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Molecular and Cellular Effects of NEDD8-Activating Enzyme Inhibition in Myeloma

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Abstract

The NEDD8-activating enzyme is upstream of the 20S proteasome in the ubiquitin/proteasome pathway and catalyzes the first step in the neddylation pathway. NEDD8 modification of cullins is required for ubiquitination of cullin-ring ligases that regulate degradation of a distinct subset of proteins. The more targeted impact of NEDD8-activating enzyme on protein degradation prompted us to study MLN4924, an investigational NEDD8-activating enzyme inhibitor, in preclinical multiple myeloma models. *In vitro* treatment with MLN4924 led to dose-dependent decrease of viability ($EC_{50} = 25–150$ nmol/L) in a panel of human multiple myeloma cell lines. MLN4924 was similarly active against a bortezomib-resistant ANBL-6 subline and its bortezomib-sensitive parental cells. MLN4924 had submicromolar activity (EC_{50} values <500 nmol/L) against primary CD138^{+} multiple myeloma patient cells and exhibited at least additive effect when combined with dexamethasone, doxorubicin, and bortezomib against MM.1S cells. The bortezomib-induced compensatory upregulation of transcripts for ubiquitin/proteasome was not observed with MLN4924 treatment, suggesting distinct functional roles of NEDD8-activating enzyme versus 20S proteasome. MLN4924 was well tolerated at doses up to 60 mg/kg 2× daily and significantly reduced tumor burden in both a subcutaneous and an orthotopic mouse model of multiple myeloma. These studies provide the framework for the clinical investigation of MLN4924 in multiple myeloma. *Mol Cancer Ther; 11(4); 942–51. ©2012 AACR.*

Introduction

Following the successful clinical development of proteasome inhibitors, such as bortezomib for injection, targeting protein homeostasis has become an attractive therapeutic strategy for multiple myeloma and other neoplasias. Cullin-ring ligases (CRL) have recently become targets of interest because they regulate degradation of proteins more selectively than the 20S proteasome in the ubiquitin/proteasome protein degradation pathway (1). Specifically, NEDD8-activating enzyme catalyzes the first step in the NEDD8 Neddylation pathway. NEDD8 modification of cullins is required for ubiquitination of CRLs and targeting NEDD8-activating enzyme or Ubc12, the downstream enzyme in the pathway, allows for inhibition

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of the entire group of proteins degraded through the NEDDylation process (1, 2).

Preclinical and clinical data from our group and others have shown the activity of proteasome inhibitors in plasma cell dyscrasias such as multiple myeloma and Waldenstroms Macroglobulinemia (WM). This activity suggests that these cancers may also be sensitive to the inhibition of the ubiquitin/proteasome pathway at other levels, such as the NEDDylation component of protein degradation. To address this hypothesis, we tested the preclinical activity of the NEDD8-activating enzyme inhibitor MLN4924 against multiple myeloma cells.

A panel of multiple myeloma cell lines were treated with MLN4924 for 72 hours and compared with the colon carcinoma line HCT116. In addition, a longitudinal assessment of viability of MM.1S showed commitment to death within less than 48 hours. We also observed that normal donor peripheral blood mononuclear cells (PBMC) were less sensitive ($EC_{50} > 1000$ nmol/L) than the multiple myeloma cell lines tested, suggesting that this compound exhibits a rapid, tumor-selective effect at clinically relevant conditions. We also evaluated primary multiple myeloma (CD138^{+}) patient bone marrow cells and observed submicromolar activity by MLN4924. In addition, we tested a series of combinations of MLN4924 with dexamethasone, doxorubicin, and bortezomib and observed additive activity or greater with MLN4924. Gene expression profiling revealed distinct signatures as well as distinct patterns of

gene expression changes that were induced by MLN4924 versus bortezomib. Last, animal studies showed that MLN4924 was tolerated and active against multiple myeloma xenografts in both subcutaneous and diffuse lesion mouse models of multiple myeloma disease.

Materials and Methods

Cell lines

We evaluated a panel of human multiple myeloma cell lines, including Dox40 (kindly proved by Lori Hazlehurst; Lee H. Moffit Cancer Center, Tampa, FL), H929 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), INA-6 (kindly provided by Renata Burger, University of Kiel, Kiel, Germany), JJN3 (DSMZ), KMS-18 [Japanese Collection of Research Bioresources (JCRB)], KMS-34 (JCRB), MM.1S (Steve Rosen, Northwestern University, Chicago, IL), OPM2 (DSMZ), and the colon carcinoma cell line HCT116 [American Type Culture Collection (ATCC)]. The bortezomib-sensitive ANBL-6 cell line and their subline ANBL-6-V5R, which exhibits decreased response to bortezomib, were kindly provided by Dr. Robert Orlowski (MD Anderson Cancer Center, Houston, TX). These lines were characterized by short tandem repeat profiling and compared with the known ATCC database and German Collection of Microorganisms and Cell Cultures databases (3) and used within 6 months from receipt and/or characterization. Cells were grown in either RPMI-1640 (Mediatech Inc.) or Dulbecco's Modified Eagle's Medium (Mediatech Inc.) media with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (GIBCO/BRL), unless stated otherwise.

Compounds

MLN4924 and bortezomib (Fig. 1A; refs. 4, 5), were provided by Millennium Pharmaceuticals, resuspended in dimethyl sulfoxide (DMSO) at 10 mmol/L and used at the concentrations indicated. Dexamethasone and doxorubicin were purchased from Sigma, resuspended in DMSO, and used at the concentrations indicated.

Correlation of *NEDD8* transcript levels with clinical outcome

We evaluated the expression levels of *NEDD8* transcript (201840_at) in the $\log_2$ transformed and median-centered

Figure 1. Levels of *NEDD8* transcript in primary multiple myeloma tumor cells correlate with clinical outcome. A, bortezomib and MNL4924 structures are shown for these studies. Kaplan–Meier curves of PFS for bortezomib-treated multiple myeloma patients with low versus high levels of *NEDD8* transcript in the phase 2 SUMMIT (B; $P = 0.0173$, log-rank test) and the phase 3 APEX trials (C; $P = 0.0482$, log-rank test). Kaplan–Meier curves of PFS for bortezomib- versus dexamethasone-treated multiple myeloma patients with low (D; $P = 0.0012$, log-rank test) versus high (E; $P = 0.6149$, log-rank test) levels of *NEDD8* transcript in the phase 3 APEX trial. Dex, dexamethasone; Bort, bortezomib.
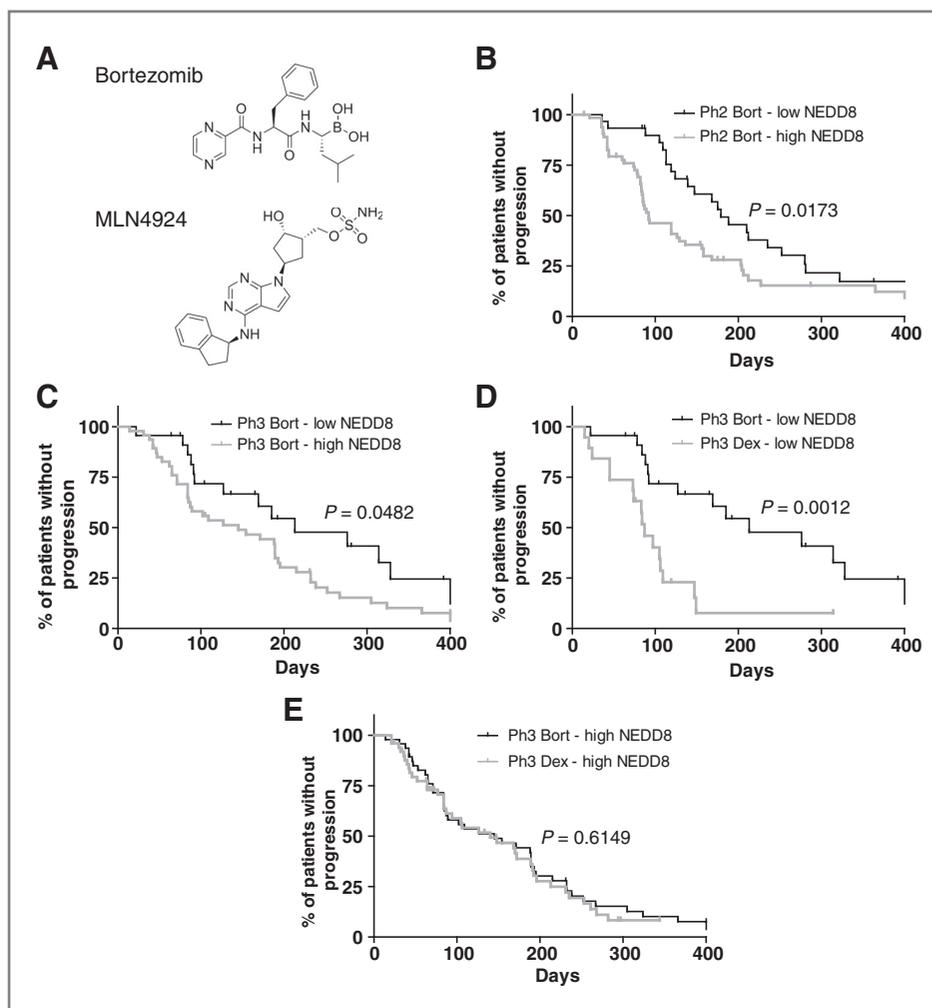
gene expression data set of tumor cells from evaluable bortezomib-treated multiple myeloma patients enrolled in phase II and III trials of relapsed/refractory multiple myeloma (6, 7). Patients were classified as having high (top quartile of expression) versus low (lower 3 quartiles of expression) levels of *NEDD8* and Kaplan–Meier survival analyses comparing the 2 groups were carried out by SPSS 17.0 and visualized with GraphPad Prism 5.0.

Cell viability assessment

Cell viability was measured by MTT (Chemicon International) colorimetric survival assay, as previously published (8) or CellTiterGlo (CTG; drug combination studies). In brief, multiple myeloma cell lines were plated in 48-well plates at a density of 20,000 cells per well. MLN4924 was added at the concentrations indicated and compared with vehicle-treated controls. Cultures were then incubated for 72 hours in a 37°C incubator with 5% CO_2. MTT was added to each well for the final 4 hours of treatment, followed by addition of isopropanol containing 0.04 N HCl to dissolve the crystals (at volume 1.5- to 3-fold of the total culture volume). Optical absorbance of the culture medium was then measured at 570/630 nm with a spectrophotometer (Molecular Devices Corp.).

Peripheral blood samples from healthy donors were collected with heparinized tubes and processed by Ficoll density gradient centrifugation (Amersham Biosciences) to isolate PBMCs, which were plated at 10,000 cells per well and prestimulated with 5 μg/mL phytohemagglutinin (PHA; Sigma-Aldrich) for 1 hour. MLN4924 was administered at the doses indicated in the respective experiments for 72 hours, followed by the addition of CTG and measurement as described above. Following consent, myeloma patient samples were collected, Ficoll gradient separated, and selected using CD138 microbeads (Miltenyi Biotec). Cells were cultured in RPMI-1640 (Mediatech Inc.) with 100 U/mL penicillin, 100 μg/mL streptomycin, and 20% fetal calf serum and treated as indicated in the figures. Viability was measured by CTG assay, as described above.

Cell death commitment assay

The minimum exposure of multiple myeloma cells to MLN4924 that is required to commit them to death was evaluated by incubating cells in 24-well plates with MLN4924 (500 nmol/L) up to 72 hours. Following incubation, the cells were washed with drug-free medium to remove any residual drug, and then incubated in drug-free medium for an additional 3 days, resulting in equal length of incubation for all experimental conditions. Multiple myeloma cell survival was quantified by CTG and expressed as percentage of the value obtained from respective controls.

Stromal cell coculture

The luciferase (luc)-expressing cell lines, MM.1S-mCherry/luc and OPM2-mCherry/luc, were generated by retroviral transduction and used for coculture experiments with the luc-negative human stromal line HS-5, as described previously (9). Briefly, HS-5 stromal cells were plated in 384-well plates and allowed to attach overnight. multiple myeloma cells were then plated and treated with MLN4924 for 48 hours at the doses indicated. Following incubation, luciferin substrate (Xenogen Corp) was added to the culture and the resulting bioluminescence signal was measured using an Envision luminometer (Perkin Elmer).

Osteoclast differentiation/coculture

PBMCs were collected from collars of normal donors, ficoll gradient separated, resuspend in α-MEM media containing 10% FBS and Pen/Strep. Cells were cultured for 16 hours to allow attachment and nonadherent cells were removed. Fresh media supplemented with MCSF (25 ng/mL; R&D Systems #216-MC-005) and RANKL (50 ng/mL; Peprotech #310-01) was added and cells were cultured for 3 to 4 weeks to differentiate mature osteoclasts. Half of media was changed 2× per week, containing fresh MCSF and RANKL. At 3 to 4 weeks, cells were trypsinized, replated, and stained with TRAP stain to determine the differentiation of osteoclasts. Mature osteoclasts were plated in 96-well plates, allowed to attach overnight, and used in coculture experiments as indicated in the figure legends.

Animal studies

The *in vivo* anti-multiple myeloma activity of MLN4924 was evaluated in a subcutaneous xenograft model of RPMI8226/S multiple myeloma cell line in CB.17-severe combined immunodeficient (SCID) mice. The antitumor activity and pharmacodynamic study in live mice was conducted at Oncodesign S.A (Dijon). In brief, 2×10^7 RPMI 8226 cells were injected into the right flank of CB.17 SCID mice and studies were initiated once tumors had formed to approximately 200 mm³. MLN4924 was dosed subcutaneously at 10, 30, and 60 mg/kg twice a day for 21 days and tumor volume monitored. To evaluate the pharmacodynamic effects of MLN4924, a single subcutaneous dose of MLN4924 was administered and the effects on the NEDD8-activating enzyme pathway were examined as described previously (4, 10).

The *in vivo* anti-multiple myeloma activity of MLN4924 was evaluated in a previously established model of diffuse GFP/luc+ multiple myeloma lesions in SCID/NOD mice. Briefly, male (6- to 8-week old) SCID/NOD mice were obtained from Charles River; housed and monitored in the Animal Research Facility of the Dana-Farber Cancer Institute; γ irradiated (150 rads) using Cs^{137} γ-irradiator source; and received (24 hours postirradiation) tail i.v. injections of 10^6 MM.1S-GFP/luc cells per mouse. Mice were monitored regularly for changes in body weight, signs of infection or paralysis, and with weekly bioluminescence imaging (11).

Immunoblotting analysis

For immunoblotting analyses, MM.1S cells (10×10^6 cells per condition) were plated in RPMI-1640 medium with 10% FBS, penicillin, and streptomycin as previously described. MLN4924 was added at a concentration of

500 nmol/L for 0 to 72 hours. Cell pellets were collected and treated with Triton X-100 lysis buffer containing $1 \times$ PBS, Triton X-100 (1% v/v), sodium deoxycholate (0.5% w/v), SDS (0.1% w/v), EDTA (1 mmol/L), 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A. The samples were cleared by centrifugation (14,000 rpm, 30 minutes, 4°C) and assessed for protein concentration by Bradford assay (Sigma). SDS-PAGE (12%) was carried out (30–50 μg of protein per lane), and proteins were electroblotted onto polyvinylidene difluoride membranes. After 1 hour incubation in blocking solution (5% milk in TBS-T buffer), membranes were exposed to primary antibody overnight at 4°C. Following washing in TBS-T, the respective secondary horseradish peroxidase–labeled antibody was added at 1:20,000 dilution for 1 hour at room temperature. The membrane was then washed with TBS-T for 45 to 60 minutes with multiple changes of the wash buffer, and the protein expression was visualized with the ECL technique. The primary antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology, Upstate Biotechnologies, or Cell Signaling. Secondary antibodies were purchased from Jackson ImmunoResearch.

Gene expression profiling of cells treated with MLN4924

Total RNA extraction and purification, cDNA synthesis and cRNA labeling, Affymetrix chip (human HG-U133 plus 2.0 array) hybridization and data analysis were carried out as previously described (12–14). Briefly, MM.1S cells were treated with 250 nmol/L MLN4924, 10 nmol/L bortezomib, or with DMSO as a control for the indicated time points; total RNA was then extracted and purified, cDNA synthesized and cRNA labeled before hybridization to the HG-U133 plus 2.0 arrays. Gene expression data have been deposited in Gene Expression Omnibus (GEO, GSE33577). Transcripts suppressed by MLN4924 were analyzed in publically available databases for relevance to clinical outcome and/or differential expression in various stages of plasma cell dyscrasias. We specifically analyzed the $\log_2$ transformed median centered values (derived from the Oncomine database) of different MLN4924-suppressed genes in the GEO (15) data sets GSE5900 [comparison of gene expression of bone marrow plasma cells from healthy donors, monoclonal gammopathy of undetermined significance (MGUS), and smoldering myeloma (SMM; ref. 16)]; GSE2658 (17); GSE2113 (comparison of gene expression profiles of purified plasma cells obtained from MGUS, newly diagnosed multiple myeloma, and from plasma cell leukemia patients; ref. 18); GSE4452 (gene expression profiles of multiple myeloma patients before treatment (19). Differential expression between normal plasma cells and MGUS and SMM; between MGUS and newly diagnosed multiple myeloma and plasma cell leukemia patients; or between patients with differential patient outcome were evaluated using 1-way ANOVA or unpaired T test, as stated in the figure legends using Prism software (GraphPad).

Results

NEDD8 transcript levels correlates with clinical outcome in multiple myeloma patients

We evaluated the expression levels of *NEDD8* transcript (201840_at) in gene expression data set of tumor cells from evaluable bortezomib-treated multiple myeloma patients enrolled in phase II and III trials of relapsed/refractory multiple myeloma (6, 7). We observed that patients with high expression of *NEDD8* transcript had significantly shorter progression-free survival (PFS) compared with patients with low *NEDD8* transcript levels. Among patients treated in the phase III trial of bortezomib versus dexamethasone, those with low levels of *NEDD8* transcript had significantly longer PFS with bortezomib than with dexamethasone however, in patients with high levels of *NEDD8* transcript, there was no significant difference in patients treated with bortezomib versus dexamethasone (Fig. 1).

In vitro activity of MLN4924 against multiple myeloma cell lines and primary patient samples

We treated a panel of multiple myeloma cell lines with MLN4924 (0–1000 nmol/L) for 72 hours and evaluated viability with MTT colorimetric survival assays. The majority of cell lines had EC_{50} values less than 500 nmol/L (Fig. 2A). The most sensitive cells lines, such as OPM2, INA-6, and KMS34, exhibited EC_{50} values less than 100 nmol/L. In addition, we evaluated the impact of MLN4924 on the colorectal carcinoma cell line HCT116, previously reported to be MLN4924 responsive (4) for comparison.

In addition to cell lines, CD138^{+} primary multiple myeloma patient samples were evaluated for sensitivity to MLN4924. Multiple myeloma patient bone marrow was collected, ficolled, and then selected for CD138^{+} cells. Cell were then plated and treated with increasing doses of MLN4924 for 72 hours. Of the 6 samples treated, we observed activity of the drug against 4 samples with EC_{50} values less than 600 nmol/L. Two samples, however, remained insensitive to the drug up to 1000 nmol/L of drug (Fig 2B).

For further understanding of the activity of MLN4924 against normal tissues, PBMCs from healthy donors were isolated, PHA stimulated to induce cycling and proliferation, then treated with MLN4924 for 72 hours (Fig. 2C). The percentage of viable PBMCs was 90% or greater following MLN4924 treatment in the absence of PHA stimulation. Sensitivity of PBMCs to MLN4924 was increased as a result of PHA stimulation, but remained less than the most sensitive multiple myeloma cell lines.

We also tested *in vitro* the activity of MLN4924 against the bortezomib-sensitive multiple myeloma cell line ANBL6 and its subline ANBL6-V5R, which exhibits decrease response to bortezomib. We did not observe increased resistance of ANBL6-V5R cells to MLN4924 compared with the parental cell line (Fig. 2D).

Cell death commitment assays were conducted on Dox40, MM.1S, OPM2, and KMS34 cells by exposing them to MLN4924 (500 nmol/L) for up to 72 hours, followed by drug washout and incubation in drug-free medium for an
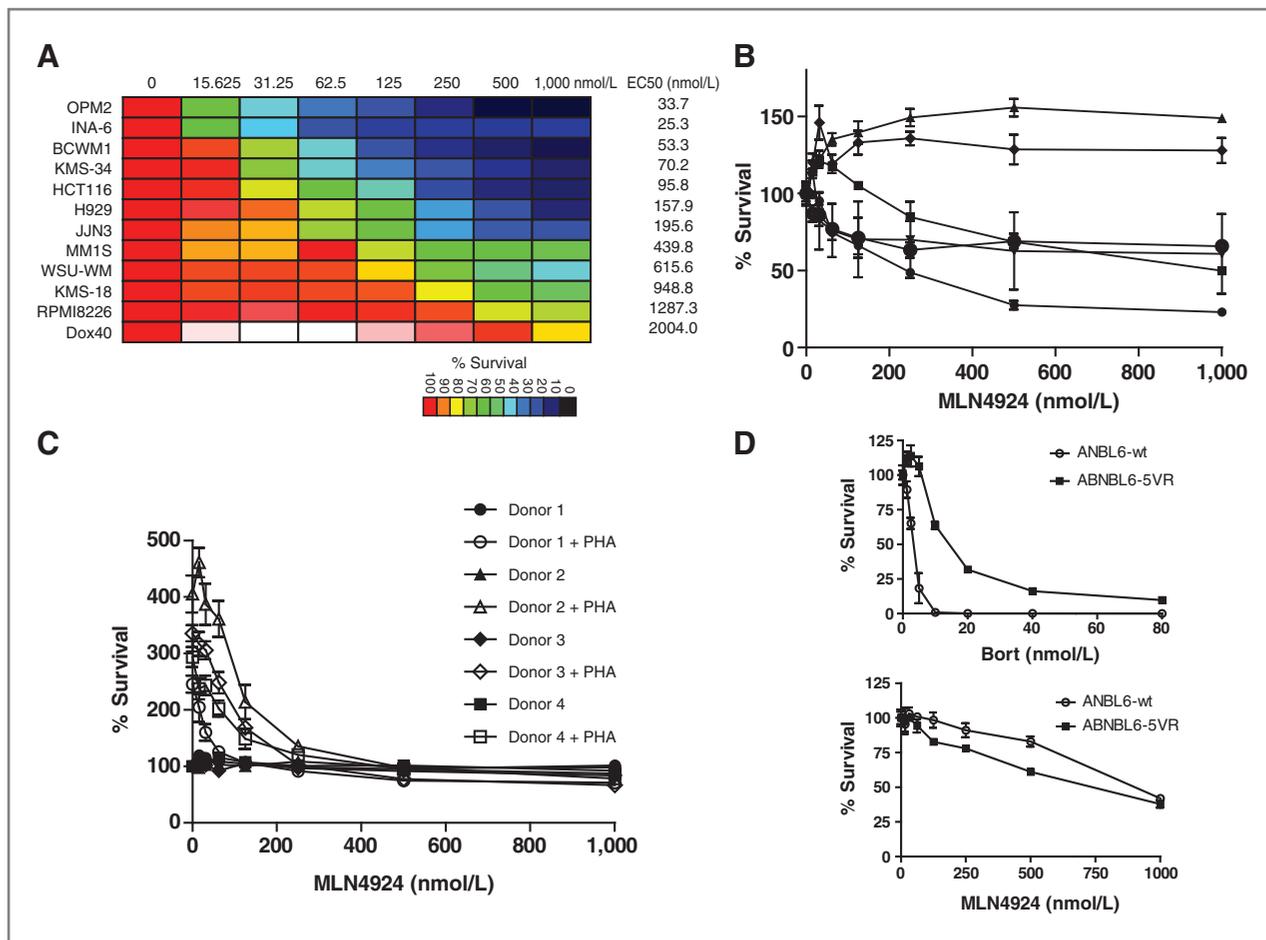
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Figure 2. Activity of MLN4924 on multiple myeloma cells. Multiple myeloma (OPM2, INA-6, KMS-34, H929, JJN3, MM.1S, KMS-18, and Dox40), WM (BCWM1 and WSU-WM), and colon carcinoma (HCT116) cell lines were treated with MLN4924 (0–1000 nmol/L for 72 hours) and viability was assessed by MTT assay. A, the most sensitive cell lines (OPM2 and INA-6) have EC_{50} values less than 40 nmol/L, whereas the most resistant multiple myeloma cell line (Dox40) has an EC_{50} value more than 2 μmol/L. Bone marrow aspirates from multiple myeloma patients were processed using Miltenyi anti-CD138 microbeads for purification of multiple myeloma cells, which were then cultured in RPMI1640 media containing 20% serum. B, $CD138^+$ selected cells were treated with increasing doses of MLN4924 for 72 hours, and viability was assessed by CTG. To access the sensitivity of nonmalignant cells, PBMCs were isolated from normal donors and screened in the presence and absence of PHA stimulation (5 ng/mL) for 72 hours. C, the number of viable cells in each condition is expressed as percentage of the non–PHA-stimulated drug-free control. The multiple myeloma cell line ANBL6 and its subline ANBL6-V5R were tested for sensitivity to bortezomib and MLN4924. D, ANBL6-V5R was more resistant to bortezomib, but not MLN4924, compared with the parental cell line. Bort, bortezomib.

additional 72 hours. MLN4924 treatment for 48 hours resulted in commitment to death in OPM2, KMS34, and MM.1S (Supplementary Fig. S1), suggesting that molecular events initiating cell death can occur in less than 48 hours of MLN4924 treatment.

MLN4924 overcomes stroma and osteoclast protection of multiple myeloma cells

Luc+ MM.1S and OPM2 cells were cultured in the presence and absence of HS-5 stromal cells or differentiated osteoclasts for 72 hours. Cell viability was normalized to each respective drug-free control. Results show that MLN49424 was similarly active both in the presence and absence of HS-5 stromal cells (Fig 3A and B) and presence and absence of osteoclasts (Fig. 3C and D), indicating that MLN4924 can overcome the protective effects of bone microenvironmental factors.

Activity of MLN4924 in animal studies

MLN4924 showed dose-dependent anti-multiple myeloma activity in the RPMI8226/S xenograft model in CB.17 SCID mice (Fig 4A). Complete inhibition of tumor growth was achieved at 60 mg/kg twice a day dose level, which is below the MTD on this schedule. In addition, MLN4924 showed dose-dependent inhibition of Nedd8-cullin levels with a concomitant increase in levels of 2 CRL substrates, Nrf-2 and pIKBa (Supplementary Fig. S2). We also evaluated the *in vivo* anti-multiple myeloma activity of MLN4924 in sublethally irradiated SCID/NOD mice injected i.v. with MM.1S-GFP/luc cells for establishment of diffuse multiple myeloma bone lesions. MLN4924 treatment was associated with decrease in tumor burden (Fig. 4B) without a significant decrease in average body weight in the mice (data not shown).

Figure 3. MLN4924 overcomes the protective effect of nonmalignant accessory cells of the bone microenvironment. MM.1S and OPM2 multiple myeloma cells were cultured in the presence and absence of HS-5 stromal cells (A and B) or differentiated osteoclasts (C and D) and treated with MLN4924 for 72 hours. The resulting cell viability was normalized to each respective no drug control ($n = 4$). Comparable activity was observed against MM.1S and OPM2 cells both in the presence or absence of these bone microenvironment cells with treatment with MLN4924.
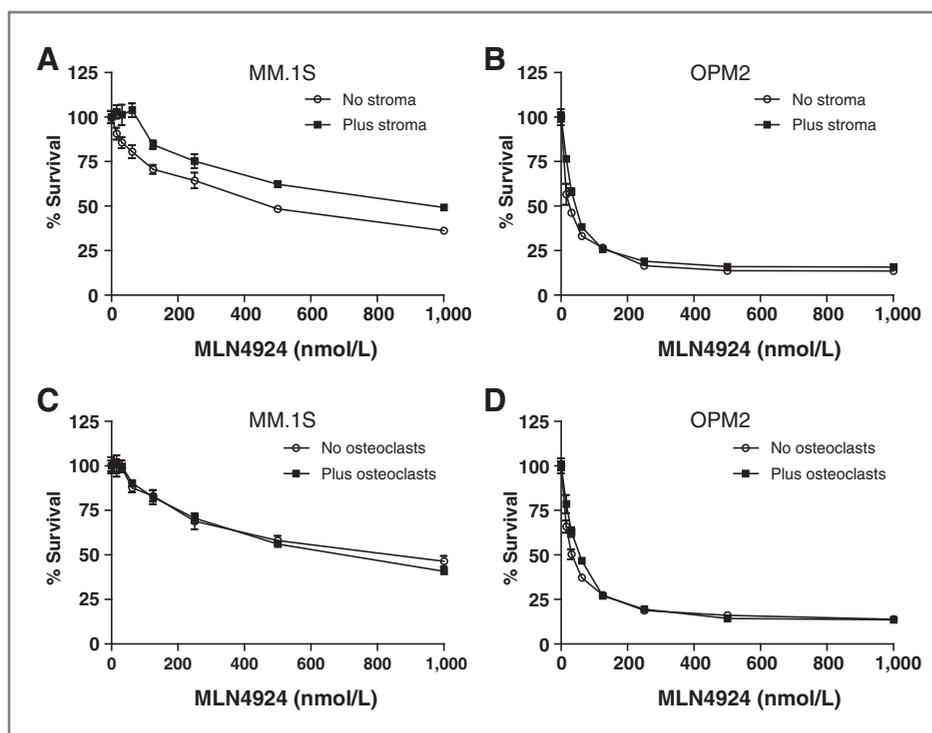
Molecular sequelae of MLN4924 treatment

Immunoblotting of MLN4924-treated MM.1S showed upregulation (as early as 8 hours) of p27, CTD1, and NRF2, which are known targets of the NEDD8 pathway, followed by markers of cell death, such as cleavage of PARP and caspase-3 at later time points (as early as 32 hours after initiation of treatment; Fig 5A). We also evaluated the gene expression profiling changes triggered in MM.1S cells treated with MLN4924 versus bortezomib. We observed that the profile of MLN4924-triggered transcriptional changes was distinct from the one induced by bortezomib exposure (Fig. 5B). Indeed, known transcriptional responses to 20S proteasome inhibition, such as upregulation of proteasome subunits (Fig. 5C) or heat shock proteins (data not shown), were minimal or absent in the MLN4924-treated cells (Supplementary Table). In addition, we observed that several MLN4924-suppressed genes were associated with higher expression in multiple myeloma patients with adverse clinical outcome or were expressed at higher levels in patients with plasma cell leukemia or multiple myeloma compared with MGUS or normal plasma cells. For example, *KLF2* (Supplementary Fig. S3A), *PHACTR1* (Supplementary Fig. S3B), and *FAM1985* (Supplementary Fig. S3C) were expressed at higher levels in SMM and MGUS compared with normal plasma cells; *FMA1985* was expressed higher in multiple myeloma patients who were deceased versus alive at 5 years (Supplementary Fig. S3D); *PSMC3IP* deceased expressed at higher levels in patients who were deceased versus alive at 1 year after diagnosis (Supplementary Fig. S3E); and *PSMC3IP* was expressed at higher levels in plasma cell leukemia versus multiple myeloma versus

MGUS patients (Supplementary Fig. S3F). These results support the notion that the molecular sequelae of MLN4924 treatment include genes with putative roles in the progression and clinical outcome of multiple myeloma patients.

MLN4924 combinations with other anti-multiple myeloma agents

We next evaluated combinations of MLN4924 with agents used for clinical management of multiple myeloma (dexamethasone, bortezomib, doxorubicin). Although no synergy was observed between MLN4924 and bortezomib (Fig. 6A), doxorubicin (Fig. 6B) or dexamethasone (Fig. 6C), there also was no antagonism observed, suggesting that MLN4924 does not adversely impact the antimultiple myeloma activity of these approved agents.

Discussion

The preclinical and clinical development of the proteasome inhibitor bortezomib for the treatment of multiple myeloma led to significant improvements in the clinical outcome of patients with this presently incurable plasma cell dyscrasia. The clinical success of bortezomib validated the notion that therapeutic targeting of ubiquitination and proteasome function is feasible without catastrophic toxicities. It also led to further efforts to identify other druggable targets in this pathway, particularly at molecular levels situated upstream of the proteasome. These targets have included the CRLs, which regulate degradation of proteins more selectively than the chymotryptic-like site of the 20S proteasome core. NEDD8-activating enzyme has been the focus of particular attention because it catalyzes the first step in the NEDD8 neddylation
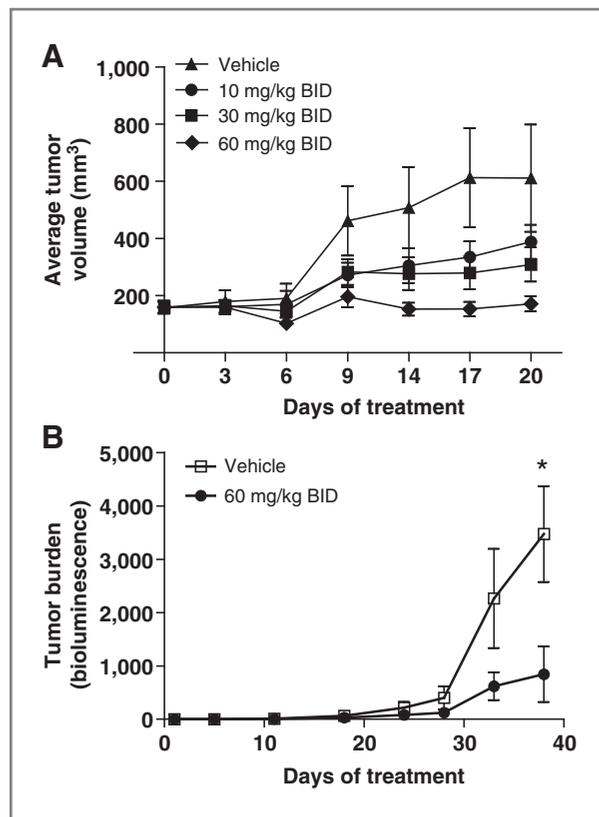
Figure 4. *In vivo* anti-multiple myeloma activity of MLN4924. RPMI8226/S cells were injected subcutaneously in CB.17-SCID mice and treatment was initiated once tumors had reached approximately 200 mm³. MLN4924 was dosed subcutaneously at 10, 30, and 60 mg/kg twice a day (BID) for 21 days and tumor volume monitored ($N = 12$ per group). There was a statistically significant difference in tumor burden only when comparing the control with 60 mg/kg group (A; $P < 0.05$; 1-way ANOVA). In addition, an orthotopic model of multiple myeloma was evaluated. Sublethally irradiated NOD/SCID mice injected i.v. with MM.1S multiple myeloma cells were randomly assigned to receive MLN4924 treatment (60 mg/kg BID i.p.) or vehicle control. B, tumor burden was evaluated by whole-body bioluminescence imaging weekly and tumor burden calculated using Living Image software. Treated mice had lower tumor burden compared with vehicle treated controls ($P = 0.1186$ day 33; $P = 0.0269$ day 38; $N = 6$ per group).

pathway. NEDD8 modification of cullins is required for ubiquitination of CRLs and targeting NEDD8-activating enzyme or Ubc12, the downstream enzyme in the pathway, allows for inhibition of the entire group of proteins degraded through the neddylation process.

Our interest on the therapeutic targeting of NEDD8-activating enzyme was further enhanced by our observation that multiple myeloma patients with high levels of *NEDD8* transcript had shorter PFS upon treatment with bortezomib. We also observed that, in the randomized phase III APEX trial which compared bortezomib with dexamethasone treatment in relapsed or refractory multiple myeloma, bortezomib-treated patients with high levels of *NEDD8* transcript did not have a significant advantage over dexamethasone-treated patients in terms of PFS. This suggests that perturbation of the NEDD8
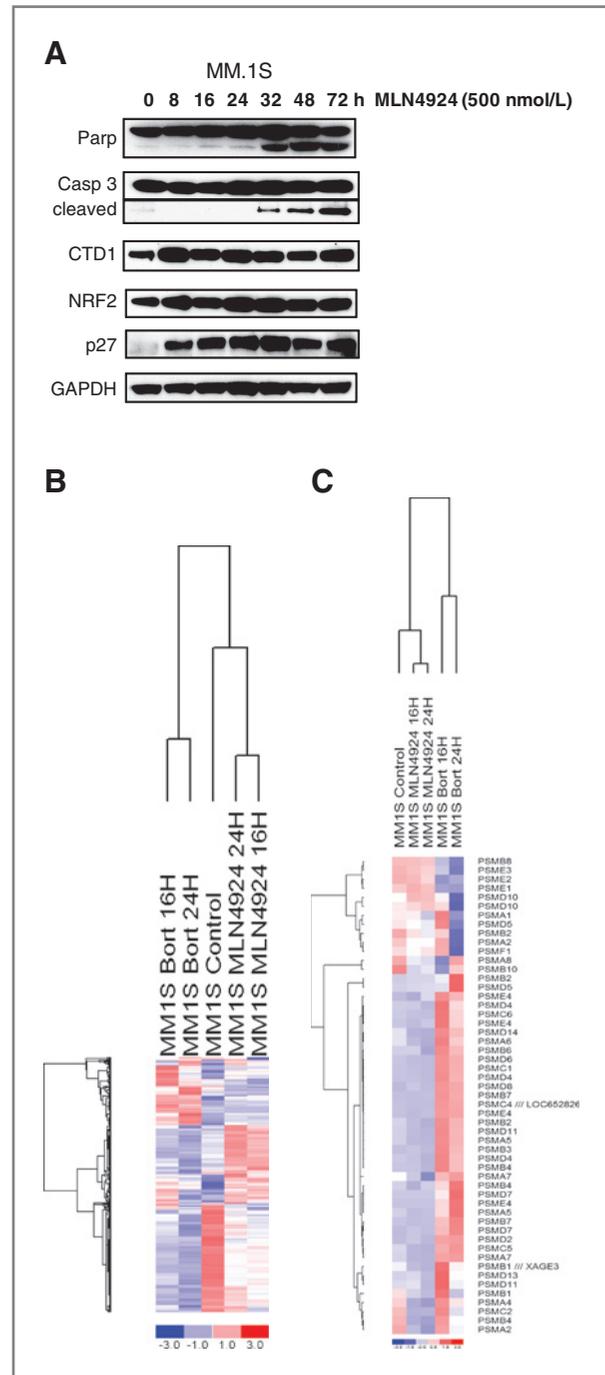

Figure 5. Immunoblotting analysis and transcriptional profiles of MLN4924-treated multiple myeloma cells. A, MM.1S cells treated with MLN4924 show upregulation (as early as 8 hours) of p27, CTD1, and NRF2, which are known targets of the NEDD8 pathway, followed by markers of cell death, such as cleavage of PARP and caspase-3 at later time points (as early as 32 hours after initiation of treatment). The gene expression profiling changes triggered in MM.1S cells by MLN4924 versus bortezomib were evaluated using the HG-U133plus2.0 oligonucleotide microarray. B, the profile of MLN4924-triggered transcriptional changes was distinct from the one induced by bortezomib exposure. C, known transcriptional responses to 20S proteasome inhibition, such as upregulation of proteasome subunits, were minimal or absent in the MLN4924-treated cells. Bort, bortezomib.

Figure 6. Combinations of MLN4924 with established anti-multiple myeloma agents. MLN4924 treatment was combined with agents used for clinical management of multiple myeloma, namely bortezomib (A), doxorubicin (B), and dexamethasone (C). Activity was observed with these combinations at drug doses relevant to those achieved clinically in the treatment of patients. Bort, bortezomib; Doxo, doxorubicin; Dex, dexamethasone.
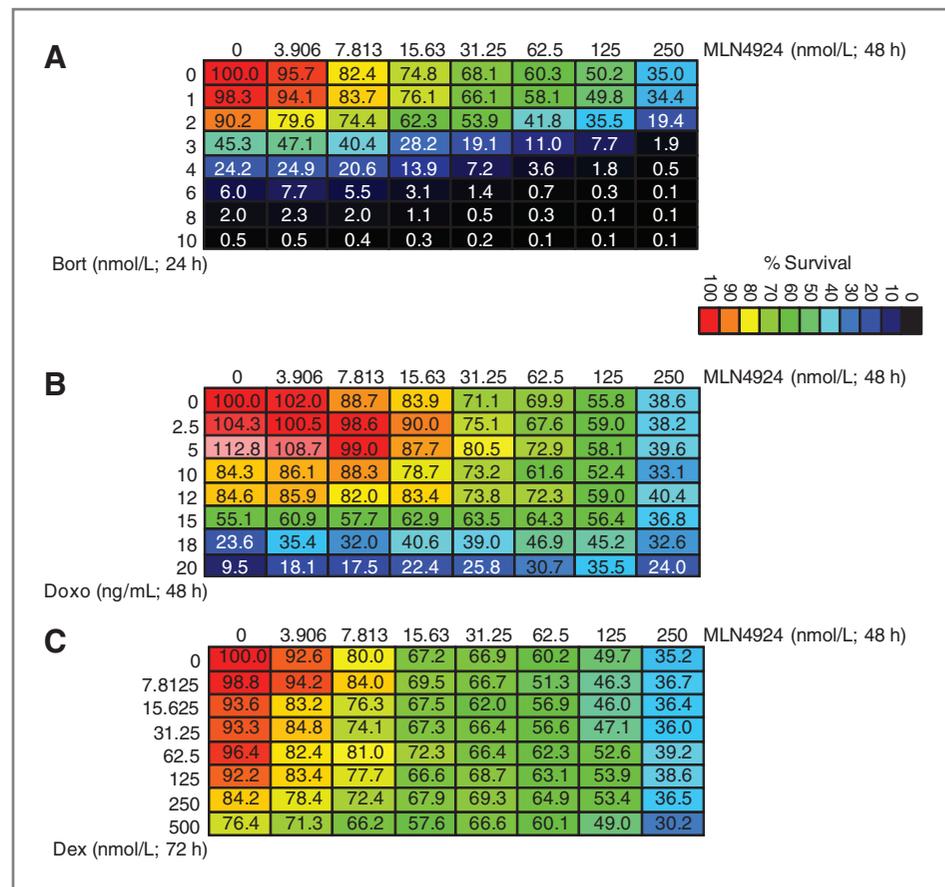
cascade may characterize a subset of multiple myeloma patients who do not receive maximum benefit from bortezomib-based therapies. For those subsets of patients, thersapeutic targeting of the NEDD8 pathway could be useful.

We evaluated the antimyeloma activity of the NEDD8-activating enzyme inhibitor MLN4924 in our *in vitro* preclinical models and observed a dose- and time-dependent reduction in viability of multiple myeloma cell lines as well as primary CD138$^+$ multiple myeloma tumor cells isolated from multiple myeloma patients. The EC$_{50}$ values of MLN4924 against multiple myeloma cell lines and primary tumor cells was comparable with or lower than the values observed in preclinical studies of other neoplasias considered to be highly responsive to NEDD8-activating enzyme inhibition (4, 10, 20). Importantly, this activity observed *in vitro* against multiple myeloma cells could be achieved with exposure to MLN4924 for 48 hours or less. Furthermore, we observed that the response of multiple myeloma cells to MLN4924 was similar when these cells were cultured alone versus cocultured with bone marrow stromal cells or osteoclasts. This indicates that NEDD8-activating enzyme inhibition can circumvent the proliferative/antiapoptotic stimulation provided to multiple myeloma cells by the interactions with non-malignant accessory cells of the bone marrow microenvironment. Consistent with this observation, MLN4924

significantly reduced the tumor burden of both subcutaneous and orthotopic multiple myeloma lesions in immunocompromised mice.

Mechanistically, NEDD8-activating enzyme inhibition with MLN4924 treatment was associated with early increase in protein levels of p27, CTD1, and NRF2. This observation is compatible with the known role of NEDD8-activating enzyme in regulating intracellular levels of these proteins. A more open-ended analysis of the transcriptional profile of MLN4924-treated multiple myeloma cells showed changes distinct from those triggered by bortezomib. For instance, although bortezomib triggers major increases in transcript levels for diverse heat shock proteins family members and proteasome subunits, MLN4924 triggered minimal, if any, such changes. This difference likely reflects the distinct functional consequences of inhibiting the β5 subunit of the 20S proteasome core versus NEDD8-activating enzyme. The β5 subunit of the proteasome core regulates the intracellular levels of a larger pool of ubiquitinated proteins. In contrast, NEDD8-activating enzyme regulates the neddylation of a more restricted set of proteins. Because the targets of NEDD8-activating enzyme regulation are enriched for proteins with critical roles in proliferation, survival, or drug resistance, MLN4924 achieves antitumor responses, even though it does not induce the pronounced intracellular proteotoxic stress

triggered by bortezomib. Interestingly, we observed that a myeloma cell line model with constitutive resistance to bortezomib and its isogenic bortezomib-sensitive parental line are both responsive to MLN4924. This suggests that resistance to inhibition of the β5 subunit of the 20S core by bortezomib does not necessarily confer cross-resistance to inhibition of upstream molecular targets, such as NEDD8-activating enzyme.

On the basis of these observations, we further explored combination studies of MLN4924 with established anti-myeloma agents, such as bortezomib, doxorubicin, and dexamethasone. These *in vitro* combinations showed no antagonism between the combined agents, suggesting that currently available regimens for multiple myeloma treatment may be combined with MLN4924 in further preclinical and potentially clinical studies.

Our results indicate that NEDD8-activating enzyme inhibition with MLN4924 has *in vitro* and *in vivo* activity in preclinical models of multiple myeloma. The molecular impact of NEDD8-activating enzyme inhibition is distinct from the sequelae triggered by bortezomib, as evidenced by the differential molecular profiles induced by MLN4924 versus bortezomib, as well as the activity of MLN4924 against bortezomib-resistant cells. These observations provide a platform for further evaluation of NEDD8-activating enzyme inhibitors to inform the design of clinical trials in multiple myeloma.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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