Eukaryotic DNA topoisomerase I (Top1p) catalyzes the relaxation of supercoiled DNA and constitutes the cellular target of camptothecin (CPT). Mutation of conserved residues in close proximity to the active site tyrosine (Tyr\textsuperscript{727} of yeast Top1p) alters the DNA cleavage religation equilibrium, inducing drug-independent cell lethality. Previous studies indicate that yeast Top1T722Ap and Top1N726Hp cytotoxicity results from elevated levels of covalent enzyme-DNA intermediates. Here we show that Top1T722Ap acts as a CPT mimetic by exhibiting reduced rates of DNA religation, whereas increased Top1N726Hp-DNA complexes result from elevated DNA binding and cleavage. We also report that the combination of the T722A and N726H mutations in a single protein potentiates the cytotoxic action of the enzyme beyond that induced by co-expression of the single mutants. Moreover, the addition of CPT to cells expressing the double top1T722A\texttildelow{}N726H mutant did not enhance cell lethality. Thus, independent alterations in DNA cleavage and religation contribute to the lethal phenotype. The formation of distinct cytotoxic lesions was also evidenced by the different responses induced by low levels of these self-poisoning enzymes in isogenic strains defective for the Rad9 DNA damage checkpoint, processive DNA replication, or ubiquitin-mediated proteolysis. Substitution of Asn\textsuperscript{726} with Phe or Tyr also produces self-poisoning enzymes, implicating stacking interactions in the increased kinetics of DNA cleavage by Top1N726Hp and Top1N726Fp. In contrast, replacing the amide side chain of Asn\textsuperscript{726} with Gln renders Top1N726Qp resistant to CPT, suggesting that the orientation of the amide within the active site is critical for effective CPT binding.

DNA topoisomerases catalyze changes in the linkage of DNA strands, playing critical roles in DNA replication, transcription, and recombination, as well as chromosome condensation and segregation (reviewed in Refs. 1–3). Alterations in DNA topology are catalyzed in reactions characterized by the formation of a covalent enzyme-DNA intermediate. Eukaryotic DNA topoisomerase I (Top1p)\textsuperscript{1} is a type IB enzyme that transiently cleaves a single strand of duplex DNA, while forming a tyrosyl linkage with the 3’-phosphoryl end of the cleaved DNA (1–3). Rotation of the non-covalently held DNA around the noncissile DNA strand allows for the relaxation of positive or negative supercoils. In a second transesterification reaction, the 5’-OH nuclease attacks the phosphotyrosyl linkage to religate the DNA.

In eukaryotes, the nuclear type IB enzyme, encoded by the TOP1 gene, is highly conserved in terms of amino acid sequence and catalytic mechanism (1–5). The nuclear enzyme is also the cellular target of several antitumor agents, such as camptothecin (CPT) and indolocarbazole analogs (reviewed in Refs. 6–9). The CPT analogs, Topotecan and SN-38 (the active metabolite of CPT-11), have shown remarkable antitumor activity against a broad range of pediatric and adult malignancies (10). These drugs target Top1p by reversibly stabilizing the covalent enzyme-DNA intermediate. During S-phase of the cell cycle, the collision of advancing replication forks with the ternary drug-Top1p-DNA complexes produces irreversible DNA lesions that trigger S-phase checkpoint activation, cell cycle arrest, and cell death (1, 5–8). Consistent with this cytotoxic mechanism, cells treated with aphidicolin to inhibit DNA replication are resistant to CPT (11). In the yeast Saccharomyces cerevisiae, strains deleted for specific DNA damage and replication checkpoint genes, such as \emph{RAD53}, \emph{MEC1}, \emph{TEL1}, \emph{RAD9}, \emph{RAD17}, \emph{RAD24}, and \emph{TOP1}, exhibit enhanced sensitivity to CPT and other Top1p poisons (12–17). Moreover, cells defective in processive DNA replication (because of alterations in Cde45p or Dpb11p) (18) or in maintaining replication fork stability (for example in cells deleted for \emph{Sgs1}) (19) are also hypersensitive to Top1p-induced DNA lesions.

Structural determinations of human Topo-70, a 70-kDa C-terminal fragment of human DNA topoisomerase I, in covalent and noncovalent complexes with duplex DNA, revealed a circumferential binding of DNA by the Top1 protein clamp (3, 20, 21). Using molecular modeling to design a reversible disulfide bond across the opposable lip domains that complete the clamp, we recently demonstrated that DNA rotation is inhibited within the locked Top1 clamp-DNA complex (22). Thus, some flexibility of Top1p protein domains is required for enzyme binding of DNA and the rotation of DNA strands necessary to effect changes in DNA topology. These studies further demonstrated that expression of the catalytically inactive Top1Y723Fp-clamp in yeast cells with elevated levels of oxidized glutathione, also sufficed to induce cell lethality independent of covalent complex formation (22).

\textsuperscript{1}The abbreviations used are: Top1p, DNA topoisomerase I; CPT, camptothecin, S.C., synthetic complete; MES, 4-morpholineethanesulfonic acid.
More subtle alterations in the DNA cleavage religation equilibrium catalyzed by Top1p have also been shown to be cytotoxic. For example, the introduction of an abasic site or a mismatch at the +1 position in the scissile DNA strand enhances the stabilization of the covalent complex (reviewed in Ref. 23). The structural distortions induced by incorporation of Ara-C at the +1 position of the complementary nonscissile strand also stabilize the covalent enzyme-DNA intermediate by reducing DNA religation (24). Mutation of conserved residues N-terminal to the active site tyrosine in the yeast and human enzymes also induces Top1p poisoning (12, 25–27). Substitution of Ala for Thr722 in yeast Top1p (or Thr718 in human Top1p) appears to mimic the action of CPT in stabilizing the covalent complex (12, 26). Based on the lack of detectable alterations in DNA binding or cleavage, a reduced rate of DNA religation by Top1T722A was inferred (28), similar to that induced by CPT (29, 30). Recent structures of the binary Topo-70 DNA and ternary topotecan-Topo-70 DNA complexes indicate the formation of a hydrogen bond between the hydroxyl of Thr718 and the nonbridging oxygen of the +1 phosphodiester of the DNA (30). Thus, substitution of Ala at this position may alter the orientation of the 5′-OH nucleophile and impede DNA religation. A distinct self-poisoning mechanism was evidenced when Asn726 in yeast Top1p (or Asn726 in human Top1p) was mutated to Gln. In this case, enhanced stabilization of the covalent enzyme-DNA complex was attributed to increased rates of DNA cleavage.

Here we report that the T722A mutation directly inhibits Top1p-catalyzed DNA religation, while Asn726 substitutions have diverse effects on enzyme sensitivity to CPT, Top1p binding to DNA, and enzyme-catalyzed DNA cleavage. Mutating Asn726 to His, Tyr, or Phe indicates that a phenolic or imidazole side chain enhanced the kinetics of DNA cleavage by Top1p, whereas the CPT resistance of Top1N726Qp suggests the orientation of the amide side chain is critical for effective drug binding. Further, the combination of the T722A and N726H mutations in a single enzyme significantly potentiated the cytotoxic activity of the double mutant beyond that induced by co-expression of the individual mutant enzymes. Biochemical studies indicate that the independent alterations in DNA cleavage and religation, evident in the double mutant, each contribute to the lethal phenotype. Genetic studies further support the notion that these self-poisoning enzymes induce distinct cytotoxic lesions in vivo. These findings suggest that more effective Top1p poisoning may be achieved with a combination of therapeutics inducing distinct alterations in Top1p catalysis.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Yeast Strains, Plasmids, and Oligonucleotides—Galactose was purchased from U.S. Biological (Swampscott, MA) and raffinose was from USB (Cleveland, OH). CPT (Sigma) was dissolved in MeSO and stored as aliquots at −20 °C. [α-32P]Cordycepin 5′-triphosphate was purchased from PerkinElmer Life Sciences (Boston, MA). S. cerevisiae strains EKY3 (Mata, ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1), MBY3 (EKY3, rad52Δ::HIS3), MMY3 (EKY3, rad6Δ::HIS3), YC11 (YcpGAL1-etop1T722AL, YcpcScTOP1L), RRY72 (top1Δ, dpb1-11), RRY71 (top1Δ, cdc45-10), and RRY72 (top1Δ, cdc54-10) and RRY79 (top1Δ, cdc54-10) were transformed with URA3 marked vectors (YcpcScTop1N726H, YcpcScTop1N726L, YcpcScTop1T722A, YcpcScTop1T722AL) and leptidase I (YcpcScTop1L, YcpcScTop1N726L, YcpcScTop1N726L, or YcpcScTop1T722AL) and plated onto SC-uracil or SC-leucine, dextrose plates, respectively. Colony number and size were assessed after 3 days incubation at 30 °C. DNA Topoisomerase I Purification and Activity—Top1p proteins were purified from EKY3 cells as described (27, 34). Top1 protein integrity and activity were assessed by immunoblotting with yeast Top1p-specific antibodies or the M2 FLAG antibody (Kodak).

**DNA Topoisomerase I Activity**—DNA cleavage was assayed by relaxation of supercoiled plasmid DNA as described (25, 27). Briefly, serial 10-fold dilutions of Top1p proteins (corrected for concentration) were incubated in 20–50 mM HEPES (pH 7.5), 2 mM dithiothreitol, 150 mM NaCl, and 150 mM KCl. Where indicated, final KCl concentrations ranged from 75 to 200 mM. After 1 h at 30 °C, reaction products were resolved in agarose gels and visualized after staining with ethidium bromide.

**DNA Cleavage Assays—Top1p sensitivity to CPT was assessed in DNA cleavage assays (25, 27). A single 3′-end-labeled DNA substrate (8,000 rpm) was incubated with Top1p proteins in 50 mM reaction volumes containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.1 mM EDTA, 50 mM KCl, 50 μg/ml gelatin, and 0 or 50 μM CPT in final 4% MeSO. After 30 min at 30 °C, the reactions were terminated with 1% SDS and heated to 75°C, treated with proteinase K, ethanol-precipitated, and resolved in 8% polyacrylamide/7 M urea gels. Cleavage products were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Suicide Cleavage Reactions—**Oligonucleotide-based assays were used to assess relative rates of DNA cleavage catalyzed by wild-type and mutant Top1p. Two approaches were taken. First, suicide substrates contained a truncated scissile strand with a high affinity cleavage site (5′-GATCTAAAAAGACTTGCTGAG-3′) (25, 27) or a CPT-sensitive cleavage site (5′-GCCGGAGACATAAGT-GTAA-3′) described in Ref. 34, where the 5′-end of the phosphodiester bond cleaved by Top1p. Suicide substrates were also generated in which a longer scissile strand contained a bridging phosphorothioate linkage at the cleavage site (indicated by “s”) in 5′-GATCTAAAAAGACATGTTGAA-AAAAAGATC-3′. Synthesis of the phosphorothioate was as described (35). Oligonucleotides were 3′-end-labeled with [32P]Cordycepin using terminal deoxynucleotidyl transferase. A modified buffer (100 mM MES, pH 6.8, 2 mM MgCl2, and 1 mM dithiothreitol) was used with phospho-
Self-poisoning Topoisomerase I Mutants

Substitution of Asn276 with His or Asp Alters Top1p Affinity for DNA—We previously reported that substitution of conserved residues immediately N-terminal to the active site tyrosine of yeast or human Top1p (Ty727 or Ty726, respectively) alters enzyme catalysis and sensitivity to CPT (12, 25–27). Mutating yeast Thr727 (or human Thr726) to Ala mimics that action of CPT in stabilizing the covalent enzyme-DNA complex (12, 26). These self-poisoning enzymes are lethal when overexpressed in yeast or human cells and induce a terminal phenotype indistinguishable from CPT-treated cells expressing wild-type Top1p (12, 31, 36). In comparisons with the wild-type enzyme, the specific activity of Top1T722Ap was slightly decreased; however, protein binding of DNA and rates of enzyme-catalyzed DNA cleavage were unaltered (12, 28). From these data, a decreased rate of DNA religation was inferred, similar to that induced by CPT. In contrast, while substitution of Asn276 in yeast Top1p with Asp or His also produced self-poisoning enzymes in yeast, the lethal phenotypes resulted from distinct effects on enzyme catalysis (25). Several lines of evidence suggested a defect in Top1N726D protein binding of DNA; however, DNA binding and religation were not directly addressed (25). The introduction of His at position Asn276 in Top1N726Hp enhanced the rate of enzyme-catalyzed DNA cleavage, although the molecular basis of this effect remained unclear.

To address these issues and further define the contribution of these residues to the DNA cleavage relaxation equilibrium, we investigated the effects of Thr727 and Asn276 mutations on Top1 protein binding of DNA and enzyme catalysis. As previously described, the introduction of an N-terminal FLAG tag allowed us to directly assess alterations in the DNA binding of purified, intact, bead bound proteins (28, 36). Increasing amounts of Top1 proteins, corrected for concentration in immunoblots with the epitope-specific M2 antibody, were first bound to streptavidin-coated cellulose beads via a biotinylated M2 antibody. After extensive washing to remove unbound protein, the bead-bound Top1p was incubated, at the indicated KCl concentrations, with a 32P-labeled DNA fragment that contains a high affinity Top1p binding site. Bead-bound Top1p-DNA complexes and unbound DNA fractions were recovered in spin columns, and the percent DNA bound was determined (25).

As previously reported (28), the relative affinities of wild-type Top1, the self-poisoning Top1T722A and the catalytically inactive Top1Y727F proteins for DNA were identical at 50 mM KCl (Fig. 1, A and B). However, consistent with the observed differences in enzyme specific activity with increasing salt concentrations (25), substitution of His or Asp for Asn276 (N726H or N726D, respectively) altered the DNA binding profiles of the single mutant enzymes. The affinity Top1N726Dp for DNA was reduced about 2.5-fold relative to that observed for wild-type Top1p at 50 or 75 mM KCl (Fig. 1A). In contrast, Top1N726Hp binding of DNA increased from 2.5-fold at 50 mM KCl to 4-fold at 100 mM KCl (Fig. 1, A and B). Mutation of the active site Tyr727 to Phe (Y727F) precludes covalent complex formation. Since the DNA binding profiles for Top1Y727Fp and Top1N726H/ Y727Fp overlapped those of Top1p and Top1N726Hp, respectively, noncovalent DNA binding was measured under these conditions. Similar results were obtained in comparisons of Top1N726Dp and Top1N726D/Y727Fp (data not shown). Thus, these data confirm the alterations in DNA binding induced by mutation of Asn276 to His or Asp, suggested by results obtained with DNA cleavage and relaxation assays.

Using suicide DNA substrates to assess DNA cleavage in the absence of DNA religation, an increased rate of DNA cleavage by Top1N726Hp, relative to Top1p and Top1T722Ap, was determined (25). Based on the increased levels of covalent enzyme-DNA complexes formed by Top1T722Ap in the absence of CPT and the DNA binding defects of Top1N726Dp, we further posited that the lethal phenotype induced by these top1 mutants was a consequence of defects in mutant enzyme-catalyzed DNA relaxation (25). As diagrammed in Fig. 2A, relative rates of DNA religation can be assessed in a DNA religation assay. Indeed, Top1N726Dp and Top1T722Ap exhibited reduced rates of DNA religation (accumulation of the 36-mer in Fig. 2B), relative to that observed with Top1p.

Top1 Asn276 Mutants Induce Different Cellular Responses—The distinct mechanisms of stabilizing Top1p-DNA complexes (increased DNA cleavage (N726H), decreased DNA religation (T722A) and defects in DNA binding (N726D)) raise the possibility that such alterations in enzyme catalysis may induce different DNA lesions and cellular responses. Toward this end, we asked if low constitutive expression of the mutant enzymes was tolerated in repair-proficient, DNA damage checkpoint competent cells. In contrast to wild-type Top1p, high levels of Top1N726Hp, Top1N726Dp, and Top1T722Ap expression induced a self-poisoning phenotype in yeast. In the presence of 5 mM 2OG, a concentration that does not affect wild-type Top1p, Top1T722Ap and Top1N726Dp proteins were stable and exhibited a self-poisoning phenotype. Unexpectedly, Top1N726Hp proteins were unstable, and a second control, Top1N726Dp, was stable. These data indicate that the lethal phenotype induced by these top1 mutants was a consequence of defects in mutant enzyme-catalyzed DNA religation (25).
achieved by the GAL1 promoter is sufficient to induce top1Δ cell death in the absence of CPT (12, 25, 28). However, low constitutive levels of Top1T722Ap, expressed from the yeast TOP1 promoter are tolerated in top1/H9004 cells, which are otherwise wild type for DNA repair and checkpoint pathways (13, 18). On the other hand, isogenic top1/H9004 strains defective in homologous recombination (because of deletion of RAD52) or the Rad9p DNA damage checkpoint (deletion of RAD9) were unable to tolerate low levels of DNA damage induced by this top1 mutant (13, 18).

In plasmid transformation assays, cells defective in homologous recombination (rad52Δ in Fig. 3; or rad50Δ, data not shown) were unable to tolerate low levels of Top1T722Ap, Top1N726Dp, or Top1N726Hp. However, cells deleted for the Rad9p DNA damage checkpoint exhibited variable sensitivity to the distinct self-poisoning enzymes. Isogenic rad9Δ cells were inviable in the presence of Top1N726Hp, exhibited a slow growth phenotype in the presence of Top1T722Ap and were unaffected by expression of Top1N726Dp. As similar levels of Top1 proteins were expressed in these isogenic top1Δ strains (data not shown), the distinct phenotypes were unlikely caused by differences in mutant enzyme levels. Rather, these results suggest two possibilities: the formation of distinct DNA lesions that trigger different checkpoint responses or a gradient of Top1p DNA damage in rank order Top1N726Hp > Top1T722-Ap > Top1N726Dp.

To further investigate potential mechanism of Top1p poisoning, additional substitutions of Asn726 were engineered, and these mutants were examined for their effects on cell viability. Based on the co-crystal structures of human Topo-70 (30, 38), the introduction of His at position Asn726 might enhance the formation of the phosphotyrosyl linkage by acting as a general base to accept the proton from the attacking hydroxyl of the active site tyrosine. Indeed, Top1N726Hp catalytic activity was unaltered over a wide range of pH values (data not shown), suggesting that His at this position is not positively charged. A series of Asn726 substitutions were engineered to address issues of charge (Lys substitution in N726K), amide side chain geometry (Gln in N726Q), and, given the imidazole ring of His, the potential for stacking interactions (Phe in N726F).

As shown in Fig. 4, substitution of Phe for Asn726 (in N726F) also induced a self-poisoning phenotype. As with Top1N726H or

![Fig. 1. Asn726 substitutions alter Top1p binding to DNA.](image-url)
GAL1-promoted expression of top1N726F induced a 4-log drop in top1/H9004 cell viability in the absence of CPT. These data were somewhat surprising and suggested the potential involvement of stacking interactions in the cytotoxic mechanism of the Top1N726H and Top1N726F enzymes. Indeed, replacing Asn726 with Tyr also produced a self-poisoning phenotype (data not shown). In contrast, overexpression of Top1N726Kp or Top1N726Qp was readily tolerated, yet failed to induce the CPT sensitivity of cells expressing wild-type Top1p.

As depicted in Table I, self-poisoning top1T722A, top1N726D, top1N726H, and top1N726F mutants, constitutively expressed at low levels from the TOP1 promoter on an ARS/CEN vector, were used in a transformation assay similar to that described in Fig. 3. However, in this case, the vectors were transformed into isogenic top1/H9004 strains harboring differences...
Self-poisoning Topoisomerase I Mutants

Table I
Transformation assay

| top1 allele       | Transformation efficiency |
|------------------|--------------------------|
| Wild-type        | +++b                     |
| top1N726D        | +++b                     |
| top1N726F        | +++b                     |
| top1N726H        | +++b                     |
| top1T722A        | +++b                     |

* Isogenic top1Δ yeast strains (wild-type or tah mutants cdc45–10, dpb11–10 or doa4–10) were individually transformed with equal amounts of the indicated YCpsSCtop1 vector (diagrammed above) that constitutively expresses low levels of Top1p, Top1N726Dp, Top1N726Fp, Top1N726H, or Top1T722Ap. Following incubation at 26°C, the number of transformed colonies was determined. In replicate experiments, + ++ indicates 200–500 large colonies, + + indicates 100–200 small colonies, + indicates 50–100 small colonies, and refers to less than 10 transformants.

+ In the shaded boxes, exponentially growing liquid cultures of individual transformants at 26°C, were adjusted to an OD595 of 0.3, serially 10-fold diluted and spotted onto SC-uracil media. Following incubation of duplicate plates at 26 and 36°C, cell growth was assessed. In shaded boxes, + ++ indicates equivalent growth at both temperatures, while + + indicates 10-fold fewer colonies at 36°C, relative to the same transformants at 26°C.

As above, serial 10-fold dilutions of exponential cultures of the indicated transformants at 26°C were plated on SC-uracil plates at 26 and 36°C. Although viable transformants were obtained at 26°C, the cultured cells failed to form colonies when plated at 36°C.

ent temperature sensitive tah strains (cdc45–10, dpb11–10, or doa4–10). The tah mutants were originally isolated in a yeast screen for enhanced sensitivity to the CPT mimetic, Top1T722Ap, at the nonpermissive temperature of 36°C (13, 18). In comparison to wild-type cells, cdc45–10 and dpb11–10 strains exhibit defects in processive DNA replication, while doa4–10 cells are defective in ubiquitin-mediated proteolysis (13, 18). As previously reported (13, 18) and shown in Fig. 3, wild-type and tah mutant strains were readily transformed with the TOP1-expressing vector and the resultant transformants were viable at 26 and 36°C. However, the tah mutants (cdc45–10, dpb11–10, and doa4–10) were inviable at 36°C in the presence of low levels of Top1T722Ap (Table I and Refs. 13 and 18). This contrasts with the pattern of tah mutant sensitivity to the Asn266 mutants (Table I). dpb11–10 cells were unable to tolerate Top1N726Dp, Top1N726Fp, or Top1N726Hp at any temperature, as no transformants were obtained. With cdc45–10 cells, Top1N726Fp transformants were viable at 26°C; however, subsequent plating at 36°C demonstrated a conditional lethal phenotype. Since the cdc45–10 and dpb11–10 mutants exhibit a synthetic lethal interaction at 36°C in the absence of Top1p poisons (13, 18), a similar pattern of enhanced sensitivity to the self-poisoning N726D, N726F, and N726H mutants was not surprising. However, the temperature-sensitive lethality of doa4–10 cells expressing Top1N726Dp or Top1T722Ap contrasts with the relative resistance to TopN726Fp and Top1N726Hp (Table I). When considered with the transformation assay of Fig. 3, these data support a model of distinct DNA lesions induced by Top1N726D and topN726H mutants and further indicate that different genetic backgrounds may be exploited to distinguish the cytotoxic action of distinct Top1 poisons.

The N726H Mutation Potentiates the Cytotoxic Activity of Top1T722Ap—As shown in Fig. 4, the self-poisoning activity of the T722A and N726H mutations was retained in the double top1T722A/N726H mutant. As with the single mutants, galactose-induced expression of Top1T722A/N726Hp produced a greater than 4-log drop in cell viability in the absence of CPT, which was dependent upon the presence of the active site tyrosine (Fig. 4) and the catalytic activity of the mutant enzyme (data not shown). However, as shown in Fig. 5A, the rate and extent of cell killing induced by expression of the double mutant Top1T722A/N726H enzyme is greatly enhanced over that induced by expression of either single mutant enzyme. After 2 h of galactose induction, cells expressing the T722A/H726H mutant exhibit a >30-fold reduction in the number of viable cells, versus the 2–3-fold reduction in viable cell number induced by expression of the single T722A or N726H mutants. The greater than additive cytotoxicity was further amplified after 4–8 h induction with galactose (a 2-log decrease in viability of cells expressing the double mutant, relative to cells expressing either single mutant enzyme). Since the pattern of Top1 protein expression was similar in all cases (data not shown), differences in cell viability could not be attributed to differences in Top1p protein levels or catalytic activity. Rather, the combination of distinct alterations in enzyme-catalyzed DNA cleavage and religation appears to enhance the formation of cytotoxic DNA lesions.

CPT co-treatment failed to enhance the cytotoxicity of the double T722A/N726H mutant, yet increased the lethal phenotype of the single T722A or N726H mutants (Fig. 5A). Clearly, a threshold of Top1-induced DNA lesions must be achieved to effect cell death, as cells expressing wild-type Top1p are less sensitive to CPT than cells expressing the self-poisoning Top1T722A or Top1N726H enzymes. However, these data also suggest an upper threshold for Top1p poisons, where further stabilization of Top1p DNA complexes may be achieved. Indeed, as shown in Fig. 5B, co-expression of the single T722A and N726H mutants proved significantly less cytotoxic than co-expression of the double Top1T722A/N726H and wild-type enzymes. Thus, the self-poisoning enzymes were dominant to wild-type Top1p and the combination of distinct Top1 poisons in a single enzyme enhanced the formation of lethal lesions.

Lethal Top1N726 Mutant Enzymes Were Catalytically Active—To define the effects of these amino acid substitutions on Top1p activity, the purified mutant enzymes, corrected for protein concentration, were assayed for plasmid DNA relaxation and in DNA cleavage assays. As seen in Fig. 6A, the
The values are an average of three independent experiments.

Number of colonies were plotted relative to that obtained at 0.1% Me₂SO alone (no drug control) was also added. At the times indicated, aliquots were serially diluted, and the number of viable cells forming colonies determined following plating on SC-uracil dextrose at 30 °C. In A, top1A cells, co-transformed with URA3- and LEU2-based YCpGAL1 vectors containing the indicated top1 alleles, were galactose-induced at t = 0, and the number of viable cells forming colonies was determined in aliquots taken at the times indicated. In A and B, the number of colonies were plotted relative to that obtained at t = 0, and the values are an average of three independent experiments.

FIG. 5. Combining the T722A and N726H mutations potentiates the cytotoxic activity of the single mutants. A, exponential cultures of top1A cells transformed with the indicated YCpGAL1-TOP1 construct, were galactose induced to express Top1, top1T722A (T722A), top1N726H (N726H), or the double mutant top1T722A/N726H (T722A,N726H) at t = 0. 100 μM CPT in 0.1% Me₂SO (CPT) or 0.1% Me₂SO alone (no drug control) was also added. At the times indicated, aliquots were serially diluted, and the number of viable cells forming colonies determined following plating on SC-uracil dextrose at 30 °C. In B, top1A cells, co-transformed with URA3- and LEU2-based YCpGAL1 vectors containing the indicated top1 alleles, were galactose-induced at t = 0, and the number of viable cells forming colonies was determined in aliquots taken at the times indicated. In A and B, the number of colonies were plotted relative to that obtained at t = 0, and the values are an average of three independent experiments.

In contrast, the CPT resistance of cells expressing Top1N726Kp or Top1N726Qp derives from distinct effects on enzyme activity. Introducing Lys at residue 726 abolished Top1p activity (Fig. 6A), while Gln at the same position had little effect on specific activity (Fig. 6, A and B). Nevertheless, in DNA cleavage assays (Fig. 7), Top1N726Qp was resistant to CPT. Whereas wild-type Top1p exhibited increased covalent enzyme-DNA complexes in the presence of CPT, substitution of Gln at position 726 abolished this effect. These data suggest that the orientation of the amide side chain at residue 726 is a critical determinant of drug sensitivity.

As previously reported (25), substitution of Asn⁷²⁶ with His increases covalent complex formation as a result of increased rates of DNA cleavage. The introduction of Phe at the same position also induced a lethal phenotype and increased the covalent complex formation in the absence of CPT (see asterisk in Fig. 7). This enzyme remained CPT-sensitive as the addition of the drug increased steady state levels of cleaved DNA, albeit at lower levels than that obtained with Top1N726Hp. In contrast, the double T722A/N726H mutant exhibited extremely high steady state covalent complexes, unaffected by the addition of CPT (Fig. 7). These data are consistent with the lack of increased cell kill in the presence of CPT in Fig. 5.

Substitution of Phe or His for Asn⁷²⁶ Enhances the Rate of Top1p-catalyzed DNA Cleavage—The results presented in Fig. 7 reflect the stabilization of covalent Top1p-DNA complexes in the presence or absence of CPT. To uncouple DNA cleavage from religation, suicide DNA substrates were used to investigate the effect of specific substitutions on the rate of DNA cleavage. As diagrammed in Fig. 8A, a 5'-bridging phosphorothioate at the preferred site of scission in a duplex oligonucleotide produces a 5'-SH in the Top1p-DNA covalent complex, which is ineffective in the transesterification that reglates the DNA (35). Consequently, the rate at which the 3'-end-labeled 22-mer accumulates approximates the rate of DNA cleavage. An alternative approach, diagrammed in Fig. 8, B and C, is to truncate the scissile DNA strand, such that Top1p cleavage at a preferred site (arrow) liberates a 5-mer. Under the reaction conditions used, dissociation of the 3'-end labeled 5-mer precludes resolution of the covalent complex, again allowing the rate of DNA cleavage to be determined. The same DNA sequences were used in Fig. 8, A and B, while in Fig. 8C, a second
CPT-sensitive cleavage site for yeast Top1p (34), indicated by an arrowhead in Fig. 7, was also synthesized as a truncated DNA oligonucleotide.

In all three cases, the rate of DNA cleavage catalyzed by Top1N726Hp was enhanced -10-fold relative to wild-type Top1p, consistent with earlier studies with the 5'-OH DNA substrate (25). The double T722A/N726H mutant also demonstrated increased rates of DNA cleavage with the canonical DNA cleavage site in Fig. 8, A and B (5-fold higher than that observed for wild-type Top1p). Thus, the contribution of His to DNA cleavage is retained in the double mutant. However, as higher levels of cleaved complexes were obtained in DNA cleavage assays with the double T722A/N726H mutant than with the single N726H mutant (in the absence of CPT), a defect in DNA religation catalyzed by the double mutant, as a consequence of the T722A substitution, may also be inferred. In panel C, the lack of enhanced DNA cleavage by the double mutant coincided with the data in Fig. 7, where complex formation at this site was only evident in the presence of CPT.

The self-poisoning activity of Top1N726Fp, evident in Fig. 4, coincided with increased DNA cleavage in Fig. 7, albeit at lower levels than that observed with Top1N726Hp, either in the presence or absence of CPT. Consistent with these findings, Top1N726Fp exhibited elevated rates of DNA scission with the 5'-OH DNA substrates in Fig. 8, B and C, -5-fold faster than wild-type Top1p. Similar kinetics of cleavage were observed with the double T722A/N726H mutant. In contrast, Top1N726Fp-catalyzed cleavage of the 5'-SH DNA substrate was reduced -2-fold below wild-type Top1p levels. Whether this reflects steric effects of the Phe side chain clashing with the larger bridging S versus O has yet to be determined. However, the increased salt optimum observed in the plasmid religation assays of Fig. 6 suggest the results are not a consequence of decreased DNA binding. Taken together, these data indicate enhanced DNA cleavage by Top1N726Fp, Top1N726Hp, and Top1T722A/N726Hp, with an added defect in DNA religation evident with Top1T722A/N726Hp.

Surprisingly, distinct alterations in the rate of DNA cleavage by Top1N726Fp were evident with the 5'-SH and 5'-OH DNA substrates. Despite the lack of detectable alterations in enzyme-catalyzed DNA relaxation (Fig. 6), the inclusion of the 5'-SH increased the cleavage rate of Top1N726Hp relative to that observed with the corresponding 5'-OH. Such an increase argues for a defect in general acid catalysis by the N726Q mutant, which is alleviated by the introduction of the bridging phosphorothiolate (39). Thus, substitution of Gln for Asn726 alters enzyme sensitivity to CPT and adversely affects the rate of enzyme-catalyzed DNA cleavage via a specific defect in general acid catalysis, possibly as a result of the altered geometry of the amide side chain within the active site of Top1p.

DISCUSSION

The self-poisoning activity of the Top1 mutant enzymes, Top1N726Hp and Top1T722Ap (12, 25, 27), results from distinct alterations in enzyme-catalyzed DNA cleavage and religation. Based on the results of suicide substrate assays, the enhanced stabilization of Top1T722Ap-DNA covalent complexes could not be attributed to alterations in DNA binding or rates of DNA cleavage (25). Rather, the data inferred a defect in DNA religation, similar to that induced by CPT. This model was supported by crystallographic data indicating the interaction of Thr158 (at the corresponding position in human Top1p) with the nonbridging oxygen of the +1 phosphodiester of the DNA in a covalent complex formed by a 70-kDa C-terminal fragment of human Top1p and duplex DNA (30, 40). Substitution of this residue with alanine might alter the optimal alignment of the 5'-OH nucleophile in the transesterification reaction that restores the integrity of the phosphodiester backbone.

In this work, we provide direct evidence for reduced catalysis of DNA religation by Top1T722Ap. We further demonstrate an increased affinity of the Top1N726H mutant enzyme for DNA (Fig. 1), suggested by the increase in salt concentration required for optimal catalytic activity in plasmid DNA relaxation assays (25) and Fig. 6. Indeed, biochemical characterizations of mutant enzymes bearing additional Asn726 substitutions, such as Top1N726Fp, indicates that the lethal phenotype of these mutants derives from increased rates of DNA cleavage and was attendant with increased salt concentrations for optimal catalytic activity.

The surprising finding (Fig. 3 and Table I) was that cell sensitivity to low, constitutive levels of distinct self-poisoning Top1 mutant enzymes varied dramatically in different genetic backgrounds. For example, cells deleted for the Rad9 DNA damage checkpoint were unable to tolerate low levels of Top1N726Hp, exhibited a slow growth phenotype in the presence of Top1T722Ap and were unaffected by expression of Top1N726Dp (Fig. 5). This contrasts with isogenic wild-type cells that easily tolerated expression of all three mutant enzymes. Based on steady state levels of covalent Top1p-DNA complexes detected in DNA cleavage assays (Refs. 12 and 25 and Fig. 7), these phenotypes may reflect a gradient of increasing DNA damage induced by these enzymes, with Top1N726Hp being the most potent self-poisoning enzyme. However, several lines of evidence support an alternative model, whereby an increase in Top1p-catalyzed DNA cleavage induces DNA lesions, some of which are intrinsically distinct from those induced by a decrease in Top1p-catalyzed DNA religation.

First, the rate of cell killing induced by Top1T722Ap or Top1N726Hp expression in top1Δ cells was almost identical (see Fig. 5A). If Top1N726Hp were a more potent poison than
Top1T722Ap, the kinetics of cell death would be faster. Second, as summarized in Table I, variable patterns of mutant Top1p cytotoxicity were observed in a panel of isogenic tah mutant strains, originally isolated in a genetic screen for enhanced sensitivity to low levels of Top1T722Ap at high temperature (13, 18). For example, cdc45-10 cells, which harbor a temperature sensitive hypomorphic allele of CDC45 (18), readily tolerate low levels of Top1T722Ap expression at 26 °C yet could not be transformed with the same vector expressing Top1N726Hp. cdc45-10 cells exhibit a transient accumulation of Okazaki fragments upon shift to the nonpermissive temperature and are defective in processive DNA replication (18). In contrast, isogenic doa4-10 cells, which are wild-type for CDC45 but contain a nonsense mutation in DOA4 (13), are relatively resistant to Top1N726Hp and Top1N726Fp expression even at 36 °C. Doa4p is a ubiquitin C-terminal hydrolase whose function is required to maintain free ubiquitin pools (13). Dosage suppression studies suggest that the enhanced sensitivity of doa4-10 cells to Top1T722Ap is a consequence of specific alterations in the DNA damage checkpoint (13). Taken together, these data indicate that select genetic alterations in otherwise isogenic genetic backgrounds profoundly alter cell sensitivity to the cytotoxic action of different self-poisoning Top1p mutants. These findings further suggest that distinct mechanisms of Top1p poisoning, such as the enhanced rate of DNA cleavage by Top1N726Hp or Top1N726Fp versus the defect in DNA religation evident with Top1T722Ap or CPT induce distinct patterns of DNA lesions. In other words, how covalent complexes form is also an important determinant of cell viability. Whether this relates to the effective sensing and/or repair of such lesions in different genetic backgrounds has yet to be determined.

The concept of independent Top1p self-poisoning mechanisms yielding distinct cytotoxic lesions was further supported by extent of cell killing achieved by expression of the double Top1T722A/N726Hp mutant. In this case, the combination of a mutation that enhances DNA cleavage with a second mutation that decreases DNA religation potently enhanced the cytotoxicity of the mutant enzyme to yield greater than additive cell death. Both defects in enzyme catalysis were evident in biochemical characterizations of the mutant enzyme. Moreover, the combination of the two mutations in a single protein induced significantly more cell death than co-expression of the single mutants. The same effect was observed with CPT, where drug treatment of cells expressing the double mutant had no effect on the rate of cell death, whereas CPT enhanced the lethal phenotype of cells expressing either single mutant enzyme. A lower threshold of DNA damage is certainly required to induce cell death, but an upper threshold may also exist above which no greater cell kill is achieved. These results might also be a consequence of decreased CPT binding of the double mutant-DNA covalent intermediate. Nevertheless, the data are consistent with distinct repair/signaling pathways affecting cell survival and suggest that two

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**FIG. 8.** 
Asn$^{726}$ substitutions alter the kinetics of DNA cleavage. Equal concentrations of Top1p, Top1N726Fp, Top1N726Hp, Top1T722A/N726Hp, Top1N726Kp, and Top1N726Qp were incubated with 3'-end-labeled suicide substrates diagrammed in A–C. At the times indicated, aliquots were treated with SDS at 75 °C, and the cleavage products were resolved in 20% polyacrylamide/7 M urea gels and visualized with a PhosphorlImage. An arrow marks the site of cleavage that liberates a 5'-SH 22-mer (in A), and the 5'-OH 5-mers (in B and C); the arrowhead indicates the position of the cleavage product in the gels. Substrates in A and B contain a high affinity cleavage site; the substrate in C contains a Top1p cleavage site induced by CPT (34).
dependent mechanisms of Top1p poisoning may be exploited to target the same enzyme.

As with the N726H mutation, amino acid substitutions with phenolic side chains (such as Phe) also enhanced the rate of DNA cleavage catalyzed by Top1p. These findings indicate that charge alone of the His side chain is not the critical determinant of the kinetics of DNA cleavage. Indeed, this is consistent with the lack of an effect of pH on Top1N726Hp activity in vitro (data not shown). Instead, stacking interactions between the imidazole ring of His, or the phenolic rings of Phe or Tyr at position 726, with the active site tyrosine at position 727 may enhance the nucleophilicity of Tyr by facilitating proton transfer to promote DNA cleavage.

Top1 Mutant Enzyme Sensitivity to CPT—The C-terminal 70-kDa fragment of wild-type human Top1p or the CPT-resistant human N722S mutant enzyme in complex with DNA and topotecan (a CPT analog) have been reported (30, 38). Although subtle differences in the geometry of a hydrogen-bonded water to Asn were reported, the introduction of Ser eliminates the water-mediated contact between Asn and the O-18 carbonyl of topotecan. Based on the orientation of the drug within the active site of the enzyme, it is tempting to speculate that replacing Asn with His or Phe might either allow for direct hydrogen bonding between O-18 and His or allow for electrostatic interactions between the positively charged edge of the aromatic ring of Phe with the O-18 of camptothecin. Such interactions might stabilize the ternary Top1p-drug-DNA complex to yield the increased CPT sensitivity of Top1N726Fp and Top1N726Hp evident in DNA cleavage assays (Fig. 7) and the ~10-fold increase in cell death induced by CPT treatment of Top1N726Hp-expressing cells (Fig. 5).

Similar structural considerations suggest that the additional methyl group within the Gln side chain in Top1N726Qp would alter the orientation of the polar amide, thereby eliminating the water-mediated contact with CPT (38). This model is consistent with the CPT resistance of Top1N726Qp in vivo and in vitro (Figs. 4 and 7, respectively). However, the restoration of DNA cleavage by Top1N726Qp with DNA substrates that yield a 5′-OH versus the lack of DNA cleavage observed with a 5′-OH DNA substrate (Fig. 8) further suggests a defect in general acid catalysis. This is based on the premise (39) that in the absence of other defects in transesterification chemistry the much lower pKa of the 5′-SH alleviates the requirement for a general acid for the effective expulsion of the 5′-OH. The altered geometry of the Gln side chain within the active site of Top1N726Qp may preclude effective interaction with such a general acid or alter the interaction with the 5′-OH end, as suggested in crystallographic data for Asn in human Top1p-DNA-topotecan complexes (38). While the N726Q substitution did not alter the specific activity of the yeast enzyme, it did abolish CPT stabilization of the covalent complex. Thus, CPT resistance and a defect in general acid catalysis may be functionally linked suggesting the same alterations in active site geometry adversely affect the expulsion of the 5′-OH from the transition state and the resultant formation of the CPT binding site within the covalent Top1-DNA complex.

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Substitution of Conserved Residues within the Active Site Alters the Cleavage and Religation Equilibrium of DNA Topoisomerase I

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