A Mutation in Yeast Topoisomerase II That Confers Hypersensitivity to Multiple Classes of Topoisomerase II Poisons*

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Jiaowang Dong, Jerrylaine Walker, and John L. Nitiss‡
From the Molecular Pharmacology Department, St. Jude Children’s Research Hospital, Memphis, Tennessee 38018

DNA topoisomerases are enzymes that catalyze changes in the topology of DNA by transiently breaking phosphodiester bonds in the DNA backbone (1). Two major classes of topoisomerases have been identified, type I enzymes, which introduce single strand breaks in DNA, and type II enzymes, which introduce transient double strand breaks. The ability of these enzymes to resolve the winding problems that arise during DNA metabolic processes including transcription, DNA replication, and chromosome segregation. Both type I and type II enzymes are ubiquitous among both prokaryotes and eukaryotes, and recent work has led to the discovery of several novel topoisomerases with novel biochemical properties and important specialized physiological roles (reviewed in Ref. 2).

In addition to their numerous biological functions, topoisomerases are important targets for both anti-bacterial agents and anti-cancer chemotherapeutic agents (reviewed in Refs. 3–5). Most of the topoisomerase targeting drugs in clinical use are topoisomerase poisons, agents that lead to enhanced levels of an intermediate of the topoisomerase reaction where the enzyme is covalently bound to DNA by a phosphotyrosine linkage and the DNA strand scission has occurred (4). Extensive evidence supports the hypothesis that topoisomerase poisons kill cells because of the DNA damage generated by the covalent complex rather than by depriving cells of an essential enzymatic activity (3, 6). Therefore, resistance to topoisomerase poisons can occur by a change in the level of protein-DNA covalent complexes that arise in the presence of drug, either by reduced activity of the enzyme or by an intrinsic change in sensitivity of the enzyme to the poison.

Eukaryotic topoisomerase I is the target of camptothecins, whereas eukaryotic topoisomerase II is the target of numerous agents including DNA intercalating agents such as the anthracycline doxorubicin and nonintercalating agents such as the epipodophyllotoxin etoposide. Prokaryotic topoisomerase II is the target of fluoroquinolones, which are important broad spectrum anti-bacterial agents (7, 8). Although most fluoroquinolones that have been described are highly specific for prokaryotic type II topoisomerases, several agents have been described that are also very active against eukaryotic topoisomerase II (9, 10). Notably, 6,8-difluoro-7-(4'-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid (CP-115,953), a fluoroquinolone closely related to ciprofloxacin, is a potent eukaryotic topoisomerase II poison (10, 11). Studies in yeast demonstrated that TOP2 is the primary physiological target for this quinolone (12).

In Escherichia coli, mutations that lead to quinolone resistance are most often found in gyrA, the structural gene for the DNA gyrase A subunit. Ser⁶⁸ of gyrA is the amino acid most frequently changed in strains with high levels of quinolone resistance. E. coli also expresses an additional fluoroquinolone sensitive type II topoisomerase, encoded by the parC and parE genes (13, 14). The parC locus is homologous to gyrA, and mutations in parC at sites analogous to quinolone-resistant gyrase mutations are also associated with fluoroquinolone resistance in E. coli and other bacteria (15–18). Proteins bearing Ser⁶⁸ mutations have essentially normal gyrase activity but have decreased affinity for fluoroquinolones (19, 20). In addition to the sites in gyrA or parC, mutations have also been found in gyrB that lead to quinolone resistance, which has led to the suggestion that domains in gyrA and gyrB collaborate to form a quinolone-binding pocket (21–23).

A wide range of mutations have been isolated in eukaryotic topoisomerase II leading to resistance to topoisomerase targeting drugs (reviewed in Ref. 24). Mutations are found in regions of eukaryotic topoisomerase II that are homologous to gyrB and gyrA as well as in other regions of the protein. However, few mutations have been described that make the enzymes more sensitive to topoisomerase poisons (25–27). Because mutations

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‡ To whom correspondence should be addressed: St. Jude Children’s Research Hospital, Dept. of Molecular Pharmacology, 332 N. Lauderdale, Memphis, TN 38018. Tel.: 901-495-2794; Fax: 901-521-1688; E-mail: john.nitiss@stjude.org.

† The abbreviations used are: CP-115,953, 6,8-difluoro-7-(4'-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid; mle, minimum lethal concentration; PCR, polymerase chain reaction; mAMSA, N-[4-(9-acridinylamino)-3-methoxy-phenyl]methanesulfonamide.
Mutation Causing Hypersensitivity in Topoisomerase Poisons

that render the enzyme more resistant to poisons can occur by a variety of ways, including reduction in enzyme activity, drug-resistant mutations may not be informative about the mechanisms of actions of drugs (28, 29). Drug-hypersensitive mutations may therefore be a uniquely useful set of tools for probing drug mechanisms. In this report, we describe a mutation in yeast topoisomerase II that renders the enzyme hypersensitive to multiple classes of topoisomerase II poisons, including intercalating agents and fluoroquinolones.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—All experiments for assessing drug sensitivity were conducted in JN362a (MATα ura3-52 leu2 trp1 his7 ade1-2 ISE2 or its isogenic rad52- and top2-4 derivatives, JN362at4-2, (MATα ura3-52 leu2 trp1 his7 ade1-2 ISE2 top2-4), JN394 (MATα ura3-52 leu2 trp1 his7 ade1-2 ISE2 rad25-LEU2), and JN394rad25-4 (MATα ura3-52 leu2 trp1 his7 ade1-2 ISE2 rad25-LEU2 top2-4). Yeast Top2p was purified using a top1 derivative of the protease-deficient strain JEL1 (30, 31).

Oligonucleotide-directed Mutagenesis—Mutagenesis was carried out in a 50 ml PCR reaction with a Perkin-Elmer GeneAmp PCR System 9600 (Norwalk, CT) using QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The formation of each mutation requires two complementary oligonucleotides containing desired mutation site. For the construction of T744P mutant, the following pair of oligonucleotides were used. The underlined nucleotides indicate the changes that occurred to the yeast Top2p-C Terminus in comparison with the S. cerevisiae Top2p-C Terminus: 5′-CAG TCA TTG GCA CCA ACT ATT ATT GGG C-3′ and 5′-GCC TAG CCC AAT AAG TTG TGC CAA TGA CTG-3′. For the construction of the T744A mutant, the following pair of oligonucleotides were used: 5′-CAG TCA TTG GCA CCA ACT ATT ATT GGG CTA GC-3′ and 5′-GCC TAG CCC AAT AAG TTG TGC CAA TGA CTG-3′. The plasmid pDEDTOP2 (32) was used as the template in PCR reactions. The reaction mixture (50 μl) contained 1× reaction buffer (supplied by Stratagene), 20 ng of template DNA plasmid, 125 ng of each primer with desired mutation nucleotide(s), 10 μM of dNTPs, and 2.5 units of Pfu DNA polymerase. The PCR amplification used the following temperature regimes: one cycle of 95 °C for 30 s and then 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min. The amplified products were treated with 10 units of Pfu DNA polymerase (supplied by Stratagene) and incubated at 30 °C for 30 min, subsequently stopped by addition of 1 μl of 0.5 M EDTA into each reaction. The reactions were loaded onto an agarose gel, and after electrophoresis, the DNA was visualized by staining with ethidium bromide and photographing under UV light.

Determination of Drug Sensitivity of Purified Topoisomerase II—The K/SDS assay was used to determine drug stabilized DNA cleavage and was performed as described previously (26, 37).

Measurement of Topoisomerase II Activity—DNA topoisomerase II assays were carried out as described previously (26, 37). Briefly, the reaction mixture contained 10 μM Tris-HCl, pH 7.5, 50 μM NaCl, 50 μM KCl, 5.0 mM MgCl2, 2.5% glycerol, 0.5 mM ATP, 200 ng of pUC18, and the indicated amount of purified Top2p protein. The reactions were incubated at 30 °C for 30 min, subsequently stopped by addition of 1 μl of 0.5 M EDTA into each reaction. The reactions were loaded onto an agarose gel, and after electrophoresis, the DNA was visualized by staining with ethidium bromide and photographing under UV light.

RESULTS

Mutation of T744P Confers Hypersensitivity to Multiple Classes of Topoisomerase II Poisons—The crystal structure of a 92-kDa wild type yeast topoisomerase II structure were obtained from the Brookhaven Protein Data Bank (accession number 1bwg). The Protein Data Bank file was edited to remove residues 410–682, which includes the disordered linker region. Threonine 744 was replaced with proline using the Biopolymer module of the Insight II package (Molecular Simulations Inc.) on a Silicon Graphics workstation. All molecular dynamics simulations and energy minimizations were carried out using the SANDER module of the AMBER program, version 5.0 (Oxford Molecular). The T744P mutant was subjected to a 250-ps simulation (1-fs time step) at 310 K following a 40-ps equilibration cycle. The resulting structures were averaged and energy minimized. All graphics were constructed using Insight II (version 2.97/95.0).

Mutation of T744P Confers Hypersensitivity to Multiple Classes of Topoisomerase II Poisons—The crystal structure of a 92-kDa fragment of yeast topoisomerase II indicated the presence of a helix-turn-helix domain (39). Wang and colleagues (39) have suggested that the helix-turn-helix domain plays a critical role in DNA binding. This model of a structure of the yTop2p protein, along with observations indicating that mutations within the helix-turn-helix altered sensitivity to topoisomerase II poisons, prompted us to construct a defined series of mutations in this domain. One mutagenesis strategy that we adopted changed residues in the helices of the domain to proline to test the effect of disrupting the secondary structures

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FIG. 1. T744P mutation of yeast TOP2 confers hypersensitivity to topoisomerase II poisons. JN394t2-4 yeast cells carrying either pDEDTOP2 plasmid or pDEDTOP2 T744P were exposed to Top2 poisons at 34 °C for the indicated times. Aliquots were removed, diluted, and plated to complete synthetic medium plates lacking uracil. Plates were incubated at 25 °C, and colonies were counted to determine viable counts. Cell survival is expressed as the percentage of surviving cells at the indicated time relative to the viable titer at the time drug was added (t = 0). A, cells carrying wild type or T744P pDEDTOP2 were treated with CP-115,953. □, wild type, no drug; ◦, wild type, 1 μM CP-115,953; ○, wild type, 4 μM CP-115,953; △, wild type, 20 μM CP-115,953; ■, T744P, no drug; ●, T744P, 1 μM CP-115,953; ●, T744P, 4 μM CP-115,953; □, T744P 20 μM CP-115,953. B, cells carrying wild type or T744P pDEDTOP2 were treated with mAMSA. □, wild type, no drug; ◦, wild type, 2.5 μM mAMSA; ○, wild type, 12.5 μM mAMSA; △, wild type, 125 μM mAMSA; ■, T744P, no drug; ●, T744P, 2.5 μM mAMSA; ●, T744P, 12.5 μM mAMSA; □, T744P, 125 μM mAMSA.

Within this domain, Thr\(^{744}\) is in the middle of helix (the helix that is thought to be in the major groove of DNA). Oligonucleotide-directed mutagenesis changed Thr\(^{744}\) to Pro in plasmid pDEDTOP2 (12). The plasmid expresses yeast TOP2 from the yeast DED1 promoter and results in 10–20-fold overexpression of the yeast enzyme. The mutagenized plasmid was transformed into strain JN394t2-4, which carries a temperature sensitive top2 allele. JN394t2-4 cells are unable to grow at 34 °C, because of a lack of topoisomerase II activity at this temperature. Transformation with pDEDTOP2 (T744P) restored normal growth of JN394t2-4 cells at 34 °C, indicating that the mutation did not eliminate top2 activity.

Because the only topoisomerase II active at 34 °C is the top2 expressed from the plasmid, we next assessed the drug sensitivity of JN394t2-4 cells carrying the T744P mutant, compared with the same strain carrying an unmutagenized plasmid. Expression of T744P mutant resulted in a strain that was hypersensitive to the quinoline CP-115,953, a fluoroquinolone that is active against eukaryotic topoisomerase II (Fig. 1A and Table I). Approximately 2.7 μM CP-115,953 was the mlc of drug with wild type yeast TOP2, and 0.6 μM was the mlc with cells expressing the T744P allele. Similar results were seen with yeast cells exposed to the intercalating topoisomerase II poison amsacrine (Fig. 1B). At 24 h, less than 1 μM mAMSA is required for cell killing when cells express the T744P TOP2 mutant. The mlc of mAMSA for the mutant is approximately 0.6 μM. By contrast, cell killing is only observed with about 5 μM amsacrine when cells express wild type TOP2.

Because the T744P mutant was hypersensitive to the fluoroquinoline CP-115,953, we were interested in determining whether the mutant was also hypersensitive to other fluoroquinolones that are principally active against prokaryotic type II topoisomerases. T744P expressing cells were hypersensitive to the antibacterial fluoroquinolones ciprofloxacin (data not shown). This result suggests that the T744P mutant is hypersensitive to quinolones generally and not just CP-115,953.

A different result was obtained with the nonintercalating topoisomerase II poison etoposide. Yeast cells expressing either wild type TOP2 or the T744P mutant enzyme had comparable sensitivity to etoposide (data not shown). Although cell survival was consistently lower when cells expressing T744P were exposed to etoposide, compared with wild type cells, the differences in survival were not statistically significant, and no difference in the mlc was observed. This result suggests that the T744P mutant does not confer etoposide hypersensitivity.

We previously showed that the level of drug sensitivity can depend on the level of active topoisomerase II protein and that an unstable topoisomerase II protein can lead to in vivo drug resistance (29). Conversely, an abnormally stable protein could lead to drug hypersensitivity. The level of topoisomerase II protein was examined by Western blot. Fig. 2 shows the level of topoisomerase II polypeptides in yeast cells expressing Top2 protein was examined by Western blot. Fig. 2 shows the level of topoisomerase II polypeptides in yeast cells expressing Top2 enzymes changed Thr\(^{743}\) and Thr\(^{744}\) to Pro in plasmid pDEDTOP2 (12). The plasmid expresses yeast TOP2 from the yeast DED1 promoter and results in 10–20-fold overexpression of the yeast enzyme. The mutagenized plasmid was transformed into strain JN394t2-4, which carries a temperature sensitive top2 allele. JN394t2-4 cells are unable to grow at 34 °C, because of a lack of topoisomerase II activity at this temperature. Transformation with pDEDTOP2 (T744P) restored normal growth of

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>mAMSA</th>
<th>CP-115,953</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>5.3</td>
<td>2.7</td>
</tr>
<tr>
<td>T744P</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>T744A</td>
<td>4.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Q743P</td>
<td>12.2</td>
<td>3.3</td>
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Minimum lethal concentrations of Top2 mutations at positions 743 and 744 to mAMSA and CP-115,953:

- Wild type: 5.3 μM mAMSA, 2.7 μM CP-115,953
- T744P: 1.8 μM mAMSA, 0.6 μM CP-115,953
- T744A: 4.6 μM mAMSA, 1.5 μM CP-115,953
- Q743P: 12.2 μM mAMSA, 3.3 μM CP-115,953
decatenation of kinetoplast DNA (data not shown).

We next examined levels of drug-dependent and drug-independent cleavage by K1/SDS precipitation. In these assays, drug-induced cleavage was compared with DNA cleavage in the absence of drug for both wild type and T744P Top2 proteins. Data for the drug-independent cleavage for this series of experiments were pooled to compare the level of drug-independent cleavage of the wild type and mutant proteins. To account for the different specific activities of the32P-labeled DNA substrate prepared on different days, we determined the ratio of drug-independent cleavage with T744P protein to the drug-independent cleavage with wild type topoisomerase II, where the measurements with both enzymes were carried out with the same batch of substrate. This ratio was 1.09 ± 0.22 (mean ± S.D., n = 11). The 95% confidence interval was 0.94–1.24. These results suggest that there was not a significant difference in drug-independent cleavage between the T744P and wild type topoisomerase II proteins.

We next compared the level of DNA cleavage obtained with wild type and Thr744 topoisomerase II in the presence of topoisomerase II poisons. Fig. 4A shows the DNA cleavage obtained at low mAMSA concentrations, and Fig. 4B shows the results obtained at higher drug concentrations. We determined the ratio of drug-independent cleavage with T744P protein to the drug-independent cleavage with wild type topoisomerase II, where the measurements with both enzymes were carried out with the same batch of substrate. This ratio was 1.09 ± 0.22 (mean ± S.D., n = 11). The 95% confidence interval was 0.94–1.24. These results suggest that there was not a significant difference in drug-independent cleavage between the T744P and wild type topoisomerase II proteins.

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A similar result was obtained with CP-115,953. DNA cleavage in the presence of CP-115,953 is shown in Fig. 5. A drug concentration of 0.3 μM doubles DNA cleavage over drug-independent levels with the T744P protein, whereas approximately 3 μM is required to double DNA cleavage with wild type Top2p. A smaller difference is seen at 10 μM drug, where the fold stimulation differs between the two proteins by a factor of two.

Results obtained from drug cytotoxicity measurements suggested that the T744P mutant would not show enhanced cleavage in the presence of etoposide. The experiment shown in Fig. 6 bears out this prediction. Etoposide-stimulated cleavage is essentially identical between the T744P and wild type Top2p at all etoposide concentrations examined. Taken together, results with the purified T744P protein demonstrate the same pattern of drug sensitivity predicted from in vivo cytotoxicity measurements.
We also examined the effect of a more potent intercalating topoisomerase II poison, mitoxantrone. Mitoxantrone, like other strong intercalating agents, shows a sharp maximum in the level of cleavage at relatively low drug concentrations. At higher drug concentrations, DNA cleavage is inhibited. Both wild type and T744P proteins show identical maxima at 0.45 μM mitoxantrone (Fig. 7). The concentration of mitoxantrone required to double DNA cleavage was about 0.1 μM with the T744P protein and about 0.2 μM with wild type Top2p. The level of maximal level of cleavage was also slightly higher with the T744P mutant protein. The T744P protein also shows slightly more inhibition of cleavage at higher mitoxantrone concentrations. This result shows that the T744P mutant was also hypersensitive to other intercalating agents.

In vivo cytotoxicity measurements showed that the T744P mutant was hypersensitive to the fluoroquinolone ciprofloxacin. We examined the levels of DNA cleavage with different fluoroquinolones that are selective for prokaryotic topoisomerase II. The results are summarized in Table II. The T744P mutant protein showed higher levels of DNA cleavage with the fluoroquinolones ciprofloxacin and norfloxacin and with the quinolone oxolinic acid. The greatest difference in potency was seen with oxolinic acid, where 76 μM oxolinic acid doubled DNA cleavage with the T744P protein, whereas the level of cleavage did not increase 2-fold with wild type topoisomerase II at 2,000 μM oxolinic acid, a greater than 26-fold difference in potency. These results show that the T744P protein is hypersensitive to multiple classes of intercalating agents and fluoroquinolones but not to all classes of topoisomerase II poisons.

Previous studies with the S740W mutant of yeast TOP2 showed an association between etoposide hypersensitivity and heat- and salt-stable covalent protein-DNA complexes. To test whether a similar mechanism of drug hypersensitivity was occurring in the T744P mutant, the heat stability of the covalent complexes formed was assessed. DNA cleavage assays were performed as above, except the reactions were incubated at 65 °C for various periods of time. The reactions were stopped by the addition of SDS, and levels of cleavage were determined. The results are shown in Fig. 8. Heat readily reverses covalent complexes formed by the T744P mutant in the presence of mAMSA, CP-115,953, or etoposide. Heat reversal with etoposide is somewhat less than observed with the other two agents, consistent with some degree of heat stability seen with the wild type protein and etoposide. For comparison, the level of heat-stable covalent complexes formed with the S740W protein in the presence of etoposide is also shown in Fig. 8. We conclude that the drug hypersensitivity associated with the T744P mutant protein is not associated with more stable covalent complexes.

The results presented above demonstrate that mutating Thr744 to Pro results in a drug-hypersensitive Top2p. To dis-
Mismatch between effects on secondary structure and the chemical nature of the side chain, Thr\textsuperscript{744} was changed to Ala, an amino acid that tends not to perturb $\alpha$ helices. As above, the mutation was constructed in pDEDTOP\textsubscript{2}, and sensitivity to mAMSA, etoposide, and CP-115,953 was determined. Table I shows the sensitivity of T744A to mAMSA and CP-115,953 after 24 h of drug exposure. Slight hypersensitivity to the fluoroquinolone CP-115,953 was observed. The minimum lethal concentration was 2.7 $\mu$M for wild type TOP\textsubscript{2} and 1.5 $\mu$M for the T744A mutant. The sensitivity of the T744A mutant to mAMSA was essentially unchanged compared with wild type TOP\textsubscript{2}. Therefore, the T744A mutant did not result in the same degree of sensitization as was observed with the T744P mutant. This result suggests that the alteration of the secondary structure because of the mutation to Pro is responsible at least in part for the phenotype seen with T744P.

A possible alteration in the secondary structure that could be caused by the mutation of Thr\textsuperscript{744} to Pro is a complete destabilization of the $\alpha$ helix. If so, then a mutation of an adjacent residue to Pro would be expected to generate a protein with similar biochemical properties. A mutation was constructed in pDEDTOP\textsubscript{2} that changes amino acid 743 to Pro. Sensitivity to mAMSA and CP-115,953 was assessed in cells expressing the 91-kDa fragment of wild type topoisomerase II that has been implicated in DNA binding, the structure fit well with the starting structure determined by Berger and colleagues (39). Fig. 11 shows the structure of 91-kDa fragment carrying a T744P substitution. The simulations of the T744P mutation, we carried out molecular dynamics simulations based on the structure reported by Berger and colleagues (39) carrying a T744P substitution. The simulations of the T744P mutant show an overall conservation of the enzyme structure. Fig. 10 shows the structure of 91-kDa fragment structure described by Berger and colleagues (39). Fig. 11 highlights the changes predicted to occur in the region around the Pro\textsubscript{744} mutant show an overall conservation of the enzyme structure. The quantitation of bound DNA from the gel shown in Fig. 9 shows a quantitation of the mobility shift shown in Fig. 9A. There is no significant difference between wild type and T744P topoisomerase II proteins. These results indicate that the T744P mutation does not lead to a quantitative alteration in DNA binding.

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Interestingly, a change in the vicinity of the active site tyrosine (Tyr\textsuperscript{782}) is also seen in the T744P mutant. Tyr\textsuperscript{782} and Arg\textsuperscript{781} in wild type Top2p (shown in Fig. 11) are separated by 3.97 Å. Work by Liu and Wang (40) has shown that Arg\textsuperscript{781} is important (but not indispensable) for catalysis by Top2p. A mutation changing R781A results in a protein that will not...
A recent new structure of topoisomerase II has led to the hypothesis that substantial movement by the enzyme during the process of cleavage and strand passage allows residues that complement a temperature sensitive top2 mutation and a protein with 10–100 fold reduction in activity in vitro. Introduction of proline at position 744 allows Ser740 to come within hydrogen bonding distance of the Arg781 and results in movement of Arg781 toward the α helix. The resulting structure increases the distance between the guanidino nitrogen of Arg781 and the hydroxyl oxygen of Tyr782 from 3.97 to 9.97 Å. An increased separation between Arg781 and Tyr782 may alter the catalytic properties of the mutant enzyme, particularly if drug binding can further increase the separation of Arg781 and Tyr782. A hypothesis that relates the position of Arg781 to drug sensitivity is presented under “Discussion.”

DNA topoisomerase II can be targeted by chemically diverse agents. The ability of a variety of agents to enhance the levels of enzyme-DNA covalent complexes has been exploited for effective anti-bacterial and anti-cancer agents. Most of these agents produce a similar effect on the enzyme, a higher level of covalent complexes. Although much information is currently available on the mechanisms of catalysis by topoisomerase II, important questions remain concerning how drugs targeting topoisomerase II exert their effects on the enzyme. Drug-binding domains for all the drugs that target topoisomerase II remain to be determined. Work by Osheroff and colleagues have demonstrated that two different processes can lead to the enhanced levels of covalent complexes that are the hallmark of topoisomerase poisons. Some agents, notably etoposide and mAMSA, inhibit the religation reaction of topoisomerase II (41, 42). Other agents, such as fluoroquinolones, do not inhibit religation and therefore stimulate covalent complex formation by increasing the rate of cleavage by the enzyme (11). Interestingly, the mutant we describe in this work can cause hypersensitivity to both classes of topoisomerase II poisons.

Several mechanisms can be envisioned that would lead to a drug-hypersensitive topoisomerase II protein. A simple mechanism is an increased affinity by the protein for the drug. A previously described mutation that confers resistance to CP-115,953 is hypersensitive to ellipticines (25). Osheroff and colleagues (43) subsequently showed that the mutant protein has a higher affinity for ellipticines in the absence of DNA. However, it is difficult to imagine an increased affinity for chemically distinct agents, as we observed for the T744P mutant protein. Nonetheless, if quinolones are able to interact with DNA in a manner similar to that of intercalating agents, then this mechanism would be a plausible interpretation for the drug hypersensitivity of the T744P mutant protein. Binding of fluoroquinolones to DNA has been suggested, but the precise mode of binding remains controversial.

A second mechanism for drug hypersensitivity is a change in the stability of the covalent protein-DNA complex. Mutation of S740W results in an etoposide-hypersensitive protein. Unlike wild type Top2p, the covalent complexes formed in the presence of etoposide are stable with respect to high salt or exposure to 65 °C, whereas covalent complexes formed in the absence of drug or the presence of a different complex stabilizing drug are heat- and salt-labile (26). We found no evidence for increased heat stability of the covalent complexes formed in the presence of any of the topoisomerase II poisons examined. Thus, the specific mechanism of drug hypersensitivity is likely to differ between the S740W mutant and the T744P mutant.

The molecular dynamics modeling of topoisomerase II suggests a simple model that may explain at least part of the drug hypersensitivity seen in the T744P mutant. The displacement of Arg781 by the T744P mutant may affect the cleavage/religation equilibrium to favor the cleaved state. It could do so in two different ways. First, if Arg781 participates in the cleavage and religation reactions, then its displacement toward Glu739 would make it less available to participate in religation. Second, the presence of DNA and drug may constrain the residue so that it remains close to Tyr782. In this case, Tyr782 when it is covalently bound to DNA, could be displaced away from the 3'-hydroxyl group, again reducing the rate of religation. Overall, this model suggests that drug sensitivity is altered by displacing amino acids that play a critical role in cleavage and religation by topoisomerase II.

A recent new structure of topoisomerase II has led to the hypothesis that substantial movement by the enzyme during the process of cleavage and strand passage allows residues that
are distant from the active site tyrosine at some points in time to affect drug sensitivity by their movement toward the active site tyrosine and their participation in the cleavage reaction (44). Because our analysis has relied mainly on the Berger and Wang structure (39), there may be other key residues that are displaced by the T744P mutation. Nonetheless, an appealing model for drug action is the displacement of key residues that are involved in catalysis. Because the ligation of DNA breaks probably involves a reversal of the cleavage reaction, residues that are important for DNA cleavage are probably also required for religation. The presence of bound drug may allow the residues to function in DNA cleavage, but after cleavage, the presence of drug may cause the residues to be displaced so that they are not properly positioned for participation in religation.

Drugs binding at different sites on the enzyme would not move in the same way. Thus, we would suggest important differences in the drug-binding sites of etoposide versus quinolones or intercalating agents. More detailed information about the sites of drug binding will provide a test for this model.

The work described here highlights the conservation of drug mechanisms between eukaryotic and prokaryotic type II topoisomerases. We have described a single amino acid change that allows quinolones that are highly specific for prokaryotic Top2 to have substantial activity against eukaryotic Top2. We suggest that the quinolone-binding domains between the eukaryotic and prokaryotic enzymes are extremely similar, with only small changes preventing quinolones active against prokaryotic enzymes from acting against eukaryotic Top2. Given the importance of quinolones as anti-bacterial agents, understanding what factors prevent the interaction of quinolones with eukaryotic Top2 will be an important element in anti-bacterial drug design.

Acknowledgment—We thank Dr. Karin Sykes for performing the Western analysis shown in Fig. 4.

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