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# The Ubiquitin-Proteasomal System and Blood Cancer Therapy

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## 1. Introduction

The ubiquitin-proteasomal system (UPS) is critical for the regulation of protein homeostasis and is composed of the protein ubiquitination system and proteasomal degradation system. Protein ubiquitination is referred to the process that the small protein ubiquitin is covalently tagged to a specific substrate protein. Once a protein is ubiquitinated, its structural conformation, cellular location, and biological function will change accordingly, or it will be delivered into the 26S proteasome complex for degradation by specific proteases. The UPS is extensively involved in nearly all the important cell biological activities, such as cell metabolism, cell proliferation, glycogen synthesis, immunological process, organogenesis, etc. (Ciechanover, 1998; Haglund and Dikic, 2005; Kirkin and Dikic, 2010).

The UPS system is also widely associated with various diseases, such as inflammation, arthritis, heart disease and cancers (Ciechanover et al., 2000). For example, the proteasome has emerged as a milestone target for cancer therapy, which was further demonstrated by the discovery of the proteasome inhibitor bortezomib for the therapy of multiple myeloma (Kisselev and Goldberg, 2001; Richardson et al., 2003). Recently, in addition to the proteasome, the protein ubiquitination pathway is also being developed as a novel target for anti-cancer drugs (Bedford et al., 2011 ; Colland, 2010). In this chapter, we will discuss the UPS system, its biological implications, and associated targeted drug discovery for hematological malignancies.

## 2. The ubiquitin-proteasomal system (UPS)

The UPS is composed by at least 6 components, including ubiquitin (Ub), ubiquitin-activating enzymes (UBA, E1), ubiquitin-conjugating enzymes (UBC, E2), ubiquitin ligases (E3), proteasomes, and deubiquitinases (Dub) (Figure 1). The substrate proteins are first tagged with a ubiquitin chain under the guidance of E1, E2 and E3, and the produced polyubiquitinated proteins are then transferred to 26S proteasomes where it is degraded by the 20S core particles.

### 2.1 Ubiquitination

Ubiquitin is a ubiquitously expressed small protein composed of 76 amino acids and it plays a central role in the UPS system. It can be linked to a substrate protein with the

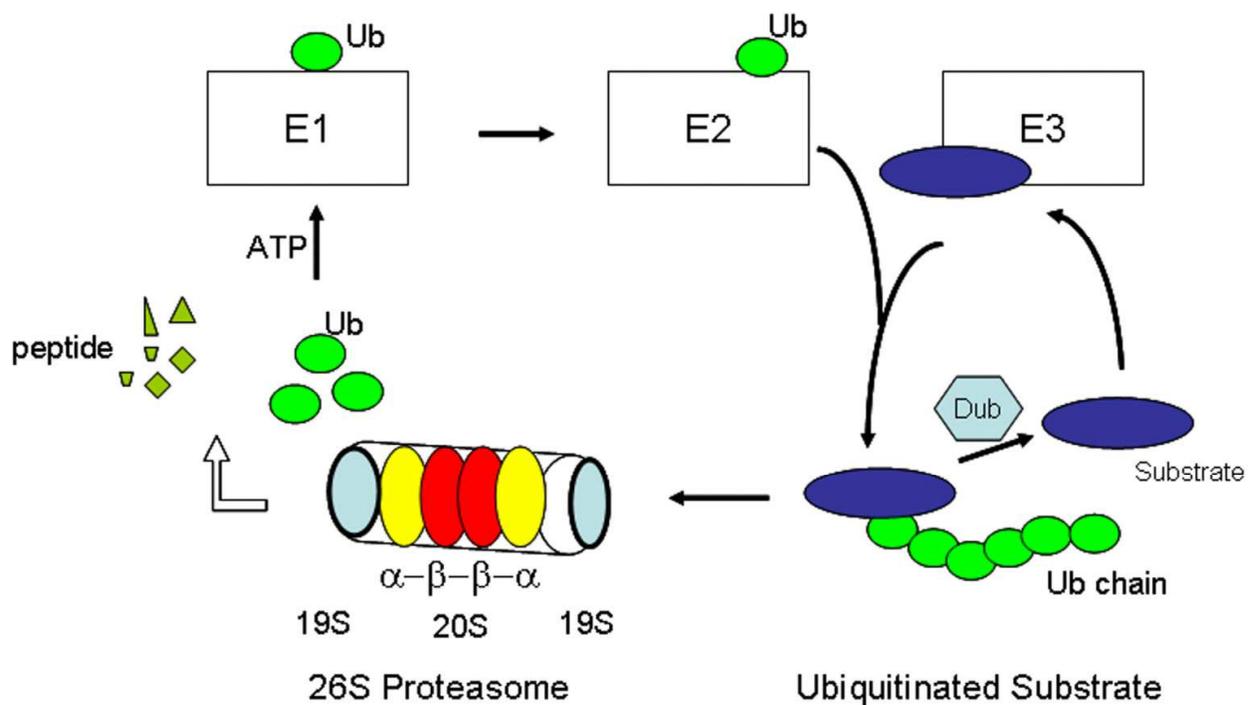


Fig. 1. The ubiquitination-proteasomal system (UPS). The UPS is composed of 6 components, including ubiquitin (Ub), ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), deubiquitinases (Dub) and proteasomes.

assistance of E1, E2, and E3, and can be removed from the target protein by Dubs. Ubiquitin is highly conserved and is expressed in most species but it is only found in eukaryotic organisms. 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 all aspects of cell biology and activities by regulating its extensive substrate proteins. Proteins will undergo turnover, translocation or conformational changes after they are covalently attached a ubiquitin, which is called ubiquitination, one of the most important post-translational modifications of proteins, where the carboxylic acid of the terminal glycine from the di-glycine motif in the activated ubiquitin forms an amide bond to the epsilon amine of the lysine in the substrate proteins (Ciechanover et al., 2000). In the 76 amino acids, there are 6 lysine residues (K) including K6, K11, K27, K29, K33, K48, and K63 as shown in Figure 2. These lysine residues are responsible for ubiquitin attachment to the target proteins. Theoretically, any lysine residues in a protein could be linked a ubiquitin, including ubiquitin itself, however, the biological function may differ and it depends on the ubiquitination status (Haglund and Dikic, 2005).

Ubiquitination can be categorized into three classes based on the tagged ubiquitin (Haglund and Dikic, 2005; Ye and Rape, 2009): i) monoubiquitination: proteins are bound to a single ubiquitin, ii) multiubiquitination or poly-monoubiquitination: proteins are tagged with several single ubiquitin molecules; iii) polyubiquitination: proteins are attached with polyubiquitin chains. These differences of ubiquitination on target proteins will regulate a variety of cellular processes, including protein degradation, signal transduction, membrane trafficking, DNA repair, chromatin remodelling, peroxisome biogenesis and viral budding (Ye and Rape, 2009). For example, polyubiquitin chain occurring at the 11th (K11) and 48th

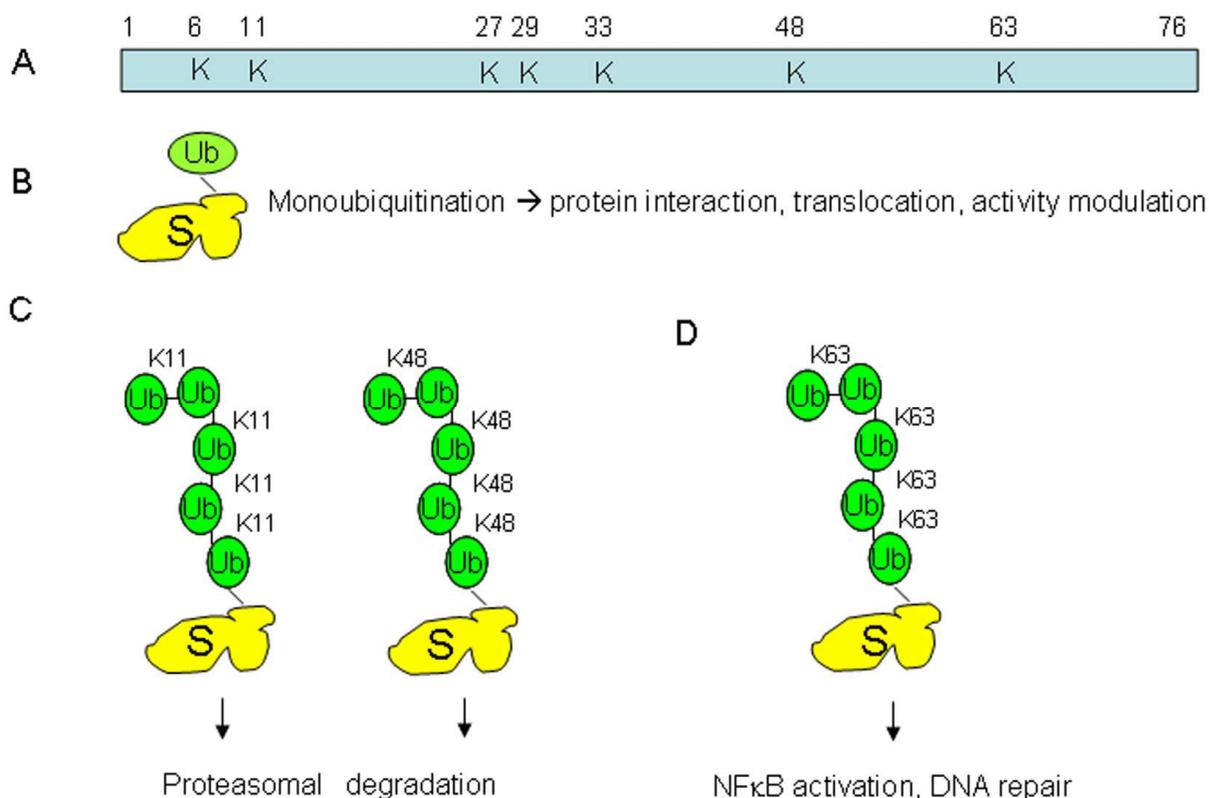


Fig. 2. Protein ubiquitination and functional modulation. There are 7 lysine (K) residues in 76 amino acids of ubiquitin and each could be further conjugated to a specific protein (A). Monoubiquitination (B) regulates protein conformation, cellular localization and protein interaction. Proteins tagged with polyubiquitin-chains occurring at K11 or K48 (C) are subject to degradation in the 26S proteasome. Polyubiquitination at K63 (D) activates NFκB function and is involved in DNA repair.

lysine (K48) of ubiquitin is mainly involved in protein degradation, but the K63 polyubiquitination is mainly responsible for modification of protein function and involved in signal transduction, including regulation of NFκB signal pathway, DNA repair and targeting to the lysosome (Ye and Rape, 2009). For other proteins polyubiquitinated at K6, 27, 29 or 33, whether they are involved in protein degradation or DNA repair is largely unknown (Ye and Rape, 2009)(Figure 2).

## 2.2 Ubiquitinating enzymes

The ubiquitination process is an ATP-dependent enzymatic reaction and requires at least 3 types of enzymes, including E1, E2 and E3 as described earlier, thus the ubiquitination process is alternatively known as the E1-E2-E3 cascade. In the process of ubiquitination, ubiquitin is first activated by E1 using ATP as an energy source to form a ubiquitin-adenylate intermediate. Subsequently, the ubiquitin is transferred to the cysteine residue, the E1 active site, resulting in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group. Secondly, the activated ubiquitin is transferred from E1 to the cysteine of an 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 (Ye and Rape, 2009).

In human genome, there are only two genes encoding E1, whilst E2 is encoded by 60-100 genes, and there are ~ 1000 different E3 genes (Deshaies and Joazeiro, 2009; Schulman and Harper, 2009). E1 activates ubiquitin at the top level, and transfers activated ubiquitin to different E2. E3s identify individual substrates and specifically ligate E2-Ub complex to a certain target protein. These enzymes form a hierarchical structure (Figure 3) and control the whole ubiquitination process. In this ubiquitination cascade, E1 binds to dozens of E2s, which bind to hundreds of E3s, and E3s specifically target thousands of substrate proteins.

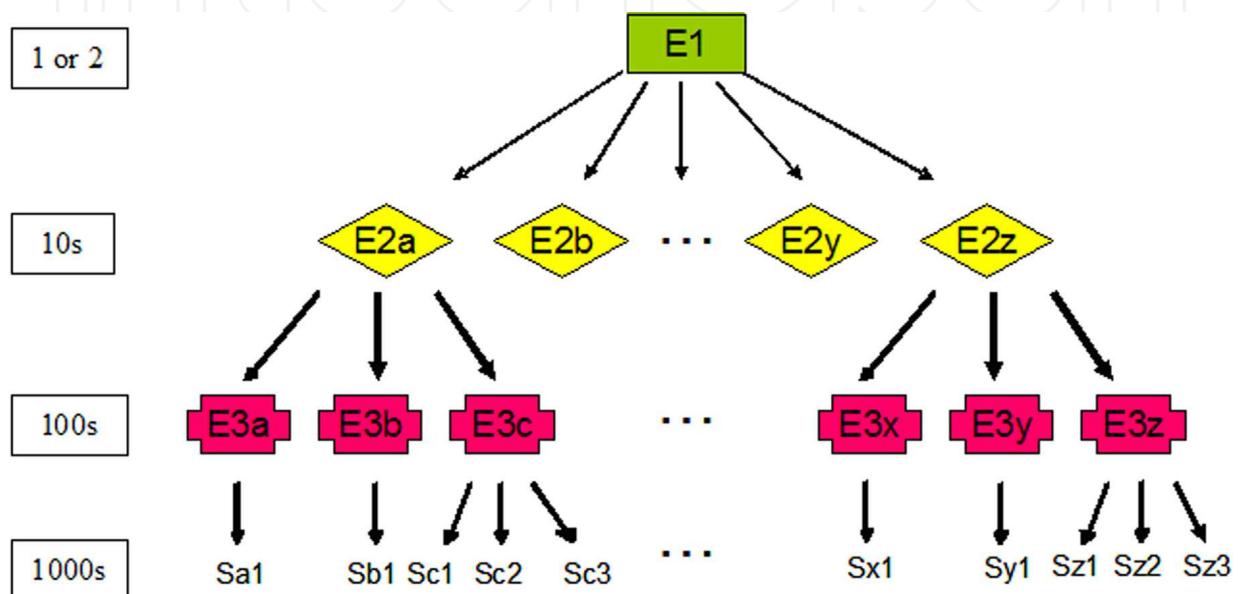


Fig. 3. E1, E2 and E3 form an enzymatic cascade for protein ubiquitination. One single E1 initiates the whole ubiquitination process, by activating Ub and transferring it to E2. There are around 100 E2s in human. Each E2 will deliver activated Ub to one or several E3s which are a large family of around 1000 members. E3s specifically identify target proteins (substrates) and attach Ub to individual proteins.

There are around 100 Dubs in human cells which cleave the ubiquitin-protein bonds thus regulating ubiquitin-dependent metabolic pathways (Colland, 2010). Polyubiquitinated proteins are deubiquitinated by Dubs immediately before degradation in the proteasome. In addition to ubiquitin recycling, Dubs are also involved in processing of ubiquitin precursors, in proofreading of protein ubiquitination and in disassembly of inhibitory ubiquitin chains.

### 2.3 The proteasome system

The 26S proteasome is a large protein complex with molecular weight more than 2000 kilodalton and it is composed of one 20S core particle and two 19S regulatory particles, where the core particle is made up of two  $\alpha$  units (at the two ends) and two  $\beta$  units (in the middle). Each of these units is composed of 7 ring-like subunits thus the total 28 subunits stack up to form a hollow cylinder (Figure 1). The  $\alpha$  subunits N-termini form a gate and serve as docking domains for the regulatory particles that block unregulated access of

substrates to the interior cavity (Smith et al., 2007). Proteins are lysed in the core particle but proteases are only found in the interior surface of the  $\beta$  units, especially  $\beta 1$ ,  $\beta 2$  and  $\beta 5$ . 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.  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$  mainly present caspase-like, trypsin-like, and chymotrypsin-like activity, respectively. Each catalytic  $\beta$  subunit also possesses a conserved lysine residue required for proteolysis. The proteasomes catalyze thousands of polyubiquitinated proteins, therefore, they are critical in regulatory protein function and cell activity.

### **3. The UPS is extensively involved in hematological malignancies**

#### **3.1 Protein ubiquitination in blood cancers**

Heavy ubiquitination levels of proteins, associated with overactivation of E1, have been observed both in leukemia cell lines and primary acute leukemia cells compared with the normal blood cells (Bedford et al., 2011; Xu et al., 2010). Additionally, blood cancer-specific proteins are also regulated by the UPS. For example, multiple myeloma (MM) tumor cells are recurrently associated with several chromosomal translocations that result in overexpression of transcription factors involved in the UPS pathway such as c-Maf, MafB, and fibroblast growth factor receptor 3 (FGFR3), which converge dysregulation of D-cyclins (Bergsagel and Kuehl, 2005). All these proteins could be poly-ubiquitinated and degraded in proteasomes. D-type cyclins are ubiquitinated under the coordination of SCF E3 ligase complex. The fibroblast growth factor receptor FGFR3 could also be ubiquitinated. In chronic leukemia, FGFR3 undergoes ubiquitination by c-Cbl, a RING finger domain-containing E3 ligase. In chronic leukemia cells, the specific BCR-ABL fusion protein is ubiquitinated by c-CBL. Targeting at c-CBL, arsenic induces degradation of BCR-ABL (Mao et al., 2010).

#### **3.2 Ubiquitination enzymes and blood cancers**

E1 is responsible for the first step of the ubiquitination process by activating ubiquitin and is overexpressed in all leukemia and MM cell lines and primary samples. When E1 is knocked down, these leukemia and MM cells will go to apoptosis (Xu et al., 2010). Several E2s have been reported to be involved in MM development. For example, CDC34, the cell cycle regulator, is highly expressed in MM patient cells and cell lines in contrast to normal cells (Block et al., 2001). CDC34 has been implicated in the ubiquitination of p27 (Kip1), I $\kappa$ B $\alpha$ , Wee1, and MyoD, thus facilitating the degradation of these proteins by 26S proteasomes and modulating cell cycle progression. Inhibition of CDC34 enzymatic activity abrogates interleukin-6-induced protection against dexamethasone-induced MM cell apoptosis.

Ubiquitin ligase E3s are the largest family in the UPS system. Various E3s are involved in leukemia, myeloma and lymphomas (Bernassola et al., 2008). For example, XIAP, the representative of the RING finger family of E3s, and Mdm2, the primary E3 ligase for p53 ubiquitination (Jones et al., 2008), are overexpressed in various leukemic and myeloma cells and contribute to cell proliferation and anti-apoptotic activity. XIAP is also the most important enzyme that inhibits caspase-3, -6, and -7 activities and confers to drug resistance. Skp2 is another important E3 ligase. In CML cells, BCR-ABL fusion oncogene frequently up-

regulate Skp2 expression via transcriptional activation, while treatment of Bcr-Abl kinase inhibitor imatinib led to G1 growth arrest accompanied with reduced Skp2 expression (Chen et al., 2011). SKP2 contributes to increased p27(Kip1) turnover, cell proliferation, and a poor prognosis in many tumor types (Zhan et al., 2007).

### 3.3 Deubiquitinases and blood cancers

Attached ubiquitin can be removed by a ubiquitin protease from targeted protein. USP9X is one of the most studied deubiquitinases and is probably involved in deubiquitination from oncoprotein MCL-1. Increased USP9X expression correlates with increased MCL1 protein in human follicular lymphomas and diffuse large B-cell lymphomas (Schwickart et al., 2010). Moreover, patients with multiple myeloma overexpressing USP9X have a poor prognosis. Knockdown of USP9X increases MCL1 polyubiquitination, which enhances MCL1 turnover and cell killing by the BH3 mimetic ABT-737, an inhibitor of MCL1 (Schwickart et al., 2010). Thus, USP9X has been identified as an effective prognostic and therapeutic target. Another important Dub is CYLD, which is a negative regulator of NFκB. CYLD is located in the 16q12 and its lower expression in MM cells is highly associated with deletion of 16q. In T cell leukemia, the Notch/Hes1 pathway sustains NFκB activation through CYLD repression. In MM cells highly expressing NFκB, both the DNA copy number and protein expression of CYLD are markedly decreased. On the other hand, when treated with proteasome inhibitors such as MG132, CYLD will be accumulated in MM cells. CYLD presents as a tumor suppressor deubiquitinase and restoration of CYLD will sensitize cancer cell apoptosis (Jin et al., 2008).

### 3.4 Proteasomes and blood cancers

Several lines of evidence have shown that proteasome subunits in both leukemia and MM cells are abnormally higher than those normal or untransformed counterparts (Kumatori et al., 1990). Immunohistochemical staining shows considerably increased concentrations of proteasomes in leukemic cells from the bone marrow of patients with various types of leukemia and the predominant localization of these proteasomes in the nuclei. Moreover, enzyme immunoassay and Northern blot analysis indicate that the concentrations of proteasomes and their mRNA levels are consistently much higher in a variety of malignant human hematopoietic cell lines than in resting peripheral lymphocytes and monocytes from healthy adults. Proteasome expression is also 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. These findings strongly suggest that proteasomes are associated with cell cycle progression. Later studies demonstrated that proteasomes regulate a serial of cell cycle proteins, including p27, pRb, cyclin D, p53, p27, pro-apoptotic Bcl-2 family members, as well as the most important transcription factor in cell proliferation, NFκB (Kisselev and Goldberg, 2001). Importantly, leukemia and myeloma cells are more sensitive to proteasome inhibitors. An early study found that the IC<sub>50</sub> to inhibit cell proliferation in lymphoma is 5 times lower than normal T cells when treating cells with lactacystin, a classic and typical inhibitor of proteasomes (Delic et al., 1998). Another study indicated that B-CLL cells are about 10 times more sensitive to lactacystin than normal peripheral B lymphocytes. These results strongly suggest that proteasomes could be used as a drug target for myeloma and lymphoma therapy.

## 4. Discovery of bortezomib and its application in MM therapy

### 4.1 Discovery of bortezomib as a treatment for myeloma

Proteasomes are critical for cancer cells, therefore they could be used as a drug target for cancer therapy. Efforts are first made to develop such kinds of inhibitors for MM therapy. The seminal 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. PS-341 alone achieved an overall remission rate of 35% in refractory and/or relapse myeloma patients, when it was used in combination with other drugs such as cyclophosphamide and dexamethasone, the ORR could reach around 90% or greater. Following several large and multi-center clinical trials, PS-341 was approved by Food and Drug Administration of USA for MM in 2003, and for mantle cell lymphoma in 2006. PS-341 is now known as its general name bortezomib based on its chemical structure and is marketed as Velcade®. Recent studies also demonstrated that bortezomib might be particularly active against the active B cell-like diffuse large B cell lymphoma (ABC-DLBCL). ABC-DLBCL has a worse survival after upfront chemotherapy and is characterized by constitutive activation of the NFκB pathway, which can inhibit chemotherapy. Although bortezomib alone has no activities on ABC-DLBCL, when administrated with chemotherapeutics, such as R-CHOP or DA-EPOCH-B, it achieved a superior overall response and survival according to a clinical study of 49 patients. Although ABC-DLBCL and GCB-DLBCL have similar poor outcome by regular chemotherapeutics, ABC-DLBCLs are more sensitive to bortezomib. Bortezomib presented a high responsive rate (83% *vs* 13%) and median overall survival (10.8 *vs* 3.4 months) in ABC compared with GCB-DLBCL, respectively (Dunleavy et al., 2009). It is predictable that bortezomib as an inhibitor of NFκB pathway will be developed for other cancer therapy.

### 4.2 Molecular mechanisms of bortezomib in the treatment of myeloma

Bortezomib is a dipeptide containing phenylalanine and leucine in which the carboxylic group is replaced by a boronic acid group (-RB(OH)<sub>2</sub>) (Figure 4).

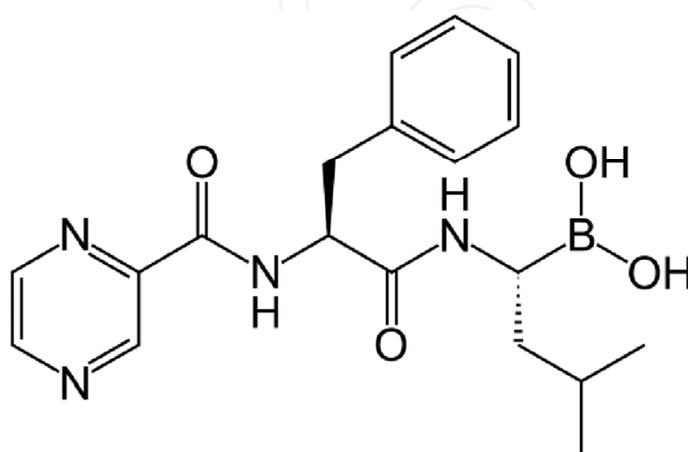


Fig. 4. The chemical structure of bortezomib. Bortezomib is a dipeptide made up of phenylalanine and leucine in which boronic acid group replaces the carboxyl group.

Bortezomib is a potent inhibitor of proteasomes. Mechanistically, its active boron acid group competitively and reversibly binds to the catalytic site of the 26S proteasome with high affinity and specificity. Specifically, the boric acid group of bortezomib binds to and blocks the catalytic threonine residue in the  $\beta$  subunits of the 20S core particle. Inhibition of proteasome results in accumulation of several important tumor suppressor proteins, including p21, p27, p53, PTEN, and I $\kappa$ B $\alpha$ . I $\kappa$ B $\alpha$  is an inhibitor of NF $\kappa$ B, the most important transcription factor in regulating cell proliferation. Normally, I $\kappa$ B $\alpha$  is bound to NF $\kappa$ B and inhibits its activity. The I $\kappa$ B $\alpha$  stability is regulated via the UPS pathway. Once I $\kappa$ B $\alpha$  is degraded, NF $\kappa$ B will be liberated and translocated into the nucleus where it binds to the promoters of various genes and initiates their transcription and expression. I $\kappa$ B $\alpha$ /NF $\kappa$ B signaling plays a critical role in bortezomib-induced cell apoptosis. Bortezomib also interrupts the interaction of Mdm2 and its substrate p53, thus restoring p53 function and leading to cell apoptosis. Moreover, bortezomib directly acts on MM cells and alters cellular interactions and cytokine secretion in the bone marrow (BM) milieu to inhibit tumor cell growth, induce apoptosis, and overcome drug resistance. Specifically, bortezomib inhibits the paracrine growth of human MM cells by decreasing their adherence to bone marrow stromal cells (BMSCs) and related NF $\kappa$ B-dependent induction of interleukin-6 secretion in BMSCs, as well as inhibiting proliferation and growth signaling of residual adherent MM cells (Hideshima et al., 2001).

### 4.3 Pitfalls of bortezomib in MM treatment

Although bortezomib has made a great success in the treatment of MM and MCL, it is not a perfect drug, and some critical features prevent its application (Kumar and Rajkumar, 2008; Oerlemans et al., 2008). Firstly, it is unstable and it retains its activity for 4-8 hrs after re-constituted, thus having to be used within 8 hours. Secondly, the drug is administered via *i.v.* injection which should be performed by a nurse at a clinic or in a hospital, which largely increases the cost of the health care system.

Thirdly, the therapeutic window of bortezomib is very narrow. The therapeutic dosage is 1.3 mg/m<sup>2</sup> body surface, it will produce dose-dependent toxicity when the dose reaches 1.5 mg/m<sup>2</sup>. These adverse effects and toxicity include myelosuppression which leads to anemia, neutropenia and thrombocytopenia, and bortezomib-induced peripheral neuropathy, which occurs in more than 30% patients and this kind of neuropathy is sometimes even worse to affect patients' daily activity (Richardson et al., 2006). Although these kinds of adverse effects are recoverable when the drug is discontinued, some patients couldn't endure the severe effects. Recent studies suggest that some important genes (such as *RHOBTB2* and *SOX8*) involved in the development of the nervous system (especially the peripheral nervous system) are upregulated by bortezomib after one cycle therapy (Cavo et al., 2010).

Lastly, drug resistance is becoming an emerging issue. Although 35% of refractory and relapsed myeloma patients are generally responsive after bortezomib treatment, there are only 4% patients with a complete remission outcome and 65% had no response. There are several underlying issues for bortezomib resistance. Firstly, the resistance to bortezomib is associated with overexpression of  $\beta$ 5 subunits of 20S core particles, which leads to impaired binding of bortezomib and decreased proteasome inhibition. For example, K562 cells with a high level of  $\beta$ 5 are more resistant to bortezomib than other cell lines such as OCI-AML2 expressing low levels of  $\beta$ 5 (Li et al., 2010). Secondly, bortezomib resistance is also associated with mutations

in  $\beta 5$  gene. A DNA sequencing analysis in bortezomib-resistant cells revealed that the G322A mutation in PSMB5 gene leads to an Alanine  $\rightarrow$  Threonine change, which largely confers resistance because threonine is the target of bortezomib (Oerlemans et al., 2008). In a Jurkat cell model, mutations such as C323T and G326A are also reported (Lu et al., 2008). Thirdly, overexpression of other anti-apoptotic genes such as PSMD4 (Shaughnessy et al., 2010), a non-ATPase subunit of the proteasomal 19S regulator, and heat shock protein 27 (HSP27) (Chauhan et al., 2003), an important gene protecting cell against apoptosis, are also found to be associated with resistance to bortezomib. Recently, an siRNA screen identified several important molecular modulators that sensitize bortezomib-induced cell apoptosis, including proteasome subunits PSMA5, PSMB2, PSMB3, and PSMB7 (Zhu et al., 2011), this is quite reasonable because these genes directly modulate the proteasome function. To be noted, the Cyclin-dependent kinase 5 (CDK5) and other 11 genes were also identified from this screen, but their detailed roles in bortezomib-induced cell death are yet to be studied. A most recent study demonstrated that impaired bortezomib binding to mutant  $\beta 5$  subunit of the proteasome is the underlying basis for bortezomib resistance in leukemia cells, while proteasome subunit overexpression is an essential compensatory mechanism for the impaired catalytic activity of these mutant proteasomes (Franke et al., 2011).

## 5. Development of novel proteasomal inhibitors

Currently, several classes of novel proteasome inhibitors have been developed and some have been moved to advanced clinical trials for the treatment of various blood cancers, such as leukemia, lymphoma, and myeloma. Although they share some common features, these novel inhibitors of proteasomes could be classified as: highly selective and irreversible, orally active, non-competitive, and natural products. The details are shown in Table 1.

### 5.1 Highly selective and irreversible novel inhibitors of proteasomes

Several promising novel proteasomal inhibitors have been extensively investigated *in vivo*, *in vitro*, and in clinical settings. Compared with bortezomib, these agents are highly selective and irreversible, such as carfilzomib, NPI-0052, and PI-083.

#### 5.1.1 Carfilzomib

Carfilzomib, or PR-171, is a tetrapeptide epoxyketone and a selective and irreversible proteasome inhibitor that primarily targets the chymotrypsin-like (CT-L) subunits in both the constitutive proteasome (c20S,  $\beta 5$ ) and the immunoproteasome (i20S, LMP7) (Parlati et al., 2009). Inhibition of proteasome-mediated proteolysis results in an accumulation of polyubiquitinated proteins, which may lead to cell cycle arrest, induction of apoptosis, and inhibition of tumor growth. Compared with bortezomib, carfilzomib displays minimal cross reactivity on off-target enzymes, good tolerability and little side effects in multiple open-label clinical trials (O'Connor et al., 2009). In patients with relapsed or refractory multiple myeloma, twice-weekly consecutive-day single-agent carfilzomib 20 mg/m<sup>2</sup>, escalating to 27 mg/m<sup>2</sup> the second cycle was associated with a 54% overall response rate in bortezomib-naive patients and a 26% overall response rate in bortezomib and immunomodulatory drug refractory patients. The overall response rate is 20% higher than that with single bortezomib treatment. The U.S. Food and Drug Administration has granted fast track designation for carfilzomib to develop as a potential treatment of patients with relapsed and refractory multiple myeloma.

Drugs	Features	R&D Stage	Institutes
<b>Highly selective and irreversible</b>			
Carfilzomib (PR-171)	Epoxomicin analog Minimal activity against off-target enzymes Lymphoid neoplasms and multiple myeloma	Phase III for MM Phase I for solid tumors	Proteolix Onyx
Marizomib (NPI-0052 Salinospormide A)	Marine product, $\beta$ -lactone- $\gamma$ -lactam family More potent than Bortezomib MM, lymphomas, leukemias and solid tumors	Phase II for myeloma	Nereus Pharmaceuticals, Inc.
PI-083	From <i>Streptomyces matensis</i> Thr21, Gly47, Ala 49 of $\beta$ 5, Asp114 of $\beta$ 6 Cancer-selective proteasome inhibitor Myeloma, lung cancer, breast cancer	Preclinical	Moffitt Cancer Center
<b>Orally active</b>			
CEP-18770	Boronic-acid based More sustained Pharmacodynamics Competes with bortezomib Few side effects during treatment	Phase II for myeloma	Cephalon
PR-047	c20S, i20S >80% inhibition in most tissues High oral bioavailability		Proteolix
<b>Non-comparative inhibitors on 20S proteasomes</b>			
Clioquinol	Binding to a subunits of 20S Non-competitive inhibition Overcome resistance to Bortezomib	Phase I	University Health Network
5-Amino-8-hydroxyl-quine (5AHQ)			
<b>Natural Products</b>			
Pristimerin	Isolated from <i>Celastrus</i> and <i>Maytenus</i> spp. Tripernoid family C6 of Pristimerin interacts with hydroxyl group of N-terminal Thr of c20S Inhibits IKK, suppresses NF $\kappa$ B, cyclin D	Preclinical	Mayo Clinic
EGCG (-)-epigallocatechin-3-gallate	From green tea Ester bond of EGCG attacked by N-terminal Thr of 20S Competitively inhibits Proteasome with Bortezomib	Preclinical	Not available

Table 1. Novel Proteasome inhibitors against blood cancers

### 5.1.2 Marizomib

Marizomib (NPI-0052 or salinosporamide A) is a structurally and pharmacologically unique  $\beta$ -lactone- $\gamma$ -lactam proteasome inhibitor produced by a marine actinomycete *Salinispora tropica* (Macherla et al., 2005). Unlike bortezomib, marizomib irreversibly binds to proteasomes and inhibits all three protease activities, including chymotrypsin-like (CT-L,  $\beta$ 5), trypsin-like (T-L,  $\beta$ 2), and caspase-like (C-L,  $\beta$ 1). This nature is responsible for its slower efflux, longer duration of action, and greater cytotoxicity (Obaidat et al.). Preclinical studies suggest that marizomib is a more potent inducer of apoptosis in myeloma cells than bortezomib, and demonstrates activity in bortezomib resistant cell lines as well. In addition to MM, marizomib has been evaluated in models for MCL, Waldenstrom's macroglobulinemia (WM), chronic and acute lymphocytic leukemia, glioma, colorectal and pancreatic cancers, and has exhibited synergistic activities in tumor models in combination with bortezomib, and various histone deacetylase inhibitors (B et al., 2011; Singh et al., 2010). Marizomib has been moved to Phase II clinical trials and achieved very good responses. In a Phase I study of 17 patients with relapsed and relapsed/refractory multiple myeloma, Drug-related adverse events have consisted principally of mild-to-moderate fatigue, nausea and diarrhea. More importantly, NPI-0052 does not appear to induce peripheral neuropathy or myelosuppression associated with bortezomib treatment (Hofmeister et al., 2009).

## 5.2 Orally active inhibitors of proteasomes: CEP-18770, PR-047 and ONX-0912

The *i.v.* administration of bortezomib largely increases the workload of the physicians and other medical staff and largely increases the healthcare budget. Therefore, orally active inhibitors of proteasomes are of great interest. Currently, several such orally active drugs have been developed, including CEP-18770, PR-047 and ONX-0912.

### 5.2.1 CEP-18770

CEP-18770 is a novel orally-active inhibitor of the chymotrypsin-like activity of the proteasome that down-modulates the NF $\kappa$ B activity (Piva et al., 2008). CEP-18770 induces apoptotic cell death in MM cell lines and in primary purified CD138-positive explant cultures from untreated and bortezomib-treated MM patients. Importantly, CEP-18770 exhibits a favorable cytotoxicity profile toward normal human epithelial cells, bone marrow progenitors, and bone marrow-derived stromal cells. Both intravenous and oral administration of CEP-18770 resulted in a sustained pharmacodynamic inhibition of proteasome activity in tumors relative to normal tissues, complete tumor regression of MM xenografts and improved overall median survival in a systemic model of human MM. In addition, CEP-18770 has a strong antiangiogenic activity *in vitro*, and potently represses RANKL-induced osteoclastogenesis. A recent study suggests that CEP-18770 enhances the anti-myeloma activity of bortezomib and melphalan which suggests a combinatorial regimen for MM therapy (Piva et al., 2008; Sanchez et al., 2010). This agent has moved to clinical trials for relapsed or refractory multiple myeloma (<http://clinicaltrials.gov/ct2/show/NCT01348919>) or for solid tumors and non-Hodgkin's lymphomas (<http://clinicaltrials.gov/ct2/show/NCT00572637>).

### 5.2.2 PR-047

PR-047 is also an orally active inhibitor that selectively inhibits CT-L activity of both the constitutive proteasome ( $\beta 5$ ) and immunoproteasome (LMP7) and demonstrated an absolute bioavailability of up to 39% in rodents and dogs. It was well tolerated with repeated oral administration at doses resulting in >80% proteasome inhibition in most tissues and elicited an antitumor response equivalent to intravenously administered carfilzomib in multiple human tumor xenograft and mouse syngeneic models (Zhou et al., 2009). The favorable pharmacologic profile supports its further development for the treatment of malignant diseases.

### 5.2.3 ONX-0912

Like carfilzomib, ONX-0912 is also an epoxyketone compound with novel selective, irreversible inhibition activity to the immunoproteasome and constituent 20S particles. ONX-0912 displays great oral activity (Chauhan et al., 2010). Primary WM cells expressing higher level of 20S are more responsive to ONX-0912 (Roccaro et al., 2010). ONX-0912 induces WM cell apoptosis through c-JNK activation, NF $\kappa$ B inhibition, caspase cleavage, and initiation of the unfolded protein response. Moreover, ONX-0912 also reduce the secretion of BM-derived interleukin-6 (IL-6) and insulin-like growth factor 1 (IGF-1), thus inhibiting BM-induced Akt phosphorylation and phosphorylated extracellular signal-related kinase activation in WM cells. In addition to blood cancers, ONX-0912 also displays potent anticancer activity in solid tumors and a Phase I study of ONX 0912 administered orally in patients with advanced refractory or recurrent solid tumors is under evaluation (<http://clinicaltrials.gov/ct2/show/NCT01129349>).

## 5.3 Non-classic inhibitors: Clioquinol and 5-amino-8-hydroxyl-quinoline

Most of the proteasomal inhibitors competitively bind to the  $\beta$  subunits of 20S proteasome, for example, MG-132, bortezomib, and carfilzomib. Recently, we found that a group of quinoline-based agents including clioquinol, chloroquine, 5-amino-8-hydroxyl quinoline (5AHQ), and metfloquinoline display potent inhibition on proteasomal catalytic activity by a non-competitive manner (Li et al., 2010; Mao et al., 2009). Further studies indicated that these agents bind to the  $\alpha$  subunits other than  $\beta$  subunits of the 20S core particle as bortezomib or MG-132 does. In analysis of its binding to purified 20S archaeal proteasomes from *Thermoplasma acidophilium* by using nuclear magnetic resonance (NMR) technology, chloroquine binds to the  $\alpha$  subunits with 260 Å distance from  $\beta$  active sites. Notably, chloroquine and MG132 can bind the proteasome simultaneously, further establishing that they exploit two completely separate binding pockets (Sprangers et al., 2008).

The interaction of 5AHQ with  $\alpha_7$ – $\alpha_7$  produced clear spectral changes localized to residues Ile159, Val113, Val87, Val82, Leu112, Val89, Val134, Val24 and Leu136, which are inside the antechamber. In contrast, MG132 which binds the proteolytic chamber produces shifts in the beta rings of the full proteasome. Binding to the  $\alpha$  subunit, 5AHQ leads to a conformational change of the core particle and displays a non-competitive inhibition on proteasome. 5AHQ preferentially induced cell death in primary myeloma and leukemia cells compared with normal hematopoietic cells. More importantly, 5AHQ overcomes the resistance to

bortezomib and is equally cytotoxic to human myelomonocytic leukemia THP1/BTZ500 cells which are 237-fold more resistant to bortezomib than wild-type THP1 cells because of the overexpression and mutation of the bortezomib-binding  $\beta 5$  subunits (Li et al., 2010). Therefore, a group of quinoline-based small molecules can inhibit proteasomal activity in a non-cannoical manner. Because of their low toxicity and novel inhibition mechanism, these compounds could be developed for MM and leukemia therapy. Currently, clioquinol has been moved to clinical trials for refractory acute myeloid leukemia.

#### 5.4 Natural proteasomal inhibitors: Pristimerin and EGCG

Except for small chemical compounds or peptide reagents, several natural products have been identified and evaluated for MM treatment in both *in vivo* and *in vitro* assays. The most promising candidate could be NPI-0052 or marizomib isolated from a marine actinomycete *Salinispora tropica* as described above. Here we discuss two more agents in this category pristimerin and (-)-epigallocatechin-3-gallate (EGCG).

##### 5.4.1 Pristimerin

Pristimerin belongs to the tripernoid family and is isolated from a traditional Chinese medicine called *Celastrus* and *Maytenus spp.* Nucleophilic susceptibility and *in silico* docking studies show that C6 of pristimerin is highly susceptible towards a nucleophilic attack by the hydroxyl group of N-terminal threonine of the proteasomal chymotrypsin subunit. This interaction leads to an inhibition of the chymotrypsin-like activity of a purified rabbit 20S proteasome (Yang et al., 2008). Pristimerin displayed similar inhibition activity in purified rabbit 20S proteasomes and in human prostate cancer cell lysates (Tiedemann et al., 2009). The  $IC_{50}$ s are 2.2 and 3.0  $\mu M$  *in vitro* and *in vivo*, respectively. The treatment of pristimerin in prostate cancer cells resulted in the accumulation of ubiquitinated proteins and three proteasome target proteins, Bax, p27 and  $I\kappa B\alpha$ . However, myeloma cells are more sensitive to pristimerin. Pristimerin potently inhibits both IKK and the proteasome in MM cells with an  $IC_{50}$  of 100 nM. Pristimerin causes overt suppression of constitutive NF $\kappa$ B activity in myeloma cells that may mediate its suppression of cyclin D, thus inducing myeloma cell apoptosis.

##### 5.4.2 (-)-epigallocatechin-3-gallate (EGCG)

EGCG is one of the polyphenols found in green tea extract and inhibits proteasomal activity (Golden et al., 2009). The ester bond of EGCG is attacked by the N-terminal threonine residue of the proteasome, forming a covalent EGCG-proteasome complex which has been confirmed by high performance liquid chromatography (HPLC) analysis. Recent studies found that EGCG competitively inhibits proteasomal activity in a same manner as bortezomib does, thus neutralizing the inhibiting activity of bortezomib and other boronic acid-based proteasome inhibitors. Therefore, green tea polyphenols block the anticancer effects of bortezomib and green tea is not encouraged for myeloma patients who are using bortezomib (Golden et al., 2009). However, a recent study didn't find antagonism of bortezomib in preclinical *in vivo* experiments, where EGCG or ascorbic acid plasma concentrations are commensurate with dietary or supplemental intake and suggest that patients receiving bortezomib treatment do not need to avoid normal dietary consumption of green tea, vitamin C-containing foods, or EGCG or vitamin C dietary supplements (Bannerman et al., 2011).

## 6. Targeting at ubiquitination and deubiquitination systems for blood cancer treatment

Proteasomes are critical components of both cancer cells and normal tissues because they determine the fate of most of the proteins and therefore inhibition of proteasome will also lead to normal cell stress and apoptosis. Thus inhibition of proteasomes indiscriminately raises levels of hundreds of proteins regardless to their anticancer effect. Thus, proteasomal inhibitors are a double-edged sword because they kill cancer cells, simultaneously, kill normal cells. Because proteasome-coupled protein ubiquitination is more specific, inhibition of certain enzymes involved in protein ubiquitination will be a more pertinent target for cancer drug development. There are four kinds of enzymes, E1, E2, E3 and Dubs which contain thousands of members in total. Currently, with the exception of E2, inhibitors of these other enzymes have been identified and are being evaluated for the treatment of hematological malignancies.

### 6.1 Targeting at E1 for hematological malignancies

E1 or Ubiquitin-activating enzyme (UBA) controls the protein ubiquitination by activating ubiquitin using ATP as an energy supplier. Knockdown of E1 by small interfering RNA (siRNA) strategy decreases the abundance of ubiquitinated proteins in leukemia and myeloma cells and induced cell death (Xu et al., 2010). Blood cancer cells including leukemia and myeloma are more sensitive to E1 inhibitors. A small molecule PYZD-4409, an inhibitor of E1, can abolish protein ubiquitination, thus inducing endoplasmic reticulum (ER) stress, and leading to cancer cell apoptosis. PYZD-4409 also displayed ideal anti-leukemia activity *in vivo* without untoward toxicity by decreasing tumor volume and weight (Xu et al., 2010). However, the same concern may arise as that already seen in bortezomib because there is only a single E1 protein in humans.

### 6.2 Targeting at E3 for blood cancer therapies

The E3 ligases are the largest family in the UPS system. E3s are the primary determinant of substrate specificity and represent the largest and most diverse class of Ub/Ub-like regulatory enzymes. There are 600-1000 potential E3s responsible for E2 binding, substrate recognition, and regulatory functions. Targeting the ubiquitin ligases promises more specificity because most E3s tag only a few proteins for destruction. Such drugs can, in theory, block degradation of its specific substrate proteins. Currently, interfering with E3-substrate interaction is one of the leading strategies for anti-cancer drugs targeting at UPS.

One of the most promising E3s is Skp2, the F-box protein that controls degradation of p27, an important tumor suppressor gene (Zhan et al., 2007). Skp2 levels are abnormally high in leukemia and myeloma cells, therefore, blocking Skp2 activity might reasonably be expected to stop cancer cell proliferation. CpdA is such an inhibitor of Skp2 by preventing incorporation of Skp2 into the SCF Skp2 ligase, CpdA induces G1/S cell-cycle arrest as well as SCF Skp2- and p27-dependent cell killing (Chen et al., 2008). In models of MM, CpdA overcomes resistance to dexamethasone, doxorubicin, and melphalan, as well as to bortezomib, and also acted synergistically with this proteasome inhibitor. Importantly, CpdA is active against patient-derived plasma cells and both myeloid and lymphoblastoid leukemia blasts, and showed preferential activity against neoplastic cells while relatively sparing other marrow components (Chen et al., 2008).

Another interesting E3 is Mdm2 which regulates p53 ubiquitination. Several inhibitors of Mdm2 have been identified, such as Nutlins (Stuhmer et al., 2005) and MI-63 (Ding et al., 2006; Samudio et al., 2010). By disrupting the interaction of Mdm2 and p53, both Nutlins and MI-63 can restore p53 which further tends to promote arrest of cell cycle and apoptosis. These drugs are effective in inducing apoptosis of MM cells which express wild-type p53, unfortunately, it won't work in cancer cells with mutated or deleted p53.

### 6.3 Targeting at deubiquitinases

Just like E3s, deubiquitinases play a tumor-suppressing or -promoting role dependent on its targeting protein. For example, USP9X is an oncoprotein enzyme that removes ubiquitin from the anti-apoptotic protein MCL-1 (Sun et al., 2011). MCL1 is overexpressed in most blood cancer cells, and is highly associated with cancer cell proliferation and protects cancer cells from apoptosis (Sun et al., 2011). MCL1 is degraded by proteasomes after poly-ubiquitination. High expression of USP9X is seen in leukemia and MM cells. A small molecule called WP1130 (Kapuria et al., 2010) has been identified as a partly selective Dub inhibitor by directly inhibiting activity of USP9x, USP5, USP14, and UCH37, which are known to regulate survival protein stability and 26S proteasome function. WP1130-mediated inhibition of tumor-activated Dubs results in downregulation of antiapoptotic and upregulation of proapoptotic proteins, such as Mcl-1 and p53, thus leading to cancer cell death (Kapuria et al., 2010).

Although large-scale inhibitors of ubiquitination enzymes are yet to fully develop, successful E3 and Dub inhibitors have established the proof-of-principle that inhibition of ubiquitinating/deubiquitinating enzymes is novel and potentially powerful strategy to develop anti-blood cancer drugs and is surely an area that will expand greatly in the future.

## 7. Summary

The ubiquitin-proteasome system has been widely investigated in the association of hematological malignancies, it is extensively involved in the development and therapy of blood cancers, including leukemia, lymphoma and multiple myeloma. Targeting at the UPS specific genes/proteins, several novel drugs have been developed including the first-approved proteasome inhibitor-bortezomib in the treatment of myeloma and mantle cell lymphoma. The upcoming years will witness the introduction of more potent and more patient-friendly next generations of UPS inhibitors such as carfilzomib and inhibitors of ubiquitinating/deubiquitinating enzymes for the treatment of blood cancer patients.

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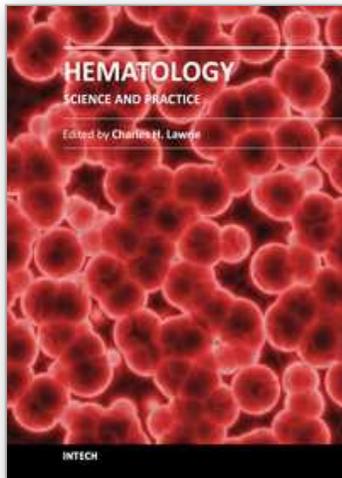
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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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