Inhibition of Topoisomerase I Cleavage Activity by Thiol-reactive Compounds

IMPORTANT OF VICINAL CYSTEINES 504 AND 505*

DNA topoisomerase I (Top1) is a nuclear enzyme that plays a crucial role in the removal of DNA supercoiling associated with replication and transcription. It is also the target of the anticancer agent, camptothecin (CPT). Top1 contains eight cysteines, including two vicinal residues (504 and 505), which are highly conserved across species. In this study, we show that thiol-reactive compounds such as N-ethylmaleimide and phenylarsines oxide can impair Top1 catalytic activity. We demonstrate that in contrast to CPT, which inhibits Top1-catalyzed religation, thiolation of Top1 inhibited the DNA cleavage step of the reaction. This inhibition was more pronounced when Top1 was preincubated with the thiol-reactive compound and could be reversed in the presence of dithiothreitol. We also established that phenylarsine oxide-mediated inhibition of Top1 cleavage involved the two vicinal cysteines 504 and 505, as this effect was suppressed when cysteines were mutated to alanines. Interestingly, mutation of Cys-505 also altered Top1 sensitivity to CPT, even in the context of the double Cys-504 to Cys-505 mutant, which relaxed supercoiled DNA with a comparable efficiency to that of wild-type Top1. This indicates that cysteine 505, which is located in the lower Lip domain of human Top1, is critical for optimal poisoning of the enzyme by CPT and its analogs. Altogether, our results suggest that conserved vicinal cysteines 504 and 505 of human Top1 play a critical role in enzyme catalytic activity and are the target of thiol-reactive compounds, which may be developed as efficient Top1 catalytic inhibitors.

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3 The abbreviations used are: Top1, DNA topoisomerase I; CPT, camptothecin; Me2SO, dimethyl sulfoxide; NEM, N-ethylmaleimide; PAO, phenylarsine oxide; DTT, dithiothreitol.

Eukaryotic DNA topoisomerase I (Top1)3 is an essential enzyme involved in the regulation of DNA topology associated with most DNA transactions, including replication, transcription, recombination, and chromatin remodeling (1–3). This role is dependent upon the ability of Top1 to introduce transient single strand breaks in duplex DNA via the formation of a covalent bond between the 3’-phosphate of the cleaved strand and a tyrosine residue of the enzyme (Tyr-723 in human) (4). Within the covalent Top1-DNA complex, rotation of the broken strand around the uncleaved strand results in DNA supercoil relaxation (5, 6). DNA continuity is then restored by Top1-catalyzed religation of the 5’-hydroxyl termini. Under normal conditions, the DNA cleavage step of the DNA cleavage/relation equilibrium is rate-limiting, which results in the detection of a small fraction of cleavage complexes at any given time (7). Top1 poisons, such as camptothecin (CPT) and its analogs, reversibly stabilize the Top1-DNA complex by inhibiting DNA religation (8, 9). Collision of the stabilized complexes with advancing replication forks leads to the formation of irreversible strand breaks that are ultimately responsible for cell death (10, 11). Conversely, catalytic inhibitors exert their cytotoxicity by preventing Top1 binding to the DNA and/or Top1 cleavage, resulting in the inhibition of DNA relaxation. This is the case for DNA binders and DNA intercalators, which presumably prevent Top1 binding by altering the structure of DNA in the vicinity of a Top1 DNA cleavage site (12, 13), and naphthoquinones, which have been suggested to directly react with the enzyme (14). Previous studies suggest that Top2poisoning by β-lapachone and related naphthoquinones may result from the alkylation of exposed thiol residues of the enzyme when it is bound to DNA (15, 16). More recently, Wang et al. (17) reported a correlation between the potency of menadione and related quinones to stimulate Top2-mediated DNA cleavage with their ability to undergo Michael-type nucleophilic addition but not with their reduction potential. Other studies in trypanosomatids and in human leukemia cell lines have also demonstrated the capability of β-lapachone to induce reactive oxygen species that could target Top1 and lead to activation of apoptosis (18–20).

Several crystal structures of a 70-kDa C-terminal fragment of human Top1 (Topo70) in complex with DNA revealed that the enzyme is a bi-lobed protein that clamps tightly around duplex DNA via protein-DNA phosphate interactions (5, 21). Top1
activity relies on two essential domains of the enzyme as follows: the C-terminal domain (residues 713–765) containing the catalytic tyrosine and the core domain (residues 215–636) that is connected to the C terminus by a linker region (22). Although the paired α-helical structure of the linker domain may be a common structural feature of eukaryotic Top1, the relative lengths and primary amino acid sequence of the linker (residues 637–712) and N-terminal domains (residues 1–215) are poorly conserved among species, and both are dispensable for Top1 activity. Human nuclear Top1 contains eight cysteines (Cys-300, -341, -386, -453, -504, -505, -630, and -733), including two vicinal residues 504 and 505. All cysteines are located in two domains that are essential for Top1 activity (23) and are highly conserved. Thus, we inferred that they may play a critical role in Top1-catalyzed DNA cleavage complex formation and could be the potential target of thiol-reactive compounds.

In this study, we investigated whether Top1 cysteines are the target of thiol-reactive compounds such as N-ethylmaleimide (NEM) and phenylarsine oxide (PAO) and whether Top1 thiolation alters enzyme activity. We show that thiolation selectively inhibited the cleavage step of the Top1 reaction, without affecting enzyme binding to DNA. This inhibition was even more pronounced when Top1 was preincubated with the thiol-reactive compound. We show that vicinal cysteine 504 and 505 play a critical role in the PAO-mediated inhibition of Top1 cleavage as this effect was abrogated by mutation of these residues to alanine. Interestingly, the combination of these Cys substitutions did not significantly alter Top1-catalyzed relaxation of supercoiled DNA but conferred resistance to the Top1 poison camptothecin, indicating that these conserved vicinal cysteines are a critical determinant of human Top1 sensitivity to thiol-reactive inhibitors as well as chemotherapeutic agents that stabilize the covalent Top1-DNA complex.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Drugs**—NEM, PAO, dimethyl sulfoxide (Me₂SO), and camptothecin (CPT) were purchased from Sigma. [α-³²P]Cordycepin was purchased from PerkinElmer Life Sciences. Terminal deoxynucleotidyltransferase was purchased from Invitrogen. Full-length human Top1 was purified as described below or purchased along with pH1 plasmid DNA from Topogen (Columbus, OH). Human recombinant Topo70 was a kind gift from Dr. Y. Pommier (NCI, Bethesda MD) (24). Proteinase K was purchased from Roche Applied Science.

**Plasmids and Yeast Strains**—Yeast strain EKY3 (MATα, top1Δ::TRP1, trp1Δ63, leu2Δ1, his3Δ200, ura3-52) has been described (25, 26). Yeast cells were transformed and cultured using standard methods.

Site-directed mutagenesis of Top1 cysteines was performed using the QuikChange site-directed mutagenesis kit from Stratagene (Amsterdam, Netherlands). Sequences of sense primers (Europgentec, Herstal, Belgium) used for mutagenesis were as follows (5’ to 3’): GCGGACACTGTTGGCGcCTGC-TCACTTTCG for C504A, GCGGACACTGTTGGCGcCTGC-TCACTTTCG for C505A, and GCGGACACTGTTGGCGcCTGC-TCACTTTCG for the double mutant. 125 ng of complementary primers were annealed to 50 ng of the pZIP GFP-top1 plasmid (kind gift from Dr. J. Tazi, Institut de Gene-tique Moleculaire de Montpellier, France) in the presence of 2.5 units of PfuTurbo DNA polymerase. PCR was performed as follows: denaturation of 30 s at 95 °C followed by 16 cycles of 30 s 95 °C, 1 min 55 °C, and a final extension of 7 min at 68 °C. Reactions were cooled down to 4 °C and incubated for 1 h at 37 °C with 10 units of DpnI. One μl of each reaction was used to transform XL1-blue super-competent cells (Stratagene). Transformants were selected on LB medium plates containing kanamycin (30 μg/ml). Purification of plasmid DNAs was performed using the Qiaprep spin miniprep kit from Qiagen according to the manufacturer’s protocol. Mutations were confirmed by DNA sequencing.

Plasmids YCPGAL1-etOP1, YCPGAL1-etop1N722S, and YCPGAL1-etop1Y723F encode FLAG epitope-tagged human wild-type Top1, the CPT-resistant Top1N722S, or the catalytically inactive Top1Y723F under the control of a galac-tose-inducible promoter (25). The h prefix, designating human TOP1, is dropped in the following discussions. The FLAG epitope, designated by the e prefix, was included in all TOP1 constructs to facilitate protein purification. The C504A mutant sequences, excised in a BamHI-Nhel DNA fragment from top1C504A sequences in pZIP GFP-top1, were first ligated into a LEU-based vector YcpGAL1-top1C504A-L and then subcloned in a BamHI-NotI DNA fragment into the URA3 marked vector YCPGAL1-top1C504A. The FLAG epitope was subsequently introduced in a BamHI-SacII DNA fragment, from YCPGAL1-etOP1, to yield YCPGAL1-e1top1C504A. The vectors YCPGAL1-etop1C505A and YCPGAL1-etop1C504A,C505A were generated by homologous recombination of PCR products amplified from pZIP vectors containing the mutant top1 CDNAS with YCPGAL1-etOP1 linearized with SphI, as described (27). In all cases, mutations were confirmed by DNA sequencing.

**Top1 Protein Purification**—Full-length human Top1, Top1C504A, Top1C505A, Top1C504A,C505A, and Top1Y723F proteins, all containing an N-terminal FLAG epitope, were partially purified as described (28) from galactose-induced cultures of top1Δ yeast cells, transformed with YCPGAL1-eTOP1 vectors. To obtain homogeneous protein preparations, Top1 fractions were applied to an anti-FLAG M2 affinity gel (Sigma), and the proteins were eluted with an excess of FLAG peptide in TBS (50 mM Tris, pH 7.4, 150 mM KCl). To remove the peptide, the fractions were bound to a phosphocellulose column, and the homogeneous Top1 proteins were eluted in TEEG buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol) plus 1.0 M KCl and protease inhibitors, diluted with 50% glycerol and stored at −20 °C. Protein integrity was assessed in immunoblots as described (25).

**Top1-catalyzed Relaxation Assays**—Top1 catalytic activity was assessed in plasmid DNA relaxation assays, using pH1 or pHC624 as substrates. pH1 reaction mixtures (10 μl each), containing 0.25 μg of supercoiled DNA in reaction buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.1% bovine serum albumin, 0.1 mM spermidine, 5% glycerol and NaCl at the indicated concentrations), were incubated with 2 units of human Top1 in the absence or in the presence of NEM for 25 min at 37 °C. For some experiments Top1 was preincubated with NEM for 1 or 5 min prior to the addition of plasmid DNA. Reactions were ter-
minated by the addition of stop buffer (5% Sarkosyl, 0.0025% bromphenol blue, 25% glycerol) and directly electrophoresed in 1% agarose gels. The reaction products were visualized by ethidium bromide staining, and relaxed DNA topoisomers were quantified using SigmaGel software (Jandel Scientific, San Rafael, CA). Plasmid pHC624 DNA relaxation was assessed as described previously (25).

Oligonucleotide Labeling—High pressure liquid chromatography-purified oligonucleotides used in this study were purchased from Eurogentec (Angers) and are shown in Fig. 2A. 3′-End labeling (Fig. 2B, A*) of the scissile strands were performed as described previously (29). Briefly, 10 pmol of oligonucleotides were incubated with 3 μl of [α-32P]corydepin and 1 μl of terminal deoxynucleotidyldtransferase in labeling buffer (100 mM potassium cacodylate, pH 7.2, 2 mM CaCl2, 200 μM DTT) for 1 h at 37 °C. The reaction mixture was passed through a G-25 Sephadex spin column by centrifugation at 1,000 rpm for 10 min. Imaging and quantitation of the cleavage products were performed using a Typhoon PhosphorImager (Amer sham Biosciences). Oligonucleotide Labeling—High pressure liquid chromatography-purified oligonucleotides used in this study were purchased from Eurogentec (Angers) and are shown in Fig. 2A. 3′-End labeling (Fig. 2B, A*) of the scissile strands were performed as described previously (29). Briefly, 10 pmol of oligonucleotides were incubated with 3 μl of [α-32P]corydepin and 1 μl of terminal deoxynucleotidyldtransferase in labeling buffer (100 mM potassium cacodylate, pH 7.2, 2 mM CaCl2, 200 μM DTT) for 1 h at 37 °C. The reaction mixture was passed through a G-25 Sephadex spin column by centrifugation at 1,000 rpm for 10 min. Imaging and quantitation of the cleavage products were performed using a Typhoon PhosphorImager (Amer sham Biosciences).

Top1 Binding to DNA—Noncovalent binding of catalytically inactive human Top1Y723F to DNA was measured by electrophoretic mobility shift assay according to previously published procedures (24). Incubation of Top1 was performed in the presence or absence of PAO for 5 min at room temperature prior to electrophoresis on a 6% nondenaturing polyacrylamide gel. Gels were dried, autoradiographed, and quantitated using a PhosphorImager.

Top1-DNA Religation Assays—Top1-catalyzed religation assays were performed as described previously using a donor-acceptor system (Fig. 2B, panel c) (24). Briefly, 3′-end Top1-linked oligonucleotides were obtained following a 10-min incubation of 0.2 nmol of Top1 with 20 flmol of unlabeled 18-mer suicide substrate where the nonscissile strands were 5′-phosphorylated to avoid recombination (29). An equivalent amount of 3′-end-labeled single-stranded 23-mer oligonucleotide, complementary to the 5′-end of the nonscissile strand, was then added to the reaction mixture in the absence or in the presence of the thiol-reactive compound. The formation of 37-mer religated products was measured as a function of time.

Plasmid DNA Nicking Assay—CPT-induced Top1 cleavage of plasmid DNA was assessed in a DNA nicking assay as described previously (30). Briefly, 0.3 μg of plasmid pbblueAK3-1 DNA was incubated with Top1 protein and increasing concentrations of CPT in a final 5% Me2SO in reaction buffer for 30 min at 37 °C. The reactions were terminated with SDS-protease K, and the nicked DNA was resolved in a 1% agarose gel containing 2 μg/ml ethidium bromide and 0.1% SDS.

Yeast Cell Viability Assays—To assess cell sensitivity to CPT, exponential cultures of top1Δ cells, transformed with the indicated YCpGAL11-eTOP1 vector, were adjusted to A600 = 0.3 and serially diluted, and 5-μl aliquots were spotted onto selective media supplemented with 25 mM HEPES, pH 7.2, 2% dextrose or galactose and 0, 0.005, or 0.05 μg/ml CPT in a final volume of 0.15% Me2SO. Colony formation was assessed following incubation at 30 °C for 3 days.
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RESULTS

NEM Inhibits Top1-catalyzed DNA Relaxation—To investigate the contribution of the conserved cysteine residues of human Top1 to enzyme catalysis, we first incubated Top1 with NEM, a compound that reacts with protein sulfhydryl groups to form thiol adducts and has been reported to inhibit eukaryotic Top1 activity (31, 32). Addition of the –SH group to the olefinic double bond of NEM to form thiol adducts and has been reported to inhibit eukaryotic Top1 activity (31, 32). Addition of the –SH group to the olefinic double bond of NEM to form a thioether is a rapid and specific reaction at NEM concentrations of 1–5 mM (Fig. 1A). We first examined the effect of NEM on Top1 catalysis. When added to a plasmid DNA relaxation assay, NEM exhibited a dose-dependent inhibition of Top1 activity (see time 0 in Fig. 1, B and C). Moreover, this inhibitory activity of NEM on Top1 was enhanced when the enzyme was preincubated with NEM, in a time-dependent manner. Top1 activity was completely inhibited by preincubating the enzyme for 5 min with a NEM concentration as low as 0.12 mM. These results suggest the inhibition of Top1 catalysis is a direct consequence of NEM thiolation of sulfhydryl groups in the purified protein.

NEM Inhibits the Cleavage Step of the Top1 Cleavage/Religation Equilibrium—To identify which step in the Top1 reaction is impaired by NEM-induced thiolation, a series of 3’-end-labeled oligonucleotide substrates that contained a unique Top1 cleavage site was used in an in vitro assay, which allowed us to assess any effects of NEM on steady-state levels of covalent DNA-Top1 complexes and on the relative rates of Top1-catalyzed DNA cleavage or religation. CPT reversibly stabilizes a covalent Top1-DNA complex by inhibiting DNA religation. The sequences of the oligonucleotides used and experimental design are diagrammed in Fig. 2, A and B. For example, in the scheme of Fig. 2B (panel a), the formation of CPT-induced Top1-DNA complexes with the 37-mer duplex DNA substrate would result in the accumulation of a cleaved 23-mer product. Thus, under steady-state conditions, we can directly assess an effect of Top1 thiolation on CPT-induced covalent complex formation by measuring 23-mer product levels. Indeed, as seen in Fig. 3, A (left panel) and B, NEM inhibited the formation of CPT-Top1-DNA complexes in a dose-dependent manner. Moreover, consistent with the relaxation experiments in Fig. 1, preincubating the enzyme with NEM completely abrogated the formation of CPT-Top1-DNA complexes (Fig. 3, A, right panel, and B). Thus, the formation of human Top1 covalent complexes is inhibited by NEM thiolation of sulfhydryl groups. A decrease in covalent complexes can either result from decreased rates of DNA cleavage by Top1 or increased rates of religation, either because of an intrinsic increase in enzyme-catalyzed DNA religation or a decrease in CPT affinity for the covalent Top1-DNA intermediate. To distinguish between these possibilities, we used a DNA substrate containing a truncated scissile strand, which acts as a suicide substrate to uncouple the DNA cleavage from religation reactions of the Top1 catalytic cycle. As diagrammed in Fig. 2B (panel b), cleavage of the 3’-end-labeled 19-mer liberates a 5-mer cleavage product that dissociates from its complementary strand and cannot be religated by the enzyme (24, 33, 34). The lower strand was phosphorylated in order to avoid intramolecular religation (29). Under these conditions, the kinetics of DNA cleavage was assessed in the absence or in the presence of increasing concentrations of NEM (Fig. 4A), by monitoring the accumulation of the 5-mer product. An approximate 2-fold reduction in the cleavage rate was observed in the presence of NEM, demonstrating that NEM thiolation of Top1 inhibits the cleavage step of the catalytic cycle. Inhibition was drastically enhanced when NEM was preincubated with Top1 prior to the addition of the DNA substrate (data not shown). We next analyzed the effect of NEM on DNA religation using the “donor-acceptor” system diagrammed in Fig. 2B (panel c). In this assay, the unlabeled

FIGURE 2. Oligonucleotide-based Top1 cleavage assays. A, sequences of full-length and partially double-stranded (suicide substrate) oligonucleotides. The 36-mer sequence is derived from a Tetrahymena rDNA hexadecameric sequence where the A was changed to a G at the +1 position relative to the Top1 cleavage site (indicated by the caret) to enhance camptothecin sensitivity. B, panel a, the 37-mer duplex substrate 3’-labeled (A*) on the scissile (upper) strand is used to measure the levels of Top1-DNA covalent complexes formed under steady-state conditions. Once equilibrium is reached, the 23-mer cleavage products are separated from the 37-mer uncleaved substrate by electrophoresis and quantified using a PhosphorImager. Panel b, the use of 3’-end-labeled suicide substrate uncouples Top1 DNA cleavage from religation as the 5-mer cleavage product is not efficiently religated under these conditions because of limited base pairing. The kinetics of 5-mer accumulation approximates the rate of DNA cleavage. Panel c, donor-acceptor system used to assess the kinetics of Top1-catalyzed religation. In this assay, an acceptor molecule (covalent Top1-DNA complex) is first generated by incubating the unlabeled suicide substrate with Top1. Then an excess of 3’-end-labeled complementary single-stranded 23-mer oligonucleotide, referred to as donor, is added to the reaction mixture, and the appearance of the 37-mer religated product is measured as a function of time. P, 5’-phosphorylated termini.
suicide substrate is incubated with Top1 for 10 min to generate "acceptor" complexes, where Top1 is covalently linked to the 3′-end of the scissile strand (Fig. 2B (panel c)). The "acceptor" complexes are then incubated with a 10-fold excess of a 3′-end-labeled, complementary single-stranded 23-mer oligonucleotide referred to as "donor" molecule, and the amount of 37-mer religated product is quantitated as a function of time. The kinetics of Top1-catalyzed DNA religation were evaluated under these conditions in the absence or in the presence of 2 and 5 mM NEM concentrations (Fig. 4B). The initial slopes (Fig. 4B, inset) did not indicate any difference in the rate of DNA religation, suggesting that NEM does not affect this step of Top1 catalysis. The final levels of religated product reflect the differences in the amount of Top1-DNA acceptor complexes generated in the presence of increasing NEM concentrations. Taken together, these results indicate that the reduction in Top1 cleavage complex formation induced by NEM results from an inhibition of Top1-catalyzed DNA cleavage. Thus, NEM thiolation acts as a Top1 catalytic inhibitor.

The Vicinal Thiol-reactive Compound Phenylarsine Oxide Inhibits Top1-catalyzed DNA Cleavage—PAO is a trivalent arsenic derivative that, at micromolar concentrations, selectively cross-links vicinal thiol groups by forming a stable ring structure (Fig. 5A) (35, 36). As two of the eight cysteine residues contained in the conserved core and C-terminal domains of human Top1 are vicinal (Cys-504 and Cys-505), we asked if PAO cross-linking of these vicinal thiol groups exerts the same effects on Top1 catalysis as NEM thiolation. Indeed, using the 3′-end-labeled “suicide” oligonucleotide as a substrate, we demonstrated that treatment of Top1 with micromolar concentrations of PAO inhibited enzyme-catalyzed DNA cleavage to the same extent as NEM (Fig. 5B), with an approximate 2-fold reduction in the rate of DNA cleavage in the presence of 0.5 or 1 μM PAO. Moreover, as observed with Top1 thiolation by NEM, inhibition of DNA cleavage was more pronounced when Top1 was preincubated with PAO, with a complete inhibition of Top1 complex formation induced by 0.25 μM PAO (data not shown). These effects were not because of an inhibition of Top1 binding to the DNA substrate as shown by electrophoretic mobility shift assays (Fig. 5B, inset). In the absence of PAO, incubation of DNA with catalytically inactive human Top1Y723F, which retains its DNA binding properties but cannot cleave DNA, resulted in a mobility shift (Fig. 5B, inset, lane 2). The intensity of this band shift was unaffected by the presence of 1 or 2 μM PAO (Fig. 5B, inset, compare lanes 3 and 4 to lane 2, respectively). Also, no significant effect of PAO on Top1-catalyzed religation was detected, even when high concentrations of the arsenic derivative were used (Fig. 5C). Together, these results suggest that the thiolation of vicinally spaced cysteines within Top1 inhibited enzyme-catalyzed DNA cleavage.

NEM- and PAO-induced Inhibition of Top1 DNA Cleavage Is Reversible—To determine whether Top1 thiolation was reversible, we used the reducing agent dithiothreitol (DTT), which reverses the inhibitory effect of thiol-reactive compounds (35, 37–40). To assess the effects of DTT on NEM- or PAO-induced alterations in Top1-DNA complex formation, the 3′-end-labeled suicide substrate (Fig. 2B (panel b)) was incubated with Top1 in the presence of 1 mM NEM or 0.5 μM PAO for 5 min at 25 °C. This led to an approximate 50% reduction in Top1-DNA complex formation (Fig. 6A, compare lane 2 with lane 6 with 5, respectively). However, further incubation with 1 mM DTT for 30 min restored the cleavage activity of Top1 in the case of NEM and partially reversed the inhibitory effects of PAO (Fig. 6A, compare lane 4 with 2 and lane 8 with 6, respectively). Thus, NEM adduction of Top1 sulphydryl groups by NEM and, to a lesser extent by PAO, could be reversed by DTT.

Vicinal Cysteines 504 and 505 Are Involved in PAO-mediated Inhibition of Top1 Cleavage—PAO specifically reacts with vicinal cysteines within the noncovalent co-crystal structure of human Topo70 bound to duplex DNA. In this configuration
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The lower salt optimum (100 mM KCl) of Top1C504A further suggests a defect in DNA binding. Surprisingly, however, the combination of the two mutations restored the specific activity and salt optimum of the double Top1C504A,C505A mutant enzyme to that observed for wild-type Top1. We then tested the effects of PAO on plasmid DNA relaxation catalyzed by the Top1 mutants. Relative to the reduction in DNA relaxation induced by PAO treatment of wild-type Top1, all three mutants exhibited resistance to the inhibitory effects of PAO (Fig. 8B). In the comparison of wild-type Top1 with Top1C504A, PAO treatment produced a much more pronounced inhibition of wild-type Top1, despite the more than 50-fold difference in enzyme activity. These data demonstrate that the conserved vicinal cysteines 504 and 505 are required for PAO-mediated inhibition of Top1 cleavage and suggest that these residues may regulate enzyme activity in response to cellular stresses.

Along these lines, we next asked if cysteine 504 and 505 affect Top1 sensitivity to camptothecin. First, the CPT sensitivity of the purified proteins was assessed in a plasmid DNA nicking assay. Equal concentrations of the proteins were incubated with negatively supercoiled plasmid DNA and increasing concentrations of CPT. The formation of ternary CPT-Top1-DNA covalent complexes was then assessed by treating the reactions with SDS and proteinase K and resolving the complexes by the Top1 mutants. Relative to that observed for wild-type Top1, Top1C504A,C505A were ~50- to 20-fold lower, respectively, than wild-type Top1. (Fig. 7B), the distance between the sulfur atoms of the vicinal Cys-504 and Cys-505 thiol groups is 5.4 Å, which is in close enough proximity to support a reaction with PAO. To test this hypothesis, alanine mutations of these two residues were engineered individually and in combination, and the mutant proteins were purified. In plasmid DNA relaxation assays (Fig. 8A), the specific activities of equal concentrations of the single mutant enzymes, Top1C504A and Top1C505A, were ~50- to 20-fold lower, respectively, than wild-type Top1.
sion of wild-type Top1 conferred yeast cell sensitivity to CPT, whereas cells expressing the CPT-resistant Top1N722S mutant (41, 42) were viable in the presence of high concentrations of the drug (Fig. 8D). Cells expressing Top1C504A exhibited more robust cell growth on galactose plates than cells expressing wild-type Top1, yet exhibited the same sensitivity to CPT. In contrast, cells expressing either the single Top1C505A or the double Top1C504A,C505A mutant enzyme exhibited a slow growth phenotype at 0.05 μg/ml CPT, such that small colonies were evident at 10- and 100-fold dilutions of cells (Fig. 8D, 2nd and 3rd columns of spots). The increased resistance of these cells to CPT is consistent with the pattern of CPT sensitivity observed in vitro (Fig. 8C). These data suggest that in contrast to the role of the vicinal cysteines 504 and 505 in mediating Top1 sensitivity to PAO, cysteine 505 alone is a determinant of enzyme sensitivity to CPT.

DISCUSSION

Sulfhydryl groups of cysteines are among the most reactive functional groups in a protein. Compounds such as NEM or PAO selectively react with sulfhydryl groups and have been used to define the importance of cysteine residues in the catalytic activity of several enzymes, including A-integrase, various phosphatases, NADPH oxidase, or Rho GTPases (36, 41–44). As discussed previously, human Top1 contains eight highly conserved cysteines, of which two are vicinal (Cys-504 and Cys-505) (23). All of the cysteines are located within the conserved core and C-terminal domains of the protein, which are both essential for Top1 activity and CPT sensitivity (5, 21, 45).

In this study, we examined the mechanistic basis upon which reactions of cysteinyl moieties of Top1 with thiol-reactive compounds impair the catalytic activity of the enzyme (31, 32). We first tested the effect of NEM on purified human Top1. In this case, the reaction involves the addition of a single –SH group to the olefinic double bond of NEM to form a thioether (46). This reaction is highly specific at low concentrations of the thiol reactant (1–5 mM). We showed that millimolar concentrations of NEM inhibited Top1-catalyzed DNA relaxation. Using oligonucleotides containing a unique Top1 cleavage site, we then showed that NEM suppressed CPT-induced Top1 poisoning. This suppression was even more pronounced when the enzyme was preincubated with NEM prior to the addition of the DNA substrate, suggesting a direct interaction of NEM with cysteinyl thiol group(s) of the free enzyme or of the enzyme bound to the DNA in its noncovalent form. It is also possible that the formation of Top1 thiol adducts decreased the affinity of the covalent Top1-DNA complex for CPT, thereby reducing the levels of cleaved DNA products under the steady-state conditions used in these assays.

These considerations were addressed using suicide oligonucleotides to uncouple the cleavage and religation reactions catalyzed by Top1 in the absence of CPT. Here the effect of NEM was to inhibit the rate of DNA cleavage by Top1, with no apparent alteration in the rate of enzyme-catalyzed DNA religation. These data refute the notion that NEM modification of reactive Top1 thiols inhibits CPT poisoning solely by suppressing...
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Drug binding to the covalent Top1-DNA complex. Although alterations in CPT binding may be a contributing factor, these results support a model whereby covalent modification of Top1 cysteine thiol groups in the catalytic activity of Top1. Cysteines were also shown to play an important role in the human Top2 catalytic activity. However, in that case, NEM had an opposite effect in stimulating covalent complex formation by interacting with sulfhydryl groups that are accessible to the thiol-reactive compound only when the enzyme was covalently bound to the DNA (17). Taken together, these data indicate that the reactivity of such a thiol-reactive agent depends on both the positioning and accessibility of the cysteinyln units in the tertiary structure of the enzyme.

Conversely to NEM, PAO is active at much lower concentrations and specifically cross-links vicinally spaced cysteines to form stable dithioarsine rings (36, 37, 47). In our studies, micromolar concentrations of PAO inhibited the Top1 cleavage of DNA without affecting protein binding to the DNA substrate, and also suppressed CPT poisoning of Top1 (data not shown). Similar to NEM, PAO inhibition of DNA cleavage by Top1 was more pronounced when the enzyme was preincubated with PAO prior to the addition of the DNA, and Top1-catalyzed DNA religation was unaffected. The involvement of vicinal thiol groups in PAO-mediated inhibition of Top1 was further evidenced by the fact that this effect could be reversed by DTT, a well known 1,4-dithiol competitor. This reversal was only partial, which may be explained by the fact that DTT competition for the dithioarsine ring is not favored as it results in a seven-member ring that is less stable than the larger nine-member ring induced by PAO cross-linking. Our results suggest PAO directly interacts with two accessible and closely spaced cysteinyln residues of the same Top1 molecule, either in its free form or when it is noncovalently bound to the DNA. Of particular relevance to this model is the fact that these two conserved, adjacent cysteines (Cys-504 and Cys-505) are located in a loop with a sulfur-to-sulfur distance between their thiol groups of 5.4 Å, which is compatible with the formation of a dithioarsine

FIGURE 8. Cysteines 504 and 505 are involved in PAO-mediated inhibition of Top1 cleavage. A, equal concentrations of purified wild-type human Top1 and the indicated mutant enzymes were serially diluted 10-fold and incubated in a plasmid DNA relaxation assay as indicated under "Experimental Procedures" in the presence of 100, 150, or 200 mM KCl (final concentrations). Reaction products were resolved in an agarose gel and subsequently visualized by ethidium bromide staining. Lane D, supercoiled DNA plasmid. B, effects of C504A and C505A mutations of Top1 on PAO-mediated inhibition of DNA relaxation. Wild-type and mutant Top1 enzymes were incubated in a plasmid DNA relaxation assay (150 mM KCl), in the presence or absence of 200 μM PAO in a final 5% Me2SO. C, CPT sensitivity of wild-type Top1 and C504A, C505A, and C504A,C505A mutant enzymes. Plasmid DNA was incubated with equal amounts of Top1 proteins for 30 min at 37 °C in the presence of indicated concentration of CPT. The reactions were terminated with SDS/proteinase K, and the CPT-induced nicked DNAs were separated from relaxed topoisomers by agarose gel electrophoresis in the presence of ethidium bromide. D, effects of Top1 mutations on CPT sensitivity in yeast. EKY3(Δtop1) yeast cells were transformed with YCpGAL1-eTOP1 or top1 mutants as indicated on the left. Exponential cultures of individual transformants were serially 10-fold diluted and spotted onto selective medium supplemented with dextrose (Dex) or galactose (Gal), 25 mM HEPES, pH 7.2, and the indicated concentration of CPT. Cell viability was assessed after a 3-day incubation at 30 °C.
nine-membered ring (Fig. 7B) (45). Further support for this model comes from studies of alanine substitutions of Cys-504 and Cys-505, where plasmid DNA relaxation induced by either TopIC505A, TopIC504A, or TopIC504A,C505A mutant enzymes was not inhibited by PAO to the same extent as for the wild-type enzyme, even though the specific enzyme activity of the double mutant was comparable with that of wild-type Top1. Our data cannot exclude the involvement of other Top1 cysteines in the reaction with PAO. A study of the hydrodynamic properties of different recombinant forms of human Top1 has demonstrated that the N-terminally truncated human Topo70 folds into a globular structure (48). Moreover, considerable structural flexibility is necessary for enzyme binding of duplex DNA and rotation of the cleaved DNA within the Top1 protein. This important notion derives from crystal structures of human Topo70 in complex with DNA (49, 50) and studies based on the reversible cross-linking of the human Top1 protein clamp (28). Thus, it is possible that distant cysteiny1 residues of the primary structure of the enzyme might come closer together and become a target for PAO. However, among the six other cysteines, the next shortest sulfur-to-sulfur distance, as measured from available crystallographic data, is 10.8 Å between the thiol groups of Cys-341 and Cys-386 (45). This distance is too long to accommodate the formation of a dithioarsine linkage, requiring unanticipated and rather substantial conformational changes to occur in the protein to shorten this distance. Intermolecular cross-linking of cysteiny1 residues from different Top1 molecules might also be envisaged. However, we consider this possibility unlikely as immunoblot experiments failed to reveal the formation of multimers when purified Top1 was incubated with increasing concentrations of PAO (data not shown).

Cysteines 504 and 505 are located near the base of a loop that includes the Lip 2 region (residues 496–505) of the Top1 DNA complex (see Fig. 7A) (21, 45, 51). The interaction of this region with an opposable loop in Lip 1 completes the circumscription of the Top1 protein clamp around duplex DNA. Because mutation of Cys-504 and Cys-505 in the double mutant did not alter enzyme catalytic activity, the two cysteines are not required for effective binding of the protein to DNA or enzyme catalysis (DNA strand cleavage, rotation, or religation). However, our data suggest adduction of PAO alters the rate of Top1-catalyzed DNA cleavage without affecting substrate binding. As there are no crystal structures of the Top1 protein alone, it is unclear whether the juxtaposition of the active site tyrosine within the catalytic pocket formed by the clamp core domain is affected by protein clamp closure around the DNA. Our findings are consistent with a model where the introduction of a bulky residue at the base of the Lip 2 loop alters the architecture of the active site so as to diminish the rate of DNA cleavage, but not DNA binding, effectively uncoupling the closure of the Top1 protein clamp around duplex DNA from the geometry of the active site tyrosine necessary for DNA strand scission.

The CPT resistance of a G365C mutant of human Top1 was suppressed by an S534C mutation in the lower Lip (28), suggesting that the functional interaction between the two Lip domains also dictates enzyme sensitivity to CPT. Lip 2 also contains glycine 503, mutation of which to a serine confers CPT resistance (51, 52). Interestingly, we found that mutation of Cys-505, but not Cys-504, to alanine also induced a CPT-resistant phenotype in yeast, with the effect being evident for both the single Top1C505A mutant and double Top1C504A,C505A mutant. It could well be that alteration of the Lip 2 region, either as a consequence of Cys-505 mutation or thiol adduction by sulfhydryl reactive agents, induces a similar shift in the orientation of aspartate 533 and subsequent drug binding as that observed for the G503S mutation (51). Thus, our results demonstrate a critical role of the two vicinal cysteines 504 and 505 in the DNA cleavage reaction catalyzed by human Top1 activity and for Cys-505 in the optimal poisoning of Top1 by the camptothecin class of chemotherapeutics.

There are two classes of Top1-targeted agents as follows: (i) Top1 poisons that stabilize covalent Top1-DNA intermediates by inhibiting the religation step of the Top1 reaction. These include camptothecin derivatives, which have significant activity against adult and pediatric solid tumors and FDA approval for the treatment of colon, ovarian, and lung cancers (53). (ii) Top1 catalytic inhibitors that inhibit binding of the enzyme to the DNA and/or the cleavage step of the Top1 reaction (54, 55). A growing number of compounds fall in this category but are often nonselective or too toxic for clinical development (54, 55). Among them, a series of naphthoquinone derivatives were shown to be active against a variety of cancer cell lines indicating their potential clinical utility (17, 56). In this study, we showed NEM and PAO mimic the effects of Top1 catalytic inhibitors. In contrast to arsenic trioxide, which was recently shown to indirectly induce the formation of Top1 cleavage complexes via the generation of reactive oxygen species (57), our data indicate Top1 inhibition by PAO is a direct consequence of cysteine 504 and 505 cross-linking. These results provide a new approach in the search for catalytic inhibitors based on the selective targeting of Top1 cysteiny1 residues, which may be exploited in the development of novel chemotherapeutics that target Top1 to complement the existing regime of Top1 poison-based cancer therapy.

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