

## Special Article - Multiple Myeloma

# Drug Resistance in Multiple Myeloma: How to Cross the Border

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## Abstract

Multiple myeloma is a hematological malignancy of plasma cells, characterized by a high level of genetic instability, with various gene mutations and chromosomal translocations. Despite the introduction of effective novel agents in the treatment of myeloma, i.e., proteasome inhibitors and Immunomodulatory Drugs (IMiDs), disease relapse and progression occur almost universally, due to innate and acquired drug resistance. We here describe molecular mechanisms and pathways of resistance so far elucidated. Due to the genetic complexity of myeloma, characterized by intra-clonal heterogeneity and progressing with branching evolutionary patterns, we believe that a single general strategy to overcome drug resistance in all patients cannot be developed. However, it is possible that in the future drug resistance will be prevented or treated with the individualized application of genomic and proteomic analyses, targeting the vulnerable pathways of a specific patient in a specific phase of the disease.

**Keywords:** Drug resistance; Multiple myeloma; Thalidomide; Lenalidomide; Pomalidomide; Bortezomib; Carfilzomib

## Introduction

Multiple myeloma (MM) is a hematological malignancy of plasma cells (PCs), characterized by lytic bone lesions, anemia, renal insufficiency, and the presence of monoclonal proteins in the blood and urine [1]. It is the second most common hematopoietic malignancy, with approximately 22,350 new cases and 10,710 deaths reported in 2013 [2]. The median age at diagnosis is approximately 70 years; 37% of patients are younger than 65 years, 26% are between the ages of 65 and 74 years, and 37% are 75 years of age or older [1]. Therapeutic advancement in recent years has increased the overall survival of myeloma patients. This is due not only to autologous Stem Cell Transplantation (SCT), but also to the availability of novel agents, such as the Immunomodulatory Drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide, and the proteasome inhibitors bortezomib and carfilzomib [3]. Despite the use of these new agents, MM relapses in virtually all patients [4].

The unique feature of myeloma cells is their intimate relationship with the bone marrow (BM) microenvironment. This consists of various Extracellular Matrix (ECM) proteins, cell components, osteoclasts, and osteoblasts [5]. The BM microenvironment provides a specialized niche that supports growth of myeloma and maintain their long-term survival by secreting growth factors such as Interleukin-6 (IL-6), Insulin Like Growth Factor-1 (IGF-1), Vascular Endothelial Growth Factor (VEGF), B-cell Activating Factor (BAFF), Fibroblast Growth Factor (FGF), Stromal cell-Derived Factor 1 $\alpha$  (SDF1 $\alpha$ ), and Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ) [6-10]. The direct interaction of the BM microenvironment with myeloma is responsible for myeloma cells growth and survival, angiogenesis, osteolytic lesions, and drug resistance [11-13]. Not surprisingly, multiple efforts have been made to target the interaction of myeloma cells to BM microenvironment for its treatment [14].

As in other malignant neoplasms, myeloma patients initially respond to chemotherapy; however, during the course of the disease, either intrinsic or acquired resistance to chemotherapy will emerge. MM is characterized by a complex genomic instability and cytogenetic constitution, which predisposes the malignant cells to intrinsic resistance [15-17]. The acquired resistance is mediated by several mechanisms, including overexpressed drug transporter proteins, such as P-glycoprotein (P-gp) -also known as Multidrug Resistance Protein 1 (MDR1)-[18], overexpression of anti-apoptotic proteins, such as B-cell leukemia protein 2 (Bcl-2), B-cell lymphoma-extra-large (Bcl-XL), Bcl2-related protein A1 (A1/Bfl1), and constitutive expression of transcription factor nuclear factor kappa B (NF- $\kappa$ B). Thus, therapeutic interventions that can induce apoptosis of tumor cells could become useful approaches to treat MM.

## Intrinsic resistance in multiple myeloma

Genomic instability and complex cytogenetic constitution are hallmarks of MM. The genetic lesions leading to myeloma have been recognized as inherited variations, translocations, copy number abnormalities, mutations, methylation, and microRNA (miRNA) abnormalities [15,16,19]. These lesions are associated with the myeloma proliferation and drug resistance [15,17]. Based on karyotype abnormalities, MM can be divided in two main groups: hyperdiploid and non-hyperdiploid. Studies suggested that only 10% of hyperdiploid group show a primary IgH translocation at 14q32 locus, whereas 70% of cases in the non-hyperdiploid group carry that [20]. Of note, patients in the hyperdiploid group have a better survival and prognosis of those in the non-hyperdiploid group. The IgH translocation usually involves juxtaposition of the immunoglobulin gene to an oncogene on partner chromosomes creating several reciprocal translocations. The more frequent ones are the t(4;14) (p16;q32) and t(11;14)(q13;q32) translocations, present in 15% and 17% of myeloma patients, respectively [21]. There are several other

translocations involving aberrant expression of oncogenes, such as c-MAF, c-Myc, and Cyclin D [21-24]. Whether these cytogenetic aberrations are directly associated with drug resistance and relapse is still debatable and warrants further investigations; nonetheless, recent studies have provided evidence that some of these cytogenetic abnormalities predispose to drug resistance and are associated to disease relapse. For example, studies of Chang-Yew et al. showed that blocking c-MAF, the oncogene overexpressed in patients with t(14;16) and t(14;20) translocations, inhibits cell proliferation and sensitizes myeloma cells to chemotherapy drugs [25,26]. Moreover, Iq21 gain, found in almost 70% of multiple myeloma patients harboring the t(14;16) or t(4;14) translocations, has been correlated with an adverse outcome [27,28].

## Acquired resistance in multiple myeloma

### Role of P-glycoprotein in acquired resistance

One of the most important players for acquiring drug resistance is the P-gp, produced by the MDR/ATP-Binding Cassette (ABC) B1 gene. This protein is the best known family member of pump transporters that mediate cellular efflux of peptides and drugs. Studies have shown that almost 75% of patients treated with doxorubicin, dexamethasone, and vincristine do increase the expression of P-gp [29]. Importantly, the expression of P-gp is found to be very low in untreated multiple myeloma patients, suggesting that the cumulative dose increases the expression of this protein in myeloma cells. A second drug-efflux protein, the Multidrug Resistance Protein-1 (MRP1), has not been found to be overexpressed in myeloma cells [30,31], making P-gp as the candidate protein in drug resistance. In untreated patients, P-gp does not seem to be expressed *de novo*. Interestingly, studies have shown that patients treated with melphalan do not increase P-gp expression [29], but increase in expression of P-gp was associated with treatment of other conventional chemotherapy agents, such as vincristine, doxorubicin, and dexamethasone [32], as well as novel agents, such as bortezomib and carfilzomib [33].

Because of the critical role of p-gp in drug resistance, inhibition of P-gp activity has become a major focus in clinical studies, and several MDR modulators have been tested to reverse drug resistance. For example, a phase I/II study was conducted using the MDR modulator verapamil, in combination with vincristine, doxorubicin, and dexamethasone, to treat patients with refractory MM [31]. This strategy induced a partial response rate of 50%, but this combination therapy resulted in excessive toxicity, and P-gp inhibitors do not constitute standard of care in the current management of MM.

### Activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and acquired resistance

Another major pathway that plays an important role in drug resistance is the NF- $\kappa$ B signaling pathway. The NF- $\kappa$ B term refers to a family of signal-responsive transcription factors that includes RelA/p65, c-Rel, RelB, NF- $\kappa$ B1/p50 and NF- $\kappa$ B2/p52 [34]. In normal resting cells, NF- $\kappa$ B transcription factors are maintained in an inactive state within the cytoplasm through binding to inhibitory proteins called I $\kappa$ B $\alpha$ , which also include the unprocessed p105 and p100 forms of NF- $\kappa$ B1 and NF- $\kappa$ B2 [34]. The activation of NF- $\kappa$ B is divided into two pathways, canonical and non-canonical. The canonical pathway is induced by physiological NF- $\kappa$ B stimuli including TNF- $\alpha$  or IL-1, and antigen receptors [35-37]. These cytokines bind and induce

Tumor Necrosis Factor Receptor 1 (TNFR1) signaling. Stimulation of TNFR1 leads to the binding of tumor necrosis factor receptor Type1-Associated Death Domain protein (TRADD), which provides an assembly platform for the recruitment of Fas-Associated protein with Death Domain (FADD) and TNF Receptor-Associated Factor 2 (TRAF2) [38]. TRAF2 couples with receptor-interacting serine/threonine-protein kinase 1 (RIP1) for I $\kappa$ B kinase (IKK) activation. IKK complex consists of two catalytically active kinases, IKK $\alpha$  and IKK $\beta$ , and the regulatory subunit IKK $\gamma$  (NEMO) [39,40]. In the canonical pathway, I $\kappa$ B $\alpha$  is phosphorylated in an IKK $\beta$ - and NEMO-dependent manner followed by ubiquitination and proteasomal degradation, which thus releases the bound NF- $\kappa$ B dimers so they can translocate to the nucleus. In contrast, the non-canonical pathway, induced by certain TNF family cytokines -such as CD40L, B-cell Activating Factor (BAFF) and lymphotoxin- $\beta$  (LT- $\beta$ )-, involves IKK $\alpha$ -mediated phosphorylation of p100 associated with RelB, which leads to partial processing of p100 and the generation of transcriptionally active p52-RelB complexes. IKK $\alpha$  activation and phosphorylation of p100 depends on Nuclear factor  $\kappa$ B-Inducing Kinase (NIK), which is tightly regulated by TRAF3, TRAF2 and additional ubiquitin ligases [35-38]. Once in the nucleus, NF- $\kappa$ B dimers are further regulated by protein phosphorylation [41] and other post-translational modifications, such as protein acetylation [42], and activate genes whose products inhibit cell death [43-46], stimulate cell proliferation [47], and promote migratory and invasive phenotypes that are associated with tumor progression [48]. The transcription factor NF- $\kappa$ B could also contribute to drug resistance in myeloma through up-regulation of some Bcl-2 anti-apoptotic family members including Bcl-XL [49].

Myeloma cells often show constitutive expression of NF- $\kappa$ B [50]. Most solid and lymphoid tumors show constitutive NF- $\kappa$ B activity [51]. Constitutive or overexpression of NF- $\kappa$ B has been associated with myeloma cell proliferation, survival, invasion and drug resistance [52,53]. Although the precise role of NF- $\kappa$ B activation in pathogenesis of myeloma has not been fully characterized, it has been shown that myeloma cell adhesion to bone marrow stromal cells induces NF- $\kappa$ B-dependent up-regulation of transcription of IL-6 [54,55], which is a major growth and survival factor for myeloma cells. Since IL-6 upregulates adherence of myeloma cells to fibronectin and it confers resistance to drug-induced apoptosis, blockade of NF- $\kappa$ B signaling represents a novel therapeutic strategy in MM. Recent studies have indicated that targeting NF- $\kappa$ B pathway in myeloma has a positive outcome [56,57]. Along these line, myeloma cells harboring TRAF3 gene mutation in NF- $\kappa$ B pathway are resistant to dexamethasone but sensitive to bortezomib [58], suggesting that apoptotic function of bortezomib is partly explained by blocking the canonical NF- $\kappa$ B pathway; however, at the same time it unexpectedly induces the alternative (non-canonical) pathway, which makes myeloma cells less responsive [59]. Moreover, recent studies have shown that though canonical NF- $\kappa$ B pathway can be successfully blocked by small-molecule inhibitors of IKK $\beta$ , in myeloma cell lines *in vitro*, the anti-MM activity *in vivo* of these IKK $\beta$  inhibitors is limited because of the compensatory activation of the non-canonical pathway [60]. Furthermore, it has been reported that myeloma cells tend to develop a bortezomib-resistant NF- $\kappa$ B phenotype through a Proteasome-Inhibitor Resistant (PIR) pathway [61]. The latter flaws of bortezomib may explain to a large extent why it should be applied in combined regimens for those myeloma patients who are refractory to it. Since

bortezomib-induced cytotoxicity cannot be fully attributed to inhibition of canonical NF- $\kappa$ B activity in myeloma cells, inhibition of both canonical and noncanonical pathways would be a desirable strategy to combat resistance.

## Cancer Stem Cells and Resistance

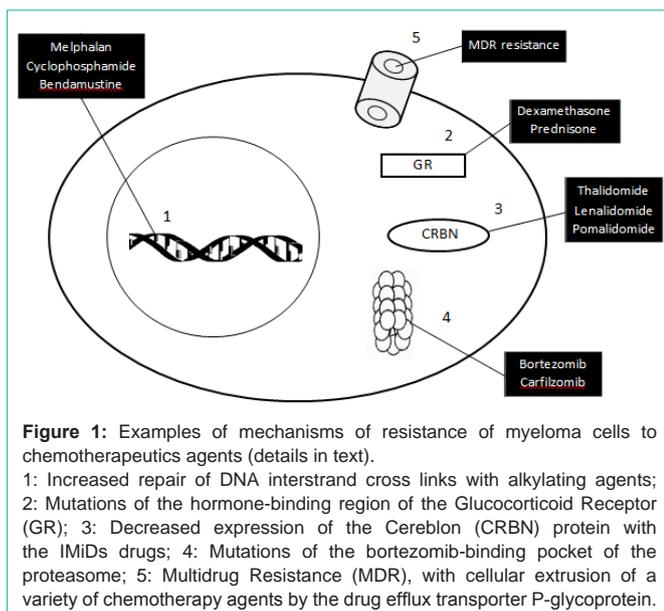
The association of cancer stem cells (CSCs) and drug resistance has been demonstrated in various types of cancer including MM [62]. The importance of targeting CSC population has been realized for many years, but this research has been limited by the fact that a specific CSC population in MM has not been properly defined yet [17]. Studies from the last decade have supported the notion that CSCs are resistant to chemotherapeutic agents. Various strategies have been proposed to target the molecular, metabolic, and epigenetic signatures, and the self-renewal signaling pathway characteristic of CSCs. These cells exhibit high levels of ABC activity, Aldehyde Dehydrogenases (ALDH1), and Retinoic Acid (RA) Receptor  $\alpha$  (RAR $\alpha$ ) that have been associated with clonogenic potential and resistance to chemotherapy [63,64]. Further research is needed to understand the molecular mechanisms that keep myeloma cells clonogenic and drive the transition between stem-like and non-stem-like states in the local bone marrow microenvironment. These studies could provide us new approaches to target drug resistance in MM.

### Drug resistance to specific antineoplastic agents

Although we are far from having a satisfactory knowledge of the different mechanisms of resistance to various chemotherapy agents, multiple studies have elucidated particular strategies and mutations developed by myeloma cells after exposure to specific drugs. We herein describe the most important published experience with the various class of agents used in the treatment of MM. A few examples are shown in Figure 1.

#### Glucocorticoids

Glucocorticoids, such as prednisone and dexamethasone, are among the most commonly used drugs in the treatment of MM. They are able to induce response rates of approximately 60% in



newly diagnosed patients, even when used in monotherapy [65]. The Glucocorticoid Receptor (GR) is a member of the nuclear hormone receptor superfamily, which consists of structurally related proteins, that function as receptors for several hormones, including estrogens, progesterone, androgens, thyroid hormone, vitamin D, retinoids, and others. These cytoplasmic receptors are activated by the binding to their ligands, translocate to the nucleus, and regulate transcription of target genes that control biologic processes important in development, cell proliferation, and differentiation [66]. The nuclear hormone receptors typically consist of 5 domains: A/B (modulating region), C [DNA-binding region, containing two zinc-fingers which specifically recognize the Hormone-Responsive Element (HRE), a DNA enhancer sequence that is located near the promoter region of target genes], D (hinge region, important for nuclear localization), E (hormone-binding region, which also contains leucine zippers, the sites of dimerization), and F (modulating region). Following ligand binding, hormone-receptor complexes dimerize with other hormone-receptor complexes and translocate to the nucleus, where they bind to and initiate transcription of HRE expressing genes. The interaction between the receptor dimer and the HRE activates transcriptional machinery, leading to the expression of a specific set of genes. Resistance to corticosteroids may be due to mutations in their receptors: in a study of a myeloma line derived from a patient who became refractory to dexamethasone, the resistance was found to be caused by a truncated mRNA transcript of the GR, which lacked the C (hormone-binding) region [67]. Studies of Sanchez-Vega and Gandhi suggest that glucocorticoid resistance can also be mediated by transcription elongation block in the glucocorticoid receptor gene NR3C1 [68]. It has been proposed that glucocorticoid resistance should be divided in two forms, primary and secondary. Primary resistance is associated with the level of GR expression and the regulation of intracellular substrate availability [69]. The secondary form develops over the period of treatment, and its mechanism is still not completely understood. Clinical surveys suggest a correlation between functional GR expression levels and primary glucocorticoid sensitivity and prognosis [69].

#### Alkylating drugs

Alkylating drugs, such as melphalan and cyclophosphamide, are chemotherapy agents commonly used in the treatment of MM. Their mechanism of action involves the formation of cross links between the two strands of DNA ("inter-strand cross linking"), with impairment of DNA synthesis and cell replication. Melphalan, a drug synthesized in 1953, has a structure that incorporates two alkylating agents, nitrogen mustard and phenylalanine. More than 60 years later, this drug is still considered standard therapy for MM, either as single agent in the preparative regimen for autologous SCT, or in combination with other agents in SCT-ineligible patients [70]. Studies have indicated that the principal mechanism of resistance to melphalan is the increased repair rate of DNA inter-strand cross links [71,72], which is mediated by the Fanconi Anemia (FA)/BRCA pathway [73]. Silencing this pathway in melphalan-resistant cells with siRNA can reverse the drug resistance, whereas its overexpression promotes cell survival following melphalan treatment [73]. It is likely that the FA/BRCA pathway is involved even in cross-resistance to other alkylating agents and radiation therapy [74].

## Proteasome inhibitors

The proteasome is a protein enzyme complex that breaks down and clears unused or misfolded proteins. Myeloma cells are particularly sensitive to inhibitors of the proteasome, presumably because they are specialized for the mass production of immunoglobulins. The increased protein load associated with this task lowers the threshold for proteotoxic stress and leaves plasma cells susceptible to toxic misfolded/unfolded proteins and pro-apoptotic signals initiated by the unfolded protein and endoplasmic reticulum stress responses [75]. Bortezomib is a dipeptide boronate that inhibits the ubiquitin-mediated proteasome degradative pathway. The 26S proteasome consists of two 19S regulatory complexes and a barrel-shaped 20S proteolytic core. The 19S regulatory complexes bind the proteins tagged with ubiquitin, and direct them to the 20S core. The 20S is a proteolytic core that consists of 2 $\alpha$ -subunit rings and 2 $\beta$ -subunit rings, each of which contains 7 different  $\alpha$  and  $\beta$  subunits. The proteolysis is mediated by the  $\beta$ -subunits:  $\beta$ 1 (caspase-like activity),  $\beta$ 2 (trypsin-like activity), and  $\beta$ 5 (chymotrypsin-like activity). Despite the efficacy of bortezomib in the treatment of myeloma, the neoplastic plasma cells invariably develop resistance to the boronic molecule. One study attributed the molecular resistance to bortezomib to a gene mutation (Ala49Thr substitution) of the proteasome  $\beta$ 5-subunit, which contains the bortezomib-binding pocket. The mutated protein was over expressed in resistant cells, and silencing of the  $\beta$ 5-subunit gene with siRNA restored bortezomib sensitivity and induced apoptosis [76]. This finding was confirmed by other studies, in which bortezomib resistance was caused by several other mutations involving the bortezomib-binding pocket of the  $\beta$ 5-subunit or its close proximity [77,78]. The development of novel proteasome inhibitors that bind to the  $\alpha$ -subunits instead of the  $\beta$  ones could overcome this mechanism of resistance.

Studies of bortezomib-resistant cell lines did not detect an abnormal composition or activity of the proteasome enzyme complex, and genomic profiling indicated the presence of other mechanisms of resistance, such as the overexpression of the Heat Shock Protein B8 (HSPB8), or cellular extrusion via the drug efflux transporter P-gp [79]. HSPB8 promotes the survival of myeloma cells by enhancing the autophagic removal of misfolded proteins through lysosomal degradation [80]. A second irreversible proteasome inhibitor, carfilzomib, has entered the clinical practice, and several other are currently in the development phase. Beside  $\beta$ 5-subunit mutations and P-gp overexpression, mechanisms of resistance to these new agents have not yet been elucidated [33].

## Immunomodulatory drugs (IMiDs)

Thalidomide and its derivatives lenalidomide and pomalidomide represent a new class of antineoplastic compounds called IMiDs, which have immune-modulatory, anti-inflammatory, and anti-angiogenic properties. Their mechanism of action was elucidated only recently, after the discovery of their receptor, the Cereblon protein (CRBN) [81]. CRBN is a substrate-recognition component of the E3 ubiquitin ligase complex, which includes the DNA Damage Binding protein-1 (DDB1), Cullin-4A (Cul4A), and Regulator of cullins (Roc1). It is now known that the therapeutic activity of IMiDs is due to a CRBN gain of function. IMiDs are able to modify the substrate specificity of CRBN, and induce the proteasome degradation of the ikaros

proteins IKZF1 and IKZF3. These are two B cell transcription factors, which can be transcriptional activators or repressors, depending on different cellular settings [82,83]. In view of these discoveries, the IMiDs should be more properly called “ubiquitin ligase modulators”. In a study, resistance to lenalidomide was mediated by the induction of the Wnt/ $\beta$ -catenin pathway [84]. Other studies indicated that the resistance was mediated to decreased expression of the CRBN protein [85,86].

## Conclusion

The introduction of novel agents in the treatment of MM, as the proteasome inhibitors and the IMiDs, has revolutionized its management, so that many patients are now a day able to remain in remission for several years. Results have been improved by the adoption of these novel agents, initially approved for the relapsing/refractory setting, and later introduced into the upfront setting, and by incorporating them into combination regimens that produced unprecedented rates of disease response. Despite these therapeutic advances, disease relapse and progression occur almost universally, due to innate or acquired drug resistance.

In this review, we described several molecular mechanisms and pathways of resistance to different anti-neoplastic agents that have been elucidated so far. MM is a genetically complex cancer, characterized by intra-clonal heterogeneity and progressing with branching evolutionary patterns, instead of a linear multistep process [87]. According to this view, disappearance of sensitive neoplastic subclones after the initial chemotherapy can be followed by the emergence of other selected subclones with different mutations, and so on. While cells of an unmutated tissue respond homogeneously to a particular chemotherapy agent, the heterogeneity of multiple myeloma subclones may express different levels of drug sensitivity. At this stage, innate mechanisms mediated by MDR leads to cross-resistance, and branching evolution with acquired mutations selects resistant clones that ultimately lead to the terminal phase of the disease. Resistance to a single drug can be eliminated by the use of combination regimens, using agents that target different receptors and molecular pathways inside the myeloma cell. Along these lines, specific cell signaling targeted therapies such as HDAC, PI3K/AKT/mTOR, Hsp90, Wnt, Notch, Hedgehog; and strategies targeting the tumor microenvironment including hypoxia, angiogenesis, CD44, CXCR4, have yielded promising results alone or in combinations in preclinical or clinical studies involving patients with relapsed/refractory MM. Based on these premises, we believe that a single general strategy to overcome drug resistance in all patients cannot be developed. However, with the rapid advances made by basic research and molecular oncology in the last decade, it is likely that the future of clinical management of myeloma patients will rely on the individualized application of genomic and proteomic analyses, targeting the vulnerable pathways of a specific patient in a specific phase of the disease. In order to cure MM, we need a better understanding of the complex drug resistance in this disease, and further development of new therapeutic agents is warranted.

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