Review

Discovery Medicine 2025; 37(201): 2072–2088
https://doi.org/10.24976/Discov.Med.202537201.178

Untangling the Mechanisms of Proteasome Inhibitor Resistance in Multiple Myeloma

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Submitted: 16 May 2025 Revised: 22 September 2025 Accepted: 28 September 2025 Published: 20 October 2025

Multiple myeloma (MM) is a hematological disease that remains largely incurable. The introduction of proteasome inhibitors (PIs) at the turn of the century led to prolonged survival of patients, though most will inevitably develop resistance over successive treatments. It is of great clinical interest, then, to resensitize MM cells to PIs. This review discusses some of the most well-established resistance pathways, such as alterations in the proteostasis network and the bone marrow microenvironment. Novel targets are highlighted and placed in the context of potential treatment strategies where applicable.

Keywords: multiple myeloma; proteasome inhibitors; therapeutic resistance; proteasome stress response

Introduction

Multiple myeloma (MM) is a largely incurable blood cancer secondary to the uncontrolled proliferation of clonal, genetically unstable plasma cells [1,2]. The American Cancer Society estimated 36,110 new cases of MM will be diagnosed in 2025 with the average adult running a 0.9% chance of developing the disease [3]. While there has been significant improvement in treatment in the past 20 years, a cure is still elusive for many patients [4,5].

One of the mainstays used in the treatment of MM is a class of drugs known as proteasome inhibitors (PIs), which inhibit protein degradation via the ubiquitin proteasome system [6]. Despite the success of PIs, intra-tumoral heterogeneity and the inevitable development of resistance over each successive round of treatment have proved to be substantial roadblocks to a cure for all [7]. This selection of resistant cells is at the basis of relapse and eventual mortality [8]. As such, it is of great clinical interest to elucidate the exact molecular mechanisms of resistance to develop more effective therapies.

In this review, we will begin with a description of the proteasome and PIs, then discuss current knowledge of PI resistance pathways in MM, and end with an analysis of select potential targets.

Myeloma Cells Depend on Proteasome Activity for Survival

The Proteasome in Multiple Myeloma

Eukaryotic cells constantly produce proteins to maintain vital functions, signal other cells, etc. Extracellular and transmembrane proteins are synthesized by ribosomes, folded in the endoplasmic reticulum (ER), and then transported along the Golgi apparatus and secretory pathway to their destination [9]. While quality control (QC) mechanisms exist within the ER, errors are common, leading to misfolded proteins which represent a potential toxic species that may lead to cytotoxicity [10–12]. To prevent buildup and maintain proteostasis, these misfolded proteins are marked for degradation via the ER associated degradation (ERAD) pathway that relies on proteasomal degradation (Fig. 1, Ref. [13]) [14,15]. Accumulation of proteins within the ER triggers the unfolded protein response (UPR), a tripartite homeostasis pathway that relies on 3 ER receptors: inositol-requiring enzyme 1 (IRE1), Activating Transcription Factor 6 (ATF6), or Protein Kinase-like ER Kinase (PERK) [16]. Initially, the IRE1 and ATF6 pathway support the selective transcription of chaperones, such as binding immunoglobulin protein (BiP), and lipid for ER biosynthesis, while the PERK pathway triggers a global reduction in protein translation with the overall results to alleviate the proteotoxic stress derived from newly synthesized proteins. Failure to resolve this stress results in the ultimate transcription of CHOP and GADD34, signaling commitment to apoptosis.

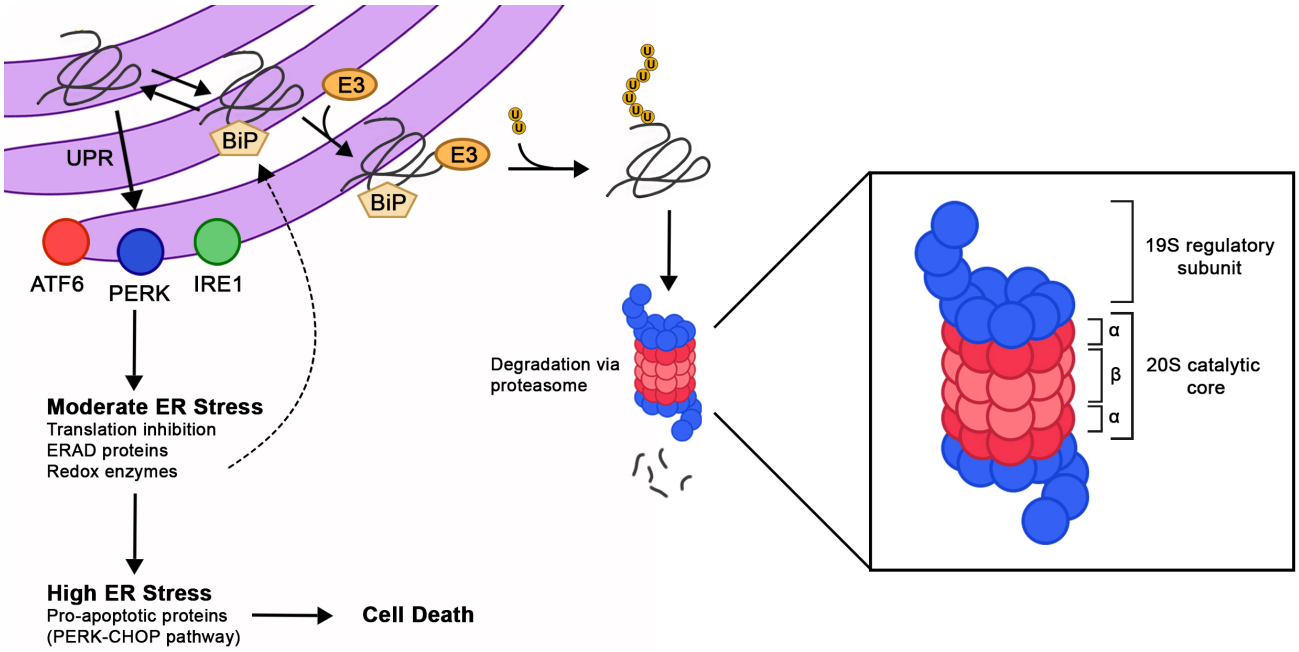

Fig. 1. A schematic of unfolded protein response (UPR) and the proteasome. Unfolded proteins in the endoplasmic reticulum (ER) trigger UPR. The three canonical branches (Activating Transcription Factor 6 (ATF6), Protein Kinase-like ER Kinase (PERK), and inositol-requiring enzyme 1 (IRE1)) are intended to restore proteostasis. An upregulation of chaperone proteins like binding immunoglobulin protein (BiP) may encourage refolding or lead to ubiquitination by E3 ubiquitin ligases. Once ubiquitinated, proteins are shuttled to the proteasome for degradation. If proteostasis is not restored, UPR may trigger apoptosis and cell death. Inset: The 26S proteasome is a barrel-shaped complex consisting of 19S regulatory subunits (blue) and a 20S catalytic core (pink/red). The 20S core consists of both structural α (red) and catalytic β (pink) components. This figure was adapted from Moscvin *et al.* [13], available under the Creative Commons Attribution (CC BY4.0) license. ERAD, ER associated degradation.

The key to proteostasis is the 26S proteasome, a complex of multiple subunits responsible for the degradation of misfolded and regulatory proteins in an adenosine triphosphate (ATP)-dependent manner [17]. The complex consists of a 19S regulatory cap for recognition of ubiquitinated protein and a barrel-shaped 20S catalytic core consisting of four rings, each containing seven distinct subunits, as shown in Fig. 1 inset [18]. The 20S core contains structural α subunits as well as catalytic β subunits, the most well-known of which are β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like) [17,19]. Proteins marked for degradation via ubiquitination are shuttled through the 19S base into the 20S core and degraded into smaller peptides while ubiquitin is recycled [9]. This particular pathway is known as the ubiquitin proteasome system (UPS).

A defining characteristic of MM is an over synthesis and secretion of immunoglobulins, creating a unique dependency of MM cells on proteostasis mechanisms, including the proteasome and UPR [9,20,21]. It has been shown that malignant cells are far more sensitive to perturbation of the proteasomal degradation pathway than healthy cells, likely due to increased protein synthesis and decreased quality control mechanisms [22,23]. The initial observation that MM cells were exquisitely sensitive to proteasomal inhibition as compared to other cancer cell lines led to the speculation that proteasome blockade would result in stabilization of IκB, leading to decreased nuclear factor κB (NF-κB) signaling, a tonic pro-survival effector in MM [24,25]. Subsequent studies demonstrated that NF-κB inhibitors could not recapitulate the cytotoxic effect of PI [26,27]. It was later demonstrated that intrinsic dependency on the proteasome for survival due to baseline proteotoxic stress stemming from sustained and inaccurate protein synthesis is at the base of PI sensitivity in MM [28]. To this effect, PIs target a biological vulnerability of MM and paved the way for targeting of proteostasis as a novel therapeutic approach to MM. Bortezomib is the first PI approved by the US Food and Drug Administration (FDA) for treatment of MM in the early 2000s, and is discussed below [29].

FDA-approved PIs

Originally designed as a tool to investigate proteasome function, PIs have now become a central component in the treatment of plasma cell dyscrasias [30]. Bortezomib (BTZ, Fig. 2a) was the first PI introduced to the clinics and its profound impact on the survival of MM patients led to its FDA approval in the early 2000s [29,31]. BTZ, administered as a subcutaneous bolus, is a reversible inhibitor of the β5 (*proteasome subunit β5* (*PSMB5*), chymotrypsin-like) and to a lesser extent the β2 (*PSMB7*, trypsin-like) subunits of the

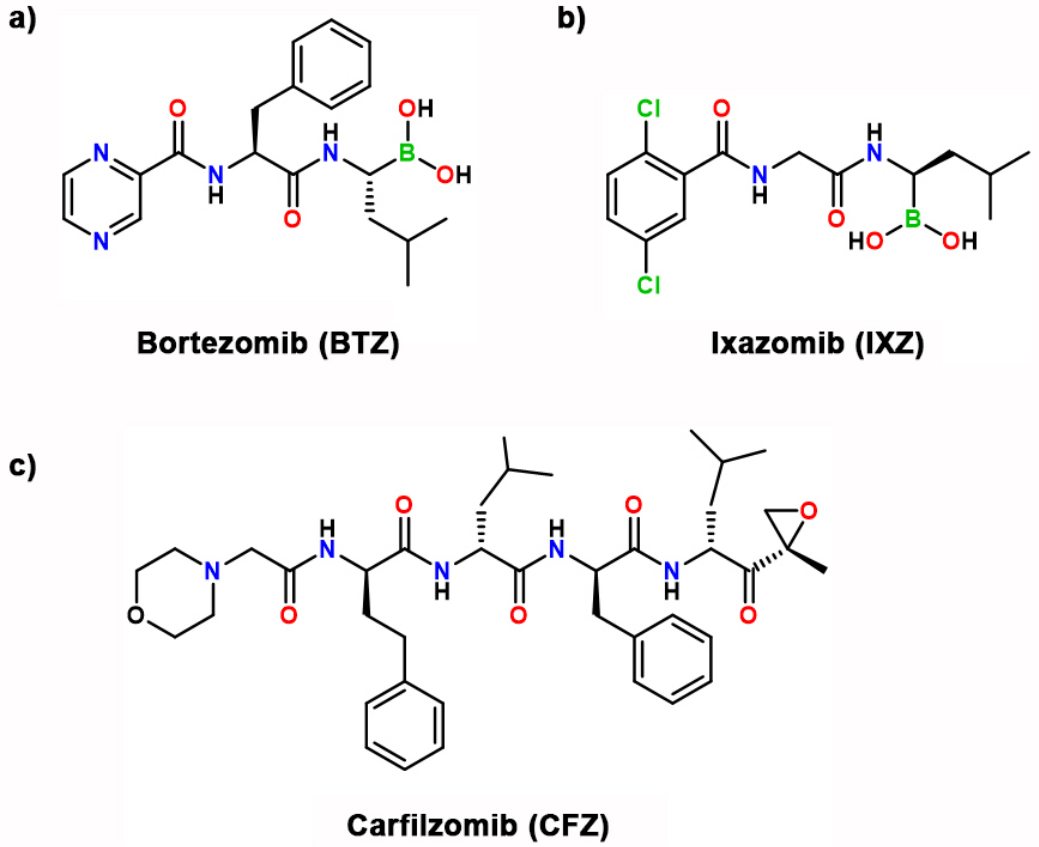

Fig. 2. Structures of proteasome inhibitors used to treat multiple myeloma. Chemical structures of (a) BTZ, (b) IXZ, and (c) CFZ. This figure was generated in ChemDraw 19.1 (Revvity Signals, Waltham, MA, USA).

proteasome, resulting in inhibition of ubiquitinated proteins awaiting proteasome degradation and resultant apoptosis. Since its approval in MM, BTZ has been proven effective against other cancers [32,33]. Initially, it was proposed that BTZ's toxicity stemmed from the inhibition of the NF-κB pathway via the stabilization of its inhibitors, though this did not fully explain the observed cytotoxicity in MM cells [27,34]. It was later discovered that BTZ exacerbates proteotoxic stress by impairing the DNA damage response and triggering UPR which is the currently agreed-upon mechanism [35,36].

In addition to BTZ, Carfilzomib (CFZ, Fig. 2b) and Ixazomib (IXZ, Fig. 2c) are also FDA-approved for use in MM patients. BTZ and IXZ are both dipeptide analogues containing boronic acid warheads while CFZ is a tetrapeptide with an epoxy ketone warhead [37]. All three are specific to the β5 subunit of the proteasome, though studies have found that co-inhibition of a second subunit, specifically β1 or β2, is necessary for cytotoxicity to occur [38–40].

CFZ, unlike BTZ and IXZ, irreversibly binds β5 and is administered by IV [41]. Possibly due to its pharmacokinetics and irreversible proteasome inhibition, CFZ has shown a signal for cardiovascular and renal adverse events that is much stronger than compared of BTZ. Careful use in patients with pre-existing renal or cardiac disease is advised and close monitoring for adverse events is required [42]. While some patients are responsive to CFZ after developing resistance to BTZ, there have been reports of cross-resistance [43]. IXZ is an orally bioavailable boronic acid PI [19,44]. There are conflicting reports on IXZ activity in BTZ/CFZ exposed and/or refractory patients [45–47].

PIs are used across the entire treatment phases of care of MM patients from induction, through consolidation, and in maintenance. Immunomodulatory drugs lenalidomide, pomalidomide and CD38-targeting antibodies daratumumab and isatuximab are common partners of PIs in combinatorial treatment regimens. Bortezomib is approved for frontline treatment of multiple myeloma, typically in a triple or quadruple combination with lenalidomide and dexamethasone (RVd) or daratumumab, lenalidomide and dexamethasone (DaraRVd) with or without autologous stem cell transplant [48,49]. CFZ is approved in second line treatment in combination with daratumumab or isatuximab plus dexamethasone (DaraKd or IsaKd), lenalidomide and dexamethasone (KRd) or dexamethasone alone (Kd) [50,51]. Finally, IXZ is approved in combinations with lenalidomide and dexamethasone (IRd) for patients who have received at least one prior line of therapy [52].

Resistance Mechanisms and Pathways

Changes in Proteasome and Proteasome Stress Response

Missense mutations in the binding domains of anticancer drugs have been shown to drive resistance by leading to abolishing drug-target binding. For example, the Epidermal Growth Factor Receptor (EGFR) may develop a T790M mutation, resulting in resistance to first- and second-generation inhibitors due to steric hindrance of the binding site [53]. As the β5 proteasome subunit is the main binding site for clinically available PIs, it has been hypothesized that mutations in the drug binding domain may lead to PI resistance [54]. Investigators have screened various *PSMB5* gene mutations in KMS-18 and KMS-27 cell lines and identified at least two single amino acid substitutions in the binding pocket that were sufficient to confer PI resistance or sensitivity [55]. Specifically, an A49V (alanine to valine) substitution sterically hindered PIs by blocking access to the substrate, while a T21A substitution opened the binding pocket, allowing for easier CFZ ingress and thus an increase in sensitivity. The A49V mutation was independently confirmed *in silico* to prevent BTZ from binding any catalytically active β5 residues [56]. Other investigators also noted an A79G substitution in the binding pocket of a BTZ-resistance version of the murine myeloma cell line 5TGM1, mimicking the effects of an A79T substitution in human patient cells [57,58]. While these mutations have been well modeled in cell lines, their biological impact is unclear as *PSMB5* mutations have not been detected at significant rates in patients with acquired PI resistance [58].

Dysregulation of Apoptotic Pathways

Dysregulation of programmed cell death is a signature of cancer. Loss of function in pro-apoptotic pathways allows cancer to propagate, modulate the microenvironment, and even evade some therapies. Consequently, there has been long-standing history in understanding dependency of cancer on specific anti-apoptotic proteins in order to develop targeted drugs [59]. While several apoptotic pathways have been identified, this review will only discuss the intrinsic pathway due to its clinical relevance in the context of BCL-2 inhibitor venetoclax. For a more comprehensive overview of apoptosis and cancer, we recommend Tian *et al*. [60].

The intrinsic apoptosis pathway occurs within the mitochondrial outer membrane (Fig. 3a, Ref. [60,61]). Under homeostatic conditions, antiapoptotic proteins such as B cell lymphoma XL (BCL-XL), Myeloid Cell Leukemia Sequence 1 (MCL1). B cell lymphoma 2 (BCL-2) suppresses the proapoptotic BH3-only proteins BCL-2 Interacting Mediator (BIM), BH3 interacting-domain death agonist (BID), and p53 upregulated modulator of apoptosis (PUMA). Intrinsic apoptosis stimuli trigger the release of BIM from antiapoptotic BCL-2 protein. Once released, BIM forces a conformational change in BCL-2 homologous antagonist killer (BAK)/BCL-2-associated X protein (BAX), thus allowing BAK/BAX to oligomerize (Fig. 3a) [62]. BAK/BAX form pores within the membrane, releasing cytochrome *c*. Apoptotic protease activating factor 1 (APAF-1) combines with cytochrome *c* and deoxyadenosine triphosphate (dATP) to form the apoptosome which in turn cleaves procaspase-9 into the active form (Fig. 3b). Caspase-9 in turn cleaves caspase-3/6/7 into their active forms, thus initiating terminal apoptosis [60]. To escape intrinsic apoptosis, cancer cells may overexpress antiapoptotic proteins like BCL-2, acquire caspase and cytochrome *c* mutations. Recent progress in overcoming cancer's resistance to apoptosis has focused on BCL-2 inhibition.

Venetoclax is a highly specific inhibitor of BCL-2 and has been approved as a front-line therapeutic for chronic lymphocytic leukemia and myeloid leukemia [63]. Venetoclax binds and inhibits BCL-2 (Fig. 3c), thus releasing BIM, BID, and PUMA. BIM activates BAX and BAK, initiating the intrinsic apoptotic pathway. It was hypothesized that the combination therapy of venetoclax with current PI-containing regimens for MM would resensitize relapsed/refractory patients to PI by promoting intrinsic apoptosis. Different from other hematologic malignancies, myeloma cells predominantly rely on MCL1 rather than BCL-2 as an anti-apoptotic protein. However, it was observed that t(11;14), a cytogenic abnormality present in about 15–20% of myeloma patients, serves as a biomarker of enhanced BCL-2 dependency in myeloma [64]. A multicentre phase 3 trial, BELLINI, evaluating BTZ and dexamethasone alone or in combination with venetoclax in relapsed and refractory MM patients, reported a significant increase in progression-free survival, particularly for patients harboring t(11;14). However, the study was put on hold due to excess mortality in the investigational arm, largely stemming from infectious signals with high-dose venetoclax [65]. Responses were variable and Gupta *et al*. [66] noted that the observed variability in patient response to venetoclax may be driven by a difference in chromatin accessibility. Specifically, venetoclax-sensitive patients demonstrated increased availability of binding sites for the B-cell transcription factor basic leucine zipper transcription factor, ATF-like, or BATF. As such, improved tools to predict vulnerability of myeloma to venetoclax, such as BH3 profiling, may be useful for treatment personalization.

Aggresome Pathway

While the UPR pathway is the most well-known degradation pathway in eukaryotic cells, alternate pathways exist. The aggresome pathway is one such example. Aggresomes are perinuclear protein aggregates formed by sequestering misfolded proteins into Vimentin cages, which are then trafficked for degradation in autolysosomes (Fig. 4) [67]. This isolation of misfolded proteins in MM thus

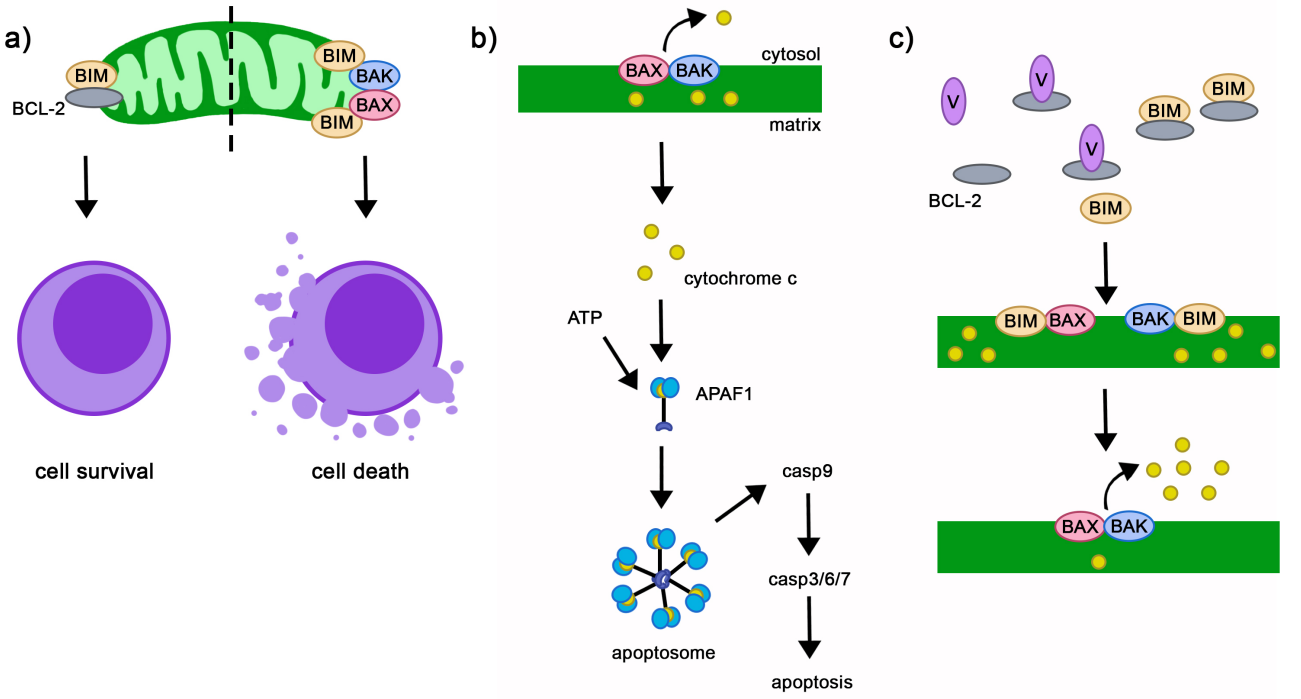

Fig. 3. Simplified representation of the intrinsic apoptosis pathway and its upregulation via inhibition of B cell lymphoma 2 (BCL-2) with venetoclax. (a) Inhibition of BCL-2 Interacting Mediator (BIM) on the outer mitochondrial membrane by BCL-2 prevents activation of BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK), leading to cell survival. Uninhibited BIM activates BAX and BAK, which then polymerize to form a pore. This pore formation initiates an apoptotic cascade, ending in cell death. (b) Upon oligomerization, BAX (red) and BAK (blue) form a pore on the mitochondrial outer membrane, releasing cytochrome *c* (yellow). Apoptotic protease activating factor 1 (APAF-1) combines with adenosine triphosphate (ATP) and cytochrome *c* to form the apoptosome, triggering a pro-apoptotic caspase cascade. (c) Venetoclax (purple, V) inhibits BCL-2 (grey), releasing BIM (yellow-orange). BIM activates BAX and BAK, allowing them to form a pore in the outer mitochondrial membrane, thus releasing cytochrome *c* (yellow). This figure was adapted from Tian *et al.* [60] and Griffioen *et al.* [61], available under the Creative Commons Attribution (CC BY4.0) license.

prevents cells from triggering UPR. Histone deacetylase 6 (HDAC6) facilitates this transport along microtubules via dynein, thus making it a target of interest in overcoming PI resistance by blocking a potential compensatory pathway that is upregulated upon PI [68].

Multiple HDAC inhibitors (HDACi) have been reported as promising therapeutics in cancer treatment, although side effects may be limiting. An HDAC6-specific drug has been evaluated in clinical trials in MM [69]. Ricolinostat (ACY-1215) is a selective HDAC6 inhibitor that was well-tolerated in combination with BTZ and dexamethasone in a 2017 phase II trial [70]. The selectivity of ricolinostat is reflected in decreased toxicity relative to *pan*-HDACi, though the overall clinical response rate was 37%. No new clinical trials for ricolinostat and MM have been filed as of the writing of this review, suggesting that development has been halted.

In addition to HDAC inhibition, other chemotherapeutic agents have been shown to affect the aggresomal pathway. For example, the topoisomerase inhibitor doxorubicin has been reported to downregulate aggresome-promoting factors like Vimentin and HDAC6 after BTZ treatment [71]. In its liposomal formulation, doxorubicin is FDA approved for use in combination with BTZ in relapsed MM patients. Furthermore, a phase I/II clinical trial suggests BTZ refractory patients will respond favorably to CFZ/doxorubicin combination therapy, suggesting that targeting the HDAC6 pathway may play a role in overcoming BTZ resistance by targeting alternative protein degradation pathways [72].

Autophagy Pathway

Autophagy is a well-conserved proteostasis mechanism wherein polyubiquitinated proteins, such as those in aggresomes, are delivered to autophagic vacuoles and subsequently degraded by lysosomes [73]. Due to its close tie to the aggresomal pathway, autophagy is crucial for proteostasis.

In MM, autophagy is an alternative method to the UPS for the removal of excess and misfolded protein, therefore contributing to protein homeostasis and cell survival [74]. Autophagic degradation of polyubiquitinated proteins relies on the fusion of vacuoles with lysosomes through

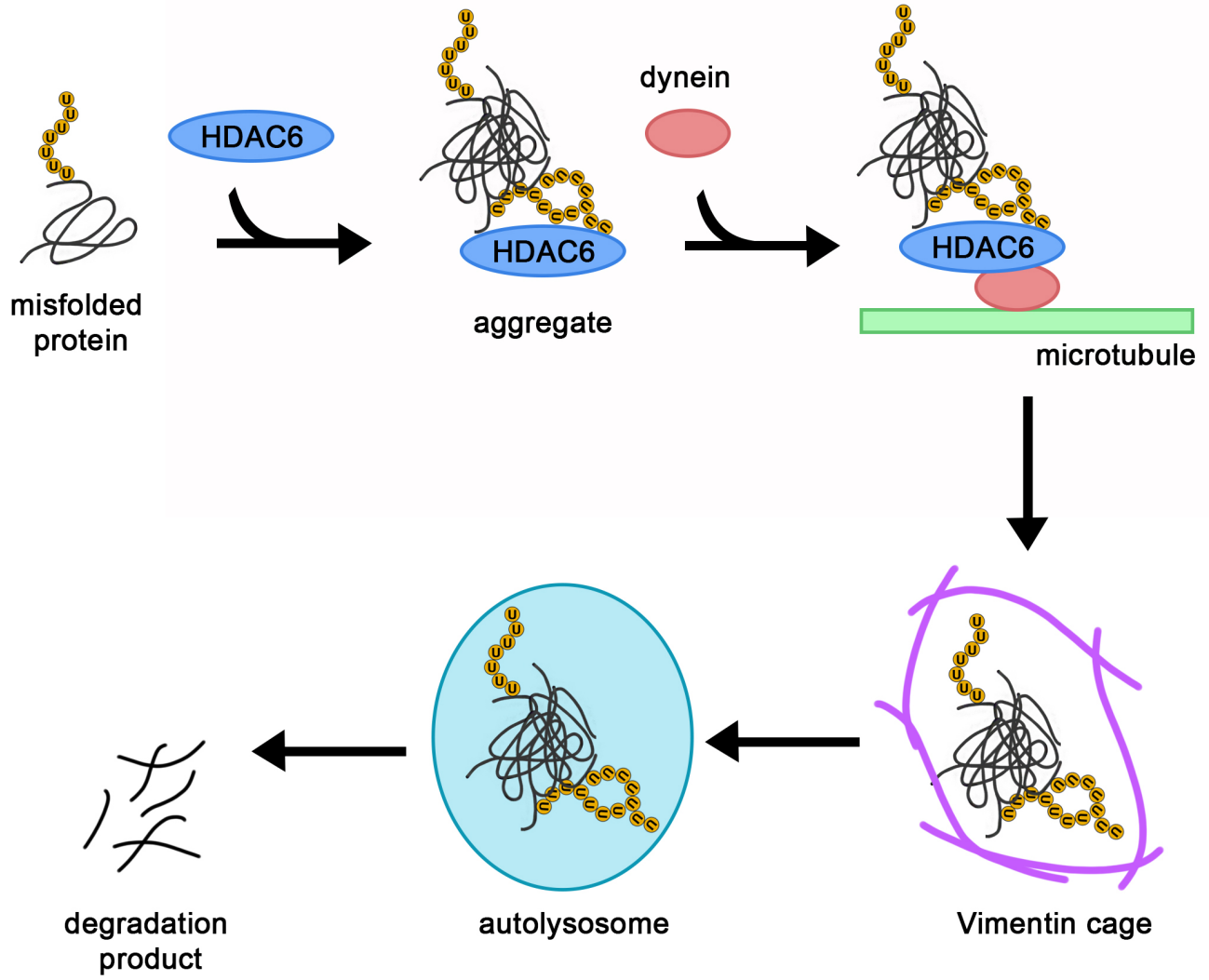

Fig. 4. A simplified schema of aggresome formation and degradation. Misfolded proteins marked for proteasomal degradation are aggregated and then facilitated toward the nucleus along microtubules by histone deacetylase 6 (HDAC6) (dark blue) and dynein (red), where they are sequestered in Vimentin cages (purple). The aggresomes are eventually shuttled to autolysosomes and degraded. This figure was generated in Adobe Photoshop 22.5.0 (Adobe, San Jose, CA, USA).

a sequestosome-1 (SQSTM1/p62)-dependent mechanism, with valosin-containing protein (p97/VCP) assisting with aggresome degradation [75,76]. A toll like receptor (TLR)-mediated increase in cargo receptor p62 expression after PI treatment suggests that autophagy is a compensatory mechanism that may contribute to PI resistance in MM [77]. In addition, a ligand specific to the ZZ-type zinc finger domain of p62 synergizes with BTZ to decrease tumor burden and inhibit osteoclasts in mouse models of MM [78]. However, beyond its role in protein homeostasis, autophagic cell death can be initiated in the face of significant stress, with autophagy thus playing a dual role. In this sense, blocking autophagy could be potentially counteractive as it will also block the autophagy-dependent cell death pathways [79].

A phase I trial of combination therapy with the autophagy inhibitor hydroxychloroquine and BTZ demonstrated better response in refractory MM patients than with BTZ alone, validating that targeting autophagy in conjunction with UPS is a viable strategy [80]. However, MM cell death was not as pronounced as anticipated, potentially due to autophagy's role in triggering cell death in conditions of stress. A new molecular target tied to both the aggresomal and autophagic pathways, Tripartate Motif-Containing protein 44 (TRIM44), will be discussed in a later section.

Metabolism and Antioxidant Response

The link between metabolic state and PI resistance has become a hot topic in the quest to overcome PI resistance in MM patients. The metabolic titan of the cell is the mitochondria, with critical roles in redox homeostasis, apoptosis, and even the degradation of unfolded proteins [81,82]. At baseline, MM and other cancer cells are constantly producing reactive oxygen species (ROS) which results in an overexpression of antioxidant proteins [83–85]. The an-

tirheumatic drug auranofin has been used as an antioxidant inhibitor to demonstrate the dependence of MM cell lines on redox homeostasis [86]. Auranofin on its own exhibited cytotoxic activity in MM and other B cell cancers and increased sensitivity to BTZ, leading to death in a caspase-dependent manner. These data provide proof of principle of the additive/synergistic effect of simultaneously targeting mitochondria and the proteasome.

On the proteostasis side, mitochondria have their own version of UPR, known as UPR^{mt}, which is known to offer protective effects to mitochondria in cancer [87,88]. Of note, MM cells characterized by an overexpression of the mitochondrial matrix protease LonP1 proved more resistant to PIs, suggesting that UPR^{mt} may offer some compensation for loss of proteasome function [89]. Alternatively, inhibition of the electron transport chain led to upregulation of the integrated stress response, resulting in decreased protein synthesis and therefore reduced proteasomal load [90]. The attenuation of protein synthesis conferred PI resistance to MM, in line with previous studies that found treating MM cells with the protein synthesis inhibitor cycloheximide reduced sensitivity to PIs [91]. In conjunction with compensation from UPR^{mt}, PI-resistant cells likely have a much greater proteasomal capacity due to a decreased protein load, suggesting that forcing an increase in protein and ROS generation may resensitize cells to PIs.

Tumor Microenvironment

Bone marrow (BM) is a complex mixture of fatty tissue and cells, including stromal cells, osteoclasts, osteoblasts, vascular cells, B cells, and plasma cells [92]. A maladaptive bone marrow milieu has been implicated in disease progression and drug-resistance in multiple myeloma patients as well as other hematological malignancies [93,94]. As such, drugs directly targeting MM cells while modulating the BM microenvironment have proven highly successful [95].

Stromal Cells

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into several non-hematopoietic cell lineages, such as fibroblasts or adipocytes [96,97]. Bone marrow stroma cells (BMSCs) produce critical survival factors for malignant plasma cells, such as interleukin 6, insulin-like growth factor 1, and stromal cell-derived factor 1α [98–101]. In addition, they also attract myeloid populations via chemokine (C-C motif) ligand 2-chemokine (C-C motif) receptor 2 (CCL2-CCR2) interactions. The myeloid population then forms a feed-forward loop with MSCs via production of IL-1β, generating a favorable milieu for MM cell growth [102]. The hypoxic environment in the bone marrow also triggers nuclear stabilization of hypoxia-inducible factor 1-α, which in turn serves to promote angiogenesis and facilitate MM growth [103]. In addition to creating a permissive environment, investigators observed transcriptomic and epigenomic remodeling in MM cell lines after exposure to BMSCs [104,105]. Ten of these MM-stromal interaction genes were labelled as prognostically significant, including A-kinase anchor protein 12 (*AKAP12*) and versican (*VCAN*) which have been implicated in the progression of other cancers [106–108]. Therefore, bone marrow stroma cells support MM growth and acquisition of drug resistance through pleiotropic mechanisms.

NK Cells

Natural killer (NK) cells are cells of the innate immune system that are capable of killing tumor cells without human leukocyte antigen engagement. Single cell studies have uncovered early and sustained alteration of NK cells in plasma cell disorders with a progressive decline in numbers during myeloma evolution [109]. BTZ has been noted to downregulate the expression of NK-inhibitory ligands, thus facilitating NK-based tumor cell lysis [110]. However, some data suggest that BTZ treatment decreases the pool of circulating NK cells, thus compromising innate immunosurveillance [111]. Still, the use of NK cells expanded *ex vivo* (eNK) is under investigation as a potential treatment for MM. Mouse xenograft studies found that pre-treating mice with a combination of daratumamab, BTZ, and dexamethasone improved the efficacy of eNK, leading to prolonged survival [112]. Early phase studies of autologous NK cells either unmodified or cultured to become cytokine induced memory like and armored with a CD38-targeting peptide have shown safety and a signal of activity in myeloma patients undergoing autologous stem cell transplant [113,114].

Osteoclasts and Osteoblasts

Osteoblasts and osteoclasts are cells responsible for the buildup and breakdown of bone tissue, respectively. This homeostasis is partially driven by extracellular Ca^{2+} levels. The Na^{+}-Ca^{2+} exchanger 1 (NCX1) is a bidirectional transporter highly expressed in many types of cancer, including MM. High extracellular Ca^{2+} levels were found to increase NCX1 expression in MM cell lines which in turn promoted osteoclast-associated genes such as receptor activator of nuclear factor-κB (RANKL) [115]. RANKL overexpression leads to increased osteoclastogenesis, thus playing a role in the development of myeloma-related osteolytic lesions. Upon NCX1 knockdown, MM cells lose viability, in turn suppressing osteoclast differentiation due to decreased RANKL secretion. Mechanistically, NXC1 promotes autophagy, thereby enhancing PI resistance in MM cell lines as well as mouse xenografts [116]. The development of highly specific NCX1 inhibitors may prove therapeutically valuable to counteract PI resistance and mitigate osteoclastogenesis.

Macrophages

The Shaker group noted that the bone marrow of mice treated with BTZ exhibited a 40% increase in macrophages and 20% increase in granulocytes compared to untreated controls, with a similar pattern reflected in patient-derived bone marrow biopsies [117]. When MM cells lines were exposed to media conditioned by naïve bone marrow-derived macrophages, MM cells were more likely to become tumor initiating cells. Similar results were observed in some MM lines grown in media conditioned by BTZ-treated bone marrow macrophages. The tumor initiating cells showed enrichment in IL1β or IL1R, suggesting that an IL1β-IL1R relationship plays a role in MM proliferation and PI resistance [117].

Fibroblast growth factors (FGF) are signaling proteins secreted by a variety of cells, including macrophages, and are involved in angiogenesis, cell proliferation, and tissue repair [118,119]. FGF binds their receptors (FGFR) and activate signaling cascades such as and mitogen-activated protein kinases and phosphatidylinositol-2/kinase/protein kinase B [120]. The Giacomini lab found that blocking FGF/FGFR signaling in MM led to mitochondrial oxidative stress, DNA damage, and cell death via degradation of c-Myc, even in primary cells derived from BTZ-resistant and relapsed/refractory (RR) patients [121]. As a result, the development of targeted FGF traps is currently under investigation in cell lines as a potential therapeutic approach in conjunction with BTZ [122].

Bone Marrow Adipose Tissue

Bone marrow adipose tissue has historically been viewed as a passive component of the bone marrow milieu but has recently received attention as a potential active player in MM progression and drug resistance [123,124]. As with BMSC, bone marrow adipocytes (BMAds) secrete pro-MM factors, such as IL-6 and leptin [125,126]. Interestingly, exosomes secreted by BMAds isolated from MM patient-derived bone marrow aspirates contain long non-coding RNAs capable of mediating drug resistance in cell lines. In return, MM cells upregulate methylation and packaging of exosomal RNA in BMAds, forming a vicious cycle [127]. Preliminary evidence suggests that MM cells receive pro-survival fatty acids from lipolysis of BMAds, providing another mechanism of adipocyte-based pro-MM effect [128]. In support of this hypothesis, treatment of MM cells with inhibitors of fatty acid binding proteins or a knockout of Fatty Acid-binding Protein 5 yielded negative metabolic consequences, including apoptosis and decreased mitochondrial respiration [129]. In this perspective, the dual protective and nurturing roles of BMAds may also play a role in mediating PI resistance by altering mitochondrial respiration and therefore ROS production. Further elucidating the effect of BMAds on ROS production and metabolism in MM may yield further treatment strategies for PI-resistant patients.

Novel Molecular Targets to Overcome PI Resistance

Targeting PSR via NRF1 and Its Regulators

Due to the dependence of MM cells on the proteasome, the proteasome stress response (PSR) has become an attractive target for drug development [13,130,131]. The master regulator of PSR is NFE2 Like BZIP Transcription Factor 1 (NFE2L1, also referred to Nuclear Respiratory Factor 1 (NRF1)), a transcription factor responsible for the biogenesis of proteasomal subunits [132]. This gene must not be confused with NRF1 which has been implicated in redox homeostasis. Of note, NRF1 is also related to redox homeostasis by means of regulating antioxidant genes, thus linking it to neurodegenerative disease [133,134]. NRF1 was recently reported to support MM cell survival upon PI by attenuating polyUb protein load on the proteasome, thereby restoring proteostasis [135].

NRF1 senses proteasomal insufficiency through an elegant mechanism. It is continuously transcribed, folded and inserted in the endoplasmic reticulum only to be subsequently ubiquitinated by the E3 ubiquitin ligase hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (HRD1), retrotranslocated to the cytosol by the AAA+ ATPase p97 and degraded by the proteasome upon binding with ubiquitin chain receptor RAD23 homolog A (RAD23A, Fig. 5, Ref. [136]). When proteasome function is impaired either due to increased workload on the proteasome or decreased proteasome activity, retrotanslocated NRF1 is deglycosylated by N-glycanase 1 (NGLY1) and then cleaved by the aspartic protease DNA damage inducible 1 homolog 2 (DDI2). The catalytically active NRF1 c-terminus is shuttled to the nucleus where it activates a transcriptional program that includes proteasome subunits, thus restoring proteasome capacity [136–138]. While there have been reports investigating the efficacy of many of these proteins as potential targets in the treatment of cancers, this review will discuss only DDI2 [139–141].

DDI2 is an aspartic protease implicated in several cellular processes, including DNA damage response, proteostasis, and inflammation, and angiogenesis [142–144]. Nedomova *et al*. [145] have also suggested the importance of DDI2 in embryonic development, with mouse embryos lacking functional DDI2 showing extreme growth retardation due to increased proteotoxic stress and type I interferon signaling. In MM cell lines, knockout of DDI2 is profoundly cytotoxic and biallelic loss of DDI2 could only be established in cells less dependent on the proteasome for survival. Even in these cells, DDI2 loss results in growth arrest and increased sensitivity to CFZ in the setting of impaired de novo proteasome biogenesis, highlighting the dependence of MM on DDI2-based PSR [146]. Structurally, DDI2 consists of an N-terminal ubiquitin-like (UBL) domain, a retroviral protease-like (RVP) domain containing the active site, and a c-terminal ubiquitin-associated do-

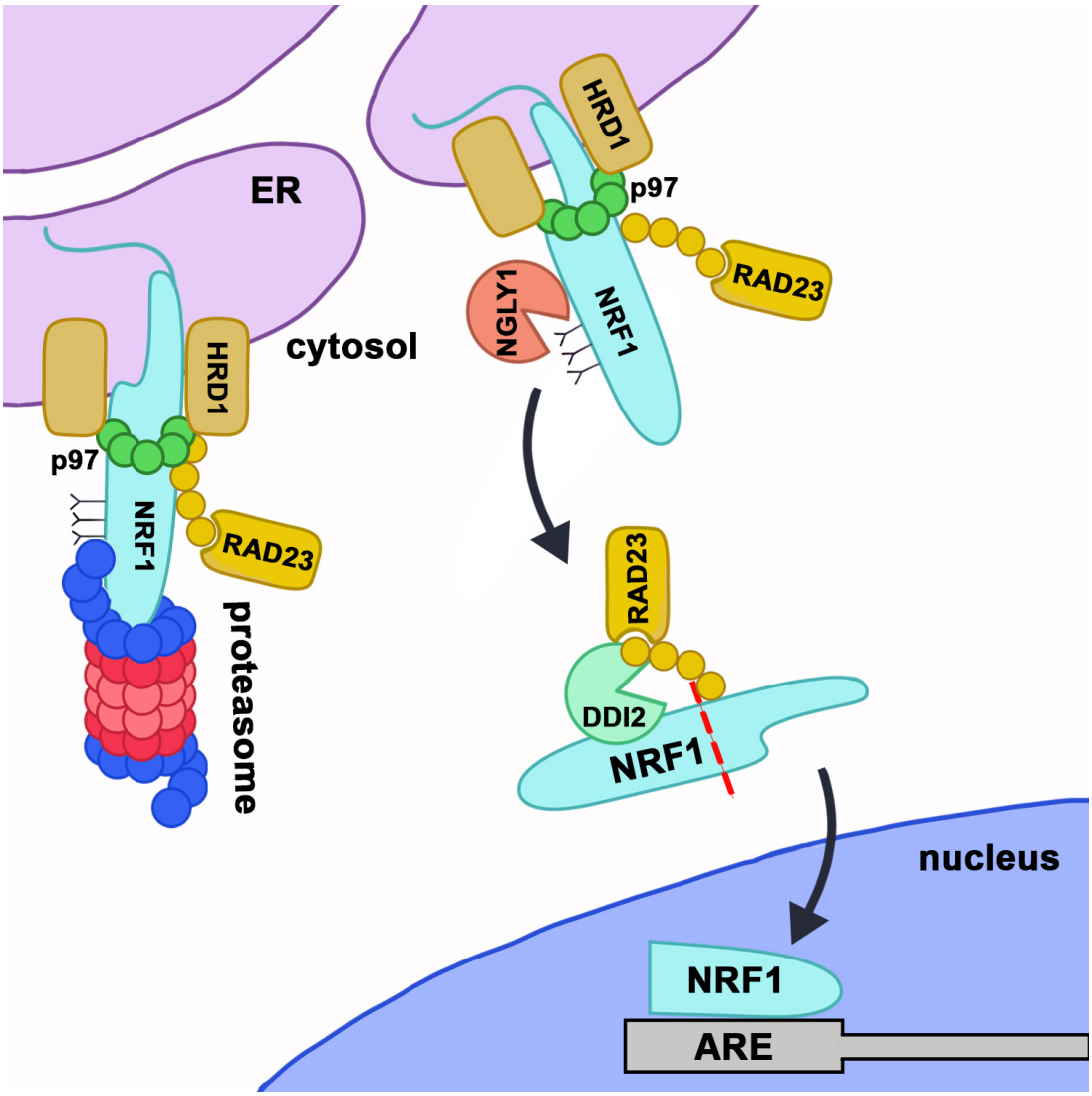

Fig. 5. Schema of the Nuclear Respiratory Factor 1/NFE2 Like BZIP Transcription Factor 1 (NRF1/NFE2L1) activation process. NRF1/NFE2L1 is constitutively degraded by the proteasome (right) under baseline conditions. Impairment of proteasome function results in NRF1 activation through deglycosylation via N-glycanase 1 (NGLY1) and cleavage by damage inducible 1 homolog 2 (DDI2) (left). NRF1 then binds antioxidant response elements in the promoter regions of proteasome subunits. This figure was adapted from Chavarria *et al*. [136], available under the Creative Commons Attribution (CC BY4.0) license. ARE, antioxidant response element; HRD1, hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1.

main [147]. Given the presence of an RVP domain, antiretroviral drugs such as nelfinavir have been suggested to potentially target DDI2, although results have been inconsistent [146,148,149]. We hypothesize that the inconsistency stems from broad, off-target effects which may be alleviated by the development of more specific inhibitors.

Recent studies have shown that DDI2-mediated processing of NFE2L1 is contingent upon its ubiquitination by HRD1 and/or Ubiquitination Factor E4A (UBE4A) in addition to the presence of the nuclear excision repair protein RAD23A [136,150,151]. Interestingly, it has also been noted that DDI2 deletion leads to a buildup of large, slow-migrating ubiquitylated proteins while NRF1 knockdown does not [150,152]. As such, it is hypothesized that DDI2 may act as a shuttling factor for K11/K48 ubiquitylated proteins, including NRF1, through its UBL domain, suggesting that targeting the UBL domain may also have a therapeutic window by broadly impacting proteostasis [152].

As of the writing of this review, there are no known DDI2-specific inhibitors, targeting neither the RVP nor the UBL domains. While some FDA-approved compounds, such as the H1 histamine receptor antagonist levocabastine and the opioid antagonist alvimopan have been identified as potential DDI2 inhibitors via *in silico* screening, there are no *in vivo* or *in vitro* data to validate these docking simulations [153].

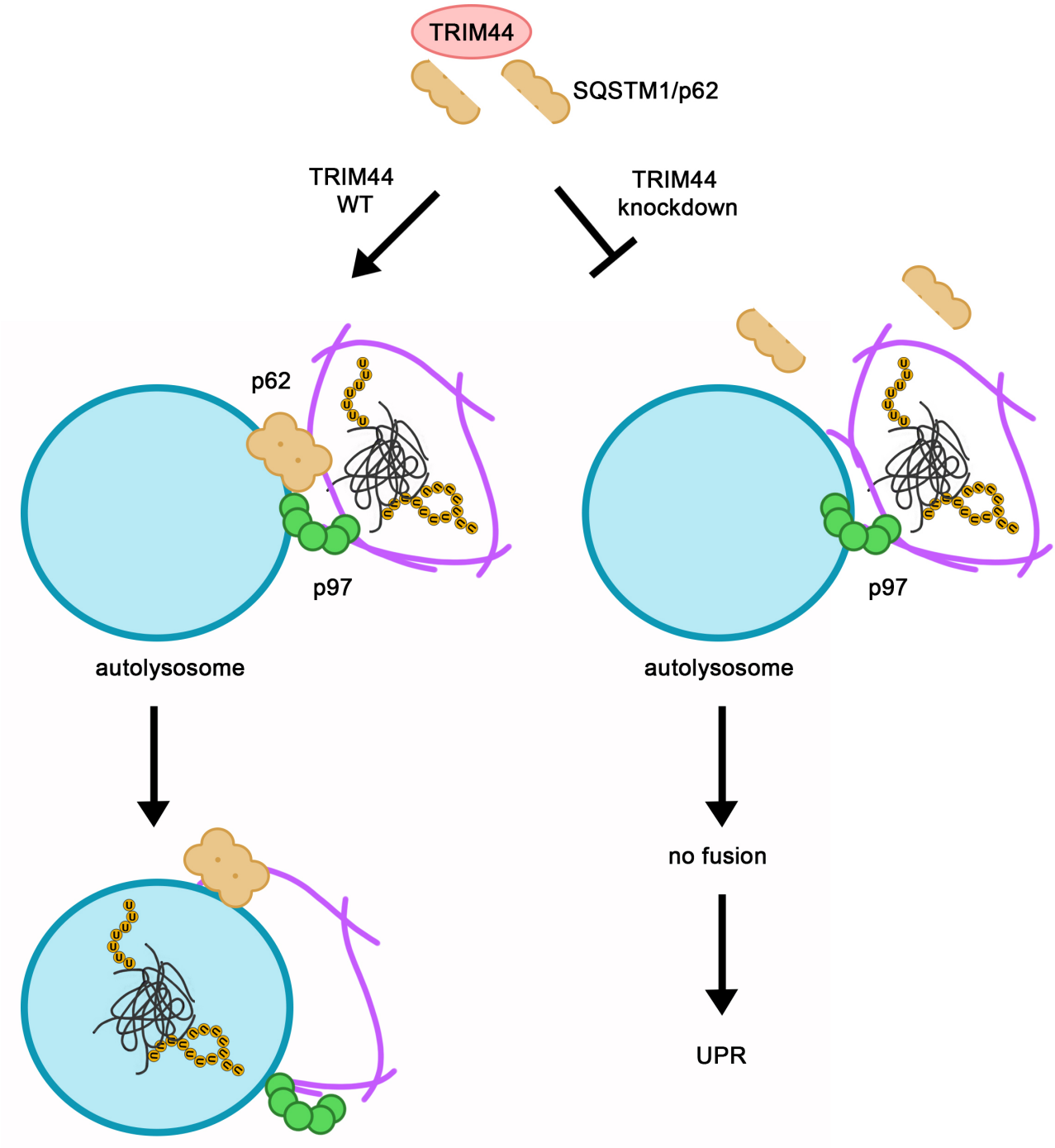

Fig. 6. Schema representing the hypothesized role of Tripartate Motif-Containing protein 44 (TRIM44) in the autophagy and aggresome pathways. Wild type (WT) TRIM44 (red) oligomerizes sequestosome-1 (SQSTM1/p62, gold). Aggresomes fuse with autolysosomes via both SQSTM1/p62 and p97 (green), leading to degradation. Upon TRIM44 knockdown, aggresomes are unable to fuse with autolysosomes, adding to increasing UPR stress. This figure was generated in Adobe Photoshop 22.5.0 (Adobe, San Jose, CA, USA).

TRIM44 Offers Specific Targeting for Aggresome and Autophagy Inhibition

Recent studies have implicated TRIM44 in PI resistance [154]. An analysis of 858 Multiple Myeloma Research Foundation patients noted high levels of TRIM44 expression in both primary and recurrent MM patients with poor prognosis [155]. Single-cell RNA sequencing from bone marrow mononuclear cells of treatment naïve patients validated TRIM44 levels as a predictive marker for response to BTZ-based treatment, with TRIM44 overexpres-

sion correlating to poor response. Of note, cells overexpressing TRIM44 showed significant upregulation of DNA repair genes, mTORC1 signaling, and the NF-κB and UPR pathways, all of which are critical to MM survival. In cell lines, TRIM44 knockdown prevented the oligomerization of SQSTM1/p62 under oxidative stress conditions, thereby minimizing activation of the autophagy pathway (Fig. 6). Additional research by Qi *et al*. [156] noted decreased proliferation as well as a downregulation of Vimentin upon TRIM44 suppression in MM cell lines, further implicating TRIM44 in the aggresome and autophagy pathways. Mouse xenograft studies verified that TRIM44 inhibition suppressed tumor growth.

While there are no TRIM44-specific inhibitors at this time, we expect to see an increase in interest in this target. A combination of proteasome and TRIM44 inhibition may effectively prevent a majority of protein degradation, thereby leading to UPR and apoptosis. Furthermore, TRIM44 may serve as a biomarker to inform personalized care of myeloma patients.

B Cell Maturation Antigen as a CAR T-Cell Target

B cell maturation antigen (BCMA) is a tumor necrosis factor receptor preferentially expressed in mature B-cells through theTNF Receptor Superfamily Member 17 (*TNFRSF17*) gene [157]. Disease progression in MM is correlated to BCMA overexpression, suggesting that BCMA is a promising therapeutic target. Indeed, Tai *et al*. [158] noted that induced overexpression of BCMA in mouse xenograft studies led to rapid tumor growth, likely due to an upregulation of pro-survival pathways activated by increased activity of both canonical and noncanonical NFκB pathways.

Recent reports demonstrated that BTZ and CFZ both increased the efficacy of BCMA-directed CAR T-cell therapy [159,160]. Specifically, BCMA has a short half-life largely determined by proteasome-mediated degradation. Pre-treatment with CFZ significantly increases BCMA expression in myeloma cells, thus enhancing CAR T cell cytotoxicity in *in vitro* and mouse studies [159]. However, Tryggestad *et al*. [161] found that toll-like receptor (TLR) signaling had a suppressive effect on BCMA expression in 50% patient primary cells, especially if CFZ was used as a PI. Additionally, TLR stimulation resulted in increased expression of SQSTM1/p62 and BCL-2 levels, thus boosting the autophagy pathway and inhibiting intrinsic apoptosis, respectively.

A single center study of four MM patients refractory to PI and immunomodulators was recently conducted to assess the lentiviral vector ESO-T01 as a clinical candidate for *in vivo* BCMA-directed CAR T-cell engineering [162]. Treatment was administered as a single intravenous infusion. Both serum and urine free light chain levels were reduced to normal limits or below two months post-infusion with at least one patient displaying eradication of extramedullary tumors, suggesting all patients successfully entered remission. While these data are exciting, the small cohort, lack of placebo controls, and short follow-up time necessitate further in-depth study.

Conclusion

Overall, there have been recent advances towards understanding the origins of PI resistance in MM and evaluating potential means of resensitizing patients to treatment. MM cells are uniquely dependent on the 26S proteasome and current first-line treatments leverage this vulnerability against the disease. However, acquisition of PI resistance over time is common, portending worse survival. Due to the intrinsic complexity and genomic heterogeneity, PI resistance can be mediated by a variety of mechanisms and this review focused on those best established.

Acquired PI resistance is a multifaceted problem, involving proteostasis and degradation pathways, antioxidant pathways, and cell-cell communication between MM and the bone marrow milieu. Over the past 2 decades, several promising targets mediating PI resistance have been discovered; however, the pathway from bench to bedside is long and no new small molecule specifically addressing PI resistance has been developed yet.

Advances in techniques such as single cell RNA sequencing are allowing us to tease apart the variability between resistance mechanisms while simultaneously highlighting previously undiscovered targets [163]. Innovations such as antibody-drug conjugates and T cell engineering have made precision treatment possible, potentially mitigating the need for harsher systemic drugs [164]. A combination of methods such as BTZ pre-treatment followed by BCMA-directed T cell and Dara infusion, may become regular clinical practice in the coming years. With time, inhibitors for promising targets like DDI2 and TRIM44 may be discovered through a drug repurposing screen or dedicated chemistry synthesis. Truly, we are in an exciting era for the advancement of MM treatment and we expect significant development in drugs targeting protein homeostasis in future years.

Availability of Data and Materials

Not applicable.

Author Contributions

Conceptualization - GB, BHS, CVC. Writing of original draft - CVC and BHS. Critical revision of the manuscript for important intellectual content - GB and CVC. Figures - CVC. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

All authors thank Dr. Zach Nilsson for the helpful discussion.

Funding

This work was supported by the National Institute of Health Training Program in Molecular Hematology [grant number 5T32HL116324-09].

Conflict of Interest

GB has acted as a paid consultant for Clearview, Karyopharm Therapeutics, MJH Life Sciences, and Pfizer. The other authors declare no competing financial interests.

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