The Nedd8-Activating Enzyme Inhibitor MLN4924 Thwarts Microenvironment-Driven NF-κB Activation and Induces Apoptosis in Chronic Lymphocytic Leukemia B Cells

J. Claire Godbersen1, Leigh Ann Humphries1, Olga V. Danilova2, Peter E. Kebbekus1, Jennifer R. Brown4, Alan Eastman3, and Alexey V. Danilov1

Abstract

Background: Stromal-mediated signaling enhances NF-κB pathway activity in chronic lymphocytic leukemia (CLL) B cells, leading to cell survival and chemoresistance. Ubiquitination of IκBα may partially account for constitutive activation of NF-κB. MLN4924 is an investigational agent that inhibits the Nedd8-activating enzyme, thereby neutralizing Cullin-RING ubiquitin ligases and preventing degradation of their substrates.

Experimental Design: We conducted a preclinical assessment of MLN4924 in CLL. Primary CLL cells were cocultured in vitro with CD40L-expressing stroma to mimic the prosurvival conditions present in lymphoid tissue. The effect of MLN4924 on CLL cell apoptosis, NF-κB pathway activity, Bcl-2 family members, and cell cycle was assessed by flow cytometry, Western blotting, PCR, and immunocytochemistry.

Results: CD40L-expressing stroma protected CLL cells from spontaneous apoptosis and induced resistance to multiple drugs, accompanied by NF-κB activation and Bim repression. Treatment with MLN4924 induced CLL cell apoptosis and circumvented stroma-mediated resistance. This was accompanied by accumulation of phospho-IκBα, decreased nuclear translocation of p65 and p52 leading to inhibition of both the canonical and noncanonical NF-κB pathways, and reduced transcription of their target genes, notably chemokines. MLN4924 promoted induction of Bim and Noxa in the CLL cells leading to rebalancing of Bcl-2 family members toward the proapoptotic BH3-only proteins. siRNA-mediated knockdown of Bim or Noxa decreased sensitivity to MLN4924. MLN4924 enhanced the antitumor activity of the inhibitors of B-cell receptor (BCR)-associated kinases.

Conclusions: MLN4924 disrupts NF-κB activation and induces Bim expression in CLL cells, thereby preventing stroma-mediated resistance. Our data provide rationale for further evaluation of MLN4924 in CLL. Clin Cancer Res; 20(6); 1576–89. ©2014 AACR.

Introduction

Inefficient B-cell apoptosis is considered the dominant defect in chronic lymphocytic leukemia (CLL; refs. 1, 2). Still, up to 2% of the circulating CLL B-cell pool is renewed daily (3). Gene expression profiling identified lymph nodes as the site of cell activation and proliferation with upregulation of NF-κB and B-cell receptor (BCR) signaling (4). Constitutive and BCR-dependent activation of NF-κB is an important feature in CLL B cells and predicts poor disease outcome (5–7). Targeting tyrosine kinases within the BCR-signaling cascade has proven a promising therapeutic strategy with novel inhibitors of Bruton’s tyrosine kinase (ibrutinib) and phosphoinositide 3-kinase δ (PI3Kδ; idelalisib, formerly CAL-101) currently in clinical trials (8, 9). However, BCR-independent activation of NF-κB may lead to tumor resistance. NF-κB activity may be driven via microenvironmental stimuli, examples of which include macrophage-medi- ed B cell–activating factor of the TNF family/a proliferation-inducing ligand (BAFF/APRIL), T cell–mediated CD40L signaling, and Toll-like receptor signaling (7, 10, 11). Thus, neutralization of NF-κB is a promising strategy in CLL, as it has the potential to target the proliferative pool of CLL cells and could hypothetically lead to sensitization to both conventional chemotherapy and BCR-targeting agents. The NF-κB pathway has been successfully targeted in CLL in vitro (12). However, lack of clinical advances with those agents necessitates the development of novel approaches.
Translational Relevance

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western Hemisphere. Successful use of purine analog-containing chemotherapy regimens has extended survival of younger patients with CLL. However, eventual progression to fludarabine-resistant disease, lack of low-risk curative strategies, and limited therapeutic options in the elderly warrant exploration of novel treatment strategies. The tumor microenvironment in the lymphatic tissues provides prosurvival and proproliferative stimuli and harbors chemoresistant CLL clones. We demonstrate that MLN4924, an investigational inhibitor of Nedd8-activating enzyme, exhibits cytotoxicity toward CLL cells incubated in the microenvironment-mimicking conditions where other drugs are ineffective. MLN4924 disrupts key pathways implicated in progressive disease—NF-κB signaling and the antiapoptotic Bcl-2 family members. Our results justify the development of MLN4924 in CLL.

MLN4924 is an investigational small-molecule inhibitor of NEDD8-activating enzyme (NAE), which has shown promising preclinical activity in hematologic malignancies, including acute myeloid leukemia and lymphoma (13, 14). NAE is necessary for activation of Cullin-RING ubiquitin ligases. In vitro MLN4294 leads to the accumulation of Cullin-RING E3 ligase (CRL) substrates, including IkBa, Nrf-2, p27, and Cdt1 (13, 15). Disrupted ubiquitination of IkB in the presence of MLN4924 results in inactivation of the NF-κB canonical pathway in several tumor types (14, 16). Because the NF-κB pathway is predominantly active in the lymphatic tissue, we propose that its pharmacologic inhibition would target CLL cells within their supportive microenvironment. Our preclinical work demonstrates for the first time that MLN4924 shows promising ex vivo activity against primary neoplastic B cells derived from patients with CLL. MLN4924 abrogates NF-κB pathway activation in CLL cells cocultured with CD40L-expressing stroma. This results in enhanced expression of the proapoptotic BH3-only proteins Bim and Noxa and circumvents stroma-mediated resistance. Furthermore, cooperation between MLN4924- and the BCR-targeting agent CAL-101 warrants exploration of its clinical activity in CLL.

Materials and Methods

Patient samples, CLL, and stromal cell cocultures

Following Institutional Review Board approval and provision of written informed consent, peripheral blood was obtained from 42 patients with B-CLL at Dartmouth-Hitchcock Medical Center (Lebanon, NH). The median time from diagnosis to study entry was 4 years; 37 patients (88%) were untreated. Blood was also obtained from 7 healthy volunteers. Standard Ficoll-Hypaque (Amersham) techniques were used to isolate the peripheral blood mononuclear cells (PBMC). Such CLL samples had more than 90% CD5+/CD19+ cells as determined by flow cytometry. CLL cells were cultured in RPMI-1640 supplemented with 15% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L l-glutamine, 25 mmol/L HEPES, 100 μmol/L minimum essential medium nonessential amino acids, and 1 mmol/L sodium pyruvate (Lonza). Ten CLL samples with 17p deletion were obtained from the CLL Center at Dana-Farber Cancer Institute (Boston, MA). All experiments were performed with freshly isolated cells except the viability assays involving the latter, which were performed with viable frozen cells.

Mouse fibroblast cell line (L cells) engineered to express CD40L (L.4.5) was given to us by Dr. Sonia Neron (Laval University, Quebec, Canada; ref. 17). Parental L cells were obtained from American Type Culture Collection. All were maintained in RPMI-1640 medium with 10% FBS and penicillin-streptomycin. CLL cells were cultured under standardized condition on stroma as previously described (18). Briefly, stromal cells were seeded to achieve 80% to 100% confluence on the following day when CLL cells were plated at a 50:1 ratio and incubated at 37°C in 5% CO2 in the presence or absence of 10 ng/mL of interleukin (IL)-4 (Cell Signaling Technology). For comparison, cells were cultured in suspension (off stroma) at the same density. Cultures were then treated with drugs for the indicated time periods. At harvest, CLL cells were gently washed off the stromal layer. When harvested for protein and mRNA analysis, CLL cells were transferred to a new plate and incubated for an additional 60 minutes for stroma reattachment to minimize contamination of CLL cells.

For BCR stimulation, CLL cells were seeded at a density of 5 × 105 per well in 24-well plates precoated with 10 μg/well of rabbit anti-human immunoglobulin M (IgM) antibody (Jackson Immunoresearch Laboratories).

Cell viability testing and drugs

CLL cell apoptosis was measured in duplicate as previously described using the ApoScreen Annexin V Apoptosis Kit (19). Briefly, cells were resuspended in 150 μL of Annexin V Binding Buffer containing 1 μL of Annexin V-PE, 1 μL of 7-aminoactinomycin D (7-AAD), and 1 μL of CD19- or CD3-FITC monoclonal antibodies (mAb; Southern Biotech) followed by flow cytometry on a FACSCalibur (Becton Dickinson). MLN4924 was provided by Millennium Pharmaceuticals, Inc. CAL-101, ibrutinib, and bortezomib were obtained from Selleck Chemicals; BMS-345541, fludarabine, chlorambucil, bendamustine, and U0126 were obtained from Sigma-Aldrich. Survival of the murine stromal cells was analyzed in a caspase-3 activity assay (Cell Signaling Technology).

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [20 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L NaF, 1 mmol/L sodium phosphate, 1 mmol/L NaVO3, 1 mmol/L EDTA, 1 mmol/L EGTA,
supplemented with protease inhibitor cocktail (Roche), and 1 mmol/L phenylmethylsulfonylfluoride (PMSF)]. Proteins were analyzed by immunoblotting as previously described (19). The following antibodies were used: Bcl-2, Bcl-xl, Bim, phospho-Bim, phospho-IκBα, cleaved PARP, Mcl-1, p65/RelA, p52/p100, XIAP, FOXO3A (Cell Signaling Technology), Bcl2-A1 (Abcam), NEDD8 (Epitomics), p27 (Santa Cruz, Biotechnology), Noxa (Imgenex), β-actin (Sigma-Aldrich), horseradish peroxidase-conjugated anti-mouse, and anti-rabbit antibodies (Bio-Rad).

Real time PCR

CLL cells were negatively selected using the B-cell Isolation Kit (Miltenyi Biotec; #130-093-660). This method achieved >99% B-cell purity as determined by flow cytometry. Total RNA was isolated using the RNaseasy Mini Kit (Qiagen). cDNA was synthesized from 500 ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed in a C1000 Thermal Cycler (Bio-Rad) using Universal PCR Master Mix, according to the manufacturer's instructions. Real-time PCR reactions (Applied Biosystems), with template cDNA and PCR Master Mix, according to the manufacturer's instructions in a C1000 Thermal Cycler (Bio-Rad) using Universal PCR Master Mix, according to the manufacturer's instructions.

Immunochemistry

A total of 3 × 10⁵ cells were adhered onto poly-L-lysine-coated coverslips (Sigma-Aldrich) during a 45-minute incubation at 37°C, fixed in 10% formalin (Fisher Scientific) and permeabilized in 1% Triton X-100 in PBS. Coverslips were blocked for 30 minutes in 5% bovine serum albumin (Sigma-Aldrich) in PBS with 0.1% Tween-20, probed with p65/RelA, p52/p100 (Cell Signaling Technology), or cleaved PARP (Thermo Scientific) antibodies, and then with Alexa Fluor 594 goat anti-mouse antibodies (Life Technologies). Coverslips were mounted with antifading Prolong Gold Solution (Life Technologies) with 4′,6-diamidino-2-phenylindole (DAPI; for nuclear counterstaining). Fluorescent images were captured with an F-view II monochrome camera (Olympus, U-CMAD3) mounted on an Olympus BX51 microscope.

NF-κB activity assay

Cells were treated as described above. Nuclear fractions were isolated and subsequently analyzed for NF-κB activity as per the manufacturer's instructions using the NF-κB p50/p65 Transcription Factor Assay Kit (Cat# ab133128; Abcam).

siRNA-mediated gene silencing

Electroporation of siRNA into CLL cells was performed using Amaxa Human B-cell Nucleofection Kit (Amaxa). A total of 1 × 10⁷ PBMCs were mixed with 100 μL of Amaxa B-cell nucleofector solution, and 2 μg of siRNA was nucleofected using program X-05. Transfection efficiency, assessed by transfection with 2 μg pMaxGFP plasmid, was 30% to 60% with cell viability of 50% to 80% at 24 hours. siRNA oligos were synthesized by Dharmacon. Sense strand against human Bim1: GACCGAGAAAGUUGACAAA IUU, Bim2: CUACCUCCCUACAGACA GAAU, Noxa8: GUAUIUAUGACACAU UUCU, Noxa9: AGUCGAGUGU GCUAACAAU, and Puma1: GCCGUAAGA IACUUGUAUA UJU.

IGHV mutation status

Following RNA and cDNA isolation (as above), IGHV mutations were analyzed using the IGH Somatic Hypermutation Assay v2.0 (Invivochrome). Briefly, PCR was performed using the supplied master mixes and AmpliTag Gold DNA polymerase (Applied Biosystems) on a Veriti Thermal Cycler Model #9902 (Applied Biosystems) to amplify the IGH sequence fragment between the leader (VH) and joining (J) regions as per the manufacturer’s instructions. To confirm amplification of a single clonal product in the expected size range, an aliquot of each sample was run on a 1.5% agarose gel and separated by gel electrophoresis. Samples were then submitted for DNA sequencing using the supplied sequencing primers. The National Center for Biotechnology Information (NCBI) IgBLAST tool was used to determine the percentage divergence of each clonal sequence. Samples which showed less than 2% divergence from germline sequence were deemed to have unmutated IGHV.

Microarray analysis

The RNA gene expression microarray experiments were carried out by the Dartmouth Genomics & Microarray Laboratory. Bead arrays with probes for all known human genes (Illumina) were used for RNA profiling. Reverse transcription using an oligo(dT) primer bearing a 17 promoter and the high yield ArrayScript reverse transcriptase were used to make cDNA. The cDNA was made double-stranded and purified to use as a template for in vitro transcription with T7 RNA polymerase and the included biotin-NTP mix. The labeled cRNA was purified and 1.5 μg used for hybridization to the bead arrays for 16 hours at 55°C. Following hybridization, the bead arrays were washed and stained with streptavidin-Cy3 (GE Healthcare). Fluorescent images were obtained with an Illumina 500GX scanner and processed with the BeadScan software (Illumina). Full results are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44864.

Statistical analysis

Statistical analysis was performed with Student t test (paired or unpaired) in GraphPad Prism software. P < 0.05 was considered to be statistically significant.
Microarray data were analyzed for functional significance using Pathway Studio software (Ariadne Genomics/Elsevier). Fisher exact test was used to identify ontology groups and pathways statistically enriched in the gene set. Data are presented as mean ± SE.

Results

**MLN4924 reduces neddylation of cullins and promotes apoptosis in CLL**

We first investigated whether NAE inhibition induced apoptosis in CLL. CLL cells incubated with 1 μmol/L MLN4924 for 24 hours demonstrated enhanced apoptosis compared with vehicle control (12.8% ± 1.7% Annexin V+ cells; Fig. 1A). Although CLL cells exhibited variable sensitivity to MLN4924, we did not find distinct responses depending on either BCR heavy-chain immunoglobulin gene (IGHV) mutational status (Fig. 1B), ZAP-70 or CD38 expression (data not shown), or cytogenetic abnormalities, including 17p deletion (Fig. 1C).

MLN4924 selectively inhibits modification of cullin proteins by NEDD8, thus preventing CRL functional activity and ultimately leading to accumulation of their substrates (15). In the presence of MLN4924, CLL cells demonstrated a dose-dependent decrease in neddylation of cullins as early as 2 hours (Fig. 1D). This effect was more pronounced at later time points when it was also seen at lower drug concentrations. We investigated the effect of MLN4924 on several CRL substrates and observed a concomitant accumulation of phospho-1κBα. CLL cell apoptosis, both spontaneous and drug-induced, was detected at 10 hours and prominent at 24 hours, but was more pronounced in the presence of MLN4924, as evidenced by PARP cleavage. The cell-cycle inhibitor p27Kip1 is another CRL substrate and is highly expressed in circulating CLL cells (20). Interestingly, MLN4924 had no effect on p27Kip1 protein levels, pointing to a relatively low turnover of this cell-cycle regulator in resting peripheral blood CLL cells (Fig. 1D).

**NAE inhibition-mediated abrogation of NF-κB reverses the stroma-mediated protection in CLL**

Given the importance of the microenvironment in sustaining CLL cell survival and proliferation in general and the NF-κB pathway activity in particular, we further studied the effects of MLN4924 in a stromal coculture system. Stroma cells present in the lymph node and bone marrow establish direct cell–cell contact with CLL cells engaging multiple...
prosurvival pathways (18), among which the TNF receptor family, including CD40, plays a prominent role. CD40L+ stroma partially rescued CLL cells from spontaneous apoptosis (Fig. 2A, white bars). We and others have previously shown that CD40L induces drug resistance in CLL (21–23). We confirmed those findings by demonstrating that the p100 Mcl-1 Bcl-2 Bcl-xL p52 β-Actin

**Figure 2.** MLN4924 abrogates the prosurvival effects of CD40L-expressing stroma in CLL cells. A, CLL cells (n = 20) were cultured on CD40L-expressing or parental L cells for 24 hours, followed by incubation with 0.25 or 1 μmol/L MLN4924 or vehicle control for 48 hours. As a reference, cells were treated off stroma. Cells were also cultured in the presence of 10 ng/mL IL-4 (N = 8). Apoptosis within CD19+ subset of cells was determined by Annexin V and 7-AAD staining. Data are the mean ± SE. *, P < 0.01 compared with untreated control; **, P < 0.05 compared with off stroma. B, CLL cells (N = 6) were cocultured with CD40L-expressing or parental L cells for 24 hours, followed by incubation with the indicated drugs or with vehicle control for 48 hours. As a reference, cells were treated off stroma. Apoptosis within the CD19+ subset of cells was determined by Annexin V and 7-AAD staining and normalized to the untreated controls. Data are the mean ± SE. *, P < 0.05 compared with off stroma or parental stroma. C, CLL cells were cocultured with CD40L-expressing (left) or parental stroma (right except for lane 5) for 2 to 24 hours. Whole-cell protein lysates were subjected to immunoblotting. Results from one of three experiments are shown. D, PBMCs from patients with CLL or healthy volunteers were cultured on CD40L-expressing stroma for 24 hours, followed by incubation with 1 μmol/L MLN4924 or vehicle control for 48 hours. Apoptosis within CD19+ and CD3+ subset of cells was determined by Annexin V and 7-AAD staining and normalized to the time-matched untreated controls (P < 0.0001).
CD40L-expressing (but not parental) stroma induced CLL cell resistance to common chemotherapy agents and CAL-101 (Fig. 2B). In agreement with earlier work (24), the CD40L-expressing stroma activated both the canonical and noncanonical NF-κB pathways in CLL cells after 16 to 24 hours of coculture. Activation of the noncanonical NF-κB pathway was readily demonstrated by the emergence of p52, a cleaved product of p100 (Fig. 2C). Canonical NF-κB pathway activation was confirmed by demonstrating nuclear translocation of p65/RelA, as discussed below. NF-κB activation was accompanied by induction of antiapoptotic proteins Mcl-1 and Bcl-xl on the CD40L-expressing stroma while Bcl-2 levels remained constant (Fig. 2C).

Unexpectedly, we found that CD40L-expressing stroma did not elicit resistance to MLN4924 (Fig. 2A, gray and black bars). Interestingly, 1 μmol/L MLN4924 induced more apoptosis on stroma (55.1% ± 4.2% CLL cells) than off stroma (42.4% ± 2.9%; P = 0.015). Thus, CD40L+ (but not parental) L cells also seemed to sensitize CLL cells toward a neddylation inhibitor (Fig. 2A). Similarly, apoptosis occurred irrespective of common genetic features albeit CLL samples carrying deletion 17p were less sensitive to MLN4924 compared with non-17p CLL (Supplementary Fig. S1A and S1B; P < 0.05). The size of a 17p− clone within each sample did not correlate with apoptotic response, indicating its susceptibility to the drug (Supplementary Fig. S1C). Neither protection from spontaneous apoptosis nor sensitivity of CLL cells to MLN4924 was altered by addition of parental L cells (Fig. 2A). Importantly, MLN4924 did not lead to caspase activation and did not impair CD40L expression in the stroma (Supplementary Fig. S2). Finally, normal B and T cells exhibited decreased sensitivity to MLN4924 compared with CLL cells (Fig. 2D).

Treatment with MLN4924 in the CD40L+ coculture system resulted in dose-dependent reversal of p100 cleavage and accumulation of phosphorylated IkBα in CLL cells (Fig. 3A). CLL cells demonstrated predominantly strong nuclear staining for both p65/RelA and p52 upon coculture with CD40L-expressing stroma, indicating that the NF-κB pathway was active (Fig. 3B). However, treatment with MLN4924 resulted in a shift toward cytoplasmic staining for p65 and p100/p52, thus indicating abrogation of both the canonical and noncanonical NF-κB pathways. Meanwhile, incubation of CLL cells with the parental control stroma did not result in nuclear translocation of either p65 or p52 (Fig. 3B). Addition of IL-4 led to a mild increase in p100 processing compared with CD40L alone, yet this was reversed by MLN4924 (Fig. 3C). We have noted no effect on IkBα under those conditions (Fig. 3C). Similarly, upon treatment with MLN4924, the p65/RelA subunit shifted into the cytoplasm in CLL cells exposed to IL-4 (Supplementary Fig. S3). We then sought to confirm that CLL cells undergoing apoptosis in response to MLN4924 had lost NF-κB activity. Indeed, cells that demonstrated cleaved PARP expression did not show nuclear expression of p65 (Supplementary Fig. S3).

We next used gene expression profiling to determine which pathways were deregulated by NAE inhibition in CLL and whether the transcriptional targets of NF-κB were affected. Of the genes incorporated in the probe set, 7,254 were expressed in CLL. Using a cutoff of at least 1.5-fold change, we identified 977 genes whose expression was significantly affected by MLN4924 (P < 0.01). A set of 559 downregulated genes was analyzed for functional significance. We determined that receptor signaling and expression target pathways involving NF-κB were most significantly associated with the downregulated genes (P < 0.0001). Of the >400 known NF-κB transcriptional targets (http://www.bu.edu/nf-kb/gene-resources/target-genes/), 181 genes were expressed in CLL (Fig. 3D and Supplementary Table S1). Chemokine receptors CXCR4 and CXCR5 are vital for CLL cell homing (25). We observed downregulation of the NF-κB transcriptional target CXCR5 (26), but not CXCR4, by RT-PCR (Supplementary Fig. S4A). Intriguingly, we detected a 7.7-fold reduction in miR-155, an oncogenic microRNA (miRNA, miR) that is predominantly expressed by proliferative CLL and regulated by NF-κB (27, 28). Finally, we confirmed that MLN4924 abrogated NF-κB activity using an assay that measures p65 and p50 binding to the DNA sequence containing the NF-κB response element. In this assay, NF-κB activity was reduced in the presence of MLN4924 in a dose-dependent manner (Supplementary Fig. S4B), consistent with previously appreciated effect on IkBα (Fig. 3B). We then analyzed several NF-κB transcriptional targets to confirm that the inhibitory effect of MLN4924 was dose-dependent (Supplementary Fig. S4C).

Thus, CLL cells exhibited NF-κB activation in stromal cocultures that was blocked by MLN4924, leading to enhanced apoptosis.

NAE inhibition rebalances Bcl-2 family members toward the proapoptotic BH3-only proteins in CLL

Although circulating CLL lymphocytes express almost exclusively Bcl-2, cells in the stromal niche have been shown to express other prosurvival Bcl-2 family members. A balance between them and their relative ratio to the proapoptotic multi-BH proteins Bax and Bak determines cell fate (29). BH3-only proteins Puma, Noxa, Bim, and others also interact with the antiapoptotic Bcl-2 family members. Recent efforts have seen emergence of Bcl-2 inhibitors and BH3-mimetics (e.g., ABT-263), which have shown promise in treatment of CLL (29). We investigated whether changes in transcription of the antiapoptotic proteins Bcl-2 and Bcl-XL as identified by gene expression profiling resulted in a significant reduction in the corresponding protein levels to explain enhanced apoptosis in response to MLN4924 on CD40L-expressing stroma.
As described above, CD40L-expressing stroma induced Bcl-xl and Mcl-1 in CLL (Figs. 2C and 4A). In addition, we observed a significant repression of Bim mRNA, but not Noxa or Puma mRNA, in cells cultured on the CD40L+ stroma versus control stroma (Fig. 4B). Furthermore, CD40L+ but not parental L cells resulted in repression of Bim protein in four of six tested CLL samples (Fig. 4C). Consistent with previous reports that unmanipulated L cells do not induce either Mcl-1 or Bcl-xl (30), we observed no change in their expression in CLL cells cocultured on the parental stroma (Figs. 2C and 4C).

Contrary to the microarray findings, Bcl-2 protein expression remained unchanged upon treatment with MLN4924. Meanwhile, of 10 CLL samples tested, modest downregulation of Bcl-xl was detected in six samples upon treatment with MLN4924 for 48 hours on CD40L+ stroma, whereas expression of Mcl-1, XIAP, and Bcl2A1 were stable (Fig. 4A). We then studied the effect of MLN4924 on the proapoptotic BH3-only proteins. Gene expression profiling experiments revealed a 2-fold upregulation of Bim transcript by MLN4924 (Supplementary Table S1). We confirmed upregulation of Bim mRNA and protein levels (Fig. 4A and B). Bim protein induction was evident by 8 hours of exposure to 1 μmol/L MLN4924. Importantly, Bim induction also occurred in the CLL samples, which showed no Bim repression by stroma and no drug-mediated change in antiapoptotic proteins. BA5-345541, an IκB kinase inhibitor, also led to induction of Bim in CLL cells, confirming the importance of NF-κB in Bim regulation (Supplementary Fig. S5). NF-κB inhibition was previously shown to upregulate other BH3-only proteins, namely Noxa, in CLL (31). We found that Noxa mRNA and protein as well as Puma mRNA were also induced by MLN4924 in CD40L-expressing stroma cocultures (Fig. 4B and D).

We further sought to confirm the role of BH3-only proteins in MLN4924-mediated apoptosis. siRNA-mediated knockdown yielded consistent reduction in baseline Bim and blunted its upregulation upon treatment with MLN4924 (Fig. 5A). Bim short isoform (S), the most cytotoxic, was no longer detected. This was accompanied by a reduction in apoptosis, despite reduced Bcl-xl expression. These findings were confirmed in an expanded cohort of CLL samples (Fig. 5B). In agreement with earlier data on the involvement of NF-κB in regulation of Noxa in CLL, apoptosis was decreased in Noxa-suppressed CLL cells, whereas manipulation of Puma had no effect (Fig. 5A and Supplementary Fig. S6). Combined knockdown of Bim and Noxa further suppressed CLL cell apoptosis (Fig. 5C).

Bortezomib was previously shown to induce Noxa in CLL cells (32). Expectedly, treatment of CLL cells with bortezomib resulted in sensitization to MLN4924 (Fig. 5D). Noxa induction was enhanced when MLN4924 was combined with bortezomib compared with either drug alone (Fig. 5D). Meanwhile, CD40L-mediated downregulation of Bim is dependent on activation of extracellular signal–regulated kinase (ERK; ref. 33). Although both MLN4924 and U0126 (a mitogen-activated protein kinase inhibitor) induced Bim in CLL cells, the increase in toxicity of the combination was not as pronounced, albeit statistically significant (P < 0.01, paired t test), possibly due to a mild cooperative effect on Bim expression. Thus, we found that Bim is frequently downregulated in a CD40L-expressing microenvironment. Meanwhile, NAE inhibition-mediated inactivation of NF-κB results in rebalancing of the Bcl-2 family members toward the proapoptotic BH3-only proteins Bim and Noxa in CLL cells, which are required for apoptosis induction in this setting.

**MLN4924 sensitizes CLL cells to the BCR-targeting agents**

Because NF-κB activation is one of the dominant pathways ensuring CLL cell survival in response to BCR signaling, we hypothesized that MLN4924 may enhance the proapoptotic effects of the novel BCR kinase inhibitors (9). We first determined whether MLN4924 had an impact on BCR signaling-mediated survival of the CLL cells. As expected, IgM stimulation resulted in CLL cell rescue from spontaneous apoptosis in a subset of patient samples (34), and this was reversed by MLN4924, once again emphasizing the importance of NF-κB activation in BCR-mediated survival (Fig. 6A). We then explored the combined effect of MLN4924- and BCR-targeting agents CAL-101 and ibrutinib (8, 9). Although CLL cells were resistant to both CAL-101 and ibrutinib in the CD40L-expressing system (but not on parental L cells—not shown), coincubation with either agent and 0.25 μmol/L MLN4924 led to an increase in cell death compared with MLN4924 alone (Fig. 6B).

**Discussion**

Gene expression profiling of peripheral blood CLL cells had initially characterized them as quiescent lymphocytes...
related to memory cells (2). Subsequently, it was established that, in addition to cell accumulation, proliferation of the neoplastic B cells provides a significant contribution to the malignant clone (3). Lymph nodes and bone marrow were identified as the preferential sites of activation and proliferation of the CLL cells (4, 5). BCR gene signature along with the NF-κB, NFAT (Nuclear Factor of Activated T-cells), and other proliferation/cell-cycle functional gene sets are over-represented in the lymph node, whereas bone marrow–resident CLL cells show decreased apoptotic priming (4, 22). Signals transduced through the BCR, CD40, and Toll-like receptors converge on NF-κB, leading to high NF-κB activity in the CLL cells resident in the microenvironment. Hewamana and colleagues demonstrated that DNA binding of the Rel A subunit is enhanced in CLL compared with normal B cells, providing evidence for constitutive NF-κB activation in CLL (5).

NF-κB activation occurs through the canonical and noncanonical pathways (35). In the canonical pathway, the IκB kinase complex phosphorylates IκB, triggering its ubiquitination and leading to nuclear translocation of the NF-κB dimers, predominantly p50/Rel A and p50/c-Rel. Noncanonical activation is based on the proteosome-assisted processing of the precursor protein p100 with nuclear translocation of p52/Rel B (35, 36). Inappropriate degradation of IκBα is among the potential reasons for constitutive activation of NF-κB in cancer (37). Thus, blocking proteosomal degradation of IκBα is a promising therapeutic approach. MLN4924 is a recently discovered inhibitor of NAE. MLN4924 binds NAE at its active site forming a covalent MLN4924–NEDD8 adduct, thus preventing the modification of cullin proteins by Neddd8 and leading to accumulation of CRL substrates (15). In this work, we demonstrate that MLN4924 efficiently reverses cullin neddylation in CLL cells in vitro, a process accomplished by the accumulation of phospho-IκBα and subsequent apoptosis. Accumulation of IκBα in CLL occurred rapidly and at concentrations of MLN4924 sufficient to induce apoptosis (14).

To study the impact of MLN4924 on the microenvironment-mediated NF-κB activation, we cocultured CLL cells with CD40L-expressing stroma. Such a strategy has been shown to activate NF-κB and counter the spontaneous apoptosis of the CLL cells in vitro (24, 38). Importantly, CLL cells exposed to CD40L-expressing stroma have decreased "priming" to undergo apoptosis and acquire resistance to both conventional and novel therapeutic agents such as ABT-737 and CAL-101 (21–23, 25). In agreement with earlier reports, we demonstrated activation of both the canonical and noncanonical NF-κB pathways in CLL cells cocultured with the CD40L-expressing stroma (but not with parental stroma), thus creating a partial recapitulation of the lymph node microenvironment (24). Importantly, we found that CD40L signaling may lead to repression of Bim.

Although CLL cells cocultured with CD40L-expressing stroma were resistant to multiple chemotherapy agents, the protective effects were abrogated by MLN4924. We further observed that NAE inhibition prevented nuclear translocation of the NF-κB pathway effectors p65/Rel A and p52 in CLL cells. Attenuated ubiquitination of phospho-IκBα resulted in an increased retention of p65 in the cytoplasm, thus leading to inactivation of the canonical pathway. Meanwhile, a noncanonical pathway effector p52 is generated when an E3 ligase induces processing of the NF-κB2 precursor protein p100 (36). Because p100 protein expression was not affected by MLN4924 in CLL, while p52 was reduced, it is likely that inactivation of the noncanonical pathway occurred because of E3 ligase inhibition and subsequent reduction in proteosomal processing of p100. It has been previously reported that both the canonical and noncanonical NF-κB pathways are activated in CLL lymph nodes, pointing to the biologic relevance of our findings (4, 24). Interestingly, MLN4924 also induced apoptosis of CLL cells cultured off stroma. Using electrophoretic mobility shift assays, it was shown that peripheral blood CLL cells also demonstrate increased NF-κB activity compared with normal B cells. It is possible that inhibition of this "baseline" NF-κB activity accounts for modest apoptosis in the presence of MLN4924 in this setting (39).

Abrogation of NF-κB activity in CLL cells by MLN4924 resulted in decreased transcription of its nuclear targets in a dose-dependent manner. Among those, we found a significant reduction in transcription of genes involved in cell cycle and CLL cell-derived chemokines mediating microenvironment dependence (CXCR5, CCL17, CCL22, etc.), some of which are induced via CD40 (40, 41). Furthermore, recent work demonstrates that NF-κB upregulates the expression of cytokines and adhesion molecules crucial for CLL survival in the stroma itself (42). Hence, MLN4924 may disrupt the chemokine network and cell–cell interactions in the protective microenvironment leading to reduced tissue homing in CLL. In addition, MLN4924 may shift the balance of CD40 signaling toward proapoptotic events (43).
Although NAE inhibition had minimal effect on expression of the antiapoptotic Bcl-2 family members, it led to the induction of Bim and Noxa. Bim plays an important role in apoptosis regulation in CLL (44). Bim is phosphorylated and ubiquitinated in response to BCR signaling, leading to CLL cell survival and disease progression (44). Bim is capable of binding to all Bcl-2 proteins with high affinity, activating the prodeath Bax and/or Bak (45). We found that MLN4924 induced all Bim splice variants (BimEL/L/S) in CLL, but the exact mechanism remains unclear. MLN4924 did not induce Forkhead box 3A transcription factor, a regulator of Bim (Supplementary Fig. S7; ref. 45). Earlier reports suggest that NF-κB neutralizes Bim via a c-Rel–dependent mechanism in B cells (46). In addition, transgenic expression of p52 led to repression of Bim and defective apoptosis of mouse lymphocytes (47). Furthermore, degradation of at least one Bim isoform, BimEL, is proposed to occur with the involvement of CRL (48). We
found that Bim transcription is upregulated by MLN4924 in CLL cells, implicating de novo synthesis in its induction. Although we did not detect Bim phosphorylation in CLL (Supplementary Fig. S7), we did not fully exclude a possibility of attenuated degradation of Bim. Meanwhile, Noxa has been designated as a putative CRL target, and therefore NAE inhibition may abrogate its degradation in CLL (49). Diminished degradation of Bim and Noxa may be contributing to the proapoptotic effect of MLN4924 in CLL cells cultured off stroma.

Because NF-κB is among the terminal effectors of BCR signaling, our findings that MLN4924 abolished the protective effect of BCR stimulation suggest that it may overcome the enhanced responsiveness to BCR signaling and the unfavorable prognosis rendered by unmutated IGHV in CLL (50). In CD40L+ stromal cocultures, CLL cells were rescued from the proapoptotic effects of the PI3K inhibitor CAL-101 and Bruton tyrosine kinase ibrutinib, an effect reversed by MLN4924. Hence, microenvironment-mediated NF-κB activation may enhance CLL cell survival independent of BCR signaling and induce resistance to BCR-targeting agents. Remarkable efficacy of MLN4924 in this setting suggests addiction to the NF-κB pathway and justifies further investigation of those drug combinations in the clinic.

In summary, we demonstrate that MLN4924 effectively inhibits cullin neddylation in CLL cells. In a model that mimics the lymph node microenvironment, this leads to inactivation of the NF-κB pathway, reexpression of Bim and Noxa, and prevents stroma-mediated drug resistance. MLN4924 shows cooperation with the BCR-targeting agents. Our data combined with the new knowledge about the indispensability of stromal NF-κB to CLL cell survival (42) justify further studies of the NAE inhibitor MLN4924 in CLL.

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

Authors’ Contributions
Conception and design: A.V. Danilov
Development of methodology: A.V. Danilov
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.C. Godbersen, L.A. Humphries, O.V. Danilova, P.E. Kebbekus, J.R. Brown, A. Eastman, A.V. Danilov
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.C. Godbersen, L.A. Humphries, O.V. Danilova, J.R. Brown, A.V. Danilov
Writing, review, and/or revision of the manuscript (e.g., statistical analysis, data interpretation, writing assistance): J.C. Godbersen, L.A. Humphries, O.V. Danilova, J.R. Brown, A. Eastman, A.V. Danilov
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.C. Godbersen, P.E. Kebbekus, A.V. Danilov
Study supervision: A.V. Danilov

Acknowledgments
The authors thank Drs. Allison Berger (Millennium Pharmaceuticals, Inc.) for the helpful discussions, and Mark Israel for use of the microscope.

Figure 6. MLN4924 enhances the activity of BCR signaling inhibitor CAL-101 and alkylating agents in CLL. A, CLL cells (N = 12) were stimulated with immobilized anti-IgM (10 μg/mL) for 1 hour or not and cultured in the presence of 0.25 or 1 μmol/L MLN4924 or vehicle control for 24 hours (in duplicates). B, CLL cells (N = 8) were cocultured with CD40L-expressing stroma for 24 hours, followed by incubation with 0.25 μmol/L MLN4924, 1 μmol/L CAL-101, 1 μmol/L ibrutinib, the drugs combined, or with vehicle control for 48 hours. Data are the mean ± SE. **, P < 0.01 compared with either single drug.
References


11. Arvanitakis S, Papatheodorou T, Drouzis J, Psallidas P, Vlahogianni M, et al. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 12, 2013; revised December 15, 2013; accepted January 3, 2014; published online March 14, 2014.

Grant Support
Support to A.V. Danilov is provided by a National Cancer Institute new faculty award (1P30CA023108-31S4) to the Norris Cotton Cancer Center. A. Eastman and A.V. Danilov are supported by a translational research award from the Leukemia & Lymphoma Society. J.R. Brown is supported by the Leukemia and Lymphoma Society and the American Cancer Society and is a Scholar in Clinical Research of the Leukemia and Lymphoma Society.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Support to A.V. Danilov is provided by a National Cancer Institute new faculty award (1P30CA023108-31S4) to the Norris Cotton Cancer Center. A. Eastman and A.V. Danilov are supported by a translational research award from the Leukemia & Lymphoma Society. J.R. Brown is supported by the Leukemia and Lymphoma Society and the American Cancer Society and is a Scholar in Clinical Research of the Leukemia and Lymphoma Society.


Downloaded from clincancerres.aacrjournals.org on October 15, 2017. © 2014 American Association for Cancer Research.


Correction: The Nedd8-Activating Enzyme Inhibitor MLN4924 Thwarts Microenvironment-Driven NF-κB Activation and Induces Apoptosis in Chronic Lymphocytic Leukemia B Cells

In this article (Clin Cancer Res 2014;20:1576–89), which was published in the March 15, 2014, issue of Clinical Cancer Research (1), there is incorrect wording in a figure title and a figure layout error. The title for Fig. 6 was incorrectly printed as "Figure 6. MLN4924 enhances the activity of BCR signaling inhibitor CAL-101 and alkylating agents in CLL." It should read as follows: "Figure 6. MLN4924 enhances the activity of BCR signaling inhibitors in CLL." The authors regret this error. In Fig. 3A, the label "CLL49" was mistakenly placed inside the image. The corrected version of Fig. 3 is below. The publisher regrets this error. The conclusions put forth in the article remain unchanged and unaffected by these errors.

Reference

Published online August 15, 2016.
doi: 10.1158/1078-0432.CCR-16-1475
©2016 American Association for Cancer Research.
The Nedd8-Activating Enzyme Inhibitor MLN4924 Thwarts Microenvironment-Driven NF-κB Activation and Induces Apoptosis in Chronic Lymphocytic Leukemia B Cells


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/20/6/1576

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/11/25/20.6.1576.DC1

Cited articles
This article cites 50 articles, 30 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/6/1576.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/6/1576.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.