AD_____

Award Number: DAMD17-00-1-0235

TITLE: A Unique Class of Topoisomerase Mutants that are Hypersensitive to Multiple Antitumor Angents

PRINCIPAL INVESTIGATOR: Erin K. O'Reilly, Ph.D.
Kenneth Kreuzer, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: March 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2001	Annual Summary (1 Mar 00 - 28 Feb 01)

4. TITLE AND SUBTITLE
A Unique Class of Topoisomerase Mutants that are Hypersensitive to Multiple Antitumor Agents

5. FUNDING NUMBERS
DAMD17-00-1-0235

6. AUTHOR(S)
Erin K O'Reilly, Ph.D.
Kenneth Kreuzer, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-Mail: oreilly@biochem.duke.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The overall goal of this research is to understand the detailed mechanism of action of antitumor drugs that target type II topoisomerases. Bacteriophage T4 topoisomerase provides a useful model system for the study of these antitumor agents. Previous analysis of drug resistant mutations in the T4 topoisomerase has fortuitously led to the discovery of a hypersensitive mutant (*Cancer Res.* **58**:1260-1267). I have purified this mutant enzyme and analyzed its drug sensitivity with DNA cleavage assays. As was expected from the previous *in vivo* data, the mutant enzyme was hypersensitive to *m*-AMSA and oxolinic acid. Additionally, the mutant was hypersensitive to a number of other cleavage-inducing inhibitors that cannot be tested *in vivo* such as VP-16, ellipticine, and mitoxantrone. I am currently using a modified filtration method to quantitate the levels of drug hypersensitivity. Surprisingly, in the absence of any inhibitors, the mutant enzyme appears to remain in the cleavage complex for a longer period of time compared to the wild type enzyme. Further, this mutation does not appear to alter the cleavage site specificity of the enzyme. Thus, this mutant seems to cause hypersensitivity in a unique manner by increasing the frequency of cleavage complexes available to inhibitors.

14. SUBJECT TERMS
drug resistance, drug hypersensitivity, antitumor agents, type II, topoisomerase, Bacteriophage T4

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The goal of this research is to understand the detailed mechanism of action of antitumor agents that target type II topoisomerases. Some of the most widely used chemotherapy drugs target type II topoisomerases including 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 5, 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 mutations are present, the G269V mutation is thought to suppress a topoisomerase negative phenotype caused by the S79F mutation alone. However, when substituted into a clean background, the G269V substitution by itself causes dramatic *in vivo* hypersensitivity (2). This result was unexpected as the G269V mutation is located in a domain of the topoisomerase that was not thought to play a role in drug sensitivity, namely, the tower domain (see 5, p.8). This is the only mutation in the tower domain of any type II topoisomerase known to affect drug sensitivity. It is unclear how a mutation in this region causes hypersensitivity. It is also unclear how this mutation causes suppression of the S79F mutation. We wondered if this mutant defined a new class of mutants that are generally hypersensitive to all drugs. We believe that the biochemical analysis of this mutant and additional hypersensitive mutants will greatly enhance our understanding of this novel mechanism of drug sensitivity and may provide us with new insights into the mechanism of antitumor drug action.

Statement of work issues. I have nearly completed Task 1 from my statement of work in which I proposed to biochemically analyze the G269V mutant (see 5, p.5). Thus far, I have purified the mutant enzyme and performed cleavage assays with it and the wild type enzyme in the presence and absence of drug. A detailed summary of the results from these experiments is outlined below.

The remaining portion of Task 1 involves quantitating the levels of drug sensitivity. This has proved to be more difficult than expected and has extended the amount of time required for completion of Task 1. The main problem with quantitation was finding a method of quantitation that was suitable and reproducible in my system. Although the simple quantitation of bands from cleavage assays (such as those shown in Figures 1, 2 and 4) can be informative, it is not very quantitative. I did attempt a previously published method of quantitating topoisomerase sensitivities, namely the

SDS/K^+ precipitation method (4). I found this method to be cumbersome and the results were quite variable. Therefore, I have taken a previously published filtration method used in studying protein-DNA interactions (6) and modified it for use in quantitating topoisomerase drug sensitivities. Unlike the other methods, this procedure is fast and simple and the results are reproducible. Appendix I outlines this procedure in detail and some preliminary results obtained utilizing this method are summarized below.

Detailed summary of data. One possible reason for the observed increase in drug sensitivity would be that the G269V enzyme remains in the cleavage-complex intermediate for a longer period of time when compared to the wild-type enzyme. This would make the G269V enzyme susceptible to drug for an extended period of time during the reaction cycle, and should allow us to observe cleavage in the absence of drug. In order to test for this, I crossed the G269V mutation into the T4 overproduction strain (see proposal) and purified the mutant enzyme. Indeed, I easily detected drug-independent cleavage with the G269V enzyme with much lower concentrations of protein than were required to observe the same amount of cleavage with the wild type protein (Figure 1, compare lane 6 to lane 7). In these experiments, the accumulation of linear products represents topoisomerase cleavage complexes that have been trapped on the DNA when the reaction is stopped with SDS. Based on this result, I expected the G269V mutant to be equally hypersensitive to all inhibitors.

To begin looking at the drug sensitivity spectrum of the mutant enzyme, I performed cleavage assays in the presence of different topoisomerase II targeting drugs. Figure 2 shows one such cleavage assay that has been performed in the presence of VM-26. Clearly large numbers of cleavage complexes are being formed with the G269V mutant enzyme at lower drug concentrations than is required for similar accumulations by the wild type enzyme (Figure 2 compare lanes 9 to lane 15). Using this method I have determined that G269V is hypersensitive to each of the drugs tested: m-AMSA, oxolinic acid, ellipticine, 2-Me-9-OH-ellipticine, VM-26, VP-16, and mitoxantrone.

In order to analyze drug hypersensitivity in a more quantitative way I have begun using the modified filter assay described in Appendix I. This type of quantitation will allow me to assign apparent K_i values for every drug/protein combination tested. For example, preliminary experiments indicate that the wild type enzyme requires three times more *m*-AMSA for half maximal cleavage when compared to the G269V mutant (K_i^{WT}= 1.5μM, K_i^{G269V}= 0.5μM, see Figure 3). Likewise, the wild type enzyme requires twice as much VP-16 than G269V for half maximal cleavage (K_i^{WT}= 9μM, K_i^{G269V}= 4μM, data not shown). This type of quantitation will allow me to biochemically define drug sensitivities and will be very useful in the future characterization of additional mutants.

Interestingly, cleavage by the G269V mutant enzyme appears to be inhibited at high levels of *m*-AMSA (Figure 3). This inhibition seems to be reproducible and may be somehow related to the altered drug sensitivity of this mutant. Oddly, I do not see this inhibition with all of the drugs. For example, there appears to be no inhibition at high levels of VP-16 (up to 160μM). However, it is possible that I will see mutant-specific inhibition with higher levels of VP-16. I plan to further explore this mutant-specific inhibition at high drug levels with the hope that it may tell us more about drug hypersensitivity.

It seemed possible that in addition to cleaving in the absence of inhibitors, the mutant enzyme might have altered cleavage site preferences in the presence of different

drugs when compared to the wild-type enzyme. This result would support the hypothesis that, in addition to stabilizing the cleavage complex, the mutation also alters the drug-binding pocket. To address this possibility, I have performed cleavage assays with end-labeled linear substrates. This procedure allows me to observe the cleavage patterns of the mutant enzyme compared to that of the wild type. As can be seen in Figure 4, the mutant enzyme predictably cleaves more readily in the presence of *m*-AMSA. However, the cleavage site preference of the mutant enzyme is not significantly altered when compared to that of the wild-type enzyme. I obtained similar results with both VP-16 and ellipticine. I also looked at the DNA site specificity in the absence of any drug and found again that the mutant enzyme cleavage pattern did not differ than that of the wild type (see Figure 4 and data not shown). These results suggest that unlike many previously studied drug-resistant mutants, the G269V mutation alters drug sensitivity in a unique manner without altering the drug binding pocket. However, I will need to confirm this by comparing the cleavage patterns in the presence of additional drugs.

Key Research Accomplishments

- 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 the purification process.
  - The specific activity of the G269V mutant enzyme was essentially identical to that of the wild type enzyme (2×10^6 U/mg and 3×10^6 U/mg respectively).
  - The G269V mutant enzyme seems to remain in the cleavage complex for a longer period of time than 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 (see Appendix I).
- In the process of quantitating the levels of drug hypersensitivity using the modified filter-binding assay.

Reportable Outcomes

Presentation: 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.

Conclusions

The G269V mutant appears to be very different than any previously identified topoisomerase mutant. For one thing, it is the only mutation in the tower domain of the protein known to affect drug sensitivity. Further, unlike other mutants, the G269V mutation causes hypersensitivity to a broad range of topoisomerase inhibitors. A probable reason for this general hypersensitivity phenotype is that this mutant appears to remain in the cleavage complex for a longer period of time than the wild type enzyme. Additionally, unlike previously isolated mutants, this mutant does not appear to have an altered drug-binding pocket. Thus, the G269V topoisomerase does seem to represent a unique class of topoisomerase mutants. As has been outlined in my proposal, I plan to isolate additional hypersensitive (and possibly drug resistant) mutants in this region of the protein. This will allow me to test the generality of the G269V mode of action and may offer new insights into the mechanism of drug action.

References

1. Beck, W.T., Danks, M.K., Wolverton, J.S., Chen, M., Granzen, B., Kim, R., & D.P. Suttle. (1994). Resistance of Mammalian Tumor Cells to Inhibitors of DNA Topoisomerase II. *Advances in Pharmacology* **29B**, 145-169.

2. Freudenreich, C.H., & Chang, C., & Kreuzer K.N. (1998). Mutations of the bacteriophage T4 type II DNA topoisomerase that alter sensitivity to antitumor agent 4'-(9-acridinylamino)methanesulfon-*m*-aniside and an antibacterial quinolone. *Cancer Res.* **58**, 1260-1267.

3. Hande, K.R. (1998). Clinical applications of anticancer drugs targeted to topoisomerase II. *Biochimica et Biophysica Acta* **1400**, 173-184.

4. Jannatipour, M., Liu, Y., & Nitiss, J.L. (1993). The top2-5 mutant of yeast topoisomerase II encodes an enzyme resistant to etoposide and amsacrine. *J. Biol. Chem.* **268**, 18586-18592.

5. 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.

6. Wong, I., & Lohman, T.M. (1993). A double-filter method for nitrocellulose-filter binding: Application to protein-nucleic acid interactions. *PNAS* USA **9**, 5428-5432.

Figure 1.

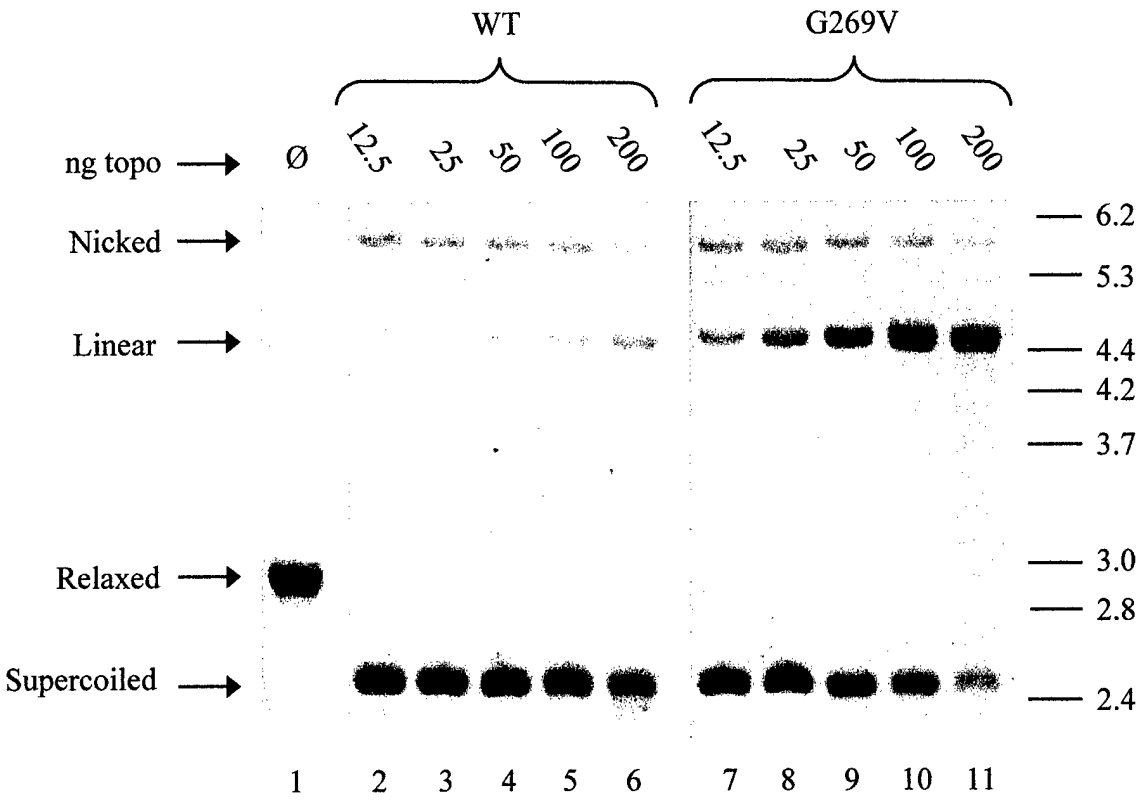


Figure 1. DNA relaxation activity of wild-type and mutant topoisomerase enzymes. Reaction mixtures of 20 µl contained 40 mM Tris-HCL (pH 7.8), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, nuclease-free bovine serum albumin (30 µg/ml), 300 ng of negatively supercoiled pBR322, and the indicated amounts of topoisomerase. 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% (wt/vol) SDS, 20% (wt/vol) 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 Na_2EDTA) overnight at 2 V/cm followed by visualization with UV illumination. T4 *XbaI*, a size scale (in kb) generated from T4 *XbaI* fragments; Ø, no enzyme and no drug, the different forms of DNA are indicated to the left of the figure.

Figure 2.

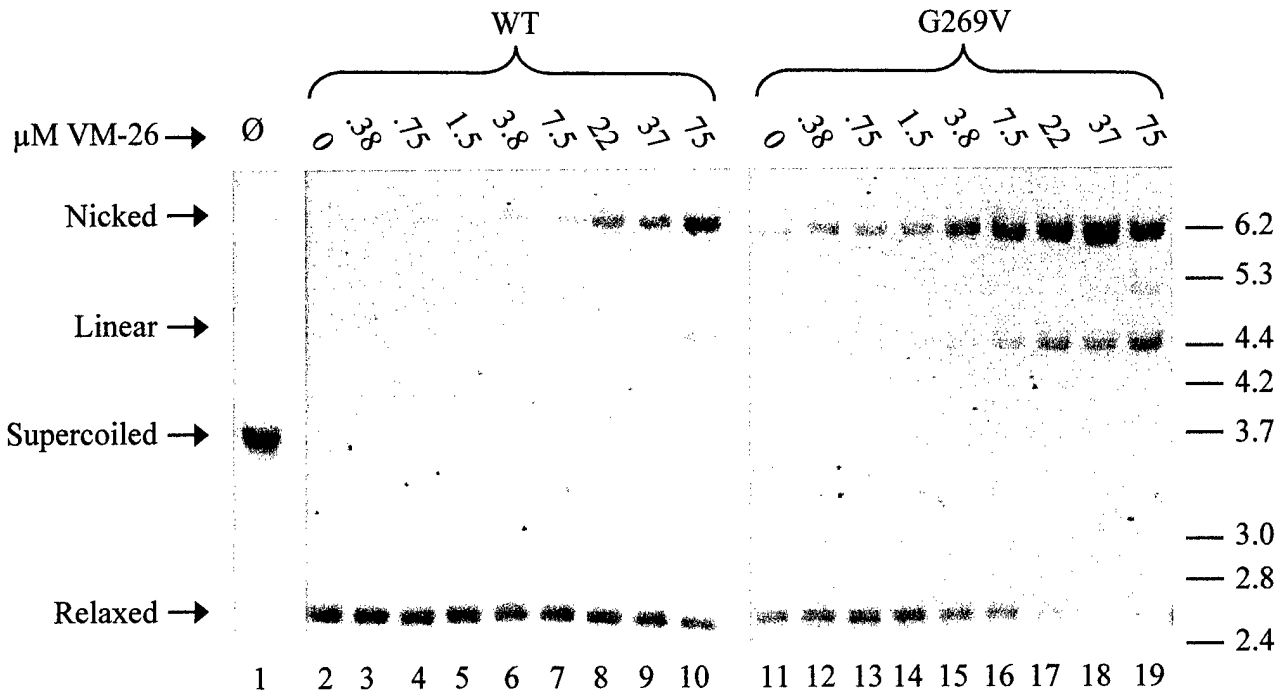


Figure 2. DNA cleavage assays in the presence of VM-26. Reaction mixtures were identical to those in Figure 1 accept that VM-26 is present at the indicated concentrations. Also, 72 ng of the indicated topoisomerase was used in these reactions for a molar ratio of 2.5 topoisomerase dimers to 1 molecule of pBR322.

Figure 3.

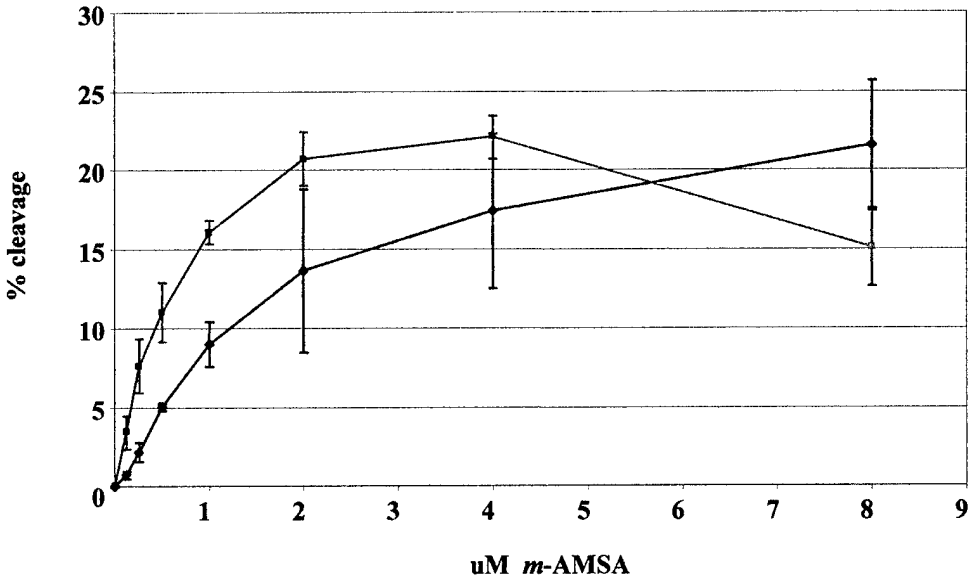


Figure 3. Quantitation of DNA cleavage in the presence of *m*-AMSA. A small amount (≈1-2 ng) of P^{33} uniquely end-labeled pBR322 linear DNA was mixed with 100 ng of similarly digested unlabeled DNA and incubated with 4.6 ng of either wild-type or mutant enzymes (final molar ratio of 0.5 topoisomerase dimers to 1 DNA molecule). Buffer conditions were identical to those described in Figure 1. The procedure was performed as discussed in Appendix I. blue line (diamonds) = WT; green line (squares) = G269V

Figure 4.

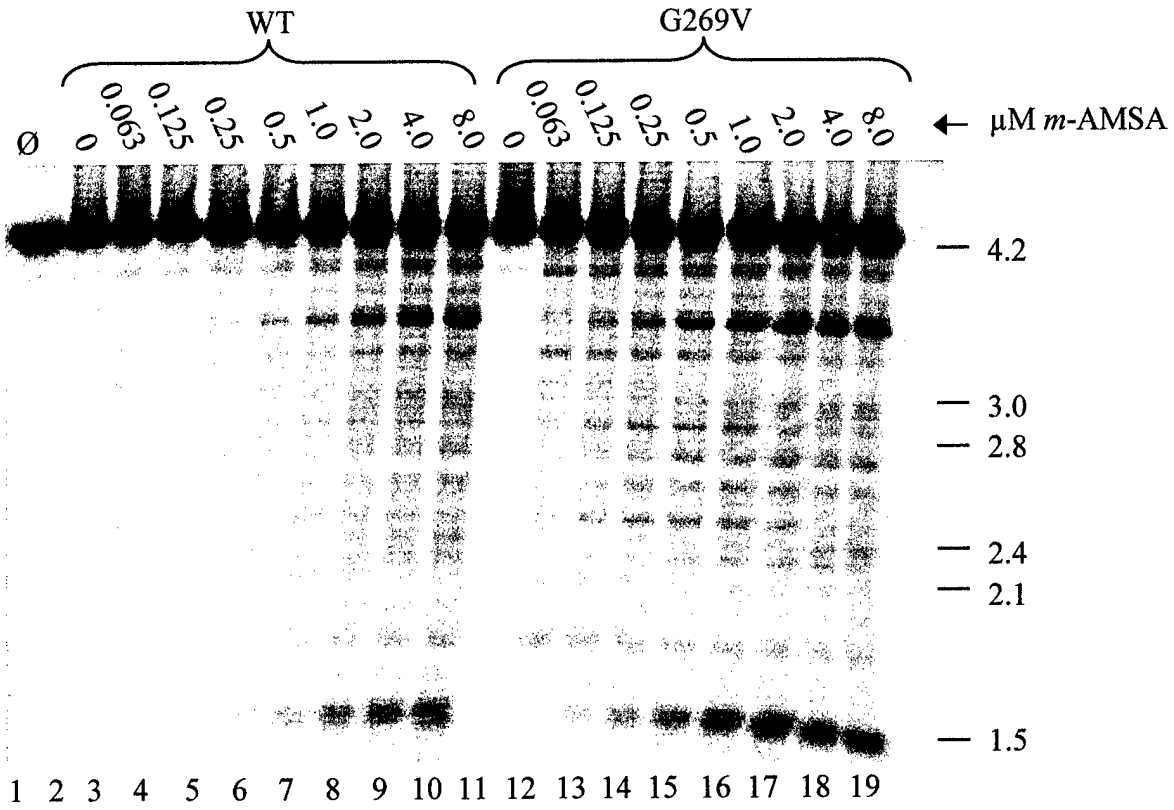
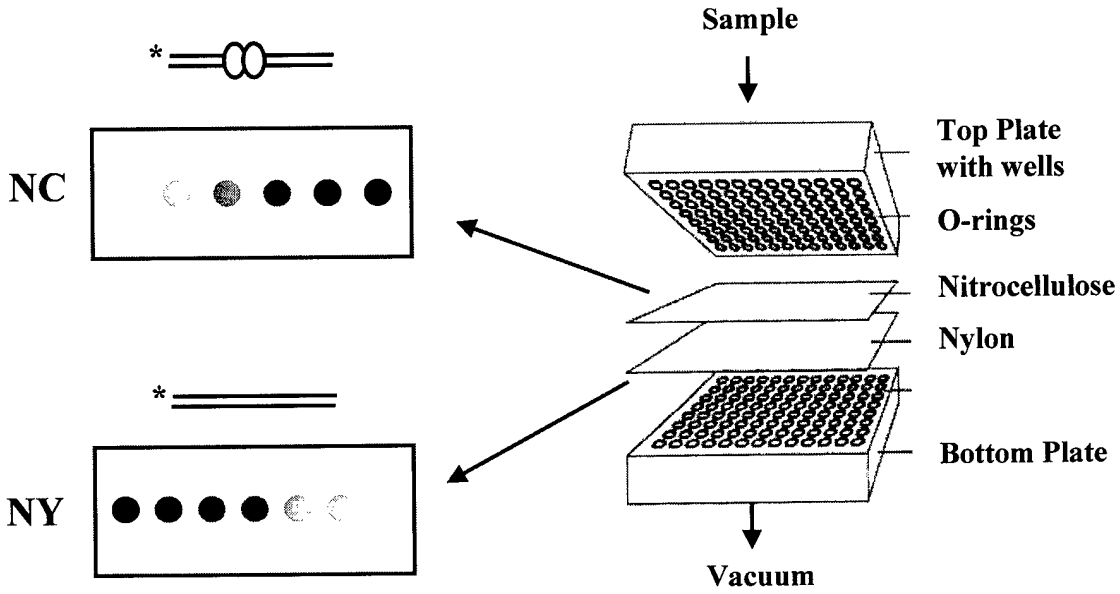


Figure 4. DNA cleavage assays of wild-type and mutant topoisomerases. A small amount (≈1-2 ng) of P^{33} uniquely end-labeled pBR322 linear DNA was mixed with 100 ng of similarly digested unlabeled DNA and incubated with 4.6 ng of either wild-type or mutant enzymes (final molar ratio of 0.5 topoisomerase dimers to 1 DNA molecule). Other buffer conditions were identical to those described in Figure 1. Amounts of *m*-AMSA are indicated above each lane. After digestion with proteinase K, the intact substrate and topoisomerase-mediated cleavage products were resolved by agarose gel electrophoresis and then visualized by autoradiography. A size scale (in kb) generated from the migration of T4 *XbaI* fragments appears to the right of the gel. Ø, no enzyme and no drug. In a longer exposure, the cleavage patterns of the wild-type and G269V enzymes did not significantly differ in the absence of drug (0 μg/mL, lanes 2 and 11).

Filtration Method for Measuring Topo DNA Cleavage

Principle: Protein-associated DNA (covalently linked in this case) binds to a nitrocellulose membrane while free DNA binds to a nylon membrane. Results can be quantitated on the Phosphorimager.



Calculation: [NC/(NC+NY)] * 100 = %NC Bound = %Cleavage

Materials:

- Schleicher and Schuell Minifold I- from VWR #28153-405
- Millipore Pump- #XX55 000 00, 115V, 60Hz
- S&S Protran BA85 4X5 ¼ inch - **Nitrocellulose membrane (NC)** (Comes Precut) from VWR 28151-760 20/pk @ $63.32/pk (includes discount)
- Genescreen Plus Hybridization Transfer Membrane- **Nylon membrane (NY)** Perkin Elmer-NEN NEF-994, 8.5 X 12.4cm, pk of 20 @ $91.00. While dry, cut off corners to fit Minifold using NC filter as guide.
- S&S Gel Blot Paper GB002 (S&S #39690) -VWR 74330-100 @ $15.72/pk. While dry, cut off corners to fit Minifold using NC filter as guide.
- Filtration Buffer: 1X = 50mM TrisHCl, pH 7.8, 200mM KCl, 10mM $MgCl_2$, 0.5mM EDTA

Reference: "A double-filter method for nitrocellulose-filter binding: Application to protein-nucleic acid interactions", Isaac Wong and Timothy M. Lohman, Proc. Natl. Acad. Sci, USA 9: 5428-5432 (1993)

Procedure:

1) Set up reactions using labeled DNA (10,000 cpm P^{33} total is sufficient) and stop with SDS to denature the topoisomerase (0.2% final is sufficient). (Do not use proteinase K to degrade the protein.)

2) Dilute samples to 100 µl final volumes using 5X Filtration Buffer (FB) and water to give 1X FB final concentration. Less that 100µl is fine but harder to load. Maximum volume approximately 400 µl. The SDS present in the sample does not inhibit protein binding to the NC filter. However, higher SDS concentrations have not been tried.

3) Label and date the nitrocellulose (NC) and nylon (NY) filters. Soak the membranes for at least 30 min in 1X FB prior to use. Immediately prior to assembling the Minifold, wet 1 sheet of the gel blotting paper. Cut the corners off before wetting the gel blotting paper.

4) Assemble Minifold with the gel blotting paper on bottom, then the nylon membrane, followed by the nitrocellulose membrane. Do not clamp Minifold too tightly or the middle wells will leak.

5) Place pump on cart. (Vibration of lab bench will cause samples to smear.)

6) Mark Minifold with tape and label to help in discerning desired wells during loading.

7) Apply one row at a time. Under vacuum prewash the first row of wells with 400 µl of 1X FB for each well. Pump should be between 500-600 mm Hg. Let all wells clear of liquid.

8) Turn off and bleed pump and load 100 µl samples into the prewashed wells. Turn vacuum on and allow wells to clear.

9) With pump still on, wash loaded wells twice with 400 µl of 1X FB allowing wells to clear between washes.

10) Repeat steps 7-9 until all rows have been loaded and washed.

11) After clearing of final wash, disassemble the minifold. Blot both NC and NY membranes gingerly between two sheets of gel blotting paper. Check each filter for counts. Check effluent for counts.

12) Wrap NC and NY filters in Saran wrap and expose to Phosphorimager screen. 10,000 cpm of P^{33} is sufficient to give a good signal. Higher numbers of counts are fine but may contaminate the Minifold.

13) Rinse Minifold with warm water and let dry on bench. Check gaskets for contamination and remove with Isoclean if necessary.