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Localization of an aminoacridine antitumor agent in a type II topoisomerase–DNA complex

(acridine/bacteriophage T4/photoactivation/quinolones)

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ABSTRACT **Type II topoisomerases are the targets of several classes of chemotherapeutic agents that stabilize an intermediate of the catalytic cycle with the enzyme covalently linked to cleaved DNA. We have used 3-azido-AMSA [4′-(3-azido-9-acridinylamino)methanesulfon-*m*-anisidide], a photoactivatible analog of the inhibitor *m*-AMSA [4′-(9-acridinylamino)methanesulfon-*m*-anisidide], to localize the inhibitor binding site in a cleavage complex consisting of an oligonucleotide substrate and the bacteriophage T4 type II DNA topoisomerase. Upon photoactivation, the inhibitor covalently attached to the substrate only in the presence of topoisomerase. Sites of inhibitor attachment were detected by primer-extension analysis and by piperidine-induced cleavage of the covalently modified substrate. 3-Azido-AMSA reacted with bases immediately adjacent to the two phosphodiester bonds cleaved by the enzyme. Therefore, topoisomerase creates or stabilizes preferential binding sites for the inhibitor precisely at the two sites of DNA cleavage.**

Type II topoisomerases are ubiquitous enzymes that catalyze important DNA topological interconversions. These interconversions are achieved by a conserved mechanism: the enzyme dimer makes a double-strand break in one helix, passes a second helix through this DNA gate, and religates the double-strand break. A key intermediate in the topoisomerase reaction, the cleavage complex, consists of each monomer of the enzyme covalently attached, via a phosphotyrosine bond, to one of the two 5′ phosphates of the 4-bp staggered DNA break (1–3). This normally short-lived intermediate is stabilized by many different inhibitors that target topoisomerases (4–8). The mammalian type II topoisomerase is a target for several classes of antitumor agents, including the aminoacridines, anthracyclines, ellipticines, and epipodophyllotoxins. Similarly, the antibacterial quinolones target the bacterial type II topoisomerase, DNA gyrase. For both the antitumor and antibacterial agents, cell death is dependent on stabilization of the cleavage complex (a form of DNA damage) rather than loss of topoisomerase activity (4–7).

The simplest general model to explain stabilization of the cleavage complex is that the inhibitors bind to DNA at or near the cleaved phosphodiester bonds, preventing DNA religation. Based on quinolone binding to single-stranded DNA, Shen *et al.* (9) proposed that quinolones bind to the 4-bp denatured region between the gyrase-cleaved bonds. For the mammalian and bacteriophage T4 enzymes, the identity of the base pairs directly adjacent to the cleaved phosphodiester bonds determines which inhibitors stabilize DNA cleavage, arguing that the inhibitors bind in the vicinity of each cleaved bond, perhaps by intercalation (7, 10–15).

The inhibitor-specific preferences for base pairs adjacent to the cleaved phosphodiester bonds provide the strongest evidence that DNA forms at least part of the inhibitor binding site. On the other hand, the protein probably also constitutes part of the drug binding site because mutational alteration of type II topoisomerases can cause drug resistance (16–18). Two previous approaches argue that both DNA and protein are involved in the inhibitor binding site. First, radioactive quinolone binding was detected with a gyrase–DNA complex but not with either DNA or gyrase alone (19, 20). Second, a single mutational alteration of the T4 type II topoisomerase simultaneously altered inhibitor sensitivity (i.e., interaction with inhibitor) and DNA cleavage site specificity (i.e., interaction with DNA) (21).

The bacteriophage T4-encoded type II topoisomerase is sensitive to many antitumor agents that inhibit the mammalian enzyme, including *m*-AMSA [4′-(9-acridinylamino)methanesulfon-*m*-anisidide], and thereby provides a good model system (22). We recently performed a mutational analysis of a *m*-AMSA-induced T4 topoisomerase cleavage site (13). We found that nucleotide residues important for enzyme recognition were in the regions flanking the cleavage site (−2 to −6 and −2′ to −6′; see Fig. 1*A*), while inhibitor-specific preferences were at the base pairs immediately surrounding the cleaved phosphodiester bonds (positions −1, −1′ and +1, +1′). Interestingly, the inhibitor-specific preferences found with the T4 enzyme are essentially identical to those found by consensus sequence analysis with the mammalian enzyme (10–15), suggesting that the same rules govern inhibitor binding specificity for evolutionarily distant topoisomerases.

In the present study, we have localized the sites of inhibitor binding in the topoisomerase–DNA complex by more direct means using 3-azido-AMSA [4′-(3-azido-9-acridinylamino)methanesulfon-*m*-anisidide], a photoactivatible analog of *m*-AMSA. 3-Azido-AMSA was synthesized by Shieh *et al.* (23) and found to have DNA binding properties similar to those of *m*-AMSA. Most importantly for our purposes, photoactivation of 3-azido-AMSA in the presence of either deoxyribonucleosides or DNA produces covalent drug–DNA adducts (23). Covalent linkage of 3-azido-AMSA to a DNA substrate during a topoisomerase cleavage reaction allowed us to precisely localize the bound inhibitor after removal of the enzyme.

MATERIALS AND METHODS

Topoisomerase and Inhibitors. T4 topoisomerase was purified as described (21, 24). 3-Azido-AMSA was a generous gift from Stephen R. Byrn (Purdue University), and *m*-AMSA was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. 3-Azido-AMSA was dissolved in

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Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; 3-azido-AMSA, 4′-(3-azido-9-acridinylamino)methanesulfon-*m*-anisidide.

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100% ethanol and *m*-AMSA was dissolved in 100% dimethyl sulfoxide. Immediately before use, each was diluted 1:10 with water and then 1:10 into the final reaction mixture (final concentrations, 12.7 μM *m*-AMSA and 11.5 μM 3-azido-AMSA unless otherwise indicated).

Oligonucleotide Substrates. Oligonucleotides were purified from a 20% denaturing polyacrylamide gel by the crush-and-soak method after visualization by UV shadowing (25, 26) and resuspended in 1× TE (10 mM Tris·HCl, pH 7.6/1 mM EDTA, pH 8.0). Concentrations of purified oligonucleotides were determined by spectrophotometry. Duplex substrates were produced by annealing top- and bottom-strand oligonucleotides at equimolar concentration and slowly cooling from 65°C to 4°C. For the direct-labeled experiments, oligonucleotides were end-labeled before annealing, either at the 5′ end by T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham) or at the 3′ end by terminal transferase (United States Biochemical) and [α-^{32}P]ddATP (Amersham).

Topoisomerase Cleavage Reactions. Topoisomerase cleavage reaction mixtures with labeled substrates (10 μl) contained 40 mM Tris·HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, 0.5 mM ATP, bovine serum albumin at 30 μg/ml, 1 pmol of duplex 38-mer, 2 pmol of T4 topoisomerase (or topoisomerase dilution buffer), and either *m*-AMSA or 3-azido-AMSA. Cleavage reaction mixtures for the primer-extension experiment (see Fig. 2) were identical except that the reaction mixtures (20 μl) contained 2 pmol of unlabeled substrate and 2 pmol of topoisomerase. Reactions with 3-azido-AMSA were set up in the dark and then incubated at room temperature either in the dark or 4 cm from the UV lamp (15-W GE F15T8; 3200 μW/cm^2 at 4 cm; peak wavelength, 360–370 nm). Reactions were terminated by adding SDS to 1% and covalently attached topoisomerase was removed with proteinase K (1 mg/ml; 30°C; 1 hr). Products destined for primer extension were extracted with phenol, and all reaction products were then purified by passing through 0.6-ml Sephadex G-25 (Pharmacia) spin columns. The reaction products were then treated in one of three ways: (*i*) *Direct analysis.* Each spin-column flow-through was brought to 16 μl with loading solution (formamide, 10 mM Na_2EDTA, dyes). (*ii*) *Primer-extension reactions.* One-fourth of each reaction product (0.5 pmol of DNA) was annealed to equimolar 5′-end-labeled 9-base primer of either the bottom-strand sequence or the top-strand sequence in 1× Klenow buffer (50 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/0.2 mM dithiothreitol) containing 25 μM (final concentration) each deoxyribonucleotide. Klenow enzyme (1 unit; Boehringer Mannheim) was added, and the extension reaction (10 μl mixture) proceeded for 5 min at 24°C before being terminated with 6 μl of loading solution. (*iii*) *Piperidine cleavage reactions (27).* Piperidine (100 μl; 1 M) was added to the purified topoisomerase reaction products (1 pmol), and the mixture was incubated at 90°C for 30 min. Piperidine was removed by three successive lyophilizations, and samples were then resuspended in 10 μl of loading solution.

Gel Electrophoresis. Samples (in loading solution) were heated at 90°C for 2 min. Half of each sample was loaded onto a denaturing (7 M urea) 20% polyacrylamide gel, which was run at 53 W for 2.5 hr in 1× TBE buffer (89 mM Tris base/89 mM boric acid/2.5 mM Na_2EDTA). Products were visualized by autoradiography. Sequencing standards were generated by the chain-termination (for Figs. 1 and 2) (25) or the Maxam–Gilbert (for Figs. 3 and 4) (27) method.

RESULTS

3-Azido-AMSA Stimulates T4 Topoisomerase-Mediated DNA Cleavage. We began by testing whether 3-azido-AMSA, the photoactivatible derivative of *m*-AMSA (23), induces cleavage complexes. The oligonucleotide substrate used in these studies contains a very strong *m*-AMSA-inducible cleavage site for the T4 topoisomerase. This cleavage site was designed based on our previous mutational analysis of a moderately strong cleavage site (13). Several base substitutions that increased the efficiency of *m*-AMSA-induced cleavage were combined to generate a new 38-bp oligonucleotide substrate (Fig. 1*A*). Topoisomerase-mediated cleavage of this substrate in the presence of *m*-AMSA was severalfold more efficient than the original substrate (data now shown).

The 38-bp 5′-end-labeled substrate was cleaved in the presence of T4 topoisomerase and either *m*-AMSA or 3-azido-AMSA, generating the expected 16- and 18-base products upon denaturation (Fig. 1*B*). Furthermore, 3-azido-AMSA induced cleavage at this site with a potency indistinguishable from that of *m*-AMSA (Fig. 1*C*). We conclude that 3-azido-AMSA is an effective inhibitor of the T4 topoisomerase, with DNA site specificity and potency similar or identical to *m*-AMSA.

Localization of the Inhibitor by Primer Extension. The first method we used to locate covalently bound 3-azido-AMSA was primer extension. Duplex 38-mer oligonucleotide was incubated with T4 topoisomerase in the presence of 3-azido-AMSA, with UV light to activate the azido group. The reaction products were purified and heat denatured, and a labeled 9-base primer with either the top-strand sequence or the bottom-strand sequence was annealed and extended with Klenow polymerase.

When the reaction products had been generated with 3-azido-AMSA and UV light but no topoisomerase, extension proceeded predominantly to full-length product (Fig. 2*A*). The faint bands present at each nucleotide position were also detected without 3-azido-AMSA and UV light (data not shown). Thus, in the absence of topoisomerase, no 3-azido-

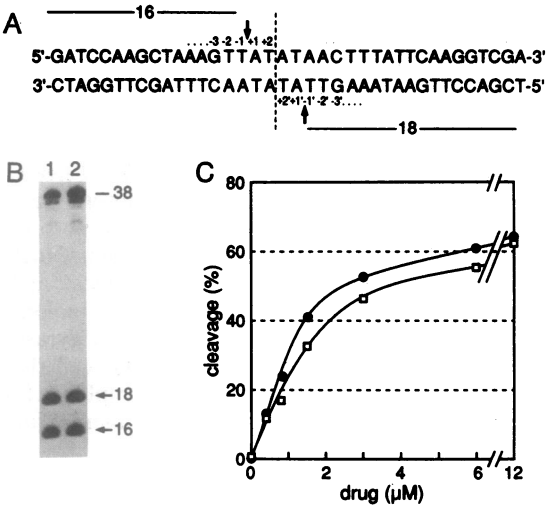

FIG. 1. Topoisomerase cleavage of a strong *m*-AMSA-inducible site. (*A*) Sequence of the duplex oligonucleotide substrate (38 bp) containing the strong *m*-AMSA cleavage site. Nomenclature for positions surrounding the cleaved phosphodiester bonds (arrows) is indicated, along with the cleavage site center of symmetry (dashed line). (*B*) The 38-bp substrate (1 pmol), labeled at the 5′ ends, was cleaved by topoisomerase (2 pmol) in the presence of either *m*-AMSA (lane 1) or 3-azido-AMSA (lane 2). Reaction mixtures were incubated for 30 min at room temperature in room light, and the products were separated on a denaturing 20% polyacrylamide gel and visualized by autoradiography. (*C*) Titration of *m*-AMSA- (□) and 3-azido-AMSA- (●) induced cleavage. Extent of cleavage (percentage total substrate) was determined by direct counting with an AMBIS imaging system (average of two experiments).

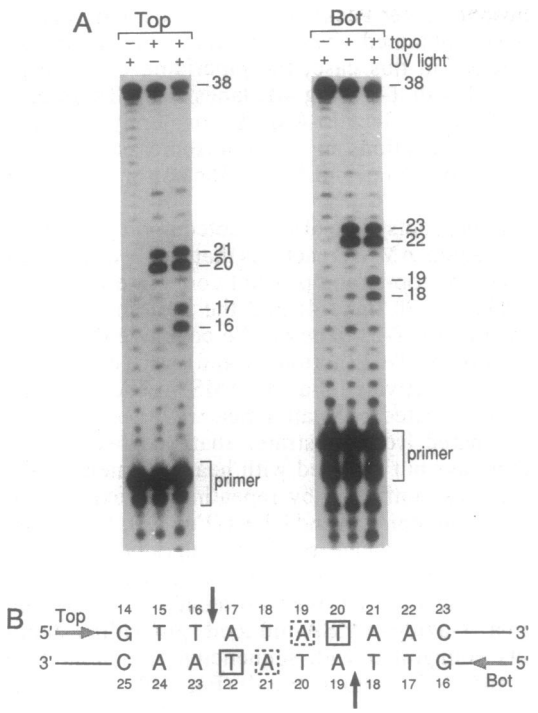

FIG. 2. Localization of 3-azido-AMSA binding sites by primer extension. (*A*) Topoisomerase cleavage reaction mixtures were incubated for 15 min in the presence of 3-azido-AMSA either under UV light (with or without enzyme) or in the dark (with enzyme). One-fourth of each reaction product was heat denatured and annealed to a 5′-end-labeled 9-base primer of either the top-strand (Top) or bottom-strand (Bot) sequence (see *B*). Primers were extended with Klenow enzyme, and one-half of each sample was analyzed by denaturing polyacrylamide gel electrophoresis. Bands corresponding to unextended primer, completely extended primer (38 bases), topoisomerase cleavage products (20 and 22 bases), and other major primer-extension stops are indicated. Exact lengths of the indicated bands were determined by comparison to sequencing standards generated by the chain-termination method. (*B*) Sequence surrounding the topoisomerase-cleaved phosphodiester bonds (vertical arrows) is shown, with bases numbered from the 5′ ends. Top-strand (Top) and bottom-strand (Bot) primers are represented by horizontal arrows, and potential sites of 3-azido-AMSA attachment are boxed.

AMSA binding sites were stable enough to be detected by this method. When the reaction products had been generated with topoisomerase and 3-azido-AMSA but no UV light, the expected runoff products due to topoisomerase cleavage were readily detected (top-strand primer, 20 bases; bottom-strand primer, 22 bases). An additional extension product formed a band 1 base larger than each of the topoisomerase runoff products. We attribute this band to untemplated base addition as described (13, 26, 28). This interpretation was confirmed by extending the top-strand primer annealed to an oligonucleotide that was synthesized to mimic the bottom-strand topoisomerase cleavage product (bottom strand, A-19–C-38); both the 20- and 21-base products were generated (data not shown).

When the reaction products had been generated with topoisomerase and 3-azido-AMSA in the presence of UV light, the runoff products due to topoisomerase cleavage were again detected, along with novel bands that apparently result from photoactivation of 3-azido-AMSA (Fig. 2*A*). Using the top-strand primer, a strong extension stop was detected at 16 bases, along with a weaker stop at 17 bases. Similarly, with the bottom-strand primer, a strong stop was detected at 18 bases and a weaker one was detected at 19 bases. Generation of these novel products was dependent on the presence of topoisomerase, 3-azido-AMSA, and UV light (Fig. 2*A*; data not shown). We interpret the strong primer-extension stop on each strand (top-strand primer, 16 bases; bottom-strand primer, 18 bases) as a block to further elongation due to covalent modification of the next template base. Thus, 3-azido-AMSA molecules apparently reacted with T-22 on the bottom strand and with T-20 on the top strand, symmetrical positions with respect to the phosphodiester bonds cleaved by topoisomerase (Fig. 2*B*).

We believe that the weaker stop on each stand (top-strand primer, 17 bases; bottom-strand primer, 19 bases) is due to an untemplated base addition, as detected above at the topoisomerase cleavage site. We cannot eliminate the possibility that the primer was blocked for further elongation due to covalent modification of the next template base (bottom strand, A-21; top strand, A-19; see Fig. 2*B*). However, a second method showed no evidence for 3-azido-AMSA modification at these positions (see below).

Photoactivation of 3-Azido-AMSA Using End-Labeled Substrates. We next sought to directly locate sites of 3-azido-AMSA modification on 5′-end-labeled 38-bp substrates. Topoisomerase reaction mixtures contained either 3-azido-AMSA (under UV light) or *m*-AMSA (as a control). Duplicate reactions were subjected to heat treatment (65°C for 10 min), which reverses most topoisomerase cleavage products (29). Both 3-azido-AMSA and *m*-AMSA induced the expected strong cleavage products, along with several faint bands representing weaker cleavage sites (Fig. 3). As expected, a large fraction of the cleavage products was reversed by heat treatment (Fig. 3, △). Two novel bands (Fig. 3, *) were detected only from the 3-azido-AMSA reactions; each ran slightly faster than one of the major cleavage products. Unlike topoisomerase cleavage products, these products were detected only with UV light and were not reversed by heat treatment (Fig. 3; data not shown). By testing substrates with only one strand labeled, the smaller photoactivation product was shown to originate from the top strand and the larger one was shown to originate from the bottom strand (Fig. 3).

The novel photoactivation products presumably do not result just from covalent attachment of 3-azido-AMSA to the

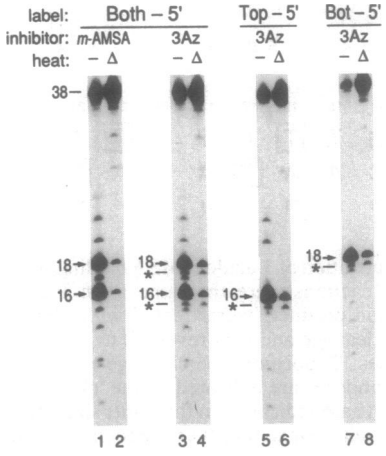

FIG. 3. Photoactivation of 3-azido-AMSA using labeled substrates. Duplex 38-mer labeled at both 5′ ends (Both) or only on the top strand (Top) or bottom strand (Bot) was incubated with topoisomerase in the presence of either *m*-AMSA or 3-azido-AMSA (3Az) and UV light for 30 min. Duplicate reaction mixtures were incubated at 65°C for 10 min after the topoisomerase reaction but before addition of SDS and proteinase K to reverse topoisomerase cleavage products (△). Uncleaved substrate (38 bases), and top-strand (16 base) and bottom-strand (18 base) cleavage products are indicated. Nonreversible bands induced by 3-azido-AMSA and UV light are indicated (*).

topoisomerase cleavage product, because the inhibitor should add both molecular weight and a positive charge and thereby decrease mobility of the oligonucleotide. Rather, we believe that the products result from covalent modification of the topoisomerase cleavage products followed by loss of the modified base. Using mass spectrometry, Shieh *et al.* (23) found that 3-azido-AMSA modification of a base can result in cleavage of the N-glycosidic bond.

Localization of the Inhibitor by Piperidine Cleavage. In Maxam–Gilbert sequencing reactions, sites of base-specific modification are cleaved by piperidine treatment (27). We reasoned that sites of 3-azido-AMSA modification could be precisely located if they are also sensitive to piperidine. Therefore, the above reactions were repeated with and without piperidine treatment, and the products were run next to Maxam–Gilbert sequencing standards of the same DNA. Upon treatment with piperidine, the nonreversible band associated with the top-strand cleavage product disappeared, and a new band appeared, comigrating with sequencing standard T-16 (Fig. 4*A*, lanes 1–4). This piperidine cleavage product required topoisomerase, was not detected in *m*-AMSA-containing control reaction mixtures, and was not reversed by prior heat treatment (Fig. 4*A*; data not shown). We conclude that a photoactivated 3-azido-AMSA modification existed at position T-16, and that piperidine cleaved the phosphodiester backbone at that position. Very similar results were obtained when the bottom strand of the substrate was labeled; in this case, the piperidine cleavage product comigrated with T-18 (Fig 4*A*, lanes 5–8). Therefore, upon photoactivation, 3-azido-AMSA covalently modified two symmetrical positions in the topoisomerase cleavage site: T-16 on the top strand and T-18 on the bottom strand (see Fig. 4*C*).

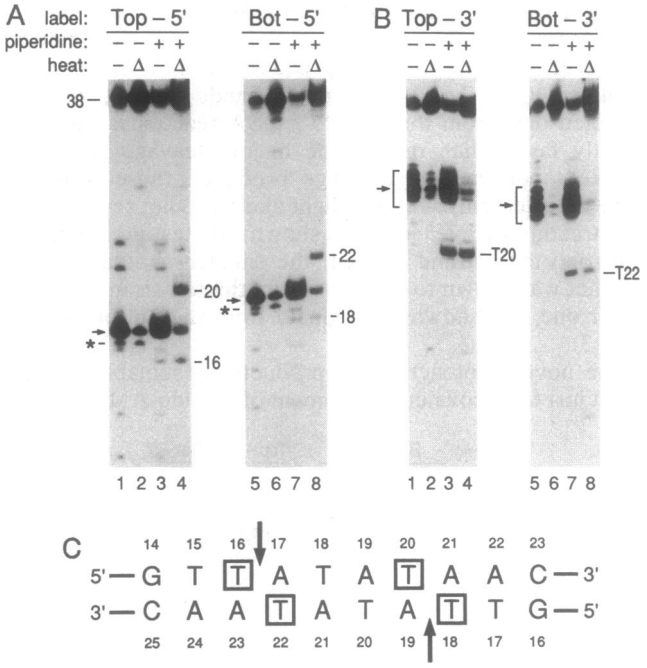

FIG. 4. Localization of 3-azido-AMSA binding sites by piperidine cleavage. (*A*) Reactions were the same as in Fig. 3, except that duplicate reaction products were treated with piperidine (+; after topoisomerase cleavage and heat reversal) to effect strand cleavage at modified bases. Topoisomerase cleavage products (arrows) and nonreversible bands (*) are indicated on the left. Sizes of piperidine cleavage products are indicated on the right; size determination was by comparison with Maxam–Gilbert sequencing products. Topoisomerase cleavage products end with a 3' hydroxyl, while piperidine cleavage products end with a 3' phosphate and thereby migrate slightly faster. (*B*) Reactions were the same as in *A*, except that the 38-bp substrate was labeled at the 3' end on either the top strand (Top) or bottom strand (Bot). Topoisomerase cleavage products are indicated by arrows. The top-strand piperidine cleavage product comigrated with top-strand sequencing standard T-20 (a 19-base product) and the bottom-strand product comigrated with sequencing standard T-22 (a 17-base product; standards generated by Maxam–Gilbert sequencing of the same 3'-end-labeled oligonucleotides). (*C*) Summary of residues cleaved by piperidine after photoactivation with 3-azido-AMSA (boxed).

Longer products were also detected upon piperidine treatment of 3-azido-AMSA reactions that had been heat reversed (Fig. 4*A*). The top-strand product comigrated with sequencing standard T-20 (lane 4) and the bottom-strand product comigrated with T-22 (lane 8). We conclude that bases T-20 (top strand) and T-22 (bottom strand) were covalently modified by photoactivated 3-azido-AMSA. These longer products were detected only after heat reversal, implying that they originated from substrates that had been cleaved by topoisomerase but resealed with heat treatment. This interpretation was confirmed by repeating the experiment with substrates containing 3' end labels (Fig. 4*B*). In this case, the topoisomerase cleavage products migrated as a series of bands, presumably because of variable amounts of residual topoisomerase that survived proteinase K treatment. The strongest 3-azido-AMSA-induced piperidine-dependent products comigrated with sequencing standards T-20 (top strand) and T-22 (bottom strand) (Fig. 4*B*). These two positions agree precisely with the assignments made from the 5'-end-labeled substrates and with those determined by primer extension (see above).

In summary, four sites of 3-azido-AMSA modification have been located by piperidine cleavage, and the two strongest of these were also detected by primer extension (Fig. 4*C*; the other two sites are not detectable by primer extension because their products would comigrate with topoisomerase cleavage bands). All four sites are within the base pairs immediately adjacent to the two phosphodiester bonds that are cleaved by topoisomerase.

DISCUSSION

The religation step is the Achilles' heel of the catalytic cycle of DNA topoisomerases. Agents that inhibit this step and stabilize the cleavage complex have been found for virtually all type I and type II topoisomerases and serve as some of the most useful chemotherapeutic agents.

This study demonstrates that the inhibitor binding site in a topoisomerase–DNA complex is in the immediate vicinity of the cleaved phosphodiester bonds. Four nucleotide residues of a strong topoisomerase cleavage site are in close contact with the inhibitor, and all four are within the base pairs immediately adjacent to the cleaved phosphodiester bonds. The same pattern of 3-azido-AMSA modification was also detected at another strong T4 topoisomerase cleavage site (unpublished data). Given the close similarities between the T4 and mammalian enzymes and the common drug-dependent sequence preferences at cleavage sites (see Introduction), the location of the drug binding site in both enzyme–DNA complexes is likely to be the same.

The photoactivatible inhibitor reacted with only one residue of each of the 4 base pairs adjacent to the cleaved phosphodiester bonds, suggesting that the orientation of the inhibitor is most favorable for the activated azido group to react with these residues. The lack of reactivity at the complementary residues is not due to the fact that they are adenines instead of thymines; substitution of either A-17 (top strand) or A-23 (bottom strand) with thymine did not result in reactivity (even though cleavage was not significantly reduced; unpublished data).

All four of the reactive bases that we detected in the cleavage complex are thymines; however, Shieh *et al.* (23)

detected covalent photoadducts of 3-azido-AMSA with free dA, dG, and dC nucleosides. To investigate the base specificity of photoactivation, we substituted the reactive T-22 (bottom strand) with A, G, or C, and monitored 3-azido-AMSA modification in the presence of topoisomerase by primer extension. We found reactivity with both G and C but not with A (unpublished data). Therefore, 3-azido-AMSA can react with 3 of the 4 possible nucleotide residues providing they are in the correct position within the cleavage complex.

Many of the type II topoisomerase inhibitors, including *m*-AMSA and 3-azido-AMSA, are DNA intercalators and therefore bind to sites in naked DNA (6, 8, 23). Nevertheless, we detected inhibitor binding sites only in the presence of topoisomerase and only at the locations where the enzyme cleaves the DNA substrate. What is the difference between these two classes of sites? We favor the possibility that the inhibitor binding sites within the cleavage complex are much more stable than those in naked DNA. An alternative possibility is that the inhibitor binds equally well to the two classes of sites but is oriented differently in the context of the cleavage complex, resulting in more efficient crosslinking.

Assuming that the inhibitor binding sites within the cleavage complex are more stable than those in naked DNA, what causes the increased stability? Perhaps the topoisomerase, in the course of its reaction cycle, creates some simple DNA structure that is a strong inhibitor binding site. However, substrates containing either a nick or a double-strand/single-strand junction at the sequence normally cleaved by the enzyme did not detectably react with 3-azido-AMSA in the absence of topoisomerase (unpublished data). Perhaps the enzyme creates some other DNA structure that is a preferred drug binding site, or, alternatively, the protein could directly stabilize inhibitor binding. The latter view is supported by the finding that simple mutational alterations in the enzyme can differentially affect sensitivity to various inhibitors, even within the same drug class (22, 30, 31).

The binding site for the quinolones probably also involves DNA at the active site of DNA gyrase (19, 20). In the model of Shen *et al.* (9), the inhibitor binding site is created by denaturation of the 4-bp region between the cleaved bonds, with the inhibitor binding preferentially to the internal bases (positions +2 and +2′). However, oxolinic acid-induced cleavage sites for DNA gyrase and for the T4 topoisomerase show base preferences at positions adjacent to the cleaved bonds rather than at the internal bases (13, 32, 33). Thus, the quinolones may also bind at the sites of phosphodiester bond cleavage, perhaps in a manner much like intercalators such as *m*-AMSA.

Finally, mammalian type I topoisomerase is the target for the antitumor drug camptothecin. Analysis of DNA cleavage sites for that enzyme revealed a strong camptothecin-induced preference for G immediately 3′ to the cleaved phosphodiester bond (34). Furthermore, photoactivation of camptothecin in the absence of topoisomerase generated lesions exclusively at G residues (35). Thus, camptothecin apparently traps cleavage complexes at sites with a 3′ G by interacting directly with the G·C base pair (35).

In summary, the inhibitor binding sites in the cleavage complexes of diverse topoisomerases are likely to be closely related. Previous studies provided many indirect arguments that the inhibitors bind to DNA at the active sites of topoisomerases, presumably inhibiting DNA religation. The results presented here provide direct evidence that localizes the inhibitor binding site within a cleavage complex. Inhibitor binding in the immediate vicinity of the cleaved phosphodiester bond, perhaps by intercalation into that internucleotide space, is likely the common mechanism for inhibition of diverse topoisomerases.

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