Preclinical Activity of the Oral Proteasome Inhibitor MLN9708 in Myeloma Bone Disease

Antonio Garcia-Gomez1,2,3, Dalia Quwaider1, Miriam Canavese5, Enrique M. Ocio1,3, Ze Tian6, Juan F. Blanco3, Allison J. Berger7, Carlos Ortiz-de-Solorzano8, Teresa Hernández-Iglesias1, Anton C.M. Martens8,9, Richard W.J. Groen6, Joaquin Mateo-Urdiales8, Susana Fraile1, Miguel Galarraga6, Dharminder Chauhan6, Jesús F. San Miguel1,2,3, Noopur Raje5, and Mercedes Garayo1,2,3

Introduction

Multiple myeloma is a hematologic malignancy resulting from the growth of plasma cells within the bone marrow (1). The presence of osteolytic lesions is a hallmark of the disease, characterized by an increase in bone-resorptive activity and number of osteoclasts and impairment of bone-forming activity and differentiation of osteoblasts (2, 3). Osteoclast hyperactivation and enhanced differentiation from precursors is primarily mediated through increased secretion of osteoclast-activating factors both by multiple myeloma cells and other cells from the bone marrow microenvironment [e.g., receptor activator of NF-κB ligand (RANKL), chemokine C-C motif ligand 3 (CCL3), interleukin (IL)-7, IL-3, activin A], whereas levels of osteoprotegerin, a soluble decoy receptor for RANKL, are reduced. At the same time, osteoclasts promote multiple myeloma cell survival and growth by the release of IL-6, CCL3, B-cell-activating factor and a proliferation-inducing ligand, creating a vicious cycle between bone lesions and tumor expansion (4, 5). On the other hand, reduced osteoblast formation and activity is in part sustained through augmented expression of Wnt and bone morphogenetic protein (BMP) signaling antagonists and other osteoblast suppressive factors (such as DKK-1, secreted frizzled-related protein 2, sclerostin, transforming growth factor-β, hepatocyte growth factor, activin A, IL-7, CCL3, and tumor necrosis factor-α; reviewed in refs. 2, 3, 5), and by blockade...

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Abstract

**Purpose:** MLN9708 (ixazomib citrate), which hydrolyzes to pharmacologically active MLN2238 (ixazomib), is a next-generation proteasome inhibitor with demonstrated preclinical and clinical anti-myeloma activity, but yet with an unknown effect on myeloma bone disease. Here, we investigated its bone anabolic and antiresorptive effects in the myeloma setting and in comparison with bortezomib in preclinical models.

**Experimental Design:** The *in vitro* effect of MLN2238 was tested on osteoclasts and osteoclast precursors from healthy donors and patients with myeloma, and on osteoprogenitors derived from bone marrow mesenchymal stem cells also from both origins. We used an *in vivo* model of bone marrow–disseminated human myeloma to evaluate MLN2238 antitymoma and bone activities.

**Results:** Clinically achievable concentrations of MLN2238 markedly inhibited *in vitro* osteoblastogenesis and osteoclast resorption; these effects involved blockade of RANKL (receptor activator of NF-κB ligand)-induced NF-κB activation, F-actin ring disruption, and diminished expression of αδβ3 integrin. A similar range of MLN2238 concentrations promoted *in vitro* osteoblastogenesis and osteoblast activity (even in osteoprogenitors from patients with myeloma), partly mediated by activation of TCF/β-catenin signaling and upregulation of the IRE1 component of the unfolded protein response. In a mouse model of bone marrow–disseminated human multiple myeloma, orally administered MLN2238 was equally effective as bortezomib to control tumor burden and also provided a marked benefit in associated bone disease (sustained by both bone anabolic and antitaboletic activities).

**Conclusion:** Given favorable data on pharmacologic properties and emerging clinical safety profile of MLN9708, it is conceivable that this proteasome inhibitor may achieve bone beneficial effects in addition to its antitymoma activity in patients with myeloma. *Clin Cancer Res; 20(6); 1542–54. ©2014 AACR.*

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Translational Relevance

Second-generation proteasome inhibitors are now being evaluated as putative alternatives to bortezomib for the treatment of patients with multiple myeloma. In this context, MLN9708 (ixazomib citrate), hydrolyzing to pharmacologically active MLN2238 (ixazomib), is an orally bioavailable proteasome inhibitor with demonstrated preclinical and clinical activity against multiple myeloma, but yet with unknown beneficial effect on myeloma bone disease (MBD).

Our in vitro data demonstrate that clinically relevant concentrations of MLN2238 promote osteoblast differentiation and function and inhibit osteoclast formation and resorption. In a mouse model of disseminated human myeloma, oral administration of MLN2238 was found to be at least equally effective as intraperitoneally administered bortezomib in the control of myeloma growth and in prevention of bone loss, sustained by both bone-forming and anticasletic activities. These data provide the rationale for clinical assessment of the bone effects of MLN9708 in patients with MBD.

of activity of the critical osteoblast transcription factor Runx2/Cbfa1 via cell–cell contact interactions of myeloma and osteoprogenitor cells (6).

The incorporation of the first in class proteasome inhibitor, bortezomib, to the antimyeloma armamentarium can be considered one of the major milestones in the treatment of patients with myeloma, greatly improving the response rates and overall survival in both front-line and relapsed/refractory settings (7). Bortezomib is a dipeptide boronic acid, primarily inhibiting the chymotrypsin-like activity (β5 subunit) of the 20S proteasome with slowly reversible kinetics (110 minutes in an in vitro enzyme assay; ref. 8). Myeloma plasma cells seem to be especially sensitive to bortezomib, and multiple effects have been reported after bortezomib treatment (e.g., blockade of NF-κB activity, cell-cycle arrest, induction of intrinsic and extrinsic apoptosis, inhibition of DNA repair enzymes, and inhibition of adhesion to bone marrow stromal cells), which likely contribute to the clinical success of this agent in multiple myeloma (reviewed in ref. 9). Moreover, bortezomib also restrains the progression of myeloma bone disease (MBD), as evidenced by changes in bone turnover markers and radiologic data favoring bone healing (10–12). This beneficial impact of bortezomib on bone metabolism is not merely secondary to its antimyeloma activity, but rather this agent acts directly interfering with osteoclastogenesis and resorption and promoting osteoblast formation and function, as shown in numerous in vitro studies (13–15), and in myelomatous and nonmyelomatous in vivo models (16, 17).

However, some patients are not sensitive to bortezomib, and main limitations associated with its use are the eventual development of resistance and the emergence of peripheral neuropathy (8). This has prompted the development of structurally different second-generation proteasome inhibitors, with the aim of retaining or improving bortezomib efficacy, overcoming bortezomib resistance, and having a safer toxicity profile. In fact, several next-generation proteasome inhibitors are currently under various stages of clinical evaluation (7).

In this context, MLN9708 was selected from a pool of bortezomib-resistant myeloma cell lines (23), suggesting that this agent, similar to bortezomib, may also display direct regulatory effects on bone remodeling. We have, therefore, conducted in vitro studies to comparatively evaluate the ability of MLN2238 (relative to that of bortezomib) to promote the osteogenic differentiation from mesenchymal progenitors from both healthy donors and patients with myeloma, and also to inhibit osteoclast formation and function. In addition, we present evidence of antimyeloma and global bone anabolic effects of orally administered MLN2238 in a bone marrow–disseminated human myeloma mouse model, demonstrating both antiresorptive and bone anabolic activities of this agent.

Materials and Methods

Reagents and immunochemicals

Bortezomib (Velcade) and MLN2238 were provided by Millennium Pharmaceuticals, Inc. Melphalan was purchased from Sigma-Aldrich. Macrophage colony-stimulating factor (M-CSF) and RANKL were obtained from Peprotech. All cell culture media and reagents were supplied by Gibco. Primary antibodies were directed against: p65, ERK (extracellular signal–regulated kinase), β-catenin, phosphorylated β-catenin, phospho-GSK3β (glycogen synthase kinase 3β), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and IRE1α.


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(inositol-requiring protein 1α) (Cell Signaling Technology), phospho-ERK (Santa Cruz Biotechnology), α-tubulin (Calbiochem), T-cell factor 4 (TCF4; TCF7L2; Millipore), and CD51/61 (R&D Systems). Rhodamine-conjugated phalloidin was purchased from Invitrogen.

**Cell cultures**

Human multiple myeloma cell lines MM.1S and MM.1R were provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). The MM.1S-luc cell line was a gift from Dr. C.S. Mitsiades (Dana-Farber Cancer Institute, Boston, MA), whereas RPMI8226 cells purchased from the American Type Culture Collection were lentivirally transduced to stably express firefly luciferase as in Groen and colleagues (24). Cell viability of multiple myeloma cells in monoculture was assessed by MTT assay as previously described (19).

The human mesenchymal stem cell line immortalized by expression of the telomerase reverse transcriptase gene (hMSC–TERT) was obtained from Dr. D. Campana (St. Jude Children’s Research Hospital, Memphis, TN). Primary MSCs derived from the bone marrow of healthy donors (n = 5), and patients with multiple myeloma with (n = 5) or without (n = 3) osteolytic lesions were generated as described by Garayoa and colleagues (25). These studies were performed with the approval of the Hospital Universitario de Salamanca Review Board, and samples were obtained after written informed consent of subjects.

**In vitro osteoclast formation, resorption pits, F-actin ring formation, and CD51/61 expression**

Peripheral blood mononuclear cells (PBMCs) from healthy donors were differentiated by culture in osteoclastogenic medium (containing 25 ng/mL M-CSF and 50 ng/mL RANKL) as described in Garcia-Gomez and colleagues (26). Alternatively, PBMCs from patients with myeloma were also used. Assays related to osteoclast formation and function included: RANKL-induced NF-kB activation, F-actin ring formation, and CD51/61 (αVβ3 integrin) expression (preosteoclasts, 14 days differentiation); resorption capacity (17 days differentiation); and osteoclast formation (21 days differentiation), and were performed as in Hurchla and colleagues (27).

**In vitro osteoblast differentiation, alkaline phosphatase activity, and mineralization assays**

Osteoblasts were generated from mesenchymal osteoprogenitors by culture in osteogenic medium (containing 5 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid, and 80 nmol/L dexamethasone) and assayed as in Garcia-Gomez and colleagues (26). Briefly, primary MSCs (passage 2–3) or the hMSC–TERT cell line were exposed to different MLN2238 or bortezomib concentrations and cultured in osteogenic medium for 11 or 21 days for alkaline phosphatase (ALP) activity and matrix mineralization assays, respectively. ALP activity was quantified by hydrolysis of p-nitrophenylphosphate into p-nitrophenol (Sigma-Aldrich), whereas mineralization was assessed by quantitative measurement of Alizarin Red staining.

**Reverse transcription real-time PCR analyses**

The hMSC–TERT cell line was cultured for 14 days in osteogenic medium (preosteoblasts) and total RNA was isolated using TRIzol reagent (Invitrogen). TaqMan Gene Expression Assays (Applied Biosystems) were performed according to the manufacturer’s instructions: Runx2, Hs01047976_m1; Osterix, Hs00541729_m1; Osteopontin, Hs00959010_m1; Osteocalcin, Hs01587814_g1 and DKK-1, Hs00183740_m1. The endogenous expression of GAPDH was used for normalization and relative quantification of target gene expression was calculated by the comparative threshold cycle method.

**TGF luciferase reporter assay**

MSCs (3 × 10⁵) were transiently cotransfected with either 400 ng/mL TOPflash or FOPflash firefly luciferase reporters (Addgene plasmids #12456-7; ref. 28) and 40 ng/mL pRL-SV40 Renilla luciferase reporter (Promega), using Nucleofector II as per the manufacturer’s instructions (Lonza). After overnight recovery, cells were treated with or without proteasome inhibitors for 24 hours and luciferase activity was evaluated using a Dual-Luciferase Reporter System (Promega). The firefly luciferase/Renilla luciferase ratio was calculated to normalize for transfection efficiency.

**Gene silencing**

hMSC–TERT cells were transfected (three times/week for 3 weeks) using SAFectin-STEM (Delveric) as per the supplier’s instructions, either with ON-TARGETplus SMARTpool siRNA targeting human IRE1 or ON-TARGETplus nontargeting pool as negative control (Dharmacon).

**Immunoblotting**

Generation of protein lysates and Western blotting were performed following standard procedures (26). A luminol detection system with p-iodophenol enhancement for chemiluminescence was used for protein detection.

**MM.1S-luc cocultures**

The hMSC–TERT cell line was plated overnight in 96-well plates (1.25 × 10⁴ cells/well) and MM.1S-luc cells (2 × 10⁴/well) were then added and cocultured for 48 hours in medium supplemented with 0.5% fetal bovine serum (FBS). Similarly, MM.1S-luc cells (4 × 10⁴/well) were added to previously generated preosteoclasts and maintained in the same low-serum medium for 5 days. MM.1S-luc growth was assessed by measurement of luciferase activity.

**Mouse model of bone marrow–disseminated human multiple myeloma**

Animal experiments were conducted according to institutional guidelines for the use of laboratory animals and after acquired permission from the local Ethical Committee for animal experimentation. Bortezomib and melphalan were solubilized in 0.9% saline, whereas MLN2238 was prepared in a 5% solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich). RPMI8226-luc cells (5 × 10⁴) were injected intravenously into 6-week-old NOD-SCID-IL-2Rγ−/− (NSG)
mice (Charles River Laboratories) and tumor development was monitored by noninvasive bioluminescence imaging (BLI) with a Xenogen IVIS 50 system (Caliper Life Sciences). After 3 weeks, animals were randomized into four groups (\( n = 6 \)/group) receiving: vehicle (5% solution of 2-hydroxypropyl-\( \beta \)-cyclodextrin, 2 times/week by oral gavage), melphalan (2.5 mg/kg, three times/week by oral gavage), bortezomib (0.8 mg/kg, three times/week by intraperitoneal injection), and MLN2238 (7.5 mg/kg, 2 times/week by oral gavage). Serum levels of human Ig \( \lambda \) (secreted by RPMI8226-luc cells) were also used as a measure of tumor burden and determined by ELISA (Bethyl Laboratories).

**Microcomputed tomography analysis**

One femur of each animal was fixed in 10% formalin and scanned using a microcomputed tomography (microCT) system (MicroCATII; Siemens) as described previously [26]. Analysis of trabecular microarchitecture in the distal femur was performed using BoneJ (29), a bone morphometry image analysis ImageJ plugin.

**Bone turnover markers**

Carboxy-terminal telopeptide collagen cross-links (CTX; indicating bone resorption) and N-terminal propeptide of type I procollagen (P1NP; indicating bone formation) were measured in mice sera using ELISA systems (Immunodiagnostic Systems).

**Histologic and immunohistochemical analyses**

The other femur from each animal was fixed in 10% formalin, decalcified, and paraffin embedded. Bone histologic evaluation on paraffin sections was performed after hematoxylin–eosin or immunohistochemical staining for TCF4 as previously described [26].

**Statistical analyses**

Each in vitro assay was performed at least three times using MSCs or PBMCs from different individuals, and duplicates (real-time PCR) or triplicates were measured for each of the tested conditions. Quantitative data are expressed as mean \( \pm \) SD or SEM, as specified. The non-parametric Mann–Whitney \( U \) test was used for comparison of vehicle control and specific treatments, and differences considered statistically significant for values of \( P < 0.05 \) (SPSS Statistics 15.0).

**Results**

MLN2238 inhibits in vitro myeloma cell growth even in the presence of microenvironment support

The antimyeloma activity of MLN2238 and bortezomib on various myeloma cell lines (dexamethasone-sensitive MM.1S, dexamethasone-resistant MM.1R and RPMI8226) was evaluated after a 48-hour culture by MTT assay (Fig. 1A and B). In agreement with previous reports, IC_{50} for MLN2238 ranged from \( \geq 10 \) to 17 nmol/L [19], whereas bortezomib presented a more potent cytotoxic effect (IC_{50}...
ranging from $\geq 1.7\text{–}3\text{ nmol/L}$ for the same myeloma cell lines (Fig. 1A and B).

MSCs and osteoclasts within the bone marrow microenvironment have been reported to support growth and survival of myeloma cells and to protect multiple myeloma cells from chemotherapy-induced apoptosis (4, 30). We therefore tested the ability of MLN2238 to decrease the growth of MM.1S luc cells when grown in coculture with MSCs (Fig. 1C) or osteoclasts (Fig. 1D). As expected, higher doses of both bortezomib and MLN2238 were necessary to inhibit MM.1S luc cell growth in the coculture as compared with monoculture conditions. Overall, although MLN2238 clearly overcame the proliferative advantage conferred by microenvironmental cells (Fig. 1C and D), higher MLN2238 concentrations were required to attain similar efficacy as bortezomib on myeloma cells.

**MLN2238 inhibits in vitro osteoclastogenesis and resorption**

To compare the relative potency of MLN2238 versus bortezomib on osteoclast formation, osteoclastogenic cultures were exposed to a wide range of concentrations of each agent along the differentiation process. As previously shown (13), bortezomib markedly inhibited the formation of TRAP$^+$ multinucleated cells ($IC_{50} \approx 1.2\text{ nmol/L}$) derived from PBMCs of healthy donors. MLN2238 was able to suppress osteoclastogenesis with similar efficacy but when added at higher concentrations ($IC_{50} \approx 10.9\text{ nmol/L}$; Fig. 2A). A higher sensitivity to MLN2238 inhibitory effect on osteoclast formation was observed when using PBMCs from patients with myeloma ($IC_{50} \approx 4.8\text{ nmol/L}$; data not shown). Noteworthy, as observed in representative micrographs of Fig. 2A, cell densities in our cultures at 10 nmol/L MLN2238 (a concentration similar to the $IC_{50}$ for this agent on osteoclast formation) were not significantly reduced, thus, suggesting a selective effect of MLN2238 on inhibition of osteoclast formation rather than an effect on cell viability.

We next examined the effect of MLN2238 on osteoclastogenic cultures established on calcium substrate-coated slides. A marked and dose-dependent reduction of the area of resorbed pits was observed with MLN2238 treatment (Fig. 2B), similarly to that already reported for bortezomib (13, 31). Specifically for MLN2238, doses required to inhibit osteoclast resorption were well below the ones required to inhibit osteoclast formation, thus suggesting that this proteasome inhibitor not only inhibits osteoclastogenesis but also has a particular effect on osteoclast resorption.

It is known that early osteoclast differentiation mediated by RANKL–RANK stimulation is primarily mediated through the adapter protein TRAF6 (TNF receptor-associated factor 6), which then leads to activation of mitogen-activated protein kinases (MAPK; i.e., c-Jun N-terminal kinase, p38, and ERK), NF-κB, and activator protein-1 (32). To gain some insight into the mechanisms mediating MLN2238 effect in inhibition of osteoclast formation and function, we first tested its effect on MAPK pathway activation. As shown in Fig. 2C, both MLN2238 and bortezomib inhibited RANKL-stimulated phosphorylation of ERK.

MLN2238 inhibits in vitro osteoclastogenesis and resorption

**MLN2238 promotes in vitro osteogenic differentiation and matrix mineralization of osteoprogenitor cells from patients with myeloma**

To check whether MLN2238 was capable of promoting osteoblast differentiation and function in vitro, primary MSCs from patients with myeloma (5/8 with bone lesions) were allowed to differentiate in osteogenic medium along with different concentrations of the agent, and ALP activity was determined as a surrogate marker of early osteoblastic activation. As observed in Fig. 3A, MLN2238 dose-dependently increased ALP activity in early-stage osteoblasts to levels similar to that attained by bortezomib. Akin to its effect on ALP activity, MLN2238 also augmented matrix mineralization and calcium deposition when present along the complete osteogenic differentiation process (Fig. 3B). MLN2238 and bortezomib were capable of increasing ALP activity and/or mineralization of MSCs both from myeloma patients with osteolytic lesions (see representative micrographs in Fig. 3B) and without osteolytic lesions, although basal levels of these parameters without treatment were generally higher in osteoblasts derived from the latter MSCs. Likewise, increased ALP activity and matrix mineralization were observed with mesenchymal progenitors derived from healthy donors (data not shown).

The relative expression of several late bone formation markers (namely, the downstream Osterix transcription factor, osteopontin, and osteocalcin) was also determined after proteasome inhibitor treatment of MSCs from patients with myeloma at concentrations yielding maximal induction of ALP activity and bone mineral formation (Fig. 3C). Both proteasome inhibitors significantly augmented the expression of the mentioned osteoblast markers. Because bortezomib has been found to suppress DKK-1 expression...
in mouse calvarial cultures and bone marrow stromal cells (14), we tested whether a similar effect could be obtained in our experimental setting. MLN2238 was also capable of achieving a modest yet significant reduction of DKK-1 mRNA levels in preosteoblasts differentiated from MSCs from patients with myeloma (Fig. 3C).

**MLN2238 activates TCF4/β-catenin signaling and the UPR response in mesenchymal precursors**

Numerous studies have demonstrated a pivotal regulatory role for the canonical Wnt signaling pathway in commitment of mesenchymal progenitors to the osteoblast lineage, in osteoblast function, and in skeletal development.
Upon binding of canonical Wnt ligands to appropriate receptors in osteoprogenitors, β-catenin is stabilized and translocated to the nucleus where it interacts with a member of the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family, to synergistically regulate the expression of osteoblast differentiating genes (i.e., Runx2; ref. 35). Because TCF4 is the most abundant TCF family transcription factor expressed in osteoblast cell lines and primary hMSCs (37), we explored whether MLN2238 treatment was able to enhance TCF4 transcriptional activity using a TOPflash reporter system. When MSCs from patients with myeloma were proteasome inhibitor treated for 24 hours, a dose-dependent increase in luciferase activity of the reporter plasmid was observed (Fig. 4A). Therefore, activation of TCF4 transcriptional activity seems to mediate, at least in part, MLN2238 promotion of osteoblastogenesis and osteoblast function.

Stabilization and nuclear translocation of β-catenin is also used as a metric of enhanced canonical Wnt signaling, and thus, we also tested whether MLN2238 could modulate β-catenin levels. As seen in Fig. 4B, MLN2238 treatment of MSCs from patients with myeloma slightly upregulated levels of dephosphorylated β-catenin, which is the stabilized active form of β-catenin able to interact with TCF/LEF transcription factors. These increased stabilized β-catenin levels may be ascribed to a modest inhibition of GSK3β activity due to increased phosphorylation (Fig. 4B; ref. 38). Taken together, our data show that MLN2238 treatment leads to increased TCF4 transcriptional activity along with modest augmented levels of active β-catenin, thus, leading to activation of TCF4/β-catenin signaling on osteoprogenitor cells.

Osteoblasts produce large amounts of extracellular matrix proteins and must activate the unfolded protein response (UPR) to handle the accumulation of misfolded proteins. In fact, the IRE1α–XBP1 (X-box binding protein 1) pathway of the UPR has been recently shown to be essential for osteoblast differentiation by driving the transcription of Osterix (39). Increased levels of IRE1α protein were readily observed after bortezomib and MLN2238 treatment of hMSC–TERT cells (Fig. 4C). Conversely, silencing of IRE1α markedly abrogated proteasome inhibitor–enhanced mineralization (Fig. 4D and E), thus underscoring the essential role of the IRE1α component of the UPR in the promotion of osteoblast function by proteasome inhibitors.

Figure 3. MLN2238 increases ALP activity, stimulates matrix mineralization, and augments the expression of bone formation markers in in vitro differentiating osteoblasts (OB). A, primary MSCs from patients with myeloma were cultured in osteogenic medium in the presence or absence of proteasome inhibitors at indicated concentrations and ALP activity was measured at day 11. Results, mean ± SEM. B, matrix mineralization was assessed by Alizarin Red staining and subsequent dye quantification in OBs differentiated from MSCs from patients with MM at day 21. Graphs, mean values ± SD. Representative images derived from MSCs of a patient with osteolytic lesions are shown. C, total RNA was isolated from primary MSCs from patients with MM differentiated toward the osteogenic phenotype for 14 days in the presence or absence of bortezomib or MLN2238. Real-time RT-PCR was performed to determine the expression of osteoblastogenic markers (Osterix, osteopontin, and osteocalcin), together with the expression of the Wnt signaling inhibitor DKK-1. Expression levels for each gene were normalized to GAPDH expression and referred to those in the absence of proteasome inhibitors. Data, mean ± SD. *, P < 0.05 versus vehicle control.
Figure 4. MLN2238 promotes OB differentiation and function through activation of TCF/ß-catenin signaling and the UPR. A, primary MSCs from patients with myeloma were transiently transfected with the TOPFlash (containing 7 TCF/LEF–binding sites) or FOPFlash (with 6 mutant TCF/LEF–binding sites) luciferase reporters. One day after transfection, MSCs in osteogenic medium were treated for 24 hours with indicated concentrations of bortezomib or MLN2238 and luciferase activity was measured in cell lysates. Results, mean ± SD. Negative control (NC) refers to luciferase activity after transfection with the FOPFlash reporter at 10 nmol/L bortezomib and 100 nmol/L MLN2238. B, MSCs from patients with MM were differentiated for 7 days in osteogenic medium and treated with 2.5 nmol/L bortezomib or 10 nmol/L MLN2238 for 72 hours. Levels of active (dephosphorylated) ß-catenin and inhibited (phosphorylated) GSK3ß were evaluated by Western blot analysis. C, proteasome inhibitor treatment (bortezomib 25 nmol/L and MLN2238 100 nmol/L) of hMSC–TERT cells over a 24-hour period in osteogenic medium augmented IRE1a levels as determined by Western blot analysis. D, transfection of hMSC–TERT cells with IRE1a targeting siRNAs (100 nmol/L, 48 hours) effectively diminished IRE1a expression at the mRNA and protein level. NC refers to IRE1a expression after transfection with nontargeting siRNAs. E, the hMSC–TERT cell line was maintained in osteogenic medium for 21 days and transfected three times per week with IRE1a or NC siRNAs. When IRE1a was silenced, matrix mineralization was abolished even in the presence of proteasome inhibitors (5 nmol/L bortezomib; 10 nmol/L MLN2238). Data, mean ± SD of at least three independent transfections for each condition, each of them run in triplicate. For A, D, and E: *P < 0.05 versus vehicle or NC.

**MLN2238 prevents tumor-associated bone loss besides reducing multiple myeloma tumor burden**

We next explored whether proosteogenic, antosteoclastogenic, and antimyeloma in vitro effects of MLN2238 could be translated into a disseminated murine model of human myeloma (27). RPMI8226-luc cells were intravenously injected to NSG mice and myeloma engraftment (as monitored by bioluminescence) occurred after 3 weeks. At this point mice were randomized to receive vehicle, melphalan, bortezomib, or MLN2238 treatments as indicated (see Materials and Methods; Fig. 5A). Melphalan was used as a control drug with expected antimyeloma activity but not a bone effect (17). Compared with the vehicle control group, both orally administered MLN2238 and intraperitoneally delivered bortezomib very efficiently controlled tumor progression, as measured by bioluminescence (Fig. 5A) or by serum levels of hIgG, secreted by RPMI8226-luc cells (Fig. 5B). Melphalan treatment, at doses used in our study, reduced myeloma burden at only about 50% of levels observed with MLN2238 or bortezomib (Fig. 5A and B).

Representative microCT images at the metaphyses of distal femurs showed evident tumor-associated bone loss in vehicle and melphalan-treated mice, in contrast with trabecular structures observed in both MLN2238 and bortezomib-treated animals (Fig. 6A). This was also reflected by bone morphometric parameters which resulted in increased trabecular bone volume and connectivity and reduced trabecular separation only in proteasome inhibitor–treated animals, as compared with vehicle control (Fig. 6B–D). In agreement with these data, histologic sections of MLN2238 and bortezomib-treated mice presented higher number of trabeculae at the growth plate of the distal femur (Fig. 6E), together with immunoreactive TCF4 osteoblasts lining the trabeculae (Fig. 6F). Of interest, TCF4-positive osteoblasts in proteasome inhibitor–treated mice presented a square or rectangular section indicative of active bone matrix secretion, whereas flat osteoblasts in vehicle and melphalan groups reflected a resting secretory state (Fig. 6G). Finally, these findings correlated with levels of bone turnover markers analyzed in serum after drug treatment. The bone resorption marker CIX was only significantly diminished in proteasome inhibitor–treated animals (Fig. 6H). P1NP, a bone formation marker, augmented significantly in the proteasome inhibitor–treated groups versus the control
group (Fig. 6I), whereas melphalan-treated animals showed no change.

Discussion

Osteolytic lesions are the most common complication of myeloma, being developed in more than 80% of patients suffering the disease. These lesions often lead to skeletal-related events (SRE), including focal lytic lesions, pathologic fractures, hypercalcemia, severe bone pain, and vertebral compression fractures (2, 40). Importantly, MBD not only negatively affects the quality of life of patients with myeloma, but has been found to be associated with decreased overall survival (41). This highlights the need for supportive bone-targeting treatments besides antimyeloma chemotherapies to reduce the risk of bone complications in multiple myeloma. Moreover, because many of the factors dysregulated in MBD are also important contributors to myeloma progression and chemoresistance, it is expected that restoring bone homeostasis would indirectly and additionally lead to tumor inhibition (42).

Bisphosphonates are anticatabolic agents representing the current standard supportive treatment for patients with myeloma with MBD. Being pyrophosphate analogs, bisphosphonates become adsorbed to mineralized matrix and cause apoptosis when osteoclasts incorporate them by endocytosis (43). Second-generation nitrogen-containing bisphosphonates now in use, such as pamidronate and zoledronic acid, have dramatically reduced the incidence of SREs and significantly improved the quality of life in patients with multiple myeloma (40). Nevertheless, adverse side effects may result from bisphosphonate therapy, including osteonecrosis of the jaw, impaired renal function, and accumulation of bone microfractures (2, 44); in addition, MBD may progress during bisphosphonate treatment, presumably because these compounds inhibit bone resorption but do not restore bone formation (3). Recent progress in the understanding of the molecular interplayers involved in MBD has stimulated the development alternative bone therapeutic compounds either focusing on osteoclast inhibition, or importantly, on osteoblastogenesis promotion (reviewed in refs. 2, 40, 42). It is likely that these agents, now at different stages of clinical development, will contribute to a more efficacious and improved management of MBD in the near future.

Within current agents used in antimyeloma chemotherapy, bortezomib is unique because of its concurrent anti-myeloma, antitumor activity, and bone anabolic activities, as evidenced in multiple preclinical studies and in bortezomib-treated patients (10, 13, 15, 17). This represents an unprecedented advantage for the treatment of both multiple myeloma and MBD either as single agent (12) or in certain combinations with other anti-myeloma drugs (11). Preclinical studies with second-generation proteasome inhibitors carfilzomib and ONX0912, have shown that in addition to their anti-myeloma properties (45, 46) and similar to bortezomib, they effectively target osteoblast and osteoclast populations to shift the bone microenvironment from a catabolic to an anabolic state (27). Akin to these proteasome inhibitors, herewith, we show that MLN2238 also combines antitumor activity with antiresorptive and bone-forming effects on bone.

As observed in our in vitro experiments, MLN2238 markedly inhibited osteoclast formation from human PBMC progenitors as well as osteoclast resorption. Mechanistically, these effects on osteoclast function were at least partially mediated through inhibition of RANKL-induced NF-κB signaling. F-actin ring disruption, and diminished expression of the αVβ3 integrin, molecular sequelae also shared by bortezomib and other proteasome inhibitor.
treatments (13, 31, 33). Proteasome inhibition has also been linked to induced in vitro differentiation of MSCs into osteoblasts and enhanced osteoblast function (14, 15, 37).

We present here that, in a similar range of concentrations affecting osteoclasts, MLN2238 was also able to stimulate the in vitro osteogenic differentiation of MSCs and to promote osteoblast function; of interest, MLN2238 effects were observed on MSCs irrespective of being derived from healthy donors or patients with myeloma (including those from patients with osteolytic lesions). As already reported for bortezomib and carfilzomib (37, 47), MLN2238 dose-dependently increased TCF transcriptional activity in reporter assays on osteoprogenitors. In relation with TCF transactivation, we also found that MLN2238 treatment was linked to modest increased levels of active dephosphorylated β-catenin (35), all of which would enhance TCF/β-catenin signaling and promote osteoblast function. Interestingly, we have also shown that MLN2238 treatment markedly augments levels of the IRE1α component of the UPR, and as evidenced by siRNA strategies, reduced levels of this protein impaired matrix mineralization even in the presence of proteasome inhibitors. This is in agreement with the results obtained with MLN2238-treated mice in our in vivo experiments.

Figure 6. MLN2238 prevented myeloma-associated bone loss. A, representative microCT cross-sections at the metaphyses of distal femurs show loss of trabecular architecture in vehicle and melphalan-treated mice, but not in mice treated with bortezomib or MLN2238. B to D, as compared with vehicle control animals, MLN2238 significantly increased bone parameters of trabecular bone volume and connectivity, whereas trabecular bone separation was decreased. Results, mean ± SD. *P < 0.05 versus vehicle control. E and F, representative femur sections after drug treatment stained with hematoxylin and eosin (E), or for TCF4 immunohistochemistry (F). Bar, 25 μm. Dotted line delimits infiltrating RPMI8226-luc cells and host tissue. OB-like cells immunostained for TCF4 can be observed lining the trabeculae (arrowheads). G, representative images at higher magnification showing flat OBs lining the trabeculae of vehicle-treated animals (arrows), whereas OBs were cuboidal in shape and presented TCF4 immunostaining in MLN2238-treated mice (arrowheads). Bar, 12.5 μm. H and I, MLN2238 significantly changed bone turnover markers in serum, increasing levels of P1NP, a bone formation marker, while decreasing levels of CTX, a bone resorption marker, as compared with vehicle-treated animals. H and I, graphs, mean values ± SEM. *P < 0.05; **P < 0.01 versus vehicle or between indicated groups.
with recent reports highlighting the critical role of the UPR, and specifically the IRE1α–XBP1 pathway, in osteoblast differentiation through promotion of Osterix transcription (39). Although both myeloma cells and MSCs/osteoblasts are protein secretory cells dependent on the UPR to eliminate misfolded proteins and to cope with endoplasmic reticulum (ER) stress, proteasome inhibitor treatment exerts apoptosis on the former and promotes osteogenic differentiation on the latter. The reason for these differential effects may reside on the lower threshold of multiple myeloma cells for proteasome inhibitor–induced terminal UPR and ER stress–induced apoptosis, because these cells already highly express prosurvival UPR components to function as secretory cells (48).

Our studies on osteoclasts and osteoblasts, as well as those on myeloma cell lines alone or in coculture with microenvironment cells, clearly showed that MLN2238, although less potent, reached a similar effect as bortezomib. This difference in potency may relate to the shorter proteasome dissociation half-life of MLN2238 compared to bortezomib (8), thus, higher concentrations of MLN2238 being needed to attain an equivalent effect. Nevertheless, clinical pharmacokinetic studies from once-weekly orally administered MLN9708 estimate plasma Cmax ~ 150 nmol/L (49), well over the required in vitro doses for the effects of MLN2238 on osteoclasts and osteoblasts in our studies. It is therefore conceivable that therapeutically administered MLN9708 will achieve antiresorptive and proanabolic bone effects (besides its antimyeloma activity) in patients with myeloma.

In fact, as observed in our in vivo murine model, MLN2238 and bortezomib treatments were very efficient in controlling the growth of RPMI8226-luc tumor cells in the bone marrow, as assessed by decreased BLI and diminished serum levels of human IgG. Increased bone trabecular architecture in MLN2238 and bortezomib-treated animals was evident on microCT images at metaphyses of distal femurs as compared with vehicle control, correlating with significantly augmented bone parameters of trabecular bone volume and connectivity and reduced trabecular separation. This global bone anabolic effect of MLN2238 and bortezomib was also associated with (i) increased trabeculae at the growth plate as observed on histologic sections, (ii) immunostaining for TCF4 and active morphologic appearance of osteoblast cells lining the trabeculae, and (iii) reduced CTX (bone resorption marker) and increased P1NP (bone formation marker) serum levels. All this evidence clearly supports, in addition to our in vitro data, that these proteasome inhibitors have a dual direct effect on bone, both promoting bone formation and inhibiting bone resorption. To exclude the possibility that the effects of MLN2238 on bone were a secondary consequence of its antimyeloma activity, we compared the in vivo effect of MLN2238 with that of melphalan, an agent with accepted antimyeloma activity but no direct effects on bone (17). Although with our specific in vivo melphalan dosing and schedule this compound was only ~50% as effective as bortezomib or MLN2238 in controlling tumor burden, its antimyeloma activity was not linked to an indirect effect on bone, with no significant changes observed with respect to vehicle control on bone turnover serum markers, trabecular architecture, osteoblast histologic appearance, or TCF4 immunostaining. These data reinforce our previous interpretation of bone effects of MLN2238 being primarily due to direct targeting of osteoblast and osteoclast populations in the bone marrow, albeit indirect bone beneficial consequences of its antimyeloma activity cannot be excluded.

Taken together, we have shown that clinically relevant concentrations of the next-generation proteasome inhibitor MLN2238, promote osteoblast differentiation and function (even in osteoprogenitor cells from multiple myeloma patients bearing osteolytic lesions) and inhibit osteoclast formation and resorption in ex vivo experiments. In a mouse model of disseminated human myeloma, oral administration of MLN2238 was found to be at least as effective as intraperitoneally administered bortezomib in the control of myeloma growth and in prevention of bone loss, sustained by both bone-forming and anticytoblastic activities. Our studies, therefore, provide rationale for the clinical evaluation of MLN9708 in the treatment of MBD and other bone pathologies. Looking at the future, it is likely that the next years will show the real value of MLN9708 and other second-generation proteasome inhibitors, such as carfilzomib and oprozomib, as both anti–multiple myeloma and bone-directed agents.

Disclosure of Potential Conflicts of Interest
J.F. San Miguel is a consultant/advisory board member for BMS, Celgene, Janssen, Millennium, MSD, Onyx, and Novartis. N. Raje is a consultant/advisory board member of Millennium. No potential conflicts of interest were disclosed by the other authors.

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References


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