In Vitro and In Vivo Selective Antitumor Activity of a Novel Orally Bioavailable Proteasome Inhibitor MLN9708 against Multiple Myeloma Cells

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

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; ref. 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 Food and Drug Administration, 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), and there is therefore a need for development of novel proteasome inhibitors with equipotent efficacy and improved safety profile.

Recent preclinical pharmacology studies showed 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 analogue that was identified by screening a large pool of boron-containing proteasome inhibitors with physicochemical properties distinct from bortezomib (10, 11). In the present study, we examined the antitumor activity of MLN2238 using both in vitro and in vivo MM models.

Materials and Methods

Cell culture

MM.1S [dexamethasone (Dex)-sensitive], MM.1R (Dex-resistant), RPMI-8226, OPM1, OPM2, Iw929, 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 mmol/L L-glutamine). ANBL-6-bortezomib–sensitive (ANBL-6.WT) and -resistant (ANBL-6.BR) were kindly provided by Dr. Robert Orlowski (M.D. Anderson Cancer Center, Houston, TX). Tumor cells from MM patients were purified (>95% purity) by CD138+ selection using the AutoMACS magnetic cell sorter (Miltenyi Biotec Inc.). Informed consent was obtained from all patients in accordance with the Helsinki protocol. Peripheral blood mononuclear cells (PBMC) from normal healthy donors were maintained in culture medium, as described earlier. Bone marrow stromal cells (BMSC) were derived from CD138+ cells obtained from MM patients and cultured in Dulbecco’s modified Eagle’s medium containing 20% FBS. Drug sources are as follows: MLN2238 from Millennium: The Takeda Oncology Company; lenalidomide, bortezomib, and suberoylanilide hydroxamic acid (SAHA) were purchased from Selleck Chemicals LLC; and Dex was obtained from Calbiochem.

In vitro proteasome activity assays

Proteasome activity assay was conducted using the 20S Proteasome Assay Kit, SDS-Activated (Calbiochem) as previously described (12, 13), with some modifications. Briefly, MM.1S cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and 20 μg (10 μL) of protein was used in a total volume of 200 μL reaction buffer (20 mmol/L HEPES, pH 7.6, 0.5 mmol/L EDTA) with 0.03% SDS except for trypsin-like (T-L) activity assay. The substrates used for measuring chymotrypsin-like (CT-L), T-L, or caspase-like (C-L) proteasome activity were Suc-Leu-Leu-Val-Try-AMC (10 μmol/L), Bz-Val-Gly-Arg-AMC (50 μmol/L), and Z-Leu-Leu-Glu-AMC (10 μmol/L), respectively. The reaction was initiated by adding 10 μL of each substrate, and free 7-amino-4-methylcoumarin (AMC) fluorescence was quantified using a 380/460-nm filter set in a SpectraMax M2e fluorometer (Bucher Biotec AG).

Cell viability, proliferation, and apoptosis assays

Cell viability was assessed by MTT (Chemicon International Inc.; ref. 14) and CellTiter-Glo (Promega) assays, according to the manufacturer’s instructions. Cell proliferation analysis in coculture experiments with patient-derived BMSCs was carried out using thymidine incorporation, as described previously (14). Apoptosis was quantified using Annexin V/propridium iodide (PI) staining kit, as per manufacturer’s instructions (R&D Systems, Inc.), and analyzed on a FACS Calibur flow cytometer (Becton Dickinson).

Immunoblotting

Western blot analysis was carried out as previously described (15), using antibodies (Ab) against PARP (BD Biosciences Pharmingen), caspase-3, caspase-8, caspase-9, p21, E2F, cyclin D1, Cdk6, p-Rb (Cell Signaling), p53, NOXA, PUMA, Bip, CHOP, eIF2-α, or β-actin (Santa Cruz Biotechnology). Blots were then developed by enhanced chemiluminescence (ECL; Amersham).

NF-κB and HtrA2/Omi activity assay

MM.1S cells were treated with MLN2238 (12 nmol/L) at various times and harvested; total cellular proteins (1 μg) were subjected to p65 and p52 nuclear factor kappa B (NF-κB) activity analysis using ELISA, as per the manufacturer’s instructions (TransAM NF-κB Transcription Factor Assay Kits; Active Motif). The effect of MLN2238 versus 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 used as previously described (13, 15, 16). CB-17 severe combined immunodeficient (SCID) mice (n = 21; Taconic) were subcutaneously inoculated with 5.0 × 10⁶ MM.1S cells (31x36)
cells in 100 μL serum-free RPMI-1640 medium and randomized to treatment groups when tumors reached 250 to 300 mm³. Mice were treated with vehicle, bortezomib (1 mg/kg; i.v.) or MLN2238 (11 mg/kg; i.v.) twice weekly for 3 weeks. Animals were euthanized when their tumors reached 2 cm³.

**In situ detection of apoptosis and assessment of angiogenesis**

Mice tumor sections were subjected to immunohistochemical (IHC) staining for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (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 VEGF receptor 2 (VEGFR2), and PECAM-α-SMA expression (13). Immunostained tissues were imaged using confocal microscopy (FV1000; Olympus).

**Statistical analysis**

Statistical significance of differences observed in MLN2238- or bortezomib-treated versus control cultures was determined using the one-way ANOVA test. The minimal level of significance was \( P < 0.05 \). Survival of mice was measured using the GraphPad Prism software (version 5). Isobologram analysis (17) was carried out using "CalcuSyn" software program (Biosoft). Combination index (CI) values of less than 1.0 indicate synergism and values more than 1.0 antagonism.

**Results and Discussion**

**Effect of MLN2238 on proteasome activity in vitro**

We first examined the ability of MLN2238 to inhibit all 3 proteasome activities in MM cells. MM.1S cells were treated with various concentrations of MLN2238 for 3 hours and harvested; cell extracts were then analyzed for CT-L, C-L, and T-L proteasome activities, using specific fluorogenic peptide substrates. MLN2238 significantly inhibited CT-L proteasome activity with an IC₅₀ at 5 nmol/L (Fig. 1A; \( P < 0.05 \)). Higher concentrations of MLN2238 showed inhibitory activity against C-L and T-L proteasome activities (Fig. 1B and C, respectively). We next compared the effects of MLN2238 versus bortezomib on CT-L proteasome activity.

**Figure 1.** Proteasome inhibitor MLN2238 inhibits proteasome activity in vitro. A–C, MM.1S cells were treated with MLN2238 at indicated concentrations for 3 hours 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 versus 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, MM.1S cells were treated with MLN2238 (12 nmol/L) 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, MM.1S cells were treated with MLN2238 for 24 hours 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 nmol/L) or MLN2238 (12 nmol/L). Data presented are means ± SD (n = 2; \( P < 0.05 \)).
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, IJ929, OPM1, and OPM2) were treated with various concentrations of MLN2238 for 48 hours, followed by assessment for cell viability by 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<sup>+</sup>/PI<sup>−</sup> apoptotic cell population in MM.1S, IJ929, 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-8226 with TP53, K-Ras, and EGFR mutations; INA-6, an IL-6-dependent cell line with N-Ras activating mutation; IJ929 with t(4;14) translocation and mutated
As seen in Figure 2D, the IC50 ratio (ANBL-6.BR/ANBL-6.WT) and -resistant (ANBL-6.BR) MM cell lines, used previously characterized (25) bortezomib-sensitive and -resistant (ANBL-6.BR) MM cell lines. To determine whether MLN2238 overcomes bortezomib resistance, we treated MM cells 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 F, 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 occurs via VEGF pathway (26, 27). To determine whether MLN2238 triggers antiangiogenic activity, we directly evaluated tumors harvested from mice by immunostaining using 2 distinct markers of angiogenesis, VEGFR2 and PECAM. As shown in Figure 3D, MLN2238 decreased the number of TUNEL-positive (green color) cells compared with vehicle treatment (Fig. 3D). In addition to the parallel treatment of MM cells obtained from bortezomib-refractory patients showed increased in vitro cytotoxicity in response to MLN2238 versus 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. To further determine whether MLN2238 overcomes bortezomib resistance, we used previously characterized (25) bortezomib-sensitive (ANBL-6.WT) and -resistant (ANBL-6.BR) MM cell lines. As shown in Figure 2D, the IC50 ratio (ANBL-6.BR/ANBL-6.WT) of MLN2238 is significantly less than bortezomib (P < 0.01; n = 3), showing 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 endoplasmic reticulum (ER) stress levels may contribute to bortezomib resistance. These issues remain to be examined in a broader context of MM cells. In the present study, MLN2238 at the IC50 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 B, respectively). 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 with 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 F, respectively). These in vivo data confirm our in vitro findings showing potent apoptotic activity of MLN2238 against MM cells.

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 PARP, a signature event during apoptosis (Fig. 4A and B). Furthermore, MLN2238 induced cleavage of caspase-3, an upstream activator of PARP (Fig. 4A and B). Mitochondria mediate apoptotic signaling via activation of cell death initiator caspase, procaspase-9 (30). Similarly, Fas-associated death-domain (FADD) protein is an essential component of the death-inducing signaling complexes (DISC), resulting in autoactivation of procaspase-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 B, 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.
In addition, pan-caspase inhibitor (Z-VAD-FMK) also attenuated MLN2238-induced cytotoxicity (Fig. 4C; $P < 0.005$). Simultaneous blockade of caspase-8 and caspase-9 led to 89/C6 4.4% attenuation of MLN2238-triggered cell death. These findings suggest that (i) MLN2238 triggers both mitochondria-dependent and -independent signaling pathways and (ii) 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 the p53 pathway to activation of mitochondrial apoptotic signaling via BH3-only proteins NOXA and PUMA (33, 34); also, 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 B). 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 F). Similarly, cyclin D1 and its target protein Cdk6 were markedly decreased upon treatment with MLN2238 (Fig. 4E and F).

**Effect of MLN2238 on ER stress pathway.** Proteasome inhibition is associated with induction of the ER stress pathway and the unfolded protein response (13, 36, 37). Analysis of proteins mediating the ER stress pathway showed that MLN2238 induces eIf2-$\alpha$ 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 cross-talk between these pathways during MLN2238-induced apoptosis in MM cells. It is conceivable that MLN2238, like bortezomib, triggers pleiotropic signaling pathways; however, because of the shorter dissociation ($t_{1/2}$) characteristics of MLN2238 than bortezomib, the kinetics of alterations in stress response signaling may vary

Figure 3. MLN2238 inhibits growth of xenografted human MM cells in CB-17 SCID mice. A, average ± SD of tumor volume (mm$^3$) is shown from mice ($n = 7$/group) versus time (days) when tumor was measured. MM.1S cells ($5 \times 10^6$ cells/mouse) were implanted in the rear flank of female mice (6 weeks of age at the time of tumor challenge). On days 28 to 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 ± SD tumor volume. A significant delay in tumor growth in MLN2238-treated mice was noted compared with vehicle-treated control mice. Bars indicate means ± 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) compared with vehicle-treated control. C, mice were treated with vehicle, MLN2238, or bortezomib (as in A); blood samples were obtained and subjected to analysis for bilirubin, creatinine, and hemoglobin levels using Quantichrom Creatinine, Bilirubin, and Hemoglobin Assay kits (BioAssay Systems). D, micrographs (top 3 panels) show apoptotic cells in tumors sectioned from untreated or MLN2238-treated mice as identified caspase-3 (Casp3) cleavage (red cells), TUNEL (TUNEL-positive cells, green cells), as well as Ki-67 staining. Dotted red/green line indicates a line between tumor and host tissue. Micrographs (bottom 2 panels) show expression of angiogenesis markers in tumors sectioned from untreated or MLN2238-treated mice, identified by VEGFR2 (green) and PECAM (red) staining. Bar scale, 100 $\mu$m (caspase-3 and TUNEL); 50 $\mu$m (Ki-67); and 10 $\mu$m (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 ± SD tumor volume. Bars indicate means ± SD. F, the Kaplan–Meier survival plot shows increase in survival of mice receiving oral doses of MLN2238 (8 mg/kg) compared with vehicle-treated control.
and this issue remains to be defined. Nonetheless, our mechanistic data show that MLN2238-induced apoptosis in MM cells is caspase dependent and correlates with activation of p53–p21, p53–NOXA–PUMA, Rb–E2F, and ER stress signaling pathways.

MLN2238 overcomes the cytoprotective effects of the MM bone marrow microenvironment and inhibits in vitro capillary-like tube formation

Interaction of MM cells with 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 BMSC-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 bone marrow 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 a decrease in the expression of angiogenesis markers. To confirm the antiangiogenic activity of MLN2238, we utilized in vitro capillary-like tube structure formation assay using human vascular endothelial cells (HUVEC), which 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 antiangiogenic effects of drugs/agents. HUVECs were pretreated with vehicle [dimethyl sulfoxide (DMSO)] or MLN2238 (10 nmol/L) for 24 hours and harvested; protein lysates were subjected to immunoblotting with indicated Abs. G, MM.1S cells were treated with MLN2238 (12 nmol/L) for 24 hours and harvested; protein lysates were subjected to immunoblotting with indicated Abs. Blots shown in the figure are representative of 3 independent experiments.

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 24 hours 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, full length; CF, 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 1 hour. MLN2238 (12 nmol/L) was then added in cultures for additional 24 hours, followed by analysis of cell viability by MTT assay. Data presented are means ± SD (n = 3; P = 0.005). Error bars represent SD. D and E, MM.1S cells were treated with MLN2238 (12 nmol/L) for 24 hours and harvested; protein lysates were subjected to immunoblotting with indicated Abs. F, MM.1R cells were treated with MLN2238 (12 nmol/L) for 24 hours and harvested; protein lysates were subjected to immunoblotting with indicated Abs. G, MM.1S cells were treated with MLN2238 (12 nmol/L) 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.
for additional 4 hours, followed by analysis of tube formation using an inverted microscope. As seen in Figure 5B, tubule formation was significantly decreased in the MLN2238-treated cells but not after treatment with DMSO alone ($P < 0.001; n = 3$). HUVEC viability was assessed using trypan blue exclusion assay: less than 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 antiangiogenic activity of MLN2238.

MLN2238 targets 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 noncanonical 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–F; $P < 0.05; n = 3$). These data suggest that MLN2238 is a potent inhibitor of both canonical and noncanonical NF-κB pathways.

Combined treatment with MLN2238 and lenalidomide, dexamethasone, or histone deacetylase 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 histone deacetylase (HDAC) inhibitors (50). Given that MLN2238, like bortezomib, is a boronic acid analogue, 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 48 hours and then analyzed for viability by MTT assay. An analysis of synergistic anti-MM activity using the Chou and Talalay method (17) showed that the combination of low concentrations of MLN2238 and lenalidomide triggered synergistic anti-MM activity, with a CI < 1.0 (Fig. 6A).

In addition to proteasomal degradation, intracellular protein catabolism also occurs via an HDAC-dependent
aggressome-autophagy 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 the 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 48 hours 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 CI < 1.0 (Fig. 6B). 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 the 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 the use of lower doses and decreased toxicity.

Collectively, our preclinical studies therefore show potent in vitro and in vivo anti-MM activity of MLN2238 at doses that are well tolerated in human MM xenograft models.
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.

**Disclosure of Potential Conflicts of Interest**

K.C. Anderson, N. Raje, and P. Richardson are advisors to Millennium Pharmaceuticals, Inc. Other coauthors have no competing financial interests.

**References**


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