In vitro and In vivo Selective Antitumor Activity of a Novel Orally Bioavailable Proteasome Inhibitor MLN9708 Against Multiple Myeloma Cells

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The favorable clinical outcome of bortezomib therapy in multiple myeloma (MM) patients provided impetus for the development of second generation small molecule proteasome inhibitors with the goals of improving efficacy of proteasome inhibition, enhancing anti-tumor activity, and reducing toxicity, as well as providing flexible dosing schedules and patient convenience. In the present study, we utilized both in vitro and in vivo MM xenograft models to show antitumor efficacy of a novel orally bioactive proteasome inhibitor MLN9708. Moreover, combination regimens of MLN9708 with bortezomib, lenalidomide, or dexamethasone induce synergistic anti-MM activity. Our preclinical data showing efficacy of MLN9708 in MM disease models provide the framework for clinical evaluation of MLN9708, either alone or in combination, to improve outcome in MM.
Abstract

**Purpose:** The success of bortezomib therapy for treatment of multiple myeloma (MM) led to the development of structurally and pharmacologically distinct novel proteasome inhibitors. In the present study, we evaluated the efficacy of one such novel orally bioactive proteasome inhibitor MLN9708/MLN2238 in MM using well-established *in vitro* and *in vivo* models.

**Experimental Design:** MM cell lines, primary patient cells, and the human MM xenograft animal model were utilized to study the antitumor activity of MN2238.

**Results:** Treatment of MM cells with MLN2238 predominantly inhibits chymotrypsin-like activity of the proteasome and induces accumulation of ubiquitinated proteins. MLN2238 inhibits growth and induces apoptosis in MM cells resistant to conventional and bortezomib therapies without affecting the viability of normal cells. In animal tumor model studies, MLN2238 is well tolerated and inhibits tumor growth with significantly reduced tumor recurrence. A head-to-head analysis of MLN2238 versus bortezomib showed a significantly longer survival time in mice treated with MLN2238 than mice receiving bortezomib. Immununostaining of MM tumors from MLN2238-treated mice showed growth inhibition, apoptosis, and a decrease in associated angiogenesis. Mechanistic studies showed that MLN2238-triggered apoptosis is associated with activation of caspase-3, caspase-8, and caspase-9; increase in p53, p21, Noxa, PUMA, and E2F; induction of ER stress response proteins Bip, phospho-eIF2-α, and CHOP; and inhibition of NF-κB. Finally, combining MLN2238 with lenalidomide, HDAC inhibitor SAHA or dexamethasone triggers synergistic anti-MM activity.

**Conclusion:** Our preclinical study supports clinical evaluation of MLN9708, alone or in combination, as a potential MM therapy.
Introduction

Normal cellular processes such as DNA replication, cell cycle, cell growth and survival, inflammation, transcription, and apoptosis are modulated by the ubiquitin-proteasome signaling pathway (UPS) (1-3), which facilitates proteolysis of key regulatory proteins. Importantly, deregulation in UPS is linked to the pathogenesis of various human diseases (3), and targeting components of UPS therefore offers great promise in novel therapeutic strategies. Bortezomib (VELCADE™) is the first-in-class proteasome inhibitor, approved by FDA for the treatment of multiple myeloma (MM) and relapsed mantle cell lymphoma (3-7). Although very effective, dose-limiting toxicities and the development of resistance limit its long-term utility (8, 9), there is therefore a need for development of novel proteasome inhibitors with equipotent efficacy and improved safety profile.

Recent preclinical pharmacology studies demonstrated that a second-generation small molecule proteasome inhibitor MLN9708 (Millennium Pharmaceuticals, Inc.) has a shorter proteasome dissociation half-life than bortezomib, as well as improved pharmacokinetics, pharmacodynamics, and antitumor activity in xenograft models (10). In contrast to bortezomib, MLN9708 is an orally bioavailable proteasome inhibitor and shows efficacy at various dosing routes and regimens. Upon exposure to aqueous solutions or plasma, MLN9708 rapidly hydrolyzes to its biologically active form MLN2238. Similar to bortezomib, MLN2238 is a boronic acid analog which was identified by screening a large pool of boron-containing proteasome inhibitors with physiochemical properties distinct from bortezomib (10, 11). In the present study, we examined the anti-tumor activity of MLN2238 using both in vitro and in vivo MM models.
Material and Methods

Cell culture MM.1S (Dexamethasone-(Dex) sensitive), MM.1R (Dex-resistant), RPMI-8226, OPM1, OPM2, H929 and INA-6 (IL-6-dependent) human MM cell lines were cultured in complete medium (RPMI-1640 media supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine). ANBL-6-bortezomib-sensitive (ANBL-6.WT) and bortezomib-resistant (ANBL-6.BR) were kindly provided by Dr. Robert Orlowski (M.D. Anderson Cancer Center, TX). Tumor cells from MM patients were purified (greater than 95% purity) by CD138 positive selection using the Auto MACS magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA). Informed consent was obtained from all patients in accordance with the Helsinki protocol. PBMCs from normal healthy donors were maintained in culture medium, as above. Bone marrow stromal cells (BMSCs) were derived from CD138- cells obtained from MM patients and cultured in DMEM medium containing 20% FBS. Drug source: MLN2238 from Millennium: The Takeda Oncology company, Cambridge, MA; lenalidomide, bortezomib, and SAHA were purchased from Selleck Chemicals LLC, Houston, TX; and Dex was obtained from Calbiochem, CA.

In vitro proteasome activity assays Proteasome activity assay was performed using 20S Proteasome Assay Kit, ADS-Activated (Calbiochem) as previously described (12, 13), with some modifications. Briefly, MM.1S cells were lysed in RIPA buffer and 20μg (10μl) of protein was used in a total volume of 200μl reaction buffer (20mmol/L Hepes, pH7.6, 0.5mmol/L EDTA) with 0.03% SDS except for trypsin-like activity assay. The substrates utilized for measuring CT-L, T-L or C-L proteasome activity were Suc-Leu-Leu-Val-Try-AMC (10μM), Bz-Val-Gly-Arg-AMC (50μM), and Z-Leu-Leu-Glu-AMC.
(10μM) respectively. The reaction was initiated by adding 10μl of each substrate, and free AMC fluorescence was quantified using 380/460 nm filter set in a SpestraMax M2e fluorometer (Bucher biotec AG, Basel Switzerland).

**Cell viability, proliferation, and apoptosis assays** Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, CA) (14) and CellTiter-Glo (Progema, Madison, WI) assays, according to the manufacturer’s instructions. Cell proliferation analysis in coculture experiments with patient-derived BMSCs was performed using thymidine incorporation, as described previously (14). Apoptosis was quantified using Annexin V/PI staining kit, as per manufacturer’s instructions (R&D Systems, Inc. Minneapolis, MN), and analysis on a FACSCalibur (Becton Dickinson, San Jose, CA).

**Immunoblotting** Western blot analysis was performed as previously described (15) using antibodies against PARP (BD Bioscience Pharmingen, San Diego, CA), Caspase-3, Caspase-8, Caspase-9, p21, E2F, Cyclin D1, Cdk6, p-Rb (Cell Signaling, Beverly, MA), p53, NOXA, PUMA, Bip, CHOP, eIF2-α, or β-actin (Santa Cruz, CA). Blots were then developed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

**NF-κB and HtrA2/Omi activity assay** MM.1S cells were treated with MLN2238 (12 nM) at various times and harvested; total cellular proteins (1 μg) were subjected to p65 and p52 NF-κB activity analysis using ELISA, as per the manufacturer’s instructions (TransAM NF-κB Transcription Factor Assay Kits, Active Motif, CA). The effect of MLN2238 vs. bortezomib on HtrA2/Omi serine protease activity was determined by
measuring cleavage of Htra2/Omi substrate β-casein in an in vitro enzyme based assay, as per manufacturer's instruction (R & D Systems).

**Human plasmacytoma xenograft model** All animal experiments were approved by and conform to the relevant regulatory standards of the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute. MLN2238 was dissolved in 5% 2-hydroxypropyl-β- cyclodextrin at 2 mg/ml concentration. The human plasmacytoma xenograft tumor model was performed as previously described (13, 15, 16). CB-17 SCID mice (n=21; Taconic, Gemantown, NY) were subcutaneously inoculated with 5.0 X10^6 MM.1S cells in 100μl serum-free RPMI-1640 medium, and randomized to treatment groups when tumors reached 250-300 mm^3. Mice were treated with vehicle, bortezomib (1 mg/kg; i.v) or MLN2238 (11mg/kg; i.v) twice weekly for 3 weeks. Animals were euthanized when their tumors reached 2 cm^3.

**In situ detection of apoptosis, and assessment of angiogenesis** Mice tumor sections were subjected to IHC staining for TUNEL, and for caspase-3 activation (13). Ki-67 was assessed by IHC staining to quantify proliferation. Tumor angiogenesis was assessed by IHC staining for VEGFR2, and Pecam-αsma expression (13). Immunostained tissues were imaged using confocal microscopy (FV1000, Olympus, Center Valley, PA).

**Statistical Analysis** Statistical significance of differences observed in MLN2238- or bortezomib-treated treated vs. control cultures was determined using the one-way ANOVA test. The minimal level of significance was P < 0.05. Survival of mice was measured by using the GraphPad Prism software (version 5; La Jolla, CA). Isobologram analysis (17) was performed using “CalcuSyn” software program (Biosoft, Ferguson,
MO and Cambridge, UK). Combination index (CI) values of < 1.0 indicate synergism and values > 1.0 antagonism.
Results and Discussion

Effect of MLN2238 on proteasome activity in vitro  We first examined the ability of MLN2238 to inhibit all three proteasome activities in MM cells. MM.1S cells were treated with various concentrations of MLN2238 for 3h and harvested; cell extracts were then analyzed for CT-L, caspase-like (C-L), and trypsin-like (T-L) proteasome activities using specific fluorogenic peptide substrates. MLN2238 significantly inhibited CT-L proteasome activity with an IC\textsubscript{50} at 5 nM (Fig 1A; P < 0.05). Higher concentrations of MLN2238 showed inhibitory activity against C-L and T-L proteasome activities (Fig 1B and 1C, respectively). We next compared the effects of MLN2238 vs. bortezomib on CT-L proteasome activity. MLN2238 triggered a significant and similar degree of CT-L inhibition as bortezomib (Fig 1D; P < 0.05). It is well established that proteasome inhibition causes stabilization and accumulation of ubiquitinated proteins; and in agreement with this observation, ML2238 induced a marked increase in ubiquitinated proteins in a time- and dose-dependent manner (Fig 1E).

A recent study showed that peripheral neuropathy associated with bortezomib therapy may be, in part, due to blockade of neuronal cell survival protease HtrA2/Omi (18). In the present study, treatment of MM cells with bortezomib inhibited HtrA2/Omi; and importantly, no significant inhibition of HtrA2/omi was noted in response to MLM2238 treatment (Fig 1F; P < 0.004). These data highlight another distinction between bortezomib and MLN2238; however, the effect of MLN2238 on other proteases remains to be examined. Nonetheless, our findings suggest that MLN2238 targets proteasomes; and importantly, retains the potency and selectivity of bortezomib against CT-L proteasome activity.
**Anti-MM activity of MLN2238 in vitro**

Human MM cell lines (MM.1S, INA-6, RPMI-8226, MM.1R, H929, OPM1 and OPM2) were treated with various concentrations of MLN2238 for 48h, followed by assessment for cell viability using MTT assays. A significant concentration-dependent decrease in viability of all cell lines was observed in response to treatment with MLN2238 (Fig 2A, P < 0.05; n = 3). Moreover, MLN2238-induced decrease in viability is due to apoptosis, as evidenced by a significant increase in the Annexin V+/PI− apoptotic cell population in MM.1S, H929, OPM1, and OPM2 cells (Fig 2B; P < 0.005, n=3). The anti-MM activity of MLN2238 was observed in MM cell lines sensitive and resistant to conventional therapies, as well as representing distinct cytogenetic profiles. For example, we examined isogenic cell lines Dex-sensitive MM.1S and Dex-resistant MM.1R with t(14;16) translocation and c-maf overexpression; RPMI-8266 with TP53, K-Ras and EGFR mutations; INA-6, an IL-6-dependent cell line with N-Ras activating mutation; H929 with t(4;14) translocation and mutated Ras; and OPM2 with t(4;14)(p16;q32) translocation, and abnormal TP53 (19-24). Thus, the variable IC\textsubscript{50} of MLN2238 observed against MM cell lines may be due to their distinct genetic background and/or drug resistance characteristics (19, 21).

To determine whether MLN2238 similarly affects purified patient MM cells, tumor cells from six MM patients, including those relapsing after multiple prior therapies such as bortezomib, lenalidomide, and Dex, were treated with MLN2238. Patients were considered refractory to specific therapy when disease progressed on therapy or relapsed within two months of stopping therapy. A significant dose-dependent decrease in viability of all patient MM cells was noted after MLN2238 treatment (Fig 2C, P < 0.001 for all patients). In addition, a parallel treatment of MM cells obtained from bortezomib-
refractory patients showed increased *in vitro* cytotoxicity in response to MLN2238 vs. bortezomib (data not shown). These findings show the ability of MLN2238 to trigger cytotoxicity even in tumor cells obtained from patient resistant to bortezomib, Dex, or lenalidomide therapies. In order to further determine whether MLN2238 overcomes bortezomib-resistance, we utilized previously characterized (25) bortezomib-sensitive (ANBL-6.WT) and bortezomib-resistant (ANBL-6.BR) MM cell lines. As seen in Figure 2D, the IC<sub>50</sub> ratio (ANBL-6.BR/ANBL-6.WT) of MLN2238 is significantly less than bortezomib (P < 0.01; n= 3), demonstrating the ability of MLN2238 to overcome bortezomib-resistance. In the context of mechanism(s) mediating bortezomib-resistance, a recent study has linked it to increased signaling through the insulin-like growth factor-1/Akt axis (25); however, involvement of other signaling cascades cannot be excluded. Furthermore, a differential proteasome content/activity profile, abnormal or mutated proteasome subunits, and/or ER stress levels may contribute to bortezomib resistance. These issues remain to be examined in a broader panel of MM cell lines and patient cells. Finally, in the present study, MLN2238 at the IC<sub>50</sub> for MM cells does not significantly affect the viability of normal PBMCs (Fig 2F), suggesting specific anti-MM activity and a favorable therapeutic index for MLN2238.

**MLN2238 inhibits human MM cell growth *in vivo* and prolongs survival in xenograft mouse model** Having shown that MLN2238 induces apoptosis in MM cells *in vitro*, we examined the *in vivo* efficacy of MLN2238 given intravenously or orally using a human plasmacytoma MM.1S xenograft mouse model (13, 16). Treatment of tumor-bearing mice with intravenous injection of MLN2238 significantly (P = 0.001) inhibited MM tumor growth and prolonged survival (87%; P < 0.001) of these mice (Fig 3A and
Bortezomib treatment also reduced tumor progression (Fig 3A), albeit to a lesser extent than MLN2238. Moreover, we found that MLN2238-treated mice survived for a longer time than mice receiving bortezomib ($P < 0.01; CI = 95\%$). In addition, blood chemistry profiles of MLN2238-treated mice showed normal levels of creatinine, hemoglobin, and bilirubin (Fig 3C). Examination of the xenografted tumor sections showed that MLN2238, but not vehicle alone, dramatically increased the number of cleaved-caspase-3 positive (Red color) cells (Fig 3D). Similarly, MLN2238 increased the number of TUNEL-positive (Green color) cells compared to vehicle treatment (Fig 3D). In agreement with these data, a significant decrease in proliferation marker Ki-67 (Red color) was noted in tumor sections from MLN2238-treated mice relative to tumors from control mice (Fig 3D). Finally, treatment of tumor-bearing mice with oral doses of MLN2238 significantly ($P = 0.001$) inhibited MM tumor growth and prolonged survival ($P < 0.01$) of these mice (Fig 3E and 3F, respectively). These in vivo data confirm our in vitro findings showing potent apoptotic activity of MLN2238 against MM cells.

Prior studies have established that MM cell growth is associated with angiogenesis, which predominantly occur via vascular endothelial growth factor (VEGF) pathway (26, 27). To determine whether MLN223 triggers anti-angiogenic activity, we directly evaluated tumors harvested from mice by immunostaining using two distinct markers of angiogenesis, VEGFR2 and Pecam. As seen in Fig 3D, MLN2238 decreased the numbers of VEGFR2- and Pecam-positive cells. These data suggest that besides inducing apoptosis, MLN2238 also inhibit tumor-associated angiogenic activity.
Taken together, our findings demonstrate that MLN2238 reduces tumor growth, prolongs survival, and is well tolerated in vivo.

**Mechanisms mediating anti-MM activity of MLN2238**

Studies to date provide evidence for activation of pleiotropic cell death signaling cascades in response to proteasome inhibition (28, 29). This is likely due to the fact that the majority of cellular proteins undergo degradation through proteasome; and, blockade of proteasome negatively affects this normal cellular process resulting in accumulation of unwanted proteins, and subsequent activation of multiple cell death signaling. In the light of this notion, we examined the effect of MLN2238 on some of the major apoptotic signaling pathways triggered by similar class of agents in MM cells.

**Effect of MLN2238 on caspases**

Treatment of H929 and MM.1S MM cells with MLN2238 triggered a marked increase in proteolytic cleavage of poly(ADP) ribose polymerase (PARP), a signature event during apoptosis (Fig 4A and 4B). Furthermore, MLN2238 induced cleavage of caspase-3, an upstream activator of PARP (Fig 4A and 4B). Mitochondria mediate apoptotic signaling via activation of cell death initiator caspase, pro-caspase 9 (30). Similarly, Fas associated death-domain (FADD) protein is an essential component of the death-inducing signaling complexes (DISCs), resulting in autoactivation of pro-caspase-8. Our data show that MLN2238 induces activation of both caspase-9 (intrinsic) and caspase-8 (extrinsic) apoptotic pathways in H929 and MM.1S and cells (Fig 4A and 4B, respectively). Studies using biochemical inhibitors showed that inhibition of either caspase-8 (IETD-FMK) or caspase-9 (LEHD-FMK) resulted in marked abrogation of MLN2238-triggered cytotoxicity (Fig 4C). Additionally, pan-caspase inhibitor (Z-VAD-FMK) also attenuated MLN2238-induced cytotoxicity (Fig
Simultaneous blockade of caspase-8 and caspase-9 led to $89 \pm 4.4\%$ attenuation of MLN2238-triggered cell death. These findings suggest that 1) MLN2238 triggers both mitochondria-dependent and –independent signaling pathways; and 2) MLN2238-induced apoptosis occurs in a caspase-dependent manner.

**Effect of MLN2238 on p53 pathway** The molecular pathways leading to caspase induction includes activation of tumor suppressor p53, which coordinates cellular response to stress signaling pathways *via* cell cycle arrest, and apoptosis (31, 32). Examination of MLN2238-treated MM cells showed an increase in both p53 and p21 (Fig 4D). The induction of p21 may account for MLN2238-induced growth arrest (data not shown). Prior studies have also linked p53 pathway to activation of mitochondrial apoptotic signaling *via* BH3-only proteins Noxa and Puma (33, 34); and, we found that MLN2238 triggered robust induction of Noxa and Puma (Fig 4D). This finding is consistent with the observed MLN2238-induced caspase-9 induction that occurs *via* mitochondria (Fig 4A and 4B). Furthermore, the p53-signaling cascade is known to communicate with retinoblastoma (Rb)-E2F axis (35). Treatment of MM.1S and MM.1R cells with MLN2238 downregulated pRb with an expected upregulation of its downstream target protein E2F (Fig 4E and 4F). Similarly, cyclin D1 and its target protein Cdk6 were markedly decreased upon treatment with MLN2238 (Fig 4E and 4F).

**Effect of MLN2238 on endoplasmic reticulum (ER) stress pathway** Proteasome inhibition is associated with induction of ER stress pathway and the unfolded protein response (13, 36, 37). Analysis of proteins mediating ER stress pathway showed that MLN2238 induces elf2-α kinase activity and protein levels of Bip and CHOP/GADD153 (Fig 4G). Of note, ER stress signaling is also linked to activation of p53-Noxa-Puma.
signaling axis (38), suggesting a potential crosstalk between these pathways during MLN2238-induced apoptosis in MM cells. It is conceivable that MLN2238, like bortezomib, trigger pleiotropic signaling pathways; however, due to the shorter dissociation (t ½) characteristics of MLN2238 compared to bortezomib, the kinetics of alterations in stress response signaling may vary and this issue remains to be defined. Nonetheless, our mechanistic data shows that MLN2238-induced apoptosis in MM cells is caspase-dependent and correlates with activation of p52-p21, p53-Noxa-Puma, Rb-E2F, and ER stress signaling pathways.

**MLN2238 overcome the cytoprotective effects of the MM bone marrow microenvironment and inhibits in vitro capillary-like tube formation** Interaction of MM cells with bone marrow stromal cells (BMSCs) triggers cytokine secretion, which mediates paracrine growth of MM cells, as well as protects against drug-induced apoptosis (28, 39, 40). To determine whether MLN2238 affects BMSCs-triggered MM cell growth, MM.1S cells were cultured with or without BMSCs in the presence or absence of various concentrations of MLN2238. A significant inhibition of BMSCs-induced proliferation of MM.1S was noted in response to MLN2238 treatment (Fig 5A). These data suggest that MLN2238 not only directly targets MM cells, but also overcomes the cytoprotective effects of the MM-host BM microenvironment.

Angiogenesis play an important role in the progression of MM (26, 41). Our in vivo data using tumor sections from MLN2238-treated mice showed decrease in the expression of angiogenesis markers. To confirm the anti-angiogenic activity of MLN2238, we utilized in vitro capillary-like tube structure formation assay using human vascular endothelial cells (HUVECs). HUVECs when plated onto Matrigel differentiate
and form capillary-like tube structures similar to in vivo neovascularization, a process dependent on cell-matrix interaction, cellular communication, and cellular motility. This assay therefore provides evidence for anti-angiogenic effects of drugs/agents. HUVECs were pretreated with vehicle (DMSO) or MLN2238 (10 nM) for 8h; washed in media and seeded in 96-well culture plates coated with Matrigel; and then incubated for additional 4h, followed by analysis of tube formation using an inverted microscope. As seen in Fig 5B, tubule formation was significantly decreased in the MLN2238-treated cells, but not after treatment with DMSO alone (P < 0.001, n=3). HUVEC cell viability was assessed using trypan blue exclusion assay: < 5% cell death was observed after MLN2238 treatment, excluding the possibility that drug-induced inhibition of capillary-tube formation was due to cell death. These in vitro data corroborate with our in vivo findings in animal model (Fig 3C) to show anti-angiogenic activity of MLN2238.

**MLN2238 targets nuclear factor-kappa B (NF-κB)** NF-κB plays a key role during MM cell adhesion-induced cytokine secretion in BMSCs, which in turn triggers MM cell growth in a paracrine manner (15, 39, 42). Reports have also linked constitutive activation of non-canonical NF-κB pathway to the genetic abnormalities/mutations (43, 44), allowing for an autocrine growth of MM cells. Importantly, constitutive NF-κB activity in primary tumor cells from MM patients renders these cells refractory to inhibition by bortezomib (45), and in fact, bortezomib induces canonical NF-κB activity (46). Given the findings from these studies, we examined whether MLN2238 affects NF-κB. ML2238, in a time-dependent manner, inhibits both constitutive and TNF-α-induced NF-κB activation in MM cells (Fig 5C-5F; P < 0.05; n =3). These data suggest that MLN2238 is a potent inhibitor of both canonical and non-canonical NF-κB pathways.
Combined treatment with MLN2238 and lenalidomide, Dexamethasone or HDAC inhibitor SAHA induces synergistic anti-MM activity

Preclinical studies (47-49) provided the basis for clinical trials of proteasome inhibitor bortezomib in combination with lenalidomide, Dex and HDAC inhibitors (50). Given that MLN2238, like bortezomib, is a boronic acid analog, we examined whether ML2238 similarly enhances the anti-MM activity of other agents. MM.1S cells were first treated with both MLN2238 and lenalidomide simultaneously across a range of concentrations for 48h, and then analyzed for viability using MTT assay. An analysis of synergistic anti-MM activity using the Chou and Talalay method (17) demonstrated that the combination of low concentrations of MLN2238 and lenalidomide triggered synergistic anti-MM activity, with a combination index (CI) < 1.0 (Fig 6A).

In addition to proteasomal degradation, intracellular protein catabolism also occurs via an HDAC-dependent aggressome-autophagic signaling pathway (51-53). Our prior study showed that inhibition of both mechanisms of protein catabolism by combining bortezomib and HDAC inhibitor induced significant cytotoxicity in MM cells (51). Recent clinical trials combining bortezomib and HDAC inhibitor vorinostat showed promising outcome in relapsed and refractory MM, including activity among some patients with prior exposure to bortezomib (54). In the light of these studies, we examined whether the combination of MLN2238 with HDAC inhibitor SAHA triggers synergistic anti-MM activity. MM.1S cells were treated with both MLN2238 and SAHA simultaneously across a range of concentrations for 48h, and then analyzed for viability. Isobologram analysis showed that the combination of low concentrations of MLN2238 and SAHA triggered synergistic anti-MM activity, with a combination index (CI) < 1.0.
These data confirm the potential for clinical trials combining MLN2238 and HDAC inhibitors.

We next examined whether MLN2238 adds to the anti-MM activity of conventional anti-MM agent Dex. As with lenalidomide and SAHA, the combination of MLN2238 with Dex triggered synergistic anti-MM activity, evidenced by a significant decrease in viability of MM.1S cells (Fig 6C). Although definitive evidence of decreased toxicity of combination therapy awaits results of clinical trials, the synergy observed in vitro may allow for use of lower doses and decreased toxicity.

Collectively, our preclinical studies therefore demonstrate potent in vitro and in vivo anti-MM activity of MLN2238 at doses that are well tolerated in human MM xenograft mouse models. These findings provide the framework for clinical trials of MLN9708 both as a single agent, and together with lenalidomide, HDAC inhibitors, or Dex to potentially increase response, overcome drug resistance, reduce side effects, and improve patient outcome in MM.
References


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Figure Legends

Figure 1. Proteasome inhibitor MLN2238 is structurally distinct from bortezomib, and inhibits proteasome activity in vitro (A-C) MM.1S cells were treated with MLN2238 at indicated concentrations for 3h and harvested; cell extracts were then analyzed for CT-L, C-L, and T-L proteasome activities. Results are represented as percent inhibition in proteasome activities in drug-treated vs. vehicle control. (D) MM.1S cells were treated with IC50 concentrations of MLN2238 or bortezomib for indicated times and harvested; cell extracts were then analyzed for CT-L proteasome activity. (E, left panel) MM.1S cells were treated with MLN2238 (12 nM) at indicated times and harvested; protein lysates were subjected to immunoblotting using anti-ubiquitin and anti-actin Abs. Blots shown are representative of 3 independent experiments. (E, right panel) MM.1S cells were treated with MLN2238 for 24h and harvested; protein lysates were subjected to immunoblotting using anti-ubiquitin and anti-actin Abs. Blots shown are representative of 3 independent experiments. (F) Recombinant human HtrA2 enzyme was incubated with its substrate β-casein in assay buffer (R&D Systems), followed by SDS-PAGE, silver staining, and quantification of cleaved β-casein. The bar graph represents percent inhibition of HtrA2-induced β-casein cleavage in the presence of bortezomib (3 nM) or MLN2238 (12 nM). Data presented are means plus or minus SD (n=2; P < 0.05).

Figure 2. Anti-MM activity of MLN2238 (A) MM cell lines were treated with or without MLN2238 at the indicated concentrations for 48h, followed by assessment for cell viability using MTT assays. Data presented are means plus or minus SD (n =3; P < 0.05 for all cell lines). (B) MM.1S, H929, OPM1, and OPM2 cell lines were treated with
MLN2238 (IC$_{50}$ concentrations) and analyzed for apoptosis using Annexin V/PI staining assay. (C) Purified patient MM cells (CD138-positive) were treated with indicated concentrations MLN2238 for 24h and 48h, and cell viability was measured using Celltiter Glo assay. Data presented are means plus or minus SD of triplicate samples (P < 0.001 for all patient samples). (D) Bortezomib-sensitive (ANBL-6.WT) and -resistant (ANBL-6.BR) MM cell lines were treated with increasing concentrations of bortezomib and MLN2238 for 48h, followed by assessment for cell viability using MTT assays. The IC$_{50}$ of MLN2238 and bortezomib for ANBL-6.WT or ANBL-6.BR was derived. The bar graph shows the IC$_{50}$ ratio (ANBL-6.BR/ANBL-6.WT) of ML2238 and bortezomib. Data presented are means plus or minus SD (n =3). (E) PBMCs from healthy donors were treated with indicated concentrations of MLN2238 for 48h, and then analyzed for viability using Celltiter Glo assay. Data presented are means plus or minus SD of quadruplicate samples (P < 0.001 for all PBMCs samples).

**Figure 3. MLN2238 inhibits growth of xenografted human MM cells in CB-17 SCID mice**  
(A) Average and standard deviation of tumor volume (mm$^3$) is shown from mice (n = 7/group) versus time (days) when tumor was measured. MM.1S cells (5 X 10$^6$ cells/mouse) were implanted in the rear flank of female mice (6 weeks of age at the time of tumor challenge). On Day 28-30, mice were randomized to treatment groups and treated intravenously with vehicle, MLN2238 (11 mg/kg), or with bortezomib (1 mg/kg) on a twice weekly schedule for 3 weeks. Data are presented as mean tumor volume ± SD. A significant delay in tumor growth in MLN2238-treated mice was noted compared to vehicle-treated control mice. Bars indicate means plus or minus SD. (B) Kaplan-Meier survival plot shows significant increase (P < 0.05) in survival of mice receiving MLN2238.
(11 mg/kg) or bortezomib (1 mg/kg) compare to vehicle treated control. (C) Mice were treated with vehicle, MLN2238, or bortezomib (as in panel A); blood samples were obtained and subjected to analysis for bilirubin, creatinine, and hemoglobin levels using Quantichrom™ Creatinine, Bilirubin, and Hemoglobin Assay kit (BioAssay Systems, Hayward, CA). (D) Micrographs (upper three panels) show apoptotic cells in tumors sectioned from untreated- or MLN2238-treated mice as identified caspase-3 cleavage (Red cells), TUNEL (TUNEL-positive cells: Green cells), as well as Ki67 staining. Dotted red/green line indicates a line between tumor and host tissue. Micrographs (lower two panels) show expression of angiogenesis markers in tumors sectioned from untreated- or MLN2238-treated mice, identified by VEGFR2 (Green) and Pecam (Red color) staining. Bar scale: 100 μm (caspase-3 and TUNEL); 50 μm (Ki67); 10 μm (VEGFR2 and Pecam). (E) Tumor-bearing mice were treated orally with vehicle, or MLN2238 (8 mg/kg) on a twice weekly schedule for 3 weeks. Data are presented as mean tumor volume ± SD. Bars indicate means plus or minus SD. (F) Kaplan-Meier survival plot shows increase in survival of mice receiving oral doses of MLN2238 (8 mg/kg) compare to vehicle treated control.

**Figure 4. Mechanisms mediating anti-MM activity of MLN2238** (A and B) H929 and MM.1S and cells were treated with MLN2238 at the indicated doses for 24h and harvested; whole cell lysates were subjected to immunoblot analysis with anti-PARP, anti-caspase-3, anti-caspase-8, anti-caspase-9, or anti-actin Abs. FL indicates full length; CF denotes cleaved fragment. (C) MM.1S cells were pretreated with biochemical inhibitors of caspase-8 (IETD-FMK), caspase-9 (LEHD-FMK), or pan-caspase (Z-VAD-FMK) for 1h. MLN2238 (12 nM) was then added in cultures for
additional 24h, followed by analysis of cell viability by MTT assay. Data presented are means plus or minus SD (n = 3; P = 0.005). Error bars represent standard deviation (SD). (D and E) MM.1S cells were treated with MLN2238 (12nM) for 24h and harvested; protein lysates were subjected to immunoblotting using indicated Abs. (F) MM.1R cells were treated with MLN2238 (12nM) for 24h and harvested; protein lysates were subjected to immunoblotting using indicated Abs. (G) MM.1S cells were treated with MLN2238 (12 nM) for indicated times and harvested; protein lysates were subjected to immunoblotting using indicated Abs. Blots shown in the figure are representative of 3 independent experiments.

**Figure 5.** MLN2238 blocks BMSCs-induced MM cell proliferation, inhibits *in vitro* capillary tubule formation, and target NF-κB

(A) MM.1S cells were treated with indicated concentrations of MLN2238 in the presence or absence of BMSCs for 48h, followed by measurement of proliferation using tritiated thymidine incorporation assay. Data presented are means plus or minus SD (n = 3; P < 0.005). (B) Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in the presence or absence of MLN2238 for 8h (cell viability > 95%); cells were then washed with plain media, and placed on the matrigel for 4h to allow for tube formation, followed by photography using inverted microscope (magnification: 4X/0.10 NA oil, media: EBM-2). The *in vitro* angiogenesis is reflected by capillary tube branch formation. Images are representative of 3 experiments with similar results. (C and D) MM.1S cells were treated with MLN2238 (12 nM) at indicated times, harvested; and subjected to NF-κB activity analysis using ELISA. Data presented are the means plus or minus SD (n = 3; P < 0.005). (E and F) MM.1S cells were treated with MLN2238 (12 nM) at indicated times, followed by addition of TNF-α.
during the last 20 mins before cells were harvested. Cells were then subjected to p65 and p52 NF-κB activity analysis using ELISA. Data presented are the means plus or minus SD (n = 3; P < 0.004). Error bars represent standard deviation.

**Figure 6. Combination of low doses of MLN2238 and lenalidomide, SAHA, or Dex trigger synergistic anti-MM activity** (A) MM.1S cells were treated for 48h with indicated concentrations of MLN2238, lenalidomide, or MLN2238 plus lenalidomide; and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of MLN2238 and lenalidomide. The graph (left panel) is derived from the values given in the Table (right panel). The numbers 1-9 in graph represent combinations shown in the Table. \( F_{a_{com}} \) = Fraction of cells showing decrease in viability with MLN2238 plus lenalidomide treatment. Combination index (CI) of < 1 indicates synergy. All experiments were performed in triplicate and mean value is shown. (B) MM.1S cells were treated for 48h with indicated concentrations of MLN2238, SAHA, or MLN2238 plus SAHA; and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of MLN2238 and SAHA. The graph (left panel) is derived from the values given in the Table (right panel). The numbers 1-9 in graph represent combinations shown in the Table. Combination index (CI) of < 1 indicates synergy. (C) MM.1S cells were treated for 48h with indicated concentrations of MLN2238, Dex, or MLN2238 plus Dex; and then assessed for viability using MTT assays. Isobologram analysis shows the synergistic cytotoxic effect of MLN2238 and Dex. The graph (left panel) is derived from the values given in the Table (right panel).
Figure 1
Figure 2

A. Graph showing viability of MM cell lines (MM.1S, MM.1R, RPMI-8226, OPM1, OPM2, INA-6) treated with different concentrations of MLN2238. Treatment time is 48 hours.

B. Flow cytometry analysis showing propidium iodide and Annexin V staining for MM.1S and H929 cells control and treated with MLN2238.

C. Graph showing viability of MM patient tumor cells (Pt#1, Pt#2, Pt#3, Pt#4, Pt#5, Pt#6) treated with different concentrations of MLN2238. Treatment time is 24 and 48 hours.

D. Bar graph comparing IC50 ratio (ANBL-6/WT vs ANBL-6/WT) for Bortezomib and MLN2238 with statistical significance P < 0.01.

E. Graph showing viability of PBMCs from healthy donors treated with different concentrations of MLN2238. Treatment time is 48 hours.
Figure 3
Figure 4
**Figure 5**
Figure 6
Clinical Cancer Research

In vitro and In vivo Selective Antitumor Activity of a Novel Orally Bioavailable Proteasome Inhibitor MLN9708 Against Multiple Myeloma Cells

Dharminder Chauhan, Ze Tian, Bin Zhou, et al.

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