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Molecular Mechanisms of Acquired Proteasome Inhibitor Resistance

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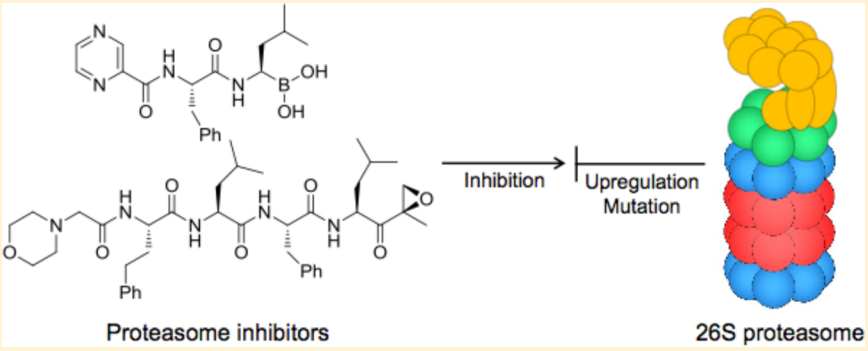
Supporting Information



ABSTRACT: The development of proteasome inhibitors (PIs) has transformed the treatment of multiple myeloma and mantle cell lymphoma. To date, two PIs have been FDA approved, the boronate peptide bortezomib and, most recently, the epoxyketone peptide carfilzomib. However, intrinsic and acquired resistance to PIs, for which the underlying mechanisms are poorly understood, may limit their efficacy. In this Perspective, we discuss recent advances in the molecular understanding of PI resistance through acquired bortezomib resistance in human cell lines and evolved salinosporamide A (marizomib) resistance in bacteria. Resistance mechanisms discussed include the up-regulation of proteasome subunits and mutations of the catalytic β-subunits. Additionally, we explore potential strategies to overcome PI resistance.

INTRODUCTION

In eukaryotes, the regulated hydrolysis of cellular proteins is mediated by a ubiquitous macromolecular enzymatic complex, the 26S proteasome.[1] The proteasome acts as the central hub of nonlysosomal cellular proteolysis, mediating processes such as cell cycle control, cell differentiation, immune response, amino acid recycling, and apoptosis; consequently, its disruption by genetic mutation or small molecule inhibitors has significant deleterious effects via multiple downstream pathways.[1] To underscore its universal role, inhibition of the proteasome has been explored in the treatment of diverse maladies such as cancer,[2−4] viruses,[2,3] stroke,[2] cardiovascular disease,[4] inflammation,[2] and transplant rejection.[5] Bortezomib (Velcade) was the first FDA approved proteasome inhibitor (PI), where it is used in the treatment of the hematological malignancies multiple myeloma (MM) and refractory mantle cell lymphoma (MCL),[6] and on July 20, 2012, carfilzomib (Kyprolis) became the second FDA-approved PI to treat advanced MM.

Despite the successes of bortezomib therapy, many patients are intrinsically resistant to bortezomib or become resistant during treatment.[7] While much effort has gone into elucidating how PIs function at the molecular level and consequently combat cancer,[4,8−10] the mechanism(s) of PI resistance is (are) currently less understood.[4,8−10] In an effort to elucidate potential mechanisms of acquired PI resistance, many recent studies have established cell lines of various malignancies that are resistant to bortezomib.[11−21] In this Perspective we summarize the different mechanisms of PI resistance that include up-regulation and/or sequence mutation of the molecular target, the 20S proteasome β5-subunit, as acquired resistance mechanisms in various cancer cell lines. While these acquired resistance mechanisms have not yet been observed in patient samples, they may foreshadow acquired resistance in the clinic. We additionally explore potential strategies to overcome such resistance mechanisms in the next generation of small molecule PIs.

UBIQUITIN−PROTEASOME SYSTEM

The 2.5 MDa eukaryotic 26S proteasome comprises a 700 kDa 20S core particle and the 19S regulatory base and lid (Figure 1).[22] The 19S structure serves as the gatekeeper of the catalytic 20S core particle for the recognition and unfolding of polyubiquitinated substrates. Proteins destined for proteasome-mediated destruction in eukaryotic cells are covalently tagged with ubiquitin (Ub), a small protein modifier. Briefly,

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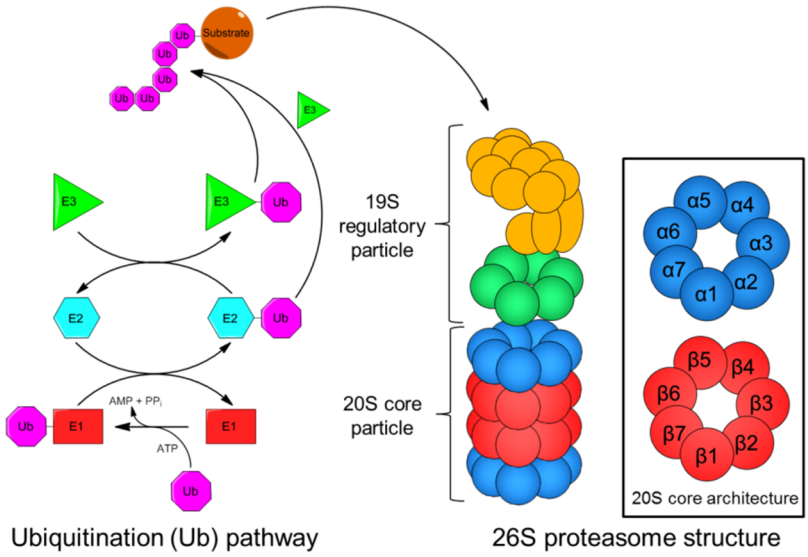

Figure 1. Ubiquitin–proteasome system. Polyubiquitination of cellular proteins by the cascade of E1, E2, and E3 enzymes assigns substrate proteins for 26S proteasomal degradation. Poly-Ub proteins are recognized and unfolded by the 19S regulatory particle and fed into the proteolytic 20S core particle for destruction by the three catalytically active subunits, β5, β2, and β1.

Ub is transferred to target protein Lys residues by a cascade of three enzymes, E1, E2, and E3 (Figure 1). Ub may also be transferred to another Ub residue forming a poly-Ub chain, which signals the substrate protein for the 19S regulatory cap and subsequent proteasomal degradation. The quantity and specificity of ubiquitinating enzymes increase from E1 to E3. Only two E1 isoforms are known in humans, while there are over 30 E2's and 300 E3's.[23] The ubiquitin–proteasome system has been well studied and extensively reviewed elsewhere.[22,24]

The 20S core particle contains four heptameric rings stacked in a cylindrical $\alpha_7\beta_7\beta_7\alpha_7$ arrangement.[1] The α-subunits act as the exterior structural scaffold, while the interior β-subunits catalyze proteolytic activity. Each α- and β-subunit per heptameric ring is unique, requiring 14 genes for the 20S core alone. Upon assembly, prosequences of the proteolytic β-subunits are autocatalytically removed yielding the N-terminal Thr1, which serves as the nucleophile for proteolytic hydrolysis. Only three of the seven β-subunits in each heptameric ring are catalytically active: the *PSMB6* encoded β1-subunits catalyze caspase-like activity (C-L); the *PSMB7* encoded β2-subunits catalyze trypsin-like activity (T-L); the *PSMB5* encoded β5-subunits catalyze chymotrypsin-like activity (CT-L). The substrate specificity of each β-subunit is determined by the interactions between the substrate amino acid side chains and the proteasome specificity pockets, including those in adjacent β-subunits, with the S1 binding pocket being the predominant driver. Mammals additionally possess γ-interferon inducible β1i, β2i, and β5i, which replace the constitutively expressed β1, β2, and β5, respectively.[1] Archaea and actinobacteria also possess a simplified 20S proteasome.[25] In contrast to eukaryotes, prokaryotic proteasome function is not essential for survival, likely because of a redundancy of proteolytic machinery.[26]

■ PROTEASOME INHIBITION IN CANCER THERAPY

Proteasome inhibitors have flourished as anticancer agents because they potently and preferentially induce apoptosis in certain malignant cell types. The natural product lactacystin was first identified to induce apoptosis in the human monoblast U937 cell line,[27] while chronic lymphocytic leukemia cells were found to be significantly more sensitive to lactacystin-induced TNFα-mediated apoptosis than were normal human lymphocytes.[28] Tumor growth was also suppressed in vivo by proteasome inhibition in mouse models of Burkitt's lymphoma, and the induction of apoptosis preferentially targeted cancerous cells.[29] Finding that malignant cells were more susceptible to PI-induced apoptosis led to speculation that malignant cells may rely more heavily on proteasomal degradation for survival.[30] Elevated proteasome expression has indeed been observed in neoplastic cells, including various types of leukemia, indicating that increased proteasome activity is required to maintain survival during rapid proliferation.[10] Basal proteasome activities have been shown to differ among cell lines and correlate to intrinsic bortezomib sensitivity[20] with cells intrinsically resistant to bortezomib displaying higher CT-L and C-L activities.[20,31,32] However, while basal proteasome activities may serve as an indicator of intrinsic resistance, there is no evidence that they serve as a predictor of acquired resistance.

The specific mechanism(s) by which proteasome inhibition translates into anticancer therapy is complex and may vary depending on the specific transformation. It was previously observed that in plasma cells undergoing late phase plasmacytic differentiation, proteasome activity decreased concomitantly with immunoglobulin accumulation, thereby overloading the proteasome, resulting in an accumulation of poly-Ub proteins followed by apoptosis.[33] It was further shown that MM cells sensitive to PI treatment were more reliant on proteasomal degradation, had lower proteasome content and activity levels, and displayed a higher proteasomal load to capacity ratio relative to PI resistant MM cells, confirming the link between proteasome workload and intrinsic PI sensitivity.[32] Many specific regulatory proteins have also been identified to be affected.[4] Inhibition of the NF-κB pathway is a frequently cited consequence of proteasome inhibition. Functional proteasomes are required to degrade IκBα, an inhibitor of NF-κB function. Proteasome inhibition allows IκBα levels to rise, thereby inhibiting NF-κB which leads to a decreased production of antiapoptotic factors, angiogenic factors, and apoptosis

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Table 1. Properties of Proteasome Inhibitors Explored for the Treatment of Malignancies

inhibitor	electrophile	developed by	P1 residue	reversibility	subunit target[a]	ref
bortezomib (**1**)	boronate	Millennium Pharmaceuticals	leucine	reversible	IC_{50} (nM): $\beta5$, 7.9; $\beta2$, 590; $\beta1$, 53	59
2	boronate	Millennium Pharmaceuticals	leucine	reversible	IC_{50} (nM): $\beta5$, 3.4; $\beta2$, 3500; $\beta1$, 31	39
delanzomib (**3**)	boronate	Teva Pharmaceutical Industries	leucine	slowly reversible	IC_{50} (nM): $\beta5$, 3.5; $\beta2$, >100; $\beta1$, NI[b]	40
carfilzomib (**4**)	epoxyketone	Onyx Pharmaceuticals, Inc.	leucine	irreversible	K_{inact}/K_i (M^{-1} s^{-1}): $\beta5$, 33 000; $\beta2$, <100; $\beta1$, < 100	41
oprozomib (**5**)	epoxyketone	Onyx Pharmaceuticals, Inc.	phenylalanine	irreversible	IC_{50} (nM):[c] $\beta5$, ~10; $\beta2$, $\beta1$, NI at 50	43
salinosporamide A (**6**)	β-lactone	Nereus Pharmaceuticals, Inc.	hydroxycyclohexenylalanine	irreversible	IC_{50} (nM): $\beta5$, 3.5; $\beta2$, 28; $\beta1$, 430	59

[a]IC_{50} values reported from the different studies cannot be directly compared because of differences in assay conditions but rather are indicative of active site specificity of a particular inhibitor. [b]NI: not inhibitory [c]Estimated from graph. Inhibition of CT-L activity includes $\beta5$ and $\beta5$i subunits.

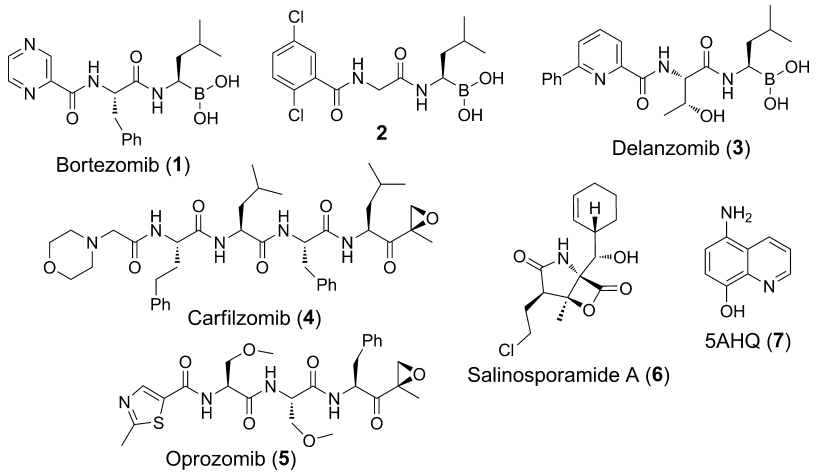

Figure 2. Chemical structures of selected proteasome inhibitors.

inhibitors.[4] As the NF-κB pathway is activated by many chemotherapeutic agents, PIs such as bortezomib may, when used in combination therapy, increase the effectiveness of such drugs.[8]

Proteasome inhibition has also been reported to cause disregulation of cyclins, cyclin-dependent kinases, and other cell cycle regulatory proteins that disrupt cell division. Such inhibition may favor apoptosis by stabilizing proapoptotic proteins such as Bax and p53 while reducing antiapoptotic proteins such as the Bcl-2-family proteins.[4,8] Additionally, antitumor activity has been attributed to the formation of reactive oxygen species and aggresomes, the unfolded protein response, the intrinsic mitochondrial apoptotic pathway, the death receptor pathway, and the ER stress response pathway.[4] For more detailed information on the mechanisms of action of PIs in cancer therapy, we suggest the following reviews: refs 4, 8, 9, 30.

■ PROTEASOME INHIBITORS

Many small molecule inhibitors of the 20S proteasome, both synthetically prepared and naturally produced, have been discovered.[2,3,6,34] The predominant structural theme of PIs is a short peptide-like substrate mimic with an electrophilic modification to covalently capture the N-terminal Thr1O$^{\gamma}$ of one or more of the catalytic β-subunits. Electrophilic warheads include the reversibly inhibiting aldehydes and boronic acids or the irreversibly inhibiting vinylsulfones and epoxyketones. Potency and selectivity for each inhibitor are determined by both the nature of the electrophile and the interactions of the inhibitor with the active site binding pockets.[6,35] While the P1/S1 interaction is often considered the primary determinant of specificity, distal binding pockets may also influence substrate selectivity and inhibition potency.[35] Six PIs, all of which primarily target Thr1O$^{\gamma}$ of the $\beta5$-subunit, are either FDA approved or currently in clinical trials for the treatment of malignancies.[36,37] A comparison of these PIs is found in Table 1, and structures are provided in Figure 2. The development status of these PIs has recently been reviewed,[36,37] and many thorough reviews of all known PI structures and catalytic mechanisms are currently available.[2,3,6,34]

The synthetic boronates are the most clinically successful class of PIs to date. Boronates act as an electron acceptor, forming reversible tetrahedral boronic esters with Thr1O$^{\gamma}$. Bortezomib (**1**) was the first PI approved by the FDA and is prescribed in the treatment of MM and MCL.[3] Bortezomib primarily inhibits the $\beta5$-subunit with low nanomolar potency but also inhibits the $\beta1$-subunit to a lesser extent.[36] Despite its high potency, **1** maintains several drawbacks such as peripheral neuropathy,[37] an inability to dose orally, and susceptibility to innate and acquired resistance of unknown mechanisms. The FDA recently approved subcutaneous administration of **1** which has been shown to be as effective as intravenous administration while reducing peripheral neuropathy.[38] Several second generation PIs are currently in development aiming to improve upon **1**'s shortcomings. For instance, the prodrug MLN9708,[39] which hydrolyzes in vivo to the active MLN2238

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Table 2. Regulatory Changes in *PSMB5* mRNA and β5-Subunit Protein Levels in Cell Lines with Acquired 1 Resistance

ref	cancer cell type	cell line	selective Btz concn (nM)	*PSMB5* regulation[a]	β5-subunit regulation[a]	proteasome CT-L activity[a]
11	Burkitt's lymphoma	Namalwa[ad]	12.5	ND	↑	(<1.5×)[b]
12	monocytic/macrophage	THP1/BTZ$_{30}$	30	(0.4×)[b]	(up to 60×)[c]	(1.3−1.4×)[d,e]
		THP1/BTZ$_{50}$	50	(0.5×)[b]	(up to 60×)[c]	(1.3−1.4×)[d,e]
		THP1/BTZ$_{100}$	100	(0.6×)[b]	(up to 60×)[c]	(1.3−1.4×)[d,e]
		THP1/BTZ$_{200}$	200	(0.9×)[b]	(up to 60×)[c]	(1.3−1.4×)[d,e]
		THP1/BTZ$_{(-100)}$	100[f]	(0.4×)[b]	(minor)	(1.3−1.4×)[d,e]
13	lymphoblastic lymphoma/ leukemia	JurkatB1	>200	↑	ND	ND
		JurkatB5	500	↑	ND	ND
14	lymphoblastic lymphoma/leukemia	JurkatB-G322A	1000	(1.7× ± 0.5×)	ND	⊗
		JurkatB-C323T	1000	(0.9× ± 0.1×)	ND	⊗
		JurkatB-G322A/C326T	1000	(1.3× ± 0.2×)	ND	⊗
15, 51	acute myeloid leukemia	HL-60a	40[g]	⊗	↑	β1/β5 (1.5×)[b,h]
	myeloma	AMO-1a	NR[g]	ND	↑	β1/β5 (4.5×)[b,h]
	plasmocytoid lymphoma	ARH-77a	NR[g]	ND	↑	⊗
16	MM	KMS-11/BTZ	NR	ND	⊗	ND
		OPM-2/BTZ	NR	ND	⊗	ND
17	MCL	HBL2-BR	100	⊗	↑	(<2×)[b]
		JEKO-BR	100	⊗	↑	(1.4×)[b]
18	MM	8226/BTZ7	7	(5×)[b]	↑	↓
		8226/BTZ100	100	(15×)[b]	↑	↓
	acute lymphoblastic leukemia	CEM/BTZ7	7	↑ (minor)	↑	↑
		CEM/BTZ200	200	↑ (minor)	↑	ND
19	HT-29 adenocarcinoma	BR100	100[i]	↑	(3−4×)	(3.3×)
		BR200	200	↑	(3−4×)	(3.5×)
20	non-small-cell lung cancer	H460BTZR$_{80}$	80	ND	(1.3−1.8×)	↓
		H460BTZR$_{200}$	200	ND	(1.3−1.8×)	↓
		A549BTZR$_{40}$	40	ND	(1.3−1.8×)	↓
		A549BTZR$_{100}$	100	ND	(1.3−1.8×)	↓
		SW1573BTZR$_{30}$	30	ND	(1.3−1.8×)	↓
		SW1573BTZR$_{150}$	150	ND	(1.3−1.8×)	↓
21	MM	8226/7B	100	↑ (large)	↑	ND

[a]Changes relative to the parental strain (1×): ↑, up-regulation; ↓, down-regulation; ⊗, no change. [b]Estimated value from figure in publication. [c]β5 level proportional to level of resistance. [d]Assayed in the absence of bortezomib. [e]Activity varied by selective concentration, but each line was not quantified. [f]6 months without bortezomib. [g]Maintained at 20 nM bortezomib. [h]β5 (CT-L) and β1 (C-L) activity could not be differentiated from each other. [i]After previous selection at 200 nM.

(**2**), and delanzomib[40] (**3**, CEP-18770) are both orally available boronate PIs currently in clinical development.

Three PIs with alternative warheads are currently in development as well. Carfilzomib[41] (**4**), which was recently FDA approved, and the orally bioactive analogue oprozomib[42,43] (**5**, ONX 0912), are highly potent, selective, and irreversible epoxyketone warhead PIs derived from the bacterial natural product epoxomicin.[2] Epoxyketones bind irreversibly to the β-subunit first by hemiacetal formation between the ketone and Thr1O$^{\gamma}$ followed by attack of the epoxide residue by the N-terminal amino group, resulting in a stable morpholino ring.[2] This intricate mechanism provides minimal cross-reactivity against other proteases such as trypsin, chymotrypsin, and cathepsins.[44] Lastly, salinosporamide A (**6**, NPI-0052 or marizomib) is a β-lactone natural product PI produced by the marine bacterium *Salinispora tropica*.[2,45] Unlike lactacystin and its β-lactone derivative omuralide, the unique mechanism of a secondary halide displacement generates a stable cyclic ether that blocks hydrolysis, thereby rendering **6** an irreversible inhibitor.

MECHANISMS OF ACQUIRED RESISTANCE TO PROTEASOME INHIBITORS

Despite **1** being more efficacious than other chemotherapeutic agents in the treatment of certain hematological malignancies, intrinsic and acquired resistance remain significant impairments to treatment.[8,37,46] Currently, the mechanisms behind PI resistance are poorly understood. In an effort to elucidate potential mechanisms of acquired PI resistance, many recent studies have elicited acquired **1** resistance in various cancer cell lines by chronic exposure to the drug.[11−21] The results of these cell line studies, while far from uniform, illustrate a common theme: up-regulation of proteasome subunits and/or mutation of the β5-subunit encoding gene *PSMB5*. These data from different resistant lines were established and analyzed by multiple investigators as summarized in Tables 2 and 3 and Supporting Information Tables S1 and S2. Proteasome subunit up-regulation at both the mRNA transcription and protein translation levels have been observed. The maximum **1** tolerance achieved and the time required to develop resistance varied widely by cell line. Cross-resistance to other β5-subunit PIs was also frequently observed (Supporting Information Table S1).

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Table 3. β5-Subunit Mutations Observed in Cancer Cell Lines with Acquired 1 Resistance[a]

ref	cancer cell type	cell line	selective Btz concn (nM)	β5-subunit mutation	cellular Btz resistance[b]
12	monocytic/macrophage	THP1/BTZ$_{30}$	30	A49T	NR
		THP1/BTZ$_{100}$	100	A49T	(79)
		THP1/BTZ$_{(-100)}$	100[c]	A49T	NR
13	lymphoblastic lymphoma/leukemia	JurkatB1	>200	A49T (het)	ND
		JurkatB2	500	A49T (het)[d]	(2.6−4)
		JurkatB3	>200	A49T (het)	ND
		JurkatB5	500	A49T (het)	ND
		JurkatB2/1000	1000	A49T (hom)	(26.8−54.6)
14	lymphoblastic lymphoma/leukemia	JurkatB-G322A	1000	A49T	(22)
		JurkatB-C323T	1000	A49V	(39.4)
		JurkatB-G322A/C326T	1000	A49T/A50V	(66.7)
16	MM	KMS-11/BTZ	NR	A49T	(24.7)
		OPM-2/BTZ	NR	A49T	(16.6)
18	MM	8226/BTZ7	7	T21A	(4.5)
		8226/BTZ100	100	A49T	(39.5)
	acute lymphoblastic leukemia	CEM/BTZ7	7	C52F	(10.4)
		CEM/BTZ200	200	A49V/C52F	(170.4)
	monocytic/macrophage	THP1/BTZ100N	100	M45V	NR
		THP1/BTZ500	500	M45I/A49T	NR
19	HT-29 adenocarcinoma	BR100	100[e]	C63F[f]	(32)[g]
		BR200	200	C63F[f]	(34)[g]
20	non-small-cell lung cancer	H460BTZR$_{80}$	80	A49T	(14)
		H460BTZR$_{200}$	200	A49T	(22)
		A549BTZR$_{40}$	40	M45V	(8)
		A549BTZR$_{100}$	100	M45V/A49T	(19)
		SW1573BTZR$_{30}$	30	C52F	(18)
		SW1573BTZR$_{150}$	150	C52F	(70)

[a]Abbreviations: Btz, bortezomib; het, heterozygous; hom, homozygous. [b]Resistance factor relative to parental cell line. [c]100 nM selection, then 0 nM for 6 months. [d]An additional silent mutation was observed. [e]Selected at 200 nM bortezomib for 7 months, then clonal selection at 100 nM. [f]An R24C prosequence mutation was also observed. [g]After 3 days in the absence of bortezomib.

Resistance by Up-Regulation of Proteasomal Subunits. Alterations in mRNA transcription of the β5-subunit encoding *PSMB5* gene (Table 2) have varied from slightly decreased[12] to unchanged,[14,17] to slightly increased,[13,15,18,19] and to substantially increased (5- to 15-fold).[18,21] In cases where transcription levels of other proteasome related genes were quantified, *PSMB6* and *PSMB7* also varied from unchanged[12] to a 5-fold increase (Supporting Information Table S2).[18] Both *PSMB5* and *PSMB7*, but not *PSMB6*, were up-regulated in **1** resistant HT-29 adenocarcinoma cells.[19] Oerlemans et al. performed microarray transcriptional analysis on their 30 and 100 nM resistant human monocytic lines as well as the 100 nM resistant line after 6 months in the absence of **1**. No discernible link between gene expression and resistance was observed.[12]

Evaluating proteasomal subunit up-regulation at the protein level serves as a more direct measurement of proteasome up-regulation. In most cases where **1** resistance was observed, β5-subunit protein levels increased (Table 2). Although many studies did not quantify the change in proteasome subunit protein levels, the degree of β5 increase has ranged from minor to as much as 60-fold.[12] However, no clear quantitative correlation between level of resistance and the extent of β5-subunit expression has been observed.

Many of these studies quantified either mRNA or protein levels but not both. In cases where both were analyzed,[12,15,17−19,21] it appears that mRNA transcription levels are not a strong indicator of protein expression levels. In one case, *PSMB5* transcription was unchanged but a 60-fold increase in β5-subunit protein was observed.[12] Silencing of *PSMB5* mRNA expression in these cells did prevent up-regulation of the β5-subunit and restored bortezomib sensitivity and induced apoptosis. In another case, *PSMB5* transcription from 7 and 100 nM bortezomib resistant lines increased by 5× and 15×, respectively, relative to the parental cells.[18] However, while both showed β5-subunit protein up-regulation relative to the parental line, there was no difference in protein concentration between these two resistant cell lines despite the 3-fold difference in mRNA transcription. On the basis of these studies, mRNA transcription levels should not be used as a proxy for proteasome content or activity.

Bortezomib resistant cell lines displayed conflicting regulation of the immunoproteasome components. One study found a complete shift in favor of the 19S−20S proteasome at the expense of the 11S and immunosubunits,[11] while another study found up-regulation of the 11S and down-regulation of the 19S regulatory particle with no change in immunosubunit expression.[15] Franke et al. observed a specific shift away from β5i toward β5 in resistant MM cells, but no alteration of β5/β5i ratio was demonstrated in resistant acute lymphoblastic lymphoma cells.[18] The 11S regulatory particle was also up-regulated in a **1** resistant acute myeloid leukemia cell line.[15] In a study of three **1** resistant non-small-cell lung cancer lines, two showed up-regulation of the immunosubunits while the third showed no change.[20] Taken together, immunoproteasome regulation appears to vary widely among and within **1** resistant cancer cell line types.

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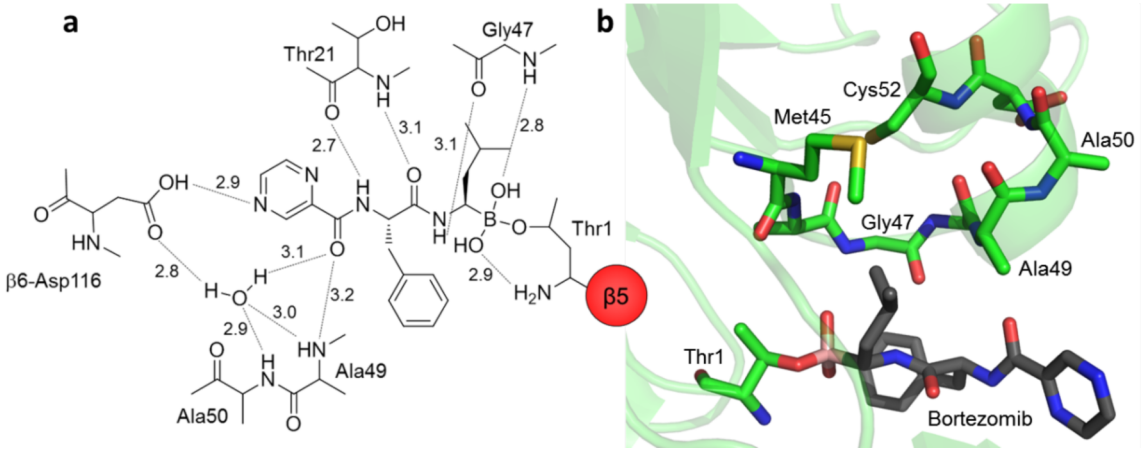

Figure 3. Substrate binding analysis of **1** and the β5-subunit of the *Saccharomyces cerevisiae* 20S proteasome. (a) Dashed lines represent H-bonding with the distance shown in Å. Mutations observed at Ala49, Ala50, and Thr21 may disrupt H-bonding and decrease PI binding. Reproduced in part from *Structure* (http://www.sciencedirect.com/science/journal/09692126), Vol. 14, Groll, M., Berkers, C. R., Ploegh, H. L., and Ovaa, H., "Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome", pp 451−456, Copyright 2006, with permission from Elsevier.[49] (b) Crystal structure of the S1 binding pocket with **1** bound. Image was created using PDB file 2F16, and chain K was rendered in PyMol.[65]

To assess proteasome subunit regulation, microarray analysis was used on over 100 patients with myeloma to identify changes in gene regulation that correlated to progression of the malignancy.[47] Several proteasome pathway genes were up-regulated 48 h after **1** was administered in combination with thalidomide and dexamethasone relative to treatment with only thalidomide and dexamethasone. These genes included *PSMD4*, encoding one of the non-ATPase 19S regulatory cap proteins, and *PSMB2*, *PSMB3*, and *PSMB4*, all encoding noncatalytic 20S β-subunits. Shaughnessy et al. suggest that this up-regulation is due to preferential killing of normal plasma cells and survival of cells with existing up-regulation as opposed to drug induced up-regulation in all cells. None of the catalytic β-subunit encoding genes were found to be differentially regulated. In another study, proteasome activity was visualized in primary cells taken from patients with chronic lymphocytic leukemia, acute lymphoblastic leukemia, and acute myeloid leukemia using fluorescent probes.[48] While the stoichiometry of the β1, β2, and β5 subunits remained the same, the activity varied even within the same cancer types but remained consistent per patient over several weeks. A correlation was observed that in myeloma and non-Hodgkin's lymphoma, cells with the lowest β1 + β5 activity levels, relative to β2, were the most sensitive to **1**.

Resistance by β5-Subunit Mutations. Many **1** resistant cell lines have been found to possess mutations in the β5-subunit encoding gene *PSMB5* (Table 3). Most of these mutations encode amino acid substitutions located in the S1 binding pocket. In particular, substitution of Ala49 with Thr or Val has been observed independently in six different studies.[12−14,16,18,20] Additional mutations in or near the S1 binding pocket include A50V, C52F, M45V, M45I, C63F, and T21A. X-ray crystallographic analysis of **1** bound to the 20S proteasome β5-subunits of the *Saccharomyces cerevisiae* previously revealed a hydrogen bonding network between **1**, a structured water molecule, and several amino acid residues of the S1 binding pocket, including Ala49, Ala50, and Thr21 (Figure 3a).[49] Although these hydrogen bonding interactions originate from backbone atoms, side chain substitutions may alter backbone positioning and disrupt the bonding network. Met45 was additionally shown to move 2.7 Å to accommodate bortezomib's P1 leucine residue.[49] Mutation of Met45 may diminish binding by constricting the S1 pocket or reducing favorable hydrophobic interactions. Cys52 is located behind the S1 binding pocket and may hinder movement of Met45. Ala49 is positioned at the entrance of the S1 binding pocket (Figure 3b). Increasing the size of the side chain may stearically hinder the binding of both inhibitors and substrates. While no crystal structures of such mutated proteasomes have yet been reported, computational modeling has shown that they should decrease both substrate and inhibitor binding.[13,18] Cleavage of the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-amc (LLVY-amc) also appears to be reduced in the resistant cell lines. However, no alternative fluorogenic substrates have been tested to check for a shift in proteolytic specificity, which could be precipitated by S1 pocket mutations.

The role of one *PSMB5* mutation in acquired PI resistance was verified in a T cell lymphoblastic lymphoma cell line. The parental line was mutated by retroviral infection to encode the same A49T seen in the **1** resistant line.[13] These cells were resistant to **1** induced apoptosis, and the inhibition of CT-L activity was decreased. The same mutation was also transfected into parental KMS-11 MM cells and shown to induce **1** resistance but not to the full extent of KMS-11/BTZ cells, suggesting that other factors also contribute to resistance.[16]

Mutations were observed in cell lines at resistance levels as low as 7 nM **1**,[18] which is below the clinically used concentration.[11] Franke et al. showed that mutations were observed in cell lines that were developed in as little as 4 months and that upon repeating the **1** desensitizing process, the same cell line developed a different set of mutations.[18] The authors argued that this observation supports de novo mutation as opposed to the selection of preexisting mutations. Although cell lines with different mutations varied greatly in their level of **1** resistance, it has not been conclusively shown in vitro that any specific mutation is fully responsible for the acquired level of resistance or that one mutation confers greater resistance than another.

Few studies have searched for mutations in other proteasome subunits. Ri et al. reported no mutations in the β1 or β6 subunit

encoding genes,[16] and the study by Franke et al. did not find mutations in *PSMB6* or *PSMB7*.[18] Suzuki et al. has reported a F50I mutation in the prosequence of the β5i encoding *LMP7* gene of the BR200 strain.[19] It should also be appreciated that not all bortezomib resistant cell lines contained *PSMB5* mutations. MM[21] and MCL[17] cell lines, each resistant to 100 nM **1**, were both found to be free of mutations.

Many potent PIs, including **6**, lactacystin, and epoxomicin, are produced by actinomycete bacteria. As the actinobacteria are the only family of eubacteria known to possess 20S proteasome machinery, in which all seven α-subunits and all seven β-subunits are identical, it raises the question of self-resistance. A recent study of the marine actinobacterium *Salinispora tropica*, which produces the drug candidate **6**, revealed that a secondary 20S proteasome β-subunit (SalI) is encoded within the biosynthetic gene cluster of **6**.[50] In vitro analysis of the heterologously expressed α/SalI complex revealed that it was markedly less susceptible than the primary *S. tropica* 20S proteasome to inhibition by both **1** and **6**. Additionally, the proteolytic specificity of the α/SalI complex activity shifted away from the CT-like LLVY-amc substrate, instead preferring a methionine P1 residue of the Z-Val-Lys-Met-amc (VKM-amc) substrate, suggesting that a more flexible P1 amino acid is accommodated in the constricted S1 binding pocket and underscoring the need to explore alternative fluorogenic substrates against mutated proteasome subunits. Genetic comparison between SalI and the housekeeping β-subunit, β_1, exposed M45F and A49V mutations located within the S1 binding pocket. Site directed mutagenesis of both β_1 and SalI revealed that position 45 played surprisingly little role in resistance, whereas alteration of position 49 significantly affected inhibitor resistance, substrate specificity, and prosequence cleavage. While the subunit topology and cellular function of the 20S proteasome in humans and actinobacteria are not identical, it is striking that equivalent mutations are utilized in both systems to achieve PI resistance.

Resistance by Efflux Pumps. One additional strategy for acquired drug resistance is achieved through multidrug resistance (MDR) efflux pumps. Resistance to the peptidyl aldehyde PI Ac-Leu-Leu-Nle-al (ALLN) in Chinese hamster ovary cells was reportedly caused by the up-regulation of P-glycoprotein (Pgp) transmembrane pump via up-regulation of the encoding multidrug resistance gene *mdr1*.[51] This verified that Pgp could export linear peptides, the primary structural scaffold of most PIs. Another MDR pump, MRP1, was later established to also export hydrophobic linear peptides, including ALLN, in various cancer cell lines.[52] Acute myeloid leukemia cell lines overexpressing Pgp were shown to display slight (~2×) **1** resistance, whereas cell lines overexpressing MRP1 were not resistant.[53] Epoxyketone inhibitors **4** and **5** were recently shown to be substrates of Pgp, resulting in 114- and 23-fold resistance, respectively, when Pgp was up-regulated in CEM/VLB cell lines.[54] However, no additional reports have attributed MDR resistance to **1** or **4**, and several studies have ruled it out,[11−14,20] suggesting that multidrug resistance is not a significant factor in PI resistance.

In most cases, acquired **1** resistance in cell lines appears to be a stable transformation. In resistant monocytic/macrophage cells transferred to **1**-free media for 7 days, *PSMB5* expression was unaltered but β5 levels decreased by 2.5-fold.[12] After 6 months, these cells still retained 35-fold **1** resistance. β5-subunit levels and the encoding mRNA both decreased over this time but were rapidly restored upon reintroduction of **1**.[12] Rüchrick et al. confirmed that the resistance phenotype was stable over 14 days, and de Wilt et al. and Lü et al. both confirmed resistance after 2 months in the absence of **1**.[14,15,20] Suzuki et al. similarly observed an increase in **1** resistance after cells were cultured for 40 days in the absence of **1**.[19] However, Pérez-Galán et al. reported that resistance to **1**, which was not caused by a β5-subunit mutation, was gradually lost over time.[17]

It is reiterated that to date, the **1** resistance mechanisms discussed here have only been observed in cancer cell lines. Although the sample size is small, no *PSMB5* mutations have yet been observed in primary patient cell samples.[16,17,55] However, as the recent cell line data indicate that such mutations are potential resistance mechanisms, the clinical relevance will remain unknown without further analysis of patients' *PSMB5* sequences.

Many of the studies reviewed here explored changes in cellular biochemistry beyond the proteasome. Several reports showed that poly-Ub proteins failed to accumulate under **1** treatment in resistant lines.[11,12,15,16,18,20,21] However, ubiquitinated proteins did accumulate when **1** levels significantly higher than the selective concentration were used.[12,18,20] No changes in growth rate or morphology were observed for most of the resistant cell lines.[12,13,15] Balsas et al. observed that resistant cells were significantly larger in size and nearly doubled in cellular DNA content.[21] Rückrich et al. observed a 75% reduction of total protein biosynthesis,[15] whereas Ri et al. observed no alteration of protein synthesis levels in bortezomib resistant lines.[16] Pérez-Galán et al. also observed that intrinsically resistance cells and those that acquired resistance were associated with plasmacytic differentiation.[17]

■ PERSPECTIVES FOR CIRCUMVENTING ACQUIRED PI RESISTANCE

Overcoming intrinsic and acquired resistance to PIs such as **1** will greatly improve efficacy in the clinic. However, the roots of clinical resistance are complex and not well understood. It is apparent that multiple mechanisms of resistance are possible with no one solution adequate to ensure effective treatment in all patients. On the basis of the mechanisms of acquired PI resistance in cancer cell lines reviewed here, we may begin to strategize methods to overcome PI resistance in the event that they are identified in patents. Possibilities include the use of irreversible β-subunit inhibitors, modifying the P1 residue to target mutated β5-subunits, targeting alternative proteasome subunits, targeting proteasome complex assembly, inhibiting upstream ubiquitination pathway enzymes, and targeting proteins outside the ubiquitin−proteasome pathway.

Several reports have identified acquired mutations of the *PSMB5* gene in bortezomib resistant cell lines. These mutations appear to alter the S1 binding pocket, which slow or prevent PI binding and often confer resistance to other PIs that target the catalytic site of the β5-subunit. As **1** and all inhibitors currently being explored in clinical trials primarily target the β5-subunit active site, they will all likely be susceptible to this mechanism of resistance. However, the administration of **3** along with **1** was shown to delay progression of MM in a patient who had become resistant to **1**.[56] As both **1**and **3** are boronates that target the β5-subunit, it is unclear how **3** was able to overcome resistance.

Irreversible inhibitors such as **4** and **6** may be less susceptible to resistance by β5-subunit mutations. While a modified S1 binding site may slow binding kinetics, as evidenced by elevated IC_{50} values, the binding event must only take place once before

permanently deactivating the catalytic site. As an example, MM 8226/BTZ100 cells, which possess an A49T mutation, showed 39.5-fold resistance to **1** but only 9.7-fold resistance to **4** and 10.1-fold resistance to **5** (Supporting Information Table S1).[18] In one study, **1** resistant MM cell lines were established by prolonged exposure to **1** to examine the ability of **4** to overcome **1** resistance. While some cross-resistance was observed for **4**, it did retain greater antiproliferative effectiveness.[57] Carfilzomib also retained antiproliferative and cytotoxic effectiveness on **1** resistant primary patient samples as well.[57] In a study of HT-29 adenocarcinoma cells, proteasome up-regulation and subunit mutations resulted in a rapid recovery of proteasome activity following exposure to **1**.[19] While some cross-resistance was observed for the irreversible inhibitor **4**, it was shown to diminish proteasome activity over a longer time period and to retain cytotoxicity toward the **1** resistant cell.[19] Epoxyketone **5** was also shown to induce apoptosis in vitro in two **1** resistant patient samples, although the specific mechanism of this resistance was not known in this case.[58] The irreversible PI **6** induced apoptosis in MM cells that were resistant to **1**, which was attributed to activation of different apoptotic pathways.[59] Substantially more clinical data will be needed to validate these anecdotal observations. Furthermore, it remains to be seen what level of resistance to these irreversible inhibitors develops when they are used as the selecting agent.

If target sequence modification is confirmed as a clinically relevant form of PI resistance, it would be ideal to develop inhibitors with specificity for the mutated proteasomes. As the same mutations, such as A49T or A49V, have been observed in several independently derived **1** resistant cell lines as well as the **6**-producing bacterium *S. tropica*,[50] a second generation of PIs tuned specifically for these active site alterations could be developed. A library of PI analogues with various P1 residues, as has been established previously when developing inhibitors for the wild-type proteasome,[2] could be assayed in vitro against a 20S complex containing a mutated β5-subunit. Proteasome inhibitors specific for other catalytic subunits such as β1, β2, or the immunosubunits could potentially be used to circumvent β5-subunit mutations. As the cell lines with acquired **1** resistance displayed conflicting regulatory changes for immunoproteasome subunits, it is unclear if immunoproteasome specific inhibitors would be of use in combating bortezomib resistance.

Several allosteric effectors of proteasome activity that bind away from the active sites have recently been reported. PR-39 is a 39 amino acid peptide found to be a reversible inhibitor of the α7 subunit of the 20S proteasome and believed to interfere with 26S assembly from 19S and 20S components.[60] PR-39 was shown to induce angiogenesis in cell cultures and mice and to possess anti-inflammatory activity.[60,61] It additionally stimulated angiogenesis by increasing cellular HIF-1α protein levels via inhibition of ubiquitin-dependent proteasomal degradation.[61] Anti-inflammatory activity resulted from inhibition of IκBα degradation that prevents activation of NFκB-dependent gene expression, yet overall proteasomal protein degradation was not impaired.[60] While not druggable, PR-39 may serve as a lead for the development of proteasome assembly inhibitors. The antimalarial drug chloroquine was reported to inhibit both eukaryotic and archaeal 20S proteasomes.[62] NMR experiments identified chloroquine as uniquely binding between the α and β subunits. Binding distal from the active sites was confirmed by the simultaneous binding of the PI MG132. However, chloroquine is clinically irrelevant, as it only inhibits the proteasome at high micromolar concentrations. A screening of compounds with the chloroquine pharmacophore identified 5-amino-8-hydroxyquinoline (**7**, 5AHQ) as a more potent inhibitor of the 20S proteasome with an IC_{50} in the low micromolar to submicromolar range.[63] 5AHQ inhibited CT-L proteasome activity (T-L and C-L activities were not tested) in both intact cells and cellular extracts of various myeloma and leukemia cell lines. Oral administration in mice was shown to inhibit tumor growth, and cell death was also preferentially induced in cancerous cells. 5AHQ was found to act as a noncompetitive inhibitor of the α7 subunit in NMR experiments with the α_7–α_7 "half-proteasome". However, it has yet to be verified that **7** does not also bind to any β-subunits or if there are other cellular targets. 5AHQ shows promising activity in many bortezomib resistant cell lines resulting from β5-subunit mutation or overexpression,[18,20] and no resistance has been observed yet to **7**, which remains effective in bortezomib-resistant cell lines.[12,18]

Multiple strategies to treat PI resistant cancers by inhibition of alternative targets have been recently reported. As inhibition of the proteasome appears to combat cancer by decreasing proteolysis of various cellular proteins, this effect could also be achieved by inhibiting the upstream ubiquitinating enzymes E1, E2, and E3 or deubiquitinating enzymes. As the specificity of E1, E2, and E3 increases, so too does the enzyme diversity. Targeting the primary E1 enzyme should prevent proteasomal degradation of most cellular proteins and therefore have a similar effect to inhibition of the proteasome. For example, the E1 inhibitor 1-(3-chloro-4-fluorophenyl)-4-[(5-nitro-2-furyl)-methylene]-3,5-pyrazolidinedione (PYZD-4409) was recently shown to preferentially induce cell death in malignant leukemia cells and delay tumor growth in a murine leukemia model, achieving a similar affect as a PI with an alternative target.[23] Inhibiting a specific E3 could target individual cellular proteins, allowing for more controlled therapy. Disruption of the ubiquitination system with small molecule inhibitors is an active area of study and has been recently reviewed.[64]

■ SUMMARY AND OUTLOOK

The emergence of PIs over the past 10 years has been a major breakthrough in the treatment of hematological malignancies. However, both intrinsic and acquired PI resistances remain major obstacles. In an effort to better understand the mechanisms of acquired PI resistance, recent investigations of various cancer cell lines that were progressively desensitized to bortezomib revealed up-regulation of the proteasome at both the mRNA and protein level as well as mutations in the β5-subunit. The data revealed that changes in mRNA transcriptional levels do not necessarily correlate to changes in protein levels. Additionally, no clear quantitative link has been established between β5-subunit up-regulation and acquired resistance. The development of mutations in cell lines was observed in as little as a few months at clinically relevant concentrations of **1**. These mutations in the S1 binding pocket appear to form de novo and may also modulate proteolytic specificity as seen in the **6** resistant actinobacterial proteasome β-subunit SalI. Therefore, analysis of proteasome activity with the fluorogenic LLVY-amc substrate may underestimate the actual proteolytic activity. A shift in proteolytic specificity could allow the continued destruction of cellular proteins with a reduced susceptibility to the PI. However, caution should be used, as no mutations have yet been confirmed in patient

samples and more patient testing will be required to establish the clinical relevance of these cell line resistance mechanisms.

It is clear that overcoming the acquired resistance mechanisms reviewed here will require the development of PIs beyond β5-subunit inhibitors. As all PIs that are currently FDA approved or under investigation in clinical trials covalently bind the N-terminal threonine of the β5-subunit, all will likely be susceptible to β5-subunit up-regulation or mutation. However, inhibitors of proteasome assembly, allosteric effectors, or ubiquitination pathway enzymes will not be susceptible to the resistance mechanisms described here. The development of Ub pathway enzyme inhibitors will achieve the same effect as PIs, the disregulation of cellular protein destruction, with an alternative target. The development of E3 inhibitors will be especially useful, as they may pinpoint treatment to specific oncogenic proteins.

■ ASSOCIATED CONTENT

Supporting Information

Proteasome inhibitor cross-resistance and a comprehensive overview of PI resistant cell lines (two tables). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Bradley S. Moore is Professor of Oceanography and Pharmaceutical Sciences at the Scripps Institution of Oceanography and the Skaggs School of Pharmacy and Pharmaceutical Sciences at University of California—San Diego. He holds degrees in Chemistry from the University of Hawaii (B.S., 1988) and the University of Washington (Ph.D., 1994), was a postdoc at the University of Zurich, Switzerland (1994–1995), and held prior faculty appointments at the University of Washington (1996–1999) and University of Arizona (1999–2005). His research interests involve exploring and exploiting microbial genomes to discover new biosynthetic enzymes, secondary metabolic pathways, and natural products for drug discovery and development.

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■ ABBREVIATIONS USED

PI, proteasome inhibitor; Btz, bortezomib; Ub, ubiquitin; CT-L, chymotrypsin-like; T-L, trypsin-like; C-L, caspase-like; MM, multiple myeloma; MCL, mantle cell lymphoma; ALLN, Ac-Leu-Leu-Nle-al; MDR, multidrug resistance; Pgp, P-glycoprotein; LLVY-amc, Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; VKM-amc, Z-Val-Lys-Met-7-amino-4-methylcoumarin

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