the immunoproteasome, ubiquitin E3 ligases, the 19S proteasome and deubiquitinases (DUBs) in MM and other cancer types. There are high hopes in the field that elucidating the mechanisms of action and resistance to proteasome inhibition will help advance future cancer management.

CONSTITUTIVE PROTEASOME, IMMUNOPROTEASOME AND CANCER

The UPS is responsible for degrading 80–90% of intracellular proteins [1–3]. Proteins targeted for proteasomal degradation are ubiquitinated by a series of enzymes, including the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and the ubiquitin E3 ligases (Fig. 1), which allow the targeted proteins to be recognized by the 26S proteasome

(MW 2,400 kDa), a multi-subunit protease complex composed of the 20S catalytic core (MW 700 kDa) and one or two 19S regulatory particles (MW 700 kDa) (Fig. 2). The 20S core is formed by two sets of identical α rings and two sets of identical β rings stacked in a symmetrical manner with the outside α rings surrounding the inner β rings (αββα). Each α or β ring contains seven different subunits, named α1-α7 or β1-β7, respectively, among which mainly β1, β2, and β5 possess proteolytic activity (Fig. 2). The β1 subunit is responsible for caspase-like (or peptidyl-glutamyl peptide-hydrolyzing-like/ PGPH-like) activity that preferentially cleaves after acidic residues (*e.g.*, aspartate and glutamate), the β2 subunit has trypsin-like activity that preferentially cleaves after basic residues (*e.g.*, arginine and lysine), and the β5 subunit has chymotrypsin- like activity preferentially cleaving after hydrophobic residues (*e.g.*, tyrosine and phenylalanine) (Fig. 2) [1–3, 19]. The 19S regulatory particle is composed of a “lid” and a “base”, and binds to both ends of the 20S core proteasome (Fig. 2). The 19S proteasome lid contains nine or more non-ATPase subunits, which recognize polyubiquitinated proteins, bind them, and remove the polyubiquitin chain from the substrate proteins by a process called deubiquitination. Meanwhile, the 19S base contains six ATPase and four non-ATPase subunits responsible for the unfolding of substrate proteins and promotion of their entry into the 20S proteasome (Fig. 2) [1–3, 19]. The 19S regulatory particle is far less well characterized than the 20S core.

As the UPS is essential for maintenance of cell homeostasis, it is not surprising that dysregulation of protein degradation plays an essential role in the cell growth, development and survival of human cancer [5, 6]. Proteasomal mRNA levels are consistently markedly increased in a variety of malignant human hematopoietic cell lines compared with peripheral lymphocytes and monocytes from healthy adults [20]. Also, increased proteasome activity is associated with malignant disease, including those of the colon [21], prostate [22], and leukemia [20]. Tumorigenic cells are more dependent upon proteasomal activity and thus more sensitive to its blockage. Indeed, researchers found that many types of actively proliferating tumor cells are more sensitive to proteasome inhibitors than non-tumorigenic cells [23, 24]. Various studies have demonstrated that pharmacological inhibition of the proteasome results in the shift in balance of pro- and anti-apoptotic proteins, including the accumulation of tumor suppressor proteins, resulting in cell cycle arrest and apoptosis [22, 25, 26]. In some tumor cell types, proteasome inhibitors exhibit more effective apoptosis-inducing capability compared to standard cytotoxic agents [27, 28]. Proteasome inhibition is found to be an effective strategy for chemosensitization and overcoming resistance [29].

In addition to the above described constitutive 20S proteasome, which contains catalytic subunits β1, β2, and β5, there also exists the immunoproteasome 20Si, which harbors different sets of catalytic subunits designated correspondingly as β1i, β2i, and β5i (Fig. 2) [30, 31]. Upon cytokine stimulation, *e.g.*, interferon-γ and tumor necrosis factor-α, the expression of β1i, β2i, and β5i dramatically increases and cooperatively assembles into the nascent 20S core particles to form immunoproteasome 20Si (Fig. 2). The 20Si core is capped on each end by 11S proteasomes, rather than 19S proteasomes (Fig. 2) [32]. The immunoproteasome has a key function related to antigen processing in the immune response [30, 31]. Compared to the constitutive 20S proteasome, the immunoproteasome 20Si

possesses enhanced chymotrypsin- and trypsin-like activities and reduced caspase-like activity. This specialized enzymatic property endows the ability of the immunoproteasome to generate peptides that are suitable for MHC class I-mediated antigen presentation [33, 34]. Increased expression of the immunoproteasome complex has been reported in myeloma [35, 36]. Both BTZ and CFZ inhibit the immunoproteasome, albeit to a lesser degree than to which they inhibit the constitutive proteasome (Table 1).

BORTEZOMIB, THE FIRST FDA APPROVED PROTEASOME INHIBITOR FOR THE TREATMENT OF CANCER

BTZ is a dipeptide boronic acid derivative containing pyrazinoic acid, phenylalanine and leucine with boronic acid in its structure (Table 1). Synthesized in 1995 by Myogenics, it was originally called MG-341, and later PS-341. The compound was later acquired by Millennium Pharmaceuticals in 1999 and renamed bortezomib. The chemical IUPAC name of BTZ is [3-methyl-1-(3-phenyl-2-pyrazin-2-ylcarbonylamino-propanoyl) amino-butyl] boronic acid, with a molecular formula of $C_{19}H_{25}BN_4O_4$. BTZ is a reversible inhibitor of the 20S proteasome. The boronic acid group of BTZ binds and forms a complex with the active site of threonine hydroxyl group in the β5-subunit, blocking the chymotrypsin-like activity of the proteasome. It also binds the β1 subunit with lower affinity, inhibiting its PGPH-like activity (Table 1) [5, 6], Proteasome inhibition results in growth suppression and apoptosis induction in tumor cells, mediated through resultant effects on NF-κB signaling under some, but not other experimental conditions [5, 37], or the balance of pro- and anti-apoptotic Bcl-2 proteins [38, 39] and other mechanisms [5, 6, 10, 11]. BTZ triggers p53-induced apoptosis which partially depends on upregulation of Noxa and functional repression of Mcl-1 in lymphoma cells [40, 41]. Cytotoxic effects of BTZ may be abrogated by induction of survival mechanisms involving Akt upregulation [42], the endoplasmic reticulum stress response, the unfolded protein response, histone deacetylase (HDAC)-mediated aggresomal degradation of misfolded proteins and autophagy [43, 44]. Proteasome inhibition in non-tumor cells, including bone marrow stromal cells [45] and osteoblasts and osteoclasts [46], as well as other tissues [47, 48] has potential clinical relevance in the treatment of plasma cell dyscrasias.

The pharmacokinetic properties of BTZ were investigated in two phase I trials in cancer patients who received combination therapy of BTZ and other anticancer agents [49, 50]. After intravenous (IV) injection, BTZ quickly distributes widely into tissues (except adipose and the central nervous system) from the plasma within 10 minutes [51]. The elimination half-life of BTZ is more than 40 hours [52]. BTZ is metabolized primarily through intracellular oxidative deboronation mediated by multiple cytochrome p450 enzymes [53]. Pharmacodynamic studies showed dose-dependent inhibition of proteasome activity by BTZ with intravenous doses ranging from 0.13 to 2.0 mg/m^2, with peak inhibition occurring within one hour after dosing [54, 55]. BTZ levels become undetectable about 72 hours after administration, accompanied by recovery of proteasome activity [56]. Pharmacokinetic properties of BTZ after subcutaneous (SC) administration are somewhat different: lower maximal plasma concentration, longer time to maximal plasma concentration, and longer time to maximal inhibition of proteasome function (*albeit* with similar levels of proteasome

inhibition eventually achieved) [57]. It is possible that these differences may account for the reduced neurotoxicity observed with SC administration compared with the IV route. Impaired renal function predictably has little-to-no effect on BTZ PK [58].

Myeloma

On the basis of impressive Phase II data from two trials (CREST [59, 60] and SUMMIT [61]), BTZ received accelerated approval by the U.S. FDA in 2003 for the treatment of patients with previously treated MM, with the brand name Velcade® [5, 6, 53]. The APEX trial was a phase III trial comparing BTZ to dexamethasone (DEX) in 669 patients with relapsed MM. A subgroup analysis showed that response rates were higher for patients receiving BTZ as second-line therapy (45%) as compared to receiving it as a later line of treatment (34%) [61]. These results essentially recapitulated those previously observed in the CREST [59, 60] and SUMMIT [62] trials. The median time to progression for patients randomized to BTZ in the APEX trial was only 6.2 months. The response rate to BTZ + DEX as first line therapy is approximately 80% [63–65]. Frontline BTZ monotherapy results are less impressive, with only 40% of patients achieving a partial response or better after 2 cycles of treatment [63, 64].

There is also extensive published clinical experience with BTZ used in combination with other anti-myeloma therapies. These BTZ-based regimens are a logical extension of pre-clinical work demonstrating synergistic anti-cancer activity when BTZ is combined with several other classes of drugs used in the treatment of multiple myeloma, including alkyating agents, anthracyclines, and immunomodulatory drugs [9, 12, 66]. This work has served as the basis for trials evaluating BTZ-based combination regimens. In general, compared to BTZ or BTZ+DEX, such regimens (Table 2) have increased anti-cancer activity, including PAD/VDD [67–69], VTD [70], VMP [71], RVD [72], CyBorD [73, 74], VMPT [75], and VTD-PACE [76]. The current labeled indications for BTZ in MM treatment include BTZ + melphalan + DEX as first-line therapy in patients who are not candidates for autologous stem cell transplant, and also BTZ + DEX + pegylated doxorubicin for previously treated MM. There is a growing experience using BTZ as maintenance therapy in patients with MM. The available data, summarized in Table 3, suggest that BTZ maintenance is tolerable and prolongs disease control [75–80].

BTZ has effects on other tissues beyond the malignant clone that are relevant for patients with MM. The drug promotes bone formation by mechanisms which may be independent of its anti-tumor effects [81]. Bone metabolism is shifted from the catabolic state typical of myeloma to an anabolic state by means of the drug's effects on both osteoblasts and osteoclasts [82–85]. Further, BTZ also has radio-sensitizing effects that ought to be considered during radiotherapy to bone lesions concomitantly with BTZ treatment [86–91]. Interstitial inflammation and progressive renal failure stemming from sustained proteinuria in myeloma (and amyloidosis) is mediated by NF-κB [92, 93]. It has been suggested that the sometimes rapid improvement in renal function seen in BTZ-treated patients [94] may be due to inhibition of NF-κB signaling [95]. Thus, BTZ may not only target the malignant plasma cells, but also help reverse some of the myeloma-associated end-organ damage commonly seen in patients. However, a preclinical study suggests that BTZ induces

canonical NF-κB activation in MM cells and therefore, BTZ-induced cytotoxicity cannot be fully attributed to NF-κB inhibition [37]. Further studies are needed to investigate whether NF-κB is the key target of BTZ in MM and other cancers under clinical setting condtions.

The side effect profile of BTZ in myeloma patients is well characterized. Neurotoxicity, fatigue, gastrointestinal effects, and modest cytopenias are common. Peripheral sensory neuropathy (PN), sometimes painful, is the most problematic side effect for many patients. The reported incidence of treatment-emergent PN in trials involving newly diagnosed myeloma patients receiving twice-weekly IV BTZ ranges from 31% with BTZ (+/−DEX) [63, 64] to 80% (including a 32% incidence of neuropathic pain) with the combination of BTZ + lenalidomide + DEX [72]. Risk factors for the development of treatment-emergent PN include pre-existing PN [96] and possibly genetic predisposition [97]. Proposed mechanisms include increased tubulin polymerization [98] and dysregulated transcription, nuclear mRNA processing, and cytoplasmic translation in dorsal root ganglion cells [99]. There may also be non-proteasomal (*i.e.*, "off-target") neuronal effects specific to BTZ that contribute to the different rates of PN observed in clinical trials involving various proteasome inhibitors [10, 18]. BTZ dose reduction [59], weekly (rather than twice-weekly) administration [75], and SC (rather than IV) administration [100] have all been shown to reduce the incidence of PN during myeloma therapy. BTZ-associated PN often improves and in many cases is completely reversible with treatment interruption [101].

AL Amyloidosis

After the successes achieved with BTZ therapy in MM, exploration of its use in the treatment of a closely related monoclonal plasma cell disorder, AL amyloidosis, soon followed. BTZ monotherapy was shown to be effective and tolerable in an international study evaluating different treatment schedules [102]. The incidence of treatment-emergent PN in this study was only 14% in patients treated with weekly or otherwise reduced dosing, which was perhaps surprising, since over 60% of these patients had a baseline history of neurologic symptoms. BTZ-containing combinations (CyBorD) [103] and melphalan + prednisone + BTZ [104] induce high-quality hematologic responses (as determined by deep reduction in serum levels of amyloidogenic light chains) in ≥80% of newly-diagnosed AL amyloidosis patients. Currently, an ongoing multicenter trial is evaluating BTZ + PEX plus the immunomodulatory drug pomalidomide as initial therapy for AL amyloidosis [105].

Other Hematologic Malignancies

Extensive preclinical work has demonstrated synergistic anti-tumor activity between BTZ and other drugs used in the treatment of non-Hodgkin lymphoma (NHL) [106, 107]. Phase I studies demonstrated the maximal tolerated dose of BTZ in previously-treated NHL patients is similar to that for MM [108, 109]. In 2006, the phase II PINNACLE trial of 155 relapsed or refractory MCL patients treated with BTZ monotherapy showed an overall response rate (ORR) of 32%, prompting the FDA to add MCL to the labeled indications [110, 111]. Phase II trials combining BTZ with rituximab and either purine analogues [112–114] or alkylating agents [115, 116] generally show overall response rates of 50–75%. When BTZ is combined with a standard regimen (Rituxan + HyperCVAD) in newly-diagnosed MCL, the response rate is 90–100% [117, 118]. The response rates reported for patients with relapsed/refractory

follicular NHL (F-NHL) seem to be similar to lower than those reported for pretreated MCL [119, 120], though the median time to response appears to be longer for F-NHL than for other subtypes of low-grade NHL - 12 weeks versus 4 weeks [121]. Phase II studies combining BTZ with rituximab and alkylator-based frontline combination regimens are tolerated; it is unclear whether the 83–90% overall response rates observed truly represent an improvement over the same regimens without BTZ added [122,123].

Waldenstrom's Macroglobulinemia (WM) is another low grade NHL against which BTZ (1.6 mg/m^2/wk IV) appears to have significant activity: 85% overall response rate when combined with rituximab as first line therapy [124]. The rate of PN in this 59-patient Phase II study was 46% with 8% of patients discontinuing BTZ due to PN. It is plausible that the dosing schedule, route of administration used, and/or baseline incidence of WM-associated PN contributed to this degree of neurotoxicity.

BTZ-based combination therapy may hold promise in the treatment of more aggressive B-cell NHL, specifically diffuse large B-cell lymphoma (DLBCL). NF-κB signaling is constitutively activated in the "activated B-Cell origin" (ABC) subtype of DLBCL, which provides a strong rationale for evaluating the potential utility of BTZ as therapy. Several studies combining BTZ with R-CHOP as initial therapy have confirmed response rates in excess of 90% [125–127]. In one study in which BTZ was combined with the R-CHOP-like regimen R-EPOCH, high response rates were observed amongst patients with the ABC subtype of DLBCL only (with little activity seen in patients with the "germinal center B-Cell origin" (GCB) type), consistent with the original rationale for incorporating BTZ into DLBCL therapy [128]. Constitutive NF-κB activation has also been described in Reed-Stemberg cells, the malignant cells in Hodgkin's lymphoma (HL) [129]. Despite this, single agent BTZ in relapsed HL was a complete failure in a Phase II study, with zero out of 30 treated patients achieving even a partial response [130]. BTZ has also been evaluated in the treatment of myeloid malignancies, specifically acute myeloid leukemia. In a small Phase II study, half of the 14 patients treated had some reduction in peripheral blood blasts or improvement in hematologic parameters, albeit with a high incidence of severe PN [131]. The drug has perhaps more promise in combination with standard cytotoxic regimens [132, 133], or with differentiating agents such as all-trans retinoic acid [134].

Solid Tumors

The results of BTZ therapy *versus* solid tumors have thus far been less impressive. To date, efficacy has not been established *versus* breast cancer alone [135,136] or combined with chemotherapy [137], hormone-resistant prostate cancer in combination with either prednisone [138] or docetaxel [139], chemotherapy-naïve advanced stage non-small cell lung cancer [140], unresectable/metastatic gastric and gastroesophageal junction adenocarcinoma [141], metastatic neuroendocrine tumors [142], or advanced renal cell carcinoma [143]. Determining whether there are mechanisms of BTZ-resistance specific to any or all of these tumor types will be instrumental in developing effective BTZ-based therapies.

Issues Identified with BTZ as Cancer Therapy

Examining the collective published experience using BTZ to treat cancer, the two main problems encountered are (i) toxicities related to proteasomal inhibition and off-target actions, and (ii) inherent and acquired resistance to the drug, as evidenced by the fact that up to half of patients with "sensitive" tumors such as myeloma may not respond to BTZ monotherapy and responses, when achieved, may be short-lived [5–11].

Cell-based studies have suggested several potential mechanisms for the emergence of BTZ resistance: (i) mutations/overexpression of the BTZ-binding proteasome β5 submit [144, 145]; (ii) overexpression/activation of downstream effectors, *e.g.*, Bcl-2 [146] or NF-κB [147]; (iii) reduced levels of proapoptotic proteins and increased levels of anti-apoptotic proteins (Bcl-2, AKT, MKP-1) [43, 44] after proteasome inhibition; or (iv) altered protein production, processing, and trafficking *via* high secretion of unfolded protein response (UPR) chaperone protein GRP-78, overexpression of a heat shock proteins, phosphorylation of eIF2α by heme-regulated inhibitor kinase (HRI) [148–151] or induction of autophagy [43, 44]. However, the relative importance of each of these alterations to clinical BTZ resistance in cancer patients remains undefined. It is known, for example, that some patients displaying clinical BTZ resistance do not harbor acquired mutations of proteasome β5 subunit [152].

It has been shown that adhesion of MM cells to extracellular matrix proteins and bone marrow stromal cells plays a role in tumor growth, survival, migration and development of drag resistance [153]. Studies of the signaling cascades mediating these events have identified multiple novel therapeutic targets in MM cells and their bone marrow microenvironment, which may provide a novel strategy to overcome BTZ resistance. Most recently, it has been reported [154] that endoplasmic reticulum to nucleus signaling 1 (ERN1), also called IRE1, is critical for BTZ response in MM cells, and suppression of the IRE-activated downstream transcription factor Xbp1 induces BTZ resistance through de-commitment to plasma cell maturation and immunoglobulin production [154]. Finally, exogenous agents such as antioxidants and green tea catechins may contribute to BTZ resistance through drug-drug interactions [155].

CARFILZOMIB: A SECOND GENERATION PROTEASOME INHIBITOR APPROVED FOR THE TREATMENT OF CHEMOTHERAPY-RESISTANT MULTIPLE MYELOMA PREVIOUSLY TREATED WITH BTZ

With the goal of overcoming the limitations of BTZ, several second-generation proteasome inhibitors (Table 1) have been developed, each with unique chemical structure and biochemical properties, binding affinity, binding reversibility, potency and selectivity. All of these factors could be expected to have an effect on the anti-cancer efficacy as well as the toxicity profiles of second-generation proteasome inhibitors [6, 14–17, 156].

Carfilzomib (CFZ, also called PR-171, marketed as Kyprolis®) is an irreversible peptide epoxyketone class proteasome inhibitor with a molecular formula of $C_{40}H_{57}N_5O_7$ (Table 1) [157]. This class includes the most specific and potent proteasome inhibitors discovered thus

far. The α,β-epoxyketone moiety interacts with both the hydroxyl group and the free α-amino group of Thr1 in the catalytic β subunits of the proteasome, leading to the formation of the morpholino adduct in an irreversible fashion [158, 159]. The irreversible binding by epoxyketone-based proteasome inhibitors leads to more sustained as activity compared to BTZ [160], as new subunit synthesis and proteasome assembly are required for restoration of proteasome activity.

CFZ has also been shown to be more specific than BTZ, with little or no off-target activity outside of the proteasome [161]. The free α-amino group required for adduct formation with CFZ is not present in serine and cysteine proteases that can be inhibited by BTZ [18]. In addition to targeting the β5 subunit in the constitutive 20S proteasome, CFZ also targets the correlated β5i subunit of the immunoproteasome 20Si, which appears to be preferentially expressed in MM [35, 162]. The affinity of CFZ for other β-subunits (β1, β2) is lower compared to BTZ, so the trypsin-like and chemotrypsin-like activities of the proteasome are less inhibited.

Rat pharmacokinetic studies showed that CFZ administered intravenously was rapidly cleared from the plasma compartment with a half-life of < 20 minutes [163, 164], Other preclinical and early clinical work with CFZ confirmed rapid clearance of CFZ from plasma with a half-life of < 30 min with widespread tissue distribution with the exception of the brain [165, 166].

Proteasome activity in blood and PBMCs was inhibited by CFZ in a dose-dependent manner, >75% inhibition at 15 mg/m^2 after the first dose, and >90% inhibition after five doses. As predicted by the binding properties of the drug, the observed proteasomal inhibition by CFZ persisted after the drug had been cleared. Proteasome function in PBMC eventually recovered > 2 wks after dosing. Like BTZ, both CFZ and another epoxyketone proteasome inhibitor, oprozomib (discussed, below), inhibit osteoclast function and promote osteoblastogenesis [167].

CFZ monotherapy has been studied in several Phase 1 and II trials involving patients with previously treated MM [168–171], In BTZ-naïve patients, the overall response rate to CFZ monotherapy was 47% with a median progression free survival of 8.2 months in patients treated at a dose of 20 mg/m^2 two consecutive days for 3 out of every 4 weeks [169]. Higher dosing (27 mg/m^2 after cycle one, same schedule otherwise) led to more responses with longer duration. The question of a dose-response association has been explored further in a Phase I dose escalation trial dosing CFZ at 56 mg/m^2/dose in combination with DEX. The response rate was 55%, with dyspnea, fatigue, and cytopenias (but not neuropathy) commonly seen [172]. This escalated dosing schedule will be formally evaluated in patients with previously treated, progressing MM not refractory to prior BTZ therapy in the ENDEAVOR trial [173]. CFZ can also induce responses in patients refractory to BTZ, *albeit* at a lower rate. In a Phase II study involving 266 patients with MM progressing on last therapy, the overall response rate was 23.7%, with a median duration of response of 7.8 months [168]. For patients progressing on BTZ as their last line of therapy prior to enrollment, the response rate was 18.6%, demonstrating that CFZ can overcome BTZ resistance in at least some cases. An integrated safety analysis of 526 patients treated with

single agent CFZ in Phase II trials revealed fatigue (55%), anemia (47%), nausea (45%), cardiac events (22%, including congestive heart failure in 12%), respiratory events 69% (dyspnea 42%), but PN in only 14% (generally grade 1–2) [174]. Essentially no patients required discontinuation or dose adjustments due to neurotoxicity, permitting long-term treatment with CFZ in responding patients [175].

A recent study comparing BTZ and CFZ further pointed out that the neurotoxicity associated with BTZ was related to its off-target inhibition of HtrA2/Omi, a protease known to be involved in neuronal survival [18]. The etiology of the cardiac and pulmonary events noted is unclear, but both are listed as part of a black-box warning on the package insert for CFZ. It remains to be seen whether these or other toxicities will be more pronounced at escalated doses. Little-to-no information is available regarding alternate dosing schedules. Currently, the medication is only administered intravenously. On July 20, 2012, CFZ was granted FDA approval for treatment of patients with MM progressing on or after treatment with BTZ and an immunomodulatory agent. Its use in the U.S. was launched on August 1, 2012 [157].

CFZ is starting to be evaluated as a substitute for BTZ in combination regimens. A handful of trials have treated patients with the combination of CFZ, lenalidomide, and DEX in relapsed and newly diagnosed patients [176–178]. In previously untreated patients, the responses are rapid and the overall response rate ultimately reaches about 100%, which is not surprising given the similarly high response rates seen with BTZ-based triplet regimens [72]. Based on these encouraging results, the pivotal phase III ASPIRE trial, a 700+ patient trial comparing CFZ + lenalidomide + DEX to lenalidomide + DEX was conducted. Enrollment was completed as of early 2013. A large EGOG-ACRIN-led study comparing CFZ/LEN/DEX to BTZ/LEN/DEX has also recently been activated.

As for other potential indications, CFZ has been studied in NHL [165, 171] with early results that appear less impressive than those seen in MM patients. There is an ongoing study of CFZ with or without DEX in patients with AL amyloidosis [179]. A large (79 patient) Phase I/II study evaluating escalated dose CFZ in patients with advanced solid tumors was conducted, with 6 or 48 response evaluable patients demonstrating reduction in tumor size (albeit not sufficiently so to meet RECIST criteria for a partial response) [180]. Side effects in this study were quite manageable, with all deaths the result of progressive disease, only one patient developing congestive heart failure, and no severe respiratory or Grade 3+ PN reported.

OTHER SECOND GENERATION PROTEASOME INHIBITORS IN CLINICAL DEVELOPMENT

Ixazomib (MLN-9708/2238)

Ixazomib (MLN-9708) (Table 1) is an orally bioavailable boronic ester prodrug [10, 11, 113, 158, 161]. Ixazomib (IXZ), a reversible proteasome inhibitor, has the advantage of being the first oral proteasome inhibitor to enter clinical investigation in MM patients. In preclinical studies, the anti-myeloma activity of the drug compared favorably to BTZ [181]. Phase I and II trials suggest that single-agent IXZ has clinical activity in heavily pretreated relapsed and/or refractory MM patients, with infrequent peripheral neuropathy [182, 183]. It

is currently being tested in many clinical trials in a wide range of clinical indications, including previously untreated MM, relapsed MM, advanced stage solid tumors, lymphoblastic leukemia, non-Hodgkin's lymphoma, and AL amyloidosis [184]. There is interest in developing the drug as maintenance therapy in MM.

Delanzomib(CEP-18770)

Delanzomib (*DLZ*) is another reversibly binding boronate-based, second-generation proteasome inhibitor (Table 1) [10, 11, 158, 161, 184] with both oral and IV bioavailability. DLZ has shown proteasome-inhibitory activity similar to that of BTZ in hematologic and solid tumor cell lines, as well as in primary cells front multiple myeloma patients. Delanzomib is currently in phase I/II clinical investigations for myeloma, lymphoma and solid tumors still using intravenous administration [185]. A recent phase I study of DLZ in patients with advanced solid tumors and MM demonstrated a favorable safety profile with minimal neurotoxicity. The half-life of the drug was rather long, 62 hrs, with linear PK. The degree of proteasome inhibition was lower than that described for BTZ or CFZ (45%), and rash occurred in over half of patients [186]. It remains to be determined whether the drug will have meaningful anti-MM activity in BTZ-resistant patients.

Oprozomib (ONX-0912, PR-047)

Oprozomib (OPZ) is an orally bioavailable peptide epoxyketone-based, irreversible proteasome inhibitor (Table 1) that showed similar potency to CFZ in cytotoxicity assays [14–16]. Orally administered oprozomib showed equivalent antitumor activity to intravenously administered CFZ in human tumor xenograft and mouse syngeneic models [187]. Early studies were hampered by significant gastrointestinal toxicity, prompting the manufacturer to develop a modified formulation. The formulation currently being investigated in Phase I and II trials for patients with hematological cancers (including multiple myeloma) or solid tumors appears more tolerable. The optimal dosing schedule of the drug is still being worked out. A Phase I/II trial combining OPZ with lenalidomide and DEX in newly diagnosed MM patients is being planned.

Marizomib (NPI-0052, Salinosporamide A)

Marizomib is an irreversible proteasome inhibitor with a β-lactone backbone (Table 1) [11, 17, 156, 158]. The carbonyl group of the β-lactone can interact with the hydroxyl group of Thrl in catalytic β subunits, leading to the formation of an acyl-ester adduct. This process is reversible, but the covalent adduct only dissociates at a very slow rate. In addition to the acyl-ester bond, marizomib also forms a cyclic tetrahydrofuran ring with the proteasome, making the reaction irreversible [17, 158, 188]. Compared to other proteasome inhibitors, marizomib produces rapid, broad and prolonged inhibition of all three 20S proteasome catalytic subunits [17]. Marizomib is the only non-peptide-based inhibitor in clinical trials [184]. It is administrated intravenously twice weekly and is being investigated for Phase Ib for recurrent MM, solid tumors, lymphoma and leukemia. Marizomib exhibits an extremely short half-life (< 5 minutes) and wide tissue distribution (including penetration of the blood brain barrier) [189]. Responses to marizomib were found in patients with BTZ-refractory MM. The safety profile of marizomib differs from BTZ, with no significant treatment-

emergent PN, myelosuppression or thrombocytopenia reported. The dose limiting toxicities include transient hallucinations, cognitive changes and loss of balance, perhaps reflecting CMS penetration.

NOVEL DRUGS INHIBITING IMMUNOPROTEASOME AND OTHER UPS COMPONENTS WITH TRANSLATIONAL FEASIBILITY

Immunoproteasome Inhibitors

It has been shown that the immunoproteasome complex plays a role in relapsed myeloma and BTZ resistance [35, 36, 162, 190, 191]. Immunoproteasome Specific Inhibitor-001 (IPSI-001; Table 4) preferably inhibits the immunoproteasome $20S_i$ activity (mainly by binding to $\beta\ 1_i$ subunit) *over* the constitutive 20S proteasome activity, potently suppressing proliferation and inducing apoptosis in MM cell lines as well as in patient samples [14]. More importantly, it was able to overcome conventional and novel drug resistance, including resistance to BTZ [192].

ONX-0914 and PR-924 are peptide epoxyketone-based immunoproteasome inhibitors (Table 4), both of which are able to selectively and irreversibly inhibit the β5i subunit ONX-0914 is 20 to 40-fold more selective towards the β5i subunit than both the β5 and β1i subunits [193]. The ability of specific immunoproteasome inhibitors to overcome BTZ resistance in the pre-clinical setting suggests that they may provide an alternative therapeutic option for cancer patients resistant to BTZ.

Inhibitors of Ubiquitin E3 Enzymes—Ubiquitin E3 ligases (Fig. 1) are a large group of ligase enzymes that facilitate the transfer of ubiquitin from E2 ubiquitin-conjugating enzyme to substrate proteins to be targeted for proteasomal degradation. Ubiquitin E3 ligases can be classified into three major groups; N-end rule ubiquitin ligases [194], HECT (Homologous to E6AP C-Terminus) [195] and RING (Really Interesting New Gene) [196, 197]. Ubiquitin E3 ligases regulate almost all cellular processes including the cell cycle, transcription, DNA repair, signal transduction, endocytosis, cellular transport and development, and their inhibition could lead to growth suppression and apoptosis of neoplastic cells. The substrate binding specificity of a given E3 ligase determines what protein(s) will be ubiquitinated. E3 ligases represent a group of potentially “druggable” targets, albeit a rather large group. Therefore, E3 ligase small molecule therapeutics might offer potential advantages over proteasomal blockade as they will target a more defined set of proteins that could further limit off-target effects currently observed with 20S proteasomal inhibitors.

One example is HDM2, the E3 ligase that regulates the degradation of p53 [198]. Inhibition of HDM2 results in accumulation of p53 and increased expression of its target gene p21, eventually leading to cell cycle arrest and apoptosis in cancer cells with wild-type p53 but not in those with mutant p53 [199]. There are at least seven HDM2 inhibitors currently in Phase I clinical trials [200, 201]. These include non-peptide small molecule HDM2 antagonists that competitively bind the deep hydrophobic p53-binding pocket in HDM2. Nutlins (Table 4), a group of cis-imidazoline analogs, are another group of HDM2 antagonists in clinical development that disrupt the interaction between HDM2 and p53 by

binding to the p53-binding pocket on HDM2. Two nutlin derivatives, RO5045337 (RG7112) and RO5503781, are currently being evaluated in clinical studies (Table 4).

IAPs (Inhibitors of Apoptosis) are a group of E3 ligases that include XIAP, cIAP1, cIAP2, and ML-IAP. IAPs function as regulators of apoptosis. Smac (Second Mitochondria-Derived Activator of Caspases, also called DIABLO) is an IAP-binding protein that regulates IAP levels and function by triggering auto-ubiquitination and destruction [202]. Several Smac mimetics (e.g., LCL161) and IAP antisense molecules (e.g., AEG 35156) are currently in Phase I/II clinical trials (Table 4) [201].

The E3 ligase Cereblon has been found to be a direct target for immunomodulatory drugs (IMiDs) thalidomide, lenalidomide and pomalidomide (Table 4). Cereblon binding appears to be necessary for the drugs to exert their cytotoxic effects. At least one report has shown a correlation between pre-treatment cereblon expression in myeloma cells and sensitivity to IMiD therapy [203].

Inhibitors to 19S Proteasome and Deubiquitinases

Proteasome Recognition Inhibitors—Ubistatin A and ubistatin B are two small molecules that disrupt substrate recognition by the 19S regulatory particle of the proteasome (Table 4) [204]. They block the binding of ubiquitinated substrates to the proteasome by targeting the ubiquitin-ubiquitin interface of K48-linked chains, the same interface recognized by ubiquitin-chain receptors of the proteasome [204]. This supports the idea that the ubiquitin chain itself could be another potential target for pharmacological intervention in the ubiquitin-proteasome pathway.

Inhibitors of 19S Proteasome-Associated Deubiquitinases—Around 100 human deubiquitinases (DUBs) have been identified, most of which belong to the cysteine protease family [205, 206]. The 19S proteasome is linked to three DUBs, UCHL5 (or UCH37), USP14 and POU1 (Rpn11) [207]. UCHL5 and USP14 can be inhibited by a small molecule called b-AP15 in a reversible manner. The drug b-AP15, which was identified in a screen for compounds that induce the lysosomal apoptosis pathway [208], inhibits UCHL5 and USP14 in a reversible manner (Table 4). Resultant accumulation of polyubiquitinated proteins induces G_2/M arrest without causing genotoxicity. Treatment with b-AP15 inhibited tumor progression in four different *in vivo* solid tumor models and inhibited organ infiltration in an acute myeloid leukemia model [208]. More importantly, although the proteasome is targeted by b-APl5, the cellular response to the drug is distinct from that of BTZ. b-AP15 induced apoptosis in tumor cells with mutant p53 or overexpressed Bcl-2, two characteristics that greatly reduce BTZ sensitivity. Experimental evidence (*e.g.*, differing cytotoxicity profiles *versus* the NCI-60 panel) supports the notion that b-AP15 may have anti-cancer efficacy against tumors resistant to BTZ [208].

Another DUB, USP7 (HAUSP), has been a focus of research due to its role in deubiquitinating and stabilizing p53 and its E3 ligase HDM2 [206]. P5091 (Table 4) actively inhibits USP7 at concentrations as low as 5–10 μM in the cellular environment, resulting in HDM2 polyubiquitination and accelerated degradation by the proteasome, and consequently increased steady-state protein levels of p53 and p21. P5091 induced apoptosis in BTZ-

resistant MM cells, supporting the concept that alternate (upstream) targeting of the UPS has potential utility in cancer therapy. In mice, P5091 was well tolerated, inhibited MM tumor growth and angiogenesis, triggered MM cell apoptosis and prolonged survival. Furthermore, combined treatment of P5091 and lenalidomide, dexamethasone, or the HDAC inhibitor vorinostat (SAHA) induced synergistic anti-multiple myeloma activity *in vitro* [209].

P22077 (Table 4) is an analog of P5091 which, in addition to decreasing levels of HDM2 and increasing levels of p53 and p21, also caused reduced levels of DNA damage proteins DDB1, RBX1, DCAF7 and DCAF11, all of which are subunits of E3 ligases. This suggests a previously unknown functional link between USP7 and enzymes involved in DNA repair [210].

HBX19818 is a recently identified selective USP7 inhibitor with an IC_{50} of 28.1 μM (211). In tumor cells, HBX19818 treatment caused accumulation of functionally active p53 in p53 wild-type cells with the expected G_1 arrest. Importantly, however, cell viability was similarly reduced even in cells missing p53, implying alternate mechanisms mediating the cellular response [211].

Other DUB inhibitors are also in development. For example, WP1130 (degrasyn) (Table 4), an inhibitor of multiple DUBs (e.g., USP5, USP9X, USP14 and UCHL5) may induce apoptosis by regulating MCL-1 and p53 [206]. Further, combination of WP1130 and BTZ showed a synergistic anti-tumor effect versus mantle cell lymphoma cells [212].

CONCLUSIONS (SEE BOX 1)

In the last decade, the proteasome has been validated as a novel, valuable molecular target in the treatment of MM and other hematologic cancers. BTZ, the first FDA-approved proteasome inhibitor, is an important treatment of MM and MCL. However, challenges remain, such as resistance, dose limiting toxicities, and low potency against solid tumors.

The successes and limitations of BTZ, the first proteasome inhibitor anticancer drug, have taught researchers useful lessons and encouraged the development of next generation proteasome inhibitors with reduced toxicities, broader anticancer activities, and - in some cases - more convenient oral dosing. Alternate chemical structures have had effects on pharmacokinetic properties, binding affinity and selectivity, breadth and potency of proteasome inhibition, efficacy spectrum and anticancer activities, *etc*. CFZ, the second FDA approved proteasome inhibitor, has increased binding specificity for the proteasome and reduced off-target effects, resulting in activity *versus* some BTZ-resistant MM with decreased neurologic toxicities. Other second generation proteasome inhibitors with improved therapeutic windows are in development as treatment for bortezomib-resistant MM, as well. The potential reversal strategies for bortezomib resistance also include identifying new targets in the UPS, such as the immunoproteasome, E3 ligases, 19S proteasome, and DUBs in bortezomib-resistant cancer cells and developing novel combinational therapies. Such novel inhibitors have demonstrated preclinical activities in cancer cells, including BTZ-resistant MM cells in some cases, which needs a confirmation under clinical settings.

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LIST OF ABBREVIATIONS

ABC	Ativated B-Cell origin
Akt	protein kinase B
AL	Amyloid Light-chain amyloidosis
ATP	Adenosine triphosphate
BTZ	Bortezomib, PS-341, Velcade
CFZ	Carfizomib PR171
CNS	Central Nervous System
DEX	Dexamethasone
CyBorD	Cyclophosphamide+BTZ+DEX
DLBCL	Dffuse large B-cell lymphoma
DLZ	Delanzomib
DUBs	Deubiquitinases
ECOG	Eastern Cooperative Oncology Group
ERN1	Edoplasmic reticulum to nucleus signaling 1, or IRE1
FDA	Food and Drug Administration
F-NHL	Fllicular NHL
GBC	Grminal center B-Cell origin
HAUSP	a ubiquitin specific protease also call USP7
HBX	Hepatitis B Virus X protein
HDAC	Histone deacetylase
HDM2	Human Double Minute 2
HECT	Homologous to E6AP C-Terminus
HL	Hodgkin’s lymphoma
HRI	Heme-regulated inhibitor kinase
IAPs	Inhibitors of Apoptosis
IC_{50}	Inhibitory Concentration 50
IMiDs	immunomodulatory drugs
IPSI	Immunoproteasome Specific Inhibitor

IV	Intravenous
IXZ	Ixazomib, MLN-9708
kDa	Kilodaltons
LEN	Lenalidomide
MCL	Myeloid Cell Lymphoma
MG	Myogenics
MLN-9708	Ixazomib
MM	multiple myeloma
MRNA	messenger Ribonucleic acid
MW	Molecular weight
NCI	National Cancer Institute
NF-κB	Nuclear Factor-KappaB
NHL	non-Hodgkin lymphoma
OPZ	Oprozomib, ONX-0912, PR-047
PAD	VDD, BTZ+DEX+Doxorubicin
PGPH	Peptidyl-glutamyl peptide-hydrolyzing
PK	Pharmacokinetics
PN	peripheral neuropathy
POU1	Rpn1
RECIST	Response Evaluation Criteria In Solid Tumors
RING	Really Interesting New Gene
RVD	BTZ+Lenalidomide+DEX
SAHA	suberoylanilide hydroxamic acid
SC	subcutaneous
Smac	Second Mitochondria-Derived Activator of Caspases
UCHL5	Ubiquitin carboxyl-terminal hydrolase L5, or UCH37
UPS	ubiquitin-proteasome system
USP7	a ubiquitin specific protease also called HAUSP
VDD	PAD, BTZ+DEX+Doxorubicin
VMP	BTZ+ Melphalan+ Prednisone
VMPT	BTZ+Melphalan+Prednisone+Thalidomide
VTD	BTZ+DEX+Thalidomide

VTD-PACE	BTZ+DEX+Thalidomide+ Cisplatin+ Adriamycin+ Cyclophosphamide+ Etoposide
WM	Waldenstrom's Macroglobulinemia
WP1130	degrasyn

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Box 1

Key points

- BTZ, the first U.S. FDA approved proteasome inhibitor for treating MM and MCL, is an established component of induction therapy for these patients but has several limitations, including emergence of resistance in MM patients, neuto-toxicities, and little efficacy in solid cancers.
- Rationally designed BTZ-based combination therapies, including BTZ + cyclophosphamide, or BTZ + lenalidomide, are associated with improved outcomes, but are still non-curative.
- CFZ, the second U. S. FDA approved proteasome inhibitor for treating relapsed MM, has increased proteasome-targeting selectivity and potency, with an excellent safety profile.
- Ixazomib, Delanzomib, Oprozomib and Marizomib are second-generation proteasome inhibitors with different pharmacologic characteristics compared to BTZ; they are currently being tested in clinical trials.
- Targeting the immunoproteasome, ubiquitin E3 ligases, 19S proteasome and DUBs may hold promise in the future as proteasome/UPS-targeted cancer therapies.

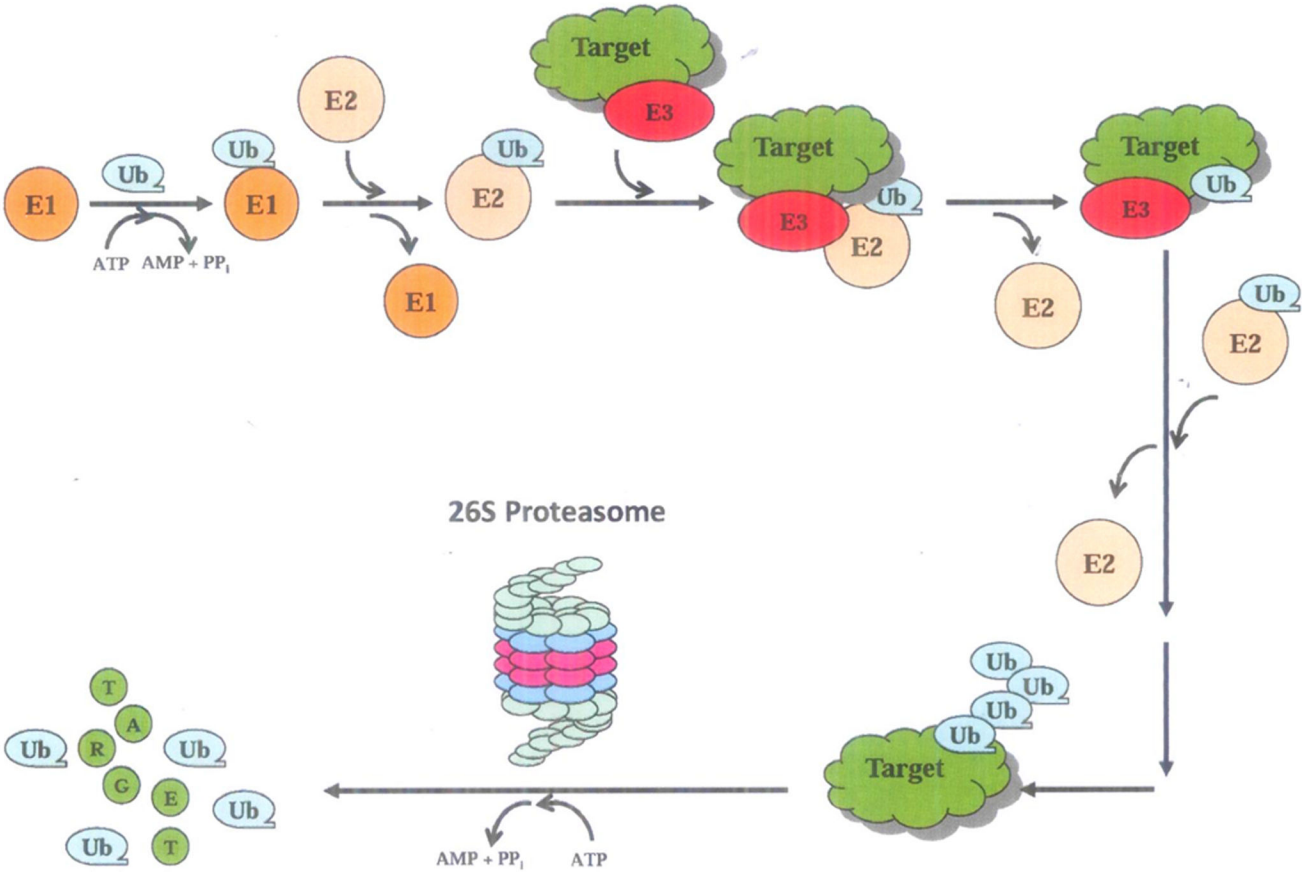


Fig. 1. The ubiquitin-proteasome system (UPS)

The UPS-mediated protein degradation is an ATP-dependent process, involving two distinct steps, ubiquitination and degradation. First, ubiquitin (Ub) is covalently linked to a target protein by a multi-enzymatic system consisting of Ub-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3). E1 activates an Ub monomer and transfers it to E2. E3 facilitates E2 to transfer the Ub to a reactive lysine residue of the target protein. The polyubiquitinated protein is then recognized by the 19S regulatory complex of the 26S proteasome and fed into the 20S catalytic core for degradation into oligopeptides and the ubiquitin molecules recycled.

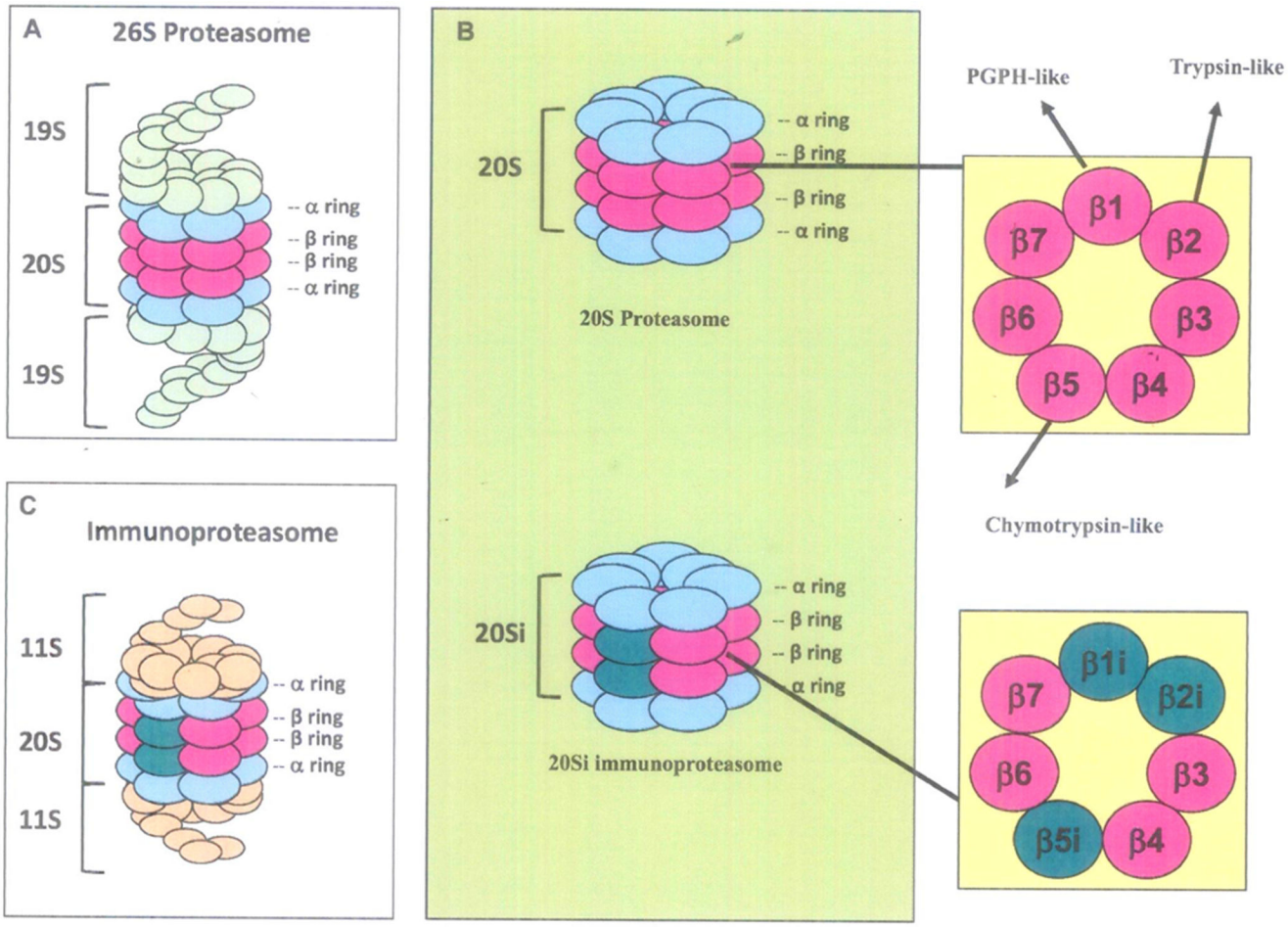


Fig 2. Proteasome structures

The 26S proteasomes is a multiple-subunit protease complex composed of the 20S catalytic core and two 19S regulatory particles (**A**). The 20S core proteasome contains four stacked rings in a αββα arrangement, each ring containing 7 subunits (1 to 7). The outer α subunits serve as a gate to regulate protein entry into the inner catalytic site, while the inner β subunits possess proteolytic chymotrypsin-like (β5), trypsin-like (β2), and the PGPH-like (β1) activities (**B** and insert). Upon cytokine (*e.g.*, interferon-γ or TNFα) stimulation, expression of β1i, β2i, and β5i dramatically increases and immunoproteasome 20Si forms, which is capped on each end by 11S proteasomes (**B**, insert, and **C**).

Table 1

Bortezomib and second generation proteasome inhibitors.

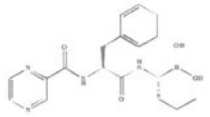
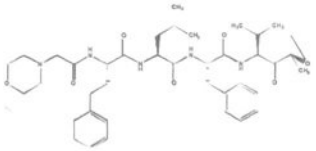
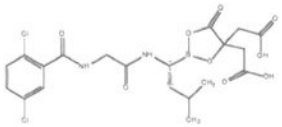
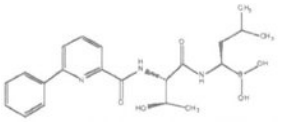
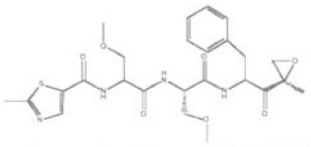
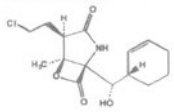
Proteasome Inhibitor	Chemical Structure	Structural Class	Inhibition Type	Inhibition Profile	Development Status	Route of Administration
Bortezomib (Velcade®, PS-341)		Peptide boronic acid	Reversible	β5 > β1 β5i	Approved	IV
Carfizomib (Kyprolis®, PR171)		Peptide epoxyketone	Irreversible	β5 β5i	Approved	IV
Ixazomib (MLN-9708/2238)		Peptide boronic acid	Reversible	β5>β1	Phase III	Oral, IV
Delanzomib (CEP-18770)		Peptide boronic acid	Reversible	β5>β1	Phase I/II	Oral, IV
Oprozomib (ONX-0912, PR-047)		Peptide epoxyketone	Irreversible	β5 β5i	Phase I/II	Oral
Marizomib (NPI-0052, Salinosporamide A)		β-Lactone-γ-lactam	Irreversible	β5> β2> β1 20Si	Phase Ib	IV

Table 2

Selected BTZ-containing multi-agent combination regimens for initial treatment of MM.

Regimen	Agents and Schedule	Notes
PAD	BTZ 1.3 mg/m^2 IV d 1,4,8,11 DEX 40 mg PO d1–4 (also d8–11,15–18 cycle 1) Doxorubicin 9 mg/m^2 continuous IV d1–4 (21-day cycle)	95% overall response rate 24% complete remission No difficulties with subsequent ASCT
VDD	BTZ 1.3 mg/m^2 IV d 1,4,8,11 DEX 40 mg PO d1,2,4,5,8,9,11,12 (20 mg cycle 2+) Pegylated doxorubicin 30 mg/m^2 d1 (21-day cycle)	92.5% overall response rate 90% incidence of Grade 1–2 PN
VTD	BTZ 1.3 mg/m^2 IV d1,4.8,11 DEX 40 mg PO d1–4,9–12 Thalidomide PO 200 mg/d (100 mg/d d1–14, cycle 1) (three 21d cycles pre-ASCT, two more 35d cycles after)	93% overall response rate 19% complete response pre-ASCT 49% CR after post-ASCT consolidation 34% PN (10% Grade 3–4)
VMP	BTZ 1.3 mg/m^2 d1,8,22,29 IV (+ d4,11,25,32 cycles 1–4) Melphalan PO 9 mg/m^2 d1−4 Prednisone PO 60 mg/m^2 d1–4 (42-day cycle)	“VISTA” study 71% overall response rate Non-transplantable patients
VMPT	BTZ IV 1.3 mg/m^2 d1,8,15,22 Melphalan PO 9 mg/m^2 d1–4 Prednisone PO 60 mg/m^2 d1–4 Thalidomide PO 50 mg/d (35-day cycle)	Original schedule of V,M, P as per “VISTA” Grade 3–4 PN dropped from 16% to 3% with modification to weekly BTZ
CyBorD	BTZ IV 1.5 mg/m^2 d 1,8,15,22 Cyclophosphamide PO 300 mg/m^2 DEX PO 40 mg d1–4,9–12, 17–20 × 2 cycles (then 40 mg/wk) (28-day cycle)	90% overall response rate RR same as d1,4,8,11 BTZ dosing, with less toxicity
RVD	BTZ 1.3 mg/m^2 IV d 1,4,8,11 Lenalidomide 25 mg PO d1–14 DEX 20 mg d1,2,4,5,8,9,11,12 (21-day cycle)	100% overall response rate 75% 18-month PFS without ASCT 80% PN Antibiotic, antiviral, aspirin prophylaxis
VTD-PACE	BTZ 1.0 mg/m^2 SC d 1,4,8,11 Thalidomide 200 mg PO d4–7 DEX 40 mg PO d4–7 Cisplatin 10 mg/m^2/day IV d4–7 Adriamycin 10 mg/m^2/d IV d4–7 Cyclophosphamide 400 mg/m^2/d IV d4–7 Etoposide 40mg/m^2/d d4–7 (up to two 56-day cycles)	At many centers, requires hospitalization Used as part of “Total Therapy 3” regimen 2 additional post ASCT cycles (lower dosing)

Table 3

BTZ-based maintenance after transplant or non-transplant induction therapy.

Maintenance Agent(s) and Schedule	Notes
BTZ 1.3 mg/m^2 IV q 2 weeks Thalidomide PO 50 mg/day (following VMPT induction, maint × 2 years)	Superior to No Maintenance 56% 3-year PFS
BTZ 1.3 mg/m^2 IV q 2 weeks (following PAD induction then ASCT, maint × 2 yrs)	Only 9% discontinuation rate for toxicity Superior to Thal maintenance 48% 3-year PFS
BTZ 1.3 mg/m^2 IV d 1,4,8,11 Thalidomide PO 100 mg/d (following ASCT, BTZ given every 3 months × 3 yrs)	Marginally superior to Thal alone or Interferon as maintenance
BTZ 1.3 mg/m^2 IV d1,4,8,11 Thalidomide PO 50 mg/d *OR* Prednisone 50 mg every other day (after non-transplant induction, BTZ given q 3 mos)	Study design made comparison of VT to VP difficult; med PFS approx. 3 yrs
BTZ SQ 1.3 mg/m^2 d1,4,8,11 Thalidomide 100 mg/d PO DEX 20 mg/wk PO (Following Total Therapy 3, VTD × 1 yr maint, then TD × 2 yr)	More recent iteration of this regimen replaces Thal with Lenalidomide 15 mg/day d1–20, 5 mg/d d21–28 q 28 d × 3 yrs

Table 4

Inhibitors of immunoproteasome, E3 ligases, 19S proteasome and DUBs.

Target	Inhibitor	Structural Class	Inhibition Property	Major biological effects	Development Stage
Immunoproteasome	IPSI-001	Aldehyde	β1i> β5i> 20S	Overcome BTZ resistance	Preclinical
Immunoproteasome	ONX-0914 PR-924	Epoxyketone	β5i> β5, β1i	Overcome BTZ resistance	Preclinical
Ubiquitin E3 ligase (HDM2)	Nutlin-3	cis-Imidazoline	p53 binding pocket on HDM2	Accumulation of p53 and increased expression of p53 targets	Prototype
Ubiquitin E3 ligase (HDM2)	RO5045337 RO5503781	cis-Imidazoline	p53 binding pocket on HDM2	Accumulation of p53 and increased expression of p53 targets	Phase I
Ubiquitin E3 ligase (IAP)	LCL161	Smac peptide mimetic	Binding and inhibiting IAP	IAP degradation	Phase I-II
Ubiquitin E3 ligase (IAP)	AEG 35156	IAP antisense	Binding and inhibiting IAP	IAP degradation	Phase I-II
Ubiquitin E3 ligase (Cereblon)	Lenalidomide Pomalidomide	Thalidomide analogs	Cereblon	Immunomodulatory and anti-tumorigenic effects	Approved
19S proteasome (proteasome recognition site)	Ubistatins	Quinolines	Ub-Ub interface	Blocking the binding of ubiquitinated substrates to the proteasome	Pre-clinical
19S proteasome-associated DUBs	b-AP15	bis-Nitrobenzylidene-piperidinone	USP14, UCH-L5	Blocking cleavage of K48-linked ubiquitin chains and accumulating polyubiquitinated proteins	Pre-clinical
DUBs	P5091 P22077	Thiophene	USP7	Deubiquitinating p53 and HDM2; overcoming BTZ resistance	Pre-clinical
DUBs	WP-1130	Tyrphostin	Multiple DUBs (USP9x, USP5, USP14, UCH-L5)	Regulating survival protein stability and 26S proteasome function; synergetic effect with BTZ	Pre-clinical