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 (CS) and cisplatin resistant (CR) 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 NBK/BIK as a regulator of therapeutic sensitivity. The *in vivo* effects of MLN4924/cisplatin on tumor burden and key pharmacodynamics endpoints were assessed in CS and CR xenograft models. **Results:** MLN4924 possessed significant activity against both CS and CR 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 CR 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 pro-apoptotic effects of the MLN4924/cisplatin combination. Administration of MLN4924 to mice bearing ovarian tumor xenografts significantly increased the efficacy of cisplatin against both CS and CR tumors. **Conclusions:** Our collective data provide a rationale for the clinical investigation of NAE inhibition
as a novel strategy to augment cisplatin efficacy in patients with ovarian cancer and other malignancies.

**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 (CS) and cisplatin-resistant (CR) models of ovarian cancer and re-sensitized CR cells to cisplatin, suggesting that aberrant protein NEDDylation may contribute to drug resistance. Increased oxidative stress and induction of the BH3-only protein NBK/BIK were key mediators of MLN4924/cisplatin activity. Administration of MLN4924 to mice bearing ovarian tumor xenografts significantly increased the efficacy of cisplatin against both CS and CR tumors. Our collective data provide a rationale for the clinical investigation of MLN4924 in combination with cisplatin for patients with ovarian cancer and other malignancies that use platinum-based therapy.
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 utilized 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 in order 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 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 (CRLs) 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, c-Myc), DNA damage (CDT1), stress responses (NRF-2, HIF-1α), and signal transduction...
(phosphorylated IκBα) (3, 4). NEDD8 activating enzyme (NAE) is the proximal regulator of the NEDDylation pathway and through transfer to the ubiquitin conjugating enzyme, Ubc12, leads to NEDDylation of the cullin family of proteins (5, 6). Given the essential functions of its protein substrates in suppressing the malignant phenotype, the NEDD8 degradation pathway is a promising new target for the treatment of many forms of cancer. MLN4924 is a potent and selective first-in-class inhibitor of NAE activity that is currently under investigation in multiple cancer clinical trials (7, 8). Our earlier work showed that induction of oxidative stress is a key event in MLN4924-induced apoptosis (9). Considering the importance of NEDD8-regulated pathways in cancer pathogenesis and that elevated antioxidant defenses are a significant factor underlying resistance to platinum-based chemotherapy, we hypothesized that targeting NAE with MLN4924 may be an effective strategy for the treatment of ovarian cancer with the potential to augment the anticancer activity of cisplatin. To test our hypothesis, we investigated the efficacy and mechanism of action of MLN4924 in cisplatin-sensitive (CS) and cisplatin-resistant (CR) models of ovarian cancer. Our results showed that MLN4924 possessed significant single agent activity and augmented the in vitro and in vivo anticancer effects of cisplatin against both CS and CR ovarian tumors. MLN4924 and cisplatin cooperated to induce DNA damage, oxidative stress, and increased expression of the BH3-only protein NBK/BIK, a critical regulator of sensitivity to the MLN4924/cisplatin combination. Our collective findings indicate that NAE inhibition is a novel and promising strategy to target cisplatin resistance.
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% fetal bovine serum at 37 °C with 5% CO₂ as previously described (10).

Chemicals and reagents. Reagents were obtained from: MLN4924 (Millennium Pharmaceuticals, Inc., Cambridge, MA), cisplatin (CTRC Pharmacy), anti-NEDD8, anti-γH2AX (Immunoblotting), anti-NRF-2, and anti-PUMA (Epitomics, Burlingame, CA), anti-p21, anti-p27, anti-WEE1, anti-CDT1, anti-γH2AX (IHC), anti-BIM, and anti-active caspase-3 (Cell Signaling, Beverly, MA), anti-NOXA (Calbiochem, Billerica, MA), anti-β tubulin and N-acetyl cysteine (Sigma, St. Louis, MO), anti-β catenin, anti-NBK/BIK, and zVAD-fmk (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-proliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark), goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Laboratories, West Grove, PA), Rat anti-mouse IgG2a-HRP antibody (Serotec, Raleigh, NC), Goat anti-mouse Alexa Fluor 488 and 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA), and sheep anti-mouse-HRP and donkey anti-rabbit-HRP (Amersham, Pittsburgh, PA).
Quantification of drug-induced cytotoxicity. Cells were treated with the indicated concentrations of MLN4924, cisplatin, or both drugs for 72 h. The effects of drug treatment on cell viability were assessed using the ATPLite OneStep assay kit (Perkin Elmer, Waltham, MA) according to the manufacturer's directions as previously described (9). Apoptosis was evaluated by PI/FACS analysis of sub-G₀/G₁ DNA content following 24 h 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 h. 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 (San Leandro, CA) gel documentation system.

NAE beta DNA sequencing. DNA from A1847, A2780, A2780/CP, 2008, 2008/C13, IGROV-1, OVCAR-5 and SKOV-3 cell lines was isolated using the DNeasy mini kit (Qiagen Inc., Valencia, CA). 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 NAEb (UBA3) exons 8, 9 and 13. Sequencing
reactions were run on an ABI 3130xl at the Nucleic Acid Core Facility at 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, Billerica, MA) 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 h. Following drug treatment, cells were incubated with a propidium iodide (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, San Jose, CA).

**Immunoblotting.** Ovarian cancer cells were incubated with MLN4924, cisplatin or the combination of both drugs for 24 h as indicated. Cells were then lysed for 1 h on ice in Triton X-100 lysis buffer (1% triton X-100, 150 mM NaCl, 25 mM Tris pH 7.5) with protease inhibitors. Approximately 50 μg of total cellular protein from each sample were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween-20 for 1 h. 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, San Leandro, CA) as previously described (13).

**Proteome Profiling.** A2780 cells were treated with MLN4924 for 24 h. Samples were harvested in a urea lysis buffer, reduced and alkylated before being digested with Lys-C (Wako, Richmond, VA). After digestion, samples were purified over SepPak C18 columns and eluted peptides were lyophilized. After lyophilization, 100 µg of each sample was labeled using TMT reagents (Thermo Fisher Pierce, Rockford, IL). Labeled samples were then combined and subjected to Strong Cation Exchange (SCX) chromatography. The six-plexes were run on the Accela high speed LC system (Thermo Fisher, Rockford, IL) over a 4.6 x 200 mm SCX column packed with polysulfoethyl aspartamide material (5 µm particle size, 200 Å-pore). A fifty-minute gradient was used to elute peptides from the column and fractions were collected at every minute for the entire gradient. The first forty fractions were combined into twenty fractions before being purified again over a C18 SepPak column. All fractions were then run individually on the Orbi-Velos using a 140-minute gradient. Peptide identification was carried out in MS² scans, while quantification of reporter ions was carried out using MS³ scans (14). In total, the twenty fractions were run consecutively for each six-plex, occupying approximately forty-seven hours of instrument time. Data generated from the MS analysis was processed using in-house software (Cell Signaling Technologies, Beverly, MA) resulting in an average of 3900 quantified proteins per six-plex.
**Immunocytochemistry.** A2780 and A2780/CP ovarian cancer cells were plated on chamber slides and treated with MLN4924, cisplatin, or both agents for 24 h. Cells were fixed with 4% paraformaldehyde, permeabilized using 0.2% Triton X-100, and incubated overnight with a γ-H2AX antibody. Goat anti-mouse Alexa 488 fluorescent secondary antibody was used to visualize γ-H2AX. DAPI was used to counterstain the nucleus. Images were captured using a Zeiss LSM 510 Meta confocal microscope with an oil 40X 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 h. Comet assays were performed using the CometAssay® kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, test samples and Alkaline Comet Control cells were mixed with molten LM agarose and spread onto well slides. Slides were chilled to 4°C, incubated in lysis solution for 30 min at 4°C, and then immersed in unwinding solution for 20 min at room temperature. Slides were electrophoresed using Trevigen's Comet ES system in cold 200 mM NaOH/1 mM EDTA solution for 30 min at 21V. 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 (ROS) generation.** Cells were treated with MLN4924, cisplatin, or the combination of MLN4924 and cisplatin for 12 h. 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, Carlsbad, CA) 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 h. The levels of 8-oxo-guanine were determined using the Oxidative DNA damage kit (Kamiya Biomedical Company, Seattle, WA) 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 non-targeted (control) or targeted shRNA directed at NBK/BIK or the NF-kB p65 subunit.
(Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s instructions. Positively infected cells were selected with puromycin treatment. Infected cells were treated with the indicated concentrations of MLN4924 and cisplatin for 24 h. Drug-induced apoptosis was quantified by PI/FACS as described above. 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 HBSS and Matrigel (BD BioSciences, San Jose, CA). 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 SC BID (n = 10), cisplatin 2 mg/kg IV Q3D (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 performed by microwaving slides in a citrate buffer for 5 min. Endogenous peroxides were blocked with a 3% hydrogen peroxide solution for 10 min. Slides were then incubated in a protein block solution (5% horse and 1% goat serum in PBS) for
20 min. PCNA, cleaved caspase-3, γH2AX, 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 h at ambient temperature. Positive reactions were visualized using 3,3′-diaminobenzidine (Dako, Glostrup, Denmark) for 10 min. The slides were rinsed with water followed by a brief counterstain with Gill's hematoxylin (Sigma, St. Louis, MO). Images were captured using an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20X objective. Image-Pro Plus software Version 6.2.1 (MediaCybernetics, Bethesda, MD) was used for image acquisition. ImageJ software was used for quantification of γH2AX and NBK/BIK levels by densitometric analysis of five random fields containing viable tumor cells. Quantification of PCNA and cleaved caspase-3 was conducted by counting the number of positive cells in five random fields as previously described (17).

**Synergy analyses.** The combination indices (CI) for MLN4924 and cisplatin were calculated based on the effect of 72 h exposure to each drug on cell viability as determined by ATPLite assay. CompuSyn software (ComboSyn, Inc. Paramus, NJ) software was utilized 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’s 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 µM – 10 µM) on the viability of a panel of 6 human ovarian cancer cell lines (A1847, A2780, IGROV-1, OVCAR-5, SKOV-3, and 2008). 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 h) exposure to sub-micromolar 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 NAE beta, a recently identified mechanism of resistance to MLN4924, as DNA sequencing of all cell lines utilized 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. In order 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. Supplemental Table 1 provides a summary of the 68 (1.7% of total number assessed) proteins whose expression was altered >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 pro-apoptotic 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, 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 CS and A2780/CP CR 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 CS and CR 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 CS counterparts A2780 and 2008. Formal synergy analyses revealed that the combination of MLN4924 and cisplatin yielded combination indices (CI) 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 be a new approach to re-sensitize CR tumors to cisplatin.

**Inhibition of NF-kB 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-kB transcriptional activity through the stabilization of the phosphorylated form of IkBα, the endogenous inhibitor of NF-kB and a well-characterized NEDD8 substrate protein (7, 9, 21). Earlier studies have demonstrated that constitutive NF-kB activity reduces sensitivity to cisplatin (22-26). In order to determine whether the NF-kB inhibitory component of MLN4924’s pharmacodynamic effects was a major factor contributing to its ability to sensitize ovarian cancer cells to cisplatin, we utilized
shRNA to target the expression of the p65 NF-kB subunit. Targeted knockdown of p65 modestly augmented the pro-apoptotic effects of cisplatin in A2780 and A2780/CP cells (Supplemental Fig. 1). This suggests that additional NF-kB-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 crosslinks 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 re-replication (7, 27). In order to determine whether inhibition of NAE with MLN4924 could augment the DNA damaging effects of cisplatin, we utilized immunocytochemistry to visualize and quantify the effects of MLN4924, cisplatin, and the combination of both drugs on the levels of $\gamma$H2AX as a marker of DNA damage in A2780 and A2780/CP cells. MLN4924 treatment induced a significant increase in $\gamma$H2AX fluorescence in both cell lines. Notably, combined treatment with MLN4924 and cisplatin triggered a dramatic induction of $\gamma$H2AX compared to either single agent in spite of the intrinsic differences in sensitivity to cisplatin between A2780 and A2780/CP cells (Fig. 3A and B). In order 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 $\gamma$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). In order 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 utilized 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 γH2AX were largely unaffected by inhibition of caspase activity, indicating that these agents trigger DNA damage in a caspase-independent manner (Supplemental Fig. 2A). 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 h was sufficient to cause significant mitochondrial depolarization (Fig. 4A). We next measured ROS levels in A2780 and the CR A2780/CP cells following treatment with MLN4924, cisplatin, or both agents using a flow cytometric method. Exposure to MLN4924 (12 h) 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 appeared to be higher in the resistant A2780/CP cells compared with their CS 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 utilized 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 CS 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 utilizing the antioxidant glutathione-mimetic N-acetyl cysteine (NAC) to quench drug-induced ROS generation. Pre-treatment 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, Supplemental Fig. 2B). 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 pro-apoptotic effects of the MLN4924/cisplatin combination.** The BH3-only protein natural born killer/bcl-2 interacting killer (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. In order to determine whether 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 demonstrated 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 utilized 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 non-targeted control or NBK/BIK-targeted shRNAs. Immunoblotting was utilized to assess knockdown efficiency and demonstrated the NBK/BIK-targeted shRNA effectively antagonized the ability of MLN4924 and cisplatin to trigger its expression (Fig. 5C). Cells expressing non-targeted control or NBK/BIK-directed shRNA were treated with MLN4924, cisplatin, or both agents for 24 h and the effects of drug-treatment on apoptosis induction were quantified by PI/FACS analyses. Our results demonstrated 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 CS and CR ovarian cancer xenografts.** Xenograft studies were carried out to investigate the *in vivo* therapeutic potential of the combination of MLN4924 and cisplatin. A2780 and CR 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 MLN4924 monotherapy against A2780/CP tumors in spite of their intrinsic resistance to cisplatin. Although the MLN4924/cisplatin combination did not yield complete tumor regressions, it produced a significant therapeutic benefit over what was achieved by either single agent and extended animal survival. The combination was also well tolerated as it exhibited only a modest, statistically insignificant loss in body weight. Immunohistochemistry was utilized to quantify the in vivo pharmacodynamic effects of MLN4924 and cisplatin in A2780 and A2780/CP tumors. The combination of MLN4924 and cisplatin was significantly more effective than single agent treatments with respect to inhibiting tumor cell proliferation (PCNA, Fig. 6B), inducing DNA damage (γH2AX, Fig. 6C), apoptosis (active caspase-3, Fig. 6D), and NBK/BIK expression (Fig. 6E). Our collective data demonstrate 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 utilize 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 utilized in salvage chemotherapy. While 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, two of the best characterized mechanisms are elevated DNA repair activity and increased glutathione (GSH) synthesis (20, 31-34). Hyperactive DNA repair pathways can result in the repair of cisplatin-DNA intrastrand crosslinks, thus diminishing its cytotoxic/anticancer 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 resistance 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 disruptions in the NEDD8 pathway may facilitate acquired resistance to anti-estrogen 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 cells and other highly proliferative malignancies (36). Similarly another investigation demonstrated 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 (CS) and resistant (CR) models of ovarian cancer. Our results showed that MLN4924 had significant and similar single agent activity in both CS and CR models, indicating that the mechanisms that underlie resistance to cisplatin and MLN4924 likely do not overlap. This is in agreement with two recently published studies that identified treatment-related hetereozygous mutations in the adenosine triphosphate binding pocket and NEDD8-binding cleft of NAEβ as a novel mechanism of resistance to MLN4924.
Additional evidence for potentially distinct mechanisms of resistance between classical chemotherapy and MLN4924 is also provided by recent studies with MLN4924 in acute myeloid leukemia (AML). Our earlier work showed that primary cells from patients that are refractory to conventional therapy retain \textit{in vitro} sensitivity to MLN4924 (9). Additionally, 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 utility 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 (Supplemental Table 1). 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, KEAP1) and the response to DNA damage (elevated p53, CDT-1, clasin). Based on these effects, we explored the potential ability of MLN4924 to re-sensitize CR ovarian cancer cells to cisplatin
treatment. Our findings showed that significant benefit was achieved by co-treatment with MLN4924 and cisplatin in two 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 re-replication stress (7, 27, 41-43). In order to elucidate the mechanisms underlying the therapeutic benefit of NAE inhibition with respect to cisplatin treatment in CS and CR 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 CS and CR cells, suggesting that NAE inhibition may overcome intrinsic mechanisms of resistance to DDR activation in CR 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 appear to be linked to the ROS stress that we previously observed following treatment of AML cells with MLN4924 (9). Our current findings demonstrate 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 pro-apoptotic 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 impose ROS stress.

The BH3-only protein NBK/BIK is a pro-apoptotic 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 pro-death properties of this therapeutic combination. Our data demonstrate that MLN4924 and cisplatin cooperate to synergistically induce the expression of NBK/BIK in both CS and CR 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 pro-apoptotic 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 demonstrated 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 (γH2AX, 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 demonstrated the greatest degree of single agent clinical activity in patients with AML (39). These studies have demonstrated 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. While 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 demonstrate 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 utilize platinum-based therapy. A clinical trial further investigating the safety and efficacy of MLN4924 in combination with platinum therapy is warranted.

References


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Figure Legends

Fig. 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 µM MLN4924 for 24 h. 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 h 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.

Fig. 2. MLN4924 augments the cytostatic and pro-apoptotic effects of cisplatin to overcome drug resistance. A, A panel of 8 human ovarian cancer cell lines including the cisplatin-resistant A2780/CP and 2008/C13 were treated with 0.3 µM MLN4924, 3 µM cisplatin, or both agents for 72 h. Cell viability was determined by ATPLite assay. N = 3 ± SD. B, Cells were treated with 3 µM MLN4924, 10 µM cisplatin, or both drugs for 24 h. Percentages of cells with sub-G_0-G_1 DNA were determined by PI/FACS. N = 3 ± SD. *P < 0.05. C, Cells were treated with 3 µM MLN4924, 10 µM cisplatin, or both drugs for 24 h. Percentages of necrotic (PI+) cells were quantified by FACS. N = 3 ± SD.
Fig. 3. MLN4924 and cisplatin cooperate to induce DNA damage. A-B, Effects of MLN4924 and cisplatin on γH2AX immunofluorescence. A2780 and A2780/CP cells were treated with 3 µM MLN4924, 10 µM cisplatin or both agents for 24 h. γH2AX immunofluorescence was determined and quantified as described in the Materials and Methods. Representative images are shown. N = 3 ± SD. *P < 0.05. C, A2780 and A2780/CP cells were treated with 3 µM MLN4924, 10 µM cisplatin or both agents for 16 h. The DNA tail moment for each experimental condition was quantified by alkaline comet assay. Representative images are shown. N = 3 ± SD. *P < 0.05.

Fig. 4. Reactive oxygen species generation is a key event in MLN4924/cisplatin-mediated apoptosis. A, Impact of MLN4924 and cisplatin on mitochondrial membrane polarization. A2780 and A2780/CP cells were treated with 3 µM MLN4924, 10 µM cisplatin or both agents for 6 h. Mitochondrial polarization was determined by Mitotracker Red CMXROS staining and flow cytometry. N = 3 ± SD. *P < 0.05. B, Impact of MLN4924 and cisplatin on ROS generation. A2780 and A2780/CP cells were treated with 3 µM MLN4924, 10 µM cisplatin or both agents for 12 h. ROS generation was determined by DCFDA staining and flow cytometry. N = 3 ± SD. *P < 0.05. C, Effects of drug treatment on oxidative DNA damage. A2780 and A2780/CP cells were treated with 3 µM MLN4924, 10 µM cisplatin or both agents for 16 h. The percentages of cells...
expressing 8-oxo-guanine were determined by flow cytometry as described in the Materials and Methods. N = 3 ± SD. *P < 0.05. D, The antioxidant NAC diminishes drug-induced apoptosis. A2780 and A2780/CP cells were pre-treated with 10 mM NAC for 2 h. Cells with and without NAC were treated with 3 µM MLN4924, 10 µM cisplatin or both agents for 24 h. Percentages of cells with sub-G₀-G₁ DNA were determined by PI/FACS. N = 3 ± SD. *P < 0.05.

Fig. 5. NBK/BIK is a critical regulator of the pro-apoptotic effects of the MLN4924/cisplatin combination. A, MLN4924 and cisplatin cooperate to induce synergistic levels of NBK/BIK. A2780 and A2780/CP cells were treated with 3 µM MLN4924, 10 µM cisplatin, or both agents for 24 h. 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 µM MLN4924, 10 µM cisplatin, or both agents in the presence and absence of the antioxidant NAC for 24 h. Protein lysates were subjected to SDS-PAGE, blotted, and probed with the relevant specific antibodies. Tubulin documented equal loading. C-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 non-targeted 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 µM MLN4924, 10 µM cisplatin, or both agents for 24 h.
Percentages of cells with sub-G₀-G₁ DNA were determined by PI/FACS. N = 3 ± SD. Immunoblotting was utilized to assess knockdown efficiency. *P < 0.05.

**Fig. 6. MLN4924 cooperates with cisplatin to reduce tumor burden in both CS and CR ovarian cancer xenografts.** A, 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 BID (n = 10), cisplatin 2 mg/kg Q3D (n = 10), or both MLN4924 and cisplatin (n = 10) for 12 days. Mice were monitored daily and tumor volumes were measured twice weekly. **B-E**, *In vivo* pharmacodynamic effects of MLN4924 and cisplatin. Immunohistochemistry was utilized to measure the levels of PCNA, γH2AX, active caspase-3, and NBK/BIK in tumor specimens collected from mice in each treatment group. Staining was quantified as described in the Materials and Methods. Representative images are shown. N = 5 ± SD. *P < 0.05. F, Schematic illustration of proposed mechanism underlying the synergy between MLN4924 and cisplatin. Inhibition of NAE with MLN4924 disrupts protein homeostasis, triggers ROS generation, and DNA damage. Cisplatin augments MLN4924-mediated ROS production and cooperates with MLN4924 to induce DNA damage. Combined treatment with MLN4924 and cisplatin triggers a dramatic increase in the levels of the BH3-only protein NBK/BIK and this event is required for maximal apoptosis.
Fig. 2

A

Viability (% Control)

Control  |  MLN4924  |  Cisplatin  |  4924 + Cis

B

% Sub-G₀/G₁ Cells

Control  |  MLN4924  |  Cisplatin  |  4924 + Cis

C

% Necrotic Cells

Control  |  MLN4924  |  Cisplatin  |  4924 + Cis

A2780  |  A2780/CP  |  IGROV-1  |  2008  |  2008/C13  |  SKOV-3  |  OVCAR-5  |  A1847
Fig. 4

A

% Mitochondrial Depolarization

Control MLN4924 Cisplatin 4924 + Cis

A2780 A2780/CP

B

ROS Generation (RFU)

Control MLN4924 Cisplatin 4924 + Cis

A2780 A2780/CP

C

% 8-oxo-G + Cells

Control MLN4924 Cisplatin 4924 + Cis

A2780 A2780/CP

D

% Sub-G0/G1 Cells

Control MLN4924 Cisplatin 4924 + Cis

A2780 NAC- A2780 NAC+ A2780/CP NAC- A2780/CP NAC+
Fig. 5

A

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- A2780/CP

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- A2780/CP

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- A2780 NT shRNA
- A2780 NBK shRNA
- A2780/CP NT shRNA
- A2780/CP NBK shRNA

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

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