

Chapter 2

Resistance to Proteasome Inhibitors in Multiple Myeloma

Francesca Cottini, Anna Guidetti, Claudia Paba Prada, Teru Hideshima, Michelle Maglio, Cindy Varga, Dharminder Chauhan, Jacob Laubach, Kenneth C. Anderson, and Paul G. Richardson

Abstract Multiple myeloma (MM) is a clonal proliferation of malignant plasma cells in the bone marrow associated with a spectrum of clinical symptoms including bone destruction, anemia, hypercalcemia, and renal failure. Although MM remains incurable, a dramatic paradigm shift in the treatment of MM has occurred over the past decade through the introduction of novel agents, including the development of small molecule inhibitors targeting the proteasome. Among the proteasome inhibitors (PIs), bortezomib (BTZ) and carfilzomib (CFZ) have been approved by the FDA for treatment of relapsed/refractory MM in 2003 and 2012, respectively. Recently, other PIs, such as ixazomib (MLN-9708), oprozomib (ONX0912), and marizomib (NPI-0052), have been under evaluation in preclinical and clinical studies. Indeed, it is now well known that malignant plasma cells are exquisitely sensitive to proteasome inhibitors due to protein overload and ER stress. Unfortunately, relapse of myeloma develops due to acquisition of resistance to proteasome inhibitors. Specifically, mutations in overexpression of proteins belonging to the proteasome complex, upregulation of transporter channels or cytochrome components, induction of alternative compensative mechanisms such as the aggresome pathway, and modulation of downstream pathways have been all reported as possible mechanisms of

Authors contributed equally.

F. Cottini, M.D. • C.P. Prada, M.D. • T. Hideshima, M.D. Ph.D. • M. Maglio • C. Varga, M.D. D. Chauhan, J.D. Ph.D. • J. Laubach, M.D. • K.C. Anderson, M.D. • P.G. Richardson, M.D. (✉)
Jerome Lipper Multiple Myeloma Center, Division of Hematologic Malignancy,
Department of Medical Oncology, Dana-Farber Cancer Institute,
450 Brookline Avenue, Boston, MA, USA
e-mail: francesca_cottini@dfci.harvard.edu; claudiap_prada@dfci.harvard.edu;
teru_hideshima@dfci.harvard.edu; michelle_maglio@dfci.harvard.edu;
cindy_varga@dfci.harvard.edu; dharminder_chauhan@dfci.harvard.edu;
jacobp_laubach@dfci.harvard.edu; kenneth_anderson@dfci.harvard.edu;
paul_richardson@dfci.harvard.edu

A. Guidetti, M.D.
Università degli Studi di Milano, Milan, Italy
e-mail: anna.guidetti@istitutotumori.mi.it

proteasome inhibitor resistance. In this chapter, we will first briefly describe the structure and function of the proteasome in normal and malignant plasma cells, and then define the major mechanisms of resistance to proteasome inhibition, and clinical approaches to overcoming these pathways in the context of both clinical application of PIs and rational combinations of them with other agents in the treatment of MM.

Keywords Proteasome inhibitor • Carfilzomib • Bortezomib • Drug resistance • Multiple myeloma

Abbreviations

BTZ	Bortezomib
CFZ	Carfilzomib
C-L	Caspase-like
CT-L	Chymotrypsin-like
CR	Complete response
HDAC	Histone deacetylase
HDACIs	HDAC inhibitors
Ig	Immunoglobulin
MM	Multiple myeloma
MTD	Maximum-tolerated dose
OS	Overall survival
PI	Proteasome inhibitor
T-L	Trypsin-like
TTP	Time to progression

2.1 Introduction

Multiple myeloma (MM) is characterized by clonal expansion of malignant plasma cells with several genomic alterations. Plasma cells differentiate from B cells, which have limited immunoglobulin (Ig) synthesis and secretory capacity. Conversely, plasma cells have increased endoplasmic reticulum (ER) content, components for protein synthesis and quality control, and secretory pathways to synthesize and secrete Ig. Malignant plasma cells secrete even higher quantities of Ig, and are hence particularly sensitive to the detrimental effects associated with accumulation of incompletely or improperly folded proteins in the ER, relying on proteasome complexes to avoid protein overload [1–4].

Proteasomes (proteasome 26S), their protein structures conserved in both prokaryotic and eukaryotic organisms, are composed of a 20S core which binds one or two 19S regulatory particles [5]. The 20S core is a barrel-like structure composed of stacked heptameric rings, each of which consists of seven related, yet distinct,

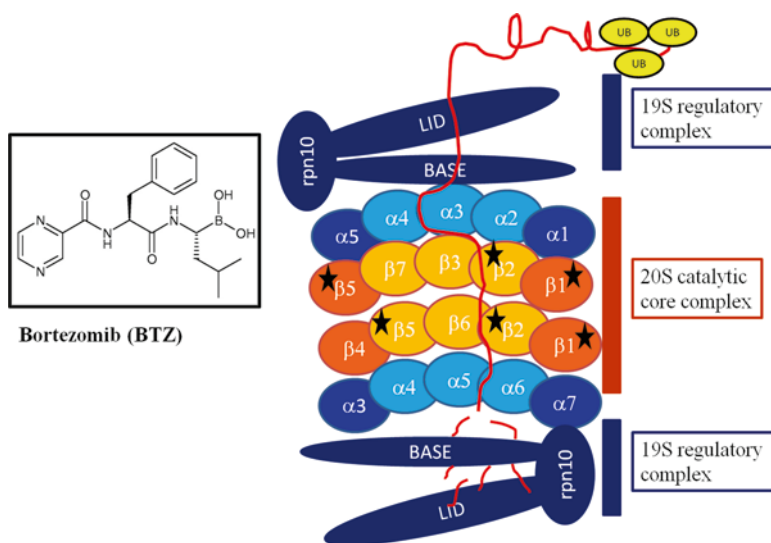


Fig. 2.1 Representative scheme of proteasome structure. 20S catalytic core and 19S regulatory complex are shown. Bortezomib (BTZ) and other proteasome inhibitors (PIs) bind to specific catalytic subunits

subunits ($\alpha 1$ – $\alpha 7$ and $\beta 1$ – $\beta 7$) organized in a fixed topological arrangement within each ring. Only a subset of β -subunits is catalytically active, and each of them preferentially cuts specific residues, such as basic, acidic, or hydrophobic amino acids. In particular, the chymotrypsin-like (CT-L), caspase-like (C-L), and trypsin-like (T-L) catalytic activities of the 20S proteasome are encoded by $\beta 5$ -, $\beta 1$ -, and $\beta 2$ -subunits, respectively. The 19S particles represent the lid or gate of the barrel and are important for substrate recognition, deubiquitination, unfolding, and translocation into the core particle. Indeed, protein degradation can occur only if the selected protein is recognized by the regulatory proteins, traverses the regulatory gate (19S structures, which bind to a ring and cause gate opening), and interacts with the catalytically active proteolytic enzymes of the core (Fig. 2.1). Additionally, in response to different biological stimuli such as interferon gamma and tumor necrosis factor-alpha, specific β -subunits can associate with a particular 11S regulatory particle, forming the so-called immunoproteasome (i20S). In the immunoproteasome, LMP7, LMP2, and MECL1, proteins represent the catalytically active subunits. 26S and i20S proteasomes are both present in MM cells: i20S is predominant in MM at diagnosis, while lower levels of i20S and increased levels of 26S proteasome are present in relapsed myelomas. Drugs targeting proteasomes are both reversible (BTZ, MLN9074, and CEP-18770) and irreversible competitive (CFZ, ONX 0912, and NPI-0052) inhibitors which differentially bind to proteasome catalytic subunits. Specifically, BTZ, MLN9074, and CEP-18770 are potent inhibitors of the C-L $\beta 5$ subunits (PSMB5), NPI-0052 is a potent inhibitor of the T-L $\beta 1$ -subunits, whereas CFZ selectively inhibits both CT-L active sites and LMP7 subunits.

2.2 Preclinical Studies on Mechanisms of Resistance to BTZ

2.2.1 Genetic Abnormalities in Proteasome Subunits

PIs induce cytotoxicity in MM cells by multiple mechanisms including: induction of terminal ER stress and unfolded protein response (UPR), activation of c-Jun NH2 terminal kinase (JNK), inhibition of nuclear factor kappa B pathway, and induction of reactive oxygen species (ROS). One of the first mechanisms of proteasome resistance is the presence of mutations or altered and/or aberrant expression of proteasome subunits, which disrupt the ability of BTZ to bind and inhibit proteasomes. The first demonstration of this mechanism of BTZ resistance was in leukemia cells [6], and the results were later applied and confirmed in MM cells. Specifically, Oerlemans and colleagues generated BTZ-resistant human myelomonocytic THP1 cells by continuous culture with stepwise increasing concentrations (2.5–200 nM) of BTZ [6]. In this study, analysis of mRNA and protein expression of various proteasome subunits showed markedly increased (up to 60-fold) protein levels of $\beta 5$ -subunit (also known as PSMB5) and only modest (<2-fold) upregulation of $\beta 1$ - and $\beta 2$ -subunits. No increased mRNA was observed in PSMB5, indicating that posttranscriptional mechanisms were responsible for increased protein expression. Of note, upregulation of PSMB5 protein expression induced by BTZ treatment was reversible. Importantly, no additional alterations in expression of genes encoding ubiquitin-conjugating enzymes, ubiquitin-specific proteases, or ubiquitin C-terminal hydrolases were recognized.

In addition to PSMB5 overexpression, a point mutation in the β -subunit of BTZ-binding pocket (G322A mutant), introducing an Ala to Thr substitution at amino acid 49, was also reported [6]. This mutation has also been described in other cellular systems, such as BTZ-resistant Jurkat human lymphoblastic T cells by Lu and colleagues [7, 8]. Lu and colleagues also identified mutations in C323T (Ala49Val) alone or C326T in combination with G322A (Ala49Thr and Ala50Val) [8]. Ri and colleagues were the first to evaluate the mechanisms of BTZ resistance in myeloma by creating two BTZ-resistant cell lines (KMS-11 and OPM-2 cells). These BTZ-resistant cells were less prone to activate apoptotic pathways than the parental sensitive cells. Moreover, the BTZ-resistant cells have reduced amounts of polyubiquitinated proteins, thereby failing to trigger the UPR and induce CHOP and NOXA expression upon BTZ treatment [9]. These resistant-clones have a point mutation (G322A) in the PSMB5 gene as well. Balsas and colleagues generated another BTZ-resistant MM cell line (RPMI-8226/7B) [10]. Interestingly, these cells were morphologically larger than the parental cells, with about twofold DNA content per cell and overexpressed PSMB5 at both mRNA and protein levels; however, no mutation was recognized. Franke and colleagues also generated BTZ resistance in RPMI-8226 (MM) and CEM (acute lymphoblastic leukemia) cell lines. As expected, these cells show resistance to different types of PIs [11]. Consistent with previous studies, these cell lines upregulate PSMB5 gene together with other constitutive catalytic β -subunits, as well as the non-catalytic $\alpha 7$ subunit. Moreover, BTZ-resistant RPMI-8226 cells have a near-complete shift from the $\beta 5$ i

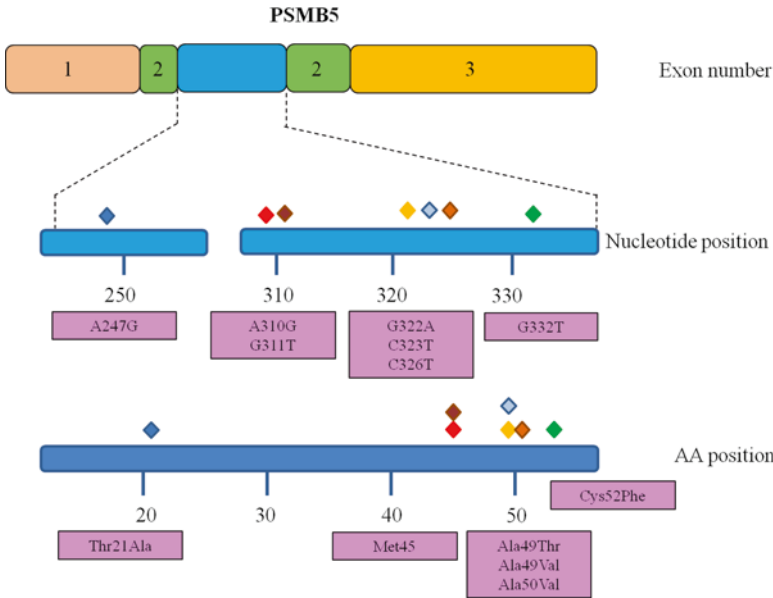


Fig. 2.2 Scheme of PSMB5 mutations identified in BTZ-resistant MM cell lines. Exons 1–3 create the S1-specific pocket of the $\beta 5$ -subunit and are the site of the most relevant mutations, which confer resistance to BTZ treatment. Both nucleotide- and amino acid-affected residues are shown

(immunoproteasome) subunit to the constitutive $\beta 5$ -subunit. To define the biologic relevance of PSMB5 mutations, they utilized a crystal structure and computer modeling system to characterize the physiological interactions between BTZ and the proteasome, showing that mutations involving PSMB5 create a functional protein. However, these mutations cluster together around the BTZ-binding pocket of proteasome, specifically around S1-specific pocket of the $\beta 5$ -subunit which recognizes the peptide bond of the substrate to be degraded [12, 13] (Fig. 2.2). In the S1 pocket, Ala49 and Ala50 residues are key positions for efficient binding of BTZ to the $\beta 5$ -subunit, and Ala49Val substitution in $\beta 5$ -subunit therefore hinders BTZ accessibility to the S1 pocket. They also identified additional mutations: G332T leading to Cys52Phe substitution, A247G to create Thr21Ala substitution, and G311T/A310G which changes Met45 residue. Thr21Ala substitution also decreases affinity of BTZ binding to the $\beta 5$ -subunit due to a loss of protein-ligand hydrogen bond. Met45 and Cys52 are not directly involved in BTZ-binding; however, Cys52Phe mutation leads to a slight repulsion of BTZ from the S1 pocket. Met45 also confers S1 pocket specificity and conformational change after BTZ binding [14]. Thus, all these mutations can alter the binding pocket's specificity and flexibility. Altogether, these studies in different cell types demonstrate two important findings that associate with PI resistance: first, the discovery of acquired mutations which decrease the affinity of the $\beta 5$ -binding pocket to proteasome inhibitors and second, compensatory upregulation of other proteasome subunits.

Although a number of in vitro studies of PI resistance have shown PSMB5 over-expression or mutations, no clinical data has identified these mechanisms in the subset of BTZ-resistant and/or refractory patients MM cells. Indeed, mutations in PSMB5 have not yet been identified by whole-genome sequencing of samples from patients with either newly diagnosed patients or relapsed MM [15]. Recently, another study tried to define more specifically the frequency and clinical relevance of PSMB5. In this study, DNA samples were isolated from MM patients ($n=16$) before and after BTZ treatment [16]. Ten patients were relatively resistant (minimal response, stable disease, or progressive disease) to BTZ monotherapy; six patients achieved PR and then subsequently relapsed. PSMB5 mutant variants were not detected in any case. Although the number of patients enrolled was small, these results suggest that clinical resistance to BTZ in patients with relapsed MM may not solely be due to PSMB5 mutations, which to date are shown only in cell lines.

Several single nucleotide polymorphisms (SNP) in the *PSMB5* gene have also been reported; however, they do not alter activity, but modulate transcription, of the proteasome [17]. Licther and colleagues identified three significant associations between SNP allelic frequencies (SNPs PSMB6 rs2304975, PSMB6 rs3169950, and PSMB9 rs241419) and overall survival (OS) or time to progression (TTP) of disease in patients treated with BTZ [16]. However, two of these variants were present only in 3–5 % patients, suggesting limited clinical relevance. In another small study, Shuqing and colleagues evaluated PSMB5 mRNA levels and DNA PSMB5 point mutations in MM cells from three patients whose MM was refractory to treatment with BADT regimen (BTZ, epirubicin, dexamethasone, and thalidomide) and normal bone marrow mononuclear cells from a healthy volunteer [18]. No mutations were present. Another single-case report from Politou and colleagues also failed to show PSMB5 mutations in one resistant patient [19].

The ubiquitin-proteasome pathway for protein degradation requires several enzymes, which could be altered and thereby mediate BTZ resistance. For example, upregulation of POMP (proteasome maturation protein) can promote BTZ resistance [20]. POMP plays a role in adding catalytically active β -subunits to the hemi-proteasome ring, which is initially composed of α -subunits. Li and colleagues identified higher mRNA level of POMP and nuclear factor erythroid-derived-2-like 2, its transcriptional regulator, in BTZ-resistant cells than in parental cells, thereby inducing higher chymotrypsin-like activity.

2.2.2 Alternative Pathways to Modulate Proteasome Activity

Another possible mechanism for acquired BTZ resistance is activation of alternative protein lysis/degradation systems, including the aggresome pathway, to degrade excessive or misfolded proteins [21]. Aggresomes or aggresomal particles form when nascent peptides do not fold correctly and create small protein aggregates in response to the presence of misfolded proteins. These structures are then transported towards the microtubule (MT) organizing center (MTOC), where they are

sequestered into a bin-like structure called the aggresome. Acetylation of α -tubulin, which is reversed by histone deacetylase 6 (HDAC6), a class IIb HDAC, modulates the structure and function of MT, thereby playing a crucial role in autophagic (or lysosomal) lysis of protein aggregates [22]. Hideshima and colleagues showed that combination strategies directed against HDAC6 and proteasome activities, using tubacin and BTZ, respectively, significantly augment accumulation of polyubiquitinated proteins, followed by cell stress and cytotoxicity [23]. Catley et al. showed that BTZ strongly induces aggresome formation. A synergistic cytotoxic effect can be achieved when BTZ is combined with LBH589, a nonselective deacetylase inhibitor [24]. Interestingly, some cell lines were more responsive to LBH589 than BTZ or vice versa, suggesting that cell lines and perhaps patients can predominantly activate one of the two pathways.

Heat shock proteins (HSPs) are molecular chaperones presented which are rapidly upregulated when cells are exposed to a stress condition, such as treatments with chemotherapeutic agents. They play significant roles in accommodation of cells to stress conditions, including endoplasmic reticulum (ER) stress that is triggered by accumulation of unfolded proteins. Shringarpure and colleagues compared gene expression profiling in BTZ-resistant (SUDHL-4) and BTZ-sensitive (SUDHL-6) diffuse large B cell lymphoma cell lines, and identified overexpression of HSPs (i.e., Hsp27, Hsp70, Hsp90) in BTZ-resistant cells [25]. Subsequently, Chauhan and colleagues showed that silencing Hsp27 in BTZ-resistant SUDHL4 cells restores sensitivity to BTZ; conversely, overexpression of Hsp27 induces BTZ resistance in BTZ-sensitive SUDHL6 cells [26]. Although the mechanism of action of Hsp27 mediating BTZ resistance has not yet been fully delineated, one possible explanation is its antiapoptotic activity due to inhibition of mitochondrial apoptotic signaling. Specifically, Hsp27 can inhibit release of cytochrome-c/Smac by modulating the integrity of the actin network responsible for controlling translocation of proapoptotic factors from the actin cytoskeleton to mitochondria. Moreover, Hsp27 can also modulate autophagic cell death through ER stress [27]. Taken together, Hsp27 can modulate autophagy and ER stress triggered by BTZ [28], thereby, promoting survival in BTZ-treated cells. Most recently, it has been shown that nicotinamide phosphoribosyltransferase (Nampt) regulates NAD, and that its inhibition depletes intracellular NAD⁺ level leading to autophagic cell death [29]. Moreover, higher Nampt mRNA levels are correlated with BTZ resistance in patient MM cells. Importantly, combining the NAD⁺-depleting agent FK866 with BTZ induces synergistic MM cell death and overcomes BTZ resistance [30].

2.2.3 Cell Dedifferentiation

One of the major steps in plasma cell differentiation from B lymphocytes is linked to the activation of control mechanisms for protein folding which permit immunoglobulin synthesis and secretion. This response is mainly orchestrated by the activation of BLIMP1 transcription factor and the blockade of PAX5 (also known as

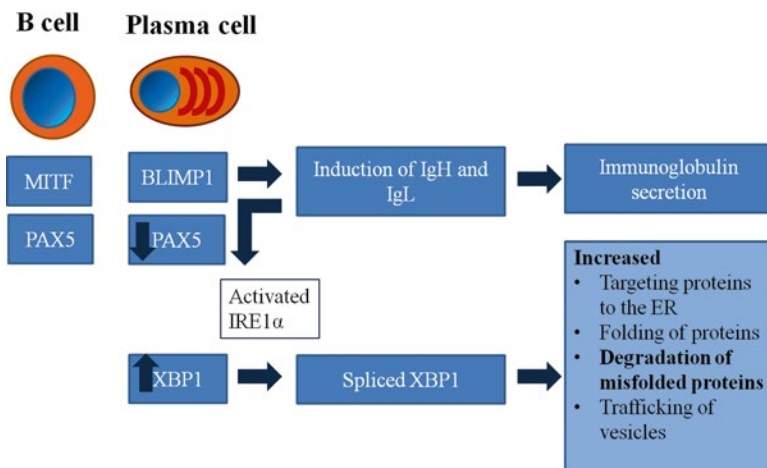


Fig. 2.3 Transition from B cell lymphocytes and plasma cells. MITF (microphthalmia-associated transcription factor) and PAX5 (paired box protein 5) transcription factors normally block plasma cell differentiation by repressing BLIMP1 (B lymphocyte-induced maturation protein 1). When PAX5 expression is downregulated, BLIMP1 represses B cell gene-expression programs and BLIMP1, XBP1 (X-box-binding protein 1), and IRF4 (interferon-regulatory factor 4) induce the expression of genes that are required for plasma cells. Specifically, there is an increase in immunoglobulin secretion and also in the control, folding, and degradation of misfolded proteins in plasma cells

BSAP, B cell-specific activator protein), thereby leading to X-box-binding protein (XBP1) transcription factor splicing and increased protein quality control (Fig. 2.3). Ling and colleagues observed that cell lines and primary MM cells which expressed low levels of XBP1 were more resistant to BTZ treatment, associated with lower levels of immunoglobulin secretion [31]. This effect was partially overcome by further inducing an UPR using alkylating agents or tunicamycin, a drug which creates ER stress by inhibiting the glycosylation of nascent proteins. Therefore, XBP1 levels can be used as a possible biomarker of response to BTZ treatment. In other study, Leung-Hagesteijn and colleagues hypothesized that BTZ resistance relies on the capabilities of MM cells to become less dependent to ER-stress control and dedifferentiate from plasma cells back to B lymphocytes [32]. In particular, they demonstrated that silencing Ire1 or XBP1, but not Atf6 or Perk (other two ER stress sensor proteins), affects response to BTZ treatment. They confirmed that XBP1 levels correlate with clinical BTZ response: patients with progressive disease express lower levels of XBP1 target genes compared to patients with complete response. Moreover, they also suggested the importance of XBP1 mutations in BTZ resistance. Indeed, whole-genome sequencing has identified two mutations in samples from patients with relapsed MM. These mutations are considered to be inactivating mutations, which inhibit correct splicing and/or transcriptional activity of XBP1 [15]. Indeed, when plasmids containing mutant XBP1 were reintroduced into MM cells with silenced XBP1, these cells were not capable of overcoming the BTZ-resistant phenotypes. Even though the most intuitive role of Xbp1 in BTZ resistance

was hyperactivation of PERK/ATF6 signaling as a compensatory mechanism, they observed that malignant plasma cells acquired a less-differentiated phenotype, characterized by decreased expression of CD138, CD38, and IL6R; upregulation of PAX5; and downregulation of heavy and light chains of Ig. These alterations lessen the high ER front loading of Ig, and so the attendant risk of harmful ER stress response. These data were shown in cell lines and confirmed in patients, since BTZ-sensitive tumors have a high proportion of mature plasma cells, while BTZ-resistant tumors contain subpopulations of plasma cell progenitor cells expressing low or absent levels of CD138, CD38, and XBP1. Moreover, when minimal residual disease was evaluated in bone marrow biopsies, patients in complete response (CR) still harbored a small subpopulation of less mature plasma cells, which might represent the principal source of relapse in responsive patients. Of note, they did not observe PSMB5 mutations or any correlation of PI resistance with expression levels of proteasome subunits. This is in contrast to in vitro models of PI resistance, where the majority of proteasome subunits including PSMB5 were upregulated. Therefore, they concluded that Ire1-XBP1 pathway is crucial to induce BTZ cytotoxicity. The Xbp-1 pathway can be shut down in malignant plasma cells without strongly affecting their survival, but reducing their dependency on protein control. A similar phenotypic change was also observed by Stessman et al. [33]. Comparing BTZ-sensitive and BTZ-resistant cells derived from tumors of the Bcl-XL/Myc mouse model of plasma cell malignancy, they identified a reduction of the levels of CD93 (a plasma cell maturation marker) and CD69 (a plasma cell activation marker) in cells with acquired PI resistance, as well as in subclones after 48 h BTZ treatment. They then evaluated CD93 mRNA expression in patient samples based on CD93 high or low expression and showed that CD93 levels correlate with better overall survival after BTZ treatment in the APEX trial. CD93, a marker of mature plasma cells in humans, also positively associates with BLIMP1 expression in MM patient samples. Thus, they proposed CD93 as a biomarker for BTZ sensitivity, together with XBP1. Hence, the loss of maturation markers is a strategy used by myeloma cells to escape BTZ-mediated apoptosis via reducing the UPR.

2.2.4 Metabolism and Drug Efflux

Another common mechanism involved in drug resistance consists in either upregulation of channel proteins/transporters which mediate expulsion of drug from the cells, or overexpression of catabolic proteins leading to rapid drug degradation. For example, overexpression of MDR-1 represents a critical mechanism of drug resistance in cancer. Rumpold and colleagues showed that both BTZ and MLN273 can be substrates of MDR-1, evidenced by sensitization of MDR-1-overexpressed K562/Dox cells to BTZ by knockdown of MDR-1 [34]. Other groups have also showed the association of activity of proteasome inhibitor with ATP-binding cassette transporter-mediated efflux expression [35]. Moreover, lymphoid CEM/VLB cells with Pgp-1 overexpression were more resistant than parental cells to

carfilzomib (114-fold), ONX-0912 (23-fold), and ONX0914 (162-fold), as well as to BTZ; conversely, a Pgp transport inhibitor P121 (reversin 121) was able to restore proteasome activity. These results therefore confirm that expression of MDR-1 can also modulate sensitivity to BTZ treatment. In addition, differential expression of genes regulating multidrug resistance and drug metabolism in BTZ-sensitive versus BTZ-resistant DLBCL cell lines was also evaluated [25]. Specifically, two genes were overexpressed in BTZ-resistant SUDHL-4 cells: ATP-binding cassette (ABC) subfamily A member 1 (ABCA1) and cytochrome P450. However, their mechanism of actions and relevance in cellular entry or metabolism of BTZ have not yet been fully elucidated [25].

Another group identified a correlation between the active Notch pathway via Dll1/Notch2 receptors and BTZ resistance [36]. Indeed, Notch upregulates CYP1A1, a cytochrome P450 enzyme involved in drug metabolism; conversely, downregulation and inhibition of CYP1A1 restore sensitivity to BTZ treatment [36]. A gamma secretase inhibitor DAPT sensitizes the cells to BTZ treatment both in vitro and in vivo in murine human MM cell xenograft models.

2.2.5 Signaling Pathways Mediating MM Cell Survival and Drug Resistance

Constitutive activation or hyperactivation due to genetic abnormalities in signaling cascades, including NF- κ B, β -catenin, IGFR, c-MET, MAF, and AKT, can also modulate sensitivity to BTZ (Fig. 2.4). Indeed, NF- κ B is constitutively activated in primary patient MM cells and in MM cell lines, and its activity is further increased in response to soluble factors including IL-6, IGF-1, TNF- α , IL-1 β , BAFF, SDF-1 α , and APRIL in the bone marrow microenvironment. Although, the NF- κ B pathway is involved in MM pathogenesis, its role in the context of sensitivity to BTZ is still controversial. Several molecules including I κ B kinase (IKK) complex trigger phosphorylation of inhibitor protein I κ B α , followed by its ubiquitination and subsequent degradation via the 26S proteasome. Degradation of I κ B α allows the NF- κ B complex to translocate to the nucleus. Importantly, I κ B α can also be degraded in a proteasome-independent manner (PIR, proteasome inhibitor resistant), relying on calcium, calmodulin, and L-type calcium channels [37]. Interestingly, BTZ triggers NF- κ B activation in MM cell lines and primary MM cells by proteasome-independent downregulation of I κ B α associated with IKK β activation, which enhances BTZ-induced cytotoxicity [38]. Two studies pointed out that primary tumor cells from MM and mantle cell lymphoma patients have relatively high constitutive NF- κ B activation due to genetic abnormalities or microenvironment modulation, and that these cells are largely resistant to BTZ [39, 40]. Indeed, as also reported by Hideshima and colleagues, they observed that a significant fraction of patient MM cells treated with BTZ do not downregulate constitutive NF- κ B activity, although BTZ effectively blocks proteasome activity. Even though the Markovina study suggests that BTZ resistance is mediated by NF- κ B activation, data from our group showed that BTZ-treated MM cells with further induction of NF- κ B activity nonetheless

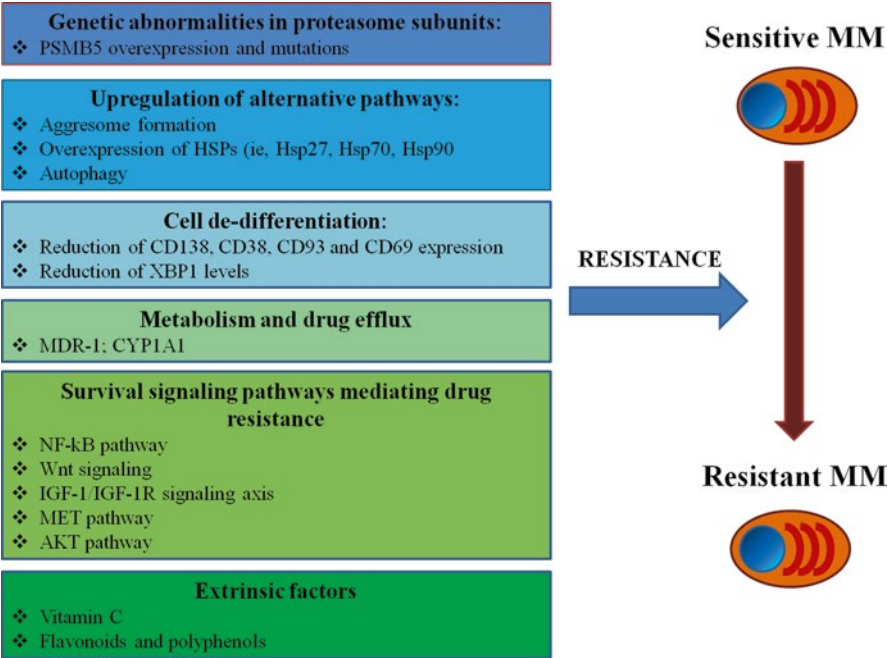


Fig. 2.4 Summary showing the state of the art in proteasome inhibitor resistance

remained responsive to BTZ treatment [38]. Hence, it remains controversial as to whether NF- κ B activation represents a resistance mechanism or a compensatory strategy which only minimally affects cytotoxicity.

Myeloma cell lines with higher β -catenin levels are also resistant to BTZ treatment [41]. These cells have higher expression of TCF-4 transcription factor, which is a central player in Wnt signaling, regulating MM-relevant target genes including cyclin D1 and c-Myc, among others. IGF-1 pathway could also play a role on BTZ resistance. Kuhn and colleagues generated BTZ-resistant cells using RPMI8226, OPM-2, ANBL-6, and KAS-6/1 cell lines [42]. They showed that the IGF-1/IGF-1R signaling axis was the most deregulated in all BTZ-resistant cell lines, according to gene set enrichment analyses. Specifically, both the levels of soluble IGF-1 in cell culture supernatants, the levels of intracellular and membrane-bound IGF-1, as well as IGF-1R activity were increased in BTZ-resistant cells. Interestingly, AKT mRNA level was also higher in resistant than parental cells. Similar findings on the role of AKT in BTZ resistance was reported by Que and colleagues [43]. Moreover, they discovered that MET silencing was capable of increasing BTZ sensitivity, even though the mechanism was not defined. Indeed, c-MET has been implicated in proliferation, migration, and invasion of MM cells, and its overexpression correlates with short overall survival in MM patients. Additionally, the proto-oncogene MAF has also been associated with BTZ resistance, since both MM cell lines and patients with high MAF levels have lower response rates to BTZ treatment [44].

2.2.6 *Extrinsic Factors*

Drugs and vitamin supplements can potentially modulate BTZ metabolism and activity, hence reducing treatment responses. Specifically, vitamins having antioxidative effects, including vitamin C and E, are often taken by cancer patients. Previous reports show that BTZ induces production of ROS, which contributes to BTZ-induced cytotoxicity [45–48]. Interestingly, vitamin C (ascorbic acid) is capable of inhibiting BTZ-mediated cytotoxicity, while *N*-acetylcysteine and vitamin E are not [49]. Indeed given its chemical structure, a boronic acid class proteasome inhibitor BTZ can bind molecules with diol functional groups, including vitamin C. This binding causes the formation of an inactive form, thus reducing BTZ activity in MM cell lines [49]. Vitamin C effects on BTZ activity were also evaluated *in vivo*. Perrone and colleagues showed that plasma isolated from healthy volunteers taking increasing doses of vitamin C supplements, when added in cultured MM cells, can reduce BTZ cytotoxicity [50]. Moreover, when combination treatment of vitamin C with BTZ was evaluated in a murine xenograft model of human MM, antitumor activity of BTZ was significantly reduced [50]. Based on these results, vitamin C supplements should be avoided in patients who are receiving BTZ, or at least should be taken at low doses at least 12 h before BTZ treatment. A similar phenotype was observed in chronic lymphocytic leukemia cells treated with BTZ in the presence of quercetin or other dietary flavonoids (myricetin, kaempferol, and apigenin), which are commonly included in vegetables, fruits, and green tea. [51] Kim and colleagues screened a group of polyphenol compounds (catechin, epicatechin, gallic acid) which are present in cocoa, red wine, vegetables, and black/green tea, and showed that these agents block BTZ activity as well. Another group also showed inhibition of BTZ activity by green tea polyphenols [52]. Taken together, these results strongly recommend avoiding the intake of herbal supplements or foods containing polyphenols in large quantities during drug peaks or before clearance of BTZ.

2.2.7 *Mechanisms to Sensitize Cells to BTZ Cytotoxicity*

BTZ works predominantly by blocking degradation of excessive or misfolded proteins, leading to lethal ER stress. A similar phenotype can be observed by inhibition of deubiquitinating enzymes (DUBs) or ubiquitinating enzymes (E1, E2, and E3). DUBs are a family of proteins which are often dysregulated in cancers, promoting stabilization of oncogenic proteins. Specifically, ubiquitin-specific protease 7 (USP7) regulates important biological signaling pathways in tumorigenesis, including FOXO4 and PTEN [53] as well as HDM2 and HDMX, resulting in the destabilization of p53 and its transactivation activity [54]. Of note, USP7 is markedly elevated in MM cells, and its expression levels inversely correlate with overall survival in MM patients. A small molecule USP7 inhibitor P5091 triggers MM cell toxicity both *in vitro* and *in vivo* in a murine human MM cell xenograft model.

Importantly, P5091 induces cytotoxicity even in BTZ-resistant ANBL-6.BR cells. USP9X is another DUB highly expressed in MM, follicular lymphoma, and diffuse large B cell lymphoma cells, which regulates Mcl-1 stabilization. Mcl-1 is a well-known antiapoptotic protein, and small molecule inhibitors against Mcl-1, including WP1130 and PR-619, can enhance BTZ-induced cytotoxicity. E1/E2/E3 ubiquitin-modifying enzymes represent a diverse group of proteins with significant roles in ubiquitin conjugation [55]. The E1 ubiquitin-activating enzyme is generally not target-specific and therefore can broadly affect protein degradation, in a similar fashion to the proteasome. E3 ligases are more selective and identify recognition signals on target proteins; for example, HMDM2 is crucial to control p53 levels, and is often overexpressed or amplified in cancers, including myeloma. Nutlin-3a is a potent non-peptide HDM2 antagonist which blocks the interaction of p53 with HDM2 and stabilizes p53 and p21. In preclinical studies, Nutlin-3a induces additive cytotoxicity with BTZ [56]. Analogues of Nutlin-3a, such as R7112, RITA, or HLI98, are currently under evaluation in preclinical models and clinical trials in patients. Silencing of E1 enzyme also results in increased cell death in MM cells. Compounds such as PYR-41 and PYZD-4409 can behave as E1 inhibitors, reducing protein ubiquitylation and sumoylation, inducing signs of ER stress, and enhancing BTZ-mediated apoptosis [57]. Finally, cereblon (CRBN) is another protein which forms an E3 ligase complex with damaged DNA-binding protein 1 (DDB1) and Cul4A and is considered a target of thalidomide and lenalidomide, suggesting one possible explanation for the synergistic MM cytotoxicity observed when immunomodulatory drugs are combined with BTZ treatment.

Other strategies can also be applied to sensitize cells to BTZ. In particular, recent studies identified a relationship between drug activity and mitochondrial or iron homeostasis. Specifically, Song et al. evaluated the role of mitochondria in BTZ resistance. Specifically, they compared indices of mitochondrial function including oxygen consumption rates and ATP and Ca^{2+} concentrations, membrane potentials (Ψ_m), and depolarization of $\Delta\Psi_m$, which trigger mitochondrial pore opening and apoptosis, in three MM cell lines, one with intrinsic resistance to BTZ treatment (KMS-20) and two which are sensitive (KMS-26 and KMS-28BM) to BTZ treatment [58]. They identified a higher stability of mitochondrial membrane potential ($\Delta\Psi_m$) and a more modest increase in mitochondrial calcium response in BTZ-resistant cells (KMS-20) in comparison to sensitive cells. These studies, indicating that BTZ-resistant cells can better control the mitochondrial Ca^{2+} pool and hence minimize BTZ-induced Ca^{2+} overload and related induction of apoptosis. Moreover, the levels of superoxide anion or ROS were lower in KMS-20 cells, suggesting that ROS induction can represent another modality through which BTZ triggers cell death. To explain this difference, several mitochondrial genes related to maintenance of $\Delta\Psi_m$, elimination of ROS, and Ca^{2+} influx into mitochondria were evaluated. For example, SOD2 (an antioxidant protein that nullifies ROS toxicity) and pyruvate dehydrogenase (PDH)-E1 α protein levels were decreased and MCU expression was increased in BTZ-sensitive cells, while CYPD, a regulator of mitochondrial permeability transition, was decreased in BTZ-resistant cells. The expression levels of the proteins mentioned above, apart from MCU (further induced by

BTZ), were not changed by BTZ, suggesting an intrinsic mechanism of resistance. Therefore, the resistance of KMS20 cells can be related to a reduced expression of CYPD, causing modifications in mitochondrial membrane potentials and a higher capacity of ROS elimination via SOD2, compared with sensitive cells. Indeed, SOD2 depletion induced cell death in KMS20 cells; and the combination of BTZ with 2-methoxyestradiol, a known SOD inhibitor, was capable of overcoming BTZ resistance in KMS-20 [59]. A similar phenotype was also observed when BTZ was combined with FCCP, which induces dissipation of $\Delta\Psi_m$. Additionally, combining BTZ with PK-1195, an antagonist to mitochondrial peripheral benzodiazepine receptors which causes loss of $\Delta\Psi_m$, generates superoxides and favors release of cytochrome-c and Smac, thereby prompting cytotoxicity even in resistant cells [60]. These data indicate that mitochondrial activity and differential expression of mitochondrial genes can be responsible for intrinsic resistance to BTZ, suggesting that some of these genes can be used to identify chemotherapeutic regimens capable of sensitizing BTZ activity.

Another mechanism to induce sensitivity to BTZ relies on the modulation of ROS by iron [61]. Indeed, the proteasome is crucial for iron homeostasis, since it mediates the degradation of iron regulatory proteins (IRP1 and IRP2) and ferritin [62–64]. Campanella and colleagues showed that BTZ abolishes the coordinated upregulation of FtH ferritin subunits and the reduction of transferrin receptor by decreasing its turnover. Interestingly, ferritin levels also positively correlated with BTZ resistance. Specifically, BTZ-resistant cells have higher levels of iron stored with ferritin to limit Fenton reaction and oxidative damage. Indeed, silencing of FtH increases sensitivity to BTZ, in comparison with control cells. Hence, interfering with iron homeostasis emerges as a potential novel synthetic lethality strategy to enhance proteasomal inhibitory effects specific for MM cells, since iron itself has no cytotoxic effects in the absence of BTZ. Approaches to overcome BTZ resistance by modulating iron in patients should focus mainly on finding strategies to selectively reduce ferritin levels in malignant plasma cells, or to specifically deliver iron supplements to cancer cells, using hepcidin antagonists or other inflammatory modulators, to relieve iron sequestration from macrophages.

2.3 Clinical Applications with a Focus on Proteasome Inhibitor-Based Combination Therapies

The introduction of proteasome inhibitors (PIs) and immunomodulatory drugs in clinical practice has revolutionized the treatment outcomes of patients with MM [65]. Bortezomib was the first proteasome inhibitor approved by the FDA for relapsed and refractory patients in 2003. Since then, this class of drug has emerged as a cornerstone of therapy for MM patients. Specifically, the use of bortezomib as part of induction, consolidation, and maintenance therapies after stem cell transplant, and then as part of salvage regimens for relapsed disease, has significantly improved the prognosis and overall survival of patients with MM.

Nevertheless, MM remains incurable, and the vast majority of patients ultimately die from their disease. In particular patients who are relapsed and/or refractory to bortezomib and immunomodulatory drugs (IMiDs) have a very poor prognosis with a median progression-free survival and overall survival of only 5 and 9 months, respectively [66].

Encouragingly, new PIs with activity to different catalytic sites within the proteasome have recently been introduced into the clinic in order to overcome resistance acquired during bortezomib and other treatments, and to so potentially improve outcome.

Carfilzomib is a selective proteasome inhibitor that has demonstrated potent anti-myeloma activity and a favorable tolerability profile with manageable toxicity as a single agent in heavily pretreated relapsed and refractory MM patients [67]. Carfilzomib has recently been approved by FDA for the treatment of MM patients who have received at least two prior lines of therapy, including bortezomib and an IMiD, and who have experienced disease progression during or within 60 days of completing their last therapy.

The family of second-generation PIs also includes marizomib (NPI-0052), oprozomib (ONX0912), and ixazomib citrate (MLN9708), each of which has demonstrated clinical activity in relapsed and refractory MM patients in phase I/II studies.

Thus, the availability of new drugs with greater efficacy than bortezomib may improve the outlook for MM patients, but nevertheless resistance to PIs remains a challenge that requires new strategies in order to obtain deeper and longer remissions in these patients, irrespective of the agents used, and typically requires a combinatorial approach, informed by preclinical studies.

The antitumor activity of PIs is a sum of various mechanisms including inhibition of the 26S subunit of the proteasome, induction of apoptosis and inhibition of NF- κ B activity that result in disruption of cell-cycle progression and control, and inhibition of proliferation and angiogenesis. Caspase-mediated apoptosis is induced by bortezomib through three different pathways: the intrinsic mitochondrial apoptotic pathway involving caspase-9, the extrinsic death receptor pathway involving caspase-8, and the endoplasmic reticulum stress response pathway involving caspase-2, caspase-4, and caspase-12 [68]. Targeting different pathways using a combination of PIs with conventional therapies as well as new drugs may therefore result in synergistic activity, so overcoming resistance, as evidenced both preclinically and now clinically.

The hypothesis that a combination of drugs could overcome resistance of bortezomib was first tested in a study adding dexamethasone to bortezomib therapy in relapsed and refractory MM patients. In this study, the addition of dexamethasone to bortezomib resulted in clinical response in patients not responding to bortezomib alone [69]. With the aim of overcoming resistance, bortezomib has since been widely tested in combination with new and old drugs as summarized in Table 2.1, and as detailed below. Preliminary results of clinical trials of carfilzomib-based combination therapies have also been published, whereas studies investigating other new PIs are still ongoing, and only relatively limited preliminary results are therefore available.

Table 2.1 Studies of proteasome inhibitors in combination with conventional and novel drugs

Class of drugs	Combination regimen	Schedule	Study	N/n	ORR (≥PR)	ORR in PI rel/ref pts	Common toxicities of combination treatment
Alkylating	Bortezomib	Mel days: 1–4	Phase III	VMP: 344	VMP: 71 %	Na	G3 neuropathy 13 %
	Melphalan [71]	Bort days: 1, 4, 8, 11, 22, 25, 29, 32	VMP vs. MP (VISTA Trial)	MP: 338	MP: 35 %		G3 neuropenia 30 %
	Bortezomib	Prednisone	Phase II	126/130 (evaluable)	VDC: 75 %		G3 thrombocytopenia 20 %
		Bort days 1, 4, 8, 11	EVOLUTION trial				VCD
	Cyclophosphamide [76]	Cyclo days 1, 8 (15 VCD-mod)	VCD, VDR, VCD-mod, VCDR	140 patients enrolled	VDC-mod: 100 % VDR: 85 % VDCR: 88 %		G3 neuropenia 30 % G3 neuropathy 9 % G3 thrombocytopenia 12 % VCD-mod G3 neuropenia 24 % G3 neuropathy 18 % G3 thrombocytopenia 10 %
Anthracycline	Bortezomib	PLD day 4	Phase III	PLD/Bor: 324	PLD/Bor: 44 %	Na	G ≥ 3 neuropenia 29 %
	Pegylated Liposomal Doxorubicin (PLD) [94]	Bortezomib days: 1, 4, 8, 11	PLD/Bor vs. Bor	Bor: 322	Bor: 41 %		G ≥ 3 thrombocytopenia 23 % G ≥ 3 diarrhea 7 %
Immu- modulatory drugs	Bortezomib	Len days 1–14	Phase I	38/36	61 %	50 %	G1–2 neuropathy 42 %
	Lenalidomide [95]	Bort days 1, 4, 8, 11					G3 neuropenia 63 %
		Dexa					G3 thrombocytopenia 45 %
	Carfilzomib	Len days 1–21	Phase II	52	76.90 %	69.20 %	G3 neuropenia 33 %
	Lenalidomide [77]	Carfilz days 1, 2, 8, 9, 15, 16					G3 thrombocytopenia 19 %
		Dexa					
	Carfilzomib	Pomalidomide days 1–14	Phase I	28/20	75 %	Na	G3 neuropenia 29 %
	Pomalidomide [79]	Bortezomib days 1, 4, 8, 11					G3 thrombocytopenia 19 %
		Dexa days 1, 2, 4, 5, 8, 9, 11, 12					
	Carfilzomib	Pomalidomide days 1–21	Phase I/II	72 (Phase I/II)	64 % (≥MR 81 %)	Na	G ≥ 3 fatigue 48 % G ≥ 3 neuropenia 20 % G ≥ 3 thrombocytopenia 20 %
	Pomalidomide [80]	Carfilzomib 1, 2, 8, 9, 15, 16					Any grade: Neuropathy: 53 % Rash: 50 % Thrombocytopenia 6 % Neutropenia 5 %
		Dexa 1, 8, 15, 22					
	Ikazomib citrate (MLN9708)	MLN9708 days 1, 4, 8, 11	Phase I/II	64/62	94 %		
	Lenalidomide [78]	Len days 1–14					
		Dexa days 1, 2, 4, 5, 8, 9, 11, 12					

2.3.1 *Proteasome Inhibitors and Conventional Drugs*

2.3.1.1 Bortezomib and Melphalan

Melphalan and prednisone (MP) were the standard of care for patients ineligible for transplant for many years. The addition of bortezomib to this regimen was the first step done to improve the response rate of this combination. A study evaluating the combination of bortezomib (also known as Velcade) and MP (VMP) showed a very encouraging overall response rate of 89 % [70]. A subsequent randomized study (the so-called VISTA Trial) compared MP versus VMP in untreated patients and confirmed the superiority of the bortezomib-containing regimen in terms of both survival and response rate [71]. The major toxicities of VMP included neuropathy and gastrointestinal symptoms. A subsequent study investigating a new schedule of VMP with weekly bortezomib in association with melphalan and prednisone or in association with thalidomide and prednisone (VTP) followed. Both arms of the study were then subject to a second randomization to maintenance with VT versus VP. No major differences between the two arms were observed, except for a lower rate of neurotoxicity in the VP arm. [72] An Italian study investigating combination of VMP plus thalidomide (VMPT) followed by bortezomib and thalidomide (VT) maintenance compared with VMP alone showed an advantage in terms of progression-free survival, overall survival, and response rate for the VMPT-VT arm. With the addition of thalidomide, a higher incidence of neuropathies was observed. This study was also amended after inclusion of the first 139 patients, and the schedule was changed to a weekly administration of bortezomib with a remarkable improvement in the incidence of grade 3–4 neuropathy. In aggregate, synergistic activity of bortezomib and melphalan results in a higher response rate in comparison with single-agent bortezomib, and this combination continues to have a role in the treatment of newly diagnosed patients not eligible to stem cell transplant.

2.3.2 *Proteasome Inhibitors and Immunomodulatory Drugs*

2.3.2.1 Bortezomib and Lenalidomide

Lenalidomide is an IMiD that exerts its anti-myeloma activity through activation of the caspase-8-mediated apoptosis pathway and inhibition of NF- κ B activity through a different mechanism than bortezomib. On this basis, the hypothesis of combining these two drugs to overcome resistance occurring with single bortezomib treatment was investigated. Synergistic activity of the combination of the two drugs was initially tested in relapsed and refractory MM, including patients who were refractory to bortezomib. [73] The MTD was lenalidomide 15 mg daily on days 1–14; bortezomib 1 mg/m² on days 1, 4, 8, and 11 of a 21-day cycle; and dexamethasone 40 or 20 mg on days 1–2, 4–5, 8–9, and 11–12. Promising activity and limited toxicity observed in this phase I and subsequent phase II studies prompted this combination to be treated in association with dexamethasone in newly diagnosed MM patients [74]. In this study, 66 patients were treated with lenalidomide, bortezomib, and

dexamethasone (RVD) for at least eight cycles. Patients achieving at least a partial response (PR) after four cycles were allowed to proceed to transplant. The percentage of patients achieving PR or better was 100 %, with more than VGPR seen in 74 %, including nCR and CR in 52 %. Main side effects observed included predominantly grade 1–2 sensory neuropathy, rare motor neuropathy, and some neuro-pathic pain, with grade 3–4 neutropenia and thrombocytopenia observed in 5–10 % of patients, and an overall rate of thrombosis (including pulmonary embolism) of 6 %. Toxicities proved generally manageable, however, and the regimen was well tolerated overall with a low rate of discontinuation.

The very high response rate observed with the combination of lenalidomide and bortezomib led to the incorporation of this regimen in the posttransplant setting in patients with high-risk MM. New studies using this combination therapy as consolidation or as long-term maintenance after autologous stem cell transplant in order to obtain deeper and longer responses are underway. A recent published paper by Nooka AK et al. evaluated the impact of a consolidation/maintenance program with lenalidomide, bortezomib, and dexamethasone (RVD) after transplant in patients with high-risk MM and showed a benefit in terms of both progression-free survival and overall survival in this poor prognosis population [75].

2.3.2.2 Bortezomib and Dexamethasone in Combination with Cyclophosphamide and Lenalidomide

Bortezomib and dexamethasone in combination with cyclophosphamide (VCD) has shown significant efficacy in patients with MM. In the randomized, phase 2, EVOLUTION study, VCD was compared to bortezomib, lenalidomide, and dexamethasone (VDR) and to the quadruple regimen of cyclophosphamide combined with VDR (VDCR). One hundred and forty patients received eight 3-week cycles of induction therapy with standard bortezomib dose with dexamethasone 40 mg on days 1, 8, and 15, with either cyclophosphamide at 500 mg/m² on days 1, 8 and lenalidomide 25 mg from days 1 to 14 (VDCR) or cyclophosphamide 500mg/m² on days 1, 8 (VCD) and lenalidomide 15 mg days 1–14 (VDR). All groups received maintenance therapy with weekly bortezomib. Following the interim analysis, the VCD arm was modified to add an additional dose of cyclophosphamide on day 15 (VDC-mod). Very good partial response (VGPR) or better was seen in 58 %, 51 %, 41 %, and 53 % of patients in the VDCR, VDR, VDC, and VDC-mod arms respectively with a 1-year progression-free survival of 86 %, 83 %, 93 %, and 100 %, respectively. No advantage was noted with VDCR over the 3-drug combinations. Although the numbers are relatively small, this trial suggests both VCD and VRD are excellent choices for newly diagnosed patients [76].

2.3.2.3 Carfilzomib and Lenalidomide

Carfilzomib is a second-generation selective proteasome inhibitor that irreversibly binds the chymotrypsin-like activity of the proteasome. Combination therapy with carfilzomib and lenalidomide in association with low dose of dexamethasone was

initially tested in relapsed or progressive MM patients. The phase Ib escalation part of the study defined the maximum planned dose of carfilzomib at 20 mg/m² on days 1 and 2 and 27 mg/mq on days 8, 9, 15, 16 thereafter, lenalidomide 25 mg on days 1 to 21, and dexamethasone 40 mg weekly on a 28-day cycle. Results of the phase 2 dose expansion in 52 patients showed an overall response rate of 77 % and duration of response of 22.1 months. Among bortezomib-refractory patients, 69 % responded, and among lenalidomide refractory patients, 70 % responded. Grade 3–4 toxicities included lymphopenia (8 %), neutropenia (33 %), thrombocytopenia (19 %), and anemia (19.2 %) [77].

More information about efficacy of this very active drug combination is expected from results of a large phase III trial comparing the activity of carfilzomib plus lenalidomide and dexamethasone versus lenalidomide and dexamethasone alone in relapsed patients (the so-called ASPIRE Trial) which was recently completed, with data on outcome anticipated soon.

2.3.2.4 Ixazomib Citrate and Lenalidomide

Ixazomib citrate (MLN9708) is an oral proteasome inhibitor that rapidly hydrolyzes to the biologically active dipeptide boronic acid MLN2238. MLN2238 preferentially binds the $\beta 5$ -site of the 20S proteasome; at higher concentrations, it also inhibits the activity of the $\beta 1$ - and $\beta 2$ -sites. In preclinical studies, MLN2238 demonstrates a faster dissociation rate from the proteasome that may result in enhanced tumor penetration, and antitumor activity, and has more prolonged tissue penetration than bortezomib. Phase I studies of MLN9708 have shown promising activity and durable responses in heavily pretreated MM patients. Ixazomib citrate in combination with lenalidomide and dexamethasone has been investigated in a phase 1/2 study in newly diagnosed patients. Sixty four patients received MLN9708 3.0 or 3.7 mg on days 1, 4, 8, 11, lenalidomide 25 mg on days 1–14, and dexamethasone 20/10 mg (cycles 1–8/9–16; days 1, 2, 4, 5, 8, 9, 11, 12) for up to 16 on a 21-day cycle, followed by MLN9708 maintenance until progression. Transplant-eligible pts could undergo stem cell collection after four cycles of therapy. In 62 response-evaluable patients, 94 % achieved greater than a PR including 76 % VGPR. Most common grade 3 adverse events were rash (16 %), hyperglycemia (8 %), pneumonia (6 %), and PN (5 %), with the regimen otherwise generally well tolerated with manageable toxicity [78].

2.3.3 Proteasome Inhibitors and Pomalidomide

2.3.3.1 Bortezomib and Pomalidomide

Pomalidomide is a new generation immunomodulatory agent that exerts its anti-myeloma activity through different ways including modulation of cytokine production, immunomodulation, and interaction with the bone marrow microenvironment.

Pomalidomide has been demonstrated to be more effective and less toxic than lenalidomide. This new IMiD has been approved by the Food and Drug Administration (FDA) for patients with relapsed/refractory MM who have received more than two prior therapies, including lenalidomide and bortezomib, and have progressive disease on or within 60 days of completion of their last line of treatment. The marked anti-myeloma activity observed in clinical trials investigating the effect of the combination of pomalidomide with dexamethasone and the known synergism between immunomodulatory drugs and bortezomib suggested a potential activity of the combination with pomalidomide and bortezomib. Preliminary data of the phase I trial testing pomalidomide in combination with bortezomib and dexamethasone in myeloma patients refractory to lenalidomide and relapsed after bortezomib have been recently published. Twenty-two out of twenty-eight planned patients were evaluable for response. Patients received escalating doses of pomalidomide (1–4 mg, days 1–14), bortezomib (1 or 1.3 mg/mq, days 1, 4, 8, 11), and dexamethasone 20 mg on days 1–2, 4–8, 8–9, 10–11. Overall response rate was 75 % (15 of 20 evaluable patients) with 30 % of VGPR or better. Most common grade 3 and 4 toxicities were neutropenia (29 %) and thrombocytopenia (19 %). No grade 3–4 peripheral neuropathy was observed [79]. The combination of pomalidomide and bortezomib seems to have a strong anti-MM activity in patients already treated with bortezomib, thus suggesting that the addition of pomalidomide may have the ability to overcome resistance acquired during treatment with PIs, and phase III studies are now underway to validate this concept.

2.3.3.2 Carfilzomib and Pomalidomide

Carfilzomib has been evaluated with pomalidomide and dexamethasone in a phase I/II study in relapsed and refractory MM patients. Dosages of the combination included carfilzomib at 20/27 mg/m², pomalidomide 4 mg, and dexamethasone 40 mg. The effect of the addition of pomalidomide to carfilzomib was evaluated in 67 patients enrolled in the phase I and II of the study. Preliminary data showed an overall response rate (PR or better) in a population of heavily pretreated patients of 64 %, and 81 % of patients achieved minimal response or better. Responses were observed also among patients with intermediate and high-risk cytogenetics. Common >grade 3 side effects included fatigue, neutropenia, anemia, thrombocytopenia, and diarrhea [80].

2.3.4 Proteasome Inhibitors and Histone Deacetylase Inhibitors

Deacetylases are a family of enzymes that exerts their activity on histone proteins and on a large number of proteins involved in intracellular functions that are dysregulated in cancer: gene expression, DNA replication and repair, cell-cycle progression, and cytoskeletal reorganization. Histone deacetylases (HDAC) remove

acetyl groups from protein and have been recognized as a target for the treatment of hematological malignancies. Although several HDAC inhibitors (HDACIs) have been investigated and are in clinical development, only two of them to date have been approved for treatment of hematological malignancies. Specifically, both vorinostat and romidepsin have been approved for treatment of T cell cutaneous lymphoma.

The first HDACI that demonstrated to have anti-myeloma activity was SAHA (vorinostat). Exposure of myeloma cells to SAHA resulted in antiproliferative and proapoptotic effect involving downregulation of transcripts for member of the insulin-like growth factor (IGF)/IGF1 receptor and IL-6 receptor signaling cascades, antiapoptotic molecules, oncogenic kinases, DNA synthesis/repair enzymes, and transcription factors. Preclinical studies of other HDACIs demonstrate that the anti-myeloma activity of this class of drugs is due to a various numbers of effect on myeloma cells and on their interactions with the microenvironment. HDACIs can induce direct cell-cycle arrest and apoptosis and also disrupt the signaling between MM cells and bone marrow stem cells. Vorinostat is able to induce expression of p21, leading to cell-cycle arrest and apoptosis. Romidepsin induces downregulation of antiapoptotic proteins BCL-2 and BCL-XL. Vorinostat suppresses the stimulation of IL-6 secretion in bone marrow stem cells by myeloma cells adhesion and also suppresses autocrine IGF production interrupting the IGF-I/IGF-IR/Akt signaling pathway. HDACIs are also involved in myeloma cell inhibition, and specifically the prevention of aggresome formation in MM cells treated with PIs. The aggresomes are an alternative way that the cell can use for catabolism of misfolded proteins when the production of misfolded protein exceeds the capacity of proteasomes, as detailed previously. Drugs like tubacin targeting HDAC6 or panHDAC inhibitors like panobinostat lead to increase ubiquitinated proteins through impairment of the transport of aggresome to lysosome [23].

Based on the important activities of HDACIs, the hypothesis of combining HDACI with bortezomib to induce synergistic activity and to overcome resistance has been investigated. As mentioned, the dual inhibition of the proteasome and aggresome pathways induces accumulation of ubiquitinated proteins, resulting in cell stress and apoptosis. In addition to this synergistic effect, the combination of the two classes of drugs also exerts an anti-myeloma activity through multiple other pathways including inhibition of NF- κ B, suppression of production of IL-6 and IGF-1, and inhibition of angiogenesis resulting in growth inhibition, apoptosis, and reduction of survival of myeloma cell.

2.3.4.1 Bortezomib and Panobinostat

The efficacy of the combination of HDACI and bortezomib has been evaluated in various clinical trials in relapsed and refractory myeloma patients and subsequently in randomized trials. The combination of panobinostat with bortezomib in refractory and relapsed myeloma patients was comprehensively tested in the multicenter phase II PANORAMA-2 study. In this study, 55 patients with relapsed and refractory MM,

all of whom were required to be refractory to bortezomib, received bortezomib with the usual biweekly schedule, panobinostat 20 mg three times per week, and dexamethasone. The overall response rate was 35 % and clinical benefit as reflected by minimal response or better was evident in 53 % of patients. Of note 40 out of the 55 patients enrolled in the study were progressing while on their last treatment with bortezomib and all of the patients being refractory to bortezomib at some point. [81] Common observed grade 3 and 4 adverse events included thrombocytopenia, fatigue, and diarrhea. Results of this study confirmed that the addition of panobinostat is able to overcome the resistance to proteasome inhibitor as demonstrated by the responses observed among patients progressing on bortezomib-based therapy. Additional information about this combination will be available from the recently completed phase III PANORAMA-1 study investigating the direct comparison between the combination of the proteasome inhibitor, dexamethasone, and panobinostat versus bortezomib, dexamethasone, and placebo in relapsed MM patients. Importantly, preliminary results of the PANORAMA-1 trial appear to strongly favor an advantage to adding panobinostat to bortezomib and dexamethasone [82].

2.3.4.2 Bortezomib and SAHA (Vorinostat)

The combination of bortezomib and vorinostat in relapsed and refractory myeloma patients was evaluated in the VANTAGE 095 study. In this study, the overall response rate was 42 %, with objective response also seen among patients refractory to bortezomib. Most common grade 3 and 4 toxicities were myelosuppression, fatigue, and diarrhea. Despite the encouraging results observed in this phase II study, the phase III VANTAGE 088 study investigating the efficacy of bortezomib plus vorinostat versus bortezomib and placebo failed to show a clinically significant difference in terms of PFS between the two groups [83]. Patients receiving the combination therapy with vorinostat and bortezomib had more objective responses, but this advantage did not result in a meaningfully longer PFS. Explanation for this unexpected result was probably related to higher toxicity observed in the vorinostat arm that required frequent dose reductions and/or treatment interruptions. In fact, in the vorinostat group the side effects were significantly higher than in the placebo group; specifically myelosuppression, fatigue, nausea, vomiting, and diarrhea were the most common drug-related toxicities. Although the evidence of synergism between HDACI and bortezomib in overcoming resistance to PIs has been demonstrated, it is also therefore clear that new less toxic HDACIs are needed in order to obtain a more efficacious combination therapy.

2.3.4.3 Bortezomib and Romidepsin

Romidepsin has been approved by the FDA in the United States for relapsed cutaneous T cell lymphoma. Preclinical studies have demonstrated its anti-MM activity through upregulation of p21, downregulation of antiapoptotic molecules, and

induction of apoptosis. Single-agent romidepsin in MM patients showed modest activity, but the synergistic effects between HDACI and bortezomib observed in preclinical studies justified a clinical trial testing the combination. Twenty-five patients were enrolled in the combination study of romidepsin dose on day 1, 8, and 15 of a 28-day cycle and bortezomib on days 1, 4, 8, and 11 with dexamethasone. The MTD for romidepsin was fixed at 10 mg/mq. At least a minimal response was seen in 72 % of patients with a median time to progression of 7.2 months. Most common adverse events included grade 3 and 4 thrombocytopenia in 64 % of patients and grade 3 and 4 peripheral neuropathy in 8 % [84].

2.3.4.4 Bortezomib and ACY1215

In order to increase activity of the combination therapy with HDACI and PIs and to reduce the toxicity related to the association of the two drugs, new more specific HDACIs have been investigated both preclinically and in clinical trials. As mentioned above, HDAC6 plays a key role in the aggresome pathway and is involved in formation and transportation of aggresome to lysosomes, thus suggesting a potential strong synergism with inhibitors of the proteasome pathway. ACY-1215 is a first-in-class selective and potent oral HDAC6 inhibitor with an anti-myeloma activity demonstrated both in vitro and in vivo animal models [85]. It has been tested in phase I/II clinical trials in combination with bortezomib and also with lenalidomide. Preliminary data presented at the ASH Meeting in 2013 reported 4 responding patients among 16 patients receiving the combination of bortezomib at 1–1.3 mg/m² and ACY-1215 at different doses with very manageable and limited toxicity [86]. More information about the feasibility and the efficacy of this combination will be available over time, but these preliminary results appear very promising.

2.3.5 Proteasome Inhibitors and Plerixafor

Interactions of MM cells with extracellular matrix proteins and bone marrow cells play a key role for survival and proliferation of malignant cells. Chemokines regulate the adhesion of the myeloma cells to their microenvironment, and cells and cytokines of the bone marrow environment activate proliferative and antiapoptotic signals. In particular the chemokine stromal-derived factor-1 (SDF-1) and its receptor CXCR4 play a central role in trafficking of myeloma cells.

The hypothesis that inhibiting CXCR4 could increase the sensitivity of myeloma cells to anti-myeloma drugs by disrupting the interaction with bone marrow has been tested in preclinical studies. Azab et al. [87] have demonstrated that AMD3100 is able to disrupt the adhesion of myeloma cells and microenvironment and induce mobilization of cells leading to increase sensitivity to bortezomib. The use of anti-CXCR4 in combination with bortezomib has been investigated in a phase I/II in relapsed and refractory MM patients with the aim to evaluate the chemosensitization

effect of AMD3100. Preliminary results of this study have been recently published. In the first part of the study, 25 relapsed/refractory MM patients were treated with AMD3100 and bortezomib in two different schedules of administration, and in the second part of the study, 11 patients received AMD3100 at the MTD dose ($320 \mu\text{g}/\text{m}^2$) on days 1, 2, 3, 6, 10, and 13 and bortezomib $1.3 \text{ mg}/\text{m}^2$ on days 3, 6, 10, and 13 every 21 days. Ten patients were evaluable for response, and more than partial remission was observed in 40 % of patients. Grade 4 toxicities included lymphopenia (10 %) and thrombocytopenia (20 %); grade 3 toxicities included anemia (10 %), thrombocytopenia (10 %), lymphopenia (20 %), hyperglycemia (10 %), and hypophosphatemia (10 %) [88]. The preliminary results of this study are hopeful, with 40 % of patients achieved a clinical benefit from the combination therapy, including a subgroup of patients refractory to bortezomib.

2.3.6 *Proteasome Inhibitors and Perifosine*

Perifosine is a synthetic novel alkylphospholipid (ALP) that targets cell membrane and inhibits Akt activation. In myeloma cells, perifosine is able to inhibit Akt activation triggered by IL-6 and IGF-1. Moreover, perifosine exerts a potent cytotoxicity against myeloma cells adherent to bone marrow stromal cells and induces apoptosis by recruitment of death receptors such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-R1/DR4 and TRAIL-R2/DR5. Myeloma cell apoptosis induced by bortezomib is associated with activation of Akt, and pre-clinical studies have demonstrated that perifosine enhances the cytotoxicity of bortezomib and dexamethasone [89].

Based on promising preclinical data, a phase I/II study was conducted in relapsed/refractory MM population; patients preexposed to bortezomib were treated with perifosine and bortezomib + dexamethasone. The dose selected for the part II of the study was perifosine 50 mg every day and bortezomib $1.3 \text{ mg}/\text{m}^2$ on days 1, 4, 8, and 11 in a 21-day cycle; addition of 20 mg of dexamethasone was permitted if progression occurred in perifosine plus bortezomib alone.

The new combination was tested in 84 patients with relapsed or relapsed/refractory MM; all have been already treated with bortezomib and majority of them were refractory to bortezomib (73 %). A minimal response or better was observed in 41 % of patients, including an overall response rate of 65 % in bortezomib-relapsed patients and of 32 % in bortezomib-refractory patients. Observed toxicities were mild and manageable; grade 3 and 4 adverse events included thrombocytopenia (23 %), neutropenia (15 %), anemia (14 %), and pneumonia (12 %). Thirty-one percent of patients had polyneuropathy of any grade. No patients experienced polyneuropathy of grade 4 [90]. Based on the results of the phase II study, a placebo randomized controlled study was conducted in relapsed/refractory MM patients previously treated with bortezomib. The study enrolled 135 patients that were randomized to receive perifosine 50 mg every day; bortezomib $1.3 \text{ mg}/\text{m}^2$ on days 1, 4, 8, and 11; and dexamethasone 20 mg, or placebo, bortezomib, and dexamethasone.

The study was discontinued after the first planned interim analysis because although well tolerated, no major benefit was observed in overall response rate and progression-free survival by adding perifosine to bortezomib and dexamethasone, as well as the emergence of major resource constraints and slow enrollment prompting a recommendation for closure [91]. Despite disappointing results of the phase III study, inhibition of Akt pathway seems to be a promising target for the treatment of relapsed/refractory MM patients, and the development of new more potent inhibitors warrants additional studies.

2.3.7 Proteasome Inhibitors and Monoclonal Antibodies, Specifically Elotuzumab

Elotuzumab is a humanized immunoglobulin G1 mAb directed against the cell surface glycoprotein CS1, expressed on normal plasma cells and myeloma cells. Elotuzumab exerts its anti-myeloma activity by blocking the adhesion of myeloma cells to bone marrow stromal cells and activating the natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC). Preclinical studies have demonstrated a synergism between elotuzumab and bortezomib. Bortezomib enhances ADCC-mediated myeloma cell death induced by elotuzumab via down-regulation of the cell surface expression of MHC class I, an inhibitor of NK activity. In vitro and in vivo studies have demonstrated that bortezomib enhanced the anti-myeloma activity of elotuzumab rendering myeloma cells more sensitive to NK cell-mediated lysis [92]. In a phase I clinical trial, 28 patients with relapsed/refractory myeloma patients were treated with escalation dosages of elotuzumab and bortezomib. The maximum-tolerated dose was not reached up to the maximum planned dose of elotuzumab of 20 mg/kg; the most frequent grade 3 and 4 adverse events were lymphopenia (25 %) and fatigue (14 %). The phase I study demonstrated clinical activity of the combination with favorable toxicity; a partial response or better was observed in 48 % of patients and a minimal response or better was observed in 63 % of patients, with the median time to progression being more than 9 months [93].

Phase III studies aimed to evaluate the anti-myeloma activity of the combination are ongoing and hopefully will confirm the synergism or at least additive effects between bortezomib and elotuzumab.

2.4 Conclusion

The introduction of PIs in the spectrum of chemotherapeutic agents used in MM patients has remarkably impacted on patient treatment and improved overall survival. BTZ as a first-in-class PI and subsequently other novel PIs (such as carfilzomib) are already and will remain a standard treatment for both newly diagnosed and relapsed/refractory MM patients, most often in combination regimens based upon

results to date. Unfortunately, BTZ and other PI resistance can be acquired in patients and so reduce treatment efficacy. Several mechanisms have been proposed for resistance including mutations and overexpression of proteasome subunits, decommitment to less-differentiated B cell stages to reduce ER overload and stress, activation of compensatory pathways such as autophagy and aggresome formation; and increase support by survival pathways, including MAF, c-MET, IGFR-1, and AKT or bone marrow stromal cells (Fig. 2.4). Hence, strategies to overcome BTZ and other PI resistance consist of combining agents capable of blocking these compensatory mechanisms, such as AKT inhibitors or IGFR inhibitors, or compounds targeting aggresome formation (HDAC6 inhibitors) or autophagy (FK866). Several biomarkers have been proposed to predict BTZ activity, such as XBP1 levels and inactivating various mutations including CD93/CD69, CYPD or SOD2, NAMPT, USP7, and MAF, but none of them has yet been validated in the clinical setting.

More broadly, synergistic combinations with IMiDs, chemotherapeutics, glucocorticoids, and more recently MoAbs have already been broadly validated with multiple clinical studies confirming the benefit of these approaches, validating that PIs are a backbone in MM therapy [97, 98]. Strategies with PIs to further improve the therapeutic index remain an area of urgent unmet medical need and hold the real promise of further improving patient outcome in this otherwise incurable disease [99, 100].

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