Stimulation of Topoisomerase II-Mediated DNA Damage via a Mechanism Involving Protein Thiolation†

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ABSTRACT: The breakage/reunion reaction of DNA topoisomerase II (TOP2) can be interrupted by DNA intercalators (e.g., doxorubicin), enzyme binders (e.g., etoposide), or DNA lesions (e.g., abasic sites) to produce TOP2-mediated DNA damage. Here, we demonstrate that thiol alkylation of TOP2 can also produce TOP2-mediated DNA damage. This conclusion is supported by the following observations using purified TOP2: (1) Thiol-reactive quinones were shown to induce TOP2-mediated DNA cleavage. (2) Thiol-reactive compounds such as *N*-ethylmaleimide (NEM), disulfiram, and organic disulfides [e.g., 2,2′-dithiobis(5-nitropyridine)] were also shown to induce TOP2-mediated DNA cleavage with similar reaction characteristics as thiol-reactive quinones. (3) TOP2-mediated DNA cleavage induced by thiol-reactive quinones was completely abolished using mutant yeast TOP2 with all cysteine residues replaced with alanine (cysteineless TOP2). These results suggest the possibility that cellular DNA damage could occur indirectly through thiolation of a nuclear protein, TOP2. The implications of this reaction in carcinogenesis and apoptotic cell death are discussed.

DNA topoisomerases are double-edged swords (*1, 2*). By catalyzing interconversion of various DNA topoisomers via DNA breakage/reunion, topoisomerases are involved in many critical functions of DNA (*1*). However, their delicate act on DNA often produces potentially lethal DNA strand breaks when their breakage/reunion reaction is interrupted by xenobiotics (*2*) or certain cellular stress conditions (*2, 3*). It is now well established that many antitumor drugs and antibiotics can interrupt the breakage/reunion reactions of topoisomerases by stabilizing the covalent topoisomerase−DNA reaction intermediates, often referred to as cleavable or cleavage complexes (*2, 3*). The cleavable complex has been shown to be the critical cellular lesion responsible for cell death (*2, 3*). So far, five topoisomerases have been identified in human cells (TOP1,[1] TOP2α, TOP2*β*, TOP3α, and TOP3*β*). Three of them (TOP1, TOP2α, and TOP2*β*) have been shown to be important molecular targets for antitumor drugs (*2, 3*).

Type II DNA topoisomerases perform double-strand DNA breakage/reunion which is particularly prone to produce potentially lethal double-strand breaks (*1*). Indeed, human TOP2 has been shown to be the molecular target for a rather large number of antitumor drugs (e.g., doxorubicin, etoposide, mitoxantrone, and m-AMSA) (*2*). The molecular basis for induction of TOP2-mediated DNA damage has been shown to result from either drug binding to DNA by an intercalative mode (e.g., doxorubicin, mitoxantrone, and m-AMSA) or drug binding to TOP2 (e.g., etoposide) (*4*). The antitumor activity of TOP2-targeting antitumor drugs is presumably due to double-strand breaks produced by interrupted TOP2 activity in tumor cells. The TOP2 breakage/reunion reaction can also be interrupted by DNA structural perturbations (e.g., abasic sites, and oxidized and alkylated DNA lesions) to produce TOP2-mediated DNA damage (*5*). It appears that the breakage/reunion reaction of TOP2 is highly vulnerable to interferences by xenobiotics and DNA structural perturbations (*3*). Accumulating evidence has also indicated that TOP2-mediated DNA damage is particularly effective in stimulating DNA sequence rearrangements which can also contribute to carcinogenesis (*6, 7*).

Quinones represent the largest class of quinoid compounds which are widely distributed in nature (*8*). A large number of them have various biological activities including antitumor and carcinogenic activities (*8*). Menadione (vitamin K3; 2-methyl-1,4-naphthoquinone) (see Table 1 for its chemical structure) has been shown to exhibit a broad spectrum of anticancer activity (*9*−*14*). Menadione, being a redox cycling quinone, has been widely used to study oxidant stress in mammalian systems (reviewed in ref *15*). The production of reactive oxygen species has been suggested to play a key role in the DNA damage and cytotoxic effect of menadione in human cultured cells (*16, 17*). However, like other

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[1] Abbreviations: TOP2, topoisomerase II; TOP1, topoisomerase I; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; AMPPNP, *β*,*γ*-imidoadenosine 5′-triphosphate; VM-26 (teniposide), 4′-demethylepipodophyllotoxin thenylidene-*β*-D-glucoside; VP-16 (etoposide), demethylepipodophyllotoxin ethylidene-*β*-D-glucoside; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

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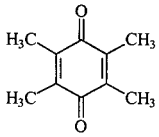
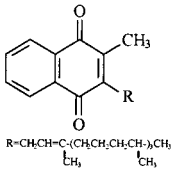
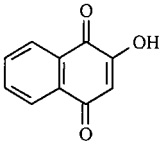
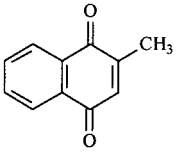
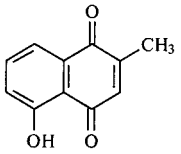
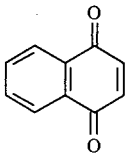
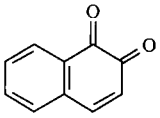
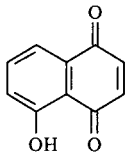
Table 1: Topoisomerase II-Mediated DNA Cleavage Activity, Cytotoxicity, and Chemical Reactivity of Menadione and Related Quinones

Quinone	Structure	Cleavage Activity[a]	Cytotoxicity[b] (IC_{50})	Chemical Reactivity: Reduction Potential[c]	Chemical Reactivity: Electrophilic Addition[d]
Tetramethyl-1,4-benzoquinone		0	8	-738	NA[e]
2-methyl-3-phytyl-1,4-naphthoquinone		0	>100	NA	NA
2-Hydroxy-1,4-naphthoquionone		0.01	230	-415	1.6×10^{-5}
2-Methyl-1,4-naphthoquionone (menadione, vit. K3)		1.0	14	-638	< 0.1
5-Hydroxy-2-methyl-1,4-naphthoquinone (plumbagin)		10	0.64	NA	NA
1,4-Naphthoquionone		50	1.3	-140	2071
1,2-Naphthoquionone		50	1.2	-473	NA
5-Hydroxy-1,4-naphthoquionone (jugalone)		100	0.52	-93	105

[a] Relative cleavage activity: The potency of menadione in stimulating TOP2-mediated DNA cleavage is arbitrarily designated 1.0. Higher relative cleavage activity indicates higher potency of the compound in stimulating TOP2-mediated DNA cleavage. [b] Cytotoxicity against human oral epidermoid carcinoma KB3-1 cells was obtained by measuring the IC_{50} (μM) of quinones at the end of 4-day continuous drug exposure using MTT assay. All measurements were done in triplicate and at least twice. [c] Reduction potentials of quinones were obtained from reference (*18*). [d] The rates of electrophilic addition to GSH (κ[GSH][Q] in M^{-1} s^{-1}) were obtained from references (*18*, *46*). [e] NA, not available.

quinones, menadione can also directly react with nucleophiles such as thiols in protein (*18*). The structural similarity of menadione to quinone moiety-containing TOP2-targeting anthracycline drugs (e.g., doxorubicin, daunorubicin, and mitoxantrone) has prompted us to investigate the potential role of TOP2 in menadione cytotoxicity. Previous studies have already demonstrated that menadione and a number of *o*-naphthoquinones (e.g., β-lapachone) can stimulate TOP2-mediated DNA cleavage in vitro (*19*, *20*). In the current studies, we provide evidence that stimulation of TOP2-mediated DNA cleavage by quinones may involve thiol modification of TOP2.

MATERIALS AND METHODS

Chemicals and Drugs. Menadione, 1,4-naphthoquinones, and 2,2′-dithiobis(5-nitropyridine) derivatives were purchased

from Aldrich Chemical Co. VM-26 (teniposide) was a gift from Bristol-Myers Co. Adriamycin (doxorubicin), ATP, AMPPNP (a nonhydrolyzable β,γ-imido analogue of ATP), disulfiram, and NEM were purchased from Sigma Chemical Co. All drugs were dissolved in DMSO (Sigma Chemical Co.) and kept frozen in aliquots at −20 °C. [α-^{32}P]dCTP (6000 Ci/mmol) was obtained from Amersham Corp.

Cell Lines. Human KB 3-1 cells (from Dr. Michael M. Gottesman, National Cancer Instititue) and human leukemia RPMI 8402 cells were cultured at 37 °C in 5% CO_2 and maintained by regular passage in Dulbecco's minimal essential medium and RPMI medium, respectively. All media were supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Enzymes and Nucleic Acids. DNA topoisomerases I and II were purified to homogeneity from calf thymus gland using published procedures (*21, 22*). Recombinant human TOP2α was purified from the protease-deficient yeast strain BCY123 harboring a human TOP2α expression plasmid, YEpWob6, according to the published procedure (*23*). Recombinant human TOP2β was similarly purified from BCY123 harboring a human DNA TOP2β expression plasmid (*24*). *Drosophila* TOP2 was a gift from Dr. T.-S. Hsieh (Duke University Medical School). The plasmid (pDAT10) encoding cysteine-less yeast TOP2 in which all nine cysteine residues were replaced with alanine was obtained from Dr. Janet Lindsley (University of Utah). YepG is a derivative of YEp24 (*25*). All plasmids were propagated in *E. coli* DH5α and purified using the Qiagen plasmid purification kit.

Preparation of End-Labeled DNA Fragments. The end-labeling procedure for plasmid DNA has been described previously (*26*). Briefly, 10 μg of YEpG DNA was first digested with *Bam*HI. Linearized YEpG DNA was then labeled at its 3′ ends with the Klenow polymerase and [α-^{32}P]dCTP. Unincorporated triphosphates were removed by two cycles of ethanol precipitation in the presence of 2.5 M ammonium acetate.

Topoisomerase Cleavage Assay. DNA topoisomerase cleavage assays were performed as described previously (*27, 28*). The reaction mixtures (20 μL each) containing 40 mM Tris, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1.0 mM ATP (for some TOP2 reactions only), 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL bovine serum albumin, 20 ng of 3′ end-labeled YEpG DNA, 10 ng of calf thymus DNA topoisomerase I or II, and various compounds were incubated at 37 °C for 30 min. The reactions were terminated by the addition of 2 μL of 5% SDS and further treated with 150 μg/mL proteinase K for 1 h at 37 °C. Following addition of sucrose (5% final concentration) and bromophenol blue (0.05 mg/mL final concentration), DNA samples were either loaded directly (for TOP2 reactions) or alkali-denatured (for TOP1 reactions) (*28*) and then loaded onto a 1.0% agarose gel in TBE buffer (0.089 M Tris−borate, 0.008 M EDTA, pH 8.0). After electrophoresis, gels were then dried onto Whatman 3MM chromatographic paper and autoradiographed at −80 °C using Kodak XAR-5 films.

For DNA sequencing, the Bluescript II SK+ DNA was digested with *Hin*dIII and end-labeled as described above. The other labeled end was removed by *Sma*I digestion. Chemical sequencing was performed as described by Maxam and Gilbert (*29*).

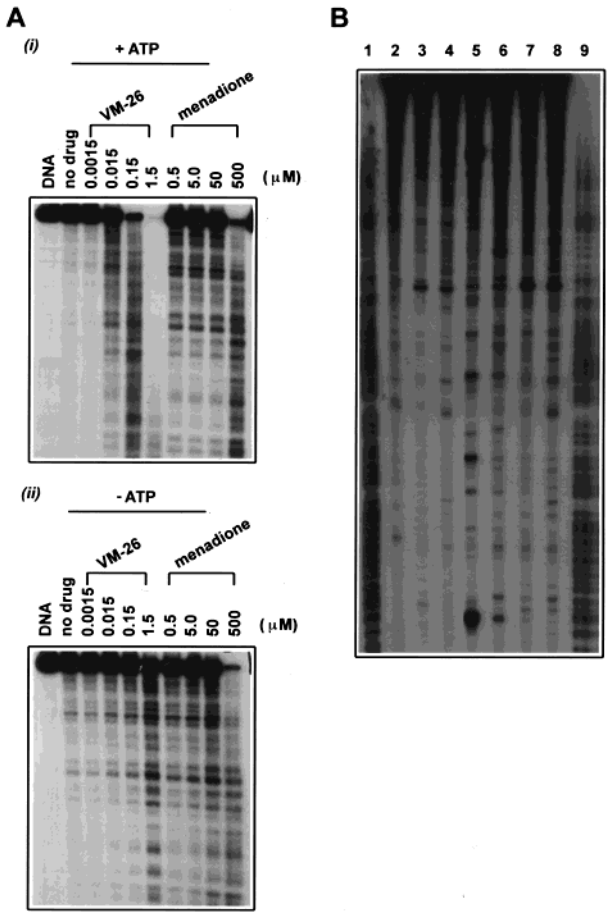


FIGURE 1: Menadione stimulates TOP2-mediated DNA cleavage. (A) TOP2-mediated DNA cleavage was performed as described under Materials and Methods using purified calf thymus TOP2. The concentrations of VM-26 and menadione were as indicated on top of the gel. ATP (1 mM) was included in reaction (i) but not reaction (ii). (B) DNA sequencing of the cleavage sites was performed as described under Materials and Methods. Equal amounts of TOP2α and TOP2β (10 ng each) were used in the cleavage assay. The concentration of menadione and nitidine used in this experiment was 100 μM. Lanes 1 and 9: the G+A ladder generated by chemical sequencing. Lane 2: DNA alone. Lane 3: with TOP2α. Lane 4: with TOP2β. Lane 5: TOP2α with nitidine. Lane 6: TOP2β with nitidine. Lane 7: TOP2α with menadione. Lane 8: TOP2β with menadione.

Cytotoxicity Assay. Cytotoxicity to human KB 3-1 and RPMI 8402 cells was determined by MTT assay at the end of a 5 day continuous exposure as described previously (*30*).

RESULTS

Quinones Stimulate TOP2-Mediated DNA Cleavage. Purified calf thymus TOP2 was used to test whether menadione can stimulate TOP2-mediated DNA cleavage. As shown in Figure 1A, menadione, like the prototypic TOP2 poison VM-26, induced dose-dependent double-strand DNA breaks in the presence of calf thymus TOP2. Interestingly, different from the cleavage pattern induced by VM-26, the DNA cleavage pattern induced by menadione is very similar to the background (no drug) DNA cleavage pattern. Furthermore, unlike VM-26-induced DNA cleavage, which was stimulated 50−100-fold by 1 mM ATP, menadione-induced DNA cleavage was only slightly (less than several fold) affected by ATP (Figure 1A). In addition to menadione, a number of 1,4-naphthoquinones (see Table 1 for their

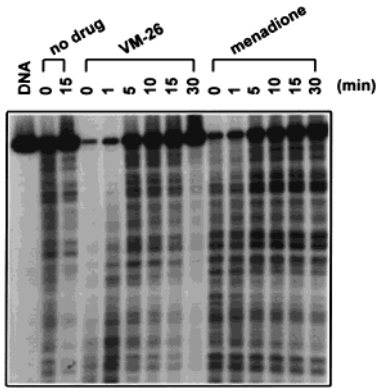


FIGURE 2: Reversibility of TOP2-mediated DNA cleavage induced by menadione and VM-26. The DNA cleavage assays were performed as described under Materials and Methods. All reactions contained 1% DMSO and were incubated at 37 °C for 30 min. The preincubated reactions were then shifted to 65 °C for various times prior to termination with SDS and proteinase K. The drug concentrations used were 0.2 and 200 μM for VM-26 and menadione, respectively.

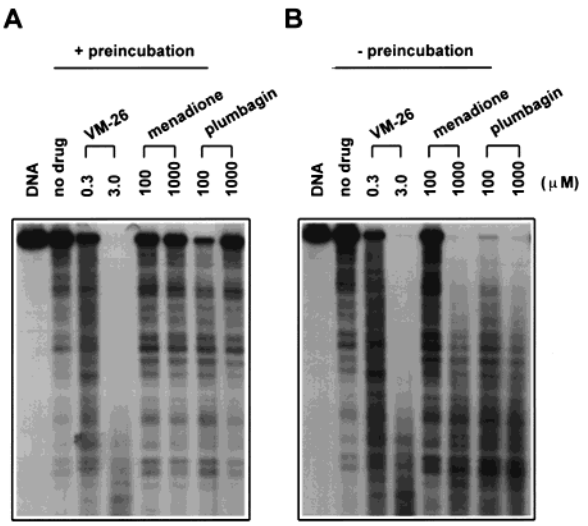


FIGURE 3: Preincubation of TOP2 with thiol-reactive quinones reduces TOP2-mediated DNA cleavage. (A) TOP2 was preincubated with menadione or plumbagin at 37 °C for 30 min in the reaction mixture as described under Materials and Methods, and then incubated with end-labeled plasmid DNA for another 30 min at 37 °C. (B) TOP2 was incubated with 1% DMSO at 37 °C for 30 min and then incubated with DNA and drugs for another 30 min.

chemical structures) were also tested and shown to exhibit variable degrees of potency in inducing TOP2-mediated DNA cleavage. The potency in inducing TOP2-mediated DNA cleavage, as measured by band intensities of the cleaved plasmid DNA, is as follows: 5-hydroxy-1,4-naphthoquinone (jugalone) > 1,2-naphthoquinone; 1,4-naphthoquinone; 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) > 2-methyl-1,4-naphthoquinone (menadione) > 2-hydroxy-1,4-naphthoquinone > 2-methyl-3-phytyl-1,4-naphthoquinone, tetramethyl-1,4-benzoquinone. Interestingly, among these menadione congeners, a correlation exists between the potency in inducing TOP2-mediated DNA cleavage and drug cytotoxicity (see IC_{50} in human KB 3-1 cells).

Human recombinant topoisomerases IIα and IIβ were also used to assess the activity of menadione (Figure 1B). The TOP2-mediated DNA cleavage in the presence of various TOP2 poisons was determined at the sequence level. As shown in Figure 1B, menadione stimulated DNA cleavage in the presence of either TOP2α or TOP2β. TOP2β appeared to be as sensitive to menadione as TOP2α. On the other hand, nitidine, which is a dual poison of both DNA topoisomerases I and II (*31*, *32*), was more effective in inducing TOP2α- than TOP2β-mediated DNA cleavage.

To test whether TOP2-mediated DNA cleavage induced by menadione is due to the formation of cleavable complexes, a standard high-temperature (65 °C) reversing procedure (*33*) was used to reverse menadione-induced DNA breaks. As shown in Figure 2, prior to terminating the cleavage reactions with SDS and proteinase K, the incubated reaction mixtures were heated to 65 °C for the indicated time periods up to 30 min. While DNA cleavage induced by VM-26 was completely reversed within 30 min, menadione-induced DNA breaks were about 80% reversed at 30 min. The reversibility of menadione-induced DNA cleavage was also demonstrated using two other standard reversing procedures for TOP2 cleavable complexes: high salt (0.5 M NaCl) and EDTA (50 mM EDTA) treatments (data not shown).

The Molecular Basis for Quinone-Induced TOP2-Mediated DNA Cleavage. Quinones are known both to undergo one-electron reduction to become semiquinones and to undergo electrophilic addition (Michael addition) to react with thiols (e.g., cysteines on proteins) (*7*). To test whether menadione may chemically modify TOP2, TOP2 was first preincubated with the quinones (menadione or plumbagin) at 37 °C for 30 min and then placed in a regular reaction containing labeled plasmid DNA (Figure 3A). Preincubation of TOP2 with menadione or plumbagin caused reduction of TOP2-mediated DNA cleavage as compared to simultaneous incubation of TOP2, DNA, and menadione/plumbagin (Figure 3, compare panels A and B). By contrast, preincubation of TOP2 with VM-26 showed no such reduction in DNA cleavage (Figure 3). The P4 unknotting assay was also used to monitor the catalytic activity of TOP2. We showed that preincubation of TOP2 with menadione or plumbagin inactivated the catalytic activity of TOP2 (data not shown). These results suggest that menadione and plumbagin probably inactivated TOP2 by chemical modification. DNA pretreated with menadione or plumbagin (30 min under the same reaction conditions followed by removal of quinones by ethanol precipitation) did not result in enhanced TOP2-mediated DNA cleavage in a subsequent incubation without menadione/plumbagin (data not shown). These results indicate that co-incubation of TOP2, DNA, and menadione/plumbagin is important for stimulating TOP2-mediated DNA cleavage. Preincubation of either DNA or TOP2 with these quinones did not lead to stimulation of TOP2-mediated DNA cleavage. In fact, preincubation of TOP2 with these quinones led to significant reduction of TOP2 catalytic activity as well as reduced stimulation of TOP2-mediated DNA cleavage. The simplest explanation for these results is that these quinones can chemically modify free TOP2 (not DNA bound), leading to enzyme inactivation. However, chemical modification of DNA-bound TOP2 may alter the breakage/reunion equilibrium favoring the formation of the cleavable complex (see Figure 7).

Thiol-Specific Alkylators Stimulate TOP2-Mediated DNA Cleavage. It is interesting to note that the potency of quinones to stimulate TOP2-mediated DNA cleavage shows no correlation with their reduction potential (Table 1).

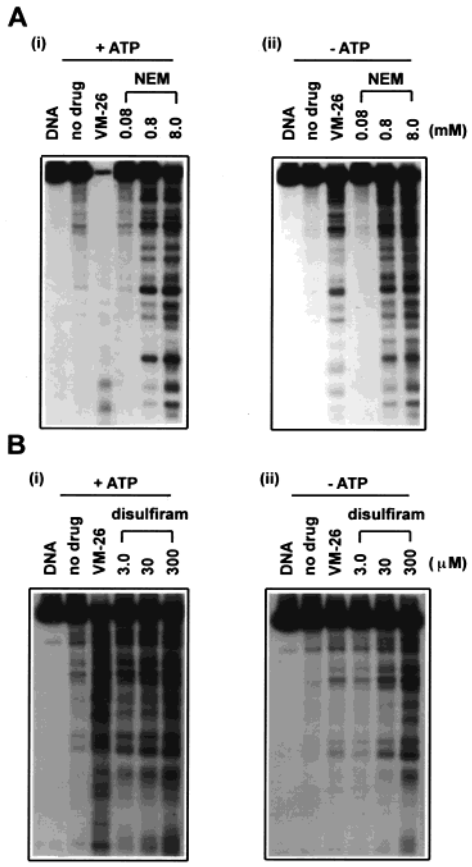


FIGURE 4: Thiol-specific alkylating agents stimulate TOP2-mediated DNA cleavage. TOP2 cleavage assays were performed as described under Materials and Methods. The concentrations of NEM and disulfiram were as marked. 1.0 μM VM-26 was used. 1 mM ATP was included in the reactions shown in panel (i) but not panel (ii). (A) NEM stimulates TOP2-mediated DNA cleavage. (B) Disulfiram stimulates TOP2-mediated DNA cleavage.

However, a correlation with their ability to undergo electrophilic (Michael) addition can be observed (Table 1). Reactions of quinones with thiols by way of Michael addition require α,β-unsaturated quinones (*34*). As shown in Table 1, the two quinones tetramethyl-1,4-benzoquinone and 2-methyl-3-phytyl-1,4-naphthoquinone, which cannot undergo Michael additions, are unable to stimulate TOP2-mediated DNA cleavage. Thus, we tested a known thiol-specific alkylating agent, NEM, which is known to undergo Michael addition with thiols, for its potential to stimulate TOP2-mediated DNA cleavage. Indeed, NEM was able to effectively stimulate TOP2-mediated DNA cleavage. In addition, like that of menadione and other thiol-reactive quinones, the cleavage pattern induced by NEM was similar to that of the background (no drug) DNA cleavage, and the cleavage reaction was only slightly stimulated by ATP (Figure 4A, compare panels i and ii).

Disulfiram, a known thiol reagent used extensively for treating alcoholism, was also tested using calf thymus TOP2 (Figure 4B). Like NEM, disulfiram was able to stimulate TOP2-mediated DNA cleavage in a dose-dependent manner. Diamide, another thiol-specific alkylating agent, was also highly active in stimulating TOP2-mediated DNA cleavage (data not shown).

To further test the hypothesis that thiol modification of TOP2 may be involved in stimulation of TOP2-mediated DNA cleavage, we have evaluated a series of 2,2′-dithiobis-(5-nitropyridine) derivatives. The ability of these compounds to stimulate TOP2-mediated DNA cleavage was correlated with the presence of the disulfide moiety (Table 2). Interestingly, their cytotoxicity against human leukemia RPMI8402 paralleled their potency in stimulating TOP2-mediated DNA cleavage (Table 2).

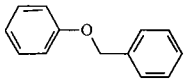
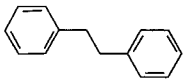
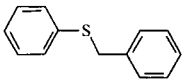
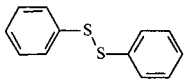
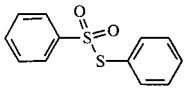
The Thiol-Reactive Jugalone Stimulates Wild-Type TOP2 but Not Cysteineless Mutant TOP2 To Cleave DNA. A yeast mutant TOP2 with all nine cysteine residues mutated to alanine (cysteineless yTOP2) has been shown to be enzymatically active (*35*). The cysteineless yTOP2 was purified from BCY123 harboring pDAT10 upon galactose induction as described (*35*). We showed that a potent thiol-reactive naphthoquinone, jugalone, was unable to stimulate cysteineless yTOP2 to cleave DNA. As a control, jugalone was shown to stimulate wild-type yTOP2 to cleave DNA, albeit at a rather low efficiency (Figure 5). In this assay, wild-type and cysteineless mutant yTOP2 were titrated to have equal catalytic activity (data not shown). By contrast, VM-26 was shown to stimulate both wild-type and cysteineless yTOP2 to cleave DNA with equal efficiency.

The Thiol Alkylator NEM Stimulates Human but Not Drosophila TOP2 To Cleave DNA. As shown in Figure 5, yeast TOP2 was only weakly stimulated by jugalone to cleave DNA. It seemed possible that TOP2 enzymes from different organisms might exhibit different degrees of sensitivity to thiol alkylators. Indeed, NEM, which readily stimulated human TOP2-mediated DNA cleavage, was completely unable to stimulate *Drosophila* TOP2-mediated DNA cleavage even at high NEM concentrations (Figure 6). At high concentrations of NEM (above 80 μM), inhibition of *Drosophila* TOP2-mediated background cleavage was observed. By contrast, NEM stimulated human TOP2α-mediated DNA cleavage even more extensively at these high concentrations (Figure 6).

DISCUSSION

We have shown that many quinones can induce TOP2-mediated DNA cleavage in vitro through the formation of reversible cleavable complexes. TOP2 cleavable complexes induced by these quinones appear qualitatively different from those induced by other known TOP2 poisons. First, the pattern of TOP2-mediated DNA cleavage sites of menadione is similar if not identical to that of the background cleavage sites. This is somewhat surprising since most if not all known TOP2 drugs (e.g., doxorubicin, etoposide, m-AMSA, and mitoxantrone) induce unique cleavage patterns characteristic of their drug classes (*2*). The characteristic DNA cleavage pattern associated with each drug class has been attributed to specific drug−DNA interactions (*2*). The lack of any detectable menadione−DNA interaction even at 100 μM (unpublished results) could explain the observed differences in cleavage patterns. Second, TOP2-mediated DNA cleavage induced by quinones is only slightly (severalfold) stimulated by ATP or AMPPNP. By contrast, TOP2-mediated DNA cleavage induced by most other TOP2-directed drugs such as etoposide, mitoxantrone, m-AMSA, and doxorubicin is 30−100-fold enhanced by ATP or nonhydrolyzable ATP analogues (*2*, unpublished results). Third, quinone-induced TOP2 cleavable complexes appear to be somewhat resistant to a brief heat treatment at 65 °C, a standard reversing

Table 2: Stimulation of Human TOP2-Mediated DNA Cleavage by 2,2′-Dithiobis(5-nitropyridine) Derivatives

	Structure	Cleavage activity[a]	Cytotoxicity (IC_{50})[b]
#1		0	> 20
#2		0	> 20
#3		1	> 20
#4		100	0.1
#5		80	1.5

[a] TOP2-mediated DNA cleavage induced by #4 was taken arbitrarily as 1. [b] IC_{50} (μM) against human leukemia RPMI 8402 cells was determined by MTT assay.

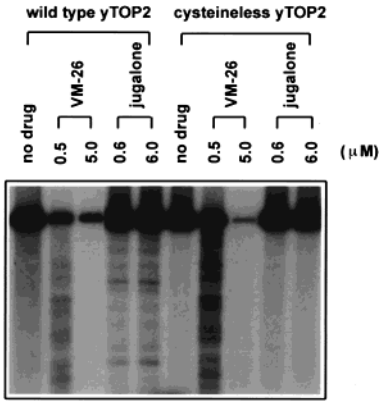


FIGURE 5: Thiol-reactive jugalone stimulates wild-type but not cysteineless mutant TOP2 to cleave DNA. TOP2 cleavage assays were performed as described under Materials and Methods except that wild-type and cysteineless mutant yeast TOP2 were used instead of calf thymus TOP2. The concentrations of VM-26 and jugalone were as marked. All reactions contained 1 mM ATP.

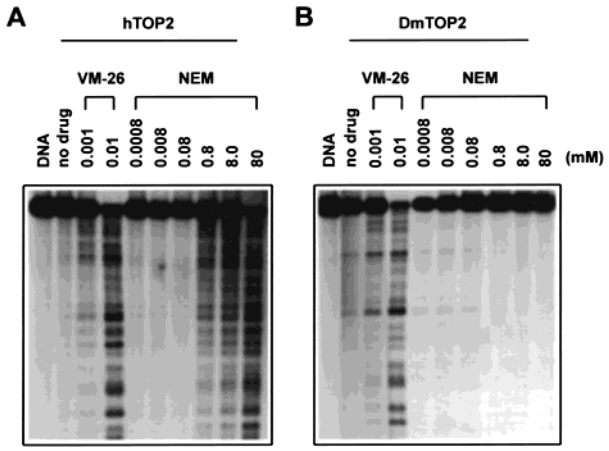


FIGURE 6: NEM stimulates human but not *Drosophila* TOP2 to cleave DNA. TOP2 cleavage assays were performed as described under Materials and Methods except that human TOP2α and *Drosophila* TOP2 were used instead of calf thymus TOP2. The concentrations of VM-26 and NEM were as marked. All reactions contained 1 mM ATP.

procedure for the reversible cleavable complexes (*33*). These differences could suggest that quinones may stimulate TOP2-mediated DNA cleavage by a mechanism not shared by other known TOP2 drugs.

Quinones are known to be chemically reactive. They can generate reactive free radicals through one-electron reduction, and can also react with thiols through Michael addition (*8*). We have shown that preincubation of TOP2 (without DNA) with quinones resulted in reduced DNA cleavage activity of TOP2 as compared to simultaneous incubation of TOP2, DNA, and quinones, suggesting that quinones may chemically inactivate TOP2 in the absence of DNA. In the presence of DNA, covalent modification of DNA-bound TOP2 by quinones may alter the breakage/reunion equilibrium, resulting in accumulation of TOP2 cleavable complexes (see Figure 7 for a working model). One possible explanation for this effect could be that a normally inaccessible cysteine residue(s) is (are) exposed upon binding of TOP2 to DNA. Modification of this (these) cysteine residue(s) by thiol-reactive quinones (or other thiol-reactive agents) alters the breakage/reunion equilibrium (see Figure 7, and discussion below).

Additional experiments have supported the notion that quinones may stimulate TOP2-mediated DNA cleavage through covalent modification of a thiol group(s) on TOP2. The clue came from the observation that the potency of quinones in poisoning TOP2 parallels their potency to undergo electrophilic addition rather than their reduction potential (Table 1). In addition, the thiol-specific alkylating agents, NEM, disulfiram, diamide, and organic disulfides [e.g., 2,2′-dithiobis(5-nitropyridine)], are also shown to poison TOP2 with similar reaction characteristics. These

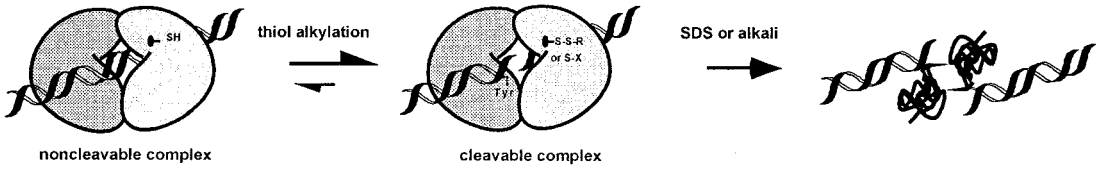


FIGURE 7: Stimulation of TOP2-mediated DNA cleavage by thiol alkylators: a working model. In this model, TOP2 is bound to DNA forming two types of complexes at equilibrium, the noncleavable and the cleavable complex. This equilibrium mixture favors the noncleavable complex. A critical sulfhydryl (-SH) group(s) on the TOP2−DNA complex was (were) exposed for modification by thiol alkylators. Modification of this (these) critical -SH group(s) shifts the equilibrium toward the TOP2 cleavable complex. Denaturation of the TOP2 cleavable complex results in the formation of a protein-linked DNA break. It should be pointed out that different cysteine residues could be exposed for thiol alkylation for unbound and DNA-bound TOP2.

results suggest that TOP2 poisoning by quinones may involve chemical modification of the TOP2−DNA complex through the electrophilic reactivity of quinones. The modification could be on DNA and/or the enzyme. It has been shown that DNA structural perturbation (e.g., abasic sites on DNA) can lead to poisoning of TOP2 (*36*). However, preincubation of DNA with quinones did not result in subsequent stimulation of TOP2-mediated DNA cleavage. It seems possible that activation of TOP2-mediated DNA cleavage by quinones may involve covalent modification of the TOP2−DNA complex through a thiolation reaction on TOP2. The experiment using yeast mutant TOP2 with all cysteine residues mutated to alanine residues (*35*) further suggests that cysteine modification may be involved.

DNA damage induced by quinones is most likely important for their cytotoxicity and carcinogenicity. However, whether TOP2-mediated DNA damage contributes to DNA damage induced by quinones in vivo is unclear. While thiol-reactive quinones primarily undergo electrophilic addition under our assay conditions for TOP2, they clearly can undergo redox cycling in vivo to generate free radicals. There is evidence that DNA damage induced by quinones involves free radicals (*8*). However, based on our current studies, it seems possible that TOP2-mediated DNA damage is also in part responsible for DNA damage induced by quinones and other thiol-alkylating compounds. It has been demonstrated that thiol-reactive compounds such as NEM, *p*-chloromercuribenzoate (PCMB), arsenite, and iodoacetate can produce protein−DNA cross-links in cells (*37*). Whether or not the cross-linked protein is TOP2 remains to be established.

Menadione is able to poison both topoisomerases IIα and IIβ. All the known antitumor drugs tested (e.g., doxorubicin, m-AMSA, and etoposide) are more active against the IIα than the IIβ isozyme (*38*, unpublished results). For example, we showed that nitidine was much more active against the IIα than IIβ isoenzyme (Figure 1B). Menadione, however, is equally active against both topoisomerases IIα and IIβ. The significance of this observation is unclear. However, tissue distribution and cell cycle regulation of topoisomerases IIα and IIβ are very different (*38*−*40*). Menadione and other TOP2-poisoning thiol alkylators may be particularly useful for certain tumors which are enriched in TOP2β. The lack of strong ATP dependence in TOP2-mediated DNA cleavage stimulated by menadione may also suggest its possible use in hypoxic solid tumors.

It has been shown that TOP2-mediated DNA damage can be effectively induced by hydrogen peroxide (*41*), acidic pH (5−7) (*21*, *42*), and calcium (*43*). It is noteworthy that these conditions often exist during certain pathological states of cells (e.g., ischemia and apoptosis) (*44*). It is now well documented that cellular oxidative stress causes the formation of glutathione−protein mixed disulfides as part of the defense mechanism associated with glutathione (*45*). Our current studies have suggested the possibility that thiolation of TOP2 through the formation of glutathione−TOP2 mixed disulfides may occur in cells undergoing oxidative stress. The formation of the putative glutathione−TOP2 mixed disulfide seems to be highly undesirable in normal cells because of its ability to induce cell death and DNA sequence rearrangements. The vulnerability of human TOP2 to thiolation compared to *Drosophila* TOP2 is intriguing. The "deleterious" effect of having thiolation-sensitive human TOP2 may be paradoxically advantageous from an evolutionary standpoint. It remains to be established that whether glutathione−TOP2 mixed disulfides are formed in cells during oxidative stress and what are the biological consequences of this reaction.

REFERENCES

1. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
2. Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351−375.
3. Li, T.-K., and Liu, L. F. (2001) *Annu. Rev. Pharmacol. Toxicol. 41*, 53−77.
4. Kingma, P. S., Burden, D. A., and Osheroff, N. (1999) *Biochemistry 38*, 3457−3461.
5. Sabourin, M., and Osheroff, N. (2000) *Nucleic Acids Res. 28*, 1947−1954.
6. Bodley, A. L., Huang, H. C., Yu, C., and Liu, L. F. (1993) *Mol. Cell. Biol. 13*, 6190−6200.
7. Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., and Rowley, J. D. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4790−4795.
8. Thomson, R. H. (1987) *Naturally Occurring Quinones*, Vol. III, Chapman and Hall, London.
9. Prasad, K. N., Edwards-Prasad, J., and Sakamoto, A. (1981) *Life Sci. 29*, 1387−1392.
10. Chlebowski, R. T., Dietrich, M., Akman, S., and Block, J. B. (1985) *Cancer Treat. Rep. 69*, 527−532.
11. Akman, S. A., Dietrich, M., Chlebowski, R., Limberg, P., and Block, J. B. (1985) *Cancer Res. 45*, 5257−5262.
12. Noto, V., Taper, H. S., Jiang, Y. H., Janssens, J., Bonte, J., and De Loecker, W. (1989) *Cancer 63*, 901−906.
13. Su, Y. Z., Duarte, T. E., Dill, P. L., and Weisenthal, L. M. (1987) *Cancer Treat. Rep. 71*, 619−625.
14. Nutter, L. M., Cheng, A. L., Hung, H. L., Hsieh, R. K., Ngo, E. O., and Liu, T. W. (1991) *Biochem. Pharmacol. 41*, 1283−1292.
15. Powis, G. (1987) *Pharmacol. Ther. 35*, 57−162.
16. Nutter, L. M., Ngo, E. O., Fisher, G. R., and Gutierrez, P. L. (1992) *J. Biol. Chem. 267*, 2474−2479.
17. Ngo, E. O., Sun, T. P., Chang, J. Y., Wang, C. C., Chi, K. H., Cheng, A. L., and Nutter, L. M. (1991) *Biochem. Pharmacol. 42*, 1961−1968.
18. Wilson, I., Wardman, P., Lin, T. S., and Sartorelli, A. C. (1987) *Chem.-Biol. Interact. 61*, 229−240.
19. Chen, A. Y., Yu, C., Lee, W.-H., Peng, L. F., and Liu, L. F. (1992) *Proc. Am. Assoc. Cancer Res. 33*, 2588.

20. Frydman, B., Marton, L. J., Sun, J. S., Neder, K., Witiak, D. T., Liu, A. A., Wang, H. M., Mao, Y., Wu, H. Y., Sanders, M. M., and Liu, L. F. (1997) *Cancer Res. 57*, 620−627.
21. Halligan, B. D., Edwards, K. A., and Liu, L. F. (1985) *J. Biol. Chem. 260*, 2475−2482.
22. Liu, L. F., and Miller, K. G. (1981) *Proc. Natl. Acad. Sci.U.S.A. 78*, 3487−3491.
23. Wasserman, R. A., Austin, C. A., Fisher, L. M., and Wang, J. C. (1993) *Cancer Res. 53*, 3591−3596.
24. Mao, Y., Yu, C., Hsieh, T. S., Nitiss, J. L., Liu, A. A., Wang, H., and Liu, L. F. (1999) *Biochemistry 38*, 10793−10800.
25. Wyckoff, E., and Hsieh, T. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6272−6276.
26. Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., and Chen, G. L. (1983) *J. Biol. Chem. 258*, 15365−15370.
27. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., and Liu, L. F. (1984) *Science 226*, 466−468.
28. Hsiang, Y. H., Hertzberg, R., Hecht, S., and Liu, L. F. (1985) *J. Biol. Chem. 260*, 14873−14878.
29. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol. 65*, 499−560.
30. Nelson, E. M., Tewey, K. M., and Liu, L. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1361−1365.
31. Wang, L. K., Johnson, R. K., and Hecht, S. M. (1993) *Chem. Res. Toxicol. 6*, 813−818.
32. Gatto, B., Sanders, M. M., Yu, C., Wu, H. Y., Makhey, D., LaVoie, E. J., and Liu, L. F. (1996) *Cancer Res. 56*, 2795−2800.
33. Hsiang, Y. H., and Liu, L. F. (1989) *J. Biol. Chem. 264*, 9713−9715.
34. Neder, K., Marton, L. J., Liu, L. F., and Frydman, B. (1998) *Cell. Mol. Biol. 44*, 465−474.
35. Lindsley, J. E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2975−2980.
36. Kingma, P. S., Corbett, A. H., Burcham, P. C., Marnett, L. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21441−21444.
37. Grunicke, H., Bock, K. W., Becher, H., Gang, V., Schnierda, J., and Puschendorf, B. (1973) *Cancer Res. 33*, 1048−1053.
38. Austin, C. A., Marsh, K. L., Wasserman, R. A., Willmore, E., Sayer, P. J., Wang, J. C., and Fisher, L. M. (1995) *J. Biol. Chem. 270*, 15739−15746.
39. Jenkins, J. R., Ayton, P., Jones, T., Davies, S. L., Simmons, D. L., Harris, A. L., Sheer, D., and Hickson, I. D. (1992) *Nucleic Acids Res. 20*, 5587−5592.
40. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth Differ. 2*, 209−214.
41. Li, T. K., Chen, A. Y., Yu, C., Mao, Y., Wang, H., and Liu, L. F. (1999) *Genes Dev. 13*, 1553−1560.
42. Zechiedrich, E. L., Christiansen, K., Andersen, A. H., Westergaard, O., and Osheroff, N. (1989) *Biochemistry 28*, 6229−6236.
43. Osheroff, N., and Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303−4309.
44. Lipton, P. (1999) *Physiol. Rev. 79*, 1431−1568.
45. Di Simplicio, P., Lupis, E., and Rossi, R. (1996) *Biochim. Biophys. Acta 1289*, 252−260.
46. Ollinger, K., and Brunmark, A. (1991) *J. Biol. Chem. 266*, 21496−21503.

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