Therapy of Multiple Myeloma and Myeloma-Related Kidney Disease

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Abstract

Multiple Myeloma (MM) is a clonal expansion of plasma cells. MM is characterized by proliferation of malignant clones producing defective monoclonal immunoglobulins in the bone marrow. MM is the second most common blood cancer following non-Hodgkin's lymphoma. It remains an incurable disease with resistance to drugs leading to relapse in most patients even if the treatments for MM have improved over the past decade. Therefore, it is necessary to understand the detailed mechanisms of MM pathogenesis and the drug resistance of MM and develop more effective therapeutic strategies. Current therapies such as Proteasome Inhibitors (PIs), aggresome inhibitors (Histone Deacetylase Inhibitors /HDACi/), Immunomodulatory Drugs (IMiDs) and anti-cell surface glycoprotein CS1, also known as SLAM Family Member 7 (SLAMF7), and CD38 monoclonal antibodies are effective and are followed by an autologous stem cell transplant for patients who are eligible. Myeloma-related End-Stage Renal Disease (ESRD) is not a contraindication for stem cell transplant. Renal insufficiency slightly contributes in transplantation-related mortality (>4% vs <1%). Coordination between the oncologist and nephrologist is important for optimal patient care.

Introduction

Multiple Myeloma (MM) is a hematological malignancy characterized by the clonal expansion of monoclonal immunoglobulin-secreting plasma cells in the Bone Marrow (BM). MM is associated with lytic bone lesions, anemia, immunodeficiency, and renal impairment. MM accounts for more than 10% of all hematologic malignancies and about 1% of all cancers [1]. In 2013, approximately 22,500 individuals in the United States and 6/100,000 in Europe were diagnosed with MM [2]. Surveillance, Epidemiology, and End Results Program (SEER) lists myeloma as the 14th most common type of cancer in the United States. There will be 30,280 new cases (17,490 men and 12,790 women) and 12,590 deaths (6,660 men and 5,930 women) this year in the United States. That is only 1.9% of all cancer deaths. MM remains mostly incurable despite conventional intensive high dose chemotherapy followed by hematopoietic stem cell transplant. The costs associated with MM are among the highest.

MM is almost exclusively diagnosed in people aged 65 or older. People under age 35 represent less than 1% of cases according American Cancer Society (ACS). The median age at MM diagnosis is 70 years, with 35% of patients younger than 65 years [3]. MM occurs in men (7 per 100, 000) at a rate 56% higher than women (4.5 per 100, 000). There has been a significant improvement in overall 5-year survival in patients with MM since the 1960s; 12% from 1960 to 1963 to 34% from 1996 to 2003. Apart from the introduction of bisphosphonates in the 1990s and the development of autologous stem cell transplantation in the mid-1980s, patients with MM were treated mainly by the alkylating agent melphalan and a synthetic corticosteroid drug prednisone or dexamethasone (Decadron). The increasing knowledge of MM biology is already contributing to more specific drug designs. It was shown that as important as the malignant cells themselves is their interaction with the microenvironment in the pathogenesis of MM [4]. Discoveries in the genetic abnormalities associated with MM [5-10] and better understanding of the bone marrow [11,12] microenvironment have led to new diagnostic, prognostic and treatment strategies. Therefore, drugs with a dual effect on the plasma cells and their bone marrow milieu in MM could be particularly valuable. The introduction of new therapies and the using of autologous stem-cell transplantation have led to increases in overall survival and progression-free survival in patients with MM. However, none of these therapies are curative and nearly all patients relapse.

Novel agents, such as thalidomide, the Immunomodulatory drugs of next generation lenalidomide (also known as CC-5013, IMiD-3 or Revlimid®) and pomalidomide (CC-4047), proteasome inhibitors (bortezomib, also known as PS-341 or VelcadeTM), histone deacetylase inhibitors (panobinostat, vorinostat and romidepsin) and anti-cell surface glycoprotein CS1, also known as SLAM Family Member 7 (SLAMF7), and CD38 monoclonal antibodies Elotuzumab and Daratumumab in various combinations can achieve responses in patients who have relapsed and refractory MM [13-32]. Furthermore, surface molecule CD38 can be used as a target for immunotherapies other than just naked monoclonal antibodies. Alternative immunotherapeutic approaches such as CD38-specific Chimeric Antigen Receptor (CAR) T cells target the same antigen

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[33]. The immune system is an important component of the tumor microenvironment in MM, and acting on the immune system is an appealing new treatment strategy. Preliminary results on Chimeric Antigen Receptor (CAR) T cells, first against CD19, and more recently against B-cell maturation antigen, have shown to induce durable responses in heavily pretreated MM patients [34]. Checkpoint inhibitors and Adoptive Cell Therapy (ACT) are 2 of the main actors, together with monoclonal antibodies and immunomodulatory agents, in the immune-oncologic approach. The aim of checkpoint inhibitors is to release the brakes that block the action of the immune system against the tumor. Anti-Programmed Death-1 (PD-1) and PD-1-Ligand, as well as anti-CTLA4 and KIR are currently under evaluation, as single agents or in combination, with the best results achieved so far with combination of anti-PD-1 and immunomodulatory agents [34]. However, the median survival remains at 6 years, with only 10% of MM patients surviving at 10 years.

Proteasome Inhibitors

Proteasomes belong to proteolytic enzymes called threonine proteases. Proteasome inhibitors were first synthesized as tools to probe the function and proteolytic activity specificity of the proteasome [35,36]. Most of the proteasome inhibitors address the chymotryptic activity of the 20S proteasome core via adduct formation with N-terminal threonine hydroxyl group as part of the catalytically active center.

Results of both preclinical and clinical studies suggest that transformed cells are more sensitive to proteasome inhibition than normal cells [37-39]. Similarly, MM cell lines were more sensitive to apoptosis induced by proteasome inhibitors than were peripheral blood mononuclear cells from healthy individuals [40]. The biologic basis for the enhanced susceptibility of cancer cells to proteasome inhibitors has not been fully elucidated. Several hypotheses have been proposed. One from them is the loss of checkpoint mechanisms for DNA repair by cancer cells. Other hypotheses are a greater sensitivity of rapidly proliferating tumor cells to proteasome inhibitors and more efficient uptake and slower inactivation of proteasome inhibitors by tumor cells [41-43]. Thus, the possibility that proteasome inhibitors could be drug candidates appeared as a new hope for cancer therapy.

There are six major classes of proteasome inhibitors: peptide aldehydes, peptide semicarbazones, peptide vinyl sulfones, peptide boronates, peptide epoxyketones (epoxomycin and eponomycin) and β -lactones (lactacystin and its derivatives), based on the pharmacophore that reacts with the threonine residue in the active site of the proteasome. In hematologic malignancies, three classes of proteasome inhibitors entered clinical trials (peptide boronates / bortezomib/, β -lactones /NPI-0052/ and epoxomycin derivatives / PR-171/). Toxicity profiles of other three classes of proteasome inhibitors (peptide aldehydes, peptide semicarbazones and peptide vinyl sulfones) prevent their use in clinical trials [44-46].

Bortezomib

Boronate inhibitors of the proteasome are more potent than structurally similar peptide aldehydes [47]. Bortezomib (VELCADE, formerly known as PS-341, pyrazinylcarbonyl-Phe-Leu-boronate) inhibits proteasome by binding reversibly to the chymotrypsin-like site in the 20S core of the proteasome [48]. Bortezomib is the first proteasome inhibitor approved by the US Food and Drug

Administration for the treatment of relapsed or relapsed and refractory Multiple Myeloma (MM) and some forms of non-Hodgkin's lymphoma, mantle cell lymphoma [49-57]. Cellular mechanisms responsible for the clinical efficacy of bortezomib include inhibition of tumor cell adhesion to stroma and disruption cytokine-dependent survival pathways, in part through suppression of the transcription nuclear factor- κ B (NF- κ B) activity, inhibition of angiogenesis, induction of aggresome (aggregates of ubiquitin-conjugated proteins) formation, endoplasmic reticulum stress, and the unfolded protein response [58-64].

Bortezomib in preclinical studies on human myeloma cell lines and primary patient multiple myeloma cells

In these studies bortezomib inhibited the proliferation in human myeloma cell lines, which were both sensitive and refractory to conventional chemotherapeutic agents (melphalan, doxorubicin, mitoxantrone and dexamethasone). Bortezomib induced caspase-dependent apoptosis of myeloma cell lines and primary patient MM cells [65]. MM cell lines were up to 40 times more sensitive to the proapoptotic effects of bortezomib than were peripheral blood mononuclear cells from healthy individuals [65]. Bortezomib also inhibited NF- κ B activation in Tumor Necrosis Factor (TNF)- α -treated MM cells by blocking the degradation of the inhibitor protein I κ B α and overcame the resistance to apoptosis in MM cells conferred by IL-6 [65-67]. Proapoptotic regulators up regulation and antiapoptotic proteins down regulation were observed [66,68]. Bortezomib stabilizes proapoptotic proteins, such as p53, Bax, Bik and Bim while reduce levels of some antiapoptotic proteins, such as Bcl-2. Bortezomib responses are also linked to the upregulation of the proapoptotic Bcl-2 protein family member Noxa [69] and this effect is independent of constitutive activity of the phosphoinositide 3-kinase/AKT (protein kinase B) and NF- κ B pathways. Bortezomib induces Noxa and the cleavage of antiapoptotic protein Mcl-1. The Myeloid Cell Leukemia-1 (Mcl-1) protein is an antiapoptotic member of the Bcl-2 family. Noxa induction allows the displacement of the direct apoptosis activator Bim, which is able to activate Bax/Bak by a "hit and run" mechanism, triggering mitochondrial dysfunction and apoptosis (Figure 1).

Bortezomib-mediated proteasome inhibition affects multiple signaling pathways, including cell cycle, growth arrest, stress response, microenvironment and apoptosis. Disruption of multiple cellular signaling by bortezomib initiates and maintains an active cell death pathway and causes apoptosis.

Binding of MM cells to BMSCs and abrogation of the NF- κ B-dependent transcription and secretion of IL-6 in BMSCs were also inhibited by bortezomib [70]. Molecular mechanisms of the antimyeloma activity of bortezomib were studied by analysis of gene expression profiles of bortezomib-treated MM cells in comparison with nontreated MM cells. Bortezomib also induced the phosphorylation of c-Jun NH2-Terminal Kinase (JNK), activating caspase-8 and, subsequently, caspase-3. The activated caspase-3 cleaved DNA protein kinase catalytic subunit and ATM/ATR proteins, and ultimately resulted in impaired DNA repair in MM cells [70]. DNA damage induced by activated caspase-3 was also observed. Subsequent phosphorylation of p53 and the degradation of Mdm2 (the product of expression of murine double minute oncogene that represses p53 transcriptional activity by p53 poly-ubiquitination and

degradation, the human analogue HDM2) are in a time and dose-dependent on the level of bortezomib which is comparable with the serum level of bortezomib used in clinical practice [70].

Bortezomib increases osteoblast number, function, and gene expression [71-73] through targeting of a multipotent population of Mesenchymal Stem Progenitor Cells (MSCs). Bortezomib induces MSCs to preferentially undergo osteoblastic differentiation, in part by modulation of the bone-specifying transcription factor Runt-Related Transcription Factor 2 (Runx-2) [74]. Bortezomib promotes bone formation in myelomatous and nonmyelomatous bones by simultaneously inhibiting osteoclastogenesis and stimulating osteoblastogenesis. As clinical and experimental studies indicate that bone disease is both a consequence and necessity of MM progression our results suggest and that bortezomib's effects on bone remodeling contribute to the antimyeloma efficacy of this drug [75].

Three other studies [76-78] showed an inhibitory effect of bortezomib on osteoclastic bone resorption in MM patients with a significant reduction in serum Dickkopf-1 (DKK1) and RANKL levels. DKK1 is an inhibitor of the Wnt signaling pathway (wingless in *Drosophila*) that is important in the growth, development, and function of osteoblasts [79].

Bortezomib in relapsed and/or refractory multiple myeloma - clinical studies

Based on preclinical studies and promising phase 1 trial, two pivotal phase 2 studies, SUMMIT [80] and CREST [81] were performed in relapsed and/or refractory MM patients. Patients were treated with bortezomib 1.3 mg/m² on days 1, 4, 8, and 11 every 3

weeks. Dexamethasone was allowed in patients with suboptimal responses to bortezomib alone. The overall response rate was 35%, including 10% complete or near complete responses with an overall survival of 17 months. The randomized CREST study [81], comparing two dosages of bortezomib (1.3 versus 1, 0 mg/m²) showed that a reduced dose was able to produce responses in up to one third of patients and it was accompanied with a lower toxicity. The addition of dexamethasone in patients with suboptimal responses to bortezomib alone resulted again in an improvement in the response degree [82].

A subsequent randomized phase 3 trial "The Assessment of Proteasome Inhibition for Extending Remissions" (APEX) including 669 patients with relapsed MM showed that bortezomib is more effective than high dose dexamethasone as demonstrated by a significant improvement in response rate (43% vs 18%), median time to progression (6.2 vs 3.4 months) and 1-year survival rate (80% vs 67%, respectively) [83]. In the updated APEX analysis (median follow-up: 22 months), survival was assessed in both arms (single-agent bortezomib versus high-dose dexamethasone), and efficacy updated for the bortezomib arm. Median survival was 29.8 months for bortezomib versus 23.7 months for dexamethasone, a 6-month benefit, despite substantial crossover from dexamethasone to bortezomib. Overall and complete response rates with bortezomib were 43% and 9%, respectively; among responding patients, 56% improved response with longer therapy beyond initial response, leading to continued improvement in overall quality of response. Higher response quality was associated with longer response duration; response duration was not associated with time to response. These data confirmed the activity of bortezomib and supported extended treatment in relapsed multiple myeloma patients tolerating therapy [84].

Bortezomib therapy has become an important part of the standard of care for patients with relapsed multiple myeloma, and preliminary clinical evidence suggests that bortezomib retreatment in patients previously treated with the drug may prolong disease control. The retrospective study [84] was designed to clarify the utility of bortezomib as a repeat therapy. Records from 3 major cancer centers that had participated in the phase 2 (SUMMIT or CREST) or phase 3 (APEX) registration studies were used to identify patients who were subsequently retreated off protocol with bortezomib-based therapy. 22 patients who received bortezomib retreatment following a 60 or more day gap between bortezomib treatments were found. Twelve patients had intervening therapy between initial bortezomib treatment and bortezomib retreatment. During retreatment, 14 of 22 patients received bortezomib in combination with another antineoplastic agent. The overall response rate for bortezomib retreatment was 50% (9% complete responses). The median length of retreatment was 5.1 months in responding patients and 2.4 months in nonresponding patients. Therapy was terminated due to unmanageable toxicity in 2 patients during retreatment, compared with 6 patients during initial treatment. During retreatment, no patients required dose reduction due to peripheral neuropathy, compared to 4 patients during their initial treatment. Thus, bortezomib retreatment appears to be safe and effective. Favorable observed response rates with bortezomib retreatment suggest that it may be a viable option for relapsed or refractory multiple myeloma, even in patients previously exposed to bortezomib [85].

An important aspect of all these studies is the toxicity profile of bortezomib, particularly when used in combinations with other agents. The most common side effects of bortezomib were gastrointestinal symptoms, fatigue, and anorexia; although these were mostly grade 1-2 [86]. Thrombocytopenia grade 3-4, due to a reversible blockage in platelet release, was found in 30% of cases, while anemia and neutropenia are uncommon (<10%). The major dose-limiting adverse effect is painful peripheral neuropathy (37% with only 9% grade 3 and more) [87]. Unusual toxicities such as tumor lysis syndrome, severe pulmonary failure, toxic hepatitis or rhabdomyolysis have been also reported [86,88,89]. Thus, the toxicity profile of bortezomib is well defined and most complications are predictable and manageable.

Combinations of Bortezomib with Conventional Anti-Multiple Myeloma Agents

Bortezomib was combined in a phase 1 trial with liposomal doxorubicin in advanced MM patients [90]. The response rate among 22 evaluable patients was 73%, including 36% of complete response. An updated analysis [91] after extended follow-up showed the time to progression 9.3 months, the median time from start of therapy to start of subsequent therapy 24.2 months and median overall survival 38.3 months. The phase 3 international study compared the efficacy and safety of a combination of Pegylated Liposomal Doxorubicin (PLD) plus bortezomib with bortezomib monotherapy in patients with relapsed or refractory multiple myeloma [92]. 646 patients were randomly assigned to receive either intravenous bortezomib 1.3 mg/m² on days 1, 4, 8, and 11 of an every 21-days cycle, or the same bortezomib regimen with PLD 30 mg/m² on day 4. Median time to progression was increased from 6.5 months for bortezomib to 9.3 months with the PLD + bortezomib combination. The 15-month survival rate for PLD + bortezomib was 76% compared with 65% for bortezomib alone. The complete plus partial response rate was 41% for bortezomib and 44% for PLD + bortezomib, a difference that was not statistically significant. Median duration of response was increased from 7.0 to 10.2 months with PLD + bortezomib. Grade 3/4 adverse events were more frequent in the combination group (80% vs 64%), with safety profiles consistent with the known toxicities of the two agents. An increased incidence in the combination group was seen of grade 3/4 neutropenia, thrombocytopenia, asthenia, fatigue, diarrhea, and hand-foot syndrome. PLD with bortezomib is superior to bortezomib monotherapy for the treatment of patients with relapsed or refractory multiple myeloma. The combination therapy is associated with a higher incidence of grade 3/4 myelosuppression, constitutional symptoms, gastrointestinal symptoms, and dermatologic toxicities.

Following the remarkably positive results of a large phase 3 trial comparing bortezomib and PLD with bortezomib monotherapy, the FDA approved the use of this combination in May 2007 for patients who have not previously received bortezomib and who have received at least one prior therapy. Bortezomib can sensitize MM cells to conventional chemotherapeutics to which a patient has previously become resistant [66,73].

Bortezomib has shown activity in relapsed and or refractory MM, even in patient's refractory to thalidomide or lenalidomide (Immunomodulatory Drug [IMiD]) treatment [94]. Combination therapies with bortezomib - thalidomide - dexamethasone [95], bortezomib-melphalan-prednisone-thalidomide [96] or with

bortezomib - lenalidomide - dexamethasone [97,98] were highly effective in advanced and refractory MM. These treatment strategies will hopefully further prolong the survival of patients with myeloma.

The Second-Generation Proteasome Inhibitors with More Potency and Fewer Side-Effects Studied in Multiple Myeloma

The good clinical results obtained with bortezomib have validated the proteasome as a therapeutic target in MM. Two second-generation proteasome inhibitors have entered phase 1 and phase 2 trials: NPI-0052 (salinosporamide A, Marizomib) and carfilzomib (Kyprolis, formerly PR-171).

Salinosporamide A (Marizomib) was discovered by William Fenical and Paul Jensen from Scripps Institution of Oceanography in La Jolla, CA. In preliminary screening, a high percentage of the organic extracts of cultured *Salinispora* strains possessed antibiotic and anticancer activities, which suggests that these bacteria are an excellent resource for drug discovery. *Salinispora* strain CNB-392 was isolated from a heat-treated marine sediment sample collected in the Bahamas and cytotoxicity-guided fractionation of the crude extract led to the isolation of salinosporamide A. Salinosporamide A shares its fused γ -lactam- β -lactone bicyclic ring structure with clasto-lactacystin- β -lactone, also called omuralide. Thus, Salinosporamide A is a natural product derived from the fermentation of the marine gram-positive actinomycete *Salinispora tropica* and is a highly cytotoxic proteasome inhibitor [99].

Preclinical studies in MM models showed that NPI-0052 induces apoptosis of MM cells, including cells resistant to conventional anti-MM therapies as well as cells isolated from patients who had developed resistance to bortezomib-based therapies [100]. NPI-0052 is distinct from bortezomib in terms of chemical structure and effects on individual proteolytic activities of the proteasome activities. While bortezomib inhibits exclusively the chymotrypsin-like activity of the 20S proteasome, NPI-0052 inhibits all three protease activities in the proteasome, the chymotrypsin-like, trypsin-like, and caspase-like activities [101]. Another important difference is that NPI-0052 is orally bioavailable, while bortezomib is not amenable to oral administration. Inhibition of all three proteolytic activities of the 20S proteasome by NPI-0052 results in activation of caspase-8. This leads to proapoptotic protein Bid cleavage and to the considerable decrease of mitochondrial transmembrane potential [102]. Mitochondrial membrane permeabilization, release of cytochrome c and activation of caspase-9 follow. Apoptosis proceeds then by activation of effector caspase-3 and consequent DNA fragmentation and increased levels of intracellular reactive oxygen species were detected [102].

Salinosporamide A potentiated the apoptosis induced by Tumor Necrosis Factor α (TNF), bortezomib, and thalidomide, and this correlated with down-regulation of gene products that mediate cell proliferation (cyclin D1, Cyclooxygenase-2 [COX-2], and c-Myc), cell survival (Bcl-2, Bcl-xL, cFLIP, TRAF1, IAP1, IAP2, and survivin), invasion (matrix metalloproteinase-9 [MMP-9] and ICAM-1), and angiogenesis (vascular endothelial growth factor [VEGF]) [103]. Salinosporamide A also suppressed TNF-induced tumor cell invasion and receptor activator of NF- κ B ligand (RANKL)-induced osteoclastogenesis. It was also found that salinosporamide A suppressed both constitutive and inducible NF- κ B activation.

Compared with bortezomib, MG-132, N-Acetyl-Leucyl-Leucyl-Norleucinal (ALLN), and lactacystin, salinosporamide A was found to be the most potent suppressor of NF- κ B activation. Further studies showed that salinosporamide A inhibited TNF-induced inhibitory subunit of NF- κ B α (I κ B α) degradation, nuclear translocation of p65, and NF- κ B-dependent reporter gene expression but had no effect on I κ B α kinase activation, I κ B α phosphorylation, or I κ B α ubiquitination [104]. Thus, overall, results indicate that salinosporamide A enhances apoptosis, suppresses osteoclastogenesis, and inhibits invasion through suppression of the NF- κ B pathway.

Combination of NPI-0052 and bortezomib induces synergistic anti-MM activity [105]. These studies therefore provide the rationale for clinical protocols evaluating NPI-0052, alone and together with bortezomib, to improve patient outcome in MM.

Another new proteasome inhibitor of next generation is PR-171 (carfilzomib, Kyprolis; Onyx Pharmaceuticals, Inc., South San Francisco, CA). PR-171 is tetrapeptide epoxyketone-based irreversible proteasome inhibitor that exhibits a high level of selectivity for a single active site in the proteasome, as well as minimal cross reactivity to other protease classes [106-116]. In models of MM, carfilzomib potently irreversibly bound and specifically inhibited the chymotrypsin-like proteasome and immunoproteasome activities, resulting in accumulation of ubiquitinated substrates. Carfilzomib induced a dose- and time-dependent inhibition of proliferation, ultimately leading to apoptosis. Programmed cell death was associated with activation JNK, mitochondrial membrane depolarization, release of cytochrome c, and activation of both intrinsic and extrinsic caspase pathways.

This agent also inhibited proliferation and activated apoptosis in patient-derived MM cells, as well as neoplastic cells from patients with other hematologic malignancies. Importantly, carfilzomib showed increased efficacy compared to bortezomib (Figure 2) and was active against bortezomib-resistant MM cell lines, and samples from patients with clinical bortezomib resistance.

Carfilzomib also overcame resistance to other conventional agents, and acted synergistically with dexamethasone to enhance cell death. Phase I and Phase II clinical studies in addition to the Phase II trial in solid tumors, carfilzomib is currently being evaluated in

two Phase II single-agent trials in multiple myeloma and a Phase I study in lymphoma. Phase I clinical studies have shown that patients with hematologic malignancies (MM and Waldenström's macroglobulinemia) who have relapsed or progressed following multiple therapies can achieve durable anti-tumor responses with carfilzomib [108, 109].

Carfilzomib was approved on July 20, 2012 by the FDA for use in patients with MM who has received at least two prior therapies, including the treatment with bortezomib and an immunomodulatory therapy and has demonstrated disease progression on or within 60 days of completion of the last therapy [112]. Results from a recent Phase 1 and 2 trials indicate that the combination of Kyprolis, Revlimid, and dexamethasone is effective in relapsed multiple myeloma patients [114,115]. Specifically, the results show that 77% of patients responded to the treatment. The investigators point out the responses seen in the trial were rapid (median time to response was one month) and robust (median duration of response was 22 months). According to the investigators, the results are particularly encouraging because one-quarter of the patients were refractory (resistant) to bortezomib and almost half were refractory to lenalidomide. The researchers also note that the side effect profile of the combination was similar to that observed in previous trials. Most of the severe side effects were blood-related. Twenty percent of the patients left the trial due to side effects a discontinuation rate the investigators describe as "moderate." Dr. Sara Brighen from the University of Torino in Italy reported results from a Phase 2 study evaluating Kyprolis, cyclo-phosphamide, and dexamethasone as initial therapy for older, newly diagnosed multiple myeloma [116,117]. After initial therapy, patients received further Kyprolis maintenance therapy. Overall 96% of study participants responded, with 64% reaching a complete or near complete response, 12% a very good partial response, and 20% a partial response. The two-year progression-free survival rate was 76%, and the overall survival rate was 87%.

A poster presented at ASH (ASH 2013 Annual Meeting Abstract 3179) by Stewart's group summarized the final results of a Phase 1/2 study of Kyprolis plus cyclophosphamide, thalidomide, and dexamethasone a combination therapy known as "CYCLONE" in newly diagnosed myeloma patients. According to the researchers, the combination is highly efficacious. After four treatment cycles, 91% of patients responded, with 76% achieving at least a very good partial response. The two-year progression-free survival was 77%, and the two-year overall survival was 98%.

MM cells are characterized by high protein synthesis resulting in chronic Endoplasmic Reticulum (ER) stress, which is adaptively managed by the unfolded protein response. Inositol-Requiring Enzyme 1 α (IRE1 α) is activated to splice X-Box Binding Protein 1 (XBP1) mRNA, thereby increasing XBP1s protein, which in turn regulates genes responsible for protein folding and degradation during the unfolded protein response. PI resistance mechanisms in MM remain controversial. Response of MM to the proteasome inhibitor bortezomib is correlated with the unfolded protein response regulator XBP1s protein [118, 119]. Leung-Hagesteijn et al. [120] reported the existence of a progenitor organization in primary MM that recapitulates maturation stages between B cells and plasma cells and that contributes to clinical PI resistance. Xbp1s tumor B cells and pre-plasmablasts with low level of Xbp1s survive therapeutic PI,

preventing cure, while maturation arrest of MM before the plasmablast stage enables progressive disease on PI treatment. Mechanistically, suppression of Xbp1s in MM is shown to induce bortezomib resistance via de-commitment to plasma cell maturation and immunoglobulin production, diminishing Endoplasmic Reticulum (ER) front-loading and cytotoxic susceptibility to PI-induced inhibition of ER-associated degradation. These results reveal the tumor progenitor structure in MM and highlight its role in therapeutic failure.

The Third-Generation of Orally Active Proteasome Inhibitors

The novel orally active proteasome inhibitor CEP-18770 /[(1R)-1-[[[(2 S,3 R)-3-hydroxy-2-[(6-phenylpyridine-2-carbonyl)amino]-1-oxobutyl]amino]-3-methylbutyl]boronic acid, Delanzomib was also studied in preclinical trials [121,122]. CEP-18770 potently induces apoptotic cell death in MM cell lines and in primary purified CD138-positive cultures from untreated and bortezomib-treated MM patients [123]. In vitro, CEP-18770 has a strong antiangiogenic activity and potently represses RANKL-induced osteoclastogenesis. CEP-18770 exhibits a favorable cytotoxicity profile toward normal human epithelial cells, bone marrow progenitors, and bone marrow-derived stromal cells. Thus, Delanzomib has a favorable tumor selectivity profile for the treatment of MM and other malignancies responsive to proteasome inhibition.

CEP-18770 combined with melphalan or bortezomib induces synergistic inhibition of MM cell viability in vitro. In MM xenograft models, the addition of CEP-18770 intravenously to melphalan completely prevented the growth of both melphalan-sensitive and melphalan-resistant tumors. The combination of CEP-18770 and bortezomib induced complete regression of bortezomib-sensitive tumors and markedly delayed progression of bortezomib-resistant tumors compared to treatment with either agent alone. Single agent CEP-18770 orally also showed marked anti-MM effects in these xenograft models. These studies provide strong preclinical rationale for further development of this novel PI in the treatment of MM as a monotherapy as well as combined with either melphalan or bortezomib [124,125].

MLN9708 rapidly hydrolyzes to the biologically active form, MLN2238 (ixazomib). Ixazomib is an investigational oral proteasome inhibitor that, compared with bortezomib, has improved pharmacokinetics, pharmacodynamics, and antitumor activity in preclinical studies. The *in vivo* activity of MLN2238 was evaluated in a variety of mouse models of hematologic malignancies, including tumor xenograft models derived from a human lymphoma cell line and primary human lymphoma tissue, and genetically engineered mouse models of plasma cell malignancies [126].

Treatment of MM cells with MLN2238 predominantly inhibits chymotrypsin-like activity of the proteasome and induces accumulation of ubiquitinated proteins. MLN2238 inhibits growth and induces apoptosis in MM cells resistant to conventional and bortezomib therapies without affecting the viability of normal cells [127]. In animal tumor model studies, MLN2238 is well tolerated and inhibits tumor growth with significantly reduced tumor recurrence. A head-to-head analysis of MLN2238 versus bortezomib showed a significantly longer survival time in mice treated with MLN2238 than mice receiving bortezomib. Immunostaining of MM tumors

from MLN2238-treated mice showed growth inhibition, apoptosis, and a decrease in associated angiogenesis. Mechanistic studies showed that MLN2238-triggered apoptosis is associated with activation of caspase-3, caspase-8, and caspase-9; increase in p53, p21, NOXA, PUMA, and E2F; induction of Endoplasmic Reticulum (ER) stress response proteins Bip, phospho-eIF2- α , and CHOP; and inhibition of nuclear factor kappa B. Finally, combining MLN2238 with lenalidomide, histone deacetylase inhibitor suberoylanilide hydroxamic acid, or dexamethasone triggers synergistic anti-MM activity.

In December 2013 at the American Society of Hematology or ASH Annual Meeting, there was an abstract 535 presented by Dr. Paul Richardson of the Dana-Farber Cancer Institute and colleagues looking at the safety, tolerability, and efficacy of the combination of ixazomib, lenalidomide, and dexamethasone in patients with newly diagnosed multiple myeloma. They found that 93% of the patients had at least a partial response including 67% of the patients achieving a very good partial response. Because there were some dose interruptions due to toxicity such as rash and peripheral neuropathy, investigators continue to refine the dosing schedule to mitigate these adverse effects as they move on to phase 3 studies.

MLN9708 in combination with the existing drugs panobinostat and dexamethasone will be studied among patients with relapsed or refractory multiple myeloma. This study will also look at the response and clinical benefit of the treatment and the progression-free survival and overall survival of study participants. The further randomized phase I/II trial studies the side effects and best dose of pomalidomide and ixazomib when given together with dexamethasone and to see how well pomalidomide and dexamethasone with or without ixazomib works in treating patients with refractory multiple myeloma. Biological therapies, such as pomalidomide and dexamethasone, may stimulate the immune system in different ways and stop tumor cells from growing. Ixazomib may stop the growth of tumor cells by blocking some of the enzymes needed for cell growth. It is not yet known whether pomalidomide and dexamethasone are more effective with or without ixazomib in treating multiple myeloma.

Oprozomib (ONX 0912, previously PR-047) is an oral, irreversible, tripeptide epoxyketone that exerts its activity via inhibition of chymotrypsin-like activity of the proteasome [128]. Biochemically, it is the oral analogue of carfilzomib, and demonstrates similar antiangiogenic and proapoptotic activity *in vitro* and *in vivo* [128]. A synergistic effect with bortezomib and with a combination of lenalidomide and dexamethasone was also seen in preclinical studies, and hypothesized to be secondary to differential effects on proapoptotic signaling pathways. The primary advantage with oprozomib is the ease of administration via the oral route. The strong preclinical data for equipotent efficacy to carfilzomib has laid the groundwork for clinical trials. Oprozomib is being evaluated as a single agent and in combination with lowdose dexamethasone in relapsed and refractory MM. Combination of pomalidomide and oprozomib or pomalidomide, oprozomib and dexamethasone are also effective [129].

The second-generation proteasome inhibitors offer benefits in terms of increased efficacy, reduced neurotoxicity as off-target effect and may overcome resistance to bortezomib because of their different chemical structure, mechanism of action and biological properties [24,

130, 131]. Novel strategies to target the different components of the Ubiquitin Proteasome System (UPS) in MM (enzymes E1, E2 and E3 ubiquitin ligases and the deubiquitinating enzymes) also exist [132]. HDACi with anti-myeloma activity include vorinostat, panobinostat and selective HDAC 6 inhibitors, ricolinostat (ACY-1215) and citarinstat (ACY-241) [24, 133,134]. Anti-cell surface glycoprotein CS1, also known as SLAM Family Member 7 (SLAMF7), and CD38 monoclonal antibodies represent the most promising group of agents with unique mechanism of action in the treatment of MM itself as well as in the treatment of bone disease accompanying most of MM patients [135-137]. The discovery and development of immune checkpoint inhibitors in cancer medicine, particularly drugs targeting Programmed Cell Death 1 (PD-1) and Programmed Cell Death Ligand 1 (PD-L1) brought great progress in solid tumor treatment. The results of monotherapy with PD-1/PD-L1 inhibitors have been unsatisfactory in MM, suggesting that a combination approach is needed[138]. The most logical partners are immunomodulatory agents as they possess many synergistic effects [138].

Immunomodulatory Drugs

At present, IMiDs include thalidomide, lenalidomide and pomalidomide. Both, pomalidomide (Pomalyst®, initially known as CC-4047, Actimid) and lenalidomide (Revlimid®, initially known as CC-5013), are a synthetic derivative of thalidomide (Thalidomid®, Immunoprin, Talidex, Talizer). Pomalidomide [4-Amino-2-(2,6-dioxopiperidin-3-yl)isoindole-1,3-dione] is a potent second-generation IMiD [139-144]. Pomalidomide has direct antiproliferative, pro-apoptotic, and antiangiogenic effects, as well as modulatory effects on bone resorption and on the immune system. Lenalidomide [3-(4-amino-1-oxo-1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione] is 4-amino-glutarimide analog of thalidomide with potent immunomodulatory, antiangiogenic and direct neoplastic cell inhibitory activity [145-152]. Chemical structure of the IMiD drugs is shown in Figure 2.

Mechanism of Immunomodulatory Drugs Action in the Treatment of Multiple Myeloma

IMiDs target both MM cells and their microenvironment, while also modulating the immune system. The exact molecular mechanisms of the antitumor effects of IMiDs remain uncertain. IMiDs affect various molecular and cellular elements within the tumor microenvironment. IMiDs change the concentration of various cytokines that support tumor cell growth [139,151,153-156]. IMiDs disrupt bone marrow stromal support for malignant MM cells, although the exact mechanisms of these actions remain unclear. IMiDs decrease the expression of adhesion molecules that facilitate the interaction between MM cells and Bone Marrow Stromal Cells (BMSCs) [157]. Importantly, the down regulation of MM cells adherence to BMSCs can overcome the cellular adhesion-mediated drug resistance by malignant MM cells. This effect of IMiDs is further increased by their ability to down regulate TNFα [151,154,158,159]. Surprisingly, in contrast to the inhibitory effect of lenalidomide in BMSCs, lenalidomide was totally ineffective in inhibiting TNFα mRNA expression in MM cells [160].

Immunomodulatory Activities of IMiDs

IMiDs are a potent co-stimulator of primary human T cells, synergizing with stimulation via T-cell receptor complex to increase

IL-2-mediated T-cell proliferation and Interferon Gamma (IFN-γ) production [161]. Secretion of IL-2 and IFN-γ increases the number of Natural Killer (NK) cells, improves their function and mediates lysis of MM cells (Figure 3). NKT cells are a heterogeneous group of T-cells that recognize lipids and glycolipids presented by CD1d molecules. NKT cells activated in presence of lenalidomide have greater ability to secrete IFN-γ. Lenalidomide enhances antigen-specific expansion of NKT cells [162]. Regulatory T-cells (Tregs) are a component of the immune system that suppresses immune response of other cells. Tregs were elevated in MM patients. IMiDs strongly inhibit Tregs proliferation via decreased FOXP3 mRNA expression [163].

Anti-Angiogenic Properties of IMiDs

All IMiDs have anti-angiogenic activity. Thalidomide has predominant anti-angiogenic activity while lenalidomide and pomalidomide have far greater immune enhancing effects [60]. It appears that anti-angiogenesis occurs via the modulation of chemotactic factors involved in endothelial cells migration including TNFα, Vascular Endothelial Growth Factor (VEGF) and basic fibroblast growth factor (bFGF) from BMSCs rather than a direct inhibition of endothelial cells proliferation [164,165]. Inhibition of VEGF and bFGF production by IMiDs is likely to have multiple other biological effects beyond that of anti-angiogenesis including inhibition of IL-6 production by BMSCs. VEGF and bFGF up-regulate IL-6 and other pro-inflammatory cytokines [166].

Inhibition of Cell Cycle and Induction of Apoptosis by IMiDs

IMiDs induce cell cycle arrest via up regulation of tumor suppressor genes (cyclin dependent kinase inhibitors p15INK4b, p16INK4a, p21Cip1, Waf1, p27Kip1; early response transcription factors /Erg1, Erg2 and Erg3/) and apoptosis by caspase activation [167,168]. The inhibition of the transcription factor NF-κB activity

by IMiDs results in reduced expression of anti-apoptotic proteins including cellular inhibitor of apoptosis protein 2 (cIAP2) [169] and FLIP [Fas-Associated Protein with Death Protein (FADD)-like Interleukin-1 β -Converting Enzyme (FLICE) inhibitor protein] [170]. These anti-apoptotic proteins inhibit caspase-8 that is on the other hand stimulated by IMiDs [168].

Disruption of Bone Marrow Stromal Support for Malignant MM Cells

IMiDs down-regulate the expression of adhesion molecules [Leukocyte Function-Associated Antigen 1 (LFA-1, CD11a), Interleukin-1 β -Converting Enzyme (FLICE) inhibitor protein] [170]. These anti-apoptotic proteins inhibit caspase-8 that is on the other hand stimulated by IMiDs [168].

Effect of IMiDs on Myeloma Cell Proliferation

The direct anti-MM effect of IMiDs was shown to occur through the induction of growth arrest of MM cells in G1 phase of cell cycle [81]. This effect is associated with a decrease in Interferon Regulatory Factor 4 (IRF4), a transcription factor that is critical for MM cell growth and survival (Figure 3) [172,173].

Raje et al. [174] showed strong synergism of anti-MM activity of rapamycin (Rapamune), a specific mTOR inhibitor, combined with lenalidomide. Importantly, this combination is able to overcome drug resistance when tested against MM cell lines resistant to conventional chemotherapy. Moreover, the combination, but not rapamycin alone, is able to overcome the growth advantage conferred on MM cells by Interleukin-6 (IL-6), Insulin-Like Growth Factor-1 (IGF-1), or adherence to Bone Marrow Stromal Cells (BMSCs). Combining rapamycin and lenalidomide induces apoptosis of MM cells. Differential signaling cascades including the Mitogen-Activated Protein Kinase (MAPK) and the Phosphatidylinositol 3'-Kinase/Akt Kinase (PI3K/Akt) pathways [175-178] are targeted by these drugs individually and in combination, suggesting the molecular mechanism by which they interfere with MM growth and survival. These studies, therefore, provide the framework for clinical evaluation of mTOR inhibitors combined with IMiDs to improve patient outcome in MM.

IMiDs down-regulate CCAAT/enhancer-binding protein- β (C/EBP β) resulting in abrogation of cell proliferation [179]. Overexpression of C/EBP β rescues MM cells from IMiD compounds-induced inhibition of proliferation, indicating that C/EBP β is critical in mediating antiproliferative effects. IMiD compounds-induced decrease of C/EBP β protein leads to impaired transcription of Interferon Regulatory Factor 4 (IRF4). Down-regulation of IRF4 by lenalidomide was confirmed by longitudinal studies of bone marrow samples from 23 patients obtained before and during lenalidomide treatment using CD138⁺/IRF4⁺ double labeling. In contrast to down-regulation of C/EBP β protein, IMiD compounds do not alter C/EBP β mRNA levels or protein stability, suggesting translational regulation of C/EBP β . Expression of the C/EBP β protein is under the Initiation Factor of Translation 4E (IF4E) control in MM cells. However, this control by IF4E was not observed in IMiD compounds-resistant MM cells. Targeting translation by inhibiting IF4E-binding protein

1 phosphorylation overcomes resistance. So, this pathway is critical and may be a target to overcome drug resistance. MM cell lines and primary MM cells strongly express C/EBP β , whereas normal B cells and plasma cells have little or no detectable levels of C/EBP β [180]. Silencing of C/EBP β leads to down-regulation of transcription factors such as IRF4, XBP1, and BLIMP1 accompanied by a strong inhibition of proliferation. Further, silencing of C/EBP β leads to a complete down-regulation of antiapoptotic B-Cell Lymphoma 2 (BCL2) expressions. In chromatin immunoprecipitation assays, C/EBP β directly binds to the promoter region of IRF4, BLIMP1, and BCL2. C/EBP β is involved in the regulatory network of transcription factors that are critical for plasma cell differentiation and survival. Targeting C/EBP β may provide a novel therapeutic strategy in the treatment of multiple myeloma.

Exposure of plasma cells to lenalidomide activates the Wnt/ β -catenin pathway and its downstream targets such as cyclin D1 and MYC [181]. The accumulation of β -catenin during treatment with lenalidomide may be the cause of drug resistance [181]. N-cadherin-based interaction between MM cells and osteoblasts blocks MM cell growth. Therefore the high levels of N-cadherin expression in osteoblasts confer strong proliferation block on MM cells. Since β -catenin associates with N-cadherin at the cell membrane, N-cadherin adhesion is disrupted, β -catenin is released and translocates to the nucleus where it regulates the transcription of target genes with effect on cell proliferation [182]. Resistance of myeloma to lenalidomide is an emerging clinical problem. Though it is associated in part with activation of Wnt/ β -catenin signaling, the mediators remain undefined. Wnt/ β -catenin inhibition by N-(2-Methyl-4-nitrophenyl)-2,5-dichlorobenzenesulfonamide (FH535), a sulfonamide based and cell-permeable compound that suppresses both Wnt/ β -catenin and Peroxisome Proliferator-Activated Receptor (PPAR) signaling, enhances the activity of lenalidomide [183].

Cereblon as the Direct Target Protein of IMiDs

Ito et al. [184-186] developed a new affinity bead technology for isolating ligand-binding proteins. Polymer-coated beads were constructed that allow single-step purification of ligand target molecules. These beads include Styrene-Glycidyl-Methacrylate (SG) beads and Ferrite-Glycidyl-Methacrylate (FG) beads. FG beads were used for the purification of thalidomide-binding proteins from various cell extracts. Thalidomide-modified beads were incubated with cell extracts and then washed with buffer. Bound proteins were eluted with free thalidomide and analyzed by gel electrophoresis. Only two specific protein bands were detected (55 kDa and 127 kDa). These proteins were identified as Cereblon (CRBN) and DNA damage binding protein 1 (DDB1). Both CRBN and DDB1 are subunits of the cullin 4 ring E3 ubiquitin ligase complex (CRL4). Auto-ubiquitination of CRBN was inhibited by thalidomide in vitro. Thus, thalidomide and its analogs enhance cullin-4 RING E3 ubiquitin ligase function [187].

The Role of Ikaros Family Proteins in IMiDs and CRBN Mechanism

Using distinct but complementary proteomic techniques and systems, three groups have recently simultaneously reported that IMiDs induce the CRBN-dependent proteasomal degradation of IKZF1 (Ikaros) and IKZF3 (Aiolos) [188-190]. Schema is shown in Figure 3. IKZF1, a zinc finger transcription factor initially discovered

as a regulator of the T cell receptor, is required for hematopoiesis, particularly lymphocyte development and plasma cell maturation. Mutations that cause loss of function of IKZF1 and IKZF3 are associated with acute lymphoblastic leukemia, consistent with a tumor suppressor function. On the other hand, IKZF1 and IKZF3 are required for the viability of many MM cell lines. IKZF1 and IKZF3 are also involved in the complex process of chromatin remodeling, and the nature of their interactions is poorly understood.

IKZF1 binds and activates the IRF4 gene promoter and loss of IKZF1 leads to decreased IRF4 and MYC expression. However, lenalidomide can also inhibit MM cell lines with high basal levels of IRF4 unchanged by drug treatment, suggesting that other IKZF1/3 targets can play a role in the therapeutic response to IMiDs. IKZF1/3 is known repressors of IL-2 gene promoter. The degradation of IKZF1/3 in response to IMiDs explains enhanced T cell IL-2 production. Hence, many of the effects of IMiDs can be explained by a unified mechanism: IMiDs re-target the cullin 4 ring E3 ubiquitin ligase activity toward IKZF1/3 in a change-of-function effect (Figure 3).

Although CRBN is an essential requirement for IMiD action, the complete molecular mechanisms responsible for lenalidomide-mediated sensitivity or resistance remain unknown. Sebastian et al. [191] have recently described that IMiDs work primarily via inhibition of peroxidase-mediated intracellular H_2O_2 decomposition in MM cells. CRBN-dependent degradation of IKZF1 and IKZF3 was the consequence of H_2O_2 -mediated oxidative stress. Lenalidomide increased intracellular H_2O_2 levels by inhibiting thioredoxin reductase in cells expressing CRBN, causing accumulation of immunoglobulin light-chain dimers. Endoplasmic reticulum stress was therefore increased and a proapoptotic protein Bim, member of the Bcl-2 family, was induced. Similar cytotoxicity was induced by inhibitors of thioredoxin reductase and thioredoxin.

Upon treatment of IMiD-sensitive MM cells with lenalidomide, the steady state levels of CRBN were significantly increased, whereas levels of CRBN-binding protein argonaute 2 were significantly decreased [192]. Argonaute 2 plays an important role for MM cell growth and survival and in microRNA maturation and function.

Multiple Myeloma -Related Kidney Disease

Spectrum of myeloma-related kidney disease includes light chain immunoglobulin cast nephropathy, monoclonal immunoglobulin deposition disease, light chain related amyloidosis, light chain proximal tubulopathy with or without Fanconi's syndrome, cryoglobulinemia, membranoproliferative Glomerulonephritis (GN), proliferative GN with monoclonal immunoglobulin deposition, fibrillary and immunotactoid GN, C3 glomerulopathy, direct plasma cell infiltration, hyperviscosity syndrome, crystal storing histiocytosis [193]. Proteasome inhibitors have also a reno-protective effect by the inhibition of transcription factor NF- κ B, which is up regulated in the mesangial cells as well as in the proximal tubule cells in Myeloma-Related Kidney Disease (MRKD).

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