LYMPHOID NEOPLASIA

# MLN4924, an NAE inhibitor, suppresses AKT and mTOR signaling via upregulation of REDD1 in human myeloma cells

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## Key Points

- Blockade of NAE and bortezomib induces phosphatidylinositol 3-kinase/mTOR inhibition.
- NAE inhibition and bortezomib combined induce synergistic plasma cell apoptosis.

**The function and survival of normal and malignant plasma cells depends on the elaborately regulated ubiquitin proteasome system. Proteasome inhibitors such as bortezomib have proved to be highly effective in the treatment of multiple myeloma (MM), and their effects are related to normal protein homeostasis which is critical for plasma cell survival. Many ubiquitin ligases are regulated by conjugation with NEDD8. Therefore, neddylation may also impact survival and proliferation of malignant plasma cells. Here, we show that MLN4924, a potent NEDD8 activating enzyme (NAE) inhibitor, induced cytotoxicity in MM cell lines, and its antitumor effect is associated with suppression of the AKT and mammalian target of rapamycin (mTOR) signaling pathways through increased expression of REDD1. Combining MLN4924 with the proteasome inhibitor bortezomib induces synergistic apoptosis in MM cell lines which can overcome the prosurvival effects of growth factors such as interleukin-6 and insulin-like growth factor-1. Altogether, our findings demonstrate an important function for REDD1 in MLN4924-induced cytotoxicity in MM and also provide a promising therapeutic combination strategy for myeloma. (*Blood*. 2014;123(21):3269-3276)**

## Introduction

Posttranslational modifications of proteins such as phosphorylation, glycosylation, ubiquitination, and acetylation regulate protein structure, activity, localization, and stability. Aberrant protein modification profiles are related to disease and cancer pathogenesis.[1-5] Among the most common posttranslational modifications of proteins, ubiquitination represents an emerging area of research interest due to its importance in so many aspects of cancer cell biology.

Ubiquitination of proteins regulates cell cycle, differentiation, and apoptosis.[6-11] Ubiquitination of target proteins is achieved via sequential enzymatic reactions mediated by E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 ligases (ubiquitin ligases). Among the known E3 ligases, cullin-ring ligases (CRLs) represent the largest and most frequently used group. The activities of CRLs are regulated by neddylation of cullin family proteins within the complex through covalent attachment of NEDD8, a 9-kDa ubiquitin-like small molecule. Protein neddylation is mediated through an enzymatic cascade in a similar manner to the ubiquitin system, which is initiated by NEDD8 activating enzyme (NAE). Thus, E3 ligases are in part regulated by neddylation, suggesting that tight regulation of ubiquitination can be imposed by enzymes upstream of the proteasome. Among all the known neddylated proteins, the cullin protein family is the major group of substrates and acts as an essential component of CRLs. MLN4924 is a newly developed NAE inhibitor with high specificity for the target enzyme.[12,13] Through its effects on protein neddylation, MLN4924 is known to have many effects including induction of DNA rereplication, apoptosis, autophagy, cell growth inhibition through p21-dependent senescence, and regulation of T-cell–mediated inflammatory response.[14-18] However, from a plasma cell biology perspective, MLN4924 can selectively inhibit the turnover of specific proteins which are targeted by CRLs.

The proteasome acts as the main lysosomal-independent protein degradation system within cells to regulate protein metabolism (turnover) which is critical in normal cell growth and survival as well as malignant cells. The phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathway integrates diverse signals to regulate cell growth, survival, metabolism, and autophagy. The interaction between protein metabolism and the PI3K/mTOR pathway is also an area of increasing interest among many cancers.[19-21] In the current report, we describe the novel effects of NAE inhibition on myeloma cell survival alone or in combination with a focus on how neddylation can impact not only growth, but more directly impact cellular metabolism and proliferation through its effects on REDD1 (regulated in development and DNA damage responses 1) and thus on the PI3K/mTOR signaling pathway. These results suggest, in aggregate, that regulation of ubiquitination and proteasome function have broad implications for malignant plasma cell metabolism and survival.

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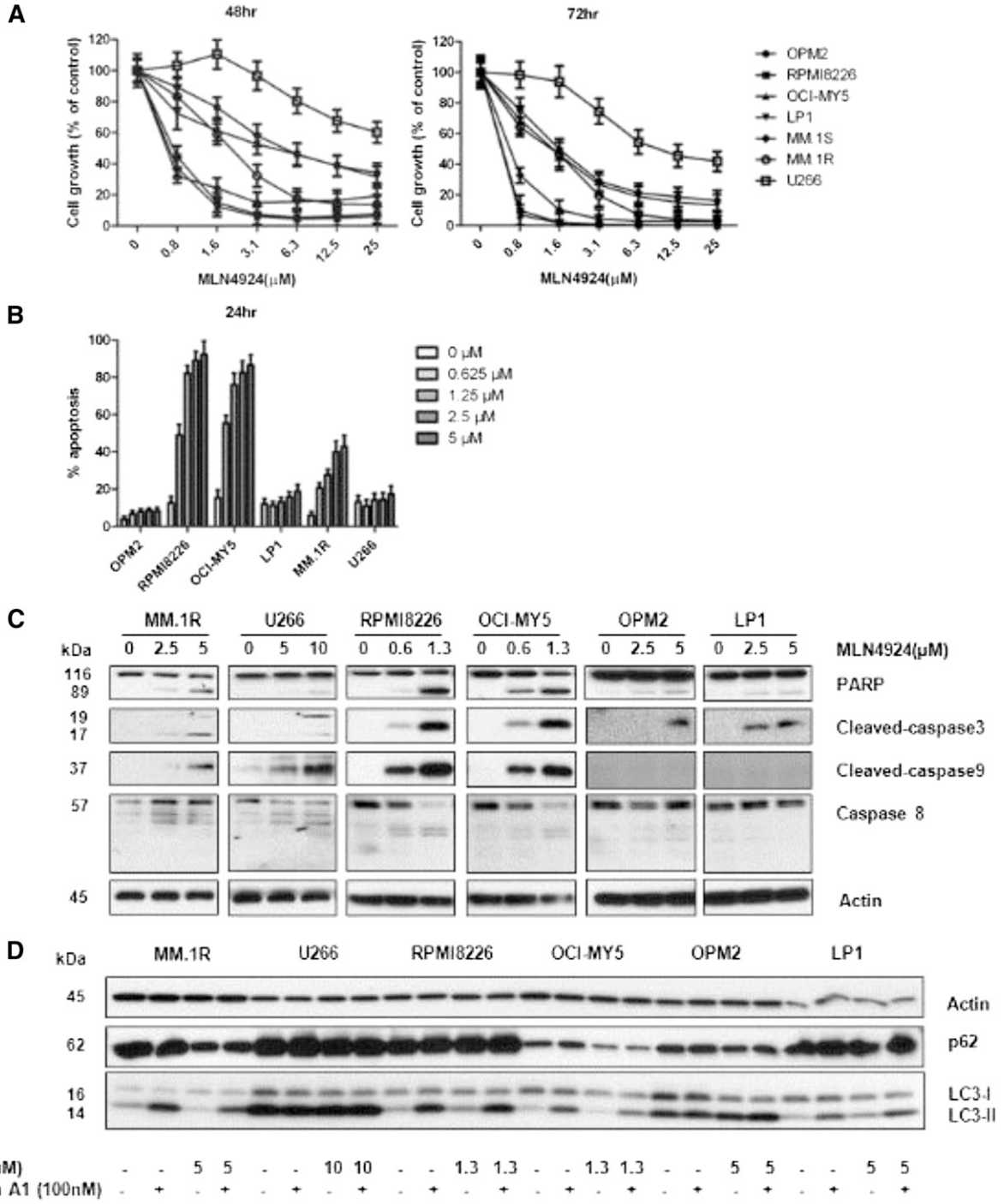


**Figure 1. MLN4924 induces cytotoxicity in human MM cell lines.** (A) HMCLs were treated with increasing concentrations of MLN4924 for 48 or 72 hours, respectively. MTT assays were performed to assess growth inhibition of MLN4924. Data represent mean ± SD derived from 3 independent experiments. (B) HMCLs were cultured with increasing doses of MLN4924 (0.625-5 μM) for 24 hours and assessed for apoptosis using flow cytometry for annexin V/PI. (C) HMCLs were treated with MLN4924 for 24 hours and cell lysates were assessed by western blotting for PARP, cleaved caspase 3, cleaved caspase 9, and cleaved caspase 8. (D) HMCLs were exposed to MLN4924 for 24 hours, before cell collection, cells were incubated with or without 100 nM bafilomycin A1 for 2 hours. HMCL, human myeloma cell line.

## Materials and methods

### Cells

Dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human MM cell lines were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). All cell lines are maintained in RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin streptomycin, 1× nonessential amino acids, 1 mM sodium pyruvate (CellGro), and 0.05 M 2-mercaptoethanol (Sigma).

### Reagents

MLN4924 and bortezomib were kindly provided by Millennium Pharmaceuticals. Recombinant human interleukin-6 (IL-6) and insulin-like growth

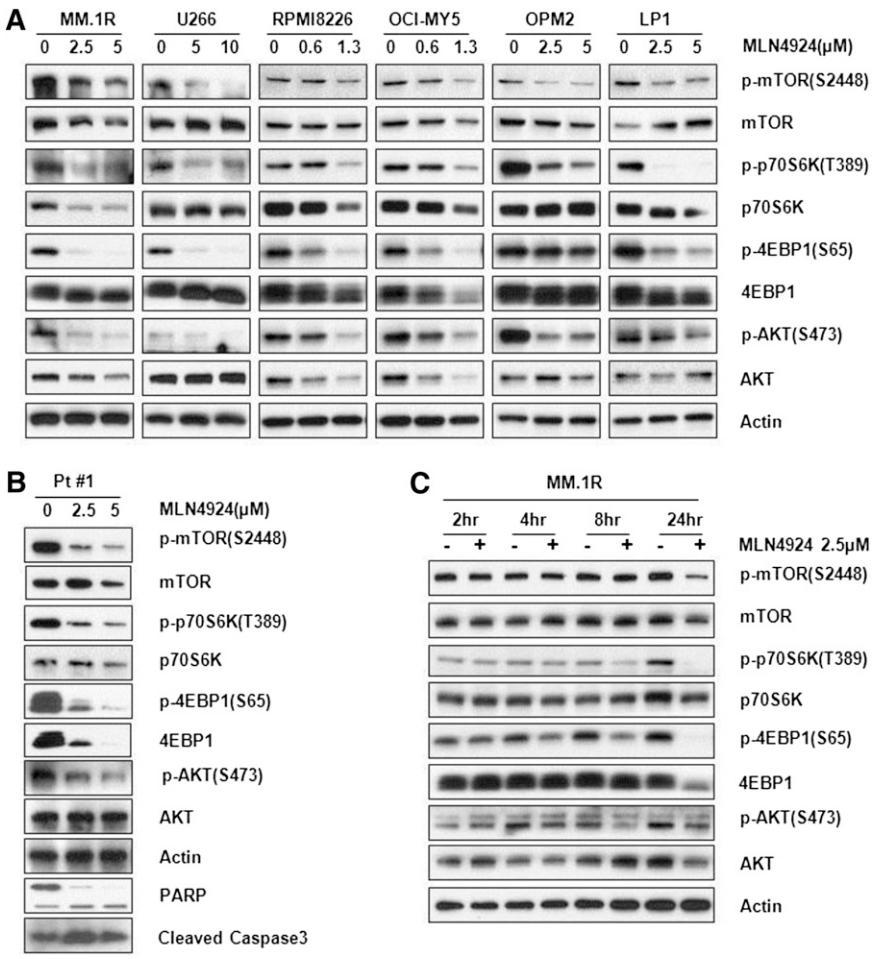


**Figure 2. MLN4924 inhibits PI3K/mTOR signaling pathway.** (A) MM.1R and U266 cells were treated as with Figure 1C and analysis of total and phosphoprotein expression was evaluated using western blot. (B) Primary human MM cells were treated with 2.5 or 5 μM MLN4924 for 24 hours; apoptosis and specific signal pathway were checked using western blot analysis. (C) The MM.1R cell was treated with 2.5 μM MLN4924 or control for the indicated time points. Total and phosphoprotein expression were again tested using western blot analysis.

factor-1 (IGF-1) were obtained from R&D Systems, Inc. Cycloheximide (CHX) and bafilomycin A1 were purchased from Sigma.

### Growth inhibition assay

The growth inhibition effect of MLN4924 alone or combination with bortezomib was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide assay (MTT kit; American Type Culture Collection) according to the manufacturer's instruction.

### Flow cytometric assay

Cell apoptosis was determined using fluorescein isothiocyanate (FITC)–annexin V/propidium iodide (PI) staining (BD Biosciences) following the manufacturer's protocol. Data were analyzed using FlowJo (TreeStar) software.

### RNA purification and reverse transcription qPCR

Total RNA was extracted from MM.1R cells using the RNeasy mini kit (Qiagen). Total RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative polymerase chain reactions (qPCRs) were carried out with the 7500 Fast Real-Time PCR System (Applied Biosystems) using Sybr Green protocol (Sigma), and the data acquired were analyzed with 7500 software (version 2.04 from Applied Biosystems). The following primers were used in the qPCR reactions: human REDD1: forward, 5′-ACGCACTTGTCTTAGCAGTT-3′; reverse, 5′-TAAGCCGTGTCTTCCTCC-3′; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-GCCACATCGCTCA GACAC-3′; reverse, 5′-GGACTCCACGACGTACTCA-3′.

### Immunoblotting

Cell lysate preparation and western blots were performed as described previously.[22] Antibodies used were as follows: actin (Sigma), REDD1 (Protein Tech Group, Inc.); all other antibodies were purchased from Cell Signaling Technology.

### Small interfering RNA (siRNA) and transfection

MM.1R cells were transiently transfected with 100 nM REDD1 On-TARGET plus SMARTpool or Nontargeting Pool (Dharmacon Research Inc.) using Nucleofector Kit V (Amaxa Biosystems).

### Patient samples

Bone marrow aspirates from multiple myeloma (MM) patients were prepared following Emory University Institutional Review Board–approved consent. This study was conducted in accordance with the Declaration of Helsinki.

Briefly, the samples were diluted with phosphate-buffered saline (PBS) and underlain with lymphocyte separation medium (Cellgro). Buffy coat cells were carefully collected and washed with PBS, then resuspended in separation buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA) and incubated with $CD138^+$ microbeads followed by plasma cell purification using MACS Cell Separation MS Columns according to the manufacturer's protocol (Miltenyi Biotec). The isolated cells were checked for purity by staining with anti-CD38-allophycocyanin, anti-CD45-allophycocyanin-Cy7, and anti-CD138-FITC antibodies (BD Biosciences) using flow cytometry. Cells were suspended in culture medium and treated with MLN4924 and/or bortezomib as indicated for western blot analysis.

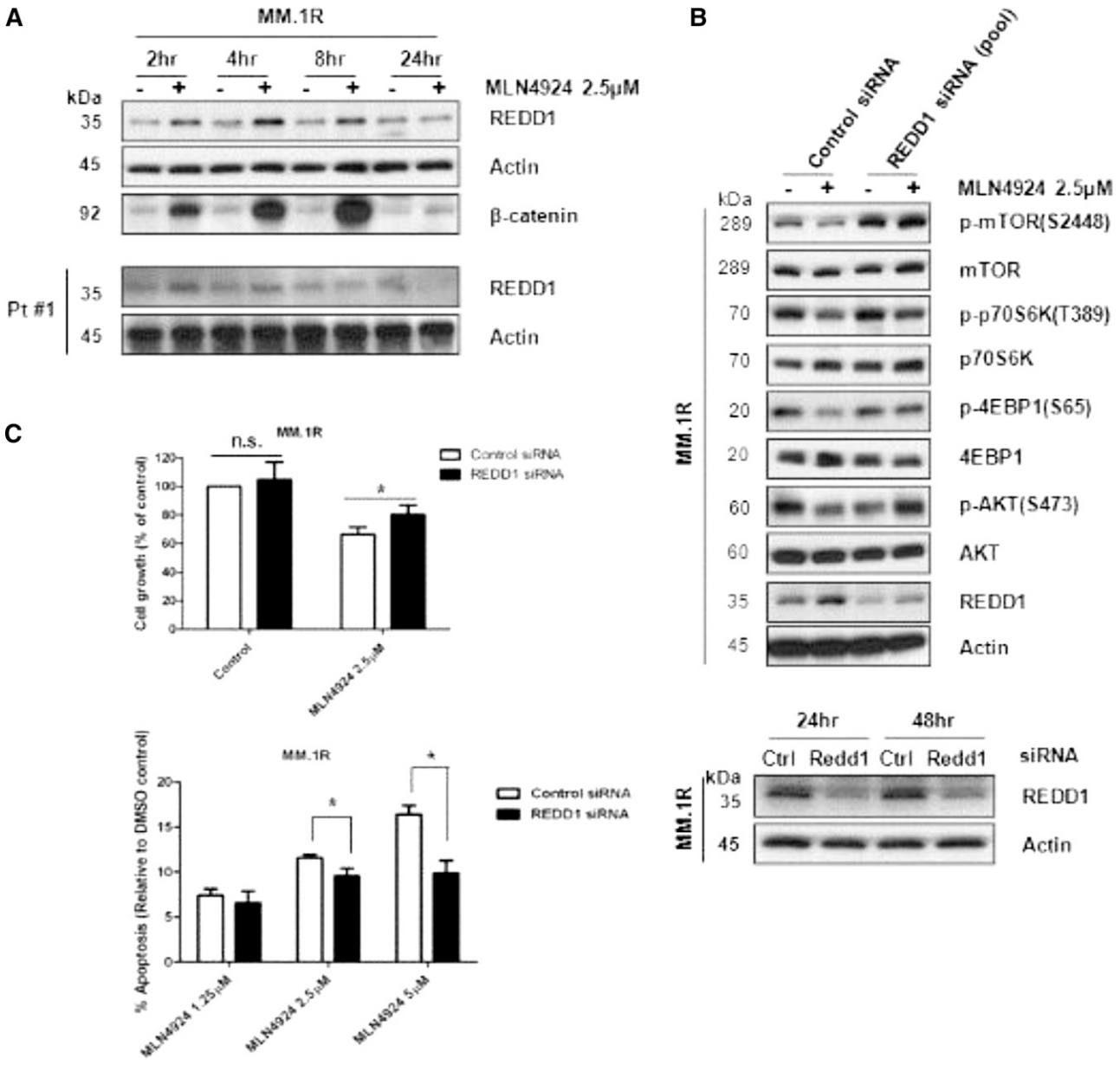


**Figure 3. Effect of REDD1 on efficacy of MLN4924.** (A) REDD1 expression by western blot with MLN4924 or control over time in MM.1R and patient samples. (B) Silencing of REDD1 by siRNA blocks the signaling effects of MLN4924. (C) Silencing of REDD1 inhibits cytotoxicity effects of MLN4924 when compared with siRNA control, $*P < .01$; paired Student *t* test.

## Results

### MLN4924 induces growth arrest and apoptosis in human myeloma cell lines

The effect of varying time and dose effects of MLN4924 was tested in a series of 7 human MM cell lines (OPM2, RPMI8226, OCI-MY5, LP1, MM.1S, MM.1R, and U266). All cell lines tested demonstrated time- and dose-dependent growth inhibition (Figure 1A). Sensitivity to MLN4924 is not related to p53 status as response was noted in p53 wild-type cell lines (MM.1S, M.1R) as well as those with mutant p53 status (LP1, OCI-MY5, RPMI8226, OPM2, and U266). The U266 cell line was more resistant with 50% inhibitory concentration of 10 μM at 72 hours, whereas OCI-MY5 and RPMI8226 were more sensitive with 50% inhibitory concentration of 0.5 μM at 72 hours. When the effect of growth inhibition on apoptosis was assessed using flow cytometry and annexin V/PI staining the effects were less clear, with 2 cell lines (RPMI and OCI) demonstrating the greatest degree of apoptosis, even with relatively lower doses of MLN4924 (Figure 1B). Apoptosis was confirmed by western blot analysis for caspase and poly ADP ribose polymerase (PARP) cleavage, further suggesting that the RPMI and OCI cell lines were the most sensitive to the proapoptotic effects of MLN4924 (Figure 1C). It has been reported that MLN4924 can induce autophagy in certain human cancer cells (breast and liver cancer). We next determined whether MLN4924 induces autophagy in MM cell lines. As shown in Figure 1D, MLN4924 did not induce an increase of LC3-II, an indicator of ongoing autophagy. Of note, MLN4924 did decrease p62 expression in some of the MLN4924-treated cell lines, including

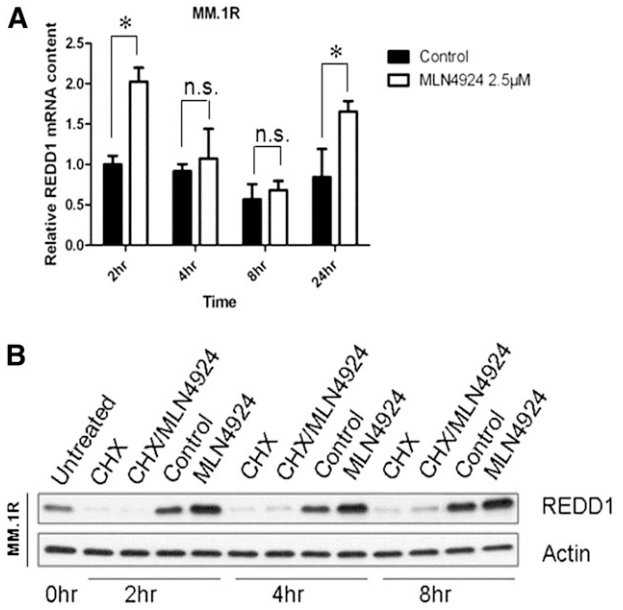


**Figure 4. MLN4924 upregulates REDD1 at mRNA and protein levels.** (A) MM.1R cells were treated with 2.5 μM MLN4924 for 2, 4, 8, and 24 hours, increasing REDD1 mRNA levels as assessed by quantitative RT-qPCR. Values represent the relative expression ratio of REDD1 to GAPDH. (B) MM.1R cells were pretreated with DMSO or 10 μg/mL CHX for 1 hour, followed by DMSO or 2.5 μM MLN4924 for 2, 4, 8 hours, demonstrating an increase in protein level if protein synthesis is blocked.

MM.1R, OCI-MY5, and OPM2. However, when bafilomycin A1 (a selective inhibitor of vacuolar-type [v-type] $H^+$ ATPase) was used to prevent autophagosome and lysosome fusion, we did not observe accumulation of p62. These data suggest that MLN4924 does not induce autophagy in MM cell lines. Based on the more uniform effect on growth inhibition as assessed by MTT assay, this led us to evaluate the impact of MLN4924 on the mTOR pathway and metabolism.

### MLN4924 inhibits AKT, mTOR signaling pathway

It has been reported in other preclinical and clinical models that the use of proteasome inhibition results in an initial activation of AKT, followed by a subsequent reduction of AKT expression,[20,23] but it is unclear whether blockade upstream of the proteasome with an agent that may not completely block proteasome function can achieve a similar effect. We began to further interrogate the impact of MLN4924 on the PI3K/mTOR cascade. Using the same 6 human myeloma cell lines, there was a dose-dependent reduction in phosphorylated mTOR, p70S6K, 4EBP1, and AKT within 24 hours of exposure to MLN4924 (Figure 2A). Additionally, total protein levels of mTOR, p70S6K, 4EBP1, and AKT were decreased in MM.1R, RPMI8226, and OCI-MY5 cells which were more sensitive to the proapoptotic effects of MLN4924 (Figure 2A). We also observed AKT and mTOR signaling pathway inhibition on freshly isolated cells from a myeloma patient (Figure 2B). To further evaluate whether the reduction in protein phosphorylation was a consequence of blocked expression or activation, we performed a short-interval time-course analysis (Figure 2C) which demonstrated a downregulation of protein phosphorylation at the 8-hour time point, while reduction of total protein expression of the same proteins was delayed until after 24 hours. This suggested 2 different effects of MLN4924 on the PI3K/mTOR signaling cascade: inhibition of protein phosphorylation as well as protein translation.

### MLN4924 induces REDD1 expression and suppresses the AKT, mTOR pathway

Based on the observation that REDD1 is a CRL substrate and is known to modulate mTOR signaling,[24] we evaluated the impact of MLN4924 on REDD1 expression as a possible explanation for the effects on signaling we had observed. Treatment with MLN4924 induced rapid REDD1 expression as early as 2 hours postexposure (Figure 3A); β-Catenin, another known CRL substrate was also rapidly upregulated following MLN4924 addition. The similar pattern of REDD1 expression was also observed on primary human MM cells. To investigate whether the effect of MLN4924 on mTOR activation was a direct effect of REDD1 expression, we used transient transfection with siRNA to silence REDD1 (Figure 3B). Transfected cells were treated with 2.5 μM MLN4924 for 8 hours. Western blot (Figure 3B) demonstrated an increase in the phosphorylation of mTOR, p70S6K, and 4EBP1 as well as AKT compared with the control siRNA, suggesting that REDD1 is necessary for the effect of MLN4924 on dephosphorylation of the PI3K/mTOR pathway.

However, it was unknown whether the cytotoxicity effects of MLN4924 were also REDD1 dependent. We then evaluated the effect of MLN4924 treatment using MTT and flow cytometry assays for apoptosis in REDD1-silenced and control cells. Among cells with REDD1 silencing, there was less growth inhibition and apoptosis when treated with MLN4924 than in the REDD1 control silenced cells (Figure 3C; supplemental Figure 1, available on the *Blood* Web site).

### REDD1 upregulation is associated with both RNA and protein levels

To further identify the mechanism responsible for MLN4924-induced REDD1 expression, we examined the REDD1 transcript level using reverse transcription (RT)–qPCR. Following exposure to MLN4924, there was rapid and sustained increase in REDD1 transcription beginning at 2 hours postexposure, and then noted again at 24 hours (Figure 4A). We then examined whether MLN4924 had an impact on REDD1 protein stability posttranslation. MM.1R cells were pretreated with CHX for 1 hour to inhibit de novo protein synthesis followed by treatment with MLN4924 or dimethylsulfoxide (DMSO). The use of 10 μg/mL CHX almost completely blocked protein translation for 8 hours, while at the 4- and 8-hour time point, we were able to detect an increase in REDD1 expression (Figure 4B). From these results it appears that MLN4924 has a significant impact on the transcription and stability of REDD1 thus leading to its effects on inhibition of mTOR signaling.

### MLN4924 and bortezomib are synergistic

Based upon prior data demonstrating the effect of bortezomib on AKT activation and mTOR signaling, we tested the combination effect of bortezomib with MLN4924. Combining MLN4924 with bortezomib induced greater cytotoxicity in MM.1S, MM.1R, and U266 (Figure 5A). The effect on apoptosis was further assessed by the observation of increased caspase and PARP cleavage over a single agent alone (Figure 5B). In addition, the suppression of the PI3K/mTOR pathway was more marked with the combination than was seen with either single agent (Figure 5B). The combination indices in MM.1R, MM.1S, OPM2, U266, and RPMI8226 cell lines are all $<1$, supporting synergistic cell death (Figure 5C, Table 1). To further determine whether the interaction worked on primary human MM cells, we performed a western blot assay on apoptosis and the AKT, mTOR pathway with patient samples (Figure 5D). Data from

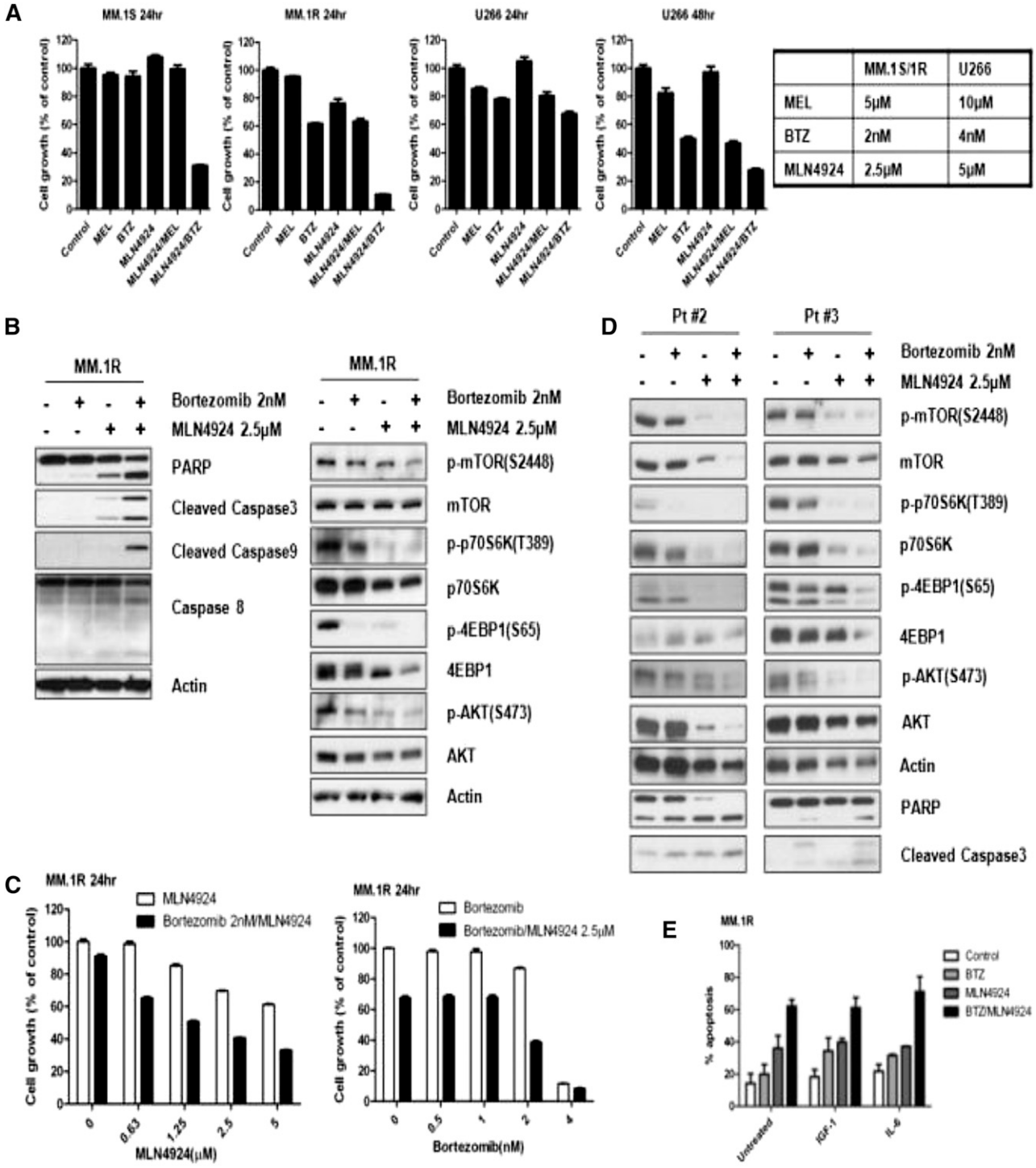


**Figure 5. Bortezomib increases MLN4924-induced cell cytotoxicity.** (A) MM.1S, MM.1R, and U266 were treated with MLN4924 (2.5 or 5 μM) separately or concurrently with MEL (5 or 10 μM) or BTZ (2 or 4 nM). MTT assay was used to evaluate growth inhibition. (B) MM.1R cells or primary human MM cells (D) were treated with 2 nM bortezomib, 2.5 μM MLN4924, or the combination for 24 hours with increased inhibition of the PI3K/mTOR pathway and increased PARP and caspase cleavage. (C) MM.1R cells were treated with serial concentrations of MLN4924 and BTZ. MTT assays were performed to assess the growth inhibition effects. Combination index values are shown in Table 1 using the Calcusyn software program. (E) MM.1R cells were treated for 24 hours with 2 nM bortezomib, 2.5 μM MLN4924 alone or in combination in the presence or absence of IGF-1 (25 ng/mL) or IL-6 (10 ng/mL). Cell death was assessed using annexin V/PI staining. BTZ, bortezomib; MEL, melphalan.

2 patient samples also showed more cell apoptosis and pathway inhibition upon the combination treatment.

IL-6 and IGF-1 are 2 important growth factors known to promote myeloma cell growth, survival, proliferation, and drug resistance. Both IL-6 and IGF-1 are known to activate the PI3K/mTOR pathway. To evaluate whether these growth factors are able to protect against MLN4924-induced cytotoxicity through constitutive activation of the PI3K/mTOR axis, MM.1R cells were treated with

**Table 1. Combination index of MLN4924 with bortezomib**

| Cell line | MLN4924 dose, μM | Bortezomib dose, nM | CI |
|---|---|---|---|
| OPM2 | 5 | 4 | 0.17836 |
| | 5 | 8 | 0.14499 |
| | 10 | 4 | 0.10951 |
| | 10 | 8 | 0.11097 |
| U266 | 2.5 | 4 | 0.42012 |
| | 5 | 4 | 0.40122 |
| | 10 | 4 | 0.39735 |
| | 20 | 4 | 0.40017 |
| MM.1S | 1.25 | 4 | 0.47348 |
| | 2.5 | 4 | 0.43892 |
| | 1.25 | 8 | 0.42433 |
| | 2.5 | 8 | 0.43257 |
| RPMI8226 | 0.625 | 4 | 0.47984 |
| | 1.25 | 4 | 0.68028 |
| | 2.5 | 4 | 0.71090 |
| | 2.5 | 2 | 0.96746 |
| MM.1R | 0.625 | 2 | 0.78764 |
| | 1.25 | 2 | 0.86669 |
| | 2.5 | 2 | 0.59118 |
| | 2.5 | 4 | 0.65250 |

CI, combination index.

bortezomib and MLN4924 either alone or in combination in the presence of IL-6 or IGF-1. Using cytotoxicity assessment from flow cytometry, neither IL-6 nor IGF-1 was able to block either single agent or combination-induced therapy (Figure 5E).

### Knockdown REDD1 decreases the cytotoxicity of bortezomib and MLN4924

Based upon the observation that REDD1 is required for MLN4924 to induce apoptosis, we sought to evaluate the effect of REDD1 silencing on the combination of bortezomib and MLN4924. As predicted, silencing REDD1 induced less growth inhibition in the combination (Figure 6A), although the combination was still more effective than either single agent, and there was less dephosphorylation seen with the combination than in the control siRNA group (Figure 6B). These data support the impact of REDD1 activity on the single agent or combination effect of MLN4924.

## Discussion

The PI3K signaling pathway plays a vital role in regulating cell growth, proliferation, and survival, though direct targeting of PI3K remains challenging due to its numerous functions in normal cell function and survival.[25] PI3K/AKT regulates many downstream signaling cascades and mTOR complex 1 (mTORC1) is one of its major effectors. There are 2 mTOR complexes which are functionally different. mTORC1 integrates signaling from growth factors such as IGF-1 and IL-6 as well as aberrant activation of the Ras/mitogen-activated protein kinase cascade.[26-28] The mTORC1 axis can activate messenger RNA (mRNA) translation through regulation of p70S6K and 4EBP1. Activation of mTORC1 also inhibits the prosurvival molecule AKT through p70S6k-IRS1–mediated negative regulation; the mTOR complex 2 (mTORC2) can act as an activator of AKT through phosphorylation of serine 473, and thus can reactivate the pathway following initial suppression mediated by AKT inhibition or mTORC1 inhibition alone.

MLN4924, an investigational NEDD8-activating enzyme inhibitor, exhibits a promising antitumor effect through both clinical and laboratory observation. The compound has active in vitro and in vivo activity in preclinical models of multiple myeloma.[29] In this report, we demonstrate that MLN4924 inhibits both AKT and mTOR signaling in MM, which can be further enhanced through the combination with bortezomib. The effect on mTOR signaling is dependent upon induced upregulation of REDD1, a known negative regulator of the mTOR pathway. However, unlike rapamycin, MLN4924 did not induce reciprocal activation of AKT. It has been reported that REDD1 inhibits mTORC2 activity in neuronal cell and skeletal muscle cell,[30,31] suggesting that through the use of MLN4924 the PI3K/mTOR axis can be inhibited without reciprocal activation. To further support this, when REDD1 was silenced, the activity of AKT and mTOR was enhanced, supporting the observation that REDD1 activity is critical for the inhibition of both mTOR and AKT. We then combined MLN4924 with bortezomib based on the hypothesis that although bortezomib initially increased activated AKT, ultimately AKT function is suppressed, suggesting possible synergy with MLN4924. In our combination studies, we demonstrated synergy with bortezomib, and more potent pathway suppression of the PI3K/mTOR axis than is seen with either agent

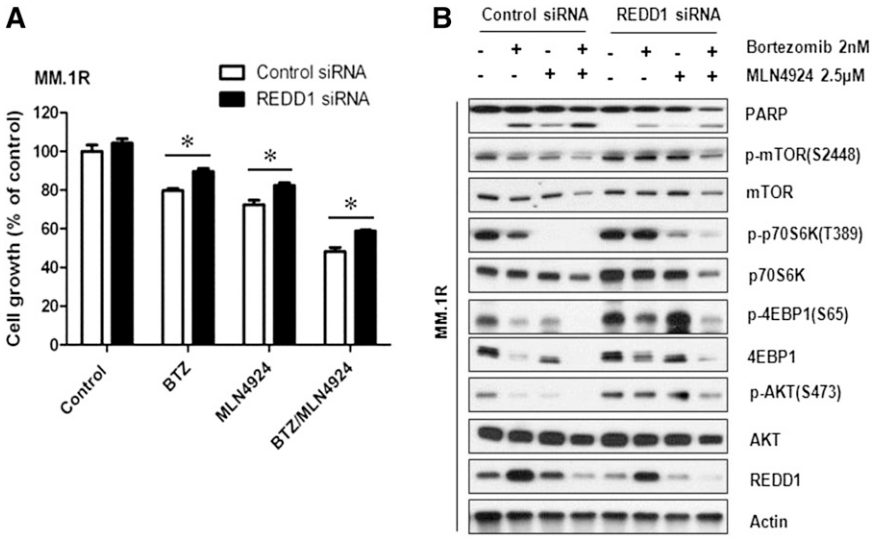


**Figure 6. Silencing REDD1 blocks bortezomib + MLN4924-induced cytotoxicity.** MM.1R was transfected with nontarget siRNA or REDD1 siRNA. Twenty-four hours after transfection, cells were treated with 2 nM bortezomib, 2.5 μM MLN4924 or the combination. Growth inhibition was assessed using MTT assay (A); western blotting was used to check specific signaling proteins (B).

alone, and this occurs independent of exogenous IL-6 or IGF-1. Furthermore, the impact of the combination effect was in part abrogated when REDD1 is silenced, suggesting that the activity of MLN4924 is dependent upon REDD1, but not bortezomib.

Conceptually, the effect of the combined proteasome inhibitor with a regulator of protein homeostasis such as MLN4924 is counterintuitive, yet their cellular effects appear to be quite different. Although proteasome inhibition is clearly important for plasma cell survival,[32] the effect from MLN4924 appears to be mediated by its effects on REDD1 and the subsequent effect on mTOR.

Taken together, the present study demonstrates that MLN4924 is a potent antitumor compound suppressing AKT and mTOR signaling in myeloma cell lines. Downregulation of AKT and mTOR signaling is associated with increased expression of REDD1. Combination of MLN4924 with the proteasome inhibitor bortezomib induces synergistic apoptosis in human myeloma cells, and suggests that combinations with other upstream regulators of protein homeostasis such as sumoylation or alternative regulators of the ubiquitin proteasome system (UPS) could have additional activity. These findings suggest that among patients with activated PI3K myelomas, combinations of MLN4924 and bortezomib may offer a mechanism for enhanced efficacy over single-agent bortezomib alone and not result in reciprocal activation of feedback loops.

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## Authorship

Contribution: Y.G., J.L.K., L.H.B., L.B., and S.L. designed the research; Y.G., C.T., S.M.M., and S.L. performed the work; Y.G., L.H.B., L.B., S.-Y.S., J.C., R.D.H., and S.L. analyzed data; and Y.G., C.T., J.L.K., L.B., J.C., R.D.H., S.M.M., L.H.B., and S.L. wrote the paper.

Conflict-of-interest disclosure: J.L.K. is a consultant for Millennium, Celgene, Onyx, and Novartis. R.D.H. and L.H.B. are consultants for Onyx. S.L. is a consultant for Millennium, Celgene, Onyx, Novartis, and Bristol-Myers Squibb. The remaining authors declare no competing financial interests.

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