The Bis(naphthalimide) DMP-840 Causes Cytotoxicity by Its Action against Eukaryotic Topoisomerase II†

John L. Nitiss,*,‡,§ Junfang Zhou,‡ Angela Rose,‡ Yuchu Hsiung,§,|| Kevin C. Gale,⊥,¶ and Neil Osheroff#

Molecular Pharmacology Department, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, Department of Biochemistry and Molecular Biology, University of Southern California Medical School, Los Angeles, California 90033, DuPont Merck Pharmaceutical Co., Experimental Station, Wilmington, Delaware 19880-0336, and Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

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ABSTRACT: DMP 840 ((*R*,*R*)-2,2′-[1,2-ethanediylbis[imino(1-methyl-2,1-ethanediyl)]-bis(5-nitro-1*H*-benz-[*de*]isoquinoline-1,3(2*H*)-dione] dimethanesulfonate) is a novel bis(naphthalimide) that has shown promising antitumor activity in a variety of preclinical model systems. The compound binds to DNA with high affinity and intercalates, but the mechanism of cell killing has not been elucidated. We have used yeast strains to test whether DMP-840 is active against either topoisomerase I or II. We found that temperature-sensitive *top2* mutants resistant to etoposide or amsacrine also confer resistance to DMP-840. In addition, cells overexpressing yeast topoisomerase II were hypersensitive to the drug. By contrast, *top1* deletions rendered cells hypersensitive to the drug. These results strongly suggest that DMP-840 acts against eukaryotic topoisomerase II and kills cells by converting the enzyme into a cellular poison. We verified that DMP-840 is active against eukaryotic topoisomerase II by demonstrating that the drug stimulates formation of a cleavage complex with purified yeast topoisomerase II in vitro. We also demonstrated that the drug is active against human topoisomerase II by showing that expression of human topoisomerase II restored sensitivity of resistant yeast cells to DMP-840. We have also directly demonstrated that DMP-840 acts as a poison against purified human topoisomerase II α. Taken together, these results indicate that DMP-840 acts like other intercalating topoisomerase II poisons; it kills eukaryotic cells by stabilizing the cleavage complex of topoisomerase II with DNA.

DNA topoisomerases are the target of a wide range of antitumor agents. Drugs that are active against topoisomerase I, topoisomerase II, or both enzymes have been identified (*1*−*3*). Most of the characterized agents that are active against topoisomerase I are derivatives of the plant alkaloid camptothecin, while a range of structurally diverse compounds are active against eukaryotic topoisomerase II. Potent intercalating agents such as doxorubicin, mitoxantrone, and amsacrine (*4*−*8*) as well as nonintercalating agents such as epipodophyllotoxins (*9*) and fluoroquinolones (*10, 11*) are active against eukaryotic topoisomerase II.

Among the intercalating topoisomerase II agents tested in clinical trials are naphthalimides, which include amonafide and mitonafide (*12*−*15*). Recently, related bis-intercalating agents have been synthesized (*16, 17*), which are more potent intercalating agents (*18*), and which have high antitumor activity in preclinical model systems (*16, 19, 20*). The bis(naphthalimides) have stimulated special interest since they have activity against tumors in model systems that are refractory to many other antitumor agents (*16, 19*). DMP-840, a bis(naphthalimide) has been the focus of several preclinical and clinical investigations. The structure of this compound is shown in Figure 1.

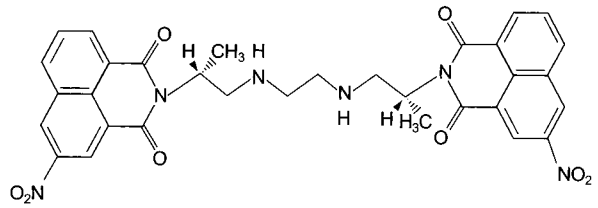

FIGURE 1: Structure of DMP-840. The structure of the bis(naphthalimide) DMP-840 is shown.

The mechanisms of cell killing by DMP-840 have remained undefined. This agent appears to have a unique pattern of cross-resistance. For example, Houghton and coworkers found only partial resistance to DMP-840 in cell lines resistant to vincristine or topotecan. In some cases, cells selected for topotecan resistance were cross-resistant

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* Author to whom correspondence should be addressed at Molecular Pharmacology Department, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38101. Telephone: 901-495-2794. Fax: 901-521-1668.

‡ St. Jude Children's Research Hospital.

§ University of Southern California Medical School.

|| Present address: Scripp's Research Institute, La Jolla, CA 92037.

⊥ DuPont Merck Pharmaceutical Co.

¶ Present address: Molecular Systems, Pharmacia Biotech, Inc., Piscataway, NJ 08855.

Vanderbilt University School of Medicine.

[1] Abbreviations: DMP-840; (*R*,*R*)-2,2′-[1,2-ethanediylbis[imino(1-methyl-2,1-ethanediyl)]-bis(5-nitro-1*H*-benz[*de*]isoquinoline-1,3(2*H*)-dione] dimethanesulfonate; SEM, standard error of the mean.

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to DMP-840, although in other topotecan-resistant cell lines, cross-resistance to DMP-840 was not observed (*19*). In addition, Kirshenbaum and colleagues failed to observe cross-resistance to DMP-840 in a CHO cell line selected for high levels of resistance to camptothecin (*21*).

Studies in *Escherichia coli* have suggested that an action of DMP 840 may be inhibition of some enzymes involved in DNA metabolism and DNA repair (*22*). While *E. coli* mutants defective in DNA ligase or the *uvrA* gene product (a subunit of the excision endonuclease) have wild-type sensitivity to DMP 840, mutants lacking both exoIII and endoIV (which both have apurinic endonuclease activity) are very hypersensitive to the drug (*43*). This result might suggest that DMP 840 forms a DNA adduct that is repairable by these enzymes. A more recent study examined *E. coli* proteins whose overexpression leads to DMP-840 resistance. One class of plasmids carried the region that includes *parC* and *parE*, the subunits of *E. coli* topoisomerase IV (*23*). Topoisomerase IV is a type II topoisomerase that is required for normal chromosome segregation (*24*) (for review, see ref *25*). The resistance to DMP-840 appears to be mediated by a gene that maps between *parC* and *parE*, rather than due to increased expression of the subunits of topoisomerase IV (*23*).

To more clearly define the in vivo targets of DMP-840, we have taken advantage of a yeast system that allows us to dissect the mechanism of action of agents that are active against topoisomerase I or topoisomerase II. We demonstrate here that DMP-840 is able to stabilize cleavage by purified yeast topoisomerase II in vitro, and that replacement of wild-type topoisomerase II with a drug-resistant allele is sufficient to abolish the cytotoxicity of DMP-840 in yeast. By contrast, *top1* mutants retain drug sensitivity. We also show that expression of human topoisomerase II α in yeast confers sensitivity to DMP-840. Taken together, our results indicate that topoisomerase II is the major target for the cytotoxic action of DMP-840 in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains. Plasmid pMJ1, which expresses human topoisomerase II α from the yeast *TOP1* promoter, has been described previously (*26*). The yeast strains JN394, JN394t1, JN394t2−4, and JN394t2−5 have all been previously described (*27, 28*). The strains are isogenic and carry the *ISE2* mutation (to increase drug permeability) and a disruption of the *rad52* gene (to increase sensitivity to anti-topoisomerase drugs). JN394t1 carries a deletion of the yeast *TOP1* gene, while JN394t2−4 and JN394t2−5 carry the *top2−4* and *top2−5* alleles of topoisomerase II, respectively, in place of wild-type *TOP2* (*27, 28*).

Determination of in Vivo Drug Sensitivity. Drug sensitivity studies in yeast cells were carried out as previously described (*27, 28*). Briefly, a logarithmically growing culture of yeast cells was diluted to 2×10^6 cells/mL, and a drug or DMSO was added. Aliquots were removed, diluted, and plated to YPDA agar. Survival is expressed relative to the number of viable colonies at the time of drug addition.

Quantitation of Drug-Stabilized Topoisomerase II−DNA Covalent Complexes. Levels of DNA covalent complexes were determined using a previously described modification of the K^+/SDS method (*28, 29*). Briefly, pUC18 DNA was linearized with the restriction endonuclease *Eco*RI and end-labeled by filling in with [α-^{32}P]dATP using the Klenow fragment of *E. coli* DNA polymerase I. The specific activity of the labeled DNA was $(1-3) \times 10^6$ cpm/μg DNA. Approximately $5-10 \times 10^5$ cpm (350 ng) of labeled DNA substrate was added per reaction. The cleavage reactions were performed in triplicate, and results are reported ±SEM. The cleavage reaction was carried out in TOP2 buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 30 μg/mL acetylated bovine serum albumin, 0.5 mM ATP) in a total volume of 50 μL, with 10 units (500 ng) of purified yeast topoisomerase II (*30*) or human topoisomerase II α (*31*) prepared as previously described. The reaction was incubated for 30 min and then stopped with 1 mL of stop buffer (1.25% SDS, 5 mM EDTA, pH 8.0, 0.4 mg/mL salmon sperm DNA). Then 250 μL of 325 mM KCl was added, and the reaction was incubated at 65 °C for 10 min. The reaction mixtures were placed on ice for 10 min, followed by centrifugation in a microcentrifuge at 15000*g* for 10 min. The supernatant was removed, and the samples were resuspended by adding 1 mL of wash buffer (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 1 mg/mL salmon sperm DNA) at 65 °C. Samples were further incubated at 65 °C for 10 min, placed on ice for 10 min, and sedimented as above. The wash procedure was carried out a total of three times. After the final wash, samples were resuspended in 400 μL of water at 65 °C. A volume of 100 μL of sample was removed and added to 4 mL of scintillation fluid (Aquasol); total radioactivity was determined by scintillation counting.

Determination of Patterns of Drug-Induced Cleavage with Purified DNA Topoisomerases. Samples for determination of cleavage patterns were treated as described for quantitation of drug-stabilized topoisomerase II−DNA covalent complexes, except that topoisomerase reactions contained 1 μg of purified topoisomerase protein (either yeast or human topoisomerase II α). The substrate DNA was digested with *Bam*HI after labeling so that the substrate for the drug-induced cleavage pattern experiments was uniquely end labeled. After all the washes, the pellet was resuspended in 400 μL of 10 mM Tris-HCl pH 7.6, 1 mM $CaCl_2$, 1.6 units proteinase K (Sigma) and was incubated overnight at 55 °C. After proteinase K digestion, the samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The pellet was resuspended in 400 μL H_2O, and an aliquot was removed for scintillation counting. A 20 μL portion was loaded onto an 8% polyacrylamide gel and subjected to electrophoresis at 220 V for 3.5−4 h. The gel was dried and exposed to film at −80 °C overnight.

RESULTS

Sensitivity of Yeast Cells to DMP-840. We first examined the sensitivity of DMP-840 in JN394, a strain carrying wild-type topoisomerase I and II. Strain JN394 carries the *ISE2* mutation for enhanced drug permeability and a disruption of the *RAD52* gene, which confers hypersensitivity to a wide range of DNA damaging agents. *rad52* mutants are hypersensitive to drugs that stabilize cleavage by either topoisomerase I or topoisomerase II (*32, 42*). JN394 cells were incubated in YPDA medium with various concentrations of

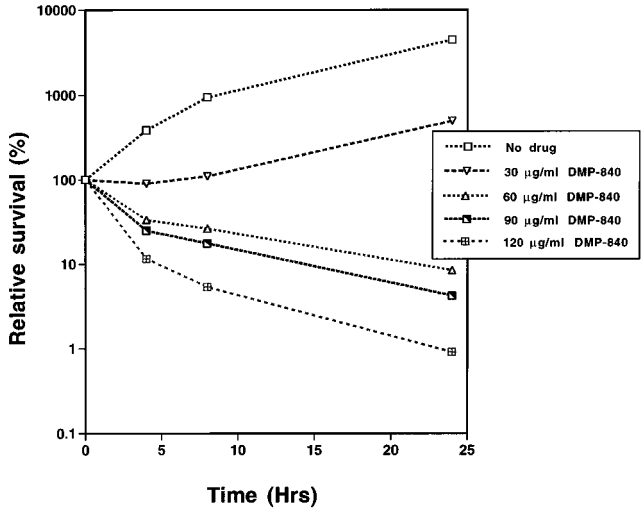

FIGURE 2: Survival of JN394 cells treated with DMP-840. JN394 (*TOP1*$^+$ *TOP2*$^+$) cells were treated with different concentrations of DMP-840. Aliquots were removed at the indicated times, diluted, and plated to YPDA agar. Survival is expressed relative to the time of drug addition. The conditions were: no drug (open squares), 30 μg/mL (down triangles), 60 μg/mL (up triangles), 90 μg/mL (half-filled squares), and 120 μg/mL (plus squares).

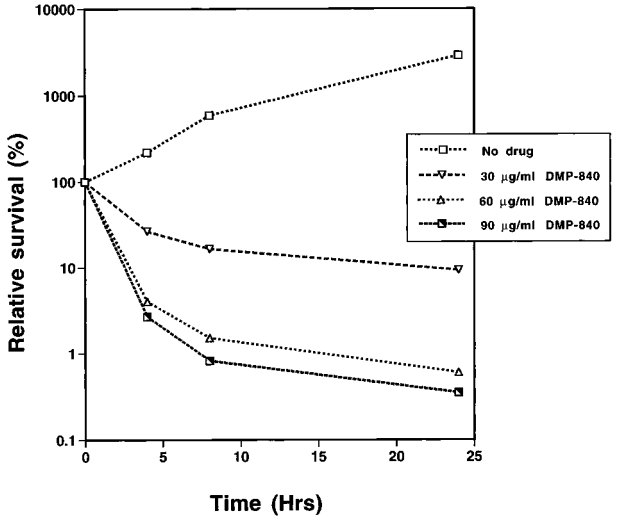

FIGURE 3: Survival of JN394 pDEDTOP2 cells treated with DMP-840. JN394 cells were transformed with pDED1TOP2, a plasmid that overexpresses yeast topoisomerase II, and sensitivity to DMP-840 was determined as in Figure 2. The conditions were: no drug (open squares), 30 μg/mL (down triangles), 60 μg/mL (up triangles), and 90 μg/mL (half-filled squares).

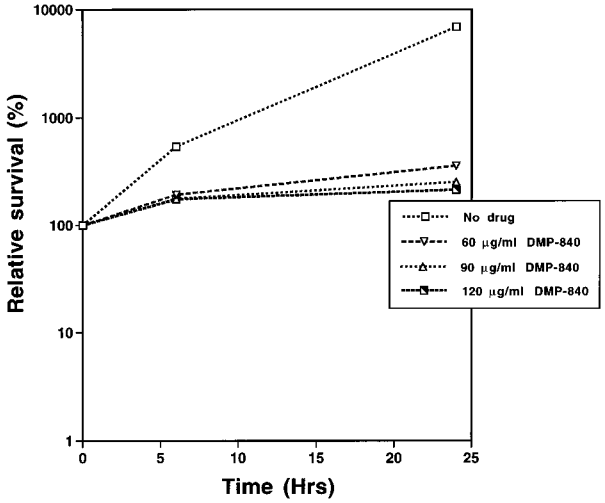

FIGURE 4: Survival of JN394t2−5 cells treated with DMP-840. JN394t2−5 (*TOP1*$^+$ *top2−5*) cells were treated with DMP-840 using the same conditions as in Figure 2. The conditions were: no drug (open squares), 60 μg/mL (down triangles), 90 μg/mL (up triangles), and 120 μg/mL (half-filled squares).

DMP-840. After incubation for 4, 8, and 24 h, aliquots were removed, diluted, and plated to YPDA plates to determine viable counts. Results of a representative experiment are shown in Figure 2. DMP-840 concentrations of 60 μg/mL or higher were cytotoxic to JN394 cells, while lower drug concentrations reduce the growth rate but did not reduce the viability of the cells below the value at the start of the experiment.

Since JN394 cells were sensitive to DMP-840, we examined the sensitivity of isogenic derivatives of JN394 that carry mutations in topoisomerase genes. We first examined JN394t1 cells, which carry a deletion of the yeast *TOP1* gene. The deletion of the *TOP1* gene renders the cells totally resistant to topoisomerase I inhibitors such as camptothecin (*32*, *33*) and confers hypersensitivity to anti-topoisomerase II agents. *top1*$^-$ cells are hypersensitive to anti-topoisomerase II agents because the topoisomerase II must perform all of the functions during replication and transcription that normally utilize topoisomerase I (*25*, *34*). JN394t1 cells have enhanced sensitivity to DMP-840, compared to JN394 cells. A drug concentration of 20 μg/mL almost completely inhibits growth, and 30 μg/mL reduces viability to <20% after 24 h incubation with drug. Higher drug concentrations lead to greater reductions in viability (data not shown). The pattern of hypersensitivity in *top1* mutants is the opposite of what is observed with drugs that stabilize covalent complexes with topoisomerase I, indicating that DMP-840 does not stabilize covalent complexes formed with the type I enzyme.

Yeast cells overexpressing topoisomerase II are hypersensitive to topoisomerase II inhibitors that stabilize cleavage, such as amsacrine and etoposide. We therefore examined the effect of topoisomerase II overexpression on the sensitivity of yeast cells to DMP-840. JN394t2−4 cells carrying pDED1TOP2 (*27*) were exposed to DMP-840. The results are shown in Figure 3. Cells overexpressing topoisomerase II had reduced viability at 30 μg/mL DMP-840, with higher drug concentrations leading to greater cytotoxicity. Cells without topoisomerase II overexpression are able to grow at a reduced rate when exposed to 30 μg/mL DMP-840 (Figure 2), hence overexpression of topoisomerase II sensitizes cells to DMP-840. Hypersensitivity to DMP-840 when topoisomerase II is overexpressed is consistent with the hypothesis that the agent is able to stabilize topoisomerase II−DNA covalent complexes (*27*).

As a further test of the hypothesis that DMP-840 stabilizes topoisomerase II−DNA covalent complexes, we determined the specificity of DMP-840 action against yeast topoisomerase II by examining drug sensitivity in a yeast strain carrying a drug-resistant topoisomerase II. We have shown previously that yeast strains carrying the *top2−5* mutation were highly resistant to amsacrine, etoposide, mitoxantrone, and other topoisomerase II inhibitors (*28*, *35*, *36*). Figure 4 shows that JN394t2−5 cells were highly resistant to DMP-840, compared to strains carrying a wild-type topoisomerase

Table 1: Relative Cleavage by DMP-840 with Purified Yeast Topoisomerase II

DMP-840 concentration (μg/mL)	relative cleavage (μg/mL)	SEM
0	1	0.020
0.5	2.2	0.28
2	4.7	0.074
5	0.68	0.050
10	0.39	0.039

Table 2: Relative Cleavage by Mitoxantrone with Purified Yeast Topoisomerase II

mitoxantrone concentration (μg/mL)	relative cleavage	SEM
0	1	0.030
0.02	2.9	0.097
0.1	6.0	0.060
0.5	5.8	0.22
2.0	0.22	0.0097

II. Cells carrying the *top2−5* mutation were able to grow in the presence of various concentrations of drug up to 120 μg/mL DMP-840, although the growth rate is reduced. The substitution of a drug-resistant allele for a drug-sensitive allele of topoisomerase II is sufficient to confer resistance to DMP-840; therefore, it appears that topoisomerase II is a major target for cell killing by this drug.

We previously showed that introduction of a wild-type topoisomerase II gene into JN394t2−5 restores sensitivity to amsacrine and etoposide, indicating that this allele confers recessive resistance to these drugs (*28*). We carried out a similar analysis with DMP-840. As was observed with etoposide and amsacrine, introduction of a wild-type yeast TOP2 gene into JN394t2−5 completely restores sensitivity to DMP-840 (data not shown). This result is consistent with DMP-840 stabilizing topoisomerase II covalent complexes, rather than acting as a catalytic inhibitor of topoisomerase II.

Action of DMP-840 against Purified Yeast Topoisomerase II. The results with the different topoisomerase mutant strains suggest that DNA topoisomerase II is the principal cytotoxic target of DMP-840 in eukaryotic cells. To further demonstrate that the target of DMP-840 is topoisomerase II, we examined the sensitivity of purified yeast topoisomerase II to this agent. A K^+/SDS cleavage assay was used with purified yeast topoisomerase II to determine the levels of topoisomerase II−DNA covalent complexes formed in the absence and presence of DMP-840. Table 1 shows the level of precipitated DNA when the K^+/SDS assay is carried out in the presence of different concentrations of DMP-840. A drug concentration of 0.5 μg/mL increases the amount of precipitated DNA by approximately 2-fold, with the maximum level of complex formation at 2 μg/mL DMP-840. As shown previously for other strong intercalating agents (*5*), higher drug concentrations reduce the level of covalent complex formed with topoisomerase II.

We compared the reduction in covalent complex observed with DMP-840 with another strong intercalating drug, mitoxantrone. Similar to what we observed with DMP-840, incubation of purified topoisomerase II with mitoxantrone causes an increased level of topoisomerase II−DNA covalent complexes, with a fairly sharp maximum. Mitoxantrone concentrations greater than about 0.5 μg/mL reduce the level of covalent complexes below the level seen in the absence of drug (Table 2). Hence, DMP-840 shows a dose/response pattern similar to that of other known intercalating topoisomerase II agents.

We also tested the ability of DMP-840 to inhibit covalent complex formation by etoposide. Strong intercalating agents have been shown to block covalent complex formation by both intercalating and nonintercalating topoisomerase II inhibitors. DMP-840 at 5 μg/mL blocks stimulation of covalent complex formation with topoisomerase II by the presence of etoposide (Figure 5). This result further shows that DMP-840 acts similarly to other intercalating topoisomerase II inhibitors.

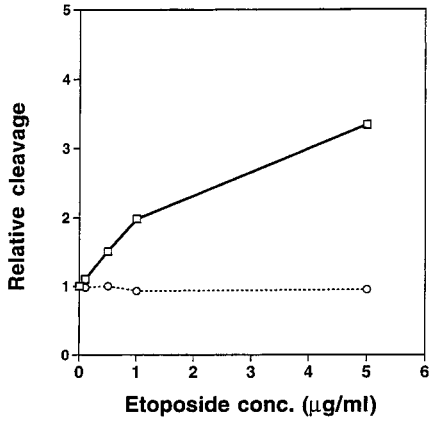

FIGURE 5: Suppression of etoposide-mediated cleavage by DMP-840. Etoposide-mediated cleavage of pUC18 DNA in the absence (open squares) or presence (open circles) of 5 μg/mL DMP-840. Cleavage was expressed relative to samples lacking drug.

We next compared the effect of DMP-840 with amonafide, a mono-intercalating isoquinoline that is chemically similar to DMP-840. We examined the patterns of cleavage using uniquely end-labeled pUC18 DNA. Figure 6A shows the patterns of drug-induced cleavage with yeast topoisomerase II and either DMP-840 or amonafide. DMP-840 shows a distinct cleavage pattern (Figure 6A, lanes 2−4) compared to either amonafide (Figure 6A, lanes 6−8) or etoposide (Figure 6A, lane 10). There are several strong cleavage sites present in the amonafide-treated samples that are absent in samples treated with DMP-840, while several cleavage sites observed with DMP-840 are absent in both the amonafide- and etoposide-treated samples. Amonafide has been previously characterized as having distinct sequence preferences for cleavage, favoring C at the −1 position, disfavoring G or T at this position, and favoring A at the +1 position (*13*). By contrast, DMP-840 preferentially binds in runs of G/C base pairs (*18*). Figure 6B shows the quantitative levels of cleavage of the samples shown in Figure 6A. While DMP-840 shows a sharp maximum level of cleavage at slightly less than 1 μM drug and strong inhibition of cleavage at 10 μM DMP-840, amonafide requires much greater concentrations to inhibit cleavage, with inhibition seen only at the highest concentration tested (357 μM, 100 μg/mL). This result is consistent with the strong intercalation seen with DMP-840 and the potent inhibition of etoposide-mediated cleavage by DMP-840 shown in Figure 6.

DMP-840 Is Active against Human Topoisomerase II α *Expressed in Yeast.* Expression of human topoisomerase II α in yeast cells can complement lethal mutations in the yeast

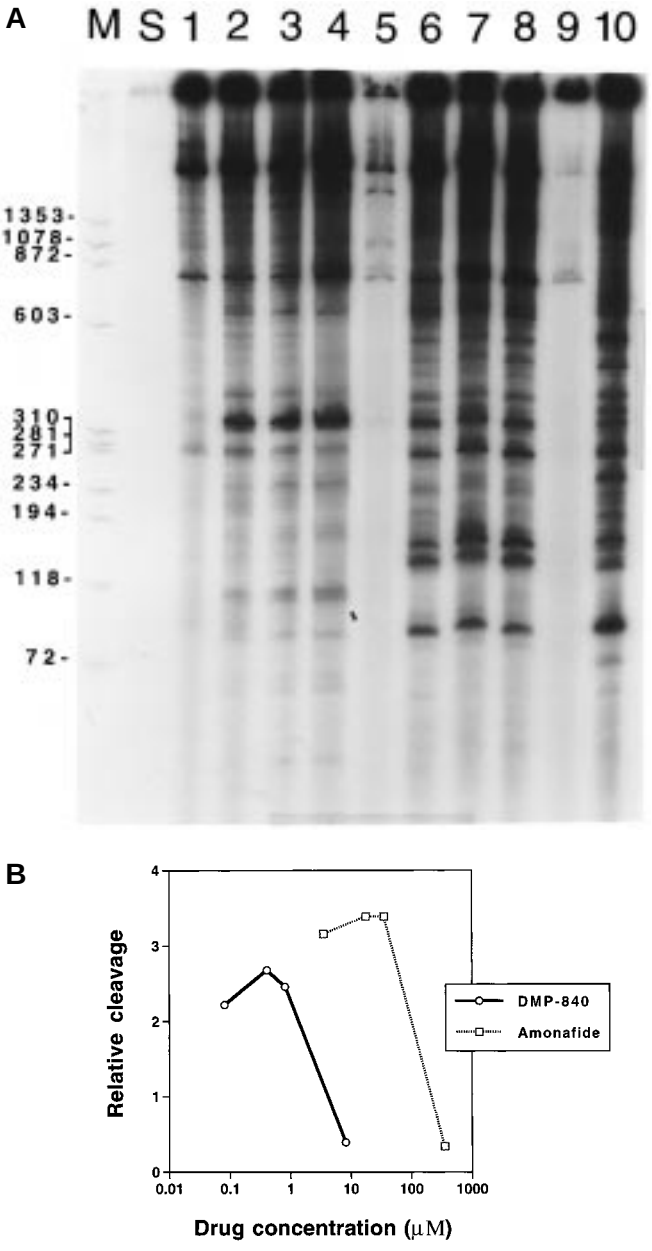

FIGURE 6: Comparison of DMP-840 and amonafide-stabilized cleavage with yeast topoisomerase II. Reactions containing yeast topoiosmerase II were performed using end-labeled pUC18 DNA in the presence of either DMP-840 or amonafide. Figure 6A shows a representative gel with cleavage reactions containing DMP-840 or amonafide. An aliquot of each reaction mixture was counted using scintillation counting, and the results are shown in Figure 6B. Note that the samples loaded on the gel were precipitated using the K^+/SDS method, so only DNA that was protein linked will be visible. Samples were treated with proteinase K prior to electrophoresis as described in Experimental Procedures. The samples in 6A were as follows: M, molecular weight markers; S, substrate alone (no added protein); lane 1, topoisomerase II only; lanes 2−10, topoisomerase II plus (lane 2) 0.1 μg/mL (0.122 μM) DMP-840, (lane 3) 0.5 μg/mL (0.613 μM) DMP-840, (lane 4) 1 μg/mL (1.22 μM) DMP-840, (lane 5) 10 μg/mL (12.2 μM) DMP-840, (lane 6) 1 μg/mL (3.53 μM) amonafide, (lane 7) 5 μg/mL (17.7 μM) amonafide, (lane 8) 10 μg/mL (35.3 μM) amonafide, (lane 9) 100 μg/mL (353 μM) amonafide, and (lane 10) 10 μg/mL etoposide. The molecular weights of the markers (in bp) are indicated to the left of the figure. Figure 6B shows the quantitation of the DMP-840-treated samples (open circles) and amonafide-treated samples (open squares) relative to samples treated in the absence of drug. A logarithmic scale is shown to facilitate comparison between the two agents.

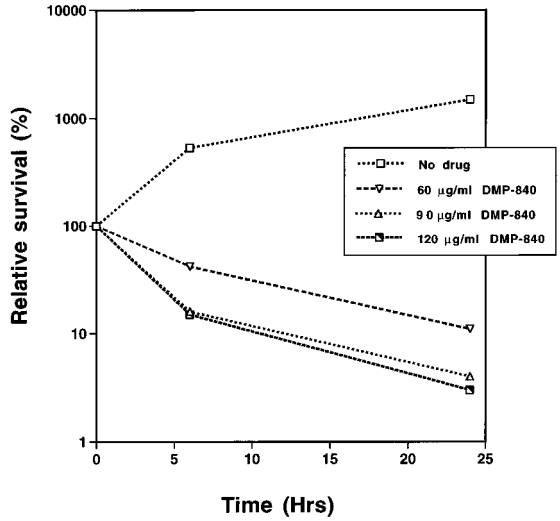

FIGURE 7: DMP-840 is active against yeast cells expressing human topoisomerase II α. JN394t2−4 cells were transformed with pMJ1, a plasmid expressing human topoisomerase II α from the yeast *TOP1* promoter. Drug sensitivity was determined at 34 °C. The conditions were the following: no drug (open squares), 30 μg/mL (down triangles), 60 μg/mL (up triangles), and 90 μg/mL (half-filled squares).

TOP2 gene (*26*, *31*). Furthermore, expression of wild-type human topoisomerase II restores drug sensitivity to yeast cells that carry a drug-insensitive topoisomerase II allele (*26*). We examined the sensitivity of human topoisomerase II α to DMP-840 using strain JN394t2−4 carrying the plasmid pMJ1. The strain carries the *top2−4* allele of yeast topoisomerase II, which is nonfunctional at 34 °C. The plasmid pMJ1 has the entire human topoisomerase II α gene under the control of the yeast *TOP1* promoter. This plasmid complements the *top2−4* mutation and allows growth at 34 °C. Drug sensitivity at 34 °C is due solely to the human topoisomerase II α. As shown in Figure 7, yeast cells expressing human topoisomerase II α were killed by 60 μg/mL DMP-840, with higher concentrations causing greater cytotoxicity. This result suggests that DMP-840 is also an effective inhibitor of human topoisomerase II α.

DMP-840 Is Active against Purified Human Topoisomerase II α. We confirmed the result suggested by the experiment shown in Figure 8 by examining cleavage using purified human topoisomerase II α. Human topoisomerase II α was purified by overexpression of the protein in yeast (*31*). The effect of DMP-840 was assessed both by examination of the cleavage pattern with human topoisomerase and quantitation of cleavage (Figures 8A and B). The pattern of drug-stabilized cleavage was similar between yeast and human topoisomerase II. Similar to the results obtained with yeast topoisomerase II, the human enzyme shows a sharp maximum at about 1 μg/mL DMP-840; however, the human enzyme is somewhat less sensitive to inhibition by this drug. Taken with the results shown in Figure 7, these experiments demonstrate that DMP-840 is a specific inhibitor of human topoisomerase II.

DISCUSSION

In this work, we have applied a yeast genetic system to determine how DMP-840 kills eukaryotic cells. Our results clearly demonstrate that DMP-840 is highly specific for

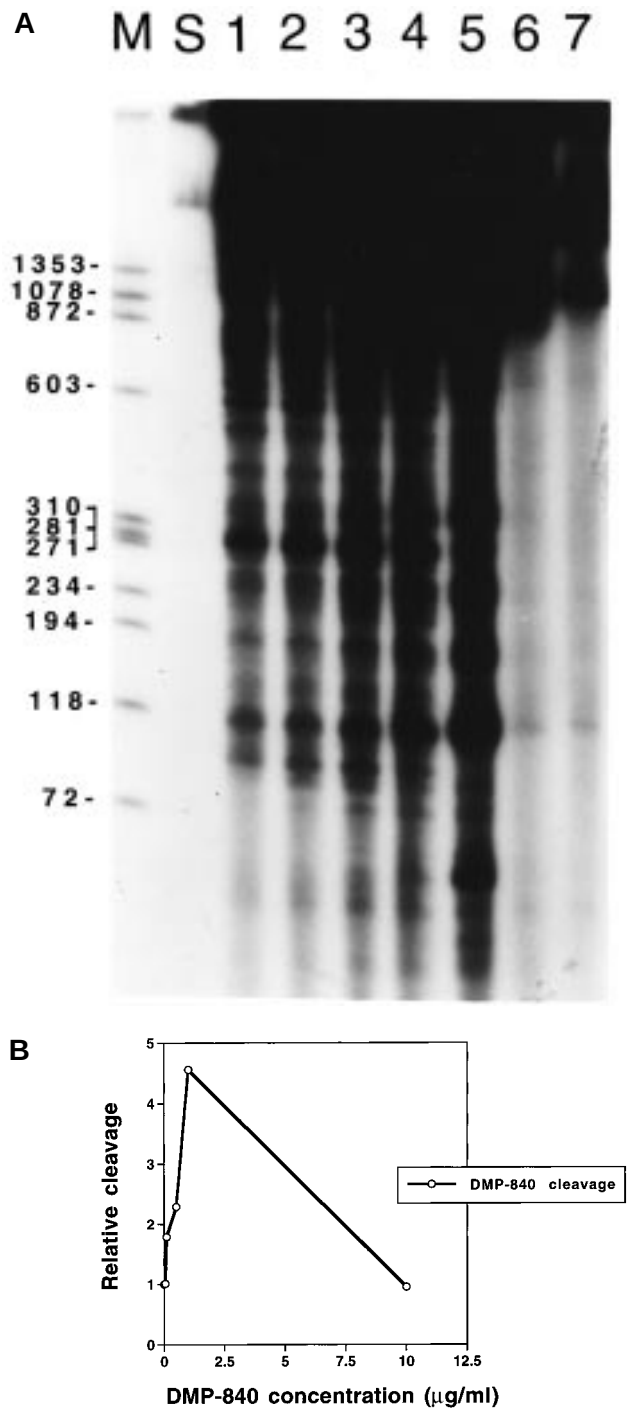

FIGURE 8: DMP-840 is active against purified human topoisomerase II α. Recombinant human topoisomerase II was used to examine whether DMP-840 could stabilize cleavage with this enzyme. Figure 8A shows an autoradiogram of a polyacrylamide gel with cleavage reactions containing DMP-840. An aliquot of each reaction was counted by scintillation counting, and the results are shown in Figure 8B. Conditions are as in Figure 6, except human topoisomerase II was substituted for yeast topoisomerase II. The samples in 8A were as follows: M, molecular weight markers; S, substrate alone (no added protein); lane 1, human topoisomerase II only; lanes 2−7, human topoisomerase II plus (lane 2) 0.05 μg/mL (0.0613 μM) DMP-840, (lane 3) 0.1 μg/mL (0.122 μM) DMP-840, (lane 4) 0.5 μg/mL (0.613 μM) DMP-840, (lane 5) 1 μg/mL (1.22 μM) DMP-840, (lane 6) 10 μg/mL (12.2 μM) DMP-840, and (lane 7) 100 μg/mL (122 μM) DMP-840. The molecular weights of the markers (in bp) are indicated to the left of the figure shows the quantitation of the samples, relative to the sample without DMP-840. For clarity, the 100 μg/mL sample is not shown in Figure 8B. The relative cleavage of the 100 μg/mL sample was 0.82.

topoisomerase II. Previous work had shown that this agent is a strong intercalating agent, and topoisomerase II-independent reduction of cell growth is likely due to the inhibition of other enzymes that act on DNA. However, the major mechanism of cell killing, and therefore the antitumor activity of this agent, is likely to be due to stabilization of topoisomerase II−DNA covalent complexes.

The yeast strains we have constructed yield a unique pattern of drug sensitivity, depending on the enzyme inhibited and the mechanism of action of the drug. The yeast cells with wild-type topoisomerase I and topoisomerase II (strain JN394) are sensitive to DMP840, indicating that the drug enters the cells and that yeast cells are sensitive to the action of the drug. For a complex stabilizing topoisomerase II inhibitor, it is expected that overexpression of topoisomerase II would increase cell killing, and as shown in Figure 3, that is what was observed. If DMP-840 was a catalytic inhibitor, such as ICRF-193, the opposite pattern would have been observed, i.e., overexpression of topoisomerase II would lead to drug resistance. The observed drug hypersensitivity observed when topoisomerase II is overexpressed favors topoisomerase II poisoning a cell killing mechanism. If the drug were a complex stabilizing topoisomerase I inhibitor like camptothecin, then a strain completely lacking *TOP1* would be resistant to the drug. Strains deleted for *TOP1* shows the opposite result: *top1*$^-$ mutants are DMP-840 hypersensitive (data not shown). We have previously observed that Δ*top1* mutants are hypersensitive to drugs targeting topoisomerase II. The sensitivity to DMP-840 observed in Δ*top1* mutants indicated that this drug does not stabilize covalent complexes between topoisomerase I and DNA. Finally, the replacement of wild-type topoisomerase II with a drug-resistant allele, *top2−5*, completely abolished cytotoxicity, although the growth rate was reduced in the presence of DMP-840. These results indicate that the major cytotoxic target of DMP-840 in yeast is topoisomerase II.

It is important to demonstrate that a topoisomerase-targeting drug has the same effect in vitro with purified enzyme as it has in vivo. The K^+/SDS assay clearly showed that DMP-840 can stimulate covalent complex formation with either yeast topoisomerase II or human topoisomerase II α. The maximal level of cleavage observed with DMP-840 was very similar to the maximal level observed with mitoxantrone, another intercalating topoisomerase II inhibitor. Like mitoxantrone, DMP-840 can stabilize covalent complexes with topoisomerase II over a fairly narrow range of concentrations. By contrast, the monointercalating drug amonafide, which is a relatively weaker intercalating agent, is active over a much broader range of concentrations. Our results with mitoxantrone and DMP-840 underline the importance of examining putative topoisomerase inhibitors, especially strong DNA binding agents, over a wide range of concentrations.

A unique tool available in yeast is the ectopic expression of human topoisomerases (*26*, *31*, *37*). Plasmids expressing human topoisomerase I, topoisomerase II α, or topoisomerase II β have been described. We have utilized a topoisomerase II α-expressing plasmid to demonstrate that DMP-840 is also active against human topoisomerase II α. In a previous publication, we demonstrated that JN394t2−4 cells, expressing topoisomerase II α from the yeast *TOP1* promoter, are about as sensitive to etoposide as yeast cells expressing wild-

type levels of yeast topoisomerase II. These results suggested that human topoisomerase II α is about as sensitive to DMP-840 as yeast topoisomerase II, a result confirmed by the in vitro sensitivity of human topoisomerase II α to DMP-840.

Interestingly, human topoisomerase II α was less sensitive to inhibition of cleavage by DMP-840, compared to yeast human topoisomerase II. We have previously observed that yeast topoisomerase II is less sensitive to the intercalating agent amsacrine than human topoisomerase II (unpublished results). Given the high homology between human topoisomerase II α and yeast topoisomerase II, it will be interesting to determine the structural features of these two enzymes that account for this difference in sensitivity.

We have also compared the effect of DMP-840 with a chemically similar mono-intercalating agent amonafide. There are several clear differences in the cleavage patterns between amonafide and DMP-840 treated samples. Amonafide has a distinct sequence preference for cleavage by topoisomerase II, favoring C at the −1 position, disfavoring G or T at this position, and favoring A at the +1 position (*13*). This pattern of cleavage presumably reflects the binding specificity of amonafide, since the patterns of drug-induced cleavage by topoisomerase II with other intercalating agents is dictated by the sequence preference for drug binding (*38*−*40*). By contrast DMP-840 preferentially binds in runs of G/C base pairs (*18*), which will lead to a distinct pattern of drug binding compared to amonafide. Some cleavage sites may be influenced by drug−protein interactions or drug−DNA−protein interactions and may be present regardless of the drug class (*41*).

The results described in this paper indicate that DMP-840 is a topoisomerase II poison, and that the drug does not poison topoisomerase I. Our results do not directly address whether DMP-840 also inhibits DNA repair, as was previously suggested (*22*). Since this work demonstrates that the major target of DMP-840 is topoisomerase II, the inhibition of DNA repair by this drug does not play a major role in cytotoxicity of the agent to eukaryotic cells. Since excision repair genes do not sensitize cells to topoisomerase poisons (*42*), any repair inhibitory activity is unlikely to be a major determinant in the antitumor properties of this agent.

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