The NEDD8-Activating Enzyme Inhibitor MLN4924 Disrupts Nucleotide Metabolism and Augments the Efficacy of Cytarabine

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Abstract

Purpose: New therapies are urgently needed for patients with acute myelogenous leukemia (AML). The novel NEDDylation inhibitor MLN4924 (pevonedistat) has demonstrated significant preclinical antileukemic activity and preliminary efficacy in patients with AML in a phase I trial. On the basis of its antimyeloid and DNA-damaging properties, we investigated the ability of MLN4924 to augment conventional cytarabine (ara-C) therapy.

Experimental Design: The effects of MLN4924/ara-C on viability, clonogenic survival, apoptosis, DNA damage, and relevant pharmacodynamic targets were determined. The efficacy and pharmacodynamics of MLN4924/ara-C were assessed in an AML xenograft model.

Results: Cotreatment of AML cell lines and primary patient specimens with MLN4924 and ara-C led to diminished clonogenic survival, increased apoptosis, and synergistic levels of DNA damage. RNAi demonstrated that stabilization of CDT-1, an event previously shown to mediate the DNA-damaging effects of MLN4924, was not a key regulator of sensitivity to the MLN4924/ara-C combination. Global metabolic profiling revealed that MLN4924 disrupts nucleotide metabolism and depletes intracellular nucleotide pools in AML cells. Subsequent experiments showed that MLN4924 promoted increased incorporation of ara-C into the DNA of AML cells. This effect as well as the therapeutic benefit of the MLN4924/ara-C combination was antagonized by supplementation with the nucleotide building block ribose. Coadministration of MLN4924 and ara-C to mice bearing FLT3-ITD+ AML xenografts stably inhibited disease progression and increased DNA damage in vivo.

Conclusions: Our findings provide strong rationale for clinical investigation of the MLN4924/ara-C combination and establish a new link between therapeutic inhibition of NEDDylation and alterations in nucleotide metabolism.

Introduction

Acute myelogenous leukemia (AML) is a multistep, multipathway malignancy that is ultimately fatal in the majority of affected patients (1). Older adults with AML have an extremely poor prognosis and derive limited benefit from intensive chemotherapy due to their prevalence of unfavorable cytogenetics predicting chemotherapy refractoriness, preexisting myelodysplastic syndromes (MDS) associated with a limited normal functioning stem cell compartment and serial genetic lesions evolving to disease manifestation, and expression of a multidrug-resistant phenotype (2–4). No standard induction approach exists for older adults, in part, due to their poor representation in clinical studies. The last prospective trial comparing intensive chemotherapy to low-dose approaches was conducted over two decades ago (5). Although induction with low-dose cytarabine (LDAC) offers a survival advantage over supportive care for patients with good/intermediate prognosis cytogenetics, patients with poorer performance status and/or unfavorable cytogenetics have a low likelihood of achieving complete responses (CR) when treated with LDAC (1). Considering these issues, new therapeutic approaches that are effective and tolerated could be transformative particularly for older patients with AML.

Protein turnover plays a critical regulatory role in many essential cellular processes, including cell-cycle progression, signal transduction, and cell death. Aberrant ubiquitin-proteasome system (UPS)-mediated protein degradation contributes to disease progression, metastasis, and drug resistance and is thus, an appealing anticancer strategy (6). The proteasome inhibitor, bortezomib (Velcade), was the first anticancer agent of this drug class to receive FDA approval (7). Bortezomib established proof of concept that targeting protein turnover is an effective anticancer strategy and is currently indicated for the treatment of multiple myeloma and mantle cell lymphoma and in combination with cytotoxic therapy, yielded high response rates in patients with AML (8, 9). Following its success, a second proteasome inhibitor (carfilzomib) also earned FDA approval for the treatment of patients with multiple myeloma who...
rupting the NEDDylation pathway has significant and logical implications for cancer progression and/or promote therapeutic resistance, disrupting the NEDDylation pathway has significant and logical therapeutic potential. MLN4924 (pevonedistat) is a first-in-class, selective small-molecule inhibitor of NEDD8-activating enzyme (NAE), the proximal regulator of the NEDDylation cascade (12–14). We previously showed that MLN4924 overcomes multiple mechanisms of prosurvival signaling, triggers DNA damage associated with cdk1 stabilization and CHK1 activation, and induces stable disease regression in an AML mouse model (15). This was confirmed in a phase I clinical trial in refractory MDS/AML patients, some of whom achieved complete responses following treatment with MLN4924 (16). The mechanistic basis for the anti-AML effects of MLN4924 remains to be fully elucidated. We conducted comprehensive metabolic profiling to further investigate the pharmacodynamic effects of MLN4924 in AML cells that contribute to its therapeutic activity and ability to augment the efficacy of ara-C.

Cell viability assay

Cells were plated in triplicate and treated with defined concentrations of MLN4924, ara-C, or both drugs for 72 hours. Viable cells were quantified using ATPLite according to the manufacturer’s instructions (PerkinElmer) as previously described (14, 15, 17).

Synergy analyses

The combination indices (CI) for MLN4924 and ara-C 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.) software was utilized to calculate CI values as previously described (18).

Analysis of drug-induced apoptosis

Drug-induced apoptosis was quantified by PI/FACS analysis of sub-G0/G1 DNA content as previously described (17, 19).

 Colony assays

AML cells were treated with the indicated concentrations of MLN4924, ara-C, or both drugs for 24 hours. Drugs were washed away and cells were seeded in Methocult methylcellulose-containing medium. Colonies were stained and scored as previously described (15, 20).

Immunoblotting

AML cells were incubated with MLN4924, ara-C, 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. Proteins were separated by SDS-PAGE and transferred...
to nitrocellulose membranes. Blots were probed with the indicated antibodies and bands were detected by enhanced chemiluminescence (Alpha Innotech) as previously described (21). β-tubulin documented equal loading.

Alkaline comet assay
AML cells were treated with MLN4924, ara-C, or both drugs for 24 hours. Comet assays were performed using the CometAssay kit (Trevigen) according to the manufacturer’s instructions as previously described (17). 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.

shRNA knockdown of CDT1
MOLM-13 human AML cells were infected with lentiviral particles containing nontargeted (control) or targeted shRNA directed at CDT1 (Santa Cruz Biotechnology) 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 ara-C for 48 hours. Drug-induced apoptosis was quantified by PI/FACS. Knockdown efficiency was assessed by immunoblotting.

Quantification of [3H]ara-C DNA incorporation
[3H]ara-C was purchased from Moravek Biochemicals. The incorporation of [3H]ara-C into AML cells was evaluated using the method developed by Kufe and colleagues (22). Briefly, cells were incubated with non-radiolabeled MLN4924, ribose, or the combination of MLN4924 and ribose in the presence and absence [3H]ara-C for 24 hours. Cells were washed twice in PBS and total DNA was isolated from cells in all experimental groups using the DNeasy kit according to the manufacturer’s instructions (Qiagen). DNA concentrations of each sample were quantified by spectrophotometry and normalized. An equivalent amount of DNA from each sample was diluted in 1 mL of scintillation fluid and [3H] counts were determined using a standard liquid scintillation method (Beckman-Coulter).

In vivo evaluation of MLN4924 and ara-C
MOLM-13 FLT3-ITD+ human leukemia cells were injected into the flanks of nude mice as previously described (23). After tumor growth reached 150 mm³, mice were randomly assigned (n = 10 per group for all regimens) to receive vehicle control, 60 mg/kg MLN4924 BID, 50 mg/kg ara-C QD, or the combination of both drugs for 21 days. Tumor growth and animal toxicity was assessed as previously described (24).

IHC
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 and probing with specific antibodies was conducted as previously described (17). Positive reactions were visualized using 3,3'-diaminobenzidine (Dako). Images were captured using an Olympus fluorescent microscope with a DP71 camera and a 20× objective. Image-Pro Plus software Version 6.2.1 (MediaCybernetics) was used for image acquisition. ImageJ software was used for quantification of γH2AX and p27 levels by densitometric analysis of five random fields containing viable tumor cells. Quantification of PCNA and cleaved caspase-3 was conducted by PI/FACS. n = 3 ± SD; *P < 0.05 indicates a significant difference from either single-agent treatment.
by counting the number of positive cells in five random fields as previously described (25).

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 $P < 0.05$ with two-sided comparisons.

Results
MLN4924 and ara-C cooperate to antagonize clonogenic survival and trigger apoptosis
To investigate inhibiting NEDDylation with MLN4924 as a novel approach to increase the anti-AML activity of ara-C, we first treated four human AML cell lines (MV4-11, MOLM-13, HL-60, and KG-1) with MLN4924, ara-C, or both drugs for 72 hours and quantified the resulting impact of drug exposure on cell viability by ATPlite assay. Pilot drug sequencing studies showed that pretreatment with one drug first for 24 hours followed by the other agent for the remainder of the assay period did not yield significantly different antileukemic effects as compared with simultaneous drug treatment (not shown). We therefore used simultaneous treatment conditions for all in vitro combination assays in this study. Combined treatment with MLN4924 and ara-C reduced cell viability significantly more effectively than either single agent (Fig. 1A). Formal synergy analyses demonstrated a CI range of 0.5 to 0.8 across all concentrations tested. CI values less than 1.0 indicate synergy. Notably, FLT3-ITD status did not appear to affect cellular sensitivity to this combination as cells with (MV4-11 and MOLM-13) and without (HL-60, KG-1) FLT3-ITD expression benefited similarly from treatment with MLN4924 and ara-C. Treatment of primary AML blasts from patients ($n = 10$) with MLN4924 and ara-C resulted in a significantly greater reduction in viability than treatment with single agents.

Figure 2. Induction of DNA damage by MLN4924 and ara-C. A, MLN4924 and ara-C activate the DNA damage response. Cells were treated with 100 nmol/L MLN4924, 100 nmol/L ara-C, or both drugs for 24 hours, lysed, subjected to SDS-PAGE, and probed with p27, pCHK1 (Ser317), total CHK1, γH2AX, and cleaved (active) caspase-3-specific antibodies. Tubulin documented equal loading. B and C, induction of DNA damage by MLN4924 and ara-C. Cells were treated with 100 nmol/L MLN4924, 100 nmol/L ara-C, or both drugs for 16 hours. The DNA tail moment for each experimental condition was quantified by alkaline comet assay. Representative images are shown. $n = 3$, $±$ SD. *, $P < 0.05$ indicates a significant difference from either single-agent treatment.
Moreover, primary cells from chemo-naive newly diagnosed (n = 4) and previously treated patients (n = 6) with AML did not differ in their sensitivity to the MLN4924/ara-c combination (Fig. 1B). Patient characteristics are detailed in Supplementary Table S1. Consistent with the results of our cell viability analyses, cotreatment with MLN4924 and ara-C resulted in significantly greater diminished clonogenic survival (Fig. 1C) and levels of drug-induced apoptosis (Fig. 1D) than exposure to either drug alone.

The combination of MLN4924 and ara-C promotes increased levels of DNA damage

The induction of DNA damage is one of the hallmark features of the anticancer mechanism of action of ara-C and other nucleoside analogs. We and others have also demonstrated that MLN4924 triggers DNA damage by promoting CDT1-dependent DNA re-replication (15, 17, 26). We hypothesized that cotreatment with MLN4924 and ara-C would yield significantly greater levels of DNA damage. Accordingly, MLN4924 and ara-C cooperated to stimulate the increased levels of the phosphorylated (active) form of the DNA damage sensor CHK1 (Ser317), and the DNA damage marker γH2AX (Fig. 2A). These effects coincided with an increase in the expression of the cyclin-dependent kinase inhibitor p27 and the active form of caspase-3, which would be expected based upon the combined effects of these agents on cell viability and apoptosis shown in Fig. 1. We next conducted alkaline comet assays to generate another readout of drug-induced DNA damage. These experiments demonstrated that the MLN4924/ara-C combination significantly increased the DNA tail moment, indicating heightened levels of DNA damage (Fig. 2B and C).

CDT1 stabilization is not essential for the proapoptotic effects of the MLN4924/ara-C combination

As mentioned earlier, CDT1 stabilization has been previously suggested to be an important pharmacodynamic effect that facilitates DNA damage and contributes to the anticancer activity of MLN4924. To investigate whether this specific effect may be responsible, at least in part, for the benefit we observed with the MLN4924/ara-C combination, we utilized shRNA to target its expression in MOLM-13 cells. Knockdown efficiency was assessed by immunoblotting (Fig. 3A). A comparison of the proapoptotic effects of the MLN4924/ara-C combination in the presence and absence of CDT1 shRNA showed that CDT1 expression levels had a modest impact on single-agent MLN4924 treatment, but had a negligible effect on the combination of both drugs (Fig. 3B). These results suggest that other aspects of the mechanism of action of MLN4924 may be mediating its ability to augment ara-C activity.

MLN4924 disrupts nucleotide metabolism and diminishes endogenous nucleotide pools

We next conducted global metabolic profiling to obtain a more comprehensive assessment of the pharmacodynamic effects of MLN4924. MV4-11 cells were treated with 100 nmol/L MLN4924 for 12 and 24 hours. A total of 268 distinct biochemicals were specifically identified and quantified in experimental samples. Heatmap analysis revealed that exposure to MLN4924 caused time-dependent alterations in multiple metabolic superpathways (Fig. 4A). Quantification of individual biochemicals revealed that the levels of 112 biochemicals (19 increased and 93 decreased) were significantly altered (P < 0.05) following 12-hour treatment with MLN4924 compared with controls. Additional exposure to MLN4924 (24 hours) resulted in 199 significant alterations (53 increased and 146 decreased, P < 0.05). The overall trend at both time-points indicated impairment rather than activation of the majority of the affected superpathways.

Additional statistical analyses revealed that the nucleotide biosynthesis superpathway was one of the most altered overall pathways following treatment with MLN4924. Heatmap analysis (Fig. 4B) and quantification of the effects of drug treatment on the levels of individual nucleotide pathway components (Supplementary Fig. S1) demonstrated the time-dependent nature of the pathway alterations. Detailed schematics illustrating the key elements of pyrimidine and purine metabolism are shown in Supplementary Figs. S2 and S3. The combined effect of drug-induced decreases in the levels of multiple key elements of nucleotide biosynthesis indicated severe pathway impairment. In particular, the dramatic diminishment of intracellular nucleotide pools following exposure to MLN4924 suggested that cells treated with MLN4924 may increase the cytotoxicity of conventional chemotherapy with nucleoside analogs such as ara-C by reducing endogenous competition for incorporation into DNA. The findings indicate that the effects of MLN4924 on nucleotide metabolism could contribute significantly to its ability to augment the efficacy of ara-C treatment.
Figure 4.
MLN4924 disrupts nucleotide metabolism in AML cells. A, cells were treated with 100 nmol/L MLN4924 for 12 and 24 hours. Cells were processed for global metabolic analyses as described in the Materials and Methods. Heatmap depicts superpathway analysis for both timepoints. Changes for each timepoint are quantified relative to control, $n = 4$ for each experimental condition. B, cells were treated with 100 nmol/L MLN4924 for 12 and 24 hours. Cells were processed for global metabolic analyses as described in the Materials and Methods. Heatmap specifically depicts nucleotide metabolism pathway analysis for both timepoints. Changes for each timepoint are quantified relative to control; $n = 4$ for each experimental condition.
MLN4924 promotes increased incorporation of ara-C into the DNA of AML cells. A, quantification of [3H]ara-C DNA incorporation. MOLM-13 and primary AML cells were treated with MLN4924, ribose, [3H]ara-C, and the indicated combinations for 24 hours. The inclusion of [3H]ara-C under each experimental condition was determined as described in the Materials and Methods. n = 3 ± SD. *P < 0.05 for the comparison between [3H]ara-C and [3H]ara-C + ribose; **P < 0.05 for the comparison between [3H]ara-C + MLN4924 and [3H]ara-C + MLN4924 + ribose. B, quantification of drug-induced apoptosis. MOLM-13 and primary AML cells were treated with 100 nmol/L MLN4924, 100 nmol/L ara-C, 10 mmol/L ribose, or the indicated combinations for 48 hours. Percentages of cells with subdiploid DNA were determined by PI/FACS. n = 3 ± SD; *P < 0.05 for the comparison between ara-C and ara-C + ribose; **P < 0.05 for the comparison between ara-C + MLN4924 and ara-C + MLN4924 + ribose.

Figure 5.

MLN4924 facilitates increased incorporation of ara-C into the DNA of AML cells

We hypothesized that the therapeutic benefit derived from combining MLN4924 with ara-C could be, in part, due to increased DNA incorporation of ara-C facilitated by reduced competition from endogenous nucleotides/nucleosides. To test this possibility, we quantified the inclusion of radiolabeled [3H]ara-C into the DNA of MOLM-13 and primary AML cells in the presence and absence of MLN4924. Notably, the quantity of [3H]ara-C was significantly higher in cells cotreated with MLN4924 in both established and primary AML cells (Fig. 5A). We next sought to determine whether this effect could be overcome by exogenous supplementation with the nucleoside building block ribose to antagonize the nucleotide depletion caused by MLN4924 treatment. As shown in Fig. 5A, the addition of ribose significantly blunted the enhancement of [3H]ara-C incorporation that we observed in the presence of MLN4924. Accordingly, quantification of drug-induced apoptosis triggered by the MLN4924/ara-C combination in the presence and absence of ribose supplementation showed that in addition to diminishing the integration of [3H]ara-C into DNA, exogenous ribose also tempered MLN4924/ara-C–induced cell death (Fig. 5B). Our collective results demonstrate that the combination benefit of MLN4924 and ara-C is at least partially due to elevated incorporation of ara-C into the DNA of AML cells.

The combination of MLN4924 and ara-C effectively inhibits the progression of FLT3-ITD+ AML xenografts in vivo

The in vivo anticancer activity of the MLN4924/ara-C combination was evaluated by administering vehicle control, MLN4924 (60 mg/kg BID), ara-C (50 mg/kg QD), or both drugs to mice implanted with MOLM-13 FLT3-ITD+ xenografts. Both MLN4924 and ara-C monotherapies significantly inhibited disease progression (Fig. 6A). Coadministration of MLN4924 and ara-C provided a significantly greater therapeutic benefit than either drug alone and resulted in stable inhibition of disease progression throughout our study. The combination was well tolerated and no notable toxicities were observed other than a very modest reduction in mean body weight. IHC analysis of specimens collected from animals in all experimental groups demonstrated superior in vivo...
growth inhibition (elevated p27 and reduced PCNA levels), augmented DNA damage (γH2AX), and increased apoptosis (cleaved caspase-3) following treatment with the MLN4924/ara-C combination compared with either single agent (Fig. 6B and Supplementary Fig. S4).

Discussion

Cytotoxic-based therapy remains the standard for the treatment of AML, and has changed little in the past four decades. New agents/strategies for AML therapy are clearly needed in general, of AML, and has changed little in the past four decades. New long-term survival (<20%) with existing therapies (4, 27, 28). Inhibition of the NEDDylation pathway with the investigational drug MLN4924 is a novel therapeutic approach that has demonstrated encouraging anti-AML activity in the preclinical and clinical setting (15, 16).

However, our understanding of the pharmacodynamic effects of MLN4924 that are responsible for its therapeutic activity in AML remains incomplete. We hypothesized that MLN4924 may augment the efficacy of ara-C based on its DNA-damaging properties that we and others have reported. Our initial data supported this hypothesis. Yet, targeted knockdown of CDT1, a chromatin licensing factor and NEDD8 substrate that has been suggested to be a key mediator of MLN4924’s DNA-damaging properties and overall anticancer activity, did not indicate that it was an important regulator of sensitivity to the MLN4924/ara-C combination. We subsequently conducted global metabolic profiling of AML cells treated with MLN4924 to identify additional pharmacodynamic effects that may promote sensitization to ara-C. Our results showed that inhibition of NEDD8-mediated protein turnover has effects on multiple metabolic superpathways, but was particularly effective at antagonizing both purine and pyrimidine nucleotide metabolism. Although several preclinical studies have reported on the pharmacodynamic characteristics of MLN4924 (29), our investigation is the first to report the specific drug-related effects on nucleotide metabolism. In addition to establishing a new link between NEDD8-mediated protein turnover and nucleotide homeostasis, our findings demonstrate the value of global metabolic profiling in the pharmacodynamic analysis of investigational anticancer agents.

The MLN4924-induced disruption of nucleotide homeostasis that we observed provided a potential mechanistic explanation underlying its ability to improve the activity of the nucleoside analog ara-C. We hypothesized that the depletion of endogenous nucleotide pools by MLN4924 would yield more efficient incorporation of ara-C into the DNA of AML cells. A series of experiments demonstrated the benefit of the MLN4924/ara-C combination, which was due, at least in part, to MLN4924-mediated heightened incorporation of ara-C into DNA. This newly discovered DNA-related pharmacodynamic effect of MLN4924 builds upon several earlier investigations, which reported that the DNA-damaging properties of MLN4924 are linked to the stabilization of the chromatin licensing factor CDT1 and subsequent DNA replication stress (14, 26, 30–32).

Another interesting aspect of our study is related to the similar sensitivity of the primary AML cells from both newly diagnosed and previously treated patients with AML to the MLN4924/ara-C combination. Although the number of specimens we analyzed is relatively small (n = 4 newly diagnosed, n = 6 previously treated), the benefit we observed in cells that were clinically refractory to the ara-C based “7+3” regimen could have important translational implications. It is possible that NEDDylation may play a previously unstudied role in ara-C resistance and that inhibiting the pathway with MLN4924 may be sufficient to counter resistance to ara-C and other anticancer agents. Further investigation is required to fully evaluate this possibility, but the complete responses of refractory AML patients that were observed in the phase I clinical trial of MLN4924 are encouraging (16). Moreover, the ability of MLN4924 to antagonize drug resistance does not appear to be restricted to AML and/or the conventional agents that are used to treat this disease. We recently showed that MLN4924 resensitizes cisplatin-resistant ovarian cancer cells to platinum chemotherapy (17). Our findings about the ability of MLN4924 to overcome platinum resistance were confirmed shortly after our initial report by an independent research team (33). These observations suggest that the mechanisms of resistance to MLN4924 do not directly overlap with those of cytotoxic chemotherapeutic agents and that NEDD8-mediated protein turnover may play a more significant role in controlling chemosensitivity than previously known.

At a minimum, these observations provide rationales to further explore potential applications for MLN4924 in the treatment of malignancies that are resistant to cytotoxic chemotherapy and to more rigorously study the mechanisms by which aberrant NEDDylation promotes malignant progression and drug resistance. The identification of a predictive biomarker of sensitivity to MLN4924 would also be a significant catalyst to its successful clinical development. Despite extensive ongoing preclinical and clinical research efforts with MLN4924, no specific predictive biomarker has been identified to date. The increasingly more powerful dataset that is developing as additional patients are treated with MLN4924 on clinical studies may help to address this issue.

In conclusion, our data establish that inhibition of NAE with MLN4924 is a novel strategy to augment the efficacy of ara-C. A clinical trial further investigating the safety and efficacy of MLN4924 in combination with ara-C therapy is warranted with ultimate comparison to LDAC monotherapy in older adults with AML. Considering the dramatic MLN4924-induced disruption of both purine and pyrimidine nucleotide metabolism we observed in this study, this approach may also have potential applications for other malignancies that use nucleoside analog-based therapy.

Disclosure of Potential Conflicts of Interest

M. Keaton is an employee of Metabolon. No potential conflicts of interest were disclosed by the other authors.

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