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NEDD8 inhibition overcomes CKS1B-induced drug resistance by upregulation of p21 in multiple myeloma

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Abstract

Purpose—CKS1B is significantly upregulated in multiple myeloma (MM) and associated with poor prognosis. The identification of novel therapies is essential for effective treatment of patients resistant to chemotherapy. The NEDD8 inhibitor MLN4924 selectively targets SCF^{Skp2} activation and offers a more specific approach to protein degradation inhibition than total proteasomal inhibition. The goal of this study was to evaluate whether MLN4924 is effective in high CKS1B conditions and identify mechanisms regulating drug potency.

Experimental Design—Bortezomib and MLN4924 sensitivity was assessed through proliferation, viability, clonogenic potential, and senescence induction in cells overexpressing CKS1B. The mechanism for MLN4924 sensitivity was elucidated by immunoblot analysis of SCF^{Skp} substrates and confirmed by shRNA knockdown. The clinical relevance of the NEDD8 pathway was examined in GEPs derived from healthy people, patients with MGUS, and MM.

Results—Cells overexpressing CKS1B were resistant to bortezomib but sensitive to MLN4924. Treatment of CKS1B-overexpressing cells with MLN4924 decreased proliferation, clonogenicity, and induced senescence. MLN4924, but not bortezomib, induced stabilization of p21 and knockdown of p21 resulted in loss of MLN4924 sensitivity. Patients with MGUS and MM exhibited increased expression of NEDD8-pathway genes relative to normal plasma cells. MM patients with high NEDD8 expression were linked to bortezomib resistance in clinical trials, and had inferior outcomes.

Conclusions—Our data demonstrate cells with elevated CKS1B expression are resistant to bortezomib but sensitive to MLN4924 and offer a mechanism through the stabilization of p21.

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Authorship Contributions

JH, YZ, ZG, YY, and HX designed and performed the research. GST designed the research, analyzed and interpreted the results, and drafted the manuscript. GT designed the research and analyzed and interpreted the data. FZ designed the research, analyzed and interpreted the results, and drafted the manuscript.

Disclosure of Conflicts of Interest

We have no conflicts of interest.

These findings provide rationale for targeting the NEDD8 pathway in MM patients exhibiting elevated expression of CKS1B.

Introduction

Multiple myeloma (MM) is a malignancy of plasma cells that accumulate in the bone marrow, secrete antibody, and interfere with the production of normal blood cells. MM is now the second most common hematological malignancy in the U.S., with a 5-year survival rate less than 50% (1). In the past decade, proteasome inhibition and immunomodulators have revolutionized MM therapies. In particular, bortezomib, which targets the 26S proteasome subunit $\beta 5$, has induced a high level of positive response rates (2, 3). However, toxicities associated with global proteasomal inhibition and resistance to bortezomib in MM are major concerns, prompting the further development of novel therapies. These improved inhibitors are designed to more specifically target critical factors in protein turnover and are now part of ongoing clinical trials. One such molecule, MLN4924, is an inhibitor of NEDD8-activating enzyme (NAE), preventing the conjugation of the small ubiquitin-like protein NEDD8 (neural precursor cell expressed developmentally down-regulated protein 8) to cullin-RING ubiquitin E3 ligases (CRL). This inhibitor has shown promise against MM *in vitro* by inhibiting the degradation of skp1/cullin-1/F-box SKP2 (SCF^{Skp2}) substrates including p27 and p21 (4).

CDC28 kinase subunit 1 (CKS1B) is a necessary cofactor of the CRL complex, SCF^{Skp2} , which regulates cellular entry into S phase and possesses anti-apoptotic activity through p27-dependent and -independent pathways, and is a critical factor in MM drug resistance (5). CKS1B is an essential protein for normal cell division and growth (6), and is expressed at a high level in various cancer tissues including hepatocellular carcinoma (7), colon (8), lung (9), oral squamous cell carcinoma (10), breast cancer (11), and others. In MM, amplification of region 1q21, which includes the *CKS1B* gene, identifies a subpopulation with poor prognosis, an aggressive clinical course, and few therapeutic options (12, 13). *CKS1B* amplification is also associated with transformation from both the benign state of monoclonal gammopathy of undetermined significance (MGUS) to MM and further progression to plasma cell leukemia (14). Previously, we identified *CKS1B* as one of 70 high-risk genes inversely associated with survival in newly diagnosed MM (15) and high nuclear expression of CKS1B is an adverse prognostic factor for relapsed/refractory MM patients (16). These findings provide compelling evidence that CKS1B represents a strong candidate target gene for therapy.

Neddylated SCF^{Skp2} represents the activated complex essential for the ubiquitination of CRL substrates (17). In this study, we test the efficacy of MLN4924 in MM cells overexpressing CKS1B. We explore how MLN4924 affects cell viability and senescence in CKS1B overexpressing MM cells. Our study identifies a novel function for MLN4921 in the upregulation of p21 expression, which is independent of the CKS1B-mediated SCF^{Skp2} complex formation and NEDD8-dependent SCF^{Skp2} activation, resulting in killing MM cells.

Materials and Methods

Gene expression profiling

The data of GEP were collected from a publicly available website which include 351 newly diagnosed patients with MM who participated in the Total Therapy 2 (TT2) clinical trial (Zhan, 2006, Shaughnessy 2007) and 264 relapsed MM in the APEX phase 3 clinical trial (Mulligan 2007). Permutation analyses were performed to correlate *NAE1*, *UBA3*, *UBC12*, and *NEDD8* expression with patient survival in the APEX trial (n = 264). The significance of gene expression with drug response in the APEX trial was analyzed by a Student t-test.

Compounds

MLN4924 and bortezomib were provided by Millenium Pahraceuticals. Each was resuspended in dimethyl sulfoxide (DMSO) to a concentration of 10mM and used at the concentrations indicated.

Cell lines and cell culture

Human MM cell lines ARP-1, OCI-My5, and KMS28PE with or without CKS1B overexpression (expression levels previously detailed (5)), were cultured in RPMI 1640 (Life Technologies). HEK-293T cells were cultured in DMEM (Life Technologies). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were cultured in humidified incubators at 37°C with 5% CO₂.

Establishment of stable MM cell lines

Overexpressing CKS1B—The construction of MM cell lines stably overexpressing CKS1B has been described previously (5). Briefly, the cDNA sequence for human and mouse CKS1B are equivalent. CKS1B was amplified from the PCDH-mouse-CKS1B-EF1-RFP vector using the following primers: CKS1B Xba1 Forward: GTGtctagaATGTCCCACAAACAAATCTACTATTTCG, CKS1B BamH1 Reverse: GTGggatccTCATTTCTTTGGCTTCTTGGGC. CKS1B cDNA was cloned into a modified pCDH vector (Systems Biology). Lentiviruses were obtained by cotransfection of the pCDH-CKS1B vector with packaging vectors into HEK-293T cells following the standard protocol of ProFection Mammalian Transfection System (Promega). Viral supernatant was harvested after 36 hours and concentrated by ultracentrifugation. ARP-1 and OCI-My5 MM cells were transfected with lentivirus containing CKS1B cDNA to yield CKS1B overexpressing MM cells. The efficiency of lentiviral transfection was determined by measuring the expression of green fluorescent protein (GFP) using flow cytometry. ~95% transduction efficiency of MM cells was consistently achieved. CKS1B-OE MM cells were screened by treatment with puromycin to select for CKS1B overexpressing cells.

p21 knockout

The lentiviral CRISPR/Cas9 GeCKO system was used for specific p21 knockout in CKS1B-overexpressing MM cells. Two pairs of p21 single guide RNAs (sgRNA) were annealed, phosphorylated, and ligated into the lentiCRISPR lentiviral sgRNA vector: p21 forward

primer, 5'-CACCG CCGCGACTGTGATGCGCTAA-3', reverse, 5' AAAGTTAGCGCATCACAGTCGCGGC-3'. The U6 forward primer: 5' GACTATCATATGCTTACCGT-3' was used for sequencing sg RNA. Lentiviruses containing the sgRNAs were obtained by cotransfection of the lentiCRISPR-p21sgRNA vector with pVSVg and psPAX2 vectors with packaging vectors into HEK-293T cells. Viral supernatant was harvested after 48 hours. ARP-1 and OCI-My5 MM cells were transduced with lentivirus containing the annealed p21 sgRNAs. All primers were purchased from Integrated DNA Technologies.

RT-PCR

mRNA was extracted using RNeasy® Mini Kit (BIO-RAD). from wild-type, empty vector-transfected, CKS1B-overexpressing, and p21 knock-out cultured cells treated with or without bortezomib, MLN4924, or vehicle for 24 hours. Reverse transcription PCR was performed with 5x iscript reverse transcription supermix (BIO-RAD) according to manufacturer protocols. Quantitative Real-Time PCR (qRT-PCR) was used to detect p21 and GAPDH expression in. Primers used for qRT-PCR were as follows: p21 forward, 5'-TGT CCG TCA GAA CCC ATG C-3', p21 reverse, 5'-AAA GTC GAA GTT CCA TCG CTC-3', and GAPDH forward, 5'-GAC ACC CAC TCC TCC ACC T-3', reverse, 5'-ATG AGG TCC ACC ACC CTG T-3'. GAPDH transcript levels were used to normalize the amount of p21 cDNA in each sample.

Cell proliferation assay

1.5×10^5 cells/ml were seeded into 6-well plates in 2mL media in triplicate. Cells were untreated, treated with 5nM bortezomib, or 1uM MLN4924. Media was replenished with fresh RPMI-1640 with or without drugs every 2 days. Cell proliferation and viability were evaluated for 7 days at the indicated timepoint using a hemocytometer paired with trypan blue exclusion. Cell viability is expressed as the ratio of live cells relative to total cells.

Soft agar clonogenic formation assay

1×10^5 ARP-1 and OCI-My5 MM cells were seeded in 12-well plates with or without bortezomib or MLN4924 at varying doses in fresh RPMI 1640. Cells were resuspended in 0.33% agar in MyeloCult H5100 cultures/10% FBS and allowed to clonally expand for 3 weeks. Cells were fed twice each week by placing two drops of medium on the layer with or without drugs. Images of all plates were taken on a Nikon Eclipse Ti microscope, and colony numbers were counted using Image J. The colony efficiency was calculated as (colony number in the image) $\times$ (well area) / (actual area the photo represent) / 10,000 $\times$ 100%.

Senescence β -Galactosidase cell staining

Senescence β -Galactosidase staining was used to detect β -galactosidase activity, a known characteristic of senescent cells. OCI-My5 and ARP 1 cells with or without the stable overexpression of CKS1B or stable knockdown of p21 were grown in 6-well plates. Cells were mock treated or treated for 48 hours with bortezomib or MLN4924, were harvested, washed with PBS, and fixed. Cells were resuspended in β -Galactosidase Staining Solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2mM $MgCl_2$, PBS up to 500 ml, pH 6.0) and

seeded into 6-well plates. Plates were sealed with paraformaldehyde and incubated at 37°C. The β -Galactosidase staining solution was removed and staining was assessed under a microscope. Stained cells were counted and expressed relative to total cells. Graphs represent the average and standard deviations from 4 separate plates. This method was also used to assess senescence in CKS1B-overexpressing MM cells treated with empty vector or stable p21 knockdown with or without the treatment of MLN4924. Representative plates are depicted in Supplemental Figure 3.

Immunoblots

Protein expression levels were determined by Western blot analysis. Briefly, cells were lysed using a Mammalian Cell Extraction Kit with protease inhibitor cocktail (Biovision). Protein concentrations were determined on a NanoDrop 1000 (Thermo) and 10 μ g of total protein lysate was separated on a Mini-PROTEAN TGX precast acrylamide gel (BIO-RAD). Gels were transferred to activated nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in Tris buffered saline (TBS) containing 0.05% Tween-20 (TBS-T). Membranes were blotted with the indicated primary antibodies. All primary antibodies were diluted according to the manufacturer's directions. CKS1B, CUL1, p27 and all secondary antibodies were purchased from Santa Cruz Biotechnology, primary antibodies p21, P-RB(S608), P-RB(S780), P-RB(S807) and β -Actin were purchased from Cell Signaling Technology. β -Actin was used to normalize the amount of protein for each sample. Protein bands were visualized using HRP-conjugated secondary antibodies and SuperSignal West Pico (Pierce). Blots were subsequently stripped and re-probed for β -actin as loading control. Uncropped blots are presented in Supplemental Figure 1.

Statistical analyses

One-way ANOVA (three groups) and Student *t* tests were used to determine significance between experimental groups. Two-way ANOVA paired with the Tukey-Kramer method for multiple comparisons was used to compare multiple groups with multiple treatment levels. Significance was defined as $p < 0.05$.

Results

Multiple myeloma cells overexpressing CKS1B are resistant to bortezomib but sensitive to MLN4924

CKS1B expression is increased in relapsed MM and confers drug resistance, including resistance to bortezomib (5). In an attempt to overcome this drug resistance and toxicities associated with pan-proteasomal inhibitors, more specifically targeted inhibitors of protein degradation have emerged, including the NAE inhibitor MLN4924. Because of the established drug-resistance against bortezomib of MM cells overexpressing CKS1B, we wanted to examine if cells overexpressing CKS1B were resistant to treatment with MLN4924.

We compared the effects of bortezomib and MLN4924 on the proliferation and survival of ARP-1 and OCI-My5 MM cell lines stably transfected with empty vector or CKS1B expression vector. We found that while cells expressing basal levels of CKS1B were

susceptible to treatment with both bortezomib and MLN4924 (Figure 1A, **left panels**), cells overexpressing CKS1B treated with bortezomib continued to proliferate in a manner comparable to untreated cells (Figure 1A, **right panels**) while cells overexpressing CKS1B treated with MLN4924 failed to proliferate appreciably (Figure 1A, **right panels, green line**). In examining cell viability, treatment with either bortezomib or MLN4924 in cells expressing basal levels of CKS1B resulted in diminished viability (Figure 1B, **left panels**). In cells overexpressing CKS1B, treatment with bortezomib resulted in viability comparable to mock treatment (Figure 1B, **right panels**) but treatment with MLN4924 yielded rapid collapse leading to total cell death within 6 days (Figure 1B, **right panels, green line**). These results highlight that cells with high CKS1B are resistant to bortezomib treatment but remain sensitive to MLN4924.

In order to assess the role of p53 on proliferation and cellular viability, we performed experiments using the p53-positive KMS28PE MM cell line with or without the stable overexpression of CKS1B. We found that KMS28PE cells exhibited very similar proliferative patterns to both OCI-MY5 and ARP1 cells when treated with MLN4924 (Supplemental Figure 2B). We also found that viability was impacted very similarly to OCI-MY5 cells of the same CKS1B background (Supplemental Figure 2C). Together, these findings suggest that p53 status does not contribute greatly to MLN4924 sensitivity.

We examined the ability of CKS1B-overexpressing cells to form colonies in soft agar in the presence of bortezomib or MLN4924. ARP-1 and OCI-My5 cell lines with or without CKS1B overexpression were seeded in the presence or absence of bortezomib or MLN4924. Treatment of either EV cell line with bortezomib or MLN4924 resulted in decreased colony formation (Figure 1C). However, upon CKS1B overexpression, colony formation significantly increased upon Bortezomib challenge. Conversely, colony formation remained very low in CKS1B overexpressing cells upon challenge with MLN4924. (Figure 1C). These results indicate that cells with basal levels of CKS1B are sensitive to both Bortezomib and MLN4924 while cells overexpressing CKS1B are resistant to Bortezomib but remain sensitive to MLN4924.

Skp2 is a critical component of the E3 ligase complex SCF^{Skp2}. Targeting Skp2 leads to accumulation of p21 and p27 and increased cellular senescence (18–20). CKS1B is an essential accessory protein for the function of SCF^{Skp2}. With a known role for CKS1B in SCF^{Skp2} function and because of the important role for SCF^{Skp2} in senescence, we examined the effects of bortezomib and MLN4924 on senescence in CKS1B-overexpressing MM cell lines. We examined the effect of CKS1B knockdown on senescence in ARP-1 and OCI-My5 MM cell lines using shRNA to deplete CKS1B expression (Figure 1D). In each cell line, knockdown of CKS1B resulted in greatly increased senescence relative to treatment with a scrambled control. These results suggest that the role for CKS1B in SCF^{Skp2} integrity relates to senescence and that a loss of CKS1B has comparable effects to the loss SCF^{Skp2} in the promotion of senescence.

Next, we examined the effects of bortezomib and MLN4924 on senescence in ARP-1 and OCI-My5 cell lines stably overexpressing CKS1B. In both cell lines, the overexpression of CKS1B slightly increased senescence in untreated cells relative to cells transfected with

empty vector (Figure 1E). Treatment of parental cell lines with either bortezomib or MLN4924 resulted in a marked increase in senescence. However, upon CKS1B overexpression, there was a significant decrease in senescence upon bortezomib treatment. This decrease was not exhibited upon MLN4924 treatment. These results indicate that MLN4924 but not bortezomib is able to induce senescence in CKS1B-overexpressing cells.

Taken together, these data illustrate that MM cells over expressing CKS1B are resistant to treatment with the proteasomal inhibitor bortezomib but sensitive to the NAE inhibitor MLN4924.

CKS1B regulates neddylation-related signaling

In order to examine the relationship between CKS1B and neddylation, we examined the neddylation of Cullin-1 in ARP-1 and OCI-My5 MM cell lines upon the modulation of CKS1B expression. In both cell lines, the overexpression of CKS1B led to increased neddylation of Cullin-1 as evidenced using an antibody targeted against NEDD8 (Figure 2A). Conversely, knockdown of CKS1B expression with shRNA led to a decrease in neddylated Cullin-1 in each cell line (Figure 2B). Taken together, these data highlight the intimate relationship between CKS1B and the neddylation of Cullin-1. We next explored the effects of MLN4924 on the neddylation of Cullin-1 in ARP-1 and OCI-My5 cells overexpressing CKS1B. Consistent with the role of MLN4924 as an NAE inhibitor, treatment with MLN4924 in each cell line led to a substantial decrease in the neddylation of Cullin-1 (Figure 2C).

Paired with our earlier findings, these results demonstrate that drug-resistant cells with increased CKS1B also have increased Cullin-1 neddylation. Because increased neddylation of Cullin-1 leads to increased activation of SCF^{Skp2}, a negative regulator of p21, p27, and senescence, we hypothesized that the drug resistance of CKS1B-overexpressing cells against bortezomib but not the NAE inhibitor MLN4924 stems from an increase in the ubiquitin-mediated degradation of downstream targets of the CRL/SCF complex. Therefore, we sought to examine the effect of drug treatment on SCF^{Skp2} regulated proteins in cells overexpressing CKS1B.

MLN4924 overcomes CKS1B-induced drug resistance by inhibiting p21 ubiquitin-mediated degradation

In order to examine the effect of drug treatment on SCF^{Skp2} regulated proteins in cells overexpressing CKS1B, we performed immunoblot analysis of known downstream targets of CRL/SCF (Figure 3A).

Consistent with Figure 2, the use of a Cullin-1-specific antibody showed the overexpression of CKS1B led to an increase in the neddylated form of Cullin-1 and treatment with MLN4924 completely blocked the neddylation of Cullin-1 (Figure 3A, **row 2**). In both cell lines, inhibition of SCF^{Skp2} activation by blocking Cullin-1 neddylation with MLN4924, independent of CKS1B expression, led to increased expression of p27 and p21 proteins relative to expression seen in cells without drug treatment (Figure 3A, **rows 7,8**). Treatment of cells with basal level CKS1B expression with bortezomib resulted in increased levels of

p27 and p21 as we expected. While we expected the overexpression of CKS1B to lead to the downregulation of p27 and p21 in bortezomib-treated cells, we observed downregulation of p21 but continued overexpression of p27 (Figure 3A, **row 7 vs. row 8**). Together, these results imply that bortezomib induced increases in p27 are insensitive to CKS1B overexpression and suggest a mechanism of p27 regulation distinct from SCF^{Skp2} regulation of p21 and p27. We further examined the effects of drug treatment in cells with basal and elevated levels of CKS1B expression on the mRNA expression of CDKN1A. Consistent with the immunoblots, MLN4924 increased the low basal level expression of p21 dramatically in both parental and CKS1B-overexpressing cells while treatment with bortezomib had negligible effects on the mRNA expression of p21 (Figure 3B).

CKS1B overexpressing cells are sensitive to MLN4924 but not bortezomib and p21 is differentially regulated from p27 in CKS1B overexpressing cells upon bortezomib. These results suggest a critical role for p21 in the regulation of CKS1B drug resistance. Accordingly, we investigated the role of p21 in drug sensitivity in MM cell lines.

p21 is necessary for MLN4924 sensitivity in CKS1B overexpressing cells

In order to investigate the role of p21 in drug sensitivity, we modulated p21 expression using cells stably expressing shRNA against p21. Because low basal expression of p21 is well-established (21, 22), we confirmed the induction of p21 expression upon treatment of cells with MLN4924, an established DNA damaging agent and activator of p21 (23, 24). CKS1B-overexpressing OCI-My5 MM cells expressing shRNA against p21 substantially knocked down p21 protein expression upon MLN4924 treatment relative to cells transfected with empty vector (Figure 4A).

We examined cell viability in OCI-My5 cells overexpressing CKS1B with and without knockdown of p21 protein expression. The knockdown of p21 decreased cell sensitivity to MLN4924 in CKS1B overexpressing cells and increased cell viability while prolonging survival relative to cells expressing p21 (Figure 4B). Importantly, we have performed comparative proliferation assays between cells with and without knockdown of p21. We find no significant differences in proliferation suggesting that changes in viability are not an artifact of altered proliferation rates (data not shown).

We further examined the clonogenic ability of p21 knockout cells to form colonies in soft agar in the presence of MLN4924 relative to cells expressing basal p21 levels. Consistent with a role for p21 in sensitivity to MLN4924, colony formation significantly increased in cells with p21 knockdown compared to cells expressing p21 upon MLN4924 treatment (Figure 4C). This increase was not seen in untreated cells. These results indicate that cells with diminished levels of p21 are less sensitive to MLN4924 and have a higher clonogenic capacity in the presence of MLN4924.

We also explored the importance of p21 in the induction of senescence in cells overexpressing CKS1B. While treatment of CKS1B-overexpressing cells with MLN4924 resulted in increased senescence relative to untreated cells, the amount of cells in senescence was dramatically reduced upon the knockdown of p21 (Figure 4D).

These results demonstrate that MLN4924 sensitivity on cell proliferation and survival, clonogenic capacity, and senescence in CKS1B-overexpressing cells is dependent upon p21 and a loss of p21 shifts CKS1B-overexpressing cells towards insensitivity to MLN4924. Taken together, these findings reveal, for the first time, a crucial role for p21 in regulating cell sensitivity to MLN4924.

Neddylation machinery is dysregulated and associated with drug resistance and poor clinical outcomes in multiple myeloma

Having demonstrated the importance of the NEDD8 pathway in regulating SCF^{Skp2} activity in MM cell lines, we investigated the clinical importance of the NEDD8 pathway in MM.

Though there is a broad distribution of gene expression in each group from Total Therapy 2 (TT2), we found that the mRNA expression of *NAE1*, *UBA3*, and *UBC12* was, on average, significantly elevated in plasma cells from patients with MGUS and patients newly diagnosed with MM (25) relative to normal plasma cells (NPCs) from healthy donors (Figure 5A).

We also examined *NEDD8* mRNA expression in patients from APEX trials that were responsive or unresponsive to standard MM therapies (i.e., bortezomib and dexamethasone) (26, 27). While great variance was seen in populations, the average expression of *NEDD8* was significantly elevated in patients exhibiting decreased response to clinical treatment ($p=0.015$) (Figure 5B). This result is further segregated into response to either bortezomib or dexamethasone. As shown in Figure 5C, average *NEDD8* expression was significantly elevated in patients exhibiting decreased clinical response to bortezomib ($p=0.0005$), but was not significantly altered in patients resistant to dexamethasone ($p=0.61$). These results identify a subset of patients with increased *NEDD8* expression who are, on average as a population, resistant to bortezomib but not to dexamethasone in the clinic.

The effect of *NAE1*, *UBA3*, *UBC12*, and *NEDD8* expression on patient outcome was further analyzed using Kaplan-Meier survival and log-rank tests. We did not find any significance in the TT2 trial which includes treatment with melphalan and thalidomide (25) (data not shown). However, patients with elevated expression of proteins involved in the neddylation and activation SCF^{Skp2} had decreased overall survival relative to patients with low expression of neddylation pathway proteins. The elevated expression of *UBA3* ($p=0.003$), *UBC12* ($p=0.001$), or *CKS1B* ($p=0.015$) each correlated with significantly poorer prognoses than patients with low expression of the same gene in the Assessment of Proteasome Inhibition for Extending Remissions (APEX) trial, a phase 3 study that compared bortezomib with dexamethasone in post-relapse MM patients (Figure 6A). Populations were further classified on two variables of *CKS1B* and *UBA3* expression (Figure 6B, **left panel**) and *CKS1B* and *UBC12* expression (Figure 6B, **right panel**). In both scenarios, patients with (high/high) expression of either *CKS1B*-*UBA3* ($p=0.029$) or *CKS1B*-*UBC12* ($p=0.006$) demonstrated significantly decreased survival from patients with heterogeneous (high/low) or (low/high) expression. In both cases, greatest survival was seen in (low/low) *CKS1B*-*UBA3* or *CKS1B*-*UBC12* populations, suggesting synergistic effects of *CKS1B* with either *UBC12* or *UBA3* in patient outcome.

Discussion

A marked increase in the frequency of high-risk designation in MM patients from 13% at diagnosis to 76% at relapse provides strong evidence for disease evolution in MM (15), suggesting the outgrowth of a drug-resistant subpopulation of MM cells. Previously, we have demonstrated CKS1B expression results in increased multidrug resistance, while, clinically, CKS1B expression is increased in relapsed MM patients (5) providing molecular evidence for CKS1B in a more drug-resistant MM disease. The existence of a subset of patients who do not receive maximum benefit from currently available treatments in MM highlight the critical need for improved therapeutic options.

The aim of this study was to evaluate if the NAE inhibitor MLN4924 is effective in treating MM in cells with increased CKS1B expression. Here we demonstrate that MM cells overexpressing CKS1B are resistant to bortezomib but remain sensitive to MLN4924. Our results demonstrate that the treatment of cells with elevated CKS1B expression with MLN4924 but not bortezomib results in decreased cellular proliferation and survival, reduced clonogenic expansion, and the induction of cellular senescence, strongly suggesting a role for NEDD8 regulation of CRL/SCF activation under conditions of CKS1B-induced drug resistance. SCF^{Skp2} is responsible for regulating the ubiquitin-mediated degradation of the cell cycle checkpoint proteins p21 and p27. We found the overexpression of CKS1B led to increased neddylation of Cullin-1 while targeted knockdown of CKS1B reduced Cullin-1 neddylation. These findings demonstrate a novel role for CKS1B in the regulation of SCF^{Skp2} ubiquitin-mediating complex and suggest a mechanism for how cells with elevated CKS1B expression may evade growth regulation. Finally, extensive immunoblot analysis of SCF^{Skp2} degradation targets upon drug treatment in CKS1B-overexpressing cells identified the differential regulation of the CRL/SCF downstream target p21, showing the degradation of p21 upon bortezomib treatment but not MLN4924 treatment. The use of targeted shRNA against p21 resulted in a loss of sensitivity to MLN4924 in cellular proliferation, clonogenic expansion, and senescence induction. These results confirm a mechanism through p21 by which CKS1B overexpressing cells exhibit drug resistance to bortezomib but sensitivity to MLN4924.

The clinical potential of MLN4924 inhibition of Cullin-1 activation through the neddylation pathway was highlighted by examining neddylation machinery expression patterns in MM patients. Consistent with GEPs derived from patient populations, a great deal of variance was exhibited in each examined population. On average, we found that the expression of the E1 Nedd8 activating enzyme *NAE1*, the E1 Nedd8-activating enzyme *UBA3*, the E2 Nedd8-conjugating enzyme *UBC12*, to be significantly upregulated in MM patients relative to patients with MGUS or normal plasma cells derived from healthy donors. In the APEX trial, we also saw a significant increase in the expression of Nedd8 in patients unresponsive to the combinatorial treatment of bortezomib and dexamethasone after relapse relative to those with response and these patients had inferior outcomes.

While our study examines MLN4924 in a drug-resistant, elevated CKS1B environment that recapitulates a portion of a known high-risk phenotype in clinical MM patients, there have been previous studies in MM systems that share consistent findings with us. In examining

the *in vitro* treatment of MM cells with MLN4924, McMillin et.al. have found MLN4924 to be effective in limiting viability a number of MM cell lines, including in the bortezomib-sensitive ANBL6wt MM cell line and the bortezomib resistant ANBL6-5VR subline (28). More recently, Gu et al. have demonstrated the utility of MLN4924 in the inhibition of both AKT and mTOR signaling in MM cell lines and primary MM cells (29). By working in p53 wild-type MM cell lines (MM.1S and MM.1R) and MM cell lines with mutant/null p53 status (LP1, OCI-MY5, RPMI8266, OPM2, and U266), their work also showed that sensitivity to MLN4924 is not related to p53 status (29). While our work was performed primarily in OCI-My5 MM cells (p53-heterozygous wt/mutant) (30, 31) and ARP-1 MM cells (p53-null, homozygous deletions) (32) cell lines, our efforts in the p53-positive KMS28PE MM cell line resulted in nearly identical effects to cellular proliferation and viability upon MLN4924 treatment as seen in the OCI-MY5 cell line, consistent with the previously published findings.

MLN4924 induction of senescence occurs in part from the accumulation of factors leading to DNA damage (e.g. CDT1) and the accumulation of cell cycle regulatory proteins (e.g. p21), the combination of which results in the robust activation of the DNA damage response, leading to senescence (33). We demonstrate the treatment of bortezomib-resistant CKS1B-overexpressing cells with MLN4924 results in an increase in senescence in a p21-dependent manner. This important role of p21 in MLN4924-induced senescence is well supported by the findings of others (20). While the consensus is that p21 is important for MLN4924-induced senescence, there still remain discrepancies between the findings of other groups. One group has found that the genetic inactivation of Skp2 was insufficient to induce senescence alone but critically dependent upon p21, p27, and ATF4 (18). Another group found that MLN4924-induced senescence is critically dependent upon p21 but independent of p53, p16, and pRb (19). A third group found that both p21 and p53 are important for initiating senescence but either factor is dispensable as SA-b-gal staining was diminished, but not absent, in p21^{-/-} and p53^{-/-} cells (20). Our results are consistent with most consistent with the second group (19). The striking differences between these studies may be accounted for in part by the different cell culture models employed by the each group.

The overexpression of CKS1B in relapsed MM patients contributes to bortezomib resistance (5) and relates to decreased survival, constituting a risk factor and highlighting the need for new therapies. We have shown here that genes in the neddylation pathway (*NAE1*, *UBA3*, *UBC12*, *NEDD8*) are upregulated leading to activation of SCF^{Skp2} and correlated with inferior survival in MM patients. These results are consistent with and expand upon the findings of McMillin, et.al. who demonstrated decreased progression-free survival (PFS) in patients with elevated expression of *NEDD8* (28). By targeting NAE, the first step in the activation of CRL complexes, MLN4924 is a more selective inhibitor of protein degradation than the 20S proteasome inhibitor bortezomib. Targets of SCF^{Skp2} are enriched for proliferation and survival and inhibition by MLN4924 achieves a disproportionately antitumoral response without the cellular toxicity associated with the pan-proteasomal inhibition of bortezomib. Therefore, inhibitors of the neddylation pathway, and MLN4924 specifically, offer a promising alternative to bortezomib in the clinical treatment of MM and other cancers.

It has been demonstrated that the treatment of MM cells with MLN4924 results in a distinct gene expression profile (GEP) from the treatment of cells with bortezomib, and that treatment with MLN4924 does not elicit the compensatory upregulation of transcripts specific for ubiquitin/proteasome (28). MLN4924 and bortezomib both inhibit proteasomal degradation, yet through distinct pathways. This excitingly suggests the possibility of a combinatorial approach between these drugs though, to date, preliminary findings remain disparate. Gu et al. have found synergy between bortezomib and MLN4924 in the induction of apoptosis (9). Conversely, in exhaustive titration experiments, McMillin et al. found no synergy but also no antagonism between bortezomib and MLN4924 in affecting the viability of MM cell lines (28).

In summary, our study has demonstrated that MLN4924 is effective at limiting cell growth and proliferation and inducing senescence in drug-resistant, high CKS1B-expressing cells. We demonstrate that p21 is critically important for cell sensitivity to MLN4924 and loss of p21 expression results in MLN4924 resistance. Our findings highlight the clinical potential of targeting the NEDD8 pathway and are supported by clinical data demonstrating MM patients with elevated expression of NEDD8 machinery have inferior clinical outcomes relative to patients with lower expression. Though we have not found a survival advantage correlated with p21 expression in MM patients (data not shown), clinical trials paired with MLN4924 have yet to be performed and analyzed. Our findings suggest that the expression of p21 may predict MLN4924 efficacy and screening patients for p21 expression may have great merit. Further examination of MLN4924 in preclinical and, possibly, in clinical combinatorial studies with currently available treatments (e.g., bortezomib, dexamethasone, Doxorubicin) for MM is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

A subpopulation of patients with overexpression of CKS1B is resistant to available therapeutics, and correlated with poor prognosis, highlighting the immediate need for novel, effective therapeutics. Current therapeutic options, including the pan-proteasomal inhibitor bortezomib, are associated with toxicity arising from non-specific proteasomal inhibition. MLN4924 inhibits the neddylation and activation of SCF^{Skp2} offering a more targeted method of inhibiting protein degradation than bortezomib. Here, we report that cells with elevated expression of CKS1B are resistant to bortezomib but remain sensitive to MLN4924 treatment, highlighting the efficacy of neddylation inhibition in high-risk MM patients. We further define the mechanistic role for p21 in MLN4924 sensitivity in elevated CKS1B environments. Examination of GEPs from patients with MM and MGUS reveal elevation of NEDD8 machinery expression and further reinforce the importance of targeting SCF^{Skp2} activation. Our data support the further examination of MLN4924 in preclinical combinatorial studies with currently available treatments for MM.

Figure 1. Multiple myeloma cells overexpressing CKS1B are resistant to bortezomib but sensitive to MLN4924

A, effects of bortezomib and MLN4924 on the *in vitro* proliferation of ARP-1 and OCI-My5 multiple myeloma cell lines with and without the overexpression of CKS1B. Cells were treated with either bortezomib (5nM) or MLN4924 (1uM) for 7 days. Proliferation was assessed by hemocytometer cell counts. B, effects of bortezomib and MLN4924 on cell viability of ARP-1 and OCI-My5 multiple myeloma cell lines with and without the overexpression of CKS1B. Cells were treated with either bortezomib (5nM) or MLN4924 (1uM) for 7 days. Cell viability is expressed as the ratio of viable cells to the total number of cells. C, effects of bortezomib and MLN4924 on the clonogenic capacity of ARP-1 and OCI-My5 multiple myeloma cell lines with and without the overexpression of CKS1B was assessed by counting colony formation in soft-agar. D, the effects of CKS1B expression on senescence in MM cell lines was assessed by β -Galactosidase staining. E, the effects of bortezomib and MLN4924 on senescence in ARP-1 and OCI-My5 cells overexpressing CKS1B was assessed by β -Galactosidase staining. In all experiments, data are means $\pm$ SD of three separate experiments.

Figure 2. CKS1B regulates neddylation-related signaling

A, protein expression was measured in ARP-1 and OCI-My5 cells stably transfected with empty vector (EV) or CKS1B (OE). 30ug/lane of whole cell lysates were resolved on an SDS-PAGE gel and analyzed by immunoblotting with the indicated antibody. B, Immunoblot of protein lysates derived from ARP-1 and OCI-My5 cells stably transduced with either scrambled control or CKS1B-targeted shRNA. 30ug whole-cell lysate was resolved using SDS-PAGE and lysates were blotted with the indicated antibodies. C, ARP-1

or OCI-My5 cells with or without the overexpression of CKS1B were immunoblotted for Cullin-1 with or without MLN4924 (1uM) treatment (1hr).

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Figure 3. MLN4924 overcomes CKS1B-induced drug resistance by inhibiting p21 ubiquitin-mediated degradation

A, immunoblot analysis of protein expression following 48 hour treatment with bortezomib (5nM) or MLN4924 (1uM) in ARP-1 and OCI-My5 multiple myeloma cell lines stably transfected with empty vector (EV) or CKS1B (OE). Whole cell lysates were resolved on an SDS-PAGE gel and analyzed by immunoblot using the indicated antibodies. B, p21 mRNA expression was determined in ARP-1 and OCI-My5 multiple myeloma cell lines stably transfected with empty vector (EV) or CKS1B (OE). Quantitative real-time PCR was performed to assess p21 mRNA expression following 48 hour treatment with bortezomib (5nM) or MLN4924 (1uM) and results were normalized to the expression of GAPDH.

Values are expressed relative to untreated cells transfected with empty vector. Data are means $\pm$ SD of four separate experiments.

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Figure 4. p21 is necessary for MLN4924 sensitivity in CKS1B overexpressing cells

A, Immunoblot analysis of p21 expression in OCI-My5-CKS1B OE multiple myeloma cells stably transfected with empty vector (EV) or p21 shRNA-expressing vector (p21KO) following 24 hour treatment with MLN4924. B, the effects of bortezomib and MLN4924 on cell viability were assessed in OCI-My5-CKS1B overexpressing cells transfected with either empty vector (EV) or shRNA targeting p21 expression (p21KO). Cells were seeded in the presence of MLN4924 (0.5 μ M, 1 μ M) and viability was monitored for 6 days. Cell viability was assessed by hemocytometer cell counts using trypan blue exclusion staining and is expressed as the ratio of viable cells to the total number of cells. Data are means $\pm$ SD of three separate experiments. C, the effect of MLN4924 (10nM, 100nM, 500nM) on the clonogenic capacity of OCI-My5-CKS1B overexpressing multiple myeloma cells transfected with empty vector or shRNA targeting p21 (p21 KO) was assessed by counting colony formation in soft-agar. Data are means $\pm$ SD of three separate experiments. D, The effects of MLN4924 (100nM) on senescence in OCI-My5-CKS1B overexpressing cells with the expression of empty vector or shRNA targeting p21 (p21 KO) was assessed by β -galactosidase staining. Data are means $\pm$ SD of three separate experiments.

Figure 5. The neddylation pathway is dysregulated and associated with drug resistance in multiple myeloma

A, examination of the mRNA expression profiles of neddylation pathway genes. Signal levels from *NAE1*, *UBA3*, *UBC12*, and *NEDD8* are shown on the y-axis. GEP signals were derived from participants in Total Therapy 2 clinical trials of different clinical backgrounds. Patients were designated as healthy donors with normal plasma cells (NPC), MGUS patients (MGUS), or multiple myeloma patients (MM) and are sorted on the x-axis. B, an examination of *NEDD8* expression in patients categorized as unresponsive (No Response) or responsive (Response) to treatment with dexamethasone and/or bortezomib. Mean signal

levels of patients and p-values of differences in expression are given. C, GEPs from 5B were further segregated by treatment with bortezomib or dexamethasone. *NEDD8* expression signal levels are plotted on the y-axis. The Mean signal level and p-value of differences are given between unresponsive and responsive patients.

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Figure 6. Dysregulation of the neddylation pathway is associated with poor prognosis in MM

A, Kaplan-Meier estimates of overall survival in patients with varied expression of neddylation pathway genes. Patients with elevated expression of UBA3 (left panel) showed poorer overall survival than those with low expression (29% vs 81%, $p=0.003$). Patients with elevated expression of UBC12 (middle panel) showed poorer overall survival than those with low expression (28% vs 48%, $p=0.001$). Patients with elevated expression of CKS1B (right panel) showed poorer overall survival than those with low expression (35% vs 47%, $p=0.015$). B, Kaplan-Meier estimates of overall survival in patients with sorted CKS1B and neddylation enzyme gene expression profiles. Left Panel: Patient survival sorted by high/low CKS1B expression and high/low UBA3 expression is depicted. Patients high in expression of CKS1B and UBA3 exhibited significantly ($p=0.029$) poorer overall survival (26%) than those either high CKS1B/low UBA3 expression (40%) or low CKS1B/high UBA3 expression (31%). Patients with low CKS1B/low UBA3 demonstrated the greatest survival rate (29%). Right Panel: Patient survival sorted by high/low CKS1B expression and high/low UBC12 expression is depicted. Patients high in expression of CKS1B and UBC12 (left panel) exhibited significantly ($p=0.006$) poorer overall survival (20%) than those either high CKS1B/low UBC12 expression (44%) or low CKS1B/high UBC12 expression (36%). Patients with low CKS1B/low UBA3 demonstrated the greatest survival rate (54%).