DNA Topoisomerases as Targets for the Anticancer Drug TAS-103: DNA Interactions and Topoisomerase Catalytic Inhibition†

John M. Fortune,‡,§ Luminita Velea,|| David E. Graves,|| Teruhiro Utsugi,⊥ Yuji Yamada,⊥ and Neil Osheroff*,‡,¶

Departments of Biochemistry and Medicine (Hematology/Oncology), Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, Department of Chemistry, University of Mississippi, University, Mississippi 38677, and Taiho Pharmaceutical Co., Ltd., Hanno Research Center, Hanno-City, Saitama, 357, Japan

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ABSTRACT: TAS-103 is a novel anticancer drug that kills cells by increasing levels of DNA cleavage mediated by topoisomerase II. While most drugs that stimulate topoisomerase II-mediated DNA scission (i.e., topoisomerase II poisons) also inhibit the catalytic activity of the enzyme, they typically do so only at concentrations above the clinical range. TAS-103 is unusual in that it reportedly inhibits the catalytic activity of both topoisomerase I and II and does so at physiologically relevant concentrations [Utsugi, T., et al. (1997) *Jpn. J. Cancer Res. 88*, 992−1002]. Without a topoisomerase activity to relieve accumulating torsional stress, the DNA tracking systems that promote the action of TAS-103 as a topoisomerase II poison would be undermined. Therefore, the effects of TAS-103 on the catalytic activity of topoisomerase I and II were characterized. DNA binding and unwinding assays indicate that the drug intercalates into DNA with an apparent dissociation constant of ~2.2 μM. Furthermore, DNA strand passage assays with mammalian topoisomerase I indicate that TAS-103 does not inhibit the catalytic activity of the type I enzyme. Rather, the previously reported inhibition of topoisomerase I-catalyzed DNA relaxation results from a drug-induced alteration in the apparent topology of the nucleic acid substrate. TAS-103 does inhibit the catalytic activity of human topoisomerase IIα, apparently by blocking the DNA religation reaction of the enzyme. The lack of inhibition of topoisomerase I catalytic activity by TAS-103 explains how the drug is able to function as a topoisomerase II poison in treated cells.

A number of drugs currently used for the treatment of human cancers are targeted to DNA topoisomerases (*1*−*9*). These agents, referred to as topoisomerase "poisons", increase the concentration of topoisomerase-generated DNA breaks that are normally short-lived intermediates in enzyme catalysis (*1*−*5*, *7*, *9*). Transient drug-induced DNA breaks are converted to permanent breaks when DNA tracking enzymes, such as those associated with replication or transcription, attempt to traverse the covalent topoisomerase-cleaved DNA reaction intermediate. This conversion triggers a series of mutagenic and recombinagenic events that ultimately initiates programmed death pathways (*2*, *3*, *10*, *11*).

Because DNA tracking enzymes separate the two strands of the double helix, they induce significant positive supercoiling in the downstream DNA (*12*−*17*). Therefore, to continue their movement along the genetic material, tracking systems require the DNA strand passage activity of a topoisomerase to alleviate the ensuing torsional stress in the chromosome (*5*, *15*−*17*). Although this role is normally performed by topoisomerase I, genetic studies indicate that topoisomerase II can substitute for the loss of type I activity (*18*, *19*). In the absence of both topoisomerase I and II, DNA tracking complexes halt, and the capacity of topoisomerase poisons to generate permanent DNA breaks and trigger cell death is greatly impaired (*5*, *7*, *9*, *17*, *19*−*21*).

At high concentrations, virtually every topoisomerase I or II poison also blocks the overall catalytic activity of its respective enzyme target (*1*, *22*−*25*). However, at clinically relevant drug concentrations, significant inhibition generally is not observed.

TAS-103 is a novel topoisomerase II poison with clinical potential (*26*−*29*). It is unusual in that it reportedly inhibits the catalytic activity of both topoisomerase I and II in the low micromolar range (*26*). The IC_{50} values for the drug are comparable to concentrations that induce 50% maximal DNA cleavage.

The reported inhibition of topoisomerase I and II activity by TAS-103 raises a potentially important issue. If the drug blocks topoisomerase catalysis, this action should stall the DNA tracking systems that are required to convert transient topoisomerase II-cleaved DNA complexes into permanent strand breaks and induce cell death. Consequently, if TAS-103 is a dual inhibitor of topoisomerase I and II, how can it function as an effective cellular topoisomerase II poison?

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* To whom correspondence should be addressed: phone (615) 322-4338; fax (615) 343-1166; e-mail osheron@ctrvax.vanderbilt.edu.

§ Trainee under National Institutes of Health Grant 5 T32 CA09582.

‡ Department of Biochemistry, Vanderbilt University School of Medicine.

¶ Department of Medicine, Vanderbilt University School of Medicine.

|| Department of Chemistry, University of Mississippi.

⊥ Hanno Research Center, Taiho Pharmaceutical Co., Ltd.

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To resolve this apparent paradox, the inhibitory properties of TAS-103 were characterized in greater detail. The present study indicates that TAS-103 intercalates into DNA and that the previously reported topoisomerase I inhibition results from drug-induced DNA unwinding rather than a specific effect on the DNA strand passage activity of the enzyme. Conversely, the drug decreases the overall catalytic activity of topoisomerase II, probably through an inhibition of enzyme-mediated DNA religation. The finding that topoisomerase I can perform its catalytic functions in the presence of TAS-103 explains how the drug can function as a competent topoisomerase II poison in the cell.

EXPERIMENTAL PROCEDURES

Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* (*30*) and purified by the protocol of Kingma et al. (*31*). Calf thymus topoisomerase I was purchased from GIBCO BRL. Negatively supercoiled pBR322 DNA was prepared as described (*32*). Etoposide, ellipticine, ethidium bromide, and calf thymus DNA were from Sigma, and [γ-^{32}P]ATP (~3000 Ci/mmol) was from Amersham. Etoposide and ellipticine were stored at 4 °C as 10 mM stock solutions in 100% DMSO. Ethidium bromide was stored at 4 °C as a 25 mM stock solution in water. TAS-103 was provided as the dichloride salt by Taiho Pharmaceuticals and was stored at 4 °C as a 10 mM stock solution in water. All other chemicals were analytical reagent grade.

TAS-103 Molecular Modeling. Molecular models of TAS-103 were constructed using Discover and the BUILDER program of INSIGHT II (Molecular Simulations, Inc.). The distribution of atomic charges was obtained using MOPAC (MNDO), and the energy minimized structure for TAS-103 was obtained using the conjugate-gradient minimization algorithm. A duplex B-DNA structure of the sequence d(CGCGCG) complexed with its complementary strand was constructed using the BIOPOLYMER program of INSIGHT II. Sodium atoms were added at a distance of 2.5 Å from each phosphate to the duplex DNA using the counterion routine of INSIGHT II. TAS-103 was manually docked within the central 5′-GpC-3′ step of the oligonucleotide to provide maximum overlap between the planar aromatic rings of the ligand and adjacent base pairs. Periodic boundary conditions were imposed on the ligand−DNA complex with an additional 3.5 Å of space added to all sides of the complex. Water molecules were added to the ligand−DNA duplex structure using the solvation routine of INSIGHT II, and atomic potentials were added to all atoms of the assembly using the AMBER force field. The hydrated ligand−DNA complex was subjected to energy minimization under periodic boundary conditions using 2000 steepest-descent iterations to reduce the maximum RMS derivative to less than 0.5, followed by 10 000 iterations using the conjugate-gradient method to reduce the maximum RMS derivative to less than 0.001. A distance-dependent dielectric constant of 1.0 was used for these calculations. Computations were performed on a Silicon Graphics Indigo Elan workstation and visualized using INSIGHT II.

TAS-103−DNA Binding. Calf thymus DNA was prepared for DNA binding studies using the method described by Chaires et al. (*33*). The DNA concentration was determined by UV absorbance at 260 nm using a molar absorptivity constant of 13 200 $M(bp)^{-1}$ cm^{-1}. Binding assays were carried out in 10 mM sodium phosphate, pH 7.9, 100 mM NaCl, and 1 mM EDTA. The buffer was degassed and filtered through a 0.45 micron filter (Millipore) prior to use.

Optical data were collected using a Cary 4 UV−visible spectrophotometer (Varian) equipped with a Lauda R61 circulating water bath. Absorbance titrations were carried out in 10 cm cylindrical quartz cells at a constant temperature of 25 °C. Molar absorptivities for the free and bound species of TAS-103 (ϵ_f and ϵ_b) were determined experimentally at the wavelength of maximal absorption. The free drug exhibited an ϵ_f of 9625 M^{-1} cm^{-1} at 485 nm. Molar absorptivities for bound species of TAS-103 (ϵ_b) were determined by titrating aliquots of concentrated calf thymus DNA into a dilute drug solution and extrapolating changes in drug absorbance to infinite DNA concentrations. The bound drug exhibited an ϵ_b of 3525 M^{-1} cm^{-1} at 491 nm.

Binding titrations were performed by adding aliquots of TAS-103 to fixed concentrations of DNA. To minimize TAS-103 aggregation, the level of free drug was maintained below 3 μM throughout all binding studies. Concentrations of bound drug were determined by monitoring changes in the absorbance of TAS-103. Theoretical fits for the binding data were calculated using a nonlinear least-squares analysis (Origin, Microcal Inc.) of the noncooperative neighbor exclusion model of McGhee and von Hippel (*34*).

Topoisomerase I DNA Unwinding Assays. The ability of TAS-103 to unwind plasmid DNA was determined as described by Fortune and Osheroff (*35*). Relaxed pBR322 plasmid DNA utilized in unwinding assays was generated by treating negatively supercoiled pBR322 with topoisomerase I in topoisomerase I reaction buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 30 μg/mL bovine serum albumin) prior to the addition of other reaction components. Assay mixtures contained 5 nM relaxed pBR322 plasmid DNA, topoisomerase I (10 units), and drug in 40 μL of topoisomerase I reaction buffer. Drugs employed in this study were TAS-103 (10 or 100 μM), etoposide (100 μM), ellipticine (100 μM), or a DMSO control (final DMSO concentration was adjusted to 5% in all samples). Following a 5 min incubation of DNA and drug, topoisomerase I was added, and reactions were incubated for 20 min at 37 °C. Reactions were stopped by adding an equal volume of phenol−chloroform. Aqueous samples (20 μL) were removed from the reactions, and 3 μL of stop solution (0.77% SDS, 77 mM NaEDTA, pH 8.0) followed by 2 μL of agarose gel loading buffer (30% sucrose, in 10 mM Tris-HCl, pH 7.9) was added to each. Samples were subjected to electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.3, 2 mM EDTA). DNA bands were stained with 1 μg/mL ethidium bromide, visualized with UV light, and photographed through Kodak 23A and 12 filters with Polaroid type 665 positive/negative film.

The concentration of TAS-103 required to unwind relaxed pBR322 DNA was determined as above over a range of TAS-103 concentrations (0−25 μM).

Topoisomerase I-Catalyzed DNA Strand Passage. The DNA strand passage activity of topoisomerase I was determined by monitoring the ability of the enzyme to relax negatively supercoiled plasmid molecules in the absence of drug (*36*) or to supercoil relaxed plasmid substrates in the presence of intercalative agents (*35*). DNA strand passage

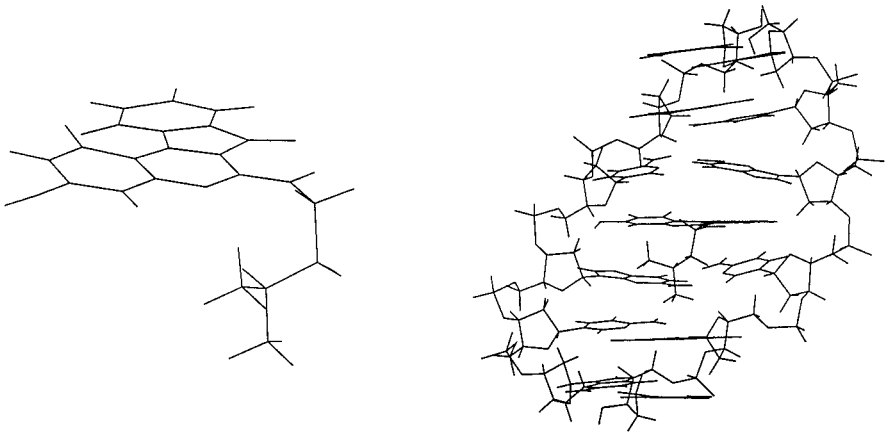

FIGURE 1: Molecular modeling of TAS-103. The energy minimized structures of TAS-103 (left) and a TAS-103/DNA complex (right) are shown. The B-form double-stranded oligonucleotide sequence d(CGCGCG) complexed with its complementary strand was used in modeling studies of the TAS-103/DNA complex.

assays contained 5 nM pBR322 plasmid DNA (relaxed or supercoiled, as above) and topoisomerase I (1 unit) in 40 μL of topoisomerase I reaction buffer. Reactions were carried out in the absence of drug or in the presence of 100 μM TAS-103 or ethidium bromide. All reaction mixtures contained 5% DMSO (final concentration). Following a 5 min incubation of DNA with drug or DMSO, topoisomerase I was added, and reactions were incubated up to 10 min at 37 °C. Reactions were stopped, processed, and subjected to gel electrophoresis as above.

Topoisomerase II DNA Strand Passage Assays. The effect of TAS-103 on the catalytic activity of human topoisomerase IIα was assessed by a DNA strand passage assay. This assay monitors the ability of the enzyme to introduce negative superhelical twists in relaxed plasmid in the presence of an intercalative drug. Reaction mixtures contained 5 nM relaxed pBR322 plasmid DNA, 7.5 nM human topoisomerase IIα, 1 mM ATP, and 0−50 μM TAS-103 in 40 μL of topoisomerase II reaction buffer (50 mM Tris-HCl, pH 7.9, 135 mM KCl, 10 mM $MgCl_2$, 0.5 mM NaEDTA, and 2.5% glycerol). Following a 5 min drug−DNA incubation, topoisomerase II was added, and samples were incubated for 15 min at 37 °C. Reactions were stopped, processed, and subjected to gel electrophoresis as described for topoisomerase I DNA unwinding assays. Relaxed pBR322 plasmid DNA used in these assays was generated by treating negatively supercoiled pBR322 with topoisomerase I in topoisomerase II reaction buffer and then heat inactivating this enzyme prior to the addition of other reaction components.

The time dependence of topoisomerase II DNA strand passage assays was examined in the presence of ethidium bromide and TAS-103. These assays were performed as above, except that drug concentration was fixed at 10 μM, and the incubation period at 37 °C was varied from 0 to 30 min.

ATP Hydrolysis. ATPase assays were performed as described by Osheroff et al. (*36*). Reaction mixtures contained 50 nM human topoisomerase IIα, 40 nM negatively supercoiled pBR322 DNA, 1 mM [γ-^{32}P]ATP, and 0−50 μM TAS-103 in 60 μL of topoisomerase II reaction buffer. Reactions were initiated by the addition of topoisomerase IIα and incubated at 37 °C. Samples (2 μL) were removed at time intervals up to 16 min and spotted on polyethyleneimine-impregnated thin layer cellulose chromatography plates (J. T. Baker). Plates were developed by chromatography in freshly made 400 mM NH_4HCO_3. Radioactive areas corresponding to inorganic monophosphate released by ATP hydrolysis were quantitated on a Molecular Dynamics PhosphorImager.

RESULTS

Although most topoisomerase poisons also inhibit the overall catalytic activity of their enzyme targets, they do so only at relatively high concentrations (*1*, *22*−*25*). In contrast, TAS-103 has been reported to inhibit the ability of topoisomerase I to relax negatively supercoiled DNA ($IC_{50} \approx 2$ μM) and topoisomerase II to decatenate kinetoplast DNA ($IC_{50} \approx 6.5$ μM) in the same concentration range required for the stimulation of enzyme-mediated DNA cleavage (*26*). Since TAS-103 kills cells by acting as a topoisomerase II poison (*29*), the apparent inhibition of both topoisomerases by this anticancer agent raises a critical issue regarding its cytotoxic mechanism. Without a topoisomerase activity to alleviate torsional stress, the DNA tracking systems that are necessary to convert transient topoisomerase II-generated DNA breaks into permanent, lethal strand breaks should be halted (*5*, *15*−*17*). Thus, if TAS-103 blocks the catalytic strand passage activities of both topoisomerase I and II, it should undermine the cellular processes that promote its action as a topoisomerase poison. To address this important issue, several approaches were utilized to characterize the inhibitory properties of TAS-103.

TAS-103 Modeling Studies. Modeling studies were carried out to provide initial insight into the underlying basis for the inhibition of topoisomerase I and II by the drug. The quinoline ring system of TAS-103 is largely planar, with a small propeller twist localized primarily to the hydroxyphenyl substituent (Figure 1, left). The planar ring structure, together with the positively charged dimethylamino moiety, suggests that TAS-103 may be a DNA intercalating agent.

This suggestion is further supported by studies that modeled the interaction between TAS-103 and the B-form DNA oligonucleotide d(CGCGCG) complexed with its complementary strand (Figure 1, right). Results predicted an intercalative interaction between TAS-103 and the double helix. In this model, there is potential for multiple hydrogen-

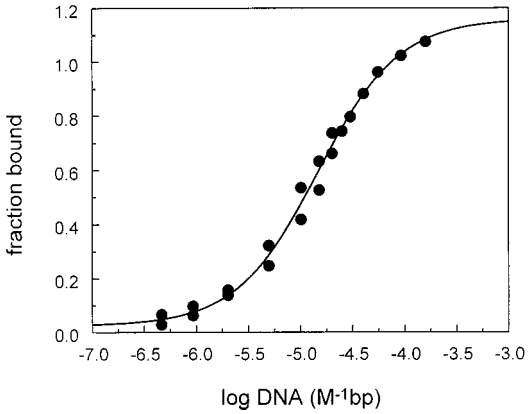

FIGURE 2: TAS-103 binds DNA. Changes in TAS-103 absorbance upon DNA binding were monitored over a range of DNA concentrations. The fraction of drug bound at different DNA concentrations (expressed as M^{-1} bp) was plotted, and an apparent K_d value was generated from a nonlinear least-squares fit. Data represent a composite of two independent experiments.

bonding interactions between the drug and its intercalation site. In addition, the dimethylaminoethylamino side chain attached to the quinoline ring is predicted to reside in the minor groove.

TAS-103 Binds DNA. As a follow up to the TAS-103 modeling studies, interactions between the drug and calf thymus DNA were characterized. A drug titration curve was generated from DNA-induced changes in the maximal absorbance of TAS-103 (Figure 2). Based on a nonlinear least-squares fit of these data, the drug binds to DNA with an apparent dissociation constant of ~2.2 μM. The binding site size (n) for TAS-103 was calculated to be ~1.9 ± 0.4, implying one drug molecule bound per two base pairs at saturation. These properties are indicative of an intercalative drug−DNA interaction (*37*, *38*). It should be noted that the drug−DNA binding isotherm deviated at TAS-103 concentrations higher than 5 μM. This result suggests that in addition to its intercalative mode of DNA binding, TAS-103 displays outside binding or stacking interactions at high concentrations.

TAS-103 Intercalates into DNA. The drug modeling and DNA binding studies presented above suggest that TAS-103 is an intercalative drug. Therefore, a DNA unwinding assay was used to further characterize drug−DNA interactions.

Since intercalative drugs locally unwind DNA, they induce compensatory unconstrained positive superhelical twists in distal regions of covalently closed circular DNA (*13*). In the presence of such compounds, a plasmid that is actually relaxed (i.e., contains no superhelical twists) appears to be positively supercoiled. Likewise, a negatively supercoiled plasmid will appear to be less supercoiled, fully relaxed, or even positively supercoiled as drug concentration increases.

Treatment of drug−DNA complexes with mammalian topoisomerase I removes the unconstrained positive DNA superhelical twists that result from drug intercalation. Following this treatment, extraction of the drug allows the local drug-induced unwinding to redistribute in a global manner and manifest itself as a net negative supercoiling of the plasmid. Thus, in the presence of an intercalative agent such as ellipticine, topoisomerase I treatment converts relaxed plasmids to negatively supercoiled molecules (Figure 3). Conversely, when a nonintercalative drug such as etoposide is included in reaction mixtures, no DNA supercoiling is observed following treatment with the type I enzyme.

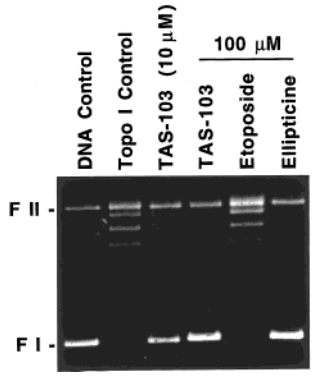

FIGURE 3: TAS-103 intercalates into DNA. Since intercalative compounds unwind circular plasmid DNA molecules by generating constrained negative superhelical twists, they induce positive supercoiling in distal regions of a relaxed plasmid substrate. Treatment with topoisomerase I removes these unconstrained positive superhelical twists, and the overall result (upon removal of drug) is the conversion of relaxed plasmid DNA to negatively supercoiled molecules. To determine whether TAS-103 is intercalative, the drug (10 or 100 μM) was incubated with relaxed pBR322 plasmid DNA in the presence of calf thymus topoisomerase I. Similar control reactions included etoposide (100 μM) or ellipticine (100 μM) in place of TAS-103. Negatively supercoiled pBR322 DNA (DNA control) and relaxed pBR322 (Topo I Control) are shown for reference. The positions of negatively supercoiled plasmid DNA (form I, FI) and nicked circular plasmid DNA (form II, FII) are indicated (also in following figures).

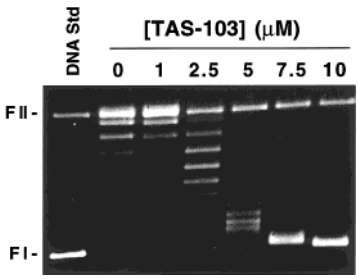

FIGURE 4: Complete unwinding of pBR322 plasmid DNA requires ~10 μM TAS-103. A DNA unwinding assay was carried out by incubating calf thymus topoisomerase I with relaxed pBR322 plasmid DNA over a range of TAS-103 concentrations. Negatively supercoiled pBR322 DNA (DNA Std) is shown for reference.

In the presence of 10 or 100 μM TAS-103, relaxed plasmid substrates were converted to negatively supercoiled molecules by treatment with topoisomerase I (Figure 3). This finding provides strong evidence that TAS-103 intercalates into DNA.

Since the plasmid was fully supercoiled at both concentrations of TAS-103 tested, DNA unwinding was assessed at lower drug concentrations (Figure 4). Complete conversion of relaxed pBR322 DNA to negatively supercoiled species required ~10 μM TAS-103. The midpoint of this transformation was ~2.5 μM TAS-103. This concentration is comparable to the apparent dissociation constant for drug−DNA interactions (see above).

TAS-103 Does Not Inhibit the Catalytic Activity of Topoisomerase I. A previous study reported that low micromolar concentrations of TAS-103 inhibited the ability of topoisomerase I to relax negatively supercoiled plasmid DNA ($IC_{50} \approx 2$ μM) (*26*). A similar drug-induced block in DNA relaxation was observed in the present study (data not

shown). These findings imply that TAS-103 is a catalytic inhibitor of topoisomerase I that blocks the DNA strand passage event of the enzyme.

Since TAS-103 is an intercalative drug, this apparent inhibition of topoisomerase I function may be explained by an alternative possibility. TAS-103 may have no direct effect on enzyme catalysis but may interfere with the DNA relaxation reaction by altering the apparent topological state of the negatively supercoiled DNA substrate. As discussed above, the presence of an intercalative drug induces constrained negative and unconstrained positive superhelical twists in plasmid DNA. Since topoisomerase I removes only the unconstrained positive supercoils, the product of such a reaction, negatively supercoiled DNA, would be identical to the topological state of the original plasmid substrate. Thus, no net relaxation would be observed, and the drug would appear to inhibit enzyme catalysis.

Support for this latter possibility comes from the fact that the IC_{50} of TAS-103 (based on DNA relaxation assays) coincides with the midpoint concentration for drug-induced DNA unwinding. Furthermore, since (1) DNA unwinding assays require active topoisomerase I, and (2) complete supercoiling of relaxed plasmid substrates was observed even at 100 μM TAS-103 (see Figure 3), it appears that the topoisomerase I can catalyze DNA strand passage even at high concentrations of the drug. However, there is a limitation to this conclusion. Since the concentration of topoisomerase I used in these assays was ~10 times higher than required to completely relax negatively supercoiled DNA, substantial enzyme inhibition by TAS-103 could have gone undetected.

Therefore, an experiment was designed to determine whether TAS-103 interferes with the DNA relaxation reaction by inhibiting topoisomerase I catalysis or by altering the apparent topological state of DNA. In this experiment, the effects of TAS-103 on enzyme-catalyzed DNA strand passage were assessed by comparing the rate of relaxation of negatively supercoiled plasmid in the absence of drug to the rate of supercoiling of relaxed plasmid in the presence of 100 μM TAS-103 or ethidium bromide (which intercalates into DNA in the same concentration range as TAS-103). It should be noted that the concentration of TAS-103 used in this assay was ~50 times higher than the reported IC_{50} for the drug.

As seen in Figure 5, the rate of topoisomerase I-catalyzed DNA relaxation in the absence of drug was identical to the rate of DNA supercoiling in the presence of ethidium bromide. If anything, rates of supercoiling were faster in the presence of TAS-103. These findings provide conclusive evidence that TAS-103 is not a catalytic inhibitor of mammalian topoisomerase I. Rather, the reported inhibition of enzyme-catalyzed DNA relaxation appears to result from a drug-induced alteration in the apparent topology of the nucleic acid substrate.

TAS-103 Is a Catalytic Inhibitor of Topoisomerase II. In a previous study, TAS-103 was reported to inhibit topoisomerase II-catalyzed DNA decatenation in human nuclear extracts ($IC_{50} \approx 6.5$ μM) (*26*). A similar drug-induced inhibition ($IC_{50} \approx 4$ μM) was noted in the present study for the decatenation of kinetoplast DNA by purified human topoisomerase IIα (data not shown).

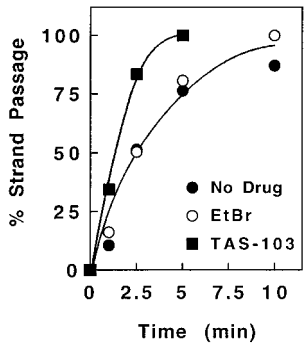

FIGURE 5: TAS-103 does not inhibit the catalytic activity of topoisomerase I. The DNA strand passage activity of calf thymus topoisomerase I was determined from the ability of the enzyme to relax supercoiled pBR322 plasmid DNA in the absence of drug or to introduce supercoiling in relaxed plasmid substrate in the presence of the intercalative agents ethidium bromide (EtBr, 100 μM) or TAS-103 (100 μM). Strand passage is expressed as the percentage of supercoiled DNA converted to relaxed (no drug reaction) or the percentage of relaxed plasmid converted to supercoiled (ethidium bromide and TAS-103 reactions). Data are representative of three independent experiments.

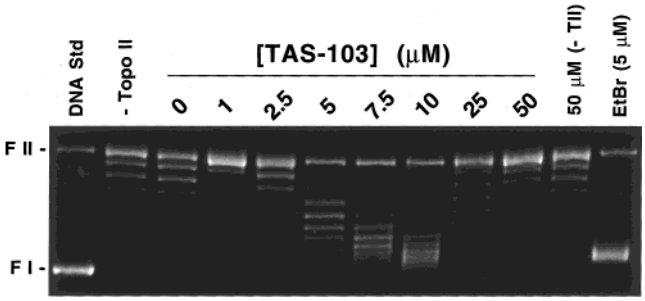

FIGURE 6: TAS-103 inhibits the catalytic activity of human topoisomerase IIα. A DNA strand passage assay (15 min) monitored the ability of topoisomerase II to introduce superhelical twists into relaxed pBR322 plasmid DNA in the presence of the intercalative drug TAS-103. The following control samples are shown: supercoiled plasmid (DNA Std); relaxed plasmid (-Topo II); 5 μM ethidium bromide (EtBr) and 50 μM TAS-103 in the absence of topoisomerase II (-TII).

The topological state of DNA can affect the ability of topoisomerase II to catenate or decatenate closed circular substrates (*39*, *40*). Therefore, a DNA strand passage assay (similar to that described above for topoisomerase I) was utilized to distinguish the effects of TAS-103 on topoisomerase II function from its effects on DNA topology. Initial experiments characterized the drug dependence of DNA supercoiling in a topoisomerase II DNA strand passage assay.

Human topoisomerase IIα converted relaxed plasmids to negatively supercoiled molecules in the presence of TAS-103 (Figure 6). However, DNA supercoiling was less than observed in topoisomerase I assays at drug concentrations up to 10 μM (compare Figures 4 and 6) and was completely blocked at drug concentrations greater than 25 μM. These findings indicate that TAS-103 impairs the catalytic DNA strand passage reaction of topoisomerase II.

To further assess this inhibition of human topoisomerase IIα, the time dependence of enzyme-catalyzed DNA supercoiling in the presence of 10 μM TAS-103 was compared to that obtained with 10 μM ethidium bromide. This concentration of either drug induces complete plasmid supercoiling with topoisomerase I, and the latter compound

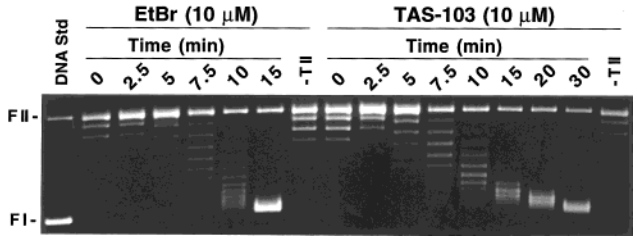

FIGURE 7: Topoisomerase II catalytic activity is decreased by TAS-103. Rates of topoisomerase II-catalyzed DNA strand passage were determined from the ability of human topoisomerase IIα to introduce superhelical twists into relaxed pBR322 plasmid DNA in the presence of 10 μM ethidium bromide (EtBr) or TAS-103. Controls shown are supercoiled DNA (DNA Std), and samples with each drug in the absence of topo II (-TII).

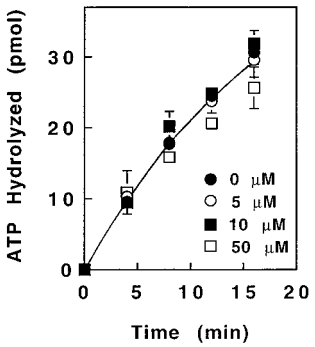

FIGURE 8: TAS-103 has little effect on the ATPase activity of human topoisomerase IIα. The products of enzyme-catalyzed ATP hydrolysis were analyzed by thin-layer chromatography. ATPase activity was determined by the amount of free phosphate released from [γ-^{32}P]ATP. TAS-103 concentrations employed were 0, 5, 10, and 50 μM. Data represent the averages of two independent experiments. Standard errors are depicted by error bars.

does not affect the DNA strand passage reaction of eukaryotic type II topoisomerases (*36*).

In the presence of ethidium bromide, human topoisomerase IIα fully supercoiled relaxed plasmid within 15 min (Figure 7). However, this process required at least 30 min when TAS-103 was present. These findings confirm that TAS-103 is a catalytic inhibitor of human topoisomerase IIα, even at low concentrations of the drug.

TAS-103 Does Not Inhibit the ATPase Activity of Topoisomerase II. As described in the accompanying paper, TAS-103 kills cells by acting as a potent topoisomerase II poison (*29*). In addition to their effects on enzyme-mediated DNA cleavage/religation, virtually every topoisomerase II poison also interferes with interactions between the enzyme and its required ATP cofactor (*25, 41, 42*).[1] These interactions are essential to the catalytic function of type II topoisomerases and are required to trigger DNA strand passage and subsequent turnover of the enzyme (*1, 7, 16, 36, 43*).

To determine whether TAS-103 inhibits the catalytic activity of human topoisomerase IIα by blocking ATP utilization, rates of ATP hydrolysis were monitored. As seen in Figure 8, concentrations of TAS-103 up to 10 μM had no effect on the ATPase activity of the enzyme. Moreover, 50 μM TAS-103, a drug concentration that completely blocks the catalytic DNA strand passage activity of topoisomerase IIα (see Figure 6), decreased rates of hydrolysis only slightly (<15%).

These findings indicate that the inhibition of topoisomerase II activity by TAS-103 does not result from alterations in enzyme−ATP interactions. Since TAS-103 is a topoisomerase II poison, it is also unlikely that the drug adversely affects either enzyme−DNA binding or DNA cleavage. As demonstrated in the accompanying paper, TAS-103 increases levels of topoisomerase II-generated DNA breaks primarily by inhibiting the ability of the enzyme to religate cleaved DNA (*29*). Taken together, these results suggest that TAS-103 enhances topoisomerase II-mediated DNA scission and inhibits topoisomerase II catalytic activity by blocking the DNA religation reaction of the enzyme.

DISCUSSION

Beyond their ability to stimulate enzyme-mediated DNA cleavage, most topoisomerase poisons also inhibit the overall catalytic activity of their respective enzyme target (*1, 22−25*). Typically, these drugs do so only at concentrations that exceed their clinically relevant range. However, TAS-103 appeared to be an atypical topoisomerase poison, because it reportedly inhibited the catalytic activity of topoisomerase I and II at drug concentrations similar to those required to enhance DNA scission (*26*).

TAS-103 binds to topoisomerase IIα and stimulates the DNA cleavage activity of the enzyme (*26, 29*). However, given the proposed inhibitory properties of TAS-103, it was not clear how the drug could function as a potent topoisomerase II poison in the cell. Topoisomerase activity is required to support the actions of polymerases and helicases, enzymes that convert transient drug-induced topoisomerase II-DNA cleavage intermediates into permanent chromosomal breaks (*5, 7, 9, 17, 19−21*). Consequently, if TAS-103 blocks the catalytic activity of both topoisomerase I and II under therapeutic conditions, the drug should impair the cellular processes that promote its cytotoxicity.

The present study offers an explanation for this apparent incongruity. Drug modeling, DNA binding, and DNA unwinding studies provide strong evidence that TAS-103 intercalates into the double helix. Although TAS-103 interferes with the overall catalytic activity of topoisomerase II independently from its intercalative properties, the reported decrease in topoisomerase I-catalyzed DNA relaxation is caused solely by a drug-induced alteration in the apparent topology of plasmid substrates. When this change in plasmid topology is taken into account, no topoisomerase I inhibition is observed. Even at drug concentrations orders of magnitude beyond its cytotoxic range, TAS-103 displays no ability to interfere with the DNA strand passage reaction catalyzed by the type I enzyme. Thus, cells treated with TAS-103 should exhibit normal levels of topoisomerase I activity, and the DNA tracking systems required to initiate drug-induced cell death pathways should not be impeded by the accumulation of topological stress in the genetic material.

TAS-103 was initially believed to be a dual topoisomerase I/topoisomerase II poison that blocked the catalytic activities of both enzymes at clinically pertinent concentrations. However, mechanistic studies presented in this and the preceding paper have altered our concepts regarding TAS-103 action. It now appears that TAS-103 kills cells primarily

[1] The only reported exception to this finding is etoposide, which has no significant effect on the ability of *Drosophila* topoisomerase II to hydrolyze its ATP cofactor (*41*). However, a subsequent study determined that this drug inhibits the ATPase activity of human topoisomerase IIα (*42*).

by acting as a topoisomerase II poison. Moreover, the drug has no discernible effect on topoisomerase I catalysis. This information clarifies the basis for TAS-103 cytotoxicity and could potentially impact future development of the drug.

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