Disrupting Protein NEDDylation with MLN4924 Is a Novel Strategy to Target Cisplatin Resistance in Ovarian Cancer

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Abstract

Purpose: Ovarian cancer has the highest mortality rate of all female reproductive malignancies. Drug resistance is a major cause of treatment failure and novel therapeutic strategies are urgently needed. MLN4924 is a NEDDylation inhibitor currently under investigation in multiple phase I studies. We investigated its anticancer activity in cisplatin-sensitive and -resistant ovarian cancer models.

Experimental Design: Cellular sensitivity to MLN4924/cisplatin was determined by measuring viability, clonogenic survival, and apoptosis. The effects of drug treatment on global protein expression, DNA damage, and reactive oxygen species generation were determined. RNA interference established natural born killer/bcl-2–interacting killer (NBK/BIK) as a regulator of therapeutic sensitivity. The in vivo effects of MLN4924/cisplatin on tumor burden and key pharmacodynamics were assessed in cisplatin-sensitive and -resistant xenograft models.

Results: MLN4924 possessed significant activity against both cisplatin-sensitive and -resistant ovarian cancer cells and provoked the stabilization of key NEDD8 substrates and regulators of cellular redox status. Notably, MLN4924 significantly augmented the activity of cisplatin against cisplatin-resistant cells, suggesting that aberrant NEDDylation may contribute to drug resistance. MLN4924 and cisplatin cooperated to induce DNA damage, oxidative stress, and increased expression of the BH3-only protein NBK/BIK. Targeted NBK/BIK knockdown diminished the proapoptotic effects of the MLN4924/cisplatin combination. Administration of MLN4924 to mice bearing ovarian tumor xenografts significantly increased the efficacy of cisplatin against both cisplatin-sensitive and -resistant tumors.

Conclusions: Our collective data provide a rationale for the clinical investigation of NEDD8-activating enzyme (NAE) inhibition as a novel strategy to augment cisplatin efficacy in patients with ovarian cancer and other malignancies.

Introduction

Ovarian cancer has the highest mortality of all forms of female reproductive malignancies with a 5-year survival rate of only 40%. Platinum-based anticancer drugs (i.e., cisplatin) are used as standard therapy for ovarian cancer and many other malignancies. However, the development of drug resistance ultimately limits their effectiveness. Novel therapeutic strategies are urgently needed to improve survival. Protein turnover plays a critical role in cellular homeostasis and is required for many essential functions, but is frequently dysregulated in cancer cells. The inappropriate degradation of proteins that regulate key transduction pathways contributes to disease progression, metastasis, and drug resistance, and thus, represents an attractive target for selective inhibition (1). The first proteasome inhibitor for cancer therapy, bortezomib (Velcade, PS-341), received U.S. Food and Drug Administration (FDA) approval for the treatment of multiple myeloma and relapsed mantle cell lymphoma (2). The success of bortezomib prompted a significant effort to develop novel agents that more specifically target critical factors involved in the regulation of protein turnover with the aim to eliminate toxicities that result from global proteasomal inhibition.

The cullin-RING ubiquitin ligases (CRL) are a subset of E3 enzymes whose activity is regulated by modification with the ubiquitin-like molecule NEDD8. The CRLs control the ubiquitination and subsequent degradation of many proteins with key roles in cell-cycle progression (such as p27, cyclin E, and c-Myc), DNA damage (CTD1), stress responses...
Translational Relevance

Drug resistance is a major cause of treatment failure for patients with ovarian cancer. Novel therapeutic strategies that circumvent mechanisms of resistance are urgently needed to improve the survivorship of women with this disease. MLN4924 is a NEDDylation inhibitor currently under investigation in multiple phase I studies. Here, we report that MLN4924 possessed significant anticancer activity against both cisplatin-sensitive and -resistant models of ovarian cancer and sensitized cisplatin-resistant cells to cisplatin, suggesting that aberrant protein NEDDylation may contribute to drug resistance. Increased oxidative stress and induction of the BH3-only protein natural born killer/bcl-2–interacting killer (NBK/BIK), a critical regulator of sensitivity to the MLN4924/cisplatin combination. Our killer/bcl-2–interacting killer (NBK/BIK), a critical regulator of sensitivity to the MLN4924/cisplatin combination.

Materials and Methods

Cells and cell culture

A1847, A2780, A2780/CP, 2008, 2008/C13, IGROV-1, OVCAR-5, and SKOV-3 human ovarian cancer cells were maintained in RPMI-1640 medium with 10% FBS at 37°C with 5% CO2 as previously described (10).

Chemicals and reagents

Reagents were obtained from: MLN4924 (Millennium Pharmaceuticals, Inc.), cisplatin (CIRC Pharmacy), anti-NEDD8, anti-γ-H2AX (Immunoblotting), anti-NRF-2 and anti-PUMA (Epitomics), anti-p21, anti-p27, anti-WEE1, anti-CDT1, anti-γ-H2AX (IHC), anti-BIM, and anti-active caspase-3 (Cell Signaling Technology), anti-NOXA (Calbiochem), anti-β-tubulin and N-acetylcysteine (NAC; Sigma), anti-β-catenin, anti-NB/BK, and zVAD-fmk (Santa Cruz Biotechnology), antiproliferating cell nuclear antigen (PCNA; Dako), goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (The Jackson Laboratory), rat anti-mouse immunoglobulin G 2a (IgG2a)-HRP antibody (S erotec), goat anti-mouse Alexa Fluor 488 and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), and sheep anti-mouse-HRP and donkey anti-rabbit-HRP (Amersham).

Quantification of drug-induced cytotoxicity

Cells were treated with the indicated concentrations of MLN4924, cisplatin, or both drugs for 72 hours. The effects of drug treatment on cell viability were assessed using the ATPLite OneStep Assay Kit (PerkinElmer) according to the manufacturer’s directions as previously described (9). Apoptosis was evaluated by propidium iodide/fluorescence-activated cell sorting (PI/FACS) analysis of sub-G0–G1 DNA content following 24 hours drug exposure (11).

 Colony assays

Cells were plated into 6-well plates and allowed to adhere overnight. Adherent cells were treated with the indicated concentrations of MLN4924 for 24 hours. After drug treatment, cells were washed twice in PBS followed by the addition of fresh media and incubated for 10 days in a humidified incubator at 37°C with 5% CO2. Colonies were then washed in PBS, fixed with methanol, and stained with crystal violet. Colonies were counted using an Alpha Innotech gel documentation system.

NAEβ DNA sequencing

DNA from A1847, A2780, A2780/CP, 2008, 2008/C13, IGROV-1, OVCAR-5, and SKOV-3 cell lines was isolated using the DNaseasy Mini Kit (Qiagen Inc.). DNA was eluted with 100 μL nuclease-free water and samples were checked for concentration and quality using a NanoDrop spectrophotometer. PCR amplifications were conducted using optimized cycling conditions per gene-exon following the previously described protocol (12). All samples were sequenced with forward and reverse primers to obtain the
complete overlapping of NALß (UBA3) exons 8, 9, and 13. Sequencing reactions were run on an ABI 3130xl at the Nucleic Acid Core Facility at The University of Texas Health Science Center at San Antonio (UTHSCSA).

Glutathione assay
The basal levels of glutathione (GSH) in A2780 and A2780/CP ovarian cancer cells were quantified using the Glutathione Assay Kit (Calbiochem) according to the manufacturer’s instructions.

Necrosis detection assay
A2780 and A2780/CP cells were exposed to the indicated concentrations of MLN4924, cisplatin, or the combination of MLN4924 and cisplatin for 24 hours. Following drug treatment, cells were incubated with a PI solution for 30 minutes. Cells were washed twice in PBS and PI uptake was quantified by flow cytometry using a BD FACS Canto II flow cytometer (BD Biosciences).

Immunoblotting
Ovarian cancer cells were incubated with MLN4924, cisplatin, or the combination of both drugs for 24 hours as indicated. Cells were then lysed for 1 hour on ice in Triton X-100 lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L Tris pH 7.5) with protease inhibitors. Approximately, 50 µg of total cellular protein from each sample was separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked with 5% nonfat milk in a TBS solution containing 0.1% Tween-20 for 1 hour. The blots were probed overnight with the indicated primary antibodies at 4°C, washed, and then probed with species-specific secondary antibodies coupled to HRP. Bands were detected by enhanced chemiluminescence (Alpha Innotech) as previously described (13).

Proteome profiling
A2780 cells were treated with MLN4924 for 24 hours. Samples were harvested in a urea lysis buffer, reduced, and alkylated before being digested with Lys-C (Wako). After digestion, samples were purified over SepPak C18 columns and eluted peptides were lyophilized. After lyophilization, 100 µg of each sample was labeled using tandem mass tag (TMT) reagents (Thermo Fisher Pierce). Labeled samples were then combined and subjected to strong cation exchange (SCX) chromatography. The 6-plexes were run on the Accela high speed LC system (Thermo Fisher) over a 4.6 mm × 200 mm SCX column packed with polyisouloethyl aspartamide material (5 µm particle size, 200-A pore). A 50-minute gradient was used to elute peptides from the column and fractions were collected at every minute for the entire gradient. The first 40 fractions were combined into 20 fractions before being purified again over a C18 SepPak column. All fractions were then run individually on the Orbit-O-Sep using a 140-minute gradient. Peptide identification was carried out in MS² scans, whereas quantification of reporter ions was carried out using MS³ scans (14). In total, the 20 fractions were run consecutively for each 6-plex, occupying approximatly 47 hours of instrument time. Data generated from the mass spectrometry (MS) analysis were processed using in-house software (Cell Signaling Technology) resulting in an average of 3,900 quantified proteins per 6-plex.

Immunocytochemistry
A2780 and A2780/CP ovarian cancer cells were plated on chamber slides and treated with MLN4924, cisplatin, or both agents for 24 hours. Cells were fixed with 4% paraformaldehyde, permeabilized using 0.2% Triton X-100, and incubated overnight with a g-H2AX antibody. Goat anti-mouse Alexa 488 fluorescent secondary antibody was used to visualize g-H2AX. DAPI was used to counterstain the nucleus. Images were captured using a Zeiss LSM 510 Meta confocal microscope with an oil ×40 objective as previously described (15). ImageJ software was used to quantify fluorescent intensity.

Alkaline comet assay
A2780 and A2780/CP human ovarian cancer cells were treated with MLN4924, cisplatin, or both agents for 24 hours. Comet assays were conducted using the CometAssay Kit (Trevigen) according to the manufacturer’s instructions. Briefly, test samples and Alkaline Comet Control cells were mixed with molten low melting point (LM) agarose and spread onto well slides. Slides were chilled to 4°C, incubated in lysis solution for 30 minutes at 4°C, and then immersed in unwinding solution for 20 minutes at room temperature. Slides were electrophoresed using Trevigen’s Comet ES system in cold 200 mmol/L NaOH/1 mmol/L EDTA solution for 30 minutes at 21 V. Following electrophoresis, slides were washed twice in water, once in ethanol, and then dried on a slide warmer. Slides were incubated with 1:30,000 SybrGold for 30 minutes at room temperature in the dark, rinsed, and then dried. Cells were imaged using fluorescent microscopy and tail moments (product of DNA amount in tail and distance of tail migration) from 50 cells per slide were calculated.

Quantification of reactive oxygen species generation
Cells were treated with MLN4924, cisplatin, or the combination of MLN4924 and cisplatin for 12 hours. Reactive oxygen species (ROS) levels were determined for each experimental condition by flow cytometry as previously described (9).

Quantification of mitochondrial membrane depolarization
A2780 and A2780/CP ovarian cancer cells were treated with MLN4924, cisplatin, or the combination of MLN4924 and cisplatin for 6 hours. The impact of drug treatment on mitochondrial membrane permeability was determined using Mitotracker Red CMXROS (Invitrogen) as previously described (16).

Oxidative DNA damage assay
A2780 and A2780/CP cells were treated with MLN4924, cisplatin, or the combination of MLN4924 and cisplatin.
for 16 hours. The levels of 8-oxo-guanine were determined using the Oxidative DNA Damage Kit (Kamiya Biomedical Company) according to the manufacturer’s instructions. Oxidative DNA damage was measured by flow cytometry.

**shRNA knockdown of NBK/BIK and p65**

A2780 and A2780/CP human ovarian cancer cells were infected with lentiviral particles containing nontargeted (control) or targeted short hairpin RNA (shRNA) directed at NBK/BIK or the NF-κB p65 subunit (Santa Cruz Biotechnology) according to the manufacturer’s instructions. Positively infected cells were selected with puromycin treatment. Infected cells were selected with the indicated concentrations of MLN4924 and cisplatin for 24 hours. Drug-induced apoptosis was quantified by PI/FACS as described earlier. Knockdown efficiency was assessed by immunoblotting.

**In vivo evaluation of MLN4924 and cisplatin**

A2780 and A2780/CP cells were harvested, washed in PBS, and suspended in a 1:1 mixture of Hanks balanced salt solution (HBSS) and Matrigel (BD BioSciences). An in vivo model of ovarian cancer was generated by injecting A2780 or A2780/CP cells into the flanks of female nude (nu/nu) mice. After tumor growth reached 150 mm³, mice were randomly assigned to receive vehicle control (n = 10), MLN4924 20 mg/kg s.c. twice a day (n = 10), or both MLN4924 and cisplatin (n = 10) for 12 days. Mice were monitored daily and tumor volumes were measured twice weekly. At the completion of the study, tumors were excised, formalin-fixed, and paraffin-embedded for immunohistochemical analysis.

**Immunohistochemistry**

Paraffin-embedded tumor sections were deparaffinized in xylene, exposed to a graded series of alcohol, and rehydrated in PBS (pH 7.5). Heat-induced epitope retrieval on paraffin-embedded sections was conducted by microwaving slides in a citrate buffer for 5 minutes. Endogenous peroxides were blocked with a 3% hydrogen peroxide solution for 10 minutes. Slides were then incubated in a protein block solution (5% horse and 1% goat serum in PBS) for 20 minutes. PCNA, cleaved caspase-3, γ-H2AX, and NBK/BIK antibodies were diluted in the protein block solution and placed at 4°C overnight. After washing with PBS, slides were incubated in appropriate secondary antibodies for 1 hour at ambient temperature. Positive reactions were visualized using 3,3’-diaminobenzidine (Dako) for 10 minutes. Slides were rinsed with water followed by a brief counterstain with Gill’s hematoxylin (Sigma). Images were captured using an Olympus fluorescent microscope with a DP71 camera and a ×20 objective. Image-Pro Plus software Version 6.2.1 (Media Cybernetics) was used for image acquisition. ImageJ software was used for quantification of γ-H2AX and NBK/BIK levels by densitometric analysis of 5 random fields containing viable tumor cells. Quantification of PCNA and cleaved caspase-3 was conducted by counting the number of positive cells in 5 random fields as previously described (17).

**Synergy analyses**

The combination indices (CI) for MLN4924 and cisplatin were calculated on the basis of the effect of 72-hour exposure to each drug on cell viability as determined by ATPLite assay. CompuSyn software (CompuSyn, Inc.) was used to calculate CI values as previously described (10).

**Statistical analyses**

Statistical significance of differences observed between samples was determined using the Tukey–Kramer comparison test or the Student t test. Differences were considered significant in all experiments at P < 0.05.

**Results**

**MLN4924 diminishes cell viability and impairs the clonogenic survival of ovarian cancer cells**

We first investigated the effects of MLN4924 (0.01–10 μmol/L) on the viability of a panel of 6 human ovarian cancer cell lines (A1847, A2780, IGROV-1, OVCAR-5, SKOV-3, and A2780/CP). MLN4924 treatment led to a dose-dependent reduction in the viability of all cell lines evaluated (Fig. 1A). We next tested the impact of MLN4924 treatment on the ability of ovarian cancer cells to form colonies. Acute (24 hours) exposure to submicromolar concentrations of MLN4924 significantly disrupted clonogenic survival (Fig. 1B). Notably, the variation in sensitivity of individual cell lines to MLN4924 was not due to mutations in NAE8B, a recently identified mechanism of resistance to MLN4924, as DNA sequencing of all cell lines used in this study failed to detect any mutations (12).

**MLN4924 alters the proteome profile of ovarian cancer cells**

NEDD8 regulates the turnover of proteins with key roles in multiple pathways that control fundamental aspects of cancer biology. To better understand the consequences of NAE inhibition in ovarian cancer cells, we conducted proteomic analyses in A2780 cells to quantify the global impact of MLN4924 treatment on protein expression levels using a multiplexed approach (14). The effects of drug exposure on 3,926 individual proteins were determined. Supplementary Table S1 provides a summary of the 68 (1.7% of total number assessed) proteins whose expression was altered more than 2-fold by treatment with MLN4924. The effects of drug treatment on the levels of selected proteins as well as the disruption of cullin NEDDylation were confirmed by immunoblotting (Fig. 1C).

**MLN4924 augments the cytostatic and proapoptotic effects of cisplatin to overcome drug resistance**

Cisplatin resistance is a prevalent problem for patients with ovarian cancer and other solid tumors and new agents/strategies that circumvent drug resistance are urgently needed. Our proteomic analyses suggested that inhibition of
NAE activity with MLN4924 may alter cellular redox status (increased levels of NFE2L2/NRF-2, HMOX1, KEAP1) and induce DNA damage (elevated p53, CDT-1, and claspin). Interestingly, increased antioxidant defenses and alterations in pathways that control the DNA damage response (DDR)/DNA repair are known to promote resistance to the standard chemotherapeutic agent cisplatin (18–20). Indeed, quantification of the basal levels of GSH in A2780 cisplatin-sensitive and A2780/CP cisplatin-resistant cells revealed that the resistant CP cells contain 2.16-fold higher levels of GSH than their cisplatin-sensitive parental cells. Considering this, we hypothesized that inhibition of NAE with MLN4924 may be a novel and effective strategy to augment the activity of cisplatin and target drug resistance. We investigated the ability of MLN4924 to augment the in vitro activity of cisplatin in a panel of 8 ovarian cancer cell lines that included 2 pairs of cisplatin-sensitive and -resistant cells (A2780 and A2780/CP, 2008 and 2008/C13, models of acquired cisplatin resistance). MLN4924 significantly enhanced the efficacy of cisplatin in all cell lines tested including A2780/CP and 2008/C13, which are both highly resistant to cisplatin treatment (Fig. 2A). Notably, we also did not observe any cross-resistance between MLN4924 and cisplatin as A2780/CP and 2008/C13 exhibited a level of sensitivity to MLN4924 that was similar to their cisplatin-sensitive counterparts A2780 and 2008. Formal synergy analyses revealed that the combination of MLN4924 and cisplatin yielded CIs less than 1.0 across all concentrations tested (range, 0.13173–0.75908), indicating true synergy. In agreement with the combined synergistic effects of MLN4924 and cisplatin with respect to reducing cell viability, the combination of both drugs induced significantly higher levels of apoptosis than either single agent (Fig. 2B). The induction of apoptosis we observed occurred without significant undesired necrotic cell death as each treatment condition contained less than 1% necrotic cells (Fig. 2C). Our results suggest that MLN4924 may itself be effective for patients that are resistant to cisplatin therapy and also may

Figure 1. MLN4924 diminishes cell viability and impairs the clonogenic survival of ovarian cancer cells. A, effects of MLN4924 on the in vitro viability of A1847, A2780, IGROV-1, OVCAR-5, SKOV-3, and 2008 human ovarian cancer cell lines. Cells were treated with the indicated concentrations of MLN4924 for 72 hours and viability was assessed by ATPLite assay. N = 3 ± SD. B, impact of MLN4924 on clonogenic survival. A2780 and IGROV-1 cells were treated with 0.03, 0.1 or 0.3, or 1.0 μmol/L MLN4924 for 24 hours. Drug was washed away and colonies were quantified on day 10 as described in the Materials and Methods. N = 3 ± SD. C, effects of MLN4924 treatment on protein expression. A2780 cells were treated for 24 hours with the indicated concentrations of MLN4924. Protein lysates were subjected to SDS-PAGE, blotted, and probed with the relevant specific antibodies. Tubulin documented equal loading.
be a new approach to resensitize cisplatin-resistant tumors to cisplatin.

Inhibition of NF-κB is not the driving event underlying the ability of MLN4924 to sensitize ovarian cancer cells to cisplatin

We have previously shown that treatment with MLN4924 disrupts NF-κB transcriptional activity through the stabilization of the phosphorylated form of 1κBα, the endogenous inhibitor of NF-κB and a well-characterized NEDD8 substrate protein (7, 9, 21). Earlier studies have shown that constitutive NF-κB activity reduces sensitivity to cisplatin (22–26). To determine whether the NF-κB inhibitory component of MLN4924’s pharmacodynamic effects was a major factor contributing to its ability to sensitize ovarian cancer cells to cisplatin, we used shRNA to target the expression of the p65 NF-κB subunit. Targeted knockdown of p65 modestly augmented the proapoptotic effects of cisplatin in A2780 and A2780/CP cells (Supplementary Fig. S1). This suggests that additional NF-κB–independent effects of MLN4924 contribute to its synergy with cisplatin.

MLN4924 and cisplatin cooperate to induce DNA damage

Cisplatin has a DNA-damaging mechanism of action and forms intrastrand cross-links with DNA. Our earlier work showed that MLN4924 also has DNA-damaging properties and activates the DDR through a different mechanism involving stabilization of the NEDD8 substrate CDT-1 and DNA rereplication (7, 27). To determine whether inhibition of NAE with MLN4924 could augment the DNA-damaging effects of cisplatin, we used immunocytochemistry to visualize and quantify the effects of MLN4924, cisplatin, and the combination of both drugs on the levels of γ-H2AX as a marker of DNA damage in A2780 and A2780/CP cells. MLN4924 treatment induced a significant increase in γ-H2AX fluorescence in both cell lines. Notably, combined treatment with MLN4924 and cisplatin triggered a dramatic induction of γ-H2AX compared with either single agent despite the intrinsic differences in sensitivity to cisplatin between A2780 and A2780/CP cells (Fig. 3A and B). To confirm the cooperative DNA-damaging properties of the MLN4924/cisplatin combination, we conducted alkaline comet assays to quantify the impact of drug treatment on the tail moment in A2780 and A2780/CP cells. Our results were consistent with those of our γ-H2AX immunocytochemistry analyses in that exposure to both MLN4924 and cisplatin induced significantly higher levels of DNA damage (greater tail moment) than single-agent treatment with either MLN4924 or cisplatin (Fig. 3C). To assess whether the DNA damage we observed following treatment with these agents preceded the onset of significant levels of apoptosis or was a phenomenon associated with late-stage apoptosis, we used the caspase-inhibitor zVAD-fmk to block caspase activation in response to treatment with MLN4924 and cisplatin in A2780 cells. Our results showed that the levels of γ-H2AX were largely unaffected by inhibition of caspase activity, indicating that these agents trigger DNA damage in a caspase-independent manner (Supplementary Fig. S2A). Our collective findings suggest that NAE inhibition may represent a new strategy to augment the DNA-damaging properties of cisplatin that is largely unaffected by differential sensitivity to cisplatin.

ROS generation is a key event in MLN4924/cisplatin–mediated apoptosis

Our earlier work showed that MLN4924 triggers a significant increase in ROS generation and this contributes to its
anticancer mechanism of action (9). Considering that previous investigations have reported that cisplatin also elevates ROS production, we hypothesized that potential cooperative redox-related effects may underlie the augmented levels of DNA damage that are triggered by the MLN4924/cisplatin combination. Given that the mitochondrial electron transport chain is a major source of intracellular ROS production and conditions that impose stress on mitochondria can lead to elevated ROS generation, we first assessed the impact of drug treatment on mitochondrial transmembrane potential. Exposure to the combination of MLN4924 and cisplatin for 6 hours was sufficient to cause significant mitochondrial depolarization (Fig. 4A).

We next measured ROS levels in A2780 and the cisplatin-resistant A2780/CP cells following treatment with MLN4924, cisplatin, or both agents using a flow-cytometric method. Exposure to MLN4924 (12 hours) caused a significant increase in ROS levels in both cell lines that was potentiated by the addition of cisplatin (Fig. 4B). Interestingly, basal ROS generation seemed to be higher in the resistant A2780/CP cells compared with their cisplatin-sensitive counterparts, suggesting that these cells may be under greater intrinsic oxidative stress. To determine whether the drug-related increased ROS generation may be linked to the DNA-damaging effects of MLN4924 and cisplatin, we used a flow-cytometric assay to quantify the levels of the 8-oxo-guanine residue in DNA, an established marker of oxidative DNA damage. As expected, cisplatin single-agent treatment had little effect in the resistant A2780/CP cells. MLN4924 significantly increased the percentages of cells expressing 8-oxo-guanine in both cell lines and was slightly more effective than cisplatin with respect to this in the cisplatin-sensitive A2780 cells. The combination of both drugs produced significantly greater levels of oxidative DNA damage than either drug alone in a manner that was consistent with what we observed in our other DNA damage assays (Fig. 4C). We confirmed the importance of redox-related effects to the anticancer mechanism of action of the
MLN4924/cisplatin combination by using the antioxidant GSH-mimetic NAC to quench drug-induced ROS generation. Pretreatment with NAC significantly diminished the ability of MLN4924 and cisplatin to induce apoptosis in both A2780 and A2780/CP cells and significantly blunted the degree of drug-induced DNA damage (Fig. 4D and Supplementary Fig. S2B). The antagonistic effects of NAC were most pronounced in cells treated with both agents. Our collective findings indicate that ROS generation is a key event that promotes both DNA damage and apoptosis in response to treatment with the MLN4924/cisplatin combination.

**NBK/BIK is a critical regulator of the proapoptotic effects of the MLN4924/cisplatin combination**

The BH3-only protein NBK/BIK has been implicated in oxidative stress-induced apoptosis (28–30). Considering that our data showed that MLN4924 and cisplatin cooperate to heighten ROS stress, we investigated the potential effects of these agents on NBK/BIK expression and the
expression of related BH3-only proteins PUMA, NOXA, and BIM by immunoblotting. Treatment with either single agent caused a modest increase in NBK/BIK expression in both A2780 and A2780/CP cells. Interestingly, the MLN4924/cisplatin combination triggered a dramatic induction of NBK/BIK expression in both sensitive and resistant cells (Fig. 5A). Although both single agents caused increased expression of NOXA, PUMA, and BIM, we did not observe the same dramatic degree of augmented expression under combination treatment conditions with any of these BH3-only proteins that we detected with NBK/BIK. Considering this, we chose to focus our investigation on the potential role of NBK/BIK as a regulator of sensitivity to the MLN4924/cisplatin combination. To determine whether

Figure 5. NBK/BIK is a critical regulator of the proapoptotic effects of the MLN4924/cisplatin combination. A. MLN4924 and cisplatin (Cis) cooperate to induce synergistic levels of NBK/BIK. A2780 and A2780/CP cells were treated with 3 μmol/L MLN4924, 10 μmol/L cisplatin, or both agents for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with specific antibodies for the BH3-only proteins NOXA, PUMA, BIM, and NBK/BIK. Tubulin documented equal loading. B, ROS generation is required for drug-induced NBK/BIK expression. A2780 and A2780/CP cells were treated with 3 μmol/L MLN4924, 10 μmol/L cisplatin, or both agents in the presence and absence of the antioxidant NAC for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with the relevant specific antibodies. Tubulin documented equal loading. C and D, NBK/BIK is a critical regulator of sensitivity to the MLN4924/cisplatin combination. A2780 and A2780/CP cells were infected with lentiviral particles expressing nontargeted control or NBK/BIK–targeted shRNAs. Positively infected cells were selected with puromycin treatment. Cells with and without targeted NBK/BIK knockdown were treated with 3 μmol/L MLN4924, 10 μmol/L cisplatin, or both agents for 24 hours. Percentages of cells with sub-G0−G1 DNA were determined by PI/FACS. N = 3 ± SD. Immunoblotting was used to assess knockdown efficiency. *, P < 0.05.
increased ROS generation is necessary for the induction of NBK/BIK by MLN4924 and cisplatin, we assessed the impact of the antioxidant NAC on the ability of these agents to increase its expression. Our results showed that ROS stress is required for NBK/BIK induction by the MLN4924/cisplatin combination as NAC treatment severely blunted the drug-related increase in its expression (Fig. 5B). We next used NBK/BIK–targeted lentiviral shRNA to ascertain the contribution of this BH3-only protein to MLN4924/cisplatin–mediated apoptosis. A2780 and A2780/CP cells were infected with lentiviral particles containing nontargeted control or NBK/BIK–targeted shRNAs. Immunoblotting was used to assess knockdown efficiency and showed that the NBK/BIK–targeted shRNA effectively antagonized the...
ability of MLN4924 and cisplatin to trigger its expression (Fig. 5C). Cells expressing nontargeted control or NBK/BIK–directed shRNA were treated with MLN4924, cisplatin, or both agents for 24 hours and the effects of drug-treatment on apoptosis induction were quantified by PI/FACS analyses. Our results showed that NBK/BIK is an important downstream effector of the MLN4924/cisplatin combination as targeted knockdown of its expression significantly diminished the ability of these agents to induce apoptosis (Fig. 5D).

**MLN4924 cooperates with cisplatin to reduce tumor burden in both cisplatin-sensitive and -resistant ovarian cancer xenografts**

Xenograft studies were carried out to investigate the in vivo therapeutic potential of the combination of MLN4924 and cisplatin. A2780 and cisplatin-resistant A2780/CP cells were injected subcutaneously into the flanks of immunodeficient nude mice. Vehicle, MLN4924, cisplatin, or the combination of MLN4924 and cisplatin were administered for 12 days as described in the Materials and Methods. As expected, cisplatin therapy had no significant activity against the resistant A2780/CP tumors. Both MLN4924 and cisplatin had substantial effects on tumor burden in A2780 xenografts and the combination resulted in significantly greater tumor growth inhibition than what was achieved by either agent alone (Fig. 6A). Notably, the MLN4924/cisplatin combination was significantly more effective than single-agent treatments with respect to inhibiting tumor cell proliferation (PCNA; Fig. 6B), inducing DNA damage (γ-H2AX; Fig. 6C), apoptosis (active caspase-3; Fig. 6D), and NBK/BIK expression (Fig. 6E). Our collective data show that inhibition of NAE with MLN4924 is a novel strategy to augment cisplatin efficacy and target cisplatin resistance in ovarian cancer with potential applications for other malignancies that use platinum-based therapy (Fig. 6F).

**Discussion**

Resistance to platinum-based chemotherapy continues to be a major problem for patients with ovarian cancer and other malignancies (31). Unfortunately, the majority of patients that develop cisplatin resistance are also cross-resistant to the related platinum-based chemotherapeutic agent oxaliplatin as well as the microtubule-disrupter paclitaxel, both of which are frequently used in salvage chemotherapy. Although new agents/strategies have the potential to benefit all patients with ovarian cancer, those with platinum-resistant disease desperately need more effective therapies to improve their long-term survival.

Although a number of different mechanisms have been associated with cisplatin resistance, 2 of the best characterized mechanisms are elevated DNA repair activity and increased GSH synthesis (20, 31–34). Hyperactive DNA repair pathways can result in the repair of cisplatin–DNA intrastrand cross-links, thus diminishing its cytotoxic/anti-cancer effects. Similarly, increased levels of GSH can facilitate the detoxification of cisplatin as well as reduce cellular apoptotic potential by buffering ROS stress, which is triggered in response to a wide variety of anticancer agents including cisplatin. The specific events that underlie the evolution of these drug-resistant mechanisms are not entirely clear. It is possible that disruption of protein homeostasis may contribute to the development of a drug resistance phenotype. The pathways that govern protein turnover often become dysregulated during malignant transformation and/or disease progression and can culminate in the inappropriate degradation of proteins that regulate key transduction cascades that control established regulators of proliferation, invasion, metastasis, and drug resistance (1). Several recent studies have reported aberrations in the NEDD8 conjugation cascade within the context of cancer. For example, one investigation showed that dysruptions in the NEDD8 pathway may facilitate acquired resistance to antiestrogen therapy in breast cancer (35). A separate study reported a direct correlation between elevated levels of NEDD8-conjugated proteins and increased rates of cell proliferation in cells from oral squamous cell carcinomas and other highly proliferative malignancies (36).
Similarly, another investigation showed abnormal patterns of CUL1 protein expression and NEDDylation in lung tumors (37). Considering this, targeting NEDD8-mediated protein degradation is a logical anticancer strategy with significant potential therapeutic benefits.

We investigated the preclinical anticancer activity and pharmacodynamic effects of MLN4924, a first-in-class inhibitor of NAE that is being evaluated in multiple phase I clinical trials, in cisplatin-sensitive and -resistant models of ovarian cancer. Our results showed that MLN4924 had significant and similar single-agent activity in both cisplatin-sensitive and -resistant models, indicating that the mechanisms that underlie resistance to cisplatin and MLN4924 likely do not overlap. This is in agreement with 2 recently published studies that identified treatment-related heterozygous mutations in the ATP-binding pocket and NEDD8-binding cleft of NAEB as a novel mechanism of resistance to MLN4924 (12, 38). Additional evidence for potentially distinct mechanisms of resistance between classical chemotherapy and MLN4924 is also provided by recent studies with MLN4924 in acute myelogenous leukemia (AML). Our earlier work showed that primary cells from patients that are refractory to conventional therapy retain in vitro sensitivity to MLN4924 (9). In addition, clinical sensitivity to MLN4924 was observed in patients with AML that failed standard therapy in an ongoing phase I study (39). These collective findings provide a basis for the further investigation of the potential use of MLN4924 for the treatment of malignancies that are resistant to cytotoxic chemotherapy.

We conducted proteome profiling analyses to investigate the consequences of NAE inhibition with MLN4924 on the global protein expression of ovarian cancer cells (Supplementary Table S1). Many of the proteins that were significantly affected by treatment with MLN4924 were known NEDD8-CUL substrates and their levels increased in a manner generally consistent with, but not identical to, what was observed in a recent investigation of the effects of MLN4924 on the proteome of melanoma cells (40). These findings suggest that there may be differences between tumor types with respect to their specific responses to NAE inhibition. Of the proteins that were significantly upregulated, we observed noteworthy changes in several factors that are associated with altered cellular redox status (increased levels of NFE2L2/NRF-2, HMOX1, and KEAP1) and the response to DNA damage (elevated p53, CDT-1, and claspin). On the basis of these effects, we explored the potential ability of MLN4924 to reestablish cisplatin-resistant ovarian cancer cells to cisplatin treatment. Our findings showed that significant benefit was achieved by cotreatment with MLN4924 and cisplatin in 2 different models of resistance (A2780/CP and 2008/C13; Fig. 2), indicating that NAE inhibition may be an effective strategy to augment the efficacy of cisplatin irrespective of differing levels of baseline sensitivity.

Several earlier studies have reported that MLN4924 has DNA-damaging properties and this has been attributed, at least in part, to the stabilization of the chromatin licensing factor CDT-1 and consequential DNA rereplication stress (7, 27, 41–43). To elucidate the mechanisms underlying the therapeutic benefit of NAE inhibition with respect to cisplatin treatment in cisplatin-sensitive and -resistant cells, we quantified the impact of drug treatment on DNA damage (Fig. 3). We observed superadditive levels of DNA damage in cells treated with the combination of MLN4924 and cisplatin as compared with either single agent. These effects were apparent in both cisplatin-sensitive and -resistant cells, suggesting that NAE inhibition may overcome intrinsic mechanisms of resistance to DDR activation in cisplatin-resistant cells. Additional studies are warranted to further explore this possibility. Of note, our study reports for the first time that inhibition of NAE with MLN4924 induces oxidative DNA damage (Fig. 4C). These effects seem to be linked to the ROS stress that we previously observed following treatment of AML cells with MLN4924 (9). Our current findings show that MLN4924 augments the ROS-inducing component of cisplatin’s mechanism of action and this effect is required for maximal apoptosis as treatment with the antioxidant NAC significantly diminishes the proapoptotic effects of these agents. The potential long-term impact of treatment with ROS-inducing agents on normal cells in the local tumor environment remains unclear. It is possible that ROS stress in malignant cells could produce a bystander effect on neighboring healthy cells and the consequences of this have not been fully characterized (44, 45). Future studies will likely improve our understanding of this phenomenon and facilitate the design of strategies that maximize the therapeutic selectivity of MLN4924, cisplatin, and other anticancer agents that induce ROS stress.

The BH3-only protein NBK/BIK is a proapoptotic factor that has been recently linked to oxidative stress-induced cell death (28–30). Considering the significant levels of ROS generation triggered by the MLN4924/cisplatin combination and the role of this phenomenon in apoptosis, we hypothesized that NBK/BIK may be a critical ROS-triggered mediator of the prodeath properties of this therapeutic combination. Our data show that MLN4924 and cisplatin cooperate to synergistically induce the expression of NBK/BIK in both cisplatin-sensitive and -resistant A2780 ovarian cancer cells (Fig. 5A). The induction of NBK/BIK was linked to ROS stress as treatment with the antioxidant NAC largely abrogated the ability of these agents to stimulate NBK/BIK expression (Fig. 5B). Targeted knockdown of NBK/BIK significantly reduced the proapoptotic effects of MLN4924/cisplatin, indicating that this BH3-only protein is a critical regulator of sensitivity to this combination (Fig. 5C and D). We are currently investigating the mechanisms that underlie the ability of MLN4924 and cisplatin to significantly increase NBK/BIK expression. Preliminary studies suggest that these agents may promote increased NBK/BIK stability in a redox-dependent manner. Additional studies will likely clarify this issue.

Our investigation of the in vivo efficacy and pharmacodynamic effects of MLN4924 and cisplatin in mice bearing A2780 and A2780/CP xenograft tumors showed that NAE inhibition is a beneficial approach to augment the
anticancer activity of cisplatin regardless of baseline cisplatin sensitivity (Fig. 6A). The in vitro pharmacodynamic effects of the MLN4924/cisplatin combination were recapitulated in vivo as tumors from mice treated with both drugs exhibited a significantly greater reduction in tumor cell proliferation (PCNA; Fig. 6B), increased level of DNA damage (γ-H2AX; Fig. 6C), elevated levels of apoptosis (active caspase-3; Fig. 6D), and synergistic induction of NBK/BIK expression (Fig. 6E).

MLN4924 has been evaluated in several phase I clinical trials to date and has shown the greatest degree of single-agent clinical activity in patients with AML (39). These studies have shown proof-of-concept that NAE inhibition is a valid approach for cancer therapy. Ongoing research aimed to identify a predictive biomarker of sensitivity to MLN4924 will help to optimize its potential clinical applications. Although the single-agent preclinical activity of MLN4924 in ovarian cancer is not as significant as what we observed in our earlier study focused in AML models, our collective data show that inhibition of NAE with MLN4924 is a valid strategy to augment cisplatin efficacy and target cisplatin resistance in ovarian cancer with potential applications for other malignancies that use platinum-based therapy. A clinical trial further investigating the safety and efficacy of MLN4924 in combination with platinum therapy is warranted.

Disclosure of Potential Conflicts of Interest
P.G. Smith is employed as Senior Scientist in Millennium Pharmaceuticals. S. Blakemore has ownership interest (including patents) in Takeda Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References


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