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13. ABSTRACT *(Maximum 200 Words)*

The goal of this research is to understand the detailed mechanism of action of antitumor drugs that target type II topoisomerases. Bacteriophage T4 provides a useful model system for the study of these antitumor agents. Previous analysis showed that a drug resistant bacteriophage T4 mutant harbored two amino acid substitutions (S79F, G269V) in topoisomerase subunit gp52. When both mutations are present, the G269V substitution suppresses a topoisomerase negative phenotype caused by the S79F substitution while the G269V substitution by itself was shown to confer hypersensitivity in vivo (*Cancer Research* **58**, 1260-1267). I purified the G269V enzyme and found it to be hypersensitive to a number of cleavage-inducing antitumor agents. The G269V enzyme displayed an apparent 10-fold increase in drug-independent DNA cleavage, suggesting a novel mechanism of sensitivity in which the enzyme equilibrium has been shifted to favor the cleavage complex. I have also purified the S79F mutant enzyme and am in the process of characterizing the defect caused by this substitution. The G269V mutant defines a new category of type II topoisomerase mutants, namely those that are hypersensitive to all inhibitors that stabilize the cleavage complex, and thereby offers new insights into the mechanism of drug action.

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Introduction

The goal of this research is to understand the detailed mechanism of action of antitumor agents that target type II topoisomerases. These include some of the most widely used chemotherapy drugs: doxorubicin (adriamycin), mitoxantrone, and the epipodophyllotoxins VP-16 (etoposide) and VM-26 (tenoposide) (3). Two of these drugs, doxorubicin and mitoxantrone, are commonly used in the treatment of breast cancer patients (3). The resistance of certain cancer cells, including some breast cancer cells, to these drugs is still a major problem in chemotherapy. One of the ways cancer cells can acquire resistance to these topoisomerase II inhibitors is by producing an altered form of the enzyme that is no longer sensitive to the drugs (1). Detailed studies of a number of these resistant forms of topoisomerase II have contributed greatly to our understanding of drug action. I am proposing to study a new class of mutants, namely those that are hypersensitive to numerous drugs. This unique class of topoisomerase mutants has never been characterized and may offer new insights into the mechanism of drug action. Additionally, understanding this hypersensitive class of enzymes may help in the design of more effective and less toxic drugs.

Body

Background. My original proposal describes in detail the genetic isolation of the G269V mutant topoisomerase strain (see ref 8, p. 8). Briefly, a T4 gene *52* drug-resistant mutant was sequenced and was found to harbor two amino acid substitutions: S79F and G269V (2). When both substitutions are present, the G269V substitution is thought to suppress a topoisomerase-negative phenotype caused by the S79F substitution alone. However, when the G269V mutation was substituted into a wild-type background, the resulting phage exhibited hypersensitivity to *m*-AMSA and oxolinic acid *in vivo* (2). This result was unexpected as the G269V substitution is located in a domain of the topoisomerase that was not thought to play a role in drug sensitivity, namely, the tower domain (see refs for a discussion of these domains). With one possible exception (a complex triple mutant of yeast topoisomerase II), this is the only mutation in the tower domain of any type II topoisomerase known to affect drug sensitivity.

To date we have shown that the G269V substitution causes hypersensitivity by increasing the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle (9). We believe this is the first type II topoisomerase mutant described that alters drug sensitivity by changing the equilibrium of the enzyme. We believe the G269V mutant enzyme defines a new class of mutants that are hypersensitive to all drugs. However, it is still unclear how a substitution in the tower domain leads to suppression of the S79F substitution located in the CAP-like domain. Further, why is the drug-resistant phenotype dominant to the hypersensitivity phenotype (recall that the double mutant is drug resistant)? We feel that an understanding of the interplay between positions S79 and G269 and the nature of the suppression could lead to a further understanding of the hypersensitivity phenotype. Further, the analysis of these two mutants and the isolation of additional tower domain mutants with altered drug

sensitivities could provide us with new insights into the mechanism of antitumor drug action.

Statement of work issues. Based on my results thus far, my statement of work has been altered and the changes are outlined below (pending approval by Dr. Kathy Moore). Although Task 1 did not change, it took longer than expected to complete. Further, our results with the G269V enzyme brought up additional questions about the S79F single and S79F/G269V double mutant enzymes. In order to address these questions we have added a new Task 2. The original Task 3 has been removed because of time constraints. A summary of the results from Tasks 1 and 2 are presented below.

REVISED STATEMENT OF WORK

The Biochemical Analysis of a Hypersensitive Topoisomerase II Mutant

Task 1. To biochemically analyze the G269V mutant enzyme (months 3-18)

- perform DNA cleavage assays with the G269V mutant and wild-type enzymes in the absence and presence of drugs in order to. . .
  - -compare their cleavage site specificity
  - -quantitate the degree of G269V drug hypersensitivity

Task 2. To biochemically analyze the S79F single and S79F/G269V double mutant enzymes (months 15-32)

- perform DNA relaxation and cleavage assays with the S79F single mutant, S79F/G269V double mutant, and wild-type enzymes in the presence and absence of drugs order to. . .
  - -characterize the nature of the defect caused by the S79F substitution
  - -understand the nature of the suppression

Task 3. Isolate additional mutants located in the tower domain (months 25-36)

- create phage strain containing an amber mutation in the tower domain (months 24-26)
- construct plasmid containing mutagenized tower domain region (months 26-28)
- perform marker rescue screen with mutagenized plasmid to isolate candidate tower domain mutants (months 29-32)
- retest and sequence mutants of interest (months 32-36)

Detailed summary of data. We have completed the biochemical analysis of the G269V mutant enzyme (Task 1) and have submitted a manuscript detailing our results to *Biochemistry* (9). Briefly, the G269V mutant appears to be very different than any previously identified topoisomerase mutant. For one thing, the substitution is located in a region of the protein that has not been previously implicated in drug or DNA binding.

Further, unlike other mutants, the G269V enzyme is hypersensitive to a broad range of topoisomerase inhibitors but does not have an altered drug binding pocket. We believe that the G269V substitution causes hypersensitivity by increasing the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle. This is the first type II topoisomerase mutant described that alters drug sensitivity by altering the equilibrium of the enzyme (9).

Recall that the G269V substitution was originally isolated in combination with S79F in a selection for drug-resistant mutants (2, 4). S79F by itself causes a topoisomerase-negative phenotype *in vivo*, which is rescued by the G269V substitution. Comparisons with the yeast enzyme place the S79 residue in the CAP-like domain of the protein, far from the G269V substitution in the tower domain (these domains are detailed in refs 8 and 9; for a picture see ref 9, Figure 6A). It is unclear how a substitution in the tower domain leads to suppression of a substitution in the CAP-like domain. What is the nature of the defect of the S79F mutant and how does G269V suppress this defect? Also, why is the drug-resistance phenotype dominant to the hypersensitivity phenotype? We feel that the answers to these questions could lead to a further understanding of the hypersensitivity phenotype (Task 2).

In order to determine the nature of the defect in the S79F containing phage strain, I purified the S79F mutant enzyme by utilizing the T4 topoisomerase overproduction strain (see proposal). Unexpectedly, the purified S79F protein was proficient at relaxing supercoiled DNA with a specific activity similar to that of the wild-type enzyme (1.7×10^6 U/mg and 2.9×10^6 U/mg, respectively). Thus, the S79F mutant enzyme is apparently proficient for DNA binding, DNA cleavage, strand passage and religation.

We next analyzed the drug sensitivity spectrum of the S79F mutant enzyme. Since, the G269V substitution causes hypersensitivity to all of the drugs, it is reasonable to assume that the S79F substitution is responsible for conferring the drug-resistance phenotype displayed by the double mutant enzyme. Therefore, we would expect the S79F single and S79F/G269V double mutant enzymes to have the same drug-sensitivity spectra. As was previously shown for the doubly substituted enzyme (5), the S79F confers resistance to *m*-AMSA when compared to the wild-type (Figure 1). At 0.75 μM *m*-AMSA the wild-type enzyme forms large amounts of linear DNA while the S79F mutant enzyme forms very little even at the highest drug concentration (Figure 1, compare lanes 3 and 11). In similar assays, the S79F substitution was shown to confer resistance to ellipticine, 2-me-9OH E^+ and oxolinic acid but was found to be partially sensitive to VP-16, VM-26 and mitoxantrone. Indeed these patterns closely mirror those previously noted for the double mutant (5).

It is somewhat difficult to compare our results with the S79F mutant protein to those previously reported for the S79F/G269V double mutant enzyme because the reaction conditions were not identical. Further, both sets of analyses were qualitative and it would be most informative to compare them directly in a quantitative manner. Will the S79F and S79F/G269V enzymes be equally sensitive/resistant to all of the drugs? Is the S79F substitution completely dominant to the G269V substitution or will the double mutant

have an intermediate phenotype? To do this, we will perform cleavage assays in the presence of different drugs and quantitate them with our filter binding method (9). We can then compare the relative levels of sensitivity by determining the drug concentrations required for half-maximal cleavage of each mutant enzyme compared to wild-type and calculate an overall drug sensitivity factor. It may be difficult to quantitate very high levels of resistance because high levels of drug can cause enzyme inhibition. However, we will attempt to overcome this by increasing the enzyme dimer-to-DNA ratio either by using more protein or less DNA.[1]

Many previously isolated topoisomerase mutants that alter drug sensitivity also alter the DNA site specificity of the enzyme. We have shown that the G269V substitution by itself does not change the DNA site specificity of the enzyme (9). On the other hand, the doubly substituted enzyme, S79F/G269V, does have an altered DNA site specificity at least in the absence of drugs (5). Therefore, it is likely that the S79F single mutant will have altered cleavage site specificity in the absence of drugs. It will also be informative to determine the cleavage site specificities of the S79F and S79F/G269V mutant enzymes in the presence of the drugs to which they are sensitive. To examine this, we will perform cleavage assays in the presence of end-labeled linear substrates as we have already done for the G269V mutant enzyme.

In the relaxation and cleavage assays described above, the S79F mutant enzyme appears to be proficient at many steps of the topoisomerase reaction pathway. These experiments were performed with supercoiled circular plasmid DNA. However, bacteriophage T4 DNA is modified with glucosylated hydroxymethyldeoxycytosines in place of deoxycytosines and the plasmid DNA does not have this modification. Thus, we wondered if the S79F mutant enzyme might be deficient for the ability to recognize modified DNA. To test this, we performed cleavage assays with the S79F mutant and wild-type enzymes in the presence of modified T4 DNA and VP-16. Although the S79F mutant is only partially sensitive to VP-16, it is clearly proficient for binding and cleaving T4 modified DNA (Figure 2). Similar levels of resistance were seen in comparable experiments performed with T4 unmodified DNA (data not shown).

Thus, under the conditions tested so far, the catalytic defect of the S79F mutant enzyme is not obvious. The mutant could be defective in something specific like the decatenation of replicated DNA. Alternatively, the mutant may have specific requirements for salt, ATP, magnesium or other cofactors and our buffers may be more permissive than the *in vivo* environment of the cell. In order to clarify the nature of the *in vivo* defect, we will perform DNA relaxation and replication assays during infections of *E. coli* containing a suppressing Phe-tRNA with an S79^{am} phage and compare them to wild-type infections. Will the mutant be partially proficient for both activities *in vivo*? What if the mutant is proficient for DNA relaxation but deficient for DNA replication?

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[1] I have not begun this quantitation because I had to purify more of the S79F/G269V mutant protein. The previous enzyme prep was over ten years old and although it was still fairly active, it had developed a nuclease activity.

The answers to these questions will influence the specific nature of further *in vitro* experiments.

Regardless of the outcome or our *in vivo* experiments, we must also consider the possibility that the nature of the S79F defect is not catalytic. One possibility is that the S79F mutant enzyme might be unstable *in vivo*. However, this is unlikely as the purification resulted in protein yields similar to those obtained from other mutant enzyme preparations. A more intriguing possibility is that the mutant protein may be defective for some essential *in vivo* protein-protein interaction. Type II topoisomerase are known to interact with a number of cellular proteins but the importance of many of these interactions is not well understood (see 7 and references therein). In the event that we cannot find a catalytic defect associated with the S79F mutant enzyme, we can screen for mutants of T4 or *E. coli* that suppress the topoisomerase-negative phenotype. This will allow us to identify essential topoisomerase protein-protein interactions and may reveal new roles for the enzyme. More importantly, the modulation of topoisomerases by other cellular proteins will have implications for topoisomerase II directed chemotherapeutics.

Key Research Accomplishments

Task 1 (Also detailed in O'Reilly and Kreuzer, 2002)

- Purified the G269V mutant topoisomerase protein from *E. coli* cells that were infected with bacteriophage T4.
    - The G269V protein behaved the same as the wild-type enzyme during all steps of the purification process.
- Determined that the specific activity of the G269V mutant enzyme was similar to that of the wild-type enzyme (2.0 X 10^6 U/mg and 2.9 X 10^6 U/mg respectively).
- Demonstrated that the G269V mutant enzyme is detected in cleavage complexes at roughly ten times the level of the wild-type enzyme.
- Determined the drug-sensitivity spectrum of the G269V mutant enzyme (qualitative).
    - The G269V mutant displayed hypersensitivity to all of the drugs tested.
- Found that the mutant enzyme does not seem to have an altered DNA sequence specificity compared to that of the wild-type enzyme.
    - This suggests a novel mechanism for altered drug sensitivity.
- Modified a published filter-binding assay for use in the quantitation of topoisomerase cleavage complexes.
- Quantitated the levels of drug sensitivity of the G269V mutant enzyme compared to the wild-type enzyme.

Task 2

- Purified the S79Fsingle and S79F/G269V double mutant enzymes from infected *E. coli* cells.
    - The S79F and S79F/G269V proteins behaved the same as the wild-type enzyme during all steps of the purification.
- Determined the specific activities of the mutant enzymes. Both similar to that of the wild-type enzyme (S79F, 1.7 X 10^6 U/mg; S79F/G269V, 2.2 X 10^6 U/mg; wild-type, 2.9 X 10^6 U/mg).
- Determined the drug sensitivity spectrum of the S79F mutant enzyme in order to compare it to the previously characterized S79F/G269V double mutant enzyme (qualitative).
    - The S79F single and S79F/G269V double mutant enzymes share similar drug sensitivity spectra.
- Found that the S79F mutant enzyme can recognize and cleave T4 modified DNA.
- Will determine the DNA cleavage site specificity of the S79F single and S79F/G269V mutant enzymes in the absence and presence of drugs.
- Will quantitate the drug sensitivity levels of the S79F single and S79F/G269V double mutant enzyme in order to compare them to the wild-type and G269V mutant enzymes.

• In the process of performing *in vivo* DNA relaxation and replication assays to clarify the nature of the S79F defect.

Task 3

• I have not begun the screen for additional tower domain mutants but expect to start in the next month.

Reportable Outcomes

Presentations

O'Reilly, Erin K., and Kenneth N. Kreuzer. "A novel mutant of T4 topoisomerase that is hypersensitive to multiple classes of antitumor drugs." The Millennial Phage Meeting. McGill University, Montreal, Canada. May 7-11, 2000.

O'Reilly, Erin K., and Kenneth N. Kreuzer. "A novel mutant of T4 topoisomerase that is hypersensitive to multiple classes of antitumor drugs." Phage Meeting 2002. The Evergreen State College, Evergreen, Washington. August, 2001.

Publications

O'Reilly, Erin K., and Kenneth N. Kreuzer. (2002). A Unique Type II Topoisomerase Mutant That Is Hypersensitive To A Broad Range of Cleavage-Inducing Antitumor Agents. *Biochemistry*. Submitted.

Conclusions

Thus far, we have described a T4 topoisomerase mutant that is hypersensitive to multiple classes of type II topoisomerase poisons. This mutation is located in a region of the protein that has not been previously implicated in drug or DNA binding. The G269V substitution increases the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle. We believe that this is the first type II topoisomerase mutant described that alters drug sensitivity by altering the equilibrium of the enzyme.

It is still unclear how the G269V substitution suppresses the topoisomerase-negative phenotype displayed by the S79F mutant strain. We are currently in the process of determining the nature of the defect as well as the nature of suppression. Understanding the relationship between these two positions of the protein could shed new light on enzyme function and lead to a further understanding of the hypersensitivity phenotype. Analysis of these and similar mutants will provide a unique perspective on the mechanism of action of topoisomerase inhibitors.

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8. O'Reilly, E.K., (1999). A unique class of topoisomerase mutants that are hypersensitive to multiple antitumor agents. Original research proposal submitted to the DOD Breast Cancer Research Program.

9. O'Reilly, E.K., & Kreuzer, K.N. (2002). A unique type II topoisomerase mutant that is hypersensitive to a broad range of cleavage-inducing antitumor agents. *Biochem*. Submitted. (See also Appendix I)

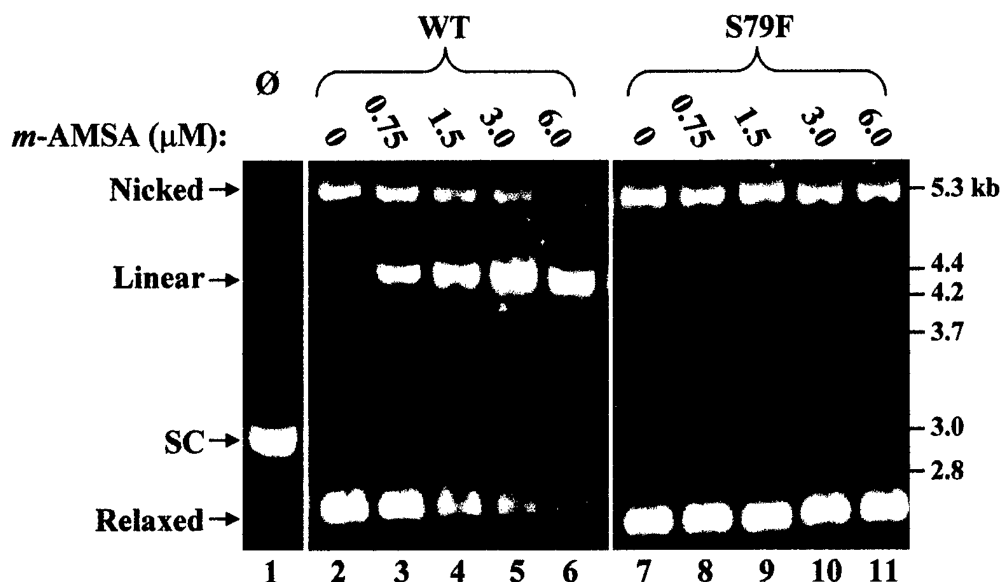

FIGURE 1. DNA cleavage assay with negatively supercoiled pBR322 DNA in the presence of *m*-AMSA. The *m*-AMSA sensitivities of the wild-type and S79F enzymes were compared in a drug titration. Reaction mixtures of 20 μl contained 40 mM Tris-HCL (pH 7.8), 60 mM KCL, 10 mM MgCl2, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.5 mM Na2EDTA, nuclease-free bovine serum albumin (30 μg/ml), 300 ng of negatively supercoiled pBR322, 25 ng of protein, 300 ng of DNA and the indicated concentrations of *m*-AMSA (μM, micromolar). The reactions were initiated by the addition of the indicated topoisomerase, incubated at 30 ° for 30 min, and then terminated by the addition of 5 μl of gel-loading buffer [5% (w/v) SDS, 20% (w/v) Ficoll, 0.1% bromophenol blue, and 0.1% xylene cyanol]. Proteinase K (final concentration 100 μg/ml) was then added and the samples were incubated for 1 h at 37 ° to permit removal of any covalently attached topoisomerase. The reaction products were then resolved by electrophoresis through 0.8% agarose containing ethidium bromide (2.5 μg/ml). Gels were run in TBE running buffer (89 mM Tris base/89 mM boric acid/ 2.5 mM Na2EDTA) overnight at 2 V/cm followed by visualization with UV illumination. A size scale (*kb*, kilobases), generated from the migration of T4dC *Xba*I restriction fragments, appears in the right hand margin, and different forms of DNA are indicated in the left hand margin. SC, supercoiled; Ø denotes a control reaction with no enzyme and no drug.

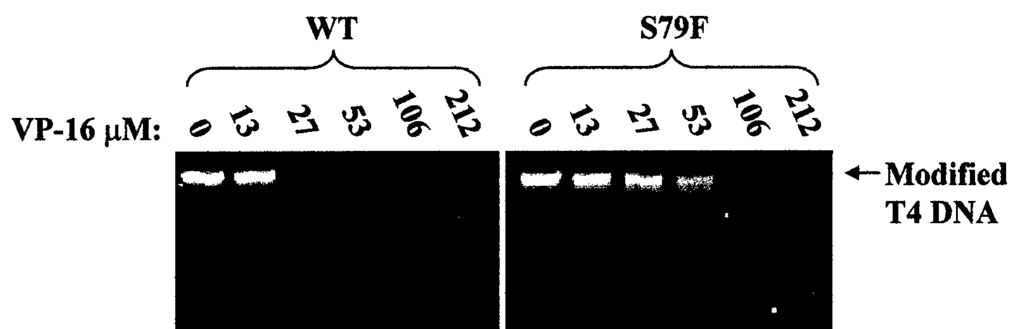

Figure 2. DNA cleavage assays with modified T4 DNA in the presence of VP-16. The abilities of the wild type and S79F mutant enzymes to cleave modified T4 DNA were compared in the presence of VP-16. Reaction conditions were identical to those in Figure 1 accept that VP-16 is present at the indicated concentrations. Also, the reactions contained 100 ng of the indicated topoisomerase and 1200 ng of DNA.

Appendix I

A Unique Type II Topoisomerase Mutant That Is Hypersensitive To A Broad Range Of Cleavage-Inducing Antitumor Agents[†]

Erin K. O'Reilly[‡] *and Kenneth N. Kreuzer*[*‡]

[†]This work was supported by research grant CA60836 to K.N.K. from the National Institutes of Health/ National Cancer Institute. E.K.O. was supported in part by the Department of Defense research grant DAMD-00-01-0235.

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[1]Abbreviations: SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide, *m*-AMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide; 2-me-9-OH-E^+, 2-methyl-9-hydroxyellipticinium acetate; VP-16, etoposide or 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); VM-26, teniposide or 4'-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); gp, gene product; NC, nitrocellulose; NY, nylon.

ABSTRACT

Bacteriophage T4 provides a useful model system for dissecting the mechanism of action of antitumor agents that target type II DNA topoisomerases. Many of these inhibitors act by trapping the cleavage complex, a covalent complex of enzyme and broken DNA. Previous analysis showed that a drug resistant T4 mutant harbored two amino acid substitutions (S79F, G269V) in topoisomerase subunit gp52. Surprisingly, the single amino acid substitution, G269V, was shown to confer hypersensitivity in vivo to *m*-AMSA and oxolinic acid [Freudenreich C.H. et al., (1998) *Cancer Research 58*, 1260-1267]. We purified this G269V mutant enzyme and found it to be hypersensitive to a number of cleavage-inducing inhibitors including *m*-AMSA, VP-16, mitoxantrone, ellipticine and oxolinic acid. While the mutant enzyme did not exhibit altered DNA cleavage site specificity compared to the wild-type enzyme, it did display an apparent 10-fold increase in drug-independent DNA cleavage. This suggests a novel mechanism of altered drug sensitivity in which the enzyme equilibrium has been shifted to favor the cleavage complex, resulting in an increase in the concentration of cleavage intermediates available to inhibitors. Mutations that alter drug sensitivities tend to cluster within two specific regions of all type II topoisomerases. Residue G269 of gp52 lies outside of these regions, and it is therefore not surprising that G269V leads to a unique mechanism of drug hypersensitivity. We believe that this mutant defines a new category of type II topoisomerase mutants, namely those that are hypersensitive to all inhibitors that stabilize the cleavage complex.

Type II DNA topoisomerases are essential enzymes required for many cellular processes such as DNA replication and transcription (for reviews, see refs *1-4*). These enzymes function by creating a double strand break in one segment of duplex DNA, passing a second duplex segment through this break, and then resealing the break. A critical intermediate in this reaction, the cleavage complex, consists of the protein linked to the 5′ ends of the staggered break via phosphotyrosine bonds.

In addition to their essential roles in cellular metabolism, type II topoisomerases are the targets of many clinically important antibacterial and antitumor agents. The prokaryotic type II topoisomerases (DNA gyrase, topoisomerase IV) are the targets of the important broad spectrum antibacterial quinolones and fluoroquinolones (*5*), while the eukaryotic type II topoisomerases are inhibited by several classes of antitumor agents, including the acridines, anthracyclines, ellipticines and epipodophyllotoxins (for reviews, see refs *3*, *6*). These drugs act by stabilizing the cleavage complex rather than by simply inhibiting strand passage (for reviews, see refs *3*, *7*, *8*). Direct binding studies from a number of systems support the existence of a stable enzyme-DNA-drug ternary complex (*9-11*, S. Neece and K. Kreuzer, unpublished data). The stabilized cleavage complex itself is relatively harmless to cells, but it can be converted into potentially lethal DNA damage by cellular processes such as DNA replication (*12-14*).

In the context of antitumor drug action, bacteriophage T4 provides a useful and valid model system. The T4 type II topoisomerase is a multisubunit enzyme composed of the products of T4 genes *39*, *52*, and *60* (*15*, *16*). Gp39 and gp60 are homologous to portions of *Escherichia coli* GyrB and to the amino-terminal half of eukaryotic topoisomerase II while gp52 contains the active site tyrosine and is homologous to *E. coli* GyrA and the

carboxy-terminal half of the eukaryotic enzymes. The phage-encoded enzyme is sensitive to many of the same antitumor agents that inhibit the eukaryotic enzyme and is partially sensitive to at least one of the antibacterial quinolones (*17*, *18*). The straightforward genetics, biochemistry, and molecular biology of the phage T4 system have facilitated studies on the mechanism of action of these topoisomerase inhibitors.

The inhibitor binding site within the cleavage complex consists of both protein and DNA. Involvement of the topoisomerase in drug binding is indicated by the fact that single amino acid changes in specific regions of the enzyme can confer altered drug sensitivities (reviewed in *19*). For example, the yeast topoisomerase S741W mutant is hypersensitive to VP-16 but resistant to the fluoroquinolone CP-115,953 (*20*). In T4, the E457K substitution in gp39 causes resistance to a number of drugs but hypersensitivity to oxolinic acid and VP-16 (*21*, *22*). The differential effects of these mutations on different drug families imply that specific amino acid residues of the protein interact with the drugs.

Involvement of DNA in the drug binding site was originally proposed based on early experiments that revealed distinct patterns of cleavage sites in the presence of different drug classes (*17*, *23-26*). Consensus sequences derived with eukaryotic enzymes suggested that the base pairs immediately flanking the cleavage site determined which drug could trap the enzyme at a particular cleavage site (*26-28*). The detailed mutational analysis of one particular T4 topoisomerase cleavage site provided direct evidence that the basepair immediately 5′ of the break confers the drug site preference (*29*). The preferred basepairs for the T4 enzyme paralleled those of the eukaryotic enzymes, suggesting that the same rules govern inhibitor binding specificity in evolutionarily

distant topoisomerases (*29*). Further, using the T4 system, the inhibitor was precisely localized to the sites of DNA cleavage using a photoactivatible analog of *m*-AMSA (*30*). Thus, the inhibitors bind directly at the sites of DNA cleavage (perhaps by intercalation) and act by preventing religation of the two strands and/or enhancing the forward rate of cleavage.

Analysis of a T4 gene *52* mutant led to some interesting and unexpected results. The mutant strain was originally isolated from a selection for *m*-AMSA resistant phage mutants (*31*). The mutant was found to harbor two amino acid substitutions: S79F and G269V (*22*). The S79F mutation was quite provocative because the corresponding residue from bacterial DNA gyrase (A84) was known to be a hotspot for drug-resistance mutations (*9*). Surprisingly, when the S79F mutation was substituted into a wild-type background, the resulting phage behaved as topoisomerase negative in vivo (*22*). It was hypothesized that the G269V mutation suppresses the defect caused by the substitution at residue S79. Indeed, when the double mutant (S79F/G269V) was substituted into a wild-type background, the resulting phage behaved as topoisomerase proficient and displayed the same drug-resistant phenotype as the original mutant.

Remarkably, the G269V single mutant phage exhibited hypersensitivity to *m*-AMSA and oxolinic acid in vivo (*22*). This hypersensitive phenotype cannot be readily explained by the location of this residue in the protein as none of the corresponding residues from the yeast or bacterial enzymes have been implicated in drug binding. Sequence comparisons with the yeast enzyme place residue G269 in the 'tower domain' of the protein, which lies outside the two domains where drug-resistance mutations cluster (see Discussion). Furthermore, there is no biochemical or structural evidence for involvement

of the tower domain in DNA binding. Therefore, it is unclear how the G269V substitution causes drug hypersensitivity or suppresses the defect at position S79.

In the present study, we have biochemically analyzed the G269V enzyme and found it to be hypersensitive to a broad range of type II topoisomerase inhibitors. While the G269V mutant enzyme did not exhibit altered cleavage site specificity compared to the wild-type enzyme, it did display elevated levels of drug-independent DNA cleavage. This suggests a novel mechanism for altered drug sensitivity, namely an alteration in the reaction pathway that leads to an increase in the frequency of cleavage complexes available to inhibitors.

EXPERIMENTAL PROCEDURES

Materials. m-AMSA (NSC 249992), ellipticine (NSC 71795), and mitoxantrone (NSC 299195) were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. 2-me-9-OH-E^+ (NSC 264137) was a generous gift of Dr. C. Paoletti (Institute Gustave-Roussy, Villejuif). VM-26 (NSC 122819) and VP-16 (NSC 141540) were kindly provided by Bristol-Myers Pharmaceutical Co. (Wallingford, CT). Oxolinic acid and additional VP-16 were purchased from Sigma. 2-me-9-OH-E^+ was dissolved in water, oxolinic acid in 50 mM NaOH, and all other drugs in DMSO. Immediately prior to use, an aliquot was diluted with water to the required concentration. Final reaction concentrations of DMSO and NaOH were $< 1.5\%$ and <0.5 µM respectively, and did not significantly affect DNA cleavage levels (data not shown).

Plasmid pBR322 was purified from *E. coli* DH5α using an alkaline lysis procedure (*32*) followed by cesium chloride/ethidium bromide isopycnic centrifugation. Restriction enzymes were obtained from New England Biolabs.

T4 topoisomerases. Wild-type and G269V mutant T4 topoisomerases were purified by adapting the T7 expression system for use in phage T4, as originally described by Singer and Gold (*33*). As will be described in more detail elsewhere, a T7 promoter was placed upstream of each T4 topoisomerase gene (*39*, *52*, and *60*) to create the overproduction phage strain for wild-type enzyme (K. Magee and K. Kreuzer, unpublished data). We then crossed the G269V mutation into this background to create the overproduction strain for the mutant enzyme. *E. coli* cells were induced for T7 RNA polymerase prior to infection with each of the topoisomerase overproduction strains and T4 topoisomerase was purified as previously described (*34*, *35*). Protein concentrations were determined using the Bradford assay (*36*) as described previously (*31*). Enzymatic activities were determined using a standard DNA relaxation assay (*35*).

End-labeling of DNA substrates. pBR322 DNA was linearized with *Eco*RI and then the recessed 3' termini were filled in using the Klenow exo$^-$ DNA polymerase I fragment in the presence of either [α-^{33}P] or [α-^{32}P] dATP and dTTP, yielding a 4361-bp substrate labeled at each *Eco*RI terminus. When indicated, the labeled substrate was further digested with *Hin*dIII to yield a 4336-bp fragment labeled at only one end.

DNA Cleavage Assays. Reaction mixtures (20 µl) contained 40 mM Tris-HCl (pH 7.8), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, nuclease-free bovine serum albumin (30 mg/ml), and the specified amounts of DNA substrate, topoisomerase, and inhibitor. Cleavage reactions were performed at an

approximate ratio of one topoisomerase dimer to one molecule of DNA except where indicated (see figure legends). The reactions were initiated by the addition of topoisomerase, incubated at 30° for 30 min, and terminated by the addition of 5 μl of gel-loading buffer [5% (w/v) SDS, 20% (w/v) Ficoll, 0.1% (w/v) bromophenol blue , and 0.1% (w/v) xylene cyanol]. Proteinase K (final concentration 100 μg/ml) was added and the samples were incubated for 1 h at 37° to remove covalently attached topoisomerase. The reaction products were resolved by electrophoresis through either 0.8% agarose gels or 6% polyacrylamide gels in TBE running buffer (89 mM Tris base/ 89 mM boric acid/ 2.5 mM Na_2EDTA). Agarose gels contained ethidium bromide (2.5 μg/ml) and were run overnight at 2 V/cm; DNA was visualized by ethidium bromide fluorescence and quantitated using an Alpha Innotech digital imaging system. Polyacrylamide gels were run at 30 watts for approximately six hours. After gel drying, the labeled DNA was visualized by autoradiography on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Filter Binding Method. The levels of DNA cleavage were quantitated using a nitrocellulose filter-binding assay similar to that described by Wong and Lohman (*37*). A Millipore vacuum pressure pump (Fisher, XX5500000) was used at 500-600 mm Hg during filtration on a 96-well dot-blot apparatus (Minifold I, Schleicher and Schuell). Nitrocellulose (NC; Schleicher and Schuell, BA-85) was used as the top filter to trap the protein-linked DNA, and nylon (NY; NEN, NEF-994) was used as the second filter to trap the protein-free DNA that does not bind to the NC filter. A single sheet of gel blot paper (Schleicher & Schuell) was used as a third layer to reduce lateral diffusion of unbound DNA. The NC and NY filters were equilibrated in 1X filter buffer (50 mM Tris-

HCl (pH 7.8), 200 mM KCl, 10 mM $MgCl_2$, 0.5 mM Na_2EDTA) for 30 min before use, and the gel blot paper was immersed in the same buffer prior to use.

Approximately 1-2 ng (10,000 cpm) of linearized DNA labeled at both ends with [α-^{33}P] dATP and 100 ng of linearized unlabeled pBR322 DNA were combined with 9.2 ng (35 fmol) of topoisomerase (molar enzyme dimer-to-DNA ratio of 1; except where indicated). The reactions were performed as described above (see DNA Cleavage Assays) except that they were terminated by the addition of 5 μl of 1% (w/v) SDS and were not treated with proteinase K. After termination, 15 μl of 5X filter buffer and 50 μl of water were added to each reaction. Immediately prior to loading, the wells were flushed with 400 μl of 1X filter buffer under vacuum. The samples were loaded without vacuum, the vacuum was reapplied, and the wells were then washed twice with 400 μl of 1X filter buffer. The two filters were then blotted dry, wrapped in plastic wrap (Mylar), and imaged by autoradiography on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The 'dots' were quantitated using the ImageQuant software provided by the manufacturer. The percent cleavage was calculated from the ratio of counts on the NC filter to the total counts (NC filter + NY filter). This percent cleavage was corrected by subtracting the small amounts (1-2%) of protein-free DNA that bind to the NC filter in control reactions. The amount of drug-dependent cleavage was determined by subtracting the amount of drug-independent cleavage observed for each enzyme, leading to no apparent cleavage at 0 μM drug. It is important to note that the G269V enzyme yields approximately 10% cleavage in the absence of drug at a molar enzyme dimer-to-DNA ratio of 1, while the wild-type enzyme generates about 1% cleavage (see below).

A final correction was also applied to account for protein that is linked to unlabeled DNA as a result of multiple cleavage events. The substrate is labeled at each end, so one cleavage event per DNA molecule results in both topoisomerase monomers being linked to a labeled DNA fragment. However, any additional cleavage events on the same molecule of DNA would not generate additional signal. Therefore, assuming that cleavage events are randomly distributed, the data were corrected with the following equation derived from the Poisson distribution

$$C_t = -ln\ (1-C_o)$$

where C_o is the experimentally observed amount of labeled DNA cleavage and C_t is the predicted amount of total DNA cleavage. For example, 30% observed cleavage ($C_o = 0.3$) is reported as 35.7% total cleavage ($C_t = 0.357$). The averages of triplicate measurements are shown with error bars indicating standard deviations.

RESULTS

The G269V substitution causes increased levels of drug-independent DNA cleavage. The G269V substitution caused dramatic hypersensitivity in vivo to both *m*-AMSA and oxolinic acid (*22*). In our system, these are the only two inhibitors that can be analyzed in vivo. We therefore purified the mutant enzyme so that we could test its sensitivity to other cleavage-inducing inhibitors. The G269V mutant topoisomerase displayed a specific activity similar to that of the wild-type protein (2.0×10^6 U/mg and 2.9×10^6 U/mg, respectively; data not shown). However, in the absence of drug, the G269V mutant protein caused the formation of much larger amounts of linear DNA from a circular

substrate than did the wild-type enzyme (Figure 1, compare lanes 2-6 with lane 7-11). At an approximate molar enzyme dimer-to-DNA ratio of 1, we estimate that 10% of the DNA was in a cleavage complex with the G269V mutant enzyme while the amount of DNA in a cleavage complex with the wild-type enzyme was too low for accurate quantitation (Figure 1, lanes 3 and 8). These cleavage products were protein linked, since the linear DNA became a broadened band of decreased electrophoretic mobility in the absence of proteinase K digestion (data not shown). Protein-linked DNA products are a normal short-lived intermediate in the topoisomerase relaxation reaction, but our results show that the G269V mutant topoisomerase accumulates higher than normal amounts of this intermediate.

The G269V substitution causes in vitro hypersensitivity to m-AMSA. We next tested whether the purified G269V enzyme displayed *m*-AMSA hypersensitivity, as was observed in vivo. As expected from the in vivo results, the G269V protein produced more linear DNA at relatively low levels of *m*-AMSA than did the wild-type enzyme (Figure 2). This conclusion is somewhat obscured by the high levels of drug-independent cleavage observed with the G269V enzyme (Figure 2, compare lane 2 to lane 11). Nonetheless, the drug-dependent percent cleavage can be determined by subtracting the drug-independent percent cleavage. Thus, at 0.125 μM *m*-AMSA, the G269V mutant enzyme yielded about 6-fold more drug-dependent cleavage than the wild-type enzyme (Figure 2, lanes 4 and 13). This difference becomes less apparent at higher drug levels presumably because the drug concentrations are reaching saturation. Additionally, at high drug levels, multiple cleavage events are evident from the smearing and minor cleavage products (Figure 2, lanes 8-10 and 15-19). All of these *m*-AMSA-induced cleavage

products were shown to be protein linked by omitting the proteinase K digestion (data not shown). Our results show that the purified G269V mutant topoisomerase is indeed hypersensitive to *m*-AMSA with respect to cleavage complex formation, mirroring the in vivo hypersensitive phenotype of the G269V mutant phage.

The G269V substitution causes in vitro hypersensitivity to multiple classes of type II topoisomerase inhibitors. We next analyzed DNA cleavage of the G269V mutant using a quantitative approach. We employed a filter-binding method similar to one described by Wong and Lohman (*37*), using a 96-well dot-blot apparatus with a double filter. After the reactions were stopped with SDS, protein-linked DNA molecules were trapped on a NC filter while free DNA passed through and bound to an underlying NY filter. This enables a direct determination of percent cleavage of the labeled substrate, with triplicate repeats of twenty different reactions analyzed on a single filter.

This filter-binding method allowed a more accurate comparison of drug-independent DNA cleavage levels between the wild-type and G269V mutant enzymes. We determined enzyme dimer-to DNA ratios that produced similar levels of total cleavage. With the G269V enzyme, 10.6% (±3.0; n=7) of the DNA was in a cleavage complex at an enzyme dimer-to DNA ratio of 1, while an enzyme dimer-to DNA ratio of 10 was needed to trap 8.0% (±0.7%, n=3) of the DNA in a cleavage complex with the wild-type enzyme. Thus, the G269V mutant enzyme is detected in cleavage complexes at roughly ten times the level of the wild-type enzyme.

Again using the filter-binding method, we compared the DNA cleavage levels of the wild-type and G269V mutant topiosomerases induced by five different drug classes. Drug-dependent cleavage levels were determined by subtracting the drug-independent

cleavage levels from each drug titration (enzyme dimer-to-DNA ration of 1). Seven topoisomerase inhibitors were tested: ellipticine and 2-me-9OH-E^+ (ellipticines), mitoxantrone diacetate (substituted anthraquinone), VM-26 and VP-16 (epipodophyllotoxins), oxolinic acid (quinolone) and the previously assayed m-AMSA (an aminoacridine).

The results obtained with m-AMSA (Figure 3) can be compared directly with the DNA cleavage assay above (Figure 2). The filter-binding assay showed that the G269V enzyme enhanced drug-dependent cleavage, especially at low m-AMSA concentrations. At 0.125 μM m-AMSA, there is almost ten times more drug-dependent cleavage with the G269V enzyme than with the wild-type enzyme (Figure 3, 7.6% versus 0.77%). As observed with the gel assays, this margin is narrowed as the drug reaches saturating levels. Thus, the filter binding method can accurately measure drug sensitivity over a broad range of drug concentrations and verifies the m-AMSA hypersensitivity of the G269V enzyme.

Somewhat surprisingly, the G269V mutant topoisomerase was found to be hypersensitive to all the drugs tested (Figure 4). All the curves follow the same general pattern, with more dramatic sensitivity at lower drug concentrations (see inserts). In order to compare the relative levels of sensitivity, we determined the drug concentrations required for half-maximal cleavage and calculated an overall hypersensitivity factor of G269V enzyme compared to wild-type (Table 1). According to this 'HS factor', the mutant enzyme was between 1.5 and 3.3-fold more sensitive to all of the drugs, with no obvious difference between intercalators (ellipticines, mitoxantrone and m-AMSA) and non-intercalators (oxolinic acid and epipodophyllotoxins). These results suggest that the G269V mutant topoisomerase is responding to all of the drugs in a similar manner.

The G269V substitution does not alter the site specificity of the enzyme in the absence or presence of drugs. The T4 type II topoisomerase cleaves duplex DNA at specific sites, generating a reproducible pattern of cleavage fragments from a given DNA substrate. Previous work has shown that each chemical class of inhibitor exerts a unique effect on the DNA cleavage site specificity of the wild-type T4 or eukaryotic enzyme (*17*, *23-26*, *29*). Further, many previously isolated topoisomerase mutants that alter drug sensitivity also alter the DNA site specificity of the enzyme, presumably because the drug binding pocket of the enzyme is very close to the DNA (*17*, *23-26*, *38*; also see Introduction). To see if the G269V mutation alters the DNA site specificity and, by implication, the drug binding pocket, we performed DNA cleavage assays in the presence and absence of drugs using a linear substrate that was labeled at one end with [α-P^{32}] dATP. The products were analyzed on a neutral polyacrylamide gel.

In the absence of drug, the wild-type cleavage pattern could only be visualized by using approximate molar enzyme dimer-to-DNA ratios of 100 or 1000 (Figure 5, lanes 4 and 5; 1000 and 10,000 ng respectively). With the G269V enzyme, the cleavage pattern could be readily visualized at a molar ratio of approximately 10 (Figure 5, lane 8; 100 ng). The drug-independent cleavage pattern of the G269V protein did not differ significantly from that of the wild-type enzyme (Figure 5, lanes 5 and 8).

To assess the cleavage patterns in the presence of drug, we used an approximate molar enzyme dimer-to DNA ratio of 1 for both enzymes (Figure 5, lanes 9-20; 10 ng), except that oxolinic acid was also tested at an approximate molar ratio of 10 in order to visualize DNA cleavage pattern of the wild-type enzyme (Figure 5, lanes 23 and 24; 100 ng). Since the observed DNA cleavage patterns are strongly influenced by the total extent of DNA

cleavage, we chose inhibitor concentrations that gave us maximal levels of cleavage (based on Figures 3 and 4). As expected, each of the inhibitors altered the cleavage site specificity of both the wild-type and G269V enzymes. More importantly, the drug-dependent cleavage patterns of the mutant and wild-type proteins did not significantly differ from one another. These results indicate that the G269V mutation alters the sensitivity of the enzyme without changing the drug binding pocket, which is thought to be in intimate contact with the DNA.

DISCUSSION

The isolation and analysis of many drug resistant mutants from bacterial, T4, and eukaryotic systems have provided a window that helps us understand the mechanism of action of topoisomerase inhibitors. In this report, we have described a new class of type II topoisomerase mutant, one that is hypersensitive to many different inhibitors that stabilize the cleavage complex. Such hypersensitive mutants should provide a different window, revealing unique aspects of the mechanism of drug action.

To facilitate this discussion, we will use the published crystal structures of the yeast type II topoisomerase (*39*, *40*) and the bacterial gyrase subunit A (*41*) as a framework for discussing G269V and other topoisomerase mutants. This approach is somewhat speculative as the structure of the T4 enzyme could differ significantly from the yeast and bacterial enzymes. However, all type IIA topoisomerases share extensive sequence homologies (*42*, *43*). Furthermore, although they represent two different enzyme conformations, the published structures of the yeast and bacterial enzymes are quite

similar. Therefore, it is likely that the T4 enzyme has the same generally conserved structure. In particular, a comparison of the predicted secondary structures in the region of the G269 residue supports the validity of a conserved structure (Figure 6B).

The crystal structure of a large fragment of the yeast type II topoisomerase homodimer is shown in Figure 6A (*39*). For reference, the A′ portion of the molecule is homologous to *E. coli* GyrA and the product of T4 gene *52*, while the B′ fragment is homologous to a portion of *E. coli* GyrB, and the products of T4 genes *39* and *60*. Two domains of interest are the CAP-like domain and the Rossmann fold (Figure 6A; see also *44*). These two domains contain several charged residues that are essential for the topoisomerase cleavage reaction (*44*, *45*). All the available structural and mutational data suggest that these two regions come together to participate in catalysis and to form a drug binding pocket (*40*, *44-48*). Not surprisingly, the vast majority of drug resistance mutations fall into one of these two regions. For example, the two quinolone-resistance determining regions (QRDR) are within the Rossmann fold and the CAP-like domain (*46*, *49*). Many of these mutations apparently alter the drug binding pocket of the enzyme because they cause differential effects on the various inhibitors (*17*, *23-26*, *38*).

One of the unique characteristics of the G269V substitution is its unusual location outside of the CAP-like and Rossmann fold regions. Sequence comparisons place the G269V mutation in the 'tower-domain' of the protein, between β-strands 12 and 13 (Figure 6B). Based on this structural alignment, residue G269 of T4 corresponds roughly to D939 and D297 of the yeast topoisomerase II and bacterial GyrA proteins, respectively. These residues are over 25 Å away from the active site tyrosine in each of the published structures. Further, the loop between β-strands 12 and 13 is located on the

opposite side of the enzyme from where DNA binding is thought to occur. It therefore seems unlikely that this region of the tower domain participates directly in the formation of the drug binding pocket. Accordingly, we did not observe a change in the cleavage site specificity of the G269V mutant. One simple model is that the G269V substitution causes drug hypersensitivity simply by increasing the concentration of cleavage complexes available to drug, that is, by shifting the equilibrium of the topoisomerase reaction cycle to favor the intermediate that drug can bind. At a molar enzyme-to-DNA ratio of 1, we find that about 10% of the G269V topoisomerase dimers are participating in cleavage complexes, much higher than with the wild-type enzyme.

Three different mechanisms could conceivably lead to higher levels of cleavage complex. First, the G269V enzyme might have a higher DNA binding affinity than wild-type, although this would presumably lead to a higher specific activity, which we did not observe. Second, once the enzyme is bound to the substrate it could have a faster forward rate of cleavage. Finally, the enzyme might have a reduced rate of religation. Regardless of the specific mechanism, the effective result is an increase in the concentration of cleavage complexes available to inhibitors. In a sense, the G269V substitution works by mimicking the activity of a cleavage-enhancing inhibitor, while at the same time acting synergistically with the inhibitors. Following this logic, we speculate that there might be novel drugs that mimic the activity of the G269V substitution (i.e. drugs that increase DNA cleavage without altering the cleavage site specificity of the enzyme and act synergistically with the classical cleavage-enhancing drugs).

The G269V enzyme responded to all of the drugs in a similar manner, suggesting that these drugs share a similar mechanism of action. If our interpretation of the cause of

hypersensitivity is correct, then these drugs must all act by inhibiting the religation step of the enzyme. If any acted by stimulating the forward rate of cleavage, as suggested for a subset of the drugs acting on eukaryotic type II topoisomerases (*50*), hypersensitivity should not result.

The only other type II topoisomerase reported to have such high DNA cleavage levels is the wild-type enzyme of the *Chlorella* virus PBCV-1, with about 30% of its dimers in a cleavage complex at any given time (*51*). However, unlike G269V, this protein was found to be generally resistant to a number of topoisomerase poisons. A peculiarity of the *Chlorella* PBCV-1 enzyme is that even with such high levels of DNA cleavage, there did not appear to be any multiple cleavage events, suggesting that the enzyme may be highly site specific. Since drug binding sites are composed of both topoisomerase and DNA, a site-specific topoisomerase would perhaps create a drug binding pocket that is specific for a very limited number of inhibitors.

The only other reported mutant in the tower domain that affects drug sensitivity is *top2-5* from *Saccharomyces cerevisiae* (*52*). This temperature sensitive mutant contains a cluster of three substitutions (R884P, R886I, and M887I) in β-strand 9 of the tower domain (Figure 6B). Unlike G269V, the *top2-5* mutant was shown to be resistant to *m*-AMSA and VP-16. However, similar to G269V, the cleavage site specificity of the *top2-5* mutant was not altered (in the presence of these two drugs). Although the authors did not comment on it, the *top2-5* mutant apparently yielded lower levels of drug-independent DNA cleavage (see Figures 5 and 6 in *52*), suggesting that this mutant might cause resistance by reducing the number of cleavage complexes available to drug.

Although other hypersensitive topoisomerase mutants have been reported, none were shown to display increased sensitivity to all inhibitors, like the G269V mutant. As described above, many other mutations cause hypersensitivity to certain drugs but resistance to others. Most of these mutations lie in the CAP-like domain or the Rossmann fold, and likely alter the enzyme portion of the drug binding site. One other mutation, H1012Y of yeast topoisomerase II, is hypersensitive to ellipticine, equally sensitive to *m*-AMSA and resistant to VP-16 and CP-115,953 (*53*), but residue 1012 lies well outside the CAP-like and Rossmann fold domains in the C-terminal portion close to the dimer interface (purple domain in Figure 6A).

Another yeast topoisomerase II mutant, T744P, showed increased sensitivity to *m*-AMSA, mitoxantrone, some quinolones and fluoroquinolones, but not to VP-16 (*54*; the ellipticines and anthracyclines, which also target the yeast enzyme, were not tested). Yeast residue T744 resides in the CAP-like domain in the region where many drug-resistance mutations have been isolated (*47*). The authors suggested that the altered drug sensitivity of T744P results from a change in the cleavage/religation equilibrium (*54*). However, the mutation did not cause increased levels of drug-independent DNA cleavage as would be expected for such a change, and so the interpretation of this mutant remains uncertain.

The G269V substitution was originally isolated in combination with S79F in a selection for drug-resistant mutants (*22, 31*). S79F by itself causes a topoisomerase negative phenotype in vivo, which is rescued by the G269V substitution. Comparisons with the yeast enzyme place the S79 residue in the CAP-like domain of the protein, far from the G269V substitution in the tower domain. What is the nature of the defect of the

S79F mutant and how does G269V suppress this defect? Also, why is the drug-resistance phenotype dominant to the hypersensitivity phenotype in the double mutant? We are currently trying to answer these questions.

In summary, we have described a T4 topoisomerase mutant that is hypersensitive to multiple classes of type II topoisomerase poisons. This mutation is located in a region of the protein that has not been previously implicated in drug or DNA binding. The G269V substitution increases the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle. We believe that this is the first type II topoisomerase mutant described that alters drug sensitivity by altering the equilibrium of the enzyme. Analysis of this and similar mutants provides a unique perspective on the mechanism of action of topoisomerase inhibitors.

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Table 1: Drug sensitivities of the wild-type and G269Vmutant enzymes.

	[drug] for half-maximal cleavage (μM)[a]		
Drug	WT	G269V	HS factor[b]
m-AMSA	2.2	0.75	2.9
Ellipticine	1.3	0.4	3.3
2-me-9-OH-E$^+$	0.3	0.19	1.6
VP-16	29.2	11.1	2.6
VM-26	27	13.3	2.0
Mitoxantrone	0.052	0.034	1.5
Oxolinic Acid	255	85	3.0

[a]Derived from Figures 3 and 4.

[b]Calculated by dividing the wild-type (WT) half-maximal [drug] by the G269V half-maximal [drug].

FIGURE LEGENDS

FIGURE 1: DNA cleavage assay with negatively supercoiled pBR322 DNA. Drug-independent DNA cleavage levels of the wild-type and G269V mutant enzyme were compared in a protein titration. Reactions contained 300 ng (107 fmoles) of DNA and the indicated amounts of protein. The asterisks indicate 96 fmoles of enzyme, for a molar enzyme dimer-to-DNA ratio of 0.9. A size scale (*kb*, kilobases), generated from the migration of T4dC *Xba*I restriction fragments, appears in the right hand margin, and different forms of DNA are indicated in the left hand margin. N, nicked DNA; L, linear DNA; SC, supercoiled DNA; R, relaxed DNA; Ø a control reaction with no enzyme.

FIGURE 2: DNA cleavage assay with negatively supercoiled pBR322 DNA in the presence of *m*-AMSA. The *m*-AMSA sensitivities of the wild-type and G269V enzymes were compared in a drug titration. Reactions contained 25 ng of protein (96 fmoles), 300 ng of DNA (107 fmoles) and the indicated concentrations of *m*-AMSA (μM, micromolar). The size scale and abbreviations are the same as described in Figure 1 except that Ø denotes a control reaction with no enzyme and no drug.

FIGURE 3: Quantitation of DNA cleavage in the presence of *m*-AMSA. A nitrocellulose-filter binding assay similar to that of Wong and Lohman (*37*) was used to assess the sensitivities of the wild-type and mutant enzymes to *m*-AMSA. Cleavage reactions

contained a small amount (≈1-2 ng) of P^{33} end-labeled pBR322 DNA, 100 ng of unlabeled linear pBR322 DNA, 9.2 ng of either wild-type or mutant enzymes (35 fmoles; molar enzyme dimer-to-DNA ratio of 1), and the indicated concentrations of *m*-AMSA. The data represent an average of three independent experiments. Percent cleavage has been corrected for drug-independent cleavage levels and for multiple cleavage events using the Poisson distribution (see Material and Methods). o, G269V; ×, wild-type.

FIGURE 4: Quantitation of DNA cleavage in the presence of six different inhibitors. Each of the six drugs was tested as described in the legend to Figure 3. o, G269V; ×, wild-type.

FIGURE 5: DNA cleavage patterns in the presence of different inhibitors. Reactions contained a small amount (≈2 ng) of end-labeled pBR322, 100 ng of unlabeled linear pBR322 DNA (35 fmoles), and the following amounts of topoisomerase: none (lane 1); increasing amounts (ng) indicated at top of gel (lanes 2-8); 10 ng (38.5 fmoles; lanes 9-22); 100 ng (385 fmoles; lanes 23 and 24). The inhibitor concentrations were as follows: *m*-AMSA, 4 μM; ellipticine, 40 μM; 2-me-9-OH-E^+, 2.5 μM; VP-16, 160 μM; VM-26, 200 μM; mitoxantrone, 0.32 μM; and oxolinic acid, 500 μM. The intact DNA substrate and topoisomerase-mediated cleavage products were resolved by 6% PAGE and visualized with a phosphorimager (Molecular Dynamics). A size scale (*bp,* base pairs), generated from the migration of pBR322 restriction fragments, appears in the right hand

margin. Ø, no enzyme and no drug; W, 10 ng wild-type enzyme; G, 10 ng G269V enzyme; W', 100 ng of wild-type enzyme; G', 100 ng of G269V enzyme.

FIGURE 6: Structural context of the G269V substitution. A ribbon diagram of a large fragment of yeast topoisomerase II is shown in (A). This figure was generated with the program RIBBONS (*55*). One protomer is colored and the domains discussed in the text are as follows: CAP-like domain, dark blue; Rossmann fold, red; tower domain, green; and the C-terminal domain that participates in dimerization, purple. The active site tyrosine is a part of the CAP-like domain and both tyrosines are shown in black. Secondary structure overlays of the tower domain from three topoisomerase proteins are shown in (B). The β-strands are indicated by arrows while the α-helices are shown as bars. The TopoII and GyrA alignments and structural nomenclature are based on published three-dimensional structures and alignments (*39*, *41*). The T4 gp52 structural elements were predicted using the PHDsec program (*56*, *57*). The tower domain mutants discussed in the text are underlined and bold.

Figure 1

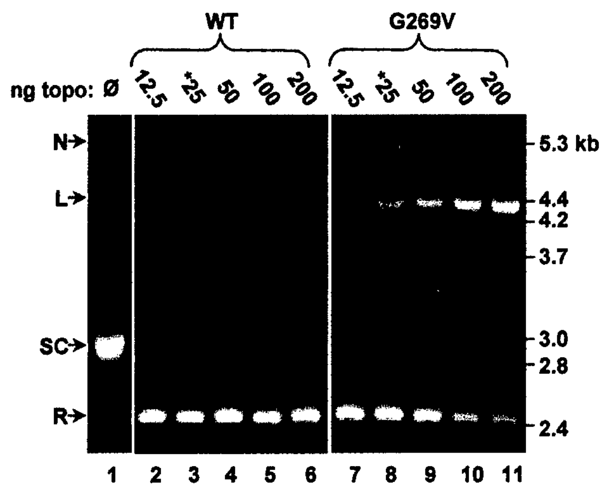

Figure 2:

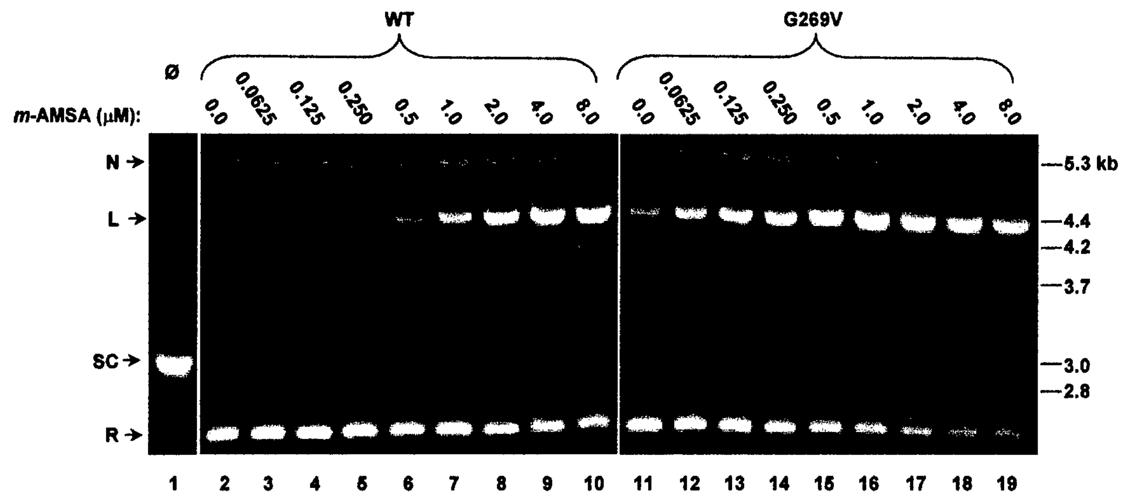

Figure 3:

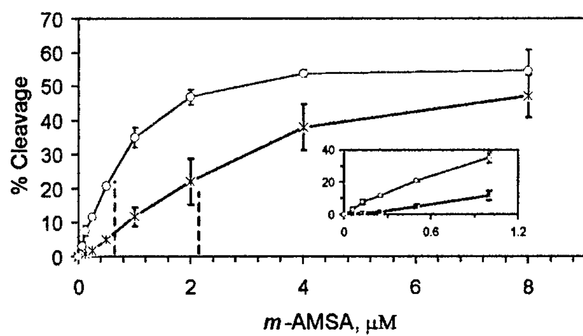

Figure 4:

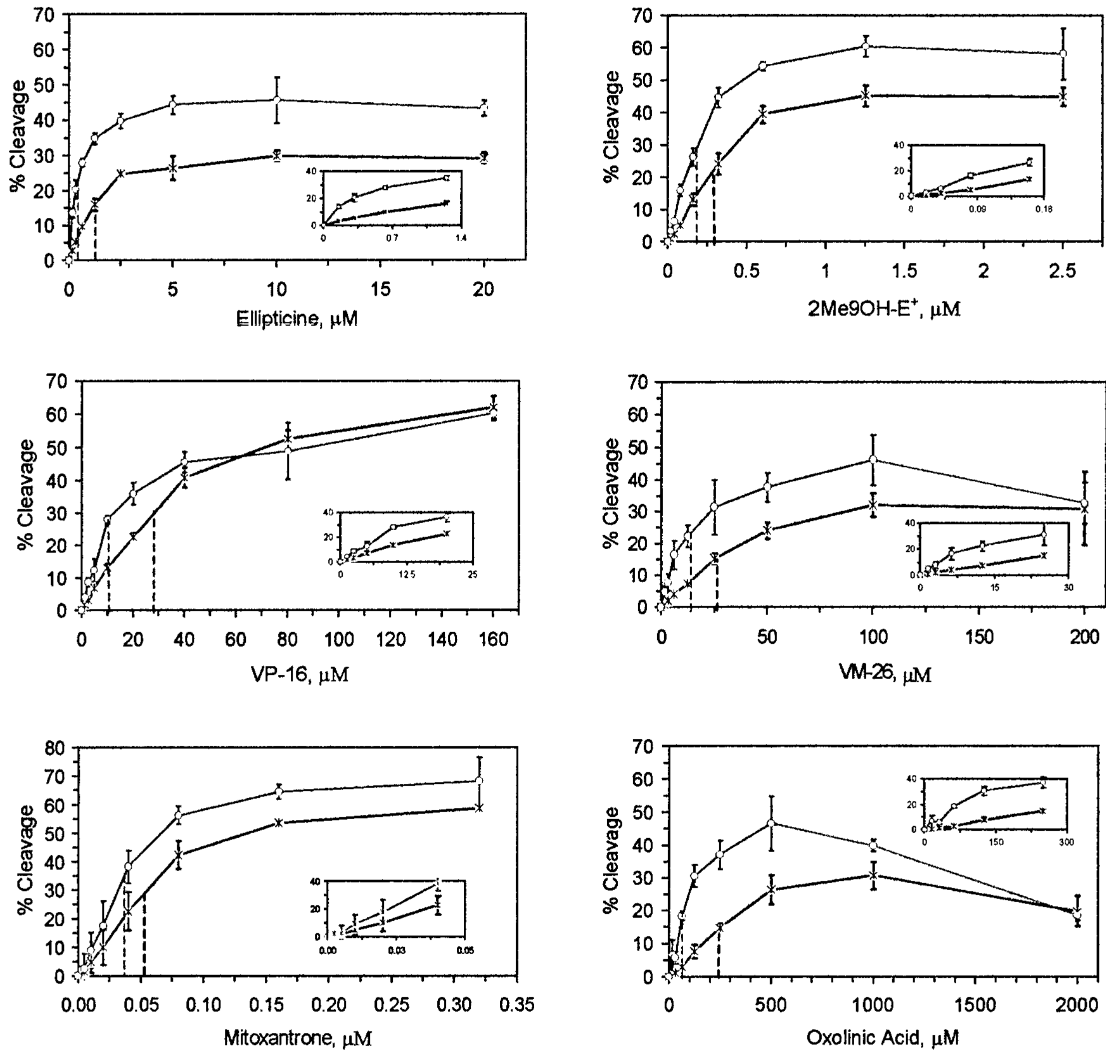

Figure 5:

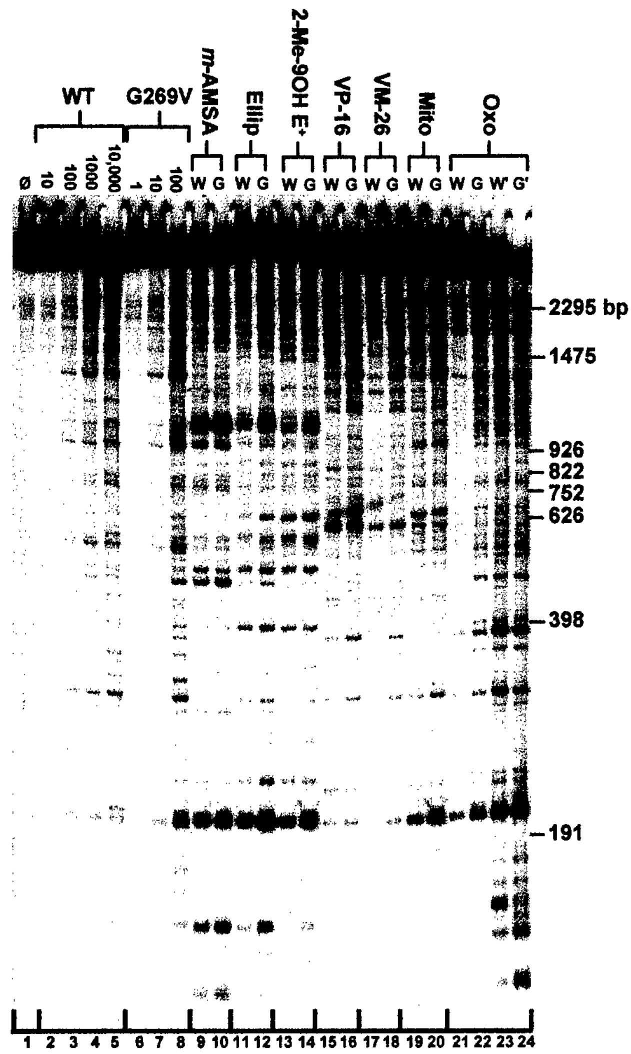

Figure 6: