Published in final edited form as:
Curr Cancer Drug Targets. 2011 March ; 11(3): 254–284.

Marizomib, a Proteasome Inhibitor for All Seasons: Preclinical Profile and a Framework for Clinical Trials

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Abstract

The proteasome has emerged as an important clinically relevant target for the treatment of hematologic malignancies. Since the Food and Drug Administration approved the first-in-class proteasome inhibitor bortezomib (Velcade®) for the treatment of relapsed/refractory multiple myeloma (MM) and mantle cell lymphoma, it has become clear that new inhibitors are needed that have a better therapeutic ratio, can overcome inherent and acquired bortezomib resistance and exhibit broader anti-cancer activities. Marizomib (NPI-0052; salinosporamide A) is a structurally and pharmacologically unique β-lactone-γ-lactam proteasome inhibitor that may fulfill these unmet needs. The potent and sustained inhibition of all three proteolytic activities of the proteasome by marizomib has inspired extensive preclinical evaluation in a variety of hematologic and solid tumor models, where it is efficacious as a single agent and in combination with biologics, che-motherapeutics and targeted therapeutic agents. Specifically, marizomib has been evaluated in models for multiple myeloma, mantle cell lymphoma, Waldenstrom's macroglobulinemia, chronic and acute lymphocytic leukemia, as well as glioma, colorectal and

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pancreatic cancer models, and has exhibited synergistic activities in tumor models in combination with bortezomib, the immunomodulatory agent lenalidomide (Revlimid®), and various histone deacetylase inhibitors. These and other studies provided the framework for ongoing clinical trials in patients with MM, lymphomas, leukemias and solid tumors, including those who have failed bortezomib treatment, as well as in patients with diagnoses where other proteasome inhibitors have not demonstrated significant efficacy. This review captures the remarkable translational studies and contributions from many collaborators that have advanced marizomib from seabed to bench to bedside.

Keywords

Proteasome inhibitor; marizomib; bortezomib; NF-κB; multiple myeloma; pharmacodynamics; combination therapy

INTRODUCTION

The ubiquitin-proteasome system (UPS) is now a well established target in cancer therapy [1, 2]. By serving as the major pathway for regulating intracellular protein degradation in eukaryotic cells, the UPS plays a critical role in maintaining cellular homeostasis, the imbalance of which may trigger pathologies associated with cancer and inflammation. The enzymatic core engine of the UPS is the 26S proteasome, which recognizes polyubiquitin-tagged proteins for degradation and hydrolyzes them into short peptides (Fig. (1)). Degradation of abnormal or misfolded proteins by the 26S proteasome provides the cell with a mechanism for protein quality control, while blocking its function results in accumulation of unwanted proteins and cell death. This is particularly relevant to cancer cells, which proliferate at a greater rate than normal cells and therefore exhibit an increased rate of protein synthesis and degradation. Importantly, proteasome substrates include not only misfolded and aged proteins, but also those that regulate signaling pathways critical for cell growth, cell cycle progression and apoptosis. Thus, downstream effects of proteasome inhibition include the stabilization of proapoptotic proteins, including p53 and Bax, and the reduction of some antiapoptotic proteins, such as Bcl-2, collectively inducing a proapoptotic state [1, 3]. The critical observation that proteasome inhibitors attenuate growth and survival signaling by inhibiting the activation of nuclear factor-kappa B (NF-κB) helped to establish the initial rationale for targeting the 26S for the treatment of cancer [4–8]. These findings culminated in the development of the first-in-class proteasome inhibitor bortezomib (Velcade®; PS-341), which received Food and Drug Administration (FDA) approvals for the treatment of relapsed, relapsed/refractory, and newly diagnosed multiple myeloma (MM), as well as mantle cell lymphoma (MCL), based on significant objective clinical responses [9–15]. However, inherent and acquired resistance, together with side effects that include peripheral neuropathy, neutropenia and throm-bocytopenia [16, 17], have led to the search for unique proteasome inhibitors with the potential to treat patients who had failed, did not respond to, or were not candidates for treatment with bortezomib [2, 18]. One such agent is marizomib (NPI-0052; salinosporamide A), a novel marine-derived β-lactone-γ-lactam natural product that is active in multiple nonclinical tumor models and is currently in clinical trials for the treatment of patients with hematologic and solid tumor malignancies [19–21].

This review offers the first comprehensive account of the preclinical and translational biology studies that provided the basis for the clinical evaluation of marizomib (Table 1). As a guide to the reader, the article commences with an introduction to the UPS pathway and the initial chemical and *in vitro* biological profiling of marizomib, followed by detailed pre-clinical findings in hematologic and solid tumor models, with descriptions of

pharmacokinetics and pharmacodynamics, and concludes with results from Phase 1 clinical trials in patients with solid tumor and hematologic malignancies, as outlined below:

- Introduction
  - Ubiquitin-Proteasome Pathway and Proteasome Inhibitors
  - Marizomib (Origins, Mechanism of Binding to the Proteasome, Proteasome Inhibition Profile and Pharmacodynamics)
- Marizomib in Hematologic Tumor Models (Preclinical Studies)
  - Multiple Myeloma
  - Waldenstrom's Macroglobulinemia
  - Chronic Lymphocytic Leukemia
  - Acute Lymphocytic Leukemia
  - Mantle Cell and Hodgkin's Lymphoma
- Marizomib in Solid Tumor Models (Preclinical Studies)
  - Colorectal Carcinoma
  - Pancreatic Carcinoma
  - Glioma
- Mechanism for reversal of tumor cell resistance to chemo- and immunotherapy
- Pharmacokinetics and Absorption, Distribution, Metabolism and Excretion
- Proteasome Activity as a Potential Biomarker
- Clinical Trials with Marizomib

This account highlights the importance of collaborative research, which fostered the evolution of marizomib from a marine-derived natural product to a promising anticancer agent in clinical trials.

The Ubiquitin-Proteasome Pathway and Proteasome Inhibitors

The UPS pathway for protein degradation in eukaryotic cells comprises: 1) a series of enzymes [E1, activation; E2, conjugation; E3, ligation] that covalently modify proteins with a polyubiquitin tag for recognition and targeted degradation; and 2) the 26S proteasome, a 2.5 MDa multicatalytic enzyme complex that hydrolyzes the polyubiquitin-tagged proteins into short (4 – 25 residue) polypeptides, typically 7–9 amino acids in length [3] (Fig. (1)). Protein degradation by the UPS is a highly regulated process that is inherent to the molecular architecture of the 26S proteasome, which consists of one or two 19S regulatory caps flanking a 20S core particle (CP) in which substrate hydrolysis is executed [22, 23] (for reviews, see [3, 24, 25]). Upon recognition by the 19S regulatory caps, the polyubiquitin-tagged protein substrate is unfolded and translocated into the hydrolytic chamber of the 20S CP for degradation. In eukaryotes, the CP houses three pairs of catalytically active subunits, β1, β2 and β5, that exhibit protein substrate cleavage preferences referred to as caspase-like (C-L), trypsin-like (T-L) and chymotrypsin-like (CT-L), respectively, and which work in concert to degrade protein substrates. Substrate hydrolysis by the 20S CP commences with recognition of amino acid side chains (P1 – Pn) by sequential binding pockets (S1 - Sn) proximal to the proteolytic active site. It is the S1 "specificity pocket" adjacent to the active site that largely confers the CT-L, T-L, and C-L sites with their preferential binding to hydrophobic, positively-, and negatively-charged residues. Once bound, hydrolysis of the

substrate peptide bond adjacent to S1 is catalyzed by the *N*-terminal threonine residue (Thr1), which employs the side chain Thr1O$^{\gamma}$ and α-amino group as the nucleophile and general base, respectively, effectively classifying the proteolytic subunits among the *N*-terminal hydrolase family of enzymes.

The above mechanism for protein substrate hydrolysis provided strong rationale for the design of proteasome inhibitors comprising peptides that are derivatized with reactive functional groups at their *C*-termini to enable both the recognition of the peptide amino acid side chains by the proteasome S1 - Sn binding pockets and the formation of stable adducts with Thr1 [1]. It was later discovered that β-lactone-γ-lactam natural products bearing P1 moieties, which are recognized by S1, and reactive β-lactones, which covalently acylate Thr1O$^{\gamma}$, are highly selective proteasome inhibitors [26]. Peptidyl and β-lactone-γ-lactam proteasome inhibitors were instrumental in elucidating features of the proteasome active site that are critical to proteolysis [22, 23]. Both classes of inhibitor are currently under clinical evaluation (for structures, development status, proteasome inhibition profiles, routes of administration and treatment schedules, see Table 2 and supporting references). The unique identities of the P1 – Pn moieties and reactive functional head groups that stabilize the ligands at the proteolytic active site(s) (e.g., see Fig. (2)) impart each agent with a unique inhibition profile against the three proteolytic subunits of the proteasome, resulting in complementary and/or overlapping functional activities. These profile differences may provide opportunities to use these agents in combination and/or to overcome resistance, as described in this review in the context of marizomib.

Marizomib is a β-Lactone-γ-Lactam Proteasome Inhibitor Derived from the Marine Actinomycete *Salinispora tropica*

Marine microorganisms are being increasingly targeted as a resource for small molecule drug discovery [27]. Although not all taxa recovered from marine samples are unique to the marine environment, a number of chemically rich and taxonomically distinct marine groups have been discovered. Key among them is the genus *Salinispora*, which was the first obligate marine actinomycete genus to be formally described [28]. Initial chemical studies of strains in this group revealed high levels of cytotoxic activity [29] and quickly led to the isolation of salinosporamide A (USAN: marizomib; NPI-0052) from *S. tropica* [19]. Subsequent studies of this and two additional species led to the identification of many other new metabolites [30]. Marizomib exhibited a GI_{50} of < 10 nM across the National Cancer Institute (NCI) panel of 60 human tumor cell lines along with potent proteasome inhibitory activity [19]. An account of the early discovery and development of marizomib has been recently reported [20]. Genome sequencing of *S. tropica* led to the elucidation of the marizomib biosynthetic pathway [31] and the discovery of a new chlorination mechanism [32], as well as a unique starter unit in polyketide biosynthesis [33]. The collective biosynthetic machinery gives rise to a densely functionalized small molecule comprising a β-lactone-γ-lactam bicyclic core that is substituted with chloroethyl, methyl, and cyclohex-2-enylcarbinol groups at C-2, C-3 and C-4, respectively (Fig. (2)). This classifies marizomib among the β-lactone-γ-lactam superfamily of proteasome inhibitors, a distinct group of natural products derived from microbial sources and their derivatives that includes omuralide, marizomib, and the cinnabaramides (for a recent review, see [25]). These densely functionalized, low molecular weight ligands exhibit remarkable specificity for the 20S proteasome [26] that rivals peptidyl inhibitors. In fact, omuralide emerged as a gold standard among small molecule non-peptide based proteasome inhibitors in over a decade of research on proteasome structure and function; subsequently, the structurally related PS-519 (Table 2) was evaluated in Phase 1 clinical trials in young male volunteers for safety, tolerability and pharmacodynamics (PD) based on preclinical data demonstrating neuroprotective efficacy in models of cerebral ischemia [34]. The discovery of the marine-derived β-lactone-

γ-lactam marizomib provided a next generation of proteasome inhibitors, which exhibit enhanced potency and increased breadth of proteasome inhibition compared to their terrestrially-derived counterparts [19, 25, 35].

The novel structure and biological activity of marizomib inspired production and analoging efforts using traditional fermentation, natural products chemistry, semi-synthesis, total synthesis, and mutasynthesis (for a recent review of the diverse methods of production and analogs, see [36]). Although over 50 analogs have been evaluated [35–45], it is the natural product that has entered clinical trials. Moreover, while the growing number of successful synthetic strategies may eventually result in the development of streamlined processes (currently, 13 published routes [36, 46]), the most efficient production of marizomib has been achieved through industrial marine microbiology, with large scale saline fermentation of *S. tropica* fueling the extensive preclinical studies and ongoing clinical trials described in this review [20, 21, 36]. Indeed, marizomib represents the first example of an active pharmaceutical ingredient (API) manufactured by saline fermentation for clinical trials. Extensive optimization of the original fermentation conditions was required to ensure consistent API production by a robust process that meets current Good Manufacturing Practice (cGMP) guidelines. The major process improvements to the original fermentation conditions for wild type strain *S. tropica* CNB476 included: 1) selection of a single colony isolate, *S. tropica* strain NPS21184, from strain CNB476, without mutation or genetic manipulation, to support higher and more selective production of marizomib; 2) extensive fermentation development to replace animal-derived media components and natural seawater with plant-derived nutrients and a chemically defined salt formulation, respectively, to be consistent with cGMP guidelines for the manufacture of APIs [47]; and 3) the addition of solid resin to the fermentation to bind, stabilize and capture marizomib from the aqueous media [48]. These and other process improvements led to > 100-fold improvement in the fermentation yield of marizomib in shake flask culture (from ~4 to 450 mg/L) and robust production of up to 360 mg/L in stainless steel fermentors. This saline fermentation process has been performed at up to 1000L scale. After purification from the crude extract, the final pharmaceutical grade cGMP API is obtained in >98% purity with overall ~50% recovery. Based on the potency of marizomib, both the production titer at fermentor scale and the recovery yield are suitable for both clinical development and commercial production [36].

Marizomib Regulates Proteasome CT-L, C-L and T-L Activities and Exhibits a Sustained Inhibition and Pharmacodynamic Profile

While marizomib shares a β-lactone-γ-lactam bicyclic core structure with omuralide and PS-519, its unique chloroethyl and cyclohex-2-enylcarbinol substituents give rise to mechanistically important interactions within the proteasome active sites that do not occur with other β-lactone inhibitors. These interactions contribute to the high affinity and specificity of marizomib for the proteasome, characterized by irreversible inhibition of all three proteolytic activities (CT-L, T-L, C-L) with IC_{50} values in the nM range [19, 35, 49]. The mechanism of inhibition of the 20S proteasome by marizomib has been well characterized *via* detailed kinetic studies [38] and crystal structures of the ligand in complex with the 20S CP [25, 50]. Binding commences with recognition of the cyclohexenyl P1 substituent by the proteasome S1 substrate specificity pocket. Once bound, the proteasome catalytic *N*-terminal Thr1O$^{\gamma}$ forms an ester linkage with the carbonyl derived from the β-lactone ring of the inhibitor. This reaction sequence is analogous to that established for omuralide [23, 26, 51]; however, in the case of marizomib, this step is followed by Thr1NH_2-catalyzed displacement of chloride by C-3O, giving rise to a tetrahydrofuran (THF) ring (Fig. (2)). Importantly, the chloride elimination step renders marizomib irreversibly bound to all proteolytic subunits [38, 50]. Irreversible binding at the molecular

level translates to sustained inhibition of proteasome activity, the duration of which is dependent upon cell/tissue type [49, 52], and correlates with greater *in vitro* efficacy compared to slowly reversible β-lactone-γ-lactam congeners that are not structurally equipped to undergo this transformation (*vide infra*) [25, 53].

Other irreversible proteasome inhibitors that are currently under clinical evaluation include the peptide epoxyketones carfilzomib (PR-171) and ONX-0912 (PR-047), which form covalent morpholine adducts upon reaction with both Thr1O$^{\gamma}$ and Thr1N, as elucidated by crystal structures of the natural product epoxomicin in complex with the 20S CP [54]. In contrast, peptide boronic acids, such as bortezomib and CEP-18770, form non-covalent adducts; their specificity for the proteasome is attributed to the high affinity of boronic acid for hard oxygen nucleophiles (i.e., Thr1O$^{\gamma}$) in contrast to soft cysteine nucleophiles, according to Lewis hard-soft acid-base principles. The ligand is further stabilized by hydrogen bonding interactions between Thr1NH_2 and B-OH, as well as non-covalent P1-P3 residue contacts with the proteasome S1-S3 binding pockets (Fig. (2)) [55], and the collective binding modality results in slowly reversible proteasome inhibition. Thus, the binding kinetics and PD profiles of the various classes of proteasome inhibitors that are currently in clinical use (Table 2) are well established at the molecular level.

Marizomib exhibits high specificity for the proteasome as compared to other proteases [21] and also inhibits immuno-proteasomes [49], a specialized form of proteasomes that are induced by cytokines such as interferon-gamma and which are involved in the generation of antigenic peptides that are loaded onto major histocompatibility complex (MHC) class I proteins for eventual participation in the initiation of the immune response and generation of cytotoxic T-cells (CTL). Most CTLs express T-cell receptors (TCRs) that can recognize a specific antigenic peptide bound to Class I MHC molecules, which are present on nucleated cells, including tumors. Interestingly, immunoproteasomes are expressed in high levels predominantly in cells of hematopoetic origin, including MM cells [56], suggesting particular relevance to proteasome inhibitor therapy in hematologic malignancies and potential targets for new proteasome inhibitors under preclinical development.

Marizomib binds to and inhibits all three proteolytic subunits (β1, β2 and β5), despite being minimally substituted with a single moiety (P1) for recognition by the proteasome S1 specificity pocket. The non-covalent P1/S1 binding interactions of marizomib have been well characterized by enzyme inhibition kinetics, structural biology, and structure-activity relationship (SAR) studies, and the collective results are in good agreement [25, 38, 50]. The crystal structure of the marizomib:CP complex revealed that the inhibitor occupies all three pairs of proteolytic subunits [50], and when evaluated against purified 20S proteasomes, the IC_{50} rank order for inhibition is CT-L < T-L < C-L [35, 49]. However, cell-based studies reveal that this profile may vary depending upon the cell type [49, 57]. Although the P1 residue presents a relatively limited surface for binding to the proteasome S1 specificity pocket, the high affinity of marizomib for the proteasome is sufficient to induce broad and potent inhibitory activity compared to some peptidyl proteasome inhibitors, despite their ability to bind to several substrate binding pockets of the proteasome (S1 - Sn) (Fig. (2)). This nicely exemplifies the binding efficiency that is inherent to the dense functionality of the β-lactone-γ-lactam inhibitor designed by nature. The inhibition profiles of the different proteasome inhibitors in clinical use (Table 2) are distinguished by their relative binding affinities for the CT-L, T-L and C-L sites (associated with noncovalent interactions) as well as the duration of inhibition against isolated proteasomes *in vitro* (reversible or slowly reversible versus irreversible binding) and their PD profiles *in vivo*. Overall, the various proteasome inhibitors exhibit different inhibition profiles for the β1,β2 and β5 and immunoproteasome subunits that ultimately impart different potencies, cellular activities, target specificities and potentially different safety profiles.

The irreversible binding properties of marizomib result in lower IC_{50} values compared to structurally related yet slowly reversible β-lactone-γ-lactam inhibitors when measured against isolated proteasomes. The potential therapeutic benefit of this property may best be gauged by understanding the downstream consequences of irreversible binding in cells and tissues. Irreversible binding by marizomib has been correlated with markedly enhanced cytotoxicity in tumor cells; a PD profile characterized by prolonged proteasome inhibition *in vivo*; and sustained inhibition in tumor tissue and packed whole blood (PWB) associated with reduced tumor growth.

With respect to cytotoxicity, SAR studies indicate that irreversible binding imparts marizomib with potent cytotoxicity relative to slowly reversible inhibitors of the same structural class. This trend is consistent across various human tumor cell lines, including those of hematologic (RPMI 8226) and solid tumor (PC-3, HCT-116) origin, and is further supported by SAR studies of more structurally diverse salinosporamides and omuralide [25, 35, 39, 41, 53, 58]. While transport across the cell membrane may contribute to cytotoxicity, cell transport studies that directly compared [^{3}H]-marizomib with the slowly reversible deschloro analog [^{3}H]-salinosporamide B concluded that both compounds exhibit similar uptake characteristics in RPMI 8226 and PC-3 cells. However, marizomib exhibits slower efflux, greater extent of proteasome inhibition at lower concentrations, prolonged proteasome inhibition, and greater cytotoxicity, all of which can be attributed to irreversible binding to the proteasome [53]. Overall, it may be concluded that high affinity irreversible binding of marizomib results in inactivation of proteasome binding sites at low ligand concentrations as well as prolonged retention in cells, allowing the downstream consequences of sustained proteasome inhibition to play out to cell death.

Prolonged inhibition of proteasome activity in cells resulting from irreversible binding at the molecular level is readily observed in non-nucleated red blood cells (RBCs) that cannot generate new proteasomes. The PD profile of marizomib in animal models [49] and in clinical trials is characterized by sustained inhibition of proteasome activity (≥ 72 hours) in packed whole blood (PWB) lysates (containing > 98% RBCs that display a half-life of 15–17 weeks in humans) after a single intravenous (IV) administration of the drug, consistent with its irreversible binding profile *in vitro*. Moreover, in PWB samples obtained from ~90 patients treated with marizomib, a dose dependent inhibition of PWB 20*S* proteasome CT-L activity was observed, with increasing inhibition upon multiple administrations and only partial recovery between consecutive doses (Fig. (3)). The PD profile of marizomib is more durable than that of bortezomib, where recovery of proteasome activity was readily observed in both PWB and peripheral blood mononuclear cell (PBMC) lysates within 24 hours [49, 59], in agreement with its reversible binding mechanism (Fig. (2)). In contrast, the peptidyl epoxyketone carfilzomib, which forms an irreversible, covalent morpholine adduct with the proteasome (*vide supra*), exhibits a sustained PD profile in PWB lysates [60] (Table 2).

In nucleated cells, restoration of proteasome activity will reflect not only the ligand off-rate from the proteasome, but also the cell half-life and the rate of *de novo* proteasome synthesis, which can restore proteasome function in cells, even after treatment with an irreversible inhibitor. Indeed, recovery of proteasome activity after administration of marizomib is more rapid in patient PBMCs (48–72 h; nucleated cells with a half-life of a few days) compared to PWB (Fig. (4)) [61]. Interestingly, PD and efficacy studies in a human MM.1S plasmacytoma xenograft murine model similarly demonstrated rapid recovery of proteasome activity in normal tissues, including liver, lung, spleen and kidney (< 24 h), but more sustained inhibition in PWB and importantly, in tumors (> 24 h) (Fig. (5)). Notably, the prolonged proteasome inhibition in tumors correlated with reduced tumor growth in this model [52] (see *Pharmacodynamics and efficacy of marizomib in a human MM.1S plasmacytoma xenograft murine model*). Thus, it is important to distinguish irreversibility at

the molecular level from the net biological effect on proteasome function in a given cell population or tissue type. The ability to monitor proteasome and immuno-proteasome inhibition and recovery profiles in different tissues may provide insights into tumor responses to proteasome inhibitors. Furthermore, initial research has shown that circulating proteasome protein levels and proteolytic activities may also be a potential biomarker that reflects the biology of the underlying disease and may serve as an independent prognostic factor for survival in MM and chronic lymphocytic leukemia (see *Implications of Monitoring Proteasome Activity as a Potential Biomarker*) [62, 63]. Studies to monitor proteasome activities in various tissues before and after marizomib treatment in the clinic are ongoing [61].

MARIZOMIB IN PRECLINICAL HEMATOLOGIC TUMOR MODELS

The FDA approved bortezomib in 2003 as a treatment for relapsed/refractory MM and in 2006 for mantle cell lymphoma (MCL). This approval has validated the use of proteasome inhibitors in hematologic malignancies, particularly in B-cell cancers, and has since fostered the development of proteasome inhibitors with specificity profiles that may overcome both the cellular resistance patterns and toxicities to bortezomib. The ability of proteasome inhibitors to regulate NF-κB activation by inhibiting the degradation of IκBα, the cytoplasmic regulator of NF-κB activation, also expands the spectrum of tumors that are potential targets for these inhibitors. Marizomib, alone or in combination with other agents, may therefore fulfill the unmet need for new approaches to treat a broader spectrum of hematologic cancers. These concepts are further explored in the following sections, which highlight nonclinical studies of marizomib in hematologic tumor models, including MM, MCL, Waldenstrom's macroglobulinemia, chronic and acute lymphocytic leukemias. These studies dissect specific mechanisms of action for marizomib, reveal synergies with bortezomib, histone deacetylase inhibitors and other agents *in vitro*, and demonstrate preclinical efficacy *in vivo,* providing the framework for ongoing clinical trials in patients with hematologic cancers.

Marizomib in Multiple Myeloma

Proteasome inhibitor therapy has proven to be a successful clinical strategy for the treatment of MM. Specifically, bortezomib is the standard of care for the treatment of relapsed, relapsed/refractory, and newly diagnosed MM [9–13]. However, clinical experience with bortezomib indicates possible off-target toxicities such as peripheral neuropathy, thrombocytopenia and neutropenia and the development of drug-resistance [16, 17]. In order to address these issues, recent research efforts have focused on the discovery and development of new proteasome inhibitors with equipotent anti-MM activity and fewer off-target activities. In this context, recent studies examined the efficacy of marizomib, which is orally active in MM models [19, 20]. Results from preclinical studies of marizomib in MM are highlighted below.

In Vitro and In Vivo Anti-Tumor Multiple Myeloma Activity of Marizomib—Initial screening of marizomib against the NCI panel of 60 human tumor cell lines showed a GI_{50} of < 10 nM for all cell lines [19]. In agreement with these observations, it was later demonstrated that 1) marizomib induces apoptosis in MM cells sensitive and resistant to both conventional and bortezomib therapies; and 2) the IC_{50} of marizomib for MM cells is within the low nanomolar concentration [49]. Examination of the effects of marizomib and bortezomib on normal PBMCs showed that marizomib does not significantly decrease normal lymphocyte viability at the IC_{50} for MM cells, with modest effects only at much higher concentrations. In contrast, bortezomib decreased the survival of PBMCs at concentrations close to the IC_{50} for MM cells. These data suggest a larger therapeutic index

for marizomib than bortezomib. Importantly, marizomib induced apoptosis in tumor cells from MM patients relapsing after various prior therapies including bortezomib and/or thalidomide. The effectiveness of marizomib against tumor cells from bortezomib-refractory patients may be due, at least in part, to its ability to inhibit all three proteasome activities, i.e., CT-L, C-L and T-L, *versus* bortezomib, which predominantly affects CT-L activity. Indeed, studies using an *in vitro* protein model system demonstrated that simultaneous inhibition of multiple proteasome activities is a prerequisite for significant (i.e., > 50%) inhibition of proteolysis [64]. Therapeutic concentrations of bortezomib primarily target CT-L proteasome activity, and C-L as a secondary target. It is likely that the remaining proteolytic activity i.e., T-L, may compensate and allow proteasome functionality to be partially maintained. In contrast to bortezomib, marizomib inhibits all three proteolytic activities, thereby achieving maximal inhibition of proteolysis. Additionally, mechanisms conferring bortezomib-resistance may not be effective against marizomib (*vide infra*).

Mechanisms Mediating Marizomib-Induced Apoptosis in Multiple Myeloma Cells—Findings in MM models revealed that marizomib-induced MM cell death is associated with: 1) decrease in mitochondrial membrane potential; 2) increase in superoxide production; 3) activation of mitochondrial apoptogenic proteins cytochrome-c and second mitochondrial activator of caspases (Smac/Diablo); and 4) activation of caspase-9, caspase-8, caspase-3, and poly (ADP-ribose) polymerase (PARP) cleavage. Importantly, in MM cells, marizomib mediates apoptosis predominantly *via* caspase-8, whereas bortezomib-induced apoptosis requires both caspase-8 and caspase-9 activation. These findings indicate that marizomib is more dependent on FAS-associated *via* death domain (FADD)-caspase-8 apoptotic signaling pathway than bortezomib, suggesting differential action of marizomib *versus* bortezomib against MM cells. Furthermore, in contrast to marizomib, bortezomib-induced apoptosis requires activation of pro-apoptotic BH3-only family member proteins Bax and Bak [49]. The BH3-only members of the Bcl-2 protein family are essential for initiation of programmed cell death and stress-induced apoptosis.

Besides induction of apoptosis, marizomib downregulates various cell growth and survival signaling pathways in MM cells. In fact, the initial rationale for the therapeutic use of proteasome inhibitors as anticancer agents was, in part, based on their ability to inhibit growth and survival signaling *via* NF-κB [4–8, 49]. Indeed, marizomib, like bortezomib, targets NF-κB; importantly, marizomib is a more potent inhibitor of NF-κB and related cytokine secretion than bortezomib [49, 65]. A detailed study on the effects of marizomib on NF-κB regulated gene products demonstrated that marizomib potentiated apoptosis induced by tumor necrosis factor-alpha (TNF-α), 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)). Marizomib also suppressed TNF-α-induced tumor cell invasion and receptor activator of NF-κB ligand (RANKL) induced osteoclastogenesis [65].

Several investigators have shown that the MM-host bone marrow microenvironment confers growth, survival, and drug resistance in MM cells [5, 66]. Adhesion of MM cells to bone marrow stromal cells (BMSCs) triggers transcription and secretion of MM cell growth and survival factor interleukin-6 (IL-6) [5, 66]. Marizomib significantly inhibits MM cell growth even in the presence of BMSCs. Furthermore, marizomib abrogates IL-6-induced proliferation of MM cells. In addition to NF-κB inhibition, marizomib overcomes survival and drug-resistance conferred by Bcl-2 in MM cells: overexpression of Bcl-2 provides more protection against bortezomib than marizomib [49]. Additional studies suggest that resistance to bortezomib, but not marizomib, involves heat shock proteins Hsp27 and Hsp70

[67, 68]. Marizomib also blocks VEGF-triggered migration of MM cells, suggesting that marizomib is an anti-migratory agent [49, 69].

Examination of the *in vivo* efficacy of marizomib using a human MM.1S plasmacytoma xenograft mouse model [70] shows potent oral anti-tumor activity [49]. Treatment of MM.1S-bearing mice with marizomib, but not vehicle, inhibits plasmacytoma growth and prolongs survival of these mice (Fig. (5)). Marizomib is well tolerated by mice, without significant weight loss or obvious neurological behavioral changes. Importantly, analysis at day 300 shows no recurrence of tumors in 57% of the marizomib-treated mice. A head-to-head examination of marizomib and bortezomib shows that both agents reduced tumor progression and prolonged survival.

Pharmacodynamics and Efficacy of Marizomib in a Human MM.1S Plasmacytoma Xenograft Murine Model—*In vivo* studies in mice using human MM.1S plasmacytoma xenografts demonstrate that IV administered marizomib is well tolerated, prolongs survival, and reduces tumor recurrence (*vide supra*). PD studies using the model described above demonstrated that marizomib: 1) rapidly leaves the vascular compartment and enters the tumors and other organs as the parent compound; 2) inhibits 20S proteasome CT-L, T-L, and C-L activities in extra-vascular tumors, PWB, liver, lung, spleen, and kidney, but not brain; and 3) triggers a more sustained (>24h) proteasome inhibition in tumors and PWB than in other organs (< 24h) [52] (Fig. (5)). These findings are consistent with earlier studies showing that marizomib targets all three 20S proteasomal activities [35, 49]. Indeed, the kinetics of proteasome inhibition differ between tumors and normal tissues. For example, the onset of marizomib-induced proteasome inhibition is rapid (within 10 min) in most tissues other than tumor, for which the onset of inhibition occurs at ~1h and is maximal at 24h. Intravenous injection of either a single or three doses of marizomib (0.15 mg/kg) blocks proteasome activities in peripheral organs, without inhibition of proteasome activity in the brain, indicating that marizomib does not cross the blood-brain barrier at this dose and schedule. The likely explanation for a sustained inhibition of proteasome activity in tumors may be the irreversible nature of marizomib; however, the cell half-life and rate of *de novo* proteasome synthesis in tumors may contribute. Importantly, marizomib-induced blockade of proteasome activity in liver, spleen, kidney, and lungs recovers by 24h, implying that *de novo* proteasome synthesis in these tissues may result in the rapid recovery of proteasome activity. An important conclusion of this study was that treatment of MM.1S bearing immunodeficient mice with marizomib reduces tumor proliferation without marked toxicity, which is associated with prolonged inhibition of proteasome activity in tumors and PWB, but not in normal tissues [52] (Fig. (5)).

Combination Studies of Marizomib with Bortezomib or the Immunomodulatory Agent Lenalidomide in Multiple Myeloma—Recent studies utilizing an *in vitro* protein model system have shown that simultaneous inhibition of multiple proteasome activities is a prerequisite for significant (i.e., > 50%) inhibition of proteolysis [64]. Since bortezomib predominantly inhibits proteasome CT-L, and more recently defined inhibition of C-L activities [49], it was hypothesized that marizomib, which blocks all three 20S proteasome activities, can be combined with bortezomib to confer a broader inhibition profile at lower and potentially safer doses. Indeed, combining marizomib and bortezomib induces synergistic anti-MM activity both *in vitro* using MM cell lines or patient bone marrow derived $CD138^+$ MM cells and *in vivo* in the human MM.1S plasmacytoma xenograft murine model [71]. Combined marizomib and bortezomib-triggered apoptosis in MM cells is associated with: 1) activation of caspase-8, caspase-9, caspase-3, and PARP cleavage; 2) induction of endoplasmic reticulum (ER) stress response and c-Jun *N*-terminal kinase (JNK); 3) inhibition of migration of MM cells and angiogenesis; 4) suppression of CT-L, C-L and T-L proteasome activities; and 5) blockade of NF-κB signaling. Animal studies

showed that administration of combined low doses of marizomib and bortezomib is well tolerated, and trigger synergistic inhibition of tumor growth, and CT-L, C-L and T-L proteasome activities in tumor cells. Histochemical analysis of the MM.1S plasmacytomas from marizomib plus bortezomib-treated mice showed growth inhibition, apoptosis, and a decrease in associated angiogenesis. Of note, it is clear from *in vivo* data that even 30–40% proteasome inhibition, albeit of all three activities, is sufficient to trigger significant anti-MM activity. The mechanisms mediating enhanced cytotoxicity of the combination regimen may likely result from greater and broader proteasome inhibition and/or differential apoptotic signaling pathways with the two-drug regimen. A similar synergistic cytotoxicity of marizomib plus bortezomib is reported in models of Waldenstrom's macroglobulinemia - an incurable low-grade B-cell lymphoma (see *Waldenstrom's Macroglobulinemia* below) [72]. Finally, the synergistic cytotoxicity of marizomib and bortezomib in lymphoma, leukemia, and solid tumor cells that are relatively resistant to bortezomib, suggests clinical applicability of this therapeutic regimen beyond MM (*vide infra*).

Combination therapeutic strategies have shown promise in reducing toxicities and overcoming drug resistance associated with bortezomib. For example, prior preclinical studies showed that lenalidomide (Revlimid®) - a novel immunomodulatory drug (IMiD) - triggered growth arrest or apoptosis in drug resistant MM cells. A Phase 1/2 clinical trial of bortezomib with lenalidomide and low dose dexamethasone demonstrated safety and remarkable efficacy in relapsed-refractory and newly diagnosed MM patients [73, 74]. The finding that the combined bortezomib and lenalidomide regimen has the ability to overcome clinical bortezomib resistance, coupled with findings that marizomib is a potent proteasome inhibitor, suggested that combining marizomib with lenalidomide may also trigger synergistic anti-MM activity. Consistent with this notion, combining low doses of marizomib and lenalidomide induced synergistic anti-MM activity [75]. Furthermore, marizomib plus lenalidomide-induced apoptosis correlates with: 1) activation of caspase-8, caspase-9, caspase-12, caspase-3, and PARP cleavage; 2) activation of BH3 protein Bim; 3) translocation of Bim to endoplasmic reticulum; 4) inhibition of migration of MM cells and angiogenesis; and 5) suppression of CT-L, C-L and T-L proteasome activities. Blockade of Bim using small interfering RNA (siRNA; sometimes known as short interfering RNA or silencing RNA, a class of double-stranded RNA molecules, 20–25 nucleotides in length) abrogated marizomib and lenalidomide-induced apoptosis. Biochemical studies demonstrate that marizomib plus lenalidomide-induced apoptosis is primarily dependent on caspase-8 signaling. The mechanistic studies suggest that the synergistic anti-MM activity of marizomib plus lenalidomide predominantly relies on the caspase-8 and Bim/ER/caspase-12 signaling axis. Importantly, low dose combinations of marizomib and lenalidomide are well tolerated, significantly inhibit tumor growth, and prolong survival in a human MM.1S plasmacytoma xenograft murine model.

Collectively, these preclinical studies demonstrate potent *in vitro* and *in vivo* anti-tumor activity of marizomib combined with either lenalidomide or bortezomib at doses that are well tolerated in a human MM.1S plasmacytoma xenograft murine model. These findings provide the framework for clinical trials of low dose combinations of marizomib with bortezomib or lenalidomide to increase response, overcome drug resistance, reduce side effects, and improve patient outcome in MM.

Marizomib in Waldenstrom's Macroglobulinemia

Waldenstrom's Macroglobulinemia (WM) is a biologically unique low grade B-cell lymphoma characterized by the presence of lymphoplasmacytic cells in the bone marrow and the secretion of immunoglobulin M (IgM) monoclonal protein in the serum, indicating that WM cells present with a high rate of protein turnover. Protein metabolism is a tightly regulated process, and inhibition of its turnover with proteasome inhibitors leads to

apoptosis in malignant cells [76, 77]. A major activity of proteasome inhibitors in certain B-cell malignancies is the targeting of the IL-6 and NF-κB signalling pathways, both of which are critical regulators of survival and proliferation in B-cell malignancies, including WM [78–80]. Based on its activity in MM, bortezomib was tested in WM and achieved a 40–80% response rate in Phase 2 trials [81]. These striking clinical responses indicate that proteasome activity is critical for the survival of WM cells. While marizomib also inhibits the proteasome, it exhibits a complementary and partially overlapping proteasome inhibition profile and ultimately, a different mechanism of action than bortezomib; for example, as discussed above, apoptosis of MM cells induced by marizomib is mediated predominately through the caspase-8 cell death cascade [49]. The role of the proteasome in WM was therefore dissected using marizomib and bortezomib. It was demonstrated that marizomib inhibits proliferation and induces apoptosis in WM cell lines and CD19+ primary WM cells at doses consistent with previous studies and achievable *in vivo* [72, 82, 83]. It was later demonstrated that the combination of marizomib and bortezomib leads to synergistic cytotoxicity on WM human cell lines (BCWM.1, WM-WSU), IgM secreting cell lines (MEC-1, Namalwa) and bone marrow derived patient primary WM cells. These two agents lead to inhibition of nuclear translocation of p65 NF-κB, with activity on the canonical and non-canonical NF-κB pathway, and synergistic induction of caspase-3, -8 and -9 cleavage, as well as PARP cleavage and induction of Smac/Diablo. These findings begin to delineate the role of the canonical and non-canonical NF-κB pathways in WM.

Studies to further dissect the mechanism of synergy of marizomib and bortezomib demonstrated differential activities on both: 1) the Akt pathway; and 2) 20S proteasome CT-L, C-L and T-L functions. Marizomib induced cytotoxicity was completely abrogated in an Akt knockdown cell line (BCWM.1; established using lentavirus infection system), indicating that its major activity is mediated through this pathway, while bortezomib modestly activated Akt activity. Previous studies have demonstrated that activation of the Akt survival pathway may be one mechanism of bortezomib resistance in malignant B cells. It was subsequently demonstrated that the major activity of marizomib is mediated through inhibition and not activation of Akt and therefore, its combination with bortezomib may overcome resistance to bortezomib *in vivo* [72].

While little is known about the role of the bone marrow microenvironment in WM, the adhesion of WM cells to cytokines and/or fibronectin present in the bone marrow milieu was found to induce NF-κB activation and IL-6 induced Akt activation, which were both down-regulated in the presence of marizomib either alone, and more significantly in combination with bortezomib. Importantly, IL-6 and NF-κB induction by adhesion are two major pathways regulated by the proteasome, and marizomib and bortezomib overcome resistance induced by mesenchymal cells and the addition of IL-6 in a co-culture *in vitro* system. The combination of the two agents overcomes the protective effect of the bone marrow niches, without affecting the growth and differentiation of normal hematopoietic components. Homing, a complex process that is regulated by migration and adhesion of malignant cells to their specific bone marrow niches, is also influenced by the two agents: marizomib and bortezomib inhibit migration and adhesion of WM cells as well as their homing *in vivo* [72]. Together, these studies provide a stronger understanding of the biological role of the proteasome pathway in WM, and provide the preclinical framework for studying in clinical trials marizomib in WM and other low-grade lymphomas.

Marizomib in Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common adult hematologic malignancy in the western world. The majority (>75%) of newly diagnosed CLL cases (about 16,000 in the US) occur in men over the age of 50. The disease is characterized by the accumulation of mature resting CD5+ B-cells in the peripheral blood and is thought to arise primarily as the

result of defects in the regulation of apoptosis rather than proliferation [84, 85]. Thus, as a result of epigenic alterations in the regulation of the *bcl-2* gene, CLL cells express very high levels of the anti-apoptotic protein Bcl-2 [86].

Chemotherapy regimens using combination approaches are effective in newly-diagnosed and relapsed CLL. Recent clinical trials have shown that combinations of the purine analog fludarabine (Fludara) with alkylating agents bendamustine (Treanda) or cyclophosphamide (Cytoxan) produce higher response rates and a longer progression-free survival than single agents. In addition, positive results have been obtained with monoclonal antibody regimens that include alemtuzumab (anti-CD52), rituximab (Campath-1H, anti-CD20), and ofatumumab (Arzerra, anti-CD20) [87, 88]. Although these approaches are effective, additional treatment regimens that are active as either a single agent or in combination with standard of care therapies are urgently needed. Preclinical studies confirmed that in CLL cells, bortezomib blocks activation of NF-κB and bypasses Bcl-2 mediated apoptosis resistance, possibly by activating the Bcl-2 inhibiting protein kinase, JNK [89, 90]. Based on these positive preclinical findings, bortezomib was evaluated in Phase 2 clinical trials in patients with refractory CLL. Unfortunately, only minimal (but suggestive) responses to bortezomib were obtained in these trials [91]. However, the preclinical biology strongly supported that a proteasome inhibitor should show clinical activity, supporting the evaluation of the second generation proteasome inhibitor marizomib in preclinical CLL models [57].

Why can marizomib succeed where bortezomib has failed to exhibit significant clinical activity in CLL? Findings using freshly isolated Ficoll-Paque fractionated peripheral blood from 37 newly diagnosed patients with CLL demonstrated some striking similarities and differences between these two inhibitors [57]. As discussed above, marizomib exhibits a more prolonged, broader proteasome inhibition profile compared to bortezomib. Although their steady-state IC_{50} values as inhibitors of proteasome CT-L activity were similar, marizomib exerted its effects more rapidly than bortezomib, and drug washout experiments showed that short exposures to marizomib (15 minutes) resulted in sustained (≥ 24 hours) proteasome inhibition. In contrast, proteasome CT-L activities recovered in CLL cells exposed to even a 10-fold higher concentration of bortezomib. In addition, prolonged exposure times with bortezomib of ≥ 8 hours were required for commitment to caspase activation and DNA fragmentation. It is currently believed that proteasome inhibitors prevent clearance of misfolded or damaged proteins leading to protein aggregation, ER stress and JNK and caspase-4 activation. Although there are differences in the activity profiles for marizomib and bortezomib, both involve caspase-4 as a central mediator of cell death.

Interestingly, there has been a recent report suggesting that plasma components, in particular the dietary flavinoid, quercetin, may inactivate bortezomib by binding to the boron moiety [92]. The findings provide an attractive potential explanation for why bortezomib did not display significant clinical activity in CLL and support testing of marizomib as a single agent or in combination with other standard of care therapies in this patient population. In relation to dietary intake, it has also been observed that the proteasome inhibitory and anticancer activity of bortezomib and other boronic acid-based proteasome inhibitors can be blocked by green tea polyphenols [93,94].

Marizomib in Acute Lymphocytic Leukemia

A Need for New Drugs and the Role of Proteasome Inhibitors in Acute Lymphocytic Leukemia—Although acute lymphocytic leukemia (ALL) boasts a cure rate of over 80%, patients that do not respond to the currently used combination chemotherapeutic regimen are left with few options [95]. In pediatric ALL cases, the cure

rate is approaching 90%, however, late effects of the currently used therapy are a significant problem for survivors of childhood leukemia, since they live longer than their adult counterparts. Specific late effects of therapy such as cardiotoxicity stemming from anthracycline exposure or diminished neurocognitive function in patients that have received cranial irradiation for central nervous system disease are problematic and can be avoided by developing less pantoxic and more selective strategies for the treatment of ALL [96].

The clinical success of proteasome inhibitors in other hematologic malignancies such as MM and MCL suggested that leukemia patients may similarly benefit from this class of drugs [97]. Validation of this concept was carried out in a 2004 Phase 1 clinical trial of bortezomib, in which fifteen adult refractory/relapsed ALL patients were enrolled and a maximum tolerated dose was identified. Importantly, significant proteasome inhibition was observed at 1.3 mg/m^2 and lower doses in peripheral blood specimens collected from the patients [98]. These data provided proof of concept that proteasome inhibitors can be utilized clinically in a leukemia population, thereby providing rationale for testing marizomib in leukemia populations.

Biochemical Effects of Marizomib in ALL—Evaluation of bortezomib and marizomib on proteasome CT-L, C-L and T-L activities in an array of leukemia cell lines indicated that marizomib more potently inhibited the CT-L and C-L activities. Importantly, the T-L activity was also inhibited by marizomib in stark contrast to bortezomib, which slightly stimulated T-L activity [83]. Another key difference between bortezomib and marizomib was in the mode of apoptosis induction in leukemia cells. Two biochemical markers of apoptosis, DNA fragmentation and caspase-3-like activity, were triggered more strongly by marizomib than bortezomib in ALL cells. Insight into the initiation of caspase activation was provided by using caspase-8 and -9 specific inhibitors. Caspase-8 inhibition significantly prevented cell death by marizomib whereas caspase-9 inhibitors had little to no effect. By using variants of the Jurkat ALL cell line that lack either FADD or caspase-8, a requirement for these two molecules in marizomib induced cell death was clearly determined for ALL cells [83].

Induction of oxidative stress has been observed in numerous cell systems after proteasome inhibition. Consistent with these reports, marizomib caused an increase in intracellular peroxide and superoxide levels. The antioxidant, *N*-acetylcysteine, abrogated cell death by marizomib but did not provide protection against caspase-8 activation by the drug. This highlighted a role for caspase-8 as upstream of oxidative stress generation by marizomib. The source of intracellular superoxide and peroxide upon treatment with marizomib remains unclear, and may arise from multiple processes which may be caspse-8 dependent or independent, since the peptide based chemical caspase-8 inhibitor, IETD-fmk, did not change levels of intracellular superoxide.

Marizomib in Combination with Histone Deacetylase Inhibitors in ALL and other Models—In leukemia therapy, as with most cancers, combining agents appears more efficacious than single agent therapies [99]. Proteasome inhibitors have been tested *in vitro* with numerous anti-leukemia drugs including nucleoside analogs [89], antibody based drugs [100], as well as alkylating agents [101]. One of the more promising combinations has been with a class of epigenetically targeted drugs: the histone deacetylases (HDAC) inhibitors [102]. One member of this class of compounds, vorinostat (suberoylanilide hydroxamic acid (SAHA), Zolinza®), has been FDA approved for the treatment of cutaneous T cell lymphoma, indicative of activity in hematologic malignancies. Bortezomib has been tested in combination with numerous HDAC inhibitors and found to exert synergistic effects [103]. Marizomib also synergizes with HDAC inhibitors in ALL [83, 104] and MM (Chauhan *et al.* unpublished results), however, quantification of the degree of

synergy using isobologram based analyses reveals that the marizomib and HDAC inhibitor combinations are more potent than bortezomib combinations in leukemia cell lines and primary acute leukemia specimens [104, 105]. Since HDAC inhibitors are divided into classes based on their chemical structure and based on which HDAC family members they inhibit [102], it was interesting to note that diverse HDAC inhibitor compounds (hydroxamic acids, benzamide and aliphatic acids) were strongly synergistic with marizomib, and more so than with bortezomib.

Investigating the mechanism of action of the synergies between proteasome inhibitors and HDAC inhibitors revealed surprising and overlapping mechanisms, as highlighted in Fig. (6). In ALL, the HDAC inhibitors were found to repress mRNA expression of the three 20S proteasomal β subunits that are responsible for the enzymatic activity. An unexpected yet distinguishing feature between bortezomib and marizomib was the ability of marizomib to cause an increase in total histone H3 and acetylation of histone H3. This histone modification is a hallmark of HDAC inhibitor action but had never before been reported as occurring as a consequence of proteasome inhibition. This epigenetic consequence of marizomib action may provide insight into the stronger synergy of marizomib with HDAC inhibitors than bortezomib and is currently under investigation. Other potential synergies between HDAC and proteasome inhibitors have been observed in pancreatic tumor cells, involving aggresome formation and HDAC6 inhibition (see *Marizomib in Pancreatic Cancer Models*). More recently, bortezomib and marizomib demonstrated synergistic effect with the class I HDAC inhibitor MGCD0103 in Hodgkin's lymphoma cell lines by inhibiting TNF-α-induced NF-κB activation [106] (see below). Moreover, regulation of NF-κB by both proteasome inhibitors and HDAC inhibitors represents a point of convergence for these drugs that may contribute to their synergistic activities in MM (Chauhan *et al.* unpublished observations) and solid tumors, such as non-small cell lung carcinoma (NSCLC) (Drabkin *et al.* unpublished observations).

The consequences of combining these classes of drugs have the potential to impact many cellular processes. In support of this point, a recent comprehensive analysis of the acetylome revealed many unexpected non-histone acetylation targets for various HDAC inhibitors including vorinostat, entinostat (SNDX-275, previously called MS-275) and romidepsin (Istodax®) [107]. The HDAC inhibitors vorinostat and romidepsin were initially approved by the FDA in 2006 and 2009, respectively, for the treatment of cutaneous T-cell lymphoma. The recent preclinical findings and increasing knowledge of the distinct and overlapping mechanisms of action for proteasome inhibitors and HDAC inhibitors paved the way for testing these drug combinations in a broader spectrum of lymphoid and solid tumor malignancies [104,108].

Marizomib in Mantle Cell Lymphoma and Hodgkin's Lymphoma

In addition to MM, CLL and WM, preclinical studies have identified additional B-cell malignancies that express constitutively activated NF-κB and are therefore considered targets for proteasome inhibitor therapy. Subsequent clinical studies lead to the FDA approval in 2006 of bortezomib as a treatment for MCL [109–111], an aggressive and one of the rarest forms of non-Hodgkin's lymphoma. Bortezomib was also tested clinically in an additional B-cell malignancy, Hodgkin's lymphoma. However in this patient population, bortezomib showed minimal single agent activity [109,110]. In light of the different proteasome inhibition profile for marizomib, it was considered of interest to evaluate its activity in preclinical models for both these B-cell malignancies.

Marizomib was shown to be active as a single agent in Hodgkin's lymphoma cell lines (HD-LM2, L-428 and KM-H2) and MCL cell lines (Jeko1, Mino and SP53) [112]. The antiproliferative activity of marizomib was observed in all cell lines tested in a time- and

concentration-dependent manner. The effect was observed in as early as 24 hours, and lasted for up to 72 hours at a dose range of 5 nM to 50 nM. The activity was comparable to bortezomib at the same conditions. The antiproliferative activity occurred through induction of apoptosis and was enhanced by treating cells in combination with the HDAC inhibitor vorinostat (SAHA) [110] or with GX15-070, a small-molecule antagonist of the BH3-binding groove of the Bcl-2 family proteins [113]. More recently, marizomib was shown to have a synergistic antiproliferative activity in combination with the class I HDAC inhibitor MGCD0103, suggesting that synergy between HDAC inhibitors and proteasome inhibitors (Fig. (6)) can also be maintained through an HDAC 6-independent mechanism [106]. The potent activity of marizomib in MCL and Hodgkin's lymphoma as a single agent and in combination with HDAC inhibitors support additional clinical testing in these diseases.

MARIZOMIB IN PRECLINICAL SOLID TUMOR MODELS

Although proteasome inhibitors have demonstrated clinical activity in hematologic, and in particular B-cell, malignancies, the clinical results with bortezomib in solid tumor malignancies have not demonstrated appreciable benefits [114–116]. Why bortezomib did not exhibit activity in patients with solid tumors may be explained in part by its proteasome inhibition profile, onset and duration of activity. Given the noted differences in mechanisms of action for bortezomib and marizomib, it is possible that marizomib may yield greater clinical efficacy in solid tumors as a single agent or in combination with clinically relevant drugs. The following sections provide preclinical findings in solid tumor models that address these issues.

Marizomib in Colorectal Carcinoma

Many early studies assessing the efficacy of proteasome inhibitors in cancer treatment were based upon the assumption that inhibition of the NF-κB pathway was the predominant anti-tumor mechanism, as discussed above in the context of various hematologic malignancies. High levels of basal NF-κB are also common in colorectal cancer (CRC) samples [117], and the clinically used chemotherapy treatments 5-fluorouracil (5-FU) and irinotecan (Camptosar, CPT-11) have been shown to activate NF-κB signaling leading to chemoresistance [118, 119]. Furthermore, increased NF-κB activity is predictive of poor response and reduced survival time in patients with CRC. These and similar studies have been used as a rationale for treating CRC with proteasome inhibitors.

Marizomib treatment blocks the activation of NF-κB by SN-38 (the active metabolite of irinotecan) in CRC cells and results in the accumulation of the phosphorylated form of IκBα (a marker of inhibited NF-κB activity). In this setting, marizomib is a 2-fold more potent inhibitor of TNFα-induced NF-κB activation than bortezomib [120]. As single agent therapies in preclinical studies, bortezomib, MG132 and marizomib have been shown to decrease proliferation and induce apoptosis in CRC cells [120–123]. While p53, p21, PUMA and Bax have all been implicated in the induction of apoptosis by proteasome inhibitors in CRC, a consensus on the mechanism behind this response is yet to be reached [118, 121, 124].

Several preclinical studies have identified targeted therapies that show synergy with bortezomib in CRC, including vorinostat (HDAC inhibitor) [125], ABT-737 (Bcl-2 inhibitor) [126] and TNF-α [127]. However, the study of bortezomib in combination with traditional cytotoxic therapies in CRC cells has been limited. Marizomib increases the apoptotic response of CRC cells to various chemotherapy combinations, including the clinically relevant drugs 5-FU, leukovorin, oxaliplatin (Eloxatin®) and SN-38 (the active metabo-lite of irinotecan) [120]. Combining marizomib with chemotherapy was also shown to increase the levels of various cell cycle regulatory proteins, including p21, p27 and p53.

This was associated with an increase in cells arrested at the G1/S cell cycle checkpoint [120]. The efficacy of marizomib as part of a combination regimen in human CRC cell lines was recapitulated in a murine subcutaneous xenograft CRC model, where reduction in tumor growth rates by combinations of 5-FU and leucovorin with or without irinotecan (CPT-11), oxaliplatin and bevacizumab (Avastin) were consistently improved by the addition of oral marizomib (Fig. (7)). Indeed, the combined treatment with 5-FU, leukovorin, irinotecan, bevacizumab and marizomib not only slowed tumor growth in mice but actually decreased tumor size over four weeks of treatment [120].

Taken together, these results clearly highlight that marizomib is able to induce apoptosis and reduce tumor burden in models of CRC, particularly when used in conjunction with traditional standard of care chemotherapeutics and biologics. Despite showing some promise in the pre-clinical setting of CRC, clinical trials using bortezomib alone [128] or in combination with irinotecan [129] or the FOLFOX-4 regimen [130] consistently failed to show any appreciable benefit of bortezomib treatment in patients with advanced colorectal disease. Given the noted differences in response of other cancer models to bortezomib and marizomib and the above highlighted efficacy of marizomib in the preclinical colon cancer setting, it is conceivable that marizomib may yield greater clinical efficacy than bortezomib in combination treatment for advanced CRC.

Marizomib in Pancreatic Carcinoma

Recent studies analyzing the molecular sequelae of marizomib treatment in models of pancreatic cancer have shown that marizomib treatment effectively induces apoptosis in pancreatic cancer cell lines [131,132]. This finding is in agreement with results for bortezomib [133]. The current standard of care for patients with advanced pancreatic cancer is the nucleoside analogue gemcitabine (Gemzar®). Marizomib has been shown to be significantly more effective at inducing apoptosis than gemcitabine both *in vitro* and in an *in vivo* xenograft model of pancreatic cancer [131]. Furthermore, addition of marizomib to either gemcitabine or a combination of gemcitabine and the anti-epithelial growth factor receptor (EGFR) monoclonal antibody cetuximab (Erbitux) significantly improved the efficacy of these regimens *in vivo*. These data suggest that marizomib may improve treatment response when combined with gemcitabine in pancreatic cancer patients.

Proteasome inhibition has been shown to sensitize many tumor types to traditional cytotoxic therapies, radiation treatment and certain targeted therapies. In pancreatic cancer models, bortezomib has been shown to increase cancer cell sensitivity to irinotecan [134], gemcitabine [135, 136] and docetaxel [137] treatment, as well as HDAC inhibitors (SAHA or trichostatin-A) or the TNF related apoptosis inducing ligand (TRAIL) [138–140] (also see "*Marizomib-induced sensitization to immunotherapy*). Analysis of the signaling pathways involved in cellular responses to proteasome inhibitor treatment has provided insight into the success of certain combination therapies. For example, HDAC inhibitors synergize with proteasome inhibitors through prevention of the formation of aggresomes (*vide supra*; Fig. (6)), an essential part of the aggresome pathway which can be utilized as an alternative to the 26S proteasome to degrade poly-ubiquitinated proteins [140]. Several other distinct anti-apoptotic responses to proteasome inhibition have been also described. In breast cancer cells, proteasome inhibitor-induced apoptosis was dependent on activation of JNK. Proteasome inhibitors also increased cellular levels of mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), a specific deactivator of pro-apoptotic JNK activity, thereby inhibiting the apoptotic response [141, 142]. In other models, including lymphoma and MM, proteasome inhibition increased the expression of the anti-apoptotic heat shock proteins Hsp27, Hsp70 and Hsp90 [68, 143–146]. Suppression of these various anti-apoptotic mechanisms by small molecule inhibitors or siRNA interference significantly increased the apoptotic response of tumor cells to proteasome inhibition [68, 141–146]. In

agreement with these findings, analysis of the molecular pathways activated in response to marizomib treatment of pancreatic carcinoma cells showed that marizomib treatment activates EGFR, extracellular signal-regulated kinase (ERK), Akt and JNK with varying kinetics [131]. Targeted inhibition of these pathways *in vitro* and *in vivo* enhanced the apoptotic response of pancreatic cancer cells to marizomib treatment.

Bortezomib as a single agent [147] or in combination with other chemotherapies [130, 148–150] has been tested in Phase 1 trials to determine the maximum tolerated dose (MTD) in patients with solid tumors. These trials formed the basis of a Phase 2 trial in which bortezomib was used for the treatment of advanced pancreatic cancer both alone and in combination with gemcitabine [151]. Despite the promising results from the various Phase 1 trials, the authors concluded that there is no significant benefit when bortezomib was combined with gemcitabine for the treatment of pancreatic cancer [151]. Due to the fundamental differences in the mechanisms between the various proteasome inhibitors, it is difficult to predict the clinical utility of second generation proteasome inhibitors currently in development. However, encouraging responses in the preclinical models of pancreatic cancer, such as increased tumoricidal response to marizomib compared to bortezomib [131], warrant further evaluation of these potent inhibitors of the proteasome in the treatment of patients with refractory solid organ malignancies.

Effects of Marizomib on Angiogenesis and Autophagy in Pancreatic Carcinoma Cells—Proteasome inhibitors have heterogeneous effects on apoptosis in human pancreatic cancer cells [133]. For example, some lines (L3.6pl, BxPC-3, and CFPac-1) are highly sensitive to low nanomolar concentrations of marizomib or bortezomib, whereas others (MiaPaCa-2, AsPC-1 and HS766T) are highly resistant [133]. Furthermore, xenografts derived from cell lines that are sensitive to proteasome inhibitor-induced apoptosis also display increased apoptosis in response to proteasome inhibitors *in vivo*, whereas xenografts derived from proteasome inhibitor-resistant lines do not [137]. Resistance to proteasome inhibitors appears to correlate with resistance to gemcitabine and other DNA damaging agents and with epithelial-to-mesenchymal transition (EMT) [152]. Based on a number of different comparisons of the effects of marizomib and bortezomib in human pancreatic cancer cell lines, it was concluded that they exhibit very similar effects at concentrations that produce equivalent effects on 20S proteasome CT-L inhibition. Specifically, it was not possible to overcome resistance to bortezomib-mediated apoptosis using either marizomib alone or in combination with low concentrations of bortezomib (R. Andtbacka, unpublished observations).

However, *in vivo* studies in orthotopic xenografts demonstrated that proteasome inhibitors also have broad, potent anti-angiogenic effects that contribute to tumor growth inhibition, even in tumors that are completely resistant to direct drug-induced apoptosis [133, 137]. These anti-angiogenic effects have also been observed in other preclinical solid tumor models [153, 154] and produce dose-dependent down-regulation of VEGF expression and tumor microvessel densities [133, 137, 153, 154] and central necrosis [133]. These observations are counterintuitive given what is known about the molecular mechanisms that control VEGF expression, which primary involve hypoxia-inducible factor-1 (and -2) alpha (HIF-1α), a transcription factor that is dynamically regulated by proteasome-mediated degradation [155]. Under conditions of normoxia (in the presence of oxygen), HIF-1α is hydroxylated by a prolyl hydroxylase, promoting recognition by a complex that includes the von Hippal-Lindau (VHL) ubiquitin ligase, resulting in polyubiquitylation and HIF-1α degradation. Because of this, one might expect proteasome inhibitors to promote HIF-1α accumulation and downstream VEGF production. However, proteasome inhibitors appear to uncouple HIF-1α from VEGF transcription *via* mechanisms that are still poorly understood

[128, 156, 157]. Thus, proteasome inhibitor-mediated accumulation of HIF-1α is not associated with increased VEGF transcription.

A second explanation for the VEGF blockade induced by marizomib and bortezomib has been identified. Both drugs actually caused HIF-1α downregulation in some cancer cell lines within 4 h of drug exposure. These effects were linked to phosphorylation of the translation initiation factor eIF2α and downregulation of HIF-1α translation. Thus, cells that displayed proteasome inhibitor-induced eIF2α phosphorylation also displayed HIF-1α downregulation, whereas other cells (including knock-in mouse embryonic fibroblasts (MEFs) expressing a phosphorylation-deficient form of eIF2α) were deficient in both responses, and proteasome inhibitors directly downregulated HIF-1α translation [69]. These results are consistent with other emerging evidence indicating that HIF-1α is also subject to tight translational control through the PI3kinase-Akt-mammalian target of ra-pamycin (mTOR) pathway [158].

From a translational perspective, the anti-angiogenic effects of proteasome inhibitors may be less important in pancreatic cancer than they are in other solid tumors. Pancreatic cancer is characterized by a dense desmoplastic response that may impair angiogenesis and overall tumor blood flow [159], and more specific anti-angiogenic agents like bevacizumab and sunitinib (Sutent, previously known as SU11248) that have produced excellent clinical activity in other models have failed to provide any benefit in pancreatic cancer patients [160]. However, there may be a major role for proteasome inhibitors in clear cell renal cell cancer (RCC), a disease that is characterized by VHL mutations, constitutive HIF-1α and VEGF expression, and high level VEGF production [158, 161]. Although direct VEGF-VEGFR inhibitors and mTOR inhibitors have excellent clinical activity in RCC patients, a significant subset displays *de novo* resistance and in others therapeutic resistance emerges over time. A Phase 2 trial has been designed to evaluating the effects of combining bortezomib with bevacizumab in patients with RCC to test this possibility (G. Falchook, Principal Investigator). There is clearly an opportunity to better understand the molecular mechanisms that govern sensitivity or resistance to proteasome inhibitor-mediated angiogenesis inhibition and then to use this information to identify the subsets of patients with RCC and other tumors that are highly dependent on VEGF-mediated angiogenesis.

Recent work has also shown that marizomib- and bortezomib-induced phosphorylation of eIF2α promotes autophagy [162], an evolutionarily conserved “recycling” system that directs protein aggregates, other macromolecules, and organelles to lysosomes for degradation and energy liberation [163, 164]. Although some studies suggested that autophagy might act as an alternative programmed cell death pathway in tumors possessing defective apoptotic control mechanisms, more recently, greater consensus has emerged implicating autophagy in tumor cell survival under conditions of low glucose and oxygen [163, 164]. Thus, the anti-angiogenic effects of proteasome inhibitors may be undermined by proteasome inhibitor-induced autophagy. Consistent with this idea, knockdown of critical autophagy pathway genes (Atg5, Atg7) or exposure to chemical inhibitors of autophagy (chloroquine, 3-methyladenine) enhanced marizomib- and bortezomib-induced tumor cell death [162]. These data support the further evaluation of proteasome and autophagy inhibitor combinations in preclinical models to determine their effects on tumor cell death, angiogenesis, and systemic toxicity.

Marizomib in Glioma

Although bortezomib has excellent anti-tumor activity in preclinical *in vitro* and *in vivo* models, it has shown minimal clinical efficacy against solid cancers as a single agent or in combination with standard of care treatment protocols [114, 115]; therefore, marizomib has opened new possibilities for the treatment of these challenging malignancies. Glioblastoma

multiforme (GBM), the most common astrocytoma and one of the most aggressive solid tumors in humans, has a median survival of about 12 months, after debulking surgery and radiotherapy. The only drug shown to be effective in combination with radiotherapy is temozolomide (Temodal®) [165], therefore, there is a clear need for novel treatment options and drug combinations for this devastating disease. Bortezomib has shown promising results *in vitro* against glioma cell lines, as a single agent or in combination with chemotheraputic drugs [166–168] however, these results did not translate into an anti-tumor effect in preclinical models of glioma [169]. A recent Phase 1 trial in glioma combining bortezomib with temozolomide and radiation did not show any additional benefit for patients treated with bortezomib [116].

The ability of marizomib to target all three activities of the 26S proteasome [35, 49] makes it a more effective inducer of cancer cell death than bortezomib [57]. Marizomib has already shown to be effective in preclinical models of solid tumors (*vide supra*) [131, 149], thus making it a good candidate for the treatment of glioma. Besides the potent cytotoxic effects of this drug, potentiated by the prolonged inhibition of all three proteolytic activities of the proteasome, one of the attractive features of marizomib is its low *in vivo* toxicity, including low toxicity to the brain [52]. Similar to bortezomib [170], a recent study on the PD of marizomib in a preclinical MM.1S murine model demonstrated that marizomib does not cross the blood brain barrier at the doses and schedule tested [52]. However, it is believed that the presence of a malignant glioma in the brain can disrupt the blood-brain barrier, thus allowing cytotoxic drugs that cannot cross a healthy blood-brain barrier, to reach the tumor [171]. Unfortunately, to date, the number of studies that have analyzed the effect of marizomib on preclinical models of glioma are limited. A recent study by Vlashi *et al.* analyzed the efficacy of marizomib as a single agent or in combination with temozolomide and radiation in a murine glioma xenograft model [167]. *In vitro*, marizomib showed a dose-dependent toxicity in five different glioma cell lines. Interestingly, its combination with temozolomide resulted in radiosensitization of only the cell lines with a mutated p53 status. It has been demonstrated that proteasome inhibitors sensitize cancer cells to radiation [172]; therefore, given that radiation remains one of the main treatment modalities in glioma, combining a proteasome inhibitor with radiation could result in the improvement of current glioma therapy. In the study by Vlashi *et al.* the effect of marizomib as a single agent on glioma xenografts *in vivo* was only modest in controlling tumor growth, and it failed to consistently radiosensitize glioma cells *in vitro* and did not synergize with radiation *in vivo* [167]. Overall, these results may indicate that while gliomas in general may be sensitive to marizomib-induced cell death, its combination with radiation may have only little effect in a subgroup of gliomas. Moreover, a recent study showed that glioma stem cells are resistant to proteasome inhibitors [173], further suggesting that proteasome inhibitors have only limited use in the radiotherapy of gliomas. However, the extent of sensitization of cells by proteasome inhibitors (possibly in combination) from cytotoxic agents may depend on the sequence of application [174] and the microenvironment [175]. Given the limited number of preclinical studies performed on this particularly challenging malignancy, it is too early to rule out the possibility that the right drug administration schedule, dose and optimal drug combinations could lead to potential improvement in glioma therapy.

MARIZOMIB SENSITIZES RESISTANT TUMOR CELLS TO APOPTOSIS BY CHEMO- AND IMMUNO-THERAPEUTIC DRUGS

Current treatments for the majority of cancers consist of chemotherapy, radiation, hormonal therapy and immunotherapy and combinations thereof. While most cancer patients initially respond to chemotherapeutic drug treatment, resulting in the inhibition of tumor cell proliferation and survival as well as induction of apoptosis, a subset of patients does not respond initially to such treatment modalities and/or develops cross-resistance following

treatment with cytotoxic agents. Several underlying molecular mechanisms have been proposed for the acquisition of tumor cell resistance that help tumor cells evade drug-induced apoptosis. For example, various anti-apoptotic mechanisms result from the hyperactivation of cell survival pathways (such as the MAPK, NF-κB and phosphatidylinositol 3-kinase (PI3K)/Akt pathways) that regulate the transcription and expression of gene products involved in the apoptotic process [176]. However, inhibition of such survival/anti-apoptotic pathways may prevent or reverse tumor cell resistance.

The activation of NF-κB is proposed to be a major pathway for the development of drug resistance. Proteasome inhibitors, which inhibit NF-κB activity (*vide supra*), may also inhibit the expression and activity of several anti-apoptotic gene products that are under the regulation of NF-κB; thus, marizomib may also sensitize resistant tumor cells to apoptosis. This hypothesis was tested using both carcinoma and lymphoma cell lines for the ability of marizomib to sensitize the resistant tumor cells to immunotherapy (TRAIL as a momodel) and chemotherapy (cisplatin (cis-diamminedichloroplatinum(II) (CDDP)) as a model).

Marizomib-Induced Sensitization to Immunotherapy

Cytotoxic cells (i.e., cytotoxic T-cells (CTL) and natural killer (NK) cells) mediate their cytotoxic activities on tumor cells by several mechanisms, including perforin/granzyme and death receptor signaling. Death receptors become activated after ligation with the corresponding ligands, which constitute TNF family members (TNF-α, FasL, TRAIL) [177]. TRAIL is a type-2 transmembrane protein and induces cell death by apoptosis against a variety of sensitive tumor cell lines after binding to functional death receptors 4 and 5 (DR4, DR5) [178]. The role of marizomib in the response to TRAIL in TRAIL-resistant tumor cell lines was examined. TRAIL was selected based on its poor toxicity to normal tissues and its current evaluation against different tumors in clinical trials [179]. However, most tumors *in vivo* are resistant to TRAIL-induced apoptosis, and resistance can be reverted by the use of sensitizing agents to modify the anti-apoptotic pathways.

TRAIL-resistant carcinoma and lymphoma tumor cell lines may be sensitized to TRAIL apoptosis *via* marizomib-induced inhibition of the constitutively activated NF-κB pathway. This hypothesis was tested in prostate (PC-3) and non-Hodgkin's B-cell lymphoma (B-NHL) tumor cell lines, where marizomib sensitized both cell lines to TRAIL-induced apoptosis in a concentration-dependent manner *via* direct inhibition of NF-κB and its downstream targets, and behaved similarly to treatment with the specific NF-κB inhibitor, dehydroxymethylepoxyquinomycin (DHMEQ). Marizomib-mediated inhibition of NF-κB activation resulted in inhibition of the NF-κB transcriptional target, Yin Yang 1 (YY1) [180]. YY1 acts as a transcriptional repressor of the TRAIL receptor, DR5, and, thus, the marizomib-mediated inhibition of YY1 resulted in upregulation of DR5 expression [181, 182]. Furthermore, the direct inhibition of YY1 by siRNA mimicked marizomib in its ability to sensitize tumor cells to TRAIL apoptosis through DR5 upregulation. In addition to marizomib-mediated activation of the extrinsic apoptotic pathway, tumor sensitization to TRAIL apoptosis by marizomib also involved activation of the intrinsic apoptotic pathway *via* increase of the mitochondrial membrane depolarization, inhibition of several anti-apoptotic gene products (such as survivin, Bcl-2, Bcl-xL, XIAP) and induction of pro-apoptotic proteins, such as Bax and Bid. Although the above gene modifications induced after single agent treatment with marizomib were not sufficient for the induction of apoptosis, the combination treatment with TRAIL resulted in a significant synergistic effect for induction of apoptosis through cooperation of both extrinsic and intrinsic apoptotic cascades [182].

An additional mechanism by which marizomib sensitizes tumor cells to TRAIL was examined. Yeung *et al.* [183, 184] reported that a novel gene product, Raf-1 Kinase Inhibitor

Protein (RKIP), inhibits the activation state of both the MAPK and NF-κB pathways. It was recently reported that RKIP transcription is under the negative regulation of Snail, a transcription factor that is positively regulated by NF-κB [185, 186]. Since RKIP induction reverses tumor cell resistance to TRAIL-mediated apoptosis [187], it was hypothesized that marizomib-mediated inhibition of NF-κB may result in the inhibition of Snail and derepression of RKIP. Therefore, RKIP induction by marizomib might play a pivotal role in tumor cell sensitization to TRAIL. Indeed, treatment of tumor cells with marizomib resulted in significant induction of RKIP mRNA and protein expression concomitant with the inhibition of both NF-κB and Snail. The direct role of RKIP induction by marizomib in TRAIL sensitization was corroborated in tumor cells overexpressing RKIP. Such cells were rendered sensitive to TRAIL apoptosis and mimicked marizomib-induced sensitization. In addition, treatment of tumor cells with Snail siRNA resulted in the upregulation of RKIP and sensitization to TRAIL [188]. These studies demonstrate that marizomib sensitizes TRAIL-resistant carcinoma and lymphoma tumor cells to TRAIL-induced apoptosis. The results also demonstrate that marizomib dysregulates the NF-κB/Snail/YY1/DR5/RKIP loop (Fig. (8)). The findings suggest that marizomib may also sensitize resistant tumor cells to ligands other than TRAIL, such as TNF-α and FasL, as well as sensitize the tumor cells to cytotoxic effector cells expressing such ligands.

Marizomib-Induced Sensitization of Tumor Cells to Chemotherapy

As discussed above for the response to immunotherapy, tumor cells develop cross-resistance to multiple apoptotic stimuli, including chemotherapy. Marizomib-mediated inhibition of major constitutively activated survival pathways, such as the NF-κB pathway, may sensitize resistant tumor cells to chemotherapy. This hypothesis was tested using *in vitro* CDDP-resistant tumor cell lines and the chemotherapeutic drug CDDP as models. Treatment with marizomib followed by treatment with CDDP resulted in sensitization of CDDP-resistant DU-145 and LNCaP prostate carcinoma and M202 melanoma cell lines to apoptosis. The sensitization and the extent of apoptosis were a function of both the concentrations used by each agent. The combination treatment resulted in synergistic cytotoxicity [188]. The molecular mechanism underlying marizomib-mediated sensitization to CDDP was examined. The direct role of marizomib-induced inhibition of NF-κB in the sensitization to CDDP was corroborated by the use of the NF-κB inhibitor DHMEQ. Cell treatment with DHMEQ sensitized the tumor cells to CDDP-apoptosis, similarly to marizomib-induced sensitization. Marizomib-induced inhibition of NF-κB and the downstream inhibition of the RKIP repressor Snail resulted in upregulation of RKIP expression. Although cell treatment with marizomib or CDDP as single agents resulted in modest activation of the type II apoptotic pathway *via* measurable inhibition of several anti-apoptotic gene products, the above apoptotic stimuli, separately, were not sufficient to induce a significant potentiation of apoptosis. In contrast, the combination treatment significantly enhanced in the activation of the type II apoptotic pathway and synergistic activity in apoptosis [188]. These and supporting studies demonstrate that marizomib, as shown above for TRAIL, dysregulates the NF-κB/Snail/RKIP circuit which plays a critical role in the regulation of apoptosis by chemotherapeutic drugs.

Mechanism for Reversal of Tumor Cell Resistance to Chemo- and Immunotherapy

The above findings clearly demonstrate that treatment of both solid and hematologic malignances with marizomib results in the reversal of tumor cell resistance to both chemo- and immunotherapy. The reversal of resistance and sensitization by marizomib result in dysregulation of the NF-κB/YY1/Snail/RKIP circuitry, as schematically diagrammed in Fig. (8). Briefly, marizomib treatment results in the inhibition of NF-κB activity through the inhibition of the proteasome and concommitant inhibition of IκBα degradation. The inhibition of NF-κB results downstream in the inhibition of both YY1 and Snail

transcription and expression, leading to RKIP induction. Since YY1 is a transcriptional repressor of the TRAIL receptor DR5, its inhibition by marizomib results in the upregulation of DR5 and sensitization to TRAIL apoptosis. In addition, the inhibition of Snail and the induction of RKIP by marizomib further result in tumor sensitization to TRAIL- and CDDP-apoptosis. Furthermore, the marizomib-mediated tumor cell sensitization to CDDP- or TRAIL-apoptosis may also result from the partial activation of the mitochondrial type II apoptotic pathway and inhibition of several anti-apoptotic gene products that play a direct role in the regulation of tumor cell sensitivity to chemo- and immunotherapy. Likewise, the inhibition of Snail and induction of RKIP sensitizes cells to CDDP. Overall, the above findings suggest the clinical application of subtoxic doses of marizomib in combination with conventional and/or new therapeutic drugs in the reversal of both chemo- and immuno-resistance of solid and hematologic tumors.

PHARMACOKINETICS AND ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME) PROPERTIES OF MARIZOMIB

During the course of the preclinical and clinical studies, the pharmacokinetics of marizomib have been described in the cynomolgus monkey and in humans. The ADME characteristics have been described in the rat and mouse, using [^{3}H]-marizomib. Marizomib is well behaved from a pharmacokinetic perspective, and due to the short half-life must be measured in whole blood rather than plasma. The following is an overview of the pharmacokinetic and ADME characteristics of marizomib following IV administration.

Pharmacokinetics

The pharmacokinetic profile of IV-administered marizomib is similar in cynomolgus monkeys and in man, and is characterized by dose-dependent increases in C_{max} and area under the total response curve (AUC_{total}), with a short half-life, rapid clearance and large volume of distribution. C_{max} and AUC_{total} have a highly significant linear correlation with dose, with similar values for both monkeys and humans within the same dose range (e.g., Fig. (9) for dosing in the cynomolgus monkey). Clinical studies indicate that exposure (maximum and total) to marizomib does not change upon repeated dosing.

The whole blood concentration-time curves for marizomib from both preclinical and clinical studies clearly distinguish distribution and elimination phases at doses ≥ 0.3 mg/m^2, with a terminal half-life in the range of 10–15 minutes. At low doses, the duration of measurable compound in the blood is too short to distinguish these phases. In parallel with the short half-life, the clearance of marizomib is very rapid (e.g. 10–20 L/min in man) and does not show any dose-related trend. The volume of distribution in both monkeys and humans is similar to, or exceeds, total body mass.

ADME

As marizomib is administered by the IV route in clinical studies, the *absorption* is considered to be 100% with T_{max} occurring at the end of the infusion (1–10 minutes, depending on dose). Preclinical studies have demonstrated the potential for an oral route of administration, with a bioavailability of approximately 30–40% in cynomolgus monkeys (when the IV formulation was administered orally).

The *distribution* of [^{3}H]-marizomib has been determined in normal male Sprague-Dawley rats (quantitative whole body autoradiography (QWBA) study) and in MM.1S tumor-bearing male CB-17 SCID-mice (also see *Pharmacodynamics and efficacy of marizomib in a human MM.1S plasmacytoma xenograft murine model*). The QWBA study demonstrated that radioactivity is rapidly distributed from the central compartment, with highest

concentrations occurring mainly in organs of excretion and metabolism. Interestingly, at the dose studied (0.6 mg/m^2), there is good penetration into bone marrow (the site of MM tumor cells) and very little penetration (well below blood levels) into brain regions, spinal cord and peripheral nerves. This latter finding is consistent with the lack of proteasome inhibition following marizomib administration to mice at doses which exhibit a clear anti-tumor activity [52] (Fig. (5)).

The human MM.1S plasmacytoma xenograft murine model study with [^{3}H]-marizomib demonstrated that the parent compound distributes rapidly from the central compartment and enters tumors within minutes of IV administration. This is followed by inhibition of proteasome activity in the tumor within an hour (Fig. (5)). In addition to the extravascular distribution, [^{3}H]-marizomib also binds to cellular blood elements, with maximum binding occurring within one minute [52]. This is consistent with the rapid inhibition of proteasome functions in RBCs and other blood elements.

The *metabolism* of marizomib has not been defined. The *excretion* of marizomib occurs *via* both urinary and fecal routes following IV administration (QWBA study in the rat), without evidence of excretion *via* expired air. The levels of urinary and fecal excretion are similar in the rat, whereas this has not been assessed in clinical studies.

MONITORING PROTEASOME ACTIVITY AS A POTENTIAL BIOMARKER

Proteasome inhibition profiles have been monitored in multiple clinical trials with marizomib, bortezomib and carfilzomib, and have demonstrated a rapid inhibition of CT-L activities in PWB using fluorogenic assays [189]. However, due to the different properties of these inhibitors and due to the long half-life and inability of RBCs to regenerate proteasomes, it has become clear that PWB while useful, may not be the only cell type of interest to assess clinical activity or response to these types of agents (also see *Marizomib Regulates Proteasome CT-L, C-L and T-L Activities and Exhibits a Sustained Inhibition and Pharmacodynamic Profile*).

In addition to the importance of the cell type, the assays utilized to determine inhibition profiles can also influence monitoring of proteasome activities. Three different assay methodologies, each with distinct advantages, have been developed to monitor proteasome activities: 1) fluorogenic assays [189]; 2) small molecule based activity assays [190, 191]; and 3) recombinant reporter based assays [192–194]. Although useful in various tissue and cell types, some small molecule and fluorogenic based assays cannot be used to probe living cells, while reporter-based assays are confined to genetically altered cells.

A multi-pronged approach was adopted to evaluate whether monitoring changes in proteasome activity has a potential use as a biomarker. The first approach utilized a fluorogenic-based assay to monitor proteasome activities in plasma and serum. The second approach utilized a fluorescent vinylsulfone based proteasome binding assay, which allows for the detection of labeled proteasome subunits in both live cells and cell extracts by SDS-PAGE, followed by scanning the gel for fluorescent emission [191]. The following describes these different methodologies and their potential usefulness to monitor proteasome activity changes as a potential biomarker.

Profiling Free Circulating Proteasome Activities in Plasma and Serum

It is frequently difficult to access patient cancer cells for analysis, especially when a pure population is desired with minimal or no dilution by normal cells. Increasing evidence suggests that in a patient with a tumor, plasma is enriched by tumor-specific DNA, RNA and protein [195–197]. This enrichment in plasma is believed to be due to the high turnover of

tumor cells [197], and may include apoptotic bodies, microvessels, protein complexes or simple debris. In contrast, the products of normal cells are systematically cleared by the reticuloendothelial system and less likely to circulate [195–199]. Despite their size and complex structure, proteasomes circulate in plasma/serum in functional forms; importantly, they can be quantified and their activities easily measured [62, 63, 200]. Furthermore, the circulating proteasomes respond to inhibitors in a fashion similar to that of cell proteasomes [200], which may have some implication for use of proteasome inhibitors in patients with high levels of circulating proteasomes. For example, it is possible that the circulating proteasomes may sequester a portion of the drug, such that higher doses might be needed for patients with high levels of circulating proteasomes.

One of the most important aspects of measuring proteasome protein levels and enzymatic activities in plasma or serum as compared to cells is the ability to express these levels in a specific quantity of plasma or serum, which helps in standardization. By measuring both proteasome protein levels and their enzymatic activities in a specific quantity of plasma or serum, the specific enzymatic activities of the individual proteasome can be determined. This can be calculated by simply dividing the level of an enzymatic activity by the level of proteasome protein in the same quantity of plasma or serum to derive three new measurements: CT-L activity/proteasome protein, C-L activity/proteasome protein, and T-L activity/proteasome protein. These values are important because absolute plasma values of enzymatic activities and proteasomes reflect tumor load, while normalizing activities to quantity of proteasome reflects the specific activity of the proteasome in the cell of origin.

Recent data have shown that circulating proteasome protein levels and their enzymatic activities can be used as biomarkers reflecting the biology of the underlying disease [62]. Therefore, studies were initiated to monitor plasma proteasome activities after treatment with marizomib. When proteasome levels and enzymatic activities were compared in plasma of patients with various cancers treated with low (0.025 or 0.05 mg/m^2) and higher doses (0.075 or 0.112 mg/m^2) of marizomib, there were significant changes in median proteasome levels at 1h and 4h. Therefore, the plasma proteasome activity was normalized to proteasome levels. Significant changes were observed in the median normalized levels of CT-L, C-L and T-L activity at 1h in low-dose and higher-dose groups that remained detectable at 4h in the higher-dose for CT-L and C-L, but not the low-dose groups. Normalized T-L activity returned to pre-therapy levels at 4h in both groups. These preliminary results support that with increasing doses of marizomib, proteasome activities for CT-L, C-L and T-L were reduced compared to pretreatment levels and that further investigation is warranted to determine the utility of these types of measurements [61].

The fact that each tissue or cell type has a specific number of proteasomes and specific enzymatic activities suggest that profiling the enzymatic activities (CT-L, T-L, and C-L), along with determining the proteasome protein levels in plasma, may be useful for the diagnosis and prediction of the clinical response of a specific cancer. Studies are needed to further explore these possibilities.

Profiling Proteasome Activity in Tissues with Fluorescent Probes

As the second approach, a fluorescent activity-based proteasome probe [191] was developed to profile proteasome activities in PWB and PBMC preparations after treatment with marizomib or bortezomib. Using this probe, we demonstrated that marizomib targeted the β1, β2 and β5 proteasome subunits in rat PBMC lysates, while bortezomib targeted only the β1 and β5 subunits. As a result, a single dose of marizomib (0.05 or 0.10 mg/kg) reduced total proteasome activity by 70–80% while a single dose of bortezomib (0.15 mg/kg) reduced activity by 40%.

The fluorescent proteasome probe was also utilized to profile PWB- and PBMC-derived preparations from cancer patients treated with marizomib. The results indicated that PWB, which contains >98% RBCs, has a different active proteasome subunit composition and recovers proteasome activity markedly slower than PBMCs, consistent with independent results of PD studies (see *Marizomib Regulates Proteasome CT-L, C-L and T-L Activities and Exhibits a Sustained Inhibition and Pharmacodynamic Profile*). Due to the high proteasome concentration in blood cells, intravenous administration of proteasome inhibitors may rapidly saturate proteasomes in the RBCs before peripheral sites are reached. In addition, studies using the fluorescent activity-based probe demonstrated that proteasome activity levels prior to treatment differed between patients, and that the initial proteasome activity level may be an important determinant of the sensitivity to proteasome inhibitors. Furthermore, it was demonstrated that PBMCs are able to rapidly change their proteasomal subunit composition in response to marizomib treatment, and that this ability may be another factor mediating sensitivity to proteasome inhibition.

In summary, the results suggest that PWB preparations probably do not fully represent the proteasome inhibition profiles in other tissues. Together, the data indicate that fluorescent proteasome probes can be used to profile patient blood samples and may enable prediction of patient responses to proteasome inhibitors, which can contribute to a more personalized proteasome inhibition strategy to improve the therapeutic potential of this class of drugs.

CLINICAL TRIALS WITH MARIZOMIB

The extensive body of preclinical data presented above suggest that marizomib, with its novel structure, produces unique signal transduction, safety and efficacy profiles compared to other proteasome inhibitors, and led to the initiation of clinical trials. The ability of marizomib to synergize with bortezomib and other chemotherapeutics (*vide supra*) and overcome bortezomib resistance, together with marizomib's greater therapeutic index and different toxicology profile (lacking neutropenia, thrombocytopenia and neuropathy) suggested that marizomib could be developed and provide unique benefits to patients, particularly those that had failed or were not candidates for treatment with bortezomib. Pre-clinical data showing efficacy in cancers such as CLL and solid tumour malignancies, where bortezomib has not shown efficacy in clinical trials, suggested additional potential.

The clinical evaluation of marizomib has consisted of 4 clinical trials, including 3 single agent Phase 1 studies in patients with solid tumors, lymphomas, leukemias and MM, and one study in combination with the HDAC inhibitor vorinostat in patients with selected advanced malignancies. Each study consists of a dose escalation to a recommended Phase 2 dose (RP2D), followed by a RP2D cohort or Phase 2 portion to gain additional data in specified indications. At the time of writing, over 150 patients have been treated with marizomib at doses ranging from 0.0125 to 0.9 mg/m^2, administered on a Days 1, 8, and 15 schedule in 28-day cycles (weekly) or Days 1, 4, 8 and 11 in 21-day cycles (twice weekly).

Clinical development of marizomib began with a Phase 1 dose escalation first-in-human study in patients with solid tumors or lymphomas [201]. As the duration of proteasome inhibition induced by marizomib in PWB is markedly longer than that of bortezomib [49], marizomib was administered once weekly instead of twice weekly. Clinical trials in patients with other diagnoses such as MM and leukemia were subsequently initiated based on preclinical and clinical data. Dose escalation was carried out through a dose of 0.9 mg/m^2, with 0.6–0.7 mg/m^2 being selected as the RP2D variously in these studies. The most common adverse events reported in marizomib studies included fatigue, nausea, headache, diarrhea, vomiting, constipation, dizziness, infusion site pain, back pain, anorexia, anemia and dyspnea [59]. Proof-of-mechanism, with proteasome inhibition levels (inhibition of CT-

L activity in PWB increasing with time and dose, up to 100%; Fig. (3)) reaching and exceeding those reported with therapeutic doses of bortezomib, was attained at lower doses than for bortezomib, supporting the potential for a significantly improved therapeutic ratio. Cumulative or new toxicities did not appear to be elicited with prolonged treatment, with most events occurring in early cycles of therapy. Importantly, marizomib did not appear to induce the limiting toxicities associated with bortezomib, such as peripheral neuropathy, neutropenia and thrombocytopenia, in spite of eliciting levels of proteasome inhibition that equal or exceed those produced by bortezomib. Improvements in tumor assessments have indicated the anti-tumor activity of marizomib in patients, including many with MM previously treated with bortezomib. Together with the preclinical data with other standard of care oncology agents, such as cytotoxic agents, immunomodulatory drugs and HDAC inhibitors, combination clinical trials have been initiated with marizomib. The combination of marizomib with vorinostat has been assessed in a clinical trial, which to date has not revealed adverse drug-drug interactions, or other significantly different safety findings [202].

Further analysis of the PD data demonstrated inhibition of all three proteasome activities in a dose, cycle and schedule dependent manner. Treatment with marizomib resulted in a dose related inhibition of proteasome CT-L activity in PWB as determined 1–4 hours after treatment on days 1 and 15 (Fig. (3)). Maximum inhibition of CT-L activity in PWB reached ~88% at Day 1 and 100% at Day 15 at the highest dose assessed; in contrast, bortezomib given at 1.3 mg/m^2 is reported to inhibit 65% of the CT-L activity. At high doses, or after multiple low doses, T-L and C-L activities are also inhibited. Interestingly, as initially reported with marizomib in preclinical models [49], minimal recovery of proteasome inhibition between doses in PWB (principally non-nucleated RBC without the ability to synthesize new proteasomes) was seen, but recovery was rapid in PBMC (nucleated cells that can regenerate proteasomes) (Fig. (4)). Thus, marizomib administered on the same schedule as bortezomib (Day 1, 4, 8 and 11) is now being assessed as well. When administered on this twice weekly schedule, low doses of marizomib administered have been seen to result in increasing inhibition of all three 20S proteasome proteolytic activities that is both dose- and cycle-dependent. No unexpected toxicities have yet been revealed with this alternate schedule, and anti-tumor activity has been seen in patients with MM. Clinical evaluation of marizomib alone and in combination with standard of care cancer therapies continues towards determining the characteristics and optimal uses of this novel agent.

CONCLUSIONS

As the demands for new targeted anti-cancer therapies have increased, it has become important to maximize drug related research and development efforts. The approach to developing marizomib involved an industrial principal, Nereus Pharmaceuticals, collaborating with university laboratories with expertise in chemistry, proteasome biology, hematologic and solid tumor malignancies, and ultimately conducting clinical trials. Marizomib, a β-lactone-γ-lactam that is structurally distinct from bortezomib and carfilzomib, answered the need for chemically and biologically distinct proteasome inhibitors. Marizomib is unique due to its broader, faster acting and durable inhibition of all three 20S proteasome proteolytic activities in various *in vitro* models, and is efficacious *in vivo* against hematologic malignancies, such as MM and WM, and solid tumor models, including colon and pancreatic carcinomas.

Marizomib and bortezomib can exhibit synergistic anti-tumor activity in models of MM and WM, which may be attributed, in part, to complementary proteasome inhibition profiles and activation of downstream signally pathways, such as caspase-8 and caspase-9. In contrast,

while these agents can synergize in these models, marizomib can overcome resistance to: 1) bortezomib in MM cells that can involve over-expression of Hsp27 and Hsp70; and 2) TRAIL and CDDP in lymphoma and prostate cancer cells, in part by regulating the NF-κB, RKIP, Snail and YY1 circuitry pathway. As in the case of bortezomib, marizomib can regulate multiple NF-κB controlled genes that mediate tumor progression, angiogenesis, metastasis, and apoptosis and that contribute to drug resistance in many tumor types. In addition to inhibiting NF-κB regulated anti-apoptotic pathways, marizomib overcomes survival and drug-resistance conferred by Bcl-2 in MM cells. Marizomib also synergizes with immunomodulatory agents such as Revlimid® in MM and with various HDAC inhibitors such as vorinostat and entinostat in MM, NSCLC and ALL.

The collective preclinical and translational biology studies from these laboratories provided the framework for ongoing clinical trials in patients with MM, lymphoma, leukemia and solid tumors. The findings have been published and/or presented in over 120 different venues and capture the remarkable translational studies that have advanced marizomib from seabed to bench to bedside.

Acknowledgments

We gratefully acknowledge the contributions of the many individuals whose research findings are captured in this review and whose efforts have contributed to the extensive body of knowledge on marizomib. We thank SR Potts for contributing Fig. (1). This work was supported by NIH grants SPORE-P50100707, PO1-CA078378, and NIH RO1CA050947 to DC and KCA; NIH R01CA98871 to JCC; F31 CA123645 to CPM; NIH R01CA115811 to JC; and NCI R37CA044848 to WF.

ABBREVIATIONS

ADME	absorption, distribution, metabolism and excretion
ALL	acute lymphocytic leukemia
API	active pharmaceutical ingredient
AUC_{total}	area under the total response curve
Bax	Bcl-2-associated X protein
Bak	Bcl-2 antagonist/killer
Bcl-2	B-cell lymphoma 2 gene product
Bcl-xl	B-cell lymphoma x gene product (long form)
BH3	Bcl-2 homology domain 3
Bid	BH3-interacting domain death agonist
BMSCs	bone marrow stromal cells
B-NHL	B-cell non-Hodgkin’s lymphoma
CD	cluster of differentiation (or cluster of designation)
CDDP	cis-diamminedichloroplatinum(II)
c-FLIP	cellular FLICE-like inhibitory protein
cGMP	current Good Manufacturing Practice
C-L	caspase-like
CLL	chronic lymphocytic leukemia

C_{max}	maximum concentration of drug observed in blood after its administration
COX-2	cyclooxygenase-2
CP	core particle
CRC	colorectal cancer
CT-L	chymotrypsin-like
CTL	cytotoxic T-cells
Diablo	direct IAP binding protein with low pI
DHMEQ	dehydroxymethylepoxyquinomycin
DR	death receptor
EGFR	epithelial growth factor receptor
EMT	epithelial-to-mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	FAS-associated *via* death domain
FDA	Food and Drug Administration
FOLFOX	folinic acid, fluorouracil and oxaliplatin chemotherapy regimen
5-FU	5-fluorouracil
GBM	glioblastoma multiforme
GI_{50}	concentration of drug required for 50% growth inhibition
HIF	hypoxia-inducible factor
Hsp	heat shock protein
IAP	inhibitor of apoptosis
IC_{50}	concentration of drug required for 50% inhibition
ICAM	inter-cellular adhesion molecule
IETD-fmk	benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone
IgM	immunoglobulin M
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL-6	interleukin-6
IMiD	immunomodulatory drug
IV	intravenous
JNK	c-Jun *N*-terminal kinase
MAPK	mitogen-activated protein kinase
MCL	mantle cell lymphoma
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex

MKP	MAPK phosphatase
MM	multiple myeloma
MMP	matrix metalloproteinase
MTD	maximum tolerated dose
mTOR	mammalian target of rapamycin
NCI	National Cancer Institute
NF-κB	nuclear factor-kappa light-chain-enhancer of deactivated B Cell
NK	natural killer
NSCLC	non-small cell lung carcinoma
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cell
PD	pharmacodynamics
PI3K	phosphatidylinositol 3-kinase
PUMA	p53 upregulated modulator of apoptosis
PWB	packed whole blood
QWBA	quantitative whole body autoradiography
RANKL	receptor activator of NF-κB ligand
RBC	red blood cell
RCC	renal cell cancer
RKIP	Raf-1 kinase inhibitor protein
RP2D	recommended Phase 2 dose
SAHA	suberoylanilide hydroxamic acid
SAR	structure-activity relationship
siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspases
TCRs	T-cell receptors
THF	tetrahydrofuran
Thr1	*N*-terminal threonine residue
T-L	trypsin-like
$\mathbf{T_{max}}$	time of maximum concentration of drug observed in blood after its administration
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAIL	TNF related apoptosis inducing ligand
Ub	ubiquitinated
UPS	ubiquitinated-proteasome system

USAN	United States adopted name
VEGF	vascular endothelial growth factor
VHL	von Hippal-Lindau
WM	Waldenstrom's macroglobulinemia
XIAP	X-linked inhibitor of apoptosis protein
YY1	Yin Yang 1

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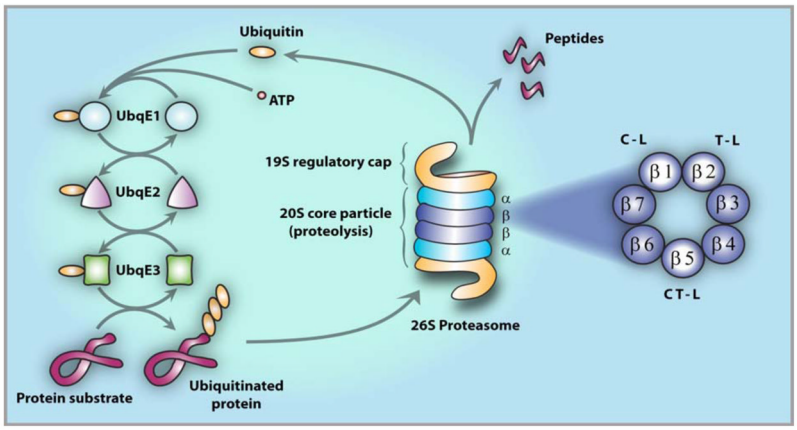


Fig. 1.

The Ubiquitin Protein System (UPS). Protein degradation by the UPS involves two distinct and successive steps: (i) polyubiquitination, i.e., covalent tagging of the target protein with multiple ubiquitin molecules; and (ii) degradation of the tagged protein by the 26S proteasome [3]. Conjugation of ubiquitin to the target protein substrate proceeds *via* a three-step mechanism, commencing with activation by ubiquitin-activating enzyme, E1, followed by transfer of ubiquitin (*via* one of several E2 enzymes) from E1 to a member of the ubiquitin-protein ligase family, E3, to which the substrate protein is specifically bound. In successive reactions, a polyubiquitin chain is synthesized by transfer of additional ubiquitin moieties to Lys48 of the previously conjugated molecule. The chain serves as a recognition marker for the 26S proteasome, which degrades the substrates to short peptides by the 20S proteasome and recycles ubiquitin *via* the action of isopeptidases. The 26S proteasome (center) comprises one or two 19S regulatory caps flanking the proteolytic 20S core particle [22, 23]. The 20S is a cylindrical structure formed by the stacking of two α-rings external to two β-rings, each of which contain seven β subunits, including catalytic subunits β1, β2 and β5 (right, expanded view).

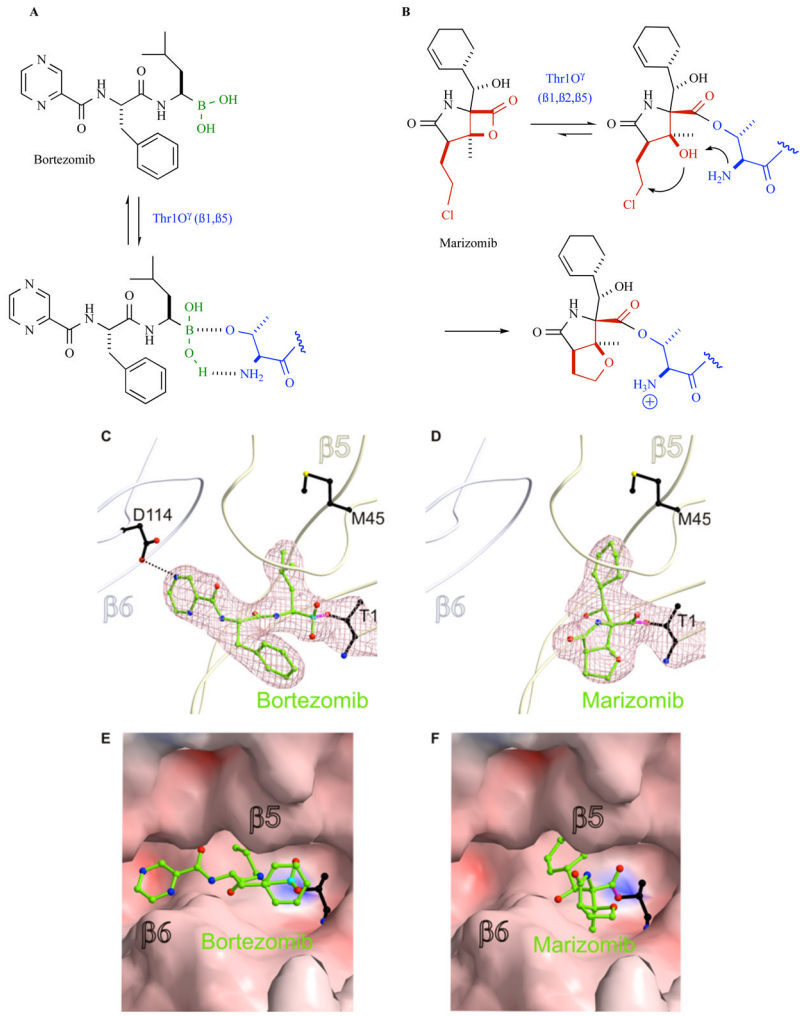


Fig. 2.

20S proteasome inhibition by bortezomib (left panels) and marizomib (right panels). **A**. Bortezomib forms a non-covalent adduct at the proteasome active site based on the high affinity of boronic acid for the hard oxygen nucleophile Thr1O$^{\gamma}$. The ligand is further stabilized by hydrogen bonding interactions between Thr1NH_2 and B-OH, as well as non-covalent P1-P3 residue contacts with the proteasome S1-S3 binding pockets (see panels **C, E**); the collective binding modality results in slowly reversible proteasome inhibition. **B**. The β-lactone of marizomib acylates Thr1O$^{\gamma}$, followed by Thr1NH_2-catalyzed nucleophilic displacement of chloride by C-3O to give a stable, irreversibly bound adduct. The binding mechanisms for these ligands were established *via* crystal structures of the yeast 20S proteasome CT-L site (subunit β5) in complex with bortezomib (**C**, **E**) and marizomib (**D**, **F**). Bortezomib residues P1 and P3 bind to the S1 and S3 pockets, respectively, while boron acts as an electron acceptor for the N-terminal threonine (T1) Thr1O$^{\gamma}$ [55]. Marizomib residue P1 binds to the S1 pocket and is covalently bound to T1 *via* an ester linkage between Thr1O$^{\gamma}$ and the carbonyl derived from the β-lactone ring [50]. T1, bortezomib and marizomib are presented as a ball and stick model. Electron density map (mesh) is contoured from 1σ around Thr1 and ligands with $2F_O$-F_C coefficients (**C**, **D**). Surface representations of the CT-L active site complex with bortezomib (**E**) and marizomb (**F**).

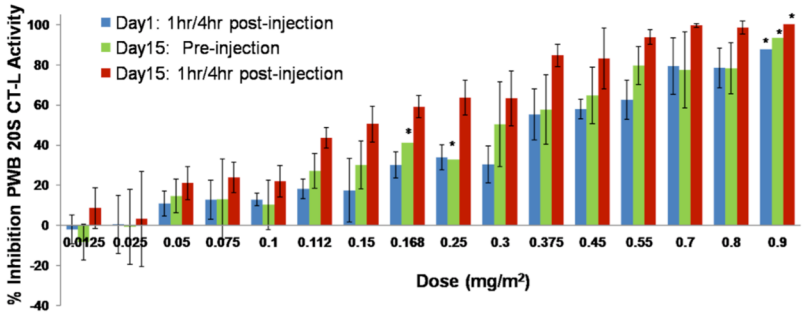


Fig. 3.

Inhibition of the packed whole blood (PWB) CT-L 20S proteasome activity in patient samples increases with dose and is more pronounced after the third administration of marizomib. Marizomib was administered IV on Days 1, 8 and 15 at the doses indicated. Proteasome activity does not restore to baseline levels, as indicated by the inhibition observed on Day 15 before the third administration of marizomib. Results are the average of 3 or more patients per cohort, except where indicated *, the average results of 2 patients is shown.

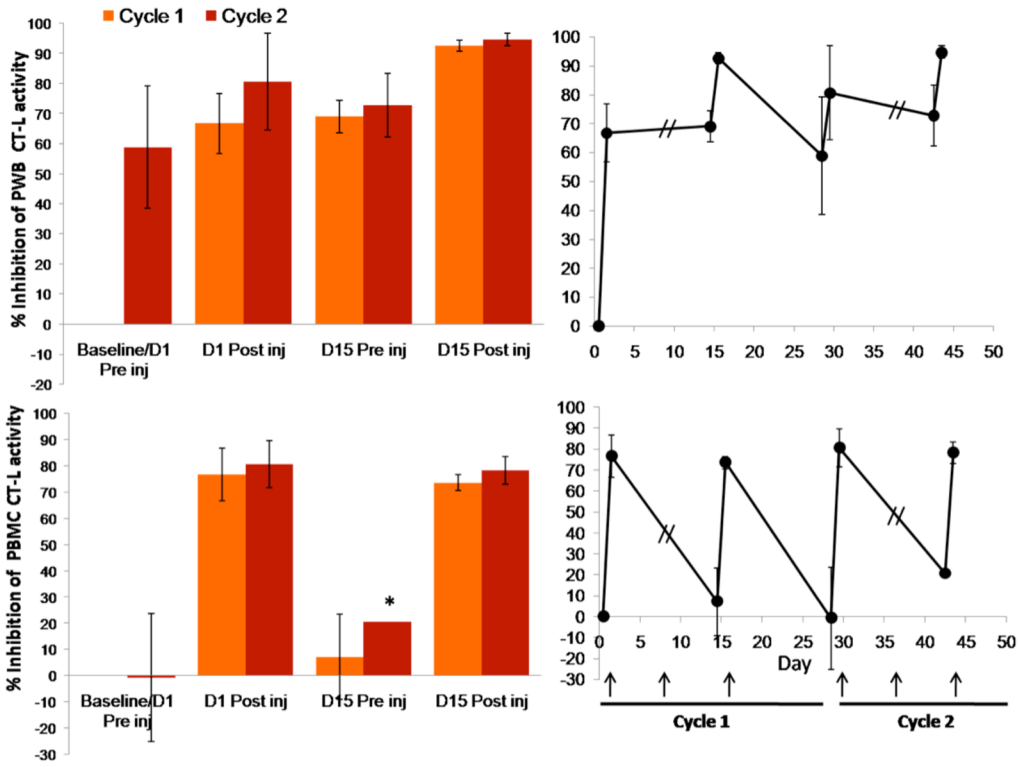


Fig. 4.

Marizomib pharmacodynamics (percent inhibition of CT-L activity), as monitored in patient PWB and PBMC lysates. Marizomib is administered IV at a dose of 0.55 mg/m^2 on Days 1, 8 and 15 of a 28 day cycle. Proteasome activity is assessed before and after the first and third marizomib administration of cycle 1 and 2. Recovery of 20S proteasome CT-L activity between consecutive marizomib administrations is more rapid in PBMCs compared to PWB. Percent inhibition is calculated relative to the Cycle 1 baseline/Day 1 preinjection proteasome activity levels. Results are the average of 3 or more patients, except where indicated *, the average results of 2 patients is shown. Arrow indicates day of marizomib administration.

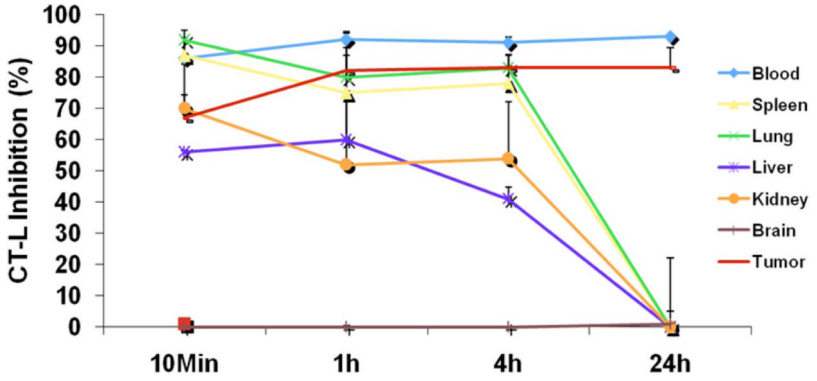


Fig. 5.

Inhibition of CT-L activity in tumors and various tissues after treatment with marizomib. [52]. MM.1S tumor-bearing mice were injected with three doses of marizomib (0.15 mg/kg, IV, Day 1, Day 4 and Day 8); euthanized at 10 min, 1, 4 and 24 hr after dosing; and PWB, liver, spleen, lung, kidney, brain and tumor were harvested. Protein extracts were prepared and analyzed for CT-L proteasome activity. Results are presented as percent inhibition compared to vehicle control. Data presented are means plus or minus SD ($n = 3$, $p < 0.05$).

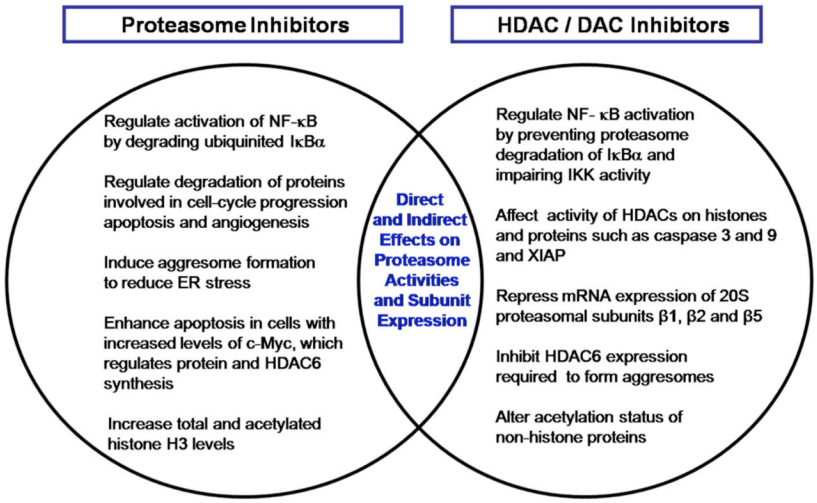


Fig. 6.

Potential distinct and overlapping mechanisms for synergies between HDAC and proteasome inhibitors.

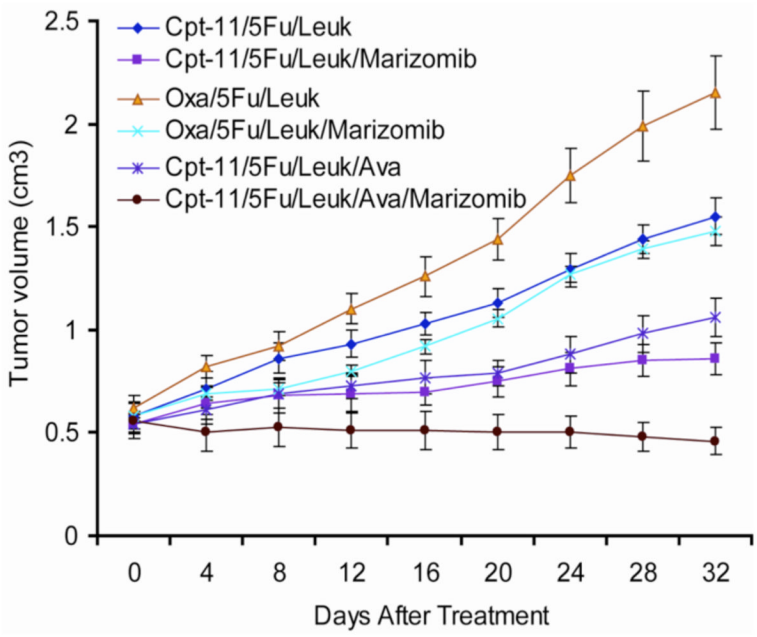


Fig. 7.

Marizomib treatment reduces tumor burden when added to three conventional colon cancer therapy regimens in a human colon carcinoma (LoVo) xenograft model (n = 6 mice per treatment group, error bars represent the standard error of the mean). CPT-11 (irinotecan); Leuk (leukovorin); Oxa (oxaliplatin); Ava (Avastin; bevacizumab). Adapted with permission from [120].

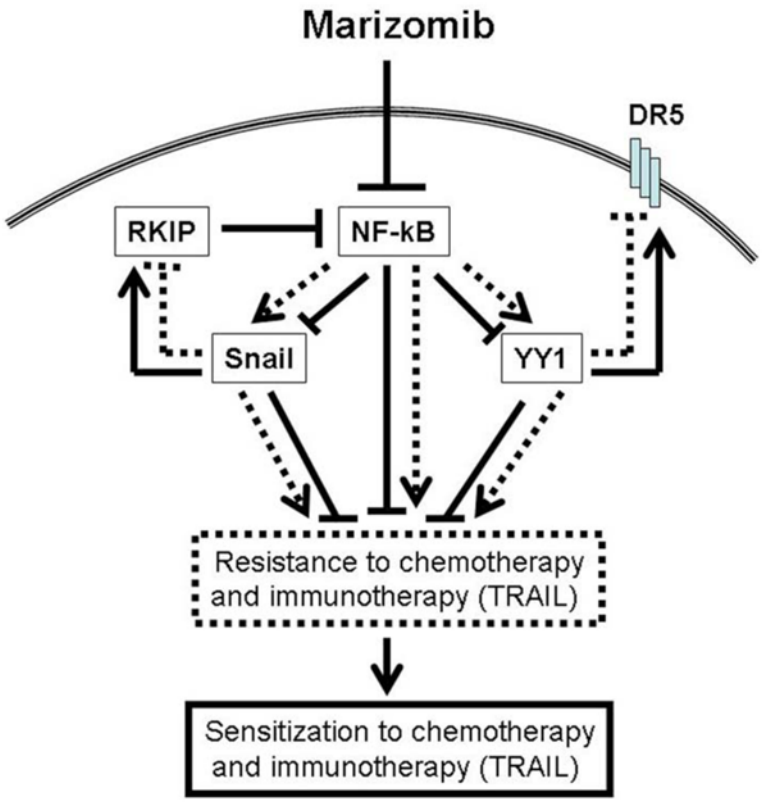


Fig. 8.

Schematic representation of the involvement of the NF-κB/YY1/Snail/RKIP/DR5 circuit in the regulation of tumor cell resistance to apoptotic stimuli in both chemotherapy and immunotherapy.

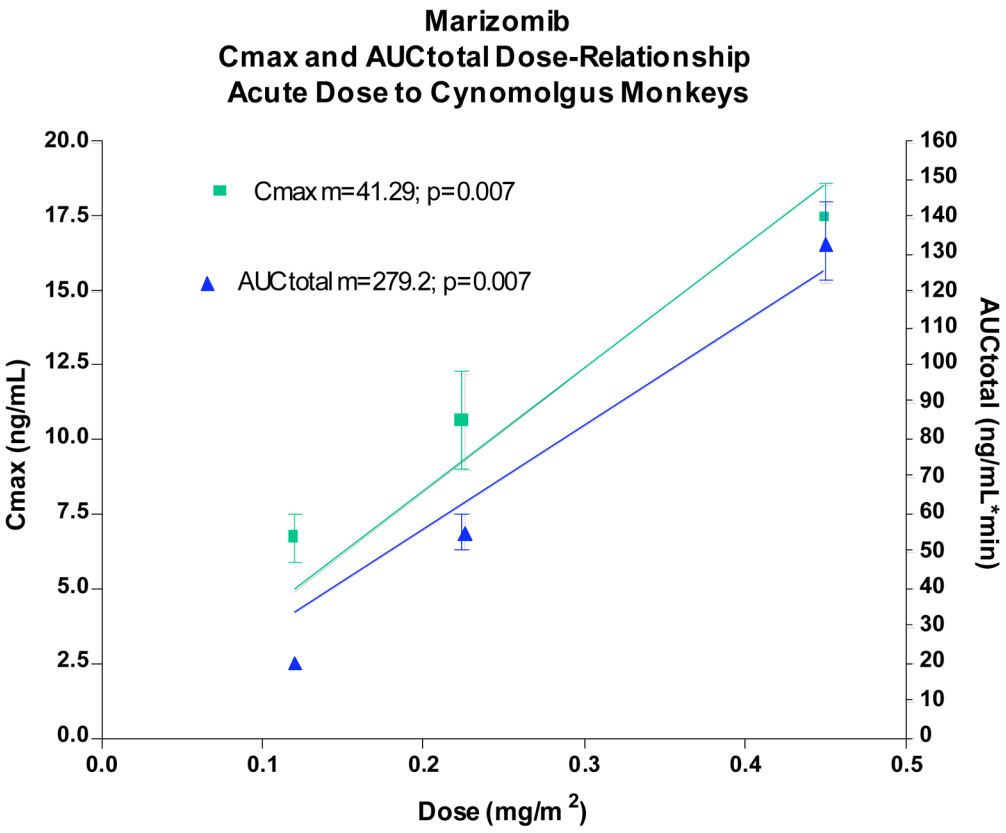


Fig. 9.

Marizomib pharmacokinetics. C_{max} and AUC_{total} dose relationship after single IV administration to cynomolgus monkeys.

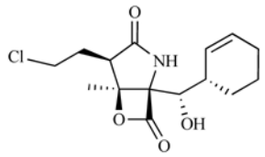
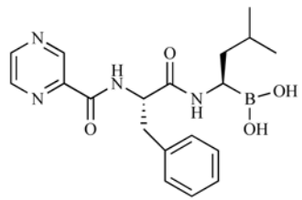
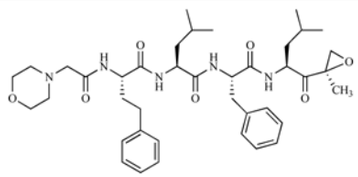
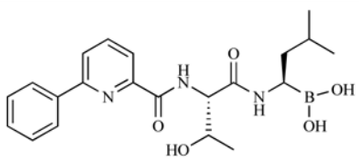
Table 1

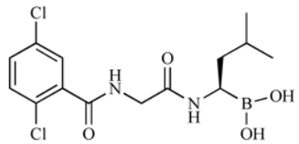
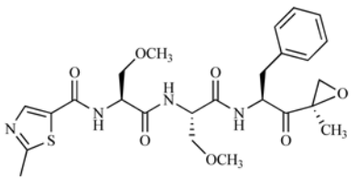
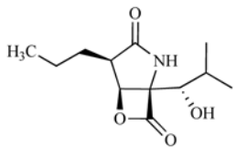
Preclinical Studies of Marizomib in Hematologic Malignancies and Solid Tumors

Preclinical Investigation	Institute (Senior Investigators)	Selected References
Hematologic Malignancies		
Multiple Myeloma: Single agent and combinations with Velcade, Revlimid, Thalomid, HDACis, Smac mimetics, thalomid. Drug resistant multiple myeloma cells.	Dana Farber Cancer Institute (Anderson/Chauhan)	[49, 71]
Waldenstrom's Macroglobulinemia and other B-cell malignancies (Burkitt's lymphoma, and low grade IgM secreting lymphoma).	Dana Farber Cancer Institute (Anderson/Ghobrial)	[72]
B-Non-Hodgkin's Lymphoma: Single agent and combination with Rituxan, CDDP in drug resistant cells. Mechanism of TRAIL resistance. Role of NF-κB, Snail, YY1, RKIP, DR5.	University of California, Los Angeles (Bonavida)	[182, 188]
Leukemias (CLL, AML and ALL): Single agent and drug combinations (HDACis, VPA, entinostat, Zolinza. Rapidity of action versus Velcade. Regulation of NF-κB and related genes.	MD Anderson Cancer Center (Chandra/ McConkey)	[57, 104]
Mantle Cell Lymphoma: Single agent, combinations with Bcl-2 and HDAC inhibitors. Regulation of NF-κB and related genes.	MD Anderson Cancer Center (Younes) MD Anderson Cancer Center (Aggarwal)	[112] [65]
Solid tumors		
Colon: Single agent and combination with CPT-11, Avastin, leucovorin, 5-FU and oxaliplatin.	Massachusetts General Hospital (Cusack)	[120]
Pancreatic: Single agent and combination with Avastin, Tarceva, Gemzar, Iressa and Erbitux. Single agent and combination with TRAIL.	Massachusetts General Hospital (Cusack) MD Anderson Cancer Center (McConkey)	[131, 132]
Prostate: Single agent and combination with docetaxel and TRAIL.	MD Anderson Cancer Center (McConkey) University of California, Los Angeles (Bonavida)	[182]
Melanoma: Single agent and combination with HDACis.	MD Anderson Cancer Center (McConkey)	Unpublished data
Glioma: Single agent and combination with radiation. Single agent	University of California, Los Angeles (McBride/ Pajonk) University of California, Irvine (Bota)	[167, 182] Unpublished data
Squamous Cell Carcinoma: Single agent.	University of California, Los Angeles (Wang)	Unpublished data
Non-Small Cell Lung Carcinoma: Combination with HDACi (Zolinza).	University of South Carolina Medical School (Drabkin/Gemmil)	Unpublished data
Renal Carcinoma: Combination with HDACi (Zolinza).	University of South Carolina Medical School (Drabkin/Gemmil)	Unpublished data

Table 2

Profiles and Treatment Regimens for Proteasome Inhibitors in Clinical Development

Proteasome Inhibitor/Company	Structural Class	Structure	Pharmacodynamic Profile			Development Status	Route of Administration	Treatment Schedule
			CT-L	T-L	C-L			
Marizomib (NPI-0052, Salinosporamide A) Nereus	β-lactone-γ-lactam		Sustained[a]	Sustained[a]	Sustained[a]	Phase 1b	IV (Oral[b] and Subcutaneous efficacy *in vivo*)	Days 1,8,15 (28 day cycle) Days 1,4,8,11 (21 day cycle)
Bortezomib (Velcade®; PS-341) Takeda/ Millennium	Peptide boronic acid		Slowly reversible[b]		Sustained[b]	Approved MM/MCL	IV (Active Subcutaneous in MM)[c]	Days 1,4,8,11 (21-day cycle)
Carfilzomib (PR-171) Onyx/Proteolix	Peptide epoxyketone		Sustained[d]			Phase 2	IV	Days 1,2,8,9,15,16 (28-day cycle)[e]
CEP-18770 Cephalon	Peptide boronic acid		Slowly reversible[f]			Phase 1	IV (Oral efficacy *in vivo*[g])	Days 1,4,8,11 (21-day cycle)[h]

Proteasome Inhibitor/Company	Structural Class	Structure	Pharmacodynamic Profile			Development Status	Route of Administration	Treatment Schedule
			CT-L	T-L	C-L			
MLN9708 (MLN2238, active form) Takeda/Millennium	Peptide boronic acid		Reversible[i]			Phase 1	IV/Oral	Days 1,4,8,11 (21-day cycle) OR Days 1,8,15 (28- day cycle)[j]
ONX-0912 PR-047 Onyx/Proteolix	Peptide epoxyketone		Sustained[k]			Phase 1	Oral	Days 1,2,3,4,5 (14-day cycle)[l]
PS-519	β-lactone-γ-lactam		Slowly reversible[m]			Discontinued after Phase 1	IV	Day 1 or Days 1,2,3 (single cycle)[n]

[a] Patient PWB, ≥ 72 h; patient PBMCs, 48–72 h [61]; > 24 h in PWB and tumors from human MM.1S plasmacytoma xenograft murine model [49, 52]; < 24 h in liver, lung, spleen, kidney from human MM.1S plasmacytoma xenograft murine model [52].

[b] Human MM.1S plasmacytoma xenograft murine model [49].

[c] [203].

[d] [60].

[e] [204].

[f] Slowly reversible in PWB and tissues (lung and liver), but sustained inhibition in tumor [205].

[g] [205].

[h] Clinicaltrials.gov: NCT00572637.

[i] [206].

[j] Clinicaltrials.gov: NCT00893464, NCT00830869, NCT00932698, NCT00963820.

[k] [207].

[l] ClinicalTrials.gov: NCT01129349.

[m] Patient PWB; maximum inhibition at 2 h post-dosing and activity generally returned to normal within 24 h [34].

[n] [34].