Abstract

Novel agents, including the proteasome inhibitor bortezomib, have significantly improved the response and survival of patients with multiple myeloma over the last decade. Despite these advances, many patients relapse or do not benefit from the currently available therapies; thus, multiple myeloma remains an incurable disease. Deacetylase inhibitors (DACi), including panobinostat and vorinostat, have recently emerged as novel agents being evaluated in the treatment of multiple myeloma. Deacetylases are a group of enzymes with effects on various intracellular proteins, including histones, transcription factors, and molecular chaperones. Although DACi inhibit cell growth and induce apoptosis in multiple myeloma cells as a single agent, synergistic activity has been observed when they were used in combination with bortezomib. The mechanistic basis of synergy is multifactorial and includes disruption of protein degradation and inhibition of the interaction of multiple myeloma cells with the tumor microenvironment. This review summarizes recent advancements in the understanding of the mechanism of action of proteasome inhibitors and DACi in multiple myeloma and examines the biological basis of their synergistic effects. Data from the studies summarized here have been used as the rationale for the implementation of phase II and III clinical trials of DACi, alone and combined with bortezomib, in relapsed and refractory multiple myeloma. Mol Cancer Ther; 10(11); 2034–42. ©2011 AACR.
cells to both the bone marrow stromal cells (BMSC) and the extracellular matrix (ECM; ref. 8). Binding of multiple myeloma cells to BMSCs occurs, at least in part, through binding of very late antigen 4 (VLA4) to vascular cell adhesion molecule 1 (VCAM1) and leukocyte function-associated antigen 1 (LFA1) to intracellular adhesion molecule 1 (ICAM1; ref. 8). The interaction between multiple myeloma cells and the ECM is mediated by the binding of syndecan 1 (CD138) to collagen and VLA4 to fibronectin (8). Importantly, the interaction of multiple myeloma cells to BMSCs activates the transcription and secretion of interleukin-6 (IL-6), which facilitates the paracrine-mediated growth and survival of multiple myeloma cells (8). IL-6 also downregulates the expression of CD138, leading to the spread of cells into the bloodstream, which may lead to the development of plasma cell leukemia (8).

Numerous cytokines, in addition to IL-6, play an important role in multiple myeloma cell proliferation, survival, migration, and drug resistance in the tumor microenvironment. Besides the direct effects of these cytokines and/or chemokines on multiple myeloma cells, several studies have examined the role of VEGF in neovascularization in the bone marrow microenvironment and disease progression in multiple myeloma (9). Myeloma cells secrete VEGF, which contributes to new blood vessel formation in vitro (10). Moreover, VEGF-mediated stimulation of microvascular endothelial cells results in increased secretion of IL-6, with continued multiple myeloma cell growth (11, 12). In addition to its proangiogenic effects in the bone marrow, VEGF has been shown to directly induce tumor cell proliferation through the Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, as well as tumor cell migration through a protein kinase C–dependent pathway (12).

TNF-α plays an important role in the biology of multiple myeloma cells within the tumor microenvironment. Although TNF-α has modest effects on multiple myeloma cell proliferation, it does lead to increased expression of adhesion molecules, including ICAM1, on both multiple myeloma cells and BMSCs. The cytokine also leads to increased heterotypic adhesion, thereby triggering NF-κB–dependent upregulation of transcription and secretion of IL-6 and resulting in paracrine multiple myeloma cell growth by BMSCs (13). Insulin-like growth factor I (IGF-I) is another cytokine that supports multiple myeloma cell proliferation and survival. IGF-I is present in both the bone marrow microenvironment and peripheral blood and stimulates multiple myeloma cell proliferation and survival (14), via activation of NF-κB and Akt, as well as increased expression of several antiapoptotic proteins, including FADD-like IL-1β–converting enzyme inhibitory protein, X-linked inhibitor of apoptosis, and survivin (14). Although IGF-I induces more prominent Akt activation than IL-6, it does not activate the Janus kinase 2/STAT3 pathway, which is commonly activated by gp130 family cytokines including IL-6.

The proliferation and survival of multiple myeloma cells within the tumor microenvironment is, therefore, dependent on their interaction with the BMSC and the ECM. These factors supporting multiple myeloma cell survival are complex and provide many mechanisms for the development of resistance to commonly used agents. Novel strategies to target or disrupt these pathways are urgently needed.

Mechanistic Basis of Antimyeloma Activity of Proteasome Inhibitors in Multiple Myeloma

Proteasomes are abundant multi-enzyme complexes that provide the main pathway for degradation of intracellular proteins and contribute to the maintenance of protein homeostasis and clearance of misfolded and/or unfolded and cytotoxic proteins (15). The 26S proteasome is a large 2.4-MDa ATP-dependent proteolytic complex, located in both the cytoplasm and nucleus. This proteasome consists of a 20S core catalytic cylindrical complex capped at both ends by 19S regulatory subunits (15). Polyubiquitination is an essential event for proteins targeted for proteasomal degradation (15). Proteins degraded by the proteasome include mediators of cell-cycle progression and apoptosis, such as the cyclins, caspases, B-cell lymphoma 2 (BCL2), and NF-κB activation (15).

It has been hypothesized that cancer cells are more dependent on the proteasome for clearance of abnormal or mutant proteins (15). In fact, several preclinical studies have shown that malignant cells are more sensitive to proteasome inhibition than normal cells (16–20). The proteasome inhibitor bortezomib is a dipeptide boronic acid analog that reversibly inhibits the chymotryptic activity of the 20S subunit of the proteasome (19). Bortezomib has been shown to directly inhibit proliferation and induce apoptosis in multiple myeloma cell lines and patient tumor cells resistant to conventional therapies (20). Furthermore, bortezomib showed enhanced anti–multiple myeloma activity with dexamethasone and overcame resistance to apoptosis conferred by IL-6 or adhesion to BMSCs (20). Significant tumor growth inhibition and increased host survival were also observed in vivo using a mouse–human multiple myeloma cell xenograft model (19).

Bortezomib showed remarkable clinical activity in patients with multiple myeloma and was rapidly approved by the FDA in 2003 to treat relapsed and refractory multiple myeloma. Clinical activity resulting in high response rates and increased progression-free and overall survival has also been observed in multiple phase III clinical trials with bortezomib. Of note, bortezomib has shown increased activity in combination with melphalan and prednisone compared with melphalan and prednisone alone in newly diagnosed patients with multiple myeloma (4), as well as increased activity as a single agent compared with dexamethasone in patients with relapsed or refractory disease (21).

The mechanism of action and target of bortezomib, leading to disruption of intracellular protein metabolism,
are well characterized. The downstream biological effects of proteasome inhibition are multifactorial, with direct effects on both multiple myeloma cells and the multiple myeloma cell microenvironment, including inhibition of cytokine secretion, suppression of adhesion molecule expression, and inhibition of angiogenesis. The initial rationale to use bortezomib in cancer was its inhibitory effect on NF-κB activity, thereby modulating transcription. Specifically, the NF-κB canonical pathway is regulated by inhibitor protein IκB, which blocks nuclear translocation of the p50 (NF-κB1)/p65 (RelA) heterodimer. Importantly, IκB is a substrate of the proteasome, and proteasome inhibition by bortezomib can, therefore, lead to an increase in the cytoplasmic level of IκB, resulting in a blockade of NF-κB translocation to the nucleus and DNA-binding activity (15). NF-κB has also been identified as a mediator of paracrine signaling between multiple myeloma cells and BMSCs within the bone marrow microenvironment. For example, NF-κB–dependent upregulation of IL-6 in BMSCs is induced by adhesion to multiple myeloma cells or via TNF-α secretion by multiple myeloma cells (13, 22). TNF-α–induced upregulation of NF-κB leads to increased expression of the adhesion molecules ICAM1 and VCAM1 on multiple myeloma cells and BMSCs, thus enhancing intercellular binding (13). Bortezomib blocks the TNF-α–induced upregulation of NF-κB, leading to decreased binding of multiple myeloma cells to BMSCs and related decreased IL-6 secretion (13, 20). Of note, the specific IκB kinase inhibitors PS-1145 and MLN120B also inhibit secretion of IL-6 and adhesion of multiple myeloma cells and BMSCs; however, these agents only lead to partial inhibition of multiple myeloma cell growth (23). Therefore, bortezomib–triggered anti–multiple myeloma activities are not solely mediated by NF-κB inhibition.

Bortezomib inhibits angiogenesis in the bone marrow microenvironment, which plays an important role in both multiple myeloma pathogenesis and disease progression. In preclinical models using multiple myeloma patient–derived endothelial cells, bortezomib inhibited cell proliferation, chemotaxis, adhesion, and capillary formation, thus supporting its angiogenic inhibitory activity in vivo (24). Bortezomib also inhibited the expression and secretion of several proangiogenic factors, including VEGF (24). In addition, VEGF-mediated migration of multiple myeloma cells was inhibited by bortezomib (12).

Inhibition of the proteasome induces accumulation of intracellular misfolded and/or unfolded proteins (20), which triggers the unfolded protein response (UPR) signaling pathway to protect cells against cellular stress (25, 26). Because multiple myeloma cells produce large amounts of immunoglobulin, a functional UPR is required for their survival (25, 26). Treatment of multiple myeloma cells with bortezomib leads to induction of proapoptotic UPR components, including growth arrest and DNA damage–inducible gene 153 (26). Proteasome inhibition also interferes with the stability of mRNA transcripts of X box–binding protein 1, a downstream transcription factor of IRE1α, regulating the UPR (25). Taken together, these data show the reliance of multiple myeloma cells on the UPR for survival and identify disruption of protein metabolism as a potential therapeutic target in multiple myeloma.

Mechanistic Basis of Antimyeloma Activity of Deacetylase Inhibitors in Multiple Myeloma

Histone deacetylases (HDAC) have emerged as a relevant clinical target in multiple myeloma. HDACs and histone acetyl transferases regulate the acetylation of target proteins (27). Specifically, HDACs remove acetyl groups from target proteins that regulate their activity (27). Eighteen HDACs have been identified in humans and divided into 4 classes based on their homology to yeast HDACs: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (SIRT family), and class IV (HDAC11; ref. 27). The different classes of enzymes also differ in their subcellular localization, with class I HDACs found in the nucleus and class II enzymes found in both nucleus and cytoplasm, and their intracellular targets (27). The name HDAC is based on the identification of histone proteins as the initial target of HDACs; however, a recent study in cancer cell lines identified 3,600 acetylation sites on 1,750 proteins associated with various intracellular functions including gene expression, DNA replication and repair, cell-cycle progression, cytoskeletal reorganization, and protein chaperone activity (28). Therefore, the term deacetylase (DAC) may be more appropriate when referring to these enzymes.

Although several deacetylase inhibitors (DACi) are in various stages of clinical development, only 2 are approved for treatment of cutaneous T-cell lymphoma, vorinostat (suberoylanilide hydroxamic acid), and romidepsin (FK228 or FR901228; refs. 29, 30). DACi differ in their structure and potency toward the HDAC enzymes. Romidepsin is a cyclic tetrapeptide with DAC inhibitory activity primarily toward class I HDACs (27). Other DACi include the benzamide class, which includes mocetinostat (MGCD103) and entinostat (MS-275) class I–specific inhibitors (27). The hydroxyacid–based DACi, vorinostat, panobinostat (LBH589), and belinostat (PXD101), are pan-DACi, with inhibitory activity against class I, II, and IV HDACs (Fig. 1; ref. 27). Panobinostat is among the most potent DACi, with nanomolar DAC inhibitory activity (31).

Preclinical studies showed that DACi have potent antimyeloma activity. The class I–specific DACi, romidepsin, along with the pan-DACi dacitinostat (LAQ824), vorinostat, and panobinostat, have been shown to inhibit proliferation and induce apoptosis in multiple myeloma cell lines in vitro and in vivo in mouse xenograft models (32–36). In many of these preclinical studies, additive or synergistic effects were observed when DACi were combined with other agents, including corticosteroids and
proteasome inhibitors, establishing the rationale for clinical studies of DACi in combination with these agents (32–36).

Clinical studies in patients with multiple myeloma have shown limited single-agent activity of DACi (37, 38). In a phase I trial of single-agent vorinostat (37, 38) in 13 patients with relapsed and/or refractory multiple myeloma, only 1 patient showed a minimal response and 9 patients showed disease stabilization (37). In a phase II trial of single-agent romidepsin in 13 patients with refractory multiple myeloma, no objective responses were observed; however, evidence of disease stabilization and resolution of disease-related symptoms were seen (38). Overall, the activity of DACi as single agents has been limited, and a clearer understanding of the biological activity of these agents will help determine the ideal combination therapies for clinical development.

Because DACi act on many intracellular targets, the biological basis of their antimalyeloma activity is due to a number of effects on multiple myeloma cells and their interaction with the tumor microenvironment. For example, LAQ824 induces upregulation of cyclin-dependent kinase inhibitor p21, leading to cell-cycle arrest followed by apoptosis through activation of caspases 8, 9, and 3 (33). Vorinostat also induced p21 expression, leading to cell-cycle arrest and apoptosis; however, no significant caspase 8, 9, or 3 cleavage was observed, suggesting caspase-independent apoptosis (39). Romidepsin has been shown to induce multiple myeloma cell apoptosis through downregulation of the antiapoptotic proteins BCL2, BCLXL, and myeloid cell leukemia sequence 1 (35).

DACi also modulate interaction of multiple myeloma cells with cellular components in the bone marrow microenvironment. LAQ824 inhibited multiple myeloma cell proliferation even in the presence of exogenous IL-6 or BMSC coculture (33). Vorinostat suppresses the stimulation of IL-6 secretion in BMSCs by multiple myeloma cell adhesion, with no effect on BMSC viability (39). Vorinostat also suppresses autocrine IGF-I production, directly interrupting the IGF-I/IGF-IR/Akt signaling pathway critical for antiapoptosis and survival of multiple myeloma cells (40). DACi, therefore, can induce direct multiple myeloma cell-cycle arrest and apoptosis, as well as disrupt signaling between multiple myeloma cells and BMSCs.

Recent studies have shown that aggresomes represent an alternative pathway for catabolism of misfolded proteins and develop when production of misfolded...
ubiquitinated proteins exceeds the capacity of proteasomes to degrade them (41). Misfolded proteins can form aggregates that are transported by microtubules via dynein motor complexes to the autophagosome, where they are degraded by lysosomes. HDAC6 belongs to the class IIb HDACs and is broadly expressed in different types of cells. HDAC6 regulates acetylation of α-tubulin and facilitates the transport of the aggresome to the lysosome (42). DACi that target HDAC6, such as the pan-DACi panobinostat and HDAC6-specific inhibitor tubacin, lead to hyperacetylation of α-tubulin, disruption of the interaction between HDAC6 and dynein, and resultant increases in ubiquitinated proteins (41, 43). Therefore, inhibition of protein degradation through targeting of the aggresome by DACi represents an attractive model for the treatment of cancers such as multiple myeloma that are reliant on efficient protein metabolism.

Mechanisms of Synergy between Proteasome Inhibitors and Deacetylase Inhibitors in Multiple Myeloma

The molecular sequelae of proteasome inhibitors and DACi in multiple myeloma are associated with key pathways vital to the proliferation and survival of multiple myeloma cells. Although some common targets and pathways are affected by each agent, some of the pathways targeted are complementary and may underlie the synergistic effects. In fact, synergistic antitumor activities between DACi and bortezomib have been observed in several preclinical studies (36, 41, 43–45). Either the pan-DACi vorinostat or panobinostat with bortezomib have synergistic effects on inhibition of cell growth and increasing apoptosis in multiple myeloma cells (41, 44). Similar effects were observed with the HDAC6-specific inhibitor tubacin combined with bortezomib, associated with a marked increase in polyubiquitinated proteins (43). These effects were observed in both multiple myeloma cell lines and primary tumor cells isolated from patients with multiple myeloma. Importantly, the class I–specific inhibitor romidepsin and the pan-DACi panobinostat have also shown antitumor effects in vivo in human multiple myeloma cell–mouse xenograft models (36, 45).

Despite the observation that synergistic effects were observed with bortezomib and a variety of DACi across several studies, the conclusions made about the biological basis of the synergy observed are varied. This variation can be partially explained by the differential potency and targets of the various DACi tested in these studies. In addition, the pleiotropic effects that these agents elicit in multiple myeloma cells, along with the experimental design of the individual studies, may have led the investigators to focus on the most relevant biological effects observed. As the data from preclinical studies have shown, numerous genes are affected by bortezomib or DACi (39, 40, 46), and it is therefore most likely that a combination of these effects leads to the synergy observed between the 2 classes of agents.

The most well-characterized model of synergy between proteasome inhibitors and DACi are the dual inhibition of the proteasome and aggresome pathways (Fig. 2; refs. 41, 43). Targeting both the proteasome with bortezomib and the aggresome with HDAC6 inhibitors in tumor cells induces greater accumulation of polyubiquitinated proteins, resulting in increased cellular stress and apoptosis (41, 43). More specifically, proteasome inhibition drives the formation of aggresomes, which are dependent on the interaction of HDAC6 with tubulin and dynein complex (41). Moreover, the proteasome inhibitor (bortezomib) and HDAC6 inhibitors (tubacin or panobinostat) lead to increased hyperacetylation of tubulin and generation of polyubiquitinated proteins, thus increasing cellular stress response (i.e., c-Jun N-terminal protein kinase activation) and leading to apoptosis, which is, in part, dependent on caspase activity (41–43).

Although disruption of protein degradation represents a major contributor to the synergistic antitumor activity observed between proteasome inhibitors and DACi, other studies have identified additional mechanisms. For example, the combination of bortezomib and vorinostat results in enhanced cytochrome-c release, caspase and PARP cleavage, and inactivation of NF-κB, followed by apoptosis (44). Conversely, antioxidative agents, including N-acetyl-L-cysteine, block these effects (44).

In addition to the synergistic effects observed when these agents are combined, it is plausible that each agent affects complementary pathways in multiple myeloma cells, thereby leading to synergistic effects on growth inhibition and apoptosis. As summarized in the preceding sections, bortezomib and DACi both affect pathways associated with the interaction of multiple myeloma cells and the microenvironment, including cytokine signaling and cell adhesion (Fig. 3). In addition, overexpression of proto-oncogenes and/or oncogenic genes is a common mechanism of resistance in cancer, and a recent study showed that bortezomib specifically downregulates the expression of class I HDACs, leading to histone hyperacetylation (45). It was also noted that exogenous overexpression of HDAC1 caused resistance to bortezomib both in vitro and in vivo, which was reversed by the class I DACi romidepsin (45). In addition, pan-DACi LAQ824 has been shown to decrease the activity of the 20S proteasome, as determined by reduced proteasome chymotrypsin-like activity (33). The ability of proteasome inhibitors to downregulate HDACs, along with the observation that DACi can decrease proteasome activity, may also contribute to the synergistic antitumor activities. Taken together, proteasome inhibitors and DACi target several relevant mechanisms in multiple myeloma biology. Further research will uncover additional mechanisms that contribute to the synergistic antitumor activities and potential avenues of resistance.
Summary and Future Directions

The synergy between proteasome inhibitors and DACi is most likely dependent on a number of mechanisms targeting multiple myeloma cell biology. Multiple myeloma cell proliferation, survival, and progression of disease are dependent on the activation of key pathways within the cell, as well as the interaction with elements in the tumor microenvironment. One of the most compelling mechanisms underlying the synergy remains the disruption of protein degradation by inhibition of the proteasome and aggresome. Because multiple myeloma cells produce abundant amounts of immunoglobulin that must be properly folded or degraded, they are more dependent on efficient processing of proteins (41–43). This mechanism clearly contributes to synergy observed between the 2 agents; however, it is unlikely to be solely responsible for the synergy observed. Of note, a recent report showed that romidepsin, a DACi with limited HDAC6 inhibitory activity, enhanced the in vitro and in vivo activity of bortezomib in HDAC6-overexpressing multiple myeloma cells, thus suggesting a role for the interaction of these agents independent of the effects on protein degradation (45). In addition, both proteasome inhibitors and DACi decrease cytokine production and expression of adhesion molecules, key factors supporting the growth and survival of multiple myeloma cells (39, 46). It is, therefore, most likely that a number of factors contribute to the synergy between proteasome inhibitors and DACi.

Although bortezomib clearly has proven activity as a single agent and in combination therapy in patients with multiple myeloma, initial trials with single-agent DACi have not led to significant clinical activity (4, 37, 38, 47). On the basis of preclinical data, combining DACi with proteasome inhibitors, such as bortezomib, represents an
attractive strategy for the treatment of patients with multiple myeloma. Preliminary data from phase I studies evaluating the pan-DACi panobinostat or vorinostat in combination with bortezomib have shown responses in patients who received bortezomib before study enrollment, including patients who failed to respond previously to bortezomib (48, 49). These preliminary observations are being evaluated further in phase II and III clinical trials. The 2 phase III trials will evaluate the role of DACi as a strategy to increase treatment efficacy in patients with relapsed multiple myeloma. The VANTAGE 088 trial (NCT00773747) is evaluating the combination of vorinostat plus bortezomib, and the PANORAMA 1 trial (NCT01023308) is evaluating the combination of panobinostat plus bortezomib and dexamethasone (50, 51). Both trials are comparing the combination with bortezomib plus placebo. The results of these trials will help to determine whether DACi can enhance the efficacy of bortezomib in patients with relapsed multiple myeloma. Two single-arm phase II studies, VANTAGE 095 (NCT00773838) and PANORAMA 2 (NCT01083602), are evaluating the combination of DACi, bortezomib, and dexamethasone in patients with relapsed and bortezomib-refractory disease. The results from these trials will determine if DACi can sensitize patients with bortezomib-resistant multiple myeloma (50, 52). The results of these trials, along with further research on other novel DACi and proteasome inhibitors in development, will help guide the ideal setting and combination partners for the treatment of patients with multiple myeloma.

Figure 3. Bortezomib and DACi inhibit key pathways associated with multiple myeloma (MM) cell growth and survival. Growth and survival of multiple myeloma cells are dependent on functioning intracellular pathways that drive proliferation of and interaction with the ECM and BMSC. The combination of bortezomib and DACi leads to inactivation of NF-κB and multiple myeloma cell apoptosis. Both DACi and bortezomib suppress the production of cytokines including IL-6 and IGF-I. Bortezomib also suppresses TNF-α, leading to inhibition of the interaction of multiple myeloma cells and BMSCs. Bortezomib has been shown to decrease the secretion of VEGF, leading to inhibition of angiogenesis. The cell-surface proteoglycan syndecan 1 is downregulated by DACi, which affects the interaction of multiple myeloma cells with the ECM.
Disclosure of Potential Conflicts of Interest

T. Hideshima is a consultant for Acetylon Pharmaceuticals. P.G. Richardson is a consultant and on advisory boards for Millennium and Celgene. K.C. Anderson is a consultant and on advisory board for Millennium, Celgene, and Novartis.

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Proteasome and Deacetylase Inhibitors in Multiple Myeloma

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Mechanism of Action of Proteasome Inhibitors and Deacetylase Inhibitors and the Biological Basis of Synergy in Multiple Myeloma

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