Mutations of the Bacteriophage T4 Type II DNA Topoisomerase That Alter Sensitivity to Antitumor Agent 4'- (9-Acridinylamino)methanesulfon-
m-anisidide and an Antibacterial Quinolone

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ABSTRACT

Various antitumor and antibacterial agents target type II DNA topoisomerases, stabilizing a cleaved DNA reaction intermediate and thereby converting topoisomerase into a cellular poison. Two 4'- (9-acridinylamino)methanesulfon-m-anisidide (m-AMSA)-resistant bacteriophage T4 topoisomerases have previously been characterized biochemically, and we have now determined the sequence of the causative mutations. In one case, a mutation (E457K) in a conserved domain of gp39 (ATPase subunit) causes resistance to antitumor agent m-AMSA but hypersensitivity to the quinolone oxolinic acid. In the second case, a combination of two amino acid substitutions (S79F and G269V) in gp52 (DNA-cleaving subunit) causes resistance to both m-AMSA and oxolinic acid. The S79F mutation is responsible for drug resistance, whereas the G269V mutation suppresses a topoisomerase deficiency caused by S79F. Surprisingly, the G269V mutation by itself causes a dramatic hypersensitivity to both inhibitors, defining a new class of topoisomerase mutants. Because S79 and the adjacent N78 are homologous to two key residues of DNA gyrase that affect quinolone sensitivity, we generated additional amino acid substitutions at these two positions. The substitutions alter sensitivity to m-AMSA and to oxolinic acid, sometimes in opposite directions. Furthermore, the quinolone sensitivities of the various mutants paralleled those of corresponding gyrase mutants. These results support models in which both quinolones and antitumor agents bind to a conserved site that overlaps the active site of the enzyme.

INTRODUCTION

Type II DNA topoisomerases catalyze DNA interconversions by making a double-strand break in one segment of DNA, passing another segment through the break, and religating the cleaved DNA (reviewed in Refs. 1–3). A key intermediate in these reactions, the cleavage complex, consists of topoisomerase covalently attached via phosphotyrosine bonds to the 5' phosphates of the broken DNA. The cleavage complex is stabilized by numerous antitumor and antibacterial agents that inhibit type II topoisomerases. Cytotoxicity of these chemotherapeutic agents is critically dependent on the stabilized cleavage complex, indicating that these agents convert a normally beneficial cellular enzyme into a destructive poison (4–6).

Recent results strongly suggest that the topoisomerase inhibitors stabilize the cleavage complex by binding to DNA at the active site of the enzyme. For example, the identity of the base pair that immediately flanks the scissile phosphodiester bond was shown to determine which chemical families of inhibitors can stabilize a cleavage complex at a particular sequence (7, 8). More directly, a photoactivatable analogue of the antitumor agent m-AMSA2 was cross-linked to the bp adjacent to the scissile bond within a cleavage complex, but cross-linking was not detected to other bp within the substrate (9).

Although the above results indicate that the inhibitors are in close contact with the DNA, the enzyme also clearly plays an important role in drug binding: (a) in the cross-linking study just mentioned, the m-AMSA derivative reacted with DNA only when topoisomerase was present; (b) a direct analysis using radiolabeled quinolone demonstrated drug binding to a gyrase-DNA complex but not to either gyrase or DNA alone (10, 11); (c) the spectrum of sensitivities to particular classes of topoisomerase inhibitors is species dependent (e.g., quinolones preferentially inhibit bacterial DNA gyrase whereas various antitumor agents inhibit the human topoisomerase); and (d) mutational alteration of a type II topoisomerase can lead to drug resistance. In Escherichia coli DNA gyrase, a high level of resistance is conferred by amino acid substitutions in the "quinolone-resistance determining region" of the GyrA subunit (residues 81–87, particularly S83; Refs. 12 and 13). Antitumor drug resistance mutations have also been analyzed in eukaryotic topoisomerases, and some are within or very near this same conserved region (see "Discussion").

The bacteriophage T4 type II topoisomerase is sensitive to many of the same antitumor agents that inhibit the mammalian enzyme, including m-AMSA, ellipticines, mitoxantrone, and the epipodophyllotoxin VP-16 (14, 15). At least in the case of m-AMSA, the topoisomerase is the physiological target in this model system because a mutation in the topoisomerase is sufficient to provide drug resistance (16, 17). The phage-encoded enzyme is also weakly sensitive to oxolinic acid, a quinolone that inhibits bacterial DNA gyrase (18, 19). The facile genetics, biochemistry, and molecular biology of the phage T4 system have made this a productive model system for analyzing the mechanism of action of these topoisomerase inhibitors (20).

Two m-AMSA-resistant T4 mutants have been previously isolated and analyzed by genetic mapping. One was shown to carry a mutation in gene 39, which encodes the ATPase subunit of the enzyme (homologous to gyrase subunit B), whereas the other was mapped to gene 52, which encodes the breakage-resolving subunit (homologous to gyrase subunit A; Refs. 16 and 17). Topoisomerase purified from each of the resistant phage mutants exhibited m-AMSA-insensitive DNA relaxation and cleavage activities (16, 17). Furthermore, both purified enzymes demonstrated cross-resistance to other antitumor agents, arguing for a common mechanism of inhibition and overlapping drug-binding sites (15). Interestingly, the gene 39-mutant enzyme displayed hypersensitivity to oxolinic acid and VP-16, suggesting a link between the mechanism of action of antibacterial and antitumor agents (15). This and other results also argue that the drug-resistance mutations subtly alter the drug binding site rather than simply destroying it.

In this communication, we report the precise amino acid substitutions in the two mutant enzymes and demonstrate that these substitutions are sufficient for m-AMSA-resistant phage growth. In addition,

Received 9/16/97; accepted 1/16/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: m-AMSA, 4'- (9-acridinylamino)methanesulfon-m-anisidide; VP-16, etoposide; I/S, insertion/substitution; MDE, mutation detection enhancement.
we generate and analyze additional mutations in the quinolone-resistance
determining region that affect sensitivity to both the antitumor
m-AMSA and the quinolone oxolinic acid. Particularly in con-
junction with recent progress in elucidating the three-dimensional
structure of type II topoisomerases (21, 22), these results provide
important insight into inhibitor-enzyme interactions relevant to both
bacterial and eukaryotic type II topoisomerases.

MATERIALS AND METHODS

Strains. *E. coli* strains MCS1 (supD araD19 3 (ara-lew^thy^, $\Delta$(lac)_Y, _galE
galK rpsL pro hsdR), B_5^B^- (nonsuppressing), and CR63 (supD) are
described by Kreuzer et al. (23). *E. coli* strain AB1157L, which is a derivative
of AB1157 (24), has a transposon insertion in the aceA gene that causes
increased permeability to m-AMSA.

T4 strain 1645 (F′ thr-1 leu25 thi-1
lacY1 supE44 thiA21 gyrAΔ^{43} parC^{K84} Tn10-tet Tn10-kan) is extremely
resistant to quinolones (25) and thus allows visualization of the T4 topoisom-
erase-dependent oxolinic acid sensitivity of a phage T4 infection. *E. coli* NalPv
(supD), which was used to test the topoisomerase-negative phenotype, was
obtained from L. Gold (University of Colorado, Boulder, CO; Ref. 26).

*E. coli* strain T4D (wild-type) was originally from the collection of B. M.
Albers (University of California, San Francisco, CA). T4 39-Δ^K (m-AMSA resistance
mutation in gene 39; otherwise wild type) is described by Huff et al. (16), and
T4 52-Δ^K (m-AMSA resistance mutation in gene 52; otherwise wild type) is
described by Huff et al. (17). T4 strain K10 carries the following mutations:
amB262 (gene 38), amS29 (gene 51), nd28 (gene denA), and rII PT8 (rII-denB
deletion; Ref. 27).

Media and Inhibitors. L broth contained 10 g NaCl, 10 g Bacto-
Tryptone, and 5 g yeast extract. Hershey agar plates contained 13 g Bacto-
Tryptone, 8 g NaCl, 2 g sodium citrate, 1.3 g glucose, and 10 g agar.
m-AMSA (NSC 249992) was provided by the Drug Synthesis and Chemistry
Branch, National Cancer Institute; it was dissolved (50 mg/ml) in DMSO and
diluted in water just prior to use. Oxolinic acid (Sigma Chemical Co.) was
dissolved (10 mg/ml) in 100 mM NaOH and diluted in water just prior to use.

Detection of Mutations by MDE Gels. Four overlapping gene 39 frag-
ments were amplified from either T4D or 39-Δ^K phage by PCR (see Fig. 1).
The PCR products from T4D, 39-Δ^K, or a mixture of the two were purified
over a CL6B column and then subjected to electrophoresis through an MDE
gel (AT Biochem) with 0.6X TBE running buffer (1X = 89 mm Tris base. 89
mm boric acid, and 1.5 mm EDTA) at 20 V/cm for about 25 h.

Introducing Mutations into T4 Phage. Mutations were generated in plas-
mids and then introduced into the phage genome by homologous recombi-
nation using the IS system (27, 28). To introduce the E457K mutation into gene
39 and the G269V mutation into gene 52, an appropriate PCR fragment was
generated with the desired mutation near the center. Each fragment was cloned
into the phage genome by homologous recombina-

RESULTS

Mapping and Reintroduction of the 39-Δ^K Mutation. Genetic
analyses indicated that one mutation conferring m-AMSA resistance
maps within gene 39, which encodes the ATPase subunit of T4
topoisomerase (16). To narrow down the region containing this 39-Δ^K
mutation, we used a high-resolution, nondenaturing MDE gel system.

MDE gels are often able to resolve two duplex DNA fragments that
differ by a single bp and can often separate a DNA homoduplex from
a heteroduplex (wild-type/mutant) containing a single mismatch. PCR
primers were designed to amplify four overlapping fragments that
spanned the length of gene 39 (Fig. 1, top). For each of the four
fragments, the PCR product amplified from wild-type DNA, from the
39-Δ^K mutant phage DNA, and a mixture of the two were subjected
to MDE gel electrophoresis. In addition, a mixture of each pair of
PCR fragments was heat denatured and renatured to look for possible
heteroduplexes with altered migration. None of the four mixtures
produced heteroduplexes with altered migration, and there was
no detectable difference in the migration of wild-type versus
mutant fragment A, B, or C (Fig. 1). However, the wild-type and 39-Δ^K
fragment D showed a clear migration difference, revealing the pres-
ence of a mutation near the 3′ end of the gene (Fig. 1). MDE gels do

\[ ^{a} J. George and K. N. Kreuzer, unpublished observations. \]
not detect all possible mutations, and therefore we cannot conclude that 39-AR fragments A, B, and C are free of mutations.

DNA sequence analysis of PCR fragment D from the 39-AR mutant phage revealed a single mutation within the gene 39 coding region. An A-to-G transition at nucleotide position 1369 would substitute lysine for glutamic acid-457 (E457K) in gp39 of the mutant phage. This amino acid substitution should cause a net +2 charge alteration in gp39, consistent with previous biochemical characterization of the purified enzyme (16).

To determine whether the E457K substitution is responsible for the m-AMSA resistance phenotype of the 39-AR phage, the mutation was substituted back into a clean genetic background using the I/S system, essentially as described above for the E457K mutation in gene 39. In addition, we introduced other potentially informative amino acid substitutions at positions 78 and 79 (equivalent to E. coli GyrA positions 83 and 84). The genome substitutions are described in detail in “Materials and Methods.”

The phenotypes of the mutant phage strains were determined by growth on gradient plates containing an appropriate bacterial host and either no drug or increasing concentrations of m-AMSA or the quinolone oxolinic acid (Fig. 3). Equal volumes of a dilute phage suspension were spotted in six drops across the gradient plate, and growth was allowed overnight at 37°C. The bacterial host for the tests of m-AMSA sensitivity was an acrA mutant, which apparently accumulates much higher intracellular levels of m-AMSA due to a defect in a multidrug efflux pump (31, 32). The host for oxolinic acid testing was the bacterial strain 1643, which contains quinolone-resistant mutations in both GyrA (S83L) and ParC (E84K; ParC is the GyrA homologue of bacterial topoisomerase IV; Refs. 25 and 33). The oxolinic acid sensitivity of phage strains could be determined with this very quinolone-resistant bacterial strain because the bacterial lawn was unaffected by high levels of the quinolone.

**Fig. 1. Localization of the 39-AR mutation.** As indicated in the gene diagram at the top, four PCR fragments of gene 39 were amplified from the wild-type and 39-AR phage. MDE gels of each fragment are labeled with the fragment name (A–D). In each case, Lane 1 contains the wild-type fragment, Lane 2 contains a mix of wild-type and 39-AR fragments, Lane 3 contains a heat-denatured/renatured mix of the two fragments, and Lane 4 contains the 39-AR fragment. Left, size markers (low molecular weight biomarkers from Bio Ventures). A photograph of the ethidium bromide-stained gel is shown.

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*In addition, an error in the published sequence (48) was detected. The reported C at position 1539 was absent in both the mutant and wild-type fragment D, and therefore the COOH terminus of gp39 should be RKNL rather than RKKSIMT. Additional sequence changes in other parts of gene 39 are incorporated in the revised T4 sequence database (E. Kutter, personal communication).*
The phage strains with mutations at position 78 of gp52 will be discussed first. One mutation replaced the asparagine with serine (N78S), thereby mimicking the bacterial GyrA residue (serine 83) known to be important in quinolone sensitivity. In addition, a tryptophan substitution (N78W) was generated because the bacterial GyrA substitution S83W leads to a high level of quinolone resistance. Finally, a cysteine substitution (N78C), which has no precedent in the literature, was easily obtained due to the method used in the construction.

Growth of the wild-type control phage was strongly inhibited, but not abolished, at the high concentration end of the m-AMSA (1 μg/ml) gradient plate (Fig. 3A). All three N78 mutants were hypersensitive to m-AMSA, with N78W being the most sensitive (Fig. 3A). The oxolinic acid phenotypes of the N78 mutant phage were even more interesting (Fig. 3B). The N78S mutant showed a marked hypersensitivity to the quinolone. Thus, by substituting the corresponding residue from DNA gyrase, we have converted the T4 topoisomerase into a more gyrase-like (i.e., quinolone-sensitive) enzyme. Furthermore, substitution at this position with tryptophan, which causes high-level oxolinic acid resistance in gyrase, also caused high-level oxolinic acid resistance in T4 (Fig. 3B). The equivalent of a gyrase S83W mutant can be imagined by comparing the N78S with the N78W mutant of T4, and the increase in oxolinic acid resistance is very dramatic. Finally, the T4 gp52 N78C substitution also showed a high level of oxolinic acid resistance.

Turning to position 79, we attempted to generate phage carrying phenylalanine (S79F; found in the 52-A<sup>R</sup> phage), proline (S79P; proline substitutions at the corresponding A84 of bacterial gyrase cause quinolone resistance; see above), leucine (S79L), and tryptophan (S79W). Unlike the above experiments with position 78 substitutions, we had difficulty isolating phages with substitutions at position 79, although the plasmids used in the I/S system had the desired mutations by DNA sequence analysis (see “Materials and Methods”). The only substitution that we obtained easily was S79L, which caused resistance to both m-AMSA and oxolinic acid (Fig. 4, A and C).

From the S79F construction, we did not find phage segregants with normal growth and an obvious drug-sensitivity or -resistance phenotype. We therefore searched for putative topoisomerase-negative mutants among the segregant phage by preferentially picking small plaques. We found one segregant (of 60) that behaved like a topoisomerase-negative mutant (see below). The S79F mutation should eliminate a BsgI restriction site in the wild-type DNA sequence, and digestion of a PCR product from this segregant revealed the expected loss of the site. Furthermore, DNA sequencing confirmed that this phage had the S79F mutation (and no other mutations in the region sequenced). Topoisomerase-negative mutants of phage T4 have a distinct phenotype of growing poorly at 37°C and not at all at 25°C (34). The S79F mutant phage displayed this characteristic phenotype (Fig. 5), arguing strongly that this mutation greatly reduces or abolishes topoisomerase activity in vivo.

Because the original 52-A<sup>R</sup> mutant phage did not show a topoisomerase-negative phenotype, we then suspected that the G269V mutation also present in that phage suppresses the defect caused by the S79F mutation. To analyze this possibility, we first substituted the G269V mutation back into a clean genetic background. The desired segregant was recognized by means of a new BbsI restriction site caused by the mutation. The single G269V mutant itself has a slight growth defect at 25°C (but normal growth at 37°C), suggesting that this mutation reduces (but does not abolish) topoisomerase activity (Fig. 5). We next used the relatively healthy G269V mutant to rescue the S79F mutation from a plasmid. As expected from the suppression model, the reconstituted double mutant phage had little or no growth defect, actually growing better at 25°C than either single mutant (Fig. 5). Clearly, the severe growth defect caused by the S79F mutation is strongly suppressed by the G269V mutation (see “Discussion”).

The drug sensitivities of both single mutants and the double S79F G269V mutant were compared using m-AMSA and oxolinic acid gradient plates (Fig. 4). The double mutant was strongly resistant to both drugs, which is the same phenotype as the original 52-A<sup>R</sup> mutant (15, 17). Thus, the phenotype of the original mutant is reconstituted by the combination of G269V and S79F. As described above, the S79F single mutant has a strong growth defect even in the absence of inhibitors. Mutants that lack topoisomerase activity display a drug-resistant phenotype, presumably because they do not accumulate...
Drug-induced cleavage complexes (35) and, therefore, the apparent drug resistance of the S79F mutant (Fig. 4) could simply be the result of the topoisomerase defect. Surprisingly, the G269V single mutant was extremely hypersensitive to both m-AMSA and oxolinic acid (Fig. 4). By comparing the G269V single mutant and the G269V S79F double mutant, it is clear that the S79F mutation causes a very strong drug resistance in the context of a topoisomerase with the G269V mutation.

DISCUSSION

We will use the recently published crystal structures of the yeast type II topoisomerase (21) and bacterial gyrase subunit A (22) as a framework for discussing drug-resistance mutations. This approach is speculative, because the T4 enzyme could have significantly different structural features. Nonetheless, comparison with the published crystal structures seems warranted: (a) the overall features of the yeast and bacterial topoisomerases are quite similar, although they do appear to represent two different enzyme conformations; and (b) all type II topoisomerases share extensive homologies throughout most of the protein sequence (36), arguing for a generally conserved structure for this family of enzymes.

Both bacterial and eukaryotic drug-resistance mutations are clustered in two distinct regions of the type II topoisomerases (roughly, residues 439–492 and 726–780 with respect to the yeast coordinates), leading to the suggestion that these regions together form a drug-binding pocket (37, 38). The T4 39-A<sup>R</sup> (E457K) and 52-A<sup>R</sup> (S79F) mutations each fall within one of these regions. These two regions roughly face each other, but are quite far apart, in the crystal structure of the yeast enzyme (21). One region is within the A' domain, very near the active site tyrosine (yeast Y783) and within a CAP-like structure thought to bind the cleaved DNA strand. The other region is within a different domain of the protein (B') that is connected by a flexible linker to the A' domain. Thus, a reasonable (but untested) scenario is that the B' domain folds down upon the DNA-binding region during the reaction cycle, juxtaposing the two regions of drug-resistance mutations.
around the bound DNA molecule, where the corresponding residues could interact with the drug molecule.

The mutation cluster in the B' domain contains two conserved oligopeptide motifs, EGDSA and PLRGKMLN, which are altered by many of the known drug-resistance mutations (38). The mutation in the 39-AK phage (E457K; E495 in the yeast enzyme) is 14 amino acids downstream from the second motif. This position has a highly conserved glutamic acid residue in nearly all type II topoisomerases. To our knowledge, no other drug-resistance mutations have been reported at this position, but mutations in nearby residues have been detected previously (39–42).

Several results suggest that this region of the B' domain interacts directly with the topoisomerase inhibitors. Mutations in this region of E. coli DNA gyrase can differentially affect sensitivity to different members of the quinolone family (37, 43), and the E457K mutation in T4 topoisomerase likewise differentially affects sensitivity to members of the ellipticine family of inhibitors (15). Furthermore, the E457K mutation causes resistance to some antimutator agents but hypersensitivity to oxolinic acid and VP-16 (15). These results indicate that the drug-resistance mutations subtly alter the drug binding site to interfere with the binding of some inhibitors but favor the binding of others. It is conceivable that these differential effects could be obtained by conformation changes that alter a more remote drug binding site, but it seems more likely that the relevant interactions are direct and local.

The second region important for inhibitor sensitivity is the putative DNA-binding region in the A' domain close to the active site tyrosine. Essentially every quinolone-resistance mutation in bacterial GyrA or ParC maps within this region, with the most commonly found mutations altering GyrA residues G81, S83, A84, D87, and Q106 (yeast residues G738, 740, 741, 744, and A767; Fig. 2). The S83W mutation blocks the binding of radiolabeled quinolone to the enzyme-DNA complex (11), and, therefore, mutations in this region almost certainly cause drug resistance by directly destabilizing the interaction between drug and enzyme.

Several antitumor-drug resistance mutations in human or yeast type II topoisomerase also map to this general region of the enzyme (41, 42, 44–46), although the mutations do not cluster as intensely as with the bacterial enzymes. To our knowledge, only two well-characterized eukaryotic drug-resistance mutations map to the region corresponding to the very strong hotspot for bacterial gyrase drug-resistance mutations (residues 83–87). These are G738D (GyrA position G81) and S741W (GyrA position A84; see Fig. 2 legend) in the yeast topoisomerase, both of which alter the sensitivities to fluoroquinolones CP-115,953 and etoposide (but not m-AMSA; see Refs 42, 44, and 45).

Our results have demonstrated the importance of T4 gp52 residues N78 and S79 in sensitivity to antimutator drugs and quinolones. The N78S substitution caused an increased sensitivity to m-AMSA and to oxolinic acid, whereas the N78C and N78W substitutions caused hypersensitivity to m-AMSA but marked resistance to oxolinic acid (Fig. 3). The S79L substitution caused resistance to both m-AMSA and oxolinic acid, as did the S79F substitution when tested in the context of the G269V mutation (see below).

The crystal structures of the yeast type II topoisomerase and E. coli gyrase subunit A greatly clarify the importance of this region of the protein in drug sensitivity (21, 22). The yeast residues equivalent to T4 gp52 N78 and S79 (Q740 and S741) are within A'α4, the second helix of a helix-turn-helix structure that is similar to the DNA-binding region of CAP and histone H5. Although there are no cocrystals of a type II topoisomerase and DNA, these structural homologies allowed Berger et al. (21) to model the topoisomerase-cleaved DNA into this region. In the topoisomerase-DNA model, A'α4 contacts the major groove of the segment of DNA just outside the cleaved phosphodiester bond. According to this model, residues Q740 and S741 of the yeast enzyme would be extremely close to the 3' hydroxyl end of the cleaved DNA. The structure by Berger et al. (21) shows an open configuration of the enzyme, with the two active site tyrosines (and the nearby CAP-like helix-turn-helix motifs) far enough apart to allow the passage of a segment of duplex DNA through the gap. The recent structure of a large fragment of E. coli gyrase subunit A, however, shows a closed configuration that is thought to be similar to that just prior to DNA cleavage (22). Once again, modeling of DNA into this active site region places the critical residues for drug resistance in close contact with the DNA. Thus, although the two crystal structures appear to reflect different enzyme conformations, both are entirely consistent with the quinolone-resistance determining region binding to DNA very near the site of DNA cleavage.

Drug cross-linking studies with a photoactivated m-AMSA derivative have placed the drug-binding site precisely at the cleaved phosphodiester bonds (9). Thus, assuming that the models of the protein-DNA complexes are accurate and that the T4 enzyme has a similar structure, T4 residues N78 and S79 are located very near or at the drug-binding site. It seems highly likely that these residues interact directly with inhibitors at the active site. Based on this inference, we can also make a strong argument that quinolones and intercalating antimutator agents such as m-AMSA bind to common or closely overlapping sites; mutations of N78 and S79 alter sensitivity to both compounds.

Our results with N78 substitutions reveal a striking parallel between the T4 topoisomerase and bacterial DNA gyrase. In gyrase, the highest level of quinolone resistance is obtained when the small polar S83 is substituted with bulky nonpolar residues (L, F, or W; Refs. 13, 29, 30, and 47). The wild-type T4 topoisomerase is much less sensitive to oxolinic acid than is DNA gyrase, arguably because the N78 residue of the T4 enzyme is bulkier than the serine of gyrase.9 We found that substitution of N78 with serine causes hypersensitivity to oxolinic acid (i.e., making the T4 topoisomerase more like gyrase). Furthermore, substitution of W at residue 78 causes dramatic resistance to oxolinic acid. These results strongly suggest that oxolinic acid interacts with this residue in the T4 topoisomerase and E. coli gyrase drug-binding pockets in a fundamentally similar manner. Interestingly, the N78C substitution in gp52 also causes resistance to oxolinic acid, but as far as we know, an S83C mutant has never been tested in bacterial gyrase.10

The S79F substitution by itself caused a topoisomerase-negative phenotype in vivo, implying that the mutant protein is either very unstable or catalytically inactive. The S79L substitution also caused poorer than normal growth at low temperature (data not shown), suggesting reduced activity, and the failure to isolate S79P and S79W suggested that these substitutions have negative consequences as well (see “Materials and Methods”). These results argue that residue 79 is critical for enzyme activity. In general, when considering which amino acid substitutions lead to drug resistance in various topoisomerases, it is thus important to realize that some substitutions may not be consistent with enzyme function.

One of the most interesting results we obtained was the suppression of the topoisomerase-negative phenotype of S79F by the G269V mutation. In the recent crystal structures, the residues corresponding to G269 in yeast topoisomerase (D939) and bacterial gyrase (M301)

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9 Mutation of S83 to N in E. coli GyrA would require a three-nucleotide substitution; hence, this substitution would not be expected in a spontaneous quinolone-resistant mutant, even if it causes quinolone resistance.

10 Mutation of S83 to C in E. coli GyrA would require a two-nucleotide substitution; hence, this substitution would not be expected in a spontaneous quinolone-resistant mutant, even if it causes quinolone resistance.
are within the “tower domain,” which sits atop the CAP-like domain discussed above (21, 22). However, these residues are too far from the residues corresponding to S79 to argue for a direct interaction. Therefore, the crystal structures do not provide a simple explanation of the mechanism of suppression. Perhaps the two residues are in close proximity in some conformation of the enzyme that has not been captured in either of the crystal structures.

Surprisingly, the G269V substitution caused a dramatic hypersensitivity to both oxolinic acid and m-AMSA. This mutant appears to define a category of topoisomerase mutants that have not been isolated previously, i.e., those that are generally hypersensitive to all inhibitors that stabilize the cleavage complex. As with the question of suppression, it is not obvious how an alteration of G269 affects drug sensitivity so dramatically when the residue appears to be distant from the cleavage site. To our knowledge, no mutation that alters drug sensitivity has ever been reported in the region corresponding to the tower domain of a type II topoisomerase, but hypersensitive mutants have never been systematically sought. It should be very interesting to explore the nature of the drug hypersensitivity in purified enzyme with the G269V substitution.

In summary, this analysis of altered drug sensitivity provides additional insight into the mechanism of action of topoisomerase inhibitors and into drug resistance. It is very likely that substitutions of N78 and S79 directly affect the binding of both the quinolone oxolinic acid and the antitumor agent m-AMSA, providing another strong link between the mechanism of action of these inhibitors. Our results, coupled with the recent crystal structures of topoisomerase, support models in which the inhibitors bind to the internucleotide space at the cleaved phosphodiester bonds and interact with residues of topoisomerase that are in close contact with the DNA at that location. We also showed that mutations in two regions quite distant from the scissile phosphodiester bond in the available crystal structures markedly affect drug action, and one of these regions has not been implicated previously in drug sensitivity. Additional studies are needed to clarify the precise chemical nature of drug-enzyme interactions at the active site and to explain the altered drug sensitivity in mutants with substitutions that are distant from the cleaved DNA.

ACKNOWLEDGMENTS

We thank Laura Wieslo for technical assistance; Jim George and Lynn Zhendrich for bacterial strains; and Steve White, James Berger, and Tony Maxwell for helpful discussions of topoisomerase structure.

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Mutations of the Bacteriophage T4 Type II DNA Topoisomerase
That Alter Sensitivity to Antitumor Agent 4′-(9-Acridinylamino)methanesulfon-\(m\)-anisidide and an
Antibacterial Quinolone

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