Introduction

In the past decade, one of the milestone achievements in cancer therapies is the discovery of bortezomib, the specific inhibitor of proteasomes [1]. Bortezomib has been approved for the treatment of multiple myeloma [2] and mantle cell lymphoma by Food and Drug Administration. Bortezomib could induce apoptosis of a variety of cancer cells, including leukemia, lymphoma, multiple myeloma, breast cancers, prostate cancers, lung cancers, and so on. However, extensive studies and overall evaluation suggested that multiple myeloma is the most sensitive and the best responsive disease which was later approved by Food and Drug Administration for bortezomib treatment. Because proteasomes are an essential component in the ubiquitin-proteasomal protein degradation pathway, the discovery of bortezomib implicates that the UPS is critical for myeloma pathophysiology. The UPS also contains ubiquitin, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), ubiquitin ligases (E3) and deubiquitinases (Dubs). In this review, we examined and analyzed the recent advancements of the UPS components in multiple myeloma and its implications in drug discovery for myeloma treatment.

Keywords: Ubiquitin-proteasomal system, deubiquitinases, bortezomib, multiple myeloma, drug discovery
The Ubiquitin-proteasomal system (UPS)

Ubiquitin and ubiquitination

The UPS is constituted by at least 6 components, including ubiquitin, ubiquitin-activating enzymes (UBA, E1), ubiquitin-conjugating enzymes (UBC, E2), ubiquitin ligases (E3), proteasomes, and deubiquitinases (Dub). Ubiquitin is the center of this system and could be linked to a substrate protein with the assistance of E1, E2, and E3 [3], while it could be removed from the target protein by Dubs any time if necessary [6].

Ubiquitin is only found in eukaryotic organisms, is expressed in most tissues and is highly conserved in most species (Figure 1a). This strong sequence conservation suggests that ubiquitin plays a very fundamental role in maintaining cell function and in species evolution. Actually, ubiquitin is involved in almost all aspects of cell biology and activities by regulating its extensively targeted proteins via ubiquitination [3]. Theoretically, any lysine residues in a protein could be linked to an ubiquitin, including ubiquitin itself. Indeed, within the 76 amino acids of ubiquitin, there are 7 lysine residues (K) including K6, K11, K27, K29, K33, K48, and K63 as shown in Figure 1b. These lysine residues are the bridge leading to ubiquitination of target proteins, however, the biological function may differ and depends on the ubiquitination types.

Ubiquitination can be categorized to 3 groups based on the tagged ubiquitins [7]: i) monoubiquitination: proteins are modified by a single ubiquitin, ii) mult ubiquitination or polyanubiquitination: proteins are tagged with several single ubiquitin molecules; iii) polyubiquitination: proteins are attached with polyubiquitin chains. This difference of ubiquitination will regulate a variety of cellular processes, including protein degradation, signal transduction, membrane traffic, DNA repair, chromatin remodeling, peroxisome biogenesis and viral budding. For example, polyubiquitination at the 11th (K11) and 48th lysine (K48) is mainly involved in protein degradation, while the K63 polyubiquitination is mainly responsible for modification of protein function and involved in signal transduction, for example regulation NFKB signal pathway, DNA repair and targeting to the lysosome [7].

Figure 1. The amino acid sequence of ubiquitin. a. Ubiquitin is highly conserved through eukaryotic organisms. b. There are 7 lysine residues out of 76 amino acids and each could be further conjugated to a specific protein based on the polyubiquitin chain.
Ubiquitin-proteasomal system in myeloma

Ubiquitination process is an ATP-dependent enzymatic reaction and requires at least 3 types of enzymes [3, 8], including E1, E2 and E3 as described earlier [3], thus the ubiquitination process is also called as E1-E2-E3 cascade. In the process of ubiquitination, ubiquitin is first activated by the E1 using ATP as an energy source to form a ubiquitin-adenylate intermediate. Subsequently, the activated ubiquitin is transferred from E1 to the active site cysteine of an ubiquitin-conjugating enzyme E2 via a trans(thio)esterification reaction. Finally, the ubiquitination cascade creates an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin with the coordination of an E3 which identifies specific recognition modules in the target protein and is capable of interaction with both E2 and substrate [3, 9].

Figure 2. E1, E2 and E3 form a enzymatic cascade for protein ubiquitination (adapted from reference 1). One single ubiquitin-activating enzyme E1 initiates the whole ubiquitination process, by activating Ub and transferring it to E2. There are around 100 E2s in human. Each E2 will deliver activated Ub to one or several E3s which are a large family of around 1000 members. E3 can specifically identify target proteins and attach Ub to individual proteins.

In human genome, there are two genes encoding E1 and 60-100 for E2s, and ~ 1000 for E3s [8, 9]. E1 activates ubiquitin at the first level, and transfers activated ubiquitin to a certain E2. E3s identify individual substrates and specifically ligate E2-Ub complex to a certain target protein. These enzymes form a hierarchical structure (Figure 2) and control the whole ubiquitination process. In this ubiquitination cascade, E1 can bind to dozens of E2s, which can bind to hundreds of E3s, and E3s specifically target thousands of substrate proteins [9].

Each E3 contains particular protein domains capable of binding the E2 conjugase, as well as a substrate-specific domain for binding the target [10], thus the E3 ligases play a critical role in the ubiquitin conjugation cascade by recruiting ubiquitin-loaded E2s, recognizing specific substrates, and facilitating or directly catalyzing ubiquitin transfer to either the Lys residues (in most cases) or the N terminus of their molecular targets. E3s are a large family and could be grouped into three subclasses based on their active and structural domains, including the homologous to E6-AP carboxyl terminus (HECT) domain containing E3s, the really interesting new gene (RING) finger domain-containing E3s, and the U box or F-box E3s [10].

The proteasome

Most ubiquitinated proteins are destined to degradation, which occur in the 26S barrel-like
complexes called proteasomes. Those proteins ubiquitinated at the 48th (K48) or 11th (K11) lysine of polyubiquitin chain will be degraded inside the cylinder [7]. The proteasome (26S) is a large protein complex with molecular weight more than 2000 kilodalton and is composed of one 20S core particle and two 19S regulatory particles. Furthermore, the core particle is made up of two β units (at the two ends) and two β units (in the middle). Each of these units is composed of 7 subunits and the total 28 subunits stack up to form a cylinder. The β units are located in both ends of the barrel and function as a regulatory partner. Each ring consists of seven α subunits, which serve as docking domains for the regulatory particles and the N-termini of β subunits form a gate that blocks unregulated access of substrates to the interior cavity [11].

To be noted, proteases are only found in the interior surface of the β subunits, especially β1, β2 and β5. Although these proteases share a common mechanism, each subunit dominates its distinctive catalytic activity due to interatomic contacts with local residues near the active sites of each subunit. For example, β1, β2, and β5 mainly present chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing (PHGH) activity, respectively [5]. Each catalytic β subunit also possesses a conserved lysine residue required for proteolysis. The proteasomes mainly catalyze those polyubiquitinated, misfolded or unwanted proteins, thus is critical in regulatory protein function and cell activity.

The ubiquitin-proteasomal system is extensively involved in multiple myeloma

Multiple myeloma (MM)

MM is a malignancy of plasma cells, featured with increased population of malignant plasma cells in the bone marrow, high level of specific immunoprotein band (M protein), damage of end organs including bone lesion, renal failure, anemia [12]. MM is the second most common hematological malignancy and constitutes 13% of blood cancers and 1% of all cancers, with a yearly incidence of 14,000 in the United States and a median survival of 3 years. MM accounts for approximately 20% of deaths from hematologic malignancy and nearly 2% of deaths from cancer [13]. Molecular genetic analysis indicated that dysregulation of oncogenes by translocation to an IgH (14q32) is a seminal event in the pathogenesis of MM, and several featured chromosomal translocations occur between IgH and specific genes including c-maf [14], MafB [15], cyclin D1 [16], D3 [17], and FGFR3/MMSET [18, 19], which play a critical role in MM development/progression [13].

Important ubiquitinated proteins in multiple myeloma

MM cells are very sensitive to proteasome inhibitors, which suggests that UPS plays a very important role in the pathophysiology of MM. For example, total ubiquitinated proteins are markedly increased in leukemia and MM cells compared with those in the normal counterparts [20]. Specifically, all MM featured proteins associated with chromosomal translocation in MM cells such as c-maf and D-cyclins could be ubiquitinated. For example, c-maf, a member of a b-HLH-ZIP transcription factor family, is expressed in more than 50% MM cell lines [21, 22] and could be ubiquitinated by dexamethasone, thus downregulating its targets genes and leading to cell cycle arrest and apoptosis [22, 23]. D-type cyclins are unifyingly overexpressed in MM cells and are indicators of prognosis [24], and these cyclins are polyubiquitinated under the direction of SCF E3 ligase complex [25-27]. The fibroblast growth factor receptor FGFR3 could also be ubiquitinated. FGFR3 undergoes autophosphorylation, and subsequently c-Cbl-dependent ubiquitination [28].

In addition to these MM specific proteins, some important proteins involved in cell proliferation and apoptosis, such as p27, p53, PTEN, IxBβ, are also found to be regulated via ubiquitin-proteasomal pathway. p27 and p53 are critical for cell cycle progress and cell proliferation, and are ubiquitinated by Skp2 and Mdm2, respectively, which will be discussed in detail later. PTEN negatively regulates the PI3K/Akt cascade and myeloma leukemogenesis [29]. Loss of PTEN expression leading to high Akt activation in human multiple myelomas [30]. PTEN is degraded in proteasomes and shuttles between cytoplasmic and nuclear compartments after ubiquitination by NEDD4-1 [31, 32]. IxBβ is a negative regulator of NFκB which is ubiquitinated and degraded in proteasomes thus activating NFκB, a transcription factor which is important for MM cell proliferation and within the bone marrow microenvironment. NFκB signaling
Ubiquitin-proteasomal system in myeloma

In stromal cells can lead to production of interleukin-6, BAFF or APRIL, known growth factors for and activators of NFκB in MM [33]. Moreover, some factors that are produced by BM cells, e.g., VEGF and IGF1, can indirectly activate the NFκB pathway in PC and MM cells [34]. Bortezomib prevents the activation of the transcription factor NFκB, while stabilizing the newly phosphorylated form of IκBα bound to NFκB [35]. CYLD exhibits deubiquitinating activity and acts as a negative regulator of NFκB and JNK signaling through its interaction with NEMO and TRAF2 [36].

The ubiquitinating enzyme cascade involved in multiple myeloma

In addition to the MM-specific and dysregulated proteins which are regulated via UPS pathway, other components of the UPS are also involved in MM, either for the development/progression or for the treatment. Ubiquitin is the center of the UPS system, and plays a critical role in the process of the protein ubiquitination [37]. We found that ubiquitin could be induced in MM cells, thus resulting in c-maf ubiquitination and degradation [22, 23]. E1 is responsible for the first step of the ubiquitination process by activating ubiquitin and is overexpressed in both leukemia and MM cell lines and primary samples [20]. When E1 is knocked down, these leukemia and MM cells will go to apoptosis [20]. Several E2s have been reported involved in MM development. For example, CDC34, the ubiquitin conjugating enzyme and cell cycle regulator, is highly expressed in MM patient cells and cell lines in contrast to normal controls [38]. Inhibition of CDC34 enzymatic activity abrogates interleukin-6-induced protection against dexamethasone-induced MM cell apoptosis [39]. CDC34 has been implicated in the ubiquitination of p27 (Kip1), IκBα, Wee1, and MyoD [38], thus facilitating the degradation of these proteins by 26S proteasomes and modulating cell cycle progression. Ubiquitin ligase E3s are the largest family in the UPS system [9] and are extensively associated with MM pathophysiology. For example, XIAP, the representative of the RING finger family of E3s, and Mdm-2, the primary E3 ligase for p53 ubiquitination, are overexpressed in MM cells and contribute to MM cell proliferation and anti-apoptotic activity [40, 41]. XIAP is also the most important enzyme that inhibits caspase-3, -6, and -7 activities [42] and confers to drug resistance in MM cells and XIAP knockdown using RNA interference enhanced bortezomib sensitivity and decreased tumor formation in NOD/SCID mice [43]. As a regulator and E3 for p53, Mdm-2 facilitates G1 to S phase transition by activation of E2F-1 and can enhance cell survival by suppressing wild-type p53 function. MDM2 protein overexpression promotes proliferation and survival of multiple myeloma cells [41]. Recently, another E3 ligase SCF(Skp2) is found associated with MM pathology and therapy [44, 45]. The SCF complex ligase consists of 4 components, including S-phase kinase-associated protein-1 (Skp1), Culin-1 (Cul1), regulator of cullins -1 (Roc1), and a variable F-box protein [44]. SCF regulates cell cycle proteins such as p27. Inhibition of SCF will sensitize bortezomib-induced MM cell death [45].

Deubiquitinases in multiple myeloma

Just like protein phosphorylation, ubiquitin conjugation is a reversible process, which is mediated by Dubs that specifically cleave the isopeptide bond at the C-terminus of ubiquitin [6]. Around 60 Dubs are predicted in human cells, some of which have been found in MM cells. USP9X is such an example and is so far an orphan deubiquitinase. Increased USP9X expression correlates with increased MCL1 protein in human follicular lymphomas and diffuse large B-cell lymphomas [46]. Moreover, patients with multiple myeloma over-expressing USP9X have a poor prognosis [46]. Knockdown of USP9X increases MCL1 polyubiquitination, which enhances MCL1 turnover and cell killing by the BH3 mimic ABT-737 [46]. Another important Dub is CYLD, which is a negative regulator of NFκB [47]. CYLD is located in the 16q12 and its lower expression in MM cells is highly associated with deletion of 16q [48]. In MM cells highly expressing NFκB, both the DNA copy number and protein expression of CYLD is markedly decreased [48]. At another hand, when treated with proteasome inhibitors such as MG132, CYLD was seen accumulated in MM cells [49]. Interestingly, as a deubiquitinating enzyme, CYLD also plays an important role in osteoclastogenesis by negatively regulating RANK signals [49].

Proteasomes in multiple myeloma

Proteasomes determine the fate of polyubiquitinated proteins, along with other proteins
Ubiquitin-proteasomal system in myeloma

such as misfolded and unwanted proteins. Several lines of evidence have shown that proteasomes subunits in MM cells are abnormally higher than those in normal or untransformed counterparts. Enzymatic immunoassay and Northern blot analysis indicated that the concentrations of proteasomes and their mRNA levels were consistently much higher in a variety of malignant human hematopoietic cell lines including MM cells than in resting peripheral lymphocytes and monocytes from healthy adults [50]. Interestingly, proteasome expression was also greatly increased in normal blood mononuclear cells during blastogenic transformation induced by phytohemagglutinin; their expression increased in parallel with induction of DNA synthesis and returned to the basal level with progress of the cell cycle [50]. These findings strongly suggest that proteasomes are associated with cell cycle progression. Actually proteasomes regulate a serial of cell cycle proteins, such as p27, pRb, cyclin D, p53, p27, pro-apoptotic Bcl-2 family members or activation of the stress kinase JNK, and the most important transcription factor in cell proliferation: IkBβ and NFκB [50]. Importantly leukemia and myeloma cells are more sensitive to proteasome inhibitors. An early study found that the IC50 to inhibit cell proliferation in lymphoma is 5 times lower than normal T cells when treating cells with lactacystin [51]; another study indicated that B-CLL cells are about 10 times more sensitive to lactacystin-induced apoptosis than normal peripheral B lymphocytes [52]. These results suggest that proteasomes could be used as a drug target for myeloma and lymphoma therapy.

The 20S core particle of proteasomes is mainly made up of 14 α and 14 β subunits. The β subunits are responsible for proteins degradation and are the primary targets of proteasomal inhibitors, such as bortezomib [5, 11]. Bortezomib competitively binds to and inhibits β subunits, especially β5 activity, thus inhibiting proteasomal enzymatic activities [53]. The β subunits are the main therapeutical target, and they also contribute to drug resistance. For instance, when β5 is mutated or overexpressed, the cells will be resistant to proteasomal inhibitor, such as bortezomib [54-57]. Thus, proteasomes are involved in MM pathophysiology as well as targeted therapy and they are good biomarkers for MM treatment and prognosis.

**Targeting the ubiquitin proteasomal system for multiple myeloma therapy**

**Discovery of bortezomib as an inhibitor of proteasomes**

Timely degradation of regulatory proteins in the proteasome is essential for maintaining normal cellular function and homeostasis. In cancer cells, the proteasomes are also essential to the mechanisms underlying tumor cell growth, apoptosis, angiogenesis and metastasis, thereby representing a novel target for cancer therapy[58]. Efforts were first made to develop such kinds of inhibitors [53]. The typical contribution came from Myogenic which developed a series of proteasome inhibitors, including MG132, one of the most common proteasome inhibitors currently used in research, and MG-341, which was renamed PS-341 and was further developed as a promising drug candidate for cancer therapy [53]. PS-341 is now known as its general name bortezomib based on its chemical structure and is marketed as Velcade®.

Bortezomib is a tripeptide containing pyrazinoic acid, phenylalanine and leucine with boronic acid instead of a carboxylic acid (Figure 3). Bortezomib is a potent inhibitor of proteasomes [59]. Mechanistically, its active boron atom binds the catalytic site of the 26S proteasome with high affinity and specificity [59]. Specifically, bortezomib highly reversibly binds to and blocks the catalytic threonine residue in the β subunits of the 20S core particle by a competi-

**Figure 3.** The chemical structure of Bortezomib. Bortezomib is a tripeptide made up of pyrazinoic acid, phenylalanine and leucine with boronic acid instead of a carboxylic acid.
Ubiquitin-proteasomal system in myeloma

There are probably several lines of mechanisms in bortezomib-selectively-induced cancer cell apoptosis [33, 59, 60]. The inhibition of proteasomes results in accumulation of several important tumor suppressor proteins, including p53, p27, p21, PTEN, IkBa, because these proteins undergo degradation in proteasomes. Bortezomib can also sensitize cancer cells to other apoptosis-inducers. Besides, bortezomib-induced MM cell apoptosis is also associated with apoptotic and autophagic pathways. Recent studies suggested that PS-341 inhibits the paracrine growth of human MM cells by decreasing their adherence to bone marrow stromal cells (BMSCs) and related NFkB-dependent induction of interleukin-6 secretion in BMSCs, as well as inhibiting proliferation and growth signaling of residual adherent MM cells [61]. These preclinical studies demonstrate that bortezomib is a good candidate for MM therapy.

Multi-center clinical trials were subsequently performed and the results suggested that as a single agent or as an active component in the combined regimens in MM treatment, bortezomib has reached remarkable responses, including complete response, partial response, or minimal responses [62]. Based on a multi-centered evaluation in both US and Europe, bortezomib can reach a more than 13% CR in MM patients resistant to other treatments. The overall rate of response to bortezomib was 35%, and those with a response included 7 patients in whom myeloma protein became undetectable and 12 in whom myeloma protein was detectable only by immunofixation. Malignant plasma cells from multiple myeloma patients were 20–40-times more sensitive to bortezomib-mediated apoptosis than blood mononuclear cells [63]. Following several large and multi-center clinical trials, PS-341 was approved by Food and Drug Administration of USA for MM in 2003 [2], and for mantle cell lymphoma in 2006.

Novel drugs beyond Bortezomib

Bortezomib has been considered as one of the most successful anti-cancer drugs and initiated a new chapter for drug discovery targeting proteasomes. Following the marketing of bortezomib, more selective and orally active drug candidates have been developed and some of them have been moved to Phase II or Phase III clinical trials, including Carfilzomib [64, 65], Marizomib [64], CEP-18770 [66], PR-047 [67], ONX-0912 [68], Clioquinol [69], pristimerin [70] and others. However, because of the ubiquitous activity of proteasomes, which is also important for normal cell activity, more specific targets in the UPS systems should be developed. The ubiquitination-associated enzymes, such as E2, E3 and Dubs are potential targets because they may only affect a small set of proteins and will not bring a disastrous effect to normal cells. Recently, efforts have been made toward to disrupt the Mdm2-p53 interaction thus stabilizing p53 [71]. Lead drugs such as Nutlins [71, 72] and MI-63 [73] have been discovered. These agents can restore the conformation and structural function of wild-type p53 in MM cells and induce apoptosis. Although these agents are only effective in wild-type p53 expressing MM cells and lose their anti-myeloma activity in p53 mutant cells, it opens another door to the UPS systems for MM drug discovery.

Conclusion

The ubiquitin-proteasomal system is composed of ubiquitin, ubiquitin-activating enzyme, ubiquitin-conjugating enzymes, ubiquitin ligases, deubiquitinases and proteasomes. All of these components are involved in MM biology and important for its treatment, thus could be developed as a therapeutic target. The success of bortezomib targeting proteasomes is an encouraging milestone for this direction. Because of the importance of proteasomes for normal cells, it is a good alternative to develop novel drugs directing to the ubiquitinating enzymes which will affect a small subset of proteins thus probably less toxic and more applicable.

Acknowledgements

This project was supported by National Natural Science Foundation of China (Grant No. 81071935), the Natural Science Foundation of Jiangsu Province (Grant No. BK2010218), by Suzhou City Science and Technology Program (Social Development Project, Grant No. SS201033), and by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Please address correspondence to: Xinliang Mao, PhD, Cyrus Tang Hematology Center, Soochow University, 199 Ren Ai Road, Suzhou Industrial Park, Suzhou, Jiangsu Province, 215123, P. R. China. Tel/Fax: +86-512-65882123, E-mail: Xinliangmao@suda.edu.cn
Ubiquitin-proteasomal system in myeloma

References


Ubiquitin-proteasomal system in myeloma


Stühmer T, Chatterjee M, Hildebrandt M, Herrmann P, Gollasch H, Gerecke C, Theurich S, Gigliano L, Manz RA, Daniel PT, Bommert K, Vassilev LT, Bargou RC. Nongenotoxic activa-
