Research Article

Cancer Therapy: Preclinical

Antitumor Activity of the Investigational Proteasome Inhibitor MLN9708 in Mouse Models of B-cell and Plasma Cell Malignancies

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**Running Title:** Preclinical Activity of MLN9708 in Hematologic Malignancies

**Key words:** MLN9708, MLN2238, proteasome, lymphoma, myeloma.
TRANSLATIONAL RELEVANCE

The success of bortezomib (VELCADE®) for the treatment of multiple myeloma and relapsed mantle cell lymphoma validated the proteasome as an oncology target. Several small-molecule proteasome inhibitors, including CEP-18770, NPI-0052 and PR-171 (carfilzomib), are in different stages of clinical development for various oncology indications. Here, we evaluated the investigational proteasome inhibitor MLN9708 in a variety of mouse models of B-cell and plasma cell malignancies, including traditional tumor xenograft models and genetically engineered mouse models. We demonstrated that its biologically active form MLN2238 has improved pharmacodynamics and antitumor activity compared to bortezomib in various B-cell lymphoma models and showed that only MLN2238 alleviated osteolytic bone disease in our plasma cell malignancy model. Our preclinical data served as the basis for the ongoing clinical development of MLN9708 in hematological malignancies. MLN9708 is being evaluated in multiple phase I and I/II studies as monotherapy or in combination with other chemotherapeutic agents.
ABSTRACT

Purpose: The clinical success of the first-in-class proteasome inhibitor bortezomib (VELCADE®) has validated the proteasome as a therapeutic target for treating human cancers. MLN9708 is an investigational proteasome inhibitor that, compared to bortezomib, has improved pharmacokinetics (PK), pharmacodynamics (PD), and antitumor activity in preclinical studies. Here, we focused on evaluating the in vivo activity of MLN2238 (the biologically active form of MLN9708) in a variety of mouse models of hematological malignancies, including tumor xenograft models derived from a human lymphoma cell line and primary human lymphoma tissue, and genetically engineered mouse (GEM) models of plasma cell malignancies (PCM).

Experimental Design: Both cell line-derived OCI-Ly10 and primary human lymphoma-derived PHTX22L xenograft models of diffuse large B-cell lymphoma were used to evaluate the PD and antitumor effects of MLN2238 and bortezomib. The iMycα/Bcl-XL GEM model was used to assess their effects on de novo PCM and overall survival. The newly developed DP54-Luc disseminated model of iMycα/Bcl-XL was used to determine antitumor activity and effects on osteolytic bone disease.

Results: MLN2238 has an improved PD profile and antitumor activity compared to bortezomib in both OCI-Ly10 and PHTX22L models. While both MLN2238 and bortezomib prolonged overall survival, reduced splenomegaly and attenuated IgG2a levels in the iMycα/Bcl-XL GEM model, only MLN2238 alleviated osteolytic bone disease in the DP54-Luc model.

Conclusions: Our results clearly demonstrated the antitumor activity of MLN2238 in a variety of mouse models of B-cell lymphoma and PCM, supporting its clinical development. MLN9708 is being evaluated in multiple phase I and I/II trials.
INTRODUCTION

The proteasome is a critical component of the ubiquitin-proteasome system, which is responsible for the regulation and degradation of the majority of intracellular proteins, including those involved in growth control, cell-cycle regulation and apoptosis (1, 2). Inhibition of the proteasome leads to stabilization and accumulation of these proteasome substrates, resulting in concomitant activation of pro- and anti-proliferative signals, disruption of cell-cycle regulation and, ultimately, activation of apoptotic pathways and cell death (3, 4). As validated by the clinical success of the first-in-class small-molecule proteasome inhibitor bortezomib (VELCADE®; Millennium Pharmaceuticals, Inc.), inhibition of the proteasome is an effective therapeutic approach for treating human cancer (5–7). Consequently, several small-molecule proteasome inhibitors, including the reversible inhibitor CEP-18770 and the irreversible inhibitors NPI-0052 and PR-171 (carfilzomib), are currently in different stages of clinical development for various oncology indications.

MLN9708 (Millennium Pharmaceuticals, Inc.) is an investigational small-molecule proteasome inhibitor currently being developed for a broad range of human malignancies (8, 9). MLN9708 is a citrate ester that immediately hydrolyzes to its biologically active form MLN2238 upon exposure to aqueous solutions or plasma in preclinical studies (Figure 1). Like bortezomib, MLN2238 is an N-capped dipeptidyl leucine boronic acid that selectively, reversibly and potently inhibits the β5 site of the 20S proteasome. However, MLN2238 has a significantly shorter proteasome dissociation half-life that we believe influences its biodistribution (9). We believe while a slowly reversible inhibitor such as bortezomib maintains a long duration of proteasome inhibition in red blood cells (RBC) and is slow to equilibrate and redistribute to tumor tissues, a more rapidly reversible inhibitor such as MLN2238 can dissociate faster from RBC proteasomes and can more readily enter tumor tissues. Consistent with this hypothesis, we have demonstrated that compared to bortezomib, MLN2238 has a greater tumor-to-blood ratio of proteasome inhibition that ultimately translated into improved tumor pharmacodynamic (PD) response and antitumor activity in several tumor xenograft models (9).
For decades, tumor xenograft models, which involve the subcutaneous inoculation of human tumor cell lines or tumor tissue fragments into immunocompromised mouse hosts, have been the mainstay of in vivo models for drug discovery in oncology. The robustness of these models allows for the rapid evaluation and prioritization of potential drug candidates into clinical trials. While virtually all new cancer therapies developed in the modern era have gone through this paradigm, these tumor xenograft models do not fully depict the biology and heterogeneity of their human disease counterpart (10–14). In contrast, the de novo diseases found in most genetically engineered mouse (GEM) models of cancer often faithfully mimic the stepwise pathological progression of human cancers: from the premalignant neoplastic stage that carries the initiating tumorigenic genetic mutations within their native tumor microenvironment, to the fully transformed cancer stage that resembles the genetic heterogeneity of their human counterparts (15, 16). However, accurate depiction of the human disease often comes with significant logistical tradeoffs, necessitating prohibitively large study cohorts for establishing statistical significance (13). In addition, disease progression and tumor onset are often slow and lengthy in these GEM models (months to years), further impeding their broader use in most fast-paced drug discovery settings.

Here, we sought to take advantage of both the robustness of the tumor xenograft models and the fidelity of the GEM models. We focused on evaluating and directly comparing the in vivo activity of MLN2238 and bortezomib in a variety of mouse models of hematological malignancies, including both tumor xenograft models derived from a human lymphoma cell line and primary human lymphoma tissue, to GEM models of plasma cell malignancies (PCM). The robustness of the tumor xenograft models allowed us to rapidly assess and relate the tumor PD profile of these molecules to their antitumor activities, while the fidelity of GEM models allowed us to preclinically assess their effects on tumor burden, osteolytic bone disease and terminal outcome analogous to the human disease. We believe this comprehensive approach will lead to a more thorough
understanding of the clinical potential of these molecules and, ultimately, better translational relevance to the clinic.
MATERIALS AND METHODS

Animal care

Strain CB17-severe combined immunodeficient (SCID) and non-obese diabetic (NOD)-SCID mice were obtained from Charles River Laboratories (Wilmington, MA). Strain C57BL/6J (B6) mice congenic for the iMyc\alpha transgene (TG) were generated by backcrossing the TG from the mixed genetic background of the original gene-targeted strain (segregating B6 and 129/SvJ alleles) to B6 for more than 10 generations (17). This strain, designated B6.iMyc\alpha, was deposited to the JAX® Strain Donation Program (The Jackson Laboratory, Bar Harbor, ME) and further bred to TG homozygosity to facilitate the generation of double-transgenic iMyc\alpha/Bcl-X\textsubscript{L} mice described below. Strain FVB/N mice heterozygous for the Bcl-X\textsubscript{L} TG were cryopreserved and expanded by the IVF-based, JAX® Speed Expansion Service (The Jackson Laboratory). A total of 90 age-matched double-transgenic iMyc\alpha/Bcl-X\textsubscript{L} mice of the hybrid (B6 x FVB/N) F\textsubscript{1} background were generated by intercrossing B6 mice homozygous for iMyc\alpha with FVB/N mice heterozygous for Bcl-X\textsubscript{L}. All mice were housed and maintained in a controlled environment and received food and water ad libitum. Mice reaching humane end points including severe weight loss (>15%), hind limb paralysis, splenomegaly, lymphadenopathy, or severe lethargy were euthanized. Veterinary care was provided in accordance with University of Iowa and Millennium Institutional Animal Care and Use Committees.

Compounds

For in vivo studies, bortezomib (dissolved in 0.9% saline) and MLN2238 (dissolved in 5% hydroxypropyl-beta-cyclodextrin [HP\betaCD]) were dosed at their maximum tolerated dose (MTD) for the specified mouse strain and model, unless otherwise specified.

Tumor xenograft models of activated B-cell diffuse large B-cell lymphoma

For the OCI-Ly10 model, freshly dissociated OCI-Ly10 diffuse large B-cell lymphoma cells of the activated B-cell subtype (ABC-DLBCL; 4 x 10\textsuperscript{6} N) suspended in IMDM:Matrigel (50:50) medium were
inoculated subcutaneously into the right flank of CB17-SCID mice as previously described (18). For the PHTX22L model, lymphoma tissue surgically isolated from a 71-year-old male with ABC-DLBCL was maintained via serial passage of the tumor fragment (~20 mg) between the right dorsal flanks of NOD-SCID mice as previously described (18). For PD studies, subcutaneous tumors (~600 mm$^3$ in size) were harvested, processed and analyzed for specific inhibition of β5 activity of the 20S proteasome as previously described (9). Efficacy studies were performed as previously described (9).

**Splenomegaly and mouse immunoglobulin levels**

Spleens harvested from iMyc$^{CA}$/Bcl-XL mice reaching humane end points were weighed as a measurement for splenomegaly. Terminal blood samples were collected by cardiac puncture, and mouse immunoglobulin and cytokine levels were determined by Searchlight® Protein Array Analysis (Aushon Biosystems, Woburn, MA).

**Cell culture and cell viability assay**

Parental DP54 cells were stably transfected with pDEST12.2-mIgK-Luc, which constitutively expresses the firefly luciferase gene (Luc) under the control of a mouse immunoglobulin kappa light-chain promoter/enhancer similar to that previously described (19). Transfection was performed using Nucleofector® Kit V (Lonza, Köln, Germany; VCA1003). One clone was selected by limited dilution in the presence of 0.8 mg/mL G418 and was designated DP54-Luc. DP54-Luc cells were maintained in RPMI-1640 media supplemented with 25 mM HEPES, 15% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 0.5 ng/mL interleukin-6, 0.8 mg/mL G418 and 50 units/mL each of penicillin and streptomycin at 37°C with 5% CO₂. ATPLite™ *in vitro* cell viability studies were performed according to the manufacturer’s instructions (Perkin-Elmer Life Sciences, Waltham, MA) as previously described (20).

**Disseminated mouse model and IVIS® bioluminescence Imaging**
For the DP54-Luc disseminated mouse model, freshly dissociated DP54-Luc cells (1 x 10^6 N) suspended in 100 μL of RPMI-1640 media were inoculated via the lateral tail vein of 8-11-week-old NOD-SCID mice. Tumor burden was monitored by IVIS® bioluminescence imaging (BLI) as previously described (9).

**Osteolytic bone disease and computed tomography imaging**

*Ex vivo* computed tomography (CT) was performed on formalin-fixed cadavers on an eXplore scanner (GE Healthcare, UK), two mice per session in a two-mouse gantry. Parameters for acquisition were the following: in-plane resolution 46 μm², 720 views, two frames/view, angle of increment 1°, exposure time 100 ms, voltage 80 kV and current 450 μA. All renderings were analyzed using Analyze 4.0 software. For analysis of the cranium, 3D renderings of the caudal aspect were cropped to include the sagittal bones, sagittal sutures and parietal sutures, before being saved as 2D images. Cranial suture widening was measured in sagittal suture separation areas (SSSA) and calculated by displaying the area of displacement between the sutures in a single-voxel layer, before being converted to mm³ by multiplying the voxel dimensions. Cranial bone erosion was assessed by bone surface roughness analysis as described previously (21).

**Statistical analyses**

Sample size determination and power analysis were calculated with the STT3 function from nQuery Adviser® (Version 7, Statistical Solutions). Simulated survival data were generated from a pseudo-random number generator under piece-wise constant hazard assumption and compared with log-rank statistics. Power was defined as the percentage of simulations in which the statistic indicates a significant outcome. For efficacy studies, survival curves and median overall survivals were generated using the Kaplan-Meier method. Hazard ratios, 95% confidence intervals (CI) and p-values were calculated based on a Cox proportional-hazard model, with treatment and gender as co-variants (22). A hazard ratio of <1 indicated an advantage of drug treatment over untreated controls. Efficacy data were analyzed using a linear mixed effect regression model as previously described (9). Statistical significance for the differences in mouse IgG2a levels, spleen weights and SSSAs.
among treatment groups were determined by one-way ANOVA and Dunnett’s adjustment to account for multiple comparisons. A $P$ value of $<0.05$ was considered significant.

**RESULTS**

**MLN2238 demonstrates antitumor activity in OCI-Ly10 subcutaneous ABC-DLBCL xenograft model**

We first evaluated and compared the *in vivo* activity of MLN2238 and bortezomib in the OCI-Ly10 subcutaneous xenograft model derived from a human cell line of ABC-DLBCL. OCI-Ly10 tumor-bearing SCID mice were administered a single intravenous (i.v.) dose of bortezomib at its twice-weekly (BIW) MTD of 0.8 mg/kg or MLN2238 at 8 mg/kg, which is below its BIW MTD of 14 mg/kg in SCID mice. Tumor PD responses were assessed by measuring the inhibition of $\beta_5$ activity of the 20S proteasome after treatment. As shown in Figure 2A, a single dose of bortezomib at MTD transiently inhibited the specific $\beta_5$ activity of OCI-Ly10 tumor for less than 24 h, whereas a single dose of MLN2238 maintained proteasome inhibition for up to 48 h even at the sub-MTD, consistent with the improved biodistribution of MLN2238 versus bortezomib in xenograft tumors. To assess whether the sustained proteasome inhibition seen with MLN2238 would translate into improved antitumor activity, efficacy studies were performed in the OCI-Ly10 model. When dosed at MTD on a twice-weekly schedule (BIW), bortezomib demonstrated moderate antitumor activity in the OCI-Ly10 model (T/C = 0.68, $P < 0.01$; Figure 2B, i). In contrast, even when dosed at sub-MTD on a once-weekly schedule (QW), MLN2238 demonstrated greater antitumor activity (T/C = 0.37, $P < 0.01$; Figure 2B, ii).

**MLN2238 demonstrates antitumor activity in PHTX22L primary human ABC-DLBCL xenograft model**

We then evaluated and compared the *in vivo* activity of MLN2238 and bortezomib in the PHTX22L primary human tumor xenograft model derived from lymphoma tissue surgically isolated from a 71-year-old male with ABC-DLBCL (18). Unlike traditional cell line-derived tumor xenograft models that often have deranged tumor architectures, primary human tumor xenograft models have been shown to retain the tissue-
specific histology seen in their clinical counterparts and maintain the dynamic interactions between the transformed cancer cells and their native tumor microenvironment (23). This provides for a useful preclinical model to evaluate MLN2238 and bortezomib, in addition to our traditional repertoire of cell line-derived tumor xenograft models.

PHTX22L tumor-bearing NOD-SCID mice were administered a single i.v. dose of bortezomib at the MTD of 0.8 mg/kg or MLN2238 at the MTD of 14 mg/kg or sub-MTDs of 7 and 3.5 mg/kg. As shown in Figure 2C, MLN2238 at all doses tested showed greater proteasome inhibition in PHTX22L tumors compared to bortezomib dosed at MTD. To test whether the greater proteasome inhibition seen with MLN2238 would translate into improved antitumor activity, efficacy studies were performed in the PHTX22L model. As shown in Figure 2Di, bortezomib dosed at the MTD did not show antitumor activity in the PHTX22L model (T/C = 0.88, \( P = 0.86 \)). In contrast, MLN2238 demonstrated potent antitumor activity at the MTD (T/C = 0.14, \( P < 0.01 \); Figure 2D, i). Importantly, MLN2238 also showed strong antitumor activity in the PHTX22L model even when dosed at sub-MTD levels of 5, 7, and 11 mg/kg on various dosing schedules (T/C ranges from 0.04 to 0.16, \( P < 0.001 \) for all; Figure 2D, ii).

**MLN2238 demonstrates antitumor activity in the iMyc\(^{Ca}\)/Bcl-X\(_L\) GEM model of de novo plasma cell malignancy**

We have previously demonstrated that intercrossing B6 mice carrying the iMyc\(^{Ca}\) TG (insertion of Myc into the immunoglobulin heavy-chain locus Igh) with FVB/N mice carrying the Bcl-X\(_L\) TG (enforced expression of Bcl-x driven by the immunoglobulin light-chain 3’\( \kappa \) enhancer and V\(_\kappa\)21 promoter) produced double-transgenic iMyc\(^{Ca}\)/Bcl-X\(_L\) mice that were genetically prone to developing de novo plasma cell tumors in multiple tissue types including the bone marrow (17, 24). These mice exhibited premalignant features such as splenomegaly, hypergammaglobulinemia and tissue plasmacytosis by 6-8 weeks of age and rapidly succumbed to PCM in less than 200 days. Importantly, the iMyc\(^{Ca}\)/Bcl-X\(_L\) de novo PCM recapitulated key features of
human multiple myeloma including elevation of serum paraproteins, manifestation of malignant plasma cells in bone marrow, occurrence of osteolytic bone disease and a myeloma-like global gene expression profile (17, 24). The short latency, full penetrance and key resemblance to the human disease provides for a great preclinical GEM model to evaluate MLN2238 and bortezomib.

Using the survival data from our earlier study (17), we simulated the hypothetical effects of different drug treatments in prolonging overall survival of iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice \textit{in silico}. As shown in Supplementary Table 1, our power analysis demonstrated that a sample size of 29 mice per treatment group was required to establish a statistical power of 0.80 ($\alpha=0.05$) for detecting an increase of 27 days in median overall survival. Accordingly, a total of 90 age-matched double-transgenic iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice were generated and randomized to three treatment groups, each consisting of 30 mice. Nine-week-old iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice with established disease were left untreated or treated with bortezomib or MLN2238 at their respective MTDs in this strain of 1.2 mg/kg i.v. BIW and 18 mg/kg i.v. BIW for 6 consecutive weeks (Figure 3). Untreated iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice ($n=30$) invariably developed \textit{de novo} PCM with short onset (median overall survival = 112 days) and pronounced splenomegaly (mean spleen weight of $0.87 \pm 0.07$ g) (Figure 3A and B). Treatment of iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice with bortezomib ($n=30$) prolonged overall survival (median overall survival = 139 days; hazard ratio = 0.162; 95% CI = 0.085 to 0.312; $P < 0.0001$) and alleviated splenomegaly (mean spleen weight of $0.59 \pm 0.05$ g; $P < 0.05$) compared to untreated controls (Figure 3A and B). Treatment of iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice with MLN2238 ($n=30$) also prolonged overall survival (median overall survival = 148 days; hazard ratio = 0.116; 95% CI = 0.057 to 0.236; $P < 0.0001$) and alleviated splenomegaly (mean spleen weight of $0.63 \pm 0.06$ g; $P < 0.05$) (Figure 3A and B). No significant difference in overall survival was observed between mice treated with MLN2238 and bortezomib in this \textit{de novo} iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} model.

In accordance with previous reports (17, 25), \textit{de novo} PCM in iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice led to elevation of serum levels of various immunoglobulins and cytokines (Supplementary Table 2). Although comparing the
average immunoglobulin and cytokine levels from mice reaching humane end points across different treatment groups has limitations, we demonstrated that both bortezomib and MLN2238 treatment attenuated the IgG2a level of iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice, underscoring the in vivo activity of these proteasome inhibitors in this GEM model of de novo PCM after only 6 weeks of treatment (P < 0.05 for both; Figure 3C).

**Osteolytic bone disease in the iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> GEM model of de novo plasma cell malignancy**

In an earlier study, we reported that iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> PCM can lead to bone lesions including focal osteolysis and putative pathological fractures (17). Here, ex vivo CT imaging similarly revealed signs of osteolytic bone disease in multiple regions of our iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice including the pelvic bone and tibiofibular complex (Figure 4A, iv and v). Targeted histopathological examination of these putative bone lesions revealed extensive infiltration of the hematopoietic bone marrow with malignant CD138<sup>+</sup> plasma cells (Figure 4B, i). Interestingly, in-depth CT analysis of the skull revealed a previously unnoticed widening of the cranial sutures, where pronounced separations and erosions of the coronal, sagittal and lambdoidal sutures were observed (Figure 4A; vi). As shown in Figure 4Bii, plasma tumor cell-induced osteolysis resulted in widening of the cranial sutures, extension of neoplastic cells through the calvarial bone and infiltration of the overlying periosteum. Bone surface roughness analysis revealed that these osteolytic bone diseases often extended beyond the cranial sutures (Figure 4C, ii), suggesting that mouse cranial sutures may represent the locus minoris resistantiae for confining plasma cell tumor cells to the bone marrow compartment and preventing the establishment of extramedullary disease in double-transgenic iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice.

**MLN2238 demonstrates antitumor activity in the DP54-Luc disseminated model of plasma cell malignancy**

To seek an alternative, less time- and resource-intensive version of the iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> PCM model to evaluate bortezomib and MLN2238, we have developed a disseminated mouse model of PCM that recapitulates key features of the de novo disease in iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice. DP54 is a mouse plasma cell tumor line derived from the bone marrow of a syngeneic F<sub>1</sub> (B6 x FVB/N) mouse previously inoculated with an iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub>
In vitro, DP54 cells express the iMyc<sup>Ca</sup> and Bcl-X<sub>L</sub> TG, various late B-cell and early plasma cell markers including CD38, CD138 and B220, and have a gene expression profile very similar to human multiple myeloma (24). ATPLite<sup>TM</sup> in vitro cell viability studies demonstrated that DP54 cells were sensitive to bortezomib and MLN2238 treatment, and yielded LD<sub>50</sub> values of 4.6 and 24.6 nM, respectively, which were comparable to those observed in a variety of human cancer cells (range 4 to 58 nM; Supplementary Table 3).

To characterize iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> tumor cells in vivo, DP54-Luc cells (which constitutively express firefly luciferase) were generated from DP54 cells and inoculated intravenously via the lateral tail vein of NOD-SCID mice. Disease progression and dissemination of DP54-Luc cells were monitored by in vivo BLI (Figure 5A). NOD-SCID mice inoculated with DP54-Luc rapidly succumbed to PCM 25 days after inoculation, with luminescent tumor nodules identified by ex vivo BLI in multiple organ compartments including lymph nodes, spleen, skull and hind limbs (Figure 5B). Similar to the de novo PCM in iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice, splenomegaly was prevalent in DP54-Luc-inoculated NOD-SCID mice. Importantly, ex vivo CT imaging (Figure 5C, i) and subsequent bone surface roughness analysis (Figure 5D) revealed widening and erosion of cranial sutures similar to those observed in de novo PCM in iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice (Figure 4A, vi and 4C, ii). Interestingly, strong ex vivo bioluminescent signals were detected from the metopic, coronal, lambdoid and sagittal sutures (Figure 5C, ii), the same locations where osteolytic bone lesions were observed (Figure 5C, i and 5D), which implied a direct effect of DP54-Luc cells infiltrating the cranium. These results demonstrated that i.v. inoculation of DP54-Luc cells resulted in a pathological phenotype that recapitulated key features of de novo PCM in double-transgenic iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice.

We then evaluated and compared the in vivo activity of MLN2238 and bortezomib in this disseminated model of PCM. DP54-Luc tumor-bearing NOD-SCID mice were treated with vehicle (5% HPβCD i.v. BIW), bortezomib (0.7 mg/kg i.v. BIW) or MLN2238 (11 mg/kg i.v. BIW) for 17 consecutive days. Tumor burden was measured by in vivo BLI (Figure 6A, ii to iv) and osteolytic bone disease as characterized by cranial suture...
widening was measured in SSSA (Figure 6B, ii to iv). While both bortezomib and MLN2238 reduced tumor burden (T/C = 0.48 and 0.22, respectively; \( P < 0.01 \) for both) (Figure 6A, i), only MLN2238 alleviated osteolytic bone disease by the end of the study (average SSSA = 0.85 vs. 1.16 mm\(^2\) in vehicle controls; \( P < 0.05 \)) (Figure 6B, i). This suggested that MLN2238 may have the potential to confer additional advantages over those seen with bortezomib in PCM including multiple myeloma. Together, our data demonstrated the improved antitumor activity of MLN2238 compared to bortezomib in a variety of mouse models of B-cell lymphoma and PCM.

**DISCUSSION**

*Antitumor activity of MLN2238 across multiple DLBCL subtypes*

DLBCL is a non-Hodgkin lymphoma that has been classified into multiple subtypes based on distinct gene expression profiles (26). The GCB-subtype likely originates from germinal center B-cells, whereas the more aggressive ABC-subtype likely arises from post-germinal center activated B-cells that are blocked from undergoing plasmacytic differentiation. A recent multicenter study with relapsed/refractory DLBCL patients \( (n = 49) \) suggested that bortezomib in combination with doxorubicin-based chemotherapy may preferentially improve the clinical outcome of patients with the ABC-subtype (27). Here, we demonstrated the improved PD and antitumor activity of MLN2238 compared to bortezomib in two mouse models of ABC-DLBCL: the OCI-Ly10 and PHTX22L models. In addition, we have recently shown that MLN2238 also has improved antitumor activity in the OCI-Ly7 model of the GCB-subtype (GCB-DLBCL) and the WSU-DLCL-2 model that was distinct from either the ABC- or GCB-subtypes (non-ABC-/non-GCB-DLBCL) (9). Together, our results suggest that MLN2238 has the potential to show broad activity across multiple DLBCL subtypes, extending beyond those seen with bortezomib.

*Manifestation of osteolytic bone disease in the cranium*
We describe for the first time the design and implementation of a preclinical study using the iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} GEM model of \textit{de novo} PCM. By combining conventional breeding strategies with existing IVF-based colony expansion technologies, we have generated a total of 90 age-matched double transgenic iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice for an efficacy study of MLN2238 and bortezomib. Like most GEM models, our original characterization of iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice was based on a small cohort with mixed genetic background (segregating B6 and 129/SvJ alleles) (17). The unprecedentedly large number of mice we have herein generated not only afforded us the ability to confirm and validate our original findings with respect to tumor penetrance, latency, and origin of the Myc- and Bcl-X\textsubscript{L}-driven \textit{de novo} malignancy, but also allowed us to validate the occurrence of osteolytic bone disease and, most importantly, discover the manifestation of cranial osteolytic lesions that were previously unnoticed. It will be of great importance for future studies to determine whether these cranial suture separations and erosions are idiosyncrasies particular to the myeloma-like bone disease in iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice, or pathologies common to metastatic bone diseases in other GEM models of human cancer. Nevertheless, our experience highlighted another important practical lesson for implementing GEM models: new tumor phenotypes may emerge and may even be susceptible to changes as mouse lines are crossed and backcrossed into different common non-segregating backgrounds (28–31), thus requiring in-depth validation before use in preclinical studies (13).

\textbf{Future use of the iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} model}

This is the first study to demonstrate the preclinical utility and translational relevance of the iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} GEM model of PCM. We showed that MLN2238 prolonged overall survival, reduced splenomegaly, and attenuated IgG2a levels of iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice after only 6 weeks of treatment. Nevertheless, comparing the average immunoglobulin and cytokine levels from mice reaching humane end points across different treatment groups has it limitations. Linden \textit{et al} have demonstrated that, similar to human multiple myeloma where elevation of serum immunoglobulin often precedes the manifestation of malignant diseases, the single Bcl-X\textsubscript{L}...
TG alone can lead to immunoglobulin elevations in the absence of PCM (25). Given the stochastic nature of tumor development and heterogeneity of immunoglobulin elevations in the iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} model (Supplementary Table 2), it is not surprising that the current cross-sectional manner of comparing the average immunoglobulin levels across different treatment groups at the end of the study did not, with the exception of IgG2a, capture changes that are inherently idiosyncratic. Future studies that longitudinally follow the serum immunoglobulin and cytokine levels of individual iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice over the clinical course of PCM (both pre- and post-drug treatment) will help provide a better understanding of drug-related changes in immunoglobulin and cytokine levels. In addition, the relationship between the observed increases in IL-6, IL-1b and MIP-1a levels and the induction of osteolytic bone disease in iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} mice compared to non-transgenic controls is also the subject of ongoing investigations (Siegfried Janz, personal communication).

While our study using the iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} model of de novo PCM yielded important terminal outcome data, this approach can nevertheless be prohibitively time consuming and logistically challenging, especially for screening potential therapeutics in today’s fast-paced drug discovery settings. We have thus developed the DP54-Luc disseminated model of PCM, which is less time- and resource-intensive than the iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} PCM model but yet still mimics key features of the de novo mouse disease. Our data showed that while both MLN2238 and bortezomib have potent antitumor activity, only MLN2238 alleviated osteolytic bone disease as indicated by reduced widening of cranial sutures. Encouraged by these results and acknowledging that a single plasma cell tumor line (e.g. DP54-Luc) would not sufficiently recapitulate the genetic heterogeneity among a diverse PCM population, we have generated more than 20 additional plasma cell tumor lines from the large number of primary iMyc\textsuperscript{Ca}/Bcl-X\textsubscript{L} tumors harvested in the current study. Our preliminary data have shown that these newly derived plasma cell tumor lines exhibit diversity in their sensitivities (all in the nM range) towards bortezomib in vitro (Brian Van Ness, personal communication). In addition, we have also isolated several bortezomib-resistant plasma cell tumor lines through in vitro dose escalation. These plasma cell tumor lines,
when fully characterized, will be used to identify the specific genetic aberrations responsible for bortezomib-resistance and may provide invaluable in vitro and in vivo tools for future drug screening and pharmacogenetic investigations. In particular, the activity of MLN9708 in these different plasma cell tumor lines is currently being investigated.

**MLN9708 in hematological malignancies**

In summary, we have demonstrated the antitumor activity of MLN2238 in a variety of mouse models of B-cell lymphoma and PCM, including tumor xenograft models derived from both a human cell line and primary human tumor tissue, and GEM models that recapitulated key features of the human diseases. Our data provide comprehensive evidence to support the clinical development of MLN9708 in hematological malignancies including lymphoma and multiple myeloma. While MLN9708 is currently being evaluated in multiple phase I and I/II clinical studies, the irreversible proteasome inhibitors NPI-0052 and PR-171 are also at their different stages of clinical development. It will be of great interest to see how these compounds, with their different proteasome dissociation kinetics, may ultimately differentiate themselves in the clinic.

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FIGURE LEGENDS

Figure 1. Chemical structure of MLN9708 and its biologically active form MLN2238

In preclinical studies, MLN9708 immediately hydrolyzes to its biologically active form MLN2238 upon exposure to aqueous solutions or plasma.

Figure 2. Proteasome inhibition and antitumor activity of bortezomib and MLN2238 in tumor xenograft models of activated B-cell diffuse large B-cell lymphoma

(A) Proteasome inhibition in OCI-Ly10 tumors 1, 24 and 48 h following acute intravenous (i.v.) administration of bortezomib (0.8 mg/kg) or MLN2238 (8 mg/kg) in OCI-Ly10 tumor-bearing SCID mice. Vertical lines represent SEM at each time point (n = 4).

(B) OCI-Ly10 tumor-bearing SCID mice (n = 10) were treated with vehicle (0.9% saline subcutaneously [s.c.] once daily [QD]) or bortezomib (0.8 mg/kg i.v. twice weekly [BIW]) for 4 consecutive weeks (i). OCI-Ly10 tumor-bearing SCID mice (n = 10) were treated with vehicle (5% HPβCD i.v. BIW) or MLN2238 (8 mg/kg i.v. once weekly [QW]) for 4 consecutive weeks (ii). Bars represent SEM at each time point.

(C) Proteasome inhibition in PHTX22L tumors 1 h following acute i.v. administration of bortezomib (0.8 mg/kg) or MLN2238 (14, 7 and 3.5 mg/kg) in PHTX22L tumor-bearing NOD-SCID mice. Vertical lines represent SEM of each treatment group (n = 4).

(D) PHTX22L tumor-bearing NOD-SCID mice (n = 10) were treated with vehicle (5% HPβCD i.v. BIW), bortezomib (0.8 mg/kg i.v. BIW) or MLN2238 (14 mg/kg i.v. BIW) for 3 consecutive weeks (i). PHTX-22L tumor-bearing NOD-SCID mice (n = 10) were treated with vehicle (5% HPβCD i.v. BIW), MLN2238 (11 mg/kg i.v. BIW), MLN2238 (7 mg/kg i.v. BIW), MLN2238 (11 mg/kg i.v. QW) or MLN2238 (5 mg/kg i.v. once daily for 2 days [QDx2] followed by 5 treatment-free days) for 3 consecutive weeks (ii). Bars represent SEM at each time point.
Figure 3. Antitumor activity of bortezomib and MLN2238 in the iMycCα/Bcl-XL GEM model of de novo plasma cell malignancy

(A) Nine-week-old double-transgenic iMycCα/Bcl-XL mice were left untreated or treated with bortezomib (1.2 mg/kg intravenously [i.v.] twice weekly [BIW]) or MLN2238 (18 mg/kg i.v. BIW) for 6 consecutive weeks (n = 30 per group).

(B) Mean spleen weights from iMycCα/Bcl-XL mice reaching humane end points in bortezomib-treated (n = 21) and MLN2238-treated (n = 18) arms were compared with those from untreated controls (n = 29). Vertical lines represent SEM in each treatment group. *P < 0.05, bortezomib-treated or MLN2238-treated versus untreated iMycCα/Bcl-XL mice.

(C) Average mouse IgG2a levels from iMycCα/Bcl-XL mice reaching humane end points in bortezomib-treated (n = 18) and MLN2238-treated (n = 13) arms were compared with those from untreated controls (n = 17) and 126-day-old non-transgenic (B6 x FVB/N) F1 mice (n = 9). Vertical lines represent SEM of each treatment group.*P < 0.05, bortezomib- or MLN2238-treated iMycCα/Bcl-XL mice versus untreated iMycCα/Bcl-XL mice.

†P < 0.05, untreated iMycCα/Bcl-XL versus 126-day-old non-transgenic (B6 x FVB/N) F1 mice.

Figure 4. Osteolytic bone disease in the iMycCα/Bcl-XL GEM model of de novo plasma cell malignancy

(A) Unlike in age-matched non-transgenic (B6 x FVB/N) F1 mice (i, ii and iii), ex vivo computed tomography (CT) imaging revealed evidence of osteolytic bone disease (red arrows) in the pelvic bone (iv), tibio-fibular complex (v) and cranium (vi) of iMycCα/Bcl-XL mice by ~110 days of age. Note separation and erosion of the coronal, sagittal and lambdoidal sutures of the skull (vi, red arrows).

(B) Histopathological examination of bone disease identified by CT imaging. A homogenous population of CD138+ plasma cell tumor cells completely effaces the normal hematopoietic elements of the marrow cavity (i).
Neoplastic plasma cell-induced osteolysis resulted in widening of the cranial sutures, invasion of neoplastic cells through the calvarial bone (red arrow) and infiltration the overlying periosteum (ii).

(C) Increases in bone surface roughness and focal osteolytic lesions, which extended beyond the area of suture widening, were observed in 110-day-old iMyc<sup>Cα</sup>/Bcl-X<sub>L</sub> mice (ii) compared to age-matched non-transgenic controls (i). The colors white and blue indicate low and intermediate regional abnormalities, respectively. The color red indicates heavily damaged and eroded areas.

**Figure 5. Disseminated mouse model of DP54-Luc iMyc<sup>Cα</sup>/Bcl-X<sub>L</sub> plasma cell malignancy**

(A) Dissemination of DP54-Luc cells and progression of disease was detected by *in vivo* bioluminescence imaging (BLI) on Days 1, 8, 15 and 23 after inoculation.

(B) Representative *ex vivo* BLI from a NOD-SCID mouse 25 days after sham (i) or intravenous inoculation of DP54-Luc cells (ii). Disseminated luminescent tumor nodules were detected in multiple enlarged lymph nodes, spleen, liver, intestine, kidney, cranium, spinal bone, and hindlimbs of DP54-Luc inoculated mice. No bioluminescent signal was detected from the blood.

(C) Representative *ex vivo* CT image of a NOD-SCID mouse 25 days after sham or i.v. inoculation of DP54-Luc cells. Pronounced separation of the coronal, sagittal and lambdoidal sutures was observed in DP54-Luc inoculated mice (i). Strong BLI signals were detected from the diseased area around the cranial sutures of DP54-Luc inoculated mice compared to sham controls (ii).

(D) Bone surface roughness analysis revealed severe bone erosions that extended beyond the metopic, coronal, sagittal and lambdoidal sutures in NOD-SCID mouse 25 days after i.v. inoculation of DP54-Luc cells compared to sham control.
Figure 6. Antitumor activity of bortezomib and MLN2238 in the disseminated mouse model of DP54-Luc iMyc<sup>Cα</sup>/Bcl-X<sub>L</sub> plasma cell malignancy

(A) NOD-SCID mice inoculated with DP54-Luc cells were treated with vehicle (5% HPβCD intravenously [i.v.] twice weekly [BIW]), bortezomib (0.7 mg/kg i.v. BIW) or MLN2238 (11 mg/kg i.v. BIW) for 17 consecutive days (n = 10 per group) (i). Bars represent SEM. Representative in vivo BLI images of DP54-Luc inoculated NOD-SCID mice after treatment with (ii) vehicle, (iii) bortezomib, and (iv) MLN2238 at the end of the study.

(B) Reduction in cranial suture widening after MLN2238 treatment. Horizontal lines represent average SSSA in each treatment group at the end of the study (n = 7) (i). *P < 0.05; MLN2238 treated versus vehicle treated. Representative computerized tomography (CT) images of the cranial region of DP54-Luc inoculated NOD-SCID mice after treatment with (ii) vehicle, (iii) bortezomib, and (iv) MLN2238 at the end of the study. By excluding the underlying bone from the field of view, these clipped 3D renderings of CT images highlighted the separation of multiple cranial sutures in each treatment group.
Figure 1
**SUPPLEMENTARY TABLES**

Supplementary Table 1. Sample size determination and power analysis on the overall survival of iMyc^Cαβ/Bcl-X_L mice

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>Median overall survival</th>
<th>Survival advantage†</th>
<th>N‡</th>
<th>Power=0.80</th>
<th>Power=0.90</th>
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<tr>
<td>Untreated Control</td>
<td>121 days§</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Treatment A</td>
<td>141 days</td>
<td>+ 20 days</td>
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<td>87</td>
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<td>+ 25 days</td>
<td>37</td>
<td>50</td>
<td></td>
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<tr>
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<td>+ 27 days</td>
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<td>38</td>
<td></td>
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<tr>
<td>Treatment D</td>
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<td>+ 30 days</td>
<td>21</td>
<td>28</td>
<td></td>
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<tr>
<td>Treatment E</td>
<td>154 days</td>
<td>+ 33 days</td>
<td>19</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

*Simulated scenarios *in silico*;

†Changes in median overall survival compared to untreated controls;

‡Number of animals required in each treatment group for specified statistical power;

§Based on data from our previous study (17).

**Note:** H_0: Survival distribution of control group = Survival distribution of treatment group; Power = Prob(Reject H_0 at α = 0.05 if a difference between the two groups truly exists).
Supplementary Table 2. Immunoglobulin and cytokine levels in iMyc<sup>Cα</sup>/Bcl-X<sub>L</sub> mice after treatment with bortezomib and MLN2238

<table>
<thead>
<tr>
<th></th>
<th>Non-transgenic</th>
<th>iMyc&lt;sup&gt;Cα&lt;/sup&gt;/Bcl-X&lt;sub&gt;L&lt;/sub&gt;</th>
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<tr>
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<td>Controls (n = 9)</td>
<td>Untreated (n = 17)</td>
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<td><strong>Immunoglobulins</strong></td>
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<td>IgA (g/dL)</td>
<td>Mean 0.0020</td>
<td>0.0097</td>
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<tr>
<td></td>
<td>S.D. 0.0006</td>
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<td>IgM (g/dL)</td>
<td>Mean 0.0038</td>
<td>0.1588*</td>
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<td>S.D. 0.0006</td>
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<td>IgG1 (g/dL)</td>
<td>Mean 0.0917</td>
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<td>S.D. 0.0227</td>
<td>0.2308</td>
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<tr>
<td>IgG2a (g/dL)</td>
<td>Mean 0.0119</td>
<td>0.3787*</td>
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<tr>
<td></td>
<td>S.D. 0.0058</td>
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<td>IgG2b (g/dL)</td>
<td>Mean 0.0789</td>
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<td></td>
<td>S.D. 0.0438</td>
<td>1.3910</td>
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<tr>
<td><strong>Cytokines</strong></td>
<td></td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>Mean 66.5</td>
<td>720.0*</td>
</tr>
<tr>
<td></td>
<td>S.D. 46.7</td>
<td>1030</td>
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<tr>
<td>IL-1b (pg/mL)</td>
<td>Mean 7.6</td>
<td>55.0*</td>
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<tr>
<td></td>
<td>S.D. 12.4</td>
<td>79.1</td>
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<tr>
<td>MIP-1a (pg/mL)</td>
<td>Mean 0.7</td>
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<tr>
<td></td>
<td>S.D. 0.3</td>
<td>10.5</td>
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*P < 0.05, untreated iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> versus 126-day-old non-transgenic (B6 x FVB/N) F<sub>1</sub> mice.

†P < 0.05, bortezomib- or MLN2238-treated iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice versus untreated iMyc<sup>Ca</sup>/Bcl-X<sub>L</sub> mice.
Supplementary Table 3. DP54 cells are sensitive to proteasome inhibition *in vitro*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue of origin</th>
<th>ATPLite™ LD$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>A375M</td>
<td>Human melanoma</td>
<td>6.5</td>
</tr>
<tr>
<td>H460</td>
<td>Human lung cancer</td>
<td>13.0</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human colorectal cancer</td>
<td>4.4</td>
</tr>
<tr>
<td>HT29</td>
<td>Human colon cancer</td>
<td>7.0</td>
</tr>
<tr>
<td>DP54</td>
<td>Mouse plasma cell tumor</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Abbreviations:** LD$_{50}$, lethal dose to 50% of the cells based on ATPLite™.
Antitumor Activity of the Investigational Proteasome Inhibitor MLN9708 in Mouse Models of B-cell and Plasma Cell Malignancies


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