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PROTEOSTASIS in health and diseases

Book **Chapter X**

Title: **Resistance to the proteasome inhibitors: Lessons from multiple myeloma and mantle cell lymphoma**

Authors: **Maria Gonzalez-Santamarta, Grégoire Quinet, Diana Reyes-Garau, Brigitte Sola, Gaël Roué and Manuel S. Rodriguez.**

Abstract

Since its introduction in the clinics in early 2000s, the proteasome inhibitor bortezomib (BTZ) significantly improved the prognosis of patients with multiple myeloma (MM) and mantle cell lymphoma (MCL), two of the most challenging B-cell malignancies in western countries. However, relapses following BTZ therapy are frequent, while primary resistance to this agent remains a major limitation for further development of its therapeutic potential. In the present chapter, we recapitulate the molecular mechanisms associated with intrinsic and acquired resistance to BTZ learning from MM and MCL experience, including mutations of crucial genes and activation of pro-survival signalling pathways inherent to malignant B cells. We also outline the preclinical and clinical evaluations of some potential druggable targets associated to BTZ resistance, considering the most meaningful findings of the past 10 years. Although our understanding of BTZ resistance is far from being completed, recent discoveries are contributing to develop new approaches to treat relapsed MM and MCL patients.

Keywords

BTZ resistance, proteasome, ubiquitin, mantle cell lymphoma, multiple myeloma.

1. Introduction

Proteolysis is tightly regulated in eukaryotes through the superposition of sophisticated molecular mechanisms to ensure protein homeostasis. One of the major proteolytic activities is driven by the 26S proteasome that holds a catalytic core particle (CP) or 20S [1]. The proteolytic activity of the 26S proteasome requires the previous ubiquitylation of protein targets mediated by a cascade of thiol-ester reactions implicating at least 3 enzymes named activating (E1), conjugating (E2) and ubiquitin ligases (E3). Removal or remodelling of ubiquitin chains conditions the stability, localization and function of the modified target proteins. The ubiquitin tagging step and the 26S mediated-proteolysis constitutes the Ubiquitin Proteasome System (UPS). Some proteins directly degraded by the CP do not require ubiquitin tagging and are therefore destroyed by an ubiquitin-independent process. The CP can also include proteasome subunits that are specifically involved in the immune response, constituting the immunoproteasome. Furthermore, the CP can be associated to other regulatory subunits such as 11S, which have specialized cellular functions [1]. In sum, the proteasome acts as a central hub of cellular proteolysis, having an impact on multiple processes such as cell cycle, DNA repair, cell differentiation, immune response, amino acid recycling or apoptosis. For this reason, the proteasome became a privileged target for drug development to treat diverse disorders including cancers, infections and inflammation-related diseases, among others [2]. The proteasome

inhibitor (PI) bortezomib (BTZ), also known as Velcade, was, in 2003 and 2006, the first Food and Drug Administration (FDA)-approved PI for the treatment of two haematological malignancies: multiple myeloma (MM) and chemotherapy-resistant mantle cell lymphoma (MCL), respectively. Despite the success of BTZ therapy, inherent and acquired resistance in patients were observed, encouraging the development of a new generation of PIs, as well as small molecules targeting enzymes of the UPS. Full understanding of the mechanisms underlying BTZ resistance in cancer is a prerequisite to design new strategies to recover sensitivity to these agents, or to use alternative treatments to lower apoptosis threshold in BTZ-resistant cells. To elucidate these mechanisms several laboratories have characterized a number of patient-derived MM and MCL cell lines with natural or acquired resistance to BTZ. In this chapter we summarize mechanisms of PI resistance reported in the last decade. Even if some of these acquired resistance mechanisms have not yet been confirmed in patients, their discovery may have an impact in upcoming clinical studies. We also review potential strategies to overcome PIs resistance mechanisms, including the use of new signalling pathways inhibitors regulating protein homeostasis.

2. Cancers treated with proteasome inhibitors

Resistance to proteasome inhibitors has been observed in various cancer types including haematological, pancreatic or breast cancer [3]. Two of the best responding cancers are MM and MCL and for this reason, more knowledge has been accumulated on BTZ resistance for these hematologic disorders [4].

2.1 Multiple myeloma

MM is a plasma cell malignancy with bone marrow (BM) infiltration of clonal cells and monoclonal immunoglobulin protein in the serum and/or urine of patients. Genomic techniques have allowed a better understanding of the genetic abnormalities of MM by providing a better landscape of this collection of diseases with a common clinical phenotype [5]. Several genetic alterations including chromosomal translocations of the immunoglobulin heavy chain (IGH) gene leading to the overexpression of D-type cyclin, have been considered as primary events. Not less important are the secondary mutations and clonal evolution. The most frequent mutations occur in *KRAS*, *NRAS*, *FAM46C*, *DIS3* and *TP53*, among others. These mutations affect multiple signalling pathways by altering the mRNA levels but also protein expression and stability. In the past decade, this knowledge has contributed to remarkable changes in the clinical practices, such as the implementation of more effective therapies including new classes of drugs like PIs. The combination of BTZ with immunomodulatory drugs (IMiDs) such as lenalidomide or dexamethasone are currently among the most effective treatments in MM (see section 4). The success of BTZ as a MM treatment underlies its broad impact on the stability and activity of vital cellular factors.

2.2 Mantle cell lymphoma

MCL is an aggressive non-Hodgkin lymphoma (NHL) arising from pre-germinal centre of mature B cells and is typically incurable due to the inevitable development of drug resistance, leading to the worst prognosis among NHL subtypes [6]. Classical MCL cells show minimal mutations in the IGH variable region gene (IGHV) and express the transcription

factor SOX11. Patients present tumours in lymph nodes or extra-nodal sites and cells overexpressing cyclin D1 prone to acquisition of additional abnormalities in cell cycle, DNA damage response, or cell survival pathways, leading to a more aggressive disease behaviour. Less typical are leukemic non-nodal MCL developed through the germinal centre with IGHV somatic hypermutation and minimal SOX11 expression. These patients present MCL cells in peripheral blood, BM and spleen. Leukemic non-nodal MCL behaves in a more indolent way with genetic stability over time. Secondary genetic abnormalities such as TP53 mutations, result in a more aggressive disease associated with poor outcome. Since BTZ approval by the FDA in 2006 for the treatment of relapsed/refractory (R/R) MCL, numerous phenomena have been described to explain innate or acquired resistance observed in more than half of patients [7]. It is known that the development of resistance to BTZ in MCL is an adaptive process, which takes place gradually and includes metabolic changes and/or deregulated (re)activation of adaptive processes like plasmacytic differentiation, autophagy, or improper activation of intracellular signalling pathways such as PI3K/AKT/mTOR axis or NF- κ B, among others.

2.3. Proteasomes and chemical inhibitors

The proteasomes are macromolecular proteolytic complexes with distinct roles under multiple physiologic or pathologic situations. The 26S proteasome is composed by a 19S regulatory particle that recognizes ubiquitin chain as degradations signals [1]. The catalytic core or 20S subunit contains 7 α and 7 β subunits of which β 5, β 2 and β 1 hold the chymotrypsin-like, trypsin-like and peptidyl-glutamyl peptide-hydrolysing activity. Alternative β subunits named β 5i, β 2i and β 1i, are expressed in haematopoietic cells in response to pro-inflammatory signals such as cytokines or γ interferon and integrate the immunoproteasome. The 20S core can also associate with 11S, another regulatory particle also known as PA28, REG or PA26 which contributes to the action of the immunoproteasome but can also drive proteolysis in other cellular compartments such as the nucleus [1]. Given the role of the proteasome in the degradation of many critical cellular factors, its potential as therapeutic target attracted the interest on many pharmaceutical companies.

Approved in the 2000s by the FDA, BTZ has been used for decades as one pivotal treatment in MM and MCL. However, its association with neuropathy and the acquisition of resistance in the clinics highlighted the need to develop new PIs that would be more effective, less toxic and will reduce the occurrence of resistance. Each of these aspects was considered for the development of second generation PIs such as marizomib (MRZ), carfilzomib (CFZ), ixazomib (IXZ) and oprozomib (OPZ) [3] (Table 1). Unlike BTZ, some of them target all the catalytic sites of the proteasome, like MRZ. They carry a different administration way and reversibility than BTZ, hence reducing off-target effects and toxicities in patients. MRZ and OPZ are in early clinical development, and CFZ and IXZ have been already approved in combined treatment for R/R MM. However, preclinical adaptation to these new agents has already been reported, strengthening the need for alternative strategies to face PI resistance [8].

3. Molecular origin of the resistance to bortezomib

In the last decade, several molecular mechanisms involved in BTZ resistance have been proposed. During the progression of the disease, complex genomic alterations promote the activation of different signalling cascades that contribute to the development of the resistant phenotype. These include defects in the initiation/regulation of cellular stress, cell differentiation, apoptosis and autophagy, in combination with mutations and alterations in the expression of the drug target. On the other hand, microenvironmental factors and epigenetics can be another source of inherent resistance mechanism, as these events can modulate the expression of critical genes, including tumour suppressors [9]. The acquired resistance to BTZ is also multifactorial including, among others alterations in the levels of expression of proteasome subunits, crosstalk with other proteolytic pathways or overexpression of efflux pumps.

3.1 Inherent resistance

In this part of the chapter, we will review available data about molecular mechanisms that have been proposed so far to be at the origin of the inherent BTZ resistant phenotype in MM and MCL.

3.1.1 Mutations in PSMB5 proteasome subunit

PSMB5 mutations are known to lower PI binding capacity and to impair the chymotrypsin-like catalytic activity of the 20S proteasome [10, 11] giving a benefit under PI stress. However, both in *in vitro* and *in vivo* settings, mutations were detected only in tumour cells having received heavy PI-based therapies, suggesting that the selected mutations emerge lately during the process of clonal selection beside the apparition of the resistant phenotype. Moreover, *in vivo*, at the time of relapse, subclones exhibiting PSMB5 mutations could partially or totally disappear, questioning the role of such mutations at late stages of the disease [12]. Finally, the relevance of *PSMB5* gene mutation in BTZ-resistance has recently been challenged. Soriano and colleagues have shown that proteasome activity is dispensable in BTZ- and CFZ-resistant MM cell lines suggesting that *PSMB5* mutations are likewise not required or involved in the development of BTZ-resistance [10].

In support, no mutations of *PSMB5* were found for years in MM primary cells, even using targeted or high-throughput sequencing techniques, of large cohorts of patients including refractory patients or in relapse. The relevance of *PSMB5* mutations and their functional impact were suggested recently. Four *PSMB5* mutations were detected in a single MM patient having received prolonged BTZ-based treatments (Table 2) [12]. According to the darwinian model of myeloma evolution, mutations evolved independently in different tumour subclones. For instance, C63Y and A27P are lost during the course of the disease, whereas A20T and M45I are maintained longer. When tested *in vitro*, all mutations were functionally relevant and provided PI-resistance but with different degrees according to both the mutation itself (A20 and M45 having a higher impact than C63 and A27), and the PI tested (BTZ, CFZ or IXZ). The response pattern was similar for BTZ and IXZ, but not for CFZ. CFZ response was less affected by *PSMB5* mutations, likely due to its unique structure and

binding [12]. No mutations of PSMB5, PSMB6 and PSMB7 were ever described in CFZ-adapted MM cell lines [10].

3.1.2 Apoptosis failure

Defective apoptosis signalling is a key oncogenic mechanism of drug resistance in haematological malignancies, mainly attributed to the deregulation of B-cell lymphoma-2 (BCL-2) family members. This family of proteins is composed by prosurvival proteins such as BCL-2, BCL-XL, MCL-1, BCLW and BFL1/A1), as well as proapoptotic factors, represented by multidomain (BAX, BAK and BOK) and BH3-only (BIM, PUMA, NOXA, BAD, BID, BMF, BIK and HRK) proteins. Once activated upon cytotoxic or stress signals, the BH3-only proteins interact with their prosurvival counterparts, leading to the release and oligomerization of BAX and BAK, permeabilization of the mitochondrial outer membrane, and the cytosolic release of apoptogenic factors, culminating in the activation of the caspase family of proteases and ultimately, cell death [13].

In MCL cells, BTZ has been described to evoke intracellular accumulation of MCL-1, which harbours a PEST sequence at the origin of its targeting to the proteasome for its degradation. As MCL-1 can physiologically interact with and block the pro-apoptotic signalling of NOXA, which is transcriptionally activated upon cell exposure to BTZ, the increase in MCL-1 levels can counteract NOXA-mediated activation of BAK, thus delaying the onset of cell death. Therefore, blocking NOXA expression or inhibiting MCL-1 was used to modulate the response to BTZ in MCL [14] (See section 4).

Despite concomitant overexpression of several antiapoptotic proteins of the BCL-2 family, MM cells depend primarily on MCL-1 for survival as demonstrated by the use of small-molecule MCL-1 inhibitor and the knockdown of MCL-1 [15, 16]. MM cells are tightly dependent on their microenvironment known to promote MCL-1 expression in plasma cells. For example, bone marrow stromal cells (BMSCs) provide survival signals such as interleukin-6 (IL-6), vascular endothelial growth factor and insulin-like growth factor. IL-6 upregulates MCL-1 transcription and induces MCL-1 dependence [17]. Recently, it has been shown that the long non-coding RNA (lncRNA) H19 is present in the serum of MM patients and that an H19/miR-29b-3p axis promotes MCL-1 translation and BTZ resistance [18]. Thus, MCL-1 is certainly an important target for coping with MM drug resistance.

3.1.3 Signalling cascades

The NF- κ B pathway is activated *via* canonical and non-canonical signalling mechanisms. The canonical pathway regulates inflammatory responses, immune regulation, and cell proliferation, whereas the non-canonical signalling cascade leads to B-cell maturation and lymphoid organogenesis. These pathways regulate the expression of genes involved in cell survival and tumour-promoting cytokines. Therefore, its activation has profound impact in tumorigenesis. The NF- κ B pathway can be potentially targeted and is expected to have a high impact on the viability of malignant B cells, due to its interplay with other crucial pathways activated during B-cell differentiation, such as B-cell receptor (BCR),

PI3K/AKT/mTOR, and toll-like receptor (TLR) signalling axes. Constitutive NF- κ B activity is often present in MCL and MM. The inhibition of NF- κ B is a primary mechanism to induce cell apoptosis after BTZ treatment and play a role in evading the effect of this treatment in BTZ-resistant phenotypes [19].

A high NF- κ B activity was found in tumour cells of BTZ refractory MM patients and in *in vitro* models of cell adhesion-mediated drug resistance (CAM-DR), reinforcing the notion that the NF- κ B pathway signals BTZ resistance [20, 21]. In MCL, this constitutive NF- κ B signalling and consequent lack of response to BTZ has been linked to a proteasome-independent degradation of the intrinsic NF- κ B inhibitor, I κ B α [19]. However, a number of studies have pointed out a lack of correlation between NF- κ B activity and BTZ resistance status [22].

NF- κ B pathway is also regulated by casein kinase 2 (CK2). CK2 is a multifaceted serine/threonine kinase involved in several cellular processes, and is overexpressed and overactive in many blood tumours. CK2 regulates signalling cascades and molecules that are targeted by BTZ. For instance, it modulates I κ B α protein turnover, p53 function, AKT activation, and has a role in the control of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) (see section 3.2.3). Inhibition of CK2 enhances BTZ cytotoxic effect in MCL cell lines by down modulating NF- κ B and signal transducer and activator of transcription 3 (STAT3) signalling cascades and by potentiating the proteotoxic effects of proteasome blockade. Altogether these results suggest that levels of CK2 are involved in MCL resistance to BTZ [23].

The BCR includes a heterodimer of CD79A/B molecules, and CD19, a key co-receptor. The upregulation of those molecules have been proposed to promote BTZ resistance in MCL cells. While BCR regulates cell survival and proliferation of MCL cells, in MM it has been only linked to monoclonal gammopathy of undetermined significance (MGUS), a premalignant phase of MM [24]. A human phospho-kinase array further pointed out an overexpression of phosphorylated BCR kinases LYN, LCK, and YES as well as a sustained downstream activation of PI3K/AKT/mTOR axis in BTZ-resistant cells. Among these kinases, LYN was functionally associated with the resistance phenotype, rendering cells more sensitive to the SRC kinase inhibitor dasatinib and allowing to synergistic activity of the dasatinib/BTZ combination *in vitro* [25].

In MCL, the redox status has also been pointed out as a crucial mediator of BTZ efficacy, as PIs lead to the generation of large amounts of reactive oxygen species (ROS), modulating at least in part the transcription of *NOXA* and thus contributing to the cytotoxic activity of proteasome inhibition [26]. The nuclear factor NF-E2 p45-related factor 2 (NRF2) was identified as a key regulator of this response. Indeed, while under physiological conditions it is sequestered by Kelch-like ECH-Associated Protein 1 (KEAP1) in the cytosol, when KEAP1 is oxidized by ROS, NRF2 is released to the nucleus where it initiates the transcription of genes involved in the adaptive oxidative stress response. Upon BTZ exposure, BTZ-sensitive MCL cells display a sharp increase in the expression of NRF2 target genes, as well as genes related to protein ubiquitylation or proteasome components, while resistant tumours show minimal changes. Accordingly, an elevated expression of NRF2 target genes at the basal level, predicted a poor sensitivity to proteasome inhibition [27]. In

line with this, a recent study has highlighted the capacity of ROS to modulate some cancer stem cells (CSCs)-like subpopulations in MCL cell lines and primary cultures and to regulate cell sensitivity to BTZ. Authors showed that O_2^- was involved in the inhibition of CSC-like cells and in the sensitization of MCL to BTZ, while H_2O_2 favoured a CSC-like phenotype, impairing BTZ-induced cell death [28]. This process was associated with transcriptional regulation of two O_2^- and H_2O_2 targets, namely MCL-1, and ZEB-1, a WNT-regulated transcription factor that interfered with MCL response to chemotherapeutics. This resulted in the activation of proliferation-associated genes including *MYC* and *CCND1* and the induction of an antiapoptotic gene signature [29].

3.2 Acquired resistance

PI-acquired resistance has multi-factorial and interconnected causes and PI-resistant cells show cross-resistant profiles. Among the main mechanisms recognized in MM and/or MCL are upregulation of 20S proteasome subunits including $\beta 5c$, downregulation of 19S proteasome subunits and overexpression of efflux pumps. Adaptive metabolic changes, modulation of the unfolded protein response, and alteration of autophagy signalling contribute also to PI-resistance in MM cells.

3.2.1 Overexpression of proteasome subunits

Beside *PMBS5* mutations, overexpression of *PMBS5* and (to a lesser extent) *PMBS6* are frequent alterations found in MM cell lines adapted to increased concentrations of BTZ [11, 30, 31]. *PSMB5* is overexpressed in one MM patient with clinical resistance to BTZ, compared to three BTZ-sensitive patients [32]. Franke and colleagues demonstrated a tight relationship between impaired proteasome activity carried on by a mutated $\beta 5c$ subunit and the $\beta 5c$ subunit overexpression. In cells harbouring homozygous *PSMB5* mutations, the upregulation of $\beta 5c$ subunit was even more important when compared to cells harbouring heterozygous mutations. The authors propose a model in which, the prolonged exposure of MM cells to BTZ leads first to the appearance of *PSMB5* mutations, resulting in decreased BTZ binding. In turn, mutant cells compensate this reduced proteasome activity by upregulation of the $\beta 5c$ subunit. Moreover, in those BTZ-resistant MM cells the upregulation of $\beta 5c$ is associated with the downregulation of $\beta 5i$ to balance the total proteasome units and impairs any possible remaining BTZ-inhibition [11].

In two recent studies, the involvement of the 19S subunits of the proteasome has been highlighted. To identify genes controlling the sensitivity and adaptation of MM cells to CFZ, Acosta-Alvear and colleagues used a new-generation shRNA library screening [33]. They found that the knockdown of several subunits of the 20S proteasome core (including $\beta 5c$) provides a strong sensitization to proteasome inhibition. Paradoxically, the genetic depletion of most of the 19S regulatory components confers a marked resistance. They further confirmed that shRNAs-mediated knockdown of *PMSC1*, *PMSC6*, *PMSD1*, *PMSD2*, *PMSD6* and *PMSD12* leads to resistance towards BTZ and CFZ in MM cell lines. Importantly, the authors showed that *PSMC2* levels in MM patients seem predictive for the response towards CFZ-based therapy. In the second report, Shi and colleagues used a genome-scale CRISPR-Cas9 library to identify genes associated with BTZ-resistance. They validated *PSMC6* depletion as the strongest hit conferring BTZ resistance in MM cells [34]. *PSMC6* deficiency

resulted in reduced BTZ ability to regulate chymotrypsin-like activity of $\beta 5c$ through changes in the proteasome structure. Mutations in other members of the PMSC group also individually impart BTZ-resistance albeit less potently. No mutation of *PSMC6* has been reported so far but the analysis was performed on a cohort of untreated MM patients [34].

Recent results from our laboratories comparing BTZ-adapted MCL cell lines and their parental counterparts revealed a reduced expression of 19S proteasome subunits in BTZ-resistant cells. Strikingly, when autophagy was blocked with inhibitors such as bafilomycin A or chloroquine, the level of those proteasome subunits increased in resistant cells only, suggesting an autophagy-mediated degradation. In MCL cell lines that naturally resist to BTZ, the accumulation of proteasome subunits after the chemical inhibition of autophagy was proportional to the level of BTZ resistance observed. The proteasome degradation by autophagy was named proteaphagy and has been observed in response to starvation or proteasome inhibition in several biologic models including human cells, [35, 36]. Quinet *et al* showed that proteaphagy can contribute to develop resistance to BTZ in MCL cells since inhibited proteasomes are degraded and BTZ reduces its impact on proteasomes and cell death. In other words, BTZ-resistant cells bypass proteasome inhibition relying on autophagy through degradation of proteasomes and perhaps other cellular proteins [37].

3.2.2 Metabolic adaptation

Metabolic reprogramming is a hallmark of cancer that has emerged as an attractive target for novel therapeutic strategies for cancer treatment. O-GlcNAcylation is an abundant, dynamic, and nutrient-sensitive posttranslational modification that corresponds to the addition of an O-linked β -N-acetylglucosamine (O-GlcNAc) moiety to the serine or threonine residues in proteins in response to changes in the hexosamine biosynthetic pathway. As this latest depends on various essential nutrients and metabolic intermediates like glucose, glutamine, acetyl-coA, and UTP, it provides an ideal machinery for cells to sense and respond to a variety of microenvironmental conditions [38]. Little was known about its role in MCL, until the recent study of Luanpitpong and collaborators who demonstrated that O-GlcNAcylation of tBID promoted apoptosis in MCL cells exposed to BTZ, and that this process could be amplified by co-treatment with the antifungal drug keniconazole, an O-GlcNAcase inhibitor that blocks tBID ubiquitylation and subsequent proteasomal degradation [39].

ABC (ATP-binding cassette) transporters such as ABCB1 (multi-drug resistance, MDR1 or P-glycoprotein, P-gp) mediate drug resistance by alterations of the absorption and elimination of xenobiotics and drugs. ABCB1 expression correlates with poor prognosis, treatment resistance and aggressiveness of the MM disease [40]. ABCB1 protein is overexpressed in CFZ-resistant compared to sensitive MM cell lines [10]. ABCB1 was expressed by circulating malignant plasma cells of MM patient at diagnosis and its expression increases along the course of CFZ-treatment [41]. Overexpressed ABCB1 protein limits the proteasome-inhibiting activity and clearance of poly-ubiquitinated proteins by CFZ and reduces its cytotoxicity. Importantly, ABCB1 overexpression affects the cytotoxic activity of epoxyketone-type PIs (CFZ) significantly stronger than non-epoxyketone PIs (BTZ). Drugs targeting ABCB1 may re-sensitize MM cells to PI, in particular CFZ.

Soriano and colleagues analysed by a combined quantitative and functional proteomics approach CFZ- and BTZ-adapted MM cell lines [10]. They found that resistance to BTZ/CFZ was independent of proteasome activity but relied on energy metabolism, redox homeostasis, protein folding and degradation. PI-resistant cells adapted themselves to a very low proteasome activity while continuing to synthesize immunoglobulins. In turn, the level of metabolic intermediates involved in oxidative glycolysis (pyruvate kinase), redox state (superoxide dismutase, glutathione peroxidase, peroxiredoxin), mitochondrial respiration (cytochrome c) were increased thus maintaining high stringent redox conditions. In agreement with previous studies, authors also confirmed that the most upregulated proteins in CFZ-resistant cells were ABCB1 [40, 41] and the heat-shock proteins HSP70 and HSP90, whose transcriptional regulator, HIF1, is involved in BTZ-resistance in MM cells [42]. Conversely, the positive apoptosis regulators BAX, CASP and DIABLO were downregulated [10].

Similarly to Soriano *et al*, Dytfeld and colleagues conducted a comparative proteomic profiling of R/R patients vs. naïve MM patients [43]. In the proteomic signature associated with BTZ-resistance, four sets of proteins were characterized including proteasomal proteins, some factors regulating the redox status, proteins signalling apoptosis, and proteins involved in the inflammation response. In particular, regulatory and catalytic components of the proteasome, including part of the 11S complex, were upregulated. The antioxidant thioredoxin, peroxiredoxin, and thioredoxin reductase were upregulated whereas annexins A1 and A2, that regulate the apoptotic process, were downregulated. PI-resistance may thus be alleviated by manipulating the redox status and the energy metabolism.

Although not revealed by the above proteomic studies, NRF2 seems to be a node for BTZ- and CFZ-resistance in MM. As it maintains redox homeostasis by inducing antioxidant and detoxification genes and by modulating energy metabolism [44], NRF2 indirectly regulates: a) chaperoning activity [45]; b) redox, metabolic and translational reprogramming [46]; and c) activation of prosurvival autophagy. These major functions are supported by clinical data showing *NRF2* upregulation in a subgroup of relapsed patients [46]. Other studies showed that high glutathione (GSH), whose levels are controlled by NRF2, dampens BTZ toxicity in MM cells [47].

In the last decade, numerous studies have indicated, that components of the BM stroma, extracellular matrix (ECM), cytokines, chemokines and growth factors, are involved in BTZ-resistance in MM cells [48]. The membrane protein myristoylated alanine-rich C-kinase substrate (MARCKS), is a protein that plays an important role in cell adhesion, spreading and invasion, and is crucial for metastasis [49]. MARCKS is overexpressed in MM cell lines and is involved in the cross-resistance to the farnesyltransferase inhibitor R115777 and BTZ, as well as in MM patients that do not reach a sustained response to BTZ therapy. In addition, the inhibition of MARCKS phosphorylation, increase cytotoxicity in BTZ-resistant cells [50].

The insulin-like growth factor IGF-1, known as a growth factor for MM cells [51], is produced by plasma cells, and is present in the BM microenvironment. IGF-1 has been proposed to promote proliferation and drug resistance in MM cells through the activation of MAPK and PI3K/AKT-signalling pathways [48]. According to these data, the IGF-1/IGF-1R

signalling axis was detected to be upregulated in three BTZ-resistant MM cell lines, compared to parental cells. Kuhn *et al* proved that a small molecule responsible for the inhibition of IGF-1R has the capacity to sensitize BTZ-resistant MM cells to the proteasome inhibitor [52].

3.2.3 Protein homeostasis

Because of their high capacity to synthesize and secrete immunoglobulins, MM cells exhibit an expanded ER network and an increased ability to cope with unfolded or misfolded proteins that accumulate in the ER. These conditions referred to as ER stress. As a consequence, MM cells activate the UPR pathway as an adaptive strategy and are rendered dependent on this mechanism for their survival [53]. ER stress upregulates three UPR signalling branches: activating transcription factor 6 (ATF6), protein kinase R (PKR)-like ER kinase (PERK)-ATF4 and inositol-requiring enzyme 1 (IRE1)-X box binding protein 1 (XBP1) which suppress global translation and promote protein folding and degradation. During ER stress, ATF6 translocates into the nucleus and activates the *XBP1* promoter allowing an upregulation of the protein. At the same time, IRE1 oligomerizes and autophosphorylates, resulting in the activation of its endonuclease activity that cleaves *XBP1* mRNA. This results in a frameshift that modifies the unspliced inactive *XBP1* form (*XBP1u*) into an active *XBP1s* form. *XBP1s* acts as transcription factor and activates genes encoding protein folding and chaperones (see section 3.2.5). Previous studies done on cohorts of MM patients and confirmed *in vitro* on BTZ-adapted cell lines, defined a “low IRE1-*XBP1*” phenotype that predicts a poor response to BTZ [54, 55]. Moreover, *XBP1* knockdown experiments in MM cell lines showed correlation with BTZ resistance, as the suppression of *XBP1* lowers both the basal ER stress and the ER stress due to proteasome inhibition [55].

High expression of deubiquitinating enzymes (DUBs) and autophagy related-proteins has been detected in MM patients resistant to BTZ. These alterations in enzymes that are involved in deubiquitinating misfolded/unfolded proteins and in the turnover of proteins by the autophagy-lysosome system (ALS), suggest an important role of ubiquitin signalling pathways in BTZ resistance. Niewerth *et al*, shown that inhibition of USP14 and UCHL5 promotes apoptosis and helps to overcome BTZ resistance in MM patients [4]. Another enzyme that can be regulated to recover sensitivity to BTZ is the ubiquitin-conjugating enzyme H10 (UbcH10). Wang *et al* proved that through the expression of hsa-miR-631, the negatively regulation of UbcH10 transcription prevents MM cells to develop resistance against proteasome inhibitors [56].

3.2.4 UPS-ALS crosstalk

Eukaryotic cells have two interconnected mechanisms for protein degradation and removal of misfolded proteins and aggregates, the UPS and the macroautophagy (here referred to as ALS). Autophagy functions by double-membrane vesicles known as autophagosomes which sequester cytosolic proteins, followed by fusion with lysosomes for degradation. Autophagy is involved in several human diseases, such as neurodegenerative diseases and cancer [57]. While it appears to be tumour-suppressive in normal cellular homeostasis, autophagy can mediate tumour cell survival under stress conditions [58]. For

many years, UPS and ALS pathways were thought to function independently, but the recent observation that impairment of either pathway impacts the other has suggested that these two proteolytic systems do collaborate [57]. It is thought that upon proteasome inhibition, autophagy is initiated as a survival mechanism to eliminate UPS substrates [59], thus upregulated autophagy could play a role in BTZ-resistance [60].

The ubiquitin-binding cargo autophagy receptor sequestrosome 1 (SQSTM1) or p62 is a critical link between UPS and ALS [61]. In CFZ-adapted MM cells, SQSTM1/p62 is elevated triggering a prosurvival autophagy however through two different mechanisms according to the settings. In the first model, the pluripotency-associated transcription factor Kruppel-like factor 4 (KLF4), is overexpressed and contributes to CFZ resistance by activating the *SQSTM1* gene [46]. In the second model, elevated levels of SQSTM1/p62 conduct CFZ resistance through both a prosurvival autophagy involving GABARAPL1 upregulation and the activation of the NRF2 pathway [46]. However, since KLF4 is also a target of NRF2, both factors could cooperate for maintaining a high level of *SQSTM1* transcription. The activation of NRF2 occurs through the activation of the PERK-eukaryotic translation initiation factor-2 α (eIF2 α) axis of the UPR [46]. As stated before (see paragraph 3.2.2), NRF2 is a major actor for PI-resistance through the reprogramming of metabolism and the control of redox status [10, 46]. In addition to eIF2 α , another NRF2 target and translation initiation factor, eIF4E3 is overexpressed in CFZ-resistant cells, and increased EIF4E3 expression was found in a subgroup of patients with chemoresistant minimal residual disease and in R/R patients [46].

Interestingly, deficiency in BIM has been shown to contribute to adaptive resistance to BTZ in MM cells, mediated by increased autophagy, and that autophagy disruption by means of chloroquine could sensitize these cells to BIM-mediated cell death [62]. Of special interest, upon exposure to pharmacological inhibitors of autophagy like chloroquine or bafilomycin A, BTZ-resistant MCL cells can undergo a blockade of proteaphagy, leading to the stabilization of proteasome subunits, and the recovery of BTZ sensitivity [37]. Importantly, sensitivity to autophagy inhibitors requires a significant degree of BTZ resistance, thus suggesting that, modulating proteaphagy with specific inhibitors may be considered as a strategy to resensitize resistant cells to PI.

3.2.5 Stress signals

Subsequent studies have been focused to determine the interplay between the deregulation of the intracellular stress machinery and MCL loss of sensitivity to BTZ. A key defect in BTZ-dependent cell death was first identified within the ER stress pathway, because its activation in MCL cells exposed to BTZ is required to elicit *NOXA* transcription [63]. ER homeostasis is controlled by the immunoglobulin heavy chain binding protein (BiP), also referred as 78-kDa glucose-regulated protein (Grp78). BiP/Grp78 forms a large multiprotein complex with a set of other ER molecular chaperones, including the Hsp90 ER homolog, Grp94, protein disulfide isomerase, calcium binding protein, and cyclophilin B [64]. Under non-stressed conditions, BiP/Grp78 binds to and maintains in an inactive monomeric state the ER transmembrane PKR-like ER kinase, IRE1, and ATF6 [65]. After proteasome inhibition, the accumulation of polyubiquitylated and misfolded proteins within the ER lumen leads to BiP/Grp78 dissociation from the luminal domains of these sensor proteins and the initiation of UPR (see section 3.2.3) [66]. This coordinated cellular response initially

promotes cell survival, but ultimately triggers apoptosis if cytoprotective mechanisms are overwhelmed. Supporting the observation that accumulation of some HSP proteins can promote cellular resistance to PIs, a correlation has been made between acquired and primary resistance to BTZ in MCL, and intracellular accumulation of BiP/Grp78 in proteasome-compromised MCL cells [67]. For this reason inhibitors of HSP90 have been used to improve the BTZ-mediated cell death induction in BZT-resistant cells (see section 4) [67].

In line with this study, deregulated expression of several cytosolic HSP70 family members has also been associated with BTZ resistance in MCL [68] and MM [69]. Supporting a role of HSP70 protein in MCL cell resistance to chemotherapeutic agents, B cells modified to overexpress cyclin D1, the genomic hallmark of MCL, presented strong alterations in their response to growth factor withdrawal [70]. Acquired BTZ resistance is also attributed to the upregulation of other HSPs such as HSP90 and HSP27, that promote NF- κ B activity [20].

3.2.6 B-cell differentiation program

It has been proposed that MM cells also achieve BTZ-resistance *via* the dedifferentiation of plasma cells. A pool of XBP1^{low/-} tumour progenitors or CSCs pre-exists drug treatment, contributing to tumour diversity [54]. CSCs recapitulate maturation stages between B cells and plasma cells. Tumour B cells and pre-plasmablasts survive PI-treatment preventing cure, while maturation arrest of MM before the plasmablast stage enables progressive disease on PI treatment. These tumour progenitors should be targeted to allow a complete cure for MM patients.

Although MCL was originally considered a neoplasm of naive lymphocytes that have not passed through the germinal centre (GC), a significant number of cases present somatic mutations in the immunoglobulin genes, suggesting that they have been in contact with the antigen in the GC. Some cases were also described, that presented evidences of plasmacytic differentiation in patients harbouring the characteristic t(11;14) translocation [71]. A couple of studies have related the resistance to BTZ with the plasmacytic differentiation program in MCL cells. Plasma cells are the final effectors of humoral immunity, which are devoted to the synthesis and secretion of immunoglobulins. BTZ-resistant MCL cells display some of the characteristics of the plasma cells, such as the over-expression of interferon regulatory factor 4 (IRF4) and elevated membrane levels of CD38 and CD138 cell surface markers, but they do not present splicing of XBP1 or increase in immunoglobulin production [6]. It is postulated that during the acquisition of resistance to BTZ, the balance between the protein load and the proteasomal activity is key. When plasmacytic differentiation is induced through stimulation of TLR9 receptor, the sensitivity to BTZ changed throughout the process of B-cell differentiation to final plasma B cell phenotype with the capacity to manage the future increase in protein loads. Since the cells do not acquire full secretory capacities, this mechanism granted them with an advantage against the anti-tumour activity of BTZ. Once MCL have completed this process of differentiation, they return to a BTZ-sensitive state [6]. Mouse xenograft models of MCL using different cell lines with different sensitivity to BTZ, including cells with acquired or primary resistance to the PI, demonstrated a tight correlation between increased tumorigenicity of BTZ-resistant tumours with a plasmacytic differentiation phenotype including upregulation of IRF4, PR domain zinc finger protein 1

(PRDM1/BLIMP-1) and CD38, and loss of the B cell markers PAX5 and CD19 [72]. Of note, beside its role as a transcription factor that represses the expression of proteins needed for B-cell identity and proliferation, and that helps to drive B cells through their final differentiation stage to become antibody-secreting cells, BLIMP-1 is also a mediator of NOXA-induced apoptosis in MCL and is required for BTZ-induced apoptosis in MCL cell lines and primary tumour samples [73]. This finding further strengthens the interplay between NOXA and plasmacytic differentiation in BTZ-mediated anticancer activity in MCL.

4. Potential targets to recover proteasome inhibitors sensitivity

Given the resistance to PIs observed in some patients, alternative drugs have been tested to overcome resistance as single or combinatorial treatments. Many agents have shown promising preclinical results in terms of safety, specificity and efficacy to treat PI-resistant MM and MCL cells. Already existent or new therapeutic drugs are used to tackle the adaptation of the cells to PIs (Table 3). Cellular mechanisms involved in PI resistance are hence targeted by a wide range of drugs, some of the most relevant are described below.

4.1 Deubiquitinases

Because proteasome degradation and UPR implicate ubiquitin signal, targeting factors regulating ubiquitin signal could potentially contribute to increase the sensitivity to BTZ and therefore be an option to overcome PI resistance. In this context, DUBs have been considered as therapeutic targets to overcome BTZ resistance. P5091 is a selective inhibitor of USP7, a DUB that targets the E3 ligase HDM2. Treatment of MM cell lines and primary cells from MM patient with P5091 inhibits growth and induces apoptosis in tumour cells including those resistant to BTZ, without affecting the viability of normal PBMCs [74]. The 19S regulatory particle inhibitor b-AP15 selectively blocks deubiquitinating activity of USP14 and UCHL5 without inhibiting proteasome activity. This leads to an activated UPR and an inhibited tumour growth, in MM xenografts resistant to BTZ [75]. The antitumoral effects of b-AP15 were also demonstrated in MCL [76]. Another USP inhibitor, SJB3-019A tackling USP1 showed synergic toxicity in MM when combined with BTZ [77]. Song *et al* found contribution of RPN11, a proteasomal deubiquitinase, in MM pathogenesis using gene expression analysis. Pharmacological inhibition of RPN11 with *O*-phenanthroline (OPA) or capzimin blocks proteasome function, induces apoptosis in MM cells and overcomes resistance to BTZ [78].

USP9X is also highly expressed in MM patients and in particular in those with as short progression-free survival. The partially selective USP9X inhibitor WP1130 induces apoptosis through the downregulation of MCL-1. However, this effect is transient due to the compensatory upregulation of USP24 who sustains MM cell survival. By contrast, a novel compound EOAI3402143 with a dual USP9X/USP24 inhibiting activity display promising anti-myeloma activity [79]. Associated with the inhibition of USP14 activity, VLX1570 lead to an extended survival of xenografts models of myeloma including BTZ-resistant cells [80].

4.2 Transport modulators

PI cellular intake is regulated by transport modulators, and treatment efficiency is directly linked to intracellular concentration of drug. Specific inhibitors of ABCB1 such as verapamil and reserpine showed increased proteotoxic stress in CFZ-resistant MM cells [10]. The two HIV inhibitors nelfinavir and lopinavir counteract ABCB1 overexpression in CFZ-resistant MM, *via* the modulation of the mitochondria transition pore. This promising preclinical results encourage the clinical evaluation of both treatments [41]. Thanks to its capacity to modulate the UPR pathway, nelfinavir has also attested a safe and promising activity in combination with BTZ and/or dexamethasone, in a phase I clinical trial involving advanced BTZ-refractory MM patients [81].

4.3 Autophagy signalling

Ubiquitin and ubiquitin-like molecules play an important role on the regulation of autophagy. In BTZ-resistant MCL cells, the use of autophagy inhibitors such as bafilomycin A and chloroquine, and the p62 inhibitor verteporfin revealed increased cytotoxicity and synergistic activity with BTZ, mediated by the reversion of the proteaphagic process [37]. Blocking autophagy also leads to multiple changes in the cell such as the accumulation of I κ B α , which prevents BTZ-induced NF- κ B activation. The combination of bafilomycin A with BTZ might therefore contribute to increase cytotoxicity in MM cells in this manner [82]. Finally, orlistat, a fatty acid synthase inhibitor that affects autophagy, sensitizes MCL cells to BTZ through the inhibition of the autophagic degradation of NOXA [83].

4.4 Oncogenes and signalling pathways

Proteasome inhibition has pleiotropic effect within the cells. It affects a wide range of cellular factors, including important signalling cascades, oncogenes or epigenetic regulators. This modulation of critical factors during BTZ treatment can hamper the apoptotic effect of PI, and leads to resistance. As described above, adaptation of signalling pathways including mTOR and NF- κ B, or modulation of important oncogenes such as BCL-2 or MYC proteins, have been directly linked to BTZ-resistance in MM and MCL. Therapeutics agents targeting these factors have been proposed to overcome BTZ resistance.

4.4.1 mTOR/AKT pathway

Modulating mTOR/AKT, key proteins of a complex signalling cascade, have successfully reverted malignant cell adaptation to BTZ in preclinical studies. The dual PI3K and mTOR inhibitor dactolisib (NVP-BEZ235) showed great results in MCL BTZ-resistant cells lines [84]. A new generation of mTOR inhibitors, such as temsirolimus and deforolimus have been also proposed for MCL treatment but limited clinical impact was obtained [85, 86]. The AKT inhibitor perifosine combined with BTZ revealed increase cytotoxicity in R/R MM patients previously treated with BTZ [87], warranting its use in BTZ-resistant cancers.

4.4.2 NF- κ B pathway

Since NF- κ B pathway is overactivated under BTZ treatment, its modulation has been used to treat PI-resistant MM and MCL. Selinexor is a reversible inhibitor of exportin 1 (XPO1) that blocks the nuclear export of NF- κ B /I κ B α complexes leading to NF- κ B pathway inactivation [88]. Selinexor associated with BTZ or CFZ overcomes acquired PI resistance in MM models and patients. MM drug resistance to CFZ and BTZ is enhanced in hypoxic conditions [89]. In such conditions, selinexor is capable to overcome PI resistance [89]. Because Transglutaminase 2 (TG2) is a calcium-dependent enzyme, calcium blockers have been proposed to hamper high NF- κ B expression in BTZ-resistant cells. The combination of such molecules with BTZ indeed improves cytotoxicity in MCL [90]. Degrasyn has been also proposed to target constitutive NF- κ B and STAT3, and combined treatment with BTZ showed a synergic apoptosis in MCL [91].

Within the BCR pathway, Bruton's tyrosine kinase (BTK) inhibitors lead to NF- κ B inactivation and downregulation of MYC. Ibrutinib (Imbruvica), a first-in class BTK inhibitor, was approved by the FDA in 2013 as second line treatment for MCL patients [92]. This promising drug leads to the best complete remission rate as a single agents when compared to the other three drugs licensed at that time for use in MCL (BTZ, temsirolimus (Torisel) and the IMiD drug, thalidomide-derivative lenalidomide (Revlimid)) [93]. Promising preclinical results were obtained combining ibrutinib and BTZ in MCL and MM BTZ-resistant cells [94]. Ibrutinib alone or in combination with dexamethasone went very recently through a phase II trial with R/R MM patients, with some positive results [95].

4.4.3 NOXA/BCL-2 proteins

BH3 mimetic compounds like obatoclax showed great results alone or in combination with BTZ in relapsed MCL. By neutralizing BTZ-induced MCL-1 accumulation, obatoclax sensitizes MCL cells to low doses of the PI [14]. A phase I/II study substantiated the tolerance of a combined treatment BTZ/obatoclax in patient with R/R MCL. However the synergism supported by the preclinical studies was not confirmed in patients [96]. Treatment of MM cell lines including BTZ-resistant cells and primary cells with cyclic adenosine monophosphate (cAMP) induces down regulation of MCL-1 and degradation of cyclin D1. Moreover, a synergy between BTZ and cAMP showed promising results in a murine xenograft model, warranting this strategy to overcome BTZ resistance [97].

4.4.4 IRF4/MYC signalling

As described before, exacerbated *de novo* IRF4 signalling has been associated with MCL resistance to BTZ *in vitro* and *in vivo*, thus supporting the preclinical/clinical evaluation of IRF4-targeting drugs. Following first observations in MM preclinical models where it efficiently suppressed IRF4-expressing cells [98], lenalidomide was found to be effective *in vitro* and *in vivo* in BTZ-resistant MCL tumours harbouring high IRF4 levels, while sparing IRF4 negative cases. Lenalidomide activity relied on a functional interaction with the component of the E3 ligase complex, cereblon (CRBN), and CRBN-dependent lowering of IRF4 expression, leading to the blocking of B-cell differentiation program, as shown by increase in PAX5 and loss of CD38 and BLIMP-1. Consequently, BTZ-lenalidomide

combination could overcome BTZ resistance [72]. Lenalidomide single agent was further validated in a phase II trial involving R/R MCL patients, including cases refractory to BTZ [99]. In contrast, in MM patients, therapy combining lenalidomide with BTZ failed in phase II trial [100]. Among the IMiD family, pomalidomide has been approved by the FDA for the treatment of R/R MM with at least two prior treatments, including BTZ. When added to BTZ or MRZ in combination with low dose dexamethasone, this agent went through successful phase I trial on heavily pre-treated, high risks relapsing MM [101, 102].

Beside these approaches, the inhibition of the IRF4 target gene, *MYC*, in BTZ-resistant MCL cultures have been studied either with siRNA-mediated gene knockdown or with treatment with an inhibitor of BRD4, a bromodomain and extraterminal domain (BET) protein. BET proteins mainly regulate epigenetics marks. They impact gene expression and in turn, participate in cancer pathogenesis. BET inhibitors (BETis) are very promising novel anticancer agents, and combinatory therapy with these inhibitors has been suggested. Because BETis target the NF- κ B pathway, their impact on BCL-2 and c-MYC proteins among others, have been tested preclinically in MCL and MM. As a proof-of-concept, inhibition of BRD4 synergistically induced cell death *in vitro* and *in vivo* when combined with lenalidomide. This confirmed that exacerbated IRF4/*MYC* signalling is associated with MCL resistance to BTZ and warranted the clinical evaluation of the IMiD-BETi combination in MCL cases refractory to the inhibition of proteasome [72].

Also, the BETi birabresib (OTX015) significantly synergizes with BTZ, CFZ, IXZ and IMiD to improve MM response and overcome resistance to PIs. It triggers the suppression of NF- κ B pathway and decrease in c-MYC signalling. The birabresib/pomalidomide combination demonstrate great results to overcome adaptive resistance in MCL [103].

Moreover, JQ1, a thieno-triazolo-1,4-diazepine has been characterized as the first indirect inhibitor c-MYC transcriptional network in MM cells [104]. Thereafter, the combination of BTZ with the JQ1 derivative, CPI203, was found to be synergistic in BTZ-resistant MM cell lines and in a primary culture from a MM patient refractory to BTZ therapy [105]. These studies supported the clinical evaluation of the IMiD-BETis combination in MM and in MCL cases refractory to PIs. An alternative approach to directly inhibit BRD4 was to promote its degradation. This was recently achieved by the use of the protein-targeting chimeric molecule (PROTAC), ARV-825, which specifically induces BRD4 ubiquitination and degradation, granting activity and overcoming PI resistance in MM [106].

4.5 Epigenetic modulators

Histone deacetylase 6 (HDAC6) mechanistically links the UPS and autophagy by facilitating the transport of protein aggregates along tubulin to juxtannuclear microtubule organizing centres [107]. Aggregated ubiquitinated proteins are transferred to lysosomes *via* autophagy, and BTZ treatment contributes to aggresome formation. Cells that lack HDAC6 were found to be defective in the removal of protein aggregates and are not able to form large aggresomes. The combination HDAC inhibitors with BTZ sensitizes MM resistant cells to proteasome inhibition [108]. Ricolinostat (ACY-1215) is a specific inhibitor of HDAC6. When ricolinostat is combined with BTZ and dexamethasone, the response rate among BTZ-refractory MM patients raised up to 20%. This combined treatment appeared well tolerated,

supporting the use of HDAC6 inhibitors to overcome PI resistance in patient [109]. In MCL, combined PI and ricolinostat treatments are still under preclinical investigations[110].

Vorinostat is an inhibitor of class I and II HDAC. In combination with BTZ, vorinostat revealed limited results in clinical phase II and I respectively for relapsing MCL and MM [111]. Others combinations involving this HDAC inhibitor are under investigation, and epigenetic regulation mechanisms such as DNA methylation have been subject of preclinical studies. Because NOXA and BCL-2 are demethylated during BTZ treatment, the DNA methyltransferase inhibitor decitabine showed synergic effect with BTZ in PI-resistant MCL [112].

6. Concluding remarks

Molecular mechanisms that explain inherent or acquired BTZ resistance have been essentially explored in MM and MCL. The identified alterations include mutations in proteasomal subunits and activation of pro-survival signalling pathways that have impact in cell cycle, cell differentiation, apoptosis, and stabilization of critical cellular factors. Two mayor proteolytic pathways inside the cell, UPS and ALS are major regulators of protein homeostasis and contribute to maintain the balance required to accomplish all protein functions, including the capacity of the cell to respond to BTZ. Beside the malignant cell itself, microenvironment is a crucial factor for impairing PI activity, especially in MM cells, which are heavily dependent on external factors for their growth and response to drugs.

New molecular mechanisms are regularly discovered that have been associated with drug resistance. Some of these mechanisms could be implicated in PI resistance, enhancing the complexity of this process. In the hypothesis of a multifactorial origin, the conception of new and more efficient approaches tackling PI resistance, should include combinatorial approaches simultaneously targeting multiple cellular mechanism. In the case of MCL and MM, the development of new types of drug such as IMiDs and BETis used in combination with PIs led to promising results *in vivo*. Nevertheless, combinatorial treatment could also increase off-target effects and for this reason a better assessment of these treatments has to be performed before been used in patients. The perfect strategy to overcome drug resistance in MM, MCL and other cancer types is far from being identified. Improving our knowledge on the molecular mechanisms implicated in resistance would also open the possibility to elaborate more efficient treatments while reducing the undesired side effects on healthy cells.

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Table 1. Proteasome inhibitors

PI	Action	Family	Target	IC ₅₀ (nM)
Bortezomib	Reversible	Boronate	β5c	7
			β5i	4
			β1c	74
Carfilzomib	Irreversible	Epoxyketone	β5c	5
			β5i	33
Oprozomib CFZ oral analog	Irreversible	Epoxyketone	β5c	36
			β5i	82
Ixazomib	Reversible	Boronate	β5c	3
			β5i	31
Marizomib	Irreversible	β-lactone	β5c	2.5
			β2c	26
			β1c	330
ONX0914	Irreversible	Epoxyketone	β5c	28
			β5i	280

Data presented in the table have been compiled from [4].

Table 2: PSMB5 mutations associated to BTZ resistance in MCL and MM

Gene	Mutation	Protein	Tumour cells	Pathology	Ref.
<i>PSMB5</i>	c.322G>A	p.A49T	KMS-11 OPM-2	MM	[113]
	c.247A>G c.322G>A	p.T21A p.A49T	8226	MM	[11]
	c.310A>G	p.M45V	8226*	MM	[4]
	c.310A>G	p.M45V	AMO	MM	[10]
	c.235G>A c.256G>C c.312G>C c.365G>A	p.A20T p.A27P p.M45I p.C63Y	Primary cells	MM	[12]

The indicated cell lines have been exposed to a continuous pressure of BTZ; except those marked with *, exposed to PR-924, a selective inhibitor of the immunoproteasome.

Table 3: Drugs currently used to recover sensitivity to BTZ

Drug Name(s)	mechanism	molecular target	pathology	ref
P5091	Ubiquitin signal	USP7	MM	[74]
b-AP15	Ubiquitin signal	USP14 and UCHL5	MM MCL	[75, 76]
SJB3-019A	Ubiquitin signal	USP1	MM	[77]
O-phenanthroline	Ubiquitin signal	RPN11	MM	[78]
Capzimin	Ubiquitin signal	RPN11	MM	[78]
WP1130	Ubiquitin signal	USP9X, USP5	MM	[79]
EOAI3402143	Ubiquitin signal	USP9X and USP24	MM	[79]
VLX1570	Ubiquitin signal	USP14	MM	[80]
Verapamil	drug transport	ABCB1	MM	[9]
Reserpine	drug transport	ABCB1	MM	[9]
Nelfinavir	drug transport	ABCB1	MM	[41, 81]
Lopinavir	drug transport	ABCB1	MM	[41]
Bafilomycin A	Autophagy	Vacuolar ATPase ATP6V1A	MM, MCL	[37, 82]
Chloroquine	Autophagy	Lysosome	MCL	[37]
Verteporfin	Autophagy	p62	MCL	[37]
Orlistat	Autophagy	fatty acid synthase	MCL	[83]
Perifosine	mTOR/ Akt	Akt, PI3K	MM	[87]
Dactolisib NVP-BEZ235	mTOR/ Akt	PI3K, mTOR	MCL	[84]
Temsirolimus (Torisel)	mTOR/ Akt	mTOR	MCL	[85]
Deforolimus (Ridaforolimus)	mTOR/ Akt	mTOR	MCL	[86]
Perillyl alcohol	NF-κB	TG2 signalling	MM MCL	[90]
Selinexor	NF-κB	XPO1	MM	[89]
Ibrutinib (Imbruvica)	NF-κB	BTK	MM MCL	[92, 94, 95]
Degrasyn	NF-κB	STAT, DUBs	MCL	[91]
Obatoclox	Bcl-2 proteins	NOXA	MCL	[14, 96]
cAMP	Bcl-2 proteins	Mcl-1	MM	[97]
Lenalidomide (Revlimid)	IMiD	CRBN , TNFSF11, CDH5, PTGS2	MCL	[99, 100]
Pomalidomide	IMiD	CRBN , TNF, PTGS2	MM	[101–103]

Dexamethasone	Glucocorticoid Immunosuppressant	NR3C1, NR0B1, ANXA1, NOS2	MM	[101, 102, 109]
JQ1	BET bromodomain inhibitors	BRD4	MM	[104, 105]
CPI203	BET bromodomain inhibitors	BRD4	MM MCL	[72, 105]
PROTACs (ARV-825)	BET specific target	BRD4 and other BET	MM	[106]
Birabresib (OTX015)	BET bromodomain inhibitors	BRD2,3,4	MM	[103]
Ricolinostat (ACY-1215)	epigenetic regulators	HDAC6	MM MCL	[109, 110]
Decitabine	epigenetic regulators	DNA methyl transferase	MCL	[112]
Vorinostat	epigenetic regulators	HDAC1	MM MCL	[111]

Figure 1: Molecular mechanisms associated to BTZ-resistance in acquired and resistant MCL and MM cell models. The pathways involved are: UPS and ALS degradation systems, UPR response, apoptosis, B-cell differentiation, cell cycle regulation and mutations in the $\beta 5$ subunit of the proteasome. Arrows indicates up, or downregulation of the different molecular mechanisms when compared with non-pathological conditions. See main text for further details.

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