DNA Topoisomerases as Targets for the Anticancer Drug TAS-103: Primary Cellular Target and DNA Cleavage Enhancement†

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ABSTRACT: TAS-103 is a novel antineoplastic agent that is active against in vivo tumor models [Utsugi, T., et al. (1997) *Jpn. J. Cancer Res. 88,* 992−1002]. This drug is believed to be a dual topoisomerase I/II-targeted agent, because it enhances both topoisomerase I- and topoisomerase II-mediated DNA cleavage in treated cells. However, the relative importance of these two enzymes for the cytotoxic actions of TAS-103 is not known. Therefore, the primary cellular target of the drug and its mode of action were determined. TAS-103 stimulated DNA cleavage mediated by mammalian topoisomerase I and human topoisomerase IIα and *β* in vitro. The drug was less active than camptothecin against the type I enzyme but was equipotent to etoposide against topoisomerase IIα. A yeast genetic system that allowed manipulation of topoisomerase activity and drug sensitivity was used to determine the contributions of topoisomerase I and II to drug cytotoxicity. Results indicate that topoisomerase II is the primary cellular target of TAS-103. In addition, TAS-103 binds to human topoisomerase IIα in the absence of DNA, suggesting that enzyme-drug interactions play a role in formation of the ternary topoisomerase II·drug·DNA complex. TAS-103 induced topoisomerase II-mediated DNA cleavage at sites similar to those observed in the presence of etoposide. Like etoposide, it enhanced cleavage primarily by inhibiting the religation reaction of the enzyme. Based on these findings, it is suggested that TAS-103 be classified as a topoisomerase II-targeted drug.

TAS-103 is a novel quinoline derivative (Figure 1) that displays marked antitumor activity in murine and human tumor models and currently is in phase I clinical trials (*1*−*3*). Although the cytotoxic mechanism of the drug has not yet been established, it is believed to act through an effect on DNA topoisomerases (*1*).

DNA topoisomerases are enzymes that control the topological state of DNA in the cell (*4*−*8*). There are two classes of topoisomerases. Type I enzymes regulate DNA under- and overwinding by generating transient single-stranded breaks in the double helix (*6, 9, 10*). Type II enzymes resolve DNA knots and tangles by creating transient double-stranded breaks in the genetic material (*7, 8, 11, 12*). In addition to their critical physiological functions, DNA topoisomerases

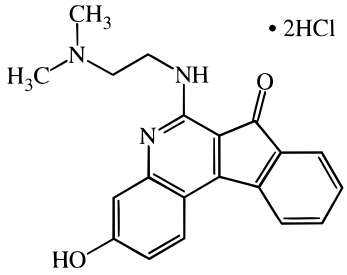

FIGURE 1: Structure of TAS-103.

are targets for several clinically important anticancer drugs (*6*−*8, 13*−*18*). Topoisomerase I is the target for a series of camptothecin-based agents, while topoisomerase II is the target for six FDA-approved drugs, including etoposide, doxorubicin, and mitoxantrone.

These anticancer drugs act in an unusual fashion; rather than depriving cells of the important catalytic activities of topoisomerases, they increase levels of topoisomerase-mediated DNA cleavage (*7, 8, 13*−*16, 19, 20*). As a result, they convert these enzymes into potent toxins that generate high levels of DNA breaks in treated cells. Consequently, they are referred to as topoisomerase "poisons".

TAS-103 has multiple effects on DNA topoisomerases. First, it is a topoisomerase poison that increases cellular levels of DNA breaks created by both the type I and II enzymes (*1*). Although the effects of TAS-103 on topoisomerase I are smaller than those observed with camptothecin, its effects

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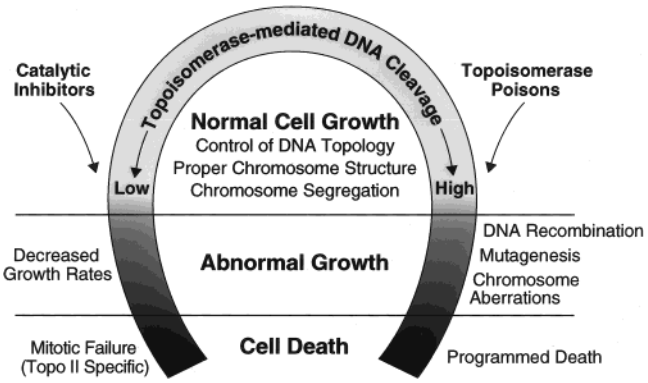

FIGURE 2: The role of topoisomerases in the life and death of cells. Topoisomerases control the topological state of DNA and are required for a number of important DNA processes (*4*−*7*). Topoisomerase II is essential for cell growth and survival, since it is required for chromosome segregation in mitosis (*4*−*7*). Despite their importance, topoisomerase I or II can become harmful if its DNA cleavage activity is abnormally high. Increased DNA cleavage can induce chromosomal damage and ultimately programmed cell death (*6*−*8*). A number of antineoplastic drugs, referred to as topoisomerase poisons, kill cells by increasing levels of topoisomerase I- or II-mediated DNA cleavage (*6*−*8*, *18*). A second class of drugs, referred to as catalytic inhibitors, kills cells by blocking topoisomerase activity (*8*, *23*). Cells treated with catalytic inhibitors of topoisomerase II die as a result of mitotic failure.

on topoisomerase II are comparable to those seen with etoposide. Second, physiologically relevant concentrations of TAS-103 have been reported to inhibit DNA relaxation and decatenation catalyzed by topoisomerase I and topoisomerase II, respectively (*1*) (addressed in the accompanying paper (*21*)). It is not clear which, if any, of these effects contributes to the clinical efficacy of TAS-103.

Each action of TAS-103 has potentially significant cellular ramifications. Treatment with topoisomerase I or topoisomerase II poisons generates permanent chromosomal breaks and triggers programmed cell death pathways (Figure 2) (*14*, *15*, *20*, *22*). Despite these similarities, drugs directed against topoisomerase I or II induce a different spectrum of DNA damage, require different physiological conditions for optimal cytotoxicity, and display different activity toward various forms of human cancers (*16*−*18*, *24*).

Inhibition of topoisomerase catalytic activity impairs critical DNA processes such as replication, transcription, and recombination (*8*, *23*, *25*) (Figure 2). Inhibition of topoisomerase II induces mitotic failure by preventing chromosome segregation (*5*, *8*, *25*−*31*). Since topoisomerase II can compensate for the loss of the type I enzyme (*26*, *27*, *32*), it is unclear whether cell death can be induced by blocking the actions of topoisomerase I. In fact, yeast strains lacking topoisomerase I display only minor phenotypic variations (*26*, *27*, *32*).

If the clinical potential of TAS-103 is to be fully exploited, the basis for its cytotoxic actions must be reconciled. Therefore, the present manuscript determined the primary cellular target of the drug and its mode of action. Results indicate that TAS-103 kills cells by increasing levels of topoisomerase II-mediated DNA cleavage. The accompanying manuscript describes the effects of TAS-103 on topoisomerase I and topoisomerase II catalysis and the relationship of these effects to drug cytotoxicity (*21*).

EXPERIMENTAL PROCEDURES

Human topoisomerase IIα and topoisomerase IIβ were expressed in *Saccharomyces cerevisiae* (*33*) and purified by the protocol of Kingma et al. (*34*). Calf thymus topoisomerase I was purchased from GIBCO BRL. Negatively supercoiled pBR322 DNA was prepared as described (*35*). Dulbecco's phosphate buffered saline was from HyClone; bacteriophage T4 polynucleotide kinase and restriction endonucleases were from New England Biolabs; [γ-^{32}P]ATP ($\sim$6000 Ci/mmol) was from Amersham; amsacrine was from Bristol-Myers Squibb; etoposide, camptothecin, and ethidium bromide, camptothecin, were from Sigma. Amsacrine, camptothecin, and etoposide were stored at 4 °C as 10 mM stock solutions in 100% DMSO. TAS-103 was provided as the dichloride salt by Taiho Pharmaceuticals and stored at 4 °C as a 10 mM stock solution in water. All other chemicals were analytical reagent grade.

The parental yeast strain used in this study was *S. cerevisiae* JN394, whose genotype is *ura3-52*, *leu2*, *trp1*, *his7*, *ade1-2*, *ISE2*, *rad52::LEU2* (*36*). Three strains derived from JN394 also were employed: JN394top1$^-$, which has a chromosomal deletion of the topoisomerase I gene (*37*); JN394t2-1, in which the wild-type topoisomerase II gene (*TOP2*+) is replaced with the temperature-sensitive *top2-1* mutant allele (*25*, *36*, *38*); and JN394t2-5, in which the wild-type topoisomerase II gene is replaced with the drug-resistant *top2-5* mutant allele (*39*, *40*). These three strains are isogenic to JN394 in all other respects.

DNA Cleavage. Topoisomerase I DNA cleavage reactions contained calf thymus topoisomerase I (14 units), 5 nM negatively supercoiled pBR322 DNA, and 0−25 μM TAS-103 (or camptothecin) in a total of 20 μL of 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. Reactions were started by the addition of drug and incubated for 6 min at 37 °C to allow the cleavage/religation reaction of the enzyme to reach equilibrium. Cleavage intermediates were trapped by adding 2 μL of 1% SDS, followed by 2 μL of 250 mM NaEDTA, pH 8.0. Proteinase K was added (2 μL of 0.8 mg/mL), and reactions were incubated 30 min at 45 °C to digest the topoisomerase I. Samples were mixed with 2 μL of agarose gel loading buffer (30% sucrose, 0.5% bromophenol blue, and 0.5% xylene cyanole FF in 10 mM Tris-HCl, pH 7.9), heated at 70 °C for 2 min, and subjected to electrophoresis in a 1% agarose gel in TBE buffer (100 mM Tris-borate, pH 8.3, 2 mM EDTA) containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled plasmid to nicked DNA. DNA bands were visualized by UV light, photographed through Kodak 23A and 12 filters with Polaroid type 665 positive/negative film, and quantitated by scanning photographic negatives with an E-C apparatus model EC910 scanning densitometer in conjunction with Hoefer GS-370 Data System software. The intensity of bands in the negative was proportional to the amount of DNA present.

Topoisomerase II DNA cleavage reactions were carried out as described previously (*41*). Assays contained 40 nM human topoisomerase IIα or topoisomerase IIβ, 10 nM negatively supercoiled pBR322 DNA, 1 mM ATP, and 0−100 μM TAS-103 (or 0−50 μM etoposide) in a total of 20 μL of topoisomerase II cleavage buffer (10 mM Tris-HCl, pH 7.9, 135 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA,

and 2.5% glycerol). Reactions were started by the addition of topoisomerase II and incubated for 6 min at 37 °C to establish DNA cleavage/religation equilibria. Cleavage intermediates were trapped by adding 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0, and proteinase K treatment was carried out as above. Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 2 min, and subjected to electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.3, 2 mM EDTA) containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled DNA to linear molecules. Gel photography and quantitation of DNA bands were as described above. Alternatively, DNA bands were quantitated using an Alpha Innotech digital imaging system.

Determination of the Primary Cellular Target of TAS-103. Sensitivity of yeast strains JN394, JN394top1$^-$, JN394t2-1, and JN394t2-5 to TAS-103 was determined as previously described (*36*−*38*, *40*). Briefly, cells were cultured in YPDA media at 25 °C. When appropriate, cells also were cultured at 30 °C. After logarithmically growing cells were adjusted to a titer of 1×10^6 cells/mL, TAS-103 (0−100 μM) was added to the cultures. Cells were incubated with drug for 24 h, then diluted into drug-free YPDA media, and plated in triplicate onto YPDA media solidified with 1.5% Bacto-agar. In experiments that utilized strain JN394top1$^-$, cells were plated in triplicate onto synthetic complete media solidified with 1.5% Bacto-agar. Plates were incubated at 25 °C, and drug sensitivy was determined by counting the number of surviving colonies. In some experiments, TAS-103 cytotoxicity was compared to that of camptothecin.

Topoisomerase II-TAS-103 Binding. Binding studies were based on fluorescence anisotropy measurements and utilized the intrinsic fluorescence of TAS-103. Assays were performed at 25 °C with an ISS PC2 spectrofluorometer. TAS-103 was excited at 470 nm with a xenon arc lamp (18 ampere current) in a 0.4 cm silica quartz glass cuvette, and emission was monitored through a 570 nm glass cutoff filter (Melles Griot). Binding assays contained 100 nM TAS-103 in 300 μL of 71% (v/v) phosphate-buffered saline (with 5 mM $MgCl_2$). Human topoisomerase IIα (0 to 200 nM) was titrated into the sample, and anisotropy values were determined for each enzyme concentration. Anisotropy binding curves were generated, and K_D values determined by Scatchard analysis.

Site-Specific DNA Cleavage Induced by TAS-103. Topoisomerase II DNA cleavage sites were determined as described by Burden et al. (*42*). A linear 564 bp fragment (*Eag* I/*Bam*H I) of pBR322 plasmid DNA was prepared such that it was labeled with ^{32}P on a single 5′ terminus. Cleavage reactions contained 1.4 nM (25 ng) labeled DNA substrate, 60 nM (1 μg) human topoisomerase IIα, 1 mM ATP, and drug in 50 μL of topoisomerase II cleavage buffer. Drugs employed were TAS-103 (0−100 μM), amsacrine (100 μM), etoposide (100 μM), or a DMSO solvent control (final DMSO concentration of 1% in control, amsacrine, and etoposide samples). Reactions were started by the addition of topoisomerase IIα and incubated for 10 min at 37 °C. Cleavage intermediates were trapped by adding 5 μL of 10% SDS followed by 5 μL of 250 mM NaEDTA, pH 8.0, and topoisomerase IIα was digested with proteinase K (5 μL of 0.8 mg/mL) for 30 min at 45 °C. Reaction products were ethanol precipitated twice, dried, and resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF. Samples were subjected to electrophoresis in an 8% sequencing gel, which was then fixed in 10% methanol/10% acetic acid for 30 min and dried. DNA cleavage products were analyzed on a Molecular Dynamics PhosphorImager.

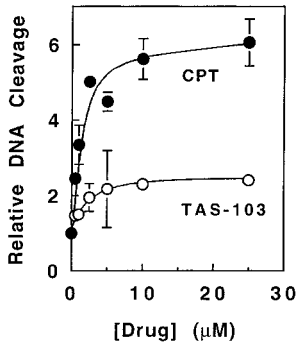

FIGURE 3: Topoisomerase I-mediated DNA cleavage is enhanced by TAS-103 in vitro. Cleavage of negatively supercoiled pBR322 plasmid DNA by calf thymus topoisomerase I was examined in the presence of camptothecin (CPT) or TAS-103. Levels of DNA cleavage are reported relative to the amount of cleavage observed in the absence of drug (~5% of the DNA substrate). Error bars represent the standard error of the mean for two independent experiments.

DNA Religation. The DNA religation reaction of human topoisomerase IIα was monitored according to the procedure of Robinson and Osheroff (*43*). Topoisomerase II DNA cleavage/religation equilibria were established as described above. Religation was initiated by shifting reactions from 37 to 0 °C. Reactions were stopped at time points following the temperature shift by adding 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Samples were treated and analyzed as described for topoisomerase II cleavage reactions. Apparent first-order religation rates were determined by quantitating the loss of linear DNA.

RESULTS

TAS-103 increases levels of DNA cleavage mediated by topoisomerase I and II in vivo and reportedly inhibits the catalytic activities of both enzymes in vitro (*1*). However, it is not known which of these properties is primarily responsible for the cytotoxicity of the drug. Therefore, experiments were carried out to investigate the mechanistic basis for TAS-103 action.

Since virtually every topoisomerase-targeted drug in clinical use kills cells by acting as a topoisomerase poison (*6*−*8*, *13*−*18*), initial studies focused on the DNA cleavage-enhancing properties of TAS-103. The inhibition of topoisomerase catalysis by TAS-103 is addressed in the accompanying paper (*21*).

TAS-103 Stimulates DNA Cleavage Mediated by Mammalian Type I and II Topoisomerases. The ability of TAS-103 to stimulate the DNA cleavage activity of purified mammalian topoisomerases was characterized to determine whether the enhanced scission observed in treated cells was due to a direct effect of the drug on enzyme−DNA interactions. As seen in Figure 3, TAS-103 enhanced topoisomerase I-mediated DNA cleavage ~2-fold. By comparison, camptothecin was much more effective, stimulating scission ~6-fold. The conclusions from these results are

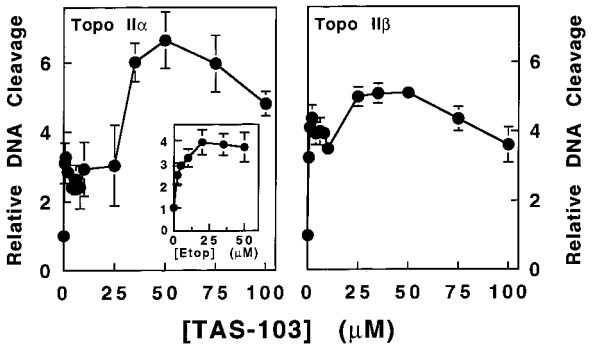

FIGURE 4: TAS-103 stimulates DNA cleavage mediated by human topoisomerase IIα (left panel) and topoisomerase II*β* (right panel) in vitro. Negatively supercoiled pBR322 was used as the substrate for these studies. The effect of etoposide (Etop) on DNA cleavage mediated by topoisomerase IIα also is shown (left panel, inset). Levels of DNA cleavage are reported relative to the amount of cleavage observed in the absence of drug (~10% of the DNA substrate). Error bars represent the standard error of the mean for two independent experiments.

comparable to those drawn from cellular studies (*1*); TAS-103 is a topoisomerase I poison, but it is not nearly as efficacious as camptothecin.

Mammalian species contain two closely related isoforms of topoisomerase II, α and *β* (*4, 8, 13, 44*−*46*). While topoisomerase IIα is dramatically upregulated during periods of rapid cell proliferation, topoisomerase II*β* levels are relatively constant across both cell and growth cycles (*46*−*49*). Both isoforms appear to play a role in mediating the effects of topoisomerase II poisons; however, topoisomerase IIα is believed to be the more important drug target (*16, 45, 50*−*53*). Although cellular studies demonstrated that TAS-103 stimulated topoisomerase II-mediated DNA cleavage (*1*), they did not distinguish between these two isozymes. Therefore, the effects of TAS-103 on the DNA cleavage activity of human topoisomerase IIα and *β* were determined (Figure 4).

TAS-103 stimulated DNA scission mediated by both isoforms. The drug displayed high potency and doubled levels of cleavage at concentrations that were less than 500 nM. Overall, cleavage increased more than 6-fold with the α isoform and ~5-fold with the *β* isoform. Maximal cleavage enhancement was observed at ~50 *μ*M TAS-103 and decreased slightly at higher drug concentrations. A characteristic trough in the cleavage enhancement profile of TAS-103 was observed at ~10 *μ*M drug. As discussed in the accompanying paper, it is believed that this trough is related to the DNA binding properties of TAS-103.[1]

The ability of TAS-103 to stimulate DNA cleavage mediated by human topoisomerase IIα was comparable to that of etoposide (Figure 4, inset). However, TAS-103 enhanced scission to an even greater extent at low drug concentrations. These findings are consistent with those from in vivo studies (*1*) and provide strong evidence that TAS-103 is a potent and efficacious topoisomerase II poison.

TAS-103 Kills Yeast Cells by Acting as a Topoisomerase II Poison. Since TAS-103 affects the DNA cleavage activity of both topoisomerase I and II, a yeast (*S. cerevisiae*) genetic system was utilized to determine whether either enzyme is the primary cytotoxic target of the drug, and whether TAS-103 kills cells by acting as a topoisomerase poison.

Initial experiments addressed the role of topoisomerase I in TAS-103 toxicity. This was accomplished by using a *top1*$^-$ strain that is devoid of topoisomerase I activity (*37*). Since topoisomerase I is not an essential enzyme in yeast, strains can be established that are completely lacking the enzyme (*25*−*27, 37*). Because topoisomerase I poisons convert the type I enzyme into a cellular toxin that generates breaks in chromosomal DNA, these drugs are ineffective when this enzyme is absent (*37*). Consequently, *top1*$^-$ strains are refractory to topoisomerase I poisons such as camptothecin (Figure 5A, inset). In contrast, no resistance was observed when *top1*$^-$ cells were treated with TAS-103 (Figure 5A). This finding provides strong evidence that TAS-103 does not kill yeast cells by acting as a topoisomerase I poison.

One caveat to this conclusion is the possibility that yeast topoisomerase I, compared to the human enzyme, is a poor target for the drug. If this were the case, cells that contained human topoisomerase I might be considerably more sensitive to TAS-103. To address this issue, the sensitivity of a *top1*$^-$ yeast strain that expressed the human type I enzyme was determined. Insertion of the human enzyme did not increase the sensitivity of yeast toward TAS-103 (data not shown). Thus, it appears unlikely that topoisomerase I is a significant cytotoxic target for TAS-103 in yeast.

When cells lose topoisomerase I activity, topoisomerase II assumes the physiological duties of the type I enzyme (*26, 27, 32*). Because of the increased importance of topoisomerase II in *top1*$^-$ strains, cells often display enhanced sensitivity toward topoisomerase II poisons (*55*). As seen in Figure 5A, the *top1*$^-$ strain was hypersensitive to TAS-103. This finding suggests that the drug may act as a topoisomerase II poison in yeast. Therefore, two additional experiments were carried out to investigate the role of topoisomerase II in the cytotoxicity of TAS-103.

In the first, a yeast strain carrying a temperature-sensitive topoisomerase II allele (*top2-1*) was employed (*36, 38*). Because of its essential nature, topoisomerase II cannot be deleted from cells (*4, 5, 26, 27*). At the semipermissive temperature of 30 °C, topoisomerase II activity is reduced to about 5−10% of that observed at the permissive temperature of 25 °C (*25*). This reduction in active topoisomerase II should result in the formation of fewer DNA breaks following treatment with topoisomerase II poisons. Thus, if TAS-103 functions as a topoisomerase II poison, *top2-1* strains should display resistance at the semipermissive temperature. Conversely, if TAS-103 functions as a catalytic inhibitor of topoisomerase II, decreasing levels of the enzyme should increase the effective drug:topoisomerase II ratio in the cell, making it easier to block residual topoisomerase II catalytic activity and inducing drug hypersensitivity. Finally, if TAS-103 functions through a target unrelated to topoisomerase II, decreasing levels of the enzyme should have little effect on drug sensitivity.

[1] Topoisomerase II can discern the topological state of DNA and preferentially cleaves negatively or positively supercoiled molecules over relaxed substrates (*54*). As determined by DNA binding and unwinding assays, TAS-103 intercalates into DNA and completely unwinds negatively supercoiled pBR322 molecules at a drug concentration of ~10 *μ*M (*21*). Thus, in the presence of 10 *μ*M TAS-103, negatively supercoiled plasmids appear to be fully relaxed and are poorer cleavage substrates for human topoisomerase IIα. At higher drug concentrations, the plasmid appears to be positively supercoiled and once again serves as an optimal DNA cleavage substrate for the type II enzyme.

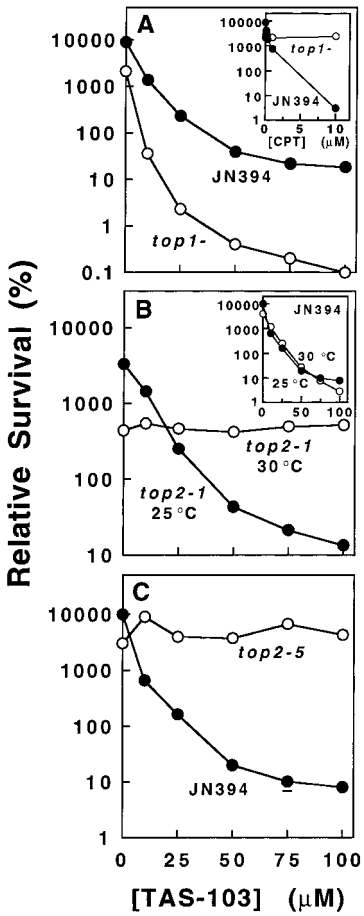

FIGURE 5: Topoisomerase II is the primary cytotoxic target for TAS-103 in yeast cells. The contributions of topoisomerase I and II to TAS-103 cytotoxicity was investigated using a yeast genetic system. The importance of topoisomerase I was examined by comparing the sensitivities of yeast strains JN394 and JN394 *top1*$^-$ toward TAS-103 (panel A). JN394 *top1*$^-$ is isogenic to JN394 except that the topoisomerase I gene is not functional. The effects of camptothecin (CPT) on these two strains also is shown (panel A, inset). The significance of topoisomerase II in mediating TAS-103 cytotoxicity was assessed using the yeast strain JN394 *top2-1*, which expresses a temperature sensitive topoisomerase II allele. Top2-1 displays greatly reduced catalytic activity at the semipermissive temperature of 30 °C. The effects of TAS-103 on JN394 top2-1 (panel B) and its parental strain JN394 (panel B, inset) at 25 °C (permissive temperature) and 30 °C are shown. The effects of TAS-103 on yeast strain JN394 *top2-5*, which encodes a mutant drug resistant type II topoisomerase, and its parental strain JN394 are shown in panel C. Data represent one of two independent experiments, each done in triplicate. Standard deviations are indicated by error bars.

At the semipermissive temperature, *top2-1* cells became highly resistant to TAS-103 (Figure 5B). Resistance was not due to a change in drug metabolism at 30 °C, since the sensitivity of the *TOP2* parental strain was identical at 25 and 30 °C (inset). These results indicate that topoisomerase II is the primary cellular target for TAS-103 and that the drug kills cells by acting as a topoisomerase II poison.

To confirm this proposed role for topoisomerase II in the cytotoxicity of TAS-103, the sensitivity of a yeast strain that expressed the *top2-5* mutant allele was determined. The *top2-5* enzyme is temperature sensitive and is inactive at 30 °C (*39*). At the permissive temperature of 25 °C, it is fully active but highly resistant to a number of topoisomerase II poisons. This lack of drug sensitivity confers considerable cellular resistance to these agents (*40*). As seen in Figure 5C, *top2-5* cells were also highly resistant to TAS-103 at 25 °C.

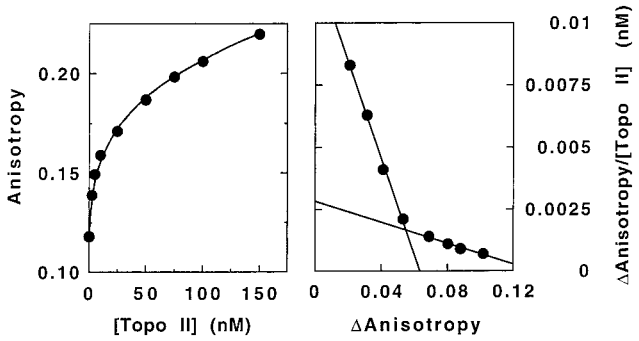

FIGURE 6: TAS-103 binds to human topoisomerase IIα in the absence of DNA. Binding assays took advantage of the intrinsic fluorescence of TAS-103. As seen in the left panel, the fluorescence anisotropy of the drug increased upon DNA binding. A Scatchard analysis of the binding is shown in the right panel. Representative data from one of three independent experiments is shown.

Results obtained with the above cell lines provide clear evidence that TAS-103 kills yeast cells by acting as a topoisomerase II poison. Although TAS-103 functions as a weak topoisomerase I poison in vitro, the type I enzyme plays no discernible role in the cytotoxicity of the drug.

TAS-103 Binds to Human Topoisomerase IIα in the Absence of DNA. Although topoisomerase II poisons (including TAS-103, see accompanying paper (*21*)) bind DNA, drugs such as etoposide and ellipticine also bind to the enzyme in the absence of nucleic acids (*42*, *56*, *57*). The role of enzyme−drug interactions is not clear, but it is believed that they play an important role in the formation of the ternary topoisomerase II·drug·DNA complex (*42*, *56*, *58*).

The ability of TAS-103 to bind human topoisomerase IIα in the absence of DNA was characterized by monitoring changes in the fluorescence anisotropy of the drug (Figure 6, left panel). TAS-103 bound to the enzyme with high affinity and in a saturable fashion. Based on Scatchard analysis (right panel), there appear to be high and low affinity binding sites for the drug on topoisomerase IIα, with K_d values of ∼5 and ∼50 nM, respectively. Although these values are lower than predicted based on DNA cleavage titrations with human topoisomerase IIα, a similar dichotomy has been observed for the interactions of ellipticine with yeast topoisomerase II (*56*).

TAS-103 Shares Sites of DNA Cleavage with Etoposide. The DNA cleavage site specificity of human topoisomerase IIα in the presence of TAS-103 was determined using a linear substrate derived from the plasmid pBR322. TAS-103 stimulated enzyme-mediated scission at a number of sites (Figure 7). Preferred TAS-103 sites corresponded to a subset of sites enhanced by etoposide and showed little overlap with those induced by amsacrine. This specificity is despite the fact that the DNA binding characteristics of TAS-103 are more similar to those of the intercalative drug amsacrine than the nonintercalative drug etoposide (see accompanying manuscript, (*21*)).

TAS-103 Strongly Inhibits DNA Religation Mediated by Human Topoisomerase IIα. There appear to be two distinct, but not mutually exclusive, mechanisms by which topoisomerase II poisons increase levels of enzyme-generated DNA breaks. Some drugs, such as etoposide and amsacrine, strongly inhibit the ability of the enzyme to religate cleaved DNA molecules (*7*, *8*, *13*, *43*, *59*, *60*). In contrast, other

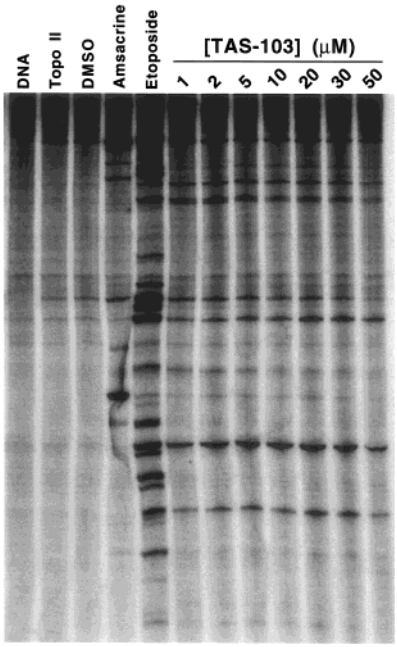

FIGURE 7: Etoposide and TAS-103 enhance DNA cleavage mediated by human topoisomerase IIα at common sites. The ability of amsacrine (100 μM), etoposide (100 μM), or TAS-103 (0−50 μM) to enhance levels of enzyme-mediated scission was determined. The DNA substrate was an end-labeled 564 bp fragment of pBR322. Controls included are DNA substrate only (*DNA*), topoisomerase II without drug (*Topo II*), and drug solvent (*DMSO*).

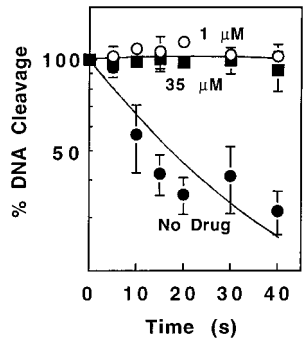

FIGURE 8: TAS-103 enhances topoisomerase II-mediated DNA cleavage primarily by inhibiting religation. The ability of human topoisomerase IIα to religate pBR322 DNA was examined in the presence of TAS-103 (1 or 35 μM). Enzyme-mediated DNA religation in the absence of drug is shown for comparison. Religation is expressed as the percent loss of linear DNA which was set to 100% at time zero. Data represent the average of two independent assays. Error bars indicate the standard error of the mean.

drugs, such as quinolones, ellipticine, azatoxin, and genistein, have little or no effect on religation rates and are believed to act primarily by enhancing the forward rate of topoisomerase II-mediated DNA scission (*7*, *8*, *13*, *61*, *62*).

As seen in Figure 8, TAS-103 is a strong inhibitor of DNA religation mediated by human topoisomerase IIα. Even at drug concentrations as low as 1 μM, virtually no religation was observed. These results indicate that TAS-103 increases levels of topoisomerase II-generated DNA breaks primarily by impairing the ability of the enzyme to carry out its DNA religation reaction.

DISCUSSION

TAS-103 is a promising new anticancer agent that is highly active against in vivo tumor models. The drug enhances cellular DNA cleavage mediated by both topoisomerase I and II, and the superior antitumor action of TAS-103 has been attributed to this dual effect (*1*).

Results of the present study demonstrate that TAS-103 stimulates DNA cleavage by both topoisomerases in purified systems. However, based on results with a yeast genetic model, it appears that drug cytotoxicity has nothing to do with its actions on topoisomerase I. Rather, topoisomerase II is the primary target of TAS-103, and the drug kills cells by acting as a topoisomerase II poison.

Although TAS-103 and etoposide are members of structurally divergent drug families, they share a number of common mechanistic features. Both drugs bind directly to topoisomerase II and increase levels of enzyme-generated DNA breaks by inhibiting the religation reaction of the enzyme (*42*, *57*, *59*). Furthermore, sites of topoisomerase II-mediated DNA cleavage induced by TAS-103 appear to be a subset of those induced by etoposide. Despite these similarities, TAS-103 displays higher antitumor activity than etoposide in a variety of mouse and human models (*1*). The basis for this improved activity is not clear; however, it may have to do with pharmacokinetic differences between these two drugs. Further in vivo and in vitro experiments will be necessary to resolve this important issue.

In summary, the present work characterized the mechanistic basis for the cytotoxic actions of TAS-103. Based on the findings of this study, we suggest that the drug be classified as a topoisomerase II poison rather than a "dual inhibitor" of the type I and II enzymes. This designation may have ramifications for the future clinical development of TAS-103.

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