Chapter 4
Mechanisms Driving Resistance to Proteasome Inhibitors Bortezomib, Carfilzomib, and Ixazomib in Multiple Myeloma

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Abstract The first clinically available proteasome inhibitor (PI) bortezomib was trialed in multiple myeloma (MM) approximately two decades ago and has since become a mainstay of myeloma therapy, significantly enhancing the overall survival of patients. However, bortezomib resistance continues to be a significant hurdle in the treatment of MM, despite the introduction of next-generation PIs such as carfilzomib and ixazomib. Unlike resistance to some other targeted therapies such as tyrosine kinase inhibitors, bortezomib resistance is highly complex and is able to arise through multiple mechanisms. This chapter discusses the current known mechanisms underlying bortezomib resistance, as well as resistance to the next-generation proteasome inhibitors carfilzomib and ixazomib.

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S. C. W. Ling, S. Trieu (eds.), *Resistance to Targeted Therapies in Multiple Myeloma*, Resistance to Targeted Anti-Cancer Therapeutics 22,
https://doi.org/10.1007/978-3-030-73440-4_4

Keywords Multiple myeloma · Proteasome inhibitors · Bortezomib · Carfilzomib · Ixazomib · Proteasome inhibitor resistance · Autophagy · Unfolded protein response

Abbreviations

ABC	ATP-binding cassette
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BiP	Binding immunoglobulin protein
BMSC	Bone marrow stromal cell
eIF2α	Eukaryotic initiation factor 2 alpha
ER	Endoplasmic reticulum
ERAD	ER-associated decay of proteins
FDA	US Food and Drug Administration
HDAC6	Histone deacetylase 6
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
MHC-1	Major histocompatibility complex class I
MM	Multiple myeloma
MSC	Mesenchymal stem cells
NF-κB	Nuclear factor kappa-B
p38MAPK	p38 mitogen-activated protein kinase
PERK	PKR-like ER kinase
PFS	Progression-free survival
PI	Proteasome inhibitor
PI3K	Phosphotidylinositol 3-kinase
RIDD	Regulated IRE1-dependent decay
RRMM	Relapsed/refractory multiple myeloma
TNF-α	Tumor necrosis factor-alpha
UPR	Unfolded protein response
XBP1	X-box binding protein 1

4.1 Introduction

Multiple myeloma (MM) is a hematological malignancy arising from plasma cells [1]. This plasma cell origin means myeloma cells often produce and secrete very high levels of nonfunctional, monoclonal immunoglobulin termed paraprotein [2].

This paraprotein production, and the subsequent endoplasmic reticulum stress and unfolded protein response activation, has been dubbed an "Achilles heel" of myeloma, which proteasome inhibitors (PI) such as bortezomib, carfilzomib, and ixazomib are able to exploit to induce myeloma cell death [2]. Although PIs have rapidly become a foundation of myeloma therapy, resistance is still a major hurdle in the treatment of patients with myeloma.

4.2 The Proteasome

The proteasome is responsible for the degradation of 70–90% of proteins that are unfolded, misfolded, or otherwise marked for degradation [3]. In eukaryotes, the 26S proteasome consists of two major subunits, a barrel-shaped core 20S subunit, and two regulatory 19S subunits bound to either end [4]. The 20S subunit is where proteolysis occurs and it contains six proteolytic centers composed of three different β subunits, β1, β2, and β5 [5]. These different subunits each have different activities; a caspase-like activity which cleaves after acidic amino acids (β1), a trypsin-like activity which cleaves after basic amino acids (β2), and a chymotrypsin-like activity which cleaves after neutral amino acids (β5) [3, 5]. Some mammalian cells also possess an immunoproteasome, where β1, β2, and β5 are replaced with β1i, β2i, and β5i, respectively [3]. The immunoproteasome is generally stimulated by γ-interferon, but can also be induced by other factors such as tumor necrosis factor α (TNF-α), and has altered substrate specificity to produce peptides optimized in size and composition for presentation to the major histocompatibility complex class I (MHC-I) on the cell surface during the immune response [6].

4.3 Endoplasmic Reticulum Stress

The extensive production of paraprotein in myeloma cells results in an increase in unfolded protein levels within the endoplasmic reticulum (ER), which in turns causes ER stress [2]. As a result of this, a cellular cascade known as the unfolded protein response (UPR) is triggered [2]. The UPR is activated by three ER transmembrane stress sensing proteins, inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [7]. Under homeostatic conditions, these ER stress sensors are kept inactive by the binding of the ER-specific chaperone binding immunoglobulin protein (BiP, also known as GRP78) to their luminal domain [8]. However, if unfolded protein levels increase, BiP is titrated away from these ER stress sensors due to its high affinity for unfolded proteins [8]. For IRE1 and PERK, the loss of BiP, as well as the direct binding of unfolded proteins to the luminal domains of IRE1 and potentially PERK, leads to their oligomerization and autophosphorylation, resulting in activation [7, 9, 10]. Meanwhile, the loss of BiP from ATF6 results in the exposure of Golgi localization

signal sequences, which results in the relocation of ATF6 to the Golgi, where it is cleaved by site-1 and site-2 proteases into its active form as a transcription factor [11, 12].

Activation of the UPR results in a complex signaling cascade, the main components of which are summarized in Fig. 4.1. This activation initially elicits a pro-survival response, aimed at restoring ER homeostasis via several mechanisms [2, 13]. However, if ER homeostasis is unable to be restored, then the UPR switches from pro-survival to pro-apoptotic signaling and induces cell death. Exactly how the

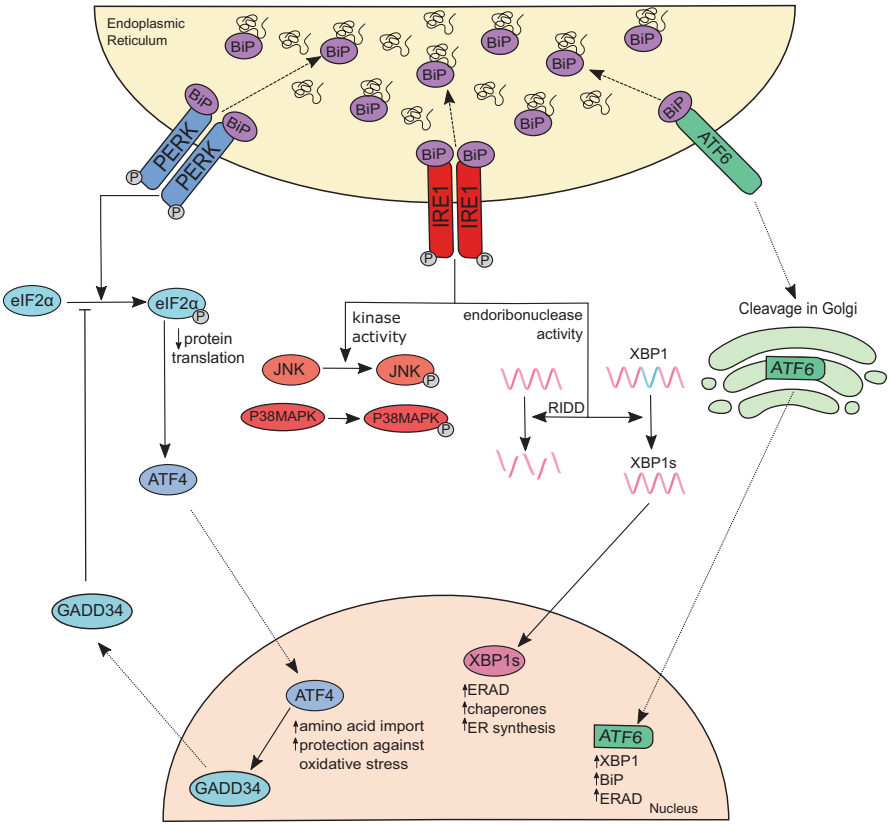

Fig. 4.1 A summary of the unfolded protein response. As unfolded protein levels increase, BiP dissociates from the luminal domain of ER stress sensors PERK, IRE1, and ATF6, resulting in their activation. IRE1 oligomerizes and autophosphorylates, activating its endoribonuclease and protein kinase activity. The endoribonuclease activity of IRE1 results in the production of the transcription factor XBP1, as well as IRE1-dependent decay (RIDD) of certain RNAs. Activation of the protein kinase activity of IRE1 results in the recruitment of binding partners and the phosphorylation of multiple targets, including p38MAPK and JNK. PERK also activates via oligomerization and autophosphorylation, resulting in reduced cap-dependent translation, and the production of transcription factor ATF4. When released by BiP, ATF6 translocates to the Golgi, where it is cleaved to form active ATF6, a transcription factor capable of upregulating several key UPR genes, including XBP1 and BiP

cell makes this decision however is still not fully understood [8, 14]. For a comprehensive review on the UPR and its role in cell fate, see Hetz and Papa (2018) [11].

Each ER stress sensor is able to activate separate but overlapping pathways [11]. Activated IRE1 is able to act as both an endoribonuclease and a protein kinase [11]. By far the most important target of the endoribonuclease activity of IRE1 is x-box binding protein 1 (XBP1), the splicing of which allows for the translation of XBP1s, a transcription factor which is important for both plasma cell differentiation and pro-survival UPR signaling [2]. XBP1s is able to upregulate several pathways, including ER membrane synthesis, ER chaperones, and ER-associated decay of proteins (ERAD) [2]. The endoribonuclease activity of IRE1 is also able to induce the degradation of certain RNAs via regulated IRE1-dependent decay, or RIDD, which is more closely associated with apoptosis [15, 16]. The protein kinase activity of IRE1 is also more closely associated with apoptosis, leading to the phosphorylation of stress-activated protein kinases such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) through association with binding partners such as TRAF2 and ASK1 [7].

The protein kinase activity of activated PERK results in the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), which suppresses cap-dependent translation, reducing the protein burden on the ER [17]. This leads to upregulation of activating transcription factor 4 (ATF4), which contributes to both cell-survival and cell-death pathways depending on how long it is present in the cell [11]. The extended presence of ATF4 results in the production of the pro-apoptotic transcription factor CHOP, as well as GADD34, which blocks eIF2α phosphorylation and thus restarts cap-dependent translation [18]. Cleavage of ATF6 into an active transcription factor also results in the upregulation of a number of UPR associated genes, including BiP and XBP1 [11].

4.4 Proteasome Inhibitors in Multiple Myeloma

Due to their high paraprotein production, myeloma cells have relatively high intrinsic levels of ER stress, and UPR is often already active in these cells as a pro-survival mechanism [2]. Proteasome inhibitors are able to take advantage of this by blocking proteasomal degradation, and therefore ERAD, further increasing unfolded protein levels and thus eliciting an apoptotic UPR [2]. The first PI bortezomib (Velcade) was approved by the US Food and Drug Administration (FDA) in 2003 for patients with relapsed/refractory multiple myeloma (RRMM) [19]. Since then, bortezomib has become one of the central drugs in myeloma treatment [20]. It is a reversible PI which acts on the 20S subunit of the proteasome, inhibiting primarily the β5 subunit (chymotrypsin-like activity), although inhibition of the β2 subunit (trypsin-like activity) and β1 subunit (caspase-like activity) also occurs, albeit with a lower affinity [21]. Proteasome inhibitors are thought to cause the death of myeloma cells through several mechanisms. One of the first proposed mechanisms was through inhibition of nuclear factor kappa-B (NF-κB), which in itself is an

inhibitor of apoptosis, although it has since been suggested that this is unlikely to be the main mechanism [21, 22]. It is now known that PIs also induce a pro-apoptotic UPR and cause changes in the bone marrow microenvironment that make it less hospitable to myeloma cells [21, 23].

Since the development of bortezomib, next-generation PIs have been developed. Of these, carfilzomib and ixazomib have both been FDA approved for the treatment of RRMM, in 2012 and 2015, respectively [24, 25]. Carfilzomib has a different active moiety to bortezomib (epoxyketone as opposed to the boronate of bortezomib) and is more specific for the chymotrypsin-like activity of the proteasome than bortezomib, which it inhibits in an irreversible manner [26]. Ixazomib, on the other hand, is based on the same structural moiety as bortezomib (boronate), and, thus, unsurprisingly is a reversible inhibitor of primarily chymotrypsin-like proteasome activity, but also trypsin- and caspase-like activity [21]. However, unlike bortezomib and carfilzomib, ixazomib is orally bioavailable, and has a better pharmacokinetic profile than bortezomib [21]. The structures of these inhibitors and their similar mode of binding to the β5 subunit of the proteasome are shown in Fig. 4.2. Both carfilzomib and ixazomib have been shown to be effective in bortezomib-resistant patients, though some cross-resistance between PIs has been observed [27–29].

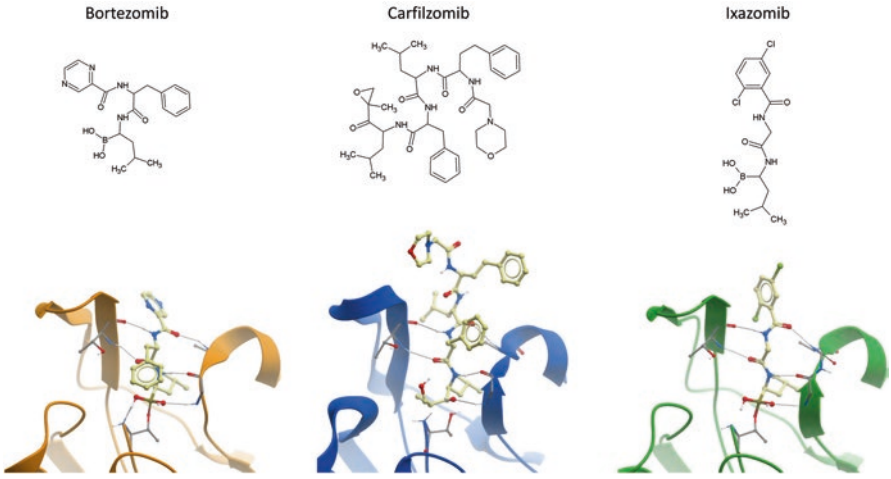

Fig. 4.2 Proteasome inhibitors and their interactions with the proteasomal subunit PSMB5. The structures of bortezomib, carfilzomib, and ixazomib are shown, along with how these drugs interact with the proteasomal subunit PSMB5. The PSMB5 protein is shown in ribbon format, and the atoms within the inhibitors are represented with different colors; red is oxygen, blue is nitrogen, white is hydrogen, green is chlorine, and brown is boron. Black dots represent hydrogen bonds between the protein and the inhibitor. Modeling was performed in Molsoft's ICM-Pro, and structures were obtained from the protein database (code 5LF3 for structure with bortezomib, 4R67 for structure with carfilzomib, and 5LF7 for structure with ixazomib)

4.5 Bortezomib Resistance Mechanisms

Almost as soon as bortezomib was FDA approved, research into bortezomib resistance and how it may potentially be overcome was already underway [30]. In the last two decades, a significant amount of research concerning the mechanisms of bortezomib resistance has been conducted.

4.5.1 Proteasome Mutation and Overexpression

One of the first proposed mechanisms of bortezomib resistance was mutation and/or overexpression of the proteasome, especially the β5 subunit (PSMB5, encoded by the *PSMB5* gene) to which bortezomib primarily binds [31]. In bortezomib-resistant cell lines, generated by exposing cells in vitro to escalating doses of bortezomib, both mutations in the bortezomib binding pocket of PSMB5 (Ala49→Thr), as well as up to 60-fold upregulation of PSMB5 protein expression, were observed [31]. The Ala49Thr mutation has since been found in independently generated bortezomib-resistant myeloma cell lines [32], as well as other bortezomib-resistant cell lines from different hematological lineages generated in a similar way [33, 34]. Furthermore, bortezomib-resistant cell lines which do not possess any PSMB5 mutations have been shown to have upregulated PSMB5 expression, although this has not always appeared to be the main mechanism of resistance [35, 36].

However, until recently, these observations from in vitro studies had not been seen in patients with myeloma. Several studies that sequenced patient samples, largely at diagnosis but also at relapse, showed no correlation between PSMB5 single nucleotide polymorphisms (SNPs) and bortezomib resistance, and no mutations within the bortezomib binding pocket [37–40]. Therefore, for a time, the idea of proteasome mutation and upregulation playing an important role in bortezomib-resistant patients fell out of favor among researchers. However, a recent study which conducted deep sequencing on a patient with relapsed myeloma found low-frequency PSMB5 mutations which correlated with resistance, and that have been confirmed to confer resistance in vitro [41]. Furthermore, overexpression of PSMB5 that correlated with bortezomib resistance has been detected in one patient [42]. Thus, these mechanisms of resistance may play an important role for some patients resistant to bortezomib.

4.5.2 Drug Efflux

The ATP-binding cassette (ABC) transporters are a superfamily of membrane transport proteins that play a well-established role in the efflux of drugs, and thus the development of drug resistance, so much so that some of them were originally

discovered and named as multidrug resistance proteins [43]. Though bortezomib efflux by multidrug resistance proteins MRP1, MRP2, MRP3 (ABCC1, ABCC2, and ABCC3), and breast cancer resistance protein (ABCG2) has been tested, only the multidrug resistance protein MDR1 (also known as ABCB1 or p-glycoprotein) has been associated with bortezomib efflux and bortezomib resistance in in vitro settings [44–46]. Although this has been largely demonstrated in overexpression systems, a recent study has shown that hypoxia increased both MDR1 and proteasome inhibitor resistance, and that this resistance could be reversed using a MDR1 inhibitor [47]. However, analysis of parental myeloma cell lines and clinical samples has found little to no association between MDR1 and bortezomib resistance, suggesting that bortezomib may be a poor substrate for MDR1, and that MDR1 is unlikely to play a significant role in bortezomib-resistant myeloma [44, 48–50].

4.5.3 Plasma Cell Differentiation

Expression of the UPR-activated transcription factor XBP1s is required for B-cells to differentiate into plasma cells and produce immunoglobulin [51]. Leung-Hagesteijn et al. found that loss of XBP1s, and thus de-commitment to plasma cell differentiation, is able to confer bortezomib resistance in myeloma [52]. Suppression of XBP1s in myeloma cell lines induced a switch from a mature plasma cell phenotype to a pre-plasmablast phenotype, including a decrease in immunoglobulin production [52]. With a lower protein production load, the pre-plasmablast-like cells showed lower basal UPR activation, and thus increased resistance to proteasome inhibitors [52]. The reverse also holds true; myeloma cells with a more mature phenotype express higher levels of XBP1 have higher immunoglobulin production, and are more sensitive to bortezomib [53]. Furthermore, loss of plasma cell maturation has also been associated with bortezomib resistance in animal models [54]. Both innate and acquired bortezomib resistance in a plasma cell malignancy in Bcl-xl/Myc transgenic mice was found to correlate with loss of plasma cell maturation markers, and induction of plasma cell maturation was able to render these cells sensitive to bortezomib [54].

Changes to XBP1s and plasma cell maturation with bortezomib resistance have also been observed in patient samples. It was found that, at diagnosis, the majority of myeloma cells were XBP1s positive plasma cells or plasmablasts [52]. However, some patients whose disease progressed on bortezomib had a large subpopulation of XBP1 negative, less differentiated myeloma cells [52]. Furthermore, it was cells of this phenotype which went on to survive bortezomib-based therapies as a minimal residual disease [52]. Other studies have also found that patients with myeloma that was sensitive to bortezomib therapy had higher paraprotein expression and higher levels of XBP1s [53]. Other studies have also identified XBP1/XBP1s levels to be a marker of bortezomib response, and overexpression of XBP1s was able to increase

the bortezomib sensitivity of a bortezomib-resistant myeloma cell line, although notably XBP1 knockdown was unable to induce bortezomib resistance in bortezomib sensitive cells [55]. Thus, there is solid evidence to suggest that plasma cell dedifferentiation contributes to bortezomib resistance, though this is unlikely to be the case for all patients.

4.5.4 Upregulation of Heat Shock Proteins

Heat shock proteins are a large family of molecular chaperones which play a key role in protein folding and trafficking, as well as degradation of unfolded proteins [56]. Thus, heat shock proteins are upregulated by the UPR as a cytoprotective mechanism and have been found to be upregulated in myeloma cells exposed to bortezomib [56]. It is therefore unsurprising that heat shock proteins may play a role in bortezomib resistance.

BiP is a member of the heat shock protein family and plays a critical role in activation of the UPR [2]. Some studies have reported that BiP expression increases with disease progression, although other studies suggest that this is not always the case [57–59]. Despite this, upregulation of BiP has been found to correlate with bortezomib resistance, and inhibition of BiP via multiple mechanisms was able to enhance cell death caused by bortezomib exposure [57, 60, 61]. To this end, an anti-BiP monoclonal antibody has been engineered, as BiP has also been observed on the surface of myeloma cells, but to date it has only been tested in one relapsed refractory patient, who achieved a partial remission before relapse [59].

HSP90 is another heat shock protein involved in the regulation of unfolded proteins in the ER and has been found to be upregulated by bortezomib treatment [62, 63]. The combination of bortezomib and HSP90 inhibition causes synergistic cell death in both myeloma cell lines and primary samples [64, 65], and has also been tested in phase I/II clinical trials, although these are yet to progress further [63, 66, 67]. Given its role in protein homeostasis, it is plausible that HSP90 not only synergizes with bortezomib but may contribute to bortezomib resistance. Although this has been shown in other hematological cancers, there is yet to be an in-depth study examining the role HSP90 plays in the development of bortezomib resistance [68, 69]. However, heat shock protein HSPB8 has been shown to play a role in bortezomib resistance, as least in vitro [70]. A myeloma cell line made resistant to bortezomib was found to have increased levels of HSPB8, and overexpression of HSBP8 in wildtype cells to a similar level to that found in their resistant counterparts was able to confer bortezomib resistance by increasing the clearance of protein aggregates [70].

4.5.5 Autophagy

Activation of the UPR has been shown to upregulate autophagy, a process by which cytosolic contents are surrounded by a double membrane to form a vesicle called an autophagosome, which then fuses with the lysosome in order to degrade its contents [71]. Autophagy has been shown to be critical for plasma cell survival, especially of long-lived plasma cells [72]. Furthermore, autophagic degradation of proteins marked for degradation can promote cell survival during proteasomal inhibition, and thus bortezomib treatment often results in upregulation of autophagy-related proteins [71, 72]. Thus, it is not surprising that autophagy has been implicated in bortezomib resistance. Indeed, the ability of both BiP and HSPB8 to confer bortezomib resistance has, in some cases, been tied to the development of autophagy [57, 61, 70].

The ability of a myeloma cell to increase autophagy has been correlated with sensitivity to bortezomib, with cells that are unable to increase their autophagic capacity having greater sensitivity to proteasome inhibition [73]. Furthermore, overexpression of autophagy-inducing proteins has been shown to cause bortezomib resistance, while inhibition of these same proteins enhances bortezomib-induced cell death [73–75]. Comparing the differential expression of microRNAs in bortezomib sensitive and resistant myeloma cells, Jagannathan et al. found that miR-29b is downregulated in bortezomib-resistant cells, and its replacement with a synthetic mimetic increased bortezomib-induced cell death through both reduction in proteasome activity and inhibition of autophagosome formation [76]. Application of an anti-β_2-microglobin (β_2M) monoclonal antibody to bortezomib-resistant myeloma cell lines and patient samples enhances bortezomib-induced cell death, which was in part due to inhibition of autophagy [77], while a phase I clinical trial of the autophagy-inducing drug hydroxychloroquine in combination with bortezomib in patients with relapsed/refractory myeloma has also been conducted, though results were modest at best [78].

Combined, the above findings would suggest a key role for autophagy in bortezomib resistance. However, as is often the case in cancer, the situation is complex [72]. Kawaguchi et al. found that inhibition of the later stages of autophagy enhanced bortezomib-induced cell death, but inhibition of early autophagy actually attenuated it [79]. Furthermore, although autophagy was upregulated in bortezomib-resistant cells, knockdown of ATG5, required for autophagosome formation, inhibited bortezomib-induced cell death of myeloma cells [80]. It has been suggested that these divergent responses may be due to what stage of autophagy is inhibited, with inhibition of late autophagy, where cellular contents have already been sequestered but are unable to be recycled, being more likely to cause cytotoxic effects [72].

4.5.6 The Bone Marrow Microenvironment

It is becoming increasingly clear that the tumor microenvironment plays a key role in resistance to therapy [81]. The bone marrow microenvironment is complex, consisting of several types of cells, including bone marrow stromal cells (BMSCs), endothelial cells, osteoblasts, osteoclasts, and many types of immune cells, as well as extracellular matrix, chemokines, and growth factors [82]. Bortezomib resistance conferred by the bone marrow microenvironment can be generally classified into two main categories, resistance generated by adhesion to various components of the microenvironment, and resistance mediated by soluble factors secreted by the microenvironment.

There are several different physical interactions between myeloma cells and their microenvironment which are able to confer drug resistance [83]. Integrin-β7 expression in myeloma cells correlates with poor patient survival and assists in myeloma cell adhesion to bone marrow stromal cells and fibronectin, the latter of which is able to convey bortezomib resistance [84]. Coculture with BMSCs also confers bortezomib resistance in myeloma cell lines, which can be prevented by inhibition of the chemokine receptor CXCR4, which blocks adhesion [85]. Furthermore, myeloma cells are able to induce a microenvironment more permissive to bortezomib resistance, for example, through inducing BMSCs to become more like cancer-associated fibroblasts [86]. Direct contact with these cells has been shown to induce bortezomib resistance in myeloma cell lines via β-catenin upregulation [86].

As well as physical contact, the bone marrow microenvironment secretes a number of soluble factors which are able to contribute to bortezomib resistance in myeloma cells [83]. For example, multiple members of the interleukin family have been found to play a role in bortezomib resistance. Interleukin (IL)-6 is very important for myeloma survival and proliferation, and BMSCs from myeloma patients have been shown to produce more IL-6 than normal BMSC [83, 87]. Furthermore, IL-6 can induce bortezomib resistance via upregulation of JunB, a transcription factor which appears to promote cell proliferation and regulate apoptosis in myeloma [88]. IL-8 is also produced at higher levels by BMSCs from myeloma patients compared to healthy controls, and this can confer bortezomib resistance via NF-κB activation [89]. Similarly, exposure to IL-10, produced by BMSCs upon exposure to the chemokine CCL27, confers bortezomib resistance, which can be reversed by an IL-10 blocking antibody [90].

In myeloma cells, there can exist cross-activation between IL-6 and insulin-like growth factor-1 (IGF-1) [91]. IGF-1 promotes myeloma proliferation through activation of pathways such as Ras and Akt, and the IGF-1 receptor has been shown to be upregulated in bortezomib-resistant cells, with inhibition restoring bortezomib sensitivity [35, 91]. Both IGF-1 and IL-6 have also been shown to activate the phosphotidylinositol 3-kinase (PI3K) pathway, and inhibition of PI3K activity reduced bortezomib resistance induced by coculturing myeloma cells with BMSCs [92]. Other factors able to influence levels of ERK1/2, Akt and/or NF-κB signaling, such as B-cell activating factor, macrophage inflammatory protein-1α, and exosomes

from BMSCs, have also been shown to play a role in bortezomib resistance, suggesting that these pathways may be common resistance mechanisms [93]. Recently, it has also been found that mesenchymal stem cells (MSCs) from bortezomib-resistant patients, but not sensitive patients, produce exosomes which can induce bortezomib resistance via increasing levels of the proteasome subunit PSMA3, which contributes to the chymotrypsin-like activity of the proteasome [94].

4.6 Resistance Mechanisms to Second Generation Proteasome Inhibitors

Second-generation PIs, including carfilzomib and ixazomib, have been clinically available for a significantly shorter period of time than bortezomib, and as such there has been less research into potential resistance mechanisms. However, it is interesting to note that many bortezomib resistance mechanisms, such as heat shock protein regulation, autophagy, and plasma cell dedifferentiation, provide the cell with ways to counteract proteasome inhibition, instead of preventing inhibition from occurring. As such, one might anticipate that these resistance mechanisms result in resistance to any proteasome inhibitor, as they are not dependent on the structure of bortezomib in the way proteasome mutations may be. Indeed, while second-generation PIs have been shown to be effective in the bortezomib-resistant setting, it has already been noted that a degree of cross-resistance does occur, with bortezomib-naïve patients more likely to respond than those who have developed bortezomib resistance [28, 29, 95].

4.6.1 Carfilzomib Resistance Mechanisms

4.6.1.1 Proteasome Mutations

Carfilzomib is based on a different active moiety, and thus interacts with slightly different residues within the binding pocket on the proteasome, as seen in Fig. 4.2 [21, 41]. A number of residues within the binding pocket of PSMB5 do however interact with both bortezomib and carfilzomib, meaning there are mutations in PSMB5 which can confer both bortezomib and carfilzomib resistance [41]. However, other unique interactions between carfilzomib and PSMB5, along with the fact that carfilzomib binds irreversibly, where bortezomib does not, means that often PSMB5 subunits bearing these mutations are less resistant to carfilzomib than they are to bortezomib [41].

4.6.1.2 Drug Efflux

Unlike bortezomib, evidence suggests that carfilzomib is much more likely to be a true MDR1/p-glycoprotein substrate [34, 50, 96, 97]. MDR1 overexpression is seen in both carfilzomib-resistant cell lines generated by long-term exposure to carfilzomib and in carfilzomib-resistant patients, and engineering myeloma cell lines to overexpress MDR1 is sufficient to convey carfilzomib resistance [34, 50, 96, 97]. Upregulation of another ABC transporter, ABCG2 (alternatively referred to as breast cancer resistance protein) has also been seen in carfilzomib-resistant patients but not carfilzomib-resistant cell lines; the significance of this is yet to be investigated [98]. Furthermore, it has been found that pharmacological inhibition of MDR1 is able to significantly increase carfilzomib-induced cell death in carfilzomib-resistant myeloma cell lines [98]. A similar result has also been seen using MDR1 peptide inhibitors in carfilzomib-resistant adenocarcinoma cell lines [96].

4.6.1.3 Autophagy

Carfilzomib is also able to upregulate autophagy in myeloma cells, and the autophagy-linked miR-29b, found downregulated in bortezomib-resistant myeloma cells, was also found to be downregulated in carfilzomib-resistant cells [76, 99]. Furthermore, myeloma cells made resistant to carfilzomib have shown an upregulation of SQSTM1, an autophagy receptor that gathers misfolded proteins into aggregates and links them to autophagic membranes [100]. Notably, overexpression of SQSTM1 is enough to convey resistance to carfilzomib [100, 101]. Inhibition of the autophagic system, both directly by chloroquine and indirectly via histone deacetylase 6 (HDAC6) inhibition (which stops unfolded proteins forming aggregates called aggresomes that can be degraded by autophagy) potentiates carfilzomib-induced cell death [99, 101, 102]. Interestingly, the combination of chloroquine and bortezomib has little to no effect in vitro, suggesting this may be a mechanism more specific to carfilzomib [99, 101].

4.6.1.4 Bone Marrow Microenvironment

As well as conferring bortezomib resistance, exposure to CCL27, which is produced by BMSCs, also confers resistance to carfilzomib [90]. Culturing myeloma cells with BMSCs is able to confer carfilzomib resistance as well as bortezomib resistance [85, 103, 104], and Azab et al. found that inhibition of PI3K was able to prevent this resistance [92]. Like bortezomib resistance, it has also been found that carfilzomib resistance can be induced by incubating cells with exosomes from bortezomib-resistant patient MSCs and that this is due to increases in PSMA3 [94]. The fact that bortezomib-resistant patient MSCs are able to directly generate carfilzomib resistance highlights the potential similarities between carfilzomib and bortezomib resistance generated by the bone marrow microenvironment.

4.6.2 Ixazomib Resistance Mechanisms

Ixazomib is the newest PI to be approved by the FDA [25]. Ixazomib has been found to increase progression free survival (PFS) of RRMM patients and is highly efficacious even in patients with high cytogenetic risk or patients who have previously been treated with a PI [105, 106]. This may be due at least partially to a more favorable pharmacokinetic profile, resulting in a higher plasma concentration and a greater distribution of ixazomib from the blood into tissue compared to bortezomib [107]. Given how new it is to the clinic, relatively little research has been conducted regarding potential resistance mechanisms to ixazomib. However, given its structural similarities to bortezomib, it is likely that there will be overlap in resistance mechanisms, despite the effectiveness of ixazomib in relapsed/refractory myeloma.

This has already been seen with proteasome mutants found during deep sequencing analysis, where mutations in PSMB5 which conferred bortezomib resistance also conferred resistance to ixazomib [41]. Given that ixazomib is much closer in structure to bortezomib than carfilzomib, as it is based on the same boronate backbone (Fig. 4.2), it is more likely that proteasome mutations which convey bortezomib resistance will also convey ixazomib resistance [21].

While looking at resistance to more commonly used PI, it was found that the autophagy-linked miR-29b, downregulated in both bortezomib and carfilzomib-resistant cells, was also found to be downregulated in myeloma cells that had been made resistant to ixazomib [76]. Using similar cell lines which had been made resistant to either bortezomib, carfilzomib, or ixazomib, Malek et al. found a high degree of cross-resistance between proteasome inhibitors, and that expression of certain long noncoding RNAs (lncRNAs) were dysregulated in all resistant cells compared to the parental cell lines [108]. These same lncRNAs were found to be dysregulated in myeloma cells from patients compared to healthy plasma cells [108]. The lncRNA which stabilizes PSMA3, along with PSMA3 itself, which increases the chymotrypsin-like activity of the proteasome, has also been found to be upregulated in ixazomib-resistant cell lines, as it is in bortezomib and carfilzomib-resistant lines, further highlighting potential similarities between bortezomib, carfilzomib, and ixazomib resistance [94].

4.7 Conclusion

Within the last two decades, PIs have become a standard-of-care in myeloma treatment. However, myeloma cells inevitably become resistant to PIs, posing a significant hurdle to the treatment of patients. Research reaching back almost as long as bortezomib has been in the clinic has demonstrated that bortezomib resistance is highly complex, and can include a variety of mechanisms such as proteasome mutations, upregulation of cellular pathways including heat shock proteins and

autophagy, plasma cell dedifferentiation, and interactions with the bone marrow microenvironment [109].

While newer generations of PIs, including carfilzomib and ixazomib, have proven to be effective in the bortezomib-resistant setting, cross-resistance is already being recognized as an issue [34, 50, 96, 97]. This is likely due to the fact that many bortezomib resistance mechanisms assist the cell in surviving proteasome inhibition, instead of preventing it, and are thus able to promote survival regardless of the structure of the proteasome inhibitor used. Thus, although proteasome inhibitors have been an important advance in myeloma pharmacotherapy, resistance to these agents represents a serious clinical problem that often requires combining more than one novel agent to target non-overlapping aspects of myeloma biology.

Acknowledgment This work was supported by an MF and MH Joyner Scholarship, the RAH Research Fund, an Australian Government Research Training Program Scholarship, a National Health and Medical Research Council of Australia (NHMRC) Peter Doherty Biomedical Early Career Fellowship (1071945), a Royal Australasian College of Physicians Research Establishment Fellowship, the Fay Fuller Foundation, and a Senior Research Fellowship from the NHMRC. The authors would also like to thank Dr. Melissa Pitman for her assistance with the protein structure analysis.

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