Nedd8-Activating Enzyme Inhibitor MLN4924 Provides Synergy with Mitomycin C through Interactions with ATR, BRCA1/BRCA2, and Chromatin Dynamics Pathways

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Abstract

MLN4924 is an investigational small-molecule inhibitor of the Nedd8-activating enzyme currently in phase I clinical trials. MLN4924 induces DNA damage via rereplication in most cell lines. This distinct mechanism of DNA damage may affect its ability to combine with standard-of-care agents and may affect the clinical development of MLN4924. As such, we studied its interaction with other DNA-damaging agents. Mitomycin C, cisplatin, cytarabine, UV radiation, SN-38, and gemcitabine demonstrated synergy in combination with MLN4924 in vitro. The combination of mitomycin C and MLN4924 was shown to be synergistic in a mouse xenograft model. Importantly, depletion of genes within the ataxia telangiectasia and Rad3 related (ATR) and BRCA1/BRCA2 pathways, chromatin modification, and transcription-coupled repair reduced the synergy between mitomycin C and MLN4924. In addition, comet assay demonstrated increased DNA strand breaks with the combination of MLN4924 and mitomycin C. Our data suggest that mitomycin C causes stalled replication forks, which when combined with rereplication induced by MLN4924 results in frequent replication fork collisions, leading to cell death. This study provides a straightforward approach to understand the mechanism of synergy, which may provide useful information for the clinical development of these combinations.

Mol Cancer Ther; 13(6); 1625–35. ©2014 AACR.

Introduction

MLN4924 is being developed as an inhibitor of the Nedd8-activating enzyme (1) and is currently in phase I clinical trials. Nedd8 is a small ubiquitin-like protein in which the best characterized role is the activation of a class of E3 ubiquitin ligases known as cullin RING ligases (CRL; ref. 2). Multiple studies have demonstrated the importance of the induction of rereplication, resulting in DNA damage, to the mechanism of cell death by MLN4924 (3–5). In addition, CRLs are known to be involved in the regulation of multiple DNA replication and repair pathways, including nucleotide excision repair, histone modification, and regulation of p21, p27, p53, Cdc25A, Wee1, claspin, and FANCM (6–10). Many standards of care in cancer chemotherapy are DNA-damaging agents and mechanistic interactions between MLN4924 and these agents may influence the clinical development of MLN4924.

Although the mechanism engaged by MLN4924 to induce DNA damage seemingly differs from other DNA-damaging agents, the pathways involved in the repair of the DNA damage may overlap. A previous siRNA screen that evaluated the genetics of sensitivity of A375 and HCT-116 cell lines to MLN4924 demonstrated an engagement of a p21-dependent intra–S-phase checkpoint in A375 cells and an Emi1-dependent G2–M checkpoint in HCT-116 cells (5). In addition, roles for p53, BRCA1/BRCA2, and nucleotide excision repair were identified. Oncogene-induced replication stress seems to play a central role in tumor progression (11, 12), suggesting that rereplication may directly challenge tumor vulnerabilities. However, it is not clear that rereplication will be the dominant mechanism of DNA damage in combination with other DNA-damaging agents and, thus, it is difficult to predict the best combination partners.

We have conducted a systematic evaluation of the effectiveness of combining MLN4924 with DNA-damaging agents across four different cancer cell types in vitro and demonstrate synergy with mitomycin C, cisplatin, UV, SN-38, melphalan, etoposide, and...
gemcitabine. Mitomycin C gave the most consistent synergistic interaction with MLN4924 across cell lines. Much work has been done to understand the mechanism of DNA repair following mitomycin C treatment (13, 14), providing a background to understand these results.

Mitomycin C alkylates DNA with about 10% of DNA modifications resulting in interstrand crosslinks, the remainder being monoadduct and intrastrand crosslinks (14). The mechanism by which the cell repairs this DNA damage is determined by how the adduct is detected, which is significantly influenced by the stage of the cell cycle in which the damage occurs. During G2, adduct can be detected by components of either global genome nucleotide excision repair or by transcription-coupled nucleotide excision repair (TC-NER) if the interstrand crosslink blocks RNA polymerase. During S-phase, encounter of the interstrand crosslink by the replication fork becomes a significant mechanism for detection of the crosslink and the repair mechanism is believed to be substantially different from that experienced during G2 (14). Notably, during S and G2 phases of the cell cycle, sister chromatids are available for exchange by homologous recombination, providing a high-fidelity mechanism of repair.

To gain a more complete understanding of the synergy between MLN4924 and mitomycin C, we used an siRNA screen to evaluate the effect of gene depletion on this synergy. Many of the genes identified in the siRNA screen underwent posttranslational modifications to their protein products upon treatment with the combination of MLN4924 and mitomycin C, confirming their likely involvement. Comet assay experiments demonstrated early induction of DNA strand breaks by the combination of MLN4924 and mitomycin C. Our results suggest that mitomycin C increases the frequency of replication fork collision induced by MLN4924 and that the BRCA1–claspin–ATR–Chk1 pathway plays an important role in the response to these collisions. These studies may provide a way of identifying promising drug combinations and understanding the mechanistic details of their synergy. Such knowledge may more efficiently guide clinical development of chemotherapeutic combinations.

Materials and Methods
Genomic characterization and authentication of cell lines
A375, A549, HCT-116, and U-2 OS were received from the American Type Culture collection (ATCC) in July 2006, February 2004, June 2009, and July 2006, respectively, and passaged twice before freezing. Genomic DNA was isolated from 2 million cells under vendor-specified protocols. Of note, 500 μg DNA per sample was then amplified, labeled, and processed on the Affymetrix SNP-6 whole genome array platform on November 7, 2011, and evaluated using Partek Genomics Suite for copy-number variation analysis. Spearman correlation was determined for each cell line against an ATCC-authenticated stock vial received at Takeda (A375 ATCC CRL-1619, A549 ATCC CCL-185, HCT-116 ATCC CCL-247, and U-2 OS ATCC HTB-96). Consistent regions of amplification and deletions were found between the cell lines of the same origin, and correlation on genotypes was greater than 0.995. Cell lines were passaged for less than 8 weeks following resuscitation.

Evaluation of cell line sensitivity to DNA-damaging agents and evaluation of combination effects
Cells were grown in their respective growth media, supplemented with 10% FBS: A375, Dulbecco’s Modified Eagle Medium; A549, Ham’s F-12K (Kaighn’s) medium; HCT-116 and U-2 OS, McCoy’s 5A medium with 1% glutamine.

A375 melanoma (800 cells/well), HCT-116 colorectal carcinoma (800 cells/well), A549 lung carcinoma (1,000 cells/well), and U-2 OS osteosarcoma (800 cells/well) were seeded on 384-well poly-D-lysine (PDL)-coated black, clear-bottom plates (BD BioCoat) and allowed to adhere for 24 hours at 37°C, 6% CO2. Cells were then treated with compounds, either alone or in combination with MLN4924, at various doses for 48 hours (HCT-116 and A375, doubling times of 18 and 16 hours, respectively) or 72 hours (A549 and U-2 OS, doubling times of approximately 24 hours). Viability was assessed with CellTiter-Glo Cell Viability reagent according to the manufacturer’s instructions (Promega). Luminescence was measured using a LEADseeker imaging system (GE Healthcare). MLN4924 will be made available to qualified researchers once a standard Material Transfer Agreement has been executed.

Calculation of combination metrics
The relationship between the normalized viability and drug concentrations was fit with a nine parameter response surface model (15). To quantify the synergy, the combination index (16) or nonlinear blending (17) was computed. Additional details can be found in Supplementary Materials and Methods.

The effect of depletion of DNA damage response genes on the synergy between MLN4924 and mitomycin C
A375 cells were cultured and reverse transfected with siRNA SMARTpools or duplexes targeting DNA damage response genes using DharmaFect4 transfection lipid (Dharmacon, GE Healthcare), as previously described (5). After 48 hours knockdown, cells were treated with DMSO (dimethyl sulfoxide; negative control), 330 nmol/L MLN4924, 1.6 or 1 μmol/L mitomycin C, or the combination of 330 nmol/L MLN4924, and 1.6 or 1 μmol/L mitomycin C, in the continued presence of siRNA oligos, and incubated for a further 48 hours, after which viability was assessed with ATPlite reagent according to the manufacturer’s instructions (PerkinElmer). HCT-116 cells were also transfected as previously described (5). After 48 hours knockdown, cells were treated with DMSO (negative control), 110 nmol/L MLN4924, 450 nmol/L...
mitomycin C, or 110 nmol/L MLN4924, and 450 nmol/L mitomycin C for an additional 48 hours.

The interaction (BI score) between MLN4924 and mitomycin C was calculated from log$_2$-transformed data as

\[ BI = V_{\text{comb}} - V_{\text{single}} \]

where \( V_{\text{comb}} \) is the viability in the presence of siRNA, MLN4924, and mitomycin C, and \( V_{\text{single}} \) is the viability in the presence of siRNA alone, \( V_{\text{single}} \) is the viability in the presence of siRNA and MLN4924, and \( V_{\text{single}} \) is the viability in the presence of siRNA and mitomycin C.

**Evaluation of synergy in the A375 xenograft model**

Female Balb/c Nude mice (Shanghai Laboratory Animal Center, Shanghai, China) were inoculated with 5 × 10$^6$ A375 tumor cells (0.1 mL in 50% Matrigel) in the right flank. After tumors grew to an average size 150 mm$^3$, mice were randomly assigned into treatment groups of 10 mice each (vehicle, MLN4924, mitomycin C, or the combination of MLN4924 and mitomycin C). Vehicle (20% HPβCD) was dosed subcutaneously on days 1, 4, 8, 11, 15, and 18 (twice weekly × 3). MLN4924 was formulated in 20% HPβCD and dosed subcutaneously twice weekly × 3 at 180 mg/kg. Mitomycin C (Intas Pharmaceuticals, Ltd.) was formulated in 0.9% saline and dosed intravenously at 2.5 mg/kg on days 1, 8, and 15 (once a week for 3 weeks). Body weight and tumor growth were monitored twice a week. All mice had access to food and water ad libitum and were housed and handled in the AAALAC-certified facility at the Medicilon Preclinical Research, LLC, in accordance with the Medicilon Institutional Animal Care and Use Committee Guidelines.

**Cell culture and reverse transfection for Western blot analysis**

A375 melanoma cells were maintained as previously described (5). For Western blot analysis of protein knockdown, A375 cells (57,000 cells/plate) were reverse transfected in 10-cm diameter PDL-coated cell culture plates (CELLCOAT; Greiner Bio-One) with DharmaFECT 4 reagent (DH4; Dharmacon) using a final concentration of 15 nmol/L siRNA (siGENOME SMARTpool; Dharmacon) using a final concentration of 15 nmol/L siRNA (siGENOME SMARTpool; Dharmacon) using a final concentration of 15 nmol/L siRNA (siGENOME SMARTpool; Dharmacon) using a final concentration of 15 nmol/L siRNA (siGENOME SMARTpool; Dharmacon). Cells were then treated with compounds, either as single agent or in combination treatments using mitomycin C and MLN4924 for 6 hours. A 6-hour treatment using etoposide was used as a positive control for induction of DNA strand breaks.

Preparation and execution of the alkaline comet assay was done according to the Trevigen CometAssay protocol for single-cell gel electrophoresis provided with the reagent kit apart from the electrophoresis protocol (catalog# 4250-050-K). Additional details can be found in Supplementary Materials and Methods.

**Results**

**MLN4924 synergizes with multiple DNA-damaging agents**

We profiled the cell lines A375 (melanoma), A549 (non–small cell lung cancer), HCT-116 (colon), and U-2 OS (osteosarcoma) to evaluate their sensitivity to 14 agents (Supplementary Fig. S2), selected across a breadth of mechanism, including cross-linking agents, topoisomerase inhibitors, nucleoside analogs, and the PARP inhibitor ABT-888 for evaluation in combination with MLN4924 in the four cancer-derived cell lines. All four cell lines were insensitive to the PARP inhibitor (LC$_{50}$ > 25 μmol/L), as expected (18–20). Paclitaxel and bortezomib were included as inhibitors that do not directly cause DNA damage, although bortezomib is likely to affect DNA repair (21).

Compound combinations were evaluated as a 10 × 10 matrix of drug concentrations in duplicate with simultaneous addition to cells. Data were fitted to a dose-response surface (15) and isobolograms were derived and plotted (Fig. 1 and Supplementary Fig. S3). Combination index (16) and nonlinear blending (17) values were calculated for each experiment, based upon the LC$_{50}$ isobologram (Fig. 2 and Supplementary Fig. S4). Both calculations use Loewe additivity as the null model (22). As expected, MLN4924 combined with itself was clearly additive (Fig. 2 and Supplementary Fig. S3 and S4).

Mitomycin C, cisplatin, clylarabine, and gemcitabine gave significant synergy (Fig. 1, concave curves). Generally, responses to the various compounds correlated between cell lines (Supplementary Fig. S3). Gemcitabine was the exception, with the observation of both significant synergy and antagonism (convex curves), depending on the cell line (Fig. 1D). Intriguingly, synergy with gemcitabine occurred in those cell lines with more extensive rereplication induction by MLN4924 ([4N DNA = 30% (U-2 OS) and 31% (HCT-116)], whereas antagonism...
occurred in the cell lines that exhibited less extensive rereplication (>4N DNA = 8% (A375) and 11% (A549); ref. 5).

As judged by both combination index and nonlinear blending values, MLN4924 demonstrated some degree of synergy with only 1 agent in A375 (mitomycin C) but with four agents in A549, four agents in U-2 OS, and seven agents in HCT-116, demonstrating a sufficient breadth of genetic background differences for our studies. Mitomycin C, cisplatin, and UV demonstrated synergy with MLN4924 in three of four cell lines. All three agents are capable of cross-linking DNA. Cytarabine, SN-38

Figure 1. Isobolograms of select DNA-damaging agents interacting with MLN4924 across A375 (melanoma), A549 (lung), HCT-116 (colon), and U-2 OS (osteosarcoma) cancer cell lines. A, evaluation of the combination of mitomycin C with MLN4924. Lines, a contour plot of the response surface from a 10 × 10 matrix, in which each line represents a decade of viability (LC10, LC20, etc.). Concave curves suggest synergy, linear curves suggest additivity, and convex curves suggest Loewe antagonism. B, isobolograms for the combination of cisplatin with MLN4924. C, isobolograms for the combination of cytarabine and MLN4924. D, isobolograms for the combination of gemcitabine with MLN4924. Isobolograms for additional combinations with MLN4924 are shown in Supplementary Fig. S3.
active metabolite of irinotecan), and gemcitabine demonstrated synergy with MLN4924 in two of four cell lines, whereas melphalan and etoposide reliably demonstrated synergy in one of the four cell lines. Melphalan was essentially inactive in HCT-116 and U-2 OS and the addition of MLN4924 did not alter this insensitivity (data not shown). Loewe antagonism was seen in at least one cell line with gemcitabine, paclitaxel, daunorubicin, trabectedin, and bortezomib.

MLN4924 and mitomycin C are synergistic in the A375 xenograft mouse model

It is often easier to achieve synergy in vitro than it is in vivo, especially because the combinations are often compromised by dose reductions due to increased toxicities and drug-drug interactions. Therefore, we wanted to test whether our most significantly synergistic example from the A375 cell line data was tolerated and synergistic in vivo. The combination of mitomycin C dosed at 2.5 mg/kg intravenously once weekly and MLN4924 dosed at 180 mg/kg subcutaneously twice weekly was well tolerated for 3 weeks. Although treatment with either single agent alone resulted in only moderate growth inhibition, the combination kept the tumor from growing for the entire time of treatment (Fig. 2B, with day 21 treated versus control tumor volume ratios of 0.7, 0.5, and 0.17 for mitomycin C, MLN4924, and the combination, respectively. Tumor regrowth was detectable 1 week after stopping treatment. Importantly, the combination of MLN4924 and mitomycin C in A375 xenograft was synergistic when assessed through day 21 ($P < 0.001$; ref. 23).

RNAi against synergistic combination of MLN4924 and mitomycin C demonstrates involvement of ATR and BRCA1 pathways

We sought to understand the role of DNA damage genes in the response of A375 cells to the combination of MLN4924 and mitomycin C. Specifically, we knocked down 320 genes using SMARTpool siRNAs targeting DNA damage responses and treated A375 cells with 330 nmol/L MLN4924 (LC36) or $1.6 \text{mol/L}$ mitomycin C (LC58) or the combination of both treatments. The observed viability for the combination with control siRNA (GL2) was 6.7%, 4-fold lower than that predicted by Bliss independence ($[1/0.36] 	imes [1/0.58] = 0.27$), giving a BI score of $-2.00 \pm 0.07$ (ref. 24). We looked for genes that when depleted would enhance or reduce the synergy, although genes enhancing synergy are expected to be more difficult to find because the viability is already low (Supplementary Table S1B). We then evaluated the four individual oligos against each gene that had demonstrated particularly large effects, to show the likelihood that these were on-target effects (Supplementary Table S1C, requiring three of four oligos to give the same phenotype to qualify as on-target), including an additional 28 genes.
from the MLN4924 screen (5). In the deconvoluted experiment, we adjusted the combination to 330 nmol/L MLN4924 (LC33) and 1.0 μmol/L mitomycin C (LC47).

The observed viability of the combination with control siRNA was 14%, giving a BI score of 1.26/C0 0.09. All reported genetic interactions were seen in at least two of four runs (Fig. 3A).

The only gene whose depletion substantially increased synergy was CDKN1A (encoding p21), suggesting that the p21-dependent intra-S-phase checkpoint helps to block some degree of synergy between MLN4924 and mitomycin C in A375. The 40 genes with the strongest reduction of synergy are classified according to their known biology, with key genes represented in the ATR, BRCA1/BRCA2, chromatin dynamics, and TC-NER pathways. A parallel experiment in HCT-116 gave similar results (Fig. 3B; Supplementary Table S1D), with about 50% of the genes reducing synergy in A375 also affecting HCT-116. Conversely, 82% of the genes reducing synergy in HCT-116 also affected A375. Greater transfection efficiency in A375 cells likely allowed for more genes to be discovered in that setting (Supplementary Table S1C and S1D).

In particular, DDB1 and EP400 depletion resulted in complete loss of synergy between MLN4924 and mitomycin C in A375. Ddb1 is a core component of the Cul4A and Cul4B E3 ubiquitin ligases, so loss of DDB1 should mimic the effect of MLN4924 on Cul4A/B activity and the addition of MLN4924 would not enhance the synergy already experienced by depletion of DDB1. Depletion of DDB1 or CUL4A and CUL4B in A375 resulted in an increase in p21 levels (Fig. 3C), suggesting that this effect may account for the loss of synergy. EP400 encodes p400, which is important for remodeling of the chromatin to allow for the recruitment of DNA damage repair proteins, including BRCA1 (25) and Rad51 (26). Finally, CDC7, MCM5, and PCNA depletion significantly reduced the synergy, suggesting that replication licensing is important for this synergy.

Figure 3. Effect on synergy between MLN4924 and mitomycin C by depletion of genes involved in DNA damage response. A, of note, 348 genes with known roles in DNA damage response and repair were knocked down and evaluated for their impact on MLN4924–mitomycin C synergy in A375. Only knockdown of CDKN1A (encoding p21) significantly increased synergy. Knockdown of 40 genes gave the most significant reduction of synergy. These genes have been classified according to their known functions. Data, mean value for the third greatest effect size of four oligos (n = 3; error bars, SEM) from a representative run, except GL2, in which only one oligo was evaluated and n = 48. Annotation of genes in which knockdown had synthetic lethal (L), epistatic (E), or suppressor (S) interactions with either MLN4924 (4) or mitomycin C (M) is given parenthetically. Data supporting Fig. 3A are included in Supplementary Table S1B and S1C. B, the same experiment as Fig. 3A, except repeated in HCT-116. Knockdown of 22 genes gave the most significant reduction of synergy. Data supporting Fig. 3B are included in Supplementary Table S1D. C, depletion of CUL4A and CUL4B or depletion of DDB1 results in increased p21 levels, both with vehicle and 330 nmol/L MLN4924 treatment.
Protein changes induced by the combination of MLN4924 and mitomycin C

Posttranslational changes to the proteins involved in the synergy of MLN4924 and mitomycin C could provide clues about the mechanism by which the combination promotes cell death. In addition, posttranslational changes specific to the combination might provide pharmacodynamic biomarkers for the combination. As such, we have evaluated 33 of the hits and associated proteins from Fig. 3A for protein changes following treatment with MLN4924, mitomycin C, or the combination. Those proteins that changed more than 2-fold are shown in Fig. 4A, those without such changes in Supplementary Fig. S5, although Rad51 (pT309) does not change. All of the proteins that were affected by mitomycin C as a single agent were also affected by MLN4924 as a single agent with generally greater effects by MLN4924 alone. Only changes in the slower migrating band of Ube2L3, the fastest migrating band of Rad17, and p-Ub-H2Ax were up more than 2-fold with the combination treatment relative to either of the single agents.

Chk1 has been shown to promote homologous recombination by phosphorylation of Rad51 (27, 28), although we do not see any changes in the phosphorylation status of Rad51 (Fig. 4A). Conversely, BRCA1 has a role in promoting ATR activation (29). Thus, it is possible that the ATR-associated genes and the BRCA1-associated genes work within a single pathway, although which effect is primary is not clear. To understand whether these two gene sets were regulating the same signal transduction pathway, we depleted ATR-associated genes (ATR, CHEK1, RFC4, and RPA2),
BRCA1-associated genes (BARD1, BRCA1, and FANCD1), and CLSPN, which is associated with both ATR and BRCA1, and treated A375 cells with vehicle or the combination of MLN4924 and mitomycin C. Chk1 phosphorylation by the combination treatment is reduced by RNAi targeting ATR, CHEK1, CLSPN, and RPA2 (Fig. 4B), as expected, but also by RNAi targeting BARD1 and BRCA1. Phosphorylation of threonine-21 on Rpa32 (encoded by RPA2) is reduced by a similar set of genes; however, ATR depletion does not reduce Rpa32 phosphorylation and, thus, ATR is not its relevant kinase.

BRCA1 is both stabilized and hyperphosphorylated in response to MLN4924 and mitomycin C (Fig. 4A), resulting in slower gel migration. Although the stabilization of BRCA1 was reduced by RNAi targeting ATR, CHEK1, CLSPN, or RPA2, its hyperphosphorylation was unaffected. These results demonstrate that BRCA1 is required for the activation of the ATR-Chk1 pathway, but that ATR and Chk1 are not responsible for the phosphorylation of BRCA1. Importantly, these results suggest that MLN4924 and mitomycin C interact at least in part through the regulation of the BRCA1–ATR-Chk1 pathway.

Combination of MLN4924 and mitomycin C results in increased DNA strand breaks

Comet assay analysis of the combination of MLN4924 and mitomycin C demonstrated increased DNA strand breaks at 6 hours compared with the single agents (Fig. 5A and Supplementary Fig. S6). Within 6 hours, it is unlikely that all cells are actively replicating their DNA. Figure 5B demonstrates that the single-agent treatments do not significantly increase comet tails over vehicle, whereas there was a significant increase in comet tails with the combination \( (P = 1.7 \times 10^{-3}) \) of 0.3 \( \mu \text{mol/L} \) MLN4924 + 1.0 \( \mu \text{mol/L} \) mitomycin C; \( P = 7.2 \times 10^{-10} \) for 1.0 \( \mu \text{mol/L} \) MLN4924 + 1.0 \( \mu \text{mol/L} \) mitomycin C. This increase compares favorably with 0.5 \( \mu \text{mol/L} \) etoposide \( (P = 3.1 \times 10^{-13}) \). In addition, phosphorylation of H2Ax Ser139 is unchanged with the single agents within 6 hours, in agreement with prior observations (5), but is increased 3- and 10-fold with the combination of 0.3 or 1 \( \mu \text{mol/L} \) MLN4924 and 1 \( \mu \text{mol/L} \) mitomycin C, respectively (Fig. 5C and Supplementary Fig. S6). The increased DNA strand breaks likely lead to the synergy between these two agents.

Discussion

In this study, we explored how MLN4924 might combine with cancer chemotherapeutic standards of care, many of which are DNA-damaging agents. Furthermore, DNA damage is an important contributor to the mechanism of MLN4924, because MLN4924 results in the stabilization of Cdt1 and the induction of rereplication (2–5). Thus, we focused on how other DNA-damaging agents might interact with MLN4924.

The four cell lines that we have used are generally sensitive to a broad array of DNA-damaging agents (Supplementary Fig. S2). This provides us with a platform to explore the interaction of MLN4924 with DNA-damaging agents across multiple genetic backgrounds. Consistent with MLN4924 inducing DNA damage on its own, MLN4924 frequently demonstrated some level of interaction with other DNA-damaging agents in at least one cell line (Fig. 2), and interestingly, this interaction could be either synergy or antagonism.

Figure 5. The combination of MLN4924 and mitomycin C leads to increased DNA strand breaks. A, alkaline comet assays were performed with the treatment of A375 with 1.0 \( \mu \text{mol/L} \) mitomycin C, 0.3 or 1.0 \( \mu \text{mol/L} \) MLN4924, or the combination for 6 hours. Etoposide (0.5 or 5.0 \( \mu \text{mol/L} \)) is included as a DNA-damaging control. B, the ratio of the comet tail to the head from Fig. 5A was calculated for each cell (minimum 189 cells) and box-and-whisker plots made. Box plots represent 25th, 50th, and 75th percentile, whereas whiskers represent 5th and 95th percentile. The \( t \) test against DMSO: *\( P < 0.01; ** \( P < 0.001 \). Dashed line, the median tail/head ratio for DMSO. C, Western blots demonstrate phosphorylation of H2Ax on Serine-139 within 6 hours of treatment by the combination of MLN4924 and mitomycin C. The arrow indicates a band consistent with monoubiquitination of the protein.
MLN4924 DNA-Damaging Agent Combinations

Figure 6. Schematic for proposed mechanism of synergy between mitomycin C and MLN4924. The following model explains the data in Figs. 3 and 5. Mitomycin C (MMC) induces DNA damage at least in part via interstrand DNA crosslinks. MLN4924 should inhibit nucleotide excision repair processes during G1 (not shown) and thereby promote detection of mitomycin C crosslinks in S-phase. Collision of the replication fork with the mitomycin C crosslink will result in stalling of the fork and recruitment of FANCM. FANCM promotes the formation of a “chicken-foot” form of the DNA, which is then stabilized by binding the BRCA1–BRCA2–Rad51 complex, which also inhibits Mre11 nuclease activity. MLN4924 induces additional origin of replication firing through its stabilization of Cdt1, leading to rereplication and increasing the likelihood that a second replication fork will collide with the stalled replication fork. The DNA fragments resulting from this head-to-tail replication fork collision then results in cell death. Blue and yellow ovals, hits from Fig. 3A. Triangles, phosphorylation sites. Yellow, changes seen within Fig. 4A. Small green circles, RPA (replication protein A); whereas small red circles, Rad51.

Pacitaxel blocks mitotic progression, whereas MLN4924 requires S-phase to induce DNA damage. Therefore, simultaneous addition of these agents may affect their activity through these cell-cycle effects. Our results in the four cell lines were consistent with this (Supplementary Fig. S4). Bortezomib may block the ability of MLN4924 to induce rereplication by its stabilization of geminin, an APC/C substrate and inhibitor of Cdt1 (30). This may explain the antagonism observed between MLN4924 and bortezomib (Supplementary Fig. S4). Agents that result in the accumulation of cells in S-phase may demonstrate synergy with MLN4924. Of note, mitomycin C was found to be the agent with the most consistent synergy with MLN4924 in this study.

Previous work on mitomycin C suggests that adducts are repaired by different mechanisms depending upon how they are detected (13, 14). MLN4924 could substantially shift their mechanism of detection, because MLN4924 causes an accumulation of cells actively replicating their DNA (3, 4). In other words, replication fork collision with the mitomycin C–induced interstrand crosslink would become the major mechanism of damage detection in the presence of MLN4924 (Fig. 6). Comet assay results support this hypothesis as the combination is leading to a substantial increase in DNA strand breaks within 6 hours. Because mitomycin C as a cross-linking agent inhibits migration of DNA into the comet tail (31), these results support an increase in MLN4924–driven DNA strand breaks.

Although depletion of DNA damage repair mechanisms would intuitively be expected to increase sensitivity to DNA damage combinations, our results (Fig. 3) suggest that depletion of ATR, BRCA1/2, and TC-NER pathways actually reduced the synergy between MLN4924 and mitomycin C. The ability of gene knockdown to reduce synergy may be due to an equivalence of function between gene depletion and drug effect. For example, both DDB1 depletion and MLN4924 treatment result in p21 stabilization, increasing engagement of the intra–S-phase checkpoint (Fig. 3C). Likewise, both ERCC1 depletion and MLN4924 treatment might be expected to inhibit TC-NER (32). Adding MLN4924 to ERCC1-depleted cells might result in reduced synergy with mitomycin C because TC-NER would already be inhibited. However, loss of synergy is not necessarily equivalent to less cell death overall, as the viability seen with the treatment of ERCC1-depleted cells by mitomycin C may already be as low as that seen with control cells treated with the combination of mitomycin C and MLN4924. Finally, cells lacking a particular DNA repair pathway may ultimately select a pathway that results in a different terminal outcome.

In contrast with TC-NER, the role of Nedd8 in ATR and BRCA1 regulation is not well characterized. Understanding the effect of gene depletion on the viability effect of the individual agents can help us better understand the effect of gene depletion on the combination. Knockdown of the ATR pathway does not seem to have a significant impact on cell death induced by MLN4924 alone, whereas knockdown of the BRCA1 pathway did (Supplementary Table S1C; ref. 5). Recent work has demonstrated a role for FANC/D2, BRCA1, BRCA2, and Rad51 in protecting
stalled replication forks from degradation by the nuclease activity of Mre11 (33–36). Although this BRCA complex is preparing the DNA for repair, the stabilization of stalled forks would increase the likelihood of fork collision under the reereplication stress induced by Cdt1 stabilization (37), here promoted by MLN4924. Importantly, siRNA oligos targeting RADS0, a subunit of the Mre11–Rad50–Nbs1 (MRN) complex, were synthetic lethal with MLN4924, whereas siRNA oligos targeting FANCM and BARD1 were suppressor (5), consistent with the BRCA1/BRCA2 complex protecting stalled forks from MRN nuclease activity and increasing the frequency of strand breaks from replication fork collision. Mre11 destabilizes the stalled replication forks, allowing for an alternative mechanism to resolve stalled forks and decreasing the likelihood of replication fork collision.

On the other hand, the BRCA1 pathway did not seem to have a significant role in sensitivity to mitomycin C alone in this setting (Supplementary Table SIC), but the ATR pathway did. Importantly, depletion of ERCC6 (CSB) was synthetic lethal with mitomycin C in A375, consistent with single-agent mitomycin C–induced damage being repaired by TC-NER in G1 (14). CSB is degraded by Cul4A/B (32) whose function is inhibited by MLN4924 (4), thereby promoting the accumulation of mitomycin C cross-linked DNA at stalled replication forks in S-phase.

Previous studies (29) have characterized a role for BRCA1 as a decision point following replication fork–blocking UV-induced DNA damage, promoting ATR activation, ERCC1/XPF recruitment, and inhibiting translesion DNA polymerases. What other proteins might be associated with BRCA1 in this decision point role is not currently known. Our data suggest that this role of BRCA1 is important for MLN4924–mitomycin C synergy. Intriguingly, we do see PCNA monoubiquitination following MLN4924 treatment (Fig. 4A), suggesting that translesion bypass (TLS) may be activated. The loss of BRCA1 may promote TLS as a DNA damage tolerance mechanism. Although RNF111-dependent neddylation has been suggested to be involved in BRCA1 regulation (38), knockdown of RNF111, RNF8, or RNF168 did not affect the synergy between MLN4924 and mitomycin C (Supplementary Table SIC and S1D).

MLN4924 has been studied for interaction with interstrand cross-linking agents (39). Kee and colleagues demonstrated that MLN4924 blocked UV-induced FANC D2 monoubiquitination and Chk1 phosphorylation in HCT-116, HeLa, MCF7, U-2 OS, and the Fanconi anemia-deficient ovarian cancer cell line 2008, as well as following cisplatin treatment in HeLa, in which MLN4924 seemed to stabilize FANCM. They observed that addition of MLN4924 increased the cytotoxicity of mitomycin C in HCT-116 and of cisplatin in the Fanconi anemia-deficient ovarian cancer cell line 2008 and its FANC D2 complemented counterpart 2008 + F. They suggest that the effects of MLN4924 on FANC D2 and Chk1 account for the observed sensitization. However, Jazaeri and colleagues did not observe similar effects in SKOV3 or ES2 cells, even though cisplatin was synergistic with MLN4924 in their setting (40).

Similarly, we do not see the described effects on FANC D2 or Chk1 in A375 (Fig. 4A and Supplementary Fig. S5), even though mitomycin C is clearly synergistic with MLN4924 in this background. In addition, although all of the known Fanconi anemia genes were included in our experiments, only knockdown of FANC M, BRCA2 (FANCD1), and PALB2 (FANC N) affected MLN4924–mitomycin C synergy. Finally, efficient knockdown of FANCI did not block Chk1 phosphorylation (Fig. 4B). Therefore, the mechanism of synergy between MLN4924 and mitomycin C seems to be independent of FANC D2/ FANC I monoubiquitination. Changes to FANC D2, FANC M, and Chk1 do not seem to be associated with synergy between MLN4924 and cross-linking agents across cell lines.

In conclusion, we have studied the interaction of MLN4924 with a broad array of DNA-damaging agents. We were able to develop an RNAi-based approach to study the mechanism of synergy between MLN4924 and mitomycin C. These results implicated the role of BRCA1 promoting the ATR–Chk1 pathway and of the BRCA2–Rad51 complex stabilizing replication forks as driving the synergistic interaction. We expect that an improved mechanistic understanding from studies such as detailed here may allow for a more informed selection of combinations for clinical development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Allison Berger, Doug Bowman, Ben Amidon, Margaret Quinlan, and John Newcomb for assistance with the article.

Grant Support
This work was financially supported by Takeda Pharmaceuticals International Co.

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Received August 9, 2013; revised March 13, 2014; accepted March 17, 2014; published OnlineFirst March 26, 2014.
Molecular Cancer Therapeutics

Nedd8-Activating Enzyme Inhibitor MLN4924 Provides Synergy with Mitomycin C through Interactions with ATR, BRCA1/BRCA2, and Chromatin Dynamics Pathways


Mol Cancer Ther 2014;13:1625-1635. Published OnlineFirst March 26, 2014.

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