The antiproliferative agent MLN944 preferentially inhibits transcription

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Abstract

MLN944 is a novel compound currently being codeveloped by Millennium Pharmaceuticals and Xenova Ltd. as a cancer therapeutic and is in a phase I clinical trial for solid tumors. Although MLN944 was originally proposed to function as a topoisomerase I and II inhibitor, more recent data has shown that it is a DNA-intercalating agent that does not inhibit the catalytic activity of topoisomerase I or II. We show here that MLN944 inhibits incorporation of radiolabeled precursors into RNA preferentially over incorporation into DNA and protein in HCT116 and H460 cells. To determine if MLN944 inhibits transcription, a human RNA polymerase II *in vitro* transcription system was used. MLN944 inhibited initiation when added before or after the formation of preinitiation complexes and inhibited elongation at higher concentrations. The preferential inhibition of initiation differentiates MLN944 from actinomycin D, which more strongly inhibits elongation. Transcription of all RNA polymerases was inhibited in nuclei isolated from HeLa cells treated with low concentrations of MLN944. Our data are consistent with transcription as the target of the potent cytotoxic effects of MLN944. [Mol Cancer Ther 2005;4(8):1260–7]

Introduction

MLN944 is a novel bis-phenazine compound that is currently being tested for anticancer activity in phase I clinical trials for solid tumors. MLN944 was developed in a drug design program intended to create topoisomerase inhibitors (1). Potent cytotoxicity of MLN944 has been seen in Jurkat and Chinese hamster ovary cell lines and has translated to human colon carcinoma xenograft models in mice (2). Recently, MLN944 was shown to be significantly more potent than doxorubicin, topotecan and paclitaxel against a variety of human tumors *ex vivo* (3). MLN944 has also been shown to have an additive potency when added sequentially with 5-fluorouracil and irinotecan to a human colon cancer cell line or xenografts (4).

The mechanism of action of MLN944 cytotoxicity is currently under investigation. MLN944 was originally reported to function as a topoisomerase I and II inhibitor due to its ability to stimulate the isolation of cleavable complexes containing topoisomerase I or II (2). Subsequently, MLN944 was found not to inhibit topoisomerase I or II catalytic activity in DNA supercoiling assays (5). Compared with the topoisomerase I and II inhibitors, camptothecin and etoposide, MLN944 only slightly stimulated the formation of nicked or linear DNA species in *in vitro* DNA cleavage assays (5). The global cellular effect of MLN944 treatment is cell cycle arrest in G_1/G_2, which is different from camptothecin and etoposide that cause a G_2-M arrest (5). Treatment of human colon carcinoma xenografts with MLN944 or the topoisomerase inhibitor CPT-11 were found to modify the expression of different subsets of genes in microarrays (5). These data strongly suggest that the mechanism of action of MLN944 is not through inhibition of either topoisomerase I or II.

Recently, the nuclear magnetic resonance structure of MLN944 binding the B-DNA oligonucleotide, d(ATGCAT)$_2$, was determined (6). The two phenazine rings of MLN944 were found to bis-intercalate between T2pG3 and C4pA5 and the carboxamide amino linker interacted with the central G3:C4 base pairs in the major groove of the DNA, causing an unwinding of the DNA at the intercalation sites, resulting in a more shallow major groove and a broader minor groove (6). Electrophoretic shift mobility assays examining the effect of MLN944 on activator protein and nuclear factor-κB found that the binding of c-Jun to the activator protein binding site was inhibited by MLN944, but nuclear factor-κB binding to its recognition sequence was not affected (6).

DNA and the processes associated with it are attractive targets for cancer therapies either directly or as the end result of signaling pathways (7). DNA-intercalating agents have inhibitory effects on transcription initiation or elongation. Actinomycin D, a monointercalator that recognizes the sequence d(pGpC), inhibits RNA synthesis (8, 9) and more specifically the elongating polymerase (9). Daunomycin, a monointercalating agent and its bis-intercalating derivative, WP631, inhibit transcription (10). WP631 also inhibits SP-1-activated transcription at lower concentrations (10). Actinomycin D has been used to treat many cancers and daunomycin is used to treat leukemia. The DNA cross-linker cisplatin is also used to treat many

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tumors, especially those of testicular and ovarian origin (11). Cisplatin bends the DNA, inhibiting transcription elongation by blocking the ability of RNA polymerase II to traverse the DNA (12, 13). Production of full-length mRNAs is also blocked by compounds, such as flavopiridol, that inhibit the kinase activity of positive transcription elongation factor b (P-TEFb; refs. 14, 15). P-TEFb phosphorylates the CTD of the largest subunit of RNA polymerase II, allowing for a transition from abortive to productive elongation (16, 17). Flavopiridol has completed phase I and II clinical trials for various cancers (18).

The DNA intercalating properties of MLN944 make transcription a strong candidate for its target. The ability of MLN944 to inhibit transcription was tested *in vivo* by examining the incorporation of radiolabeled precursors into RNA and DNA and with nuclear run-ons. The effects of MLN944 on initiation and elongation was examined separately in *in vitro* transcription assays and compared with another bis-intercalator, actinomycin D.

Materials and Methods

Compounds

MLN944 dimesylate salt was synthesized at Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand (2). DMSO stocks of MLN944 of 5 or 10 mmol/L were prepared and stored at −80°C. Actinomycin D was purchased from Sigma-Aldrich (St. Louis, MO) and 1 mmol/L stocks in DMSO or 10 mmol/L stocks in methanol were prepared. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was purchased from Sigma-Aldrich and a 10 mmol/L stock in ethanol was prepared.

Trichloroacetic Acid Precipitation Assays

HCT116 and H460 cells (American Type Culture Collection, Manassas, VA) were plated overnight at a density of 5×10^5 per well in a 6-well dish. HCT116 cells were maintained in McCoys 5A media (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% L-Glutamine (Life Technologies). H460 cells were maintained in RPMI 1640 (Life Technologies) media supplemented with 10% fetal bovine serum and 1% L-Glutamine. Cells were cultured under standard conditions (37°C in 5% CO_2). Cells were treated with MLN944 or actinomycin D for 1 hour before labeling while maintaining DMSO below 2% of the final volume. Cells were then pulse labeled for 1 hour with [5-6, ^{3}H] uridine, [Methyl-^{3}H] thymidine, or L-[U-^{14}C] leucine (Amersham, Arlington Heights, IL) to measure RNA, DNA, and protein synthesis, respectively. The percent incorporation was measured by precipitating each sample in 5 volumes of 20% trichloroacetic acid (Perkin-Elmer, Norwalk, CT) for 30 minutes at 40°C and recovered by filtration onto glass fiber filters (VWR, Chester, PA). Filters were washed twice with 5 mL of 5% trichloroacetic acid followed by a single wash with 5 mL of 95% ethanol, air dried, and counted in 5 mL Optiphase Super Mix scintillation fluid (Perkin-Elmer) on a scintillation counter (Beckman Coulter LS 6500).

Transcription Assays

The RNA polymerase II *in vitro* transcription assays were done as described previously (14, 19). Transcription reactions were carried out by incubating 20 ng/μL template DNA, a PCR product containing the cytomegalovirus (CMV) promoter, in the presence of HeLa nuclear extract (20, 21) under standard transcription conditions of 60 mmol/L KCl, 20 mmol/L HEPES, and 7 mmol/L $MgCl_2$ for 10 minutes. Reactions contained 50 μmol/L DRB (Sigma, St. Louis, MO), or indicated amounts of MLN944 while maintaining a final concentration of 0.5% DMSO. Transcription was initiated by the addition of 0.5 mmol/L ATP, CTP, and GTP and 10 μCi α-^{32}P-UTP (ICN). After 45 seconds, the UTP concentration was raised to 1 mmol/L UTP to generate run-off transcripts. Transcription was stopped by the addition of Sarkosyl stop solution [1% Sarkosyl, 100 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 10 mmol/L EDTA, and 100 μg/mL tRNA]. The RNA was isolated and resolved on a 6% urea gel. Quantitation was done using an InstantImager (Packard, Meriden, CT) and data was normalized to the DMSO control and curve fit with Table Curve (Jandel Scientific, San Rafael, CA) to determine the IC_{50}. Transcription initiation assays were done in a similar manner, except 45 seconds after the addition of 0.5 mmol/L ATP, CTP, and GTP and 10 μCi α-^{32}P-UTP (ICN) transcription was stopped by the addition of the Sarkosyl stop solution. The initiation assays were also done with 30 ng/μL Bgl II–linearized C<6> plasmid (22) DNA containing the SV40 promoter as the template DNA. The amounts of MLN944 or actinomycin D and order of addition are as indicated in the figure legends.

Kinase Assay

The *in vitro* kinase assays were done as previously described (17) except that recombinant P-TEFb, composed of human Cdk9 and cyclin T2a (23) was used, and the substrate was recombinant human DSIF (21). Kinase reactions were carried out with 2.5 μCi α-^{32}P-ATP (Amersham), 30 μmol/L ATP, 20 mmol/L HEPES, 7 mmol/L $MgCl_2$, and 30 mmol/L KCl for 20 minutes at 30°C. The kinase reactions were stopped by the addition of SDS loading buffer and resolved on a 7.5% SDS polyacrylamide gel. Label incorporation into the largest subunit of DSIF was quantitated with an InstantImager (Packard).

Immobilized Template Transcription Assay

The immobilized template *in vitro* transcription assays were done as outlined previously (14, 24). Transcription reactions were carried out in the presence of HeLa nuclear extract at 60 mmol/L KCl, 20 mmol/L HEPES, 7 mmol/L $MgCl_2$, and 50 μmol/L DRB for 10 minutes using a biotinylated template DNA that had been conjugated to streptavidin paramagnetic beads. Transcription was initiated by the addition of 0.5 mmol/L ATP, CTP, and GTP and 10 μCi α-^{32}P-UTP for 45 seconds. Transcription was stopped by addition of EDTA to a final concentration of 30 mmol/L to produce early elongation complexes that were isolated with a magnetic concentrator. The early elongation complexes were washed twice with 1 mol/L KCl and 1% Sarkosyl to remove all factors except for the engaged

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polymerase, and equilibrated by washing once with transcription buffer (60 mmol/L KCl, 20 mmol/L HEPES, 7 mmol/L $MgCl_2$, and 0.2 μg/mL bovine serum albumin) before suspension in transcription buffer. The isolated early elongation complexes were then incubated with increasing amounts of MLN944 or actinomycin D for 10 minutes and then chased for 5 minutes in transcription buffer containing nucleotide triphosphates. The RNA was isolated and resolved on a 6% UREA gel and subjected to autoradiography.

Nuclear Run-ons

HeLa S3 cells were cultured in suspension under standard conditions to a concentration of 3.5 to 4×10^5 cells/mL in DMEM/F-12 media (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (Hyclone) and 1× penicillin/streptomycin (Invitrogen). Cells were treated with 0, 30, 100, or 300 nmol/L MLN944 for 1 hour. Nuclei were isolated by isotonic homogenization of the cells in the presence of 0.1% Triton X-100 and spinning them through a 1.9 mol/L sucrose cushion (25). The nuclei were stored at −80°C in storage buffer [25% glycerol, 10 mmol/L Tris (pH 7.5), 5 mmol/L $Mg(Ac)_2$, and 1 mmol/L DTT]. Nuclear run-on experiments were carried out in 18-μL reactions containing 12.5% glycerol, 120 mmol/L KCl, 7.5 mmol/L $Mg(Ac)_2$, 15 mmol/L Tris (pH 7.5), 0.5 mmol/L DTT, 0.5 mmol/L A/G/CTP, 5 μmol/L UTP, 20 μCi α-^{32}P-UTP, ± 2 μg/mL α-amanitin (Sigma), and 20 mmol/L HEPES. Reactions were incubated at room temperature for 0, 10, or 20 minutes and stopped with 82 μL of Sarkosyl stop solution, spotted on Whatman DE81 filters and allowed to air dry. Filters were washed four times for 5 minutes with at least 10 mL of 5% K_2HPO_4 and 0.3% Na_4PO_7 per filter, or until no more counts were detected in the wash, and dried under a strong lamp. Counts were analyzed with an InstantImager and by scintillation counting.

Results

MLN944 Inhibits RNA Synthesis

To begin to understand the mechanism of cellular toxicity of MLN944, its effect on RNA, DNA, and protein synthesis was evaluated in two human tumor cell lines. H460 and HCT116 cells were treated with increasing amounts of MLN944 for 1 hour and pulsed with radiolabeled precursors for 1 hour followed by trichloroacetic acid precipitation to monitor their incorporation. The transcription of new RNA, as indicated by [5-6, ^{3}H] uridine incorporation, was inhibited with IC_{50}s of 0.3 and 3 nmol/L for H460 (Fig. 1A) and HCT116 (Fig. 1B) cells, respectively. DNA synthesis, as indicated by [methyl-^{3}H] thymidine incorporation, was inhibited with an IC_{50} of 300 nmol/L in both cell lines (Fig. 1A and B). Protein synthesis, as indicated by L-[U-^{14}C] leucine incorporation, remained unaffected in both cell lines at all concentrations of MLN944 that were tested (Fig. 1A and B). The results indicate that the primary target of MLN944 is transcription, because inhibition of RNA synthesis occurs at two to three orders of magnitude lower concentrations than inhibition of DNA synthesis. The time course of this experiment is such that protein synthesis should be unaffected by inhibition of transcription. The inhibition of RNA synthesis also occurs in the same concentration range in which the potent cytotoxicity of MLN944 was seen previously in Jurkat and Chinese hamster ovary cells lines, with IC_{50}s in the range of 0.04 to 0.4 nmol/L (2).

MLN944 Inhibits RNA Polymerase II Transcription *In vitro*

To develop an understanding of how MLN944 inhibits RNA synthesis, its effect on transcription by RNA polymerase II was examined in an *in vitro* system. Transcription was carried out using HeLa nuclear extract and a template containing the CMV promoter. The incorporation of a radiolabeled nucleotide into a 600-nucleotide run-off transcript was used detect the level of transcription. As expected, inhibition of P-TEFb by the inclusion of 50 μmol/L DRB (16, 26) reduced the amount of runoff compared with the control reaction (Fig. 2A). Addition of increasing amounts of MLN944 (0.01–10 μmol/L) caused a decrease in the amount of run-off transcripts starting at 0.3 μmol/L, with a complete loss of run-off transcripts by 3 μmol/L (Fig. 2A). The amount of runoff was quantitated (Fig. 2B) and an IC_{50} of 0.6 μmol/L

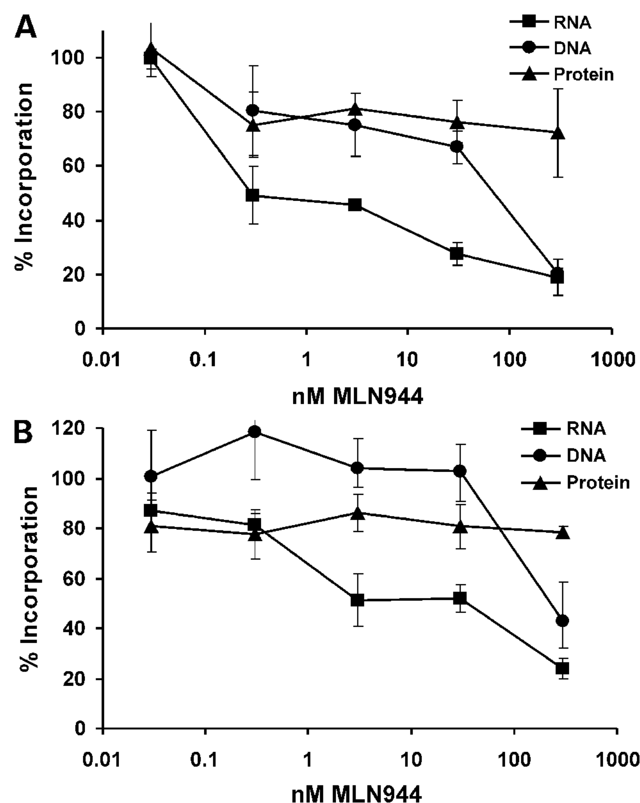

Figure 1. MLN944 inhibits RNA synthesis. RNA, DNA, and protein synthesis was examined in H460 (**A**) and HCT116 (**B**) cells treated with the indicated amounts of MLN944 for 1 h followed by pulse labeling with [5-6,^{3}H] uridine, [methyl-^{3}H] thymidine, or L-[U-^{14}C] leucine (for RNA, DNA, and protein synthesis, respectively) for 1 h. Trichloroacetic acid precipitation was done and the incorporation of label was quantitated with a scintillation counter.

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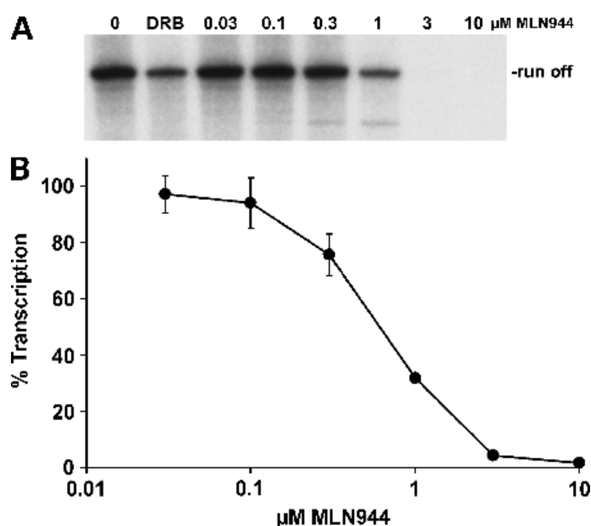

Figure 2. MLN944 inhibits RNA polymerase II transcription *in vitro*. **A,** RNA polymerase II transcription using HNE and a template containing the CMV promoter was measured by generation of 600-nucleotide run-off transcripts in an *in vitro* transcription assay. Reactions contained a constant amount of DMSO to normalize for any slight effects it may have on transcription and were carried out with DMSO alone, 50 μmol/L DRB, or the indicated amounts of MLN944. The RNA was isolated, resolved on a 6% urea gel, quantitated with an InstantImager, and subjected to autoradiography. **B,** the data from three independent experiments was normalized to the run-off transcripts produced in the DMSO control and plotted.

was calculated. These results indicate that MLN944 inhibits transcription by RNA polymerase II but do not differentiate between inhibition of initiation and an effect on elongation.

MLN944 Does Not Inhibit P-TEFb

To determine if the effect of MLN944 on transcription was due to the inhibition of the elongation control factor P-TEFb, an *in vitro* kinase assay was done. The incorporation of labeled phosphate from γ-^{32}P-ATP into the largest subunit of DSIF (27) was examined in the presence of increasing amounts of MLN944 (0.01–10 μmol/L). No inhibition was seen at any concentration tested (Fig. 3). We conclude that the effects of MLN944 in the *in vitro* transcription assay (Fig. 2) are not due to inhibition of P-TEFb.

MLN944 Inhibits RNA Polymerase II Elongation

To determine if MLN944 affects elongation by inhibiting RNA polymerase II directly, an *in vitro* transcription assay using an immobilized template was done. Transcription was initiated using HeLa nuclear extract and a CMV template conjugated to paramagnetic beads. Early elongation complexes containing RNA polymerase II and short transcripts were isolated by washing with 1 mol/L KCl and 1% Sarkosyl to strip away all factors. Increasing amounts of MLN944 (0.3–10 μmol/L) were added to the early elongation complexes before the complexes were chased for 5 minutes in the presence of 0.5 mmol/L nucleotide triphosphates. The first effects on elongation were seen at 1 μmol/L as indicated by the generation of slightly shorter transcripts compared with the control reaction (Fig. 4). Elongation was further hindered, as the amount of MLN944 was increased (Fig. 4). Clearly, MLN944 inhibited transcription elongation by RNA polymerase II directly; however, the concentration of MLN944 needed to inhibit elongation was significantly greater than that needed to inhibit the generation of run-off transcripts (Fig. 2A). This suggests that the effects of MLN944 on elongation may be secondary to its effects on initiation.

MLN944 Inhibits Initiation

To examine the ability of MLN944 to inhibit initiation by RNA polymerase II, short pulse labeling reactions were used. MLN944 has been shown to block the binding of a transcription factor, c-Jun (6). Because MLN944 might be more effective at blocking the binding of the basal transcription factors than inhibiting initiation of preformed preinitiation complexes, the compound was added to reactions before, at the same time, or after formation of preinitiation complexes. Transcription was initiated in the presence of a limiting radiolabeled nucleotide during a 45-second pulse that predominately produced a 15-nucleotide transcript (Fig. 5). As expected the P-TEFb inhibitor, DRB, had no effect on initiation compared with the control under all three conditions tested (Fig. 5). Increasing amounts of MLN944 (0.03–10 μmol/L) caused a decrease in the amount transcripts produced regardless of when the compound was added (Fig. 5). The results were quantitated (Fig. 5E) and transcription initiation from the CMV promoter under all three conditions was inhibited IC_{50}s of ~0.4 μmol/L. This is similar to 0.6 μmol/L calculated earlier for inhibition of the generation of run-off transcripts from the CMV promoter (Fig. 2). Inhibition of initiation was seen at concentrations of MLN944 that had no effect on elongation in the immobilized template transcription assay (Fig. 4), indicating that MLN944 inhibits RNA polymerase II transcription initiation from the CMV promoter before elongation is affected.

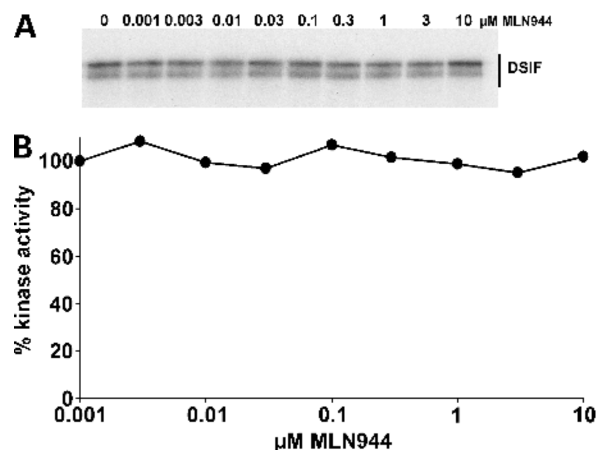

Figure 3. MLN944 is not a P-TEFb inhibitor. **A,** a P-TEFb *in vitro* kinase assay with DSIF as the substrate was done in the presence of the indicated amounts of MLN944. The reactions were resolved on a 7.5% SDS-PAGE gel and subjected to autoradiography. **B,** incorporation of γ-^{32}P-ATP into the largest subunit of DSIF, in three independent experiments, was quantitated with an InstantImager, normalized to the DMSO control, and plotted.

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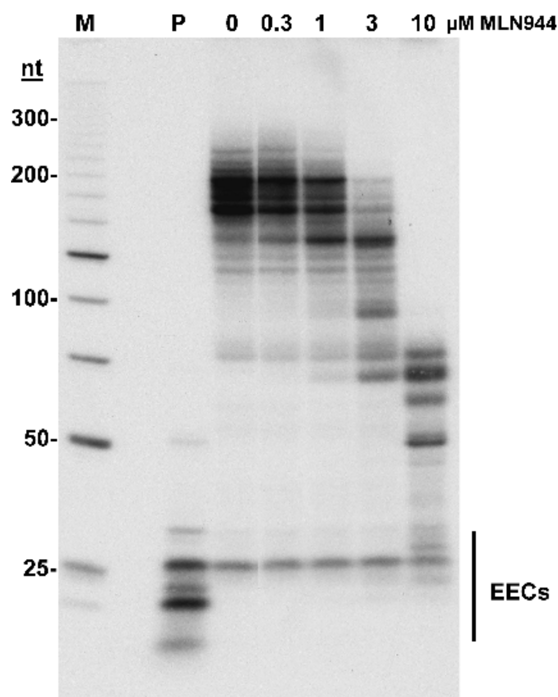

Figure 4. MLN944 inhibits elongation by RNA polymerase II. Isolated early elongation complexes (*EEC*) obtained from a pulse (*P*) labeling were incubated with the indicated amounts of MLN944 for 10 min and allowed to extend transcripts for 5 min. Markers (*M*), 25-bp ladder.

To determine if the effect of MLN944 on initiation was promoter specific, transcription from another template containing the SV40 promoter was analyzed. MLN944 was added after the formation of preinitiation complexes and initiation was allowed to occur during a 45-second pulse. Initiation was strongly inhibited between 1 and 3 μmol/L MLN944 (Fig. 5D). Addition of MNL944 before formation of preinitiation complexes on the SV40 promoter exhibited an identical dose response (data not shown). Transcription initiation from the SV40 promoter was quantitated and an IC_{50} of ~0.9 μmol/L was calculated. The reaction carried out in the presence of 0.1 μmol/L MLN944 was excluded from this calculation, due the poor recovery of the transcripts. The IC_{50} of ~0.9 μmol/L is about 2-fold higher than that found when a CMV template was used. For this experiment, the SV40 template was a restriction digested plasmid rather than a PCR product as was used when the CMV promoter was tested. To compensate for the lower SV40 promoter concentration, more template DNA was added into SV40 transcription reactions and because of this more MLN944 was needed to inhibit transcription to the same level. Supporting this idea, we found that twice as much MLN944 was required to achieve the same level of inhibition of initiation from the CMV promoter, when the total amount of DNA was doubled by addition of plasmid DNA lacking an RNA polymerase II promoter (data not shown). These data strongly suggest that inhibition of initiation by MLN944 is not promoter specific.

Mechanism of Action of MLN944 Is Novel

The effects of MLN944 on transcription initiation and elongation were compared with actinomycin D, a mono-intercalating agent that inhibits RNA polymerase II (8). The generation of short transcripts was examined with increasing amounts of actinomycin D (0. 3–10 μmol/L actinomycin D) added to the template before or after formation of preinitiation complexes. When the compound was added before the formation of preinitiation complexes, inhibition of initiation was first seen at 3 μmol/L and was complete by 10 μmol/L (Fig. 6A). When it was added after the formation of preinitiation complexes similar, but not identical results were obtained (Fig. 6B). Both experiments were quantitated (Fig. 6C), and IC_{50}s were calculated for addition of actinomycin D before (1.6 μmol/L) or after (2.5 μmol/L) preinitiation complex formation. Actinomycin D was more inhibitory when added to the template before formation of preinitiation complexes, in contrast to MLN944 which inhibited equally well whether it was added before, at the same time, or after preinitiation

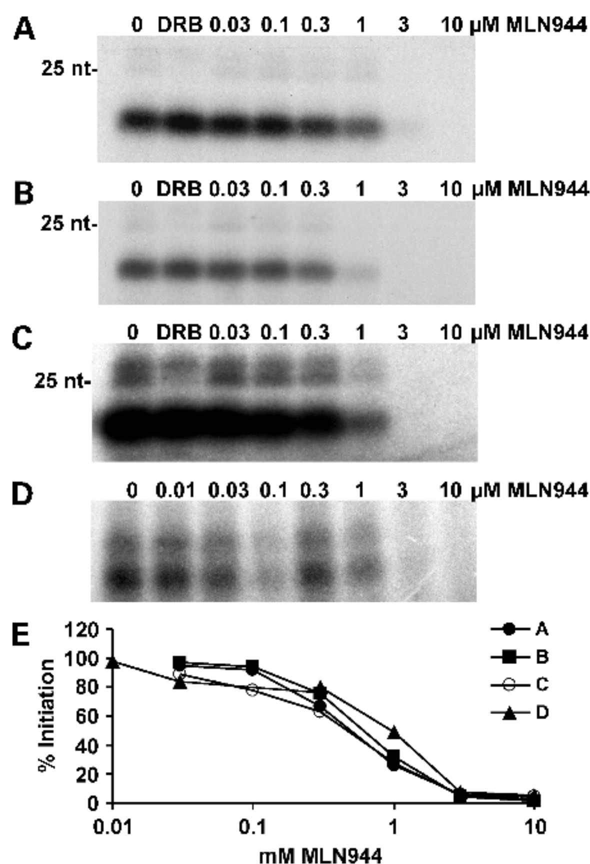

Figure 5. MLN944 inhibits RNA polymerase II transcription initiation. RNA polymerase II transcription initiation was examined by measuring the generation of short transcripts from the CMV promoter. Reactions were carried out in the presence of 50 μmol/L DRB or the indicated amounts of MLN944 added either before (**A**), during (**B**), or after (**C**), formation of preinitiation complexes. **D,** transcription initiation from the SV40 promoter was also examined in the presence of the indicated amounts of MLN944. The RNA was resolved on a 6% urea gel. **E,** short transcripts were quantitated and plotted.

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complex formation. The effect of actinomycin D on RNA polymerase II elongation was examined using an immobilized template assay. Isolated early elongation complexes were incubated for 10 minutes with increasing amounts of actinomycin D (0.01–30 μmol/L) and then chased for 5 minutes. The first of effects of actinomycin D on transcript length were seen at 0.3 μmol/L and became more dramatic as the concentration was increased (Fig. 6D). Actinomycin D inhibited initiation with an IC_{50} of 1.6 to 2.5 μmol/L and began to inhibit elongation at 0.3 μmol/L compared with MLN944 that inhibited initiation with an IC_{50} of 0.4 μmol/L and began to inhibit elongation at 1 μmol/L. Overall, the data indicate that MLN944 preferentially inhibits initiation and that actinomycin D preferentially inhibits elongation.

MLN944 Inhibits Transcription *In vivo*

The ability of MLN944 to inhibit transcription *in vivo* was tested with nuclear run-on assays. Nuclei were isolated from HeLa S3 cells after undergoing a 1-hour treatment of 0, 30, 100, or 300 nmol/L MLN944. Nuclear run-ons were carried out and the amount of radiolabeled UTP incorporated after 10 or 20 minutes was quantitated. Total nuclear transcription was inhibited to below 50% after treatment with 30 nmol/L MLN944 (IC_{50} ~20 nmol/L; Fig. 7A). Nuclear run-ons were also carried out in the presence of 2 μg/mL α-amanitin to inhibit RNA polymerase II allowing for examination of transcription by RNA polymerases I and III. Again, over half of the RNA polymerase I and III transcription was inhibited by 30 nmol/L (IC_{50} ~10 nmol/L; Fig. 7B). Transcription by RNA polymerase II was calculated by subtracting the α-amanitin insensitive counts from the total counts. A dose-dependent decrease of RNA polymerase II transcription was seen with increasing concentrations of MLN944, with approximately half of transcription inhibited at 30 nmol/L (IC_{50} ~30 nmol/L; Fig. 7C). A comparison of the 20-minute time points from run-ons looking at amanitin-resistant and amanitin-sensitive counts suggests that RNA polymerases I and/or III are inhibited at slightly lower concentrations than RNA polymerase II (Fig. 7D). This slight difference may not be significant, but it is clear that MLN944 inhibits cellular transcription at very low concentrations, even after only a short time of treatment.

Discussion

The data presented here clearly show that the bis-intercalator MLN944 inhibits transcription and suggest that this is its primary mechanism of action. Incorporation of radiolabeled precursors into RNA was inhibited by MLN944 in H460 and HCT116 cells with IC_{50}s of 0.3 and 3 nmol/L, respectively. MLN944 inhibited both initiation and elongation by RNA polymerase II during *in vitro* transcription reactions using the CMV promoter and HeLa nuclear extract. Nuclear run-ons determined that all RNA polymerases are inhibited by a short treatment with MLN944 *in vivo*. Although MLN944 and actinomycin D are both intercalators and both inhibit transcription, differences were found in the stage of transcription preferentially affected by the two compounds.

In vitro transcription assays were used to examine the effect of MLN944 on initiation and elongation by RNA polymerase II. MLN944 inhibited initiation by RNA polymerase II with an IC_{50} of 0.4 μmol/L and at higher concentrations directly inhibited elongation of polymerases initiated before drug addition. It is impossible to calculate an IC_{50} for an effect on elongation, but it is clear that concentrations of MLN944 that completely blocked initiation of transcription did not stop elongation. The patterns of transcripts resulting from elongation in the presence of MLN944 suggested that the drug was preferentially binding to specific sequences of the DNA because the pattern was different to that caused by intrinsic pause sites normally found in the template. The mechanism of MLN944 inhibition of initiation was not revealed by our data; however, because the drug had the same effect if added either before or after

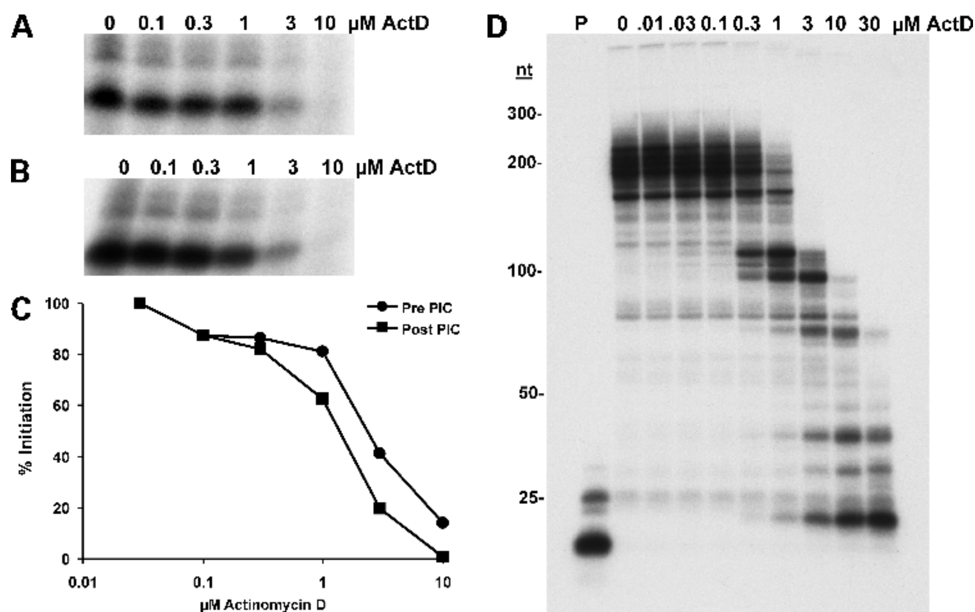

Figure 6. Actinomycin D inhibits transcription elongation. The generation of short transcripts was examined in a transcription initiation assay. **A,** template DNA was incubated with the indicated amounts of actinomycin D for 10 min before HeLa nuclear extract was added for an additional 10-min incubation. **B,** template DNA was incubated with HeLa nuclear extract for 10 min before the indicated amounts of actinomycin D were added and incubated for another 10 min. **C,** quantitation of short transcripts. **D,** isolated elongation complexes obtained from a pulse (*P*) labeling were incubated with the indicated amounts of actinomycin D for 10 min and allowed to extend transcripts for 5 min. **A, B,** and **D,** RNA was isolated, resolved on a 6% urea gel, and subjected to autoradiography. **D,** transcript sizes are indicated.

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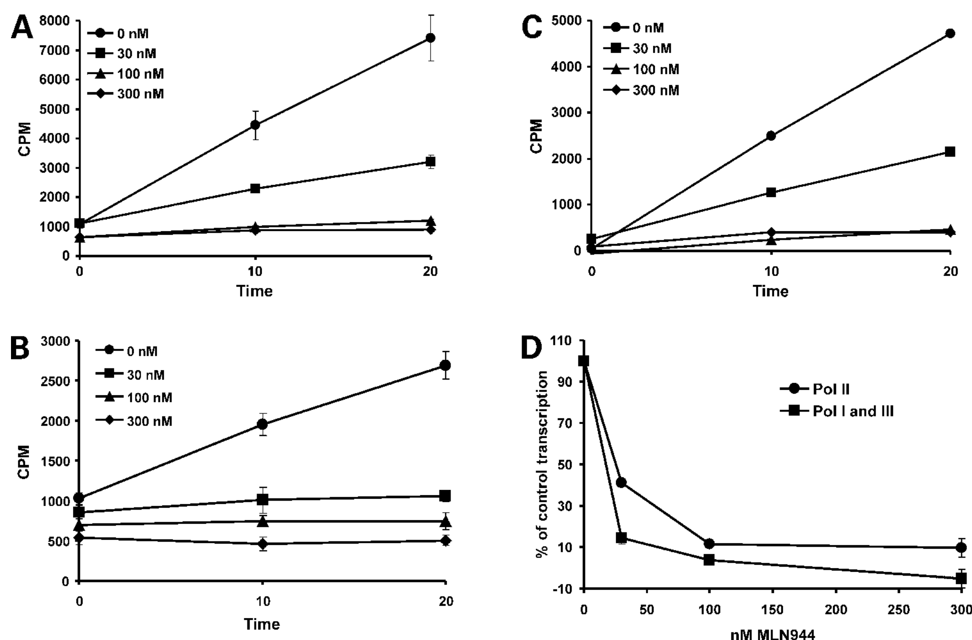

Figure 7. MLN944 inhibits transcription *in vivo*. Nuclei were isolated from HeLa S3 cells that had been treated with increasing amounts of MLN944 (0, 30, 100, or 300 nmol/L) for 1 h. Nuclear run-ons were done, with or without 2 μg/mL α-amanitin, and incorporation of ^{32}P-UTP was quantitated. Inhibition of total (**A**), α-amanitin insensitive (RNA polymerase I and III; **B**), and α-amanitin sensitive (RNA polymerases II; **C**) was examined. **D**, comparison of the effect of MLN944 on transcription by RNA polymerase II with RNA polymerases I and/or III.

preinitiation complex formation, it is not likely that formation of the preinitiation complex is affected. Perhaps the sequences or structures used by RNA polymerase II during abortive initiation, promoter escape, or very early stages of elongation are preferential binding sites for the compound. It is possible that other promoters might have different sensitivities to MLN944, but the SV40 promoter was inhibited in the same manner.

IC_{50}s determined for MLN944 inhibition of transcription *in vivo* and *in vitro* varied widely, but this can be explained by the compound's ability to intercalate DNA. As the ratio of DNA to MLN944 present in each experiment increased, the IC_{50} also increased. The concentration of DNA in the *in vitro* transcription reactions is much higher than in the *in vivo* experiments, and higher concentrations of MLN944 are required for inhibition of transcription *in vitro*. At the IC_{50} for inhibition of transcription *in vitro*, there is one molecule of MLN944 for every 80 bp of DNA, and at the IC_{50} for inhibition of cellular transcription, there is one molecule of MLN944 present for every 50 bp of DNA while the cells are being treated in culture. The MLN944/DNA ratio would suggest that MLN944 is slightly more effective *in vitro*. Although MLN944 is taken up by the cells and concentrated in nuclei, as indicated by their yellow color, it is likely that not all of the compound is removed from the growth medium. Taken together, these results suggest that caution should be taken when interpreting results from different types of experiments. Because the compound is concentrated by cells during long-term treatment, it would take less MLN944 to achieve similar inhibitory effects, especially if the medium containing fresh compound was periodically replaced. This would explain why LD_{50}s were very low (0.04–0.4 nmol/L) in initial experiments with Chinese hamster ovary and Jurkat cells that received long-term treatments (2).

The effects of actinomycin D on initiation and elongation by RNA polymerase II were compared with those of MLN944. Actinomycin D had its first effect on elongation at 0.3 μmol/L, whereas MLN944 did not have an effect until 1 μmol/L. Comparison of the pattern of transcripts obtained with both compounds indicated that the induced pause sites were different from each other and different from the intrinsic sites on the template (compare Fig. 4 with Fig. 6D). Therefore, the effect of either compound on elongation will likely be dependent on the sequence of the template. Using the CMV promoter MLN944 was about five times more effective at blocking initiation than actinomycin D (IC_{50}s of 0.4 and 2 μmol/L, respectively). At other promoters, the difference between the effect of MLN944 and actinomycin D might be more or less pronounced.

Our results support a model in which MLN944 could generally inhibit transcription; however, it is likely that at low concentrations the compound might alter expression of specific genes. This could occur through sequence specific binding of MLN944 leading to decreased initiation, an effect on chromatin structure, or to an effect on activation or repression of transcription by blocking interaction of regulatory molecules with their target sequences. This latter possibility is supported by the finding that MLN944 can block c-Jun binding to activator protein, but not nuclear factor-κB binding to its target sequence *in vitro* (6). Supporting the idea that MLN944 might alter expression of a subset of genes, Sappal et al. found that in mice treated with the compound, xenographic colon tumors expressed a different pattern of genes than those in untreated mice or mice given the topoisomerase inhibitor, CPT-11 (5). In this way, MLN944 may selectively inhibit transcription of rapidly dividing tumor cells before the general effects on transcription are seen in noncancerous cells.

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